G protein-coupled receptor-mediated transcriptional regulation in pathological cardiac remodelling

by

Ryan Douglas Martin

Department of Pharmacology and Therapeutics,

McGill University, Montréal, Québec, Canada

May 2020

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Abstract

Pathological cardiac remodelling is an adaptive response to various stressors placed on the heart, such as sustained hypertension or following myocardial infarction. The remodelling attempts to preserve cardiac function and contractility through increased left ventricular wall thickness via a hypertrophic response in cardiomyocytes. At the same time, cardiac fibroblasts are activated and mediate a fibrotic response in damaged areas to maintain structural integrity and aid in wound healing. While initially adaptative, chronic activation of these processes can lead to left ventricle dilation and heart failure, where the heart is no longer able to maintain the necessary cardiac output. This remodelling is predominantly mediated through neurohormonal activation of multiple G protein-coupled receptors (GPCRs), as well as their Gα and Gβγ partner proteins, to elicit intracellular signalling cascades in the different cell types. Currently, the primary therapies are aimed at blocking these receptors. Despite modest clinical success of these therapies, heart failure remains a leading cause of mortality in Canada. Therefore, development of new approaches are required to understand and impact disease progression and improve patient prognosis.

GPCR signalling pathways converge on the transcriptional machinery to regulate gene expression changes critical for the development of pathological cardiac remodelling. Due to the integration of multiple pathological signals by the transcriptional machinery, therapies targeting these processes are an attractive prospect that may have greater efficacy than current options. This thesis describes how GPCR signalling pathways alter the activity of the transcriptional machinery to regulate the gene expression changes underlying pathological cardiac remodelling. Herein, we identify the differential activation of Gαs/cAMP/PKA signalling between two GPCRs that drive hypertrophy, the α_1-adrenergic receptor and the endothelin-1 receptor, in both primary rat neonatal cardiomyocytes and a heterologous HEK 293 cell system. Furthermore, we demonstrate the implications of the differential signalling between the α_1-adrenergic and endothelin-1 receptors on positive transcription elongation factor b (P-TEFb) recruitment mechanisms employed by either receptor to promote cardiomyocyte hypertrophy. Additionally, we demonstrate the regulatory role of an interaction between Gβγ and RNA polymerase II on fibrotic gene expression downstream of the angiotensin II type I receptor in primary rat neonatal cardiac fibroblasts. Overall, this thesis expands our understanding of how GPCR signalling regulates the transcriptional machinery and proposes important considerations for the use and development of therapies for heart failure.

Resumé

Le remodelage cardiaque pathologique est un phénomène qui affecte le coeur en réponse aux différents stress qu'il peut subir, tels que l'hypertension ou un infarctus du myocarde. Ce remodelage tente de préserver la fonction cardiaque et sa contractilité en augmentant l'épaisseur de la paroi du ventricule gauche suite à une réponse hypertrophique des cardiomyocytes. Au même moment, l'activation des fibroblastes cardiaques déclenche une réponse fibrotique qui permet de maintenir l'intégrité structurelle et la réparation des blessures dans les régions affectées. Ces processus sont considérés au départ comme une adaptation aiguë, mais ils peuvent devenir chroniques et mener ainsi à la dilation du ventricule gauche et à l'insuffisance cardiaque, le cœur n'étant plus en mesure de maintenir sa fonction. Ce remodelage est médié de manière prédominante par l'activation neurohormonale de nombreux récepteurs couplés aux protéines G (RCPGs) et leurs partenaires protéiques Gα et Gβγ déclenchant des voies de signalisation intracellulaires dans différents types de cellules. À ce jour, les traitements cliniques principaux ont pour but de bloquer ces récepteurs et leur efficacité est modeste. Linsuffisance cardiaque étant actuellement une des causes principales de la mortalité au Canada, l'élaboration de nouvelles études est donc nécessaire pour comprendre et cibler la progression de la maladie afin d'améliorer le pronostic du patient.

Les mécanismes de signalisation des RCPGs semblent être associés à la machinerie transcriptionnelle régulant les changements d'expression génique qui influencent le remodelage cardiaque pathologique. Les thérapies ciblant ces processus sont une approche intéressante par rapport aux traitements actuels car elles incluent de nombreux signaux pathologiques reliés à cette machinerie transcriptionnelle. Cette étude décrit le niveau auquel les mécanismes de signalisation des RCPGs affectent l'activité de la machinerie transcriptionnelle lors des changements de la régulation de l'expression génique reliés au remodelage cardiaque pathologique. Dans un premier temps, le mécanisme de signalisation de Gαs/cAMP/PKA semble être activé différemment par deux RCPGs qui contrôlent l'hypertrophie, soit le récepteur α_1-adrenergique et le récepteur endotheline-1, dans les cardiomyocytes néonatales de rat et les cellules HEK 293 exprimant ces récepteurs de façon hétérologue. Par la suite, nous démontrons l'implication des différents mécanismes de signalisation des récepteurs α_1-adrenergique et le récepteur endotheline-1 sur les mécanismes de recrutement du facteur positif d'élongation de la transciption b (PTEF-b) utilisés par chaque récepteur afin de promouvoir l'hypertrophie des cardiomyocytes. De plus, l'interaction

entre Gβγ et l'ARN polymerase II semble avoir un rôle de régulation sur l'expression génique fibrotique en aval du récepteur d'angiotensine de type I dans les fibroblastes cardiaques primaires de rats nouveaux-nés. En conclusion, cette thèse cette thèse étend nos connaissances au niveau de la régulation de la signalisation des RCPGs qui affecte la machinerie transcriptionnelle en proposant le développement de nouvelles thérapies pour les maladies cardiovasculaires.

Table of Contents

Acknowledgements

I would like to thank my supervisors Dr. Jason Tanny and Dr. Terry Hébert. Thank you for taking a chance and seeing my potential six years ago when I was applying to graduate school. Your guidance and mentorship over the years has been instrumental in my development towards becoming the independent and curious scientific researcher I am today. I am grateful each of you allowed me the opportunity to develop the path forward for my project and provided a safe environment for me to experience and learn from failure when those paths did not work out. Thank you for understanding the importance of gaining experiences outside of the lab throughout graduate school and your encouragement to participate in student council, workshops and competitions. I am certain the skills I developed under your guidance will be critical for my future successes. Lastly, thank you for creating the amazing, open learning and working environments in each of your labs.

To the members of my advisory committee (Dr. Jean-Francois Trempe, Dr. Bruce Allen, Dr. Francois Robert), thank you for your insights and guidance as my project progressed. Each of you were instrumental in developing and molding this thesis to what it is today.

I want to thank all the people I have met throughout my time in graduate school, without you graduate school would not have been the same and I would not be the person I am today. While I can't name everyone, there are a few people I would like to give specific thanks to.

- To Jace Jones-Tabah, thank you for the many conversations about our respective projects over the years, all the nights of intense political debates (in which I don't think we ever came to an agreement), and for making life outside of the lab enjoyable over the years.
- To Kyla Bourque, thank you for all the insightful discussions about our projects and for all the not-so-insightful conversations blowing off steam and getting things off our chest.
- To Jen Chen and Sarah MacKinnon, thank you for all the science discussions and input in my project, for making Jason's lab an amazing environment over the years, and for making my time in graduate school as fun and exciting as it was.
- To Anne-Sophie Pépin, thank you for being my CEEHRC conference partner and travel buddy, for all the days in Café St-Barth drinking lattes and coding, and for being the amazing, supportive friend you are.

- To Courtney Smith, thank you for all the afternoon lab chats and for the many enjoyable nights over the years.
- To Viviane, thank you for all your support in the lab and for translating my abstract to French (and Madeleine Hébert for editing).
- To Dr. Shahriar Khan, thank you for taking me under your wing when I started graduate school and for all your support and encouragement in my personal life over the years.
- To Phan Trieu, Darlaine Pétrin, and Dr. Dominic Devost, thank you for all the training and support over the years and making sure the lab ran smoothly.
- To Yalin Sun and Luca Cuccia, thank you for your dedication and assistance in making this thesis into what it is today.
- To Rory Sleno, Jen Sun, Celia Bouazza, Daniel Pinto, and all the other members of Jason and Terry's labs over the years, thank you for making these labs a great environment to work.
- To Dr. Thomas Nardelli, thank you for being my rock throughout my PhD. Thank you for supporting and encouraging me in times of stress and anxiety and celebrating with me in times of success, regardless of how small they may have been. I could not have done this without you, and I look forward to our future adventures together.

Lastly, none of this would have been possible without the encouragement, support and love from my family. I want to thank my brothers and my sister-in-laws for the support and encouragement over the years. Thank you to all my nieces and nephews for making trips home so enjoyable and helping to get my mind off the stress of graduate school without even knowing how important it was to me. To my mom, thank you for everything you have done and continue to do for me. Thank you for always supporting me and encouraging me to push myself just a little bit further and for helping to pick myself up again when I fall. You are a continuous source of inspiration to me and are the strongest person I know. To my dad, I am forever grateful you developed and encouraged my scientific curiosity at each stage of my life. Your support and encouragement throughout my life was instrumental to my success and desire to obtain my PhD. I know you would be proud of who I am today and all that I have accomplished.

I dedicate this thesis to my dad.

Author Contributions

Chapter 1

Parts of Chapter 1 are from sections I wrote for a review published in 2020 (Martin *et al.*, 2020). I performed the literature review and wrote the chapter and both Dr. Terry Hébert and Dr. Jason Tanny edited the text.

Chapter 2

I designed, performed, and analyzed the experiments in Figure 2.1-2.5 and wrote the initial draft of the manuscript. Yalin Sun performed and analyzed experiments in Figures 2.2-2.5. Kyla Bourque validated the HEK 293 Gαs knockout cell line in Supplemental Figure 2.1. Nicolas Audet developed the cytoplasmic and nuclear BRET-based EPAC biosensors used for Figure 2.5. Asuka Inoue developed the HEK 293 Gαs knockout cell line used in Figure 2.3-2.4 and Supplemental Figure 2.1. Dr. Jason Tanny and Dr. Terry Hébert supervised the project and designed experiments. Dr. Jason Tanny, Dr. Terry Hébert and I edited and wrote the final version of the manuscript.

Chapter 3

I designed, performed, and analyzed the experiments in Figures 3.1-3.9 and wrote the initial draft of the manuscript. Yalin Sun performed and analyzed the experiment in Figure 3.8. Sarah MacKinnon contributed spike-in chromatin for the ChIP experiments in Figures 3.6 and 3.9. Luca Cuccia and Viviane Pagé assisted with experimental and technical aspects of the manuscript. Dr. Jason Tanny and Dr. Terry Hébert supervised the project and designed experiments. Dr. Jason Tanny, Dr. Terry Hébert and I edited and wrote the final version of the manuscript.

Chapter 4

I designed, performed, and analyzed experiments in Figures 4.5 and 4.6, Supplemental Figure 4.8 and Table 4.1. Shahriar Khan designed, performed and analyzed experiments in Figures 4.1-4.5 and Supplemental Figures 4.1-4.7. Sarah Gora was responsible for initially identifying the Gβγ-RNAPII interaction. Celia Bouazza performed experiments in Supplemental Figures 4.5 and

4.8. Jace Jones-Tabah and Andy Zhang produced the AAV_1-FLAG-$G\beta_1$ used for ChIP and co-immunoprecipitation experiments. Sarah MacKinnon contributed spike-in chromatin for the ChIP experiments. Phan Trieu assisted with experimental and technical aspects of the manuscript. Shahriar Khan wrote an initial draft with the signalling data and preliminary transcription data, which I expanded and rewrote to incorporate additional RT-qPCR-based fibrosis arrays and ChIP-seq experiments. Dr. Paul Clarke edited the manuscript. Dr. Jason Tanny, Dr. Terry Hébert and I edited and rewrote the final version of the manuscript.

Chapter 5

I wrote the chapter and Dr. Jason Tanny and Dr. Terry Hébert edited the text.

List of Figures

List of Tables

List of Supplemental Figures

List of Supplemental Tables

Abbreviations

ΔGαs	Gαs knockout
AC	Adenylyl cyclase
ACE	Angiotensin converting enzyme
ACII	Adenylyl cyclase II
AEBP1	Adipocyte enhancer-binding protein
AF2	Activation function 2
AF9	ALL1-fused gene from chromosome 9
AFF	AF4/FMR family
AHD	ANC1 homology domain
AKAP	A kinase anchoring protein
Akt	Rac-alpha serine/threonine-protein kinase
Ang II	Angiotensin II
AP-1	Activator protein 1
ARB	Angiotensin II receptor blockers
AT1R	Angiotensin II type I receptor
AT2R	Angiotensin II type II receptor
ATR	Angiotensin II receptor
BAF	Brg1/Brm1-associated factor
BD	Bromodomain
BET	Bromodomain and extra-terminal
BID	Basic residue-enriched interaction domain
Brd4	Bromodomain containing protein 4
BRET	Bioluminescent resonance energy transfer
Brg1	Brahma-related gene 1 (Brg1)
Brm	Brahma
CaaX	Cysteine-aliphatic-aliphatic-X
CAM	Constitutively active mutant
CDK	Cyclin dependent kinase
CDK1	Cyclin dependent kinase 1
CDK9	Cyclin dependent kinase 9

CFR	Corticotropin releasing factor
CHD	C-terminal homology domain
ChIP-seq	Chromatin immunoprecipitation followed by next-generation sequencing
ChIRP	Chromatin isolation by RNA purification
CKII	Casein kinase II
cMyBP-C	Cardiac myocyte binding protein-c
Col1a1	Collagen type I alpha 1 chain
CPA	Cleavage and polyadenylation
CREB	Cyclic AMP-responsive element-binding protein
CREM	cAMP responsive element modulator
CTD	C-terminal domain
CTIP2	COUP-TF-interacting protein 2
CTM	C-terminal motif
CTR1	C-terminal repeat region 1
Cyro-EM	Cryogenic electron microscopy
D2R	Dopamine D2 receptor
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's medium
DRB	5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
DSIF	DRB sensitivity inducing factor
EAF	ELL associated factor
ECC	Excitation-contraction coupling
ECM	Extracellular matrix
Egr1	Early growth response protein 1
ELL	Eleven-nineteen Lys-rich leukaemia
EndMT	Endothelial-mesenchymal transition
ENL	Eleven-nineteen leukaemia
ERK1/2	Extracellular signal regulated kinases 1/2
ET	Extraterminal
ET-1	Endothelin-1
ET_AR	ET_A receptor

ET_BR	ET_B receptor
ETR	Endothelin-1 receptor
FRET	Fluorescent resonance energy transfer
GATA4	GATA binding protein 4
GPCR	G protein-coupled receptor
GR	Glucocorticoid receptor
GRE	Glucocorticoid response genes
GRK2	G protein-coupled receptor kinase 2
GRK2ct	GRK C-terminal peptide
GTF	General transcription factor
HAT	Histone acetyltransferase
HBSS	Hank's balanced salt solution
HDAC	Histone deacetylase
HDAC3	Histone deacetylase 3
HDAC4	Histone deacetylase 4
HDAC5	Histone deacetylase 5
HDM2	Human double minute-2 protein
HEK 293-AT1R	HEK 293 cell line with heterologous AT1R expression
HEXIM1/2	Hexamethylene bisacetamide-induced protein 1/2
HIV-1	Human immunodeficiency virus 1
HMBA	Hexamethylene bisacetamide
HMGA1	High mobility group protein HMG-I/HMG-Y
hnRNP	Heterologous nuclear ribonucleoprotein
Hsp	Heat shock protein
HTLV-1	Human T-lymophotropic virus type 1
ICE	Interacts with carboxyl terminus of ELL
IFN-γ	Interferon-γ
IKKα	Iκβ kinase α
IL-2	Interleukin-2
IP_3	Inositol trisphosphate
IP_3R	Inositol trisphosphate receptor

iPSC	Induced pluripotent stem cell
JMJD6	Jumonji domain containing 6
KAP1	Kruppel-associated interacting protein 1
KOW	Kyrpides, Ouzounis, Woese
LARP7	La-related protein 7
LEC	Little elongation complex
LLPS	Liquid-liquid phase separation
LTCC	Voltage-gated L-type Ca^{2+} channels
LTR	Long terminal repeat
M3-MAChR	M3 muscarinic acetylcholine receptor
MAPK	Mitogen activated protein kinase
MEF2	Myocyte enhancer-factor 2
MEF2A	Myocyte enhancer factor 2A
MEF2C	Myocyte enhancer factor 2C
MePCE	Methylphosphate capping enzyme
MHC	Myosin heavy chain
MLL	Mixed lineage leukaemia
MMP	Matrix metallopeptidase
N-CoR	Nuclear receptor co-repressor 1
NELF	Negative Elongation factor
NES	Nuclear export sequence
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NFAT1	Nuclear factor of activated T cells 4
NFAT2	Nuclear factor of activated T cells 2
NFATc4	Nuclear factor of activated T cells 4
NIS	Sodium iodide symporter
NLS	Nuclear localization sequence
Nppa	Natriuretic peptide A
Nppb	Natriuretic peptide B
NPS	N-terminal phosphorylation sites
NRCM	Neonatal rat cardiomyocyte

NTP	Nucleotide triphosphate
P-TEFb	Positive transcription elongation factor b
PAFc	RNA polymerase-associated factor complex
PARP	Poly [ADP-ribose] polymerase
PAS	PolyA signal
PCAF	p300/CBP-associated factor
PCR	Polymerase chain reaction
PDE4	Phosphodiester 4
PDID	Phosphorylation-dependent interaction domain
PE	Phenylephrine
PEST	Rich in proline, glutamate, serine, and threonine
PI3K	Phosphoinositide 3-kinase
PIC	Preinitiation complex
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PITALRE	Pro-Ile-Thr-Ala-Leu-Arg-Glu
PKA	Protein kinase A
PKC	Protein kinase C
PKD	Protein kinase D
PL	Parental line
PLB	Phospholamban
PLCβ	Phospholipase Cβ
PMA	Phorbol myristyl acetate
PNUTS	Phosphatase 1 nuclear targeting subunit
PolyA	Polyadenylation
Postn	Periostin
PP1α	Protein phosphatase 1α
PP2B	Ca^{2+}–calmodulin–protein phosphatase 2B
PPM1A	Protein phosphatase, Mg^{2+}/Mn^{2+} dependent 1A
PPM1G	Protein phosphatase, Mg^{2+}/Mn^{2+} dependent 1G
pSer2	Phosphorylated serine 2 of the Rpb1 CTD
pSer5	Phosphorylated serine 5 of the Rpb1 CTD

RAAS	Renin-angiotensin aldosterone system
RAR	Retinoic acid receptor
RNAPI	RNA polymerase I
RNAPII	RNA polymerase II
RNAPIII	RNA polymerase III
RyR2	Ryanooide receptors
SE	Standard error
SEC	Super elongation complex
SEC-L	SEC-like complex
SERCA2a	Sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SIRT2	Sirtuin 2
SNP	Single nucleotide polymorphism
snRNP	Small nuclear ribonucleoprotein
SR	Sarcoplasmic reticulum
SRSF1/2	Serine and arginine rich splicing factor 1 or 2
STAT	Signal transducer and activator of transcription
STAT3	Signal transducer and activator of transcription 3
STAT5B	Signal transducer and activator of transcription 5B
SUMO	Small ubiquitin-like modifier
TAC	Transverse aortic constriction
TAF	TBP-associated factor
TAP	Tandem affinity purification
TAR	Trans-activation response
TBP	TATA-binding protein
TCR	T cell receptor
TF	Transcription factor
TGF-β1	Transforming growth factor β 1
TIMP	Tissue inhibitors of metalloproteinases
TM5	Transmembrane domain 5

TM6	Transmembrane domain 6
TRIM28	Tripartite motif containing 29
TRM	Tat-TAR recognition motif
TSHR	Thyroid stimulating hormone receptor
UBE2O	Ubiquitin conjugating enzyme E2 O
UTR	Untranslated region
Val-HeFT	Valsartan heart failure trial
α_1-AR	α_1-adrenergic receptor
β-AR	β-adrenergic receptor
β_1-AR	β_1-adrenergic receptor
β_2-AR	β_2-adrenergic receptor
β_3-AR	β_3-adrenergic receptor

CHAPTER 1: General Introduction

1.1. Cardiac Remodelling and Heart Failure

Heart failure is a complex, multifactorial disease that develops following structural or functional impairments to cardiac function [1]. Heart failure with left ventricular dysfunction reflects the inability of the heart to maintain sufficient cardiac output for the body. The lack of blood and oxygen supply to peripheral tissues results in symptoms such as shortness of breath and fatigue [2]. Heart failure remains a main cause of mortality and morbidity in Canada with 600 000 people (~1.5% of the population) as of 2019 living with heart failure and 50 000 new cases diagnosed each year. These rates are expected to continue to rise due to the aging population and as current treatments enable patients to live longer following cardiovascular insults such as myocardial infarctions [3]. While medical advances have improved disease management for these conditions, they are not effective in preventing progressive cardiac remodelling processes which lead to heart failure. The failure of effective therapies has dire complications for these patients as the 5 year mortality rate following heart failure is currently between 25-50% [2]. New therapies are required to prevent, delay or reverse pathological cardiac remodelling leading to heart failure and ultimately improve patient prognosis.

Cardiac remodelling occurs secondary to other cardiovascular diseases such as chronic hypertension, coronary artery disease, myocarditis, hypertrophic cardiomyopathy, and myocardial infarction [2, 4]. Although initiating processes differ, similar changes in systemic and cellular processes function in concert to remodel the myocardium and initially maintain cardiac output [2]. The various stressors on the myocardium affect diverse populations of cells, including cardiomyocytes, fibroblasts, immune and endothelial cells. For the purposes of this thesis, I will focus on the regulation of cardiomyocyte and fibroblast function in cardiac remodelling. The morphological changes are mediated by local increases in inflammatory cytokines as well as systemic increases in neurohormones, such as catecholamines, capable of activating G protein-coupled receptors (GPCRs). The stress-induced remodelling is initially adaptive, with increased left ventricular wall thickness due to cardiomyocyte hypertrophy. Chronic cardiomyocyte stimulation by neurohormones can also lead to cardiac arrhythmias and cardiomyocyte apoptosis. The decrease in healthy cardiomyocyte numbers puts greater contractile demand on the remaining cardiomyocytes, creating a positive feedback loop with further apoptosis [2]. Furthermore, there is an activated fibrotic response altering the extracellular matrix (ECM) in order to maintain structural integrity in areas of damage. The resulting accumulation of interstitial collagen decreases

contractility and increases left ventricular wall stiffness. Fibrosis reduces oxygen diffusion through the myocardium leading to further cardiomyocyte apoptosis [5, 6]. Heart failure develops when the heart is unable to maintain cardiac output due to cardiomyocyte loss and dilation of the left ventricle wall (Figure 1.1). A desirable therapeutic strategy is to target these processes to prevent progression to heart failure and reverse the pathological remodelling.

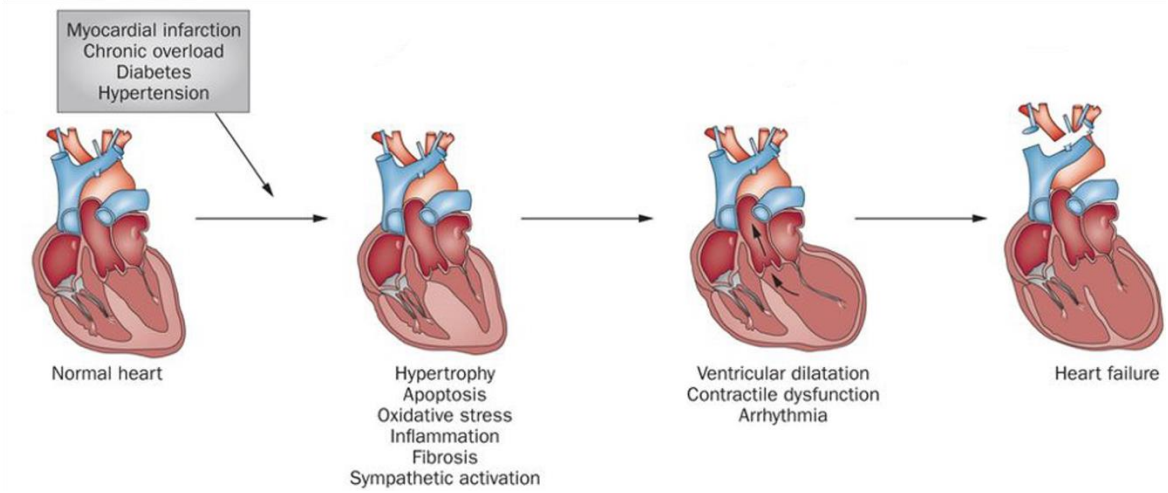


Figure 1.1. Progression of pathological cardiac remodelling.

In response to various stressors, the healthy heart undergoes a response characterized by increased sympathetic activation and inflammation which increase left ventricle hypertrophy and fibrosis. These processes lead to increased oxidative stress and cellular apoptosis, which can result in the development of ventricular dilatation, contractile dysfunction, arrhythmias and eventual heart failure. Figure adapted from [7].

1.1.1. Hypertrophy

In the face of chronic stress imposed by cardiovascular disease, the myocardium undergoes compensatory hypertrophic growth in the left ventricular wall to maintain cardiac output and reduce wall stress and oxygen consumption. The growth of the left ventricle is due to hypertrophy of cardiomyocytes, as these cells are terminally differentiated and unable to proliferate. The local and systemic increase in neurohormonal factors (i.e. catecholamines, angiotensin II, endothelin-1) modulate intracellular signalling events through GPCRs that culminate in a hypertrophic phenotype [8]. Cardiomyocyte hypertrophy also reflects the reactivation of a fetal-like gene expression program. That is, the gene expression in the adult cardiomyocytes begins to resemble the expression profile of cardiomyocytes early in development. For example, two common

markers of the fetal gene expression program are the natriuretic peptide A (Nppa) and natriuretic peptide B (Nppb), which show increased expression in hypertrophic cardiomyocytes [9]. These peptides are secreted into the blood to regulate blood pressure and volume through vasodilation, sodium removal in the kidney, and to inhibit cardiomyocyte hypertrophy through direct actions on cardiomyocytes [10, 11]. Furthermore, circulating BNP (protein encoded by the Nppb gene) and N-terminal proBNP are considered primary biomarkers of heart failure in the clinic [12]. The transition to a fetal gene expression program alters several aspects of cardiomyocyte function, including calcium handling [13, 14], sarcomere structure [15], and metabolism [16-20].

Cardiomyocyte contractile function depends on the proper mobilization of intracellular Ca^{2+} regulated by a process termed excitation-contraction coupling (ECC). When a cardiomyocyte becomes depolarized by an action potential during systole, voltage-gated L-type Ca^{2+} channels (LTCC) open and allow extracellular Ca^{2+} entry into the cell. The intracellular Ca^{2+} activates ryanodine receptors (RyR2) in the sarcoplasmic reticulum (SR) leading to the release of SR Ca^{2+} into the cytoplasm. The large increase in cytosolic Ca^{2+} concentration activates contractile proteins in the sarcomere leading to the ATP-dependent cardiomyocyte contraction. The Ca^{2+} is then removed from the cytoplasm to facilitate relaxation and diastole by several calcium channels, such as back into the SR by the sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) [21]. While cardiomyocyte SERCA2a expression increases throughout development, expression and activity are decreased in cardiac remodelling [13]. The decreased expression results in impaired removal of Ca^{2+} from the cytoplasm and consequently an increased cytoplasmic Ca^{2+} concentration and decreased SR Ca^{2+} concentration during diastole. The altered Ca^{2+} handling reduces systolic contraction and increases the time of diastolic relaxation in failing human myocardium [14].

Reversion to the fetal gene expression program in hypertrophic cardiomyocytes leads to functional changes to the contractile sarcomeres. A common marker for cardiac remodelling, among other sarcomere proteins with altered expression, is the transcriptional switch between two myosin heavy chain (MHC) ATPase isoforms [15]. In rodent models, the β-MHC is more abundant early in development, with a switch to predominantly α-MHC expression occurring throughout aging [22]. Hypertrophic cardiomyocytes revert back to predominantly β-MHC expression, and therefore the ratio of these two isoforms is a commonly used marker of heart failure [23-25]. Although there is not a developmental isoform switch in humans, the ratio of α-MHC to β-MHC still decreases in myocardium from failing human hearts [25, 26]. The small alterations in MHC

composition have profound effects on cardiomyocyte contractility as a 12% increase in α-MHC expression in rat cardiomyocytes increased power output by 52% [27]. The increased relative amount of β-MHC, which has low ATPase activity, is part of an adaptive response to reduce energy consumption that also alters expression of genes controlling metabolic activity and energy use [28].

The heart is one of the largest energy consumers in the body and requires a constant supply of ATP to continue contracting. Fetal cardiac tissue utilizes carbohydrates that are metabolized by the glycolytic pathway, such as glucose and lactate, as the predominant energy source [29, 30]. Glycolysis is favoured as there are high levels of the appropriate substrates, and it is more suitable for the low oxygen environment in the uterus. Furthermore, the fetal cardiomyocyte predominantly expresses metabolic enzymes involved in glycolysis, switching to lipid metabolism for energy in postnatal development [31]. In the adult heart, lipid oxidation is predominant as there is high lipid concentration and oxygenation of the blood [32]. Cardiac hypertrophy alters the balance of energy production from long-chain fatty acid oxidation back to anaerobic glycolysis as the myocardium becomes hypoxic [2, 20]. This metabolic adaption is accompanied by reactivation of the fetal gene expression program with reduced lipid oxidation enzyme and increased glycolytic enzyme expression and activity [16-19].

1.1.2. Fibrosis

The ECM is a common, non-cellular component in all organs that provides structural support and regulates changes in cellular phenotypes through interactions with cell surface proteins [33]. The ECM is a dynamic structure, with a network of structural proteins continually being remodelled to adapt to the current status of the tissue. The particular ECM components differ between each organ to match specific requirements, with the cardiac ECM predominantly composed of structural proteins, such as collagen, and other non-structural proteins [34]. During cardiac remodelling, myofibroblasts regulate a fibrotic response involving extensive ECM remodelling and collagen deposition. Fibrosis occurs in a localized manner, termed replacement or reparative fibrosis, following myocardial infarction that replaces the damaged tissue with fibrotic tissue to maintain structural integrity during healing. Another form involving diffuse ECM remodelling, termed interstitial or reactive fibrosis, occurs at sites distal to the area of damage following myocardial infarction and throughout the myocardium in many other cardiovascular

diseases, such as hypertensive heart disease [35, 36]. Again, fibrosis is initially an adaptive response to preserve the structural integrity of the myocardium, but a prolonged response leads to excess deposition of ECM proteins and increased myocardial stiffness. The excess ECM impairs cardiac output by disrupting electrical coupling between cardiomyocytes, which can lead to the development of arrhythmias, and reduces the diffusion of oxygen and nutrients into the tissue leading to cardiomyocyte death [6, 37, 38].

In the healthy heart, fibroblasts are responsible for maintaining the ECM through continuous turnover of structural proteins. Fibroblasts achieve this through both enzymatic and non-enzymatic mechanisms. First, fibroblasts secrete matrix metallopeptidases (MMPs) capable of breaking down collagen fibres and tissue inhibitors of metalloproteinases (TIMPs) to inhibit degradation [39, 40]. Second, fibroblasts secrete collagen type I and collagen type III, which form collagen fibres, and other structural proteins, such as fibronectin [36]. In the damaged heart, fibroblast differentiation to myofibroblasts alters the balance between collagen degradation and secretion to increase ECM components. Myofibroblasts are primarily characterized by the expression of α-smooth muscle actin and are not found in the healthy heart [41, 42]. Pertinent to this thesis is the role of fibroblasts and their differentiation to myofibroblasts, although several other cell types differentiate to cardiac myofibroblasts [43].

Reparative fibrosis following myocardial infarction is regulated through a sequential process from injury to fibroblast differentiation to scar tissue formation. In the infarct region, extensive cell death elicits an initial immune response to remove the cellular debris and extracellular matrix. The immune response creates space for fibroblasts to migrate to and proliferate in the infarct area. Inflammatory cytokines released in damaged areas, such as transforming growth factor β1 (TGF-β1), lead to the differentiation of fibroblasts into myofibroblasts [44, 45]. Furthermore, TGF-β1 increases the local production of angiotensin II (Ang II), which signals in an autocrine manner through the angiotensin II type 1 receptor (AT1R) to elicit a positive feedback loop further increasing TGF-β1 production [43]. Concurrently, cardiac injury increases systemic levels of catecholamines to overcome reduced cardiac function by increasing heart rate and inotropy [44, 46]. The increased systemic catecholamine levels promote the secretion of renin and angiotensin II from the kidneys into the circulation, which further elevates AT1R signalling in the myocardium [44]. The activated myofibroblasts secrete and deposit collagen to form scar tissue in the infarct area preventing wall rupture. Alongside collagen deposition, myofibroblasts secrete inflammatory

and pro-fibrotic cytokines, eliciting autocrine and paracrine signalling and further activating myofibroblasts [43]. After reparation of the infarct, myofibroblasts persist in the area of damage and continue cytokine secretion and diffusion into the surrounding tissue. These cytokines facilitate the further transition of fibroblasts into myofibroblasts in distal areas of the heart and subsequent development of interstitial fibrosis, continuing the positive feedback loop in these areas [36, 47]. In order to develop effective therapies to prevent the adverse aspects of fibrosis, we must understand how signalling mechanisms elicit the transition to myofibroblasts and their function in an active secretory state.

1.2. G protein-coupled receptors

GPCRs comprise a family of transmembrane proteins that transduce extracellular signals across the cell membrane to elicit intracellular signalling events. This family of proteins responds to a diverse range of stimuli, including peptides, lipids, odorants and light [48]. The range of diversity is reflected in the more than 800 genes encoding members of the GPCR family. GPCRs are expressed in many cell types throughout the body and while initially thought to localize only to the cell surface, recent evidence has indicated GPCRs localize and signal from intracellular organelles, such as the nucleus [49]. The pathological role of GPCRs has led to many drug development programs focusing on these receptors, with ~40% of current drug therapies on the market targeting a small subset of these receptors [50].

The GPCR family is characterized structurally by the presence of seven transmembrane helices connected by three intracellular and three extracellular loops, an extracellular N-terminal domain and an intracellular C-terminal tail. Despite these gross structural similarities, there is significant diversity in primary sequence, structure and function within the GPCR family. Phylogenetic analysis of the human GPCRs led to the ‘GRAFS’ classification system comprised of five main subfamilies: Glutamate, Rhodopsin, Adhesion, Frizzled/taste2 and Secretin [51]. The rhodopsin family, with ~670 members, is the largest family with intrafamily diversity predominantly found within the transmembrane regions [52]. Compared to the other families, the rhodopsin family lacks an extended N-terminal that contains the ligand-binding domains of the secretin and glutamate families, the diverse functional domains of the adhesion family or the large, cysteine-rich domain of frizzled receptors [52]. Across the five families, ligand binding elicits a

conformational change to the receptor leading to activation of intracellular signalling cascades (Figure **1.2**). For the purposes of this thesis, I will focus on GPCRs in the rhodopsin family.

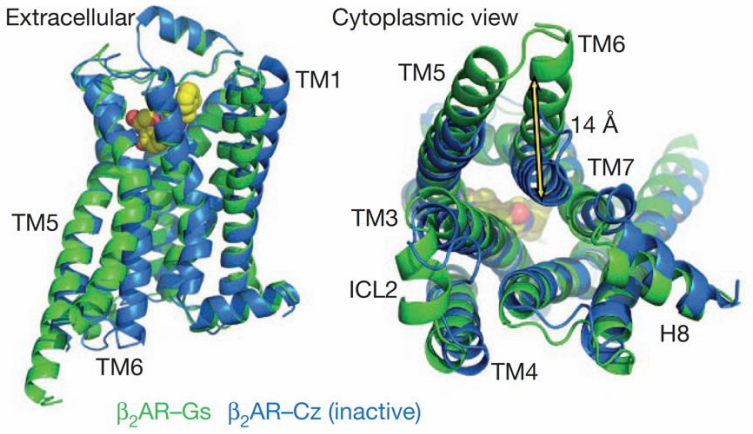


Figure 1.2. Crystal structure the β_2-adrenergic receptor.

Side (left) and cytoplasmic (right) view of the active β_2-AR (green) structure in complex with the heterotrimeric G protein complex (removed in the picture) or the inactive receptor bound by the inverse agonist carazolol (blue). Overlap of the two structures illustrates the extension of TM5 and 14 Å outward movement of TM6 in the active structure. The agonist BI-167107 occupies the binding pocket on the extracellular side (yellow). Figure adapted from [53].

Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature, "Crystal structure of the β_2 adrenergic receptor-Gs protein complex," Rasmussen SG et al., Jul 19;477(7366):549-55, Copyright 2011. See Appendix.

1.2.1. An overview of G protein signalling

Since the first crystal structure of rhodopsin in complex with 11-cis-retinal two decades ago, there have been tremendous technological advances enabling structural analysis of many other receptors, predominantly in the rhodopsin family, in a variety of states [54, 55]. Crystal structures have identified a common activation mechanism following ligand binding, as illustrated by the β_2-adrenergic receptor (β_2-AR) crystal structure in Figure **1.2**. The ligand binds a site typically formed within the bundle of transmembrane helices, which leads to an outward movement of transmembrane domain 6 (TM6) and an extension of transmembrane domain 5 (TM5) on the cytoplasmic side of the receptor (Figure **1.2**) [53, 55]. The structural rearrangement reveals an intracellular pocket required for recruitment of G proteins and other signalling partners. Additional

biophysical approaches have revealed a highly dynamic nature to GPCR conformation. For example, spectroscopy-based methods identified two, rapidly interchanging, inactive states of the unbound β_2-AR. In the presence of an agonist, the receptor conformation shifted towards an activated state capable of interacting with the G protein. The transition to an active state was incomplete, as a population of intermediate states were also detectable, requiring the presence of the G proteins for a complete shift [55]. Importantly, similar requirements were identified for the μ-opioid receptor indicating these are general features of the GPCR family [56, 57].

A common characteristic of GPCR subfamilies, except for frizzled, is the activation of heterotrimeric G proteins. Discovered over 40 years ago, the heterotrimer consists of a Gα subunit and an obligate heterodimer of a Gβ and Gγ subunit [58, 59]. Numerous genes encode isoforms of the three subunits and many combinations between isoforms are capable of being formed. The abundance of isoforms and their diverse combinations enable GPCRs to regulate numerous signalling pathways depending on the G protein activated.

1.2.1.1. Gα subunits

The Gα family is encoded by 16 different genes in mammals, and consists of at least 21 protein isoforms, which can be classified into four different families: Gαq/11, Gαi/o/t, Gαs/olf and Gα12/13 [60]. The classification is based on sequence similarity of isoforms as well the primary canonical effector protein regulated. The Gαq/11 family activates phospholipase Cβ (PLCβ), leading to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol trisphosphate (IP_3) [61]. The Gαi/o and Gαs families inhibit or activate adenylyl cyclase activity and cAMP production, respectively [62]. Lastly, the Gα12/13 family canonically activates the Rho-signalling pathway [63]. The isoforms within each family are expressed to varying degrees in different cell types throughout the body and are critical intracellular signalling mediators following GPCR activation.

Gα contains two distinct domains, a Ras-like domain with GTPase activity and an α-helical domain. Interactions with the Ras-like domain and the receptor binding pocket formed by translocation of TM5 and TM6 recruit the heterotrimeric G protein complex to a receptor (Figure 1.3A) [53]. In the inactive state, Gα contains a GDP molecule in a binding pocket formed by both the Ras-like and α-helical domain. The inactive state of Gα interacts with Gβγ through two interaction sites comprised of the Gα N-terminal α-helix and the side of the Gβ β propeller and the

Gα switch II domain and the top of the Gβ propeller (Figure 1.3B) [64-66]. The open Gα state formed upon receptor binding is rapidly converted to an active state by the binding of a GTP molecule, displacing the GDP. The binding of GTP leads to functional dissociation from the receptor and of the A otrimeric complex, with the Gα and Gβγ free to modulate effector protein activity. Gα remains active until the hydrolysis of the bound GTP to GDP. The GTPase activity of each Gα subunit varies, serving as a molecular clock regulating the duration of G protein activation (Figure 1.3C) [67]. Individual GPCRs only activate a small subset of the available Gα proteins. Although the mechanisms of selective Gα activation are not fully understood, bioinformatic approaches have identified specific residues in the GPCR and Gα interacting regions important for specificity [68].

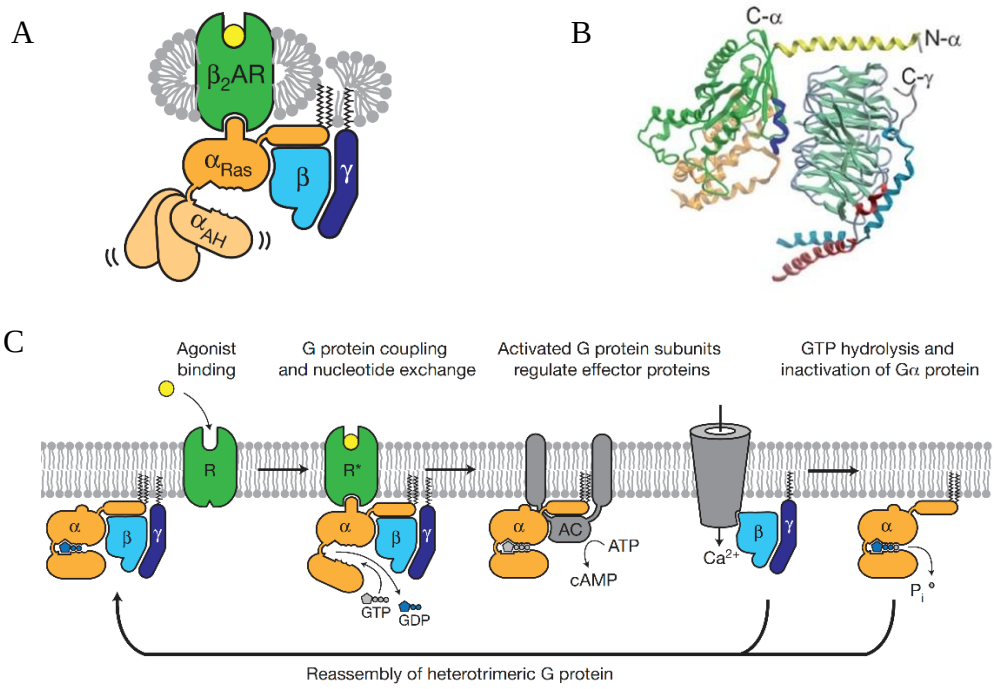


Figure 1.3. Structure and activation of heterotrimeric G proteins.

(A) Illustration of the β_2-AR in complex with the G protein heterotrimer through interactions of the Gα Ras-like domain and the binding pocket on the receptor. Figure adapted from [53].

(B) Crystal structure of heterotrimeric G$\alpha i\beta_1\gamma_2$ complex. The Gα subunit's Ras-like domain is green, the α-helical domain is beige, the N-terminal α-helix is yellow, and the switch II domain is dark blue. The Gβ β propeller structure is teal and the N-terminal helix is red. Lastly, the Gγ subunit is light blue. The interactions between the Gα switch II and N-terminal α-helix with the end or side of the Gβ β propeller, respectively, are evident. Figure adapted from [65]

(C) Agonist binding induces a conformational change in the GPCR (R) to an activated state (R*) enabling the binding of the heterotrimer G proteins. R* functions as a guanine exchange factor, promoting exchange of a GTP for the GDP bound to the Gα subunit and subsequent dissociation of the G proteins from the receptor. The Gα and Gβγ dissociate to regulate respective effector proteins, such as Gα regulation of adenylyl cyclases (ACs) and Gβγ regulation of Ca^{2+} channels, as illustrated. GTP hydrolysis by the Gα subunit leads to reassembly of the heterotrimer and inactivation, beginning the cycle again. Figure adapted from [53].

1.2.1.2. Gβ and Gγ subunits

As mentioned, the other component of the heterotrimeric G protein complex is the obligate heterodimer comprised of a Gβ and Gγ subunit. Gβ and Gγ require dimer formation for proper expression, as evident by formation of aggregates due to misfolding when a single subunit was expressed in the Sf9 insect cell line [69]. When the heterotrimeric G protein complex was discovered, the Gβγ dimer was thought to function solely as a negative regulator of Gα-mediated signalling pathways [67]. The discovery that Gβγ activated the muscarinic acetylcholinergic receptor-activated inwardly rectifying K^+ channel changed our understanding of the dimer's function [70]. Since then, the list of effector proteins regulated by Gβγ subunits has expanded dramatically and now includes adenylyl cyclases, phospholipase Cβ, voltage-gated calcium channels, and other effectors, which are typically referred to as the canonical pathways [71, 72].

The mammalian genome encodes five Gβ isoforms ($G\beta_{1-5}$) and twelve Gγ isoforms ($G\gamma_{1-5, 7-13}$). Within the Gβ family, $G\beta_{1-4}$ are highly similar with between 79-80% sequence similarity, whereas $G\beta_5$ is more divergent, with only 52% sequence similarity to the other four isoforms [72]. Furthermore, endogenous $G\beta_5$ does not dimerize efficiently with Gγ, although heterologous overexpression drove heterodimer formation with $G\gamma_2$ [73]. For the purposes of this thesis,

discussion of Gβγ refers to $G\beta_{1-4}$ isoforms only, unless otherwise stated. On the other hand, the Gγ family has more diversity with isoforms possessing 26-76% sequence similarity [72]. Despite the sequence diversity within each family, the majority of Gβ and Gγ subunits form stable complexes with similar structure, although interaction affinities between pairs vary [74]. The first crystal structures of the heterotrimeric G protein complex revealed Gβ belongs to the WD40 repeat protein family. The Gβ subunit forms a circular, seven-bladed β propeller structure and an α-helical N-terminus. The Gγ subunit has an extended α-helical structure and forms a coiled-coiled structure with the N-terminal α-helix of Gβ (Figure 1.4A) [75-77]. Furthermore, Gα interacts with the dimer through Gβ only (Figure 1.3A). A comparison between the Gα-bound (inactive) and free (active) dimer demonstrated that the Gβγ dimer does not undergo a conformational change upon activation. Instead, the release from Gα unmasks Gβγ surfaces required for interaction with effector proteins [71]. Gβγ does not possess any intrinsic catalytic activity and instead mediates cellular processes through protein-protein interactions. The top of the Gβ propeller consists of a 'hot spot' for interactions with effector proteins (Figure 1.4B), however, the N-terminus also mediates specific interactions [78-80].

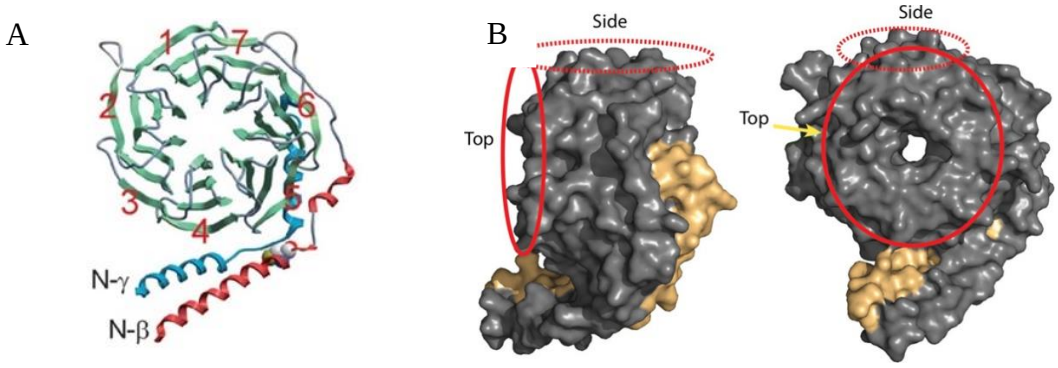


Figure 1.4. Protein binding surfaces on structure of Gβγ.

(A) Ribbon model of $G\beta_1\gamma_2$ heterodimer with the 7 blades of the Gβ β propeller structure indicated and the N-terminal α-helix in red. The Gβ N-terminal α-helix forms a coiled-coiled with the Gγ subunit (light blue).

Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Cellular and Molecular Life Sciences, "G protein βγ subunits: Central mediators of G protein-coupled receptor signaling," Smrcka, AV., May 19;65(14):2191-214, Copyright 2008. See Appendix.

(B) Space-filled model of the $G\beta_1$ (grey) and $G\gamma_2$ (beige) heterodimer with the two surfaces involved with protein-protein interactions identified. Gβ interacts with the N-terminal α-helix of Gα on the side and the switch II domain of Gα and downstream effector proteins on the top. Figure adapted from [71]

As Gβγ does not contain any catalytic activity, small molecule inhibitors must disrupt the relevant protein-protein interactions. The first Gβγ inhibitor required the expression of the Gβγ interacting region of G protein-coupled receptor kinase 2 (GRK2) comprising the C-terminal 192 residues (GRK2ct) [81]. The peptide selectively inhibited Gβγ signalling, with no observed effect on Gα signalling [82, 83]. A crystal structure of Gβγ and GRK2 revealed the C-terminus interacted with the top of the Gβ β propeller, which is masked by the Gα switch II domain in the heterotrimer [84]. Subsequent peptide-based approaches identified two inhibitors: a short sequence from adenylyl cyclase II (ACII) termed QEHA, and a short peptide referred to as the SIRK peptide [78, 85]. Crosslinking studies with QEHA and characterization of the $G\beta_1\gamma_2$-SIGK (an analogue of SIRK) structure revealed these peptides also interacted with the Gβ region masked by the Gα switch II domain [86, 87]. The identification of a common Gβ surface mediating interactions with effector proteins led to the characterization of a Gβ 'hot spot'. The 'hot spot' comprises a core set of residues with which effector proteins have distinct dependencies [88]. For example, mutagenesis of Gβ 'hot spot' residues revealed differential effects on PLCβ2, PLCβ3, and ACII regulation, with PLCβ and ACII requiring residues on opposing sides of the β propeller [89]. Similarly, SIRK blocked Gβγ-dependent activation of PLCβ and phosphoinositide 3-kinase (PI3K), but not Gβγ-dependent inhibition of voltage-gated calcium channels or ACI activity stimulated by Gαs [78]. The differential inhibition by SIRK was the first evidence that Gβγ signalling pathways could be selectively inhibited [88].

The success of Gβγ inhibitory peptides led to a computational screen to identify small molecules able to bind to the Gβ 'hot spot'. The relative affinities of identified hits were assessed by the ability to compete with SIRK for binding to $G\beta_1\gamma_2$ at a range of doses. A subsequent search

looked for structurally similar molecules to the high affinity hits. The screen identified M119 and M201, which were validated by further functional experiments to confirm their ability to inhibit the GRK2-Gβγ interaction. M119 prevented Gβγ interactions with PLCβ2/3 and PI3Kγ, whereas M201 potentiated the binding with PLCβ3 and PI3K, but not PLCβ2, *in vitro* [90]. A subsequent study identified the structurally related small molecule gallein and characterized its ability to inhibit Gβγ-dependent activation of PI3Kγ and Rac1 [91]. Lastly, the structurally distinct, Gβ 'hot spot' targeting small molecule 12155 was identified. In contrast to the other inhibitors, 12155 disrupted the interaction between Gβγ and GDP-bound Gαi independently of Gα nucleotide exchange. Furthermore, 12155 treatment in a neutrophil cell line led to receptor-independent activation of PLCβ, PI3K and extracellular signal regulated kinases 1/2 (ERK1/2). The identification of 12155 provides a tool to directly activate Gβγ independently of a GPCR and its many signalling pathways [92]. The development of these small molecules has enabled interrogation of Gβγ signalling in disease states, such as pathological cardiac remodelling, which I will discuss in Section 1.2.2.3.

Whereas Gβ mediates interactions with various effector proteins, the Gγ subunit is required to tether the complex to the membrane. The Gγ C-terminus contains a cysteine-aliphatic-aliphatic-X (CaaX) motif, which is modified by post-translational isoprenylation [71]. The isoprenyl modification is not required for dimer formation but is essential to retain the ability to regulate effector proteins [93]. Furthermore, variation in the length of the isoprenyl group altered the dimer's membrane affinity and interactions with effector proteins [94, 95]. Although initially thought to tether to the plasma membrane solely, it is now evident that Gβγ can translocate from the plasma membrane to the membranes of intracellular organelles [96, 97]. Furthermore, non-canonical Gβγ signalling pathways and interactors have recently been discovered in intracellular organelles such as the ER, Golgi apparatus, mitochondria and nucleus [98]. For example, cellular fractionation coupled with tandem affinity purification (TAP) followed by mass spectroscopy of TAP-tagged $G\beta_1$ identified numerous nuclear interactors including heterologous nuclear ribonucleoprotein (hnRNP) family members, proteins which facilitate translocation between the nucleus and cytoplasm such as importin 7 and exportin 1, and the transcription factor (TF) NF-κB [99]. Of particular importance to this thesis is the increasing evidence of Gβγ-dependent transcriptional regulation, which will be discussed further in Section 1.3.5.

1.2.2. GPCR signalling in pathological cardiac remodelling

Among the more than 800 GPCRs in the genome, expression of approximately 200 of them has been detected in the heart [100]. Many cell types in the heart express GPCRs that are activated by local or systemic increases of their endogenous ligands. Importantly, GPCRs' intracellular signalling cascades regulate many aspects of pathological cardiac remodelling. Here I will focus on GPCRs that regulate cardiomyocyte hypertrophy and fibrosis in animal models and how this has been translated to the clinic, with a focus on GPCRs relevant to chapters contained in this thesis.

Three common animal models, among others that will be referred to, are mice with cardiac-specific overexpression of a transgene, transverse aortic constriction (TAC) and systemic infusion of GPCR agonists [101]. First, placing a transgene downstream of the α-MHC promoter drives cardiac-specific expression at high levels in adult cardiomyocytes. Therefore, these studies are limited to identifying *in vivo* regulatory functions in cardiomyocytes. Throughout this thesis, cardiac-specific overexpression refers to α-MHC driven expression unless otherwise stated. Transgenes under the control of a periostin (Postn) promoter can target expression to myofibroblasts but not in a tissue-specific manner, adding a potential confound to these experiments [102]. Second, the pressure-overload TAC model is a surgical method that increases left ventricle afterload resulting in pathological remodelling of the left ventricle and, ultimately, heart failure. A main limitation is the rapid onset of pressure overload following surgery compared to the slow progression in a clinical setting. Lastly, surgical implantation of an osmotic pump provides continuous, systemic infusion of hypertrophic inducers. A drawback is the effects on other organ systems due to elevated systemic levels that may confound the direct effects on cardiac cells [101].

1.2.2.1. Gαs-coupled GPCRs

GPCR signalling through Gαs activation exerts positive effects on the force (inotropy), frequency (chronotropy), and relaxation rate (lusitropy) of cardiomyocyte contractility [103]. Gαs signalling results in activation of ACs, elevating intracellular cAMP levels and subsequent activation of protein kinase A (PKA) [104]. In the cardiomyocyte, PKA regulates contractility through phosphorylation of critical proteins involved in excitation-contraction coupling. PKA phosphorylation potentiates LTCC activity [105, 106], enhances RyR2 Ca^{2+} sensitivity [107], and

reduces phospholamban (PLB) inhibition of SERCA2a [108]. Furthermore, PKA phosphorylates the sarcomeric proteins cardiac myocyte binding protein-c (cMyBP-C) and troponin I [109, 110]. Altogether, these PKA-mediated phosphorylation events increase calcium release during systole, increasing inotropy, and the removal of calcium during cytosol, increasing lusitropy. Furthermore, PKA is also an important regulator of gene expression through phosphorylation of TFs, such as cyclic AMP-responsive element-binding protein (CREB) and histone deacetylase 5 (HDAC5) [111].

While acute PKA activation has positive effects, long term activation can lead to detrimental cardiac remodelling. Prolonged PKA substrate phosphorylation impairs calcium handling, negatively impacting contractility and cardiac output [21]. The pathological effects of chronic PKA activation are evident in transgenic mice with cardiac-specific Gαs overexpression. These mice developed the hallmarks of cardiac remodelling late in life, including fibrosis and cardiomyocyte hypertrophy [112]. Similarly, transgenic mice with cardiac-specific overexpression of PKA showed impaired contractility and cardiomyocyte hypertrophy, followed by the development of dilated cardiomyopathy and hyperphosphorylation of RyR2 and PLB [113]. Furthermore, transgenic mice with myofibroblast-specific overexpression of the PKA catalytic subunit α (PKAcα) also develop cardiac hypertrophy due to increased secretion of paracrine factors [114]. Depending on the substrate assessed, there are reports of enhanced or decreased PKA activity in cardiac tissue from failing human hearts. For example, cardiac tissue from patients with ischemic cardiomyopathy or idiopathic dilated cardiomyopathy exhibited RyR2 hyperphosphorylation [115]. Furthermore, human failing myocardium also exhibited increased LTCC currents, suggesting hyperphosphorylation by PKA [116]. Other studies have shown that cardiac tissue from human heart failure patients exhibits decreased phosphorylation of cMyBP-C [117], troponin I [118], and PLB [119]. The differential phosphorylation status of PKA targets may be due to the highly compartmentalized regulation of cAMP signalling.

A-kinase anchoring proteins (AKAPs) assemble and localize components required for cAMP production and degradation and effector proteins to specific subcellular sites [120]. The localized nature of PKA activation has differential impacts on cardiomyocyte function. For example, cytoplasmic PKA activation in neonatal rat cardiomyocytes enhanced contractility, whereas nuclear activation led to hypertrophy [121]. As neonatal rat cardiomyocytes can be cultured *in vitro* for a limited time before losing their cardiomyocyte phenotype, the lack of a

hypertrophic effect may be due to insufficient length of PKA activation to see such effects. Conversely, PKA-activated TFs CREB and HDAC5 do not promote adverse cardiac remodelling and instead appear to be cardioprotective [122, 123]. While activation of these two TFs is not pro-hypertrophic, activation of nuclear PKA is, suggesting additional pro-hypertrophic targets in the nucleus, one of which I will discuss further in Chapter 3. Furthermore, the localization of PKA signalling also differs between non-failing and failing hearts. In a rabbit model of heart failure, the balance of PKA signalling shifted from the sarcolemma to the myofilaments [124]. While localization of phosphorylated PKA substrates in human heart failure patients is opposite (decreased myofilament protein and increased SR protein phosphorylation), the results indicated that the temporal and spatial dynamics of PKA signalling are dependent on the cardiomyocyte's health status.

1.2.2.1.2. β-adrenergic receptors (β-AR)

The β-AR family is composed of three different isoforms: β_1-AR, β_2-AR, and β_3-AR. The β_1-AR and β_2-AR are the important mediators of cardiac function expressed at a ~4:1 ratio in cardiomyocytes, whereas the β_3-AR is expressed at low levels [125]. In non-myocyte cells, β_2-AR and β_3-AR are abundant and there is low β_1-AR expression [126, 127]. All three receptors signal through activation of Gαs, with the β_2-AR and β_3-AR also coupling with Gαi [127]. These receptors respond to the catecholamines epinephrine and norepinephrine released by the sympathetic nervous system, altering cardiomyocyte contractility through PKA signalling as previously described [128]. In cardiovascular disease, there is both a local and systemic increase in catecholamine levels leading to chronic receptor activation [129]. The sustained signalling decreases β_1-AR expression and cell surface receptor density, thereby altering the ratio of β_1-AR to β_2-AR [130-132].

A variety of animal models have demonstrated the pathological response to β-AR activation. Chronic infusion of the β-AR specific ligand isoproterenol in mice led to the development of pathological cardiac remodelling [133] and isoproterenol treatment of neonatal rat cardiomyocytes *in vitro* reactivated the fetal gene program and elicited a hypertrophic phenotype [134]. In cardiac fibroblasts, β_2-AR knockout decreased migration and secretion of pro-fibrotic factors in response to isoproterenol, indicating the β_2-AR promotes fibrosis in wild-type conditions [114]. Transgenic mice with cardiomyocyte specific overexpression of the β-AR isoforms revealed isoform specific

roles. Cardiac-specific β_1-AR overexpression in mice initially enhanced cardiac function, but progressed to pathological cardiomyocyte hypertrophy, apoptosis and interstitial fibrosis [135]. On the other hand, low levels of cardiac-specific β_2-AR overexpression enhanced cardiac function, with pathological remodelling observed only at very high levels of expression [136, 137]. The differential effect of β_1-AR and β_2-AR suggests the increased levels of cardiomyocyte β_2-AR in heart failure patients is a cardioprotective mechanism [125]. The fact that β-AR antagonists are still used in the clinic to treat end-stage heart failure exemplifies the critical role of the β-ARs in heart failure.

The clinical benefit of β-AR antagonists in reducing hypertension was identified almost 60 years ago, with propranolol as the first approved small molecule [138, 139]. Since then several β-AR antagonists have been developed and used in clinical trials for a variety of cardiovascular diseases such as hypertension, ischemic heart disease, and heart failure (although initially contraindicated for heart failure) [140]. Antagonist treatment prevents overactivation of β-AR signalling, thereby reducing desensitization and normalizing the surface expression of the receptor family [141]. β-AR antagonists with varying degrees of β_1-AR selectivity were developed as animal studies indicated this subtype as the family member predominantly driving pathological remodelling. Both β_1-AR-selective antagonists such as metoprolol, and non-selective antagonists, such as carvedilol, demonstrated reduced mortality and improved cardiac function in patients with heart failure in clinical trials [142-144]. Meta-analysis comparing the clinical benefit of multiple β-AR antagonists revealed that carvedilol treatment had the highest reduction in mortality, although this may be potentially due to properties other than β-AR antagonism [145]. Although β-AR antagonists are an important tool in the clinical setting, they only target one aspect of the complex signalling network in cardiovascular diseases.

1.2.2.2 Gαq-coupled GPCRs

Gαq-coupled GPCR signalling is a strong driver of cardiomyocyte hypertrophy and fibroblast proliferation through the sustained mobilization of intracellular calcium. Gαq signals through activation of PLCβ, which hydrolyzes PIP_2 into DAG and IP_3. Increased IP_3 activates the IP_3R on the SR membrane, releasing Ca^{2+} into the cytoplasm. The increased Ca^{2+} activates protein kinase C (PKC), various TFs (i.e. NFAT) and other pro-growth pathways. Alongside IP_3 pathways, DAG directly activates PKC. A variety of transgenic mice models have demonstrated the role of

Gαq in pathological cardiac remodelling. Transgenic mice with four-fold greater cardiac-specific expression of Gαq developed several characteristics of pathological cardiac remodelling: increased heart weight due to cardiomyocyte hypertrophy, reactivation of fetal gene expression, and impaired contractility [146]. Furthermore, mice expressing constitutively active Gαq developed severe cardiomyopathies, potentially due to pronounced cardiomyocyte apoptosis [147, 148]. On the other hand, cardiac-specific Gαq (and Gα11) knockout in mice prevented pathological cardiac remodelling in response to aortic banding, another model of pressure overload-induced heart failure [149]. These studies have altered Gαq activity solely in cardiomyocytes, but Gαq signalling is also an important regulator of fibrosis. Assessing signalling pathways regulating fibrosis has predominantly been done with primary cultures and activation of Gαq-GPCRs, which will be discussed in the upcoming sections. The Gαq-GPCRs associated with the pathological cardiac remodelling in cardiomyocytes and cardiac fibroblasts, and pertinent to this thesis, include the angiotensin II receptors (ATR), endothelin-1 receptors (ETR) and α_1-adrenergic receptors (α_1-AR).

1.2.2.2.1. Angiotensin II Receptors (ATR)

Two isoforms of the angiotensin II receptor, the angiotensin II type I receptor (AT1R) and type II receptor (AT2R), are expressed in cardiac tissue of rodents and humans, with rodents expressing two AT1R isoforms [150-152]. Both the AT1R and AT2R are expressed in cardiomyocytes and cardiac fibroblasts [153, 154]. The development of heart failure leads to different changes in isoform expression between rodent models and humans. Whereas in rodent heart failure models there is increased AT1R and AT2R expression, cardiac tissue from human heart failure patients displayed decreased AT1R and stable or increased AT2R expression [151, 155-157]. In pathological cardiac remodelling, there is enhanced receptor signalling through increased levels of the endogenous ligand angiotensin II (Ang II) following systemic and local increase in activity of the renin-angiotensin aldosterone system (RAAS).

Several models have indicated that the RAAS exerts pathologic effects through the AT1R subtype. First, the AT1R-specific antagonist losartan prevented cardiac remodelling in mice following TAC surgery [158]. The pathological effects of AT1R were further demonstrated by the development of cardiomyocyte hypertrophy, fibrosis and reactivation of the fetal gene expression program in mice with cardiac-specific AT1R expression [159]. The AT1R is also the functional

isoform regulating cardiac fibroblast differentiation to myofibroblasts, proliferation, and secretion of cytokines and collagen [160-162]. Conversely, transgenic mice with cardiac-specific AT2R expression showed reduced cardiac remodelling following myocardial infarction, indicating a cardioprotective role of this isoform [163]. Alternatively, several studies have shown that Ang II-mediated cardiomyocyte hypertrophy may be due to alterations in paracrine signalling between fibroblasts and cardiomyocytes and not direct actions on cardiomyocytes *per se*. For example, TGF-β1 knockout mice did not develop signs of pathological cardiac remodelling observed in wildtype mice following chronic Ang II infusion [164]. Furthermore, Ang II-mediated hypertrophy of neonatal rat cardiomyocytes required co-culture with cardiac fibroblasts. The Ang II effect was prevented by co-treatment with an ETR antagonist, suggesting ET-1 paracrine signalling from cardiac fibroblasts is also required [165].

Small molecules targeting elements of the RAAS are commonly employed as therapies for patients with cardiovascular disease. Two common treatments are angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARBs), specifically AT1R antagonists. The therapeutic strategies target the RAAS at two distinct parts of the pathway: the conversion of Ang I to Ang II by ACE by ACE inhibitors or Ang II interactions with the receptor by ARBs. A meta-analysis of 32 heart failure clinical trials assessing ACE inhibitors identified a significant reduction in risk of death and hospitalization [166]. Although there were significant benefits to ACE inhibitors, the reduction in risk of death is minimal with one clinical trial identifying a 16% reduction compared to placebo in patients with heart failure [167] and another found a 29% reduction in asymptomatic patients with reduced left ventricular ejection fraction [168]. Similar decreases in mortality were observed in clinical trials for ARBs, with one identifying a 13.2% reduction in mortality and morbidity [169]. However, a meta-analysis of 22 ARB clinical trials for heart failure identified a nonsignificant reduction in mortality and hospitalizations [170, 171]. The moderate benefits of targeting the RAAS system highlights again the difficulties in preventing disease progression when targeting a single aspect of the complex regulatory signalling network.

1.2.2.2.2. Endothelin-1 Receptors (ETR)

Endothelin-1 (ET-1) is the predominant endothelin isoform (of four) regulating cardiovascular function through activation of the ET_A receptor (ET_AR) or ET_B receptor (ET_BR). Fibroblasts from the rat left ventricle exhibit similar isoform expression, whereas cardiomyocytes

predominantly express the ET_AR isoform [172]. Following myocardial infarction in rats, ET-1, ET_AR and ET_BR expression increased in the left ventricle [173]. Similar altered expression was observed in the left ventricle of patients with heart failure due to idiopathic dilative cardiomyopathy, although no change or decreased ET_BR expression was observed [174, 175]. The increased ET-1 expression corresponds to its role in promoting pathological cardiac remodelling. ET-1 primarily functions in a paracrine manner and is produced locally by cardiomyocytes, endothelial cells and fibroblasts [176]. The pathological effect of ET-1 was demonstrated in transgenic mice with cardiac-specific ET-1 overexpression. These mice developed left ventricle dilation, cardiomyocyte hypertrophy and increased expression of inflammatory cytokines [177].

The pathological effects of ET-1 are mediated through activation of the ET_AR subtype. In primary rat and human cardiac fibroblasts, ET_AR signalling increased collagen synthesis and proliferation [178, 179]. In neonatal rat cardiomyocytes, the hypertrophic response to ET-1 was inhibited by co-treatment with an ET_AR-specific antagonist [180]. Corresponding to this, systemic ET_AR-specific antagonist treatment prevented pathological cardiac remodelling following aortic banding in rats [181, 182]. Interestingly, a time-dependent effect of ET_AR antagonist treatment was observed in a myocardial infarction model. Antagonist treatment beginning within 24 h of the MI surgery did not alter survival, impaired scar healing and led to greater left ventricle dilation [183]. Conversely, antagonist treatment starting ten days after MI surgery improved survival and cardiac function [182]. These studies suggest ET-1 is initially cardioprotective, with the detrimental effects requiring chronically elevated levels. Although ET_AR antagonists reduce pathological cardiac remodelling in animal models, clinical trials with these compounds for heart failure have not been successful [184].

1.2.2.2.3. α_1-adrenergic receptors (α_1-AR)

The α_1-AR family, composed of the α_{1A}-AR, α_{1B}-AR, and α_{1D}-AR isoforms, is activated by the endogenous catecholamines epinephrine and norepinephrine. Cardiomyocytes express the α_{1A}-AR and α_{1B}-AR, with predominant α_{1B}-AR expression, whereas cardiac fibroblasts do not express any α_1-AR isoforms [185]. Similarly, the human heart expresses the α_{1A}-AR and α_{1B}-AR isoforms, with the α_{1B}-AR the predominant isoform [186]. In the healthy heart, the α_1-AR comprise a small proportion of the total adrenergic receptors (which also includes the β-AR). The relative proportion increases in human heart failure as there is a slight increase in α_1-AR expression and a substantial

decrease in the β_1-AR expression. It has been postulated that the increased relative α_1-AR expression functions as a reserve to sustain the positive inotropic effects of catecholamines in the heart [185].

Animal models assessing the functional role of the α_1-ARs suggest the α_{1A}-AR isoform is cardioprotective and the α_{1B}-AR isoform promotes pathological cardiac remodelling. Transgenic mice with overexpression of an α_{1B}-AR constitutively active mutant (CAM) under a heart-specific or its endogenous promoter develop cardiac hypertrophy [187, 188]. At three months of age, transgenic mice with cardiac-specific overexpression of wild-type α_{1B}-AR exhibited impaired left ventricular function without the development of cardiac remodelling. By nine months of age, these transgenic mice died with the hallmarks of dilated cardiomyopathy, including decreased left ventricle function, chamber dilation and reactivated fetal gene expression [189-191]. On the other hand, transgenic mice with cardiac overexpression of wild-type α_{1A}-AR did not develop cardiac hypertrophy up to six months of age and displayed enhanced contractility [192]. Lastly, α_{1A}-AR and α_{1B}-AR double knockout mice developed more severe dilated cardiomyopathy and had higher mortality rates following TAC surgery than wild-type [193]. Replacing a single subtype in the cardiomyocytes from the double knockout mice revealed that the α_{1A}-AR, and not the α_{1B}-AR, mediated pro-survival signalling in cardiomyocytes [194]. Furthermore, hearts were protected from ischemic injury in transgenic mice with an α_{1A}-AR CAM, and not an α_{1B}-AR CAM, under the endogenous promoter [195, 196]. The various transgenic mouse models suggest the α_{1A}-AR mediates cardioprotective signalling, whereas the α_{1B}-AR is involved with pathological cardiac remodelling. While the animal models suggest isoform specific effects in the heart, clinical trials clearly demonstrated a cardioprotective role of the α_1-AR family. Clinical trials with pan-α_1-AR antagonists demonstrated that antagonist treatment led to increased heart failure rates in patients with hypertension and increased mortality in heart failure patients [197, 198].

Although the pathological role of the α_1-AR is not clear, specific agonists are used to model cardiomyocyte hypertrophy with neonatal rat cardiomyocytes. The hypertrophic effect of the α_1-AR specific agonist phenylephrine (PE) on neonatal rat cardiomyocytes was observed almost 40 years ago and is still used to model hypertrophy [199, 200]. Activation of the α_1-AR elicits an increase in cell size and similar reactivation of the fetal gene expression observed with *in vivo* models, allowing for a defined system to assess specific cellular processes [185].

1.2.2.3. Gβγ signalling

The functional role of the receptors previously discussed is typically associated with their activation of a specific Gα isoform, with less known about Gβγ-mediated effects. Initial *in vivo* studies with cardiac-specific overexpression of GRK2ct, an inhibitor of Gβγ, prevented remodelling in a genetic mouse [201] and a rabbit heart failure model [202]. Development of Gβγ small-molecule inhibitors enabled the functional role of Gβγ to be further assessed without transgenic expression of peptide inhibitors. The systemic, daily treatment of the inhibitor M119 prevented cardiac remodelling in response to chronic infusion of the β-AR agonist isoproterenol in mice. In the same study, daily injections of the chemically related inhibitor gallein prevented the progression of pre-established heart failure in a mouse genetic model [203]. A subsequent study investigated the functional pathways targeted by Gβγ inhibition to prevent cardiac remodelling following TAC surgery. Gallein restored β-AR surface expression in the heart and the adrenal gland, reducing catecholamine secretion and plasma levels in the mice. Therefore, systemic Gβγ treatment prevents the progression of heart failure through direct actions on the heart as well as other organs [204].

Further studies using primary cardiac fibroblasts and cardiomyocytes addressed the direct role of Gβγ on the heart. In mouse cardiac fibroblasts, gallein reduced myofibroblast activation in response to TGF-β, which was associated with increased intracellular cAMP due to restored β-AR density. A similar reduction in myofibroblast activation and increased cAMP occurred in human cardiac fibroblasts from heart failure patients treated with gallein [205]. Lastly, Gβγ signalling positively regulated hypertrophy of neonatal rat cardiomyocytes and increased fetal gene expression in adult rat cardiomyocytes following ETR activation. Both gallein and Golgi-targeted GRK2ct demonstrated that Gβγ signalling at the Golgi apparatus is required to activate PLCε signalling and a subsequent hypertrophic response [206]. In general, canonical Gβγ signalling pathways proximal to the receptor have been implicated in cardiac remodelling. As already discussed, extensive work has identified nuclear Gβγ-dependent regulation of RNAPII transcription through interactions with TFs. In Chapter 4, I will describe a new role for Gβγ in regulating RNAPII transcription.

1.3. RNA Polymerase II-mediated Transcription

1.3.1. Discovery of eukaryotic RNA polymerases

RNA polymerase activity in eukaryotes was first identified in rat liver homogenates in 1959 [207]. At the time, a single bacterial RNA polymerase was known to produce the three major classes of RNA: rRNA, tRNA, and mRNA, while little was known about the eukaryotic polymerase [208]. A decade after identification of eukaryotic RNA polymerase activity, three distinct RNA polymerases were purified by chromatography from sea urchin embryos, rat liver and yeast [209]. The three polymerases were termed RNA polymerase I (RNAPI), RNA polymerase II (RNAPII) and RNA polymerase III (RNAPIII) based on the order of elution. Unlike bacteria, the three eukaryotic RNA polymerases each transcribe a different class of RNA from a DNA template. Using the toxin α-amanitin, which has differential effects on the activity of each polymerase *in vitro*, RNAPI, RNAPII and RNAPIII were determined to transcribe rRNA, mRNA, and tRNA, respectively [210-214]. Subsequent identification of the components comprising each polymerase, revealed a set of shared proteins and polymerase-specific components required for specific transcriptional functions [208]. RNAPI, RNAPII, and RNAPIII contain 14, 12, and 17 subunits, respectively, with five subunits shared among the polymerases [215]. These five core subunits are conserved from bacteria, archaea and eukaryotes [216], reflecting the highly conserved mechanism employed for transcription throughout evolution. While the RNAPII-specific subunits were initially discovered in yeast [217], these subunits are conserved across eukaryotes [218].

1.3.2. Structure of RNA polymerase II

Various structural methods have enabled generation of highly detailed structures of the large multi-subunit RNAPII in complex with DNA, RNA and a variety of different transcriptional regulators. These structures have defined core features conserved across species and provided insights into how transcription regulators alter RNAPII function. While low resolution structures had previously been determined [219], the first high resolution structures of RNAP defined our mechanistic understanding of transcription. The first structure was derived from the bacterial *Thermus aquaticus* RNAP core enzyme [220], which was quickly followed by structures of the *Saccharomyces cerevisiae* 10-subunit core RNAPII without [221, 222] and with DNA [223], the

complete 12 subunit *S. cerevisiae* RNAPII [224, 225] and human RNAPII [226]. The various structures revealed a general architecture creating the catalytic core of the RNAPII defined by the conserved five subunits: Rpb1, Rpb2, Rpb3, Rpb6, Rpb11. The remaining unique RNAPII subunits provide additional functional and regulatory features.

The architecture is similar to a crab claw, with four large flexible regions termed the core, clamp, shelf and jaw (Figure 1.5A) [227]. The jaw region maintains contact with downstream DNA throughout transcription, orienting entry of DNA into the active site [228, 229]. During transcription, the negatively charged DNA template enters the active site through the positively charged cleft. The clamp region undergoes an extensive conformational change upon DNA binding to secure the template DNA in the active site (Figure 1.5B) [221-223, 229]. The polymerase melts the double-stranded DNA as it enters the cleft and inserts the template strand into the active site. In order to facilitate nucleotide catalysis at the active site, RNAPII requires Mg^{2+} and the entry of an appropriate nucleotide triphosphate (NTP) through the pore. Downstream of the active site, a nine base pair RNA-DNA hybrid extends perpendicular to the entry DNA due to the wall domain near the active site, which along with regions of Rpb1 and Rpb2 stabilizes the open transcription bubble [223].

The RNA-DNA hybrid is melted through the coordinated actions of the lid, rudder and fork loop 1. The fork loop contacts and stabilizes the hybrid, the lid functions as a wedge to separate the RNA and DNA, and the rudder's interaction with the released single-stranded DNA prevents the hybrid from reannealing [230]. Additionally, the lid guides the RNA into the polymerase's RNA exit channel, placing the RNA close to a critical regulatory domain of co-transcriptional RNA processing (Figure 1.5C). Rpb1 contains a C-terminal domain (CTD) near the exit site that is comprised of multiple repeats, from 26 in *S. cerevisiae* to 52 in mammals, of the consensus Tyr^{1}-Ser^{2}-Pro^{3}-Thr4-Ser^{5}-Pro^{6}-Ser^{7} heptad repeat [231]. Although not identified in structural studies due to its intrinsic disorder, the CTD undergoes dynamic changes in phosphorylation throughout the transcription cycle that is required for the recruitment of transcription co-factors at specific stages of transcription [232, 233]. The role of specific phosphorylation events will be discussed further in the subsequent sections.

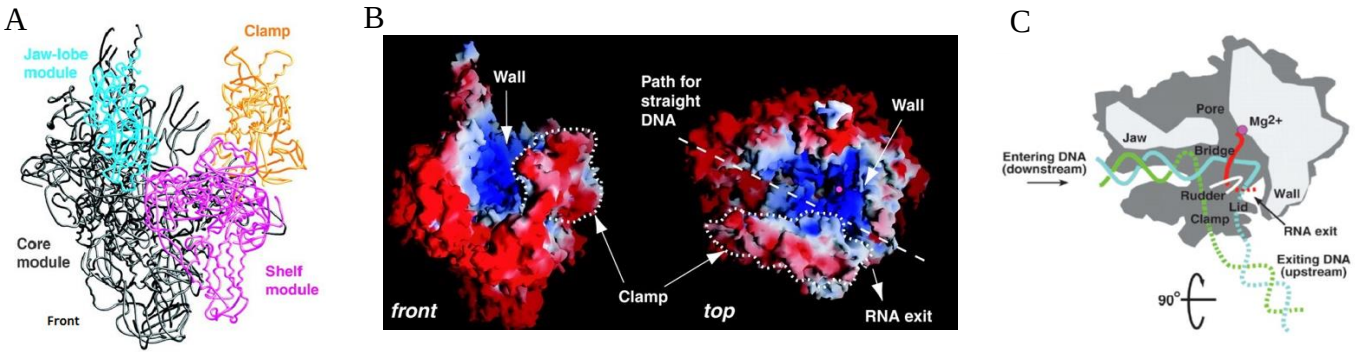


Figure 1.5. Structure of RNAPII.

(A) Ribbon model of core 10-subunit RNAPII structure with the core (grey), jaw (blue), shelf (pink) and clamp (orange) module indicated.

(B) Space filled model of RNAPII coloured according to the surface charge. The majority of the RNAPII surface area is negatively charged (red), with the cleft positively charged (blue) to guide negatively charged DNA towards the active site.

(C) Diagram of transcribing RNAPII with DNA template strand (blue), non-template strand (green), RNA (red) and Mg^{2+} (purple). The rudder is shown melting the RNA-DNA hybrid as it leaves the active site, with the lid guiding the RNA to the exit groove. Figure adapted from [230].

From Cramer, P., D.A. Bushnell, and R.D. Kornberg, Structural basis of transcription: RNA polymerase II at 2.8 angstrom resolution. Science, 2001. 292(5523): p. 1863-76. Reprinted with permission from AAAS. See Appendix.

Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer, Nature Structural & Molecular Biology, "Structure and mechanism of the RNA polymerase II transcription machinery," Hahn, S., 11(5):394-403, Copyright 2004. See Appendix.

The initial RNAPII structures provided detailed mechanistic information on the enzymatic activity involved in transcription. However, many of these initial structures were of RNAPII alone, a state that does not reflect the highly coordinated transcription that occurs in the cellular environment. This process is highly regulated at various stages to ensure proper gene expression and to elicit adaptive responses to the many forms of cellular stress. A host of other proteins are recruited to RNAPII in a spatiotemporally regulated manner to ensure proper gene expression under a variety of conditions. Advances in structural biology have enabled the generation of RNAPII in complex with a variety of different transcriptional regulators, which, along with biochemical data, has expanded our knowledge of the regulatory stages.

1.1.3. Regulatory stages of RNA Polymerase II transcription

RNAPII transcription occurs in several stages termed initiation, elongation and termination (Figure 1.6). The initiation stage recruits RNAPII to a gene promoter in the proper orientation, establishes the transcription bubble and promotes promoter-clearance, enabling RNAPII to begin transcription (Step 1 and 2). At a plurality of genes (30-90% in metazoans depending on the organism and cell type in question), this transcript elongation phase is interrupted ~30-50 bp downstream of the transcription start site, where it enters an intermediate stage of promoter-proximal pausing (Step 3) [234]. Following release from the paused state, productive elongation begins along the gene body where the nascent RNA transcript is produced and co-transcriptionally processed (Step 4). At the 3' end of the gene, termination releases the RNA transcript from the polymerase and the polymerase from the DNA (Step 5 and 6). Regulation of each stage requires the recruitment of transcriptional regulators to ensure proper gene expression. The Rpb1 CTD is a critical regulator of the transcription cycle, serving as a scaffold for the proper spatiotemporal recruitment of transcriptional regulators. Dysregulation of transcription occurs in a variety of human diseases, either through aberrant signalling or mutation in critical proteins leading to improper recruitment of transcriptional machinery and alterations in gene expression.

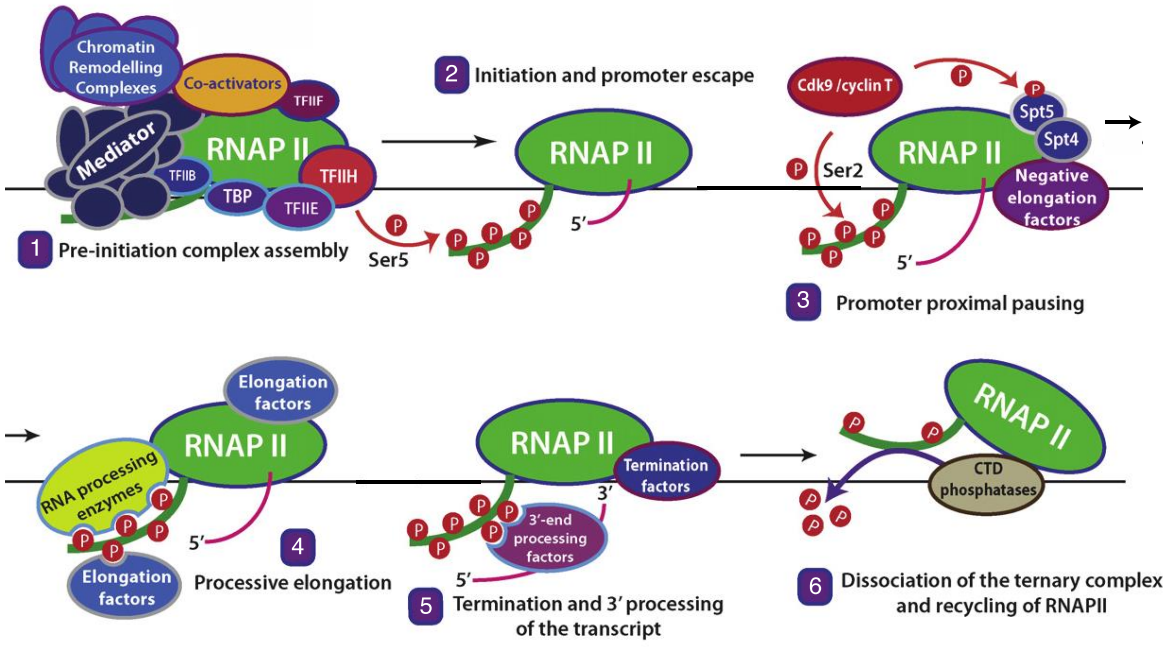


Figure 1.6. Simplified schematic of RNAPII transcription cycle stages.

(1) The concerted actions of general transcription factors, Mediator, chromatin remodelling complexes and other co-activators form the preinitiation complex

(2) Phosphorylation of serine 5 in Rpb1's CTD heptad repeat promotes dissociation of RNAPII from the preinitiation complex. RNAPII transcribes ~30-50 base pairs before promoter proximal pausing.

(3) P-TEFb, a dimer of Cdk9 and cyclin T, releases paused RNAPII through phosphorylation of transcriptional machinery.

(4) RNAPII enters the elongation stage throughout the gene body, recruiting RNA processing factors and elongation factors in a spatiotemporal manner through phosphorylation of Rpb1's CTD.

(5 and 6) At the 3' end of the gene, the RNA transcript is processed and RNAPII dissociated from the DNA.

Reprinted from DNA Repair, 9(3), Aygün, O., Svejstrup, JQ., RECQL5 helicase: connections to DNA recombination and RNA polymerase II transcription, 345-353, Copyright (2010), with permission from Elsevier. See Appendix.

1.3.3.1. Initiation

Initiation is the first rate-limiting regulatory stage of RNAPII transcription whereby the polymerase is loaded onto the DNA template and begins transcribing. Initiation consists of several intricate steps to form a preinitiation complex (PIC) composed of RNAPII and the general TFs (GTFs) TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH. PIC formation is regulated at individual genes by the presence of local cis-regulatory DNA sequences termed promoters and distal regions termed enhancers. Genome-wide analysis identified sequence motifs commonly found within promoter regions required for GTF recruitment [235]. Furthermore, promoters and enhancers contain sequence motifs capable of recruiting sequence-specific TFs (Figure 1.7A) [236]. [236]. TFs promote PIC formation through a complex set of mechanisms involving multiple interactions. For example, TFs promote local and long-range effects on chromatin structure and chromosome architecture. TFs interact with chromatin remodellers and modifiers, to alleviate nucleosome barriers and create accessible DNA for the transcriptional machinery (Figure 1.7B) [237]. [237]. For example, TFs promote dynamic alterations in histone acetylation by histone acetyltransferases (HAT) and HDACs at promoters and enhancers. Increased histone acetylation corresponds to

increased DNA accessibility at these genomic loci and correlates with increased TF binding and transcriptional activity [238, 239]. Functional cooperation between TFs and chromatin-modifying factors is an important feature of transcriptional responses following environmental changes to the cell.

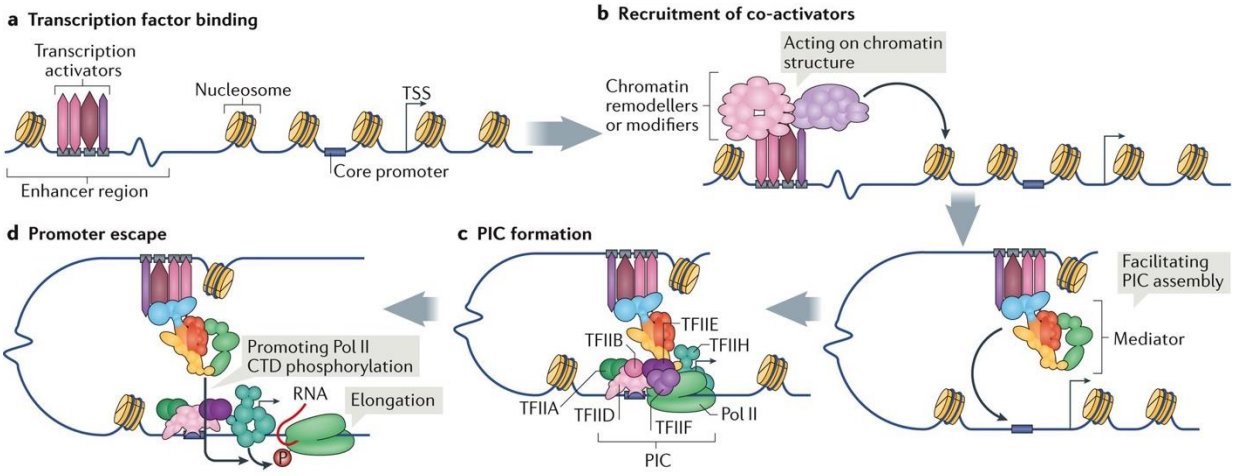


Figure 1.7. Schematic of RNAPII initiation steps.

(A and B) Transcription factors recognize their sequence motifs in enhancers and recruit chromatin remodellers to promote accessible DNA for the recruitment of transcriptional machinery.

(C) Mediator and GTFs recruit RNAPII to the developing preinitiation complex.

(D) Phosphorylation of serine 5 of Rpb1 CTD heptad repeat promotes RNAPII promoter escape.

Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Reviews Molecular Cell Biology, "Transcription regulation by the Mediator complex," Soutourina, J., Dec 6;19(4):262-274, Copyright 2017. See Appendix.

TFs can also function through the Mediator complex. Mediator is a multi-subunit complex (up to 30 subunits in humans) that is bound to RNAPII not engaged in RNA synthesis [237]. The ~1.4 mDa complex serves as a bridge between gene-specific TFs bound at promoters and enhancers and the RNAPII transcription machinery [240]. Most gene-specific TFs do not directly contact RNAPII and instead interact with Mediator through activation domains, enabling integration of multiple TF signals to modulate RNAPII recruitment [241]. Beyond direct interactions with RNAPII, Mediator also promotes RNAPII recruitment by regulating GTF function and recruitment (Figure 1.7C). Furthermore, Mediator facilitates the long-range

interactions required for enhancer transcriptional regulation by altering the 3D organization of the chromatin, forming enhancer-promoter gene loops [241].

TFIID, a complex consisting of TBP and TAFs 1-13, interactions with regulatory elements at the core promoter, such as TBP recognition of the TATA box, initiate PIC formation [242-244]. However, additional core promoter elements are recognized by TAFs that are essential at non-TATA box promoters [245]. Recruitment of TFIIA and TFIIB stabilizes the TBP-promoter complex and assists with proper orientation, although transcription *in vitro* does not require TFIIA [246-249]. In the intact PIC, TFIIB extends into the catalytic site of RNAPII through the RNA exit channel to modulate catalytic activity and transcription start site selection [250-252]. The growing PIC recruits the TFIIF-RNAPII complex, followed by recruitment of TFIIE and TFIIH to form the complete initiation complex [230, 253]. TFIIH consists of a core domain, which contains the helicase subunit XBP and a kinase domain that includes the cyclin-dependent kinase CDK7 [254]. The ATP-dependent helicase XPB is required to melt the promoter DNA, transitioning the PIC to an open complex with the template DNA in the active site of RNAPII [255, 256].

The open initiation complex begins transcribing RNA as it undergoes promoter clearance, an inefficient process *in vitro* with RNAPII undergoing multiple rounds of abortive transcription resulting in the production of short (~10 nt) nascent RNA transcripts [257, 258]. The growing RNA transcript displaces TFIIB from the RNA exit channel of RNAPII, promoting release from the GTFs [259, 260]. Phosphorylation of Ser5 of the Rpb1 CTD (pSer5) by the TFIIH subunit CDK7 disrupts the RNAPII-Mediator interaction, further promoting RNAPII release (Figure 1.7D) [261]. The phosphorylation also facilitates the recruitment of capping enzyme to modify the 5’ end of the nascent RNA with a methyl-guanosine cap. Continued RNAPII transcription disrupts the interactions with other GTFs, although TFIIF can also interact with elongating complexes [262]. A subset of GTFs and Mediator remain at the promoter forming a reinitiating scaffold to maintain a higher rate of initiation at some genes [263-265]. After transcribing 30-50 bp, RNAPII enters a promoter-proximal paused state mediated by the recruitment of several negative elongation factors [266].

1.3.3.2. Promoter-Proximal Pausing

The first evidence of promoter-proximal RNAPII pausing in mammalian cells was found at *Drosophila melanogaster* heat shock protein (Hsp) genes and subsequently at other immediate

early genes, such as c-Fos and c-Myc [267-270]. The advent of next-generation sequencing enabled the development of genome-wide methodologies that have identified promoter-proximal RNAPII at a large number of genes, with estimates ranging between 30-90% of genes with paused RNAPII depending on the method and criteria [271-273]. These genome-wide methods demonstrated that promoter-proximal pausing is abundant at genes involved with development, differentiation and response to extracellular stimuli [274]. The paused RNAPII complex is highly stable with a mean half-life of 6.9 min. The stability was proposed to facilitate integration of multiple signals and create a transcriptional checkpoint for co-transcriptional processing [275]. Furthermore, researchers have proposed pausing exerts important regulatory functions on the synchronization of gene induction and speed of gene induction [266, 276]. Promoter-proximal pausing requires the actions of the negative elongation factors 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) Sensitivity Inducing Factor (DSIF) and Negative Elongation Factor (NELF) [234, 277, 278].

Following promoter clearance, negative elongation factors are recruited to the actively transcribing RNAPII to establish pausing. First, DSIF is recruited and forms extensive interactions with RNAPII, upstream DNA and RNA [279, 280]. The recruitment of DSIF requires many of the GTFs to be released as these factors share several interaction sites with RNAPII (Figure 1.8A) [280]. For example, competition between TFIIE and DSIF for binding to the clamp domain of RNAPII has been observed [279, 281]. The Kyrpides, Ouzounis, Woese (KOW) 4 and KOW5 domains of Spt5 are required to establish RNAPII pausing, forming a clamp around the RNA exiting RNAPII (Figure 1.8B) [280, 282]. DSIF recruits and cooperatively regulates RNAPII pausing with the four subunit NELF complex [277, 283]. NELF restrains RNAPII mobility through interactions with the face opposite of the cleft domain [284]. Of the four subunits, the N-terminal domain of NELF-A regulates RNAPII pausing and interactions with the DSIF-RNAPII complex [284, 285]. Initial studies indicated that the depletion of DSIF or NELF led to reduced promoter-proximal paused RNAPII at the hsp90 gene promoter in *D. melanogaster* [286]. Chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq) for NELF-A and Spt5 demonstrated genome-wide co-occupancy of these factors with promoter-proximal paused RNAPII [271]. While the majority of NELF resides at genomic loci with paused RNAPII, DSIF also occupies gene bodies with another peak at the 3’ end of the gene

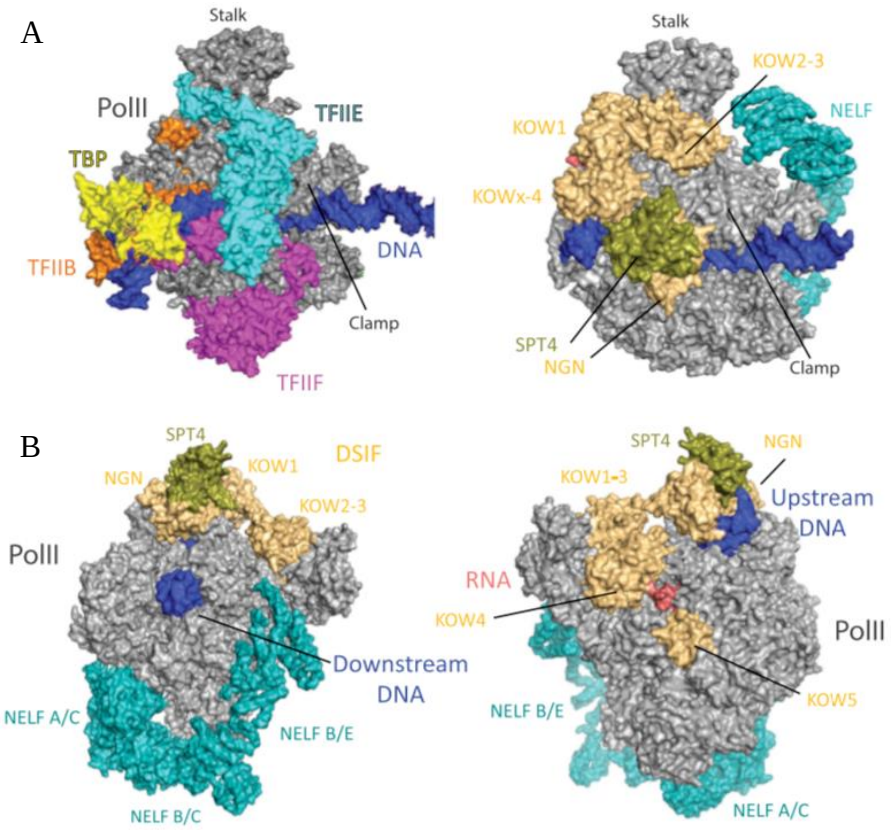


Figure 1.8. Spaced-filled model of partial preinitiation or paused RNAPII complex.

(A) RNAPII in complex with TBP, TFIIB, TFIIF and TFIIE (left) or DSIF and NELF (right). A comparison of the two structures illustrates the common RNAPII binding surfaces required for GTF and DSIF. Figure from [287].

(B) Two views of RNAPII in complex with NELF and DSIF rotated 180°. NELF binds to RNAPII on the opposite side of the cleft where DNA enters. DSIF interacts with upstream DNA and the Spt5 KOW4 and KOW5 domains localize around the RNA exit channel. Figure from [287].

Positive transcription elongation factor b (P-TEFb), a heterodimer of cyclin-dependent kinase 9 (CDK9) and cyclin T, phosphorylates and releases the transcriptional machinery from the paused state. TFs, bromodomain-containing protein 4 (Brd4), and the multi-subunit Super Elongation Complex (SEC) recruit P-TEFb to the chromatin., which will be discussed further in Section 1.3.4. The genome-wide role of P-TEFb in promoter-proximal pause release was initially demonstrated in mouse embryonic fibroblasts using the CDK9 inhibitor flavopiridol and RNAPII ChIP-seq. Following flavopiridol treatment, RNAPII occupancy increased at the promoter-proximal pause site and decreased throughout the gene body, leading to an increased pausing index

(Pausing Index = $\frac{\text{Promoter Proximal Occupacy}}{\text{Gene Body Occupancy}}$) [271]. P-TEFb stimulates the transcriptional machinery through phosphorylation of NELF, DSIF and RNAPII. Although not every gene contains paused RNAPII, transcription of >95% of actively transcribed genes required P-TEFb activity [275].

P-TEFb phosphorylates the NELF subunits NELF-A and NELF-E [285, 288]. The NELF-A phosphorylation sites reside within the region required for RNAPII pausing, with alanine mutagenesis of these residues preventing P-TEFb-dependent pause release [284, 289]. Furthermore, phosphorylation of NELF-E released NELF from the transcriptional machinery and relieved its transcriptional repression [288]. P-TEFb also phosphorylates the DSIF subunit Spt5 within the linker region between the KOW4 and KOW5 domains and at threonine 4 in the first C-terminal repeat region 1 (CTR1) [290, 291]. Phosphorylation of the KOW linker is thought to open the RNA clamp formed by KOW4 and KOW5 and assist with pause relief [284]. Similar to Rpb1 CTD, the Spt5 CTR1 provides an important scaffold to recruit transcriptional regulators, histone modifiers and RNA processing enzymes [292, 293]. Lastly, P-TEFb activity has canonically been ascribed to RNAPII CTD Ser2 phosphorylation (pSer2), a mark of elongating polymerase [294]. The initial characterization was mainly due to indirect studies that correlated CDK9 depletion with decreased pSer2 [295], whereas direct assessment of Cdk9 substrate-specificity identified preferential phosphorylation of Ser5 [294, 296]. Although a recent study suggested phosphorylation of tyrosine 1 of the CTD heptad repeat alters the specificity to Ser2 [297]. The identification of CDK12 and CDK13 as Ser2 kinases *in vivo* adds another regulatory layer to pSer2 [298]. Following the P-TEFb dependent pause-release, RNAPII enters into productive elongation to transcribe the gene body.

1.3.3.3. Elongation

As RNAPII transcribes through the gene body, the coordinated spatiotemporal alterations in the transcriptional machinery are regulated predominantly through the phosphorylation status of the RNAPII CTD and the Spt5 CTR1. The numerous repeats with varying combinations of post-translational modifications in each domain serve as a functional code. Alterations in the code as RNAPII transcribes enables properly timed recruitment of transcriptional machinery as they ‘read’ the code, such as the already mentioned capping enzyme recruitment to pSer5 [231]. These factors

typically promote transcription, with elongation rates accelerating through the gene body and ranging from 0.5 to 4 kb/min [275]. For example, P-TEFb phosphorylation of DSIF and RNAPII facilitates the recruitment of the RNA polymerase-associated factor complex (PAFc). PAFc further promotes the P-TEFb dependent ejection of NELF from the transcriptional machinery [299, 300]. The multi-subunit PAFc localizes with RNAPII and promotes transcription through the chromatin template and deposition of co-transcriptional histone modifications [301, 302]. Furthermore, the PAFc regulates pSer2 throughout the gene body by recruitment of CDK12, increasing pSer2 as RNAPII proceeds to the 3' end [303, 304]. The pSer2 facilitates the recruitment of RNA processing factors, such as the spliceosome, to co-transcriptionally produce the mature RNA [305]. The elongating complex continues through the gene body until reaching the polyadenylation (polyA) site, a signal for RNAPII to terminate transcription.

1.3.3.4. Termination

Termination requires dissociation of the nascent RNA from RNAPII and the subsequent release of the polymerase from the DNA template [305]. An essential polyA signal (PAS) at the 3' end of a gene begins termination by recruiting the polyA complex [306]. This complex adds an adenosine tail to the RNA transcript that improves RNA stability and regulates downstream translation [307]. Two models were proposed to explain the mechanism behind the promoted termination by recruitment of the polyA complex. The first, referred to as the allosteric model, suggests these factors alter the RNAPII elongation complex conformation to a state more prone to termination [308]. The second, referred to as the torpedo model, involves the recruitment of the 5' to 3' exonuclease Xrn2 to the newly formed, uncapped 5' end of the RNA tethered to RNAPII following cleavage of the transcript. Xrn2 degrades the nascent transcript remaining tethered to RNAPII, eventually catching up to the polymerase and displacing it from the DNA [308, 309]. Recently, a study proposed a unified model of the two hypotheses referred to as the sitting duck torpedo mechanism (Figure 1.9). Here, the cleavage and polyA complex recruits phosphatase 1 nuclear targeting subunit (PNUTS)-PP1 to dephosphorylate DSIF, aligning with the decreased DSIF phosphorylation immediately downstream of the PAS identified by ChIP-seq. DSIF dephosphorylation slows RNAPII transcription to 0.1-0.9 kb/min through an allosteric conformational change of the complex [310]. The slow rate of RNAPII leads to the accumulation of polymerase immediately downstream of the PAS, a common signal in RNAPII ChIP-seq [311].

The decreased RNAPII speed leaves it as a ‘sitting duck’ for Xrn2, the ‘torpedo,’ to quickly catch up to, which aligns with previous evidence that slower RNAPII complexes terminate sooner [310, 312]. The released RNAPII remains functional and can begin another round of transcription, potentially at the same gene as it is released close to the promoter through looping mechanisms connecting the 5’ and 3’ end of the gene [305].

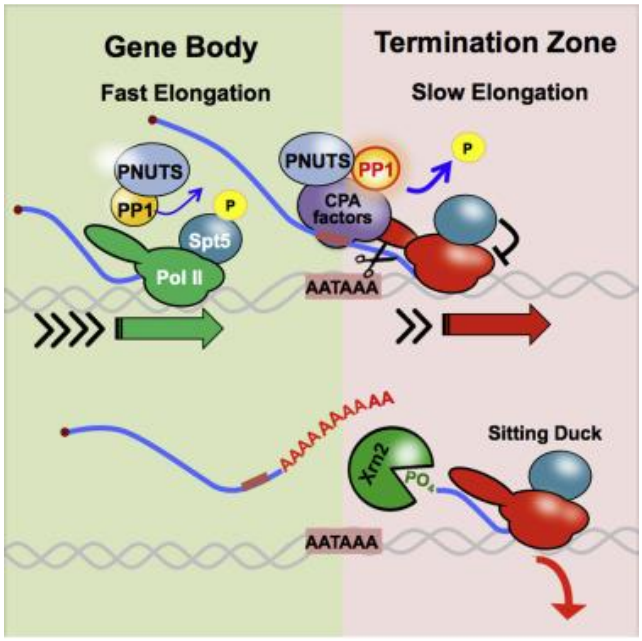


Figure 1.9. “Sitting duck torpedo” mechanism of RNAPII termination.
Recognition of the PAS by cleavage and polyadenylation (CPA) factors recruits the PNUTS-PP1 phosphatase to the transcriptional machinery. PNUTS-PP1 dephosphorylates the Spt5 CTR1, decreasing RNAPII’s elongation rate. The slow elongation rate converts RNAPII to a “sitting duck” for the “torpedo” Xrn2 to catch up with and terminate transcription.
Reprinted from Molecular Cell, 76(6), Cortazar, MA., et al., Control of RNA Pol II Speed by PNUTS-PP1 and Spt5 Dephosphorylation Facilitates Termination by a “Sitting Duck Torpedo” Mechanism, 896-908, Copyright (2019), with permission from Elsevier. See Appendix.

1.3.4. Positive transcription elongation factor b (P-TEFb)

Cyclin-dependent kinases (CDKs) canonically regulate specific stages of the cell cycle. As a member of the transcriptional CDK family, P-TEFb does not have a pronounced regulatory role in the cell cycle and instead is critical for RNAPII regulation. P-TEFb is a heterodimer comprised of CDK9 and its cyclin partner cyclin T1 or T2 [294]. P-TEFb is predominantly localized

throughout the nucleus, with a small proportion also identified in the cytoplasm [313]. P-TEFb was independently identified and characterized in humans and *D. melanogaster* around 30 years ago. The sequence of CDK9 was first cloned from the human genome through a search for CDKs related to the cell-cycle regulatory cyclin-dependent kinase 1 (CDK1) [314]. The identification of a Pro-Ile-Thr-Ala-Leu-Arg-Glu (PITALRE) motif in the N-terminus, highly similar to the PSTAIRE box conserved in the CDK family, led to the original protein name PITALRE. The *in vitro* kinase activity differed from CDK1 indicating a different target site preference and, surprisingly at the time, it's activity did not oscillate during cell cycle progression like other kinases in the family [314].

Around the same time, researchers working with *D. melanogaster* identified P-TEFb. The P-TEFb dimer, comprised of a 43 and 124 kDa subunit, was identified as a complex which stimulated RNAPII elongation *in vitro* in a manner that was sensitive to inhibition by the small molecule DRB [315]. A subsequent study demonstrated that P-TEFb phosphorylation of the Rpb1 CTD led to increased productive elongation *in vitro* [316]. Following cloning of the 43 kDa *D. melanogaster* subunit, a search for homologous proteins revealed 72% sequence similarity to the human PITALRE protein. Furthermore, P-TEFb was able to rescue the ability to produce DRB-sensitive transcripts from HeLa nuclear extract depleted of PITALRE [317]. Similar to other CDKs, and as the names suggest, P-TEFb requires the presence of cyclin T for its function [318]. The cyclin partner discovered soon after was cyclin T in *D. melanogaster* and either cyclin T1 or T2 in the human dimer [318, 319]. Lastly, a larger, second CDK9 isoform with an extended N-terminus and similar *in vitro* kinase activity was identified [320]. Although the presence of two isoforms has been known for many years, most functions have been associated with the smaller CDK9 and will be the isoform discussed unless otherwise stated [294].

A prominent role for P-TEFb in human immunodeficiency virus 1 (HIV-1) infection was identified soon after the heterodimer was discovered [321]. This model has been used to understand many aspects of P-TEFb regulation, and therefore a basic introduction is required before delving into the various regulatory mechanisms. The HIV-1 genome encodes the transactivator Tat protein that is responsible for driving expression from the viral genome's long terminal repeat (LTR) promoter. The HIV-1 LTR promoter recruits RNAPII transcriptional machinery that beings transcribing before entering promoter-proximal pausing. The 5' end of the RNA transcript contains a stem-loop structure referred to as the trans-activation response (TAR)

element. Tat interacts with P-TEFb and the TAR element, bringing P-TEFb to the viral promoter and stimulating RNAPII pause release (Figure 1.10) [322].

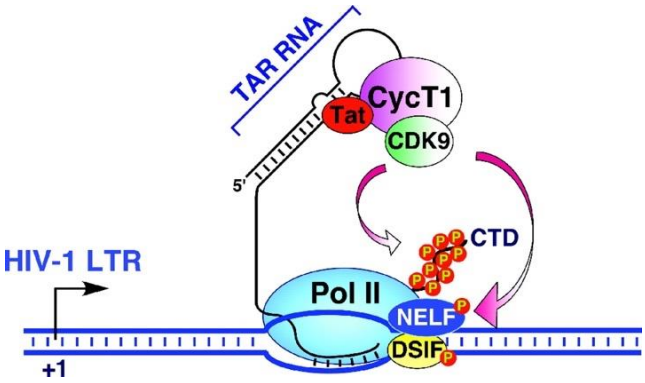


Figure 1.10. Transcription from the HIV-1 LTR by Tat and P-TEFb.
RNAPII transcription from the HIV-1 LTR promoter generates a nascent RNA transcript with a hairpin loop structure referred to as the TAR element. The transactivator Tat interacts with the TAR element, bringing P-TEFb with it to phosphorylate the transcriptional machinery and promote RNAPII pause release. Figure adapted from [323].

1.3.4.1. Structure of P-TEFb

X-ray crystallography of the human cyclin T1 N-terminal, CDK9/cyclin T1 heterodimer and free cyclin T2 provided important structural information for P-TEFb [324, 325]. The Cyclin T1 and T2 tertiary structures consist of two canonical cyclin box motifs with five α-helices and additional N-terminal and C-terminal α-helices (Figure 1.11A, green and red, respectively) [325]. In cell-cycle cyclins, the N-terminal makes important regulatory interactions with the kinase that are absent in cyclin T. In contrast, the cyclin T C-terminal helix is required for interactions with regulatory proteins, whereas this helix does not have important functions in cell cycle cyclins. The tertiary structure of CDK9 is typical of protein kinases with the active site between the N-terminal and a C-terminal lobe and an activation T-loop near the entry to the ATP-binding site [294]. The N-terminal cyclin fold of cyclin T mediates the interaction between cyclin T (Figure 1.11A, yellow) and the N-terminal lobe of CDK9, with a large rotation of cyclin T and less contact area between the two subunits than cell-cycle cyclins. Comparison between free cyclin T1 and the cyclin T1-CDK9 dimer demonstrated minimal conformational changes to the cyclin fold. A common mechanism of CDK activation is a conformational change to an activated state by T-loop phosphorylation [326]. Analysis of the P-TEFb structure revealed that phosphorylation of CDK9

at threonine 186 in the T-loop (Figure 1.11B, red dot) promoted an active conformation. However, the conformational changes are currently unknown due to the lack of an unphosphorylated structure [325, 327]. Successful P-TEFb crystallization enabled the detailed understanding of the binding mechanism of CDK9 inhibitors, such as flavopiridol, and *in silico* small-molecule docking to identify new lead molecules [325, 328].

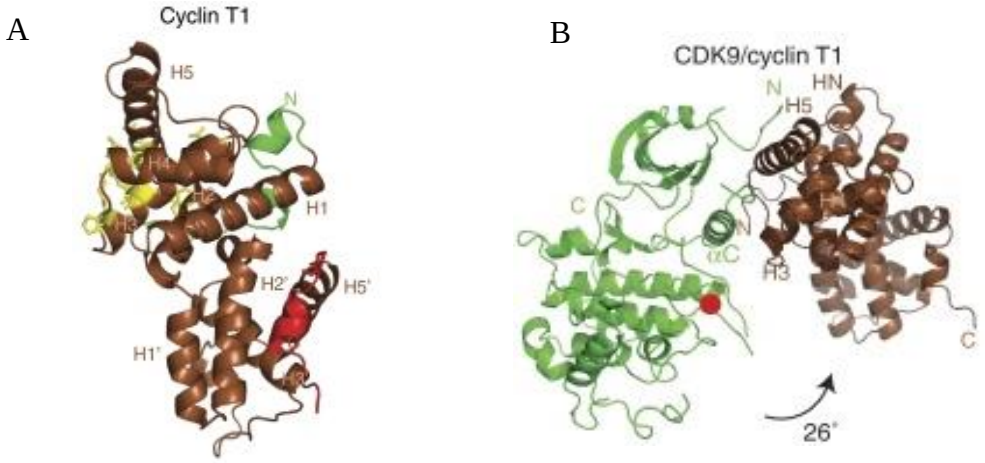


Figure 1.11. Ribbon model of (A) cyclin T1 or (B) P-TEFb structure.

(A) Cyclin T1 structure with the five α-helices in each cyclin fold identified, N-terminal helix in green, C-terminal helix in red and CDK9 interacting residues in yellow.

(B) CDK9 (green) and cyclin T1 (brown) structure with the T-loop threonine 186 marked by a red dot. Figure reprinted from [325] with permission. *See Appendix.*

1.3.4.2. Sequestering P-TEFb in the 7SK snRNP

The main mechanism regulating P-TEFb activity involves the assembly of the multi-subunit 7SK small nuclear ribonucleoprotein (snRNP). P-TEFb is dynamically and reversibly assembled in the complex to inhibit P-TEFb kinase activity, with approximately half of cellular P-TEFb sequestered in unstimulated cells [329, 330]. The small, non-coding 7SK RNA serves as a scaffold to assemble the multiple components necessary to sequester P-TEFb. The 7SK snRNP is comprised of a hexamethylene bisacetamide-induced protein 1 or 2 (HEXIM1/2) dimer, methylphosphate capping enzyme (MePCE) and La-related protein 7 (LARP7) (Figure 1.12A) [331-333]. MePCE protects the 7SK RNA from exonuclease degradation through the co-transcriptional addition of a 5' monomethyl γ-phosphate cap modification [334, 335]. The addition of LARP7 at a U-rich region within the 7SK RNA 3' stem loop improves the stability of 7SK RNA

and inhibits MePCE methyltransferase activity to prevent 7SK RNA de-capping [336, 337]. The stable complex formed by 7SK RNA, MePCE and LARP7 is insufficient to inhibit P-TEFb activity and requires the dynamic association of a homo- or heterodimer comprised of HEXIM1 and/or HEXIM2 [327, 338-340]. HEXIM1 interactions with the 7SK snRNA promotes a conformational change enabling P-TEFb inhibition [341]. In fact, while MePCE and LARP7 are required for 7SK RNA stability *in vivo* they are dispensable for P-TEFb recruitment to a HEXIM1-7SK RNA complex *in vitro* [331, 342, 343]. P-TEFb is recruited to the 7SK snRNP by a HEXIM1 interaction with cyclin T N-terminal helix and a LARP7 interaction with CDK9 [332, 344, 345]. Within the 7SK snRNP, P-TEFb is inhibited due to HEXIM1 interactions with the CDK9 catalytic site interfering with substrate binding [345]. Furthermore, phosphorylation of CDK9 at threonine 186 within the T-loop, a hallmark of activated CDKs, is required for recruitment to the 7SK snRNP [327, 346]. The removal of P-TEFb coincides with Hexim1 removal from the 7SK snRNP and association of hnRNPs with the remaining 7SK snRNP components [347].

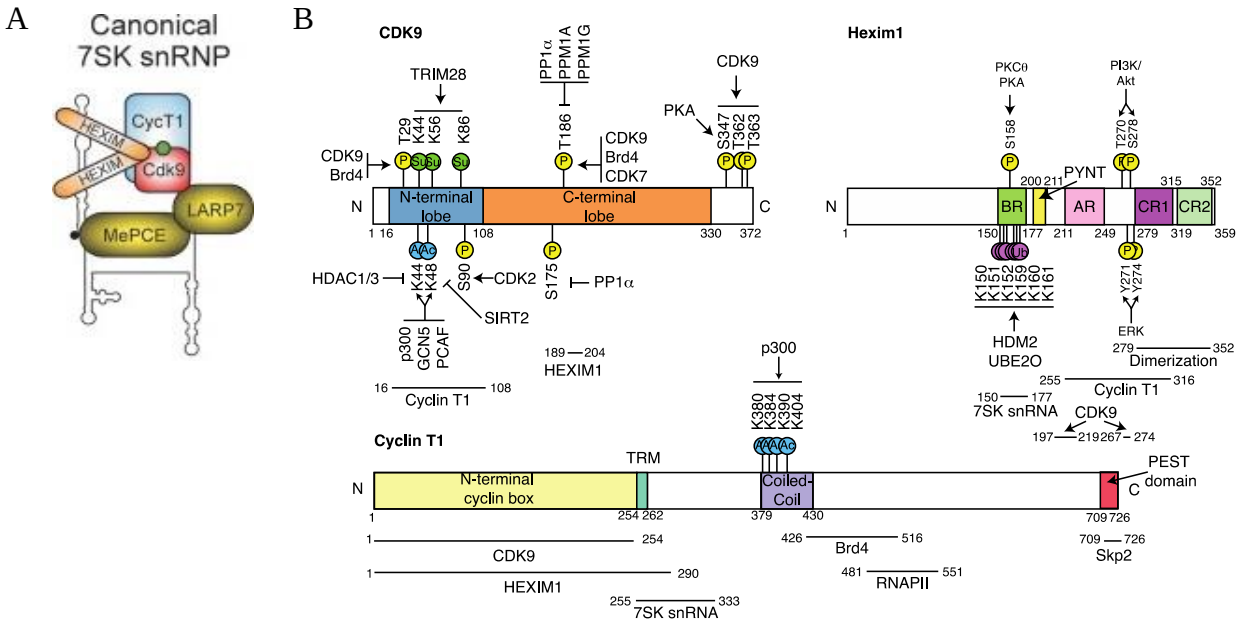


Figure 1.12. 7SK snRNP components and identified post-translational modifications.

(A) Diagram of 7SK snRNP complex comprised of a HEXIM dimer, CDK9, cyclin T, MePCE and LARP7. *C Quaresma AJ., et al., Cracking the control of RNA polymerase II elongation by 7SK snRNP and P-TEFb, Nucleic Acids Research, 2016, 44, 16, 7527-39, by permission of Oxford University Press. See Appendix.*

(B) Schematic of CDK9, cyclin T1 and HEXIM1 domains, enzymes responsible for post-translational modifications, and interacting regions. ⊣ indicates the enzyme negatively regulates the post-translational modification and → indicates positive regulation. The lines underneath and the corresponding protein indicate regions required for interacting with the respective protein. BR – basic region, AR – acidic region, CR1/2 – coiled region 1/2, TRM – Tat-TAR recognition motif. Figure adapted from [348].

1.3.4.3. P-TEFb post-translational modifications

Cellular signals regulate P-TEFb activity through a variety of post-translational modifications, including phosphorylation, acetylation, ubiquitination, and sumoylation. Although the numerous post-translational modifications were studied in response to select receptor activation, these protein kinases and enzymes respond to a plethora of stimuli suggesting these mechanisms may be utilized in other contexts as well.

P-TEFb is regulated by phosphorylation of three residues in the C-terminal domain (S347, T362, T363), T186 in the T-loop, and T29 (Figure 1.12B) [325, 349]. These residues were initially identified as autophosphorylation sites, but other kinases have since been identified for some of the residues. First, phosphorylation of the C-terminal residues was required for the interaction of Tat-P-TEFb with the TAR element and activation of the HIV-1 LTR promoter [350]. Although primarily characterized as CDK9 autophosphorylation sites, PKA-mediated S347 phosphorylation also promoted the formation of the P-TEFb-Tat-TAR complex [350]. As mentioned, T186 phosphorylation is required for incorporation into the 7SK snRNP and also increases P-TEFb activity [327, 351]. To transition between the inactive complex and free active P-TEFb, T186 is dephosphorylated and subsequently re-phosphorylated. Following cellular stress, Ca^{2+}–calmodulin–protein phosphatase 2B (PP2B) facilitates a conformational change of the 7SK snRNP followed by protein phosphatase 1α (PP1α) mediated T186 dephosphorylation [348]. The dephosphorylated P-TEFb is recruited to the PIC and remains inactive until RNAPII has transcribed a short RNA [352]. P-TEFb at the PIC is proposed to be activated through phosphorylation of T186 by the TFIIH subunit CDK7 or Brd4 [353-355]. Other T186 phosphatases identified include protein phosphatase, Mg^{2+}/Mn^{2+} dependent 1A (PPM1A) and 1G (PPM1G) [356, 357]. Furthermore, P-TEFb T29 autophosphorylation inhibits kinase activity. Similar to Tat regulation of the HIV-1 genome, the human T- lymphotropic virus type 1 (HTLV-1) genome

contains the transactivator Tax. Tax stimulates T29 phosphorylation and recruits the phosphorylated P-TEFb to the viral promoter. As Tax also requires P-TEFb for transactivation, it was proposed that T29 is dephosphorylated once recruited to the PIC [349]. Brd4 was later demonstrated to either stimulate autophosphorylation or directly phosphorylate T29 at the PIC to inhibit HIV-1 transcription, which was removed following Brd4 release from the transcription complex [355, 358]. While these residues (T29, T186, S347, T362, T363) are currently the only auto-phosphorylated sites with known *in vivo* functions, *in vitro* autophosphorylation identified a total of seven phosphorylated forms indicating other potential regulatory sites [325].

Two other CDK9 phosphorylation sites with characterized regulatory roles are S90 and S175 (Figure 1.12B). First, CDK2 mediated S90 phosphorylation increased Tat-dependent HIV-1 transcription, indicating a link between cell-cycle and P-TEFb activity [359]. Lastly, conflicting studies stated S175 phosphorylation either did not alter [327] or increased CDK9 activity [346] *in vitro*. Whereas an *in vivo* study indicated a CDK9- S175A mutant was active and the phosphomimetic S175D mutant was inactive. Furthermore, PP1α was required to dephosphorylate S175 and increased expression from an HIV-1 LTR reporter gene [360]. Although the role in regulating kinase activity is unclear, phosphorylation-dependent changes in P-TEFb interactions are evident. CDK9 phosphorylated at S175 was excluded from the 7SK snRNP and promoted the interaction with Brd4, potentially through eliciting a conformational change of P-TEFb [360-362].

Acetylation of CDK9 and cyclin T regulate P-TEFb activity and incorporation into the 7SK snRNP (Figure 1.12B). The concerted actions of acetyltransferases (p300, GCN5, and p300/CBP-associated factor (PCAF)) and HDAC1 and HDAC3 regulate the acetylation of CDK9 K44 [363, 364]. Nuclear receptor co-repressor 1 (N-CoR) interacts with HEXIM1 and recruits HDAC3 to maintain the deacetylation of CDK9 K44. Disruption of the complex through knockdown of N-CoR increased CDK9 K44 acetylation and activity [363]. In contrast, CDK9 K48 acetylation, mediated by p300, GCN5 and PCAF acetyltransferases and the deacetylase sirtuin 2 (SIRT2), inhibits kinase activity [364, 365]. K48 is critical for the proper orientation of ATP and Mg^{2+} in the active site, with acetylation disrupting the interaction between ATP and CDK9 and thereby reducing kinase activity [348, 364]. Additionally, the deacetylase sirtuin 7 (SIRT7) was shown to positively regulate release of P-TEFb from the 7SK snRNP through deacetylating CDK9. The limited effect on 7SK snRNP formation by CDK9 K44 and K48 acetylation previously observed suggests SIRT7 deacetylates a currently unidentified residue critical for complex formation [366].

With regards to cyclin T post-translational modifications, acetylation of K380, K386, K390, and K404 by p300 acetyltransferase dissociates P-TEFb from the 7SK snRNP. Acetylation of these cyclin T residues was necessary for NF-κB-mediated interleukin-8 expression, but not for Tat transactivation of the HIV-1 genome [367]. This differential effect was potentially due to cyclin T acetylation promoting the interaction between P-TEFb and Brd4, an activation complex for NF-κB-dependent transcription and not Tat transactivation [348, 368].

The addition of ubiquitin and small ubiquitin-like modifier (SUMO) to the CDK9 subunit regulates P-TEFb degradation and activity (Figure 1.12B). Cellular signals dynamically regulate P-TEFb ubiquitination and subsequent CDK9 degradation by the proteasome. Cyclin T1 interacts with the E3 ubiquitin ligase Skp2 through its PEST (rich in proline, glutamate, serine, and threonine) domain to facilitate the ubiquitination of an unidentified residue on CDK9 and subsequent degradation by the proteasome. Interferon-γ (IFN-γ) negatively regulates P-TEFb degradation to increase transcription from the class II major histocompatibility complex promoter. IFN-γ increased cellular P-TEFb levels through decreasing Skp2 expression and consequently decreasing P-TEFb degradation [369]. Alternatively, the formation of the P-TEFb-Tat-TAR element complex and activation of the HIV-1 genome required Skp2 in mouse embryonic fibroblasts. CDK9 ubiquitination did not alter cyclin T1 binding, kinase activity or Tat recruitment to the HIV-1 promoter. Instead it was suggested ubiquitination relieved autoinhibitory intramolecular interactions within cyclin T1, although an exact mechanism was not determined [370]. On the other hand, sumolyation negatively regulates P-TEFb activity. CDK9 sumoylation prevented the formation of P-TEFb by disrupting the interaction between CDK9 and cyclin T [371, 372]. The SUMO E3 ligase tripartite motif containing 28 (TRIM28) mediated sumoylation of CDK9 K44, K56 and K86 [372]. Overexpression of the oncogenic TF MYC prevented CDK9 sumoylation in order to elicit its transcriptional regulatory role [271, 371]. In addition to those discussed, mass spectrometry-based methods have identified other phosphorylated, acetylated and methylated P-TEFb residues with undetermined functional significance [361].

1.3.4.4. 7SK snRNP post-translational modifications

In addition to directly modifying the P-TEFb heterodimer, cellular signals alter the stability of the 7SK snRNP to indirectly alter P-TEFb activity. Phosphorylation of the 7SK snRNP subunit HEXIM1 regulates the ability to sequester P-TEFb in the inactive complex (Figure 1.12B) [337].

Hexamethylene bisacetamide (HMBA) increased expression of the HIV-1 genome through activation of the PI3K/Akt signalling cascade in Jurkat T cells. Akt phosphorylated HEXIM1 at T270 and S278, two residues within the cyclin T binding domain, to disrupt the interaction between P-TEFb and HEXIM1, releasing the kinase and enabling recruitment to the viral genome [373]. Along with PI3K-Akt signalling, HMBA promoted HIV-1 expression through PKCμ signalling, although the phosphorylation target was not identified [374]. TCR signalling pathways in T cells also modulated HEXIM1 S158, Y271 or Y274 phosphorylation. PKCθ activation by phorbol myristyl acetate (PMA) or following TCR activation increased HEXIM1 S158 phosphorylation, preventing HEXIM1 interaction with the 7SK snRNP and inhibition of P-TEFb [375]. PKA signalling in autosomal dominant polycystic kidney disease also disrupts 7SK snRNP through S158 phosphorylation [376]. Interestingly, these authors did not observe disruption of the 7SK snRNP with a phosphomimetic mutation (S158E), whereas the initial study found an S158A mutation prevented PCKθ-mediated 7SK snRNP disruption [375, 376]. TCR activation in T cells also disrupts the 7SK snRNP complex via ERK-dependent phosphorylation of HEXIM1 Y271 and Y274, another two residues within the cyclin T interaction domain. Mutation of these residues to phenylalanine prevented the release of P-TEFb from the 7SK snRNP and expression of the HIV-1 genome [377, 378].

HEXIM1 activity is also regulated by ubiquitination, independently of degradation. One E3 ubiquitin ligase regulating HEXIM1 is human double minute-2 protein (HDM2). HDM2-mediated ubiquitination increased the inhibitory action of HEXIM1 on P-TEFb, reducing Tat transactivation of the HIV-1 genome [379]. The hybrid E2-E3 ubiquitin ligase ubiquitin-conjugating enzyme E2 O (UBE2O) also mediated HEXIM1 ubiquitination. Interestingly, UBE2O was recruited to HEXIM1 by Tat in the cytoplasm. Ubiquitinated HEXIM1 was sequestered in the cytoplasm and released P-TEFb to translocate to the nucleus and activate HIV-1 genome expression. While the mechanism was assessed in the context of Tat transactivation, treatment with DRB also led to similar P-TEFb translocation suggesting this regulatory mechanism is used in other contexts as well [380].

1.3.4.5. Chromatin recruitment of P-TEFb

P-TEFb release from the 7SK snRNP is insufficient for selective gene expression in response to cellular signals. Other proteins must recruit active P-TEFb to chromatin in order to

phosphorylate its substrates, and release promoter-proximal paused RNAPII into productive elongation. The recruitment occurs through four mechanisms that will be discussed: sequence-specific TFs, Brd4, SEC and 7SK snRNP (Figure 1.13).

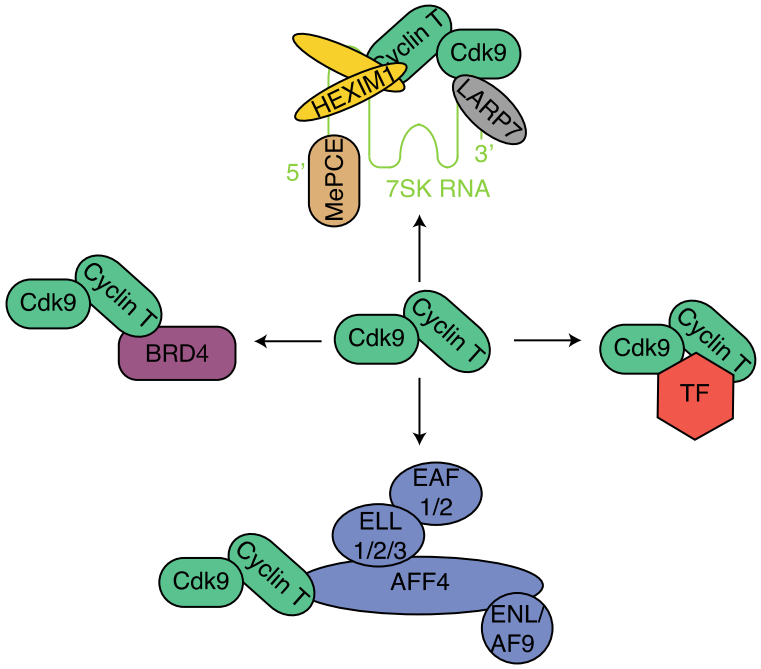


Figure 1.13. Mechanisms of P-TEFb chromatin recruitment.

TFs (right), the SEC (bottom), Brd4 (left) or the 7SK snRNP (top) recruit P-TEFb to the chromatin.

1.3.4.5.1. Transcription factors

TFs recognize specific sequence elements within genomic regulatory regions. The recruited TFs regulate gene expression by altering the chromatin landscape and assembly of the transcription complex through enzymatic activity or protein-protein interactions with other factors. The human genome contains over 1600 TFs, expressed in a cell-type specific manner, that are critical in regulating all manner of physiological and pathological cellular processes [381]. For example, GPCR signalling in cardiac cell types alters the activity of numerous TFs (i.e. nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), GATA binding protein 4 (GATA4), myocyte enhancer-factor 2 (MEF2)) to reprogram gene expression patterns [382, 383]. Similar to the many signalling cascades converging on P-TEFb and the 7SK snRNP, a range of post-translational modifications modify each TF, enabling the integration of inputs from multiple cellular signalling pathways. Particular to this thesis, one mechanism employed by these TFs to activate transcription

is the recruitment of P-TEFb. For example, NF-κB recruits P-TEFb to the transcriptional machinery following TNF-α stimulation. An *in vitro* GST pull-down experiment with recombinant GST-tagged cyclin T1 and NF-κB subunit RelA demonstrated a direct interaction between the two proteins, which also occurred in COS cells with heterologous overexpression of each protein. Furthermore, CDK9 inhibition with DRB increased promoter-proximal and decreased gene body RNAPII occupancy at IL8 in response to TNF-α [384]. Similarly, recombinant GATA4 and CDK9 co-immunoprecipitated in an *in vitro* GST pull down experiment. In neonatal rat cardiomyocytes, PE increased expression of ANP through increased recruitment of a Cdk9/GATA4/p300 complex to the ANP promoter [385]. In another study, GST-tagged Mef2 family members Mef2A, B and C co-immunoprecipitated P-TEFb from cell lysates. Decreased P-TEFb activity through knockdown or increased activity through overexpression of cyclin T1 led to decreased or increased expression, respectively, from a MEF2 reporter gene in the mouse myoblast cell line C2C12 [386]. While these examples are from a select few TFs, they highlight the ability of TFs to recruit P-TEFb to the chromatin to stimulate gene expression.

1.3.4.5.2. Bromodomain-containing protein 4

Brd4 is a member of the bromodomain and extra-terminal (BET) family of proteins consisting of Brd2, Brd3 and the testis-specific BrdT (Figure 1.14A) [387]. This family is characterized by the presence of two tandem bromodomains (BD1 and BD2) and an extraterminal (ET) domain. The BDs mediate the interaction with acetyl-lysine residues on target proteins, such as TFs and histones, whereas the ET domain mediates protein-protein interactions [388, 389]. BDs are a highly conserved domain abundant in chromatin-associated proteins. The domain is formed by a left-handed four-helix bundle with two long loops, the ZA loop located between helices α_Z and α_A and the BC loop located between α_B and α_C (Figure 1.14B). The loops form a hydrophobic pocket at the end of the bundle where the acetyl-lysine residue is inserted (Figure 1.14C). While the four helices are highly conserved across bromodomains, the divergent sequences in the ZA and BC loops confer substrate specificity [388]. Although BDs can interact with a single acetyl-lysine, the presence of multiple modifications greatly enhances the binding affinity. For example, BDs in Brd2 bind monoacetylated H4K12ac with a K_D of 2.9 mM, decreasing almost 10-fold to 360 μM when binding to the diacetylated H4K5acK12ac [390]. Similarly, Brd4 BD1 binding affinity for H4K5ac9ac was 30-times greater than its affinity for H4K5ac [390]. Structural studies for Brd4

and BrdT suggested the synergistic effect is due to the recognition of two acetylated residues by a single bromodomain [390, 391]. The two BET BDs differ in their affinities for acetylated peptides, with BD1 preferentially interacting with a Kac-X-X-Kac consensus sequence and BD2 displaying minimal selectivity for di- and tri-acetylated peptides [390].

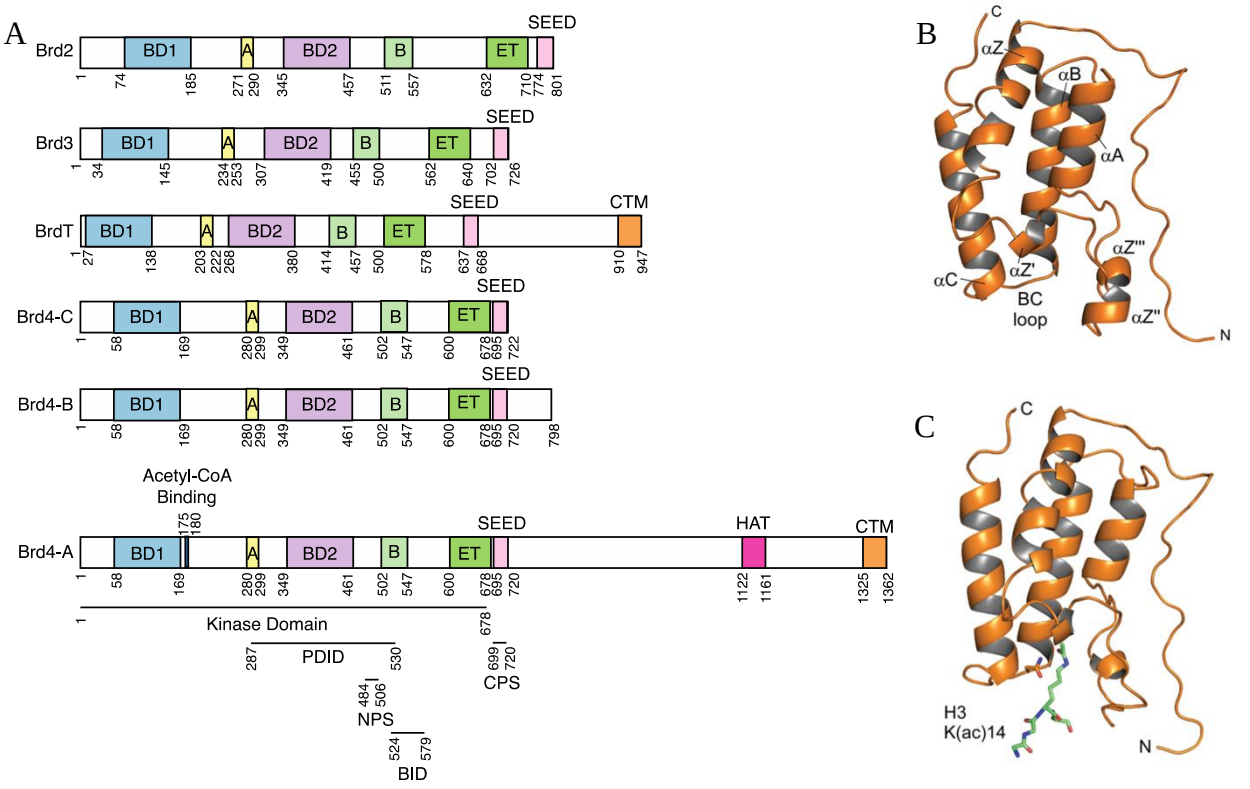


Figure 1.14. BET family and structure the characteristic bromodomain.

(A) Human BET family members and their respective domains. A and B are two conserved regions among the family members. BD1 – bromodomain 1, BD2 – bromodomain 2, ET – extraterminal domain, CTM – C-terminal motif, SEED - Ser (S)/Glu (E)/ Asp (D) motif, PDID – phosphorylation-dependent interaction domain, NPS – N-terminal phosphorylation sites, BID – basic-residue enriched interaction domain, CPS – C-terminal cluster of phosphorylation sites [392, 393].

(B) Ribbon model of Brd4 BD1 with the BC loop indicated and the AZ loop comprised of αZ', αZ'', αZ'''. Figure adapted from [394].

(C) Ribbon model of Brd4 BD1 in complex with a histone H3 peptide acetylated at K14 inserted into BD binding pocket. Figure adapted from [394].

Among the BET family members, Brd4 has been the most extensively studied due to its prominent roles in cancer, inflammation, and cardiovascular disease [395]. The functional roles of the other family members have been described as well. For example, an *in vitro* transcription assay indicated Brd2 and Brd3 contain histone chaperone activity enabling them to promote RNAPII transcription through chromatin [396]. BrdT, expressed in the testis and aberrantly activated in some cancers, regulates chromatin compaction in sperm [397]. Brd4 was initially identified as a member of the multi-subunit Mediator complex, although its function was not determined [398]. A few years later, it was observed that Brd4 maintained associated with mitotic chromosomes and was critical for cell-cycle progression [399]. The chromatin-bound Brd4 is thought to impart memory to the post-mitotic cell, facilitating re-activation of the proper genes [400].

Three Brd4 isoforms have been discovered which are functionally distinct: Brd4-A (also referred to as Brd4L), Brd4-B, and Brd-C (also referred to as Brd4-S) (Figure 1.14A). First, Brd4-B is comprised of the Brd4L N-terminal and a unique 75 residue C-terminus. This isoform inhibited the DNA damage response in an osteosarcoma cell line through recruitment of the condensing II complex to promote chromatin compaction [401]. Brd4-C also contains the Brd4L N-terminus and three unique residues at the C-terminus. Brd4-C promoted formation of liquid-liquid phase separation (LLPS) condensates that contain transcriptional activators, such as RNAPII, P-TEFb and Mediator, to promote gene expression [402]. Finally, the transcriptional activation by Brd4-A is primarily dependent on its ability to interact with and recruit P-TEFb to chromatin [362, 403]. For the purposes of this thesis, I will focus on the role of Brd4-A in regulating transcription and this will be the isoform I am referring to when discussing Brd4.

Among the BET family, Brd4 and BrdT are the only members that interact with P-TEFb. The common mechanism requires a short P-TEFb interacting motif at the C-terminal of the respective protein termed the C-terminal motif (CTM), which interacts with CDK9 and cyclin T (Figure 1.14A) [404]. As already discussed, Brd4 also interacts with cyclin T acetylated at K380, K386, and K390 in a BD2-dependent manner [368]. The interaction between P-TEFb and Brd4 serves two functions: P-TEFb removal from the 7SK snRNP and recruitment to chromatin. Brd4-dependent transcriptional activation of P-TEFb responsive genes requires both modes of interaction between Brd4 and P-TEFb. In the absence of the CTM, the Brd4 BD2-cyclin T interaction mediated a Brd4-P-TEFb interaction, but the complex remained associated with 7SK snRNP. The CTM was required to remove P-TEFb from the inhibitory complex, with

overexpression of this domain alone leading to P-TEFb dissociation but not transcriptional activation [368]. In the absence of a Brd4-P-TEFb structure, the mechanism of P-TEFb removal is unclear. The proposed model involves cyclin T acetylation recruiting Brd4, which elicits a conformational change in Brd4, exposing the CTM to promote removal of P-TEFb from the complex [368].

Despite Brd4's important regulatory role in steady-state transcriptional programs, small molecule inhibitors preferentially reduce disease state transcription. The selective effect is primarily due to the prevalence of super-enhancers in regulating these disease states. Super-enhancers were first characterized in mouse embryonic stem cells, which was followed by the identification in a broad range of human cell types [405, 406]. Super-enhancers are large genomic regions (>20 kb on average) abundant in key transcriptional activators, such as Mediator, Brd4, TFs, p300, and active enhancer histone marks, such as H3K27ac and H3K4me1 [407]. In somatic cells, super-enhancers regulate genes critical for cell-identity and cell-specific biological processes [405, 407]. Furthermore, disease-associated single nucleotide polymorphisms (SNPs) are enriched in these genomic regions [405]. The abundance and cooperativity of transcriptional regulators at super enhancers confers a high sensitivity of gene expression to slight perturbations in protein occupancy along these regions [408]. Therefore, these regions are perturbed with low concentrations of BET inhibitors, while genes regulated by typical enhancers and promoters are not affected.

In addition to their role in initiating PIC formation and P-TEFb recruitment, TFs regulate recruitment of Brd4 to specific genomic loci either directly or indirectly. Through these mechanisms, signalling pathways regulate Brd4 recruitment in a context dependent manner [387]. First, TFs interact with Brd4 in a non-BD dependent manner through the ET domain of Brd4 and a phosphorylation-dependent manner with N-terminal phosphorylation site (NPS) region of Brd4 (Figure 1.15) [389, 409]. The NPS, which contains seven casein kinase II (CKII) consensus sequences, resides in the phosphorylation-dependent interaction domain (PDID) along with the basic residue-enriched interaction domain (BID) (Figure 1.14A). In the unphosphorylated state, the NPS prevents BD2's interaction with acetyl-lysine residues. CK2 phosphorylation increases the negative charge of the NPS, shifting its interaction from BD2 to the BID and releasing the inhibitory effect on BD2. The conformational change also promotes interactions between the NPS and TFs, such as p53 and NF-κB [410]. Although CK2 and CK1δ are the only two kinases currently

shown to directly phosphorylate Brd4 *in vivo*, PKA and IKKε phosphorylated the PDID in an *in vitro* kinase assay [409, 411]. Furthermore, the protein kinases p38 and CDK9 regulated Brd4 chromatin occupancy, although the mechanism was undetermined [412, 413]. Second, BD-dependent interactions with acetylated lysine residues on TFs recruit Brd4. For example, Brd4 interacts with the NF-κB RelA subunit acetylated at lysine 310 to promote transcription of its target genes [414]. Lastly, TFs recruit acetyltransferases, such as p300, to the chromatin to acetylate proximal histones at enhancers and promoters, which recruits Brd4 in a BD-dependent manner [387, 415, 416]. Altogether, these mechanisms enrich Brd4 at regulatory genomic regions to promote assembly of the RNAPII transcriptional machinery and release into productive elongation.

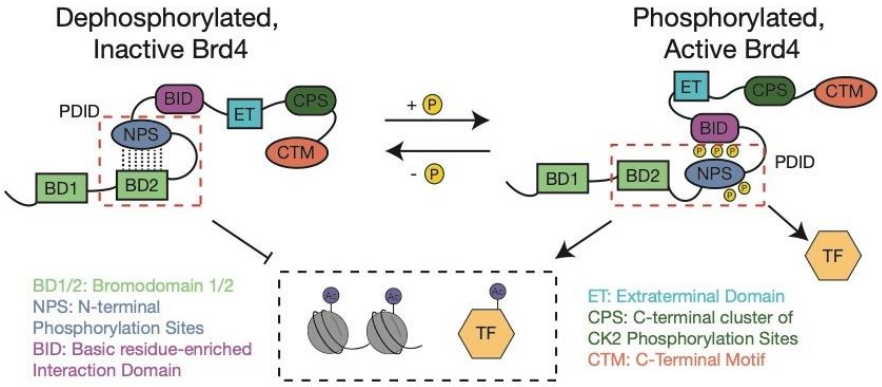


Figure 1.15. Phosphorylation-dependent conformational change of Brd4 regulates BD2 and TF interactions.

NPS phosphorylation by CK2 leads to a conformational change removing the inhibitory intramolecular interactions between the NPS and BD2. NPS phosphorylation also promotes interactions between the NPS and TFs. Figure adapted from [410].

Reprinted from Drug Discovery Today: Technologies, 19, Chiang, CM., Phospho-BRD4: transcription plasticity and drug targeting, 17-22, Copyright (2016), with permission from Elsevier. See Appendix.

Although typically associated with P-TEFb dependent transcriptional regulation, Brd4 also regulates transcription through P-TEFb-independent mechanisms. First, Brd4 co-localizes with the Mediator complex along cis-regulatory genomic regions to coordinate PIC formation. Displacement of Brd4 from chromatin leads to reduced Mediator occupancy as well, suggesting Brd4 is an important regulator of Mediator recruitment [417]. Second, the Brd4 N-terminal is an

atypical kinase that is able to phosphorylate the RNAPII CTD Ser2 *in vitro* and *in vivo* and was proposed to stimulate transcription in a kinase-dependent manner [418]. A subsequent study demonstrated kinase-dependent crosstalk amongst Brd4, CDK9 and CDK7. At low concentrations, Brd4 activated P-TEFb through phosphorylation of CDK9 T186, whereas at high concentrations, Brd4 repressed P-TEFb through CDK9 T29 phosphorylation. Furthermore, Brd4 relieved CDK7 and CDK9 inhibition by the TFIID subunit TAF7 in a phosphorylation-dependent manner [355]. Third, Brd4 histone chaperone activity stimulated RNAPII transcription through hyperacetylated histones [419]. Fourth, Brd4 acetylated histone H3 and H4 through its C-terminal HAT domain. Brd4 HAT activity destabilized nucleosomes through acetylation of H3K122, promoting chromatin decompaction at cis-regulatory regions [420]. Lastly, Brd4 interacts with the splicing machinery in a BD-independent manner, modulating exon usage in T cell acute lymphoblastic leukemia [421].

The BET family has proven to be highly conducive to small-molecule drug discovery programs with numerous molecules entering clinical trials for various types of cancer [422]. The inhibition strategy centers around competitive inhibition of the BD-acetyl-lysine interaction, thereby blocking chromatin recruitment. Two first in-class pan-BET inhibitors developed were JQ1 and I-BET, which targeted BET BDs with nanomolar affinities [423, 424]. The initial characterization of JQ1 demonstrated its ability to reduce proliferation of NUT midline carcinoma cell lines and patient-derived xenograft models driven by a Brd4-NUT fusion protein [424]. I-BET prevented LPS-induction of inflammatory genes in activated macrophages [423]. The anti-proliferative and anti-inflammatory effect of BET inhibitor treatment has since expanded to include hematological cancers (i.e. multiple myeloma [425]), solid tumours (i.e. neuroblastoma [426]), rheumatoid arthritis [427], and heart failure [428] to name a few. In recent years, new small molecule inhibitors have been developed with specificity for BD1 or BD2, which have enabled the identification of BD specific functions. For example, inhibition of BD1 disrupted BET protein chromatin occupancy and reduced gene expression in cancer cell lines similar to pan-BET inhibitors. However, BD2 inhibition did not displace chromatin-bound BET proteins or alter established gene expression programs in cancer cell lines. Instead, BD2 inhibition prevented gene induction following treatment with the pro-inflammatory factor IFNγ and improved disease status in a variety of rat inflammatory disease models [429]. Importantly, these inhibitors still targeted

all members of the BET family and it remains to be determined whether Brd4 specific inhibitors, such as the recently designed Brd4 BD1 specific ZL0580, will elicit similar responses [430].

1.3.4.5.3. Super elongation complex (SEC)

The SEC is another multi-subunit complex that promotes RNAPII transcription through several mechanisms, including the recruitment of P-TEFb. The SEC name refers to a family of complexes with different combinations of an AF4/FMR family (AFF) member 1, 2, 3, or 4, eleven-nineteen Lys-rich leukemia (ELL) 1, 2, or 3, ELL associated factor (EAF) 1 or 2, eleven-nineteen leukemia (ENL) or ALL1-fused gene from chromosome 9 (AF9), and P-TEFb (Figure 1.16). The complex promotes various aspects of RNAPII transcription, such as pause release and elongation rate [431]. SEC-mediated regulation of these processes is critical for the rapid upregulation of gene expression, such as following heat shock or differentiation signals to embryonic stem cells [432, 433]. Furthermore, the SEC is a driver of oncogenic gene expression in various forms of leukemia and MYC-driven cancers [434].

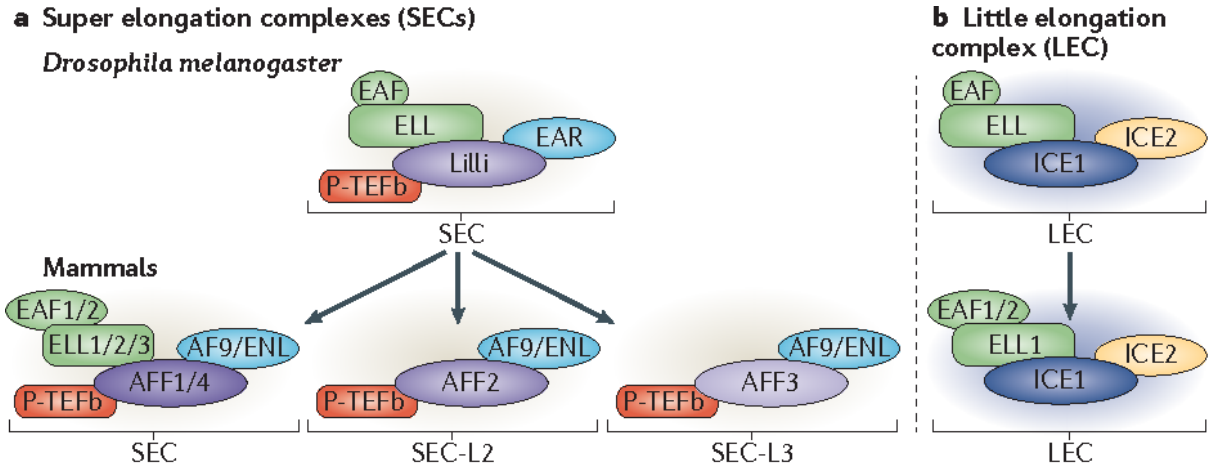


Figure 1.16. SEC composition in *Drosophila melanogaster* and mammals.

(A) In *Drosophila melanogaster*, a single isoform for each subunit assemble into a single SEC. In mammals, there are four AFF isoforms, two EAF isoforms, three ELL isoforms and the mutually exclusive AF9 and ENL. The multiple isoforms form many distinct complexes referred to as the SEC (with AFF1/4 and all subunits) or SEC-L2 or SEC-L3 with AFF2 or AFF3, respectively, and lacking an ELL and EAF isoform.

(B) A separate ELL complex has been identified in *Drosophila melanogaster* referred to as the little elongation complex (LEC). This complex contains ELL, EAF, and the unique proteins ICE1 and ICE2. In mammals, ELL1 forms this complex to regulate snRNA expression.
Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Reviews Molecular Cell Biology, "The super elongation complex (SEC) family in transcriptional control," Luo, Z., et al., Aug 16;13(9):543-7, Copyright 2012. See Appendix.

Several of the subunits, such as AFF1, AF9, and ENL, were initially identified as partner genes of translocated mixed lineage leukemia (MLL) that formed oncogenic drivers of leukaemia [435]. In MLL translocations, MLL loses its histone H3K4 methyltransferase domain and retains its DNA binding ability. The DNA binding activity enables the aberrant recruitment of the fusion partners to MLL target genomic loci. In the case of fusion with SEC components, the aberrant recruitment promotes SEC assembly and transcription of MLL target genes. The aberrant transcription leads to the transformation of healthy haematological stem/progenitor cells to leukemic blasts [436]. Before characterization of the full complex, functions of individual subunits had been described to better understand the MLL-translocations. For example, purified ELL was shown to stimulate RNAPII elongation in an *in vitro* transcription assay [437]. The term super elongation complex was coined a decade ago in a study assessing the common interactors of these MLL fusion proteins by affinity purification mass spectrometry [433]. Around the same time as identification of the SEC, Tat transactivation was demonstrated to recruit P-TEFb as a member of the SEC through interactions with the AFF4 subunit, providing further functional significance to the complex [438, 439].

In the SEC, the AFF1/4 subunits function as the central scaffold holding the various subunits together [433]. The AFF1/4 C-terminal contains a conserved C-terminal homology domain (CHD) required for the formation of AFF4 homodimers or AFF1/4 heterodimers and that mediates an interaction with RNA and DNA *in vivo* [440, 441]. Furthermore, P-TEFb phosphorylated the CHD *in vitro*, which was required for proper assembly of the complex [440, 442]. Although AFF1/4 heterodimers are observed and correlate with the MLL-fusion protein's oncogenic potential *in vivo*, the two proteins form separate and distinct SECs with a single AFF protein, suggesting a separate function of the heterodimers [441, 443]. The AFF1-SEC and AFF4-SEC are thought to regulate distinct sets of genes, as evident by AFF1 or AFF4 knockdown in HEK 293 cells followed

by RNA-seq. The gene-specificity was also evident by the requirement of the AFF4-SEC for Hsp70 upregulation following heat shock, whereas AFF1-SEC was more efficient in Tat stimulated removal of P-TEFb from the 7SK snRNP [443]. In contrast, AFF1 and AFF4 ChIP-seq exhibit similar occupancy patterns throughout the genome, although the comparison of gene expression changes between respective knockdowns was not performed in the study [444, 445]. A model with sequential AFF1-SEC and AFF4-SEC recruitment to facilitate P-TEFb phosphorylation of distinct substrates has been proposed [289]. The different gene expression changes following AFF1 or AFF4 knockdown may indicate differential requirement for P-TEFb target phosphorylation at specific genes. Lastly, AFF2 and AFF3 form multi-subunit complexes, termed SEC-like complex (SEC-L) 2 or 3, respectively, which lack an ELL and EAF subunit [431]. The presence of these complexes provides further gene specific regulatory control. For example, AFF4-SEC, and not SEC-L2 or SEC-L3, was recruited to Hsp70 following heat shock and knockdown of the specific AFF led to different gene expression changes [434].

AFF1/4 contain an intrinsically disordered N-terminal that mediates interactions with the various components through short hydrophobic regions [446]. First, AFF1/4 recruits P-TEFb through interactions between its N-terminus and cyclin T [447]. AFF4 also contains an ELL interacting domain that contacts the C-terminus of ELL2 [448]. The ELL family promotes RNAPII elongation through their ability to maintain the 3’ end of nascent RNA in the polymerase’s catalytic site [437, 449-451]. In addition to interactions with AFF4 and RNAPII, interactions with EAF1 or EAF2 stimulate ELL activity [452]. Outside of the SEC, ELL1 has been identified in the little elongation complex (LEC) comprised of EAF1/2 and ELL1 and interacts with carboxyl terminus of ELL (ICE) 1 and 2 that LEC regulates snRNA expression [453]. Lastly, the ENL and AF9 ANC1 homology domains (AHD) interact with AFF4 in a mutually exclusive manner [454, 455]. AF9/ENL are also a part of a separate complex containing the H3K79 methyltransferase Dot1L, which competes with AFF4 for interaction with the AHD domain [454]. The N-terminus of ENL/AF9 contains a YEATS domain responsible for an interaction with the PAFc and H3K9ac [454, 456]. While biochemical methods have identified complexes containing the full subunits, whether they are recruited together and remain together throughout the gene body remains to be determined. For example, ELL2 and AFF4 ChIP-seq identified differential enrichment of ELL2 and AFF4, with greater ELL2 at the TSS and AFF4 downstream of the paused RNAPII. Furthermore, there was only 50% overlap between AFF4 and ELL2 peaks, suggesting dynamic

assembly of the various subunits and their isoforms at specific stages of RNAPII transcription [432].

SEC recruitment in MLL-fusion leukemia and to the HIV-1 genome has been extensively studied, but its gene-specific recruitment in a physiological state is less well understood. In a native context, the SEC is recruited in a gene-specific manner suggesting TFs are able to mediate recruitment of the complex. For example, in response to retinoic acid the SEC was recruited to retinoic acid receptor (RAR) target genes in mouse embryonic stem cells. The authors suggested this was due to RAR targeting the SEC, although an interaction was not assessed [432]. TF-directed recruitment is potentially mediated through intermediates (i.e. Mediator) which, as already discussed, interact with TFs. First, the Mediator MED26 subunit recruits the SEC to promote RNAPII pause-release through interactions with the EAF1/2 subunit [457]. Through interactions with Mediator, the TF T-bet promotes recruitment of a P-TEFb-containing SEC to super-enhancers [458]. The SEC is also recruited through ENL/AF9 subunits' YEATS domain. The YEATS domain interacts with PAFc and was required for the Tat-independent activation of an HIV-1 luciferase gene reporter. Furthermore, knockdown of the PAFc subunit PAF1 reduced the recruitment of P-TEFb to the chromatin [454]. The YEATS domain also interacts directly with acetylated chromatin, which was critical for the maintenance of the oncogenic state driven by other MLL translocations [456, 459]. Lastly, AF9 interacts with TFIID's TAF subunits through a poly-Ser domain adjacent to the YEATS domain, which mediates recruitment of the full SEC to chromatin [460].

Small molecule inhibitors targeting specific functions of the SEC have recently been developed. As a potential therapeutic for MLL-fusion and non-MLL-fusion leukemias, small molecule competitive antagonists towards the ENL YEATS domain-acetylated histone interaction were developed [459, 461-463]. For example, the inhibitor XL-13m reduced ENL chromatin recruitment and oncogenic gene expression in a leukemia cell line [463]. More pertinent to this thesis, is the development of small molecules targeting SEC recruitment of P-TEFb. A high throughput screen for small molecules that disrupt the AFF4-cyclin T1 interaction identified the small molecules KL-1 and KL-2. The small molecules reduced AFF1/4 chromatin occupancy, increased RNAPII pausing index, and slowed RNAPII elongation rate in HEK 293T cells. These compounds also prevented rapid gene upregulation following heat shock and Myc-dependent gene expression, two responses with established SEC dependency [445].

1.3.4.5.4. Recruitment of 7SK snRNP

While the majority of 7SK snRNP is diffuse throughout the nucleoplasm, a chromatin-bound 7SK snRNP fraction was identified through co-immunoprecipitation of 7SK snRNP subunits and components of the RNAPII PIC [464, 465]. The co-immunoprecipitation of these proteins led to the identification of 7SK snRNP occupancy at the HIV-1 LTR promoter [464]. Subsequent CDK9, HEXIM1, and LARP7 ChIP-seq identified Kruppel-associated interacting protein 1 (KAP1)-mediated recruitment of the 7SK snRNP to promoter-proximal genomic regions through a KAP1-LARP7 interaction [466]. A genome-wide method analogous to ChIP for RNA, chromatin isolation by RNA purification (ChIRP)-seq, identified 7SK RNA chromatin occupancy along transcribed genes, similar to RNAPII, and cis-regulatory genomic regions, including promoters and super-enhancers, in mouse and human embryonic stem cells. Overlap with HEXIM1 ChIP-seq revealed that the canonical 7SK snRNP occupies promoters and gene bodies but not super-enhancers, with subsequent experiments identifying 7SK RNA P-TEFb-independent functions at these sites [467]. The inhibitory complex is also recruited by a direct interaction between 7SK RNA and the repressive histone modification H4K3me$^{2(s)}$ along certain enhancers [468] and via a 7SK snRNP, COUP-TF-interacting protein 2 (CTIP2), and high mobility group protein HMG-I/HMG-Y (HMGA1) complex to the HIV-1 and human gene promoters [469]. The chromatin-bound 7SK snRNP is thought to increase local P-TEFb levels rapidly in order to accelerate the kinetics of gene activation by TFs [465].

Chromatin-bound P-TEFb-7SK snRNP is inactive and requires further factors to release P-TEFb in an active form. The 7SK snRNP is recruited to anti-pause enhancers, occupied by Brd4 and the demethylase Jumonji domain containing 6 (JMJD6), by a 7SK snRNA interaction with the repressive H4K3me$^{2(s)}$. Brd4 recruits JMJD6 to demethylate H4K3 and remove the methyl cap on 7SK RNA, leading to dissociation of HEXIM and the activation of P-TEFb [468]. The Brd4 dependent activation of chromatin-bound P-TEFb was also identified using a chemical degrader of BET proteins, dBET6. The chemical degrader is a bifunctional small molecule, interacting with both the BET BD and the E3 ubiquitin ligase cereblon. The small molecule mediated proximity between the two target proteins induces ubiquitination and degradation of the BET proteins [470]. BET degradation reduced pSer2 levels and RNAPII elongation independently of altered P-TEFb recruitment. The authors proposed the effect was due to Brd4 regulating formation of the

transcriptional machinery as there was reduced Spt5, NELF and MED1 chromatin occupancy [471]. The results also suggest Brd4 was required for P-TEFb release from chromatin-bound 7SK snRNP in order to phosphorylate RNAPII, although additional experiments are required to address this possibility. The mechanisms identified at promoters rely on a different repertoire of proteins including PPM1G, the DEAD-box RNA helicase DDX21, and serine and arginine rich splicing factor 1 or 2 (SRSF1/2). With regards to PP1MG, TFs recruit the protein phosphatase to dephosphorylate CDK9's T-loop leading to the release of P-TEFb [472]. PPM1G remains bound to the 7SK snRNP following P-TEFb release to further promote transcription by preventing reassembly of P-TEFb [356]. DDX21 is recruited to promoters and disassembles the 7SK snRNP through alterations to the 7SK RNA secondary structures via its helicase activity [473]. Lastly, SRSF1/2 has also been identified as a component of promoter 7SK snRNP that is recruited to nascent RNA once RNAPII begins transcription and promotes P-TEFb release from the inhibitory complex [474].

1.3.5. Regulation of transcription by Gβγ

While the canonical role of Gβγ involves modulating activity of effector proteins in the cytoplasm, it also has important functional roles in the nucleus. Specifically, Gβγ regulates RNAPII transcription through a variety of mechanisms upon GPCR activation in several cell types. First, Gβγ indirectly impacts RNAPII transcription through modulating receptor proximal signalling pathways that alter TF activity (Figure 1.17). For example, activation of the dopamine D2 receptor (D2R) increased IP_3 receptor (IP_3R)-1 expression via a Gβγ-PLC-Ca^{2+} signalling pathway in primary mouse cerebral cortical neurons. Here, D2R activation led to Gβγ-dependent translocation of the TF nuclear factor of activated T cells 4 (NFATc4) from the cytoplasm to the nucleus and recruitment to the IP_3R-1 promoter alongside the TF activator protein 1 (AP-1) [475]. D2R-mediated Gβγ signalling also regulates the TF early growth response protein 1 (Egr1), also referred to as Zif268, in the differentiated dopaminergic-like SH-SY5Y cell line and rat midbrain slices. In response to D2R activation, Egr1 recruitment to the glial cell line-derived neurotrophic factor (GDNF) promoter and GDNF expression increased in a Gβγ- and ERK1/2-dependent manner [476]. Additionally, Gβγ activation of MAPK signalling was implicated in phosphorylation of the TF CREB in striatal neurons. Corticotropin-releasing factor (CFR) receptor 1 activation led to Gβγ- and MAPK-dependent CREB phosphorylation, independent of canonical

PKA-dependent CREB phosphorylation [477]. Similarly, $GABA_B$ receptor activation in cerebellar granule neurons led to Gβγ- and ERK1/2-dependent CREB phosphorylation [478]. Furthermore, Gβγ-dependent regulation of calcium signalling regulates transcription of the interleukin-2 (IL-2) gene following T cell receptor (TCR) activation in $CD4^+$ T helper cells. $G\beta_1$ knockdown and Gβγ inhibition with the small molecule gallein, but not $G\beta_2$ knockdown, potentiated TCR-mediated Ca^{2+} signalling, increasing translocation of the TFs nuclear factor of activated T cells 1 (NFAT1) and nuclear factor of activated T cells 2 (NFAT2) into the nucleus to positively regulate IL-2 expression [479]. Lastly, Gβγ signalling negatively regulates thyroid stimulating hormone receptor (TSHR)-induced transcription in the process of thyroid differentiation. TSHR signalling through Gαs-cAMP increased recruitment of the TF Pax8 to the sodium iodide symporter (NIS) promoter to increase NIS expression. Conversely, the THSR negatively regulated Pax8 through increased Gβγ-dependent PI3K/rac-alpha serine/threonine-protein kinase (Akt) signalling which excludes Pax8 from the nucleus [480].

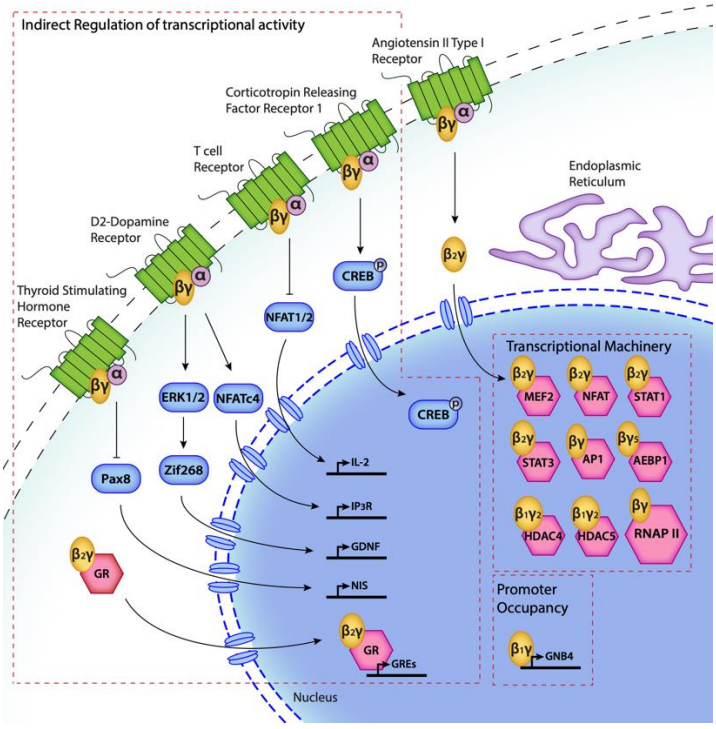


Figure 1.17. Indirect or direct regulation of transcription factors by Gβγ.

Gβγ signalling modulates TF activity (indirect), or Gβγ interacts with TFs altering their activity (direct). Figure adapted from [481].

Reprinted from GPCRs, Martin, RD., Bouazza CA., Hébert, TE., Organellar Gβγ signalling-GPCR signalling beyond the cell surface, 257-267, Copyright (2020), with permission from Elsevier. See Appendix

Other studies have demonstrated activation of the signal transducer and activator of transcription (STAT) family of TFs by Gβγ. For example, of 48 different Gβ and Gγ combinations co-expressed in HEK 293 cells, 13 dimers increased the levels of active, phosphorylated STAT3. This study did not determine the Gβγ-dependent mechanism for STAT3 activation [482]. A separate study identified a potential Gβγ-dependent mechanism for the activation of STAT5B downstream of the δ-opioid receptor heterologously expressed in HEK 293 cells. Here, Gβγ interacted with STAT5B to form a constitutive complex at the δ-opioid receptor, and functions as a scaffold to recruit c-Src to the receptor and phosphorylate STAT5B [483]. Interestingly, this study characterized Gβγ-dependent activation of TFs at the plasma membrane instead of the nucleus, as in the other examples. These studies demonstrate that Gβγ indirectly regulates TF activity through its canonical pathways.

Several studies have identified negative regulation of TF activity through interactions with Gβγ (Figure 1.17). One of the first identified was the direct interaction, as determined by a yeast-two hybrid assay, between $G\beta\gamma_5$ and adipocyte enhancer-binding protein (AEBP1) to negatively regulate its activity in adipocyte-like 3T3-L1 cells [484]. $G\beta_1\gamma_2$ interacts with histone deacetylase 4 (HDAC4)/histone deacetylase 5 (HDAC5) and relieves their inhibitory effect on transcription in response to α_{2A}-adrenergic receptor activation in HEK 293A cells. Under basal conditions, HDAC5 interacted with and inhibited the TF myocyte enhancer factor 2C (MEF2C). Following receptor activation, $G\beta_1\gamma_2$ disrupted the HDAC5-MEF2C interaction enabling MEF2C activity, as assessed by increased MEF2C reporter gene expression [485]. Similar Gβγ-dependent disruption of the HDAC5-MEF2 interaction occurred following EP_3 receptor stimulation in neonatal rat cardiomyocytes. Although here the authors suggested the disruption was due to Gβγ-dependent activation of protein kinase D (PKD) and its phosphorylation of HDAC5 leading to exclusion from the nucleus [486]. In contrast, the ability of Gβγ to interact with HDACs negatively regulates the TF AP-1 in HEK 293 cells and LβT2 gonadotrope cells. In response to stimuli, $G\beta_1\gamma$ recruited an HDAC to the AP-1 dimer, inhibiting transcriptional activation of an AP-1 reporter gene [487]. Lastly, Gβγ also inhibits glucocorticoid receptor (GR)-mediated activation of glucocorticoid

response genes (GREs) through direct interaction with the GR. Following GR activation with dexamethasone, $G\beta_2\gamma$ translocated with the GR into the nucleus and co-occupied GREs, preventing the function of the GR activation function 2 (AF2) domain [488].

A role for Gβγ as an activator of TF activity has also been identified (Figure 1.17). In HEK 293 cells with heterologous AT1R expression (HEK 293-AT1R), $G\beta_2\gamma$ translocated to the nucleus in response to receptor activation with Ang II. Activation of endogenous AT1R in human aortic smooth muscle cells, neonatal rat cardiomyocytes and adult rat ventricular cardiomyocytes led to similar $G\beta_2\gamma$ translocation. In HEK 293-AT1R cells, nuclear Gβγ interacted with HDAC5, aligning with the previously observed Gβγ-HDAC5 interaction. Furthermore, $G\beta_2\gamma$ interacted with myocyte enhancer factor 2A (MEF2A), histone H3 and H4, TATA-binding protein (TBP) and the TBP-associated factor (TAF) complex. $G\beta_2\gamma$ mediated the synergistic activation of a MEF2 reporter gene by MEF2 and the TBP/TAF complex at the promoter. Furthermore, the authors identified a -LLTPPG- motif in MEF2A which interacted with the $G\beta_2$ WD repeat. This repeat was also present in the $G\beta_2\gamma$ interacting TFs NFAT and STAT1/3 (another potential mechanism of Gβγ regulation of the STAT family). Similar to the MEF2 reporter gene, transcriptome microarray analysis revealed a reduction in AT1R mediated gene expression of these TFs' target genes when $G\beta_2$ was knocked down [489]. Additionally, a ChIP-on-chip experiment of HEK 293 cells identified $G\beta_1$ occupancy on more than 700 promoters [490].

1.3.6. Regulation of RNAPII in pathological cardiac remodelling

The adaptive response to stress in the heart requires dynamic alterations in gene expression networks in cardiac fibroblasts and cardiomyocytes. As previously described, a defining characteristic is the reactivation of a fetal-like gene expression program. These changes require the altered expression and activity of cardiac TFs whose DNA sequence-motifs reside within cis-regulatory regions of differentially expressed genes [491]. Knockout and gain-of-function studies have identified several critical TFs for cardiomyocyte hypertrophy and fibrotic gene expression in cardiac fibroblasts, such as NFAT, MEF2, NF-κB, and GATA4 [492, 493]. Cardiac TFs co-occupy many genomic regions, interact with each other and regulate RNAPII transcription synergistically [494-496]. The combinatorial recruitment of TFs has been proposed to be required for specific cardiac gene expression program, as these factors are also individually expressed in other tissues [497]. As discussed earlier, TFs primarily activate transcription through the recruitment of GTFs

and other co-activators to their target genomic loci. While the interactions with the general transcriptional machinery have not been clearly defined in cardiac cell types for many of the TFs, interactions have been identified in other systems. For example, human NFATp interacts with and recruits the TFIID subunit TAF4 to target assembly of the TFIID complex in Cos-1 cells [498]. In a human liver hepatoma cell line, GATA4 was demonstrated to interact with the Mediator complex [499]. Studies in the embryonic myocardium and in models of heart failure have identified interactions of TFs with various transcriptional co-activators. For example, GATA4 and NF-κB interact with and are acetylated by the acetyltransferase p300, which enhanced their respective transcriptional activation activity [500]. Furthermore, GATA4 chromatin recruitment of p300 increased the level of proximal histone acetylation in the embryonic mouse heart [501]. These are a few examples of how TFs interface with GTFs and co-activators to regulate assembly of the PIC or alterations in the chromatin, promoting recruitment of other transcriptional activators.

The role of GTFs involved with initiation in the development of pathological cardiac remodelling has not been extensively studied. One study assessed the role of TFIIB in regulating gene expression changes in the TAC model. TFIIB was constitutively bound at housekeeping and RNAPII paused genes, whereas it was dynamically recruited to TAC-induced genes regulated by *de novo* RNAPII recruitment. TFIIB knockdown prevented the upregulation of stress-induced genes and cardiac remodelling in neonatal rat cardiomyocytes treated with ET-1 and an *in vivo* TAC model [502]. While the role of other GTFs has not been extensively studied in cardiac remodelling, SNPs in the TFIID subunit TAF10 were associated with primary familial hypertrophic cardiomyopathy [503]. Although the functional significance is currently unknown, the association suggests alterations in TFIID may lead to the development of a hypertrophic phenotype.

On the other hand, positive regulation of RNAPII promoter-proximal pausing in pathological cardiac remodelling was identified almost two decades ago. The initial study pursued the kinase responsible for the increased hyperphosphorylated RNAPII following treatment of cardiomyocytes with the α_1-AR agonist PE and in cardiac tissue from the *in vivo* TAC model [504]. Through a combination of *in vivo* mouse models and *in vitro* kinase assays with protein extracts from hypertrophic cardiac tissue, the authors identified Gαq and calcineurin-dependent RNAPII hyperphosphorylation through increased P-TEFb activity. The role of P-TEFb was also observed in neonatal rat cardiomyocytes treated with ET-1. The CDK9 small molecule inhibitor DRB or

overexpression of a dominant negative CDK9 prevented hypertrophy of neonatal rat cardiomyocytes. Furthermore, transgenic mice with cardiac-specific overexpression of cyclin T1 developed pathological cardiac remodelling and exhibited increased cardiomyocyte hypertrophy in response to stress through alterations in the expression of mitochondrial proteins [505, 506].

P-TEFb activation was dependent on removal from the inhibitory 7SK snRNP in cardiac hypertrophy [505], potentially through JAK/STAT and calcium signalling [352, 507]. Mice with disrupted 7SK snRNP due to heterozygous HEXIM1 knockout developed enhanced cardiac hypertrophy in an *in vivo* pressure overload model [508]. When bred with transgenic mice with cardiac specific overexpression of cyclin T1, the double transgenic mice developed synergistic compensatory hypertrophy [509]. Similar enhanced cardiac remodelling was observed when the HEXIM1 heterozygous mice were bred with transgenic mice with cardiac-specific overexpression of angiotensinogen, which increased local Ang II levels due to the elevated precursor expression. Interestingly, HEXIM1 heterozygous cardiac fibroblasts had enhanced transcriptional responses to TGF-β1 and Ang II treatment, as measured by α-smooth muscle actin and Nox4, indicating the important role of P-TEFb in fibrotic gene expression. The increased transcription in cardiac fibroblasts correlated with increased fibrosis and fibrotic gene expression *in vivo* [510].

P-TEFb recruitment to the chromatin by Brd4 is required for pathological cardiac remodelling. Two studies independently characterized the requirement of Brd4 in cardiomyocyte hypertrophy. The first was through a BET-focused approach and the second identified the BET inhibitor JQ1 through a high-throughput screen [511, 512]. BET inhibitors reduced hypertrophy of primary neonatal rat cardiomyocytes treated with PE and the PKC activator PMA *in vitro* [511, 512]. Brd4 knockdown with short hairpin RNA (shRNA) recapitulated the inhibitor effect of JQ1 on PE-induced hypertrophy *in vitro*, indicating BET inhibitors were blocking hypertrophy through Brd4 inhibition [511]. Furthermore, JQ1 improved left ventricle hemodynamic function and reduced cardiomyocyte hypertrophy and fibrosis in the *in vivo* TAC model [511, 512]. In TAC hearts, increased RNAPII pause release was observed as there was a decrease in RNAPII pausing index compared to sham operated hearts. Systemic treatment with JQ1 in TAC mice prevented the decrease in RNAPII pausing index and reduced total pSer2 levels [511]. ChIP experiments also demonstrated reduced pSer2 following Brd4 inhibition at Brd4 target gene TSSs following PE treatment [513]. Together these results indicate Brd4 inhibition prevented cardiac remodelling due to the reduced recruitment of P-TEFb Additionally, genome-wide gene expression methods from

in vitro and *in vivo* models identified enrichment of inflammatory and fibrotic biological processes in the JQ1 attenuated genes [428, 511].

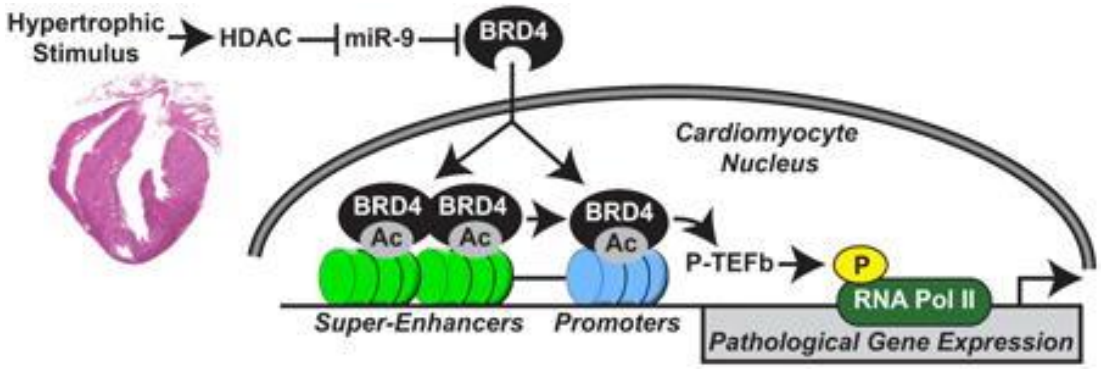


Figure 1.18. Model of Brd4 regulation in cardiomyocyte hypertrophy.
Hypertrophic stimuli increase HDAC activity to repress expression of the Brd4 targeting miR-9. Decreased miR-9 expression enables increased Brd4 protein expression and increased recruitment to super-enhancers and promoters of pro-hypertrophic genes. Chromatin-bound Brd4 recruits P-TEFb to activate the pathological gene expression through phosphorylation of the transcriptional machinery. Figure adapted from [513].

Brd4 ChIP-seq with neonatal rat cardiomyocytes identified increased Brd4 abundance at promoters and super-enhancers of PE upregulated genes. The authors proposed that the increased Brd4 occupancy was due to increased protein production through stimulus-dependent downregulation of miR-9a, which targets the 3' untranslated region (UTR) of Brd4 (Figure 1.18) [513]. In order to understand how Brd4 was selectively recruited, potential TF-mediated recruitment of Brd4 was assessed in several studies. First, TF motif enrichment of super-enhancer sequences identified enrichment of AP-1 motifs. Interestingly, overexpression of a dominant negative AP-1 reduced Brd4 chromatin occupancy, indicating a causal role in Brd4 recruitment. Second, transcriptome analysis of human induced pluripotent stem cells (iPSCs) treated with ET-1 and *in vivo* heart failure models identified enrichment of NF-κB and GATA4 target genes in JQ1 attenuated genes, suggesting a co-regulatory role with Brd4 [428, 511].

Brd4 regulates signal-dependent processes in other cardiac cell types as well. Brd4 has a critical role in regulating cardiac fibroblast activation in response to TGF-β. TGF-β signalling, potentially through p38 kinase, increased Brd4 occupancy at promoters and super-enhancers. The increased Brd4 occupancy corresponded with increased RNAPII elongation and expression of

genes critical for cardiac fibroblast activation. Importantly, JQ1 treatment prevented TGF-β and TAC-induced gene expression changes leading to reduced cardiac fibroblast activation [413]. Lastly, TAC-induced Brd4 expression was also identified in cardiac endothelial cells. Brd4 activity was required for endothelial cell conversion to fibroblasts through the process of endothelial-mesenchymal transition (EndMT) [514]. Altogether, a critical role for Brd4 in regulating pathological gene expression changes in several cardiac cell types and a variety of pre-clinical models has been identified [511, 515].

The therapeutic potential of BET inhibition was further addressed in a more clinically relevant model of heart failure, where the therapy commonly begins after the patient has established pathological cardiac remodelling. Here, the authors began systemic JQ1 treatment following development of cardiac remodelling in the TAC and myocardial infarction mouse models. The delayed JQ1 treatment retained its ability to improve left ventricle hemodynamic function, reduce cardiomyocyte hypertrophy and fibrotic area, and attenuate upregulated gene expression [428]. The success of BET inhibitors in relevant pre-clinical models has led to an increased interest of BET inhibitor clinical trials for cardiovascular disease. A phase III clinical trial with the BET BD2 selective inhibitor apabetalone assessed the time until a major adverse cardiovascular event in patients with type 2 diabetes and low HDL-C after an acute coronary syndrome (ClinicalTrials.gov, NCT02586155). RVX 208 did not significantly reduce time until a major adverse cardiovascular event, although there was a trend for improvement (10% RVX-208 vs 12.4% placebo, $p = 0.11$). Furthermore, RVX 208 reduced hospitalizations for congestive heart failure [516]. Although the clinical trial was performed in a form of cardiovascular disease not relevant to this thesis, it demonstrates the potential and interest for BET inhibition in cardiovascular diseases.

As BET proteins regulate critical processes throughout the body, there is a risk for off-target side effects. Although initial mouse models did not identify cardiac toxicity to systemic JQ1 treatment, subsequent work in healthy male mice and rats identified functional and metabolic alterations in the heart with systemic treatment of the BET inhibitor I-BET-151 [511, 517]. Furthermore, other side effects in mice include reduced weights of the thymus and spleen, altered hematopoiesis and reduced small intestine stem cell population. Lastly, BET inhibitor clinical trials for various cancers have reported thrombocytopenia, fatigue, nausea, vomiting and diarrhea as common toxicities to drug treatment [518]. It is thought that current efforts to improve drug

specificity for the different BET family members or one of the BDs will reduce the side effects and increase efficacy of these therapies [518].

1.4. Rational and objective of study

For decades, the common therapeutic approach for heart failure has been to inhibit extracellular ligands from eliciting intracellular signalling cascades. As discussed, targeting GPCR signalling in particular has provided modest success in modifying disease progression and preventing patient mortality. Due to the multifactorial nature of pathological cardiac remodelling, targeting a single signalling pathway has limited beneficial effects. An emerging therapeutic strategy is to target molecular processes downstream of GPCR activation instead. The pronounced transcriptional alterations in cardiac cell types in response to stress suggests altering this response could have beneficial outcomes. Transcriptional responses integrate numerous signalling cascades through post-translational modifications of TFs, P-TEFb, 7SK snRNP components, and Brd4, among other transcriptional regulators. The signalling integration by transcriptional regulators suggests that therapies targeting these processes will be effective in altering disease progression in a broad range of patients. Developing successful treatments requires a deeper understanding of how transcription is regulated during the development of pathological cardiac remodelling in cardiac cell types to identify potential therapeutic targets. Furthermore, understanding how various stimuli modulate gene expression will enable identification of commonalities and differences in employed mechanisms, indicating how broadly the treatment will be effective.

The overall objective of this thesis was to determine mechanisms employed by GPCRs and their signalling partners to modulate gene expression leading to cardiomyocyte hypertrophy and the fibrotic response in fibroblasts. We hypothesized that different GPCRs modulate transcription through distinct mechanisms due to their unique signalling profile. The specific objectives are:

1. To characterize a non-canonical signalling pathway downstream of the α_1-AR. Transcriptome analysis of neonatal rat cardiomyocytes following activation of the canonically Gαq-coupled α_1-AR and ETR indicated activation of a cAMP pathway following α_1-AR activation. We assessed the potential for α_1-AR to increase intracellular cAMP in HEK 293 cells with heterologous expression of either receptor using various genetically encoded biosensors.

2. To determine the role of P-TEFb containing complexes in the development of cardiomyocyte hypertrophy following activation of distinct GPCRs. Here, we assessed the role of Brd4 and SEC mediated P-TEFb recruitment in regulating cardiomyocyte hypertrophy following activation of the α_1-AR or ETR. Comparison of the two receptors identified a novel mechanism of Brd4 activation required for the hypertrophic response.
3. To determine the role of Gβγ recruitment to RNAPII in regulating gene expression in cardiac fibroblasts. AT1R signalling activates pro-fibrotic gene expression and an interaction of Gβγ with RNAPII. We addressed the regulatory role of the Gβγ-RNAPII interaction on fibrotic gene expression.

CHAPTER 2: Receptor- and cellular compartment-specific activation of the cAMP/PKA pathway by α_1-adrenergic and ETA endothelin receptors

Ryan D. Martin[1], Yalin Sun[1], Kyla Bourque[1], Nicolas Audet[1], Asuka Inoue[2], Jason C. Tanny[1]*, and Terence E. Hébert[1]*

[1] Department of Pharmacology and Therapeutics, McGill University, Montréal, Québec, Canada.

[2] Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Miyagi, Japan.

Reprinted from Martin, R.D., Sun, Y., Bourque, K., Audet, N., Inoue, A., Tanny, J.C., and Hebert, T.E. (2018). Receptor- and cellular compartment-specific activation of the cAMP/PKA pathway by alpha1-adrenergic and ETA endothelin receptors. Cell Signal *44*, 43-50.

2.1. Preface

This study was initiated to compare signalling profiles of two GPCRs, the ETR and α_1-AR, commonly used to study hypertrophy of neonatal rat cardiomyocytes. The hypertrophic response following receptor activation has been ascribed to their ability to stimulate intracellular signalling cascades through Gαq. In the literature, we noticed few studies with direct comparisons of their signalling profiles able to identify unique pathways and mechanisms employed to induce cardiomyocyte hypertrophy. Furthermore, previous studies assessing GPCR regulation of P-TEFb activity and recruitment used ligands for the ETR or the α_1-AR, assuming the signalling profiles to be highly similar. We hypothesized that these distinct receptors had unique signalling profiles that need to be considered when assessing transcriptional regulation in cardiomyocytes. Therefore, in order to identify signalling differences between these receptors in an unbiased manner, we assessed transcriptome changes following 1.5 h or 24 h receptor activation by RNA-seq. From our transcriptome analysis, we identified a unique signalling pathway activated by the α_1-AR not previously thought to be activated by this receptor. Throughout the study, we used a variety of genetically encoded biosensors to further understand the α_1-AR subtype and compartment specificity in activating the novel pathway. This chapter expands our understanding of signalling pathways activated by the α_1-AR with direct implications for its regulation of cardiomyocyte function.

2.2. Abstract

The signalling functions of many G protein-coupled receptors (GPCRs) expressed in the myocardium are incompletely understood. Among these are the endothelin receptor (ETR) family and α_1-adrenergic receptor (α_1-AR), which are thought to couple to the G protein Gαq. In this study, we used transcriptome analysis to compare the signalling networks downstream of these receptors in primary neonatal rat cardiomyocytes. This analysis indicated increased expression of target genes of cAMP responsive element modulator (CREM) after 24h treatment with the α_1-AR agonist phenylephrine, but not the ETR agonist endothelin-1, suggesting a specific role for the α_1-AR in promoting cAMP production in cardiomyocytes. To validate the difference observed between these two GPCRs, we used heterologous expression of the receptors and genetically encoded biosensors in HEK 293 cell lines. We validated that both α_{1A}- and α_{1B}-AR subtypes were

able to lead to the accumulation of cAMP in response to phenylephrine in both the nucleus and cytoplasm in a Gαs-dependent manner. However, the ETR subtype ETA did not affect cAMP levels in either compartment. All three receptors were coupled to Gαq signalling as expected. Further, we showed that activation of PKA in different compartments was α_1-AR subtype specific, with α_{1B}-AR able to activate PKA in the cytoplasm and nucleus and α_{1A}-AR only able to in the nucleus. We provide evidence for a pathway downstream of the α_1-AR, and show that distinct pools of a receptor lead to differential activation of downstream effector proteins dependent on their cellular compartment.

2.3. Introduction

G protein-coupled receptors (GPCRs) comprise a diverse family of seven transmembrane domain-containing receptors represented by over 800 genes in humans. GPCRs respond to a range of stimuli, including peptides, hormones, growth factors, lipids, odorants, and light [1]. Upon ligand binding, GPCRs activate heterotrimeric G proteins, consisting of an α, β, and γ subunits, which subsequently activate downstream effectors and signalling cascades. Cardiovascular tissues (heart, aorta and smooth muscle) express more than 150 GPCRs [2], but in many cases their signalling and physiological roles remain incompletely understood.

Two GPCR subtypes of interest in the myocardium are the endothelin receptor (ETR) and the α_1-adrenergic receptor (α_1-AR). Upon ligand binding, these receptors canonically activate the Gαq protein leading to activation of phospholipase C, hydrolysis of phosphatidylinositol 4,5-bisphosphate into diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP_3), and a subsequent increase in intracellular Ca^{2+} levels and protein kinase C (PKC) activation. ETR and α_1-AR, in response to endothelins or the endogenous catecholamines epinephrine and norepinephrine respectively, mediate signalling events important for cardiac function and pathology (reviewed in [3]).

The ETR family contains two subtypes, ETA and ETB, that are expressed at similar levels in the heart [4-7]. ETR subtypes are able to regulate multiple signalling pathways including phospholipase D, phospholipase A2, Na^+/H^+ exchangers, cAMP and cGMP production, mitogen activated protein kinase (MAPK) pathways, and tyrosine kinases [8-15]. In the heart, ETR signalling has inotropic and chronotropic effects [16, 17] and mediates cardiac remodelling in

hypertrophy, myocardial infarction, and congestive heart failure [18-20]. In these various cardiac pathologies, ETR signalling through endothelin-1 is increased and the associated cardiac hypertrophy can be blocked with an ET_AR-specific antagonist [21-24].

The α_1-AR family consists of multiple subtypes, including the α_{1A}-, α_{1B}- and α_{1D}-ARs [25, 26]. A role for α_1-ARs has been demonstrated in the regulation of phospholipase D, phospholipase A2, MAPK pathways, Na^+/H^+ exchangers, tyrosine kinases, as well as cAMP and cGMP production [10, 14, 15, 27-32]. Cardiac α_1-ARs have some inotropic and chronotropic effects and also regulate cardiac remodeling in hypertrophy and following myocardial infarctions (reviewed in [33, 34]). All three subtypes are expressed in cardiomyocytes but only α_{1A}-AR and α_{1B}-AR are detectable at the protein level [35]. Differences between the receptor subtypes in mediating cardiac pathologies have been identified through the use of transgenic mice. Cardiac specific overexpression of α_{1B}-AR led to an exacerbated hypertrophic response to pressure overload and dilated cardiomyopathy, whereas overexpression of α_{1A}-AR did not affect the response [36-39].

Initially, studies of GPCRs predominantly assessed the signalling pathways downstream of receptors on the cell surface. There is now an understanding that GPCRs can localize to and signal from various intracellular compartments, such as the nucleus (reviewed in [40]). These intracellular pools of receptors can lead to distinct signalling pathways from those activated by the same receptor at the cellular surface. In adult rat ventricular cardiomyocytes, ET_BR localizes along the nuclear membrane, whereas ET_AR is mainly found at the cell surface [41]. In the α_1-AR family, both subtypes found in adult cardiomyocytes localize to the nucleus and to a lesser extent the cell surface [42]. The nuclear GPCR population activates proximal signalling pathways similar to those on the cell membrane, but also have more direct effects on nuclear activities such as transcription initiation and gene expression (reviewed in [40, 43]). Studies assessing these nuclear specific events have used both the ETR and α_1-AR interchangeably as both are thought to predominantly couple to Gαq. Furthermore, the receptor subtype-specific signalling that occurs in distinct cellular compartments has not been addressed.

Here, we have used transcriptome analysis of primary neonatal rat cardiomyocytes treated with either the ETR agonist endothelin-1 or the α_1-AR agonist phenylephrine to assess differences in their respective signalling networks, and further probed these differences using a panel of fluorescent resonance energy transfer (FRET)- and bioluminescent resonance energy transfer (BRET)-based biosensors. We also used genetically-encoded biosensors targeted to specific

cellular compartments to compare differential signalling by distinct GPCR populations. These experiments revealed unexpected specificity in signalling function, both among the receptor subtypes tested and between subcellular compartments.

2.4. Methods

2.4.1. Constructs

Wild type ETA receptor in pcDNA3.1+, wild-type α_{1A}-adrenergic receptor in pCAGGS [44], wild-type α_{1B}-adrenergic receptor in pcDNA3.1+ (Missouri S&T cDNA Resource Center), GFP10-EPAC-RlucII in pcDNA3.1 [45] were a generous gift from Dr. Michel Bouvier (Université de Montréal). Mammalian wild-type Gαs construct was a generous gift from Dr. Peter Chidiac (Western University). The Gαq biosensor was described by Namkung *et al.* (2016) [46]. The AKAR4-NLS construct, as described in [47], was a generous gift from Dr. Jin Zhang (UCSD). The AKAR3EV-NES construct [48] was a generous gift from Dr. Michiyuki Matsuda (Kyoto University).

To generate the GFP10-EPAC-RLucII-NLS and GFP10-EPAC-RLucII-NES constructs, we first excised the stop codon at the end of RLucII with XhoI and PmeI restriction enzymes and replaced it with polymerase chain reaction (PCR) amplified linkers (SGPIESSILAQRRLINPGLNS) containing MfeI and PmeI restriction sites at the 3' end. Then, two oligonucleotides were annealed together to create a double stranded DNA coding for the nuclear localization sequence of SV40 (PKKRKVENA) or the nuclear export sequence of Heat Stable Inhibitor of cAMP-dependent protein kinase (LALKLAGLDI) bordered by a 5' MfeI restriction site and a 3' PmeI restriction site. Finally, the annealed oligonucleotides were introduced in the new GFP10-EPAC-RLucII-linker vector using MfeI and PmeI digestion.

2.4.2. RNA-seq Analysis

RNA was isolated with the RNeasy® Mini Kit (Qiagen) according to manufacturer's instructions. Library preparation with the KAPA Stranded RNA-Seq kit with RiboErase (HMR) and paired-end 125bp sequencing on the Illumina HiSeq 2500 was performed at the *McGill University and Génome Québec Innovation Centre, Montréal, Canada.* Illumina adapter sequences were removed with Cutadapt [49] and reads were aligned to the rat genome (Rnor.6)

with STAR [50] (default settings). Counts were obtained with featureCounts (-Q 3) [51] and differential gene expression analyzed with DESeq2. Upstream regulators were predicted through the use of Ingenuity Pathway Analysis (IPA, QIAGEN Inc., *https://www.qiagenbio-informatics.com/products/ingenuity-pathway-analysis) [52]*.

2.4.3. Cardiomyocyte Isolation and Culture

Unless otherwise stated, all reagents were obtained from Sigma. Cardiomyocytes were isolated from 1-3 day old Sprague-Dawley pups (Charles River Laboratories, St-Constant, Quebec) as previously described by Colombo *et al.* (2013). After isolation, cardiomyocytes were plated on 10 cm dishes coated with 10 μg/mL fibronectin and 0.1% gelatin in low glucose DMEM, 7% (v/v) fetal bovine serum, 1% (v/v) pencillin and streptomycin and 10 μM cytosine β-D-arabinofuranoside for 24h. Media was changed to maintenance media [low glucose DMEM and 1% (v/v) insulin/selenium/transferrin] and 10 μM cytosine β-D-arabinofuranoside. After 24h, media was replaced with fresh maintenance media and experiments completed 24h later. Cardiomyocytes were treated with 100 nM endothelin-1 (Bachem), 10 μM phenylephrine, or vehicle (0.001% acetic acid) with DMSO for 1.5h or 24h prior to RNA isolation. Cardiomyocytes were maintained in a controlled environment at 37°C and 5% CO_2.

2.4.4. Cell Culture and Transfection

HEK 293SL, HEK 293 parental (PL) and CRISPR-mediated Gαs knockout (ΔGαs) [53] cell lines were maintained in Dulbecco's Modified Eagle's medium (DMEM) high glucose, 5% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin. All cell lines were grown in a controlled environment at 37°C and 5% CO_2. For transfection, cells were plated at a density of $2x10^5$ cells per well in 6-well plates (Thermo Scientific, 140675) for HEK 293SL and HEK 293PL and $2.1x10^5$ cells per well for HEK 293ΔGαs cells. Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions 24h after plating. For each well, 1 μg of the respective biosensor DNA and 0.5 μg of the receptor DNA was transfected. For the Gαs rescue, 0.2 μg of receptor DNA was transfected and 0.3 μg of wild type Gαs or pcDNA3.1(-). After 24h incubation, cells were detached with 0.25% trypsin-EDTA (Wisent) and plated at a density of $4x10^4$ cells/well in a poly-L-ornithine (Sigma-Aldrich)-coated 96-well white bottom plate

(Thermo Scientific, 236105) for BRET or a similar black bottom plate (Costar, 3916) for FRET. Cells were incubated for another 24h prior to biosensor experiments.

2.4.5. BRET Biosensors

After 24h incubation in a 96-well plate, the media was removed and cells washed once with Krebs buffer (146 mM NaCl, 42 mM $MgCl_2$, 10 mM HEPES pH 7.4, 1g/L D-glucose) and incubated for 2h at room temperature in Krebs buffer. Coelenterazine 400A (Cedarlane) was added, to a final concentration of 5 μM, to each well and incubated for 5 min prior to basal reading. For pretreatment with 1 μM alprenolol, the β-adrenergic receptor (βAR) antagonist was added 30 min prior to the addition of coelenterazine 400A. Each agonist was added to the indicated final concentration and the plate was read after a 15 min stimulation. BRET ratios were calculated as the emission at 515 nm/emission at 400 nm. For all experiments, ΔBRET refers to: (Stimulated Agonist BRET Ratio – Basal Agonist BRET Ratio) – (Stimulated vehicle BRET Ratio – Basal vehicle BRET Ratio). The average of three technical replicates was taken for all treatments. BRET experiments were performed using a Victor X Light plate reader (Perkin Elmer). For validation of the HEK 293ΔGαs cell line, experiments were performed using a TriStar2 multimode plate reader (Berthold Technologies). The normalized BRET ratio was computed by dividing the fluorescence by the luminescence for the stimulated cells divided by the basal BRET; $\frac{BRET_{stimulated}}{BRET_{basal}}$.

2.4.6. FRET Biosensors

After 24h incubation in a 96-well plate, the media was removed and replaced with Krebs solution and incubated for 1h at 37°C. For pretreatment with 1 μM alprenolol, the βAR antagonist was added 30 min prior to the basal FRET reading. Following the basal FRET readings, each agonist was added to the indicated final concentration and a reading taken after 15 min. Each well was excited with 420 nm light and emissions read at 485 nm and 528 nm separately. The FRET ratio was calculated by emission at 485 nm/emission at 528 nm. For all experiments, ΔFRET refers: (Stimulated Agonist FRET Ratio – Basal Agonist FRET Ratio) – (Stimulated vehicle FRET Ratio – Basal FRET Ratio). The average of three technical replicates was taken for all treatments. All FRET experiments were performed using a Synergy2 plate reader (Biotek) at 37°C.

2.4.7. Immunofluorescence

HEK 293SL cells expressing EPAC-NLS or EPAC-NES were fixed with 4% paraformaldehyde at room temperature for 10 min and subsequently stained with Draq5 for 10 min at room temperature. Images were obtained with an Opera Phenix high content microscope (Perkin Elmer) using the confocal setting with 63x magnification. The GFP10 channel was obtained with a 375 nm excitation laser and emission filter at 500-550 nm and Draq5 channel with a 650 nm excitation and emission filter at 650-760 nm.

2.4.8. Statistical Analysis

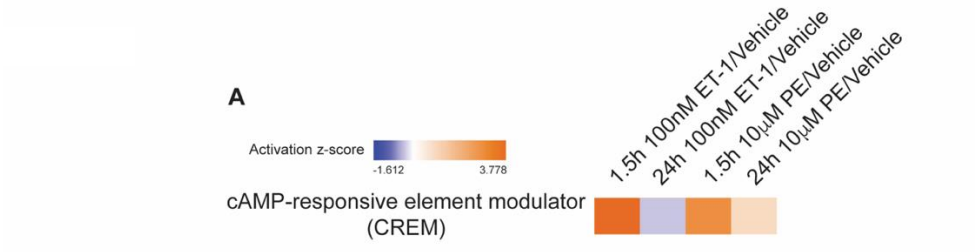
All statistical analysis was performed using GraphPad Prism 6.0 software. Data is represented as mean ± standard error (SE). Dose response curves were plotted using a four-parameter (variable slope) non-linear regression and $\log EC_{50}$ values were compared with an extra sum-of-squares F test. The activation of Gαq, EPAC-NES and EPAC-NLS were evaluated with a one-sample t test (Figure 2.2, Figure 2.5). One-way analysis of variance was performed followed by Bonferroni-corrected t-tests for validation of HEK 293ΔGαs (Supplemental Figure 2.1). Two-way analysis of variance was performed followed by Bonferroni-correct t-tests for Gαs rescue experiments (Figure 2.3E,F). For the upstream analysis prediction (Figure 2.1A), the p-value was obtained by a Fisher's Exact Test completed by the Ingenuity Pathway Analysis software package.

2.5. Results

2.5.1. Transcriptome analysis suggests cAMP signalling may be regulated by the α_1-adrenergic receptor but not the endothelin receptor

In order to compare the responses induced by distinct hypertrophic stimuli in neonatal rat cardiomyocytes, RNA-seq analysis was performed to assess the differential gene expression following treatment with 100 nM ET-1, 10 μM PE or vehicle for 1.5h or 24h. In this regard, we used conditions similar to those used in previous studies of gene expression in the hypertrophic response [54]. Pathway analysis of genes differentially expressed between treatment groups at 24h revealed a significant increase in cAMP responsive element modulator (CREM) target genes in cells treated with PE, suggesting that CREM activity was increased (activation z-score=0.894, p-

value=5.58x10^{-8}) (Figure 2.1A). In contrast, ET-1 treatment was associated with a slight decrease in apparent activity of CREM (activation z-score=-0.355, p-value=5.46x10^{-6}). CREM mRNA expression was strongly upregulated by PE but not ET-1 at the earlier 1.5h time point, consistent with subsequent increase in CREM activity (Figure 2.1B), whereas neither treatment affected CREM expression at 24h (Figure 2.1C). Transcription of CREM mRNA and its subsequent transcriptional activity is regulated by cAMP, which is not a canonical downstream signalling pathway of either the α_1-AR or ET_AR.



B

	Phenylephrine	Endothelin-1
Gene	Log2FC	Log2FC
CREM	2.45*	0.17

C

	Phenylephrine	Endothelin-1
Gene	Log2FC	Log2FC
CREM	0.30	0.12

Figure 2.1. Transcriptome analysis of neonatal rat cardiomyocytes suggests cAMP signalling may be downstream of α_1-adrenergic receptor activation.

A) Upstream regulator prediction by Ingenuity Pathway Analysis. A positive z-score predicts an increase in activity of the transcription factor, whereas a negative z-score predicts a decrease in activity. B) CREM expression changes in response to phenylephrine or endothelin-1 after 1.5h. C) CREM expression changes in response to phenylephrine or endothelin-1 after 24h. Significant gene expression was determined by DESeq2 from two independent biologic replicates. (* $p < 0.05$).

2.5.2. α_{1A}- and α_{1B}-adrenergic receptor activation lead to accumulation of cAMP

To validate the predicted cAMP signalling pathway activity from our transcriptome analysis, a panel of genetically-encoded biosensors and the relevant GPCRs were co-expressed in HEK 293 cells. First, a BRET-based Gαq sensor (Figure 2.2A) was used to validate that the different receptors activated their canonical G protein partner. The α_{1A}- and α_{1B}-ARs as well as ET_AR efficiently activated Gαq (Figure 2.2B). We next assessed the accumulation of cAMP following

receptor activation using a BRET-based whole-cell EPAC biosensor. Binding of cAMP to this biosensor induces a conformational change resulting in a decrease in BRET signal, thus a decrease in ΔBRET indicates an increase in total cellular cAMP concentration. The α_{1A}- and α_{1B}-ARs both lead to a dose-dependent accumulation of cAMP in response to phenylephrine (Figure 2.3A), whereas endothelin-1 stimulation of the ET_AR did not affect cAMP levels (Figure 2.3B).

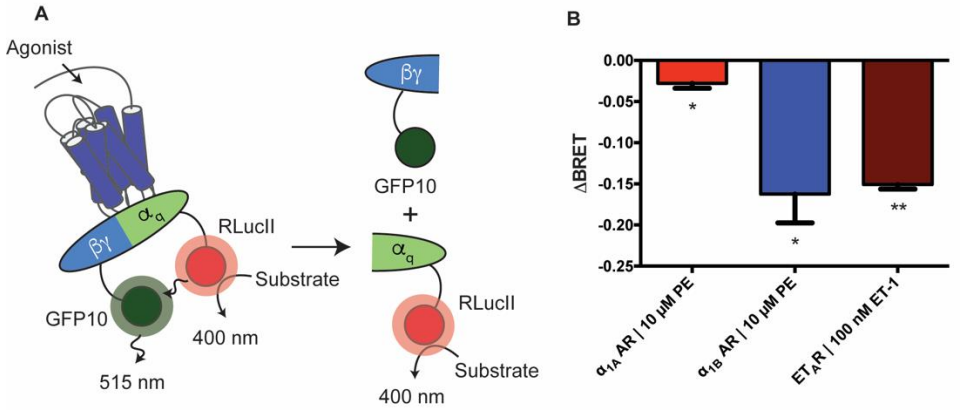


Figure 2.2. α_1-adrenergic and endothelin-A receptors activate Gαq.

A) Schematic illustrating principal of the Gαq BRET biosensor. B) Activation of Gαq in response to receptor activation. HEK 293SL cells were transiently transfected with ET_AR, α_{1A}-AR or α_{1B}-AR and the vector encoding the BRET-based Gαq biosensor. All readings were made using a Victor X Light microplate reader (Perkin Elmer) 1min after activation and are expressed as ΔBRET. Data are presented as mean ± SE for three independent experiments. One sample t-test was performed to determine if response was significantly different than control (* $p < 0.05$, ** $p < 0.01$).

Although it is considered a specific α-AR agonist, phenylephrine is known to have effects on the βAR at high concentrations [55]. In order to determine that the observed effect was not through off-target activation of the βAR, cells expressing α_{1A}- and α_{1B}-ARs were treated with the β-blocker alprenolol prior to activation with phenylephrine. In the presence of alprenolol, both the α_{1A}- and α_{1B}-AR still led to accumulation of cAMP following treatment with phenylephrine (Figure 2.3C,D). There was a slight rightward shift in the responses measured in the presence of alprenolol (α_{1A}-AR, control 6.4 +/- 0.1 versus 5.9 +/- 0.1 log EC_{50}; α_{1B}-AR, control 6.7 +/- 0.2 versus 6.2 +/- 0.1 log EC_{50}) suggesting that coupling to both βAR (as an off-target effect of phenylephrine) and α_1-AR occurs.

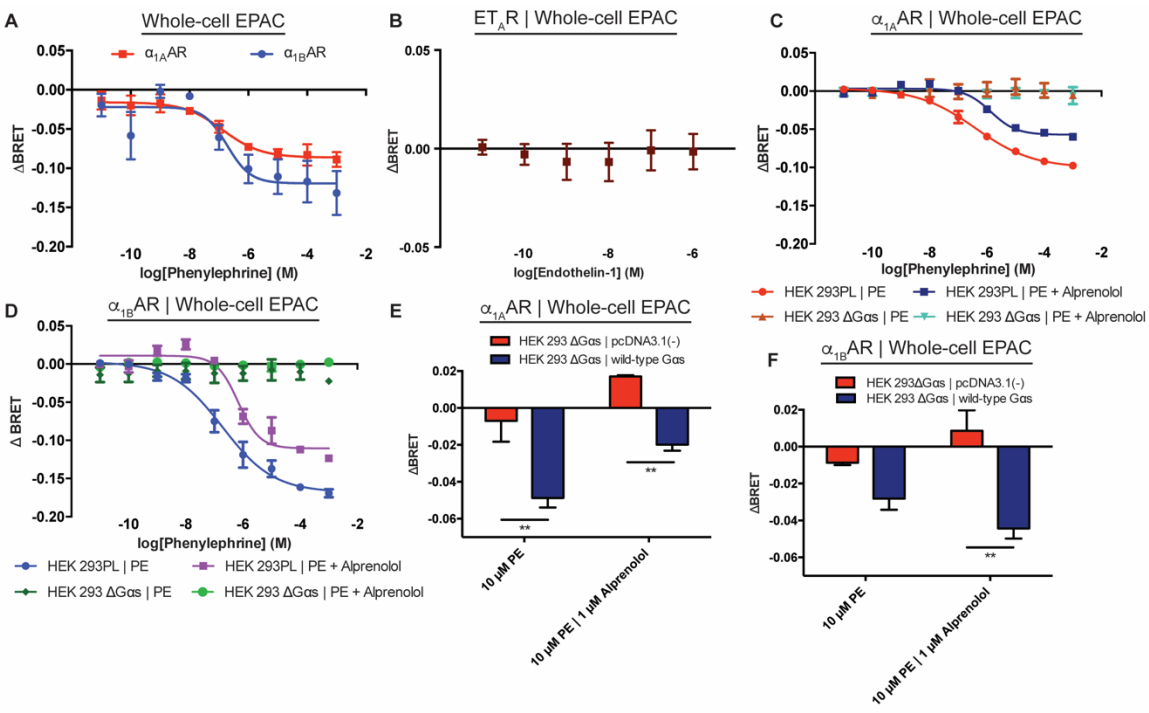


Figure 2.3. α_1-adrenergic receptors, but not the endothelin-A receptor, increase cAMP production in a dose-dependent and Gαs-dependent manner.

HEK 293SL cells were transiently transfected with α_{1A}-AR (A), α_{1B}-AR (A) or ET_AR (B) with a vector encoding a global whole cell BRET-based EPAC biosensor. C and D) HEK 293PL or ΔGαs cells were transfected with α_{1A}-AR (C) or α_{1B}-AR (D), respectively, and the global BRET-based EPAC biosensor. E and F) HEK 293ΔGαs cells were transfected with pcDNA3.1- or wild-type Gαs alongside α_{1A}-AR (E) or α_{1B}-AR (F). Cells were pretreated with 1 μM alprenolol or vehicle for 30 min prior to stimulation with phenylephrine. All readings were made using a Victor X Light microplate reader (Perkin Elmer) 15 min after treatment and are expressed as ΔBRET. Data are presented as mean ± SE for three independent experiments. Two-way analysis of variance was performed followed by Bonferroni corrected t-tests (** $p < 0.01$).

Accumulation of cAMP mediated by adenylyl cyclase activation is a prototypical consequence of Gαs signalling. In order to assess the dependence on Gαs for the α_{1A}- and α_{1B}-ARs, a CRISPR-mediated Gαs knockout HEK 293 line was used (validated in Supplemental Figure 2.1). The BRET-based EPAC biosensor was transfected into the HEK 293 parental line (PL) or Gαs knockout (ΔGαs) with either the α_{1A}- or α_{1B}-AR. Accumulation of cAMP downstream

of either receptor was dependent on the presence of Gαs (Figure 2.3C,D). Lastly, we performed a rescue experiment in the HEK 293ΔGαs cells by reintroducing wild-type Gαs. When Gαs was reintroduced, α_{1A}- and α_{1B}-AR activation again led to an accumulation of cAMP (Figure 2.3E,F).

2.5.3. Differential activation of PKA by receptor subtypes in specific cellular compartments

As both the α_{1A}- and α_{1B}-AR lead to accumulation of cAMP, we next assessed their ability to activate PKA in specific cellular compartments. We utilized a FRET-based PKA sensor engineered with either a nuclear export sequence (NES) or a nuclear localization sequence (NLS) to determine compartment-specific PKA activation by the ET_AR, α_{1A}- and α_{1B}-AR in response to agonist. In these assays, an increase in ΔFRET indicates an increase in PKA activity. Again, the ET_AR did not activate PKA in either the cytoplasm or the nuclear compartment (Figure 2.4A,D). There was again a slight rightward shift in the responses measured in the presence of alprenolol (AKAR-NES- α_{1A}-AR, control 6.8 +/- 0.9 versus 6.5 +/- 1.0 log EC_{50}; α_{1B}-AR, control 6.0 +/- 0.3 versus 6.7 +/- 0.2 log EC_{50}, AKAR-NLS- α_{1A}-AR, control 6.3 +/- 0.3 versus 6.1 +/- 0.4 log EC_{50}; α_{1B}-AR, control 7.4 +/- 0.2 versus 6.8 +/- 0.4 log EC_{50}) suggesting off-target coupling to endogenous βAR in response to phenylephrine. Stimulation of the α_{1A}-AR increased PKA activity in the nucleus but not the cytoplasm, and the nuclear PKA activation was again dependent on the presence of Gαs (Figure 2.4B,D). Activation of the α_{1B}-AR led to Gαs-dependent increases in PKA activity in both the cytoplasm and the nucleus (Figure 2.4C,E). Thus, there is a localized specificity to PKA signalling in response to activation of different α_1-AR subtypes.

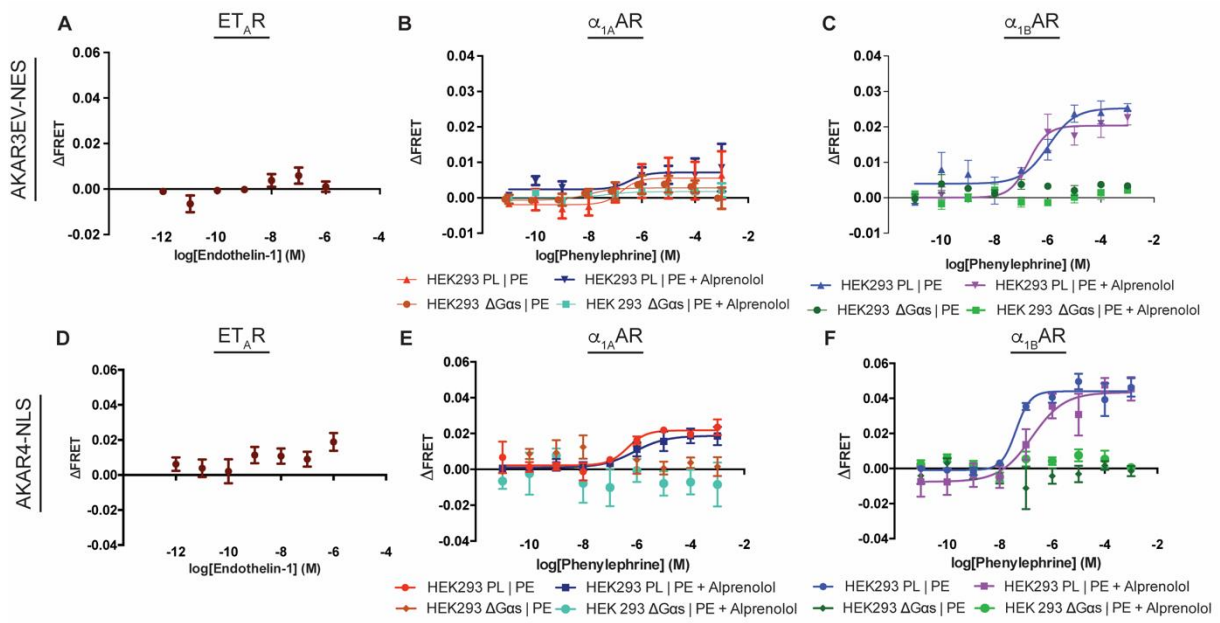


Figure 2.4. α_1-adrenergic receptors show subtype specific activation of PKA in different cellular compartments.

A) The vector encoding an AKAR3EV-NES biosensor was transiently transfected alongside the ET_AR into HEK 293PL. AKAR3EV-NES was transiently expressed alongside the α_{1A}-AR (B) or α_{1B}-AR (C) into HEK 293PL or ΔGαs cells. D) HEK 293SL were transiently transfected with AKAR4-NLS biosensor and ET_AR and stimulated with endothelin-1. HEK 293PL or ΔGαs cells were transiently transfected with AKAR4-NLS biosensor and α_{1A}-AR (E) or α_{1B}-AR (F). Cells were pretreated with 1 μM alprenolol or vehicle for 30 min prior to stimulation with phenylephrine. All readings were obtained using a Synergy2 microplate reader (Biotech) 15 min after treatment and are expressed as ΔFRET. Data are presented as mean ± SE for three (α_{1A}-AR and α_{1B}-AR) or four (ET_AR) independent experiments.

2.5.4. cAMP accumulates in the nucleus and cytoplasm following activation of the α_{1A}- and α_{1B}-adrenergic receptors

In order to determine if the localized activation of PKA was due to localized cAMP production, we used BRET-based EPAC cytoplasmic and nuclear-localized biosensors (Figure 2.5A). α_{1A}-AR activation led to an accumulation of cAMP in both the nucleus and cytoplasm following stimulation with phenylephrine for 15min (Figure 2.5B,C). We also found that the α_{1B}-AR increased cAMP levels after 15 min in response to phenylephrine (Figure 2.5B,C). Thus,

although both the α_{1A}- and α_{1B}-AR are able to increase cAMP production in the nucleus and cytoplasm, downstream PKA activation is compartmentalized.

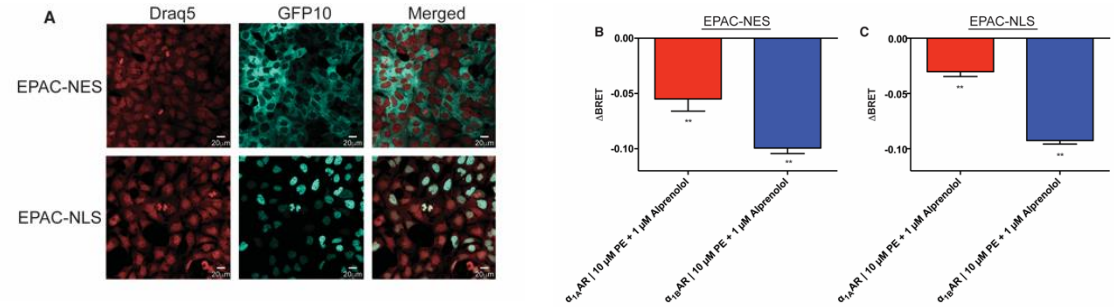


Figure 2.5. cAMP increases in the cytoplasm and nucleus upon activation of the α_1-adrenergic receptors.

A) Immunofluorescent images of HEK 293SL cells expressing BRET-based EPAC biosensors targeted to the cytoplasm (EPAC-NES, top panel) or nucleus (EPAC-NLS, lower panel). Images for Draq5 (left image) and GFP (middle iamged) were captured and presented as merged images (left image). HEK 293SL cells were transiently transfected wit α_{1A}-AR or B and α_{1B}-AR with a BRET-based EPAC-NES (B) or EPAC-NLS (C) biosensor. HEK 293SL cells were pretreated for 30 min with vehicle or 1 μM alprenolol prior to stimulation with 10 μM phenylephrine. All readings were taken on a Victor X Light microplate reader (Perkin Elmer) 15 min after treatment and are expressed as ΔBRET. Data are presented as mean ± SE for 3-5 independent experiments. One sample t-test was performed to determine if response was significantly different than control (* $p < 0.05$, ** $p < 0.01$).

2.6. Discussion

Here we have shown that two GPCR subtypes thought to trigger similar signalling events by coupling to Gαq in fact regulate different signalling networks via coupling to distinct G proteins. Thus global effects regulated by both receptors in events such as cardiac hypertrophy should be assessed independently. We first demonstrated distinct signalling pathway responses activated by the α_1-AR compared to the ETR in hypertrophic cardiomyocytes. The upregulation of CREM expression after 1.5h stimulation of the α_1-AR suggested a concomitant increase in cAMP levels following receptor activation, as some CREM isoforms are upregulated in response to cAMP [56, 57]. We explored this potential signalling pathway further as upregulation of cAMP synthesis by

catecholamines, the endogenous ligands for α_1-AR, is usually associated with βAR receptor activation. We used a heterologous expression system with a panel of FRET- and BRET-based biosensors in HEK 293SL cells and the pertinent subtypes of the α_1-AR and ETR that regulate cardiac hypertrophy. Whereas ET_AR stimulation did not increase cAMP production or PKA activity, both α_{1A}-AR and α_{1B}-AR were able to generate cellular cAMP accumulation and PKA activation in a Gαs-dependent manner. This expands the current view of the α_1-AR subfamily, which is classically associated with Gαq, to include regulation of cAMP and PKA through Gαs.

Previous studies have demonstrated the ability of the α_1-AR to lead to accumulation of cAMP through different mechanisms. Such increases were found to be secondary to activation of protein kinase C [58, 59] or through direct activation of Gαs [27, 28, 30, 60]. These studies assessed cAMP production in the whole cells and determined signalling pathways using small molecule inhibitors or co-immunoprecipitation of G proteins. Here we show directly that the increase in cAMP downstream of the α_1-AR is dependent on the presence of Gαs. On the other hand, heterologous ET_AR expression in Chinese hamster ovary cells showed the ability of the receptor to activate Gαs [61, 62], whereas ET_AR activated PKA in a cAMP-independent manner in HeLa cells [63]. We have demonstrated that in HEK 293, ET_AR does not activate PKA, either in a Gαs-dependent or -independent manner. Therefore, depending on the cellular context and the complements of G proteins, ET_AR may be able to functionally couple to distinct signalling pathways. Increases in α_1-AR densities have been noted during the progression to heart failure, especially in patients treated with β-blocking agents [64, 65]. This leads to an increase of the α_1-AR to βAR ratio. It has been suggested that α_1-AR may therefore assume a greater functional role in the failing heart by acting as a secondary inotropic system when β-adrenergic signalling is compromised by drugs or downregulation of βAR.

Although we observed increases in α_1-AR mediated cAMP production separately in the nucleus and cytoplasm, compartment specificity was observed for PKA activation. GPCRs and their effector proteins are commonly found in multiprotein signalosomes with A-kinase anchoring proteins (AKAPs) serving as scaffolds [66]. These AKAPs bring the components of signalling cascades into close proximity with one another, including the GPCR, adenylyl cyclases, cAMP phosphodiesterases, PKA, as well as different substrates [67, 68]. These complexes contain both positive and negative regulators of cAMP synthesis, which allows for discrete localized signalling and activation of specifically-localized subsets of PKA near their substrates [69]. PKA substrate

phosphorylation following α_1-AR stimulation was observed to be highly compartmentalized within the cell, and was delocalized by microtubule disruption [27]. More recently, an AKAP-Lbc signaling complex was shown to regulate α_1-AR signalling through RhoA [70]. The nuclear-specific activation of PKA by the α_{1A}-AR, despite cAMP production in both the cytoplasm and nucleus suggests interaction with different AKAP complexes. Multiple AKAPs have been shown to interact with the same GPCR. For example, the βAR interacts with both AKAP250 and AKAP150. To determine the compartment-specific AKAP interactions, future experiments with isoform specific AKAP disrupting peptides could be performed [71].

PKA signalling in the nucleus was thought to be due to the translocation of the catalytic subunit upon activation from the cytoplasm to the nucleus via diffusion [72]. However, a new understanding has emerged, as both the regulatory and catalytic subunits have been identified in the nucleus and functionally separate from the cell surface [73-75]. Functional differences between the two pools of PKA have been identified in cardiomyocytes, with cytoplasmic PKA exerting inotropic effects and the nuclear pool regulating hypertrophic responses [47]. The compartment specific activation of PKA by different subtypes of the α_1-AR adds another dimension to their differential physiological and pathological roles. Subtype selective agonists or antagonists could be used to assess these differences in cardiomyocytes.

2.7. Conclusion

In conclusion, we have provided evidence that the α_1-AR family activates the cAMP/PKA pathway in a Gαs-dependent manner. Within this subfamily, there is subtype specific activation of PKA in various cellular compartments. Furthermore, the inability of the ET_AR to activate PKA highlights that when studying global effects in cardiac hypertrophy, agonists for GPCRs that canonically couple to Gαq need to be assessed independently for additional signalling phenotypes.

2.8. Acknowledgements

This work was supported by a grant from the Heart and Stroke Foundation of Canada to TEH and JCT. RM was supported by a scholarship from the Canadian Institutes of Health Research (CIHR). NA and RM were supported by a fellowship and studentship, respectively, from the McGill-CIHR Drug Development Training Program and from the Mathematics of Information Technology and Complex Systems (MITACS). KB was supported by a Faculty of Medicine

Doctoral Scholarship and YS received a summer bursary from the Groupe d'étude des protéines membranaires (GEPROM). The authors thank Viviane Pagé for administrative and technical support.

2.9. References

1. Hermans, E., *Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors.* Pharmacol Ther, 2003. **99**(1): p. 25-44.
2. Hakak, Y., et al., *Global analysis of G-protein-coupled receptor signaling in human tissues.* FEBS Lett, 2003. **550**(1-3): p. 11-7.
3. Salazar, N.C., J. Chen, and H.A. Rockman, *Cardiac GPCRs: GPCR signaling in healthy and failing hearts.* Biochim Biophys Acta, 2007. **1768**(4): p. 1006-18.
4. Arai, H., et al., *Cloning and expression of a cDNA encoding an endothelin receptor.* Nature, 1990. **348**(6303): p. 730-2.
5. Sakurai, T., et al., *Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor.* Nature, 1990. **348**(6303): p. 732-5.
6. Davenport, A.P., *International Union of Pharmacology. XXIX. Update on endothelin receptor nomenclature.* Pharmacol Rev, 2002. **54**(2): p. 219-26.
7. Molenaar, P., et al., *Characterization and localization of endothelin receptor subtypes in the human atrioventricular conducting system and myocardium.* Circ Res, 1993. **72**(3): p. 526-38.
8. Liu, Y., B. Geisbuhler, and A.W. Jones, *Activation of multiple mechanisms including phospholipase D by endothelin-1 in rat aorta.* Am J Physiol, 1992. **262**(4 Pt 1): p. C941-9.
9. Husain, S. and A.A. Abdel-Latif, *Role of protein kinase C alpha in endothelin-1 stimulation of cytosolic phospholipase A2 and arachidonic acid release in cultured cat iris sphincter smooth muscle cells.* Biochim Biophys Acta, 1998. **1392**(1): p. 127-44.
10. Ballard, C. and S. Schaffer, *Stimulation of the Na+/Ca2+ exchanger by phenylephrine, angiotensin II and endothelin 1.* J Mol Cell Cardiol, 1996. **28**(1): p. 11-7.
11. Hilal-Dandan, R., K. Urasawa, and L.L. Brunton, *Endothelin inhibits adenylate cyclase and stimulates phosphoinositide hydrolysis in adult cardiac myocytes.* J Biol Chem, 1992. **267**(15): p. 10620-4.

12. Fujitani, Y., et al., *A selective agonist of endothelin type B receptor, IRL 1620, stimulates cyclic GMP increase via nitric oxide formation in rat aorta.* J Pharmacol Exp Ther, 1993. **267**(2): p. 683-9.
13. Simonson, M.S. and W.H. Herman, *Protein kinase C and protein tyrosine kinase activity contribute to mitogenic signaling by endothelin-1. Cross-talk between G protein-coupled receptors and pp60c-src.* J Biol Chem, 1993. **268**(13): p. 9347-57.
14. Sadoshima, J., et al., *Angiotensin II and other hypertrophic stimuli mediated by G protein-coupled receptors activate tyrosine kinase, mitogen-activated protein kinase, and 90-kD S6 kinase in cardiac myocytes. The critical role of Ca(2+)-dependent signaling.* Circ Res, 1995. **76**(1): p. 1-15.
15. Bogoyevitch, M.A., P.E. Glennon, and P.H. Sugden, *Endothelin-1, phorbol esters and phenylephrine stimulate MAP kinase activities in ventricular cardiomyocytes.* FEBS Lett, 1993. **317**(3): p. 271-5.
16. Namekata, I., et al., *Intracellular mechanisms and receptor types for endothelin-1-induced positive and negative inotropy in mouse ventricular myocardium.* Naunyn Schmiedebergs Arch Pharmacol, 2008. **376**(6): p. 385-95.
17. Ishikawa, T., et al., *Positive chronotropic effects of endothelin, a novel endothelium-derived vasoconstrictor peptide.* Pflugers Arch, 1988. **413**(1): p. 108-10.
18. Doggrell, S.A., *The endothelin system and its role in acute myocardial infarction.* Expert Opin Ther Targets, 2004. **8**(3): p. 191-201.
19. Higazi, D.R., et al., *Endothelin-1-stimulated InsP3-induced Ca2+ release is a nexus for hypertrophic signaling in cardiac myocytes.* Mol Cell, 2009. **33**(4): p. 472-82.
20. Spieker, L.E., et al., *Endothelin receptor antagonists in congestive heart failure: a new therapeutic principle for the future?* J Am Coll Cardiol, 2001. **37**(6): p. 1493-505.
21. Francis, G.S., et al., *Comparison of neuroendocrine activation in patients with left ventricular dysfunction with and without congestive heart failure. A substudy of the Studies of Left Ventricular Dysfunction (SOLVD).* Circulation, 1990. **82**(5): p. 1724-9.
22. Serneri, G.G., et al., *Selective upregulation of cardiac endothelin system in patients with ischemic but not idiopathic dilated cardiomyopathy: endothelin-1 system in the human failing heart.* Circ Res, 2000. **86**(4): p. 377-85.

23. Yorikane, R., et al., *Increased production of endothelin-1 in the hypertrophied rat heart due to pressure overload.* FEBS Lett, 1993. **332**(1-2): p. 31-4.
24. Ito, H., et al., *Endothelin ETA receptor antagonist blocks cardiac hypertrophy provoked by hemodynamic overload.* Circulation, 1994. **89**(5): p. 2198-203.
25. Michel, M.C., B. Kenny, and D.A. Schwinn, *Classification of alpha 1-adrenoceptor subtypes.* Naunyn Schmiedebergs Arch Pharmacol, 1995. **352**(1): p. 1-10.
26. Graham, R.M., et al., *alpha 1-adrenergic receptor subtypes. Molecular structure, function, and signaling.* Circ Res, 1996. **78**(5): p. 737-49.
27. Gallego, M., et al., *alpha1-Adrenoceptors stimulate a Galphas protein and reduce the transient outward K+ current via a cAMP/PKA-mediated pathway in the rat heart.* Am J Physiol Cell Physiol, 2005. **288**(3): p. C577-85.
28. Ruan, Y., et al., *Alpha-1A adrenergic receptor stimulation with phenylephrine promotes arachidonic acid release by activation of phospholipase D in rat-1 fibroblasts: inhibition by protein kinase A.* J Pharmacol Exp Ther, 1998. **284**(2): p. 576-85.
29. Spector, M., et al., *Activation of mitogen-activated protein kinases is required for alpha1-adrenergic agonist-induced cell scattering in transfected HepG2 cells.* Exp Cell Res, 2000. **258**(1): p. 109-20.
30. Shibata, K., et al., *alpha 1-Adrenergic receptor subtypes differentially control the cell cycle of transfected CHO cells through a cAMP-dependent mechanism involving p27Kip1.* J Biol Chem, 2003. **278**(1): p. 672-8.
31. Burch, R.M., A. Luini, and J. Axelrod, *Phospholipase A2 and phospholipase C are activated by distinct GTP-binding proteins in response to alpha 1-adrenergic stimulation in FRTL5 thyroid cells.* Proc Natl Acad Sci U S A, 1986. **83**(19): p. 7201-5.
32. Stojkov, N.J., et al., *In vivo blockade of alpha1-adrenergic receptors mitigates stress-disturbed cAMP and cGMP signaling in Leydig cells.* Mol Hum Reprod, 2014. **20**(1): p. 77-88.
33. O'Connell, T.D., et al., *Cardiac alpha1-adrenergic receptors: novel aspects of expression, signaling mechanisms, physiologic function, and clinical importance.* Pharmacol Rev, 2014. **66**(1): p. 308-33.
34. Woodcock, E.A., et al., *Cardiac alpha 1-adrenergic drive in pathological remodelling.* Cardiovasc Res, 2008. **77**(3): p. 452-62.

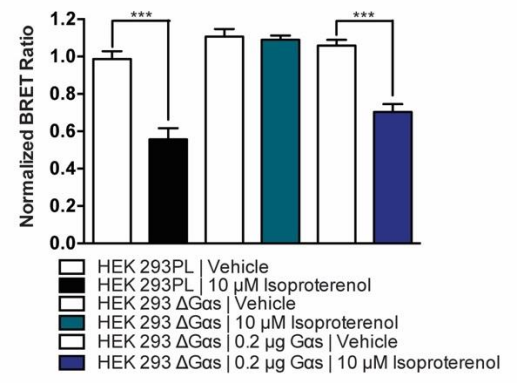
35. O'Connell, T.D., et al., *The alpha(1A/C)- and alpha(1B)-adrenergic receptors are required for physiological cardiac hypertrophy in the double-knockout mouse.* J Clin Invest, 2003. **111**(11): p. 1783-91.
36. Lin, F., et al., *Targeted alpha(1A)-adrenergic receptor overexpression induces enhanced cardiac contractility but not hypertrophy.* Circ Res, 2001. **89**(4): p. 343-50.
37. Du, X.J., et al., *Genetic enhancement of ventricular contractility protects against pressure-overload-induced cardiac dysfunction.* J Mol Cell Cardiol, 2004. **37**(5): p. 979-87.
38. Wang, B.H., et al., *Adverse effects of constitutively active alpha(1B)-adrenergic receptors after pressure overload in mouse hearts.* Am J Physiol Heart Circ Physiol, 2000. **279**(3): p. H1079-86.
39. Lemire, I., et al., *Cardiac-directed overexpression of wild-type alpha1B-adrenergic receptor induces dilated cardiomyopathy.* Am J Physiol Heart Circ Physiol, 2001. **281**(2): p. H931-8.
40. Boivin, B., et al., *G protein-coupled receptors in and on the cell nucleus: a new signaling paradigm?* J Recept Signal Transduct Res, 2008. **28**(1-2): p. 15-28.
41. Boivin, B., et al., *Functional endothelin receptors are present on nuclei in cardiac ventricular myocytes.* J Biol Chem, 2003. **278**(31): p. 29153-63.
42. Wright, C.D., et al., *Nuclear alpha1-adrenergic receptors signal activated ERK localization to caveolae in adult cardiac myocytes.* Circ Res, 2008. **103**(9): p. 992-1000.
43. Vaniotis, G., B.G. Allen, and T.E. Hebert, *Nuclear GPCRs in cardiomyocytes: an insider's view of beta-adrenergic receptor signaling.* Am J Physiol Heart Circ Physiol, 2011. **301**(5): p. H1754-64.
44. Inoue, A., et al., *TGFalpha shedding assay: an accurate and versatile method for detecting GPCR activation.* Nat Methods, 2012. **9**(10): p. 1021-9.
45. Leduc, M., et al., *Functional selectivity of natural and synthetic prostaglandin EP4 receptor ligands.* J Pharmacol Exp Ther, 2009. **331**(1): p. 297-307.
46. Namkung, Y., et al., *Quantifying biased signaling in GPCRs using BRET-based biosensors.* Methods, 2016. **92**: p. 5-10.
47. Yang, J.H., et al., *PKA catalytic subunit compartmentation regulates contractile and hypertrophic responses to beta-adrenergic signaling.* J Mol Cell Cardiol, 2014. **66**: p. 83-93.

48. Komatsu, N., et al., *Development of an optimized backbone of FRET biosensors for kinases and GTPases.* Mol Biol Cell, 2011. **22**(23): p. 4647-56.
49. Martin, M., *Cutadapt removes adapter sequences from high-throughput sequencing reads.* EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data Analysis, 2011.
50. Dobin, A., et al., *STAR: ultrafast universal RNA-seq aligner.* Bioinformatics, 2013. **29**(1): p. 15-21.
51. Liao, Y., G.K. Smyth, and W. Shi, *featureCounts: an efficient general purpose program for assigning sequence reads to genomic features.* Bioinformatics, 2014. **30**(7): p. 923-30.
52. Kramer, A., et al., *Causal analysis approaches in Ingenuity Pathway Analysis.* Bioinformatics, 2014. **30**(4): p. 523-30.
53. Stallaert, W., et al., *Purinergic Receptor Transactivation by the beta2-Adrenergic Receptor Increases Intracellular Ca2+ in Nonexcitable Cells.* Mol Pharmacol, 2017. **91**(5): p. 533-544.
54. Anand, P., et al., *BET bromodomains mediate transcriptional pause release in heart failure.* Cell, 2013. **154**(3): p. 569-82.
55. Zawilska, J.B., et al., *Characterization of beta-adrenergic receptors in duck cerebral cortex.* Acta Neurobiol Exp (Wars), 2000. **60**(3): p. 301-7.
56. Herdegen, T. and J.D. Leah, *Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins.* Brain Res Brain Res Rev, 1998. **28**(3): p. 370-490.
57. Bodor, J., et al., *Differential inducibility of the transcriptional repressor ICER and its role in modulation of Fas ligand expression in T and NK lymphocytes.* Eur J Immunol, 2002. **32**(1): p. 203-12.
58. Schwinn, D.A., et al., *The alpha 1C-adrenergic receptor: characterization of signal transduction pathways and mammalian tissue heterogeneity.* Mol Pharmacol, 1991. **40**(5): p. 619-26.
59. Perez, D.M., M.B. DeYoung, and R.M. Graham, *Coupling of expressed alpha 1B- and alpha 1D-adrenergic receptor to multiple signaling pathways is both G protein and cell type specific.* Mol Pharmacol, 1993. **44**(4): p. 784-95.

60. Horie, K., H. Itoh, and G. Tsujimoto, *Hamster alpha 1B-adrenergic receptor directly activates Gs in the transfected Chinese hamster ovary cells.* Mol Pharmacol, 1995. **48**(3): p. 392-400.
61. Takagi, Y., et al., *Structural basis of G protein specificity of human endothelin receptors. A study with endothelinA/B chimeras.* J Biol Chem, 1995. **270**(17): p. 10072-8.
62. Kawanabe, Y., et al., *Molecular mechanism for endothelin-1-induced stress-fiber formation: analysis of G proteins using a mutant endothelin(A) receptor.* Mol Pharmacol, 2002. **61**(2): p. 277-84.
63. Dulin, N.O., et al., *Cyclic AMP-independent activation of protein kinase A by vasoactive peptides.* J Biol Chem, 2001. **276**(24): p. 20827-30.
64. Mugge, A., C. Reupcke, and H. Scholz, *Increased myocardial alpha 1 adrenoceptor density in rats chronically treated with propranolol.* Eur J Pharmacol, 1985. **112**(2): p. 249-52.
65. Vago, T., et al., *Identification of alpha 1-adrenergic receptors on sarcolemma from normal subjects and patients with idiopathic dilated cardiomyopathy: characteristics and linkage to GTP-binding protein.* Circ Res, 1989. **64**(3): p. 474-81.
66. Diviani, D., et al., *A-kinase anchoring proteins: scaffolding proteins in the heart.* Am J Physiol Heart Circ Physiol, 2011. **301**(5): p. H1742-53.
67. Bauman, A.L., et al., *Dynamic regulation of cAMP synthesis through anchored PKA-adenylyl cyclase V/VI complexes.* Mol Cell, 2006. **23**(6): p. 925-31.
68. Dodge-Kafka, K.L., et al., *The protein kinase A anchoring protein mAKAP coordinates two integrated cAMP effector pathways.* Nature, 2005. **437**(7058): p. 574-8.
69. Zaccolo, M. and T. Pozzan, *Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes.* Science, 2002. **295**(5560): p. 1711-5.
70. Appert-Collin, A., et al., *The A-kinase anchoring protein (AKAP)-Lbc-signaling complex mediates alpha1 adrenergic receptor-induced cardiomyocyte hypertrophy.* Proc Natl Acad Sci U S A, 2007. **104**(24): p. 10140-5.
71. Wang, Y., et al., *Isoform-selective disruption of AKAP-localized PKA using hydrocarbon stapled peptides.* ACS Chem Biol, 2014. **9**(3): p. 635-42.

72. Harootunian, A.T., et al., *Movement of the free catalytic subunit of cAMP-dependent protein kinase into and out of the nucleus can be explained by diffusion.* Mol Biol Cell, 1993. **4**(10): p. 993-1002.
73. Zippin, J.H., et al., *Bicarbonate-responsive "soluble" adenylyl cyclase defines a nuclear cAMP microdomain.* J Cell Biol, 2004. **164**(4): p. 527-34.
74. Meoli, E., et al., *Protein kinase A effects of an expressed PRKAR1A mutation associated with aggressive tumors.* Cancer Res, 2008. **68**(9): p. 3133-41.
75. Sample, V., et al., *Regulation of nuclear PKA revealed by spatiotemporal manipulation of cyclic AMP.* Nat Chem Biol, 2012. **8**(4): p. 375-82.

2.10. Supplemental Figures



Supplemental Figure 2.1. Functional validation of ΔGαs cells.

Gαs-mediated signaling in HEK 293 parental cells was compared to the CRISPR-Cas9 genome edited ΔGαs cells to validate the loss of Gαs in the engineered cells. Cells were transiently transfected with a BRET-based EPAC biosensor. HEK 293ΔGαs were also transiently transfected with wild-type Gαs. Cells were stimulated with vehicle (ascorbic acid) or 10 µM isoproterenol and readings taken on Tristar microplate reader (Berthold Technologies) 15 min after treatment. Readings are expressed as normalized BRET ratios. Data represent mean ± SE of three independent experiments. One-way analysis of variance was performed followed by Bonferroni-corrected t-tests (*** $p < 0.001$).

CHAPTER 3: Differential activation of P-TEFb complexes in the development of cardiomyocyte hypertrophy following activation of distinct GPCRs

Ryan D. Martin[a], Yalin Sun[a], Sarah MacKinnon[a], Luca Cuccia[a], Viviane Pagé[a], Terence E. Hébert[a#], Jason C. Tanny[a#]

[a] Department of Pharmacology and Therapeutics, McGill University, Montréal, Québec, Canada

Reprinted from Martin, R.D., Sun, Y., MacKinnon, S., Cuccia, L., Page, V., Hebert, T.E., and Tanny, J.C. (2020). Differential activation of P-TEFb complexes in the development of cardiomyocyte hypertrophy following activation of distinct GPCRs. Mol Cell Biol *40*(14).

3.1. Preface

In Chapter 2, we demonstrated that α_1-AR subtypes coupled to Gαq and increased intracellular cAMP levels and PKA activity through Gαs, whereas the ET_AR coupled with Gαq and not Gαs. Previous studies have stimulated the α_1-AR or ETR to assess P-TEFb function in cardiomyocytes, assuming similar signalling and therefore similar P-TEFb regulation. In this chapter, we describe the functional implication of the identified signalling difference in Chapter 2 on P-TEFb regulation in cardiomyocytes. We utilized a variety of small molecules to inhibit P-TEFb activity, P-TEFb recruitment, and receptor signalling pathways following activation of the α_1-AR or ETR in primary neonatal rat cardiomyocytes. With these pharmacological tools, we demonstrated the α_1-AR specific requirement for Brd4-mediated, and the common requirement for SEC-mediated, P-TEFb recruitment to induce cardiomyocyte hypertrophy. Transcriptome analysis revealed BET inhibition selectively attenuated pathways involved with cardiomyocyte hypertrophy and inflammation following α_1-AR activation. Furthermore, we demonstrated Brd4 recruitment in response to α_1-AR activation required PKA signalling. In this chapter, we propose the addition of PKA signalling shifts the balance of P-TEFb complexes required for hypertrophy.

3.2. Abstract

Pathological cardiac hypertrophy is driven by neurohormonal activation of specific G protein-coupled receptors (GPCRs) in cardiomyocytes and is accompanied by large-scale changes in cardiomyocyte gene expression. These transcriptional changes require activity of positive transcription elongation factor b (P-TEFb), which is recruited to target genes by the bromodomain protein Brd4 or the *S*uper *E*longation *C*omplex (SEC). Here we describe GPCR-specific regulation of these P-TEFb complexes and a novel mechanism for activating Brd4 in primary neonatal rat cardiomyocytes. The SEC was required for the hypertrophic response downstream of either the α_1-adrenergic receptor (α_1-AR) or the endothelin receptor (ETR). In contrast, Brd4 inhibition selectively impaired the α_1-AR response. This was corroborated by the finding that activation of α_1-AR, but not ETR, increased Brd4 occupancy at promoters and super-enhancers of hypertrophic genes. Transcriptome analysis demonstrated that activation of both receptors initiated similar gene expression programs, but that Brd4 inhibition attenuated hypertrophic genes more robustly following α_1-AR activation. Finally, we show that protein kinase A (PKA) is required for α_1-AR

stimulation of Brd4 chromatin occupancy. The differential role of the Brd4/P-TEFb complex in response to distinct GPCR pathways has potential clinical implications as therapies targeting this complex are currently being explored for heart failure.

3.3. Introduction

The heart undergoes extensive remodelling in response to various mechanical and hormonal stressors during the progression to heart failure following myocardial infarction and/or sustained hypertension (1, 2). This includes hypertrophy of terminally differentiated cardiomyocytes in order to sustain cardiac output (3). While initially adaptive, prolonged cardiomyocyte hypertrophy leads to cardiomyocyte death, fibrosis and progression to chronic heart failure (4). Cardiomyocyte hypertrophy is initiated in part by neurohormonal activation of G protein-coupled receptors (GPCRs), such as the α_1-adrenergic receptor (α_1-AR), endothelin-1 receptor (ETR), and β-adrenergic (β-AR) families (5, 6). Upon ligand binding, GPCRs activate heterotrimeric G proteins comprised of a Gα subunit and the obligate heterodimer Gβγ. Both the α_1-AR and ETR canonically activate Gαq signalling, whereas the β-AR activates Gαs signalling. Cardiac-specific overexpression of Gαq and Gαs isoforms in mice leads to cardiomyopathy phenotypes, including cardiomyocyte hypertrophy (7, 8). The Gα isoforms elicit distinct signalling pathways involving calcium release and cyclic AMP (cAMP) formation respectively, which are capable of activating transcription factors and a gene expression program culminating in cardiomyocyte hypertrophy (9, 10). These pathological gene expression changes also require the coordinated interplay between dynamic alterations in chromatin structure, various master transcription factors and general transcriptional regulators, such as positive transcription elongation factor b (P-TEFb) (9, 11, 12).

P-TEFb, a heterodimer consisting of cyclin-dependent kinase 9 and cyclin T, positively regulates the release of RNA polymerase II (RNAPII) from a promoter proximal paused state into productive elongation. P-TEFb phosphorylates multiple proteins in the RNAPII elongation complex, including the C-terminal repeat domain of RNAPII itself, DRB sensitivity inducing factor (DSIF), and negative elongation factor (NELF) (13). In cardiomyocytes, P-TEFb activity is regulated by Gαq signalling as evidenced by cardiac-specific overexpression of Gαq in mice and ETR activation in primary neonatal rat cardiomyocytes (11). P-TEFb is a critical regulator for cardiomyocyte hypertrophy, with inhibition preventing the gene expression and cell size changes characteristic of cardiomyocyte hypertrophy (11). These transcriptional events require recruitment

of the active P-TEFb complex to chromatin. P-TEFb recruitment is predominantly regulated through interactions with the bromodomain and extra-terminal (BET) protein Brd4 or through interactions with the super elongation complex (SEC) (14, 15).

Like other BET family members (Brd2, Brd3, and testis specific BrdT), Brd4 contains two N-terminal bromodomains, which bind to acetylated lysines on histone proteins leading to recruitment of Brd4 to chromatin, as well as an extra-terminal domain, which interacts with multiple transcriptional regulators (16). Brd4 and BrdT contain an additional domain that interacts with P-TEFb (17). The importance of Brd4 as a regulator of P-TEFb and transcription elongation in cardiomyocyte hypertrophy has been demonstrated using small molecule inhibitors of the BET bromodomain/acetyl lysine interaction such as JQ1 (18-21). JQ1 treatment reduced stress-induced gene expression and cardiomyocyte hypertrophy in primary culture models and in mice subjected to pressure overload via transverse aortic constriction (TAC), a potent inducer of cardiac hypertrophy *in vivo* (18, 21). Brd4 inhibition was also able to partially reverse pre-established signs of heart failure in a mouse model of myocardial infarction and pressure overload (20). These effects are correlated with the loss of Brd4 from super-enhancers and promoters of hypertrophic genes in cardiomyocytes, as well as reduced RNAPII elongation.

Multiple forms of SEC have been found in mammalian cells, comprised of P-TEFb, AF9, ENL, the three ELL family members (ELL1/2/3), EAF1/2, AFF1 and AFF4 (22). The SEC positively regulates release of RNAPII from promoter proximal-pausing to productive elongation (23). Aberrant targeting and activity of the SEC underlies development of various cancers and developmental diseases. For example, mixed lineage leukemia 1 (MLL) is fused to various SEC subunits in certain types of acute leukemias (24) and a germline Aff4 gain-of-function mutation leads to the developmental syndrome CHOPS (*C*ognitive impairment and coarse facies, *H*eart defects, *O*besity, *P*ulmonary involvement and *S*hort stature and skeletal dysplasia) (25). Although RNAPII promoter proximal pausing is dysregulated in cardiac hypertrophy, the role of the SEC in regulating the hypertrophic gene expression program in cardiomyocytes has not been investigated.

How diverse signaling pathways involved in cardiac remodelling cooperate to orchestrate the hypertrophic gene expression program *in vivo* remains poorly understood. Although neurohormonal signals induce similar hypertrophic responses in primary cardiomyocytes, distinct signalling pathways are initiated through activation of their cognate GPCRs (26). Such effects are generally attributed to differential G protein coupling, however receptor-specific differences in

downstream effector protein activation of the same Gα subunit have also been demonstrated (27). The coordination between these signalling pathways, and the fact that each activates a unique combination of transcription factors, suggests that there may be differences in how they regulate gene expression. However, comparison of changes in gene expression have only been assessed for a limited repertoire of genes (28, 29). How different receptors alter global transcriptional regulation has not been systematically compared. Such differences may have important therapeutic implications for treating patients with heart disease stemming from varied clinical origins.

In this study we focused on the differential impact of cardiomyocyte GPCR signalling pathways on mechanisms regulating transcription. We investigated the role of P-TEFb and its interacting partners, Brd4 and SEC, in cardiomyocyte hypertrophy caused by activation of either of two GPCRs, the α_1-AR or ETR. These receptors are canonically thought to elicit their hypertrophic responses through Gαq activation, with both receptors also able to activate additional Gα subunits which has not been thoroughly assessed. We found that P-TEFb activity and the SEC are required for cardiomyocyte hypertrophy induced by activation of either GPCR. However, only the α_1-AR response was attenuated by Brd4 inhibition. Transcriptome analysis after Brd4 inhibition indicated attenuation of α_1-AR upregulated genes that were enriched for pathways involved in the pathophysiology of cardiomyocyte hypertrophy. Brd4 chromatin occupancy at promoters and super-enhancers of hypertrophic genes was specifically induced by α_1-AR activation, an effect that was dependent on the activity of PKA. Lastly, we demonstrated that the hypertrophic response downstream of another receptor known to signal through PKA, the β-AR, was also attenuated by Brd4 inhibition. Our study suggests receptor-specific regulation of P-TEFb function and expands the currently known cellular repertoire of protein kinases capable of regulating Brd4 function. Further, our findings suggest that the clinical efficacy of BET inhibitors for heart failure may depend on patients' specific neurohormonal signalling patterns.

3.4. Methods

3.4.1. Primary neonatal rat cardiomyocyte isolation, tissue culture, transfection and treatments

Unless otherwise stated, all reagents were obtained from Sigma. Primary rat cardiomyocytes were isolated from 1-3 day old Sprague-Dawley rats (Charles River Laboratories, St-Constant,

Quebec) as previously described with minor modifications (81). Following isolation, cardiomyocytes were seeded at a density of 40 000 cells/cm^2 on tissue culture dishes coated with 0.1% gelatin and 10 μg/mL fibronectin in DMEM low glucose (Wisent) supplemented with 7% FBS (Wisent) (v/v), 1% (v/v) penicillin/streptomycin (P/S), and 10 μM cytosine-β-d-arabinoside (MP Biomedicals). After 24 h, plates were washed twice with DMEM low glucose and media changed to cardiomyocyte maintenance media (DMEM low glucose, 1% (v/v) insulin/selenium/transferrin (Wisent), and 1% (v/v) P/S) with 10 μM cytosine-β-d-arabinoside. Twenty-four hours later, media was replaced with fresh cardiomyocyte maintenance media and experiments were initiated 24h later. Cardiomyocytes were maintained at 37°C with 5% CO_2 and typical cultures contained >90% cardiomyocytes. Cardiomyocytes were treated with endothelin-1 (Bachem), phenylephrine, iCdk9 (Novartis), KL-2 (ProbeChem Biochemicals), JQ1, alprenolol, KT5720, forskolin, 3-isobutyl-1-methylxanthine (IBMX), or isoproterenol.

For small interfering RNA (siRNA) transfection (siGENOME SMARTPool, Horizon Discover), cardiomyocytes were pelleted at 400 g for 5 min at 4°C after isolation, resuspended in DMEM low glucose supplemented with 2.5% (v/v) FBS and plated at a density of 60 000 cells/cm^2. Cardiomyocytes were transfected with 50 nM siRNA for the specified target gene with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 5h incubation, the media was replaced with DMEM low glucose supplemented with 7% FBS (v/v), 1% (v/v) penicillin/streptomycin (P/S), and 10 μM cytosine-β-d-arabinoside and cultured as previously described.

3.4.2. Immunofluorescence and measurement of cell area

Cardiomyocytes were plated in 96-well plates and cultured as described. Following indicated treatment, cells were fixed with methanol for 5 min at -20°C, permeabilized with 0.2% Triton X-100 (v/v) in PBS for 5 min at room temperature and blocked with 10% horse serum (Wisent) in PBS for 1 h at room temperature. Primary anti-α_2-actinin antibody (Sigma, A7811; 1/200) in 10% horse serum/PBS was incubated with cardiomyocytes overnight at 4°C. The following day, the fixed cardiomyocytes were incubated with anti-mouse Alexa Fluor 488 secondary antibody (Invitrogen, A-11029; 1/500) in 10% horse serum/PBS for 1 h at room temperature and 10 min with Hoechst dye (Invitrogen) (1 μg/μL) in PBS at room temperature. Stained cardiomyocytes were imaged with an Operetta high-content screening system (PerkinElmer) with 20X

magnification and analyzed with Columbus Image Analysis System (PerkinElmer). Hoechst dye was excited with a 360-400 nm filter and emissions detected at 410-480 nm and Alexa 488 was excited with a 460-490 nm filter and emissions detected at 500-550 nm. The average of two technical replicates was taken for all treatments.

3.4.3. AKAR4-NLS AAV transduction and FRET experiments

The AKAR4-NLS construct was a gift from Dr. Jin Zhang (UCSD) and the pAAV-CAG-GFP was a gift from Dr. Karel Svoboda (Addgene plasmid #28014) (82, 83). To generate the AKAR4-NLS biosensor for adeno-associated virus (AAV) production, AKAR4-NLS biosensor was excised and cloned into the pAAV backbone using BamHI and EcoRI (New England Biolabs) at the 5' and 3' end, respectively. For AAV production, HEK 293T cells were maintained in DMEM high glucose supplemented with 10% (v/v) FBS and 1% (v/v) P/S in a controlled environment of 37°C and 5% CO_2. Adeno-associated viruses were produced as previously described (84).

Twenty-four hours after plating cardiomyocytes, media was changed to cardiomyocyte maintenance media with AAV9-packaged AKAR4-NLS biosensor at a multiplicity of infection (MOI) of 5000. Following 24 h transduction, media was changed to cardiomyocyte maintenance media and changed every 24 h until experiment. After 48 h incubation, cardiomyocyte maintenance media was removed and cells were washed with Krebs solution (146 mM NaCl, 4.2 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES pH 7.4, 1 g/L glucose) and incubated for 1 h at 37°C with 5% CO_2 in Krebs solution prior to FRET readings. All cardiomyocyte FRET experiments were performed using the Opera Phenix™ High Content Screening System (PerkinElmer) with the confocal setting at 40X magnification at 37°C and 5% CO_2 and analyzed with Columbus Image Analysis System (PerkinElmer). Each well was excited with 425 nm light and emissions detected at 434-515 nm for CFP and 500-550 nm for YFP. Basal FRET images were obtained prior to addition of agonist and stimulated FRET images were obtained 15 minutes after addition of agonist to indicated final concentration. For experiments requiring a β-AR antagonist, 1 μM alprenolol was added to cardiomyocytes 30 minutes prior to obtaining basal FRET images. The FRET ratio was calculated as YFP emission/CFP emission. For all experiments, ΔFRET refers to: (Stimulated Agonist FRET Ratio – Basal Agonist FRET Ratio) – (Stimulated vehicle FRET Ratio – Basal FRET Ratio). The average of three technical replicates was taken for all treatments.

Following FRET experiments, cardiomyocytes were stained with 5 μM Draq5 at room temperature for 5 minutes. Images were obtained on the Opera Phenix™ High Content Screening System (PerkinElmer) using the confocal setting at 40X magnification. Draq5 was imaged using 640 nm excitation and emissions detected at 650-760 nm, YFP using 425 nm excitation and emission detected at 434-515 nm, and CFP using 425 nm excitation and emission detected at 500-550 nm.

3.4.4. RT-qPCR

Following indicated treatments of cardiomyocytes, cells were lysed in TRI reagent and RNA was extracted following the manufacturer's protocol. Reverse transcription was performed with random hexamer primers using an MMLV-RT platform (Promega) according to the manufacturer's protocol. Subsequent qPCR analysis was performed with BrightGreen 2x qPCR Master mix (Applied Biological Materials Inc.) on a Bio-Rad 1000 Series Thermal Cycling CFX96 Optical Reaction module. Ct values were normalized to U6 snRNA and fold change over respective control was calculated using $2^{-\Delta\Delta Ct}$ method. Primer sequences were the following: Nppb (5' CAATCCACGATGCAGAAGCTG 3' and 5' TTTTGTAGGGCCTTGGTCCTTT 3'), Nppa (5' CCTGGACTGGGGAAGTCAAC 3' and 5' ATCTATCGGAGGGGTCCCAG 3'), Serpine1 (5' TCCTCGGTGCTGGCTATGCT 3' and 5' TGGAGAGCTTTCGGAGGGCA 3'), and U6 snRNA (5' TGGAACGATACAGAGAAGATTAG 3' and 5' GAATTTGCGTGTCATCCTTG 3').

3.4.5. RNA-seq analysis

RNA was isolated with the RNeasy® Mini Kit (Qiagen) according to manufacturer's instructions. Libraries were prepared using the NEBNext® rRNA-depleted (HMR) stranded library kit and single-read 50bp sequencing completed on the Illumina HiSeq 4000 at the McGill University and Génome Québec Innovation Centre, Montréal, Canada. Reads were trimmed with TrimGalore (0.6.0) (85, 86) using the following settings*:* --phred33 --length 36 -q 5 --stringency 1 -e 0.1. Following processing, reads were aligned to the Ensembl rat reference genome (Rattus_norvegicus.Rnor_6.0.94) (87) with STAR (2.7.1a) (88). Transcripts were assembled with StringTie (1.3.4d) (89) and imported into R (3.6.1) with tximport (1.12.3) (90). Differential gene

expression was assessed with DESeq2 (1.24.0) (91) with the independent hypothesis weighting (IHW) library for multiple testing adjustment (92). Heatmaps and K-means clustering was completed with pheatmap and the removeBatchEffect function from limma (3.40.6) (93) was used prior to data visualization. Pathway analysis was completed with Ingenuity Pathway Analysis *(IPA, QIAGEN Inc., https://www.qiagenbio- informatics.com/products/ingenuity-pathway-analysis*) (94). The data is available at the NCBI Gene Expression Omnibus (GEO) with the accession GSE147402. Our code to analyze the RNA-seq data is available at https://github.com/tannylab/Cardiomyocyte-RNA-seq.git.

3.4.6. Chromatin immunoprecipitation-qPCR

Preparation and immunoprecipitation of cardiomyocyte chromatin was performed as previously described, with minor modifications (95). Following the indicated treatments, cardiomyocytes were crosslinked with 1% formaldehyde in DMEM low glucose for 10 min at room temperature with slight agitation. Crosslinking was quenched by addition of glycine to 125 mM final concentration and incubated for 5 min at room temperature with slight agitation. Cardiomyocytes were placed on ice following fixation, washed once with cold PBS, scraped into PBS with 1 mM PMSF and pelleted at 800 g for 5 min at 4°C. The pellet was resuspended in lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100, 1 mM PMSF, 1x protease inhibitor cocktail) and incubated for 10 min at 4°C on a nutator. Nuclei were pelleted at 800 g for 5 min at 4°C and resuspended in nuclei lysis buffer (50 mM TrisHCl pH 8.0, 10 mM EDTA, 1% SDS, 1 mM PMSF, 1x protease inhibitor cocktail). Nuclei were incubated for 15 min on ice followed by sonication with a BioRuptor (Diagenode) (18 cycles, 30 s on/off, high power). Insoluble cellular debris was removed by centrifugation at 14 000 g for 10 min at 4°C. A small aliquot was taken for quantification and the remaining sample stored at -80°C until use. The aliquot was incubated at 65°C overnight to reverse crosslinks, treated with RNase A (50 μg/mL) for 15 min at 37°C, and then treated with proteinase K (200 μg/mL) for 1.5 h at 42°C. Protein was removed by phenol/chloroform extraction and DNA precipitated at -80°C with 0.3M sodium acetate pH 5.2, 2.5 volumes of 100% ethanol, and 20 μg of glycogen. Samples were centrifuged for 20 min at 16 000 g, the pellet was washed with 70% ethanol, resuspended with ddH_2O and

quantified using a NanoDrop spectrophotometer (Thermo Fisher) to determine concentration of chromatin for each sample.

For immunoprecipitations, 10 μg of chromatin was diluted 9x with dilution buffer (16.7 mM Tris-HCl pH 8.0, 1.2 mM EDTA, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1x protease inhibitor cocktail). *S. pombe* chromatin, prepared as previously described (96), was spiked-in to each sample for normalization. A rabbit anti-Brd4 antibody (Bethyl, A301-985A; 5ug) or rabbit IgG antibody (Millipore, 12-370; 5 μg), as well as anti-*S. pombe* H2B antibody (Abcam, ab188271), were added to respective IPs and 1% input sample taken for subsequent analysis. Each IP was incubated at 4°C overnight on a nutator, followed by addition of 15 μL Protein G Dynabeads (Invitrogen) in dilution buffer for 4 h. Beads were washed 2X with low salt buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100), 2X with high salt buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 500 mM NaCl, 0.1% SDS, 1% Triton X-100), 1X with LiCl buffer (10 mM Tris pH 8.0, 1 mM EDTA, 0.25M LiCl, 1% NP-40, 1% deoxycholate), 1X with TE buffer (10mM Tris-HCl pH 8.0, 1 mM EDTA) at 4°C. Beads were resuspended in elution buffer (200 mM NaCl, 1% (w/v) SDS) and heated at 65°C for 20 min to elute chromatin. The eluted chromatin was incubated at 65°C overnight to reverse crosslinks and then incubated with proteinase K (200 μg/mL) for 2 h at 37°C. DNA was purified and quantified as described above.

Localization was assessed by qPCR with primers for specific genomic loci; a primer pair amplifying *S. pombe cdc2*$^{+}$ was used for normalization. All qPCR reactions were performed using a Bio-Rad 1000 Series Thermal Cycling CFX96 Optical Reaction module and iQ SYBR Green Supermix (Bio-Rad). For each primer pair in a given experimental condition, percent input for IgG control IP was subtracted from the percent input for the Brd4 IP, followed by normalization to the percent input of *S. pombe cdc2*$^{+}$. Primer sequences were the following: Nppb SE (chr5:164778453-164778528, 5’ AGGTGGCACCCCCTCTTCTAC 3’ and 5’ TTGGGGGAGTCTCAGCAGCTT 3’), Nppb TSS (chr5:164796330-164796402, 5’ TTTCCTTAATCTGTCGCCGC 3’ and 5’ GGATTGTTCTGGAGACTGGC 3’), Nppa TSS (chr5:164808403-164808456, 5’ GTGACGGACAAAGGCTGAGA 3’ and 5’ ATGTTTGCTGTCTCGGCTCA 3’), Serpine1 SE #1 (chr12:22636488-22636538, 5’ TCCCCCGCTAACTCGAACGC 3’ and 5’ TTGTTTGGAGAGCCACCAGGC 3’), Serpine1 SE #2 (chr12:22634466-22634539, 5’ TTGAGTGGCAGACAGCCGACA 3’ and 5’ GGCGGCCTCCAACATTCCTC 3’), Serpine1

TSS (chr12:22640931-22641011, 5’ AGCCCCACCCACCTTCTAACTC 3’ and 5’ TACTGGGAGGGAGGGAAGGAGA 3’), Ctgf SE #1 (chr1:21871291-21871393, 5’ AGCCCTGGAATGCTGTTT 3’ and 5’ ACCGCATGATATCTCCTAAACC 3’), Ctgf SE #2 (chr1:21984665-21984753, 5’ AGTGAGTCAGGGAGGAAGAA 3’ and 5’ CTCCTGCAGCCTGTGATTAG 3’), Ctgf TSS (chr1:21854660-21854725, 5’ CAGACCCACTCCAGCTCCGA 3’ and 5’ GTGGCTCCTGGGGTTGTCCA 3’), Fos TSS (chr6:109300463-109300526, 5’ GACTGGATAGAGCCGGCGGA 3’ and 5’ CAGAGCAGAGCTGGGTGGGA 3’), *S. pombe cdc2*$^{+}$ (II:1500254-1500328, 5’ ATCATTCTCGCATCTCTATTA 3’ and 5’ ATTCTCCATTGCAAACCACTA 3’).

3.4.7. Protein extraction and western blot

Treated cardiomyocytes were lysed in RIPA buffer (1% NP-40, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 0.5% sodium deoxycholate) and protein quantified by Bradford assay. Proteins were denatured at 65°C for 15 min in Laemmilli buffer and protein expression was assessed by western blot. Western blots were probed with anti-Brd4 (Bethyl, A301-985A; 1:1000) or anti-Hsp90 (Enzo Life Sciences, AC88; 1:1000) in 5% milk overnight at 4°C. The following day, blots were visualized with peroxidase-conjugated secondary antibodies and an Amersham^{TM} Imager 600.

3.4.8. Statistical Analysis

All statistical analysis was performed using GraphPad Prism 8 software. Two-way analysis of variance was performed followed by post-hoc t-tests with Bonferroni correction (Figure 3.1B, Figure 3.2B/C/E, Figure 3.3B/C, Figure 3.5B/C, Figure 3.9A/C). Unpaired t-test was completed for validation of gene knockdown by siRNA (Figure 3.2D, Figure 3.5A) and for Brd4 ChIP with forskolin and IBMX (Figure 3.9B). One-way analysis of variance followed by Dunnett’s post-hoc comparison was performed for Brd4 ChIP (Figure 3.6A) and Brd4 protein expression (Figure 3.6C) following 24 h receptor activation. For DAVID GO term enrichment (Figure 3.7G), the false discover rate (FDR) was calculated by a Fisher’s Exact Test completed within DAVID. For the upstream regulator prediction (Figure 3.7H), the p-value was obtained by a Fisher's Exact Test completed within the Ingenuity Pathway Analysis software.

3.5. Results

3.5.1. Evidence for receptor-specific P-TEFb regulation in cardiomyocyte hypertrophy

We first revisited the requirement of P-TEFb activity for the hypertrophic response in primary neonatal rat cardiomyocytes (NRCMs). Previous experiments used the ATP analog 5,6-dichlorobenzimidazone-1-β-D-ribofuranoside (DRB), a cyclin-dependent kinase inhibitor that affects Cdk9, to implicate P-TEFb activity in the hypertrophic response (11). To confirm these results, we repeated this experiment using iCdk9, a Cdk9 inhibitor that is ~1000 times more potent and ~100 times more selective than DRB (30). Following 24 h treatment, agonists for the ETR (endothelin-1; ET-1) or α_1-AR (phenylephrine; PE) increased cardiomyocyte surface area by 35-40% relative to control, as assessed by analysis of α_2-actinin immunostaining using high-content microscopy (Figure 3.1A and Figure 3.1B). Simultaneous treatment with 0.2 µM iCdk9 completely abolished the increase in cardiomyocyte size elicited by either agonist, confirming a stringent requirement for P-TEFb activity in cardiomyocyte hypertrophy (Figure 3.1A and Figure 3.1B).

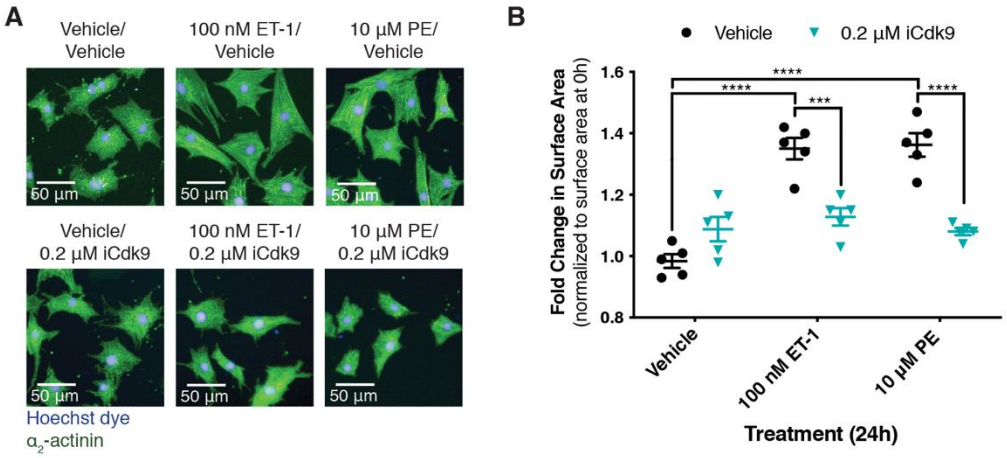


Figure 3.1. Inhibition of the P-TEFb kinase subunit Cdk9 prevents cardiomyocyte hypertrophy in response to α_1-AR or ETR activation.

(A) NRCMs were treated with PE or ET-1 for 24 h as indicated. Cardiomyocytes were stained with Hoechst dye and identified by staining for the cardiomyocyte-specific marker α2-actinin. (B) Fold change in cardiomyocyte surface area following 24 h treatment over the surface area of cardiomyocytes from the same biological replicate at 0 h. Data is presented as mean ± S.E.M with

each point representing a biological replicate. Two-way ANOVA followed by post-hoc t-tests with Bonferroni correction was performed (***p<0.001, ****p<0.0001).

To further characterize P-TEFb function in the hypertrophic response, we assessed the roles of the SEC and Brd4, two key P-TEFb-interacting proteins (31). To perturb the P-TEFb/SEC interaction, we used KL-2, which prevents the interaction between the cyclin T component of P-TEFb and the SEC scaffolding subunit Aff4 (32). KL-2 treatment blocked the increase in cell size in response to both ETR and α_1-AR activation, similar to the effect of Cdk9 inhibition (Figure 3.2A and Figure 3.2B). To determine whether disruption of the P-TEFb/SEC interaction also affected the expression of hypertrophic genes, we monitored changes in mRNA levels for established hypertrophy marker genes Nppa, Nppb, and Serpine1 using RT-qPCR. Whereas mRNA levels for these genes were robustly increased in response to activation of either receptor, induction of Nppb and Nppa was blocked by KL-2 co-treatment (Figure 3.2C). Interestingly, Serpine1 induction was unaffected by KL-2 treatment indicating a gene-specific aspect to SEC function. To confirm the observed effect was due to disruption of SEC function, we next reduced Aff4 levels using siRNA and verified knockdown by RT-qPCR (Figure 3.2D). Similar to KL-2 treatment, knockdown of Aff4 blocked the increase in cell size following activation of either receptor (Figure 3.2E).

We next tested the role of Brd4 using the pan-BET small-molecule inhibitor JQ1. JQ1 targets the BET family bromodomains, acting to competitively inhibit their interaction with acetylated lysine residues (33). Interestingly, BET inhibition attenuated the hypertrophic response in a receptor-specific manner: the response to α_1-AR activation was decreased, whereas there was no effect on ETR-mediated increase in cell size (Figure 3.3A and Figure 3.3B). We also observed that JQ1 treatment more strongly reduced expression of hypertrophic marker genes in cells stimulated with the α_1-AR agonist compared to cells stimulated with an ETR agonist (Figure 3.3C). One possible explanation for the receptor-specific effect of JQ1 is that the dose of ET-1 used to drive hypertrophy was sufficiently high to overcome JQ1 inhibition. To address this, we tested the effect of JQ1 on the ET-1-driven hypertrophic response over a wide range of ET-1 doses. The ET-1 response was insensitive to JQ1 at all doses tested (Figure 3.4). Thus, receptor-specific differences in JQ1 sensitivity likely reflect intrinsic differences in the respective GPCR signalling outcomes.

These data suggest that the SEC is generally required for P-TEFb function in the hypertrophic response, whereas the P-TEFb/Brd4 complex mediates receptor-specific functions.

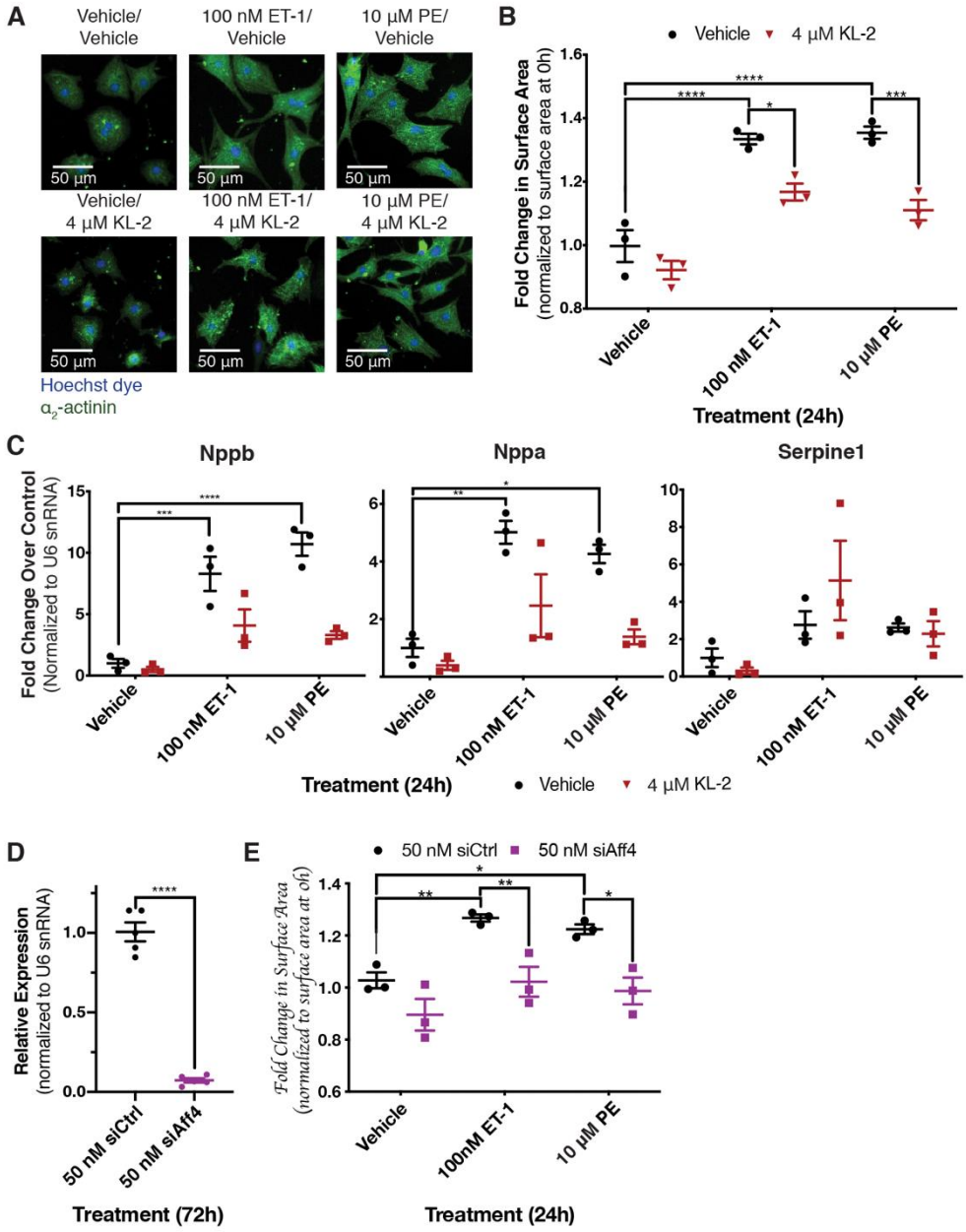


Figure 3.2. Disruption of SEC-P-TEFb interaction blocks the hypertrophic response following activation of either receptor.

(A) NRCMs were treated for 24 h as indicated. Cardiomyocytes were stained with Hoechst dye and identified by staining of the cardiomyocyte-specific marker α_2-actinin. **(B)** Fold change in surface area of identified cardiomyocytes over siRNA control-transfected cardiomyocytes from the same biological replicate at 0 h. Two-way ANOVA followed by post-hoc t-tests with

Bonferroni correction was performed. **(C)** Expression of three genes previously identified as upregulated in hypertrophic cardiomyocytes, Nppb, Nppa and Serpine1, was determined by RT-qPCR. Two-way ANOVA followed by post-hoc t-tests with Bonferroni correction was performed. **(D)** Aff4 knockdown in cardiomyocytes 72 h after transfection with Aff4 targeted siRNA was validated by RT-qPCR. An unpaired t-test was performed. **(E)** Fold change in surface area of identified cardiomyocytes over the surface area of siRNA control-transfected cardiomyocytes from the same biological replicate at 0 h. Cardiomyocytes were transfected 72 h prior to treatment with 50 nM of the specified siRNA. Data is presented as mean ± S.E.M with each point representing a biological replicate. Two-way ANOVA followed by post-hoc t-tests with Bonferroni correction was performed (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

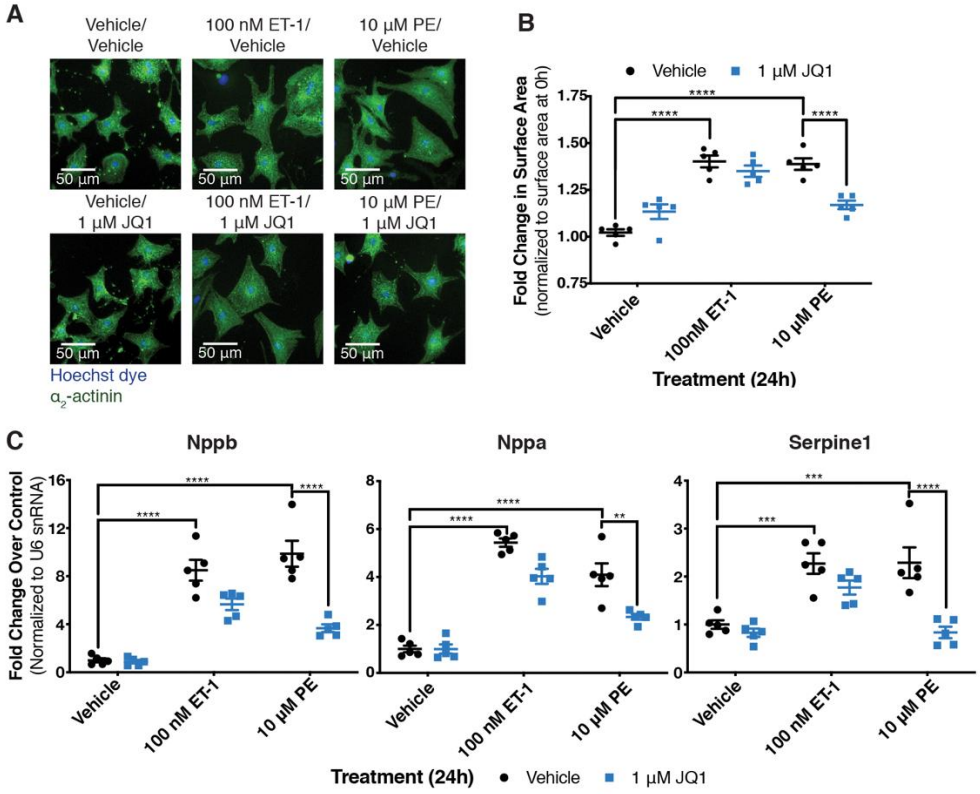


Figure 3.3. Effects of BET inhibitor JQ1 on cardiomyocyte hypertrophy are specific to the receptor driving the response.

(A) NRCMs treated for 24 h were fixed and stained with Hoechst dye and identified by staining for the cardiomyocyte-specific marker α_2-actinin. **(B)** Fold change in surface area of cardiomyocytes over surface area of cardiomyocytes at 0 h from the same biological replicate. Two-way ANOVA followed by post-hoc t-tests with Bonferroni correction was performed. **(C)**

Expression of genes previously demonstrated to be upregulated in hypertrophic cardiomyocytes was determined by RT-qPCR. Data is presented as mean ± S.E.M with each point representing a biological replicate. Two-way ANOVA followed by post-hoc t-tests with Bonferroni correction was performed (**p<0.01, ***p<0.001, ****p<0.0001).

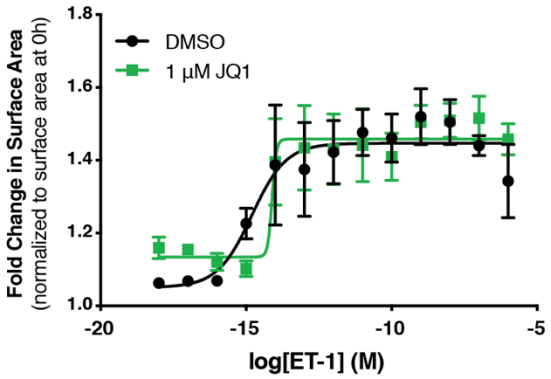


Figure 3.4. ETR-mediated hypertrophy is insensitive to BET inhibition independent of ET-1 concentration.

Dose response curves were generated to assess the effect of JQ1 on cardiomyocyte surface area at a range of ET-1. Cardiomyocytes were treated for 24 h as indicated followed by fixation and staining with Hoechst dye and for α_2-actinin to identify NCRMs. Fold change in surface area over cardiomyocytes from the same biological replicate at 0 h was determined. Data is presented as mean ± S.E.M for n=3-4 independent experiments. Dose response curves were plotted using sigmoidal dose response (variable slope) curves by non-linear regression.

As JQ1 inhibits all members of the BET family of bromodomain proteins, we confirmed the effects on hypertrophy were mediated by Brd4 and not Brd2 and/or Brd3. Brd2, Brd3, and Brd4 were individually depleted in NRCMs using siRNA (Figure 3.5A). Hypertrophic responses were then induced through activation of the α_1-AR or ETR. Brd2 and Brd3 knockdown reduced the basal size of NRCMs relative to control siRNA (Figure 3.5B), but the response following activation of either receptor was comparable to that observed with control siRNA (Figure 3.5C). In contrast, Brd4 knockdown recapitulated the effects observed with JQ1 in that it attenuated the response to α_1-AR activation but did not affect ETR-mediated hypertrophy (Figure 3.5B and Figure 3.5C). These data argue that Brd4 inhibition accounts for the receptor-specific effects of JQ1 on cardiomyocyte hypertrophy.

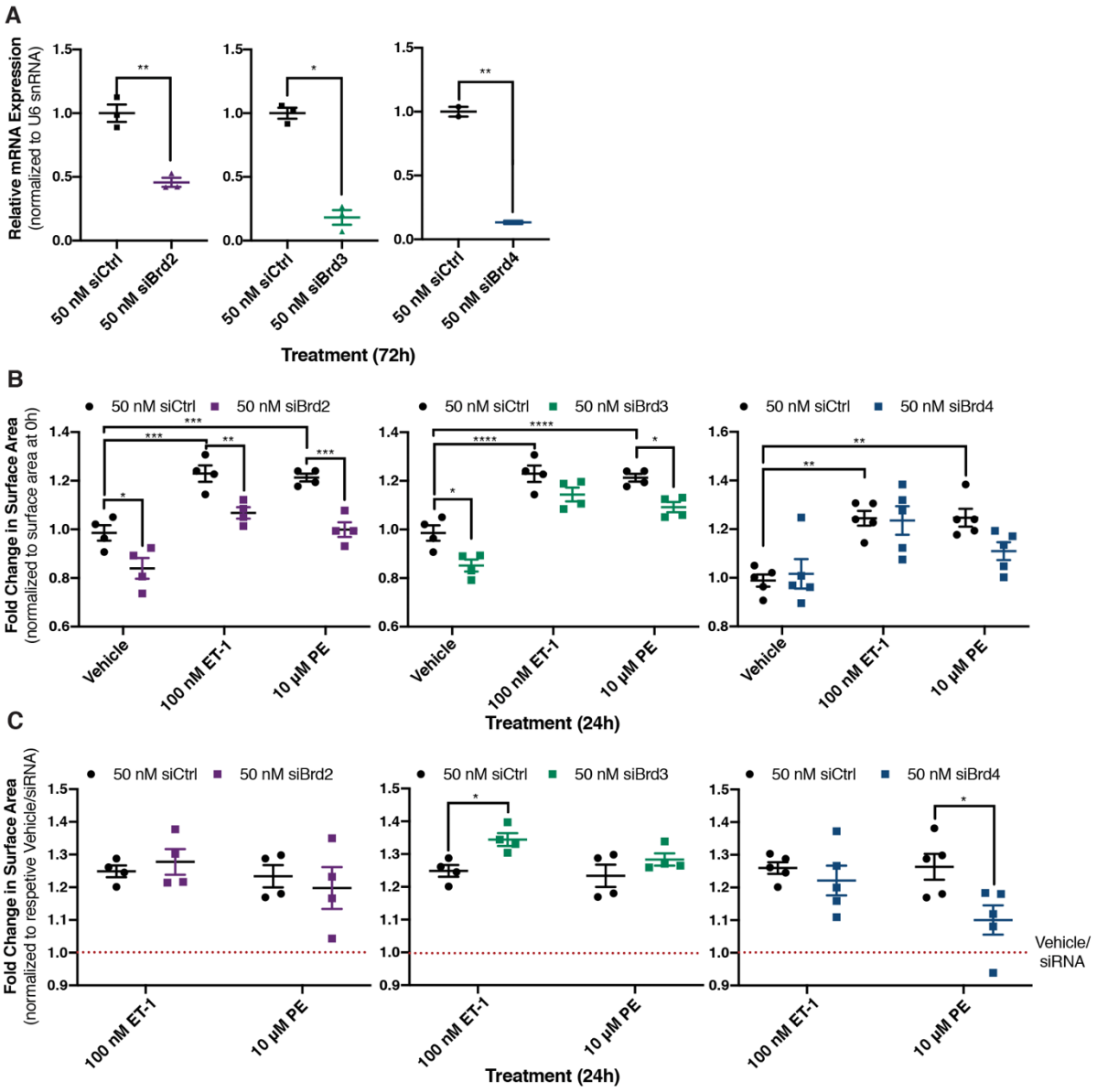


Figure 3.5. Role of individual BET family members expressed in cardiomyocytes assessed after siRNA-mediated knockdown.

(A) Brd2, Brd3, and Brd4 knockdown efficiency in NRCMs 72 h after transfection with targeted siRNA was determined by RT-qPCR. An unpaired t-test was performed. **(B)** Fold change in surface area over cardiomyocytes transfected with control siRNA from the same biological replicate at 0 h. Following 72 h knockdown with indicated siRNA, cardiomyocytes were treated for 24 h as indicted. Cardiomyocytes were fixed and identified by staining for the cardiomyocyte specific marker α_2-actinin. Two-way ANOVA followed by post-hoc t-tests with Bonferroni correction was performed. **(C)** Change in cardiomyocyte size from (B) is presented relative to

respective vehicle/siRNA treatment to normalize for the difference in basal size. Data is presented as mean ± S.E.M with each point representing a biological replicate. Two-way ANOVA followed by post-hoc t-tests with Bonferroni correction was performed (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

To gain further insight into how Brd4 function is differentially affected by distinct GPCR signalling pathways, we assessed gene-specific Brd4 localization to chromatin using chromatin immunoprecipitation coupled to qPCR (ChIP-qPCR). ChIP was performed using an antibody recognizing endogenous Brd4 and was quantified by qPCR using primer pairs near the transcription start sites of Nppb, Nppa, and Serpine1. Occupancy at previously defined super-enhancers for Nppb and Serpine1 in cardiomyocytes was also assessed (19). At all genomic loci tested, 24 h α_1-AR activation increased Brd4 chromatin occupancy compared to vehicle treatment (Figure 3.6A). We observed no change in Brd4 occupancy compared to vehicle following ETR treatment, despite the fact that mRNA levels for the same genes were similarly induced by PE and ET-1 (Figure 3.2C and Figure 3.3C). The effects on Brd4 occupancy were not simply a reflection of altered Brd4 protein levels, as immunoblots performed on cell extracts from NRCMs treated with PE or ET-1 revealed slight decreases in expression compared to vehicle controls (Figure 3.6B and Figure 3.6C). This suggests that signalling through α_1-AR, but not ETR, triggers recruitment of Brd4, making gene expression changes downstream of this receptor more sensitive to Brd4 inhibition by JQ1.

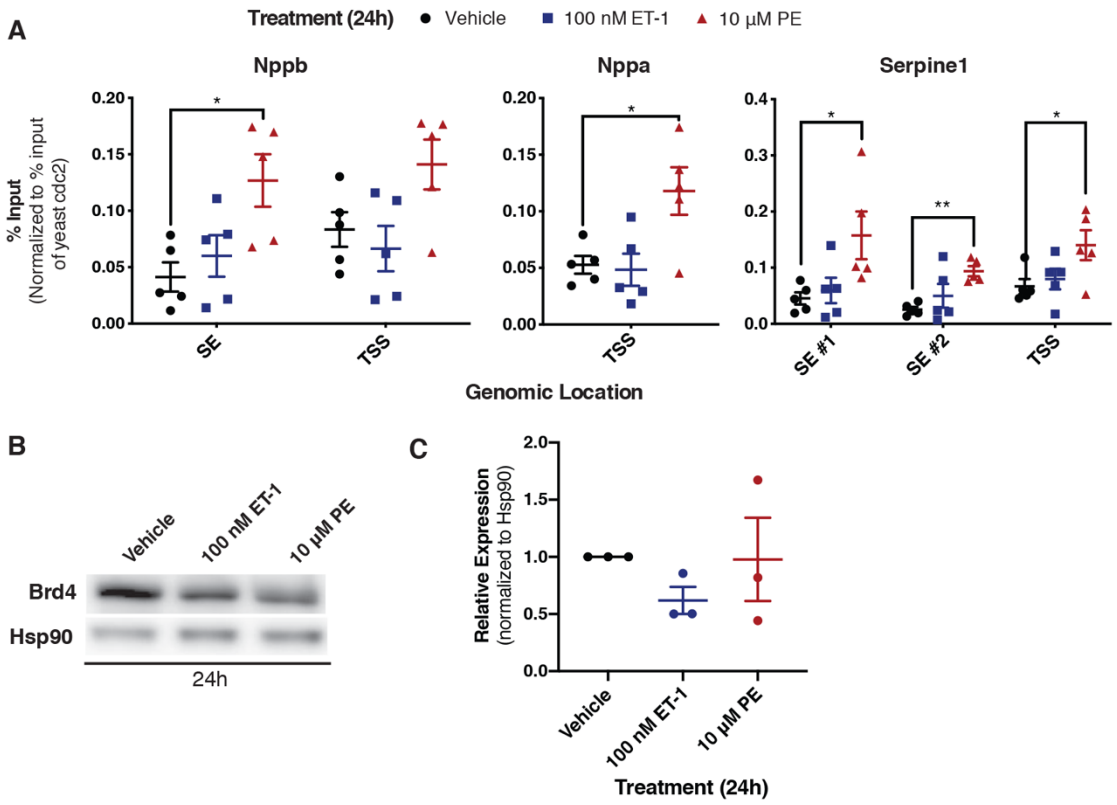


Figure 3.6. Brd4 chromatin occupancy increases in response to α_1-AR but not ETR activation.

(A) Cardiomyocytes were treated for 24 h as indicated. Following treatment, crosslinked chromatin was immunoprecipitated with an anti-Brd4 antibody, followed by DNA purification and quantification by qPCR using primers at the indicated loci. Each immunoprecipitation was normalized to the % input for exogenous *S. pombe* spike-in DNA at the *cdc2*$^+$ loci. Data was analyzed by one-way ANOVA followed by Dunnett's post-hoc comparison. **(B)** A western blot of whole cell lysates to assess changes in Brd4 protein expression following the indicated treatment for 24 h. **(C)** Densitometry based quantification of Brd4 normalized to Hsp90 expression. Data is presented as mean ± S.E.M with each point representing a biological replicate. Data was analyzed by one-way ANOVA followed by Dunnett's post-hoc comparison (*p<0.05, **p<0.01).

3.5.2. RNA-seq reveals differences in GPCR-dependent signalling between hypertrophic agonists

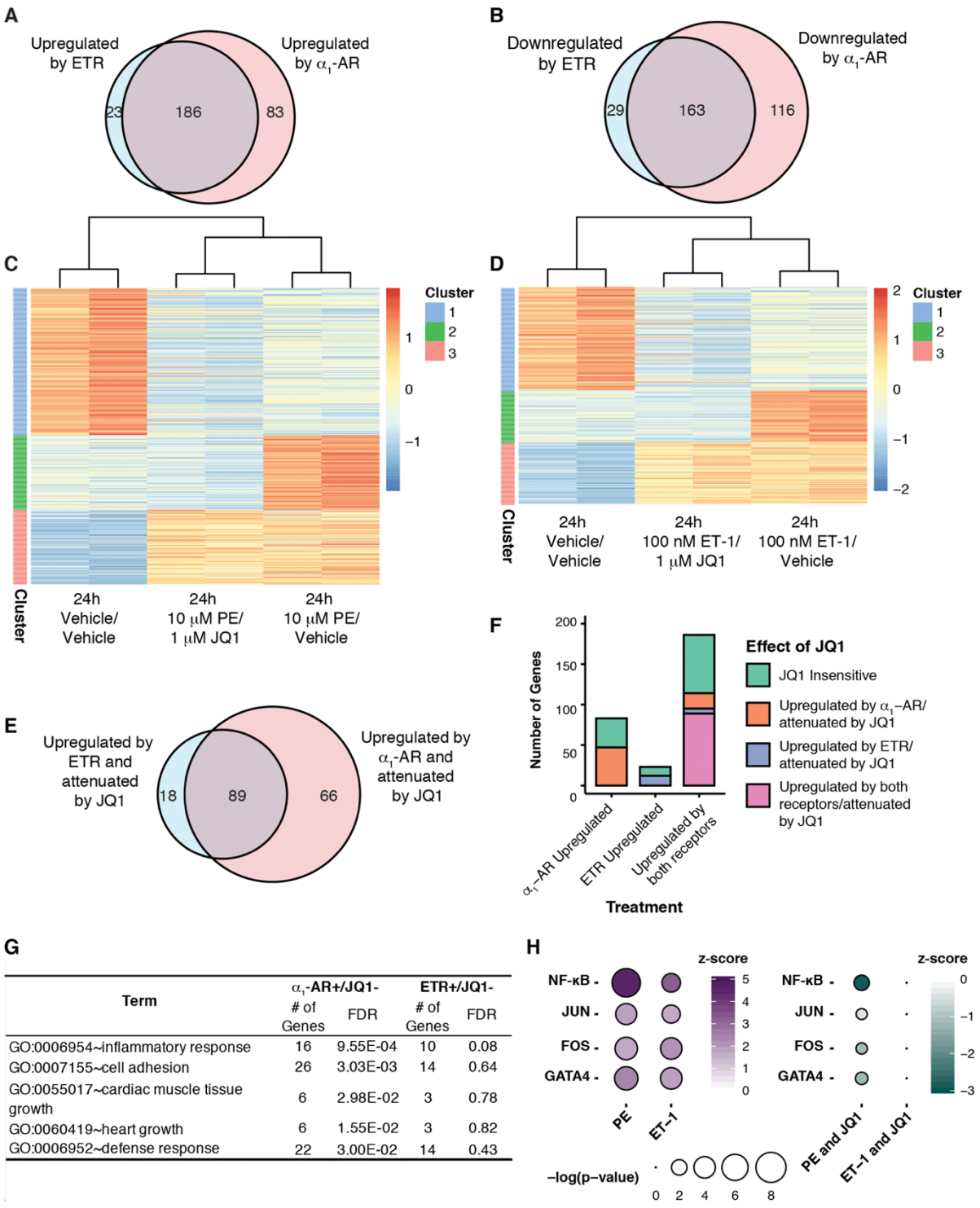
To comprehensively profile receptor-specific effects on cardiomyocyte hypertrophy, we performed RNA-seq on NRCMs following activation of either receptor for 24 h in the presence or

absence of JQ1 (**Supplemental Table 1**). When comparing agonist versus vehicle conditions, we observed robust gene expression changes [$\log_2$(Fold Change) > ±1 and p-value < 0.05] for hundreds of genes following ETR (209 upregulated, 192 downregulated) or α_1-AR activation (269 upregulated, 279 downregulated). The genes regulated by either receptor overlapped significantly, although there were more genes uniquely regulated by α_1-AR activation than by ET-1 activation (Figure 3.7A and Figure 3.7B). The combined effect of receptor agonists and JQ1 on differential expression was visualized by performing K-means clustering (Figure 3.7C and Figure 3.7D). This analysis identified three major gene clusters that were similarly regulated by agonist and JQ1: one in which genes were repressed by agonist in the presence or absence of JQ1 (cluster 1), one in which genes were activated by agonist and attenuated by JQ1 (cluster 2), and one in which genes were activated by agonist in the presence or absence of JQ1 (cluster 3). The observation that the primary effect of JQ1 was to dampen expression of genes regulated by receptor activation aligns with the known roles of Brd4 in recruiting P-TEFb to regulate pause-release and activate transcription (15), and is consistent with the effect of JQ1 on cardiac stress-induced genes previously characterized (20).

We focused on groups of genes for which increased expression caused by activation of either receptor was attenuated by JQ1 [$\log_2$(Fold Change) < -0.5 compared to agonist alone; p-value < 0.05]. JQ1 attenuated expression of 107 ETR-induced genes and 155 α_1-AR-induced genes (termed receptor+/JQ1-) (Figure 3.7E). Roughly equal proportions of genes induced by activation of either receptor were JQ1-sensitive, irrespective of whether they were induced by one receptor or both (Figure 3.7F). Gene ontology term analysis of α_1-AR+/JQ1- and ETR+/JQ1- gene sets revealed that the terms inflammatory response, defense response, cell adhesion, cardiac muscle tissue growth and heart growth, which correspond to the pathophysiology of cardiomyocyte hypertrophy and align with those previously identified to be affected by JQ1, were significantly enriched among the α_1-AR+/JQ1- genes (Figure 3.7G) (20). In contrast, none of these terms were significantly enriched among ETR+/JQ1- genes, consistent with the selective effect of JQ1 on the α_1-AR response.

Previous studies have identified multiple transcription factors that are required to activate pro-hypertrophic genes in cardiomyocytes (9, 34). Some of these transcription factors have been associated with Brd4 activity in cardiomyocytes, either through motif enrichment in genomic loci with high Brd4 occupancy or various gene set enrichment methods for JQ1-sensitive genes. The

pathway-specific effect of Brd4 inhibition suggests that specific transcription factors may be dependent on Brd4 to activate transcription. We initially focused on those transcription factors that were previously implicated including, NF-κB, GATA4, and the AP-1 subunits Jun and Fos (18-20). To determine the effect of JQ1 on these transcription factors, we used Ingenuity Pathway Analysis software (IPA; Qiagen) to predict changes in their activity (Figure 3.7H). IPA upstream regulator analysis provides a z-score, to indicate the predicted change in activity between the two treatment groups, and a Fisher's exact test p-value, to indicate whether particular upstream regulator's target genes are significantly enriched in the gene expression program. Interestingly, IPA predicted increased activity of these transcription factors following activation of either receptor (positive z-score, agonist/vehicle vs vehicle/vehicle), but activity was specifically attenuated by JQ1 following α_1-AR activation (negative z-score, agonist/JQ1 vs agonist/vehicle) (Figure 3.7H). This suggests that receptor-specific activation mechanisms for these transcription factors dictate their dependence on Brd4 activity. When we expanded the analysis to include all activated transcription factors a more ubiquitous effect of Brd4 inhibition was observed. We identified 78 transcription factors with enhanced activity following α_1-AR activation of which activity of 39 (50%) were attenuated by co-treatment with JQ1. In contrast, ETR activation was predicted to enhance activity of 50 transcription factors and only eight (16%) were attenuated by JQ1. Thus, although specific transcription factors may function to recruit Brd4 to specific loci, the more ubiquitous effect of Brd4 inhibition on α_1-AR-mediated transcription factor activity suggests that α_1-AR signalling may increase the pool of active Brd4.



Term	α1-AR+/JQ1- # of Genes	α1-AR+/JQ1- FDR	ETR+/JQ1- # of Genes	ETR+/JQ1- FDR
GO:0006954~inflammatory response	16	9.55E-04	10	0.08
GO:0007155~cell adhesion	26	3.03E-03	14	0.64
GO:0055017~cardiac muscle tissue growth	6	2.98E-02	3	0.78
GO:0060419~heart growth	6	1.55E-02	3	0.82
GO:0006952~defense response	22	3.00E-02	14	0.43

Figure 3.7. Transcriptome analysis of gene expression programs mediated by receptor activation and the effect of Brd4 inhibition.

(A) Venn diagram of significantly upregulated genes (log2FC > 1 for Agonist/Vehicle vs Vehicle/Vehicle, p-value < 0.05) or **(B)** downregulated genes (log2FC < -1 for Agonist/Vehicle vs

Vehicle/Vehicle, p-value < 0.05) following 24 h receptor activation. **(C and D)** Heat maps were generated for genes differentially regulated following activation of the specified receptor for 24 h. Each row was normalized, with the color representing the z-score for the specific row. K-means clustering was performed to identify subsets of genes with distinct patterns following Brd4 inhibition. **(E)** Venn diagram of genes upregulated by respective receptor activation (log2FC > 1 for Agonist/Vehicle vs Vehicle/Vehicle, p-value < 0.05) and attenuated by Brd4 inhibition from the activated state (log2FC < -0.5 for Agonist/JQ1 vs Agonist/Vehicle, p-value < 0.05). (**F**) Number of genes uniquely upregulated by either agonist or both and whether expression was attenuated by JQ1. Genes were first categorized as uniquely upregulated by α_1-AR or ETR or upregulated by both receptors (log2FC > 1 for Agonist/Vehicle vs Vehicle/Vehicle, p-value < 0.05). Within each category, genes were further characterized as JQ1 sensitive if they were attenuated by Brd4 inhibition from the activated state (log2FC < -0.5 for Agonist/JQ1 vs Agonist/Vehicle, p-value < 0.05). In the category of genes upregulated by both receptors, genes were categorized if they were attenuated by Brd4 inhibition when upregulated by either receptor (Upregulated by both receptors/attenuated by JQ1) or if the effect of Brd4 inhibition was unique to a specific receptor. **(G)** Gene ontology enrichment for genes attenuated by JQ1 following receptor activation (from E) performed with DAVID. The false discovery rate (FDR) indicates whether the pathway was significantly enriched in the gene list. **(H)** Changes in transcription factor activity predicted by Ingenuity Pathway Analysis (IPA). The z-score represents the predicted change in transcription factor activity between the two treatment groups and the p-value indicates whether the transcription factor's targets are significantly enriched in the gene set. The purple dots (left side) indicate the change in activity following agonist treatment alone (log2FC > 1 for Agonist/Vehicle vs Vehicle/Vehicle, p-value < 0.05). The green dots (right side) indicate the JQ1 dependent decrease in activity from the activated state (log2FC < -0.5 for Agonist/JQ1 vs Agonist/Vehicle, p-value < 0.05).

3.5.3. Signalling pathway regulating Brd4 recruitment to chromatin involves PKA

We hypothesized that a distinct signalling pathway activated by the α_1-AR determines differential recruitment of Brd4 and inhibitory effect of JQ1 on transcription factor activity. Brd4 is activated following phosphorylation of its phosphorylation-dependent interaction domain (PDID). An *in vitro* kinase assay demonstrated that PKA was able to phosphorylate this region,

although the functional significance was not determined (35). We have previously demonstrated that α_1-AR, but not ETR activation, led to Gαs-dependent activation of cAMP/PKA signalling in HEK 293 cells (36). We thus hypothesized that PKA, a protein kinase activated by cAMP, regulates the specific effects of α_1-AR signalling on Brd4 in cardiomyocytes.

We confirmed that α_1-AR and not ETR signalling activated PKA in NRCMs. Cardiomyocytes were transduced with a nuclear-localized Förster resonance energy transfer (FRET)-based PKA biosensor (AKAR4-NLS) to monitor PKA activity (37). When phosphorylated, the biosensor undergoes a conformational change that moves the two fluorophores into closer proximity, leading to an increased FRET ratio. Nuclear localization of the AKAR biosensor was confirmed by fluorescence microscopy (Figure 3.8A). We then generated a dose-response relationship for PKA activity following stimulation with ET-1 and PE. Alprenolol was included in the PE experiments to prevent off-target effects on the β-AR at high concentrations. We observed a dose-dependent increase in FRET, indicating an increase in PKA activity, following 15 min α_1-AR activation. In contrast, no change in activity was observed following 15 min ETR activation (Figure 3.8B). This demonstrated that the α_1-AR uniquely activates PKA in the nucleus of cardiomyocytes, similar to what we detected in HEK 293 cells (36).

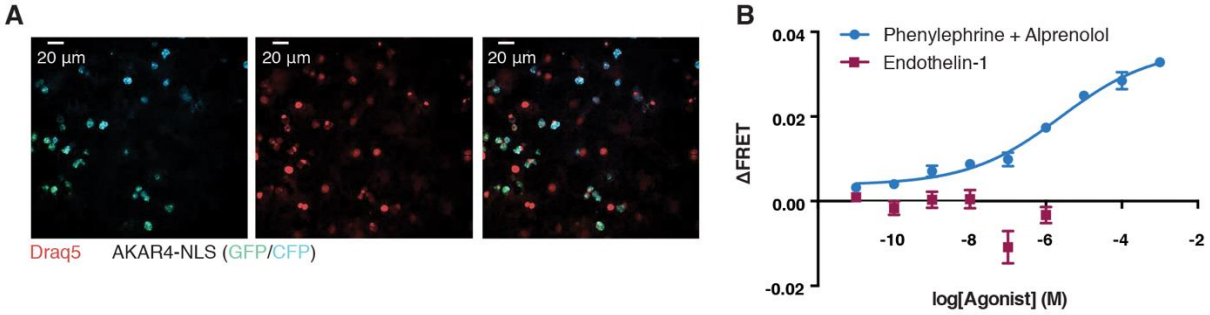


Figure 3.8. α_1-AR activation leads to increased nuclear PKA signalling.

(A) Cardiomyocytes were transduced with AAV9-AKAR4-NLS virus at a MOI of 5000 and imaged 72 h later. Nuclei were visualized by staining live cells with Draq5. **(B)** Dose-response curves for PKA activation following activation of the ETR or α_1-AR were generated. Alprenolol was included to prevent off-target β-AR activation by high concentrations of PE. Data is presented mean ± S.E.M for three biological replicates. Dose response curves were plotted using sigmoidal dose response (variable slope) curves by non-linear regression.

In order to determine if PKA activity downstream of the α_1-AR regulates Brd4 function in cardiomyocytes, we performed ChIP-qPCR after inhibition or activation of PKA. To inhibit PKA, we used the competitive inhibitor KT5720 (38). As we anticipated that long-term PKA inhibition could cause other changes in cellular physiology that would complicate interpretation of the experiments, we used a treatment time of 1.5 h. We quantified Brd4 localization using primer pairs near the transcription start site of c-Fos and Ctgf as well as previously defined super-enhancer regions of Ctgf (19). Activation of the α_1-AR for 1.5 h enhanced Brd4 occupancy near the transcription start sites of both genes and along the previously defined Ctgf super-enhancers, whereas ETR stimulation had no effect (Figure 3.9A and Figure 3.9B). These data suggest that the specific effect of α_1-AR signalling on Brd4 chromatin occupancy is maintained at the shorter treatment time. Pre-treatment with KT5720 abrogated the increase in Brd4 occupancy following α_1-AR activation, consistent with a requirement for PKA activity for chromatin recruitment of Brd4 downstream of α_1-AR signalling (Figure 3.9A and Figure 3.9B). PKA inhibition also increased the basal occupancy of Brd4 at these sites, perhaps reflecting a repressive function for PKA in unstimulated NRCMs. We also stimulated activation of PKA in NRCMs by increasing intracellular cAMP levels with forskolin and IBMX, an adenylyl cyclase activator and phosphodiesterase inhibitor, respectively (39). Sustained PKA activation (1.5 h) increased Brd4 chromatin association at two of the four genomic loci assessed, reinforcing the key role of PKA in Brd4 activation (Figure 3.9C).

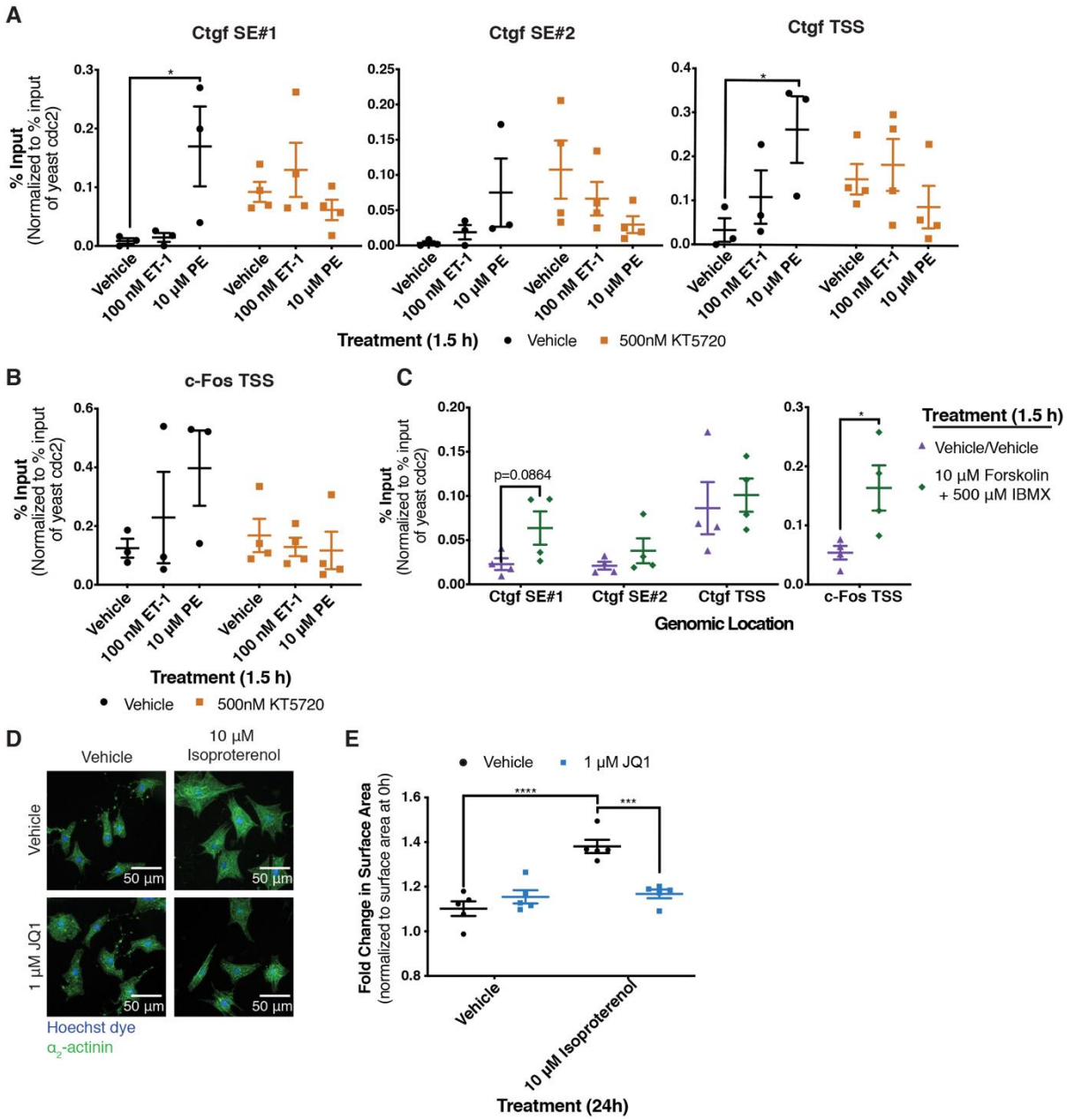


Figure 3.9. PKA signalling regulates recruitment of Brd4 to chromatin.

(A and B) Effect of PKA inhibition with the small-molecule inhibitor KT5720 on receptor-mediated increases in Brd4 occupancy. Cardiomyocytes were pre-treated for 30 min with the PKA inhibitor prior to receptor activation for 1.5 h with the indicated agonists. Two-way ANOVA followed by post-hoc t-test comparisons with Bonferroni correction was performed. **(C)** PKA was activated for 1.5 h by increasing intracellular cAMP levels with forskolin and IBMX, an adenylyl cyclase activator and phosphodiesterase inhibitor respectively. Following indicated treatment, cardiomyocytes were fixed, and ChIP was performed with an anti-Brd4 antibody. ChIP was

quantified by qPCR using primers at the indicated loci. An unpaired t-test was performed. **(D)** After 24 h of the indicated treatments, NRCMs were fixed and stained with Hoechst dye and for the cardiomyocyte specific marker α_2-actinin. **(E)** Fold change in surface area after 24 h of the indicated treatment over cardiomyocytes fixed at 0 h from the same biological replicate. Two-way ANOVA followed by post-hoc t-test comparisons with Bonferroni correction was performed. Data is presented as mean ± S.E.M with each point representing a separate biological replicate. (*p<0.05, ***p<0.001, ****p<0.0001).

To test whether a regulatory link between PKA and Brd4 could be detected in response to other GPCRs coupled to Gαs, we examined the role of Brd4 downstream of the β-AR, activation of which is strongly pro-hypertrophic in cardiomyocytes (40, 41). The primary signalling pathway downstream of this receptor in cardiomyocytes (and other cell types as well) involves adenylyl cyclase activation, cAMP production, and increased protein kinase A activity (41). We thus predicted that hypertrophy mediated by the β-AR would also be attenuated by inhibition of Brd4 with JQ1. Following 24 h treatment with the agonist isoproterenol, we observed a ~25% increase in surface area which was completely blocked by co-treatment with JQ1 (Figure 3.9D and Figure 3.9E). This demonstrates that the observed connection between PKA and Brd4 is not unique to α_1-AR signalling and may reflect a general Gαs-coupled GPCR-dependent pathway for Brd4 activation. Taken together, our results point to PKA and Brd4 as central players underlying receptor-specific gene regulatory mechanisms in hypertrophic cardiomyocytes (Figure 3.10).

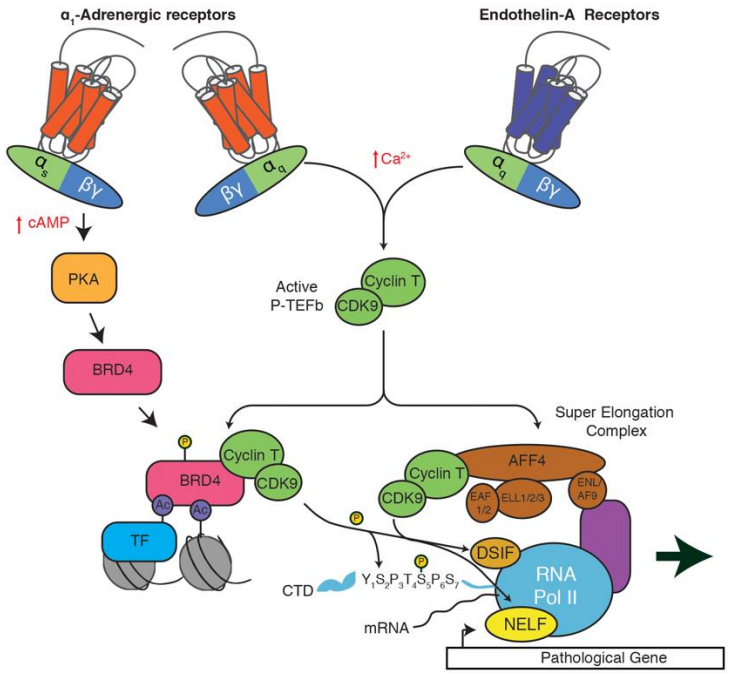


Figure 3.10. Model of P-TEFb complex activation following activation of the α_1-AR or ETR. Both the ETR and α_1-AR activate a signalling cascade which increases active P-TEFb and requires subsequent recruitment through the SEC. The α_1-AR activation also leads to Brd4-dependent recruitment due to the activation of a PKA signalling pathway.

3.6. Discussion

In this study, we showed that activation of distinct GPCRs in cardiomyocytes can result in hypertrophic responses and gene expression programs that are qualitatively similar, but that operate through different transcriptional regulatory mechanisms. We also identified a novel mechanism regulating Brd4 in the development of cardiomyocyte hypertrophy that expands our understanding of how specific signalling pathways regulate the recruitment of general transcription regulators and that may have relevance in other physiological contexts.

Chromatin occupancy of Brd4 undergoes extensive redistribution in order to positively regulate expression of the cardiomyocyte hypertrophic gene program. These changes lead to enhanced Brd4 occupancy on specific super-enhancers and promoter regions. Previous Brd4 ChIP-seq experiments have used primary cardiomyocytes treated with PE (in accord with our results), or cardiac tissue isolated from mice subjected to transverse aortic constriction (TAC) (19, 20). The increased genomic loading of Brd4 in heart failure models has been attributed to increases in Brd4

expression (19), similar to proposed mechanisms in various forms of cancer (42-44). In heart failure, increased Brd4 protein expression is thought to occur due to decreased expression of the Brd4 targeting microRNA miR-9 (21, 45). These reports assessed whole cardiac tissue not enriched for cardiomyocytes or *in vitro* cardiomyocyte studies which reported conflicting evidence regarding changes in Brd4 expression (18, 21). Following 24 h activation of the α_1-AR or ETR in cardiomyocytes, we did not observe a significant change in Brd4 protein expression (Figure 3.6B). Instead, the increased Brd4 recruitment following α_1-AR activation was dependent on cAMP/PKA signalling pathway.

Phosphorylation of Brd4 is critical for its activation and also mediates alterations in its interactome. Brd4 hyperphosphorylation correlates with its oncogenic potential and increased phosphorylation levels lead to development of BET inhibitor resistance in certain types of cancer (46, 47). At present, casein kinase 2 (CK2) and CK1δ are the only protein kinase demonstrated to directly phosphorylate Brd4 *in vivo*, although others have been shown to regulate Brd4 activity (35, 46, 48, 49). The CK2 and CK1δ phosphorylation sites reside within the PDID domain, where phosphorylation results in a conformational change that unmasks the second bromodomain and enables interactions with acetyl-lysine residues (35). An *in vitro* kinase screen of the PDID domain identified PKA as a potential Brd4 kinase, aligning with the PKA regulatory effect on Brd4 we observed (35). This suggests that in cardiomyocytes, enhanced PKA activity could increase PDID phosphorylation and drive the conformational change required for Brd4 to interact with chromatin. Further work remains to identify the putative phosphorylated sites and elucidate their functional role(s). Conversely, we also observed an increase in basal Brd4 occupancy following PKA inhibition (Figure 3.9A). Such increased Brd4 occupancy may be related to PKA's known role in regulating histone deacetylases (52). The balance between these two opposing processes regulated by PKA is likely linked to the highly localized nature of PKA signalling through interactions with A-kinase anchoring proteins (AKAPs) (53).

RNA-seq analyses revealed that the transcriptional programs triggered by activation of α_1-AR or ETR were highly overlapping, consistent with the similar hypertrophic response downstream of either receptor. The mechanistic difference between the two pathways was instead linked to the fact that specific groups of genes relevant to the hypertrophic response were differentially sensitive to JQ1 downstream of α_1-AR activation compared to ETR. Previous reports have identified inflammatory pathways enriched in JQ1-attenuated genes using both *in vitro* and

in vivo models of cardiomyocyte hypertrophy (20). We observed JQ1-selective attenuation of these inflammatory pathways following α_1-AR activation (Figure 3.7G). We argue that this difference stems from a greater role for Brd4 downstream of α_1-AR, consistent with our Brd4 ChIP results. Such inflammatory responses are characteristic of heart failure, and the production of several cytokines is increased during cardiac remodelling (54, 55). Importantly, inflammation is a driver of cardiomyocyte hypertrophy and inhibition of those pathways prevents the progression of heart failure (56, 57). Although specific pathways were not enriched among genes attenuated by JQ1 follow ETR activation, a large number of genes did exhibit JQ1 sensitivity. We suspect the observed effects are likely due to functions of Brd2 and/or Brd3 in regulating these genes, although further work is required to confirm this.

Further evidence for the effect of JQ1 on the inflammatory response is evident by the negative effect on inflammatory transcription factors such as NF-κB and AP-1 (Figure 3.7H) (34, 58, 59). We focused on these transcription factors for two reasons: their gene expression signatures were previously identified in JQ1-sensitive, TAC-induced genes, or a causal role in regulating Brd4 recruitment in cardiomyocytes has been identified (18, 19). Importantly, these transcription factors are also directly implicated in driving pathological cardiomyocyte hypertrophy in various *in vitro* and *in vivo* models (60-64). Despite the fact that similar transcription factors were activated downstream of α_1-AR and ETR, JQ1 only attenuated the α_1-AR response. The receptor-specific attenuation of transcription factor activity may be due to distinct signalling mechanisms required for transcription factor activation, creating a differential dependence on Brd4 activity (65-68). However, the greater number of transcription factors attenuated by JQ1 following α_1-AR activation suggests that it leads to an active form of Brd4 that more readily binds to chromatin and promotes the activity of transcription factors. Although we predict that PKA activates Brd4 directly, indirect activation through a downstream factor is also possible. Notably, a direct role for PKA activity in regulating P-TEFb has also been observed. For example, PKA-dependent phosphorylation of Cdk9 promotes its association with the viral transactivator Tat and phosphorylation of hexamethylene bisacetamide inducible protein 1 (HEXIM1) by PKA releases P-TEFb from the inhibitory 7SK sRNP complex (50, 51). Thus, P-TEFb itself could be an important PKA target, although how these phosphorylation events impact its interaction with Brd4 is not known. Further investigation is required to determine the effects of JQ1 on Brd4's interactome and phosphorylation status following α_1-AR or ETR activation.

Receptor-specific activation of Brd4 we identified may have implications for the clinical use of Brd4 inhibitors in cardiovascular disease. Efficacy will be dependent on the specific neurohormonal signalling pathways altered in a particular patient. Specifically, we would expect a negative correlation with a patient's ET-1 levels. Therefore, more extensive characterization of signalling molecules in patients might be important predictors of drug efficacy. Importantly, we expect that Brd4 inhibition will be less effective in severe and/or late stages of heart failure as PKA activity is reduced (41) and levels of endothelin-1 or its precursors (69-71) increases. Chronic infusion of these neurohormones with mice is required to determine how the receptor-specific effects of Brd4 inhibition effects cardiac remodelling. Furthermore, although JQ1 has been demonstrated to reverse established heart failure in mouse TAC and myocardial infarction mouse models (20), we expect JQ1 efficacy would decrease as heart failure progresses.

Our finding that JQ1 sensitivity is dependent on activation of PKA signalling raises the question of whether Brd4 inhibition is also an effective therapeutic for other pathologies associated with enhanced PKA signalling. For example, chronic activation of PKA is a hallmark of dopamine-dependent neuronal pathologies such as cocaine addiction and L-DOPA induced dyskinesia (LID) (72, 73). Recent studies have implicated Brd4 in regulating the neuronal transcriptional programs and behavioural effects driven by dopamine signalling in these contexts. Systemic administration of JQ1 reduces reward seeking behaviour in addiction models and prevents LID development in Parkinson's models (74, 75). The correlation between PKA and Brd4 activity in these cases suggests that the regulation of Brd4 chromatin occupancy by PKA might be a common regulatory mechanism for other GPCRs and cell types. Furthermore, certain adrenocortical adenomas are driven by enhanced basal PKA activity due to activating mutations in Gαs or the catalytic subunit of PKA (76, 77). We expect these adenomas would be highly sensitive to Brd4 inhibition, although further work is required to establish the requirement of Brd4 in progression of these cancers.

Dysregulated activation and recruitment of P-TEFb is an underlying cause of several diseases and developmental disorders (78). While recruitment of P-TEFb is regulated either by Brd4 or the SEC, little is known about the functional relationship between these two complexes. It has been suggested that these complexes work together to target P-TEFb to different substrates whereas others have shown that Brd4 assists in recruiting the SEC (79, 80). Our results indicate the cooperative nature of these two complexes is dependent on the signalling pathway employed to activate transcriptional responses. Following ETR activation, the SEC alone is sufficient to elicit

a gene expression program required for cardiomyocyte hypertrophy, whereas the activation of the Gαs/cAMP/PKA pathway by the α_1-AR leads to an additional dependence on Brd4. This suggests that the SEC form of P-TEFb may have a general transcriptional regulatory role whereas the form associated with Brd4 may have a more restricted signal-responsive role. The lack of an effect on Serpine1 expression by the small molecule KL-2, which is attenuated by JQ1, suggests these complexes do regulate different gene expression programs (as proposed) leading to similar phenotypic changes to cardiomyocytes. Further genome-wide investigation is required to assess whether these complexes have distinct or overlapping functions in cardiomyocytes. Furthermore, expanding our understanding of how signalling pathways activate these complexes is an important step to improve therapeutic approaches in diseases with dysregulated P-TEFb.

3.7. Acknowledgements

This work was supported by a grant from the Heart and Stroke Foundation of Canada (G-15-0008938) to T.E.H and J.C.T, a grant from Canadian Institute of Health Science (CIHR) (MOP 130-362) to J.C.T. and a grant from CIHR (PJT 159687) to T.E.H. R.M. was supported by a studentship from the McGill-CIHR Drug Development Training Program and McGill Faculty of Medicine. We thank Dr. Jin Zhang (UCSD), Dr. Karel Svoboda, and Novartis Institutes for Biomedical Research (Emeryville, CA) for providing materials instrumental to this study. We thank the McGill Imaging and Molecular Biology Platform for assistance with our high-content microscopy experiments. Lastly, we thank all the members of Dr. Hébert and Dr. Tanny's labs for discussions and critical reading of the manuscript.

3.8. References

1. Sutton MG, Sharpe N. 2000. Left ventricular remodeling after myocardial infarction: pathophysiology and therapy. Circulation 101:2981-8.
2. Nadruz W. 2015. Myocardial remodeling in hypertension. J Hum Hypertens 29:1-6.
3. Gerdes AM. 2002. Cardiac myocyte remodeling in hypertrophy and progression to failure. J Card Fail 8:S264-8.
4. Hill JA, Olson EN. 2008. Cardiac plasticity. N Engl J Med 358:1370-80.

5. Heineke J, Molkentin JD. 2006. Regulation of cardiac hypertrophy by intracellular signalling pathways. Nat Rev Mol Cell Biol 7:589-600.
6. van Berlo JH, Maillet M, Molkentin JD. 2013. Signaling effectors underlying pathologic growth and remodeling of the heart. J Clin Invest 123:37-45.
7. D'Angelo DD, Sakata Y, Lorenz JN, Boivin GP, Walsh RA, Liggett SB, Dorn GW, 2nd. 1997. Transgenic Gαq overexpression induces cardiac contractile failure in mice. Proc Natl Acad Sci U S A 94:8121-6.
8. Iwase M, Bishop SP, Uechi M, Vatner DE, Shannon RP, Kudej RK, Wight DC, Wagner TE, Ishikawa Y, Homcy CJ, Vatner SF. 1996. Adverse effects of chronic endogenous sympathetic drive induced by cardiac GS α overexpression. Circ Res 78:517-24.
9. Akazawa H, Komuro I. 2003. Roles of cardiac transcription factors in cardiac hypertrophy. Circ Res 92:1079-88.
10. Ho MK, Su Y, Yeung WW, Wong YH. 2009. Regulation of transcription factors by heterotrimeric G proteins. Curr Mol Pharmacol 2:19-31.
11. Sano M, Abdellatif M, Oh H, Xie M, Bagella L, Giordano A, Michael LH, DeMayo FJ, Schneider MD. 2002. Activation and function of cyclin T-Cdk9 (positive transcription elongation factor-b) in cardiac muscle-cell hypertrophy. Nat Med 8:1310-7.
12. Papait R, Cattaneo P, Kunderfranco P, Greco C, Carullo P, Guffanti A, Vigano V, Stirparo GG, Latronico MV, Hasenfuss G, Chen J, Condorelli G. 2013. Genome-wide analysis of histone marks identifying an epigenetic signature of promoters and enhancers underlying cardiac hypertrophy. Proc Natl Acad Sci U S A 110:20164-9.
13. Zhou Q, Li T, Price DH. 2012. RNA polymerase II elongation control. Annu Rev Biochem 81:119-43.
14. Lin C, Smith ER, Takahashi H, Lai KC, Martin-Brown S, Florens L, Washburn MP, Conaway JW, Conaway RC, Shilatifard A. 2010. AFF4, a component of the ELL/P-TEFb elongation complex and a shared subunit of MLL chimeras, can link transcription elongation to leukemia. Mol Cell 37:429-37.
15. Jang MK, Mochizuki K, Zhou M, Jeong HS, Brady JN, Ozato K. 2005. The bromodomain protein Brd4 is a positive regulatory component of P-TEFb and stimulates RNA polymerase II-dependent transcription. Mol Cell 19:523-34.

16. Shi J, Vakoc CR. 2014. The mechanisms behind the therapeutic activity of BET bromodomain inhibition. Mol Cell 54:728-36.
17. Bisgrove DA, Mahmoudi T, Henklein P, Verdin E. 2007. Conserved P-TEFb-interacting domain of BRD4 inhibits HIV transcription. Proc Natl Acad Sci U S A 104:13690-5.
18. Anand P, Brown JD, Lin CY, Qi J, Zhang R, Artero PC, Alaiti MA, Bullard J, Alazem K, Margulies KB, Cappola TP, Lemieux M, Plutzky J, Bradner JE, Haldar SM. 2013. BET bromodomains mediate transcriptional pause release in heart failure. Cell 154:569-82.
19. Stratton MS, Lin CY, Anand P, Tatman PD, Ferguson BS, Wickers ST, Ambardekar AV, Sucharov CC, Bradner JE, Haldar SM, McKinsey TA. 2016. Signal-Dependent Recruitment of BRD4 to Cardiomyocyte Super-Enhancers Is Suppressed by a MicroRNA. Cell Rep 16:1366-1378.
20. Duan Q, McMahon S, Anand P, Shah H, Thomas S, Salunga HT, Huang Y, Zhang R, Sahadevan A, Lemieux ME, Brown JD, Srivastava D, Bradner JE, McKinsey TA, Haldar SM. 2017. BET bromodomain inhibition suppresses innate inflammatory and profibrotic transcriptional networks in heart failure. Sci Transl Med 9.
21. Spiltoir JI, Stratton MS, Cavasin MA, Demos-Davies K, Reid BG, Qi J, Bradner JE, McKinsey TA. 2013. BET acetyl-lysine binding proteins control pathological cardiac hypertrophy. J Mol Cell Cardiol 63:175-9.
22. Luo Z, Lin C, Shilatifard A. 2012. The super elongation complex (SEC) family in transcriptional control. Nat Rev Mol Cell Biol 13:543-7.
23. Lin C, Garrett AS, De Kumar B, Smith ER, Gogol M, Seidel C, Krumlauf R, Shilatifard A. 2011. Dynamic transcriptional events in embryonic stem cells mediated by the super elongation complex (SEC). Genes Dev 25:1486-98.
24. Biswas D, Milne TA, Basrur V, Kim J, Elenitoba-Johnson KS, Allis CD, Roeder RG. 2011. Function of leukemogenic mixed lineage leukemia 1 (MLL) fusion proteins through distinct partner protein complexes. Proc Natl Acad Sci U S A 108:15751-6.
25. Izumi K, Nakato R, Zhang Z, Edmondson AC, Noon S, Dulik MC, Rajagopalan R, Venditti CP, Gripp K, Samanich J, Zackai EH, Deardorff MA, Clark D, Allen JL, Dorsett D, Misulovin Z, Komata M, Bando M, Kaur M, Katou Y, Shirahige K, Krantz ID. 2015. Germline gain-of-function mutations in AFF4 cause a developmental syndrome functionally linking the super elongation complex and cohesin. Nat Genet 47:338-44.

26. Wang J, Gareri C, Rockman HA. 2018. G-Protein-Coupled Receptors in Heart Disease. Circ Res 123:716-735.
27. Clerk A, Sugden PH. 1997. Regulation of phospholipases C and D in rat ventricular myocytes: stimulation by endothelin-1, bradykinin and phenylephrine. J Mol Cell Cardiol 29:1593-604.
28. Galvez AS, Brunskill EW, Marreez Y, Benner BJ, Regula KM, Kirschenbaum LA, Dorn GW, 2nd. 2006. Distinct pathways regulate proapoptotic Nix and BNip3 in cardiac stress. J Biol Chem 281:1442-8.
29. Hong HM, Song EJ, Oh E, Kabir MH, Lee C, Yoo YS. 2011. Endothelin-1- and isoproterenol-induced differential protein expression and signaling pathway in HL-1 cardiomyocytes. Proteomics 11:283-97.
30. Lu H, Xue Y, Yu GK, Arias C, Lin J, Fong S, Faure M, Weisburd B, Ji X, Mercier A, Sutton J, Luo K, Gao Z, Zhou Q. 2015. Compensatory induction of MYC expression by sustained CDK9 inhibition via a BRD4-dependent mechanism. Elife 4:e06535.
31. Li Y, Liu M, Chen LF, Chen R. 2018. P-TEFb: Finding its ways to release promoter-proximally paused RNA polymerase II. Transcription 9:88-94.
32. Liang K, Smith ER, Aoi Y, Stoltz KL, Katagi H, Woodfin AR, Rendleman EJ, Marshall SA, Murray DC, Wang L, Ozark PA, Mishra RK, Hashizume R, Schiltz GE, Shilatifard A. 2018. Targeting Processive Transcription Elongation via SEC Disruption for MYC-Induced Cancer Therapy. Cell 175:766-779 e17.
33. Filippakopoulos P, Qi J, Picaud S, Shen Y, Smith WB, Fedorov O, Morse EM, Keates T, Hickman TT, Felletar I, Philpott M, Munro S, McKeown MR, Wang Y, Christie AL, West N, Cameron MJ, Schwartz B, Heightman TD, La Thangue N, French CA, Wiest O, Kung AL, Knapp S, Bradner JE. 2010. Selective inhibition of BET bromodomains. Nature 468:1067-73.
34. Gordon JW, Shaw JA, Kirshenbaum LA. 2011. Multiple facets of NF-κB in the heart: to be or not to NF-κB. Circ Res 108:1122-32.
35. Wu SY, Lee AY, Lai HT, Zhang H, Chiang CM. 2013. Phospho switch triggers Brd4 chromatin binding and activator recruitment for gene-specific targeting. Mol Cell 49:843-57.

36. Martin RD, Sun Y, Bourque K, Audet N, Inoue A, Tanny JC, Hébert TE. 2018. Receptor- and cellular compartment-specific activation of the cAMP/PKA pathway by α1-adrenergic and ETA endothelin receptors. Cell Signal 44:43-50.
37. Depry C, Allen MD, Zhang J. 2011. Visualization of PKA activity in plasma membrane microdomains. Mol Biosyst 7:52-8.
38. Kase H, Iwahashi K, Nakanishi S, Matsuda Y, Yamada K, Takahashi M, Murakata C, Sato A, Kaneko M. 1987. K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. Biochem Biophys Res Commun 142:436-40.
39. Boularan C, Gales C. 2015. Cardiac cAMP: production, hydrolysis, modulation and detection. Front Pharmacol 6:203.
40. Morisco C, Zebrowski DC, Vatner DE, Vatner SF, Sadoshima J. 2001. B-adrenergic cardiac hypertrophy is mediated primarily by the β(1)-subtype in the rat heart. J Mol Cell Cardiol 33:561-73.
41. de Lucia C, Eguchi A, Koch WJ. 2018. New Insights in Cardiac β-Adrenergic Signaling During Heart Failure and Aging. Front Pharmacol 9:904.
42. Tan Y, Wang L, Du Y, Liu X, Chen Z, Weng X, Guo J, Chen H, Wang M, Wang X. 2018. Inhibition of BRD4 suppresses tumor growth in prostate cancer via the enhancement of FOXO1 expression. Int J Oncol 53:2503-2517.
43. Lu L, Chen Z, Lin X, Tian L, Su Q, An P, Li W, Wu Y, Du J, Shan H, Chiang CM, Wang H. 2020. Inhibition of BRD4 suppresses the malignancy of breast cancer cells via regulation of Snail. Cell Death Differ 27:255-268.
44. Dai X, Gan W, Li X, Wang S, Zhang W, Huang L, Liu S, Zhong Q, Guo J, Zhang J, Chen T, Shimizu K, Beca F, Blattner M, Vasudevan D, Buckley DL, Qi J, Buser L, Liu P, Inuzuka H, Beck AH, Wang L, Wild PJ, Garraway LA, Rubin MA, Barbieri CE, Wong KK, Muthuswamy SK, Huang J, Chen Y, Bradner JE, Wei W. 2017. Prostate cancer-associated SPOP mutations confer resistance to BET inhibitors through stabilization of BRD4. Nat Med 23:1063-1071.
45. Stratton MS, McKinsey TA. 2015. Acetyl-lysine erasers and readers in the control of pulmonary hypertension and right ventricular hypertrophy. Biochem Cell Biol 93:149-57.

46. Wang R, Cao XJ, Kulej K, Liu W, Ma T, MacDonald M, Chiang CM, Garcia BA, You J. 2017. Uncovering BRD4 hyperphosphorylation associated with cellular transformation in NUT midline carcinoma. Proc Natl Acad Sci U S A 114:E5352-E5361.
47. Shu S, Lin CY, He HH, Witwicki RM, Tabassum DP, Roberts JM, Janiszewska M, Huh SJ, Liang Y, Ryan J, Doherty E, Mohammed H, Guo H, Stover DG, Ekram MB, Brown J, D'Santos C, Krop IE, Dillon D, McKeown M, Ott C, Qi J, Ni M, Rao PK, Duarte M, Wu SY, Chiang CM, Anders L, Young RA, Winer E, Letai A, Barry WT, Carroll JS, Long H, Brown M, Liu XS, Meyer CA, Bradner JE, Polyak K. 2016. Response and resistance to BET bromodomain inhibitors in triple-negative breast cancer. Nature 529:413-417.
48. Stratton MS, Bagchi RA, Felisbino MB, Hirsch RA, Smith HE, Riching AS, Enyart BY, Koch KA, Cavasin MA, Alexanian M, Song K, Qi J, Lemieux ME, Srivastava D, Lam MPY, Haldar SM, Lin CY, McKinsey TA. 2019. Dynamic Chromatin Targeting of BRD4 Stimulates Cardiac Fibroblast Activation. Circ Res 125:662-677.
49. Penas C, Maloof ME, Stathias V, Long J, Tan SK, Mier J, Fang Y, Valdes C, Rodriguez-Blanco J, Chiang CM, Robbins DJ, Liebl DJ, Lee JK, Hatten ME, Clarke J, Ayad NG. 2019. Time series modeling of cell cycle exit identifies Brd4 dependent regulation of cerebellar neurogenesis. Nat Commun 10:3028.
50. Sun Y, Liu Z, Cao X, Lu Y, Mi Z, He C, Liu J, Zheng Z, Li MJ, Li T, Xu D, Wu M, Cao Y, Li Y, Yang B, Mei C, Zhang L, Chen Y. 2019. Activation of P-TEFb by cAMP-PKA signaling in autosomal dominant polycystic kidney disease. Sci Adv 5:eaaw3593.
51. Garber ME, Mayall TP, Suess EM, Meisenhelder J, Thompson NE, Jones KA. 2000. CDK9 autophosphorylation regulates high-affinity binding of the human immunodeficiency virus type 1 tat-P-TEFb complex to TAR RNA. Mol Cell Biol 20:6958-69.
52. Ha CH, Kim JY, Zhao J, Wang W, Jhun BS, Wong C, Jin ZG. 2010. PKA phosphorylates histone deacetylase 5 and prevents its nuclear export, leading to the inhibition of gene transcription and cardiomyocyte hypertrophy. Proc Natl Acad Sci U S A 107:15467-72.
53. Lehmann LH, Worst BC, Stanmore DA, Backs J. 2014. Histone deacetylase signaling in cardioprotection. Cell Mol Life Sci 71:1673-90.
54. Kuusisto J, Karja V, Sipola P, Kholova I, Peuhkurinen K, Jaaskelainen P, Naukkarinen A, Yla-Herttuala S, Punnonen K, Laakso M. 2012. Low-grade inflammation and the phenotypic expression of myocardial fibrosis in hypertrophic cardiomyopathy. Heart 98:1007-13.

55. Erten Y, Tulmac M, Derici U, Pasaoglu H, Altok Reis K, Bali M, Arinsoy T, Cengel A, Sindel S. 2005. An association between inflammatory state and left ventricular hypertrophy in hemodialysis patients. Ren Fail 27:581-9.
56. Huang S, Frangogiannis NG. 2018. Anti-inflammatory therapies in myocardial infarction: failures, hopes and challenges. Br J Pharmacol 175:1377-1400.
57. Samak M, Fatullayev J, Sabashnikov A, Zeriouh M, Schmack B, Farag M, Popov AF, Dohmen PM, Choi YH, Wahlers T, Weymann A. 2016. Cardiac Hypertrophy: An Introduction to Molecular and Cellular Basis. Med Sci Monit Basic Res 22:75-9.
58. Rogatsky I, Adelman K. 2014. Preparing the first responders: building the inflammatory transcriptome from the ground up. Mol Cell 54:245-54.
59. Fiordelisi A, Iaccarino G, Morisco C, Coscioni E, Sorriento D. 2019. NFκB is a Key Player in the Crosstalk between Inflammation and Cardiovascular Diseases. Int J Mol Sci 20.
60. Freund C, Schmidt-Ullrich R, Baurand A, Dunger S, Schneider W, Loser P, El-Jamali A, Dietz R, Scheidereit C, Bergmann MW. 2005. Requirement of nuclear factor-κB in angiotensin II- and isoproterenol-induced cardiac hypertrophy in vivo. Circulation 111:2319-25.
61. Purcell NH, Tang G, Yu C, Mercurio F, DiDonato JA, Lin A. 2001. Activation of NF-κ B is required for hypertrophic growth of primary rat neonatal ventricular cardiomyocytes. Proc Natl Acad Sci U S A 98:6668-73.
62. Oka T, Maillet M, Watt AJ, Schwartz RJ, Aronow BJ, Duncan SA, Molkentin JD. 2006. Cardiac-specific deletion of Gata4 reveals its requirement for hypertrophy, compensation, and myocyte viability. Circ Res 98:837-45.
63. Liang Q, De Windt LJ, Witt SA, Kimball TR, Markham BE, Molkentin JD. 2001. The transcription factors GATA4 and GATA6 regulate cardiomyocyte hypertrophy in vitro and in vivo. J Biol Chem 276:30245-53.
64. Omura T, Yoshiyama M, Yoshida K, Nakamura Y, Kim S, Iwao H, Takeuchi K, Yoshikawa J. 2002. Dominant negative mutant of c-Jun inhibits cardiomyocyte hypertrophy induced by endothelin 1 and phenylephrine. Hypertension 39:81-6.
65. Chen LF, Williams SA, Mu Y, Nakano H, Duerr JM, Buckbinder L, Greene WC. 2005. NF-κB RelA phosphorylation regulates RelA acetylation. Mol Cell Biol 25:7966-75.

66. Huang B, Yang XD, Zhou MM, Ozato K, Chen LF. 2009. Brd4 coactivates transcriptional activation of NF-κB via specific binding to acetylated RelA. Mol Cell Biol 29:1375-87.
67. Garg R, Caino MC, Kazanietz MG. 2013. Regulation of Transcriptional Networks by PKC Isozymes: Identification of c-Rel as a Key Transcription Factor for PKC-Regulated Genes. PLoS One 8:e67319.
68. Katanasaka Y, Suzuki H, Sunagawa Y, Hasegawa K, Morimoto T. 2016. Regulation of Cardiac Transcription Factor GATA4 by Post-Translational Modification in Cardiomyocyte Hypertrophy and Heart Failure. Int Heart J 57:672-675.
69. Zhang CL, Xie S, Qiao X, An YM, Zhang Y, Li L, Guo XB, Zhang FC, Wu LL. 2017. Plasma endothelin-1-related peptides as the prognostic biomarkers for heart failure: A PRISMA-compliant meta-analysis. Medicine (Baltimore) 96:e9342.
70. Wei CM, Lerman A, Rodeheffer RJ, McGregor CG, Brandt RR, Wright S, Heublein DM, Kao PC, Edwards WD, Burnett JC, Jr. 1994. Endothelin in human congestive heart failure. Circulation 89:1580-6.
71. Rodeheffer RJ, Lerman A, Heublein DM, Burnett JC, Jr. 1992. Increased plasma concentrations of endothelin in congestive heart failure in humans. Mayo Clin Proc 67:719-24.
72. Feyder M, Bonito-Oliva A, Fisone G. 2011. L-DOPA-Induced Dyskinesia and Abnormal Signaling in Striatal Medium Spiny Neurons: Focus on Dopamine D1 Receptor-Mediated Transmission. Front Behav Neurosci 5:71.
73. Lynch WJ, Kiraly DD, Caldarone BJ, Picciotto MR, Taylor JR. 2007. Effect of cocaine self-administration on striatal PKA-regulated signaling in male and female rats. Psychopharmacology (Berl) 191:263-71.
74. D AF, Standaert DG. 2017. Dysregulation of BET proteins in levodopa-induced dyskinesia. Neurobiol Dis 102:125-132.
75. Sartor GC, Powell SK, Brothers SP, Wahlestedt C. 2015. Epigenetic Readers of Lysine Acetylation Regulate Cocaine-Induced Plasticity. J Neurosci 35:15062-72.
76. Weigand I, Ronchi CL, Rizk-Rabin M, Dalmazi GD, Wild V, Bathon K, Rubin B, Calebiro D, Beuschlein F, Bertherat J, Fassnacht M, Sbiera S. 2017. Differential expression of the protein kinase A subunits in normal adrenal glands and adrenocortical adenomas. Sci Rep 7:49.

77. Calebiro D, Hannawacker A, Lyga S, Bathon K, Zabel U, Ronchi C, Beuschlein F, Reincke M, Lorenz K, Allolio B, Kisker C, Fassnacht M, Lohse MJ. 2014. PKA catalytic subunit mutations in adrenocortical Cushing's adenoma impair association with the regulatory subunit. Nat Commun 5:5680.
78. Lee TI, Young RA. 2013. Transcriptional regulation and its misregulation in disease. Cell 152:1237-51.
79. Lu X, Zhu X, Li Y, Liu M, Yu B, Wang Y, Rao M, Yang H, Zhou K, Wang Y, Chen Y, Chen M, Zhuang S, Chen LF, Liu R, Chen R. 2016. Multiple P-TEFbs cooperatively regulate the release of promoter-proximally paused RNA polymerase II. Nucleic Acids Res 44:6853-67.
80. Dawson MA, Prinjha RK, Dittmann A, Giotopoulos G, Bantscheff M, Chan WI, Robson SC, Chung CW, Hopf C, Savitski MM, Huthmacher C, Gudgin E, Lugo D, Beinke S, Chapman TD, Roberts EJ, Soden PE, Auger KR, Mirguet O, Doehner K, Delwel R, Burnett AK, Jeffrey P, Drewes G, Lee K, Huntly BJ, Kouzarides T. 2011. Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. Nature 478:529-33.
81. Calderone A, Thaik CM, Takahashi N, Chang DL, Colucci WS. 1998. Nitric oxide, atrial natriuretic peptide, and cyclic GMP inhibit the growth-promoting effects of norepinephrine in cardiac myocytes and fibroblasts. J Clin Invest 101:812-8.
82. Yang JH, Polanowska-Grabowska RK, Smith JS, Shields CWt, Saucerman JJ. 2014. PKA catalytic subunit compartmentation regulates contractile and hypertrophic responses to β-adrenergic signaling. J Mol Cell Cardiol 66:83-93.
83. Mao T, Kusefoglu D, Hooks BM, Huber D, Petreanu L, Svoboda K. 2011. Long-range neuronal circuits underlying the interaction between sensory and motor cortex. Neuron 72:111-23.
84. Burger C, Nash KR. 2016. Small-Scale Recombinant Adeno-Associated Virus Purification. Methods Mol Biol 1382:95-106.
85. Krueger F. Trim Galore! http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/.
86. Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. 2011 17:3.
87. Zerbino DR, Achuthan P, Akanni W, Amode MR, Barrell D, Bhai J, Billis K, Cummins C, Gall A, Giron CG, Gil L, Gordon L, Haggerty L, Haskell E, Hourlier T, Izuogu OG, Janacek

SH, Juettemann T, To JK, Laird MR, Lavidas I, Liu Z, Loveland JE, Maurel T, McLaren W, Moore B, Mudge J, Murphy DN, Newman V, Nuhn M, Ogeh D, Ong CK, Parker A, Patricio M, Riat HS, Schuilenburg H, Sheppard D, Sparrow H, Taylor K, Thormann A, Vullo A, Walts B, Zadissa A, Frankish A, Hunt SE, Kostadima M, Langridge N, Martin FJ, Muffato M, Perry E, et al. 2018. Ensembl 2018. Nucleic Acids Res 46:D754-D761.

88. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29:15-21.
89. Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL. 2015. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nat Biotechnol 33:290-5.
90. Soneson C, Love MI, Robinson MD. 2015. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. F1000Res 4:1521.
91. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:550.
92. Ignatiadis N, Klaus B, Zaugg JB, Huber W. 2016. Data-driven hypothesis weighting increases detection power in genome-scale multiple testing. Nat Methods 13:577-80.
93. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. 2015. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 43:e47.
94. Kramer A, Green J, Pollard J, Jr., Tugendreich S. 2014. Causal analysis approaches in Ingenuity Pathway Analysis. Bioinformatics 30:523-30.
95. Bolli P, Vardabasso C, Bernstein E, Chaudhry HW. 2013. Chromatin immunoprecipitation of adult murine cardiomyocytes. Curr Protoc Cell Biol Chapter 17:Unit17 14.
96. Mbogning J, Tanny JC. 2017. Chromatin Immunoprecipitation of Histone Modifications in Fission Yeast. Methods Mol Biol 1528:199-21

CHAPTER 4: An interaction between Gβγ and RNA polymerase II regulates transcription in cardiac fibroblasts

Shahriar M. Khan[1†], **Ryan D. Martin**[1†], Sarah Gora[1], Celia Bouazza[1], Jace Jones-Tabah[1], Andy Zhang[1], Sarah MacKinnon[1], Phan Trieu[1], Paul B.S. Clarke[1], Jason C. Tanny[1]*, and Terence E. Hébert[1]*

[1] Department of Pharmacology and Therapeutics, McGill University, Montréal, Québec, H3G 1Y6, Canada

†These authors contributed equally to the study.

Manuscript under review at Science Signalling.

4.1. Preface

In the previous chapters we characterized regulatory mechanisms utilized by GPCRs to recruit P-TEFb to the chromatin and activate pathological gene expression in cardiomyocytes. During this time, we were also interested in the P-TEFb-dependent transcription following AT1R activation in cardiac fibroblasts, which is a critical driver of the fibrotic response in pathological cardiac remodelling. Specifically, we identified a novel interaction between Gβγ and RNAPII following AT1R activation with unknown function, although the signalling mechanism regulating the interaction had been extensively characterized. Interestingly, the Gβγ-RNAPII interaction required P-TEFb activity, as P-TEFb inhibition ablated both the basal and agonist-stimulated interaction. In this chapter, we describe the role of specific Gβγ dimers in regulating fibrotic gene expression in response to AT1R activation using RT-qPCR-based gene arrays and ChIP-seq of Gβγ and RNAPII. We identify the role of Gβγ in negatively regulating RNAPII to supress fibrotic transcription following AT1R activation.

4.2. Abstract

Gβγ subunits are involved in many different signalling processes in various compartments of the cell, including the nucleus. To gain insight into the functions of nuclear Gβγ, we investigated the functional role of Gβγ signalling in regulation of GPCR-mediated gene expression in primary rat neonatal cardiac fibroblasts. Following activation of the angiotensin II type I receptor in these cells, Gβγ dimers interact with RNA polymerase II (RNAPII). Our findings suggest that $G\beta_1\gamma$ recruitment to RNAPII negatively regulates the fibrotic transcriptional response, which can be overcome by strong fibrotic stimuli. The interaction between Gβγ subunits and RNAPII expands the role for Gβγ signalling in cardiac fibrosis. The Gβγ-RNAPII interaction was regulated by signaling pathways in HEK 293 cells that diverged from those operating in cardiac fibroblasts. Thus, the interaction may be a conserved feature of transcriptional regulation although such regulation may be cell specific.

4.3. Introduction

In recent years, study of the role of paracrine interactions between cardiomyocytes and cardiac fibroblasts in modulating the response to cardiac damage has expanded dramatically.

Cardiac fibroblasts, in particular, respond dynamically following damage to the myocardium which is characterized by differentiation into myofibroblasts, increased proliferation and migration to areas of damage (1-3). This fibrotic response is modulated by the renin-angiotensin system, acting predominantly through the peptide ligand angiotensin II (Ang II) (4, 5). Ang II drives changes in fibroblast function both directly and indirectly by increasing expression of other pro-fibrotic growth factors, such as transforming growth factor β1 (TGF-β1) (6). Collectively, these factors regulate alterations in cardiac architecture required for tissue repair by modulating the expression of genes encoding extracellular matrix proteins and proteases (7, 8). Ang II also promotes cytokine secretion, thereby triggering autocrine and paracrine signalling to elicit further responses (9, 10). These signalling events create a feedforward loop, amplifying the fibrotic response from the initial area of damage to more distal regions of the heart (11). While the process initially aids in wound healing, a prolonged, activated fibrotic response worsens adverse cardiac remodelling and accelerates progression to heart failure (1, 12). Inhibiting aspects of the fibrotic response reduces adverse cardiac remodelling (2, 13). Hence, deciphering how Ang II signalling regulates pro-fibrotic gene expression is an important step towards understanding how these processes might be targeted therapeutically.

Cardiac fibroblasts respond to increased Ang II levels through the Ang II type I receptor (AT1R), a G protein-coupled receptors (GPCRs), and Ang II type II receptor (AT2R). Of these, the AT1R is responsible for positively regulating the fibrotic response in cardiac fibroblasts (1). The AT1R couples to multiple heterotrimeric G proteins composed of specific combinations of Gα and Gβγ subunits (14). G proteins serve as signal transducers to relay extracellular ligands bound to GPCRs into activation of different intracellular signalling pathways (15). Gβγ subunits, like the more extensively studied Gα subunits, modulate a wide variety of canonical GPCR effectors at the cellular surface such as adenylyl cyclases, phospholipases and inwardly rectifying potassium channels (15-17). However, compared with Gα-mediated events, Gβγ-mediated signalling is relatively understudied and is complicated by the existence of 5 Gβ and 12 Gγ subunits which can combine in multiple ways to form obligate dimers. Gβγ subunits also regulate a variety of non-canonical effectors in distinct intracellular locations, and a number of studies have described roles for Gβγ signalling in the nucleus (15, 18). Nuclear Gβγ subunits modulate gene expression through interactions with a variety of transcription factors, such as adipocyte enhancer binding protein 1 (AEBP1), the AP-1 subunit c-Fos, HDAC5 and MEF2A (19-22). Furthermore,

we have detected $G\beta_1$ occupancy at numerous gene promoters in HEK 293 cells (23). While canonical $G\beta\gamma$ signalling has been implicated in both cardiac fibrosis and heart failure (24, 25), how nuclear $G\beta\gamma$ signalling impacts these events is currently unknown.

Here, we describe a novel interaction between $G\beta\gamma$ subunits and RNA polymerase II (RNAPII) which regulates the cardiac fibrotic response to Ang II activation of AT1R. We characterize the GPCR-dependent, signalling pathway-specific regulation of this interaction in primary neonatal rat cardiac fibroblasts and in HEK 293 cells. To understand the potential role of individual $G\beta\gamma$ subunits, we knocked down $G\beta_1$ and $G\beta_2$ as exemplars of $G\beta$ subunits highly expressed in these cells and characterized how nuclear $G\beta_1$, in particular, is a key regulator of AT1R-driven transcriptional changes.

4.3. Methods

4.3.1. Reagents

The following were all purchased from Sigma-Aldrich: carbachol, angiotensin II, BAPTA-AM, KN-93, Gö6983, PTX, U0126, calyculin A, cyclosporin A, TRI reagent, isopropyl thiogalactopyranoside (IPTG), protease inhibitor cocktail, triton X-100, bovine serum albumin, ethylenediaminetetraacetic acid (EDTA), 70% NP-40 (Tergitol), sodium deoxycholate, magnesium chloride, lithium chloride, anti-rabbit IgG (whole molecule)-agarose antibody, anti-mouse IgG (whole molecule)-agarose antibody, goat anti-rabbit IgG (whole molecule) conjugated to peroxidase secondary antibody, goat anti-mouse IgG (Fab specific) conjugated to peroxidase secondary antibody, anti-FLAG M2 antibody, and rabbit IgG (St. Louis, MO, USA). U71322 pan-PKC inhibitor was purchased from Biomol International (Plymouth Meeting, PA, USA). Lysozyme (from hen egg white) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Roche Applied Sciences (Laval, QC, Canada). Ethylene glycol bis (2-aminooethyl ether) N,N,N',N' tetraacetic acid (EGTA) and HEPES were purchased from BioShop (Burlington, ON, Canada). Sodium chloride, glutathione (reduced form), dithiothreitol (DTT) and Dynabeads protein G were purchased from Fisher Scientific (Ottawa, ON, Canada). Dulbecco's modified Eagle's medium (DMEM) (supplemented with 4.5 g/L glucose, L-glutamine and phenol red), DMEM low glucose (supplemented with 1.0 g/L glucose, L-glutamine and phenol red), Hank's Balanced salt solution (HBSS), HBSS (with no phenol), Penicillin/Streptomycin solution, Tris base buffer, ampicillin sodium salt, and fetal bovine serum were purchased from Wisent (St.

Bruno, QC, Canada). Glutathione sepharose 4B GST beads was purchased from GE Healthcare (Mississauga, ON, Canada). Lipofectamine 2000 and Alexa Fluor 488 goat anti-mouse IgG were purchased from Invitrogen (Burlington, ON, Canada). Enhanced chemiluminescence (ECL) Plus reagent was purchased from Perkin Elmer (Woodbridge, ON, Canada). Moloney murine leukemia virus reverse transcriptase (MMLV-RT) enzyme and recombinant RNasin® ribonuclease inhibitor were purchased from Promega (Madison, WI, USA). Evagreen 2X qPCR MasterMix was purchased from Applied Biological Materials Inc. (Vancouver, BC, Canada) and iQ SYBR Green Supermix was purchased from Bio-Rad Laboratories (Mississauga, ON, Canada). Anti-Gβ1-4 (T-20) antibody, anti-RNA Polymerase I Rpa194 (N-16) antibody, anti-ERK1/2 antibody, anti-Gαq antibody and anti-Rpb1 (N20) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-RNA polymerase II clone CTD4H8 (Rpb1) antibody was purchased from EMD Millipore (Temecula, CA, USA). Anti-*Schizosaccharomyces pombe* histone H2B (ab188271) antibody was purchased from Abcam Inc. (Toronto, ON, Canada). Polyclonal anti-$G\beta_1$ and anti-$G\beta_2$ were a generous gift of Professor Ron Taussig (UT Southwestern). THZ1 was a gift from Nathanael S. Gray (Harvard University) and iCdk9 was a gift from James Sutton (Novartis). FLAG-$G\beta_1$, FLAG-$G\beta_2$, FLAG-$G\beta_3$, FLAG-$G\beta_4$ and FLAG-$G\beta_5$ plasmids were obtained from UMR cDNA Resource (www.cdna.org).

4.3.2. Tissue culture, transfection and treatments

Human embryonic kidney 293 (HEK 293), HEK 293T cells and CRISPR/Cas9 generated ΔGαq/11/12/13 knockout HEK 293 cells (quadKO cells) (56), a generous gift from Dr. Asuka Inoue (Tohuku University, Sendai, Japan), were grown at 37°C in 5% CO_2 in DMEM supplemented with 5% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin (P/S). HEK 293 cells were transiently transfected with FLAG-Gβ1-5 using Lipofectamine 2000 as per the manufacturer's recommendations. Primary rat neonatal cardiac fibroblasts were isolated from 1-3 day old Sprague-Dawley rat pups (Charles River Laboratories, St-Constant, Quebec) as previously described (57). All procedures using animals were approved by the McGill University Animal Care Committee, in accordance with Canadian Council on Animal Care Guidelines. Two days after isolation, cells were detached with trypsin/EDTA and plated at a density of ~8×10^3 cells/cm^2 in fibroblast growth medium for 48h. For siRNA transfection, cardiac fibroblasts were plated at a density of ~20×10^3 cells/cm^2 and transfected using Lipofectamine 2000 as per the manufacturer's

instructions. For treatment of HEK 293F cells, HEK 293F quadKO cells or cardiac fibroblasts, cells were serum-deprived for 6 h with DMEM or overnight (~12 h) with DMEM low glucose (with no FBS and no P/S) respectively, and subsequently treated with pathway inhibitors, 1 mM carbachol or 1 μM Ang II for the treatment lengths indicated in the various assays.

4.3.3. RT-qPCR

Reverse transcription of RNA isolated from rat neonatal cardiac fibroblasts was performed as previously described (23). Briefly, cells were lysed in TRI reagent and RNA was extracted using a protocol adapted from Ambion (Burlington, ON, Canada). Reverse transcription was performed on 1 μg of total RNA using an MMLV-RT platform according to the manufacturer's protocol. Subsequent qPCR analysis was performed with Evagreen Dye qPCR master-mixes using a Corbett Rotorgene 6000 thermocycler or Bio-Rad 1000 Series Thermal Cycling CFX96 Optical Reaction module. mRNA expression data were normalized to housekeeping transcripts for U6 snRNA. Ct values obtained were analyzed to calculate fold change over respective control values using the $2^{-\Delta\Delta Ct}$ method. Primer sequences for all primers used are listed in **Supplemental Table 4.1**.

4.3.4. Ca^{2+} mobilization

Cardiac fibroblasts were cultured as previously described following transfection with respective siRNA. Cardiac fibroblasts were washed and media replaced with HBSS (no phenol) and incubated for 1 h at 37°C and 5% CO_2. Media was replaced with Fura 2-AM in HBSS and incubated for another 1 h at 37°C and 5% CO_2. Fura 2-AM containing media was replaced with HBSS and Cardiac fibroblasts incubated for another 30 min at 37°C and 5% CO_2 prior to recordings. Baseline recordings were obtained every 0.7 s for 10 s followed by injection of Ang II to a final concentration of 1 μM and recordings obtained every 0.7 s for a total of 1 min. A control well with no Fura-2 AM was included in order to control for background fluorescence. Fluorescence intensity was recorded using Bio-Tek Synergy 2 Multi-Mode Microplate Reader with fluorescence excitation at 340 nm or 360 nm and fluorescence emission at 516 nm. Data is presented as the ratio of fluorescence emission at 516 nm following 340 nm excitation over 360 nm excitation. The ratio was normalized to the mean baseline ratio from control cells.

4.3.5. Nuclear isolation

Nuclei from HEK 293 cells and cardiac fibroblasts were isolated as previously described (26). Briefly, cells seeded in T175 flasks (Corning) were treated as indicated, washed three times with 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), and harvested in 1X PBS by centrifugation. Pelleted cells were lysed in lysis buffer (320mM sucrose, 10 mM HEPES, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 1% Triton X-100), added gently on top of a high-sucrose buffer (1.8 M sucrose, 10 mM HEPES, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF), and centrifuged at 4600 g for 30 min at 4°C, separating unlysed nuclei from the cytosolic fraction. Pelleted nuclei were then resuspended in resuspension buffer (320 mM sucrose, 10 mM HEPES, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF), pelleted at 300 g for 5 min and subsequently lysed in 1X RIPA buffer.

4.3.6. Immunoprecipitation and western blotting

Immunoprecipitation (IP) assays of Gβ and Rpb1 pull downs were performed as previously described, with minor alterations (20). Protein extracts from treated HEK 293 cells and cardiac fibroblasts lysed in RIPA (1% NP-40, 50 mM Tris-HCl ph 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 0.5% sodium deoxycholate) were quantified by Bradford assay and 500 µg of protein lysate was precleared with 15 µl of anti-rabbit IgG-agarose beads. Precleared lysates were then incubated with 1 µg anti-$G\beta_{1\text{-}4}$, 2 µg of anti-Rpb1 or anti-$G\beta_1$ serum or anti-$G\beta_2$ serum overnight at 4°C with end-over mixing. The next day, 40 µl of washed agarose beads were added to each lysate/antibody mixture, incubated for 3.5 hours at 4°C with end-over mixing, and then beads were washed 3X with RIPA. Proteins were eluted off the beads by the addition of 4X Laemmli buffer followed by denaturation at 65°C for 15 min. Protein immunoprecipitation and co-IP were then assessed by western blot as previously described (23). Resulting western blot images were quantified using ImageJ 1.48v.

4.3.7. Rat Fibrosis qPCR arrays

Fibrosis qPCR arrays were performed as per the manufacturer's instructions (Qiagen, Toronto, ON, Canada). Briefly, 0.5 µg of isolated total RNA from siRNA transfected and vehicle or Ang II treated cardiac fibroblasts was subject to genomic DNA elimination using mixes supplied

with the array kit for 5 mins at 42°C. DNA eliminated RNA was then subject to reverse transcription reactions using Qiagen RT^2 First Strand Kits with protocols according to the manufacturer's instructions. Qiagen RT^2 SYBR Green MasterMix was added to the cDNA and subsequently dispensed in wells of a 96-well plate containing pre-loaded lyophilized primers provided by the manufacturer. Quantitative PCR reactions were then run on an Applied Biosystems ViiA 7 thermocycler according to the manufacturers cycle recommendations. Each sample was run on separate individual 96 well plates and Ct values for each gene assessed were collected and analyzed; Ct values greater than 35 were eliminated from the overall analysis. Expression data was normalized to levels of two housekeeping genes contained on each plate – Ldha1 and Hprt.

4.3.8. AAV Production and transduction of cardiac fibroblasts

FLAG-$G\beta_1$ and FLAG-$G\beta_2$ were PCR amplified from a pcDNA3.1+ plasmid and BamHI and EcoRI restrictions sites added to the 5' and 3' end, respectively. These restrictions sites were used to insert each FLAG-Gβ into the pAAV-CAG plasmid. Adeno-associated viruses were produced as previously described (58). Cells were transduced with AAV1-FLAG-$G\beta_1$ (MOI of 10^3 or $5x10^4$) in DMEM low glucose for 6h. Additional media was added to obtain a final 7% FBS concentration and incubated for another 24 h. At this point, the cells were detached with trypsin/EDTA and plated as described for respective experiments.

4.3.9. ChIP-qPCR

Immunoprecipitation in cardiac fibroblasts was performed as previously described, with minor modifications (59). Isolated nuclei were sonicated with a Diagenode BioRuptor™ UCD-200 (18 cycles, 30 s on/off, high power) to shear chromatin. FLAG-$G\beta_1$ immunoprecipitation was performed with 10 μg sheared rat chromatin alongside 5 μg of *Schizosaccharomyces pombe* yeast chromatin, obtained as previously described (60). Chromatin was immunoprecipitated with an anti-FLAG M2 antibody (2 μg) or equivalent amount of rabbit IgG alongside an anti-*Schizosaccharomyces pombe* H2B antibody. RNAPII immunoprecipitation was performed with 20 μg of sheared rat chromatin alongside 0.2 μg of *Schizosaccharomyces pombe* yeast chromatin. Chromatin was immunoprecipitated with an anti-Rpb1 (8WG16) antibody. Localization was

assessed by qPCR with primers for specific genomic loci (**Supplemental Table 4.1**). All qPCR reactions were performed using a Bio-Rad 1000 Series Thermal Cycling CFX96 Optical Reaction module and iQ SYBR Green Supermix. Data analysis included subtracting the % Input of IgG control for each treatment from the respective IP, followed by normalization to the % Input yeast *cdc2*$^{+}$ of each FLAG IP or *act1*$^{+}$ for each RNAPII IP to account for differences in IP efficiencies.

4.3.10. ChIP-seq immunoprecipitation and data analysis

Immunoprecipitation in Cardiac fibroblasts was performed as previously described, with minor modifications (59). Isolated nuclei were sonicated with a Diagenode BioRuptor^{TM} UCD-200 (18 cycles, 30 s on/off, high power) to shear chromatin. FLAG-Gβ_1 immunoprecipitation was performed with 40 μg of sheared rat chromatin alongside 0.4 μg of chromatin from a *S. pombe* strain expressing FLAG-Bdf2. RNAPII immunoprecipitation was performed using 20 μg sheared rat chromatin alongside 0.2 μg wild-type *S. pombe* chromatin. Chromatin was immunoprecipitated with an anti-FLAG M2 antibody (2 μg) or anti-Rpb1 (8WG16) antibody (2 μg). Two biological replicates of FLAG-Gβ_1 immunoprecipitation and three biological replicates of Rpb1 immunoprecipitation were included. Following immunoprecipitation and DNA cleanup, libraries were prepared with the NEBNext® Ultra^{TM} II DNA Library Prep kit for Illumina and 50 bp single end reads obtained with an Illumina HiSeq 4000 *at the McGill University and Génome Québec Innovation Centre, Montréal, Canada*.

Reads were trimmed with TrimGalore (0.6.0) (61, 62) using the following settings: --phred33 --length 36 -q 5 --stringency 1 -e 0.1. A Bowtie2 genome comprised of the Ensembl rat reference genome (Rattus.norvegicus.Rnor.6.0.94) (63) and *S. pombe* reference genome (Schizosaccharomyces_pombe.ASM294v2) was built with the bowtie2-build function. Processed reads were aligned to the custom combined rat and *S. pombe* genome with Bowtie2 (v2.3.5), followed by removal of low-quality mapped reads (MAPQ < 10) and reads mapped to non-standard chromosomes with SAMtools (v1.9) (64). Duplicate reads were removed with Picard tools (v2.20.6, Broad Institute). Aligned reads were separated into individual files for the rat or *S. pombe* genome respectively. For RNAPII ChIP, peaks were called using macs2 (v2.1.1) with settings --broad and --broad-cutoff 0.1 (41), reads extended by the fragment length determined by phantompeakqualtools (v1.14) (65, 66), a scaling factor estimated using the NCIS R package (67) and potential misassembled regions of the rat genome blacklisted (68). RNAPII peaks were

annotated with HOMER (v4.11) (40) and those protein-coding genes with RNAPII peaks in two of three replicates in any treatment were used for subsequent analysis. BAM files of treatment replicates were combined, input reads subtracted with the deepTools (69) function bamCompare (--scaleFactorsMethod SES) and negative values set to 0. Lastly, values were converted to counts per million mapped reads with library size adjusted by the total number of reads aligned to the *S. pombe* genome. K-means clustering for genes with identified RNAPII peaks and data visualization was performed with the deepTool's computeMatrix and plotProfile functions. Gene ontology enrichment was performed using the R package topGO (v2.36.0). The data is available at the NCBI Gene Expression Omnibus (GEO) with the accession GSE147416. All code used to analyze the ChIP-seq is available upon request.

4.3.11. Statistical Analysis

Statistical tests were performed using GraphPad Prism 8.0 software. For quantifications of immunoprecipitation experiments, two-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test was used on quantifications of western blot bands, with all multiple comparisons being made to vehicle-vehicle conditions. To analyse Ca^{2+} release experiments, the dependent measure was the area under the curve (AUC), computed from release-time data sets. AUC data were subjected to ANOVA and Dunnett's tests, using as a point of comparison the siRNA control condition. For the FLAG-Gβ and RNAPII interaction in HEK 293F cells, one-sample t-tests were performed with a Bonferroni correction. Summary gene expression of the fibrosis array qPCR was compared with a two-way ANOVA followed by post-hoc t-tests with a Bonferroni correction. Individual gene expression from the fibrosis qPCR array and Ctgf gene expression with in-house primers was assessed with a two-way ANOVA followed by Bonferroni corrected post-hoc t-tests at individual time points. For validation of $G\beta_1$ and $G\beta_2$ knockdown in cardiac fibroblasts, fold changes over siRNA control were compared to siRNA control using paired Student's t-tests. For FLAG-$G\beta_1$ ChIP-qPCR, independent paired Student t-tests with a Bonferroni post-hoc correction were performed. A Fisher's exact test was used to compare the proportion of cluster 1 genes in the list of genes with RNAPII peaks following Ang II treatment in control or $G\beta_1$ knockdown conditions and to assess GO Term enrichment in cluster 1 genes. Alpha was set at $p<0.05$ (2-tailed). All results are expressed as mean ± S.E.M, and data are represented as pooled experiments whose sample sizes are indicated in figure legends.

4.4. Results

4.4.1. Gβγ interaction with RNAPII following activation of Gαq-coupled GPCRs

As Gβγ interacts with transcription factors and occupies gene promoter regions, we hypothesized that Gβγ subunits interact with a protein complex ubiquitously involved in transcription, and we initially focused on RNAPII. We assessed the potential Gβγ-RNAPII interaction following endogenous M3-muscarinic acetylcholine receptors (M3-mAChRs) activation with carbachol in HEK 293F cells. An initial co-immunoprecipitation time course experiment revealed a carbachol-induced interaction between endogenous Gβγ subunits ($G\beta_{1\text{-}4}$ detected with a pan-Gβ antibody) and Rpb1, the largest subunit of RNAPII, peaking between 45 and 120 mins (Supplemental Figure 4.1A, B). Immunoprecipitation of Rpb1 with two different antibodies also co-immunoprecipitated $G\beta_{1\text{-}4}$ in an agonist-dependent manner (Supplemental Figure 4.1C). Further, we observed no basal or carbachol-dependent interaction of Rpb1 with Gαq/11 or ERK1/2 (Supplemental Figure 4.1D, E) suggesting that Gβγ was not in complex with these proteins when it was associated with RNAPII in the nucleus. Under similar conditions, we observed no basal or carbachol-dependent interaction of Gβγ subunits with the A194 subunit of RNA polymerase I (Supplemental Figure 4.1F), suggesting Gβγ is not recruited to all RNA polymerases.

We next assessed the whether the Gβγ-RNAPII interaction also occurred in primary rat neonatal cardiac fibroblasts following treatment with Ang II. A time-course co-immunoprecipitation experiment revealed an agonist induced Gβγ-RNAPII interaction with a major peak interaction observed 75 minutes post stimulation (Figure 4.1A, B). As cardiac fibroblasts express both AT1R and AT2R, we next examined which receptor subtype regulated the response, by pre-treatment with the AT1R-specific antagonist losartan. Pre-treatment of cells with losartan prior to Ang II treatment abolished the agonist-induced interaction, but preserved the basal interaction, suggesting that AT1R, and not AT2R, is primarily responsible for mediating the interaction (Figure 4.1C, D).

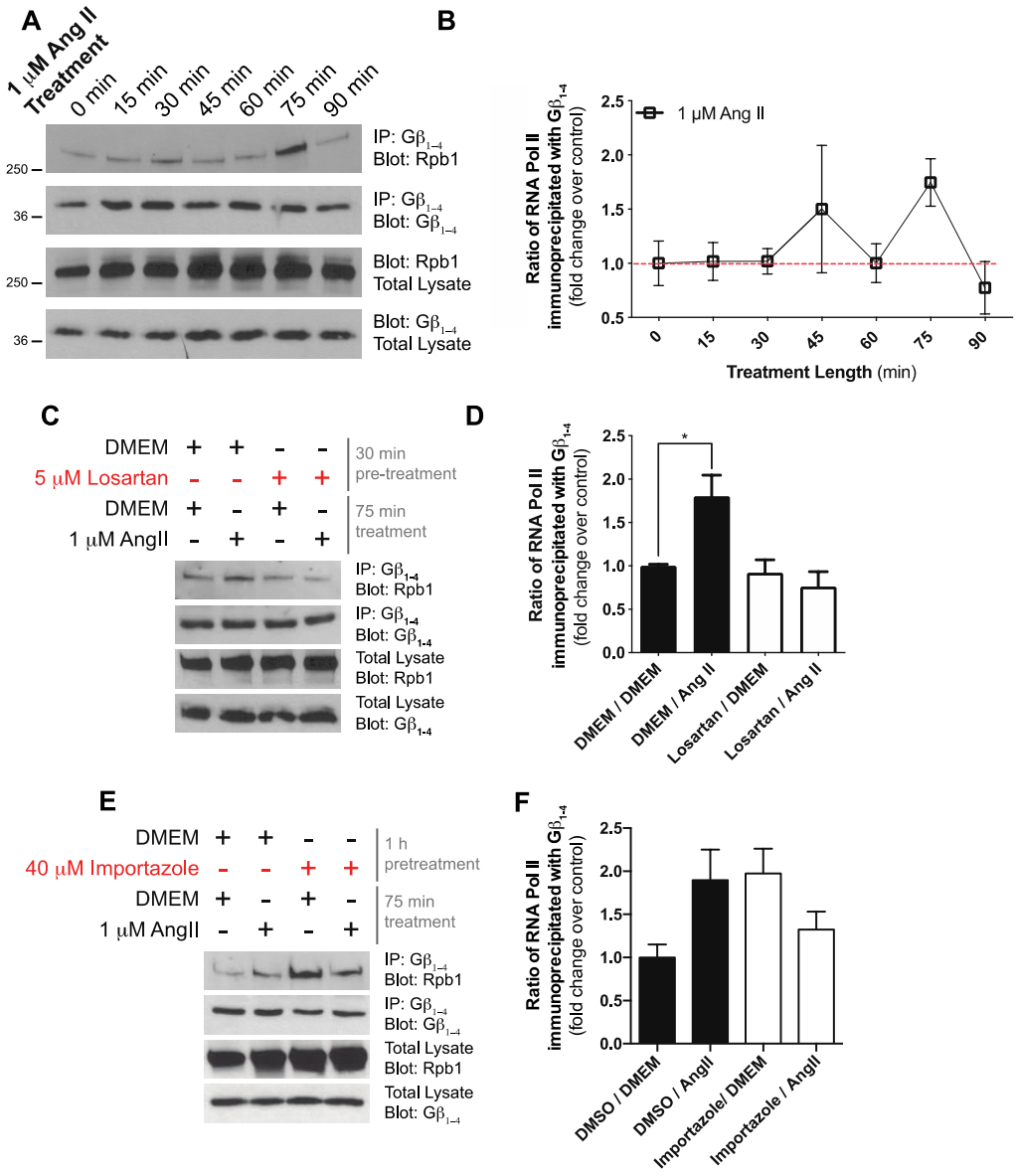


Figure 4.1. Characterization of Gβγ-RNAPII in rat neonatal cardiac fibroblasts.

(A) Time course of the Ang II-stimulated interaction between Gβγ and Rpb1. The ratio of Rpb1 co-immunoprecipitated with $G\beta_{1\text{-}4}$ upon treatment of 1 μM Ang II treatment at the indicated timepoints in cardiac fibroblasts was assessed. **(B)** Densitometry-based quantification of panel A was used to determine the ratio of Rpb1 to $G\beta_{1\text{-}4}$ immunoprecipitated at each time point. The fold change over the 0 min time point was then calculated. Data is representative of four independent experiments. One-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test was performed. **(C)** Effect of AT1R antagonist losartan pre-treatment on the Ang II-mediated interaction, demonstrating angiotensin receptor subtype selectivity. **(D)** Densitometry-based

quantification of AT1R antagonist effect on Ang II-induced interaction. The ratio of Rpb1 immunoprecipitated with $G\beta_{1-4}$ was determined for each condition and fold change over DMSO/DMEM was calculated. Data is representative of three independent experiments. Two-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test was performed. **(E)** Assessment of the necessity of Gβγ import into the nucleus for interaction to occur upon AT1R stimulation with Ang II. Cardiac fibroblasts were pretreated for 1 h with importazole prior to Ang II stimulation. Data are representative of four independent experiments. **(F)** Densitometry-based quantification of the Ang II induced interaction and the effect of nuclear import inhibition. The ratio of Rpb1 to $G\beta_{1-4}$ immunoprecipitated was determined and normalized to fold change over DMSO/DMEM treatment. Two-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test was performed. In all panels, data represents mean ± S.E.M, * indicates $p<0.05$, ** indicates $p<0.01$.

Although several Gβγ isoforms have been detected in the nucleus (22, 26, 27), the mechanisms leading to entry of Gβγ into the nucleus remain unknown. Using subcellular fractionation following M3-mAChR activation in HEK 293F cells, we observed importin-β dependent translocation of Gβγ into the nucleus (data not shown). In addition, the agonist-dependent interaction of $G\beta_{1-4}$ and RNAPII was blocked by importazole pre-treatment, suggesting that nuclear import of $G\beta_{1-4}$ is required for the interaction with RNAPII in these cells (Supplemental Figure 4.2A, B). Next, we determined the effect of importazole pre-treatment on the Ang II-mediated Gβγ-RNAPII interaction in cardiac fibroblasts. The Gβγ-RNAPII interaction was also ablated when nuclear import via importin-β was inhibited, suggesting again that Gβγ subunits must translocate to the nucleus for the interaction with RNAPII to occur (Figure 4.1E, F).

4.4.2. Signalling pathways regulating Gβγ-RNAPII interaction are cell-specific

We next examined signalling events downstream of receptor activation that could mediate the interaction between Gβγ subunits and RNAPII. To this end, we pursued a pharmacological and genetic approach using both cardiac fibroblasts (Figure 4.2A-H) and HEK 293F cells (Figure 4.3A-G). Our data indicated that the pathways responsible for promoting the Gβγ-RNAPII interaction are cell type specific. Since AT1R couples to both Gq/11 and Gi/o G proteins (28), we used FR900359 to inhibit Gαq/11 (29) and pertussis toxin (PTX) to inhibit Gαi/o. The agonist-induced

response was markedly (~80%) decreased by the Gαq/11 inhibitor, and also decreased (~30%) by the Gαi/o inhibitor, demonstrating that AT1R signalling through Gαq is the primary pathway leading to increased Gβγ-RNAPII interaction (Figure 4.2A-B, Supplemental Figure 4.3A-B). We next used U71322 to inhibit the activity of phospholipase Cβ (PLCβ), downstream of both Gq/11 and Gi/o (the latter via Gβγ signalling). In cardiac fibroblasts, pre-treatment of U71322 blocked the agonist-induced Gβγ-RNAPII interaction with no effect on the basal interaction, suggesting a pivotal role for PLCβ (Figure 4.2C, Supplemental Figure 4.3C). Chelation of Ca^{2+} using BAPTA-AM in cardiac fibroblasts also abrogated the Ang II-induced Gβγ-RNAPII interaction (Figure 4.2D, Supplemental Figure 4.3D), as did treatment with the PKC inhibitor Gö6983 and the CaMKII inhibitor KN-93 (Figure 4.2E, F, Supplemental Figure 4.3E, F). Conversely, the MEK1 inhibitor U0126 led to an increased basal Gβγ-RNAPII interaction but abrogated the Ang II-induced interaction (Figure 4.2G, Supplemental Figure 4.3G). Lastly, the calcineurin inhibitor cyclosporin A lead to an increased basal interaction did not prevent further Ang II-dependent increase in interaction (Figure 4.2H, Supplemental Figure 4.3H).

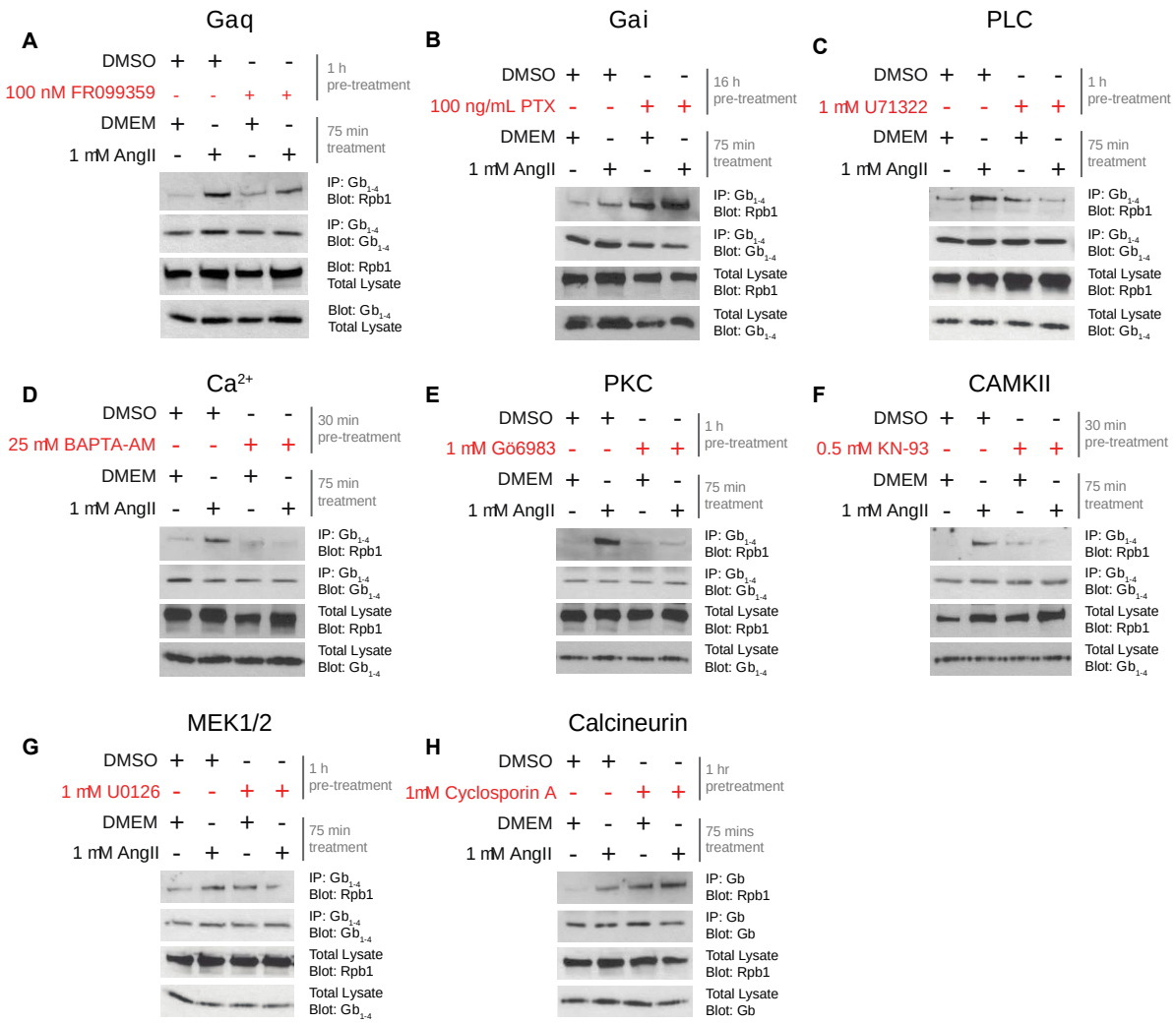


Figure 4.2. Mechanistic analysis of Gβγ interactions with Rpb1 in rat neonatal cardiac fibroblasts.

(A-H) Assessment of the effect of inhibition of signalling molecules and effectors implicated in AT1R signalling on the induction of the Gβγ-RNAPII interaction in cardiac fibroblasts. Concentrations of inhibitors and lengths of pre-treatment are indicated in each panel. In all experiments, Ang II treatment was applied at a concentration of 1 μM for 75 min in order to induce the interaction. Data shown is representative of (A) 3, (B) 6, (C) 3, (D) 4, (E) 5, (F) 5, (G) 4 or (H) 3 co-immunoprecipitation and western blot experiments. Corresponding quantification analyses of inhibitor co-IP experiments are depicted in Supplemental Figure 4.3.

Extending these studies to HEK 293F cells, we observed a similar reliance on Gαq signalling for the agonist-induced Gβγ-RNAPII interaction. The carbachol-induced Gβγ-RNAPII interaction was prevented by pre-treatment with the Gαq inhibitor FR900359 (Figure 4.3A and Supplemental Figure 4.4A) and also by CRISPR/Cas9-mediated knockout of Gαq/11/12/13 (Figure 4.3B and Supplemental Figure 4.4B). However, except for this common event, the signalling pathways in cardiac fibroblasts and HEK 293F cells diverged substantially. In HEK 293F cells, U71322 also blocked the carbachol-induced Gβγ-RNAPII interaction but there was a pronounced increase in the basal interaction (Figure 4.3C, Supplemental Figure 4.4C). Further differences were observed following chelation of calcium with BAPTA-AM which increased basal levels of the Gβγ-RNAPII interaction but did not block further carbachol-induced stimulation of the interaction (Figure 4.3D, Supplemental Figure 4.4D), suggesting a modulatory role for calcium in HEK 293F cells rather than the direct role seen in cardiac fibroblasts. HEK 293F cells employed different regulatory mechanisms involving protein kinases activated downstream of Gαq/11-coupled GPCRs compared to cardiac fibroblasts. For example, the PKC inhibitor Gö6983 and the CaMKII inhibitor KN-93 both increased basal levels of interaction but did not block carbachol-induced interactions between Gβγ and Rpb1 (Figure 4.3E, F, Supplemental Figure 4.4E, F). Indeed, inhibition of calcineurin with cyclosporin A blocked the carbachol-mediated increase in interaction between Gβγ and Rpb1, suggesting a role for this phosphatase in mediating the interaction in response to M3-mAChR activation (Figure 4.3G and Supplemental Figure 4.4G). While the requirement for activation of Gαq is common for the Gβγ-RNAPII interaction in both cell types, the regulation by downstream signalling pathways diverges.

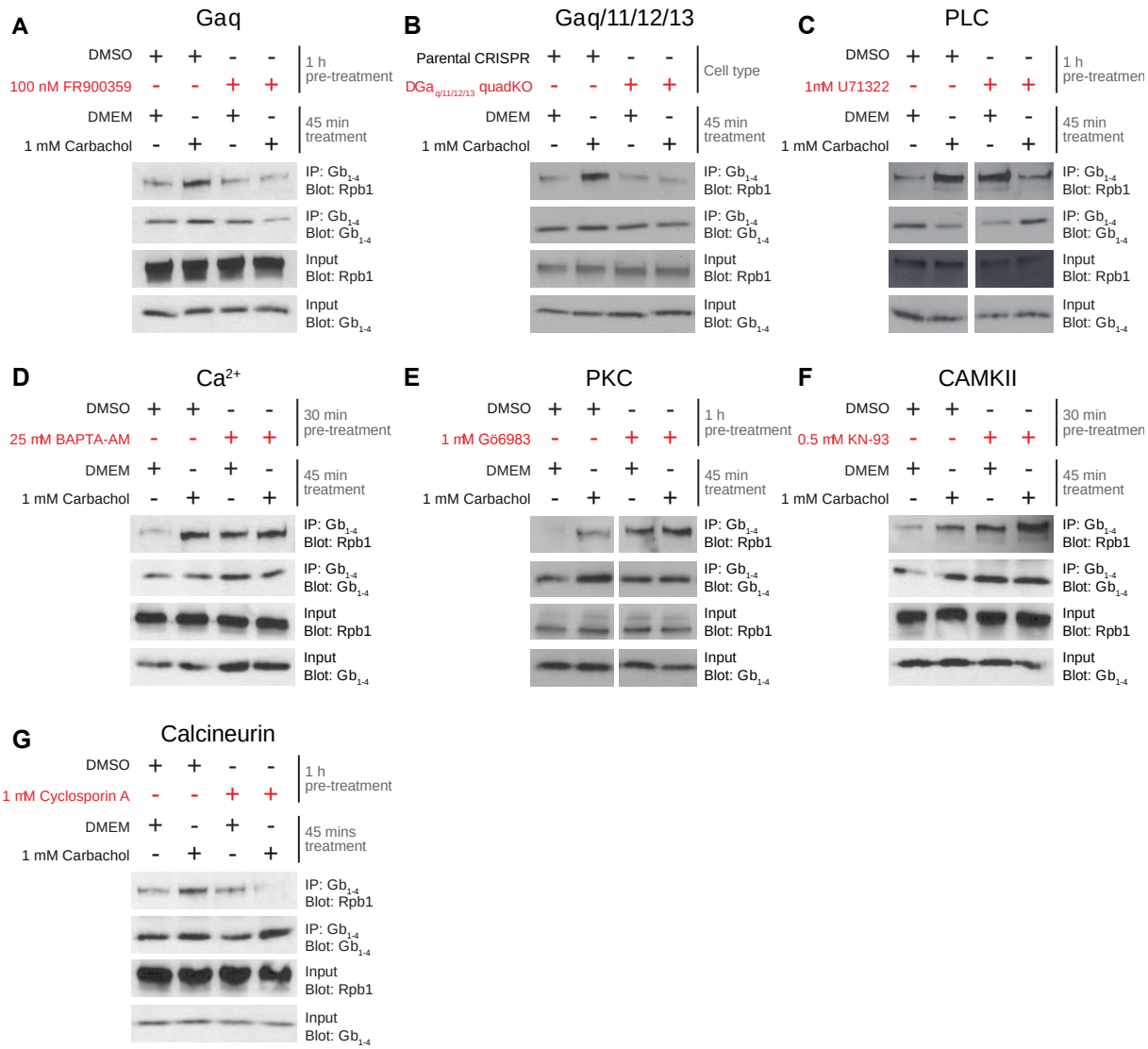


Figure 4.3. Mechanistic analysis of carbachol-induced Gβγ interaction occurs in HEK 293 cells.

(A-G) HEK 293 cells were starved for 10-12 hours in DMEM without FBS and were then pre-treated with the indicated inhibitors for the indicated times. Cells were subsequently treated with 1 mM carbachol for 45 min, and the amount of Rpb1 co-immunoprecipitated with $G\beta_{1-4}$ was assessed by western blot. Data shown is representative of (A) 3, (B) 4, (C) 5, (D) 3, (E) 3, (F) 6 or (G) 4 independent co-immunoprecipitation and western blot experiments. The associated quantifications of the co-IPs are represented in Supplemental Figure 4.4.

4.4.3. Roles of individual Gβ subunits in regulating the angiotensin II-activated fibrotic response in rat neonatal cardiac fibroblasts

The Gβ family is comprised of five members which, with the exception of $G\beta_5$, exhibit high levels of sequence and structural similarity (15). Despite these similarities, Gβ isoforms differ considerably with respect to their associated receptors and signalling pathways (23, 30, 31). As our above-reported characterization used a pan-$G\beta_{1-4}$ antibody, we next sought to examine the specificity of Gβ isoforms interacting with Rpb1 in cardiac fibroblasts. We initially focused on $G\beta_1$ and $G\beta_2$ as they exhibit the highest expression in cardiac fibroblasts determined by RNA-seq (32) and RT-qPCR (Supplemental Figure 4.5A). Immunoprecipitation with a $G\beta_1$ specific antibody revealed an increase in the amount of Rpb1 co-immunoprecipitated in response to Ang II treatment, whereas immunoprecipitation of $G\beta_2$ indicated a basal interaction with Rpb1 that was lost in response to Ang II treatment (Supplemental Figure 4.5B). We also assessed Gβ isoform specificity in HEK 293F cells through heterologous expression of FLAG-tagged versions of each Gβ subunit. In response to M3-mAChR activation, FLAG-$G\beta_1$ was the only isoform that showed an increased interaction with Rpb1 (Supplemental Figure 4.5C, D). Hence, an increased interaction between $G\beta_1$ and Rpb1 was seen in both cell types, suggesting that our earlier observations using the pan-$G\beta_{1-4}$ antibody likely reflected increased interactions with $G\beta_1$.

As we observed isoform-specific roles in RNAPII interactions, we next assessed how knockdown of either Gβ isoform affected the interaction. We first validated knockdown conditions for each Gβ subunit by siRNA at the mRNA and protein levels (Supplemental Figure 4.6A, B). We observed a reduction in the Ang II-induced Gβγ-RNAPII interaction upon knockdown of $G\beta_1$, supporting $G\beta_1$ as the isoform involved in the increased interaction with Rpb1. Surprisingly, knockdown of $G\beta_2$ also prevented the Ang II-mediated increase in the Gβγ-RNAPII interaction (Figure 4.4A, B). The loss of Gβγ-RNAPII interaction after $G\beta_2$ knockdown, despite it not being involved in the Ang II-dependent increase, suggested that AT1R signalling could be altered by loss of $G\beta_2$ subunits.

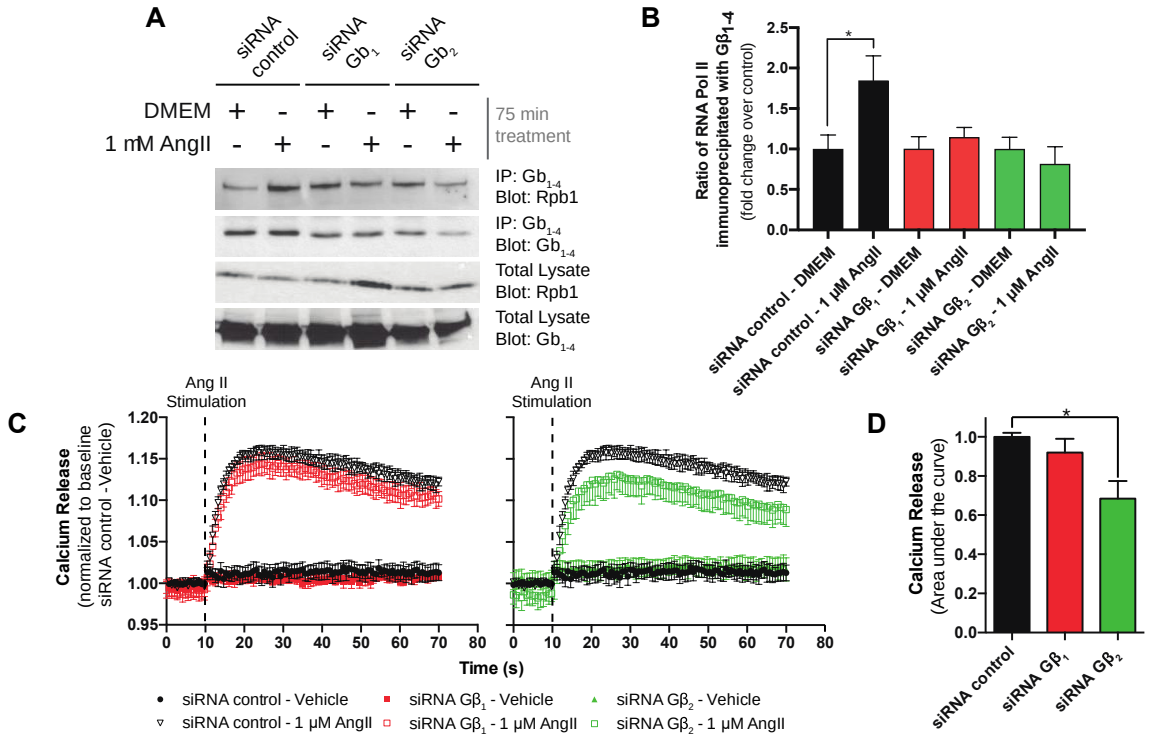


Figure 4.4. Gβ subunit-specific effects on Ang II signalling and induction of Rpb1 interaction. **(A)** Assessment of the effect of Gβ subunit knockdown by siRNA on the Gβγ-RNAPII interaction upon AT1R stimulation. Cardiac fibroblasts were transfected with siRNA control or siRNA to knockdown $G\beta_1$ or $G\beta_2$ and were then serum-deprived overnight before treatment with Ang II for 75 min. Cells were assessed for Gβγ-RNAPII interaction by co-immunoprecipitation and western blots. Data represents mean ± S.E.M. of 6-7 independent experiments. **(B)** Densitometry-based quantification of knockdown experiments in (C) were normalized as fold change over the respective siRNA-DMEM condition; data represents mean ± S.E.M. of six independent experiments. Two-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test was performed. **(C)** Traces of calcium release upon AT1R stimulation with Ang II at the 10 s time point, with or without knockdown of either $G\beta_1$ or $G\beta_2$. Data represents mean ± S.E.M. of fluorescence ratios of 340/516 emission readings to 360/516 emissions readings normalized to basal ratios of three independent experiments. **(D)** Area under the curve analysis of the data obtained in panel A. One-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test was performed. * indicates $p<0.05$.

We thus determined whether specific Gβ isoforms were required to initiate signalling cascades proximal to AT1R activation. Following receptor activation, Gβγ subunits regulate intracellular Ca^{2+} mobilization through activation of PLCβ (33). As we have previously demonstrated Gβ isoform specificity for PLCβ signalling in HEK 293F cells (23), we assessed the relative roles of $Gβ_1$ and $Gβ_2$ in AT1R-dependent Ca^{2+} mobilization. To assess AT1R-dependent intracellular Ca^{2+} mobilization, we used the cell-permeable Ca^{2+} dye Fura 2-AM. Following AT1R activation, we observed a rapid increase in intracellular Ca^{2+} mobilization (Figure 4.4A, black, empty triangles) and the quantified area under the curve (Figure 4.4B, black bar). Knockdown of $Gβ_1$ did not alter Ca^{2+} mobilization following stimulation with Ang II (8.1 ± 7.0% decrease, red bar). However, knockdown of $Gβ_2$ resulted in a significant 31.6 ± 9% decrease in Ca^{2+} release (Figure 4.4A, B, green bar), suggesting a role for $Gβ_2$-containing Gβγ dimers in mediating receptor-proximal signalling downstream of AT1R activation. This suggests $Gβ_2$ knockdown prevented the Ang II-dependent increase in Gβγ-RNAPII interaction through disruption to AT1R Ca^{2+} signalling, aligning with the observed effect of Ca^{2+} chelation with BAPTA-AM. These results highlight the complex interplay between cell surface receptors and multiple Gβγ subunits, in modulating both basal and ligand stimulated RNAPII/Gβγ interactions.

4.4.4. Gβγ interacts with transcribing RNAPII

As we demonstrated that Gβγ is recruited to RNAPII following AT1R activation, which also activates a transcriptional program in fibroblasts, we assessed the relationship between the transcriptional response and Gβγ recruitment (32, 34). To assess this potential relationship, we disrupted the transcription cycle at two different regulatory points through inhibition of Cdk7, a component of the general transcription factor TFIIH, and Cdk9, the protein kinase subunit of P-TEFb (35). Following RNAPII recruitment, Cdk7 activity stimulates promoter clearance of RNAPII to begin transcription. Soon after RNAPII pauses at a promoter-proximal region and requires the activity of Cdk9 in order to be released into productive elongation (36). We assessed involvement of both Cdk7 and Cdk9 on the Ang II-induced Gβγ-RNAPII interaction using the selective inhibitors THZ1 and iCdk9, respectively (37, 38). THZ1 abrogated the Ang II-stimulated Gβγ-RNAPII interaction (Figure 4.5A, Supplemental Figure 4.7A) while iCdk9 resulted in a loss of both the basal and Ang II-stimulated Gβγ-RNAPII interaction (Figure 4.5B, Supplemental Figure 4.7B). This suggests that the Gβγ-RNAPII interaction requires the transcriptional response

to Ang II in cardiac fibroblasts. As with cardiac fibroblasts, in HEK 293F cells disruption of the transcriptional cycle through inhibition of Cdk7 and Cdk9 with DRB also blocked the increased interaction between RNAPII and Gβγ (data not shown), showing that the Gβγ/RNAPII interaction is dependent on an active transcriptional response in both cell types.

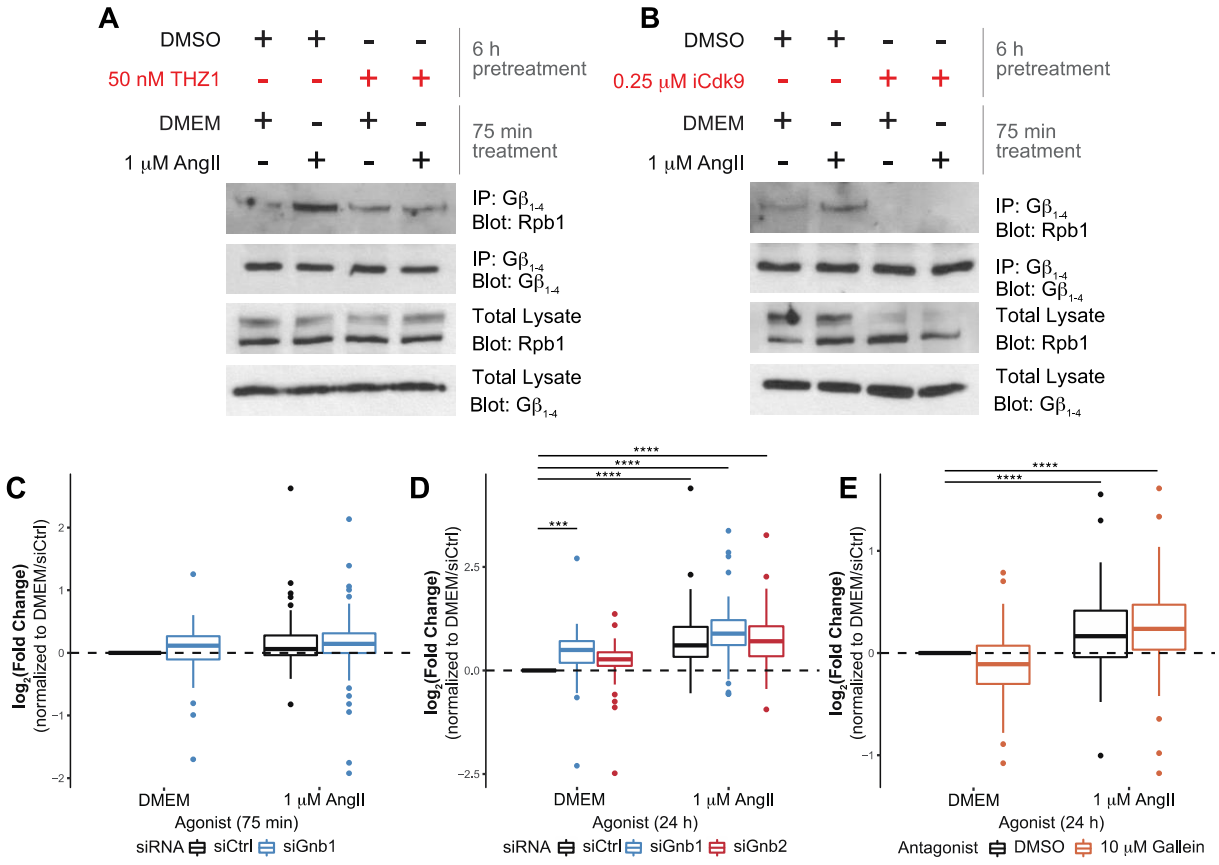


Figure 4.5. Requirement of RNAPII transcription for Gβγ-RNAPII interaction in rat neonatal cardiac fibroblasts.

Effect of Cdk7 inhibition with THZ1 **(A)** or Cdk9 inhibition with iCdk9 **(B)** on Ang II-induced Gβγ-RNAPII interaction. Length of inhibitor pre-treatment is indicated in each respective panel, and the extent of Gβγ-RNAPII interaction was assessed by co-immunoprecipitation coupled to western blot analysis. Data is representative of three independent experiments. Corresponding quantification analyses of inhibitor co-immunoprecipitation experiments are depicted in Supplemental Figure 7. Cumulative $\log_2$(Fold Change) of all genes detected by qPCR-based fibrosis array following treatment with 1 μM Ang II lasting either 75 min **(C)** or 24 h **(D and E)**. Cardiac fibroblasts were transfected with 50 nM of the indicated siRNA, and were serum-deprived for 12 h before Ang II treatment for the indicated times. Cardiac fibroblasts were pre-treated for

30 min with 10 μM gallein prior to Ang II. Ct values were normalized to the housekeeping genes Hprt1 and Ldha and the $\log_2$(fold change) over control was determined. For each gene, the average $\log_2$(fold change) across three independent experiments was plotted. Two-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test was performed. *** indicates $p<0.001$, **** indicates $p<0.0001$.

4.4.5. The role of Gβγ subunits in fibrotic gene expression

In order to understand the role of Gβγ in Ang II-regulated gene expression, we examined changes in the levels of 84 genes involved in the fibrotic response using the Qiagen RT^2 Profiler^{TM} PCR array platform. Gene expression changes were assessed following 75 min or 24 h Ang II treatment alongside Gβ1 or Gβ2 knockdown. These two time points were selected to investigate the effect of disrupting the Gβγ-RNAPII interaction or, in the longer term, upstream signalling, respectively. We assessed gene expression changes across all 67 genes remaining after excluding genes below our chosen threshold of detection (i.e. Ct > 35). After 75 min of Ang II treatment, we observed a similar upregulation of fibrotic genes in both control and $G\beta_1$ knockdown conditions (Figure 4.5C, Table 1). However, $G\beta_1$ knockdown increased both basal expression and the total number of genes altered by AT1R stimulation (Figure 4.5C, Table 1). Following 24 h Ang II treatment, this effect became more pronounced. $G\beta_1$ knockdown led to increases in basal gene expression, expression regulated by Ang II treatment and the overall number of genes upregulated (Figure 4.5D, Table 1). The increased expression following $G\beta_1$ knockdown suggests the Gβγ-RNAPII interaction negatively modulates the Ang II transcriptional response.

Table 4.1. Summary of fibrosis RT-qPCR array results.

This table summarizes gene expression changes measured using the Qiagen RT^2 Profiler^{TM} PCR Array at 75 min and 24 h Ang II stimulation. Genes were considered to have altered expression with fold changes ≥ 1.5 or ≤ 0.5 compared to DMEM/siRNA control conditions at the respective time point. In parenthesis are the number of genes with a significant ($p<0.05$) change in expression compared to DMEM/siRNA control at the respective time point. Two-way ANOVA followed by post-hoc t-test comparisons with Bonferroni correction was performed for each gene individually. Data is representative of three independent biological replicates.

Time	siRNA	Treatment	Upregulated	Downregulated
75 min	Control	DMEM	0	0
		1 μM Ang II	7 (5)	0
	Gnb1	DMEM	4 (1)	1
		1 μM Ang II	10 (6)	2
24 h	Control	DMEM	0	0
		1 μM Ang II	37 (7)	0
	Gnb1	DMEM	26 (2)	1
		1 μM Ang II	53 (13)	0
	Gnb2	DMEM	7	1
		1 μM Ang II	44 (11)	0

Whereas Gβ1 knockdown altered the transcriptional response to Ang II treatment, disruption of AT1R signalling by $G\beta_2$ knockdown did not significantly alter basal fibrotic gene expression or the overall response to 24 h Ang II treatment (Figure 4.5D, Table 1). The lack of effect of $G\beta_2$ knockdown suggests that Gβγ signalling through Ca^{2+} is not required for AT1R-mediated transcriptional changes. To further address the role of Gβγ signalling, we utilized the small-molecule pan-Gβγ inhibitor gallein (39). As with $G\beta_2$ knockdown, pre-treatment with gallein did not significantly alter the transcriptional response following 24 h Ang II treatment (Figure 4.5E). This suggests that Gβγ-dependent signalling downstream of the AT1R is not a key driver of transcriptional changes. Instead, Gβγ is required to modulate processes driven by other signalling pathways and dampen the fibrotic response until such signals rise above a threshold.

4.4.6. Genome-wide recruitment of $G\beta_1$ and the effect on RNAPII occupancy following Ang II treatment

To assess the possibility of genome-wide $G\beta_1$ recruitment and changes in RNAPII occupancy following 75 min Ang II treatment in cardiac fibroblasts, we performed chromatin immunoprecipitation followed by next generation sequencing (ChIP-seq) for heterologously expressed FLAG-$G\beta_1$ and endogenous Rpb1. We confirmed that, like endogenous $G\beta_1$, the interaction of Rbp1 with heterologously expressed FLAG-$G\beta_1$ increased following AT1R activation (Supplemental Figure 4.8A, B). We focused on genes with RNAPII peaks identified by

the peak calling software macs2 and annotated with HOMER (40, 41). The same $G\beta_1$ knockdown conditions that increased the number of genes upregulated in response to Ang II (above) also increased the number of genes occupied by RNAPII following Ang II treatment (Figure 4.6A). To identify groups of genes with similar FLAG-$G\beta_1$ and RNAPII occupancy patterns, we performed K-means clustering with genes that RNAPII peaks were identified in any treatment condition. Two K-means clusters were identified (98 genes in cluster 1 and 806 in cluster 2) with distinct occupancy patterns (Figure 4.6B, C). In cluster 1, FLAG-$G\beta_1$ occupancy increased within the gene body in response to Ang II. A similar but weaker tendency was also observed in cluster 2 (Figure 4.6B). The increased FLAG-$G\beta_1$ occupancy in cluster 1 corresponded to $G\beta_1$-dependent changes to the Ang II-induced RNAPII occupancy alterations. First, Ang II treatment led to increased RNAPII occupancy throughout the gene body under siRNA control conditions (Figure 4.6C). In the absence of Ang II, $G\beta_1$ knockdown increased RNAPII occupancy near transcription start sites (TSSs) which corresponds with increased gene expression under these conditions (Figure 4.6C). Lastly, there was greater RNAPII occupancy when Ang II treatment was combined with $G\beta_1$ knockdown than in the absence of knockdown (Figure 4.6C). Similar RNAPII occupancy patterns were observed in cluster 2, suggesting that $G\beta_1$ also plays a regulatory role along these genes and our FLAG-$G\beta_1$ ChIP-seq was not sensitive enough to reliably detect $G\beta_1$. We also assessed the functional pathways enriched in cluster 1, through gene ontology (GO) term enrichment. The top four significant GO terms identified (corresponding to cellular processes such as inflammation, fibroblast activation and apoptosis) indicate that $G\beta_1$ is recruited to genes involved in processes essential to fibrosis (Figure 4.6D).

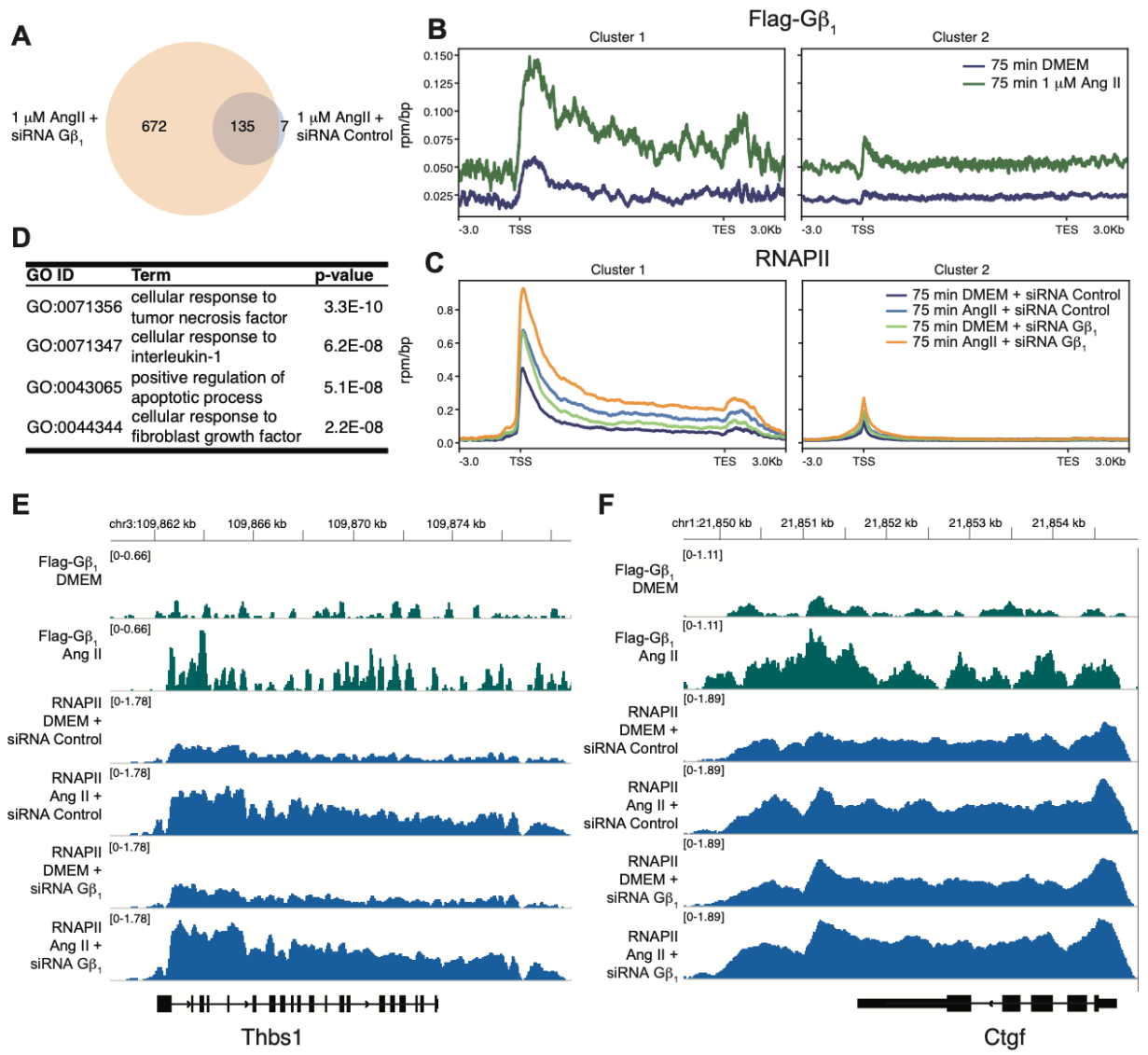


Figure 4.6. ChIP-seq for FLAG-Gβ$_1$ and Rpb1 following 75 min Ang II treatment in cardiac fibroblasts.

Cardiac fibroblasts were transduced with AAV1-FLAG-Gβ$_1$ or transfected with the indicated siRNA followed by Ang II treatment (1 μM for 75 min). **(A)** Comparison of genes with annotated RNAPII peaks following Ang II treatment and siRNA control or Gβ$_1$. FLAG-Gβ$_1$ (**B**) or the Rpb1 subunit of RNAPII **(C)** were immunoprecipitated from crosslinked and sonicated chromatin, followed by DNA purification and next-generation sequencing. Reads were normalized to an exogenous *S. pombe* chromatin spike-in. Genes with a RNAPII peak annotated by HOMER in two of the three replicates were used to identify two K-means clusters. **(D)** Top four significant GO

terms enriched in cluster 1. Individual FLAG-Gβ_1 or RNAPII tracks for two genes from cluster 1, **(E)** Thbs1 or **(F)** Ctgf.

The increased number of genes with RNAPII occupancy in the Ang II and Gβ_1 knockdown condition suggested that Gβ_1 occupancy impairs RNAPII recruitment. As such, we would expect cluster 1 genes to be more enriched in genes with RNAPII occupancy under Ang II and Gβ_1 knockdown condition than Ang II and siRNA control conditions. Therefore, we performed a Fisher's exact test to compare the proportion of cluster 1 genes in these treatment conditions, which demonstrated a significant (p-value < 0.01) enrichment in the Ang II and Gβ_1 knockdown condition gene list compared to Ang II and siRNA control condition. This again suggests Gβ_1 functions to suppress RNAPII transcription following AT1R activation.

In order to assess the relationship between Gβ_1 occupancy and transcription, we focused on genes from our fibrosis qPCR array that were also found in cluster 1. Eight genes from the fibrosis array were identified in cluster 1, which included five of the seven genes upregulated after 75 min of Ang II treatment such as thrombospondin 1 (Thbs1) and connective tissue growth factor (Ctgf) (Figure 4.6E, F). We confirmed the Ang II-dependent increase in Gβ_1 occupancy along Ctgf by ChIP-qPCR (Supplemental Figure 4.8C). We also assessed the effect of Gβ_1 knockdown on AT1R-dependent changes of RNAPII occupancy along Ctgf by ChIP-qPCR. Similar to our ChIP-seq analysis, we observed a greater increase in RNAPII along the gene in response to Ang II under siRNA GNB1 knockdown compared to siRNA control, where we observed a slight decrease (Supplemental Figure 4.8D). We also validated the change in expression of Ctgf by RT-qPCR using primers designed in-house (Supplemental Table 4.1). Under control conditions Ang II had a minor effect on Ctgf expression, however in the absence of Gβ_1 Ang II treatment resulted in a significant upregulation of Ctgf mRNA. (Supplemental Figure 4.8E). Taken together, our results demonstrate Gβ_1 recruitment negatively regulates expression of genes involved in the fibrotic response to Ang II by inhibiting early stages of the RNAPII transcription cycle.

4.5. Discussion

The functional specificity of Gβ and Gγ subunits has been mostly investigated in the context of signalling proximal to GPCR activation (i.e., the regulation of effector activity downstream of receptor stimulation) (15). In contrast, our findings provide new insights regarding non-canonical

roles of specific Gβγ dimers in more distal events in the nucleus, particularly in the regulation of gene expression. Here, we demonstrate for the first time an interaction between Gβγ and RNAPII and investigate the regulatory signalling mechanisms in transformed cell lines (HEK 293 cells) and in primary cells (neonatal rat cardiac fibroblasts). The interaction of Gβγ and RNAPII represents a significant addition to the expanding list of Gβγ interactors, and our findings suggest that regulatory mechanisms impacting the interaction are dependent on cellular context. We also show that Gβγ signalling is a critical regulator of the fibrotic response in cardiac fibroblasts.

Our findings suggest that following acute treatment with Ang II, $G\beta_1$ is transiently recruited to pro-fibrotic genes to negatively regulate RNAPII recruitment, thereby limiting the fibrotic response following transient fluctuations in local Ang II concentrations likely seen in vivo. This negative RNAPII regulation may potentially occur through direct interactions with RNAPII, preventing its recruitment or other aspects of initiation, or else via an indirect mechanism in which Gβγ would form part of a larger RNAPII-containing complex altering the local chromatin landscape. We cannot currently distinguish between these two possibilities, given that our co-immunoprecipitation assay was performed using whole-cell lysates. On the other hand, chronic stress or damage to the heart leads to a sustained increase of Ang II concentrations in cardiac tissue (42, 43). We propose that such sustained AT1R signalling overcomes the transient $G\beta_1$ "brake" to elicit a robust fibrotic response. Alternatively, pro-fibrotic factors that are upregulated and secreted following AT1R activation may elicit autocrine signalling pathways that overcome the $G\beta_1$ transcriptional repression (11, 44). Our gene expression data at 75 min, and more especially at 24 h, begins to identify the increased number and greater gene expression in the absence of the proposed negative regulatory mechanism when $G\beta_1$ is knocked down. Further analysis of the kinetics of the interaction and how this changes the dynamics of chromatin occupation or gene expression are required as well. Future experiments assessing nascent RNA production are required to accurately determine gene expression changes at early time points.

We demonstrated that $G\beta_2$, and not $G\beta_1$, was important for proximal signalling downstream of AT1R activation similar to the requirement of specific Gβ isoforms for activation of PLCβ in HEK 293 cells (23). Our data suggest that $G\beta_2$ plays a minimal role in regulating AT1R-dependent gene expression *per se*. Rather, our findings using the broad-spectrum Gβγ inhibitor gallein suggest that receptor-proximal Gβγ signalling in general is not required for the transcriptional response and instead it is dependent on Gαq signalling and more distal $G\beta_1$-dependent events.

Knockdown of $G\beta_2$ also compromised Ang II-mediated interactions between Gβγ and RNAPII even though $G\beta_2$ had a limited role in the fibrotic transcriptional response. This suggests $G\beta_2$ knockdown does not prevent the response but rather alters the kinetics of Gβγ-RNAPII interactions, which then translates into different fibrotic responses over time. Further, the roles of specific Gγ subunits in mediating proximal signal transduction must also be considered as for other Gβγ effectors (23), and should be the subject of future studies. Taken together, our findings suggest that in fibrosis and potentially in other diseases, the indiscriminate targeting of Gβγ signalling (e.g. with compounds such as gallein) will result in outcomes that differ considerably from those obtained by targeting particular Gβγ combinations (45-47).

Analysis of the signalling networks regulating the Gβγ/RNAPII interaction yielded four main conclusions: (1) different GPCR signalling systems in distinct cell types lead to different kinetics of the Gβγ-RNAPII interaction, (2) different signalling pathways downstream of GPCR activation act to both induce or modulate the interaction, (3) Gαq-coupled GPCRs regulate the interaction in both cell types examined, and (4) signalling ultimately converged on activation of transcription. Indeed, our results suggest that the cell context plays a critical role in determining the mechanism by which the Gβγ-RNAPII interaction is regulated. First, in cardiac fibroblasts, the Gβγ/RNAPII interaction depended on a Gq-PLCβ-Ca^{2+}-CaMKII/PKC/MEK-dependent pathway downstream of AT1R activation, whereas calcineurin acted as a basal negative regulator (summarized in Figure 4.7). On the other hand, in HEK 293 cells, we observed that the interaction was reliant on a Gq-PLCβ-Ca^{2+}-calcineurin pathway downstream of M3-mAChR activation, whereby PKC and CaMKII both negatively regulate this interaction under basal conditions (summarized in Figure 4.7). The involvement of Ca^{2+}, PKC and ERK1/2 in the induction of the Gβγ/RNAPII interaction in fibroblasts is supported by previous reports that demonstrate their involvement in Ang II-induced fibrosis (48, 49).

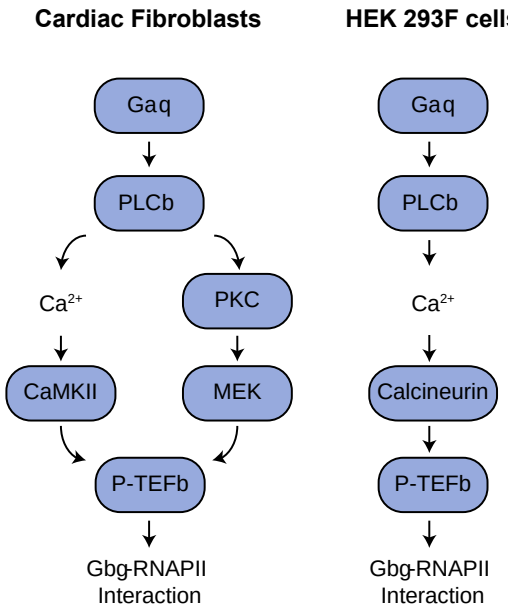


Figure 4.7. Schema summarizing signalling events regulating the agonist induced Gβγ interaction with RNAPII.

Signalling cascade downstream of AT1R in cardiac fibroblasts or M3 muscarinic receptors in HEK 293F cells regulating the interaction. Signalling pathways were determined by assessing Gβγ-RNAPII interactions by co-immunoprecipitation and western blot as shown in Figure 4.2 and Supplemental Figure 4.3 for cardiac fibroblasts and Figure 4.3 and Supplemental Figure 4.4 for HEK 293F cells.

The different signalling pathways promoting the Gβγ-RNAPII interaction appear to converge at the point of Cdk7 and Cdk9 activation. In particular, we found that the Cdk7 and Cdk9 inhibitors (DRB, THZ1 and iCdk9, respectively) inhibited both carbachol-induced Gβγ-RNAPII interaction in HEK 293 cells and the analogous Ang II-induced interaction in cardiac fibroblasts. This suggests the differential regulatory signalling pathways identified are due to cell type- and receptor-specific activation pathways of both Cdk7 and Cdk9. The recruitment of Gβγ serves as a common negative regulatory mechanism regardless of the pathway leading to transcriptional activation. Furthermore, a strong connection has been established between the control of transcriptional pausing and pathological cardiac remodelling, although primarily in the cardiomyocyte (50-55). Our results indicate that regulation of the early stages of the RNAPII transcription cycle is also an important checkpoint in the fibrotic response mediated by cardiac fibroblasts.

Taken together, the Gβγ-RNAPII interaction identifies a new mechanism by which Gβγ modulates gene expression. Our study highlights the complex interplay of different Gβγ subunit combinations at the cell surface and in the nucleus initiated upon stimulation of Gαq-coupled receptors. Since $G\beta_1\gamma$ dimers play an important role in regulating the expression of fibrotic genes in cardiac fibroblasts, the development of selective $G\beta_1\gamma$ inhibitors hold some promise for preventing the pathological consequences of myocardial damage.

4.6. Acknowledgements

We thank Dr. Ron Taussig (UT Southwestern), Dr. Nathanael S. Gray, and Novartis for providing materials instrumental to this study. We thank all the members of the Hébert and Tanny labs for discussion and critical reading of the manuscript.

4.7. Funding

This work was supported by a grant from the Heart and Stroke Foundation of Canada (G-15-0008938) to T.E.H and J.C.T, a grant from Canadian Institute of Health Science (CIHR) (MOP 130362) to J.C.T. and a grant from CIHR (PJT-159687) to T.E.H. R.M. and S.M.K were supported by studentships from the McGill-CIHR Drug Development Training Program and the McGill Faculty of Medicine.

4.8. Data Availability

The data is available at the NCBI Gene Expression Omnibus (GEO) with the accession GSE147416. All code used to analyze the ChIP-seq is available upon request.

4.9. References

1. Travers JG, Kamal FA, Robbins J, Yutzey KE, Blaxall BC. Cardiac Fibrosis: The Fibroblast Awakens. Circ Res. 2016;118(6):1021-40.
2. Fu X, Khalil H, Kanisicak O, Boyer JG, Vagnozzi RJ, Maliken BD, et al. Specialized fibroblast differentiated states underlie scar formation in the infarcted mouse heart. J Clin Invest. 2018;128(5):2127-43.

3. Dobaczewski M, Bujak M, Li N, Gonzalez-Quesada C, Mendoza LH, Wang XF, et al. Smad3 signaling critically regulates fibroblast phenotype and function in healing myocardial infarction. Circ Res. 2010;107(3):418-28.
4. Murphy AM, Wong AL, Bezuhly M. Modulation of angiotensin II signaling in the prevention of fibrosis. Fibrogenesis Tissue Repair. 2015;8:7.
5. Kawano H, Do YS, Kawano Y, Starnes V, Barr M, Law RE, et al. Angiotensin II has multiple profibrotic effects in human cardiac fibroblasts. Circulation. 2000;101(10):1130-7.
6. Campbell SE, Katwa LC. Angiotensin II stimulated expression of transforming growth factor-β1 in cardiac fibroblasts and myofibroblasts. J Mol Cell Cardiol. 1997;29(7):1947-58.
7. Rosenkranz S. TGF-β1 and angiotensin networking in cardiac remodeling. Cardiovasc Res. 2004;63(3):423-32.
8. Gao X, He X, Luo B, Peng L, Lin J, Zuo Z. Angiotensin II increases collagen I expression via transforming growth factor-β1 and extracellular signal-regulated kinase in cardiac fibroblasts. European Journal of Pharmacology. 2009;606(1–3):115-20.
9. Cheng T-H, Cheng P-Y, Shih N-L, Chen I-B, Wang DL, Chen J-J. Involvement of reactive oxygen species in angiotensin II-induced endothelin-1 gene expression in rat cardiac fibroblasts. Journal of the American College of Cardiology. 2003;42(10):1845-54.
10. Ahmed MS, Øie E, Vinge LE, Yndestad A, Øystein Andersen G, Andersson Y, et al. Connective tissue growth factor—a novel mediator of angiotensin II-stimulated cardiac fibroblast activation in heart failure in rats. Journal of Molecular and Cellular Cardiology. 2004;36(3):393-404.
11. Ma ZG, Yuan YP, Wu HM, Zhang X, Tang QZ. Cardiac fibrosis: new insights into the pathogenesis. Int J Biol Sci. 2018;14(12):1645-57.
12. Weber KT, Sun Y, Bhattacharya SK, Ahokas RA, Gerling IC. Myofibroblast-mediated mechanisms of pathological remodelling of the heart. Nat Rev Cardiol. 2013;10(1):15-26.
13. Weber KT, Diez J. Targeting the Cardiac Myofibroblast Secretome to Treat Myocardial Fibrosis in Heart Failure. Circ Heart Fail. 2016;9(8).
14. Namkung Y, LeGouill C, Kumar S, Cao Y, Teixeira LB, Lukasheva V, et al. Functional selectivity profiling of the angiotensin II type 1 receptor using pathway-wide BRET signaling sensors. Sci Signal. 2018;11(559).

15. Khan SM, Sleno R, Gora S, Zylbergold P, Laverdure J-P, Labbé J-C, et al. The Expanding Roles of Gβγ Subunits in G Protein–Coupled Receptor Signaling and Drug Action. Pharmacological Reviews. 2013;65(2):545-77.
16. Dupré DJ RM, Rebois RV, Hébert TE. The role of Gβγ subunits in the organization, assembly and function of GPCR signaling complexes. Annu Rev Pharmacol Toxicol. 2009;49:31-56.
17. Smrcka AV. G protein βγ subunits: central mediators of G protein-coupled receptor signaling. Cell Mol Life Sci. 2008;65.
18. Campden R, Audet N, Hébert TE. Nuclear G Protein Signaling: New Tricks for Old Dogs. Journal of Cardiovascular Pharmacology. 2015;65(2):110-22.
19. Park JG, Muise A, He GP, Kim SW, Ro HS. Transcriptional regulation by the γ5 subunit of a heterotrimeric G protein during adipogenesis. EMBO J. 1999;18(14):4004-12.
20. Robitaille M, Gora S, Wang Y, Goupil E, Petrin D, Del Duca D, et al. Gβγ is a negative regulator of AP-1 mediated transcription. Cellular signalling. 2010;22(8):1254-66.
21. Spiegelberg BD, Hamm HE. Gβγ binds histone deacetylase 5 (HDAC5) and inhibits its transcriptional co-repression activity. J Biol Chem. 2005;280(50):41769-76.
22. Bhatnagar A, Unal H, Jagannathan R, Kaveti S, Duan ZH, Yong S, et al. Interaction of G-protein βγ complex with chromatin modulates GPCR-dependent gene regulation. PLoS One. 2013;8(1):e52689.
23. Khan SM, Min A, Gora S, Houranieh GM, Campden R, Robitaille M, et al. Gβ4γ1 as a modulator of M3 muscarinic receptor signalling and novel roles of Gβ1 subunits in the modulation of cellular signalling. Cellular signalling. 2015;27(8):1597-608.
24. Kamal FA, Travers JG, Schafer AE, Ma Q, Devarajan P, Blaxall BC. G Protein-Coupled Receptor-G-Protein βγ-Subunit Signaling Mediates Renal Dysfunction and Fibrosis in Heart Failure. J Am Soc Nephrol. 2017;28(1):197-208.
25. Travers JG, Kamal FA, Valiente-Alandi I, Nieman ML, Sargent MA, Lorenz JN, et al. Pharmacological and Activated Fibroblast Targeting of Gβγ-GRK2 After Myocardial Ischemia Attenuates Heart Failure Progression. J Am Coll Cardiol. 2017;70(8):958-71.
26. Campden R, Pétrin D, Robitaille M, Audet N, Gora S, Angers S, et al. Tandem Affinity Purification to Identify Cytosolic and Nuclear Gβγ-Interacting Proteins. In: Allen BG,

Hébert TE, editors. Nuclear G-Protein Coupled Receptors. Methods in Molecular Biology. 1234: Springer New York; 2015. p. 161-84.

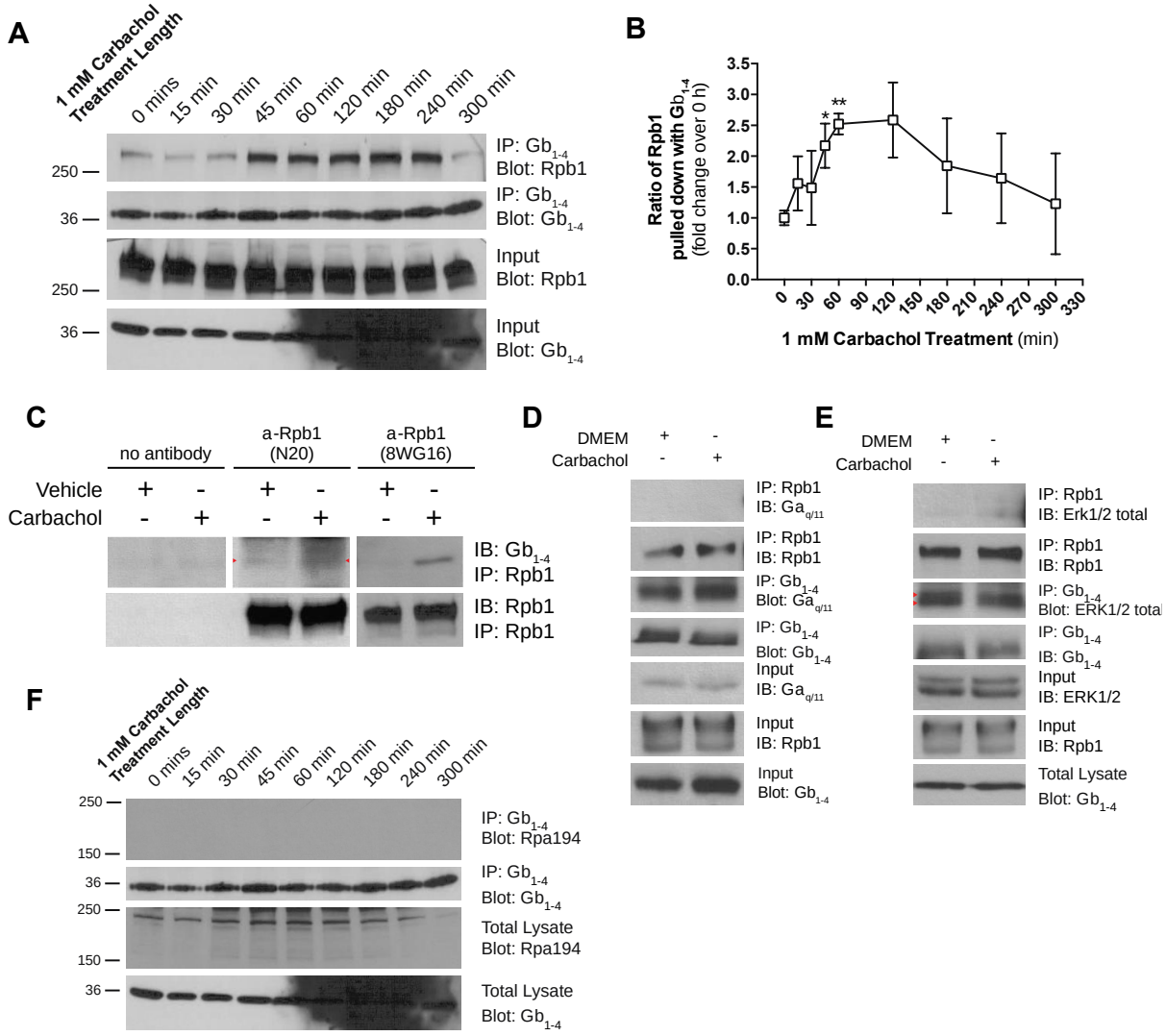
27. Zhang JH, Barr VA, Mo Y, Rojkova AM, Liu S, Simonds WF. Nuclear localization of G protein β 5 and regulator of G protein signaling 7 in neurons and brain. J Biol Chem. 2001;276(13):10284-9.
28. Sauliere A, Bellot M, Paris H, Denis C, Finana F, Hansen JT, et al. Deciphering biased-agonism complexity reveals a new active AT1 receptor entity. Nat Chem Biol. 2012;8(7):622-30.
29. Schrage R, Schmitz AL, Gaffal E, Annala S, Kehraus S, Wenzel D, et al. The experimental power of FR900359 to study Gq-regulated biological processes. Nat Commun. 2015;6:10156.
30. Yim YY, Betke KM, McDonald WH, Gilsbach R, Chen Y, Hyde K, et al. The in vivo specificity of synaptic Gβ and Gγ subunits to the alpha2a adrenergic receptor at CNS synapses. Sci Rep. 2019;9(1):1718.
31. Greenwood IA, Stott JB. The Gβ1 and Gβ3 Subunits Differentially Regulate Rat Vascular Kv7 Channels. Front Physiol. 2019;10:1573.
32. Shu J, Liu Z, Jin L, Wang H. An RNAsequencing study identifies candidate genes for angiotensin IIinduced cardiac remodeling. Mol Med Rep. 2018;17(1):1954-62.
33. Park D, Jhon DY, Lee CW, Lee KH, Rhee SG. Activation of phospholipase C isozymes by G protein β γ subunits. J Biol Chem. 1993;268(7):4573-6.
34. Dang MQ, Zhao XC, Lai S, Wang X, Wang L, Zhang YL, et al. Gene expression profile in the early stage of angiotensin II-induced cardiac remodeling: a time series microarray study in a mouse model. Cell Physiol Biochem. 2015;35(2):467-76.
35. Zhou Q, Li T, Price DH. RNA polymerase II elongation control. Annu Rev Biochem. 2012;81:119-43.
36. Liu X, Kraus WL, Bai X. Ready, pause, go: regulation of RNA polymerase II pausing and release by cellular signaling pathways. Trends Biochem Sci. 2015;40(9):516-25.
37. Lu H, Xue Y, Yu GK, Arias C, Lin J, Fong S, et al. Compensatory induction of MYC expression by sustained CDK9 inhibition via a BRD4-dependent mechanism. Elife. 2015;4:e06535.

38. Kwiatkowski N, Zhang T, Rahl PB, Abraham BJ, Reddy J, Ficarro SB, et al. Targeting transcription regulation in cancer with a covalent CDK7 inhibitor. Nature. 2014;511(7511):616-20.
39. Lehmann DM, Seneviratne AM, Smrcka AV. Small molecule disruption of G protein β γ subunit signaling inhibits neutrophil chemotaxis and inflammation. Mol Pharmacol. 2008;73(2):410-8.
40. Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, et al. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol Cell. 2010;38(4):576-89.
41. Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, et al. Model-based analysis of ChIP-Seq (MACS). Genome Biol. 2008;9(9):R137.
42. Sun Y, Weber KT. Angiotensin converting enzyme and myofibroblasts during tissue repair in the rat heart. J Mol Cell Cardiol. 1996;28(5):851-8.
43. Passier RC, Smits JF, Verluyten MJ, Daemen MJ. Expression and localization of renin and angiotensinogen in rat heart after myocardial infarction. Am J Physiol. 1996;271(3 Pt 2):H1040-8.
44. Lee AA, Dillmann WH, McCulloch AD, Villarreal FJ. Angiotensin II stimulates the autocrine production of transforming growth factor-β 1 in adult rat cardiac fibroblasts. J Mol Cell Cardiol. 1995;27(10):2347-57.
45. Lin Y, Smrcka AV. Understanding molecular recognition by G protein βγ subunits on the path to pharmacological targeting. Mol Pharmacol. 2011;80(4):551-7.
46. Kamal FA, Smrcka AV, Blaxall BC. Taking the heart failure battle inside the cell: small molecule targeting of Gβγ subunits. J Mol Cell Cardiol. 2011;51(4):462-7.
47. Smrcka AV, Lehmann DM, Dessal AL. G protein βγ subunits as targets for small molecule therapeutic development. Comb Chem High Throughput Screen. 2008;11(5):382-95.
48. Chintalgattu V, Katwa LC. Role of protein kinase C-δ in angiotensin II induced cardiac fibrosis. Biochemical and Biophysical Research Communications. 2009;386(4):612-6.
49. Olson ER, Shamhart PE, Naugle JE, Meszaros JG. Angiotensin II-Induced Extracellular Signal-Regulated Kinase 1/2 Activation Is Mediated by Protein Kinase Cδ and Intracellular Calcium in Adult Rat Cardiac Fibroblasts. Hypertension. 2008;51(3):704-11.

50. Yang J, Tian B, Brasier AR. Targeting Chromatin Remodeling in Inflammation and Fibrosis. Adv Protein Chem Struct Biol. 2017;107:1-36.
51. Sayed D, He M, Yang Z, Lin L, Abdellatif M. Transcriptional regulation patterns revealed by high resolution chromatin immunoprecipitation during cardiac hypertrophy. J Biol Chem. 2013;288(4):2546-58.
52. Anand P, Brown JD, Lin CY, Qi J, Zhang R, Artero PC, et al. BET bromodomains mediate transcriptional pause release in heart failure. Cell. 2013;154(3):569-82.
53. Duan Q, McMahon S, Anand P, Shah H, Thomas S, Salunga HT, et al. BET bromodomain inhibition suppresses innate inflammatory and profibrotic transcriptional networks in heart failure. Sci Transl Med. 2017;9(390).
54. Stratton MS, Lin CY, Anand P, Tatman PD, Ferguson BS, Wickers ST, et al. Signal-Dependent Recruitment of BRD4 to Cardiomyocyte Super-Enhancers Is Suppressed by a MicroRNA. Cell Rep. 2016;16(5):1366-78.
55. Sano M, Abdellatif M, Oh H, Xie M, Bagella L, Giordano A, et al. Activation and function of cyclin T-Cdk9 (positive transcription elongation factor-b) in cardiac muscle-cell hypertrophy. Nat Med. 2002;8(11):1310-7.
56. Devost D, Sleno R, Petrin D, Zhang A, Shinjo Y, Okde R, et al. Conformational Profiling of the AT1 Angiotensin II Receptor Reflects Biased Agonism, G Protein Coupling, and Cellular Context. J Biol Chem. 2017;292(13):5443-56.
57. Calderone A, Thaik CM, Takahashi N, Chang DL, Colucci WS. Nitric oxide, atrial natriuretic peptide, and cyclic GMP inhibit the growth-promoting effects of norepinephrine in cardiac myocytes and fibroblasts. J Clin Invest. 1998;101(4):812-8.
58. Burger C, Nash KR. Small-Scale Recombinant Adeno-Associated Virus Purification. Methods Mol Biol. 2016;1382:95-106.
59. Bolli P, Vardabasso C, Bernstein E, Chaudhry HW. Chromatin immunoprecipitation of adult murine cardiomyocytes. Curr Protoc Cell Biol. 2013;Chapter 17:Unit17 4.
60. Mbogning J, Tanny JC. Chromatin Immunoprecipitation of Histone Modifications in Fission Yeast. Methods Mol Biol. 2017;1528:199-210.
61. Krueger F. Trim Galore! [Available from: http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/.

62. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. 2011. 2011;17(1):3.
63. Zerbino DR, Achuthan P, Akanni W, Amode MR, Barrell D, Bhai J, et al. Ensembl 2018. Nucleic Acids Res. 2018;46(D1):D754-D61.
64. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25(16):2078-9.
65. Landt SG, Marinov GK, Kundaje A, Kheradpour P, Pauli F, Batzoglou S, et al. ChIP-seq guidelines and practices of the ENCODE and modENCODE consortia. Genome Res. 2012;22(9):1813-31.
66. Kharchenko PV, Tolstorukov MY, Park PJ. Design and analysis of ChIP-seq experiments for DNA-binding proteins. Nat Biotechnol. 2008;26(12):1351-9.
67. Liang K, Keles S. Normalization of ChIP-seq data with control. BMC Bioinformatics. 2012;13:199.
68. Ramdas S, Ozel AB, Treutelaar MK, Holl K, Mandel M, Woods LCS, et al. Extended regions of suspected mis-assembly in the rat reference genome. Sci Data. 2019;6(1):39.
69. Ramirez F, Dundar F, Diehl S, Gruning BA, Manke T. deepTools: a flexible platform for exploring deep-sequencing data. Nucleic Acids Res. 2014;42(Web Server issue):W187-91.

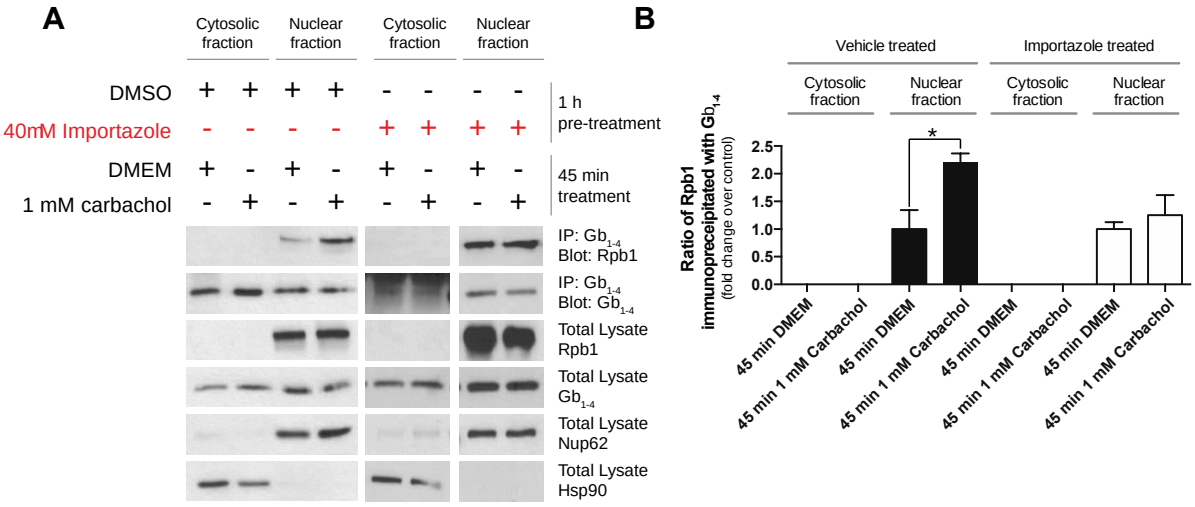
4.10. Supplemental Figures and Tables



Supplemental Figure 4.1. Induction of the Gβγ-RNAPII interaction in HEK 293 cells.

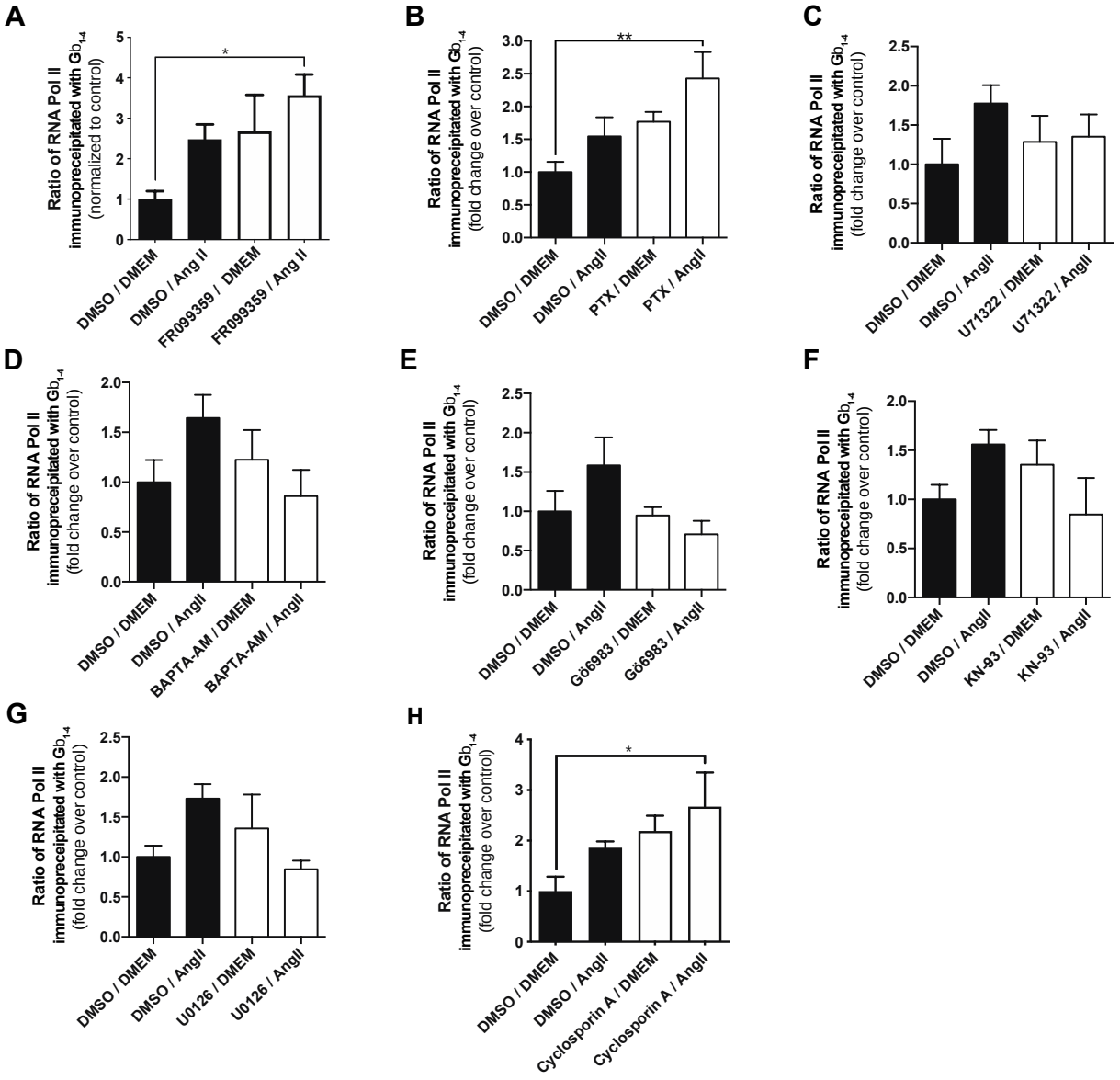
(A) Time-course analysis of the induction of the Gβγ-RNAPII interaction. The amount of Rpb1 co-immunoprecipitated with $G\beta_{1-4}$ from HEK 293 cells treated for the indicated times with 1 mM carbachol was assessed by western blot for each time point. Data is representative of three independent experiments. **(B)** Quantification of Gβγ-RNAPII time-course co-immunoprecipitation. Densitometry-based analysis of bands corresponding to Rpb1 at each timepoint was normalized to the band intensity of the amount of $G\beta_{1-4}$ immunoprecipitated to yield ratios of Rpb1 pulled down with $G\beta_{1-4}$. Two-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test was performed. **(C)** Assessing the Gβγ and Rpb1 interaction by immunoprecipitation of Rpb1 with two different antibodies. Western blots are representative of at

least two independent experiments. Immunoprecipitation experiments demonstrating that carbachol treatment does not induce interaction of Rpb1 with **(D)** $G\alpha_{q/11}$ nor **(E)** ERK1/2 in HEK 293 cells, and also does not alter the amount of $G\alpha_{q/11}$ or ERK1/2 interacting with Gβγ under such conditions. **(F)** Assessment of interaction between $G\beta_{1\text{-}4}$ and Rpa194, the largest subunit of RNA polymerase I. Data represents analysis of a time course experiment western blot performed as in **Supplemental Figure 4.1A**. Data represents mean ± S.E.M; * indicates $p<0.05$, ** indicates $p<0.01$.



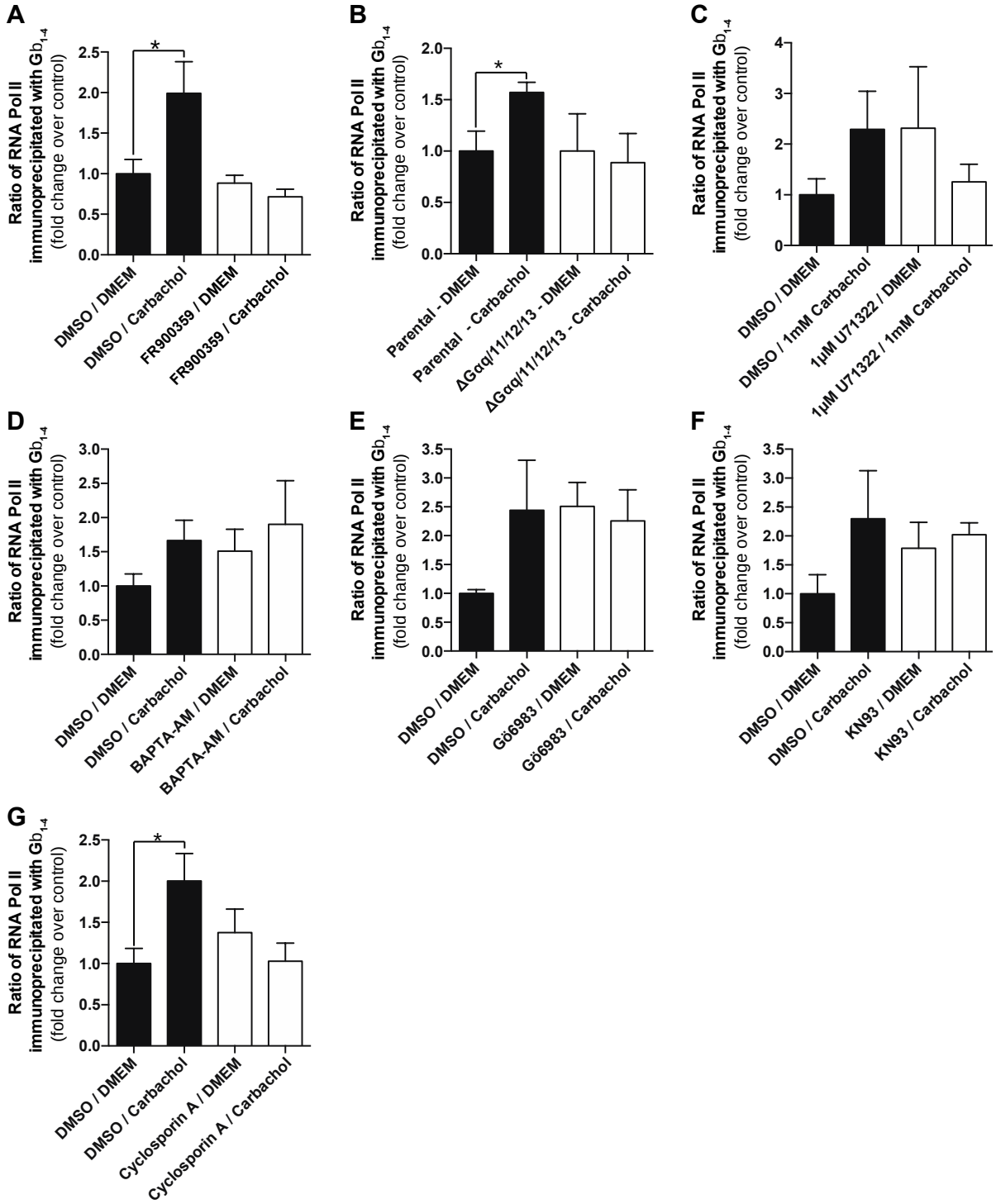
Supplemental Figure 4.2. Requirement for Gβγ nuclear transport for RNAPII interaction in HEK 293 cells.

(A) Representative experiment assessing the requirement of importin-β inhibition on the Gβγ-RNAPII interaction by sub-cellular fractionation and co-immunoprecipitation. **(B)** Densitometry-based quantification of the carbachol induced interaction and the effect of nuclear import inhibition on interaction induction. Data represents mean ± S.E.M. of three independent experiments for black bars, and two independent experiments for white bars (nuclear import inhibition conditions). Two-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test was performed. * indicates $p<0.05$.



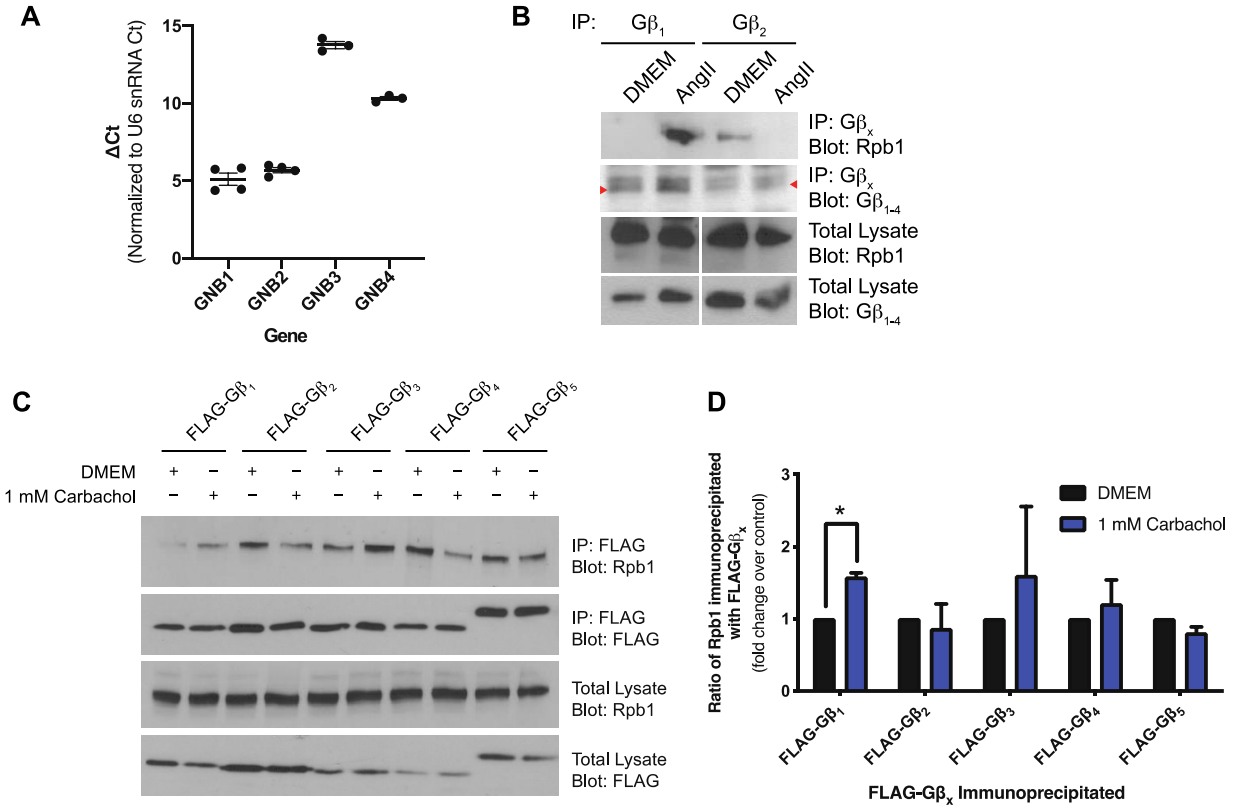
Supplemental Figure 4.3. Quantitative analysis of the effects of inhibition of signalling molecules downstream of AT1R activation.

(A-H) The relative quantities of Rpb1 co-immunoprecipitated with $G\beta_{1\text{-}4}$ under different conditions depicted in Figure 4.2 were quantified using ImageJ and normalized to DMSO/DMEM control conditions. Data shown is representative of (A) 3, (B) 6, (C) 3, (D) 4, (E) 5, (F) 5, (G) 4 or (H) 3 independent co-immunoprecipitation and western blot experiments. Data is represented as fold change over respective controls and error bars represent S.E.M. Two-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test was performed. * indicates $p<0.05$, ** indicates $p<0.01$.



Supplemental Figure 4.4. Quantitative analysis of the effects of inhibition of signalling molecules downstream of M3-mAChR activation in HEK 293 cells.

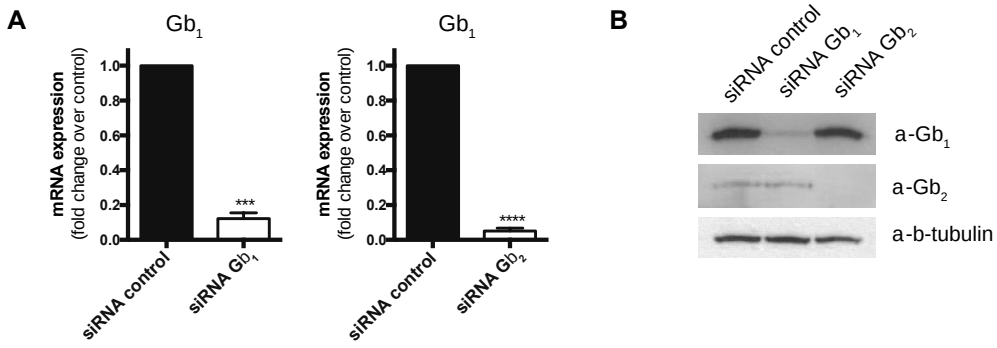
(A-G) The relative quantities of Rpb1 co-immunoprecipitated with $G\beta_{1-4}$ under different conditions depicted in Figure 4.3 were quantified using ImageJ and were normalized to amounts pulled down in DMSO/DMEM control conditions. Data shown is representative of (A) 3, (B) 4, (C) 5, (D) 3, (E) 3, (F) 6 or (G) 4 independent co-immunoprecipitation and western blot experiments. Data is represented as fold change over respective controls and error bars represent S.E.M. Two-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test was performed. * indicates $p<0.05$.



Supplemental Figure 4.5. Assessment of specific Gβ subunits interacting with RNAPII upon agonist stimulation in rat cardiac fibroblasts or in HEK 293 cells.

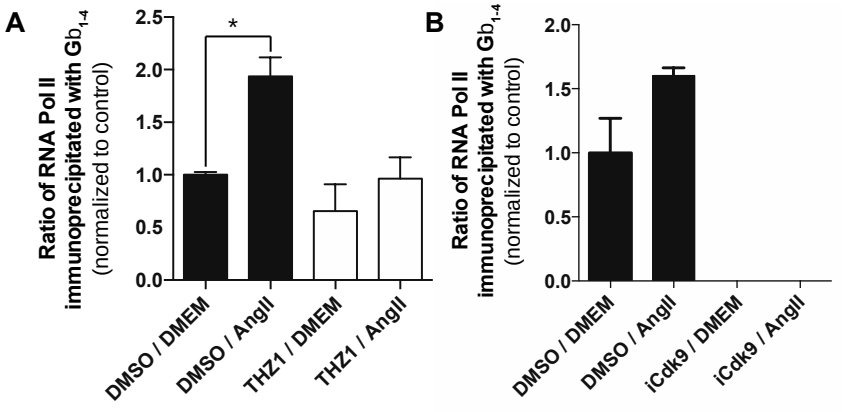
(A) Transcript levels for GNB1, GNB2, GNB3, and GNB4 were assessed in cardiac fibroblasts by RT-qPCR. The Ct values for each gene transcript were normalized to the house keeping U6 snRNA gene transcript for comparison. Data represents mean ± S.E.M for 3-4 independent experiments. **(B)** $G\beta_1$ and $G\beta_2$ were immunoprecipitated with isoform specific antibodies from cardiac fibroblasts lysates treated with 1 μM Ang II for 75 min. The amount of Rpb1 pulled down with either Gβ isoform was assessed by western blot. **(C)** Assessment of specific FLAG-tagged Gβ isoforms interaction with Rpb1 under conditions of M3-mAChR stimulation with carbachol in

HEK 293 cells. The amount of Rpb1 interacting with each Gβ isoform was assessed by western blot following FLAG immunoprecipitation. **(D)** Densitometry-based quantification of the ratio of Rpb1 co-immunoprecipitated with the indicated FLAG-tagged Gβ subunit. The ratio of Rpb1 to FLAG-$G\beta_x$ immunoprecipitated was determined and normalized to fold change over DMEM treatment. Data represents mean ± S.E.M for four independent replicates. One-sample t-tests were performed with a Bonferroni correction. * indicates $p<0.01$.



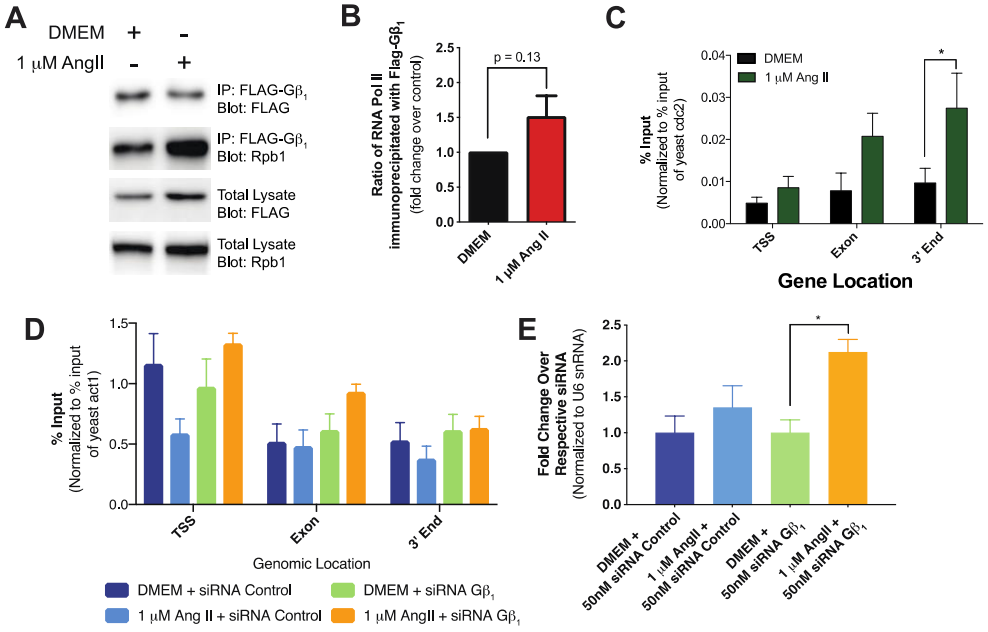
Supplemental Figure 4.6. Validation of RNAi knockdown of Gβ1 and Gβ2.

Validation of $G\beta_1$ and $G\beta_2$ mRNA **(A)** and protein **(B)** knockdown with siRNA in rat neonatal cardiac fibroblasts. Rat neonatal cardiac fibroblasts were transfected with 50 nM siRNA control, $G\beta_1$ or $G\beta_2$ for 72 hours, serum-deprived for 12 h and RNA or protein collected as described in *Methods*. Data in (A) represents mean ± S.E.M for four independent experiments; * Ct values were normalized to the housekeeping U6 snRNA transcript and fold change over siRNA control determined using the $2^{-\Delta\Delta Ct}$. Paired Student's t-tests were performed. ** indicates $p<0.001$ and **** indicates $p<0.0001$.



Supplemental Figure 4.7. Quantitative analysis of the effect of transcriptional regulator inhibition on the Gβγ-RNAPII interaction in cardiac fibroblasts.

(A-B) The relative quantities of Rpb1 co-immunoprecipitated with $G\beta_{1\text{-}4}$ under conditions depicted in Figure 5A (THZ1) and B (iCdk9) were quantified and normalized to DMSO/DMEM control conditions. Data shown is representative of between three to six independent co-immunoprecipitation and western blot experiments. Data represents mean ± S.E.M. Two-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test was performed. * indicates $p<0.05$.



Supplemental Figure 4.8. Validation of heterologously expressed FLAG-tagged Gβ1 in rat neonatal cardiac fibroblasts.

(A) Assessment of Rpb1 co-immunoprecipitated with FLAG-Gβ_1 following 75 min treatment of 1 μM Ang II in rat neonatal cardiac fibroblasts. Cardiac fibroblasts were transduced with AAV1-FLAG-Gβ_1 prior to treatment with 1 μM Ang II. **(B)** Densitometry-based quantification of the ratio of Rpb1 co-immunoprecipitated with FLAG-Gβ_1. The ratio of Rpb1 to FLAG-Gβ_1 was calculated and normalized as fold change over DMEM condition. Data is represented as mean ± S.E.M for four independent experiments. One-sample t-tests were performed with a Bonferroni correction. Assessing changes in FLAG-Gβ_1 **(C)** or **(D)** Rpb1 occupancy along Ctgf following 75 min treatment with 1 μM Ang II. FLAG-Gβ_1 or Rpb1 was immunoprecipitated from crosslinked and sonicated chromatin, DNA purified and quantified by qPCR. Data is represented as mean ± S.E.M for 4-6 independent experiments, * indicates $p<0.05$. For (C), paired Student t-tests with a Bonferroni post-hoc correction were performed. For (D), two-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test were performed for each genonic loci independently. **(E)** Validation of Ctgf gene expression with primers distinct from those used in the Qiagen RT^2 Profiler^{TM} PCR array. Data represents mean ± S.E.M for four independent experiments. Two-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test was performed. * indicates $p<0.05$.

Supplemental Table 4.1. List of primers used to assess gene expression by RT-qPCR and ChIP-qPCR in cardiac fibroblasts.

Forward and reverse primers were used at a concentration of 300 nM for each qPCR reaction. Primer sequences were designed using NCBI's Primer-BLAST tool and validated by analysis of standard curve qPCR assays performed in-house.

Target	Forward (5' -> 3')	Reverse (5' -> 3')
U6 snRNA	TGGAACGATACAGAGAAGATTAG	GAATTTGCGTGTCATCCTTG
Gβ_1	CTCATGACCTACTCCCATGA	TCAGCTTTGAGTGCATCC
Gβ_2	CAGCTACACCACTAACAAGG	CTCTCGGGTCTTGAGACTAT
Gβ_3	CTCCTTAGGGTCAGTCTTCTAT	AAAGGCACACTCCCATAATC
Gβ_4	GGTGGTCAAAGAAACAATCAAG	GTCTGTCGGGATAGGGATAA
Ctgf	TGCATCCTCCTACCGCGTCC	GAGGCTGATGGGACCTGCGA
Ctgf TSS	CAGACCCACTCCAGCTCCGA	GTGGCTCCTGGGGTTGTCCA

Ctgf Exon	TCAAGCTGCCCGGGAAATGC	GCGGTCCTTGGGCTCATCAC
Ctgf 3' End	AATGGCTTGCTCAGGGTAACTGG	AACTGCCTCCCAAACCAGTCATAG
$cdc2^{+}$	ATCATTCTCGCATCTCTATTA	ATTCTCCATTGCAAACCACTA
$act1^{+}$	GGTTGCTCAATGTTATCCGTTTC	TGATAAAGCCACACACAGCGTTA

CHAPTER 5: General Discussion

5.1. Contributions to scientific understanding

This thesis describes how GPCR signalling effectors regulate RNAPII transcription to elicit pathological gene expression programs. In order to develop therapeutic strategies targeting transcriptional regulators, a deeper understanding of how signalling pathways converge on these regulators is required. A nuanced view of how different GPCRs or effector isoforms regulate transcription will enable personalized therapies with specific inhibitor classes and perhaps spur the development of isoform-specific inhibitors. This body of work expands our understanding of regulatory links between GPCRs and the transcriptional machinery in cardiomyocytes and cardiac fibroblasts. First, comparison of transcriptome changes in cardiomyocytes following activation of two canonical Gαq-coupled GPCRs identified a non-canonical signalling pathway that differentially activates the P-TEFb/Brd4 complex. This indicates that a patient's neurohormonal profile may be an important criterion in determining the efficacy of BET inhibitors in cardiovascular disease. Second, assessment of the role of $G\beta_1\gamma$ dimers following AT1R activation in cardiac fibroblasts elucidated a negative regulatory role for the non-canonical Gβγ-RNAPII interaction in transcription. These findings highlight the potential utility of developing Gβ isoform- or effector-specific inhibitors to preserve the negative transcriptional regulation by $G\beta_1\gamma$.

In Chapter 2 (Martin *et al*., 2018), we described the subtype and compartment specific non-canonical signalling pathway downstream of α_1-AR activation. We aimed to identify signalling differences between the ETR and α_1-AR, two receptors whose pro-hypertrophic effects in cardiomyocytes are typically associated with Gαq activation. To uncover signalling differences in an unbiased manner, we assessed transcriptome changes following 1.5 h or 24 h receptor activation in neonatal rat cardiomyocytes. Pathway enrichment identified CREM target genes enriched in the differentially expressed genes following 24 h α_1-AR activation, and not ETR activation (Figure 2.1A). This corresponded to an increase in CREM expression at the 1.5 h time point (Figure 2.1B). As some CREM isoforms' expression and activity are regulated by cAMP, we hypothesized that the α_1-AR activated cAMP signalling in cardiomyocytes. To further characterize the non-canonical α_1-AR signalling pathway, we used a heterologous expression system with the α_1-AR and ETR isoforms pertinent to cardiomyocyte hypertrophy alongside genetically encoded FRET- and BRET-based biosensors in HEK 293SL cells. In this system, the α_{1A}-AR and α_{1B}-AR increased cAMP production in the cytoplasm and nucleus, whereas the ET_AR did not (Figure 2.5B). We also identified subtype and compartment specific activation of the cAMP-sensitive kinase PKA. The

α_{1B}-AR increased PKA activity in the cytoplasm and nucleus, whereas the α_{1A}-AR only activated PKA in the nucleus (Figure 2.4). Finally, cAMP production and PKA activation by α_1-AR subtypes was ablated in a Gαs knockout HEK 293 cell line (Figure 2.3, Figure 2.4), with cAMP subsequently rescued by heterologous Gαs expression (Figure 2.3). The identification of compartment specific, Gαs-dependent α_1-AR signalling expanded our understanding of cardiomyocyte signalling pathways and raised questions about the potential implications for regulating pathological gene expression in cardiomyocytes.

In Chapter 3 (Martin *et al*., 2020), we described the roles of two active P-TEFb complexes in the cardiomyocyte hypertrophic response following GPCR activation. As in Chapter 2, we focused on the canonically Gαq-coupled α_1-AR and ETR as Gαq signalling is implicated in P-TEFb activation in cardiomyocytes. With the identification of additional Gαs coupling by the α_1-AR, we hypothesized the additional signalling pathway would elicit a different requirement for specific P-TEFb complexes compared to the ETR. To assess the functional role of each complex, we used the small-molecules KL-2 and JQ1 to disrupt the AFF4-P-TEFb interaction or BET protein acetyl-lysine interaction, respectively. We also confirmed on-target effects of both inhibitors through siRNA knockdown of AFF4 or the individual BET family proteins. We demonstrated that the cardiomyocyte hypertrophic response following ETR activation required P-TEFb recruitment by the SEC (Figure 3.2). On the other hand, α_1-AR-mediated cardiomyocyte hypertrophic response required both SEC- and Brd4-mediated P-TEFb recruitment (Figure 3.2, Figure 3.3). Transcriptome analysis indicated that JQ1 attenuated processes implicated in pathological cardiomyocyte hypertrophy following α_1-AR, and not ETR, activation (Figure 3.7G). The differential effect of JQ1 corresponded with the PKA-dependent Brd4 recruitment to cis regulatory genomic regions of pathological genes in response to α_1-AR activation only (Figure 3.9A/B). To further assess the requirement for Brd4 activity when PKA signalling is activated, we also examined the effect of JQ1 following activation of the Gαs-coupled β-AR. JQ1 also prevented the increased cardiomyocyte surface area following β-AR activation (Figure 3.9D/E), suggesting signalling through Brd4 was a common feature of Gαs-coupled GPCRs.

In Chapter 4 (Khan and Martin *et al*., 2020), we described the $G\beta_1\gamma$-dependent regulation of AT1R-induced RNAPII transcription in cardiac fibroblasts. Here, we aimed to identify a regulatory role of a novel interaction identified between Gβγ and RNAPII. This was assessed through two methods: a RT-qPCR based fibrotic gene expression array and ChIP-seq. We

observed an overall increase in fibrotic gene expression following 75 min (the time point with peak Gβγ-RNAPII interaction) and 24 h AT1R activation. At both 75 min and 24 h, $G\beta_1$ knockdown with siRNA increased basal gene expression, potentiated AT1R-mediated gene expression, and increased the number of genes upregulated in response to AT1R activation. This trend was more strongly observed following 24 h AT1R activation (Figure 4.5C/D, Table 4.1). To determine the genome-wide recruitment of $G\beta_1$ and changes in RNAPII, we performed ChIP-seq of RNAPII and heterologously expressed FLAG- $G\beta_1$ following 75 min AT1R activation. We identified two gene clusters with different $G\beta_1$ and RNAPII occupancy patterns (Figure 4.6B/C). In cluster 1, $G\beta_1$ was recruited predominantly to TSSs following AT1R activation. With regards to RNAPII, we observed occupancy patterns that correlated with the fibrotic gene expression changes we previously characterized. First, cluster 1 genes had increased basal RNAPII occupancy at TSSs following $G\beta_1$ knockdown, similar to the increased gene expression observed under these conditions. Second, cluster 1 genes displayed increased RNAPII occupancy following AT1R activation that was potentiated by $G\beta_1$ knockdown conditions, similar to the potentiated gene expression observed under these conditions. Third, we identified a greater number of genes with RNAPII peaks following AT1R activation under $G\beta_1$ knockdown conditions, similar to the greater number of genes upregulated. Furthermore, the majority of upregulated genes we identified following 75 min AT1R activation resided within cluster 1. Altogether, these results indicated that $G\beta_1$ is recruited to the chromatin in response to AT1R to negatively regulate RNAPII transcription.

5.2. Compartmentalized PKA signalling by α_1-AR subtypes

Diverse extracellular signals alter cellular function through common second messenger molecules, such as via modulating the production of cAMP. The ability for a single small molecule to regulate a range of cellular processes is in part due to the formation of localized microdomains. For cAMP, these domains are formed by the scaffolding AKAPs, which bring together the proteins required to transmit a signal linking cAMP production to effector protein regulation. In Chapter 2, we described distinct compartmentalized Gαs-cAMP-PKA signalling by specific α_1-AR subtypes. The α_{1B}-AR increased cAMP and PKA in the nucleus and cytoplasm, whereas the α_{1A}-AR increased PKA only in the nucleus despite cAMP production in both compartments (Figure 2.3, Figure 2.4). While the mechanism for the selective PKA activation was not determined, insights from other GPCRs offer suggestions. For example, similar disconnect between cAMP and PKA

signalling in the cytoplasm and nucleus was observed for the β_1-AR and β_2-AR subtypes in cardiomyocytes. Both receptors increased cAMP levels in the cytoplasm and nucleus and increased PKA activity in the cytoplasm. However, nuclear PKA activity was only increased by the β_1-AR through uptake of the protein kinase from the cytoplasm. For the β_2-AR, phosphodiesterase 4 (PDE4) prevented activation of muscle AKAP β (mAKAPβ)-tethered PKA and its subsequent diffusion to the nucleus [519]. The compartment-specific PKA activation by the α_{1A}-AR and α_{1B}-AR suggests the subtypes signal through different AKAP complexes, enabling distinct trafficking of cAMP and PKA from the cytoplasm to the nucleus. Alternatively, the α_1-AR subtypes may directly activate the nuclear pool of PKA through localization to the nuclear envelope. Many GPCRs, including the α_1-AR subtypes, have been identified at the nuclear envelope [520]. A previous study identified a small percentage of intracellular α_{1A}-AR and α_{1B}-AR in a heterologous HEK 293 expression system, indicating overexpressed receptors may localize to the nucleus in these cells [521]. In this scenario, the α_{1A}-AR could signal through nuclear AKAPs that interact with PKA and cytoplasmic AKAPs that do not. Further work is required to assess differences in the receptor interactomes in specific compartments in order to identify potential mechanisms for the subtype specific signalling.

5.3. Compartmentalized GPCR signalling in pathological cardiac remodelling

In cardiomyocytes, compartmentalized GPCR signalling enables two receptors to elicit different functional outcomes even with activation of the same G protein. For example, a previous study sought to identify mechanisms underlying the different alterations in cardiomyocyte function following activation of the Gαq-coupled AT1R or α_1-AR. Comparison of AT1R and α_1-AR gene expression programs in adult mouse ventricular cardiomyocytes indicated that AT1R activation elicited a subset of the α_1-AR gene expression program. Here, it was proposed that transcriptional differences were due to distinct subcellular localization of the AT1R and α_1-AR. The AT1R was identified predominantly at the plasma membrane with a small population on the inner nuclear membrane, whereas the α_1-AR localized solely to the inner nuclear membrane. The authors proposed the unique transcriptional profiles were due to the nuclear α_1-AR signalling through PLCβ1 that led to the export of HDAC5 from the nucleus, relieving its inhibitory effect and promoting transcription. The authors suggested the unique receptor localization accounts for the cardioprotective role of the α_1-AR and the pathological role of the AT1R [522].

Of interest is how different α_1-AR subtypes are localized in cardiomyocytes and whether they differentially activate signalling pathways as described in Chapter 2. Similar to the opposing effects of the β_1-AR and the β_2-AR, differential compartmentalized signalling may explain opposing effects of the α_{1A}-AR or α_{1B}-AR. Cardiac specific overexpression of the α_{1A}-AR in mice improved contractility, whereas the α_{1B}-AR led to impaired left ventricle function and dilated cardiomyopathy [191, 192]. The opposing functions of the β-AR subtypes is thought to be due to differentially localized signalling patterns of the receptors. The β_1-AR, which promotes pathological cardiac remodelling and cardiomyocyte apoptosis, is localized along the entire plasma membrane and activation leads to a diffuse increase in cAMP levels throughout the cardiomyocyte. On the other hand, the β_2-AR, activation of which is initially cardioprotective, is localized to transverse tubules and increases cAMP levels in a highly localized manner [523, 524]. In failing cardiomyocytes, β_2-AR localization shifts to the plasma membrane and produces a diffuse cAMP signal, potentially promoting pathological cardiac remodelling [523]. The results from Chapter 2 indicate that α_1-AR subtypes also exhibit compartment-specific signalling, although whether the distinct Gαs-cAMP-PKA signalling transfers to cardiomyocytes remains to be determined. Further work is required to assess if the different cardiac outcomes from overexpressing specific α_1-AR subtypes is regulated by activation of signalling pathways in a localized manner.

5.4. Activation of P-TEFb complexes by distinct signalling cascades

As mentioned in the introduction, hypertrophic cardiomyocytes exhibit increased P-TEFb activity in cell culture and *in vivo* models [505]. The central role of P-TEFb in cardiomyocyte hypertrophy was further corroborated in Chapter 3, where we demonstrated P-TEFb inhibition with the small molecule iCdk9 prevented both ETR and α_1-AR mediated hypertrophy. In cardiomyocytes, it is thought that P-TEFb's release from the 7SK snRNP is dependent on the Ca^{2+} activated phosphatase calcineurin (also referred to as PP2B), a signalling effector activated by both the ETR and α_1-AR [352]. On the other hand, P-TEFb recruitment mechanisms employed depend on other aspects of receptor signalling profiles. Gαq-coupled receptors have a common requirement for the AFF4-SEC to increase pathological gene expression and cardiomyocyte surface area. For receptors that also couple to Gαs, P-TEFb recruitment involves a balance between the AFF4-SEC and Brd4. We also showed that the β-AR, which elicits a Gαs-dependent hypertrophic response, requires Brd4. The correlation between signalling profiles and P-TEFb

recruitment mechanisms suggests that SEC recruitment requires Gαq signalling, although assessing the effect of SEC inhibition on cardiomyocyte hypertrophy following activation of the Gαs-coupled β-AR is required to confirm this. Further work is required to determine how Gαq signalling converges on the SEC and leads to gene specific recruitment of the complex. Furthermore, various possibilities are raised to explain the differential requirement for P-TEFb complexes following activation of distinct GPCRs: different requirement for phosphorylation of specific P-TEFb substrates, different TF activation profiles, or receptors elicit different types of hypertrophy dependent on different gene expression programs.

5.4.1. P-TEFb complexes regulate phosphorylation of different P-TEFb substrates

The hypertrophic gene expression changes require release of RNAPII from a promoter-proximal paused state into productive elongation through increased activity of P-TEFb [505, 525]. As mentioned in the introduction, P-TEFb phosphorylates the Rpb1 CTD, the Spt5 subunit of DSIF, and the NELF-A and NELF-E subunits of NELF to promote pause-release [284, 288, 294]. It has been proposed that each substrate could be targeted by a distinct P-TEFb complex. In this model, Brd4 regulates Spt5 phosphorylation, AFF1-SEC regulates NELF-A phosphorylation, AFF1/4-SEC regulates NELF-E phosphorylation, and AFF4-SEC regulates Rpb1 CTD phosphorylation [289]. The differential P-TEFb complex activation by each GPCR would then predict that the gene expression changes require phosphorylation of different P-TEFb substrates. According to this model, the α_1-AR critical genes require Brd4-P-TEFb phosphorylation of DSIF for pause-release or downstream RNA processing.

The requirement for DSIF phosphorylation is further suggested by the prevalence of inflammatory pathways and TFs identified in the α_1-AR transcriptome data. Previous studies have identified an important regulatory role of a potential Brd4-DSIF pathway on inflammatory gene expression. First, NF-κB transcriptional activation requires Brd4-mediated recruitment of P-TEFb [414], aligning with the strong attenuation of NF-κB gene expression by JQ1 following α_1-AR activation (Figure 3.7H). Furthermore, previous studies have identified a role for DSIF in NF-κB regulated inflammatory gene expression. Spt5 knockdown or treatment with small molecules that inhibit the Spt5-RNAPII interaction reduced NF-κB target gene expression following TNFα treatment [526, 527]. Combined, these studies are consistent with the notion that NF-κB-dependent recruitment of Brd4 to target genes increases gene expression through Spt5 phosphorylation.

Further work is required to address whether a Brd4-Spt5 phosphorylation cascade is triggered by the α_1-AR, and a different P-TEFb substrate is phosphorylated downstream of the ETR. First, Spt5 knockdown or Spt5 inhibitor treatment will assess the Spt5 requirement for the ETR and α_1-AR-mediated hypertrophic response. From the proposed model, it is hypothesized that these experiments would selectively attenuate α_1-AR-mediated cardiomyocyte hypertrophy and gene expression. The potential toxicity of long term Spt5 knockdown and off-target effects of the Spt5 inhibitors may limit the ability to associate the observed effects solely on Spt5 function. Instead, ChIP of the relevant components (i.e. Brd4, SEC, total and phosphorylated Spt5 and NELF) along JQ1 attenuated genes would provide a direct assessment of how these factors are altered in response to ETR or α_1-AR activation.

5.4.2. Distinct hypertrophic signalling leads to differential recruitment of Brd4 by TFs

In silico prediction of TF activity revealed similar TFs activated in response to either receptor, with JQ1 predominantly attenuating activity following α_1-AR activation (Figure 3.7H). This suggests the selective effect of JQ1 is due to divergent ETR and α_1-AR signalling pathways that activate TFs through different mechanisms. To illustrate this, I will focus on NF-κB and GATA4, two TFs activated by both receptors and selectively attenuated by JQ1 following α_1-AR activation (Figure 3.7H). Links between PKA signalling, these transcription factors and Brd4 recruitment have previously been identified. First, a well characterized signalling cascade links PKA, NF-κB and Brd4 activity. PKA phosphorylation of the NF-κB subunit RelA promotes its interaction and acetylation by CBP/p300 acetyltransferase [528, 529]. The acetylated RelA recruits Brd4 in a BD-dependent manner to enhance transcriptional activation of NF-κB target genes [414]. The unique activation of PKA by the α_1-AR, and not the ETR, may function through this pathway to promote NF-κB activity in a Brd4-dependent manner. On the other hand, ETR signalling through PKCβ may activate NF-κB by disrupting the interaction of NF-κB with the inhibitory Iκβα subunit. PKCβ phosphorylates and activates Iκβ kinase α (IKKα), which subsequently promotes Iκβα degradation and NF-κB activation [530]. This aligns with a study demonstrating overexpression of a mutant Iκβα resistant to degradation prevented increased NF-κB reporter gene and Nppa expression following activation of Gαq coupled GPCRs in cardiomyocytes [531]. These examples highlight how PKA signalling may activate a NF-κB-Brd4-P-TEFb complex, whereas PKC signalling downstream of Gαq increases free, active NF-κB.

Similarly, a potential mechanism linking PKA signalling to Brd4-dependent activation of GATA4 target genes has been demonstrated. Phosphorylation of GATA4 serine 261, initially identified as an ERK1/2 substrate, was required for GATA4-dependent increase in gene expression. Overexpression of a GATA4-S261A mutant prevented the erythropoietin-dependent increase in cell size of the rat cardiac H9c2 cell line, indicating the importance of this phosphorylation event for GATA4 transcriptional activation. Furthermore, GATA4 S261 phosphorylation was also required for GATA4 acetylation [532]. Mutation of the acetylated GATA4 residues prevented the α_1-AR-mediated hypertrophy and increased pro-hypertrophic gene expression in cardiomyocytes [533]. Lastly, GATA4 acetylation was critical for its interaction with CDK9 in cardiomyocytes, although whether this occurred through Brd4, similar to other members of the GATA transcription factor family, remains to be determined. [385, 534, 535]. Although the identified pathway required ERK1/2 activity, PKA has also been demonstrated to phosphorylate S261 and enhance GATA4 activity gonadal cells [536]. Altogether, the proposed mechanism involves PKA phosphorylation of GATA4 S261 promoting GATA4 acetylation to recruit the Brd4-P-TEFb complex to GATA4 target genes. On the other hand, PKC signalling enhances GATA4 activity through phosphorylation of S419/S420. For example, AT1R activation of PKC enhanced GATA4 DNA binding activity and reporter gene expression [537]. These examples illustrate how PKA signalling regulates a TF-Brd4 complex able to promote transcription, which is absent in PKC signalling pathways. While these TFs may explain part of the differential effect of Brd4 inhibition, *in silico* analysis predicted a majority of TFs were attenuated by JQ1. The ubiquitous effect of JQ1 suggests α_1-AR signalling alters TF activity through a common mechanism and not unique post translational modifications for each TF.

5.4.3. Receptor activation leads to different types of hypertrophy with unique requirements for P-TEFb complexes

Cardiomyocytes respond to various stressors through increased transcription of a gene regulatory network enabling the adaptive hypertrophic phenotype. In Chapter 3, we proposed the hypertrophic response to α_1-AR and ETR activation was the same and that Brd4 had a unique role in α_1-AR-mediated hypertrophy. An alternative hypothesis is that these receptors lead to different types of cardiomyocyte hypertrophy with unique gene expression programs, which impart a differential requirement for Brd4 activity. Transcriptome analysis indicated that the ETR

transcriptional program represented a subset of the α_1-AR program, as ~87% of ETR-mediated differentially expressed genes (up and down regulated) were also modulated by α_1-AR activation. Conversely, ~36% of α_1-AR differentially expressed genes were uniquely modulated by this receptor (Figure 3.7A/B). This suggests that the ETR mediates a type of hypertrophy which requires a minimal gene expression program. The attenuation of ETR-mediated hypertrophy by inhibition of the SEC, and not Brd4, suggests these genes are solely regulated by the SEC. In contrast, the α_1-AR activates a type of hypertrophy requiring a broader gene expression program, with the uniquely modulated genes leading to a requirement for Brd4 activity. Two aspects of our transcriptome analysis support this hypothesis. First, there is a population of genes upregulated by both receptors whose expression is attenuated by JQ1 independent of receptor activation (Figure 3.7F). As ETR activation still leads to cardiomyocyte hypertrophy in the presence of JQ1, these commonly upregulated genes must not impart sensitivity to BET inhibition. In Chapter 3, we proposed these genes were regulated by Brd2 or Brd3, although further work is required to confirm this hypothesis. Importantly, genes were placed in these categories by a binary requirement, leaving the possibility that subsets of these genes have differential sensitivity that is not evident. Second, ~30% of genes attenuated by JQ1 when upregulated by the α_1-AR were not upregulated by the ETR (Figure 3.7F). This suggests that these additional genes alter the type of cardiomyocyte hypertrophy mediated by the α_1-AR to one dependent on Brd4 activity. We would expect that similar transcriptome analysis following β-AR activation would identify similarities with the α_1-AR transcriptome, as β-AR-mediated hypertrophy also required Brd4 activity.

Further experiments are required to assess the hypothesis that P-TEFb complexes regulate distinct gene expression programs. First, comparing the effect of KL-2 and JQ1 treatment on gene expression changes will identify clusters of genes sensitive to one or both inhibitors. In Chapter 3, we determined Serpine1 was insensitive to KL-2 and attenuated by JQ1, providing preliminary evidence that P-TEFb complexes regulate separate groups of genes. Second, assessing Brd4 and AFF4 occupancy patterns across genes attenuated by either inhibitor will provide potential mechanistic details on how the two complexes regulate gene expression. Overall, these experiments will enable categorization of SEC and Brd4-dependent gene expression changes and help characterize the differential requirement of the two complexes by distinct GPCRs.

5.5. Regulation of P-TEFb complex components directly by PKA

In Chapter 3, we proposed PKA signalling leads to the selective attenuation of α_1-AR-mediated hypertrophy by BET inhibition. We proposed PKA activates Brd4 through direct phosphorylation of the Brd4 PDID, comprising BD2 and NPS. As mentioned in the introduction, NPS phosphorylation leads to a conformational change that relieves the intramolecular inhibition of BD2 by the NPS. PKA phosphorylated the PDID in an *in vitro* kinase assay, although the functional implications were not identified [409]. Furthermore, *in silico* prediction of phosphorylated PDID residues identified S324 and S325 as potential PKA substrates (unpublished data). Alternatively, PKA signalling also regulates other aspects of P-TEFb activity which may have functionally significance in cardiomyocytes. For example, PKA phosphorylation of CDK9 S347 led to increased affinity of the HIV Tat-P-TEFb complex for the TAR element and HEXIM1 S158 phosphorylation led to disruption of the 7SK snRNP complex. Further work is required to assess whether PKA phosphorylation of CDK9 S347 is increased following α_1-AR activation and whether this modification alters the interaction with Brd4. On the other hand, HEXIM1 S158 phosphorylation is less likely to promote a specific complex as it simply increases levels of free, active P-TEFb without directly modifying the heterodimer.

Further work is required to identify the PKA phosphorylated residues of Brd4 and the functional role of the phosphorylated residues. To identify the phosphorylated residues, neonatal rat cardiomyocytes will be transduced with an adenovirus containing a FLAG-tagged Brd4 construct. Following treatment with an α_1-AR agonist, ETR agonist, or forskolin and IBMX, affinity purification LC-MS/MS will be performed to identify phosphorylated residues. Following identification of phosphorylated residues, α_1-AR-mediated recruitment of Brd4 constructs with the identified residues mutated to alanine or a phosphomimetic residue will assess the functional role of the phosphorylated residues. The hypothesis is that mutation to alanine will block Brd4 recruitment and a phosphomimetic mutation would increase basal Brd4 occupancy.

5.6. Gβγ regulation of RNAPII transcription

In Chapter 4, we described the negative regulation of AT1R-mediated gene expression by $G\beta_1\gamma$ in cardiac fibroblasts. The FLAG-$G\beta_1$ and RNAPII ChIP-seq profiles suggests $G\beta_1\gamma$ negatively regulates RNAPII initiation (Figure 4.6B/C). First, FLAG-$G\beta_1$ occupancy increased at

the TSS in response to AT1R activation, indicating it is recruited to the early RNAPII complex. Second, $G\beta_1$ knockdown under basal conditions led to increased RNAPII occupancy at the TSS, suggesting increased recruitment to these genes. While we did not assess the mechanism underlying the negative regulation in this chapter, a previous study assessing the interactome of a TAP-tagged $G\beta_1\gamma_7$ by LC-MS/MS provide hints of a potential mechanism [99]. The study identified an interaction between $G\beta_1\gamma_7$ and the Brg1/Brm1-associated factor (BAF) complex subunits Brg1 and BAF57 in response to M3 muscarinic acetylcholine receptor (M3-MAChR) activation in HEK 293 cells. Preliminary cell fractionation and co-immunoprecipitation experiments confirmed that the M3-MAChR stimulated interaction between heterologously expressed FLAG-$G\beta_1$ and GFP-Brg1 in HEK 293 cells occurred in the nucleus (Celia Bouazza, unpublished data). Furthermore, prediction of transcriptional regulatory activity (as performed in Figure 3.7H) from an RNA-seq experiment with two HEK 293 $G\beta_1$ knockout cell lines predicted increased Brg1 activity in the knockout cells compared to control (unpublished data).

In vertebrates, BAF complexes are comprised of a central Brahma (Brm) or Brahma-related gene 1 (Brg1) ATPase subunit and up to 15 subunits. The large complex exists in diverse assemblies of the many subunits and their paralogues, leading to numerous distinct assemblies of the complex [538]. One function of the complex is to increase or decrease chromatin accessibility through nucleosome remodelling by the ATPase subunit at cis regulatory genomic regions. The increased chromatin accessibility promotes assembly of the RNAPII PIC and subsequent gene expression [539]. Furthermore, the diverse assembly of subunits enables the BAF complex to regulate cell-type specific processes, with several studies characterizing its role in cardiac cell types. For example, Brg1-containing BAF complexes are critical for proper cardiac differentiation during development. In cardiac precursor cells, Brg1 is recruited to TSSs and active enhancer regions through interactions with TFs to maintain an open chromatin state and expression of genes involved in cardiovascular developmental and cardiac tissue morphogenesis [540]. Throughout development, Brg1 expression decreases in cardiomyocytes, but is reactivated following cardiac stress to promote transition to the fetal gene expression program. Brg1 regulates the switch in expression from the adult α-MHC isoform to the fetal β-MHC isoform in cardiomyocyte hypertrophy. At the α-MHC promoter, a Brg1-HDAC-poly [ADP-ribose] polymerase (PARP) complex inhibits expression, whereas at the β-MHC promoter, a Brg1-PARP complex promotes gene expression [541].

While several studies have expanded our understanding of the roles of the BAF complex in cardiac development and cardiomyocyte hypertrophy, less is understood about its role in cardiac fibroblasts and in the fibrotic response. Brg1 was identified at the promoter and as a positive regulator of the profibrotic osteopontin gene in mouse cardiac tissue following TAC, suggesting a role potential role in regulating fibrosis [542]. Studies in other cell systems provide further evidence for the role of Brg1 in fibrosis. First, Brg1 positively regulated expression of the genes encoding two fibrotic proteins, α-SMA and collagen type I alpha 1 chain (Col1a1), in the liver [543]. Second, Brg1 was required for TGFβ-induced expression of the profibrotic Ctgf gene in HaCaT keratinocytes. Knockdown of Brg1 prevented RNAPII initiation at the Ctgf promoter and reduced Ctgf gene expression induced by TGFβ [544]. While these studies suggest a role for Brg1 in fibrotic gene expression, a potential function in cardiac fibroblasts still needs to be verified experimentally.

My proposed model for AT1R-mediated transcription in cardiac fibroblasts is that $G\beta_1\gamma$ recruitment to promoter bound RNAPII complexes negatively regulates Brg1-dependent chromatin remodelling. Following sustained signalling, $G\beta_1\gamma$ dissociates from the complex, relieving the negative regulation and allowing robust transcriptional activation. First, this model explains the increased RNAPII TSS occupancy in $G\beta_1$ knockdown conditions. $G\beta_1$ knockdown relieves the negative regulation of Brg1 to promote accessible chromatin and subsequent RNAPII initiation. Furthermore, this model is supported by preliminary data that indicates the interaction between $G\beta_1\gamma$ and Brg1 is conserved in cardiac fibroblasts (Celia Bouazza, unpublished data). Further work is required to determine if $G\beta_1\gamma$ regulation of Brg1 activity is responsible for negative regulation of AT1R-mediated gene expression by $G\beta_1\gamma$ in cardiac fibroblasts. First, *in vitro* interaction experiments with purified $G\beta_1\gamma$ and cardiac fibroblast BAF complexes or RNAPII are essential to determine if $G\beta_1\gamma$ directly interacts with either component of the transcriptional machinery. If $G\beta_1\gamma$ is found to interact with the Brg1-containing BAF complex, *in vitro* nucleosome remodelling assays to assess regulation of remodelling activity should be performed. Following *in vitro* validation, regulation of the BAF complex activity in cardiac fibroblasts should be assessed. Changes in BAF complex activity can be determined indirectly by assessing chromatin accessibility changes by ATAC-seq following AT1R activation under control or $G\beta_1$ knockdown conditions. While this model explains the mechanism for negative regulation of RNAPII transcription by $G\beta_1\gamma$, it does not explain if $G\beta_1\gamma$ is recruited to specific genomic loci as

a component of the BAF complex or through interactions with TFs. Further work assessing the $G\beta_1\gamma$ interactome in cardiac fibroblasts is required to address this question.

5.7. Implications for targeting Gβγ signalling in pathological cardiac remodelling

As mentioned in the introduction, Gβγ signalling is a driver of pathological cardiac hypertrophy in various models of heart failure [203, 204]. Systemic treatment with the small molecule Gβγ inhibitors M119 or gallein disrupted the Gβγ-GRK2 interaction, preventing β-AR desensitization and normalizing β-AR surface expression [204]. While inhibiting the Gβγ-GRK2 interaction is cardioprotective, therapeutic approaches should aim to preserve the $G\beta_1\gamma$-RNAPII interaction to maintain the negative regulation of fibrotic transcription. Therefore, small molecules which differentially inhibit these Gβγ functions could be clinically useful. To enable the targeted drug discovery, a more detailed understand of how $G\beta_1\gamma$ interacts with the RNAPII complex is required. The first approach involves obtaining structural information of $G\beta_1\gamma$ in complex with the transcriptional components it directly interacts with, as determined by the previously described *in vitro* immunoprecipitation experiments with purified complexes. Comparison of these structures with the $G\beta_1\gamma$ effector structures will enable identification of different Gβγ 'hot spot' residues required for each interaction and where small molecules should target. Alternatively, alanine mutagenesis of Gβγ 'hot spot' residues followed by co-immunoprecipitation experiments from whole cell lysates would identify differentially required residues for GRK2 and RNAPII. Interestingly, the small molecule gallein did not alter the transcriptional response to AT1R (Chapter 4), suggesting it does not affect the $G\beta_1\gamma$-RNAPII interaction although assessing the interaction by co-immunoprecipitation is required to confirm this. Therefore, 'hot spot' regions required for the $G\beta_1\gamma$-RNAPII interaction may be assumed to be different than those targeted by gallein; the latter could be inferred by docking gallein to the $G\beta_1\gamma_2$ structure. Altogether, these various approaches may enable a strategy for generation of a selective Gβγ inhibitor for the signalling pathways which promote pathological cardiac remodelling, while sparing those that inhibit it.

5.8. Gβγ signalling in cancer and neurodevelopmental diseases

While we focused on Gβγ signalling in pathological cardiac remodelling, the heterodimer is also dysregulated in other diseases. For example, several mutations have been identified in GNB1, the gene encoding $G\beta_1$. GNB1 mutations were initially identified in various types of leukemia that clustered along the Gβ protein surface interacting with the Gα subunit [489]. Subsequently, heterozygous GNB1 mutations were identified in patients with GNB1 encephalopathy, a neurological disease characterized by developmental and intellectual delay, hypotonia and seizures [545]. Surprisingly, there is overlap between mutations leading to cancer or neurodevelopmental disorders, suggesting the development of a specific pathology is not driven by specific mutations but rather the timing or location of such mutations. In fact, the development of a specific pathology appears to depend on whether it is a somatic or germline mutation, leading to cancer or GNB1 encephalopathy, respectively. To date, the focus has been on how these mutations alter G protein heterotrimer formation and interactions with canonical signalling pathways, such as PI3K, MAPK and PLCβ [546]. For example, mass spectrometry of TAP-tagged $G\beta_1$ with mutations identified in various leukemias identified reduced association with all Gα isoforms. The decreased association with Gα led to a Gβγ-dependent increase in PI3K, MAPK and PLCβ activity independent of receptor activation [546]. Of particular interest going forward is how these mutations affect the Gβγ-RNAPII interaction and the implications for pathological gene expression changes. Within leukemias, this interaction may serve to suppress dysregulated transcription, a hallmark of cancer [547], and reduce oncogenic transformation. Preliminary data indicates many somatic GNB1 mutations disrupt the Gβγ-RNAPII interaction (Iulia Pirvulescu, unpublished data) although further work to assess transcriptome changes is required.

5.9. Conclusion

This work expands our understanding of how GPCR signalling pathways converge on transcription to alter the state of cardiac cell types and regulate pathological cardiac remodelling. In cardiomyocytes, we identified the novel regulation of Brd4 by the cAMP/PKA pathway to promote gene expression and hypertrophy. In cardiac fibroblasts, we have identified a novel role for $G\beta_1\gamma$ in the negative regulation of RNAPII to suppress fibrotic gene expression in response to AT1R. Furthermore, we highlight potential considerations in developing and administering

therapeutics for pathological cardiac remodelling. As clinical development of BET inhibitors advances, the findings in this thesis indicate it is essential to characterize specific neurohormonal profiles in patients to assess the potential efficacy of this class of small molecules. Our work in cardiac fibrosis highlights the importance of targeting specific Gβγ-effector interactions as some have negative regulatory functions (i.e. Gβγ-RNAPII) which should be preserved. The chapters contained in this thesis add another piece to our understanding of pathological cardiac remodelling in the hopes of developing better therapeutic approaches and improving disease prognosis for patients.

References

1. Yancy, C.W., et al., *2013 ACCF/AHA guideline for the management of heart failure: executive summary: a report of the American College of Cardiology Foundation/American Heart Association Task Force on practice guidelines.* Circulation, 2013. **128**(16): p. 1810-52.
2. Tanai, E. and S. Frantz, *Pathophysiology of Heart Failure.* Compr Physiol, 2015. **6**(1): p. 187-214.
3. *(Dis)connected: How unseen links are putting us at risk*. 2020 30 March 2020]; Available from: https://www.heartandstroke.ca/-/media/pdf-files/canada/2019-report/heartandstrokereport2019.ashx.
4. Dick, S.A. and S. Epelman, *Chronic Heart Failure and Inflammation: What Do We Really Know?* Circ Res, 2016. **119**(1): p. 159-76.
5. Creemers, E.E. and Y.M. Pinto, *Molecular mechanisms that control interstitial fibrosis in the pressure-overloaded heart.* Cardiovasc Res, 2011. **89**(2): p. 265-72.
6. Piek, A., R.A. de Boer, and H.H. Sillje, *The fibrosis-cell death axis in heart failure.* Heart Fail Rev, 2016. **21**(2): p. 199-211.
7. Melaku, L., *Angiotensin-converting enzyme 2 and its potential protective effect upon heart.* Archives of Medicine and Health Sciences, 2018. **6**(2): p. 238-246.
8. Heineke, J. and J.D. Molkentin, *Regulation of cardiac hypertrophy by intracellular signalling pathways.* Nat Rev Mol Cell Biol, 2006. **7**(8): p. 589-600.
9. Cameron, V.A. and L.J. Ellmers, *Minireview: natriuretic peptides during development of the fetal heart and circulation.* Endocrinology, 2003. **144**(6): p. 2191-4.
10. Nishikimi, T., N. Maeda, and H. Matsuoka, *The role of natriuretic peptides in cardioprotection.* Cardiovasc Res, 2006. **69**(2): p. 318-28.
11. Nakagawa, Y., T. Nishikimi, and K. Kuwahara, *Atrial and brain natriuretic peptides: Hormones secreted from the heart.* Peptides, 2019. **111**: p. 18-25.
12. Zhang, Z.L., et al., *Natriuretic peptide family as diagnostic/prognostic biomarker and treatment modality in management of adult and geriatric patients with heart failure: remaining issues and challenges.* J Geriatr Cardiol, 2018. **15**(8): p. 540-546.
13. Periasamy, M., P. Bhupathy, and G.J. Babu, *Regulation of sarcoplasmic reticulum Ca2+ ATPase pump expression and its relevance to cardiac muscle physiology and pathology.* Cardiovasc Res, 2008. **77**(2): p. 265-73.
14. Schmidt, U., et al., *Contribution of abnormal sarcoplasmic reticulum ATPase activity to systolic and diastolic dysfunction in human heart failure.* J Mol Cell Cardiol, 1998. **30**(10): p. 1929-37.
15. Machackova, J., J. Barta, and N.S. Dhalla, *Myofibrillar remodeling in cardiac hypertrophy, heart failure and cardiomyopathies.* Can J Cardiol, 2006. **22**(11): p. 953-68.
16. Tian, R., et al., *Increased adenosine monophosphate-activated protein kinase activity in rat hearts with pressure-overload hypertrophy.* Circulation, 2001. **104**(14): p. 1664-9.
17. Keller, A., et al., *Differential expression of alpha- and beta-enolase genes during rat heart development and hypertrophy.* Am J Physiol, 1995. **269**(6 Pt 2): p. H1843-51.
18. Barger, P.M. and D.P. Kelly, *Fatty acid utilization in the hypertrophied and failing heart: molecular regulatory mechanisms.* Am J Med Sci, 1999. **318**(1): p. 36-42.
19. Sack, M.N., et al., *Fatty acid oxidation enzyme gene expression is downregulated in the failing heart.* Circulation, 1996. **94**(11): p. 2837-42.
20. Allard, M.F., et al., *Contribution of oxidative metabolism and glycolysis to ATP production in hypertrophied hearts.* Am J Physiol, 1994. **267**(2 Pt 2): p. H742-50.

21. Gorski, P.A., D.K. Ceholski, and R.J. Hajjar, *Altered myocardial calcium cycling and energetics in heart failure--a rational approach for disease treatment.* Cell Metab, 2015. **21**(2): p. 183-194.
22. Lompre, A.M., B. Nadal-Ginard, and V. Mahdavi, *Expression of the cardiac ventricular alpha- and beta-myosin heavy chain genes is developmentally and hormonally regulated.* J Biol Chem, 1984. **259**(10): p. 6437-46.
23. Kameyama, T., et al., *Mechanoenergetic alterations during the transition from cardiac hypertrophy to failure in Dahl salt-sensitive rats.* Circulation, 1998. **98**(25): p. 2919-29.
24. Gidh-Jain, M., et al., *Alterations in cardiac gene expression during ventricular remodeling following experimental myocardial infarction.* J Mol Cell Cardiol, 1998. **30**(3): p. 627-37.
25. Reiser, P.J., et al., *Human cardiac myosin heavy chain isoforms in fetal and failing adult atria and ventricles.* Am J Physiol Heart Circ Physiol, 2001. **280**(4): p. H1814-20.
26. Nakao, K., et al., *Myosin heavy chain gene expression in human heart failure.* J Clin Invest, 1997. **100**(9): p. 2362-70.
27. Herron, T.J. and K.S. McDonald, *Small amounts of alpha-myosin heavy chain isoform expression significantly increase power output of rat cardiac myocyte fragments.* Circ Res, 2002. **90**(11): p. 1150-2.
28. Krenz, M. and J. Robbins, *Impact of beta-myosin heavy chain expression on cardiac function during stress.* J Am Coll Cardiol, 2004. **44**(12): p. 2390-7.
29. Hay, W.W., Jr., *Recent observations on the regulation of fetal metabolism by glucose.* J Physiol, 2006. **572**(Pt 1): p. 17-24.
30. Lopaschuk, G.D., M.A. Spafford, and D.R. Marsh, *Glycolysis is predominant source of myocardial ATP production immediately after birth.* Am J Physiol, 1991. **261**(6 Pt 2): p. H1698-705.
31. Lopaschuk, G.D. and J.S. Jaswal, *Energy metabolic phenotype of the cardiomyocyte during development, differentiation, and postnatal maturation.* J Cardiovasc Pharmacol, 2010. **56**(2): p. 130-40.
32. Piquereau, J. and R. Ventura-Clapier, *Maturation of Cardiac Energy Metabolism During Perinatal Development.* Front Physiol, 2018. **9**: p. 959.
33. Hinderer, S. and K. Schenke-Layland, *Cardiac fibrosis - A short review of causes and therapeutic strategies.* Adv Drug Deliv Rev, 2019. **146**: p. 77-82.
34. Rienks, M., et al., *Myocardial extracellular matrix: an ever-changing and diverse entity.* Circ Res, 2014. **114**(5): p. 872-88.
35. Jellis, C., et al., *Assessment of nonischemic myocardial fibrosis.* J Am Coll Cardiol, 2010. **56**(2): p. 89-97.
36. Talman, V. and H. Ruskoaho, *Cardiac fibrosis in myocardial infarction-from repair and remodeling to regeneration.* Cell Tissue Res, 2016. **365**(3): p. 563-81.
37. de Jong, S., et al., *Fibrosis and cardiac arrhythmias.* J Cardiovasc Pharmacol, 2011. **57**(6): p. 630-8.
38. Travers, J.G., et al., *Cardiac Fibrosis: The Fibroblast Awakens.* Circ Res, 2016. **118**(6): p. 1021-40.
39. Liu, P., M. Sun, and S. Sader, *Matrix metalloproteinases in cardiovascular disease.* Can J Cardiol, 2006. **22 Suppl B**: p. 25B-30B.
40. Moore, L., et al., *Tissue inhibitor of metalloproteinases (TIMPs) in heart failure.* Heart Fail Rev, 2012. **17**(4-5): p. 693-706.

41. Leslie, K.O., et al., *Cardiac myofibroblasts express alpha smooth muscle actin during right ventricular pressure overload in the rabbit.* Am J Pathol, 1991. **139**(1): p. 207-16.
42. Willems, I.E., et al., *The alpha-smooth muscle actin-positive cells in healing human myocardial scars.* Am J Pathol, 1994. **145**(4): p. 868-75.
43. Bomb, R., et al., *Myofibroblast secretome and its auto-/paracrine signaling.* Expert Rev Cardiovasc Ther, 2016. **14**(5): p. 591-8.
44. Eadie, A.L., A.J. Titus, and K.R. Brunt, *Getting to the heart of myofibroblast differentiation: implications for scleraxis in ECM remodeling and therapeutic targeting.* Am J Physiol Heart Circ Physiol, 2018. **315**(5): p. H1232-H1235.
45. Frieler, R.A. and R.M. Mortensen, *Immune cell and other noncardiomyocyte regulation of cardiac hypertrophy and remodeling.* Circulation, 2015. **131**(11): p. 1019-30.
46. Foster, A.J., et al., *Central-acting therapeutics alleviate respiratory weakness caused by heart failure-induced ventilatory overdrive.* Sci Transl Med, 2017. **9**(390).
47. Sun, Y., et al., *Infarct scar as living tissue.* Basic Res Cardiol, 2002. **97**(5): p. 343-7.
48. Hermans, E., *Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors.* Pharmacol Ther, 2003. **99**(1): p. 25-44.
49. Boivin, B., et al., *G protein-coupled receptors in and on the cell nucleus: a new signaling paradigm?* J Recept Signal Transduct Res, 2008. **28**(1-2): p. 15-28.
50. Stevens, R.C., et al., *The GPCR Network: a large-scale collaboration to determine human GPCR structure and function.* Nat Rev Drug Discov, 2013. **12**(1): p. 25-34.
51. Fredriksson, R., et al., *The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints.* Mol Pharmacol, 2003. **63**(6): p. 1256-72.
52. Lagerstrom, M.C. and H.B. Schioth, *Structural diversity of G protein-coupled receptors and significance for drug discovery.* Nat Rev Drug Discov, 2008. **7**(4): p. 339-57.
53. Rasmussen, S.G., et al., *Crystal structure of the beta2 adrenergic receptor-Gs protein complex.* Nature, 2011. **477**(7366): p. 549-55.
54. Palczewski, K., et al., *Crystal structure of rhodopsin: A G protein-coupled receptor.* Science, 2000. **289**(5480): p. 739-45.
55. Hilger, D., M. Masureel, and B.K. Kobilka, *Structure and dynamics of GPCR signaling complexes.* Nat Struct Mol Biol, 2018. **25**(1): p. 4-12.
56. Sounier, R., et al., *Propagation of conformational changes during mu-opioid receptor activation.* Nature, 2015. **524**(7565): p. 375-8.
57. Manglik, A., et al., *Structural Insights into the Dynamic Process of beta2-Adrenergic Receptor Signaling.* Cell, 2015. **161**(5): p. 1101-1111.
58. Coffino, P., H.R. Bourne, and G.M. Tomkins, *Somatic genetic analysis of cyclic AMP action: selection of unresponsive mutants.* J Cell Physiol, 1975. **85**(3): p. 603-10.
59. Gill, D.M. and R. Meren, *ADP-ribosylation of membrane proteins catalyzed by cholera toxin: basis of the activation of adenylate cyclase.* Proc Natl Acad Sci U S A, 1978. **75**(7): p. 3050-4.
60. Anantharaman, V., et al., *Comparative genomics uncovers novel structural and functional features of the heterotrimeric GTPase signaling system.* Gene, 2011. **475**(2): p. 63-78.
61. Sanchez-Fernandez, G., et al., *Galphaq signalling: the new and the old.* Cell Signal, 2014. **26**(5): p. 833-48.
62. Hurley, J.H., *Structure, mechanism, and regulation of mammalian adenylyl cyclase.* J Biol Chem, 1999. **274**(12): p. 7599-602.

63. Kelly, P., P.J. Casey, and T.E. Meigs, *Biologic functions of the G12 subfamily of heterotrimeric g proteins: growth, migration, and metastasis.* Biochemistry, 2007. **46**(23): p. 6677-87.
64. Lambright, D.G., et al., *Structural determinants for activation of the alpha-subunit of a heterotrimeric G protein.* Nature, 1994. **369**(6482): p. 621-8.
65. Smrcka, A.V., *G protein betagamma subunits: central mediators of G protein-coupled receptor signaling.* Cell Mol Life Sci, 2008. **65**(14): p. 2191-214.
66. Flock, T., et al., *Universal allosteric mechanism for Galpha activation by GPCRs.* Nature, 2015. **524**(7564): p. 173-179.
67. Neer, E.J., *Heterotrimeric G proteins: organizers of transmembrane signals.* Cell, 1995. **80**(2): p. 249-57.
68. Flock, T., et al., *Selectivity determinants of GPCR-G-protein binding.* Nature, 2017. **545**(7654): p. 317-322.
69. Schmidt, C.J. and E.J. Neer, *In vitro synthesis of G protein beta gamma dimers.* J Biol Chem, 1991. **266**(7): p. 4538-44.
70. Logothetis, D.E., et al., *The beta gamma subunits of GTP-binding proteins activate the muscarinic K+ channel in heart.* Nature, 1987. **325**(6102): p. 321-6.
71. Smrcka, A.V. and I. Fisher, *G-protein betagamma subunits as multi-functional scaffolds and transducers in G-protein-coupled receptor signaling.* Cell Mol Life Sci, 2019. **76**(22): p. 4447-4459.
72. Khan, S.M., et al., *The expanding roles of Gbetagamma subunits in G protein-coupled receptor signaling and drug action.* Pharmacol Rev, 2013. **65**(2): p. 545-77.
73. Slepak, V.Z., *Structure, function, and localization of Gbeta5-RGS complexes.* Prog Mol Biol Transl Sci, 2009. **86**: p. 157-203.
74. Dingus, J., et al., *G Protein betagamma dimer formation: Gbeta and Ggamma differentially determine efficiency of in vitro dimer formation.* Biochemistry, 2005. **44**(35): p. 11882-90.
75. Wall, M.A., et al., *The structure of the G protein heterotrimer Gi alpha 1 beta 1 gamma 2.* Cell, 1995. **83**(6): p. 1047-58.
76. Sondek, J., et al., *Crystal structure of a G-protein beta gamma dimer at 2.1A resolution.* Nature, 1996. **379**(6563): p. 369-74.
77. Lambright, D.G., et al., *The 2.0 A crystal structure of a heterotrimeric G protein.* Nature, 1996. **379**(6563): p. 311-9.
78. Scott, J.K., et al., *Evidence that a protein-protein interaction 'hot spot' on heterotrimeric G protein betagamma subunits is used for recognition of a subclass of effectors.* EMBO J, 2001. **20**(4): p. 767-76.
79. Bonacci, T.M., et al., *Regulatory interactions between the amino terminus of G-protein betagamma subunits and the catalytic domain of phospholipase Cbeta2.* J Biol Chem, 2005. **280**(11): p. 10174-81.
80. Brand, C.S., et al., *Adenylyl Cyclase 5 Regulation by Gbetagamma Involves Isoform-Specific Use of Multiple Interaction Sites.* Mol Pharmacol, 2015. **88**(4): p. 758-67.
81. Ayoub, M.A., *Small molecules targeting heterotrimeric G proteins.* Eur J Pharmacol, 2018. **826**: p. 169-178.
82. Inglese, J., et al., *Functionally active targeting domain of the beta-adrenergic receptor kinase: an inhibitor of G beta gamma-mediated stimulation of type II adenylyl cyclase.* Proc Natl Acad Sci U S A, 1994. **91**(9): p. 3637-41.

83. Koch, W.J., et al., *Cellular expression of the carboxyl terminus of a G protein-coupled receptor kinase attenuates G beta gamma-mediated signaling.* J Biol Chem, 1994. **269**(8): p. 6193-7.
84. Lodowski, D.T., et al., *Keeping G proteins at bay: a complex between G protein-coupled receptor kinase 2 and Gbetagamma.* Science, 2003. **300**(5623): p. 1256-62.
85. Chen, J., et al., *A region of adenylyl cyclase 2 critical for regulation by G protein beta gamma subunits.* Science, 1995. **268**(5214): p. 1166-9.
86. Weng, G., et al., *Gbeta subunit interacts with a peptide encoding region 956-982 of adenylyl cyclase 2. Cross-linking of the peptide to free Gbetagamma but not the heterotrimer.* J Biol Chem, 1996. **271**(43): p. 26445-8.
87. Davis, T.L., et al., *Structural and molecular characterization of a preferred protein interaction surface on G protein beta gamma subunits.* Biochemistry, 2005. **44**(31): p. 10593-604.
88. Smrcka, A.V., D.M. Lehmann, and A.L. Dessal, *G protein betagamma subunits as targets for small molecule therapeutic development.* Comb Chem High Throughput Screen, 2008. **11**(5): p. 382-95.
89. Li, Y., et al., *Sites for Galpha binding on the G protein beta subunit overlap with sites for regulation of phospholipase Cbeta and adenylyl cyclase.* J Biol Chem, 1998. **273**(26): p. 16265-72.
90. Bonacci, T.M., et al., *Differential targeting of Gbetagamma-subunit signaling with small molecules.* Science, 2006. **312**(5772): p. 443-6.
91. Lehmann, D.M., A.M. Seneviratne, and A.V. Smrcka, *Small molecule disruption of G protein beta gamma subunit signaling inhibits neutrophil chemotaxis and inflammation.* Mol Pharmacol, 2008. **73**(2): p. 410-8.
92. Surve, C.R., D. Lehmann, and A.V. Smrcka, *A chemical biology approach demonstrates G protein betagamma subunits are sufficient to mediate directional neutrophil chemotaxis.* J Biol Chem, 2014. **289**(25): p. 17791-801.
93. Iniguez-Lluhi, J.A., et al., *G protein beta gamma subunits synthesized in Sf9 cells. Functional characterization and the significance of prenylation of gamma.* J Biol Chem, 1992. **267**(32): p. 23409-17.
94. Myung, C.S., et al., *Role of isoprenoid lipids on the heterotrimeric G protein gamma subunit in determining effector activation.* J Biol Chem, 1999. **274**(23): p. 16595-603.
95. Senarath, K., et al., *Ggamma identity dictates efficacy of Gbetagamma signaling and macrophage migration.* J Biol Chem, 2018. **293**(8): p. 2974-2989.
96. Ajith Karunarathne, W.K., et al., *All G protein betagamma complexes are capable of translocation on receptor activation.* Biochem Biophys Res Commun, 2012. **421**(3): p. 605-11.
97. O'Neill, P.R., et al., *G-protein signaling leverages subunit-dependent membrane affinity to differentially control betagamma translocation to intracellular membranes.* Proc Natl Acad Sci U S A, 2012. **109**(51): p. E3568-77.
98. Khan, S.M., J.Y. Sung, and T.E. Hebert, *Gbetagamma subunits-Different spaces, different faces.* Pharmacol Res, 2016. **111**: p. 434-441.
99. Campden, R., et al., *Tandem affinity purification to identify cytosolic and nuclear gbetagamma-interacting proteins.* Methods Mol Biol, 2015. **1234**: p. 161-84.
100. Salazar, N.C., J. Chen, and H.A. Rockman, *Cardiac GPCRs: GPCR signaling in healthy and failing hearts.* Biochim Biophys Acta, 2007. **1768**(4): p. 1006-18.

101. Riehle, C. and J. Bauersachs, *Small animal models of heart failure.* Cardiovasc Res, 2019. **115**(13): p. 1838-1849.
102. Kanisicak, O., et al., *Genetic lineage tracing defines myofibroblast origin and function in the injured heart.* Nat Commun, 2016. **7**: p. 12260.
103. Boularan, C. and C. Gales, *Cardiac cAMP: production, hydrolysis, modulation and detection.* Front Pharmacol, 2015. **6**: p. 203.
104. Halls, M.L. and D.M.F. Cooper, *Adenylyl cyclase signalling complexes - Pharmacological challenges and opportunities.* Pharmacol Ther, 2017. **172**: p. 171-180.
105. De Jongh, K.S., et al., *Specific phosphorylation of a site in the full-length form of the alpha 1 subunit of the cardiac L-type calcium channel by adenosine 3',5'-cyclic monophosphate-dependent protein kinase.* Biochemistry, 1996. **35**(32): p. 10392-402.
106. Sculptoreanu, A., et al., *Voltage-dependent potentiation of the activity of cardiac L-type calcium channel alpha 1 subunits due to phosphorylation by cAMP-dependent protein kinase.* Proc Natl Acad Sci U S A, 1993. **90**(21): p. 10135-9.
107. Xiao, B., et al., *Functional consequence of protein kinase A-dependent phosphorylation of the cardiac ryanodine receptor: sensitization of store overload-induced Ca2+ release.* J Biol Chem, 2007. **282**(41): p. 30256-64.
108. Chen, Z., B.L. Akin, and L.R. Jones, *Mechanism of reversal of phospholamban inhibition of the cardiac Ca2+-ATPase by protein kinase A and by anti-phospholamban monoclonal antibody 2D12.* J Biol Chem, 2007. **282**(29): p. 20968-76.
109. McNamara, J.W., R.R. Singh, and S. Sadayappan, *Cardiac myosin binding protein-C phosphorylation regulates the super-relaxed state of myosin.* Proc Natl Acad Sci U S A, 2019. **116**(24): p. 11731-11736.
110. Rao, V., et al., *PKA phosphorylation of cardiac troponin I modulates activation and relaxation kinetics of ventricular myofibrils.* Biophys J, 2014. **107**(5): p. 1196-1204.
111. Muller, F.U., et al., *Activation and inactivation of cAMP-response element-mediated gene transcription in cardiac myocytes.* Cardiovasc Res, 2001. **52**(1): p. 95-102.
112. Iwase, M., et al., *Adverse effects of chronic endogenous sympathetic drive induced by cardiac GS alpha overexpression.* Circ Res, 1996. **78**(4): p. 517-24.
113. Antos, C.L., et al., *Dilated cardiomyopathy and sudden death resulting from constitutive activation of protein kinase a.* Circ Res, 2001. **89**(11): p. 997-1004.
114. Imaeda, A., et al., *Myofibroblast beta2 adrenergic signaling amplifies cardiac hypertrophy in mice.* Biochem Biophys Res Commun, 2019. **510**(1): p. 149-155.
115. Marx, S.O., et al., *PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts.* Cell, 2000. **101**(4): p. 365-76.
116. Schroder, F., et al., *Increased availability and open probability of single L-type calcium channels from failing compared with nonfailing human ventricle.* Circulation, 1998. **98**(10): p. 969-76.
117. Jacques, A.M., et al., *Myosin binding protein C phosphorylation in normal, hypertrophic and failing human heart muscle.* J Mol Cell Cardiol, 2008. **45**(2): p. 209-16.
118. Wijnker, P.J., et al., *Protein phosphatase 2A affects myofilament contractility in non-failing but not in failing human myocardium.* J Muscle Res Cell Motil, 2011. **32**(3): p. 221-33.
119. Dash, R., et al., *Gender influences on sarcoplasmic reticulum Ca2+-handling in failing human myocardium.* J Mol Cell Cardiol, 2001. **33**(7): p. 1345-53.

120. Diviani, D., et al., *A-kinase anchoring proteins: scaffolding proteins in the heart.* Am J Physiol Heart Circ Physiol, 2011. **301**(5): p. H1742-53.
121. Yang, J.H., et al., *PKA catalytic subunit compartmentation regulates contractile and hypertrophic responses to beta-adrenergic signaling.* J Mol Cell Cardiol, 2014. **66**: p. 83-93.
122. Ha, C.H., et al., *PKA phosphorylates histone deacetylase 5 and prevents its nuclear export, leading to the inhibition of gene transcription and cardiomyocyte hypertrophy.* Proc Natl Acad Sci U S A, 2010. **107**(35): p. 15467-72.
123. Watson, P.A., et al., *Cardiac-specific overexpression of dominant-negative CREB leads to increased mortality and mitochondrial dysfunction in female mice.* Am J Physiol Heart Circ Physiol, 2010. **299**(6): p. H2056-68.
124. Barbagallo, F., et al., *Genetically Encoded Biosensors Reveal PKA Hyperphosphorylation on the Myofilaments in Rabbit Heart Failure.* Circ Res, 2016. **119**(8): p. 931-43.
125. Woo, A.Y. and R.P. Xiao, *beta-Adrenergic receptor subtype signaling in heart: from bench to bedside.* Acta Pharmacol Sin, 2012. **33**(3): p. 335-41.
126. Myagmar, B.E., et al., *Adrenergic Receptors in Individual Ventricular Myocytes: The Beta-1 and Alpha-1B Are in All Cells, the Alpha-1A Is in a Subpopulation, and the Beta-2 and Beta-3 Are Mostly Absent.* Circ Res, 2017. **120**(7): p. 1103-1115.
127. de Lucia, C., A. Eguchi, and W.J. Koch, *New Insights in Cardiac beta-Adrenergic Signaling During Heart Failure and Aging.* Front Pharmacol, 2018. **9**: p. 904.
128. Triposkiadis, F., et al., *The sympathetic nervous system in heart failure physiology, pathophysiology, and clinical implications.* J Am Coll Cardiol, 2009. **54**(19): p. 1747-62.
129. Najafi, A., et al., *beta-adrenergic receptor signalling and its functional consequences in the diseased heart.* Eur J Clin Invest, 2016. **46**(4): p. 362-74.
130. Bristow, M.R., et al., *Beta 1- and beta 2-adrenergic-receptor subpopulations in nonfailing and failing human ventricular myocardium: coupling of both receptor subtypes to muscle contraction and selective beta 1-receptor down-regulation in heart failure.* Circ Res, 1986. **59**(3): p. 297-309.
131. Bristow, M.R., et al., *Reduced beta 1 receptor messenger RNA abundance in the failing human heart.* J Clin Invest, 1993. **92**(6): p. 2737-45.
132. Bristow, M.R., et al., *Beta 1- and beta 2-adrenergic receptor-mediated adenylate cyclase stimulation in nonfailing and failing human ventricular myocardium.* Mol Pharmacol, 1989. **35**(3): p. 295-303.
133. Chang, S.C., et al., *Isoproterenol-Induced Heart Failure Mouse Model Using Osmotic Pump Implantation.* Methods Mol Biol, 2018. **1816**: p. 207-220.
134. Lu, J., et al., *SIRT6 suppresses isoproterenol-induced cardiac hypertrophy through activation of autophagy.* Transl Res, 2016. **172**: p. 96-112 e6.
135. Engelhardt, S., et al., *Progressive hypertrophy and heart failure in beta1-adrenergic receptor transgenic mice.* Proc Natl Acad Sci U S A, 1999. **96**(12): p. 7059-64.
136. Milano, C.A., et al., *Enhanced myocardial function in transgenic mice overexpressing the beta 2-adrenergic receptor.* Science, 1994. **264**(5158): p. 582-6.
137. Liggett, S.B., et al., *Early and delayed consequences of beta(2)-adrenergic receptor overexpression in mouse hearts: critical role for expression level.* Circulation, 2000. **101**(14): p. 1707-14.
138. Prichard, B.N., *Hypotensive Action of Pronethalol.* Br Med J, 1964. **1**(5392): p. 1227-8.

139. Prichard, B.N. and P.M. Gillam, *Use of Propranolol (Inderal) in Treatment of Hypertension.* Br Med J, 1964. **2**(5411): p. 725-7.
140. Baker, J.G., *The selectivity of beta-adrenoceptor antagonists at the human beta1, beta2 and beta3 adrenoceptors.* Br J Pharmacol, 2005. **144**(3): p. 317-22.
141. Wang, J., C. Gareri, and H.A. Rockman, *G-Protein-Coupled Receptors in Heart Disease.* Circ Res, 2018. **123**(6): p. 716-735.
142. Kukin, M.L., et al., *Prospective, randomized comparison of effect of long-term treatment with metoprolol or carvedilol on symptoms, exercise, ejection fraction, and oxidative stress in heart failure.* Circulation, 1999. **99**(20): p. 2645-51.
143. Packer, M., et al., *The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. U.S. Carvedilol Heart Failure Study Group.* N Engl J Med, 1996. **334**(21): p. 1349-55.
144. *Effect of metoprolol CR/XL in chronic heart failure: Metoprolol CR/XL Randomised Intervention Trial in Congestive Heart Failure (MERIT-HF).* Lancet, 1999. **353**(9169): p. 2001-7.
145. Barrese, V. and M. Taglialatela, *New advances in beta-blocker therapy in heart failure.* Front Physiol, 2013. **4**: p. 323.
146. D'Angelo, D.D., et al., *Transgenic Galphaq overexpression induces cardiac contractile failure in mice.* Proc Natl Acad Sci U S A, 1997. **94**(15): p. 8121-6.
147. Adams, J.W., et al., *Enhanced Galphaq signaling: a common pathway mediates cardiac hypertrophy and apoptotic heart failure.* Proc Natl Acad Sci U S A, 1998. **95**(17): p. 10140-5.
148. Mende, U., et al., *Transient cardiac expression of constitutively active Galphaq leads to hypertrophy and dilated cardiomyopathy by calcineurin-dependent and independent pathways.* Proc Natl Acad Sci U S A, 1998. **95**(23): p. 13893-8.
149. Wettschureck, N., et al., *Absence of pressure overload induced myocardial hypertrophy after conditional inactivation of Galphaq/Galpha11 in cardiomyocytes.* Nat Med, 2001. **7**(11): p. 1236-40.
150. Sechi, L.A., et al., *Characterization of angiotensin II receptor subtypes in rat heart.* Circ Res, 1992. **71**(6): p. 1482-9.
151. Haywood, G.A., et al., *AT1 and AT2 angiotensin receptor gene expression in human heart failure.* Circulation, 1997. **95**(5): p. 1201-6.
152. Dasgupta, C. and L. Zhang, *Angiotensin II receptors and drug discovery in cardiovascular disease.* Drug Discov Today, 2011. **16**(1-2): p. 22-34.
153. Tadevosyan, A., et al., *Nuclear-delimited angiotensin receptor-mediated signaling regulates cardiomyocyte gene expression.* J Biol Chem, 2010. **285**(29): p. 22338-49.
154. Tadevosyan, A., et al., *Intracellular Angiotensin-II Interacts With Nuclear Angiotensin Receptors in Cardiac Fibroblasts and Regulates RNA Synthesis, Cell Proliferation, and Collagen Secretion.* J Am Heart Assoc, 2017. **6**(4).
155. Meggs, L.G., et al., *Regulation of angiotensin II receptors on ventricular myocytes after myocardial infarction in rats.* Circ Res, 1993. **72**(6): p. 1149-62.
156. Nio, Y., et al., *Regulation of gene transcription of angiotensin II receptor subtypes in myocardial infarction.* J Clin Invest, 1995. **95**(1): p. 46-54.
157. Tsutsumi, Y., et al., *Angiotensin II type 2 receptor is upregulated in human heart with interstitial fibrosis, and cardiac fibroblasts are the major cell type for its expression.* Circ Res, 1998. **83**(10): p. 1035-46.

158. Rockman, H.A., et al., *ANG II receptor blockade prevents ventricular hypertrophy and ANF gene expression with pressure overload in mice.* Am J Physiol, 1994. **266**(6 Pt 2): p. H2468-75.
159. Paradis, P., et al., *Overexpression of angiotensin II type I receptor in cardiomyocytes induces cardiac hypertrophy and remodeling.* Proc Natl Acad Sci U S A, 2000. **97**(2): p. 931-6.
160. Wu, M., et al., *17beta-estradiol inhibits angiotensin II-induced cardiac myofibroblast differentiation.* Eur J Pharmacol, 2009. **616**(1-3): p. 155-9.
161. Schorb, W., et al., *Angiotensin II is mitogenic in neonatal rat cardiac fibroblasts.* Circ Res, 1993. **72**(6): p. 1245-54.
162. Campbell, S.E. and L.C. Katwa, *Angiotensin II stimulated expression of transforming growth factor-beta1 in cardiac fibroblasts and myofibroblasts.* J Mol Cell Cardiol, 1997. **29**(7): p. 1947-58.
163. Xu, J., et al., *Effects of cardiac overexpression of the angiotensin II type 2 receptor on remodeling and dysfunction in mice post-myocardial infarction.* Hypertension, 2014. **63**(6): p. 1251-9.
164. Schultz Jel, J., et al., *TGF-beta1 mediates the hypertrophic cardiomyocyte growth induced by angiotensin II.* J Clin Invest, 2002. **109**(6): p. 787-96.
165. Harada, M., et al., *Significance of ventricular myocytes and nonmyocytes interaction during cardiocyte hypertrophy: evidence for endothelin-1 as a paracrine hypertrophic factor from cardiac nonmyocytes.* Circulation, 1997. **96**(10): p. 3737-44.
166. Garg, R. and S. Yusuf, *Overview of randomized trials of angiotensin-converting enzyme inhibitors on mortality and morbidity in patients with heart failure. Collaborative Group on ACE Inhibitor Trials.* JAMA, 1995. **273**(18): p. 1450-6.
167. Investigators, S., et al., *Effect of enalapril on survival in patients with reduced left ventricular ejection fractions and congestive heart failure.* N Engl J Med, 1991. **325**(5): p. 293-302.
168. Investigators, S., et al., *Effect of enalapril on mortality and the development of heart failure in asymptomatic patients with reduced left ventricular ejection fractions.* N Engl J Med, 1992. **327**(10): p. 685-91.
169. Cohn, J.N., G. Tognoni, and I. Valsartan Heart Failure Trial, *A randomized trial of the angiotensin-receptor blocker valsartan in chronic heart failure.* N Engl J Med, 2001. **345**(23): p. 1667-75.
170. Heran, B.S., et al., *Angiotensin receptor blockers for heart failure.* Cochrane Database Syst Rev, 2012(4): p. CD003040.
171. Bisping, E., et al., *Targeting cardiac hypertrophy: toward a causal heart failure therapy.* J Cardiovasc Pharmacol, 2014. **64**(4): p. 293-305.
172. Fareh, J., et al., *Endothelin-1 and angiotensin II receptors in cells from rat hypertrophied heart. Receptor regulation and intracellular Ca2+ modulation.* Circ Res, 1996. **78**(2): p. 302-11.
173. Picard, P., et al., *Coordinated upregulation of the cardiac endothelin system in a rat model of heart failure.* J Cardiovasc Pharmacol, 1998. **31 Suppl 1**: p. S294-7.
174. Pieske, B., et al., *Functional effects of endothelin and regulation of endothelin receptors in isolated human nonfailing and failing myocardium.* Circulation, 1999. **99**(14): p. 1802-9.

175. Zolk, O., et al., *Expression of endothelin-1, endothelin-converting enzyme, and endothelin receptors in chronic heart failure.* Circulation, 1999. **99**(16): p. 2118-23.
176. Kedzierski, R.M. and M. Yanagisawa, *Endothelin system: the double-edged sword in health and disease.* Annu Rev Pharmacol Toxicol, 2001. **41**: p. 851-76.
177. Yang, L.L., et al., *Conditional cardiac overexpression of endothelin-1 induces inflammation and dilated cardiomyopathy in mice.* Circulation, 2004. **109**(2): p. 255-61.
178. Hafizi, S., et al., *Profibrotic effects of endothelin-1 via the ETA receptor in cultured human cardiac fibroblasts.* Cell Physiol Biochem, 2004. **14**(4-6): p. 285-92.
179. Piacentini, L., et al., *Endothelin-1 stimulates cardiac fibroblast proliferation through activation of protein kinase C.* J Mol Cell Cardiol, 2000. **32**(4): p. 565-76.
180. Cheng, T.H., et al., *Role of mitogen-activated protein kinase pathway in reactive oxygen species-mediated endothelin-1-induced beta-myosin heavy chain gene expression and cardiomyocyte hypertrophy.* J Biomed Sci, 2005. **12**(1): p. 123-33.
181. Ito, H., et al., *Endothelin ETA receptor antagonist blocks cardiac hypertrophy provoked by hemodynamic overload.* Circulation, 1994. **89**(5): p. 2198-203.
182. Sakai, S., et al., *Inhibition of myocardial endothelin pathway improves long-term survival in heart failure.* Nature, 1996. **384**(6607): p. 353-5.
183. Nguyen, Q.T., et al., *Endothelin A receptor blockade causes adverse left ventricular remodeling but improves pulmonary artery pressure after infarction in the rat.* Circulation, 1998. **98**(21): p. 2323-30.
184. Rich, S. and V.V. McLaughlin, *Endothelin receptor blockers in cardiovascular disease.* Circulation, 2003. **108**(18): p. 2184-90.
185. O'Connell, T.D., et al., *Cardiac alpha1-adrenergic receptors: novel aspects of expression, signaling mechanisms, physiologic function, and clinical importance.* Pharmacol Rev, 2014. **66**(1): p. 308-33.
186. Jensen, B.C., et al., *{alpha}1-Adrenergic receptor subtypes in nonfailing and failing human myocardium.* Circ Heart Fail, 2009. **2**(6): p. 654-63.
187. Milano, C.A., et al., *Myocardial expression of a constitutively active alpha 1B-adrenergic receptor in transgenic mice induces cardiac hypertrophy.* Proc Natl Acad Sci U S A, 1994. **91**(21): p. 10109-13.
188. Zuscik, M.J., et al., *Hypotension, autonomic failure, and cardiac hypertrophy in transgenic mice overexpressing the alpha 1B-adrenergic receptor.* J Biol Chem, 2001. **276**(17): p. 13738-43.
189. Akhter, S.A., et al., *Transgenic mice with cardiac overexpression of alpha1B-adrenergic receptors. In vivo alpha1-adrenergic receptor-mediated regulation of beta-adrenergic signaling.* J Biol Chem, 1997. **272**(34): p. 21253-9.
190. Grupp, I.L., et al., *Overexpression of alpha1B-adrenergic receptor induces left ventricular dysfunction in the absence of hypertrophy.* Am J Physiol, 1998. **275**(4): p. H1338-50.
191. Lemire, I., et al., *Cardiac-directed overexpression of wild-type alpha1B-adrenergic receptor induces dilated cardiomyopathy.* Am J Physiol Heart Circ Physiol, 2001. **281**(2): p. H931-8.
192. Lin, F., et al., *Targeted alpha(1A)-adrenergic receptor overexpression induces enhanced cardiac contractility but not hypertrophy.* Circ Res, 2001. **89**(4): p. 343-50.
193. O'Connell, T.D., et al., *Alpha1-adrenergic receptors prevent a maladaptive cardiac response to pressure overload.* J Clin Invest, 2006. **116**(4): p. 1005-15.

194. Huang, Y., et al., *An alpha1A-adrenergic-extracellular signal-regulated kinase survival signaling pathway in cardiac myocytes.* Circulation, 2007. **115**(6): p. 763-72.
195. Rorabaugh, B.R., et al., *alpha1A- but not alpha1B-adrenergic receptors precondition the ischemic heart by a staurosporine-sensitive, chelerythrine-insensitive mechanism.* Cardiovasc Res, 2005. **65**(2): p. 436-45.
196. Shi, T., R.S. Papay, and D.M. Perez, *alpha1A-Adrenergic receptor prevents cardiac ischemic damage through PKCdelta/GLUT1/4-mediated glucose uptake.* J Recept Signal Transduct Res, 2016. **36**(3): p. 261-70.
197. Piller, L.B., et al., *Validation of Heart Failure Events in the Antihypertensive and Lipid Lowering Treatment to Prevent Heart Attack Trial (ALLHAT) Participants Assigned to Doxazosin and Chlorthalidone.* Curr Control Trials Cardiovasc Med, 2002. **3**(1): p. 10.
198. Cohn, J.N., *The Vasodilator-Heart Failure Trials (V-HeFT). Mechanistic data from the VA Cooperative Studies. Introduction.* Circulation, 1993. **87**(6 Suppl): p. VI1-4.
199. Yamamura, S., et al., *Cardiomyocyte Sirt (Sirtuin) 7 Ameliorates Stress-Induced Cardiac Hypertrophy by Interacting With and Deacetylating GATA4.* Hypertension, 2020. **75**(1): p. 98-108.
200. Simpson, P., *Stimulation of hypertrophy of cultured neonatal rat heart cells through an alpha 1-adrenergic receptor and induction of beating through an alpha 1- and beta 1-adrenergic receptor interaction. Evidence for independent regulation of growth and beating.* Circ Res, 1985. **56**(6): p. 884-94.
201. Rockman, H.A., et al., *Expression of a beta-adrenergic receptor kinase 1 inhibitor prevents the development of myocardial failure in gene-targeted mice.* Proc Natl Acad Sci U S A, 1998. **95**(12): p. 7000-5.
202. Li, Z., et al., *Effects of two Gbetagamma-binding proteins--N-terminally truncated phosducin and beta-adrenergic receptor kinase C terminus (betaARKct)--in heart failure.* Gene Ther, 2003. **10**(16): p. 1354-61.
203. Casey, L.M., et al., *Small molecule disruption of G beta gamma signaling inhibits the progression of heart failure.* Circ Res, 2010. **107**(4): p. 532-9.
204. Kamal, F.A., et al., *Simultaneous adrenal and cardiac g-protein-coupled receptor-gbetagamma inhibition halts heart failure progression.* J Am Coll Cardiol, 2014. **63**(23): p. 2549-2557.
205. Travers, J.G., et al., *Pharmacological and Activated Fibroblast Targeting of Gbetagamma-GRK2 After Myocardial Ischemia Attenuates Heart Failure Progression.* J Am Coll Cardiol, 2017. **70**(8): p. 958-971.
206. Malik, S., et al., *G protein betagamma subunits regulate cardiomyocyte hypertrophy through a perinuclear Golgi phosphatidylinositol 4-phosphate hydrolysis pathway.* Mol Biol Cell, 2015. **26**(6): p. 1188-98.
207. Weiss, S.B. and L. Gladstone, *A MAMMALIAN SYSTEM FOR THE INCORPORATION OF CYTIDINE TRIPHOSPHATE INTO RIBONUCLEIC ACID1.* Journal of the American Chemical Society, 1959. **81**(15): p. 4118-4119.
208. Roeder, R.G., *50+ years of eukaryotic transcription: an expanding universe of factors and mechanisms.* Nat Struct Mol Biol, 2019. **26**(9): p. 783-791.
209. Roeder, R.G. and W.J. Rutter, *Multiple forms of DNA-dependent RNA polymerase in eukaryotic organisms.* Nature, 1969. **224**(5216): p. 234-7.

210. Weinmann, R. and R.G. Roeder, *Role of DNA-dependent RNA polymerase 3 in the transcription of the tRNA and 5S RNA genes.* Proc Natl Acad Sci U S A, 1974. **71**(5): p. 1790-4.
211. Weinmann, R., H.J. Raskas, and R.G. Roeder, *Role of DNA-dependent RNA polymerases II and III in transcription of the adenovirus genome late in productive infection.* Proc Natl Acad Sci U S A, 1974. **71**(9): p. 3426-39.
212. Roeder, R.G., R.H. Reeder, and D.D. Brown, *Multiple Forms of RNA Polymerase in Xenopus laevis: Their Relationship to RNA Synthesis in vivo and Their Fidelity of Transcription in vitro.* Cold Spring Harbor Symposia on Quantitative Biology, 1970. **35**: p. 727-735.
213. Seifart, K.H. and C.E. Sekeris, *Alpha-amanitin, a specific inhibitor of transcription by mammalian RNA-polymerase.* Z Naturforsch B, 1969. **24**(12): p. 1538-44.
214. Lindell, T.J., et al., *Specific inhibition of nuclear RNA polymerase II by alpha-amanitin.* Science, 1970. **170**(3956): p. 447-9.
215. Vannini, A. and P. Cramer, *Conservation between the RNA polymerase I, II, and III transcription initiation machineries.* Mol Cell, 2012. **45**(4): p. 439-46.
216. Ebright, R.H., *RNA polymerase: structural similarities between bacterial RNA polymerase and eukaryotic RNA polymerase II.* J Mol Biol, 2000. **304**(5): p. 687-98.
217. Sentenac, A., et al., *Yeast RNA Polymerase Subunits and Genes.* Cold Spring Harbor Monograph Archive, 1992. **22**: p. 27-54.
218. Young, R.A., *RNA polymerase II.* Annu Rev Biochem, 1991. **60**: p. 689-715.
219. Darst, S.A., et al., *Three-dimensional structure of yeast RNA polymerase II at 16 A resolution.* Cell, 1991. **66**(1): p. 121-8.
220. Zhang, G., et al., *Crystal structure of Thermus aquaticus core RNA polymerase at 3.3 A resolution.* Cell, 1999. **98**(6): p. 811-24.
221. Cramer, P., et al., *Architecture of RNA polymerase II and implications for the transcription mechanism.* Science, 2000. **288**(5466): p. 640-9.
222. Cramer, P., D.A. Bushnell, and R.D. Kornberg, *Structural basis of transcription: RNA polymerase II at 2.8 angstrom resolution.* Science, 2001. **292**(5523): p. 1863-76.
223. Gnatt, A.L., et al., *Structural basis of transcription: an RNA polymerase II elongation complex at 3.3 A resolution.* Science, 2001. **292**(5523): p. 1876-82.
224. Armache, K.J., H. Kettenberger, and P. Cramer, *Architecture of initiation-competent 12-subunit RNA polymerase II.* Proc Natl Acad Sci U S A, 2003. **100**(12): p. 6964-8.
225. Bushnell, D.A. and R.D. Kornberg, *Complete, 12-subunit RNA polymerase II at 4.1-A resolution: implications for the initiation of transcription.* Proc Natl Acad Sci U S A, 2003. **100**(12): p. 6969-73.
226. Kostek, S.A., et al., *Molecular architecture and conformational flexibility of human RNA polymerase II.* Structure, 2006. **14**(11): p. 1691-700.
227. Woychik, N.A. and M. Hampsey, *The RNA polymerase II machinery: structure illuminates function.* Cell, 2002. **108**(4): p. 453-63.
228. Barnes, C.O., et al., *Crystal Structure of a Transcribing RNA Polymerase II Complex Reveals a Complete Transcription Bubble.* Mol Cell, 2015. **59**(2): p. 258-69.
229. Bernecky, C., et al., *Structure of transcribing mammalian RNA polymerase II.* Nature, 2016. **529**(7587): p. 551-4.
230. Hahn, S., *Structure and mechanism of the RNA polymerase II transcription machinery.* Nat Struct Mol Biol, 2004. **11**(5): p. 394-403.

231. Eick, D. and M. Geyer, *The RNA polymerase II carboxy-terminal domain (CTD) code.* Chem Rev, 2013. **113**(11): p. 8456-90.
232. Corden, J.L., *RNA polymerase II C-terminal domain: Tethering transcription to transcript and template.* Chem Rev, 2013. **113**(11): p. 8423-55.
233. Buratowski, S., *Progression through the RNA polymerase II CTD cycle.* Mol Cell, 2009. **36**(4): p. 541-6.
234. Zhou, Q., T. Li, and D.H. Price, *RNA polymerase II elongation control.* Annu Rev Biochem, 2012. **81**: p. 119-43.
235. Roy, A.L. and D.S. Singer, *Core promoters in transcription: old problem, new insights.* Trends Biochem Sci, 2015. **40**(3): p. 165-71.
236. Consortium, E.P., et al., *Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project.* Nature, 2007. **447**(7146): p. 799-816.
237. Soutourina, J., *Transcription regulation by the Mediator complex.* Nat Rev Mol Cell Biol, 2018. **19**(4): p. 262-274.
238. Gorisch, S.M., et al., *Histone acetylation increases chromatin accessibility.* J Cell Sci, 2005. **118**(Pt 24): p. 5825-34.
239. Spitz, F. and E.E. Furlong, *Transcription factors: from enhancer binding to developmental control.* Nat Rev Genet, 2012. **13**(9): p. 613-26.
240. Ansari, S.A. and R.H. Morse, *Mechanisms of Mediator complex action in transcriptional activation.* Cell Mol Life Sci, 2013. **70**(15): p. 2743-56.
241. Allen, B.L. and D.J. Taatjes, *The Mediator complex: a central integrator of transcription.* Nat Rev Mol Cell Biol, 2015. **16**(3): p. 155-66.
242. Burley, S.K. and R.G. Roeder, *Biochemistry and structural biology of transcription factor IID (TFIID).* Annu Rev Biochem, 1996. **65**: p. 769-99.
243. Albright, S.R. and R. Tjian, *TAFs revisited: more data reveal new twists and confirm old ideas.* Gene, 2000. **242**(1-2): p. 1-13.
244. Juven-Gershon, T. and J.T. Kadonaga, *Regulation of gene expression via the core promoter and the basal transcriptional machinery.* Dev Biol, 2010. **339**(2): p. 225-9.
245. Warfield, L., et al., *Transcription of Nearly All Yeast RNA Polymerase II-Transcribed Genes Is Dependent on Transcription Factor TFIID.* Mol Cell, 2017. **68**(1): p. 118-129 e5.
246. Imbalzano, A.N., K.S. Zaret, and R.E. Kingston, *Transcription factor (TF) IIB and TFIIA can independently increase the affinity of the TATA-binding protein for DNA.* J Biol Chem, 1994. **269**(11): p. 8280-6.
247. Tyree, C.M., et al., *Identification of a minimal set of proteins that is sufficient for accurate initiation of transcription by RNA polymerase II.* Genes Dev, 1993. **7**(7A): p. 1254-65.
248. Kays, A.R. and A. Schepartz, *Virtually unidirectional binding of TBP to the AdMLP TATA box within the quaternary complex with TFIIA and TFIIB.* Chem Biol, 2000. **7**(8): p. 601-10.
249. Tolic-Norrelykke, S.F., et al., *Stepwise bending of DNA by a single TATA-box binding protein.* Biophys J, 2006. **90**(10): p. 3694-703.
250. Bushnell, D.A., et al., *Structural basis of transcription: an RNA polymerase II-TFIIB cocrystal at 4.5 Angstroms.* Science, 2004. **303**(5660): p. 983-8.
251. Hawkes, N.A. and S.G. Roberts, *The role of human TFIIB in transcription start site selection in vitro and in vivo.* J Biol Chem, 1999. **274**(20): p. 14337-43.

252. Fairley, J.A., et al., *Core promoter-dependent TFIIB conformation and a role for TFIIB conformation in transcription start site selection.* Mol Cell Biol, 2002. **22**(19): p. 6697-705.
253. Buratowski, S., et al., *RNA polymerase II-associated proteins are required for a DNA conformation change in the transcription initiation complex.* Proc Natl Acad Sci U S A, 1991. **88**(17): p. 7509-13.
254. Coin, F. and J.M. Egly, *Ten years of TFIIH.* Cold Spring Harb Symp Quant Biol, 1998. **63**: p. 105-10.
255. Fishburn, J., et al., *Double-stranded DNA translocase activity of transcription factor TFIIH and the mechanism of RNA polymerase II open complex formation.* Proc Natl Acad Sci U S A, 2015. **112**(13): p. 3961-6.
256. Holstege, F.C., U. Fiedler, and H.T. Timmers, *Three transitions in the RNA polymerase II transcription complex during initiation.* EMBO J, 1997. **16**(24): p. 7468-80.
257. Luse, D.S. and G.A. Jacob, *Abortive initiation by RNA polymerase II in vitro at the adenovirus 2 major late promoter.* J Biol Chem, 1987. **262**(31): p. 14990-7.
258. Pal, M. and D.S. Luse, *Strong natural pausing by RNA polymerase II within 10 bases of transcription start may result in repeated slippage and reextension of the nascent RNA.* Mol Cell Biol, 2002. **22**(1): p. 30-40.
259. Kostrewa, D., et al., *RNA polymerase II-TFIIB structure and mechanism of transcription initiation.* Nature, 2009. **462**(7271): p. 323-30.
260. Luse, D.S., *Promoter clearance by RNA polymerase II.* Biochim Biophys Acta, 2013. **1829**(1): p. 63-8.
261. Svejstrup, J.Q., et al., *Evidence for a mediator cycle at the initiation of transcription.* Proc Natl Acad Sci U S A, 1997. **94**(12): p. 6075-8.
262. Cheng, B. and D.H. Price, *Analysis of factor interactions with RNA polymerase II elongation complexes using a new electrophoretic mobility shift assay.* Nucleic Acids Res, 2008. **36**(20): p. e135.
263. Jiang, Y. and J.D. Gralla, *Uncoupling of initiation and reinitiation rates during HeLa RNA polymerase II transcription in vitro.* Mol Cell Biol, 1993. **13**(8): p. 4572-7.
264. Yudkovsky, N., J.A. Ranish, and S. Hahn, *A transcription reinitiation intermediate that is stabilized by activator.* Nature, 2000. **408**(6809): p. 225-9.
265. Zawel, L., K.P. Kumar, and D. Reinberg, *Recycling of the general transcription factors during RNA polymerase II transcription.* Genes Dev, 1995. **9**(12): p. 1479-90.
266. Adelman, K. and J.T. Lis, *Promoter-proximal pausing of RNA polymerase II: emerging roles in metazoans.* Nat Rev Genet, 2012. **13**(10): p. 720-31.
267. Rougvie, A.E. and J.T. Lis, *The RNA polymerase II molecule at the 5' end of the uninduced hsp70 gene of D. melanogaster is transcriptionally engaged.* Cell, 1988. **54**(6): p. 795-804.
268. Rasmussen, E.B. and J.T. Lis, *In vivo transcriptional pausing and cap formation on three Drosophila heat shock genes.* Proc Natl Acad Sci U S A, 1993. **90**(17): p. 7923-7.
269. Plet, A., D. Eick, and J.M. Blanchard, *Elongation and premature termination of transcripts initiated from c-fos and c-myc promoters show dissimilar patterns.* Oncogene, 1995. **10**(2): p. 319-28.
270. Gilmour, D.S. and J.T. Lis, *RNA polymerase II interacts with the promoter region of the noninduced hsp70 gene in Drosophila melanogaster cells.* Mol Cell Biol, 1986. **6**(11): p. 3984-9.

271. Rahl, P.B., et al., *c-Myc regulates transcriptional pause release.* Cell, 2010. **141**(3): p. 432-45.
272. Min, I.M., et al., *Regulating RNA polymerase pausing and transcription elongation in embryonic stem cells.* Genes Dev, 2011. **25**(7): p. 742-54.
273. Day, D.S., et al., *Comprehensive analysis of promoter-proximal RNA polymerase II pausing across mammalian cell types.* Genome Biol, 2016. **17**(1): p. 120.
274. Muse, G.W., et al., *RNA polymerase is poised for activation across the genome.* Nat Genet, 2007. **39**(12): p. 1507-11.
275. Jonkers, I., H. Kwak, and J.T. Lis, *Genome-wide dynamics of Pol II elongation and its interplay with promoter proximal pausing, chromatin, and exons.* Elife, 2014. **3**: p. e02407.
276. Gaertner, B. and J. Zeitlinger, *RNA polymerase II pausing during development.* Development, 2014. **141**(6): p. 1179-83.
277. Yamaguchi, Y., et al., *NELF, a multisubunit complex containing RD, cooperates with DSIF to repress RNA polymerase II elongation.* Cell, 1999. **97**(1): p. 41-51.
278. Wada, T., et al., *DSIF, a novel transcription elongation factor that regulates RNA polymerase II processivity, is composed of human Spt4 and Spt5 homologs.* Genes Dev, 1998. **12**(3): p. 343-56.
279. Compe, E., et al., *TFIIE orchestrates the recruitment of the TFIIH kinase module at promoter before release during transcription.* Nat Commun, 2019. **10**(1): p. 2084.
280. Bernecky, C., J.M. Plitzko, and P. Cramer, *Structure of a transcribing RNA polymerase II-DSIF complex reveals a multidentate DNA-RNA clamp.* Nat Struct Mol Biol, 2017. **24**(10): p. 809-815.
281. Grohmann, D., et al., *The initiation factor TFE and the elongation factor Spt4/5 compete for the RNAP clamp during transcription initiation and elongation.* Mol Cell, 2011. **43**(2): p. 263-74.
282. Qiu, Y. and D.S. Gilmour, *Identification of Regions in the Spt5 Subunit of DRB Sensitivity-inducing Factor (DSIF) That Are Involved in Promoter-proximal Pausing.* J Biol Chem, 2017. **292**(13): p. 5555-5570.
283. Missra, A. and D.S. Gilmour, *Interactions between DSIF (DRB sensitivity inducing factor), NELF (negative elongation factor), and the Drosophila RNA polymerase II transcription elongation complex.* Proc Natl Acad Sci U S A, 2010. **107**(25): p. 11301-6.
284. Vos, S.M., et al., *Structure of paused transcription complex Pol II-DSIF-NELF.* Nature, 2018. **560**(7720): p. 601-606.
285. Narita, T., et al., *Human transcription elongation factor NELF: identification of novel subunits and reconstitution of the functionally active complex.* Mol Cell Biol, 2003. **23**(6): p. 1863-73.
286. Wu, C.H., et al., *NELF and DSIF cause promoter proximal pausing on the hsp70 promoter in Drosophila.* Genes Dev, 2003. **17**(11): p. 1402-14.
287. Schier, A.C. and D.J. Taatjes, *Structure and mechanism of the RNA polymerase II transcription machinery.* Genes Dev, 2020. **34**(7-8): p. 465-488.
288. Fujinaga, K., et al., *Dynamics of human immunodeficiency virus transcription: P-TEFb phosphorylates RD and dissociates negative effectors from the transactivation response element.* Mol Cell Biol, 2004. **24**(2): p. 787-95.
289. Lu, X., et al., *Multiple P-TEFbs cooperatively regulate the release of promoter-proximally paused RNA polymerase II.* Nucleic Acids Res, 2016. **44**(14): p. 6853-67.

290. Sanso, M., et al., *P-TEFb regulation of transcription termination factor Xrn2 revealed by a chemical genetic screen for Cdk9 substrates.* Genes Dev, 2016. **30**(1): p. 117-31.
291. Yamada, T., et al., *P-TEFb-mediated phosphorylation of hSpt5 C-terminal repeats is critical for processive transcription elongation.* Mol Cell, 2006. **21**(2): p. 227-37.
292. Wier, A.D., et al., *Structural basis for Spt5-mediated recruitment of the Paf1 complex to chromatin.* Proc Natl Acad Sci U S A, 2013. **110**(43): p. 17290-5.
293. Lenasi, T. and M. Barboric, *P-TEFb stimulates transcription elongation and pre-mRNA splicing through multilateral mechanisms.* RNA Biol, 2010. **7**(2): p. 145-50.
294. Paparidis, N.F., M.C. Durvale, and F. Canduri, *The emerging picture of CDK9/P-TEFb: more than 20 years of advances since PITALRE.* Mol Biosyst, 2017. **13**(2): p. 246-276.
295. Zhou, M., et al., *Tat modifies the activity of CDK9 to phosphorylate serine 5 of the RNA polymerase II carboxyl-terminal domain during human immunodeficiency virus type 1 transcription.* Mol Cell Biol, 2000. **20**(14): p. 5077-86.
296. Czudnochowski, N., C.A. Bosken, and M. Geyer, *Serine-7 but not serine-5 phosphorylation primes RNA polymerase II CTD for P-TEFb recognition.* Nat Commun, 2012. **3**: p. 842.
297. Mayfield, J.E., et al., *Tyr1 phosphorylation promotes phosphorylation of Ser2 on the C-terminal domain of eukaryotic RNA polymerase II by P-TEFb.* Elife, 2019. **8**.
298. Kohoutek, J. and D. Blazek, *Cyclin K goes with Cdk12 and Cdk13.* Cell Div, 2012. **7**: p. 12.
299. Chen, Y., et al., *DSIF, the Paf1 complex, and Tat-SF1 have nonredundant, cooperative roles in RNA polymerase II elongation.* Genes Dev, 2009. **23**(23): p. 2765-77.
300. Vos, S.M., et al., *Structure of activated transcription complex Pol II-DSIF-PAF-SPT6.* Nature, 2018. **560**(7720): p. 607-612.
301. Kim, J., M. Guermah, and R.G. Roeder, *The human PAF1 complex acts in chromatin transcription elongation both independently and cooperatively with SII/TFIIS.* Cell, 2010. **140**(4): p. 491-503.
302. Van Oss, S.B., C.E. Cucinotta, and K.M. Arndt, *Emerging Insights into the Roles of the Paf1 Complex in Gene Regulation.* Trends Biochem Sci, 2017. **42**(10): p. 788-798.
303. Yu, M., et al., *RNA polymerase II-associated factor 1 regulates the release and phosphorylation of paused RNA polymerase II.* Science, 2015. **350**(6266): p. 1383-6.
304. Komarnitsky, P., E.J. Cho, and S. Buratowski, *Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription.* Genes Dev, 2000. **14**(19): p. 2452-60.
305. Guo, J. and D.H. Price, *RNA polymerase II transcription elongation control.* Chem Rev, 2013. **113**(11): p. 8583-603.
306. Connelly, S. and J.L. Manley, *A functional mRNA polyadenylation signal is required for transcription termination by RNA polymerase II.* Genes Dev, 1988. **2**(4): p. 440-52.
307. Nicholson, A.L. and A.E. Pasquinelli, *Tales of Detailed Poly(A) Tails.* Trends Cell Biol, 2019. **29**(3): p. 191-200.
308. Rosonina, E., S. Kaneko, and J.L. Manley, *Terminating the transcript: breaking up is hard to do.* Genes Dev, 2006. **20**(9): p. 1050-6.
309. West, S., N. Gromak, and N.J. Proudfoot, *Human 5' --> 3' exonuclease Xrn2 promotes transcription termination at co-transcriptional cleavage sites.* Nature, 2004. **432**(7016): p. 522-5.

310. Cortazar, M.A., et al., *Control of RNA Pol II Speed by PNUTS-PP1 and Spt5 Dephosphorylation Facilitates Termination by a "Sitting Duck Torpedo" Mechanism.* Mol Cell, 2019. **76**(6): p. 896-908 e4.
311. Davidson, L., L. Muniz, and S. West, *3' end formation of pre-mRNA and phosphorylation of Ser2 on the RNA polymerase II CTD are reciprocally coupled in human cells.* Genes Dev, 2014. **28**(4): p. 342-56.
312. Fong, N., et al., *Effects of Transcription Elongation Rate and Xrn2 Exonuclease Activity on RNA Polymerase II Termination Suggest Widespread Kinetic Competition.* Mol Cell, 2015. **60**(2): p. 256-67.
313. Liu, H. and C.H. Herrmann, *Differential localization and expression of the Cdk9 42k and 55k isoforms.* J Cell Physiol, 2005. **203**(1): p. 251-60.
314. Grana, X., et al., *PITALRE, a nuclear CDC2-related protein kinase that phosphorylates the retinoblastoma protein in vitro.* Proc Natl Acad Sci U S A, 1994. **91**(9): p. 3834-8.
315. Marshall, N.F. and D.H. Price, *Purification of P-TEFb, a transcription factor required for the transition into productive elongation.* J Biol Chem, 1995. **270**(21): p. 12335-8.
316. Marshall, N.F., et al., *Control of RNA polymerase II elongation potential by a novel carboxyl-terminal domain kinase.* J Biol Chem, 1996. **271**(43): p. 27176-83.
317. Zhu, Y., et al., *Transcription elongation factor P-TEFb is required for HIV-1 tat transactivation in vitro.* Genes Dev, 1997. **11**(20): p. 2622-32.
318. Peng, J., N.F. Marshall, and D.H. Price, *Identification of a cyclin subunit required for the function of Drosophila P-TEFb.* J Biol Chem, 1998. **273**(22): p. 13855-60.
319. Peng, J., et al., *Identification of multiple cyclin subunits of human P-TEFb.* Genes Dev, 1998. **12**(5): p. 755-62.
320. Shore, S.M., et al., *Identification of a novel isoform of Cdk9.* Gene, 2003. **307**: p. 175-82.
321. Gold, M.O., et al., *PITALRE, the catalytic subunit of TAK, is required for human immunodeficiency virus Tat transactivation in vivo.* J Virol, 1998. **72**(5): p. 4448-53.
322. Gatignol, A., *Transcription of HIV: Tat and cellular chromatin.* Adv Pharmacol, 2007. **55**: p. 137-59.
323. Zhou, Q. and J.H. Yik, *The Yin and Yang of P-TEFb regulation: implications for human immunodeficiency virus gene expression and global control of cell growth and differentiation.* Microbiol Mol Biol Rev, 2006. **70**(3): p. 646-59.
324. Anand, K., et al., *Cyclin box structure of the P-TEFb subunit cyclin T1 derived from a fusion complex with EIAV tat.* J Mol Biol, 2007. **370**(5): p. 826-36.
325. Baumli, S., et al., *The structure of P-TEFb (CDK9/cyclin T1), its complex with flavopiridol and regulation by phosphorylation.* EMBO J, 2008. **27**(13): p. 1907-18.
326. Brown, N.R., et al., *The structural basis for specificity of substrate and recruitment peptides for cyclin-dependent kinases.* Nat Cell Biol, 1999. **1**(7): p. 438-43.
327. Li, Q., et al., *Analysis of the large inactive P-TEFb complex indicates that it contains one 7SK molecule, a dimer of HEXIM1 or HEXIM2, and two P-TEFb molecules containing Cdk9 phosphorylated at threonine 186.* J Biol Chem, 2005. **280**(31): p. 28819-26.
328. Sancineto, L., et al., *Computer-aided design, synthesis and validation of 2-phenylquinazolinone fragments as CDK9 inhibitors with anti-HIV-1 Tat-mediated transcription activity.* ChemMedChem, 2013. **8**(12): p. 1941-53.
329. Nguyen, V.T., et al., *7SK small nuclear RNA binds to and inhibits the activity of CDK9/cyclin T complexes.* Nature, 2001. **414**(6861): p. 322-5.

330. Yang, Z., et al., *The 7SK small nuclear RNA inhibits the CDK9/cyclin T1 kinase to control transcription.* Nature, 2001. **414**(6861): p. 317-22.
331. Jeronimo, C., et al., *Systematic analysis of the protein interaction network for the human transcription machinery reveals the identity of the 7SK capping enzyme.* Mol Cell, 2007. **27**(2): p. 262-74.
332. Markert, A., et al., *The La-related protein LARP7 is a component of the 7SK ribonucleoprotein and affects transcription of cellular and viral polymerase II genes.* EMBO Rep, 2008. **9**(6): p. 569-75.
333. He, N., et al., *A La-related protein modulates 7SK snRNP integrity to suppress P-TEFb-dependent transcriptional elongation and tumorigenesis.* Mol Cell, 2008. **29**(5): p. 588-99.
334. Xue, Y., et al., *A capping-independent function of MePCE in stabilizing 7SK snRNA and facilitating the assembly of 7SK snRNP.* Nucleic Acids Res, 2010. **38**(2): p. 360-9.
335. Muniz, L., S. Egloff, and T. Kiss, *RNA elements directing in vivo assembly of the 7SK/MePCE/Larp7 transcriptional regulatory snRNP.* Nucleic Acids Res, 2013. **41**(8): p. 4686-98.
336. Brogie, J.E. and D.H. Price, *Reconstitution of a functional 7SK snRNP.* Nucleic Acids Res, 2017. **45**(11): p. 6864-6880.
337. AJ, C.Q., A. Bugai, and M. Barboric, *Cracking the control of RNA polymerase II elongation by 7SK snRNP and P-TEFb.* Nucleic Acids Res, 2016. **44**(16): p. 7527-39.
338. Yik, J.H., et al., *Inhibition of P-TEFb (CDK9/Cyclin T) kinase and RNA polymerase II transcription by the coordinated actions of HEXIM1 and 7SK snRNA.* Mol Cell, 2003. **12**(4): p. 971-82.
339. Byers, S.A., et al., *HEXIM2, a HEXIM1-related protein, regulates positive transcription elongation factor b through association with 7SK.* J Biol Chem, 2005. **280**(16): p. 16360-7.
340. Dulac, C., et al., *Transcription-dependent association of multiple positive transcription elongation factor units to a HEXIM multimer.* J Biol Chem, 2005. **280**(34): p. 30619-29.
341. Li, Q., et al., *HEXIM1 is a promiscuous double-stranded RNA-binding protein and interacts with RNAs in addition to 7SK in cultured cells.* Nucleic Acids Res, 2007. **35**(8): p. 2503-12.
342. Krueger, B.J., et al., *LARP7 is a stable component of the 7SK snRNP while P-TEFb, HEXIM1 and hnRNP A1 are reversibly associated.* Nucleic Acids Res, 2008. **36**(7): p. 2219-29.
343. Michels, A.A., et al., *Binding of the 7SK snRNA turns the HEXIM1 protein into a P-TEFb (CDK9/cyclin T) inhibitor.* EMBO J, 2004. **23**(13): p. 2608-19.
344. Michels, A.A., et al., *MAQ1 and 7SK RNA interact with CDK9/cyclin T complexes in a transcription-dependent manner.* Mol Cell Biol, 2003. **23**(14): p. 4859-69.
345. Kobbi, L., et al., *An evolutionary conserved Hexim1 peptide binds to the Cdk9 catalytic site to inhibit P-TEFb.* Proc Natl Acad Sci U S A, 2016. **113**(45): p. 12721-12726.
346. Chen, R., Z. Yang, and Q. Zhou, *Phosphorylated positive transcription elongation factor b (P-TEFb) is tagged for inhibition through association with 7SK snRNA.* J Biol Chem, 2004. **279**(6): p. 4153-60.
347. Krueger, B.J., et al., *The mechanism of release of P-TEFb and HEXIM1 from the 7SK snRNP by viral and cellular activators includes a conformational change in 7SK.* PLoS One, 2010. **5**(8): p. e12335.

348. Cho, S., S. Schroeder, and M. Ott, *CYCLINg through transcription: posttranslational modifications of P-TEFb regulate transcription elongation.* Cell Cycle, 2010. **9**(9): p. 1697-705.
349. Zhou, M., et al., *Tax interacts with P-TEFb in a novel manner to stimulate human T-lymphotropic virus type 1 transcription.* J Virol, 2006. **80**(10): p. 4781-91.
350. Garber, M.E., et al., *CDK9 autophosphorylation regulates high-affinity binding of the human immunodeficiency virus type 1 tat-P-TEFb complex to TAR RNA.* Mol Cell Biol, 2000. **20**(18): p. 6958-69.
351. Kim, J.B. and P.A. Sharp, *Positive transcription elongation factor B phosphorylates hSPT5 and RNA polymerase II carboxyl-terminal domain independently of cyclin-dependent kinase-activating kinase.* J Biol Chem, 2001. **276**(15): p. 12317-23.
352. Chen, R., et al., *PP2B and PP1alpha cooperatively disrupt 7SK snRNP to release P-TEFb for transcription in response to Ca2+ signaling.* Genes Dev, 2008. **22**(10): p. 1356-68.
353. Larochelle, S., et al., *Cyclin-dependent kinase control of the initiation-to-elongation switch of RNA polymerase II.* Nat Struct Mol Biol, 2012. **19**(11): p. 1108-15.
354. Nekhai, S., M. Petukhov, and D. Breuer, *Regulation of CDK9 activity by phosphorylation and dephosphorylation.* Biomed Res Int, 2014. **2014**: p. 964964.
355. Devaiah, B.N. and D.S. Singer, *Cross-talk among RNA polymerase II kinases modulates C-terminal domain phosphorylation.* J Biol Chem, 2012. **287**(46): p. 38755-66.
356. Gudipaty, S.A., et al., *PPM1G Binds 7SK RNA and Hexim1 To Block P-TEFb Assembly into the 7SK snRNP and Sustain Transcription Elongation.* Mol Cell Biol, 2015. **35**(22): p. 3810-28.
357. Wang, Y., et al., *Phosphatase PPM1A regulates phosphorylation of Thr-186 in the Cdk9 T-loop.* J Biol Chem, 2008. **283**(48): p. 33578-84.
358. Zhou, M., et al., *Bromodomain protein Brd4 regulates human immunodeficiency virus transcription through phosphorylation of CDK9 at threonine 29.* J Virol, 2009. **83**(2): p. 1036-44.
359. Breuer, D., et al., *CDK2 regulates HIV-1 transcription by phosphorylation of CDK9 on serine 90.* Retrovirology, 2012. **9**: p. 94.
360. Ammosova, T., et al., *Protein phosphatase-1 activates CDK9 by dephosphorylating Ser175.* PLoS One, 2011. **6**(4): p. e18985.
361. Mbonye, U.R., et al., *Phosphorylation of CDK9 at Ser175 enhances HIV transcription and is a marker of activated P-TEFb in CD4(+) T lymphocytes.* PLoS Pathog, 2013. **9**(5): p. e1003338.
362. Yang, Z., et al., *Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4.* Mol Cell, 2005. **19**(4): p. 535-45.
363. Fu, J., et al., *Regulation of P-TEFb elongation complex activity by CDK9 acetylation.* Mol Cell Biol, 2007. **27**(13): p. 4641-51.
364. Sabo, A., et al., *Acetylation of conserved lysines in the catalytic core of cyclin-dependent kinase 9 inhibits kinase activity and regulates transcription.* Mol Cell Biol, 2008. **28**(7): p. 2201-12.
365. Zhang, H., et al., *SIRT2 directs the replication stress response through CDK9 deacetylation.* Proc Natl Acad Sci U S A, 2013. **110**(33): p. 13546-51.
366. Blank, M.F., et al., *SIRT7-dependent deacetylation of CDK9 activates RNA polymerase II transcription.* Nucleic Acids Res, 2017. **45**(5): p. 2675-2686.

367. Cho, S., et al., *Acetylation of cyclin T1 regulates the equilibrium between active and inactive P-TEFb in cells.* EMBO J, 2009. **28**(10): p. 1407-17.
368. Schroder, S., et al., *Two-pronged binding with bromodomain-containing protein 4 liberates positive transcription elongation factor b from inactive ribonucleoprotein complexes.* J Biol Chem, 2012. **287**(2): p. 1090-9.
369. Kiernan, R.E., et al., *Interaction between cyclin T1 and SCF(SKP2) targets CDK9 for ubiquitination and degradation by the proteasome.* Mol Cell Biol, 2001. **21**(23): p. 7956-70.
370. Barboric, M., et al., *Ubiquitylation of Cdk9 by Skp2 facilitates optimal Tat transactivation.* J Virol, 2005. **79**(17): p. 11135-41.
371. Yu, F., et al., *SUMO suppresses and MYC amplifies transcription globally by regulating CDK9 sumoylation.* Cell Res, 2018. **28**(6): p. 670-685.
372. Ma, X., et al., *TRIM28 promotes HIV-1 latency by SUMOylating CDK9 and inhibiting P-TEFb.* Elife, 2019. **8**.
373. Contreras, X., et al., *HMBA releases P-TEFb from HEXIM1 and 7SK snRNA via PI3K/Akt and activates HIV transcription.* PLoS Pathog, 2007. **3**(10): p. 1459-69.
374. Choudhary, S.K., N.M. Archin, and D.M. Margolis, *Hexamethylbisacetamide and disruption of human immunodeficiency virus type 1 latency in CD4(+) T cells.* J Infect Dis, 2008. **197**(8): p. 1162-70.
375. Fujinaga, K., et al., *PKC phosphorylates HEXIM1 and regulates P-TEFb activity.* Nucleic Acids Res, 2012. **40**(18): p. 9160-70.
376. Sun, Y., et al., *Activation of P-TEFb by cAMP-PKA signaling in autosomal dominant polycystic kidney disease.* Sci Adv, 2019. **5**(6): p. eaaw3593.
377. Mbonye, U.R., et al., *Phosphorylation of HEXIM1 at Tyr271 and Tyr274 Promotes Release of P-TEFb from the 7SK snRNP Complex and Enhances Proviral HIV Gene Expression.* Proteomics, 2015. **15**(12): p. 2078-86.
378. Kim, Y.K., et al., *T-cell receptor signaling enhances transcriptional elongation from latent HIV proviruses by activating P-TEFb through an ERK-dependent pathway.* J Mol Biol, 2011. **410**(5): p. 896-916.
379. Lau, J., et al., *Ubiquitination of HEXIM1 by HDM2.* Cell Cycle, 2009. **8**(14): p. 2247-54.
380. Faust, T.B., et al., *The HIV-1 Tat protein recruits a ubiquitin ligase to reorganize the 7SK snRNP for transcriptional activation.* Elife, 2018. **7**.
381. Lambert, S.A., et al., *The Human Transcription Factors.* Cell, 2018. **172**(4): p. 650-665.
382. Kohli, S., S. Ahuja, and V. Rani, *Transcription factors in heart: promising therapeutic targets in cardiac hypertrophy.* Curr Cardiol Rev, 2011. **7**(4): p. 262-71.
383. Ye, R.D., *Regulation of nuclear factor kappaB activation by G-protein-coupled receptors.* J Leukoc Biol, 2001. **70**(6): p. 839-48.
384. Barboric, M., et al., *NF-kappaB binds P-TEFb to stimulate transcriptional elongation by RNA polymerase II.* Mol Cell, 2001. **8**(2): p. 327-37.
385. Sunagawa, Y., et al., *Cyclin-dependent kinase-9 is a component of the p300/GATA4 complex required for phenylephrine-induced hypertrophy in cardiomyocytes.* J Biol Chem, 2010. **285**(13): p. 9556-68.
386. Nojima, M., et al., *The positive transcription elongation factor b is an essential cofactor for the activation of transcription by myocyte enhancer factor 2.* J Mol Biol, 2008. **382**(2): p. 275-87.

387. Donati, B., E. Lorenzini, and A. Ciarrocchi, *BRD4 and Cancer: going beyond transcriptional regulation.* Mol Cancer, 2018. **17**(1): p. 164.
388. Zeng, L. and M.M. Zhou, *Bromodomain: an acetyl-lysine binding domain.* FEBS Lett, 2002. **513**(1): p. 124-8.
389. Rahman, S., et al., *The Brd4 extraterminal domain confers transcription activation independent of pTEFb by recruiting multiple proteins, including NSD3.* Mol Cell Biol, 2011. **31**(13): p. 2641-52.
390. Filippakopoulos, P., et al., *Histone recognition and large-scale structural analysis of the human bromodomain family.* Cell, 2012. **149**(1): p. 214-31.
391. Moriniere, J., et al., *Cooperative binding of two acetylation marks on a histone tail by a single bromodomain.* Nature, 2009. **461**(7264): p. 664-8.
392. Kvaratskhelia, M., et al., *Molecular mechanisms of retroviral integration site selection.* Nucleic Acids Res, 2014. **42**(16): p. 10209-25.
393. Devaiah, B.N., A. Gegonne, and D.S. Singer, *Bromodomain 4: a cellular Swiss army knife.* J Leukoc Biol, 2016. **100**(4): p. 679-686.
394. Vollmuth, F., W. Blankenfeldt, and M. Geyer, *Structures of the dual bromodomains of the P-TEFb-activating protein Brd4 at atomic resolution.* J Biol Chem, 2009. **284**(52): p. 36547-56.
395. Duan, Y., et al., *Targeting Brd4 for cancer therapy: inhibitors and degraders.* Medchemcomm, 2018. **9**(11): p. 1779-1802.
396. LeRoy, G., B. Rickards, and S.J. Flint, *The double bromodomain proteins Brd2 and Brd3 couple histone acetylation to transcription.* Mol Cell, 2008. **30**(1): p. 51-60.
397. Bourova-Flin, E., et al., *The Role of Bromodomain Testis-Specific Factor, BRDT, in Cancer: A Biomarker and A Possible Therapeutic Target.* Cell J, 2017. **19**(Suppl 1): p. 1-8.
398. Jiang, Y.W., et al., *Mammalian mediator of transcriptional regulation and its possible role as an end-point of signal transduction pathways.* Proc Natl Acad Sci U S A, 1998. **95**(15): p. 8538-43.
399. Dey, A., et al., *A bromodomain protein, MCAP, associates with mitotic chromosomes and affects G(2)-to-M transition.* Mol Cell Biol, 2000. **20**(17): p. 6537-49.
400. Zhao, R., et al., *Gene bookmarking accelerates the kinetics of post-mitotic transcriptional re-activation.* Nat Cell Biol, 2011. **13**(11): p. 1295-304.
401. Floyd, S.R., et al., *The bromodomain protein Brd4 insulates chromatin from DNA damage signalling.* Nature, 2013. **498**(7453): p. 246-50.
402. Han, X., et al., *Roles of the BRD4 short isoform in phase separation and active gene transcription.* Nat Struct Mol Biol, 2020. **27**(4): p. 333-341.
403. Jang, M.K., et al., *The bromodomain protein Brd4 is a positive regulatory component of P-TEFb and stimulates RNA polymerase II-dependent transcription.* Mol Cell, 2005. **19**(4): p. 523-34.
404. Bisgrove, D.A., et al., *Conserved P-TEFb-interacting domain of BRD4 inhibits HIV transcription.* Proc Natl Acad Sci U S A, 2007. **104**(34): p. 13690-5.
405. Hnisz, D., et al., *Super-enhancers in the control of cell identity and disease.* Cell, 2013. **155**(4): p. 934-47.
406. Whyte, W.A., et al., *Master transcription factors and mediator establish super-enhancers at key cell identity genes.* Cell, 2013. **153**(2): p. 307-19.

407. Jia, Y., W.J. Chng, and J. Zhou, *Super-enhancers: critical roles and therapeutic targets in hematologic malignancies.* J Hematol Oncol, 2019. **12**(1): p. 77.
408. Niederriter, A.R., et al., *Super Enhancers in Cancers, Complex Disease, and Developmental Disorders.* Genes (Basel), 2015. **6**(4): p. 1183-200.
409. Wu, S.Y., et al., *Phospho switch triggers Brd4 chromatin binding and activator recruitment for gene-specific targeting.* Mol Cell, 2013. **49**(5): p. 843-57.
410. Chiang, C.M., *Phospho-BRD4: transcription plasticity and drug targeting.* Drug Discov Today Technol, 2016. **19**: p. 17-22.
411. Penas, C., et al., *Time series modeling of cell cycle exit identifies Brd4 dependent regulation of cerebellar neurogenesis.* Nat Commun, 2019. **10**(1): p. 3028.
412. Wang, R., et al., *Uncovering BRD4 hyperphosphorylation associated with cellular transformation in NUT midline carcinoma.* Proc Natl Acad Sci U S A, 2017. **114**(27): p. E5352-E5361.
413. Stratton, M.S., et al., *Dynamic Chromatin Targeting of BRD4 Stimulates Cardiac Fibroblast Activation.* Circ Res, 2019. **125**(7): p. 662-677.
414. Huang, B., et al., *Brd4 coactivates transcriptional activation of NF-kappaB via specific binding to acetylated RelA.* Mol Cell Biol, 2009. **29**(5): p. 1375-87.
415. Field, A. and K. Adelman, *Evaluating Enhancer Function and Transcription.* Annu Rev Biochem, 2020.
416. Wang, Z., et al., *Combinatorial patterns of histone acetylations and methylations in the human genome.* Nat Genet, 2008. **40**(7): p. 897-903.
417. Bhagwat, A.S., et al., *BET Bromodomain Inhibition Releases the Mediator Complex from Select cis-Regulatory Elements.* Cell Rep, 2016. **15**(3): p. 519-530.
418. Devaiah, B.N., et al., *BRD4 is an atypical kinase that phosphorylates serine2 of the RNA polymerase II carboxy-terminal domain.* Proc Natl Acad Sci U S A, 2012. **109**(18): p. 6927-32.
419. Kanno, T., et al., *BRD4 assists elongation of both coding and enhancer RNAs by interacting with acetylated histones.* Nat Struct Mol Biol, 2014. **21**(12): p. 1047-57.
420. Devaiah, B.N., et al., *BRD4 is a histone acetyltransferase that evicts nucleosomes from chromatin.* Nat Struct Mol Biol, 2016. **23**(6): p. 540-8.
421. Uppal, S., et al., *The Bromodomain Protein 4 Contributes to the Regulation of Alternative Splicing.* Cell Rep, 2019. **29**(8): p. 2450-2460 e5.
422. Alqahtani, A., et al., *Bromodomain and extra-terminal motif inhibitors: a review of preclinical and clinical advances in cancer therapy.* Future Sci OA, 2019. **5**(3): p. FSO372.
423. Nicodeme, E., et al., *Suppression of inflammation by a synthetic histone mimic.* Nature, 2010. **468**(7327): p. 1119-23.
424. Filippakopoulos, P., et al., *Selective inhibition of BET bromodomains.* Nature, 2010. **468**(7327): p. 1067-73.
425. Delmore, J.E., et al., *BET bromodomain inhibition as a therapeutic strategy to target c-Myc.* Cell, 2011. **146**(6): p. 904-17.
426. Puissant, A., et al., *Targeting MYCN in neuroblastoma by BET bromodomain inhibition.* Cancer Discov, 2013. **3**(3): p. 308-23.
427. Klein, K., et al., *The bromodomain protein inhibitor I-BET151 suppresses expression of inflammatory genes and matrix degrading enzymes in rheumatoid arthritis synovial fibroblasts.* Ann Rheum Dis, 2016. **75**(2): p. 422-9.

428. Duan, Q., et al., *BET bromodomain inhibition suppresses innate inflammatory and profibrotic transcriptional networks in heart failure.* Sci Transl Med, 2017. **9**(390).
429. Gilan, O., et al., *Selective targeting of BD1 and BD2 of the BET proteins in cancer and immuno-inflammation.* Science, 2020.
430. Niu, Q., et al., *Structure-guided drug design identifies a BRD4-selective small molecule that suppresses HIV.* J Clin Invest, 2019. **129**(8): p. 3361-3373.
431. Luo, Z., C. Lin, and A. Shilatifard, *The super elongation complex (SEC) family in transcriptional control.* Nat Rev Mol Cell Biol, 2012. **13**(9): p. 543-7.
432. Lin, C., et al., *Dynamic transcriptional events in embryonic stem cells mediated by the super elongation complex (SEC).* Genes Dev, 2011. **25**(14): p. 1486-98.
433. Lin, C., et al., *AFF4, a component of the ELL/P-TEFb elongation complex and a shared subunit of MLL chimeras, can link transcription elongation to leukemia.* Mol Cell, 2010. **37**(3): p. 429-37.
434. Luo, Z., et al., *The super elongation complex family of RNA polymerase II elongation factors: gene target specificity and transcriptional output.* Mol Cell Biol, 2012. **32**(13): p. 2608-17.
435. Rowley, J.D., *The critical role of chromosome translocations in human leukemias.* Annu Rev Genet, 1998. **32**: p. 495-519.
436. Chan, A.K.N. and C.W. Chen, *Rewiring the Epigenetic Networks in MLL-Rearranged Leukemias: Epigenetic Dysregulation and Pharmacological Interventions.* Front Cell Dev Biol, 2019. **7**: p. 81.
437. Shilatifard, A., et al., *An RNA polymerase II elongation factor encoded by the human ELL gene.* Science, 1996. **271**(5257): p. 1873-6.
438. He, N., et al., *HIV-1 Tat and host AFF4 recruit two transcription elongation factors into a bifunctional complex for coordinated activation of HIV-1 transcription.* Mol Cell, 2010. **38**(3): p. 428-38.
439. Sobhian, B., et al., *HIV-1 Tat assembles a multifunctional transcription elongation complex and stably associates with the 7SK snRNP.* Mol Cell, 2010. **38**(3): p. 439-51.
440. Chen, Y. and P. Cramer, *Structure of the super-elongation complex subunit AFF4 C-terminal homology domain reveals requirements for AFF homo- and heterodimerization.* J Biol Chem, 2019. **294**(27): p. 10663-10673.
441. Yokoyama, A., et al., *A higher-order complex containing AF4 and ENL family proteins with P-TEFb facilitates oncogenic and physiologic MLL-dependent transcription.* Cancer Cell, 2010. **17**(2): p. 198-212.
442. Kuzmina, A., S. Krasnopolsky, and R. Taube, *Super elongation complex promotes early HIV transcription and its function is modulated by P-TEFb.* Transcription, 2017. **8**(3): p. 133-149.
443. Lu, H., et al., *Gene target specificity of the Super Elongation Complex (SEC) family: how HIV-1 Tat employs selected SEC members to activate viral transcription.* Nucleic Acids Res, 2015. **43**(12): p. 5868-79.
444. Liang, K., et al., *Therapeutic Targeting of MLL Degradation Pathways in MLL-Rearranged Leukemia.* Cell, 2017. **168**(1-2): p. 59-72 e13.
445. Liang, K., et al., *Targeting Processive Transcription Elongation via SEC Disruption for MYC-Induced Cancer Therapy.* Cell, 2018. **175**(3): p. 766-779 e17.
446. Chou, S., et al., *HIV-1 Tat recruits transcription elongation factors dispersed along a flexible AFF4 scaffold.* Proc Natl Acad Sci U S A, 2013. **110**(2): p. E123-31.

447. Schulze-Gahmen, U., et al., *The AFF4 scaffold binds human P-TEFb adjacent to HIV Tat.* Elife, 2013. **2**: p. e00327.
448. Qi, S., et al., *Structural basis for ELL2 and AFF4 activation of HIV-1 proviral transcription.* Nat Commun, 2017. **8**: p. 14076.
449. Elmendorf, B.J., et al., *Transcription factors TFIIF, ELL, and Elongin negatively regulate SII-induced nascent transcript cleavage by non-arrested RNA polymerase II elongation intermediates.* J Biol Chem, 2001. **276**(25): p. 23109-14.
450. Shilatifard, A., et al., *ELL2, a new member of an ELL family of RNA polymerase II elongation factors.* Proc Natl Acad Sci U S A, 1997. **94**(8): p. 3639-43.
451. Miller, T., et al., *Identification, cloning, expression, and biochemical characterization of the testis-specific RNA polymerase II elongation factor ELL3.* J Biol Chem, 2000. **275**(41): p. 32052-6.
452. Kong, S.E., et al., *ELL-associated factors 1 and 2 are positive regulators of RNA polymerase II elongation factor ELL.* Proc Natl Acad Sci U S A, 2005. **102**(29): p. 10094-8.
453. Smith, E.R., et al., *The little elongation complex regulates small nuclear RNA transcription.* Mol Cell, 2011. **44**(6): p. 954-65.
454. He, N., et al., *Human Polymerase-Associated Factor complex (PAFc) connects the Super Elongation Complex (SEC) to RNA polymerase II on chromatin.* Proc Natl Acad Sci U S A, 2011. **108**(36): p. E636-45.
455. Leach, B.I., et al., *Leukemia fusion target AF9 is an intrinsically disordered transcriptional regulator that recruits multiple partners via coupled folding and binding.* Structure, 2013. **21**(1): p. 176-183.
456. Li, Y., et al., *AF9 YEATS domain links histone acetylation to DOT1L-mediated H3K79 methylation.* Cell, 2014. **159**(3): p. 558-71.
457. Takahashi, H., et al., *Human mediator subunit MED26 functions as a docking site for transcription elongation factors.* Cell, 2011. **146**(1): p. 92-104.
458. Hertweck, A., et al., *T-bet Activates Th1 Genes through Mediator and the Super Elongation Complex.* Cell Rep, 2016. **15**(12): p. 2756-70.
459. Wan, L., et al., *ENL links histone acetylation to oncogenic gene expression in acute myeloid leukaemia.* Nature, 2017. **543**(7644): p. 265-269.
460. Yadav, D., et al., *Multivalent Role of Human TFIID in Recruiting Elongation Components at the Promoter-Proximal Region for Transcriptional Control.* Cell Rep, 2019. **26**(5): p. 1303-1317 e7.
461. Hetzner, K., et al., *The interaction of ENL with PAF1 mitigates polycomb silencing and facilitates murine leukemogenesis.* Blood, 2018. **131**(6): p. 662-673.
462. Christott, T., et al., *Discovery of a Selective Inhibitor for the YEATS Domains of ENL/AF9.* SLAS Discov, 2019. **24**(2): p. 133-141.
463. Li, X., et al., *Structure-guided development of YEATS domain inhibitors by targeting pi-pi-pi stacking.* Nat Chem Biol, 2018. **14**(12): p. 1140-1149.
464. D'Orso, I. and A.D. Frankel, *RNA-mediated displacement of an inhibitory snRNP complex activates transcription elongation.* Nat Struct Mol Biol, 2010. **17**(7): p. 815-21.
465. Egloff, S., C. Studniarek, and T. Kiss, *7SK small nuclear RNA, a multifunctional transcriptional regulatory RNA with gene-specific features.* Transcription, 2018. **9**(2): p. 95-101.

466. McNamara, R.P., et al., *KAP1 Recruitment of the 7SK snRNP Complex to Promoters Enables Transcription Elongation by RNA Polymerase II.* Mol Cell, 2016. **61**(1): p. 39-53.
467. Flynn, R.A., et al., *7SK-BAF axis controls pervasive transcription at enhancers.* Nat Struct Mol Biol, 2016. **23**(3): p. 231-8.
468. Liu, W., et al., *Brd4 and JMJD6-associated anti-pause enhancers in regulation of transcriptional pause release.* Cell, 2013. **155**(7): p. 1581-1595.
469. Eilebrecht, S., et al., *HMGA1 recruits CTIP2-repressed P-TEFb to the HIV-1 and cellular target promoters.* Nucleic Acids Res, 2014. **42**(8): p. 4962-71.
470. Winter, G.E., et al., *DRUG DEVELOPMENT. Phthalimide conjugation as a strategy for in vivo target protein degradation.* Science, 2015. **348**(6241): p. 1376-81.
471. Winter, G.E., et al., *BET Bromodomain Proteins Function as Master Transcription Elongation Factors Independent of CDK9 Recruitment.* Mol Cell, 2017. **67**(1): p. 5-18 e19.
472. McNamara, R.P., et al., *Transcription factors mediate the enzymatic disassembly of promoter-bound 7SK snRNP to locally recruit P-TEFb for transcription elongation.* Cell Rep, 2013. **5**(5): p. 1256-68.
473. Calo, E., et al., *RNA helicase DDX21 coordinates transcription and ribosomal RNA processing.* Nature, 2015. **518**(7538): p. 249-53.
474. Ji, X., et al., *SR proteins collaborate with 7SK and promoter-associated nascent RNA to release paused polymerase.* Cell, 2013. **153**(4): p. 855-68.
475. Mizuno, K., K. Kurokawa, and S. Ohkuma, *Regulation of type 1 IP(3) receptor expression by dopamine D2-like receptors via AP-1 and NFATc4 activation.* Neuropharmacology, 2013. **71**: p. 264-72.
476. Ahmadiantehrani, S. and D. Ron, *Dopamine D2 receptor activation leads to an up-regulation of glial cell line-derived neurotrophic factor via Gbetagamma-Erk1/2-dependent induction of Zif268.* J Neurochem, 2013. **125**(2): p. 193-204.
477. Stern, C.M., et al., *Corticotropin releasing factor-induced CREB activation in striatal neurons occurs via a novel Gbetagamma signaling pathway.* PLoS One, 2011. **6**(3): p. e18114.
478. Tu, H., et al., *Dominant role of GABAB2 and Gbetagamma for GABAB receptor-mediated-ERK1/2/CREB pathway in cerebellar neurons.* Cell Signal, 2007. **19**(9): p. 1996-2002.
479. Yost, E.A., et al., *Inhibition of G-protein betagamma signaling enhances T cell receptor-stimulated interleukin 2 transcription in CD4+ T helper cells.* PLoS One, 2015. **10**(1): p. e0116575.
480. Zaballos, M.A., B. Garcia, and P. Santisteban, *Gbetagamma dimers released in response to thyrotropin activate phosphoinositide 3-kinase and regulate gene expression in thyroid cells.* Mol Endocrinol, 2008. **22**(5): p. 1183-99.
481. Martin, R.D., C.A. Bouazza, and T.E. Hébert, *Chapter 13 - Organellar Gβγ signaling—GPCR signaling beyond the cell surface*, in *GPCRs*, B. Jastrzebska and P.S.H. Park, Editors. 2020, Academic Press. p. 257-267.
482. Yuen, J.W., et al., *Activation of STAT3 by specific Galpha subunits and multiple Gbetagamma dimers.* Int J Biochem Cell Biol, 2010. **42**(6): p. 1052-9.
483. Georganta, E.M., A. Agalou, and Z. Georgoussi, *Multi-component signaling complexes of the delta-opioid receptor with STAT5B and G proteins.* Neuropharmacology, 2010. **59**(3): p. 139-48.
484. Park, J.G., et al., *Transcriptional regulation by the gamma5 subunit of a heterotrimeric G protein during adipogenesis.* EMBO J, 1999. **18**(14): p. 4004-12.

485. Spiegelberg, B.D. and H.E. Hamm, *G betagamma binds histone deacetylase 5 (HDAC5) and inhibits its transcriptional co-repression activity.* J Biol Chem, 2005. **280**(50): p. 41769-76.
486. Toth, A.D., et al., *Inflammation leads through PGE/EP3 signaling to HDAC5/MEF2-dependent transcription in cardiac myocytes.* EMBO Mol Med, 2018. **10**(7).
487. Robitaille, M., et al., *Gbetagamma is a negative regulator of AP-1 mediated transcription.* Cell Signal, 2010. **22**(8): p. 1254-66.
488. Kino, T., et al., *G protein beta interacts with the glucocorticoid receptor and suppresses its transcriptional activity in the nucleus.* J Cell Biol, 2005. **169**(6): p. 885-96.
489. Bhatnagar, A., et al., *Interaction of G-protein betagamma complex with chromatin modulates GPCR-dependent gene regulation.* PLoS One, 2013. **8**(1): p. e52689.
490. Khan, S.M., et al., *Gbeta4gamma1 as a modulator of M3 muscarinic receptor signalling and novel roles of Gbeta1 subunits in the modulation of cellular signalling.* Cell Signal, 2015. **27**(8): p. 1597-608.
491. Akazawa, H. and I. Komuro, *Roles of cardiac transcription factors in cardiac hypertrophy.* Circ Res, 2003. **92**(10): p. 1079-88.
492. Alexanian, M., et al., *Epigenetic therapies in heart failure.* J Mol Cell Cardiol, 2019. **130**: p. 197-204.
493. Manabe, I., T. Shindo, and R. Nagai, *Gene expression in fibroblasts and fibrosis: involvement in cardiac hypertrophy.* Circ Res, 2002. **91**(12): p. 1103-13.
494. Morin, S., et al., *GATA-dependent recruitment of MEF2 proteins to target promoters.* EMBO J, 2000. **19**(9): p. 2046-55.
495. Kinnunen, S.M., et al., *Cardiac Actions of a Small Molecule Inhibitor Targeting GATA4-NKX2-5 Interaction.* Sci Rep, 2018. **8**(1): p. 4611.
496. Schlesinger, J., et al., *The cardiac transcription network modulated by Gata4, Mef2a, Nkx2.5, Srf, histone modifications, and microRNAs.* PLoS Genet, 2011. **7**(2): p. e1001313.
497. Nemer, G. and M. Nemer, *Regulation of heart development and function through combinatorial interactions of transcription factors.* Ann Med, 2001. **33**(9): p. 604-10.
498. Kim, L.J., et al., *Human Taf(II)130 is a coactivator for NFATp.* Mol Cell Biol, 2001. **21**(10): p. 3503-13.
499. Enane, F.O., et al., *GATA4 loss of function in liver cancer impedes precursor to hepatocyte transition.* J Clin Invest, 2017. **127**(9): p. 3527-3542.
500. Gordon, J.W., J.A. Shaw, and L.A. Kirshenbaum, *Multiple facets of NF-kappaB in the heart: to be or not to NF-kappaB.* Circ Res, 2011. **108**(9): p. 1122-32.
501. He, A., et al., *Dynamic GATA4 enhancers shape the chromatin landscape central to heart development and disease.* Nat Commun, 2014. **5**: p. 4907.
502. Sayed, D., et al., *Acute targeting of general transcription factor IIB restricts cardiac hypertrophy via selective inhibition of gene transcription.* Circ Heart Fail, 2015. **8**(1): p. 138-48.
503. Nykamp, K., et al., *Sherloc: a comprehensive refinement of the ACMG-AMP variant classification criteria.* Genet Med, 2017. **19**(10): p. 1105-1117.
504. Abdellatif, M., et al., *A Ras-dependent pathway regulates RNA polymerase II phosphorylation in cardiac myocytes: implications for cardiac hypertrophy.* Mol Cell Biol, 1998. **18**(11): p. 6729-36.
505. Sano, M., et al., *Activation and function of cyclin T-Cdk9 (positive transcription elongation factor-b) in cardiac muscle-cell hypertrophy.* Nat Med, 2002. **8**(11): p. 1310-7.

506. Sano, M., et al., *Activation of cardiac Cdk9 represses PGC-1 and confers a predisposition to heart failure.* EMBO J, 2004. **23**(17): p. 3559-69.
507. Espinoza-Derout, J., et al., *Pivotal role of cardiac lineage protein-1 (CLP-1) in transcriptional elongation factor P-TEFb complex formation in cardiac hypertrophy.* Cardiovasc Res, 2007. **75**(1): p. 129-38.
508. Huang, F., M. Wagner, and M.A. Siddiqui, *Ablation of the CLP-1 gene leads to down-regulation of the HAND1 gene and abnormality of the left ventricle of the heart and fetal death.* Mech Dev, 2004. **121**(6): p. 559-72.
509. Espinoza-Derout, J., et al., *Positive transcription elongation factor b activity in compensatory myocardial hypertrophy is regulated by cardiac lineage protein-1.* Circ Res, 2009. **104**(12): p. 1347-54.
510. Mascareno, E., et al., *Cardiac lineage protein-1 (CLP-1) regulates cardiac remodeling via transcriptional modulation of diverse hypertrophic and fibrotic responses and angiotensin II-transforming growth factor beta (TGF-beta1) signaling axis.* J Biol Chem, 2012. **287**(16): p. 13084-93.
511. Anand, P., et al., *BET bromodomains mediate transcriptional pause release in heart failure.* Cell, 2013. **154**(3): p. 569-82.
512. Spiltoir, J.I., et al., *BET acetyl-lysine binding proteins control pathological cardiac hypertrophy.* J Mol Cell Cardiol, 2013. **63**: p. 175-9.
513. Stratton, M.S., et al., *Signal-Dependent Recruitment of BRD4 to Cardiomyocyte Super-Enhancers Is Suppressed by a MicroRNA.* Cell Rep, 2016. **16**(5): p. 1366-1378.
514. Song, S., et al., *Inhibition of BRD4 attenuates transverse aortic constriction- and TGF-beta-induced endothelial-mesenchymal transition and cardiac fibrosis.* J Mol Cell Cardiol, 2019. **127**: p. 83-96.
515. Sun, Y., J. Huang, and K. Song, *BET protein inhibition mitigates acute myocardial infarction damage in rats via the TLR4/TRAF6/NF-kappaB pathway.* Exp Ther Med, 2015. **10**(6): p. 2319-2324.
516. Agasthi, P.A.L. *Effect of BET Protein Inhibition With Apabetalone on Cardiovascular Outcomes in Patients With Acute Coronary Syndrome and Diabetes*. 2020 29 March 2020]; Available from: https://professional.heart.org/professional/ScienceNews/UCM_505223_BETonMACE-Clinical-Trial-Details.jsp.
517. Piquereau, J., et al., *The BET Bromodomain Inhibitor I-BET-151 Induces Structural and Functional Alterations of the Heart Mitochondria in Healthy Male Mice and Rats.* Int J Mol Sci, 2019. **20**(7).
518. Padmanabhan, A. and S.M. Haldar, *Drugging transcription in heart failure.* J Physiol, 2019.
519. Bedioune, I., et al., *PDE4 and mAKAPbeta are nodal organizers of beta2-ARs nuclear PKA signalling in cardiac myocytes.* Cardiovasc Res, 2018. **114**(11): p. 1499-1511.
520. Cattaneo, F., et al., *Intranuclear Signaling Cascades Triggered by Nuclear GPCRs.* Journal of Cell Signaling, 2016. **01**.
521. Hague, C., et al., *Cell surface expression of alpha1D-adrenergic receptors is controlled by heterodimerization with alpha1B-adrenergic receptors.* J Biol Chem, 2004. **279**(15): p. 15541-9.

522. Dahl, E.F., et al., *Subcellular compartmentalization of proximal Galphaq-receptor signaling produces unique hypertrophic phenotypes in adult cardiac myocytes.* J Biol Chem, 2018. **293**(23): p. 8734-8749.
523. Nikolaev, V.O., et al., *Beta2-adrenergic receptor redistribution in heart failure changes cAMP compartmentation.* Science, 2010. **327**(5973): p. 1653-7.
524. Nikolaev, V.O., et al., *Cyclic AMP imaging in adult cardiac myocytes reveals far-reaching beta1-adrenergic but locally confined beta2-adrenergic receptor-mediated signaling.* Circ Res, 2006. **99**(10): p. 1084-91.
525. Yoshikawa, N., et al., *Cardiomyocyte-specific overexpression of HEXIM1 prevents right ventricular hypertrophy in hypoxia-induced pulmonary hypertension in mice.* PLoS One, 2012. **7**(12): p. e52522.
526. Bahat, A., et al., *Targeting Spt5-Pol II by Small-Molecule Inhibitors Uncouples Distinct Activities and Reveals Additional Regulatory Roles.* Mol Cell, 2019. **76**(4): p. 617-631 e4.
527. Diamant, G., A. Bahat, and R. Dikstein, *The elongation factor Spt5 facilitates transcription initiation for rapid induction of inflammatory-response genes.* Nat Commun, 2016. **7**: p. 11547.
528. Zhong, H., R.E. Voll, and S. Ghosh, *Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300.* Mol Cell, 1998. **1**(5): p. 661-71.
529. Chen, L.F., et al., *NF-kappaB RelA phosphorylation regulates RelA acetylation.* Mol Cell Biol, 2005. **25**(18): p. 7966-75.
530. Saijo, K., et al., *Protein kinase C beta controls nuclear factor kappaB activation in B cells through selective regulation of the IkappaB kinase alpha.* J Exp Med, 2002. **195**(12): p. 1647-52.
531. Purcell, N.H., et al., *Activation of NF-kappa B is required for hypertrophic growth of primary rat neonatal ventricular cardiomyocytes.* Proc Natl Acad Sci U S A, 2001. **98**(12): p. 6668-73.
532. Jun, J.H., et al., *Erythropoietin-activated ERK/MAP kinase enhances GATA-4 acetylation via phosphorylation of serine 261 of GATA-4.* J Cell Physiol, 2013. **228**(1): p. 190-7.
533. Takaya, T., et al., *Identification of p300-targeted acetylated residues in GATA4 during hypertrophic responses in cardiac myocytes.* J Biol Chem, 2008. **283**(15): p. 9828-35.
534. Chaytor, L., et al., *The Pioneering Role of GATA2 in Androgen Receptor Variant Regulation Is Controlled by Bromodomain and Extraterminal Proteins in Castrate-Resistant Prostate Cancer.* Mol Cancer Res, 2019. **17**(6): p. 1264-1278.
535. Lambert, J.P., et al., *Interactome Rewiring Following Pharmacological Targeting of BET Bromodomains.* Mol Cell, 2019. **73**(3): p. 621-638 e17.
536. Tremblay, J.J. and R.S. Viger, *Transcription factor GATA-4 is activated by phosphorylation of serine 261 via the cAMP/protein kinase a signaling pathway in gonadal cells.* J Biol Chem, 2003. **278**(24): p. 22128-35.
537. Wang, J., et al., *Convergence of protein kinase C and JAK-STAT signaling on transcription factor GATA-4.* Mol Cell Biol, 2005. **25**(22): p. 9829-44.
538. Alfert, A., N. Moreno, and K. Kerl, *The BAF complex in development and disease.* Epigenetics Chromatin, 2019. **12**(1): p. 19.
539. Young, R.S., et al., *Bidirectional transcription initiation marks accessible chromatin and is not specific to enhancers.* Genome Biol, 2017. **18**(1): p. 242.

540. Hota, S.K., et al., *Dynamic BAF chromatin remodeling complex subunit inclusion promotes temporally distinct gene expression programs in cardiogenesis.* Development, 2019. **146**(19).
541. Hang, C.T., et al., *Chromatin regulation by Brg1 underlies heart muscle development and disease.* Nature, 2010. **466**(7302): p. 62-7.
542. Han, P., et al., *A long noncoding RNA protects the heart from pathological hypertrophy.* Nature, 2014. **514**(7520): p. 102-106.
543. Li, H., et al., *Brg1 promotes liver fibrosis via activation of hepatic stellate cells.* Exp Cell Res, 2018. **364**(2): p. 191-197.
544. Xi, Q., et al., *Genome-wide impact of the BRG1 SWI/SNF chromatin remodeler on the transforming growth factor beta transcriptional program.* J Biol Chem, 2008. **283**(2): p. 1146-55.
545. Revah-Politi, A., et al., *GNB1 Encephalopathy*, in *GeneReviews((R))*, M.P. Adam, et al., Editors. 1993: Seattle (WA).
546. Yoda, A., et al., *Mutations in G protein beta subunits promote transformation and kinase inhibitor resistance.* Nat Med, 2015. **21**(1): p. 71-5.
547. Bradner, J.E., D. Hnisz, and R.A. Young, *Transcriptional Addiction in Cancer*. Cell, 2017. **168**(4): p. 629-643.

Appendix

Subject: Re: Request for permission to use manuscripts in PhD thesis
Date: Monday, April 20, 2020 at 4:05:46 PM GMT-04:00
From: Terry Hebert, Dr.
To: Ryan Martin

I so confirm!

TH

Terry Hébert
Professor
Department of Pharmacology and Therapeutics
Assistant Dean, Biomedical Science Education
McGill University

Email : terence.hebert@mcgill.ca
http://www.mcgill.ca/pharma/facultystaff/faculty/terry-hebert
http://www.medicine.mcgill.ca/pharma/hebertlab

Find us on Facebook: https://www.facebook.com/pages/Hébert-Lab/364089560357222?ref=hl
Follow me on Twitter: @THebertMcGill

Phone info:
514 398-1398 (Office)
514 398-8803 (Lab)
Fax: 514 398-2045

Mailing address:
Department of Pharmacology and Therapeutics
McGill University
Room 1303 McIntyre Medical Sciences Building
3655 Promenade Sir William Osler
Montréal, Québec
H3G 1Y6

From: Ryan Martin <ryan.martin@mail.mcgill.ca>
Sent: April 20, 2020 3:46 PM
To: Terry Hebert, Dr. <terence.hebert@mcgill.ca>
Subject: Request for permission to use manuscripts in PhD thesis

Hello Terry,

I hope this email finds you well. I am writing to you regarding the following manuscripts:

Receptor- and cellular compartment-specific activation of the cAMP/PKA pathway by a_1-adrenergic and ETA endothelin receptors
Ryan D. Martin, Yalin Sun, Kyla Bourque, Nicolas Audet, Asuka Inoue, Jason C. Tanny, and Terence E. Hébert,
Published in Cellular Signalling (doi: 10.1016/j.cellsig.2018.01.002.)

Differential activation of P-TEFb complexes in the development of cardiomyocyte hypertrophy following activation of distinct GPCRs
Ryan D. Martin, Yalin Sun, Sarah MacKinnon, Luca Cuccia, Viviane Pagé, Terence E. Hébert, Jason

C. Tanny
Accepted to Molecular and Cellular Biology.

An interaction between Gβγ and RNA polymerase II regulates transcription in cardiac fibroblasts
Shahriar M. Khan†, Ryan D. Martin†, Sarah Gora, Celia Bouazza, Jace Jones-Tabah, Andy Zhang, Sarah MacKinnon, Phan Trieu, Paul B.S. Clarke, Jason C. Tanny, and Terence E. Hébert
Manuscript in preparation.

One of the requirements to include these manuscripts as data chapters in my thesis is the written permission from all authors.

As you are an author, please reply confirming permission for use of the manuscripts at your soonest convenience.

Thank you!

Best,

Ryan Martin
Ph.D. Candidate
Department of Pharmacology and Therapeutics
3655 Promenade Sir William Osler
McIntyre Medical Sciences Building Rm 1303
McGill University
Montreal, Quebec
H3G1Y6
Work: (514) 398-8803

Subject: Re: Request for permission to use manuscripts in PhD thesis
Date: Monday, April 20, 2020 at 3:56:32 PM GMT-04:00
From: Jason Tanny
To: Ryan Martin

Confirmed!

Sent from my iPhone

On Apr 20, 2020, at 3:46 PM, Ryan Martin <ryan.martin@mail.mcgill.ca> wrote:

Hello Jason,

I hope this email finds you well. I am writing to you regarding the following manuscripts:

Receptor- and cellular compartment-specific activation of the cAMP/PKA pathway by a_1-adrenergic and ETA endothelin receptors
Ryan D. Martin, Yalin Sun, Kyla Bourque, Nicolas Audet, Asuka Inoue, Jason C. Tanny, and Terence E. Hébert,
Published in Cellular Signalling (doi: 10.1016/j.cellsig.2018.01.002.)

Differential activation of P-TEFb complexes in the development of cardiomyocyte hypertrophy following activation of distinct GPCRs
Ryan D. Martin, Yalin Sun, Sarah MacKinnon, Luca Cuccia, Viviane Pagé, Terence E. Hébert, Jason C. Tanny
Accepted to Molecular and Cellular Biology.

An interaction between Gβγ and RNA polymerase II regulates transcription in cardiac fibroblasts
Shahriar M. Khan†, Ryan D. Martin†, Sarah Gora, Celia Bouazza, Jace Jones-Tabah, Andy Zhang, Sarah MacKinnon, Phan Trieu, Paul B.S. Clarke, Jason C. Tanny, and Terence E. Hébert
Manuscript in preparation.

One of the requirements to include these manuscripts as data chapters in my thesis is the written permission from all authors.

As you are an author, please reply confirming permission for use of the manuscripts at your soonest convenience.

Thank you!

Best,

Ryan Martin
Ph.D. Candidate
Department of Pharmacology and Therapeutics
3655 Promenade Sir William Osler
McIntyre Medical Sciences Building Rm 1303
McGill University

Montreal, Quebec
H3G1Y6
Work: (514) 398-8803

Subject: Re: Request for permission to use manuscripts in PhD thesis
Date: Monday, April 20, 2020 at 4:00:06 PM GMT-04:00
From: Yalin Sun
To: Ryan Martin

Hello Ryan,

No objections, please go ahead and include them. Good luck with your thesis!

Best,
Yalin

From: Ryan Martin <ryan.martin@mail.mcgill.ca>
Sent: Monday, April 20, 2020 3:56 PM
To: Yalin Sun <yalin.sun@mail.utoronto.ca>
Subject: Request for permission to use manuscripts in PhD thesis

Hello Yalin,

I hope this email finds you well. I am writing to you regarding the following manuscripts:

Receptor- and cellular compartment-specific activation of the cAMP/PKA pathway by a_1-adrenergic and ETA endothelin receptors
Ryan D. Martin, Yalin Sun, Kyla Bourque, Nicolas Audet, Asuka Inoue, Jason C. Tanny, and Terence E. Hébert,
Published in Cellular Signalling (doi: 10.1016/j.cellsig.2018.01.002.)

Differential activation of P-TEFb complexes in the development of cardiomyocyte hypertrophy following activation of distinct GPCRs
Ryan D. Martin, Yalin Sun, Sarah MacKinnon, Luca Cuccia, Viviane Pagé, Terence E. Hébert, Jason C. Tanny
Accepted to Molecular and Cellular Biology.

One of the requirements to include these manuscripts as data chapters in my thesis is the written permission from all authors.

If you do not object to this request, please reply confirming permission for use of the manuscripts at your soonest convenience.

Thank you!

Best,

Ryan Martin
Ph.D. Candidate
Department of Pharmacology and Therapeutics
3655 Promenade Sir William Osler
McIntyre Medical Sciences Building Rm 1303
McGill University
Montreal, Quebec
H3G1Y6
Work: (514) 398-8803

Subject: Re: Request for permission to use manuscript in PhD thesis
Date: Monday, April 20, 2020 at 4:10:38 PM GMT-04:00
From: Kyla Bourque
To: Ryan Martin

Hi Ryan,

You have my permission!

Best,

Kyla

On Apr 20, 2020, at 3:28 PM, Ryan Martin <ryan.martin@mail.mcgill.ca> wrote:

Hi Kyla,

I hope this email finds you well. I am writing to you regarding the following manuscript published in Cellular Signalling.

Receptor- and cellular compartment-specific activation of the cAMP/PKA pathway by a_1-adrenergic and ETA endothelin receptors
Ryan D. Martin, Yalin Sun, Kyla Bourque, Nicolas Audet, Asuka Inoue, Jason C. Tanny, and Terence E. Hébert,
(doi: 10.1016/j.cellsig.2018.01.002.)

One of the requirements to include this manuscript as a data chapter in my thesis is the written permission from all authors.

As you are an author, please reply confirming permission for use of the manuscript at your soonest convenience.

Thank you!

Best,

Ryan Martin
Ph.D. Candidate
Department of Pharmacology and Therapeutics
3655 Promenade Sir William Osler
McIntyre Medical Sciences Building Rm 1303
McGill University
Montreal, Quebec
H3G1Y6
Work: (514) 398-8803

Subject: RE: Request for permission to use manuscript in PhD thesis
Date: Monday, April 20, 2020 at 3:50:47 PM GMT-04:00
From: Nicolas Audet, Mr
To: Ryan Martin

Hi Ryan,

You have my permission to use this manuscript in your thesis.

There is no form to sign?

Best,

Nicolas

Nicolas Audet, PhD
Academic Associate
Imaging and Molecular Biology Platform
Department of Pharmacology and Therapeutics, McGill University
Room 1333 McIntyre Medical Sciences Building
3655 Promenade Sir William Osler, Montréal, Québec, H3G 1Y6
nicolas.audet2@mcgill.ca
514-398-4455 ext. 09036

De : Ryan Martin <ryan.martin@mail.mcgill.ca>
Envoyé : 20 avril 2020 15:29
À : Nicolas Audet, Mr <nicolas.audet2@mcgill.ca>
Objet : Request for permission to use manuscript in PhD thesis

Hi Nico,

I hope this email finds you well. I am writing to you regarding the following manuscript published in Cellular Signalling.

Receptor- and cellular compartment-specific activation of the cAMP/PKA pathway by α_1-adrenergic and ETA endothelin receptors
Ryan D. Martin, Yalin Sun, Kyla Bourque, Nicolas Audet, Asuka Inoue, Jason C. Tanny, and Terence E. Hébert,
(doi: 10.1016/j.cellsig.2018.01.002.)

One of the requirements to include this manuscript as a data chapter in my thesis is the written permission from all authors.

As you are an author, please reply confirming permission for use of the manuscript at your soonest convenience.

Thank you!

Best,

Ryan Martin
Ph.D. Candidate
Department of Pharmacology and Therapeutics
3655 Promenade Sir William Osler
McIntyre Medical Sciences Building Rm 1303
McGill University
Montreal, Quebec
H3G1Y6
Work: (514) 398-8803

Ryan Martin
Ph.D. Candidate
Department of Pharmacology and Therapeutics
3655 Promenade Sir William Osler
McIntyre Medical Sciences Building Rm 1303
McGill University
Montreal, Quebec
H3G1Y6
Work: (514) 398-8803

Subject: Re: Request for permission to use manuscript in PhD thesis
Date: Monday, April 20, 2020 at 8:46:20 PM GMT-04:00
From: 井上 飛鳥
To: Ryan Martin
CC: Terry Hebert, Dr.

Dear Ryan,

Sure, I am totally fine with the manuscript being included as a part of your PhD thesis. Congratulations on your PhD degree (soon)!
Best wishes,

Aska

****
Asuka Inoue, Ph.D., Associate Professor
Graduate School of Pharmaceutical Sciences
Tohoku University
6-3, Aoba, Aramaki, Aoba-ku, Sendai, Miyagi, 980-8578 Japan
Tel 81-22-795-6861
Email iaska@tohoku.ac.jp
****

送信元: Ryan Martin <ryan.martin@mail.mcgill.ca>
日付: 2020年4月21日 火曜日 4:34
宛先: "iaska@m.tohoku.ac.jp" <iaska@m.tohoku.ac.jp>
Cc: "Terry Hebert, Dr." <terence.hebert@mcgill.ca>
件名: Request for permission to use manuscript in PhD thesis

Hello Dr. Inoue,

I hope this email finds you well. I am a PhD candidate in Dr. Terry Hébert's lab at McGill University currently in the process of writing my thesis. I am writing to you regarding a manuscript we published in Cellular Signalling of which you are an author. One of the requirements to include this manuscript as a data chapter in my thesis is the written permission from all authors.

If you have no objection to this request, please reply to this email to that effect at your soonest convenience.

Receptor- and cellular compartment-specific activation of the cAMP/PKA pathway by α_1-adrenergic and ETA endothelin receptors
Ryan D. Martin, Yalin Sun, Kyla Bourque, Nicolas Audet, Asuka Inoue, Jason C. Tanny, and Terence E. Hébert,
(doi: 10.1016/j.cellsig.2018.01.002.)

Thank you!

Best,

Ryan Martin
Ph.D. Candidate

Department of Pharmacology and Therapeutics
3655 Promenade Sir William Osler
McIntyre Medical Sciences Building Rm 1303
McGill University
Montreal, Quebec
H3G1Y6
Work: (514) 398-8803

Subject: Re: Request for permission to use manuscripts in PhD thesis
Date: Monday, April 20, 2020 at 3:48:54 PM GMT-04:00
From: Sarah MacKinnon
To: Ryan Martin

Permission granted

Sarah

Get Outlook for iOS

From: Ryan Martin <ryan.martin@mail.mcgill.ca>
Sent: Monday, April 20, 2020 3:48:10 PM
To: Sarah MacKinnon <sarah.mackinnon@mail.mcgill.ca>
Subject: Request for permission to use manuscripts in PhD thesis

Hello Sarah,

I hope this email finds you well. I am writing to you regarding the following manuscripts:

Differential activation of P-TEFb complexes in the development of cardiomyocyte hypertrophy following activation of distinct GPCRs
Ryan D. Martin, Yalin Sun, Sarah MacKinnon, Luca Cuccia, Viviane Pagé, Terence E. Hébert, Jason C. Tanny
Accepted to Molecular and Cellular Biology.

An interaction between Gβγ and RNA polymerase II regulates transcription in cardiac fibroblasts
Shahriar M. Khan†, Ryan D. Martin†, Sarah Gora, Celia Bouazza, Jace Jones-Tabah, Andy Zhang, Sarah MacKinnon, Phan Trieu, Paul B.S. Clarke, Jason C. Tanny, and Terence E. Hébert
Manuscript in preparation.

One of the requirements to include these manuscripts as data chapters in my thesis is the written permission from all authors.

As you are an author, please reply confirming permission for use of the manuscripts at your soonest convenience.

Thank you!

Best,

Ryan Martin
Ph.D. Candidate
Department of Pharmacology and Therapeutics
3655 Promenade Sir William Osler
McIntyre Medical Sciences Building Rm 1303
McGill University
Montreal, Quebec
H3G1Y6
Work: (514) 398-8803

Subject: Re: Request for permission to use manuscript in PhD thesis
Date: Monday, April 20, 2020 at 3:30:37 PM GMT-04:00
From: Luca Cuccia
To: Ryan Martin

Hi Ryan,

I confirm permission for use of the manuscript.

Thanks,

Luca

On 4/20/2020 3:24 PM, Ryan Martin wrote:

> Hi Luca,
>
> I hope this email finds you well. I am writing to you regarding the following manuscript accepted to Molecular and Cellular Biology.
>
> **Differential activation of P-TEFb complexes in the development of cardiomyocyte hypertrophy following activation of distinct GPCRs**
> Ryan D. Martin, Yalin Sun, Sarah MacKinnon, Luca Cuccia, Viviane Pagé, Terence E. Hébert, Jason C. Tanny
>
> One of the requirements to include this manuscript as a data chapter in my thesis is the written permission from all authors.
>
> As you are an author, please reply confirming permission for use of the manuscript at your soonest convenience.
>
> Thank you!
>
> Best,
>
> Ryan Martin
> Ph.D. Candidate
> Department of Pharmacology and Therapeutics
> 3655 Promenade Sir William Osler
> McIntyre Medical Sciences Building Rm 1303
> McGill University
> Montreal, Quebec
> H3G1Y6
> Work: (514) 398-8803

Subject: RE: Request for permission to use manuscript in PhD thesis
Date: Tuesday, April 21, 2020 at 9:46:42 AM GMT-04:00
From: Viviane Pagé, Ms.
To: Ryan Martin

Hi Ryan,

I agree on your request to use that manuscript in your PhD thesis.

Have a nice day,

Viviane Pagé

De : Ryan Martin <ryan.martin@mail.mcgill.ca>
Envoyé : lundi 20 avril 2020 15:26
À : Viviane Pagé, Ms. <viviane.page@mcgill.ca>
Objet : Request for permission to use manuscript in PhD thesis

Hi Viviane,

I hope this email finds you well. I am writing to you regarding the following manuscript accepted to Molecular and Cellular Biology.

Differential activation of P-TEFb complexes in the development of cardiomyocyte hypertrophy following activation of distinct GPCRs
Ryan D. Martin, Yalin Sun, Sarah MacKinnon, Luca Cuccia, Viviane Pagé, Terence E. Hébert, Jason C. Tanny

One of the requirements to include this manuscript as a data chapter in my thesis is the written permission from all authors.

As you are an author, please reply confirming permission for use of the manuscript at your soonest convenience.

Thank you!

Best,

Ryan Martin
Ph.D. Candidate
Department of Pharmacology and Therapeutics
3655 Promenade Sir William Osler
McIntyre Medical Sciences Building Rm 1303
McGill University
Montreal, Quebec
H3G1Y6
Work: (514) 398-8803

Subject: Re: Request for permission to use manuscript in PhD thesis
Date: Monday, April 27, 2020 at 12:30:12 PM GMT-04:00
From: Shahriar Khan
To: Ryan Martin

Hi Ryan,

You have my permission to use this manuscript as a data chapter in your thesis.

Best,

Shahriar

On Mon, Apr 20, 2020 at 3:51 PM Ryan Martin <ryan.martin@mail.mcgill.ca> wrote:

> Hello Shar,
>
> I hope this email finds you well. I am writing to you regarding the following manuscript:
>
> **An interaction between Gβγ and RNA polymerase II regulates transcription in cardiac fibroblasts**
>
> Shahriar M. Khan†, Ryan D. Martin†, Sarah Gora, Celia Bouazza, Jace Jones-Tabah, Andy Zhang, Sarah MacKinnon, Phan Trieu, Paul B.S. Clarke, Jason C. Tanny, and Terence E. Hébert
>
> Manuscript in preparation.
>
> One of the requirements to include this manuscripts as a data chapter in my thesis is the written permission from all authors.
>
> If you do not object to this request, please reply confirming permission for use of the manuscript at your soonest convenience.
>
> Thank you!
>
> Best,
>
> Ryan Martin
> Ph.D. Candidate
> Department of Pharmacology and Therapeutics
> 3655 Promenade Sir William Osler

McIntyre Medical Sciences Building Rm 1303
McGill University
Montreal, Quebec
H3G1Y6
Work: (514) 398-8803

Subject: Re: Request for permission to use manuscript in PhD thesis
Date: Friday, May 1, 2020 at 9:07:45 AM GMT-04:00
From: Sarah GORA
To: Ryan Martin

Dear Ryan,

My congratulations on the completion of your doctorate.
You have my permission to include the article mentioned below in your thesis report.

An interaction between Gβγ and RNA polymerase II regulates transcription in cardiac fibroblasts
Shahriar M. Khan†, Ryan D. Martin†, Sarah Gora, Celia Bouazza, Jace Jones-Tabah, Andy Zhang, Sarah MacKinnon, Phan Trieu, Paul B.S. Clarke, Jason C. Tanny, and Terence E. Hébert
Manuscript in preparation.

I would be delighted to learn more about your recent developments on the subject.
Sarah

Sarah GORA, PhD
Tel: 514-701-2901

" Toute science commence comme philosophie et se termine en art."
Will Durant

De : Ryan Martin <ryan.martin@mail.mcgill.ca>
Envoyé : 27 avril 2020 15:37
À : sarah.gora@hotmail.com <sarah.gora@hotmail.com>
Objet : Request for permission to use manuscript in PhD thesis

Hello Sarah,

I hope this email finds you well. I am writing to you regarding the following manuscript:

An interaction between Gβγ and RNA polymerase II regulates transcription in cardiac fibroblasts
Shahriar M. Khan†, Ryan D. Martin†, Sarah Gora, Celia Bouazza, Jace Jones-Tabah, Andy Zhang, Sarah MacKinnon, Phan Trieu, Paul B.S. Clarke, Jason C. Tanny, and Terence E. Hébert
Manuscript in preparation.

One of the requirements to include this manuscript as a data chapter in my thesis is the written permission from all authors.

If you do not object to this request, please reply confirming permission for use of the manuscript at your soonest convenience. If you have any questions about the manuscript, I'd be happy to provide more information!

Thank you!

Best,

Ryan Martin
Ph.D. Candidate
Department of Pharmacology and Therapeutics
3655 Promenade Sir William Osler
McIntyre Medical Sciences Building Rm 1303
McGill University
Montreal, Quebec
H3G1Y6

Subject: Re: Request for permission to use manuscripts in PhD thesis
Date: Monday, April 20, 2020 at 4:11:29 PM GMT-04:00
From: Célia Bouazza
To: Ryan Martin

I'll allow it.

Celia

On Apr 20, 2020, at 3:52 PM, Ryan Martin <ryan.martin@mail.mcgill.ca> wrote:

Hello Celia,

I hope this email finds you well. I am writing to you regarding the following manuscript:

An interaction between Gβγ and RNA polymerase II regulates transcription in cardiac fibroblasts
Shahriar M. Khan†, Ryan D. Martin†, Sarah Gora, Celia Bouazza, Jace Jones-Tabah, Andy Zhang, Sarah MacKinnon, Phan Trieu, Paul B.S. Clarke, Jason C. Tanny, and Terence E. Hébert
Manuscript in preparation.

One of the requirements to include this manuscript as a data chapter in my thesis is the written permission from all authors.

If you do not object to this request, please reply confirming permission for use of the manuscript at your soonest convenience.

Thank you!

Best,

Ryan Martin
Ph.D. Candidate
Department of Pharmacology and Therapeutics
3655 Promenade Sir William Osler
McIntyre Medical Sciences Building Rm 1303
McGill University
Montreal, Quebec
H3G1Y6
Work: (514) 398-8803

Subject: Re: Request for permission to use manuscript in PhD thesis
Date: Wednesday, April 22, 2020 at 1:42:26 PM GMT-04:00
From: Jace Jones-Tabah
To: Ryan Martin

Permission granted!

Jace

From: Ryan Martin <ryan.martin@mail.mcgill.ca>
Sent: April 20, 2020 3:52 PM
To: Jace Jones-Tabah <jace.jones-tabah@mail.mcgill.ca>
Subject: Request for permission to use manuscript in PhD thesis

Hello Jace,

I hope this email finds you well. I am writing to you regarding the following manuscript:

An interaction between Gβγ and RNA polymerase II regulates transcription in cardiac fibroblasts
Shahriar M. Khan†, Ryan D. Martin†, Sarah Gora, Celia Bouazza, Jace Jones-Tabah, Andy Zhang, Sarah MacKinnon, Phan Trieu, Paul B.S. Clarke, Jason C. Tanny, and Terence E. Hébert
Manuscript in preparation.

One of the requirements to include this manuscript as a data chapter in my thesis is the written permission from all authors.

If you do not object to this request, please reply confirming permission for use of the manuscript at your soonest convenience.

Thank you!

Best,

Ryan Martin
Ph.D. Candidate
Department of Pharmacology and Therapeutics
3655 Promenade Sir William Osler
McIntyre Medical Sciences Building Rm 1303
McGill University
Montreal, Quebec
H3G1Y6
Work: (514) 398-8803

Subject: Re: Request for permission to use manuscript in PhD thesis
Date: Tuesday, April 21, 2020 at 9:40:25 AM GMT-04:00
From: Andy Zhang
To: Ryan Martin

Hi Ryan,

You have my permission to use the manuscript in your thesis.

Thanks,

Andy

From: Ryan Martin <ryan.martin@mail.mcgill.ca>
Sent: April 21, 2020 9:08 AM
To: Andy Zhang <andy.zhang@mail.mcgill.ca>
Subject: Request for permission to use manuscript in PhD thesis

Hello Andy,

I hope this email finds you well. I am writing to you regarding the following manuscript:

An interaction between Gβγ and RNA polymerase II regulates transcription in cardiac fibroblasts
Shahriar M. Khan†, Ryan D. Martin†, Sarah Gora, Celia Bouazza, Jace Jones-Tabah, Andy Zhang, Sarah MacKinnon, Phan Trieu, Paul B.S. Clarke, Jason C. Tanny, and Terence E. Hébert
Manuscript in preparation.

One of the requirements to include this manuscript as a data chapter in my thesis is the written permission from all authors.

If you do not object to this request, please reply confirming permission for use of the manuscript at your soonest convenience.

Thank you!

Best,

Ryan Martin
Ph.D. Candidate
Department of Pharmacology and Therapeutics
3655 Promenade Sir William Osler
McIntyre Medical Sciences Building Rm 1303
McGill University
Montreal, Quebec
H3G1Y6
Work: (514) 398-8803

Subject: Re: Request for permission to use manuscript in PhD thesis
Date: Monday, April 20, 2020 at 4:23:25 PM GMT-04:00
From: Phan Trieu, Ms.
To: Ryan Martin

Hi Ryan,

Permission granted.

Take care,

Phan Trieu
Research Technician
McGill University
Department of Pharmacology & Therapeutics
McIntyre Medical Sciences Building - Room 1303
3655 Promenade Sir William Osler
Montréal, QC. H3G 1Y6
Tel.: (514) 398-8803
Fax.: (514) 398-6690
Email: phan.trieu@mcgill.ca

From: Ryan Martin <ryan.martin@mail.mcgill.ca>
Sent: Monday, April 20, 2020 3:53 PM
To: Phan Trieu, Ms. <phan.trieu@mcgill.ca>
Subject: Request for permission to use manuscript in PhD thesis

Hello Phan,

I hope this email finds you well. I am writing to you regarding the following manuscript:

An interaction between Gβγ and RNA polymerase II regulates transcription in cardiac fibroblasts
Shahriar M. Khan†, Ryan D. Martin†, Sarah Gora, Celia Bouazza, Jace Jones-Tabah, Andy Zhang, Sarah MacKinnon, Phan Trieu, Paul B.S. Clarke, Jason C. Tanny, and Terence E. Hébert
Manuscript in preparation.

One of the requirements to include this manuscript as a data chapter in my thesis is the written permission from all authors.

If you do not object to this request, please reply confirming permission for use of the manuscript at your soonest convenience.

Thank you!

Best,

Ryan Martin
Ph.D. Candidate
Department of Pharmacology and Therapeutics
3655 Promenade Sir William Osler
McIntyre Medical Sciences Building Rm 1303
McGill University

Montreal, Quebec
H3G1Y6
Work: (514) 398-8803

Subject: RE: Request for permission to use manuscript in PhD thesis
Date: Monday, April 20, 2020 at 4:53:30 PM GMT-04:00
From: Paul Clarke, Dr.
To: Ryan Martin

Hi Ryan
Of course, please go ahead!
Best
Paul

From: Ryan Martin <ryan.martin@mail.mcgill.ca>
Sent: 20-Apr-20 3:54 PM
To: Paul Clarke, Dr. <paul.clarke@mcgill.ca>
Subject: Request for permission to use manuscript in PhD thesis

Hello Paul,

I hope this email finds you well. I am writing to you regarding the following manuscript:

An interaction between Gβγ and RNA polymerase II regulates transcription in cardiac fibroblasts
Shahriar M. Khan†, Ryan D. Martin†, Sarah Gora, Celia Bouazza, Jace Jones-Tabah, Andy Zhang, Sarah MacKinnon, Phan Trieu, Paul B.S. Clarke, Jason C. Tanny, and Terence E. Hébert
Manuscript in preparation.

One of the requirements to include this manuscript as a data chapter in my thesis is the written permission from all authors.

If you do not object to this request, please reply confirming permission for use of the manuscript at your soonest convenience.

Thank you!

Best,

Ryan Martin
Ph.D. Candidate
Department of Pharmacology and Therapeutics
3655 Promenade Sir William Osler
McIntyre Medical Sciences Building Rm 1303
McGill University
Montreal, Quebec
H3G1Y6
Work: (514) 398-8803

SPRINGER NATURE LICENSE
TERMS AND CONDITIONS

May 24, 2020

This Agreement between Mr. Ryan Martin ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number	4835551273293
License date	May 24, 2020
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Nature
Licensed Content Title	Crystal structure of the β2 adrenergic receptor–Gs protein complex
Licensed Content Author	Søren G. F. Rasmussen et al
Licensed Content Date	Jul 19, 2011
Type of Use	Thesis/Dissertation
Requestor type	academic/university or research institute

Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	3
High-res required	no
Will you be translating?	no
Circulation/distribution	1 - 29
Author of this Springer Nature content	no
Title	G protein-coupled receptor-mediated transcriptional regulation in pathological cardiac remodelling
Institution name	McGill University
Expected presentation date	Aug 2020
Portions	Figure 1A, Figure 1B, Figure 3A
Requestor Location	Mr. Ryan Martin 3655 Promenade Sir William Osler Room 1303 Montreal, QC H3G1Y6

Canada
Attn: Mr. Ryan Martin

Total 0.00 USD

Terms and Conditions

Springer Nature Customer Service Centre GmbH
Terms and Conditions

This agreement sets out the terms and conditions of the licence (the **Licence**) between you and **Springer Nature Customer Service Centre GmbH** (the **Licensor**). By clicking 'accept' and completing the transaction for the material (**Licensed Material**), you also confirm your acceptance of these terms and conditions.

1. Grant of License

1. 1. The Licensor grants you a personal, non-exclusive, non-transferable, world-wide licence to reproduce the Licensed Material for the purpose specified in your order only. Licences are granted for the specific use requested in the order and for no other use, subject to the conditions below.

1. 2. The Licensor warrants that it has, to the best of its knowledge, the rights to license reuse of the Licensed Material. However, you should ensure that the material you are requesting is original to the Licensor and does not carry the copyright of another entity (as credited in the published version).

1. 3. If the credit line on any part of the material you have requested indicates that it was reprinted or adapted with permission from another source, then you should also seek permission from that source to reuse the material.

2. Scope of Licence

2. 1. You may only use the Licensed Content in the manner and to the extent permitted by these Ts&Cs and any applicable laws.

2. 2. A separate licence may be required for any additional use of the Licensed Material, e.g. where a licence has been purchased for print only use, separate permission must be obtained for electronic re-use. Similarly, a licence is only valid in the language selected and does not apply for editions in other languages unless additional translation rights have been granted separately in the licence. Any content

owned by third parties are expressly excluded from the licence.

2. 3. Similarly, rights for additional components such as custom editions and derivatives require additional permission and may be subject to an additional fee. Please apply to Journalpermissions@springernature.com/bookpermissions@springernature.com for these rights.

2. 4. Where permission has been granted **free of charge** for material in print, permission may also be granted for any electronic version of that work, provided that the material is incidental to your work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.

2. 5. An alternative scope of licence may apply to signatories of the STM Permissions Guidelines, as amended from time to time.

3. Duration of Licence

3. 1. A licence for is valid from the date of purchase ('Licence Date') at the end of the relevant period in the below table:

Scope of Licence	Duration of Licence
Post on a website	12 months
Presentations	12 months
Books and journals	Lifetime of the edition in the language purchased

4. Acknowledgement

4. 1. The Licensor's permission must be acknowledged next to the Licenced Material in print. In electronic form, this acknowledgement must be visible at the same time as the figures/tables/illustrations or abstract, and must be hyperlinked to the journal/book's homepage. Our required acknowledgement format is in the Appendix below.

5. Restrictions on use

5. 1. Use of the Licensed Material may be permitted for incidental promotional use and minor editing privileges e.g. minor adaptations of single figures, changes of format, colour and/or style where the adaptation is credited as set out in Appendix 1 below. Any

other changes including but not limited to, cropping, adapting, omitting material that affect the meaning, intention or moral rights of the author are strictly prohibited.

5. 2. You must not use any Licensed Material as part of any design or trademark.

5. 3. Licensed Material may be used in Open Access Publications (OAP) before publication by Springer Nature, but any Licensed Material must be removed from OAP sites prior to final publication.

6. Ownership of Rights

6. 1. Licensed Material remains the property of either Licensor or the relevant third party and any rights not explicitly granted herein are expressly reserved.

7. Warranty

IN NO EVENT SHALL LICENSOR BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL OR INDIRECT DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND
WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

8. Limitations

8. 1. ***BOOKS ONLY:*** Where **'reuse in a dissertation/thesis'** has been selected the following terms apply: Print rights of the final author's accepted manuscript (for clarity, NOT the published version) for up to 100 copies, electronic rights for use only on a personal website or institutional repository as defined by the Sherpa guideline (www.sherpa.ac.uk/romeo/).

9. Termination and Cancellation

9. 1. Licences will expire after the period shown in Clause 3 (above).

9. 2. Licensee reserves the right to terminate the Licence in the event that payment is not received in full or if there has been a breach of this agreement by you.

Appendix 1 — Acknowledgements:

For Journal Content:
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

For **Advance Online Publication papers:**
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].)

For Adaptations/Translations:
Adapted/Translated by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

Note: For any republication from the British Journal of Cancer, the following credit line style applies:

Reprinted/adapted/translated by permission from [**the Licensor**]: on behalf of Cancer Research UK: : [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

For **Advance Online Publication** papers:
Reprinted by permission from The [**the Licensor**]: on behalf of Cancer Research UK: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM])

For Book content:

Reprinted/adapted by permission from [**the Licensor**]: [**Book Publisher** (e.g. Palgrave Macmillan, Springer etc) [**Book Title**] by [**Book author**(s)] [**COPYRIGHT**] (year of publication)

Other Conditions:

Version 1.2

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

SPRINGER NATURE LICENSE
TERMS AND CONDITIONS

May 24, 2020

This Agreement between Mr. Ryan Martin ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number	4835551156741
License date	May 24, 2020
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Cellular and Molecular Life Sciences
Licensed Content Title	G protein βγ subunits: Central mediators of G protein-coupled receptor signaling
Licensed Content Author	A. V. Smrcka
Licensed Content Date	May 19, 2008
Type of Use	Thesis/Dissertation
Requestor type	academic/university or research institute

Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	2
Will you be translating?	no
Circulation/distribution	1 - 29
Author of this Springer Nature content	no
Title	G protein-coupled receptor-mediated transcriptional regulation in pathological cardiac remodelling
Institution name	McGill University
Expected presentation date	Aug 2020
Portions	Figure 1B, Figure 1C
Requestor Location	Mr. Ryan Martin 3655 Promenade Sir William Osler Room 1303 Montreal, QC H3G1Y6 Canada Attn: Mr. Ryan Martin

Total 0.00 CAD

Terms and Conditions

Springer Nature Customer Service Centre GmbH
Terms and Conditions

This agreement sets out the terms and conditions of the licence (the **Licence**) between you and **Springer Nature Customer Service Centre GmbH** (the **Licensor**). By clicking 'accept' and completing the transaction for the material (**Licensed Material**), you also confirm your acceptance of these terms and conditions.

1. Grant of License

1. 1. The Licensor grants you a personal, non-exclusive, non-transferable, world-wide licence to reproduce the Licensed Material for the purpose specified in your order only. Licences are granted for the specific use requested in the order and for no other use, subject to the conditions below.

1. 2. The Licensor warrants that it has, to the best of its knowledge, the rights to license reuse of the Licensed Material. However, you should ensure that the material you are requesting is original to the Licensor and does not carry the copyright of another entity (as credited in the published version).

1. 3. If the credit line on any part of the material you have requested indicates that it was reprinted or adapted with permission from another source, then you should also seek permission from that source to reuse the material.

2. Scope of Licence

2. 1. You may only use the Licensed Content in the manner and to the extent permitted by these Ts&Cs and any applicable laws.

2. 2. A separate licence may be required for any additional use of the Licensed Material, e.g. where a licence has been purchased for print only use, separate permission must be obtained for electronic re-use. Similarly, a licence is only valid in the language selected and does not apply for editions in other languages unless additional translation rights have been granted separately in the licence. Any content owned by third parties are expressly excluded from the licence.

2. 3. Similarly, rights for additional components such as custom editions and derivatives require additional permission and may be subject to an additional fee.

Please apply to Journalpermissions@springernature.com/bookpermissions@springernature.com for these rights.

2. 4. Where permission has been granted **free of charge** for material in print, permission may also be granted for any electronic version of that work, provided that the material is incidental to your work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.

2. 5. An alternative scope of licence may apply to signatories of the STM Permissions Guidelines, as amended from time to time.

3. Duration of Licence

3. 1. A licence for is valid from the date of purchase ('Licence Date') at the end of the relevant period in the below table:

Scope of Licence	Duration of Licence
Post on a website	12 months
Presentations	12 months
Books and journals	Lifetime of the edition in the language purchased

4. Acknowledgement

4. 1. The Licensor's permission must be acknowledged next to the Licenced Material in print. In electronic form, this acknowledgement must be visible at the same time as the figures/tables/illustrations or abstract, and must be hyperlinked to the journal/book's homepage. Our required acknowledgement format is in the Appendix below.

5. Restrictions on use

5. 1. Use of the Licensed Material may be permitted for incidental promotional use and minor editing privileges e.g. minor adaptations of single figures, changes of format, colour and/or style where the adaptation is credited as set out in Appendix 1 below. Any other changes including but not limited to, cropping, adapting, omitting material that affect the meaning, intention or moral rights of the author are strictly prohibited.

5. 2. You must not use any Licensed Material as part of any design or trademark.

5. 3. Licensed Material may be used in Open Access Publications (OAP) before publication by Springer Nature, but any Licensed Material must be removed from OAP sites prior to final publication.

6. Ownership of Rights

6. 1. Licensed Material remains the property of either Licensor or the relevant third party and any rights not explicitly granted herein are expressly reserved.

7. Warranty

IN NO EVENT SHALL LICENSOR BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL OR INDIRECT DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND
WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

8. Limitations

8. 1. ***BOOKS ONLY:*** Where '**reuse in a dissertation/thesis**' has been selected the following terms apply: Print rights of the final author's accepted manuscript (for clarity, NOT the published version) for up to 100 copies, electronic rights for use only on a personal website or institutional repository as defined by the Sherpa guideline (www.sherpa.ac.uk/romeo/).

9. Termination and Cancellation

9. 1. Licences will expire after the period shown in Clause 3 (above).

9. 2. Licensee reserves the right to terminate the Licence in the event that payment is not received in full or if there has been a breach of this agreement by you.

Appendix 1 — Acknowledgements:

For Journal Content:
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

For **Advance Online Publication papers:**
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].)

For Adaptations/Translations:
Adapted/Translated by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

Note: For any republication from the British Journal of Cancer, the following credit line style applies:

Reprinted/adapted/translated by permission from [**the Licensor**]: on behalf of Cancer Research UK: : [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

For **Advance Online Publication** papers:
Reprinted by permission from The [**the Licensor**]: on behalf of Cancer Research UK: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM])

For Book content:
Reprinted/adapted by permission from [**the Licensor**]: [**Book Publisher** (e.g. Palgrave Macmillan, Springer etc) [**Book Title**] by [**Book author**(s)] [**COPYRIGHT**] (year of publication)

Other Conditions:

Version 1.2

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

SPRINGER NATURE LICENSE
TERMS AND CONDITIONS

May 24, 2020

This Agreement between Mr. Ryan Martin ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number	4835551381195
License date	May 24, 2020
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Cellular and Molecular Life Sciences
Licensed Content Title	G-protein βγ subunits as multi-functional scaffolds and transducers in G-protein-coupled receptor signaling
Licensed Content Author	Alan V. Smrcka et al
Licensed Content Date	Aug 21, 2019
Type of Use	Thesis/Dissertation
Requestor type	academic/university or research institute

Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Will you be translating?	no
Circulation/distribution	1 - 29
Author of this Springer Nature content	no
Title	G protein-coupled receptor-mediated transcriptional regulation in pathological cardiac remodelling
Institution name	McGill University
Expected presentation date	Aug 2020
Portions	Figure 2
Requestor Location	Mr. Ryan Martin 3655 Promenade Sir William Osler Room 1303 Montreal, QC H3G1Y6 Canada Attn: Mr. Ryan Martin

Total 0.00 CAD

Terms and Conditions

Springer Nature Customer Service Centre GmbH
Terms and Conditions

This agreement sets out the terms and conditions of the licence (the **Licence**) between you and **Springer Nature Customer Service Centre GmbH** (the **Licensor**). By clicking 'accept' and completing the transaction for the material (**Licensed Material**), you also confirm your acceptance of these terms and conditions.

1. Grant of License

1. 1. The Licensor grants you a personal, non-exclusive, non-transferable, world-wide licence to reproduce the Licensed Material for the purpose specified in your order only. Licences are granted for the specific use requested in the order and for no other use, subject to the conditions below.

1. 2. The Licensor warrants that it has, to the best of its knowledge, the rights to license reuse of the Licensed Material. However, you should ensure that the material you are requesting is original to the Licensor and does not carry the copyright of another entity (as credited in the published version).

1. 3. If the credit line on any part of the material you have requested indicates that it was reprinted or adapted with permission from another source, then you should also seek permission from that source to reuse the material.

2. Scope of Licence

2. 1. You may only use the Licensed Content in the manner and to the extent permitted by these Ts&Cs and any applicable laws.

2. 2. A separate licence may be required for any additional use of the Licensed Material, e.g. where a licence has been purchased for print only use, separate permission must be obtained for electronic re-use. Similarly, a licence is only valid in the language selected and does not apply for editions in other languages unless additional translation rights have been granted separately in the licence. Any content owned by third parties are expressly excluded from the licence.

2. 3. Similarly, rights for additional components such as custom editions and derivatives require additional permission and may be subject to an additional fee.

Please apply to Journalpermissions@springernature.com/bookpermissions@springernature.com for these rights.

2. 4. Where permission has been granted **free of charge** for material in print, permission may also be granted for any electronic version of that work, provided that the material is incidental to your work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.

2. 5. An alternative scope of licence may apply to signatories of the STM Permissions Guidelines, as amended from time to time.

3. Duration of Licence

3. 1. A licence for is valid from the date of purchase ('Licence Date') at the end of the relevant period in the below table:

Scope of Licence	Duration of Licence
Post on a website	12 months
Presentations	12 months
Books and journals	Lifetime of the edition in the language purchased

4. Acknowledgement

4. 1. The Licensor's permission must be acknowledged next to the Licenced Material in print. In electronic form, this acknowledgement must be visible at the same time as the figures/tables/illustrations or abstract, and must be hyperlinked to the journal/book's homepage. Our required acknowledgement format is in the Appendix below.

5. Restrictions on use

5. 1. Use of the Licensed Material may be permitted for incidental promotional use and minor editing privileges e.g. minor adaptations of single figures, changes of format, colour and/or style where the adaptation is credited as set out in Appendix 1 below. Any other changes including but not limited to, cropping, adapting, omitting material that affect the meaning, intention or moral rights of the author are strictly prohibited.

5. 2. You must not use any Licensed Material as part of any design or trademark.

5. 3. Licensed Material may be used in Open Access Publications (OAP) before publication by Springer Nature, but any Licensed Material must be removed from OAP sites prior to final publication.

6. Ownership of Rights

6. 1. Licensed Material remains the property of either Licensor or the relevant third party and any rights not explicitly granted herein are expressly reserved.

7. Warranty

IN NO EVENT SHALL LICENSOR BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL OR INDIRECT DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND
WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

8. Limitations

8. 1. ***BOOKS ONLY:*** Where '**reuse in a dissertation/thesis**' has been selected the following terms apply: Print rights of the final author's accepted manuscript (for clarity, NOT the published version) for up to 100 copies, electronic rights for use only on a personal website or institutional repository as defined by the Sherpa guideline (www.sherpa.ac.uk/romeo/).

9. Termination and Cancellation

9. 1. Licences will expire after the period shown in Clause 3 (above).

9. 2. Licensee reserves the right to terminate the Licence in the event that payment is not received in full or if there has been a breach of this agreement by you.

Appendix 1 — Acknowledgements:

For Journal Content:
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

For **Advance Online Publication papers:**
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].)

For Adaptations/Translations:
Adapted/Translated by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

Note: For any republication from the British Journal of Cancer, the following credit line style applies:

Reprinted/adapted/translated by permission from [**the Licensor**]: on behalf of Cancer Research UK: : [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

For **Advance Online Publication** papers:
Reprinted by permission from The [**the Licensor**]: on behalf of Cancer Research UK: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM])

For Book content:
Reprinted/adapted by permission from [**the Licensor**]: [**Book Publisher** (e.g. Palgrave Macmillan, Springer etc) [**Book Title**] by [**Book author**(s)] [**COPYRIGHT**] (year of publication)

Other Conditions:

Version 1.2

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

ELSEVIER LICENSE
TERMS AND CONDITIONS

May 24, 2020

This Agreement between Mr. Ryan Martin ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	4835551471558
License date	May 24, 2020
Licensed Content Publisher	Elsevier
Licensed Content Publication	Elsevier Books
Licensed Content Title	GPCRs
Licensed Content Author	Ryan D. Martin,Célia A. Bouazza,Terence E. Hébert
Licensed Content Date	Jan 1, 2020
Licensed Content Pages	11
Start Page	257

End Page	267
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier chapter?	Yes
Will you be translating?	No
Title	G protein-coupled receptor-mediated transcriptional regulation in pathological cardiac remodelling
Institution name	McGill University
Expected presentation date	Aug 2020
Portions	Figure 1
Requestor Location	Mr. Ryan Martin 3655 Promenade Sir William Osler Room 1303 Montreal, QC H3G1Y6 Canada Attn: Mr. Ryan Martin

Publisher Tax ID	GB 494 6272 12
Total	0.00 CAD

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions

shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors:** the following clauses are applicable in addition to the above:

Preprints:

A preprint is an author's own write-up of research results and analysis, it has not been peer-

reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
    - via their non-commercial person homepage or blog
    - by updating a preprint in arXiv or RePEc with the accepted manuscript
    - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
    - directly by providing copies to their students or to research collaborators for their personal use
    - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
    - via non-commercial hosting platforms such as their institutional repository
    - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing,

formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's posting policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions

You can publish open access with Elsevier in hundreds of open access journals or in nearly

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. **Other Conditions**:

v1.9

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

THE AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE LICENSE TERMS AND CONDITIONS

May 24, 2020

This Agreement between Mr. Ryan Martin ("You") and The American Association for the Advancement of Science ("The American Association for the Advancement of Science") consists of your license details and the terms and conditions provided by The American Association for the Advancement of Science and Copyright Clearance Center.

License Number	4835560136908
License date	May 24, 2020
Licensed Content Publisher	The American Association for the Advancement of Science
Licensed Content Publication	Science
Licensed Content Title	Structural Basis of Transcription: RNA Polymerase II at 2.8 Ångstrom Resolution
Licensed Content Author	Patrick Cramer,David A. Bushnell,Roger D. Kornberg
Licensed Content Date	Jun 8, 2001
Licensed Content	292

Volume	
Licensed Content Issue	5523
Volume number	292
Issue number	5523
Type of Use	Thesis / Dissertation
Requestor type	Scientist/individual at a research institution
Format	Print and electronic
Portion	Figure
Number of figures/tables	2
Title	G protein-coupled receptor-mediated transcriptional regulation in pathological cardiac remodelling
Institution name	McGill University
Expected presentation date	Aug 2020
Portions	Figure 6, Figure 7
	Mr. Ryan Martin

	3655 Promenade Sir William Osler Room 1303
Requestor Location	Montreal, QC H3G1Y6 Canada Attn: Mr. Ryan Martin
Total	0.00 CAD

Terms and Conditions

American Association for the Advancement of Science TERMS AND CONDITIONS

Regarding your request, we are pleased to grant you non-exclusive, non-transferable permission, to republish the AAAS material identified above in your work identified above, subject to the terms and conditions herein. We must be contacted for permission for any uses other than those specifically identified in your request above.

The following credit line must be printed along with the AAAS material: "From [Full Reference Citation]. Reprinted with permission from AAAS."

All required credit lines and notices must be visible any time a user accesses any part of the AAAS material and must appear on any printed copies and authorized user might make.

This permission does not apply to figures / photos / artwork or any other content or materials included in your work that are credited to non-AAAS sources. If the requested material is sourced to or references non-AAAS sources, you must obtain authorization from that source as well before using that material. You agree to hold harmless and indemnify AAAS against any claims arising from your use of any content in your work that is credited to non-AAAS sources.

If the AAAS material covered by this permission was published in Science during the years 1974 - 1994, you must also obtain permission from the author, who may grant or withhold permission, and who may or may not charge a fee if permission is granted. See original article for author's address. This condition does not apply to news articles.

The AAAS material may not be modified or altered except that figures and tables may be modified with permission from the author. Author permission for any such changes must be secured prior to your use.

Whenever possible, we ask that electronic uses of the AAAS material permitted herein

include a hyperlink to the original work on AAAS's website (hyperlink may be embedded in the reference citation).

AAAS material reproduced in your work identified herein must not account for more than 30% of the total contents of that work.

AAAS must publish the full paper prior to use of any text.

AAAS material must not imply any endorsement by the American Association for the Advancement of Science.

This permission is not valid for the use of the AAAS and/or Science logos.

AAAS makes no representations or warranties as to the accuracy of any information contained in the AAAS material covered by this permission, including any warranties of

merchantability or fitness for a particular purpose.

If permission fees for this use are waived, please note that AAAS reserves the right to charge for reproduction of this material in the future.

Permission is not valid unless payment is received within sixty (60) days of the issuance of this permission. If payment is not received within this time period then all rights granted herein shall be revoked and this permission will be considered null and void.

In the event of breach of any of the terms and conditions herein or any of CCC's Billing and Payment terms and conditions, all rights granted herein shall be revoked and this permission will be considered null and void.

AAAS reserves the right to terminate this permission and all rights granted herein at its discretion, for any purpose, at any time. In the event that AAAS elects to terminate this permission, you will have no further right to publish, publicly perform, publicly display, distribute or otherwise use any matter in which the AAAS content had been included, and all fees paid hereunder shall be fully refunded to you. Notification of termination will be sent to the contact information as supplied by you during the request process and termination shall be immediate upon sending the notice. Neither AAAS nor CCC shall be liable for any costs, expenses, or damages you may incur as a result of the termination of this permission, beyond the refund noted above.

This Permission may not be amended except by written document signed by both parties.

The terms above are applicable to all permissions granted for the use of AAAS material. Below you will find additional conditions that apply to your particular type of use.

FOR A THESIS OR DISSERTATION

If you are using figure(s)/table(s), permission is granted for use in print and electronic versions of your dissertation or thesis. A full text article may be used in print versions only of a dissertation or thesis.

Permission covers the distribution of your dissertation or thesis on demand by ProQuest / UMI, provided the AAAS material covered by this permission remains in situ.

If you are an Original Author on the AAAS article being reproduced, please refer to your License to Publish for rules on reproducing your paper in a dissertation or thesis.

FOR JOURNALS:
Permission covers both print and electronic versions of your journal article, however the AAAS material may not be used in any manner other than within the context of your article.

FOR BOOKS/TEXTBOOKS:
If this license is to reuse figures/tables, then permission is granted for non-exclusive world rights in all languages in both print and electronic formats (electronic formats are defined below).

If this license is to reuse a text excerpt or a full text article, then permission is granted for non-exclusive world rights in English only. You have the option of securing either print or electronic rights or both, but electronic rights are not automatically granted and do garner additional fees. Permission for translations of text excerpts or full text articles into other languages must be obtained separately.

Licenses granted for use of AAAS material in electronic format books/textbooks are valid only in cases where the electronic version is equivalent to or substitutes for the print version of the book/textbook. The AAAS material reproduced as permitted herein must remain in situ and must not be exploited separately (for example, if permission covers the use of a full text article, the article may not be offered for access or for purchase as a stand-alone unit), except in the case of permitted textbook companions as noted below.

You must include the following notice in any electronic versions, either adjacent to the reprinted AAAS material or in the terms and conditions for use of your electronic products: "Readers may view, browse, and/or download material for temporary copying purposes only, provided these uses are for noncommercial personal purposes. Except as provided by law, this material may not be further reproduced, distributed, transmitted, modified, adapted, performed, displayed, published, or sold in whole or in part, without prior written permission from the publisher."

If your book is an academic textbook, permission covers the following companions to your textbook, provided such companions are distributed only in conjunction with your textbook at no additional cost to the user:

- Password-protected website
- Instructor's image CD/DVD and/or PowerPoint resource
- Student CD/DVD

All companions must contain instructions to users that the AAAS material may be used for non-commercial, classroom purposes only. Any other uses require the prior written permission from AAAS.

If your license is for the use of AAAS Figures/Tables, then the electronic rights granted herein permit use of the Licensed Material in any Custom Databases that you distribute the electronic versions of your textbook through, so long as the Licensed Material remains within the context of a chapter of the title identified in your request and cannot be downloaded by a user as an independent image file.

Rights also extend to copies/files of your Work (as described above) that you are required to provide for use by the visually and/or print disabled in compliance with state and federal laws.

This permission only covers a single edition of your work as identified in your request.

FOR NEWSLETTERS:
Permission covers print and/or electronic versions, provided the AAAS material reproduced as permitted herein remains in situ and is not exploited separately (for example, if permission covers the use of a full text article, the article may not be offered for access or for purchase as a stand-alone unit)

FOR ANNUAL REPORTS:
Permission covers print and electronic versions provided the AAAS material reproduced as permitted herein remains in situ and is not exploited separately (for example, if permission covers the use of a full text article, the article may not be offered for access or for purchase as a stand-alone unit)

FOR PROMOTIONAL/MARKETING USES:
Permission covers the use of AAAS material in promotional or marketing pieces such as information packets, media kits, product slide kits, brochures, or flyers limited to a single print run. The AAAS Material may not be used in any manner which implies endorsement or promotion by the American Association for the Advancement of Science (AAAS) or Science of any product or service. AAAS does not permit the reproduction of its name, logo or text on promotional literature.

If permission to use a full text article is permitted, The Science article covered by this permission must not be altered in any way. No additional printing may be set onto an article copy other than the copyright credit line required above. Any alterations must be approved in advance and in writing by AAAS. This includes, but is not limited to, the placement of sponsorship identifiers, trademarks, logos, rubber stamping or self-adhesive stickers onto the

article copies.

Additionally, article copies must be a freestanding part of any information package (i.e. media kit) into which they are inserted. They may not be physically attached to anything, such as an advertising insert, or have anything attached to them, such as a sample product. Article copies must be easily removable from any kits or informational packages in which they are used. The only exception is that article copies may be inserted into three-ring binders.

FOR CORPORATE INTERNAL USE:
The AAAS material covered by this permission may not be altered in any way. No additional printing may be set onto an article copy other than the required credit line. Any alterations must be approved in advance and in writing by AAAS. This includes, but is not limited to the placement of sponsorship identifiers, trademarks, logos, rubber stamping or self-adhesive stickers onto article copies.

If you are making article copies, copies are restricted to the number indicated in your request and must be distributed only to internal employees for internal use.

If you are using AAAS Material in Presentation Slides, the required credit line must be visible on the slide where the AAAS material will be reprinted

If you are using AAAS Material on a CD, DVD, Flash Drive, or the World Wide Web, you must include the following notice in any electronic versions, either adjacent to the reprinted AAAS material or in the terms and conditions for use of your electronic products: "Readers may view, browse, and/or download material for temporary copying purposes only, provided these uses are for noncommercial personal purposes. Except as provided by law, this material may not be further reproduced, distributed, transmitted, modified, adapted, performed, displayed, published, or sold in whole or in part, without prior written permission from the publisher." Access to any such CD, DVD, Flash Drive or Web page must be restricted to your organization's employees only.

FOR CME COURSE and SCIENTIFIC SOCIETY MEETINGS:
Permission is restricted to the particular Course, Seminar, Conference, or Meeting indicated in your request. If this license covers a text excerpt or a Full Text Article, access to the reprinted AAAS material must be restricted to attendees of your event only (if you have been granted electronic rights for use of a full text article on your website, your website must be password protected, or access restricted so that only attendees can access the content on your site).

If you are using AAAS Material on a CD, DVD, Flash Drive, or the World Wide Web, you must include the following notice in any electronic versions, either adjacent to the reprinted AAAS material or in the terms and conditions for use of your electronic products: "Readers may view, browse, and/or download material for temporary copying purposes only, provided

Original Authors may post the 'Accepted Version' of their full text article on their personal or on their University website and not on any other website. The 'Accepted Version' is the version of the paper accepted for publication by AAAS including changes resulting from peer review but prior to AAAS's copy editing and production (in other words not the AAAS published version).

FOR MOVIES / FILM / TELEVISION:
Permission is granted to use, record, film, photograph, and/or tape the AAAS material in connection with your program/film and in any medium your program/film may be shown or heard, including but not limited to broadcast and cable television, radio, print, world wide web, and videocassette.

The required credit line should run in the program/film's end credits.

FOR MUSEUM EXHIBITIONS:
Permission is granted to use the AAAS material as part of a single exhibition for the duration of that exhibit. Permission for use of the material in promotional materials for the exhibit must be cleared separately with AAAS (please contact us at permissions@aaas.org).

FOR TRANSLATIONS:
Translation rights apply only to the language identified in your request summary above.

The following disclaimer must appear with your translation, on the first page of the article, after the credit line: "This translation is not an official translation by AAAS staff, nor is it endorsed by AAAS as accurate. In crucial matters, please refer to the official English-language version originally published by AAAS."

FOR USE ON A COVER:
Permission is granted to use the AAAS material on the cover of a journal issue, newsletter issue, book, textbook, or annual report in print and electronic formats provided the AAAS material reproduced as permitted herein remains in situ and is not exploited separately

By using the AAAS Material identified in your request, you agree to abide by all the terms and conditions herein.

Questions about these terms can be directed to the AAAS Permissions department permissions@aaas.org.

Other Terms and Conditions:

v 2

SPRINGER NATURE LICENSE
TERMS AND CONDITIONS

May 24, 2020

This Agreement between Mr. Ryan Martin ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number	4835560311053
License date	May 24, 2020
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Nature Structural & Molecular Biology
Licensed Content Title	Structure and mechanism of the RNA polymerase II transcription machinery
Licensed Content Author	Steven Hahn
Licensed Content Date	Apr 27, 2004
Type of Use	Thesis/Dissertation
Requestor type	academic/university or research institute

Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Will you be translating?	no
Circulation/distribution	1 - 29
Author of this Springer Nature content	no
Title	G protein-coupled receptor-mediated transcriptional regulation in pathological cardiac remodelling
Institution name	McGill University
Expected presentation date	Aug 2020
Portions	Figure 3A
Requestor Location	Mr. Ryan Martin 3655 Promenade Sir William Osler Room 1303 Montreal, QC H3G1Y6

Canada
Attn: Mr. Ryan Martin

Total 0.00 CAD

Terms and Conditions

Springer Nature Customer Service Centre GmbH
Terms and Conditions

This agreement sets out the terms and conditions of the licence (the **Licence**) between you and **Springer Nature Customer Service Centre GmbH** (the **Licensor**). By clicking 'accept' and completing the transaction for the material (**Licensed Material**), you also confirm your acceptance of these terms and conditions.

1. Grant of License

1. 1. The Licensor grants you a personal, non-exclusive, non-transferable, world-wide licence to reproduce the Licensed Material for the purpose specified in your order only. Licences are granted for the specific use requested in the order and for no other use, subject to the conditions below.

1. 2. The Licensor warrants that it has, to the best of its knowledge, the rights to license reuse of the Licensed Material. However, you should ensure that the material you are requesting is original to the Licensor and does not carry the copyright of another entity (as credited in the published version).

1. 3. If the credit line on any part of the material you have requested indicates that it was reprinted or adapted with permission from another source, then you should also seek permission from that source to reuse the material.

2. Scope of Licence

2. 1. You may only use the Licensed Content in the manner and to the extent permitted by these Ts&Cs and any applicable laws.

2. 2. A separate licence may be required for any additional use of the Licensed Material, e.g. where a licence has been purchased for print only use, separate permission must be obtained for electronic re-use. Similarly, a licence is only valid in the language selected and does not apply for editions in other languages unless additional translation rights have been granted separately in the licence. Any content

owned by third parties are expressly excluded from the licence.

2. 3. Similarly, rights for additional components such as custom editions and derivatives require additional permission and may be subject to an additional fee. Please apply to Journalpermissions@springernature.com/bookpermissions@springernature.com for these rights.

2. 4. Where permission has been granted **free of charge** for material in print, permission may also be granted for any electronic version of that work, provided that the material is incidental to your work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.

2. 5. An alternative scope of licence may apply to signatories of the STM Permissions Guidelines, as amended from time to time.

3. Duration of Licence

3. 1. A licence for is valid from the date of purchase ('Licence Date') at the end of the relevant period in the below table:

Scope of Licence	Duration of Licence
Post on a website	12 months
Presentations	12 months
Books and journals	Lifetime of the edition in the language purchased

4. Acknowledgement

4. 1. The Licensor's permission must be acknowledged next to the Licenced Material in print. In electronic form, this acknowledgement must be visible at the same time as the figures/tables/illustrations or abstract, and must be hyperlinked to the journal/book's homepage. Our required acknowledgement format is in the Appendix below.

5. Restrictions on use

5. 1. Use of the Licensed Material may be permitted for incidental promotional use and minor editing privileges e.g. minor adaptations of single figures, changes of format, colour and/or style where the adaptation is credited as set out in Appendix 1 below. Any

other changes including but not limited to, cropping, adapting, omitting material that affect the meaning, intention or moral rights of the author are strictly prohibited.

5. 2. You must not use any Licensed Material as part of any design or trademark.

5. 3. Licensed Material may be used in Open Access Publications (OAP) before publication by Springer Nature, but any Licensed Material must be removed from OAP sites prior to final publication.

6. Ownership of Rights

6. 1. Licensed Material remains the property of either Licensor or the relevant third party and any rights not explicitly granted herein are expressly reserved.

7. Warranty

IN NO EVENT SHALL LICENSOR BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL OR INDIRECT DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND
WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

8. Limitations

8. 1. ***BOOKS ONLY:*** Where **'reuse in a dissertation/thesis'** has been selected the following terms apply: Print rights of the final author's accepted manuscript (for clarity, NOT the published version) for up to 100 copies, electronic rights for use only on a personal website or institutional repository as defined by the Sherpa guideline (www.sherpa.ac.uk/romeo/).

9. Termination and Cancellation

9. 1. Licences will expire after the period shown in Clause 3 (above).

9. 2. Licensee reserves the right to terminate the Licence in the event that payment is not received in full or if there has been a breach of this agreement by you.

Appendix 1 — Acknowledgements:

For Journal Content:
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

For **Advance Online Publication papers:**
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].)

For Adaptations/Translations:
Adapted/Translated by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

Note: For any republication from the British Journal of Cancer, the following credit line style applies:

Reprinted/adapted/translated by permission from [**the Licensor**]: on behalf of Cancer Research UK: : [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

For **Advance Online Publication** papers:
Reprinted by permission from The [**the Licensor**]: on behalf of Cancer Research UK: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM])

For Book content:

Reprinted/adapted by permission from [**the Licensor**]: [**Book Publisher** (e.g. Palgrave Macmillan, Springer etc) [**Book Title**] by [**Book author**(s)] [**COPYRIGHT**] (year of publication)

Other Conditions:

Version 1.2

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

ELSEVIER LICENSE
TERMS AND CONDITIONS

May 24, 2020

This Agreement between Mr. Ryan Martin ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	4835560838218
License date	May 24, 2020
Licensed Content Publisher	Elsevier
Licensed Content Publication	DNA Repair
Licensed Content Title	RECQL5 helicase: Connections to DNA recombination and RNA polymerase II transcription
Licensed Content Author	Ozan Aygün,Jesper Q. Svejstrup
Licensed Content Date	Mar 2, 2010
Licensed Content Volume	9
Licensed Content Issue	3

Licensed Content Pages	9
Start Page	345
End Page	353
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Title	G protein-coupled receptor-mediated transcriptional regulation in pathological cardiac remodelling
Institution name	McGill University
Expected presentation date	Aug 2020
Portions	Figure 2
	Mr. Ryan Martin

Requestor Location	3655 Promenade Sir William Osler Room 1303 Montreal, QC H3G1Y6 Canada Attn: Mr. Ryan Martin
Publisher Tax ID	GB 494 6272 12
Total	0.00 CAD

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which

permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any

purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to

bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors:** the following clauses are applicable in addition to the above:

Preprints:

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
    - via their non-commercial person homepage or blog
    - by updating a preprint in arXiv or RePEc with the accepted manuscript
    - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
    - directly by providing copies to their students or to research collaborators for their personal use
    - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
    - via non-commercial hosting platforms such as their institutional repository
    - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI

- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's posting policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of

SPRINGER NATURE LICENSE
TERMS AND CONDITIONS

May 24, 2020

This Agreement between Mr. Ryan Martin ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number	4835560972543
License date	May 24, 2020
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Nature Reviews Molecular Cell Biology
Licensed Content Title	Transcription regulation by the Mediator complex
Licensed Content Author	Julie Soutourina
Licensed Content Date	Dec 6, 2017
Type of Use	Thesis/Dissertation
Requestor type	academic/university or research institute

Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Will you be translating?	no
Circulation/distribution	1 - 29
Author of this Springer Nature content	no
Title	G protein-coupled receptor-mediated transcriptional regulation in pathological cardiac remodelling
Institution name	McGill University
Expected presentation date	Aug 2020
Portions	Figure 1
Requestor Location	Mr. Ryan Martin 3655 Promenade Sir William Osler Room 1303 Montreal, QC H3G1Y6 Canada

Attn: Mr. Ryan Martin

Total 0.00 CAD

Terms and Conditions

Springer Nature Customer Service Centre GmbH
Terms and Conditions

This agreement sets out the terms and conditions of the licence (the **Licence**) between you and **Springer Nature Customer Service Centre GmbH** (the **Licensor**). By clicking 'accept' and completing the transaction for the material (**Licensed Material**), you also confirm your acceptance of these terms and conditions.

1. Grant of License

1. 1. The Licensor grants you a personal, non-exclusive, non-transferable, world-wide licence to reproduce the Licensed Material for the purpose specified in your order only. Licences are granted for the specific use requested in the order and for no other use, subject to the conditions below.

1. 2. The Licensor warrants that it has, to the best of its knowledge, the rights to license reuse of the Licensed Material. However, you should ensure that the material you are requesting is original to the Licensor and does not carry the copyright of another entity (as credited in the published version).

1. 3. If the credit line on any part of the material you have requested indicates that it was reprinted or adapted with permission from another source, then you should also seek permission from that source to reuse the material.

2. Scope of Licence

2. 1. You may only use the Licensed Content in the manner and to the extent permitted by these Ts&Cs and any applicable laws.

2. 2. A separate licence may be required for any additional use of the Licensed Material, e.g. where a licence has been purchased for print only use, separate permission must be obtained for electronic re-use. Similarly, a licence is only valid in the language selected and does not apply for editions in other languages unless additional translation rights have been granted separately in the licence. Any content owned by third parties are expressly excluded from the licence.

2. 3. Similarly, rights for additional components such as custom editions and derivatives require additional permission and may be subject to an additional fee. Please apply to Journalpermissions@springernature.com/bookpermissions@springernature.com for these rights.

2. 4. Where permission has been granted **free of charge** for material in print, permission may also be granted for any electronic version of that work, provided that the material is incidental to your work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.

2. 5. An alternative scope of licence may apply to signatories of the STM Permissions Guidelines, as amended from time to time.

3. Duration of Licence

3. 1. A licence for is valid from the date of purchase ('Licence Date') at the end of the relevant period in the below table:

Scope of Licence	Duration of Licence
Post on a website	12 months
Presentations	12 months
Books and journals	Lifetime of the edition in the language purchased

4. Acknowledgement

4. 1. The Licensor's permission must be acknowledged next to the Licenced Material in print. In electronic form, this acknowledgement must be visible at the same time as the figures/tables/illustrations or abstract, and must be hyperlinked to the journal/book's homepage. Our required acknowledgement format is in the Appendix below.

5. Restrictions on use

5. 1. Use of the Licensed Material may be permitted for incidental promotional use and minor editing privileges e.g. minor adaptations of single figures, changes of format, colour and/or style where the adaptation is credited as set out in Appendix 1 below. Any other changes including but not limited to, cropping, adapting, omitting material that

affect the meaning, intention or moral rights of the author are strictly prohibited.

5. 2. You must not use any Licensed Material as part of any design or trademark.

5. 3. Licensed Material may be used in Open Access Publications (OAP) before publication by Springer Nature, but any Licensed Material must be removed from OAP sites prior to final publication.

6. Ownership of Rights

6. 1. Licensed Material remains the property of either Licensor or the relevant third party and any rights not explicitly granted herein are expressly reserved.

7. Warranty

IN NO EVENT SHALL LICENSOR BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL OR INDIRECT DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND
WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

8. Limitations

8. 1. ***BOOKS ONLY:*** Where **'reuse in a dissertation/thesis'** has been selected the following terms apply: Print rights of the final author's accepted manuscript (for clarity, NOT the published version) for up to 100 copies, electronic rights for use only on a personal website or institutional repository as defined by the Sherpa guideline (www.sherpa.ac.uk/romeo/).

9. Termination and Cancellation

9. 1. Licences will expire after the period shown in Clause 3 (above).

9. 2. Licensee reserves the right to terminate the Licence in the event that payment is not received in full or if there has been a breach of this agreement by you.

Appendix 1 — Acknowledgements:

For Journal Content:
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

For **Advance Online Publication papers:**
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].)

For Adaptations/Translations:
Adapted/Translated by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

Note: For any republication from the British Journal of Cancer, the following credit line style applies:

Reprinted/adapted/translated by permission from [**the Licensor**]: on behalf of Cancer Research UK: : [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

For **Advance Online Publication** papers:
Reprinted by permission from The [**the Licensor**]: on behalf of Cancer Research UK: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM])

For Book content:

Reprinted/adapted by permission from [**the Licensor**]: [**Book Publisher** (e.g. Palgrave Macmillan, Springer etc) [**Book Title**] by [**Book author**(s)] [**COPYRIGHT**] (year of publication)

Other Conditions:

Version 1.2

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

ELSEVIER LICENSE
TERMS AND CONDITIONS

May 24, 2020

This Agreement between Mr. Ryan Martin ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	4835561481934
License date	May 24, 2020
Licensed Content Publisher	Elsevier
Licensed Content Publication	Molecular Cell
Licensed Content Title	Control of RNA Pol II Speed by PNUTS-PP1 and Spt5 Dephosphorylation Facilitates Termination by a “Sitting Duck Torpedo” Mechanism
Licensed Content Author	Michael A. Cortazar,Ryan M. Sheridan,Benjamin Erickson,Nova Fong,Kira Glover-Cutter,Kristopher Brannan,David L. Bentley
Licensed Content Date	Dec 19, 2019
Licensed Content Volume	76

Licensed Content Issue	6
Licensed Content Pages	17
Start Page	896
End Page	908.e4
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Title	G protein-coupled receptor-mediated transcriptional regulation in pathological cardiac remodelling
Institution name	McGill University
Expected presentation date	Aug 2020

Portions	Graphical Abstract
Requestor Location	Mr. Ryan Martin 3655 Promenade Sir William Osler Room 1303 Montreal, QC H3G1Y6 Canada Attn: Mr. Ryan Martin
Publisher Tax ID	GB 494 6272 12
Total	0.00 CAD

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE

SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors:** the following clauses are applicable in addition to the above:

Preprints:

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
    - via their non-commercial person homepage or blog
    - by updating a preprint in arXiv or RePEc with the accepted manuscript
    - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
    - directly by providing copies to their students or to research collaborators for their personal use
    - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
    - via non-commercial hosting platforms such as their institutional repository
    - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's posting policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of

JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS

May 24, 2020

This Agreement between Mr. Ryan Martin ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	4835570205956
License date	May 24, 2020
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	The EMBO Journal
Licensed Content Title	The structure of P−TEFb (CDK9/cyclin T1), its complex with flavopiridol and regulation by phosphorylation
Licensed Content Author	Louise N Johnson, Stefan Knapp, Judit É Debreczeni, et al
Licensed Content Date	Jun 19, 2008
Licensed Content Volume	27

Licensed Content Issue	13
Licensed Content Pages	12
Type of use	Dissertation/Thesis
Requestor type	University/Academic
Format	Print and electronic
Portion	Figure/table
Number of figures/tables	2
Will you be translating?	No
Title	G protein-coupled receptor-mediated transcriptional regulation in pathological cardiac remodelling
Institution name	McGill University
Expected presentation date	Aug 2020
Portions	Figure 1, Figure 2A

Requestor Location	Mr. Ryan Martin 3655 Promenade Sir William Osler Room 1303 Montreal, QC H3G1Y6 Canada Attn: Mr. Ryan Martin
Publisher Tax ID	EU826007151
Total	0.00 CAD

Terms and Conditions

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a"Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a particular work (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your RightsLink account (these are available at any time at http://myaccount.copyright.com).

Terms and Conditions

- The materials you have requested permission to reproduce or reuse (the "Wiley Materials") are protected by copyright.

- You are hereby granted a personal, non-exclusive, non-sub licensable (on a stand-alone basis), non-transferable, worldwide, limited license to reproduce the Wiley Materials for the purpose specified in the licensing process. This license, **and any CONTENT (PDF or image file) purchased as part of your order,** is for a one-time use only and limited to any maximum distribution number specified in the license. The first instance of republication or reuse granted by this license must be completed within two years of the date of the grant of this license (although copies prepared before the end date may be distributed thereafter). The Wiley Materials shall not be

used in any other manner or for any other purpose, beyond what is granted in the license. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher. You shall also duplicate the copyright notice that appears in the Wiley publication in your use of the Wiley Material. Permission is also granted on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Wiley Material. Any third party content is expressly excluded from this permission.

- With respect to the Wiley Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Wiley Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication), translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Wiley Materials without the prior permission of the respective copyright owner.**For STM Signatory Publishers clearing permission under the terms of the STM Permissions Guidelines only, the terms of the license are extended to include subsequent editions and for editions in other languages, provided such editions are for the work as a whole in situ and does not involve the separate exploitation of the permitted figures or extracts,** You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Wiley Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Wiley Materials on a stand-alone basis, or any of the rights granted to you hereunder to any other person.

- The Wiley Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc, the Wiley Companies, or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Wiley Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Wiley Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto

- NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU.

- WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.

- You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.

- IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

- Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

- The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

- This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.

- Any fee required for this permission shall be non-refundable after thirty (30) days from receipt by the CCC.

- These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.

- In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.

- WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

- This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.

- This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state's conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.

WILEY OPEN ACCESS TERMS AND CONDITIONS

Wiley Publishes Open Access Articles in fully Open Access Journals and in Subscription journals offering Online Open. Although most of the fully Open Access journals publish open access articles under the terms of the Creative Commons Attribution (CC BY) License only, the subscription journals and a few of the Open Access Journals offer a choice of Creative Commons Licenses. The license type is clearly identified on the article.

The Creative Commons Attribution License

The Creative Commons Attribution License (CC-BY) allows users to copy, distribute and transmit an article, adapt the article and make commercial use of the article. The CC-BY license permits commercial and non-

Creative Commons Attribution Non-Commercial License

The Creative Commons Attribution Non-Commercial (CC-BY-NC)License permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.(see below)

Creative Commons Attribution-Non-Commercial-NoDerivs License

The Creative Commons Attribution Non-Commercial-NoDerivs License (CC-BY-NC-ND) permits use, distribution and reproduction in any medium, provided the original work is properly cited, is not used for commercial purposes and no modifications or adaptations are made. (see below)

Use by commercial "for-profit" organizations

Use of Wiley Open Access articles for commercial, promotional, or marketing purposes requires further explicit permission from Wiley and will be subject to a fee.

Further details can be found on Wiley Online Library
http://olabout.wiley.com/WileyCDA/Section/id-410895.html

Other Terms and Conditions:

v1.10 Last updated September 2015

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

OXFORD UNIVERSITY PRESS LICENSE
TERMS AND CONDITIONS

May 24, 2020

This Agreement between Mr. Ryan Martin ("You") and Oxford University Press ("Oxford University Press") consists of your license details and the terms and conditions provided by Oxford University Press and Copyright Clearance Center.

License Number	4835570323690
License date	May 24, 2020
Licensed content publisher	Oxford University Press
Licensed content publication	Nucleic Acids Research
Licensed content title	Cracking the control of RNA polymerase II elongation by 7SK snRNP and P-TEFb
Licensed content author	C. Quaresma, Alexandre J.; Bugai, Andrii
Licensed content date	Jul 1, 2016
Type of Use	Thesis/Dissertation

Institution name	
Title of your work	G protein-coupled receptor-mediated transcriptional regulation in pathological cardiac remodelling
Publisher of your work	McGill University
Expected publication date	Aug 2020
Permissions cost	0.00 CAD
Value added tax	0.00 CAD
Total	0.00 CAD
Title	G protein-coupled receptor-mediated transcriptional regulation in pathological cardiac remodelling
Institution name	McGill University
Expected presentation date	Aug 2020
Portions	Figure 2
Requestor Location	Mr. Ryan Martin 3655 Promenade Sir William Osler Room 1303 Montreal, QC H3G1Y6

Canada
Attn: Mr. Ryan Martin

Publisher Tax ID GB125506730

Total 0.00 CAD

Terms and Conditions

STANDARD TERMS AND CONDITIONS FOR REPRODUCTION OF MATERIAL FROM AN OXFORD UNIVERSITY PRESS JOURNAL

1. Use of the material is restricted to the type of use specified in your order details.

2. This permission covers the use of the material in the English language in the following territory: world. If you have requested additional permission to translate this material, the terms and conditions of this reuse will be set out in clause 12.

3. This permission is limited to the particular use authorized in (1) above and does not allow you to sanction its use elsewhere in any other format other than specified above, nor does it apply to quotations, images, artistic works etc that have been reproduced from other sources which may be part of the material to be used.

4. No alteration, omission or addition is made to the material without our written consent. Permission must be re-cleared with Oxford University Press if/when you decide to reprint.

5. The following credit line appears wherever the material is used: author, title, journal, year, volume, issue number, pagination, by permission of Oxford University Press or the sponsoring society if the journal is a society journal. Where a journal is being published on behalf of a learned society, the details of that society must be included in the credit line.

6. For the reproduction of a full article from an Oxford University Press journal for whatever purpose, the corresponding author of the material concerned should be informed of the proposed use. Contact details for the corresponding authors of all Oxford University Press journal contact can be found alongside either the abstract or full text of the article concerned, accessible from www.oxfordjournals.org Should there be a problem clearing these rights, please contact journals.permissions@oup.com

7. If the credit line or acknowledgement in our publication indicates that any of the figures,

images or photos was reproduced, drawn or modified from an earlier source it will be necessary for you to clear this permission with the original publisher as well. If this permission has not been obtained, please note that this material cannot be included in your publication/photocopies.

8. While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by Oxford University Press or by Copyright Clearance Center (CCC)) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and Oxford University Press reserves the right to take any and all action to protect its copyright in the materials.

9. This license is personal to you and may not be sublicensed, assigned or transferred by you to any other person without Oxford University Press's written permission.

10. Oxford University Press reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

11. You hereby indemnify and agree to hold harmless Oxford University Press and CCC, and their respective officers, directors, employs and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

12. Other Terms and Conditions:

v1.4