Gβγ dimers: Understanding their evolutionary divergence, specificity in signalling and non-canonical nuclear functions

by

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Abstract

Heterotrimeric combinations of Gα, Gβ and Gγ subunits interact with G protein-coupled receptors (GPCRs) as signal transducers. The presence of G proteins with specific subunit compositions as well as distinct effector molecules within particular GPCR signalling complexes suggests that there might be correspondingly unique structural determinants important in specific signal transduction events. Given the existence of diverse Gβ and Gγ subunits, understanding the individual roles that these distinct subunits play in signal transduction becomes important. Previously, various biochemical and gene-silencing techniques have been used to elucidate Gβγ function and to demonstrate specificity conferred by different Gβγ combinations in GPCR signalling. However, a systematic approach to understand Gβ and Gγ subunit diversity and both canonical and non-canonical Gβγ functions has never been undertaken. Our objectives for this thesis were to (1) perform a species-wide phylogenetic analysis of Gβ and Gγ subunits (2) employ a Gβ and Gγ RNAi screen to understand their roles in GPCR activated second messenger systems and (3) characterize nuclear functions of Gβγ in the context of a novel interaction between these dimers and RNA polymerase II. Using heterologous cellular systems (HEK 293 cells) and primary cell models (rat neonatal cardiac fibroblasts) and lessons learnt from an analysis of evolutionary divergence patterns of Gβ and Gγ subunits, this thesis demonstrates that specific combinations of Gβγ regulate the activities of signalling entities downstream of M3-muscarinic receptors and angiotensin II type I receptors, and also interact with different subunits of RNA polymerase II in response to GPCR activation. Overall, this work expands our understanding of Gβγ dimers in the context of their specific combinations in cellular signalling, and describes a new interaction with RNA polymerase II.

Resumé

Les protéines G hétérotrimériques interagissent avec les récepteurs couplés aux protéines G (RCPG) et transduisent leurs signaux à l'intérieur des cellules. Ces protéines G sont composées de différentes combinaisons des sous-unités Gα, Gβ et Gγ. La présence de compositions déterminées de sous-unités aussi bien que d'effecteurs distincts à l'intérieur de complexes de signalisation de RCPG suggère qu'il pourrait exister des déterminants structurels uniques menant à l'activation d'évènements de transduction spécifiques. Étant donnée la grande diversité des sous-unités Gβ et Gγ, il devient important de mieux comprendre les différents rôles de ces sous-unités distinctes dans la transduction des signaux. Précédemment, une grande variété de techniques biochimiques et de silençages génétiques ont été utilisés dans le but d'élucider les fonctions du dimère Gβγ et de démontrer la spécificité conférée par différentes combinaisons de Gβγ dans la signalisation des RCPG. Toutefois, aucune de ces études n'a utilisé une approche systématique dans le but de comprendre l'impact de la diversité des sous-unités Gβγ au niveau de la signalisation canonique et non canonique des RCPG. Les objectifs de cette thèse sont, premièrement, d'effectuer une analyse phylogénique des sous-unités Gβ et Gγ. Deuxièmement, dépister à l'aide d'ARN interférant (ARNi) les différents rôles des sous-unités Gβ et Gγ dans les systèmes d'activation de second messager par les RCPG. Finalement, caractériser des fonctions nucléaires des Gβγ dans le contexte d'une nouvelle interaction entre ces dimères et l'ARN polymérase II. En utilisant un système cellulaire hétérologue (cellules HEK 293) et des cultures primaires de rat (fibroblastes cardiaques néonataux de rat) ainsi que les leçons retenues de l'analyse des divergences évolutionnaires des sous-unités Gβ et Gγ, cette thèse démontre que des combinaisons déterminées du dimère Gβγ régulent l'activité de signalisation en aval du récepteur muscarinique M3 et du récepteur de l'angiotensine II de type I. Cette thèse démontre également

que ces combinaisons peuvent interagir avec différentes sous-unités de l'ARN polymérase II en réponse à l'activation de RCPG. Pris dans son ensemble, ce travail élargit notre compréhension des différentes combinaisons de dimères de Gβγ dans le contexte de signalisation cellulaire et décrit pour la première fois l'interaction entre l'ARN polymérase II et des dimères précis de Gβγ.

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Author Contributions

Chapter 1

Parts of Chapter 1 can be found in reviews published in 2013 (Khan et al, 2013) and 2016 (Khan et al, 2016). I wrote the sections taken from the review and the final version was elaborated with Dr. Terry Hébert. Rory Sleno and I designed and created Figures 1.6 and 1.8, whereas Dr. Peter Zylbergold and I designed Figure 1.5.

Chapter 2

The data presented in Chapter 2 can be found as a part of a review published in 2013 (Khan et al, 2013). I designed and collected data for the comparison of human Gβ and Gγ subunits (Tables 1, 2) while the phylogenetic analysis was performed in collaboration with Dr. Terry Hébert, Dr. Jean Claude Labbé and Dr. Jean-Philippe Laverdure. Dr. Jean-Philippe Laverdure performed the phylogenetic analysis and I performed the data analysis (Figure 2.1-2.2). For the structural mapping analysis, I collected data while Dr. Gregory Miller performed the structural mapping with PyMOL (Figure 2.3). Dr. Terry Hébert, Dr. Jean-Claude Labbé and myself undertook final deliberations and framed conclusions for both analyses. I wrote the first draft of the manuscript and the final version was re-written by Dr. Terry Hébert and myself. Rory Sleno, Dr. Sarah Gora and Dr. Peter Zylbergold were co-authors on the manuscript and wrote sections of the review that were not used or reproduced in this Chapter.

Chapter 3

I designed and performed the experiments for Figures 3.1-3.5, 3.7 and 3.8C-D. I designed the experiments and Adam Min performed the experiments in Figure 3.6. Dr. Sarah Gora and Geeda Houranieh designed and performed the data presented in Figure 3.8A-B, while I re-analyzed this data. Ashley Jacobi and Mark Behlke designed siRNAs used in this study and performed the validation experiments shown in Supplemental Figure 3.2a, b. Rhiannon Campden designed and Dr. Melanie Robitaille performed the LC/MS experiments (in Dr. Stephane Angers' lab) mentioned in the text (data not shown). Darlaine Pétrin assisted in setting up the aequorin assay and Phan Trieu assisted in setting up siRNA knockdown of $G\beta_1$ and $G\beta_4$. I wrote the first draft of the manuscript and the final version was re-written by Dr. Terry Hébert and myself.

Chapter 4

I designed and performed the experiments shown in Figures 4.1(A-C, E-G), 4.2, 4.3(A-B, D-I), 4.4-4.6 and Supplemental Figures 4.1(B-F), 4.2-4.4. Phan Trieu designed and performed experiments shown in Figures 4.1D and 4.3C, for which I prepared purified proteins for. Dr. Sarah Gora designed and performed the experiments shown in Supplemental Figure 4.1A. Ryan Martin assisted in the isolation cardiac fibroblasts from neonatal rat hearts used for experiments shown in Figures 4.3-4.5. Dr. Alan Smrcka provided the purified $G\beta\gamma$ proteins mentioned in the text. I wrote the first draft of the manuscript and the final version was re-written by Dr. Terry Hébert and myself.

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Abbreviations

AC	Adenylyl cyclase
AEBP1	Adipocyte enhancer-binding protein
AMP	Adenosine monophosphate
AngII	Angiotensin II
AP-1	Activator protein 1
AT1R	Angiotensin II type 1 receptor
AT2R	Angiotensin II type II receptor
BAPTA-AM	Glycine, N,N'-[1,2-ethanediylbis(oxy-2,1-phenylene)]bis[N-[2-[(acetyloxy)methoxy]-2-oxoethyl]]-, bis[(acetyloxy)methyl] ester
CalyA	CalyculinA
cAMP	Cyclic adenosine monophosphate
CCT	Chaperonin containing TCP-1 protein
ChIP	Chromatin immunoprecipitation
CK2	Casein kinase 2
CsA	CyclosporinA
D2-R	Dopamine receptor D2
DAG	Diacyl glycerol
DHHC	Aspartate-histidine-histidine-cysteine palmitoytransferase
DMEM	Dubelco's modified eagle medium
DRiP78	Dopamine receptor interacting protein 78
DsiRNA	Dicer-substrate short interfering RNAs
DTT	Dithiothreitol

EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
Epac	Exchange protein directly activated by cAMP
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase
ET-1	Endothelin-1
ET-R	Endothelin receptor
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
G protein	Guanine-nucleotide binding protein
GAP	GTPase-activating proteins
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
GIRK	G protein-coupled inwardly rectifying potassium channels
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GST	Glutathione sepharose transferase
GTP	Guanine triphosphate
Gα	Guanine-nucleotide binding protein, α subunit
Gβ	Guanine-nucleotide binding protein, β subunit
Gγ	Guanine-nucleotide binding protein, γ subunit
HA-tagged	Human influenza hemagglutinin-tagged
HBSS	Hank's balanced salt solution

HEK 293	Human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hnRNP	Heterogeneous nuclear ribonucleoprotein
IC_{50}	Inhibitory concentration 50
IgG	Immunoglobulin G
IP	Immunoprecipitation
IP_3	Inositol triphosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JNK	c-Jun N-terminal kinase
kDa	Kilo daltons
Kir3	G protein-coupled inwardly rectifying potassium channels
KN-93	(2-[N-(2-Hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]-amino-N-(4-chlorocinnamyl)-N-methylbenzylamine)
M1-mAChR	M1-muscarinic acetylcholine receptor
M2-mAChR	M2-muscarinic acetylcholine receptor
M3-mAChR	M3-muscarinic acetylcholine receptor
mAKAP	m-A-kinase anchoring protein
MAPK	Mitogen activated protein kinase
MEK1	Mitogen-activated protein kinase kinase
mGluR5	Metabotropic glutamate receptor 5
MMLV-RT	Moloney murine leukemia virus – reverse transcriptase
NaCl	Sodium chloride
NaF	Sodium fluoride

NIS	Sodium-iodine transporter
OPL	Outer plexiform layer
OR	Opioid receptor
PCR	Polymerase chain reaction
pERK1/2	Phosphorylated-extracellular signal regulated kinase 1/2
$PhLP_1$	Phosducin-like protein 1
PI3K	Phosphoinositide-3 kinase
PI_4P	Phosphatidylinositol-4 phosphate
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PKD	Protein kinase D
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PtdIns	Phosphatidylinositol
PTX	Pertussis toxin
quadKO	$G\alpha_{q/11/12/13}$ knockout HEK 293 cells
RGS	Regulator of G protein signalling protein
RIPA	Radioimmunoprecipitation assay buffer
RNAi	RNA interference
RNAPII	RNA polymerase II
RNCF	Rat neonatal cardiac fibroblasts
RT-PCR	Reverse transcription polymerase chain reaction
RTK	Receptor tyrosine kinase

SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
snRNA	Small nuclear RNA
TSH	Thyroid stimulating hormone
WD40	Tryptophan-aspartic acid dipeptide repeat
β-ARK-ct	β-adrenergic receptor kinase, c-tail
β_2-AR	β_2-adrenergic receptor
μ-OR	μ-opioid receptor

CHAPTER 1: General Introduction

1.1 Preface

Cellular signalling encompasses a large variety of means by which cells, tissues and organs communicate with one another within a multicellular organism. Signals may be received by means of ligands presented locally to the surface of a cell or soluble molecules generated proximally (for example, synaptic transmission) or distally (for example, hormone signalling) [1]. Cells, in turn, decode and interpret these signals into biological responses by detecting signals via various receptors and proteins expressed at their surfaces. Classes of such cell surface receptors and proteins include G protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs), ligand-gated or voltage-gated ion channels, cytokine receptors and nuclear hormone receptors. Of particular interest for this thesis are GPCRs, of which a multitude of sub-families are expressed throughout the human body and represent the largest class of signalling receptors studied to date [2]. The central mediators that transduce extracellular stimuli received by GPCRs into intracellular signalling events are G proteins, heterotrimeric complexes of Gα, Gβ and Gγ subunits. In this thesis, I describe the diversity, phylogeny, mechanistic actions and functions served by Gβγ subunits, dimeric protein complexes that modulate signalling downstream of GPCR activation.

1.2 G Protein-Coupled Receptors

GPCRs are membrane receptors that transduce extracellular signals, interpreted in the form of ligand binding, into activation of various intracellular signalling pathways. The initial cloning of the hamster β_2-adrenergic by Brian Kobilka and Robert Lefkowitz in 1986 led to a rapid expansion of the number of receptors cloned, representing a family of more than 800 different GPCRs [3]. From a pharmacological perspective, GPCRs carry significant importance as they serve as targets of more than 50% of currently available drugs on the market, although these drugs only target 50-60 different receptors [4].

1.3 GPCR structure, function and activation

GPCRs are expressed in a multitude of tissues and serve roles in several biological processes and physiological functions, ranging from neurotransmission, release of hormones from endocrine and exocrine glands, cardiac muscle contraction and blood pressure regulation, to name a few [5]. Through autocrine, intracrine, paracrine or endocrine signalling modes, these receptors respond to a large array of cellular modulators that include hormones, neurotransmitters, lipids, nucleotides, ions and photons [6]. GPCRs were thought to be expressed predominantly at the cell surface, however, recent evidence has suggested that these receptors also reside and signal from in intra-cellular locations, most notably at the nuclear membrane [7]. From a structural viewpoint, almost all GPCRs contain 7 transmembrane domains, 3 intracelullar and 3 extracellular loops and N terminal domains and C-terminal domains that flank

transmembrane domain 1 and transmembrane domain 7, respectively. The structural and sequence diversity of GPCRs has allowed for the phylogenetic classification of this family of receptors into 5 different families based on their conserved features and structural motifs, according to the "GRAFS" nomenclature system – the *G*lutamate receptor-like family (22 members), the *R*hodopsin receptor family (672 members), the *A*dhesion receptor family (33 members), the *F*rizzled/Taste2 receptor family (11 members) and the *S*ecretin receptor family (15 members) [8]. The most noticeable differences between these different families of GPCRs lie in their N-terminal domains, ranging from large N-terminal ectodomains in the Glutamate receptor and Secretin receptor families [9, 10] to cysteine-rich N-terminal domains in the Frizzled receptor family [11]. Furthermore, the combinatorial three-dimensional arrangements of the N-terminus and extracellular loops of GPCRs form distinct binding sites for a variety of ligands capable of activating these receptors.

The activation of GPCRs by ligands leads to conformational changes in the structure of these receptors that are further relayed to the activation of G proteins and subsequent modulation of downstream signalling pathways. The clearest understanding of how ligand binding leads to GPCR activation and subsequent activation of the G protein has come from efforts in solving the crystal structure of GPCRs, in particular the β_2-adrenergic receptor (β_2-AR), for which structures have been obtained for both inactive and active states (Figure 1.1). In particular, studies on un-liganded and inverse-agonist bound β_2-AR reveal that these receptors exist in predominantly two inactive states that exchange between one another within hundreds of microseconds [12]. Binding of agonists to β_2-ARs results in large conformational outward movements of the cytoplasmic end of transmembrane domain 6 (TM6) and α-helical extension of transmembrane

domain 5 (TM5) (Figure 1.1) [13]. Similar structural rearrangements have been identified in other GPCRs, namely from the structure of agonist-bound M2-muscarinic acetylcholine receptors [14]. It has been found that binding of either high affinity agonists or low affinity natural agonists, such as adrenaline, stabilizes similar conformational rearrangements, although through different chemical interactions [15]. It must also be noted that while agonist binding destabilizes the inactive state, it alone does not stabilize a fully active conformation of β_2-ARs [16]. Instead, agonist binding is linked to conformational heterogeneity that allows for the β_2-AR to interact with multiple regulatory or signalling proteins, such as G proteins [16]. Importantly, the transition of a GPCR from its inactive state to an active state requires the coupling to a G protein [16]. Being more pertinent to this thesis, the activation of G proteins as a result of agonist binding to GPCRs is discussed in a section below.

1.4 G Proteins and G Protein Signalling

1.4.1 History and discovery of G proteins

Guanine-nucleotide binding proteins, or G proteins, are heterotrimeric combinations of Gα, Gβ and Gγ subunits, of which Gβ and Gγ subunits form obligate dimers. These proteins act as transducers that link GPCR activation to the regulation of various effectors such as adenylyl cyclases, phospholipases and ion channels. The discovery of G proteins stems from two parallel studies regarding cAMP production. First, Gill & Meren demonstrated that the treatment of pigeon erythrocyte lysates with the entotoxin produced by *Vibrio cholera* produced a sustained

generation and release of cAMP via its action as a mono-ADP-ribosyltransferase – an enzyme that transfers the ADP-ribose element of nicotinamide adenine dinucleotide (NAD^+) to a substrate protein [17, 18]. Second, work done by Coffino *et al* on mouse lymphoma S49 cell lines further corroborated the existence of G proteins. Mutant cells (which would otherwise die under conditions of sustained cAMP elevation due to β-adrenergic receptor simulation) were selected based on the lack of their ability to generate cAMP in response to isoproterenol, a β-adrenergic receptor agonist. Intriguingly, these cells retained the ability to bind radiolabelled β-adrenergic receptor ligands and still expressed adenylyl cyclase (the enzyme directly responsible for the production of cAMP). Experiments with wild-type S49 cells using cholera toxin and [^{32}P]-NAD+ revealed incorporation of radioactivity in a 45 kDa protein entity that was not seen in mutant S49 cells [18, 19]. Subsequent work to purify this 45kDa protein by Alfred Gilman's group in 1980 revealed that this protein co-purified with a 35 kDa and a 8-10 kDa protein entity. These 45, 35 and 8-10 kDa proteins would go on to be identified as the α-, the β- and γ-subunits of the heterotrimeric adenylyl cyclase stimulatory G protein Gs [18, 20]. Similar work was performed by Martin Rodbell's group to show that a 41 kDa protein was responsible for a reduction in cAMP in response to α2-adrenergic receptor stimulation in islet cells – a protein identified as the α-subunit of Gi, a protein co-purified with the 35 and 8-10 kDa β and γ subunits of Gs, respectively. As a result of their work on the identification of G proteins, their structures and functions, Gilman and Rodbell were awarded the Nobel Prize in Physiology or Medicine in 1994.

1.4.2 Gα subunits – structure, diversity and functions

Gα subunits are the catalytic components of G proteins that contain two domains – a GTPase or Ras-like domain and a α-helical domain [21]. The GTPase domain consists of 3 flexible loops called switch regions that change conformation when bound by GTP whereas the helical domain consists of 6 α-helices that capture nucleotides by forming a lid over the nucleotide-binding pocket [22]. Gα proteins remain bound to GDP in their inactive states, but when acted on Guanine Exchange Factors (GEFs; such as GPCRs) a switch occurs between this GDP-bound state for a GTP-bound state, eventually resulting in an active-state Gα [6]. 20 different subtypes of Gα subunits corresponding to 16 different genes have been described, and can be further grouped into 4 different classes – $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ or $G\alpha_{12}$ [23]. These different subtypes of Gα subunits share around 20% amino acid sequence similarity [24] and are differentially myristoylated or palmitoylated at their N-termini to facilitate anchoring to the cell membrane [25]. These differences in amino acid sequences and post-translational modifications reveal clues as to the distinct roles and functions that they serve in cellular signalling, in addition to the specific GPCRs, Gβ and Gγ subunits they are capable of interacting with. Indeed, a great deal of work has shown that different types of GPCRs couple specifically to different types of Gα containing G proteins, resulting in various signalling and biological outcomes. For example, β_2-adrenergic receptors couple to both Gαi (G_i) and Gαs (G_s) G proteins [26], whereas M1-, M3- or M5-muscarinic receptors show selectivity for $G\alpha_{q/11}$ containing G proteins (G_q) [27]. While a great deal of work has elucidated the specificities of Gα subunits coupling to specific GPCRs, far less has been done to delineate similar specificities for Gβ and Gγ subunits.

With regard to Gα subunit function, these proteins are known to regulate a wide variety of effectors. From a classical viewpoint, members of the G_s family stimulate adenylyl cyclases to increase cAMP production, while members of the G_i family act to inhibit the activity of adenylyl cyclases [24]. Furthermore, members of the $G_{q/11}$ family activate phospholipase C β to stimulate the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacyl glycerol and inositol triphosphate [28], whereas $G_{12/13}$ is known to regulate Rho-dependent signalling [29]. Given their central roles in GPCR signalling, it is not surprising that many Gα subunits have been implicated in a variety of diseases such as heart failure and cancer [21, 30, 31].

1.4.3 Gα subunit activation

As mentioned previously, the exchange of GDP for GTP renders Gα subunits in an active state. However, the exact mechanism of Gα activation had not been elucidated until the co-crystal structure of the β_2-adrenergic receptor with an active G_s protein was solved. Work done by the Kobilka group showed that interactions between β_2-AR and G_s involves both the amino- and carboxy-terminals α-helices of G_s, with conformational changes being relayed to the nucleotide binding pocket (Figure 1.2). More specifically, as observed from the crystal structure of $G\alpha_s$-GTPγS, the binding of guanine nucleotide to the nucleotide-binding pocket formed by the Ras-like domain and α-helical domain stabilizes the interaction between these two domains [13, 32]. Agonist binding to the GPCR confers a conformational change to the Gα subunit such that there is a large outward displacement of α-helical domain relative to the Ras-like domain – nearly a 127° rotation about the junction between the domains [13]. This rotation allows for an "opening" of the nucleotide binding domain and the subsequent exchange of GDP for GTP,

driving the G protein into an active state (Figure 1.3). This in turn activates both Gα subunit and Gβγ dimer mediated regulation of effector activity – models of how G protein activation leads to the initiation of Gβγ dimer activity are discussed in a section below.

1.5 Gβγ Dimers

1.5.1 Gβ and Gγ subunits: An introduction

Gβ and Gγ subunits form obligate dimers that constitute the other major component of G proteins (apart from Gα subunits). To date, 5 different subtypes of Gβ subunits ($G\beta_{1\text{-}5}$) and 12 different types of Gγ subunits ($G\gamma_{1\text{-}5,\ 7\text{-}13}$) have been described in mammals. Of the different Gβ and Gγ subtypes, $G\beta_{1\text{-}4}$ share the most amino acid sequence similarity, with $G\beta_5$ being the most dissimilar subtype from the rest, whereas Gγ subunits differ significantly from one another. The reasons why Gβ are so similar and Gγ subunits so different from one another have not been extensively described. Chapter 2 of this thesis presents an analysis of Gβ and Gγ diversity and their phylogeny across a wide range of species, and as such, will not be expanded on further in this chapter.

As previously mentioned, Gβ and Gγ subunits were initially discovered as 35 kDa and 8-10 kDa protein entities that were co-purified with a 45 kDa protein, the Gα subunit, as regulatory subunits of adenylyl cyclase [20, 33]. Originally, the Gβγ dimer was thought to be necessary primarily for inactivation of Gα subunits, allowing them to re-associate with the receptor for

subsequent rounds of signalling. In this sense, Gβγ was viewed as a negative regulator of Gα signalling, and was thought to decrease the signal-to-noise ratio by preventing spontaneous Gα activation in the absence of receptor stimulation [34]. However, since then, it has been found that Gβγ dimers possess functional roles of their own, both dependent and independent of Gα subunit mediated signalling [35]. Indeed, the first evidence for a direct role of Gβγ dimers in cellular signalling came in 1987, when it was shown that purified Gβγ subunits from bovine brain were able to activate a cardiac potassium channel normally activated by muscarinic receptors following acetylcholine release [36]. A large body of work subsequently revealed that Gβγ subunits can also modulate many other effectors, via direct interaction including some that are also regulated by Gα subunits, including phospholipase Cβ [37], adenylyl cyclase isoforms [38], and voltage-gated calcium channels[39, 40] – roles that may be viewed as canonical functions (expanded on in a later section of this chapter). In addition to these roles, many studies have elucidated that Gβγ subunits possess non-canonical roles that range from the regulation of microtubule dynamics of the cytoskeletal structure to the modulation of transcriptional events in the nucleus to regulate gene expression.

1.5.2 Structural analysis and posttranslational modifications of Gβ and Gγ subunits

The crystal structure of Gβγ structure was first elucidated in 1995 in complex with $G\alpha_{i1}$ using purified bovine $G\beta_1$ and $G\gamma_2$ [41]. Gβ, a member of the β-propeller family of proteins, consists of a 7-bladed β-propeller of 7 WD40 domains where each blade consists of 4 antiparallel β-sheets, with a α-helical domain at the N-terminal of the protein [41, 42]. In contrast, Gγ

subunits are much smaller and simpler in structure, containing two α-helices connected by a short linker. These two subunits form obligate dimers by forming a tight parallel coiled-coil structure at their respective N-termini via extensive hydrophobic interactions [42, 43]. Additionally, it has been found that when recombinant Gβ or Gγ subunits are expressed alone in Sf9 cells, they form unstable aggregates due to misfolding [44, 45]. The importance of their obligatory dimerization is further corroborated by the fact that these dimers can only be separated under denaturing conditions [44].

With respect to posttranslational modifications of Gβ and Gγ subunits, modifications to Gγ subunits have been extensively studied. Gγ subunits contain a CAAX motif at their C-terminal that can be prenylated by a 15-carbon farnesyl group or a 20-carbon geranylgeranyl group at the carboxy-terminal cysteine of a CAAX motif [46]. Moreover, interaction with Gβ has been found to be necessary in order for Gγ subunits to be properly processed [47]. Such lipid modifications allow Gβγ subunits to be targeted and anchored at the plasma membrane and increase their ability to interact with Gα subunits and GPCRs such as rhodopsin [48-50]. In addition, it has been shown that $G\gamma_{12}$ subunits can be phosphorylated by PKC that facilitates its interactions with both Gα subunits and adenylyl cyclase [51]. Taken together, the importance posttranslational modifications on Gβγ dimers translate to their increased ability to perform their functions in heterotrimeric G protein activity as well as modulation of effectors.

1.5.3 Tissue distribution and cellular localization of Gβγ dimers

While Gβγ subunits play pivotal roles in GPCR signalling and biology, little is known about their expansive tissue distribution. Data derived from numerous studies listed in the Human Protein Atlas (http://www.proteinatlas.org/) have however provided some clues regarding where these subunits are expressed. $G\beta_{1\text{-}4}$ subtypes are expressed at the protein level in varying quantities in a variety of cell types and tissues across the human body that include examples such as neurons in the cerebral cortex, Leydig cells in the testes, and cardiomyocytes and cardiac fibroblasts in the heart. In contrast, $G\beta_5$ displays a more restricted expression profile with its expression being limited to fewer tissues and organs compared to its $G\beta_{1\text{-}4}$ counterparts. Similarly, Gγ subunit subtypes display differential mRNA expression in a variety of tissues. As rudimentary as knowledge of Gβ and Gγ tissue distribution may be, numerous efforts have revealed their expression patterns in specific tissues. In particular, using a targeted proteomics approach coupled with subcellular fractionation of mouse cortex, Betke *et al* explored Gβ and Gγ subunit expression in the brain regions [52]. Their studies revealed that $G\beta_1$, $G\beta_2$ and $G\beta_4$ were all detected at differing levels both pre- and post-synaptically in the cortex, striatum, cerebellum and hippocampus, with $G\beta_5$ only detected in the striatum [52]. With respect to Gγ subunits, $G\gamma_2$, $G\gamma_3$, $G\gamma_4$, $G\gamma_7$, $G\gamma_{12}$, and $G\gamma_{13}$ were detected in all four aforementioned brain regions [52]. Similar to this tissue specific analysis of subunit expression, although $G\gamma_1$ displays a broad tissue expression pattern (it has been detected in the placenta, muscle, liver, kidney, pineal gland, and uterus), the majority of research on this subunit has been focused on its roles in the eye [35]. $G\gamma_2$ exhibits a ubiquitous tissue expression pattern, and has been shown to be the most abundant Gγ subunit in the brain [53, 54]. $G\gamma_7$ is expressed almost exclusively in the striatum of the brain,

although significant expression is also observed in the neocortex and hippocampus [53].

Much more is known about Gβ and Gγ subcellular localization compared to their tissue distributions. From a classical standpoint, since Gβγ dimers are integral components in the transduction of signals received by GPCRs, these dimers exert their canonical functions at the inner cytoplasmic surface of the plasma membrane. However, Gβγ dimers also perform functions non-canonically in various intracellular compartments such as the mitochondria, Golgi apparatus, endoplasmic reticulum and nucleus (described in a later section). Functions at these subcellular locations also vary. Depending on the Gγ subunit contained in a Gβγ dimer, these dimers have been found to be able to translocate from the plasma membrane to different endomembranes such as the Golgi where they have been found to also regulate the anterograde trafficking of vesicles from the trans-Golgi to the plasma membrane [55, 56]. Furthermore, Gβγ subunits have been found in the ER where they play roles in receptor/G-protein/effector complex formation in the ER and cis-golgi where they act to assemble these different components of a GPCR signalling complex before their trafficking to the plasma membrane [57, 58]. Gβγ has also been suggested to be involved in the maturation of inwardly rectifying potassium channels at the ER and Golgi apparatus [59]. Moreover, Gβγ subunits have also been detected at the mitochondria where they are involved in mitochondrial fusion [60]. Of more importance to this thesis, Gβγ has recently been identified to play roles in the nucleus. An in-depth review of these roles is explained in later sections of this chapter. Taken together, these distinct expression profiles in different tissues provide clues regarding the roles played by individual subunits and their localization in different subcellular compartments expands our understanding of the diverse roles played by Gβγ in cellular signalling.

1.5.4 Assembly and specificities of formation of Gβγ dimers

While it remains to be identified what controls the expression of Gβ and Gγ subunits, the assembly of these dimers is a tightly regulated process that occurs in conjunction with molecular chaperones (Figure 1.5). The central proteins involved in the assembly of Gβγ dimers are cytosolic chaperonin complex (CCT), phosducin-like protein 1 (PhLP1) and DRiP78. In this process, newly synthesized $G\beta_{1\text{-}4}$ subunits are folded by interacting in a complex in the cytosol with CCT to which $PhLP_1$ also binds to accelerate folding [61], whereas DRiP78 acts to fold Gγ subunits correctly in the ER [62]. Phosphorylation of PhLP1 by casein kinase 2 faciliates the release of the Gβ–PhLP1 complex from CCT [63], further providing space for Gγ to assemble with Gβ in the ER. As a final step, PhLP1 exits the Gβγ complex via an unknown mechanism, leaving the newly formed dimer at the ER. $G\beta_5$ acts to dimerize with RGS proteins in a similar manner that does not require DRiP78 [61].

While PhLP1 does not discriminate between which Gβ subtypes it interacts with [61], DRiP78 seems to regulate only a certain subset of Gγ subunits [62]. The significance of this finding may translate into the regulation of which Gβ subunits dimerize with specific Gγ subunits. Indeed, yeast two-hybrid screens and immunoprecipitation studies have shown that certain Gβ subunits exhibit preference to which Gγ subunits they dimerize with [64, 65]. These studies have shown that $G\beta_1$ and $G\beta_4$ subunits display little to no selectivity for which Gγ subunits they interact with, $G\beta_2$ and $G\beta_3$ exhibit low affinity for $G\gamma_{1/8/11/13}$ and $G\gamma_{1/4/9/10}$, respectively, while $G\beta_5$ does not form any significant functional dimers with any Gγ subunits [64, 65]. These findings suggest that different subtypes of Gβ and Gγ subunits may indeed not be

redundant, but rather selective to their choice of cognate signalling partners. Such patterns of selectivity for Gβγ dimerization coupled to differential tissue expression suggests levels of specificity of Gβγ in cellular signalling that are yet to be completely determined. The significance of the existence of specific Gβγ dimers and the implications of such functional specificity in GPCR biology are further elaborated in Chapter 3.

1.5.5 Activation of Gβγ signalling

Whether Gβγ dissociates from Gα subunits or remains in a heterotrimer complex upon G protein activation remains a widely disputed attribute of G protein signalling. Different models of G protein activation have been proposed. With respect to the subunit dissociation model of G protein activation, the Gα subunit is believed to dissociate from its cognate Gβγ partner, allowing effector binding surfaces to be exposed and subsequent downstream signalling by the Gβγ subunit [66]. Indeed, evidence presented from the co-crystalization of G_s with β_2-adrenergic receptors suggests uncoupling of Gβγ from Gα subunits upon the exchange of GDP for GTP at the Gα subunit [13]. This implies that different Gα subunits might share a pool of diverse Gβγ dimers, and that hetero-trimerization at the cell surface (especially after receptor stimulation) would occur via collisional coupling. However, an alternate " clamshell" model has been described wherein GPCR activation induces conformational changes in the G protein subunits and exposes otherwise hidden surfaces at the Gα and Gβγ interface [67]. This leads to interaction with effectors, without the Gα and Gβγ unhinging completely from one another, effectively remaining associated throughout activation [67]. This model has been supported by studies that

have used resonance energy transfer techniques [68, 69], but may not apply to all the possible permutations of G protein complexes that form [70, 71]. Such paradigms make sense when considering that certain canonical effectors regulated by Gα subunits are also regulated by Gβγ dimers upon G-protein activation, implying that these dimers must exhibit selectivity for association with Gα subunits and effectors contained in a GPCR signalling complex. This notion calls to mind again the idea that all Gβγ dimer combinations are not equal, may have distinct functions, and that different cellular pools of Gβγ control a great deal of the architecture of cellular signalling.

1.6 $G\beta_5$ – The odd man out of the Gβ Family

$G\beta_5$ subunits are not similar to the rest of the Gβ subunit family, sharing under 50% sequence similarity. This subunit plays a multitude of roles that $G\beta_{1\text{-}4}$ do not. While *in vitro* studies have demonstrated $G\beta_5$ to be capable of coupling to Gγ subunits, such demonstrations of coupling have yet to be described in any endogenous or *in vivo* context. This section will serve to describe the diversity displayed by $G\beta_5$ subunits, and they distinct roles in cellular signalling.

1.6.1 $G\beta_5$ is an atypical Gβ subunit

As mentioned previously, $G\beta_5$ displays lower sequence similarity to the other Gβ subunits than $G\beta_{1\text{-}4}$. Mammalian $G\beta_5$ exists as two isoforms, a long splice variant ($G\beta_5$ -L) and a

short splice variant ($G\beta_5$-S), the former being expressed exclusively in retinal photoreceptor cells. Unlike the other Gβ subunits, this subunit has only been shown to reconstitute with $G\gamma_2$ under *in vitro* conditions [72, 73]. Instead, $G\beta_5$ preferentially forms obligate dimers with the G-γ-like (GGL) domain containing the R7-RGS family of proteins [72, 73]. Indeed, it has been observed that in mice lacking $G\beta_5$ ($G\beta_5$ knockout mice), levels of the R7-RGS family (RGS6, RGS7, RGS9 and RGS11) are downregulated in the retina and striatum of these mice below levels of detection, while their respective mRNA levels remain unchanged, suggesting posttranscriptional mechanisms of stability [74]. Similarly, knockout of RGS9 in mice results in reduced $G\beta_5$-L protein expression, although $G\beta_5$-L mRNA levels remain normal, leading to the notion that RGS9 is required for the maintenance of normal levels of $G\beta_5$-L protein *in vivo* [75]. This suggests that the levels of $G\beta_5$ and RGS proteins are not regulated by the level of their respective transcripts, but instead confer mutual stability upon each other at the protein level, further supporting the nature of their obligatory dimerization. The co-crystal structure of $G\beta_5$ with R7-RGS revealed that $G\beta_5$ folds into an identical seven-bladed β-propeller structure as $G\beta_5$ [76]. The structure of $G\beta_5$ contains a conserved Gα binding surface; although the $G\beta_5$-R7-RGS dimer has been suggested to act as a GTPase activating protein (GAP) to support nucleotide exchange on Gα subunits under conditions of agonist-stimulation of GPCRs, the function of $G\beta_5$ in these possible heterotrimers remains unknown [76]. Given the divergence from the rest of the Gβ subunits, $G\beta_5$ signalling has been an active area of study, and extensive studies have been carried out to understand its roles.

1.6.2 Roles of Gβ$_5$-R7-RGS in GPCR signalling

Although Gβ$_5$ displays atypical dimerization patterns and does not interact with Gγ subunits, the cellular functions of Gβ5-RGS7 proteins are not so dissimilar. Initial *in vitro* studies demonstrated that Gβ$_5$-RGS7 dimers act as GAPs exclusively for Gα$_5$ proteins, and not Gα$_q$ or Gα$_i$ [77]. However, it has since then been found that Gβ$_5$-RGS7 indeed does not act to activate Gα$_q$ proteins, but binds it directly to inhibit its activity [78]. Similar to its conventional Gβγ counterparts, Gβ$_5$-RGS7 dimers have been shown to modulate the activities of the same canonical effectors downstream of GPCR activation. Indeed, *in vitro* studies have shown Gβ$_5$ containing Gβγ dimers act to inhibit currents generated by neuronal G protein-coupled inwardly rectifying potassium channels (GIRK, or Kir3) via a mechanism that involves Gα$_{q/11}$ and phospholipase Cβ activation [79]. In addition, Gβ$_5$-RGS7 has specifically been found to bind to GIRK channels, facilitate their coupling to GABA$_B$ receptors, and regulate channel responses to receptor activation [80]. Similarly, it has been demonstrated that Gβ$_5$-RGS7 acts to inhibit carbachol-stimulated M3-muscarinic acetylcholine receptor (M3-mAChR) mediated Ca^{2+} release from intracellular stores and promote M3-mAChR-mediated calcium influx via nifedipine-sensitive Ca^{2+} channels [81]. However, Gβ$_5$ containing Gβγ does not inhibit adenylyl cyclase type VII downstream of μ-opioid receptor activation, while their typical Gβ$_{1-4}$ containing Gβγ counterparts did [82]. Interestingly, in studies using Gβ$_5$ knockout mice, it was noted that loss of Gβ$_5$ led to the dysregulation of multiple genes in the brains these mice— expression of 150 genes in the cerebellum and 228 genes from noncerebellar regions was altered [83]. These changes might also point to a direct role for Gβ$_5$ in transcriptional regulation. Insights into roles played by Gβ$_5$-R7-RGS in transcriptional regulation are discussed in a later section. Overall, it

would seem apparent that the functions served by $G\beta_5$-RGS7 dimers oppose those carried out by conventional $G\beta_{1\text{-}4}\gamma$ dimers, providing further evidence for the functional consequences of the divergence of $G\beta_5$ from $G\beta_{1\text{-}4}$.

1.6.3 Functions of Gβ5 in the Central Nervous System and Visual System

$G\beta_5$ is expressed primarily in brain and neuronal tissues and as a result, a great deal of work has been done to elucidate their functions in the central nervous system and visual system. Termination of light responses in retinal rods requires GTP hydrolysis by transducin, composed of $G\alpha_t$, $G\beta_5$-L, and RGS9-1. Loss of $G\beta_5$-L in these rods does not alter the activation of its cognate G protein cascade, but rather slows its deactivation and altered the rate of incremental dim flashes during light adaptation, implying that $G\beta_5$-L is essential for normal G protein deactivation and rod function [84]. With regard to the $G\beta_5$-S isoform, it was shown that $G\beta_5$-S and RGS11 colocalize with $G\alpha_o$ at the tips of ON (as opposed to OFF)-bipolar cell dendrites, and morphologic analysis of rod bipolar cells revealed that the retinal outer plexiform layer (OPL) of $G\beta_5$ knockout mice was disorganized with shorter dendrites [85]. A decrease in the number of synaptic triads in the OPL in these mice was also observed, suggesting a role for $G\beta_5$-S in OPL synaptic development.

In regions outside the retina, knockout of $G\beta_5$ leads to impaired neurobehavioral development as knockout mice displayed tiptoe walking with motor learning and coordination deficiencies [83]. These mice also exhibited impaired neuronal development in the cerebellum

and hippocampus. Based on these findings, it might be speculated that alterations in the overall development of $G\beta_5$ knockout mice may, in part, be a result of the aforementioned subsequent changes in gene expression due to the loss of $G\beta_5$. Apart from their roles in the brain and visual system, $G\beta_5$ has been implicated to play roles in metabolism. Homozygous $G\beta_5$ knockouts have been found to have smaller body size -- concentrations of triglycerides, free fatty acids, and glucose were decreased, whereas levels of insulin were increased, and these mice also had impaired glucose clearance [74, 86]. On the other hand, in heterozygous $G\beta_5$ knockout mice, instead of exhibiting partial reduction in body weight compared to homozygous knockout mice and wild-type mice, these animals became heavier, displayed higher adiposity, and increase in leptin levels [86]. These phenotypes suggest that heterozygous mice show characteristics reminiscent of obesity in humans, which in turn is associated with type 2 diabetes and metabolic syndrome. Thus, $G\beta_5$ may play a role in the progression of these disease phenotypes.

1.7 Gβγ Dimers in Cellular Signalling

Over the past two decades, the roles played by Gβγ dimers downstream of GPCR activation have expanded extensively and it is now appreciated that these dimers are responsible for the regulation of many effectors beyond their once thought solo-role as a negative regulator of Gα subunits. Mounting evidence has suggested that the effects of these dimers are not only proximal to GPCR and G protein activation, but can also be distal in a spatio-temporal sense. Such findings have guided new thinking and suggested a broadening in the description of Gβγ function – canonical roles/functions such as those functions served by Gβγ dimers in the

regulation of classical effectors, versus emerging non-canonical roles that are beginning to redefine the true spectrum of Gβγ dimer function. This section will serve to describe the current understandings and positions of the field in terms of overall Gβγ dimer function in cellular signalling.

1.7.1 Canonical functions of Gβγ in cellular signalling

Independent roles for Gβγ subunits that are distinct from Gα subunits have emerged and been extensively described. A sizeable proportion of the work done to describe these roles has focused on the regulation of effector molecules by direct binding by Gβγ downstream of GPCR activation. These classical effector molecules, or rather, canonical effectors, include ion channels such as inwardly rectifying potassium channels and voltage gated calcium channels, and enzymes that regulate the formation of second messenger molecules such as cyclic AMP and Ca^{2+}, such as adenylyl cyclases and phospholipase C, respectively. Here, I describe the diversity, function, and regulation by Gβγ of these canonical effectors (See Figures 1.6, 1.7).

1.7.1 (i) Inwardly Rectifying Potassium (Kir3) Channels

As previously mentioned, Kir3 channels, also known as G protein coupled inwardly rectifying potassium channels (GIRK), were the first direct effectors identified to be regulated by Gβγ subunits in cardiac cells [36]. Since then, the mechanistic basis of Gβγ regulation of Kir3 channels has been extensively characterized. Kir3 channels are a family of heterotetramic

channels that contain four distinct subtypes (Kir3.1 through 3.4) encoded on separate genes that can be directly regulated by binding by Gβγ subunits [87]. These channels are expressed in various tissues including the heart, and are widely expressed throughout the brain [88, 89]. PIP_2 is directly involved in the maintenance of activity of these channels such that hydrolysis of PIP_2 results in current inhibition [90]. Activation of these channels results in hyperpolarization of excitable cells because of the outflow of potassium ions under physiological conditions [35]. It has been reported that $G\beta_{1\text{-}4}$-containing Gβγ subunits are capable of activating these channels [91], and it is believed that Gβγ binds Kir3 channels at 4 distinct intersubunit binding sites with multiple points of contact [92-94]. The mechanism by which Gβγ dimers regulate Kir3 channels is such that when bound concurrently with PIP_2 to the channel, both the helix loop gate and G loop gate in the pore of the channel open, allowing for the flux of potassium ions, whereas if Gβγ does not bind the channel, only the G loop gate opens and there is no flow of ions through the channel [35]. Gβγ binding to these channels has been found to be dependent on the α-helical domain of PTX-sensitive Gα subunits [95]. Furthermore, due to the speed and specificity observed in signal transduction and channel activation, it is believed that Gβγ dimers and Kir3 channels interact and assemble well before being trafficked to the plasma membrane, and remain together as a signalling unit during the maturation of these channels from their biosynthesis [59, 96].

1.7.1 (ii) Voltage-gated Ca^{2+} Channels

Voltage-gated Ca^{2+} channels (Ca_v) mediate the flow of calcium ions across the plasma membrane; at resting membrane potential, these channels are inactive, but are opened upon depolarization of the cell membrane. Ca_v channels are hetero-oligomeric channels that consist of three subunits – a pore-forming α1 subunit, a cytoplasmic β subunit, and a membrane-associated α2δ [97]. The α1 subunit can be classified into forming three subtypes of channels: Ca_v1, Ca_v2 and Ca_v3 [98]. It has been determined that the α1 subunit contains Gβγ binding sites [99] and that different isoforms of this subunit determine the distinct properties of the different channels they form [100]. In particular, while all of these different Ca_v channels are able to bind Gβγ to differing degrees, the Ca_v2 channel is the best characterized of the three. Ca_v2 channels, which can be further classified into the N-, P/Q- and R-type channels, are localized at presynaptic terminals in different regions of the brain and their main function is to induce release of synaptic vesicles containing neurotransmitters upon their voltage-gated activation [101, 102].

The first implication of the modulatory roles of G proteins on calcium channel function came from efforts in 1981 that demonstrated that G proteins induce an inhibitory effect on norepinephrine-stimulated Ca^{2+} current amplitude. Since then, the roles of GPCRs and G proteins in controlling channel function have been further expanded. It has been determined that Gβγ dimers directly bind Ca_v channels to impart an inhibitory effect on their function, a phenomenon called voltage-dependent inhibition, and this effect manifests as a decrease in the peak-amplitude of the calcium current [103]. Such an inhibition results in slower channel activation kinetics, although a depolarizing prepulse can remove the inhibition, cause Gβγ to

unbind, and subsequently restore channel kinetics [35]. Indeed, in HEK 293T cells, Gβγ slows $Ca_v2.2$ channel current kinetics upon activation by oxotremorine-M stimulated M1-muscarinic acetylcholine receptors. Moreover, it was shown that this inhibition of channel activation was relieved when β-adrenergic receptor kinase-C-tail (βARK-ct; a protein that acts as a Gβγ scavenger) inhibited the actions of Gβγ dimers. Similarly, Gβγ dimers have been shown to inhibit the activation of both $Ca_v2.2$ and $Ca_v2.3$ channels upon stimulation of μ-, δ, and κ-opioid receptors [104]. These examples are only a few of the many instances that demonstrate the inhibition of Ca_v channels by Gβγ upon activation of the GPCRs they couple to, establishing such inhibition as a canonical function of Gβγ dimers. However, it should be noted that inhibition is not the sole action of Gβγ on Ca_v channels; it has previously been reported that upon activation of Angiotensin II Type I Receptor (AT1R) that couples specifically to the $G\alpha_{12}\beta_1\gamma_3$ heterotrimer in rat portal vein myocytes, $G\beta_1\gamma_3$ was found to transduce the activation of these receptors to stimulate $Ca_v1.3$ channels to increase cytoplasmic Ca^{2+} [105].

1.7.1 (iii) Adenylyl Cyclases (ACs)

As previously mentioned, Gβ and Gγ subunits were discovered as a result of the research to understand the regulation of adenylyl cyclases (ACs) upon GPCR activation. Activation of adenylyl cyclases leads to the intracellular production of adenosine-3',5'-monophosphate (cyclic AMP, or cAMP) from ATP [106]. ACs are integral membrane protein consisting of 12 transmembrane-spanning domains that can further be envisioned as two transmembrane regions of 6 transmembrane helices each (M1 and M2) and a catalytic domain (C1 or C2 domains)

located on the cytoplasmic side of the membrane whereby the C1 and C2 domains form a catalytic core [107]. Molecular cloning efforts have revealed nine mammalian genes that encode for different membrane-bound isoforms of AC (AC1-AC10) and one additional gene that encodes a soluble isoform of AC (sAC) [106, 108]. The membrane-bound isoforms are further dividied into subclasses based on their amino acid sequence similarities and functional characteristics, namely into four different types – Type I (AC1, AC3, AC8), Type II (AC2, AC4, AC7), Type III (AC5, AC6) and Type IV (AC9) [108]. Different regulatory molecules modulate AC activity; the types of regulation of ACs include modulation by Gα subunits, Gβγ subunits or Ca^{2+}.

While $G\alpha_s$ and $G\alpha_i$ subunits act to stimulate or inhibit different adenylyl cyclases, respectively, Gβγ subunits are known to regulate ACs in a subtype-specific manner [109-111]. Of particular interest, Type II ACs are stimulated by Gβγ (although $G\alpha_s$ co-activation is necessary), while Type I ACs are inhibited by Gβγ [38, 82, 106, 112]. Although Gβγ has been found to lower levels of cAMP in cells transfected with Type III ACs, the direct effect of Gβγ on these types of AC is stimulation [113]. A stretch of sequence in the middle of C2 corresponding to amino acids 956 to 982 has been identified as a Gβγ binding region on ACs stimulated by Gβγ. This Gβγ binding motif to was identified to be QXXER [106]. AC that do not contain this motif correspond are not modulated by Gβγ, and disruption of this region with peptidomimetics has been shown to abrogate Gβγ action [114]. In addition, a second site of regulation that corresponded to a PFAHL motif on C1 was identified via deletion mutant analysis [112]. Similar to the QXXER motif, this motif was present only on AC types that are stimulated by Gβγ. Furthermore, recent work has identified that Gβγ in inactive G_s heterotrimers bind a region spanning amino acids 66-173 on the N terminus of AC5 (AC5NT) and also that binding to this

region is not necessary for Gβγ stimulation of AC5 [115]. Conversely, it was found that Gβγ required residues 77-151 in the N-terminus of AC6 (AC6NT) for its stimulatory effects [113]. These findings suggest multiple points of contact by Gβγ dimers on ACs according to the subtype they modulate. In contrast, regions of Gβγ that are required for binding to AC have been mapped. It was found that a Gβγ "hotspot" (area on structure of Gβ where the turns of the WD40 repeats/blades intersect) was required to stimulate AC5 and was different from the Gβγ utilizes to interact with AC5NT. Furthermore, it has been shown that amino acid residues 23-27 are required for the stimulation of AC5 and AC6, and that Gβγ binds the C1 and C2 regions of the catalytic core of these two ACs in a hotspot dependent manner [116]. More particularly, mechanisms of AC inhibition by Gβγ are less characterized and less well understood. Gβγ binding to these ACs have been identified in regions in the C1 and C2 domains [117]. Interestingly, it has been found that inhibition of ACs is dependent on the specific subtypes of Gβ and Gγ subunits that comprise a Gβγ dimer [118, 119].

1.7.1 (iv) Phospholipase C

As previously mentioned, phospholipase C (PLC) is a family of enzymes that is responsible for the enzymatic catalysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). Upon generation of these two intermediate signalling molecules, IP_3 diffuses to the endoplasmic reticulum to bind its receptor (IP_3-receptor) to initiate the release of calcium into the cytosol from its intracellular stores, while membrane-bound DAG goes on to activate distal signalling molecules downstream of PLC activation. To

date, 13 different isoforms of PLC have been described, and these are further classified into six groups – PLC-β, PLC-γ, PLC-δ, PLC-ε, PLC-ζ, and PLC–η [35]. In particular, there are four different subtypes of the PLCβ isoform that differ in expression pattern and regulation [120]. Structurally, these enzymes consist of conserved domains that include an N-terminal pleckstrin homology domain, a series of tandem EF hands, a catalytic TIM (triose phosphate isomerase) barrel domain, and a C-terminal C2 domain [121, 122]. In addition, PLCβ in particular also contains a ~400 C-terminal amino acid extension that is involved in autoinhibitory functions along with the X-Y linker within the TIM barrel domain [28, 120].

Of these groups of different PLC isoforms, the roles of G proteins, and in particular, the functions of Gβγ in the activation and modulation of these enzymes has been described extensively for PLCβ. Initial implications of the actions of Gβγ on PLCβ came from two concurrent studies. *In vitro* studies demonstrated that purified Gβγ subunits isolated from transducin were able to stimulate both $PLC\beta_1$ and $PLC\beta_2$, with greater efficacy for the former [37]. Similar results were obtained in transfection assays showing a role for Gβγ subunits from G_i whereby coexpression of cDNA coding for Gβ, Gγ, M2-muscarinic acetylcholine receptors and $PLC\beta_2$ resulted in an increase in carbachol stimulated PIP_2 turnover that was inhibited by PTX [123]. Moreover, numerous studies dating back to 1994 have described the selectivity of specific Gβ and Gγ subunits for different subtypes of PLCβ. One study demonstrated that specific combinations of Gβ and Gγ were more potent stimulators of PLCβ that was purified from different sources (turkey erythrocytes, bovine brain, rat brain) whereby $G\beta_1\gamma_2$, $G\beta_1\gamma_3$, $G\beta_1\gamma_5$, $G\beta_2\gamma_2$ were potent stimulators while $G\beta_2\gamma_3$ and $G\beta_1\gamma_1$ were poor stimulators [124, 125]. Others have also demonstrated that purified $G\beta_1\gamma_2$ interacts with $PLC\beta_{1\text{-}3}$, with a stronger

preference for PLCβ_2 [126]. Recently, in a overexpression study that systematically analyzed the stimulatory capabilities of 48 different dimer permutations of G$\beta_{1\text{-}4}$ and G$\gamma_{1\text{-}13}$, it was shown that all Gβ_1 and Gβ_2 containing G$\beta\gamma$ dimers stimulated PLC$\beta_{1\text{-}3}$, with preference in the order of PLC$\beta_2 \geq$PLCβ_3>PLCβ_1 [127] In addition, the same study showed that Gβ_3 and Gβ_4 containing dimers were poor activators of PLCβ_1 and demonstrated preference for PLCβ_3 and PLCβ_2, respectively [127]. These findings indicate the importance of the composition of G$\beta\gamma$ dimers that translate to the selectivity of not only which PLCβ isoforms they modulate, but perhaps for which effectors they regulate in general.

The binding site of G$\beta\gamma$ to PLCβ has been extensively studied. Efforts by the Smrcka group in 1998 first identified using crosslinking studies of two overlapping peptides of derived from the catalytic domain of PLCβ_2 (N20K that corresponded to Asn564-Lys583; E20K that corresponded to Glu574-Lys593) that were able to inhibit G$\beta\gamma$-dependent activation of PLCβ_2, thus identifying an α-helical region in this catalytic domain that binds G$\beta\gamma$ [128]. A similar crosslinking study where cysteine mutated Gβ_1 subunits were used identified the corresponding region on Gβ_1 that bound PLCβ – this study demonstrated Cys25 in the amino-terminal coiled-coil region of Gβ_1 as a PLC-binding site, that was distinct from PLCβ's Gα interaction site [129]. This finding corroborates the suggestion that Gα and G$\beta\gamma$ dimers modulate PLCβ independent of one another [126]. Roles of Gγ subunits in the modulation of PLCβ have also been explored. In particular, C-terminal mutants of purified Gγ_5 (deletion mutations, insertion mutations or point mutations) displayed a diminished ability of Gγ_5 to modulate PLCβ compared to wild-type purified Gγ_5 [130]. Conversely, a recent study has suggested a model with a dual mode of PLCβ binding to G$\beta\gamma$ whereby one the interaction between the catalytic domain of PLCβ and N-

terminal coiled-coiled domain is inhibitory for function, while another interaction of the Gα subunit switch II binding surface on Gβγ with PLCβ which is stimulatory [131].

While the regulation of PLCβ by Gβγ subunits is extensively described, PLCε and PLCη are also modulated by Gβγ. PLCε has been implicated in the modulation of β-adrenergic receptor dependent cardiac contractility and inhibition of cardiac hypertrophy [132]. It was discovered to contain additional functional domains in the N-terminus (EF hands and PH domains), and it was shown that it is through these new PH domains that Gβγ binds PLCε [133]. Analysis of the specificity of Gβ and Gγ subunit binding to PLCε revealed that $G\beta_1$, $G\beta_2$ and $G\beta_4$ subunits that were contained in dimers with $G\gamma_1$, $G\gamma_2$, $G\gamma_3$ and $G\gamma_{13}$ all activated PLCε to similar levels while $G\beta_3$ and $G\beta_5$ contained in dimers with the same Gγ subunits produced no PLCε activation [133]. Further insight regarding the roles of the modulatory roles of Gβγ on PLCε is discussed in a section below. More recently, Gβγ has been found to also activate PLCη. Two isoforms of this PLC have recently been identified due to the effort of four separate groups – PLCη1 and PLCη2 [134-136]. It is believed that PLCη2 is a neuron specific isoform that has roles in neuronal Ca^{2+} signalling because of their sensitivity to Ca^{2+} [137, 138]. Gβγ was shown to activate this novel PLC isoform using purified PLCη reconstituted in PIP_2 containing phospholipid vesicles, although their site of interaction with PLCη has yet to be determined [139].

1.7.1 (v) Mitogen-Activated Protein Kinases (MAPKs)

Mitogen Activated Protein Kinases, or MAPKs, are a family of serine-threonine kinases that regulate a variety of cellular processes such as differentiation, transformation, proliferation, survival and cell death [140]. MAPK pathways comprise of a series of multiple kinases that sequentially phosphorylate one another to be activated: MAPK kinase kinase (MAP3K) activates MAPK kinase (MAP2K) that in turn dually phosphorylates the MAPK at a Thr-X-Tyr consensus sequence (where X represents any amino acid). 14 distinct types of MAPKs are expressed in mammalian cells and these include ERK1/2, the p38 MAPK family (comprises four isoforms - p38α, p38β, p38γ and p38δ), the Jun N-terminal kinase family (JNK; comprises three isoforms – JNK1, JNK2 and JNK3), ERK3, ERK4, ERK5, ERK7 and NLK [141]. The classical MAPKs are the extracellular signal-regulated kinases 1 (ERK1 or p44) and ERK2 (or p42), that are downstream of activation by MAP2Ks MKK1 and MKK2, which are themselves activated by MAP3K Raf in response to antigen receptor or growth factor stimulation of this pathway [141, 142]. The four isoforms of p38 MAPKs, which are encoded on separate genes, act to regulate two groups of proteins that include transcription factors such as p53 and protein kinases such as MAPK activated kinase-2 (MK2) [143, 144]. The three subtypes of the JNK family of MAPKs can be alternatively spliced into ten different isoforms [145], and are activated by MKK4 and MKK7 [141]. These kinases are ubiquitously expressed (JNK1 and JNK2) or expressed primarily in the brain (JNK3) [146], and act to target their actions to transcription factors such as AP-1 [147]. With respect to the remaining MAPK subtypes, much less is known about their physiological roles and regulation [141].

Roles of Gβγ in the activation of different MAPK signalling pathways have been elaborately elucidated. Regarding JNK activation, it has been shown that M2-muscarinic acetylcholine receptor (M2-mAChR) mediated activation of JNK is Gβγ dependent, and that the M1-mAChR-, M2-mAChR- and β-AR-mediated activation of p38 MAPK pathways are also Gβγ dependent [148, 149]. However, the roles of Gβγ in the activation of ERK1/2 have been described in greater detail. Efforts in the mid-1990s revealed direct roles for Gβγ in activating ERK1/2. It was first demonstrated that when $G\beta_1$, $G\beta_2$, $G\gamma_1$ or $G\gamma_2$ are overexpressed in COS-7 cells either alone or in combination, only dimers of $G\beta_1\gamma_1$, $G\beta_1\gamma_2$ and $G\beta_2\gamma_2$ (but not $G\beta_2\gamma_1$) stimulated phosphorylation of ERK1 while the Gβ and Gγ subunits expressed on their own did not [150]. Similar studies revealed isoprenylation-deficient mutants of Gγ expressed alone, or with $G\beta_1$ also did not stimulate ERK2 phosphorylation [151]. This showed that a functional Gβγ dimer is required to activate ERK1/2. Studies examining G_i-coupled GPCR activation of MAPK pathway activation provided further evidence for roles of Gβγ in this process. More specifically, it was shown that overexpression of β-ARK-ct to "antagonize" the effects of Gβγ attenuated the lysophosphatidic acid receptor-, α_2-adrenergic receptor- and M2-muscarinic acetylcholine receptor-mediated activation (all G_i-coupled GPCRs) of ERK2, but not that of α_1-adrenergic receptors, a G_q-coupled GPCR [152]. Moreover, when considering the roles of Gα subunits in the process of G-protein mediated MAPK activation, it has been determined that Gβγ dimers activate MAPK pathways under conditions where constitutively active mutants of different Gα proteins do not activate either ERK1 or ERK2 [150, 151, 153].

Three different mechanisms of how Gβγ dimers activate MAPK signalling cascades have been suggested and described. The first mechanism involves direct activation of secondary

intracellular effectors such as the Src family of proteins, PI3K (discussed below), activation of PLCβ or direct activation of nonreceptor tyrosine kinases [154-157]. A second mechanism describes the formation of a scaffold for MAPK activation whereby Gβγ dimers recruit G protein coupled receptor kinase (GRK) isoforms that in turn recruit β-arrestins that function as adapters for Src recruitment [158]. Another mechanism includes the transactivation of receptor tyrosine kinases or assembly of focal adhesion as scaffolds for the assembly of complexes that in turn activate classical MAPK signalling pathways [159]. With respect to regions of Gβγ required for activation of MAPK pathways, mutational analyses Gβ subunits have revealed that the C-terminal region of Gβ is important for participation in the activation of ERK2 [160].

1.7.1 (vi) Phosphoinositide 3 Kinases

Phosphoinositide 3-kinases (PI3Ks) are a group of kinases that act to phosphorylate intracellular membrane inositol lipids that in turn modulate various intracellular protein effectors that cause diverse signalling processes [161]. These kinases were discovered during the era of research that focused on elucidating the roles of PLCs. They were first identified as viral oncoproteins that acted as lipid kinases that phosphorylated phosphatidylinositols (PtdIns; PI3K substrates) at their 3-hydroxy position to generate phosphatidylinositol-3-phosphate [161-163]. A great deal of work has described the functions of PI3Ks, of which eight mammalian isoforms have been described, and it has been shown that these kinases can be activated by the activity of receptor tyrosine kinases or GPCRs [164]. These enzymes contain a catalytic subunit and a

regulatory subunit; the protein sequence and domain structures of the eight different mammalian catalytic subunits of these enzymes allows for the classification of PI3Ks into three different classes – Class I (four p110 catalytic subunits), Class II (three C2 catalytic subunits) and Class III (one catalytic subunit, VPS34) [165]. Class I PI3Ks can further be grouped into Class IA and Class IB PI3Ks whereby Class IA PI3Ks contain a p110α, p110β or p110δ catalytic subunit constitutively bound to one of five isoforms of a p85 regulatory subunit [161, 165]. On the other hand, Class IB PI3Ks contain a p110γ catalytic subunit bound to regulatory subunits p101 or p84 and it is this class of PI3Ks that are activated by Gβγ subunits upon GPCR activation [161, 165].

Roles for G proteins in the activation of PI3Ks was first described in 1994 where it was demonstrated using purified Gβγ subunits and PI3K from crude platelet extracts or purified GST-p110γ that Gβγ dimers regulate a PI3K distinct from receptor tyrosine kinase regulated PI3Ks [166-168]. Stimulation of PI3K by Gβγ leads to downstream activation of a variety of process that include activation of vascular L-type Ca^{2+} channels [169, 170], nuclear translocation of PI3Kγ [171], NFκB activation [172], regulation of thyrotropin induced gene regulation in thyroid cells [173], and membrane translocation of P-Rex1, a guanine-nucleotide exchange factor (GEF) [174]. Of the Class IB PI3Ks, roles of Gβγ in the stimulation of the p110γ-p101, or PI3Kγ, combination PI3K are the best characterized [166, 175]. Gβγ has been found to directly bind PI3Kγ [176], whereby p101 binds Gβγ at its C-terminal domain to lead to the recruitment of p110γ to the plasma membrane [177]. It has also been demonstrated that Gβγ binding to PI3Kγ is inhibited by direct interactions with phosphopleckstrin, a product of PI3Kγ activity, suggesting a inhibitory feedback regulation [178]. An analysis of the individual roles played by the catalytic and regulatory subunits of PI3K has revealed that the p101 subunit determines the substrate

specificity of p110γ in both Gβγ-stimulated and non-stimulated states, and also a preference of Gβγ for the full form of PI3Kγ over p110γ alone [179]. Moreover, the specificities of Gβγ dimer combinations on PI3Kγ stimulation have been characterized. It has been shown that all Gβ subtypes, with the exception of $G\beta_5$, activate PI3K equally while the Gγ subunit contained in a specific Gβγ dimer and its prenylation status dictated the extent to which specific Gβγ dimers could activate PI3K [180]. Such findings provide more evidence for the functional selectivity of different Gβγ dimer combinations for the effectors they regulate.

1.7.2 Emerging non-canonical functions of Gβγ in cellular signalling

Distinct from their roles of modulation of effectors in GPCR-dependent signalling at the cell surface, many recent findings regarding Gβγ signalling have revealed that these dimers regulate a large number of signalling molecules that cannot be classified as classical or canonical effectors. These novel, non-canonical Gβγ roles have reshaped our understanding of how these dimers function. Indeed, beyond their functions of regulating canonical effectors, Gβγ dimers have been identified as regulators of signalling complex formation, anterograde and retrograde trafficking, regulators of microtubule dynamics and modulators of second messenger molecule generation at intracellular organelles. This section will describe and summarize recent advances and findings of these novel non-canonical roles of Gβγ. (See Figure 1.6, 1.7).

1.7.2 (i) Gβγ-mediated translocation events

Due to the capability of Gγ subunits to be isoprenylated, it was believed once that Gβγ subunits were localized strictly to the plasma membrane. Recent studies of Gβγ subcellular localization have shown that they are also present on various endomembranes and compartments that include the Golgi apparatus, ER, mitochondria and nucleus [6, 56, 60, 181-186]. Gβγ translocation was previously thought to be limited to certain combinations of Gβγ with particular Gγ subunits, but it has been found that all 12 Gγ subunits are capable of supporting Gβγ translocation, albeit with varying kinetics under basal and GPCR stimulation conditions [185, 187]. This suggests Gβγ translocation may be a general phenomenon following receptor

activation and may provide explanations for the many of the non-canonical roles Gβγ dimers play in cellular signalling.

With respect to how Gβγ might be involved in the translocation of other signalling proteins and complexes, translocation of ERK1/2 to the nucleus serves as an excellent example. A role for Gβγ subunits in this translocation event was described in a study describing an unique auto-phosphorylation event of ERK1/2 at Thr188 which results in phosphorylation of nuclear targets [188]. This novel regulatory event was found to be induced by Gβγ signalling, downstream of the activation of the Raf-Mek-ERK cascade whereby stimuli induced interaction of Gβγ with Raf1 and ERK1/2 that was dependent on ERK2 dimerization, resulting in auto-phosphorylation of ERK1/2 and subsequent nuclear localization of ERK1/2 [188, 189]. What happened to Gβγ itself in this process (i.e whether it shuttles to the nucleus alongside the Thr188-phosphorylated ERK1/2) remains unknown.

1.7.2 (ii) Gβγ functions in the endoplasmic reticulum (ER)

Upon their biosynthesis and assembly with the aid of the aforementioned chaperones PhLP1 and DRiP78 in the endoplasmic reticulum, Gβγ dimers have been found to interact with a multitude of signalling proteins as a part of their journey to the plasma membrane before they subserve canonical signalling roles. These interactions formed by Gβγ, namely those with Gα subunits, GPCRs and effectors, help form the eventual signalling machines that are believed to be trafficked as complete signalling complexes from the ER to the plasma membrane. Taken

together, such interactions may provide the basis for a non-canonical role for Gβγ in orchestrating the formation and assembly of pre-formed signalling complexes prior to being trafficked to the plasma membrane (Figure 1.8).

Our lab first suggested the notion that Gβγ dimers form "precocious" interactions with GPCRs and effectors in the ER. The first indication of such phenomena came from studies that regarding the formation of signalling complexes formed by β_2-adrenergic receptors and Gβγ dimers. Through the use of dominant negative Rabs and Sars to induce alternate trafficking itineraries in conjunction with confocal microscopy and bioluminescent resonance energy transfer (BRET) studies, it was found that receptor-Gβγ interactions form initially at the ER whereas Gα subunits interact later in the trafficking itinerary, being added to the complex during the ER-Golgi apparatus transit [190]. Similar results were observed from studies regarding GABA-B receptor and Kir3 channel trafficking. GABA-B receptors are comprised of GABA-B1 and –B2 subunits, whereas as Kir3 channels contain hetero-tetrameric combinations of any of the Kir3.1-Kir3.4 subunits. GABA-B1 receptors contain a carboxy terminal ER retention signal [191] and Kir3.1 channels lack forward ER trafficking signals [192], meaning that neither of these proteins can be trafficked to the ER alone. It was observed that both Gβγ and Kir3 channels form interactions with GABA-B1 in the ER, irrespective of the presence of GABA-B2 [193]. Furthermore, in addition to its role of regulating Kir3 channels at the cell surface, our lab has also suggested that Gβγ dimers play roles in the trafficking and maturation of Kir3 channels during early biosynthesis. Gβγ dimers were shown to interact with Kir3 channels while still present at the ER, with the interaction remaining intact throughout anterograde trafficking to the cell surface [58, 96]. Moreover, similar observations have been noted with regard to adenylyl

cyclases and Gβγ, where again, the the initial sites of interaction occur at the ER [96] and signalling complexes containing adenylyl cyclase II are pre-formed with Gβγ and β_2-AR at the ER prior to their trafficking to the plasma membrane [57]. Interestingly, it has been suggested that Gα subunits interact with the Gβγ-receptor complex at the Golgi apparatus [57, 190]. Taken together, the evidence of all these initial, precocious interactions formed by Gβγ dimers suggests a non-canonical role for these dimers as organizers and orchestrators of receptor-G-protein-effector (R/G/E) complex formation and assembly (Figure 1.8). Moreover, the presence of these early pre-formed R/G/E signalling complexes suggests that assembly of these complexes occur prior to ever being exposed to agonists, refuting the claims that these signalling complexes form at the plasma membrane upon receptor stimulation.

In addition to their roles as regulators of R/G/E assembly, Gβγ dimers have also been found to directly regulate Ca^{2+} release at the ER. As previously described, under conditions of G_q-coupled GPCR stimulation, Gβγ dimers modulate PLCβ activity at the cellular surface to eventual result in Ca^{2+} release via activation of IP_3-receptors at the ER by IP_3 generated from PIP_2. In addition to this regulatory activity, Gβγ dimers have been found to directly bind IP_3-receptors at the ER to stimulate Ca^{2+} release as effectively as IP_3, with these observations being independent of GPCR activation, and thus, independent of PLCβ activity [194]. Such findings suggest alternative routes of Ca^{2+} immobilization influenced by Gβγ subunits and are of particular interest for G_i-coupled GPCR mediated Ca^{2+} release.

1.7.2 (iii) Roles of Gβγ in the Golgi apparatus

Roles for Gβγ in regulating trafficking from the Golgi apparatus have been described whereby Gβγ regulates anterograde trafficking (by means of Golgi fragmentation) from the trans-Golgi network to the plasma membrane. One instance of such a role is the regulation of a Golgi-resident protein, protein kinase D (PKD) activity by Gβγ [195, 196]. It has been found that Gβγ dimers bind the PH domains contained within PKD and such binding events are necessary for the induction of PKD activity [196]. In particular, Gβγ subunits have been found to regulate the organization of pericentriolarly localized Golgi stacks through whereby "free" Gβγ added to permeablized mammalian cells resulted in a disruption of these stacks in a Gα subunit independent manner, whereas reformation of the heterotrimer resulted in inhibition of this Golgi vesiculation [195]. Moreover, it was found that $G\beta_1\gamma_2$ and $G_3\beta\gamma_2$ activate PKD in a PKC-η dependent manner (via activation by $PLC\beta_3$) to result in Golgi fragmentation [197]. Such a model has been further corroborated with recent evidence that shows endogenous Gβγ subunits capable of mediating such a PLC- and PKD-dependent Golgi fragmentation phenomena [198]. With respect to specific Gβ and Gγ subunits that regulate PKD, an analysis of Gβ and Gγ subunit specificity for activation of PKD reveals that $G\beta_1$ dimers with γ_2, γ_3, γ_4, γ_5, γ_7, and γ_{10} effectively activate PKD whereas the remaining Gγ subunits do not. It remains to be identified what the roles of the other Gβ subunits are in regulating PKD activity [199]. Jensen et al have recently elucidated a role for Gβγ and PKD in the agonist-induced trafficking of intracellular Protease-activated-receptor 2 (PAR2) [200]. Here, they demonstrate that activation of PAR2 by its agonists trypsin and 2-Furoyl-LIGRLO-NH2 results in the translocation of Gβγ to the Golgi apparatus where it activates PKD whereby inhibition of Gβγ with gallein resulted in inhibition of

PKD activity [200]. These findings corroborate other examples of agonist-induced plasma membrane to Golgi apparatus Gβγ shuttling that include Gβγ translocation upon activation of the M3-mAChR [55, 56, 187]. Furthermore, it was revealed that inhibition of PKD with CRT0066101 resulted in a loss of trypsin-stimulated translocation of PAR2 from Golgi apparatus to the plasma membrane, diminishing the mobilization of intracellular stores of PAR2 to rapidly replenish the plasma membrane with signalling-competent receptors [200]. It remains to be identified what the roles of the other Gβ subunits are in regulating PKD activity.

In addition to PKD, RTKG (Raf kinase trapping to the Golgi apparatus) or PAQR3 (Progestin and AdipoQ Receptor 3) have been found to interact with Gβ at the Golgi apparatus whereby it acts in a sequestering manner resulting in the inhibition of canonical Gβγ functions at the plasma membrane [201]. Indeed, this Golgi resident membrane protein has been found to bind the N-terminal region of Gβ attaching Gβγ to the Golgi. Such interactions have been found to decrease Gβγ-dependent signalling by inhibiting Akt phosphorylation, abrogating the GPCR-stimulated subcellular localizations of GRK2 and inhibiting Gβγ translocation to the Golgi [201]. A recent article demonstrates that PAQR3 also acts to promote Gβγ signalling in the Golgi apparatus [202]. Gβ binding-deficient PAQR3 mutants display an inability to cause fragmentation of the Golgi compared to wild type PAQR3. Golgi fragmentation is also inhibited by βARK-ct, gallein and overexpression of a dominant negative PKD [202]. Furthermore, the Gβ binding deficient PAQR3 mutant results in an inhibition of the constitutive transport of VSV-G cargo protein from the Golgi to the plasma membrane [202]. All in all, these findings suggest a new role for PAQR3 in regulating the functions of Gβγ at the Golgi and the transport of Gβγ from the Golgi to the plasma membrane via the Gβγ-PKD pathway.

1.7.2 (iv) Novel roles of Gβγ in perinuclear regions

Gβγ dimers have been recently implicated in the regulation of PLCε in perinuclear regions. PLCε is a novel form of PLC that has been shown to be activated downstream of receptor tyrosine kinases (RTKs) and GPCRs via its regulation by Ras, Rho, Rap and Gβγ [203]. Knockdown of PLCε results in a loss of endothelin-1 (ET-1), norepinephrine and isoproterenol induced cardiac hypertrophy and it has been shown that PLCε scaffolds with mAKAP in the nuclear envelope whereby inhibition of such an interaction prevents agonist induced hypertrophy [204]. A recent study by Zhang et al aimed to elucidate the mechanisms and functional consequences of PLCε activation in cardiac failure [205]. In this study, it was identified that PLCε is recruited to the perinucleus in complex with the nuclear envelope scaffolding protein mAKAP, Epac1 (to which PLCε directly binds), and PKD that PLCε acts to activate. Furthermore, it was found that PI_4P is enriched at the nuclear envelope, and that PI_4P, not PIP_2, is the substrate for PLCε. PLCε was found to generate DAG from PI_4P in close proximity to the perinucleus required for the activation of nuclear PKD [205]. As PKD activity is regulated by Gβγ binding and activation, it has been suggested that Gβγ-dependent activation of PLCs to generate DAG is required for Golgi PKC and PKD activity. These studies show that the ET-1-mediated PI_4P hydrolysis to DAG and subsequent PLCε activation is a Gβγ regulated process, leading to PKD activation and eventual development of cardiac hypertrophy [206].

1.7.2 (v) Emerging roles for Gβγ in mitochondria

In comparison to their roles in other organelles such as the Golgi apparatus and the ER, far less is known regarding the functions of Gβγ in mitochondria. With respect to Gα function, it has been demonstrated that $G\alpha i_{1\text{-}3}$ and $G\alpha_{12}$ are present at the mitochondrial outer membrane [207, 208], the latter of which was shown to regulate mitochondrial morphology and dynamics. Interestingly, it was recently demonstrated $G\alpha_{q/11}$, $G\beta_1\gamma_2$ and $G\beta_4\gamma_2$ also localize at the mitochondrial outer membrane, with the former localized to the mitochondrial inner membrane as well. Here, it was shown that $G\alpha_q$ acts to regulate mitochondrial fusion/fission and organization of respiratory functions and energy production via Dynamin-like protein (DLP1/Drp1)- and optic atrophy 1 (OPA)-dependent mechanisms [209]. While roles for Gβγ in these processes remain to be elucidated, it has been demonstrated that $G\beta_2$ interacts with mitofusin1 (a mitochondrial GTPase) via its WD40 domains [60]. This non-canonical $G\beta_2$ interaction has been suggested to regulate mitofusin1's mobility along the outer mitochondrial membrane and mitochondrial fragmentation, affecting mitochondrial fusion overall [60]. Furthermore, analysis of a recent tandem affinity purification proteomics screen suggests that Gβγ dimers may also have functions in regulating oxidative phosphorylation [184]. Indeed, this screen identified Gβγ to interact with 18 proteins involved in the oxidative phosphorylation process that include complexes I, II and IV, as well as ATP synthase with and without activation of M3-mAChRs (unpublished data). While it has been demonstrated that abrogation of $G\alpha_{q/11}$ causes decreased dimerization of ATPase and thus reduced ATP production efficiency [209], the roles for Gβγ in regulating such processes remains to be elucidated.

1.7.2 (iv) Gβγ, chemotaxis and the cytoskeleton

Cell migration is a basic cellular process by which a cell migrates in response to a spatial cue received in the form of a chemoattractant. One particular process by which this occurs is the binding of these chemoattractants, which are known to bind various GPCRs [210]. Gβγ dimers have been implicated in these processes whereby activation of these chemoattractant receptors leads to their activation to result in alterations of cellular motility and directional polarization by modulation of F- actin towards the source of chemoattractant [211]. One mechanism by which this occurs has been shown to involve ElmoE, a Gβγ effector, that relays signals from these receptors to actin polymerization at the leading edge of cells via induction of RacB, a small G protein [212]. Specificities of Gγ interactions have also been described in this regard; $G\gamma_{12}$ was shown to association with F actin in C6 glioma cells and Swiss 3T3 [213], whereas $G\gamma_5$ was not found to associate actin, but with vinculin [214], a protein involved in cell-cell and cell-matrix adhesion junctions [215]. Studies have shown that small molecule inhibitors of Gβγ function such as gallein and M119 (discussed in a later section in detail) block N-formylmethionyl-leucyl-phenylalanine (fMLP) mediated chemotaxis in human promyelocytic leukemia cells (HL60) and human primary neutrophils by abolishing the interaction between Gβγ and PI3K. Further examples of roles of Gβγ in regulating chemotaxis include interactions of Gβγ with RACK1 via PI3K and PLCβ to control cell motility [216] and the Gβγ mediated activation of cell division cycle protein 42 (Cdc42) via p21-activated kinase 1 (PAK1) and its associated GEF, PIXα, which in turn acts to regulate F actin localization and directional polarization [217]. In addition to their interactions with actin, Gβγ dimers have also been shown to interact with microtubules. Microtubules consist of heterodimeric α and β tubulin subunits that bind GTP to polarize a plus

end; this tubulin GTP can further be transferred to Gα of specific G protein subunit composition (G_s or G_i), subunits leading to their activation [218]. Further studies have demonstrated that both Gα and Gβγ subunits interact with tubulin to activate tubulin GTPase and regulate microtubule turnover, and promote polymerization and stability of microtubules, respectively [219-222].

1.8 Emerging Nuclear Functions of Gβγ Subunits

Increasing evidence suggests that GPCRs reside on the nuclear envelope where they have distinct signalling profiles compared to their counterparts at the cell surface [7]. Similar to these plasma membrane GPCRs, nuclear GPCRs have been found to regulate the production of second messenger molecules and signalling proteins in the nucleus; examples include regulation of nitric oxide synthesis by β_3-adrenergic receptors and endothelin type B receptors [223], regulation of metabotropic glutamate receptor 5 (mGluR5)-mediated nuclear Ca^{2+} release [224] and modulation of nuclear protein kinases such as ERK1/2 and JNK [225]. Although Gβγ subunits have been discovered to reside in the nucleus as well, roles for distinct Gβγ in the nuclear compartments are not fully defined and are mostly unknown [226]. Nuclear effects of Gβγ dimers are novel in concept and are only beginning to be understood [186]. In this section, I describe the current understanding of recently described Gβγ nuclear functions.

1.8.1 Gβ$_5$ in the nucleus

Although Gβ$_5$ is the most distinct subunit in the Gβ family, it is also capable of nuclear translocation. Cellular distribution and nuclear targeting of Gβ$_5$-R7-RGS is believed to involve the R7-binding protein (R7BP) [227-229]. Palmitoylation of R7BP anchors it to the plasma membrane, however, a recent study demonstrates that mutant R7BP lacking the N-terminal Disheveled, EGG-10, pleckstrin homology domain displays marked decreases in nuclear localization [230]. Gβ$_5$ nuclear localization was assessed in neurons and brains from R7BP knockout mice and it was found that Gβ$_5$-R7-RGS displays 50-70% less localization [230]. This suggests that R7BP is central to the nuclear localization of Gβ$_5$-R7-RGS. Indeed, R7BP has been further suggested to shuttle heterotrimers consisting of Gα$_{i/o}$ and Gβ$_5$/R7-RGS to and from the nucleus whereby palmitoylation of R7BP by DHHC (a palmitoyltransferase) leads to nuclear exit and Gα$_{i/o}$ signalling inhibits depalmitoylation [231]. These atypical Gβ$_5$γ dimers have also been found to regulate transcriptional activity. RGS6, a member of the R7 RGS family that also interacts with Gβ$_5$ [232], interacts with Dmnt1-associated protein 1 (DMAP1) in a region distinct from its Gβ$_5$ binding region to inhibit DMAP1's transcriptional repressor activity [233]. Taken together, it is evident that atypical Gβ$_5$ containing dimers have clear and distinct nuclear functions.

1.8.2 Conventional Gβγ dimers and their roles in the nucleus

Recent advances in the field of Gβγ biology have revealed that, similar to Gβ$_5$/R7-RGS, Gβ$_{1-4}$ containing Gβγ dimers also localize to the nucleus and possess nuclear functions. These

conventional Gβγ dimers modulate an array of processes that range from regulation of signalling pathways that converge on transcription, regulation of co-modulatory proteins that affect transcription indirectly, or the direct interaction and regulation of nuclear proteins that regulate the process of gene expression. While exact mechanisms of their localization remain to be identified, various studies described below demonstrate that Gβγ dimers can be transported as cargo with other proteins known to shuttle to the nucleus and are present in a variety of cell types that in particular include primary rat adult cardiomyocytes [183], suggesting that "pools" of nucleus-resident and nucleus-shuttling Gβγ might exist as well. Indeed, using a tandem affinity purification-based proteomics screen, we noted that Gβγ subunits change their interactions with partner proteins in response to GPCR activation [184, 186]. Examples of such newly defined interactors include members of the heterologous nuclear ribonucleoprotein family, proteins involved in nuclear import and export such as importin 7 and exportin 1, and transcription factors such as NFκB [184, 186]. While these interactions remain to be validated in future research projects, identification of interactors that vary in function provides for a solid indication that we are just at the tip of the iceberg with respect to why Gβγ is present in the nucleus, and what these dimers are responsible for function in this organelle.

1.8.2 (i) Regulation of transcriptional activity by Gβγ-dependent signalling pathways

GPCR signalling pathways and the effectors modulated by G proteins have previously been shown to converge on the regulation of gene expression [5]. In particular, Gβγ dimers are also involved in modulating pathways that affect gene expression. Gβγ subunits have been

implicated in thyroid differentiation [173]. Activation of thyrotropin receptor by TSH causes $G\alpha_s$ activation, increases in intracellular cAMP and a subsequent increase in gene transcription of the gene for the sodium-iodide transporter (NIS) via binding of Pax8 to the NIS promoter [173]. Inhibition of Gβγ by sequestration using CD8-βARK causes inhibition of NIS transcription whereas overexpression of Gβγ led to increases in NIS promoter activity. Mechanisms underlying these signalling events were found to be phosphoinositide 3-kinase (PI3K)-mediated whereby inhibition of Gβγ led to exclusion of Pax8 from the nucleus [173]. Gβγ dimers have also been implicated in the modulation of interleukin-2 (IL-2) levels in CD4+ T helper cells. Knockdown of $G\beta_1$ (but not $G\beta_2$) and gallein-mediated inhibition of Gβγ resulted in increased levels of the T cell receptor-mediated IL-2 mRNA production in human naïve and memory T helper cells and Jurkat cells, whereby inhibition of Gβγ resulted in increased nuclear localization of NFATc1 and increased NFAT mediated transcriptional activity [234]. In addition, activation of M2-muscarinic acetylcholine receptors (M2-mAChR) results in a Gβγ-, ERK- and JNK-dependent activation of the cFos promoter in human embryonic kidney 293 cells (HEK 293), a process that was inhibited using β-ARK-ct and that was found to be dependent on Ras- and Rho-dependent signalling pathways [235]. Moreover, Gβγ subunits have been implicated in the regulation of GDNF levels in SH-SY5Y cells and rat midbrain slices whereby stimulation of D2-R with quinpirole results in a Gβγ- and ERK1/2-dependent increase in Zif268, a transcription factor that was also found to bind GDNF promoters, resulting in increased expression of GDNF [236]. Similarly, treatment of striatal neurons with corticotropin release factor was found to result in a Gβγ-dependent increase in phosphorylated levels of CREB that is also thought to occur through a MAPK-dependent pathway [237]. Furthermore, other studies have suggested nuclear action for Gβγ downstream of angiotensin II type 1 receptor (AT1R) signalling. In

particular, it was demonstrated Ang II-mediated activation of AT1R results in the nuclear translocation of $G\beta_2$ subunits where it was found to interact with core histones and proteins that modulate transcription [238]. Knockdown of $G\beta_2$ led to a repression of AT1R-stimulated MEF2 transcriptional activity, via a specific interaction motif of Gβ2 found on various transcription factors [238]. This latter study added significantly to our growing understanding of nuclear roles of Gβγ in GPCR-mediated regulation of gene transcription via interaction with chromatin-bound transcription factors.

1.8.2. (ii) Gβγ interactions with proteins that regulate transcription

Gβγ dimers regulate the activities of transcriptional modulators by mechanisms that either relieve transcriptional repression or inhibit transcription factor mediated transcriptional activity. Via a yeast two-hybrid screen, adipocyte enhancer-binding protein (AEBP1), a transcriptional repressor, was found to interact with $G\beta\gamma_5$ [239]. This interaction was localized in the nucleus where $G\beta\gamma_5$ acts to inhibit of AEBP1's transcriptional repression activity [239]. Similary, Class II histone deacetylases, HDAC5 and HDAC4 have been found to interact with $G\beta_1\gamma_2$ via their C terminal domains [240]. In a basal state, HDAC5 interacts with myocyte enhancer factor 2 (MEF2C), inhibiting MEF2C function. The authors of this studies demonstrated that α_2A-adrenergic receptor activation activation resulted in increased Gβγ interaction with HDAC5, resulting in increased activation of MEF2C transcriptional activity; here, relief of interaction mediated inhibition results in increased transcriptional activity [240]. Futhermore, Gβγ dimers have been implicated in glucocorticoid receptor (GR) function.

Activation of this nuclear receptor results in its homodimerization and nuclear import upon which the GR dimer directly binds glucocorticoid response elements (GRE) on DNA to initate transcription [241]. Interestingly, it has been demonstrated that Gβγ binds GR dimers prior to nuclear import, co-translocates to the nucleus and represses GR mediated transcriptional activity [242, 243], acting in a similar sense compared to its interactions with AEBP1 and HDAC5.

In contrast to these repressive transcriptional roles, our lab has previously shown that Gβγ interacts with AP-1 transcription factors [183]. We demonstrated that Gβγ regulates AP-1 mediated transcriptional activity via direct interaction with cFos resulting in co-localization of Gβγ and AP-1 in the nucleus, recruitment of HDACs and subsequent inhibition of AP-1 mediated gene transcription ([183]). More recently, Mizuno et al have investigated mechanisms of IP_3-R1 upregulation as a result of D_2-R activation whereby inhibition of Gβγ led to abrogation of the D_2 dopamine receptor-mediated increase in IP_3-R1 mRNA expression [244]. Receptor activation by quinpirole, a selective D2 dopamine receptor agonist, resulted in increased cFos and Jun protein expression, increased nuclear transport of NFATc4 and increased binding of AP-1 and NFATc4 to the IP_3R-1 promoter, and it has been suggested that these events occur after Gβγ activation and are thus Gβγ-dependent [244]. It remains to be determined whether Gβγ was also recruited to the IP_3-R promoter, where it can be speculated that Gβγ acts as a direct transcriptional co-modulator via interactions with transcription factors.

Members of the signal transducer and activator of transcription protein (STAT) protein family are also regulated by G protein signalling. STATs are integral components of the JAK-STAT pathway whereby JAK-mediated tyrosine phosphorylation of STAT proteins results in

their nuclear translocation [245]. Previous studies have shown that $G\alpha_{q/11}$, $G\alpha_{16}$ and $G\alpha_{14}$ are capable of stimulating STAT3 and STAT1 [246]. Recent studies have investigated possible roles of STAT activation by Gβγ dimers – a comprehensive Gβγ overexpression screen of 48 possible dimers in HEK 293 cells demonstrated that 13 specific dimer pairs stimulated STAT3 phosphorylation to varying degrees according to the specific Gβ and Gγ subunits assessed [246]. However, it was not determined whether Gβγ dimers directly interacted with STAT3. Subsequent studies to describe mechanisms that lead to these phosphorylation events focused on δ-opioid receptor (δ-OR)-mediated regulation and activation of STAT5B [247]. It was shown that STAT5B constitutively interacts with δ-OR and is released upon δ-OR activation, resulting in STAT5B activation in a c-SRC-mediated mechanism. Interestingly, this study demonstrates that Gβγ subunits directly bind STAT5B, serving as a scaffold to facilitate recruitment of c-Src to the δ-OR [247]. These findings provide further evidence of roles played by Gβγ in the regulation of transcriptional events.

1.9 Gβγ subunits: Implications in Disease and Pharmacological Interventions

As the largest class of cell surface receptors, GPCR dysfunction has been linked to many diseases that include, but are not limited to, heart failure, various types of cancer, and inflammatory diseases [5, 248]. Biological causes for development of such diseases that GPCRs are implicated in include loss-of-function mutations and gain-of-function mutations that can result in altered sensitivity to agonists or allosteric ligands, altered basal activities or broadened signalling pathway specificities [248]. Being directly downstream of receptor activation, such genetic alterations and subsequent pathological outcomes would affect G protein activity and in particular, Gβγ function, and further alter signalling cascades. In turn, a growing amount of evidence suggests that effectors regulated by Gβγ and these dimers themselves contribute to the pathogenesis of different diseases. As such, it is not surprising that they are implicated in pathologies of diseases such as cancer and heart failure. The first indication of the effects of Gβγ in pathology came from a series of studies that demonstrated that inhibition of Gβγ using adenovirus delivered β-ARK-ct resulted in a 37% decrease in intimal hyperplasia in rabbits undergoing carotid artery bypass grafts with the jugular vein [249, 250]. Furthermore, several effectors that are regulated by Gβγ subunits such as PI3K and MAPKs are known to contribute to the pathogenesis of disease [251, 252]. Therefore, Gβγ dimers present themselves as an attractive target for therapeutic intervention for disease treatment. This section will serve to discuss such findings, their impact on our understanding of Gβγ subunits implicated in disease and the development of pharmacological interventions targeted against Gβγ dimers.

1.9.1 Implications of Gβγ in cancer

In simplistic terms, cancer is disease characterized by the loss of cell cycle control leading to uncontrolled proliferation, a loss of differentiation, increased cell invasiveness and metastasis [253]. While close to 20% of all tumours harbor mutations in GPCRs leading to irregular GPCR activity, and activating mutations of Gα subunits are observed in 4-5% of all cancers [254], little is known about oncogenic alterations in Gβ and Gγ sunbunits. As mentioned above, the roles of the Gβγ effector PI3K and its downstream pathways in the development of cancer have been extensively characterized, and a great deal of effort has turned components of this signalling cascade into an attractive targets for cancer therapeutics [252, 255]. Since activation of PI3K results in the transduction of cellular survival signals, the regulation of this protein by Gβγ in cancer becomes interesting. Activation of P-Rex1, a Rac-selective GEF whose activation is dependent on PI3K and Gβγ, has been shown to be a important player in mediating lysophosphatidic acid (LPA) induced lung epithelial cancer cell migration [256]. Gβγ mediated activation of P-Rex1 has been implicated in prostate cancer as well whereby its activation causes cancer cell metastasis [257]; inhibition of Gβγ with β-ARK-ct was shown to abrogate prostate cancer cell growth in vitro and tumor formation in vivo [258]. Furthermore, WDR26, a WD40 protein that binds Gβγ and promotes its signalling [259], has been found to be act as a scaffold between Gβγ, PI3Kβ and its downstream target, AKT2 in breast cancer cells; downregulation of WDR26 was found to alleviate GPCR mediated PI3K signalling and tumor cell growth, migration and invasion in highly malignant cells [260].

Roles for Gβγ in regulating Epac mediated cell migration in melanoma cell lines have been studied. mSIRK, a Gβγ activating peptide, and overexpression of $G\beta_1$ and $G\gamma_2$ both acted to inhibit the Epac induced cell migration, whereas Gβγ sequestration using β-ARK-ct abrogated this inhibitory effect [261]. Furthermore, mSIRK also inhibited the 8-(4-Methoxyphenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8-pMeOPT; direct Epac activator) mediated increase in Ca^{2+} from extracellular space, suggesting that cell migration in melanoma is a process that is regulated by Gβγ, Ca^{2+} and Epac [261, 262]. It has also been demonstrated that inhibition of Gβγ using the Gα subunit of transducin, $G\alpha_t$, results in a decrease in breast cancer tumor cell migration [263] and other studies have corroborated these findings showing that Gβγ signalling promotes malignant breast cancer tumor growth and metastatis, whereas Gβγ inhibition led to blockage of tumor angiogenesis as well [263, 264]. Taken together, it is evident that Gβγ plays important roles in the regulation of cellular components that act to themselves regulate cellular processes that are central to cancer pathology.

Interestingly, mutations in Gγ subunits have been reported that lead to the development of cancer phenotypes. In a study that assessed the significance of $G\gamma_2$ in human malignant melanoma cells, it was found that $G\gamma_2$ overexpression in these cells results in decreased c-Src and PI3K dependent AKT activity, while $G\gamma_2$ depletion led to increased activities of these proteins and cell proliferation [265]. Moreover, overexpression $G\gamma_2$ in vivo resulted in decreased mean tumor size [265]. In a follow-up study, the same group demonstrated that $G\gamma_2$ overexpression results in suppression of migration and invasion, and decreased focal adhesion kinase (FAK) activity in human malignant melanoma cells [266]. FAK a key mediator of

signalling that is overexpressed and pro-metastatic in cancer [267, 268], and taken together, these results suggest that $G\gamma_2$ acts in a tumor-suppressive manner.

Mutations in Gβ subunits have also been described. Using a functional screen from cytokine-dependent cells and patient derived cDNA libraries, several missense, nonsense and frameshift mutations were identified in gene loci encoding for $G\beta_1$ and $G\beta_2$ and that these mutations occurred in multiple cancers [269]. Intriguingly, it was discovered that the recurrent mutations that affected both $G\beta_1$ and $G\beta_2$ occurred at residues on the surface that makes contact with Gα subunits; these mutants displayed decreased ability to bind all Gα subunits ($G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_{11}$ in particular), but retained their ability to bind Gγ subunits [269]. Moreover, $G\beta_1$ mutants at positions Lys57, Lys89 and Iso80 displayed increased PI3K-AKT-mTOR and MAPK activity in human erythroleukaemia (TF-1) cells [269]. $G\beta_1$ mutants also promoted myeloid dendritic cell neoplasms and interestingly, both $G\beta_1$ and $G\beta_2$ mutants conferred resistance to kinase inhibitors [269]. Taken together, these findings point to a potential pivotal role that Gβ subunit mutants play in cancer cell transformation and disease progression.

1.9.2 Functions of Gβγ in cardiovascular disease

Cardiovascular diseases (CVDs) comprise a multitude of pathologies that affect blood vessels and the heart. Given that the incidence of CVDs is highly prevalent in the global population, studies over the past few decades have provided insights into the mechanisms of how these diseases develop. Coronary heart disease, cardiac hypertension, atherosclerosis, and

myocardial infarction are examples of various stimuli that ultimately lead to deficiencies in the heart to effectively perform its functions [270]. It is now understood that most these stimuli initially induce compensatory cardiac hypertrophy that involves increasing the size of cardiomyocytes as a means of acutely improving the pumping action of the heart; however, chronic stimulatory conditions lead to eventual heart failure [270-272]. In addition to cardiac hypertrophy, it is well accepted that patients with CVD also exhibit cardiac fibrosis, a tissue repair program that acts to synthesize and remodel the extracellular matrix upon cardiac insult [273]. This section will serve to understand the functions and roles of Gβγ in the development of cardiac hypertrophy and cardiac fibrosis. GPCRs and their downstream signalling pathways that control and contribute to the development of cardiac hypertrophy have been well defined.

1.9.2 (i) Gβγ and Cardiac Hypertrophy

Cardiac hypertrophy is can be classified into two types – physiological hypertrophy and pathological hypertrophy. Physiological cardiac hypertrophy is understood to be growth in the heart that occurs through aerobic conditioning through exercise and is adaptive in the long-term; this type of hypertrophy is not detrimental to cardiac function [270]. Pathological hypertrophy occurs in response to conditions such as hypertension and myocardial infarction that are the drivers of ventricular remodeling, fibrosis and decreased cardiac output [274] and is characterized by increased size of cardiomyocytes and ventricular remodeling of the extracellular matrix that harmfully affects cardiac function, leading to increased cardiomyocyte cell death [270, 275, 276]. Mechanisms of how hypertensive heart disease leads to a left ventricular

hypertrophy and thickening is believed to be as a due compensatory increases in blood pressure that increases LV wall stress, leading to LV dilation, decrease cardiac output, and ultimately reduced cardiac function [277].

Roles for GPCRs in the development of hypertrophy are well-defined; it is understood that catecholamines such as adrenaline and noradrenaline bind α- and β-adrenergic receptors (α-AR and β-AR, respectively), angiotensin II type I and endothelin receptors to play pivotal roles in pathological cardiac hypertrophy [270]. In particular, activation of these receptors in cardiomyocytes by their natural ligands leads to increased expression of phenotypic hypertrophy markers such as β-myosin heavy chain protein upon endothelin receptor (ET-R) activation [278], and skeletal α-actin and atrial natriuretic factor expression upon Angiotensin II type I receptor activation (AT1R) [279]. As a consequence of their involvement in the development of left ventricular hypertrophy, AT1R inhibition using Angiotensin Receptor Blockers (ARBs) such as valsaltran and candesartan are used as pharmacological interventions to counter these cardiac phenotypes [280, 281]. Furthermore, both AT1Rs and ET-Rs are coupled to $G\alpha_{q/11}$ containing G proteins [282, 283]. Interestingly, $G\alpha_{q/11}$ coupling to GPCRs has been identified as a necessary prerequisite for the development and induction of pathological hypertrophy; transgenic overexpression of wild-type $G\alpha_q$ in murine hearts has been shown to cause increased hypertrophy, apoptotic cardiomyocyte cell death and dilated cardiomyopathy [284-287]. The mechanisms of how $G\alpha_q$ overexpression causes the hypertrophy phenotype have been suggested to involve the activities of PKCε [284], whereas dilated cardiomyopathy was suggested to involve PLCβ [287].

In comparison to specific GPCRs and Gα proteins, less is known regarding the contributions of Gβγ dimers in cardiac hypertrophy. However, recent evidence suggests that Gβγ dimers play important roles in the progression this disease. The Gβγ mediated dimerization and autophosphorylation of ERK1/2 and its subsequent translocation to the nucleus has been implicated in the development of cardiac hypertrophy [188]. Transverse aortic constriction (TAC) mouse models reveal stable interactions between Raf1/ERK1/2 with Gβγ, and a five-fold increase Thr188 autophosphorylation in failing hearts [188]; cardiac $G\alpha_q$ overexpression does not induce increased MAPK activity [284]. Taken together, this suggests a direct role for Gβγ in inducing cardiac hypertrophy. A parallel study by the same group demonstrated that the stimulus required to induce this Gβγ-mediated hypertrophy was activation of G_s-coupled β-adrenergic receptors [189]. TAC mouse models have also been demonstrated to display increased PI3K activity [288]. TAC mice in which β-ARK-ct was overexpressed specifically in cardiac tissue, demonstrated that Gβγ sequestration completely abolished the aforementioned increased PI3K activity, suggesting further roles for Gβγ in signalling pathways present in hypertrophied hearts [288]. Furthermore, it has been demonstrated that inhibition of Gβγ in TAC pressure overload mice improves cardiac function and attenuates cardiac remodeling [289]. Such studies on these canonical Gβγ effectors and the previously described non-canonical role of Gβγ-regulated PLCε action in cardiac hypertrophy elegantly demonstrate the contribution of Gβγ function in regulating this disease phenotype.

Interestingly, a $G\beta_3$ polymorphism that is implicated in cardiac disease has been described. In a study that sought to determine causes of increased sodium-proton exchanger activity in patients with essential hypertension, it was discovered that ion exchange enhanced

activity was due to increased intracellular signal transduction via PTX-sensitive G proteins [290]. Further investigation revealed that the observed augmentation of G protein activity was not due to mutations in $G\beta_1$ or $G\beta_2$, but a cytosine/thymine (C/T) polymorphism at nucleotide position 825 of GNB3 cDNA in hypertensive subjects [291]. Characterization of this polymorphism, $G\beta_3$-S (S depicting 'short'), revealed that while this mutation did not affect the amino acid sequence of $G\beta_3$, it encodes for a splice variant with a 123 nucleotide in-frame deletion that results in deletion of the last four amino acids of the third WD repeat and a large portion of the fourth WD repeat in $G\beta_3$'s β-propeller structure [291]. $G\beta_3$-S was found to be a biologically active variant of $G\beta_3$ capable of dimerizing with Gγ subunits and was found to enhance Gα activity as measured by GTPγS binding Gα upon mastoparan-7 stimulation of Gα and carbachol-mediated stimulation of M2 muscarinic acetylcholine receptors [292]. With respect to regulation of effector activity, $G\beta_3$-S has been found to lack the ability to modulate calcium channels and Kir3 channels [293], although their inability to modulate Kir3 channels has been contested [294]. Physiologically, this polymorphism has been associated with higher diastolic blood pressure [295], lower renin levels [295], impaired left ventricular diastolic filling [296], an increased risk of left ventricular hypertrophy [297], a decreased risk for atrial fibrillation [298], higher serum potassium and cholesterol levels [299], enhanced epinephrine-induced platelet activation [300] and arterial hypertension but not myocardial infarction [301]. Taken together, it is evident that this Gβ subunit splice variant is involved in the progression of features of cardiac disease. The existence of other polymorphisms of other Gβ or Gγ subunits is a question that remains to be answered, waiting to be uncovered through future deep sequencing efforts.

1.9.2 (ii) Gβγ in cardiac fibrosis?

While mechanisms of how GPCRs and Gβγ dimers contribute to cardiac hypertrophy is well known, much less is known about the formation of fibrosis in a diseased heart. In general, fibrosis is the scarring of tissue characterized by the accumulation of fibroblasts and increased deposition of extracellular matrix proteins that in turn result in altered organ structure [302, 303]. Similarly, in cardiac fibrosis, increased fibrotic extracellular matrix (ECM) proteins results in increased stiffness and altered signalling, leading to a pathological cardiomyocyte phenotype that results in a failing heart [303]. The cells responsible for secretion and deposition of ECM proteins are cardiac fibroblasts; these fibroblasts and their differentiated derivatives, myofibroblasts [304], are known to contribute significantly to cardiac fibrosis [305]. Indeed, it is believed that fibrosis occurs due to the unrestrained tissue repair process that is controlled predominantly by myofibroblasts [306]. Furthermore, cardiac fibroblasts have been described to directly contribute to the development of cardiac hypertrophy in a paracrine-signalling manner [307, 308].

Cardiac fibroblasts, in essence, serve major roles as modulators of ECM homeostasis. In response to several stimuli such as growth factors (e.g. transforming growth factor β, TGF-β), cytokines (e.g. IL-6) and mechanical stimulation such as stretch, fibroblasts produce collagens, laminins and elastins that contribute to ECM build-up, and metalloproteinases that act to degrade the ECM [309, 310]. Furthermore, cardiac fibroblasts secrete active biomolecules that act in paracrine and autocrine fashion on other cardiac cells, contribute to changes in cardiac electrophysiology and the homeostasis of cardiac angiogenesis [303]. Of particular interest to

this thesis is the functions of angiotensin II type I receptors in cardiac fibroblasts. Angiotensin II (Ang II) is believed to play a dominant role in fibrotic responses and in the differentiation of cardiac fibroblasts to myofibroblasts [311]. Ang II contributes to the fibrotic response by acting upstream of TGF-β whereby it induces TGF-β expression and expression of fibrotic proteins such as collagen [308, 312]. Furthermore, Ang II has been found to promote the formation of αSMA stress fibers and contractile properties in fibroblasts, a process that is suggested to contribute to myofibroblast transformation [313]; the roles of myofibroblasts in Ang II-mediated fibrosis remain to be fully elucidated [306].

Activation of AT1R in cardiac fibroblasts has been shown to induce hypertrophy in cardiomyocytes in a paracrine manner via the release of TGF-β1 and endothelin-1 [308]. Interestingly, has been demonstrated that inhibition of Gβγ subunits using β-ARK-ct in fibroblasts decreases the activation of ERK1/2, a process that is necessary for expression of collagen I in fibroblasts [314, 315]. However, these studies did not directly demonstrate that inhibition of Gβγ results in decreased expression of the previously mentioned secretory active molecules. That said, the roles of Gβγ in the normal physiological function of cardiac fibroblasts downstream of AT1R activation and in the progression of cardiac fibrosis remains largely uncharacterized. In my thesis, I describe possible roles for Gβγ in such capacities.

1.9.3 Gβγ as drug targets for therapeutic intervention

Given their numerous roles in both GPCR dependent and independent signalling and what seems to be an ever-expanding list of functions they serve in cellular biology, it should not

come as a surprise that Gβγ dimers have been explored as a potential therapeutic target. Having been implicated in the aforementioned diseases and other pathophysiologies such as pain and inflammation, a number of Gβγ-specific small molecule and peptidomemtic inhibitors have been developed. In this section, I describe the mechanisms of action and consequences of these inhibitory molecules on Gβγ action.

1.9.3 (i) Gβγ peptide inhibitors and peptidomimetics

The carboxy-terminal tail of β-adrenergic receptor kinase (β-ARK-ct) can be considered a classical Gβγ inhibitor. Gβγ subunits were first described to interact with the 125 amino acid carboxy terminal tail of β-ARK via GST-purifcation interaction assays whereby it was demonstrated *in vitro* that Gβγ inhibited the pertussis toxin catalyzed ADP-ribosylation of Gαo mediated by β-ARK [316]. Further validation of this interaction between β-ARK-ct and Gβγ demonstrated that β-ARK-ct inhibits the Gβγ mediated activation of receptor-stimulated adenylyl cyclase type II, and Gαi mediated activation of ACII and PLCβ (but not Gαq mediated)[317, 318]. Demonstrations of the inhibitory potential of β-ARK-ct in physiological settings have also been described. As previously mentioned, inhibition of Gβγ using adenovirus delivered β-ARK-ct reduces intimal hyperplasia in rabbits undergoing carotid artery bypass grafts with the jugular vein [249], and was found to prevent restenosis and vascular smooth muscle hyperplasia upon injury to rat carotid arteries [319]. Furthermore, inhibition of Gβγ action using β-ARK-ct in different types of cancer have been described whereby such inhibitions reduced cell proliferation

of a human prostate cancer cell line (PC3) and decreased prostate tumor formation in xenograft mouse models [258].

Other peptidomimetic inhibitors for Gβγ were identified in a study that demonstrated that a QEHA, peptide sequence corresponding to residues 956-982 of adenylyl cyclase II (ACII), inhibited Gβγ-mediated activation of ACII but did not affect basal activity or forskolin mediated activation [320]. In attempt to identify key residues on Gβ subunits that were responsible for effector binding, using molecular modeling, it was shown that a peptide corresponding QEHA docks on to surfaces on Gβ that correspond to residues 86-105 and 115-135 of $G\beta_1$ [321]. Based on these findings, an unbiased approach for Gβγ binding-peptides using a peptide phage display screen for purified $G\beta_1\gamma_2$ led to the discovery of a series of peptide sequences, bound a single protein-protein interaction region on Gβ; these regions were dubbed to be Gβγ protein-protein interaction "hotspots", the existence of which was used to explain the single site of action of all these peptides despite their sequence diversities [322]. A peptide identified as SIRK (SIRKALNILGYPDYD) was found to share homology with peptide sequences from $PLC\beta_2$ [131], and was found to inhibit the activities of Gβγ dependent activation of $PLC\beta_2$ and PI3K, but not adenylyl cyclases or N-type Ca^{2+} channels [322]. SIRK was found to enable nucleotide exchange-independent dissociation of Gα from Gβγ but intriguingly also activate ERK1/2 via a Gβγ-dependent mechanism [323, 324]. Crystal structure studies of SIGK (SIGKAFKILGYPDYD), a derivative of SIRK, bound to $G\beta_1\gamma_2$ reveal that SIGK is a structural analog of the switch II region of Gα that occupies a region on Gβ bound by several Gβγ interactors [325].

1.9.3 (ii) Small molecule inhibitors of Gβγ

In order to identify small molecule inhibitors of Gβγ, Bonacci et al used FlexX virtual screening software in conjunction with molecular modeling packages to identify inhibitors that competed with SIGK binding to Gβγ; 9 compounds that inhibited SIGK binding with varying inhibitory concentration values were identified [326]. Of these compounds, M119 (cyclohexanecarboxylic acid [2-(4,5,6-trihydroxy-3-oxo-3*H*-xanthen-9-yl)-(9Cl)]) was identified to bind Gβγ with high affinity, to attenutate Gβγ-dependent PLCβ and PI3K activation, but not promote Gα dissociation as SIGK does [326]. In contrast, the same screen yielded another small molecule, M201, that was shown to enhance the binding of Gβγ and activities of PLC β3 and PI3Kγ, but not PLCβ2 [326]. Moreover, M119 has been identified as a potential therapeutic for μ–OR-mediated analgesia. Opioid receptors (ORs) are known to control various aspects of the development of pain and represent important therapeutic targets in analgesia [327]. In order to elucidate the benefits of inhibiting Gβγ in analgesia, intracerebroventricular injections and systemic administration of of M119 in mice demonstrated marked increases in the potencies of morphine-mediated analgesia and also attenuated acute antinociceptive tolerance and dependence in mice, implying Gβγ inhibition serves as a attractive target for enhancement of opioid analgesia [328]. Furthermore, gallein, a Gβγ inhibitor structurally similar to M119 [329], was shown to potentiate morphine-induced antinociception in mice as measured by the 55°C warm-water tail-removing test, but not morphine-induced respiratory depression, hyperlocomotion or constipation, suggesting selective Gβγ-mediated potentiation of analgesia without matched increases in the adverse effects caused by morphine [330]. In the context of heart disease, both M119 and gallein have been found to enhance β-AR mediated cardiac

contractility in human cardiomyocytes and reduce GRK2 levels [331], a protein whose overexpression potentiates heart failure [332] and whose genetic deletion has been found to be cardioprotective [333, 334]. Using isoproterenol-induced mouse models of heart failure, both M119 and gallein prevented the isoproterenol-induced hypertrophy, left ventricular wall size and volume and cardiac contractility [331]. These results indicate the value of using small molecule inhibitors such as M119 or gallein in inhibiting Gβγ function in heart disease and pain. While these inhibitors are general Gβγ inhibitors, the value and significance of inhibiting dimers of specific Gβ and Gγ isoforms remains to be identified.

1.10 Rationale and Objectives of Study

Over the past two decades, a substantial amount of work has been done to identify signalling functions of GPCRs and their cognate signalling partners, G proteins. Our understanding of the roles played by components of G proteins, Gα and Gβγ subunits, has expanded vastly. Gβγ subunits have been shown to play pivotal roles in the regulation of canonical effectors in GPCR signalling pathways and in the modulation of non-canonical signalling partners in intracellular organelles [6, 335]. While we can now appreciate that Gβγ no longer can only be regarded as negative regulators of Gα function, the field must also shift away from its tendencies to consider $G\beta_1\gamma_2$ as the eponymous Gβγ and use this particular combination to study "roles of Gβγ". We must also realize that specific combinations of dimers may not be, or are rather not completely redundant. Many questions remain unanswered regarding specificities of Gβγ signalling and roles they serve beyond their known canonical functions. The primary interest of this project was to study Gβγ dimers in the context of their evolutionary divergence patterns and functions in endogenous signalling systems, both in canonical and non-canonical regards.

We hypothesized that specific combinations of Gβ and Gγ dimers are responsible for imparting levels of selectivity in the canonical signalling pathways they modulate and the non-canonical signalling roles they serve. The central aim of my thesis was to attribute roles for specific Gβ and Gγ subunits in the context of their roles in endogenous signalling pathways. Our objectives were:

1) To study the significance of Gβ and Gγ subunit diversity. Here, we phylogenetically characterized Gβ and Gγ subtype divergences within and between various species, and generated Gβγ structural maps to understand their putative structure-function relationships.
2) To study the roles of specific Gβ and Gγ subunits in the modulation of effectors activated downstream of GPCRs endogenously expressed in model cellular systems. In this objective, we coupled the use of a Gβ-specific and Gγ-wide RNAi screen to assess the effects of knockdown on M3-mAChR mediated signalling events in HEK 293 cells.
3) To study non-canonical functions of Gβγ dimers as regulators of gene expression. Here, we characterized a novel interaction between Gβγ dimers and RNA polymerase II that is induced in response to GPCR stimulation.

1.10 Figures for Chapter 1

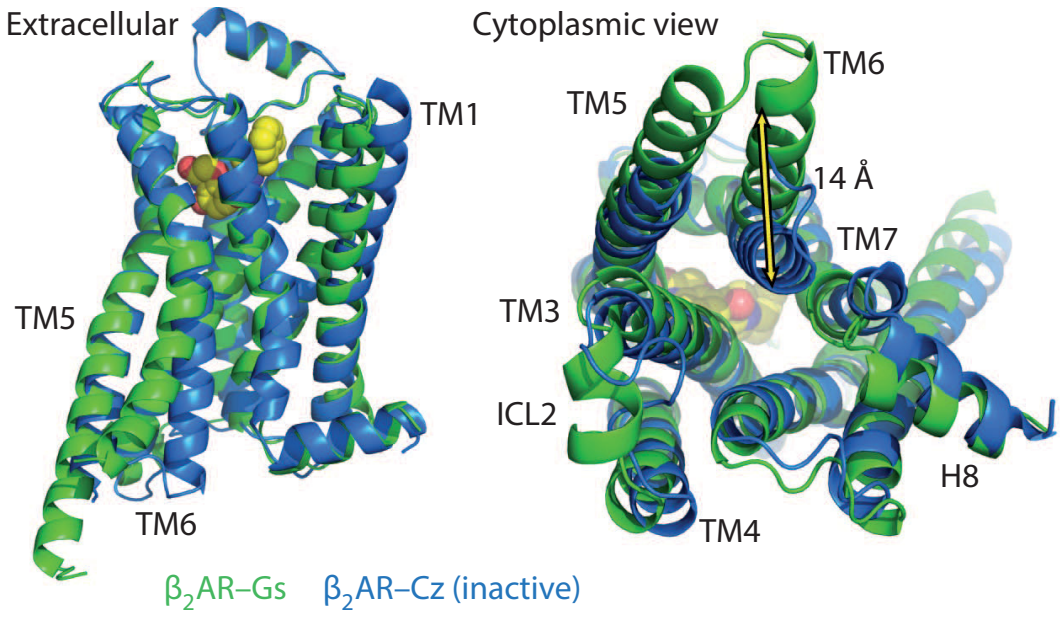

Figure 1.1 Representative crystal structure of a GPCR -- Crystal structures of β_2-adrenergic receptors

Side and cytoplasmic view representations of active (green) and inactive antagonist bound (blue) structure of β_2-adrenergic receptors. α-helices represent transmembrane domains of the receptor. Comparison of the two structures reveals a 14 Å outward movement of TM6 and an extension of TM5. Figure taken from [13].

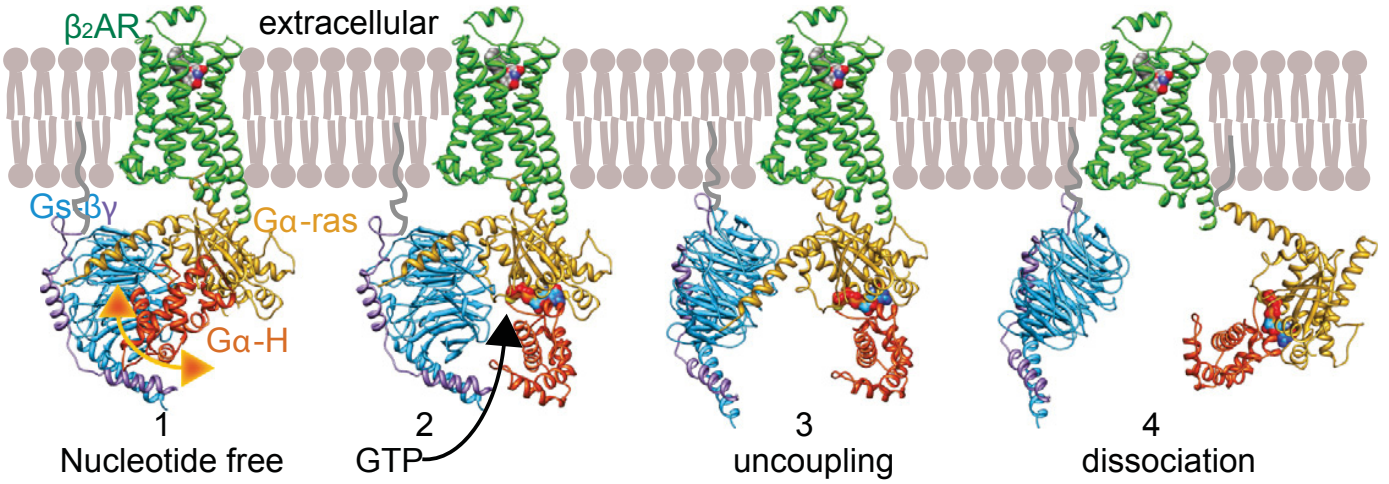

Figure 1.2 Structural depiction of Gα activation derived from the β_2-AR-Gs structure

A schematic representation of GPCR-G protein complex; β_2-AR (green) coupled to Gαs (ras-like domain in yellow, α-helical domain in red) and Gβ (blue) in a dimer with Gγ (purple). The flexibility of the Gα-α-helix domain (1) is stabilized by nucleotide binding, (2). Binding of GTP is believed to lead to uncoupling of the Gα from the GPCR, (3) and subsequent (4) dissociation of the Gα and βγ dimer. Figure taken from Westfield GH et al, 2011 [14].

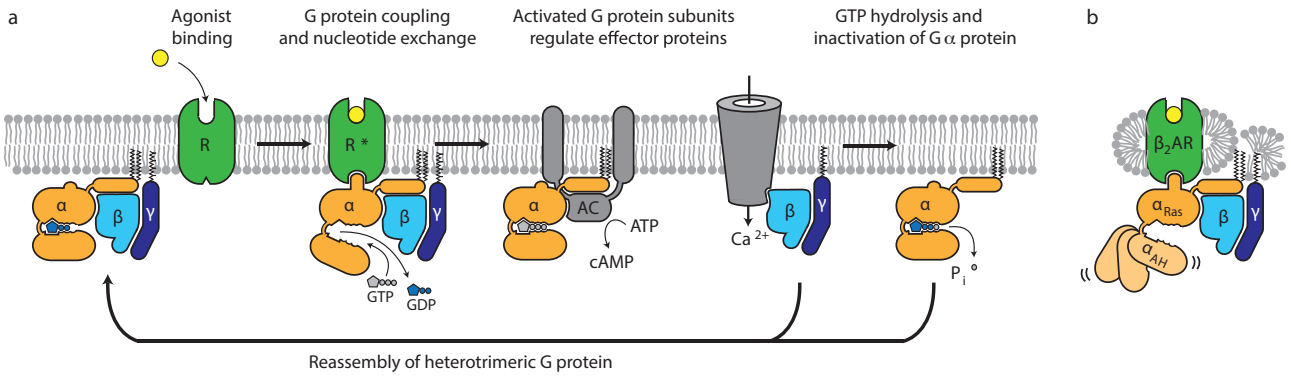

Figure 1.3 Model of the G protein cycle derived from the β_2-AR-Gs structure

(a) Binding of agonist to a GPCR on its extracellular face causes a conformational change in the receptor that is transmitted to the cytoplasmic ends of the transmembrane domains. This allows for G protein coupling, and subsequent transmission of conformational change to the Gα subunit such that there is a large outward displacement of the α-helical domain relative to the Ras-like domain. This allows for the opening of the nucleotide binding pocket and exchange of GDP for GTP, activating the subunit. As shown in this model, the activated G protein stimulates effectors. Hydrolysis of GTP then leads to the inactivation of the G protein, which can then partake in subsequent rounds of activation. (b) Cartoon depiction of the arrangement of a GPCR and G protein. The Ras-like domain and α-helical domains of the Gα subunit are distinguished in the figure. Figure taken from [13].

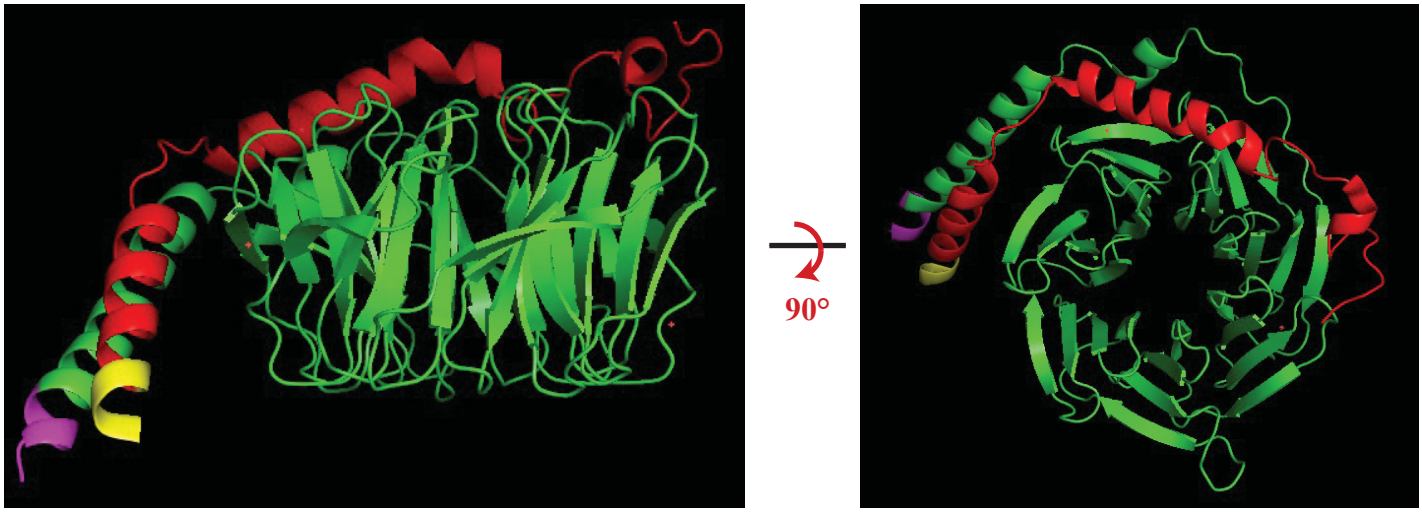

Figure 1.4 Structure of Gβγ

Depicted in this figure is a cartoon depiction of Gβ (green) and Gγ (red) as arranged in a dimer. The N-termini of Gβ and Gγ are depicted in purple and yellow, respectively. The structure on the left depicts a side view of the dimer, whereas the one on the right potrays a top view, looking down in the in barrel formed by the seven-bladed β propeller structure of Gβ. It can be seen that the α-helices of Gγ interacts with the N-terminus α-helix of Gβ along the top of the structure. This image was generated in PyMOL using a protein structure of the bovine GRK2-Gβγ interaction published by Thal et al, 2011 (PDB ID: 3psc) [43]; the structure of GRK2 was removed from the image.

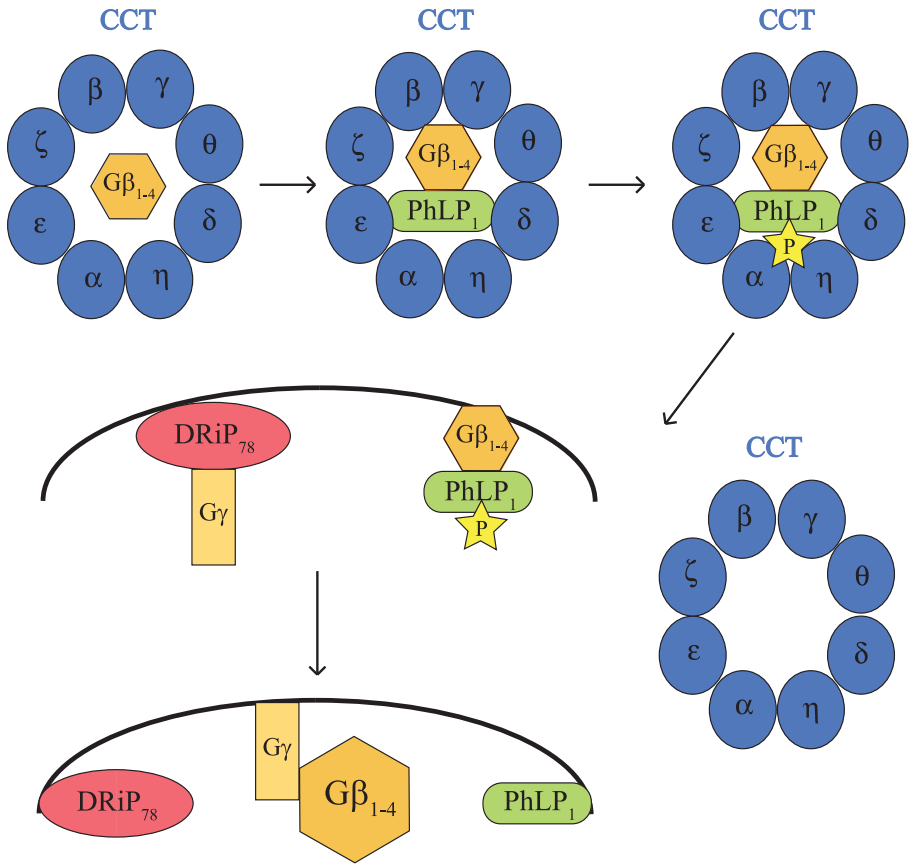

Figure 1.5 Assesmbly of Gβγ dimers upon biosynthesis

$G\beta_{1\text{-}4}$ subunits first interact with CCT to be properly folded into the seven bladed β propeller conformation. This process is accelerated by the binding of $PhLP_1$, which stabilizes the $G\beta_{1\text{-}4}$-CCT interaction. Gγ folding is facilitated by DRiP78. Upon correct folding of $G\beta_{1\text{-}4}$, $PhLP_1$ is phosphorylated by CK2, l eading to the dissociation of $G\beta_{1\text{-}4}$ from CCT. $PhLP_1$ leaves the complex via an unknown mechanism, allowing for the assembly of Gγ with $G\beta_{1\text{-}4}$. Figure taken from [35].

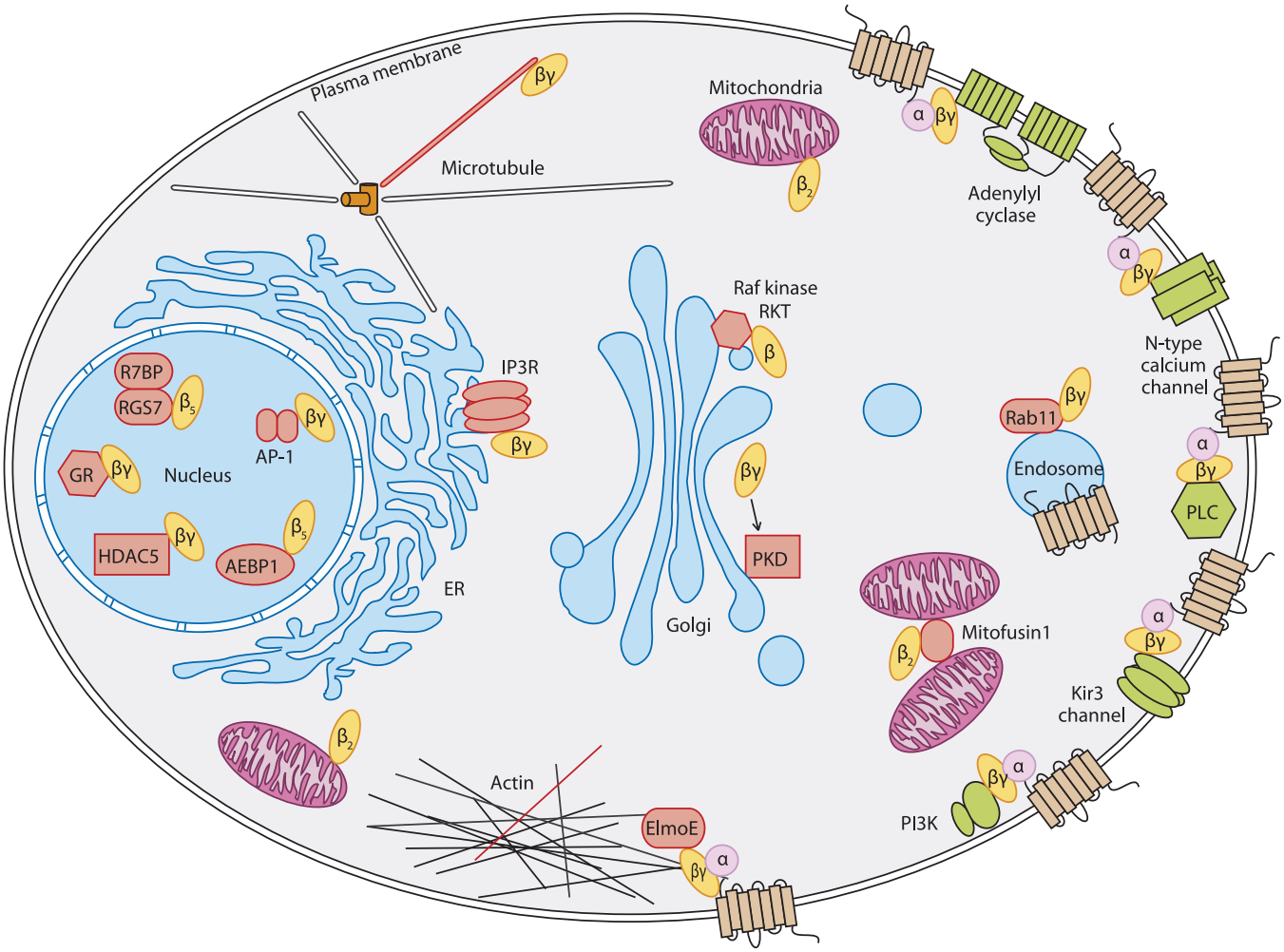

Figure 1.6 Gβγ canonical and non-canonical effectors (as of 2014)

Depicted in this figure is a collective summary of known canonical and non-canonical effectors described up until 2014. Canonical effectors are shown in green, whereas non-canonical effectors are shown in red. Specifics of the roles Gβγ dimers play in regulating these effectors is described in the Introduction. Figure taken from [35].

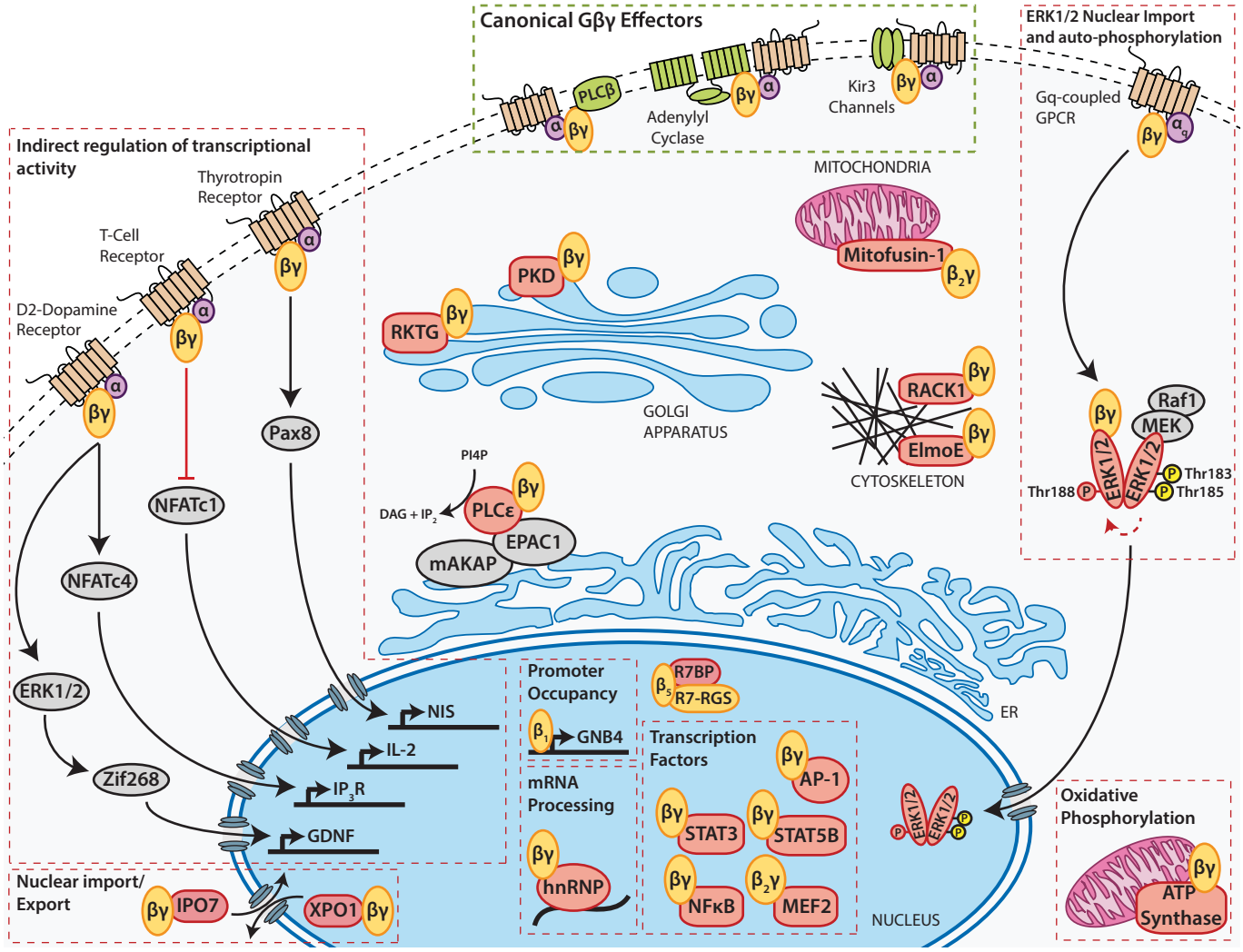

Figure 1.7 Gβγ canonical and non-canonical effectors (as of 2016)

Depicted in this figure is a collective summary of known canonical and non-canonical effectors described up until 2016. Canonical effectors are shown in green, whereas non-canonical effectors are shown in red. Specifics of the roles Gβγ dimers play in regulating these effectors is described in the Introduction. Figure taken from *Khan SM et al, 2016 Pharmacological Research* [499].

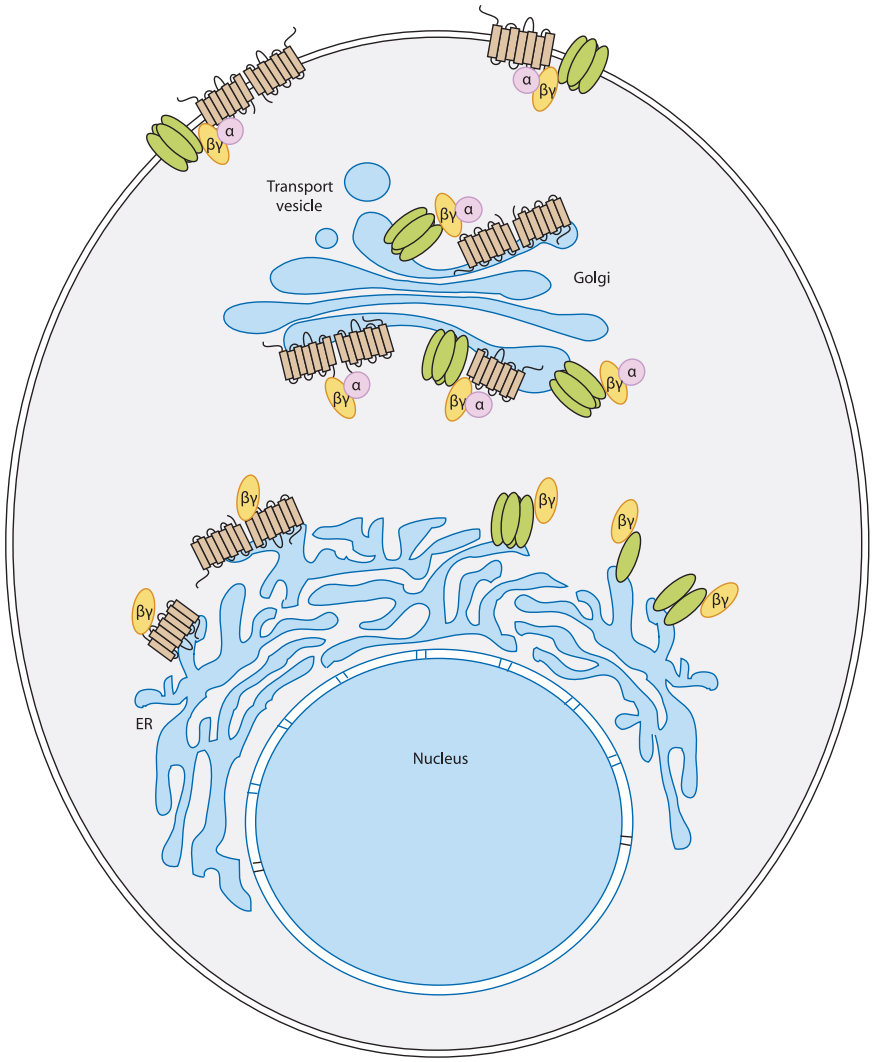

Figure 1.8 Putative roles for Gβγ dimers in the assembly of signalling complexes

Gβγ dimers have been suggested to orchestrate the formation of receptor/G protein/effector complexes. Gβγ dimers have been found to form precocious interactions with both receptors and effectors in the ER [57,96, 190] , while interactions between Gβγ and Gα have been found to initially occur in the Golgi apparatus [57, 190]. Assesmbled signalling complexes are therefore thought to form earlier than their eventual trafficking to the plasma membrane. Figure taken from [35].

CHAPTER 2: Deciphering evolutionary divergence patterns of Gβ and Gγ subunits

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2.1 Preface

Prior to pursuing this study, little was known about Gβ and Gγ subunit diversity beyond their similarities and differences between a handful of species, let alone why mammals express so many different subtypes of these G protein subunits. It was well accepted that $G\beta_{1\text{-}4}$ subtypes were more similar to one another, with $G\beta_5$ known to be an atypical subtype of the family. On the other hand, much less was known about Gγ subunits and the significance of their diversity remained largely uncharacterized. In attempt to further our understanding of these subunits from an evolutionary standpoint, we engaged in collaboration with Dr. Jean-Claude Labbé and Dr. Jean-Philippe Laverdure (Université de Montréal) to design and perform a species-wide phylogenetic analysis of the divergence patterns of Gβ and Gγ subunits. As described in this chapter herein, our analysis uncovered intricate patterns of subunit divergence between species for both Gβ and Gγ subunits whereby we uncover possible reasons for why we express so many different types of Gβ and Gγ subunits and how they relate to different species. Furthermore, in collaboration with Dr. Gregory Miller (formerly at McGill University, now at The Catholic University of America), we performed a structural mapping study of Gβγ subunits to identify clues of how the structure of these dimers relate to their function. Here, we identified that the greatest degree of structural diversity is exhibited by Gγ subunits on exposed surfaces, suggesting that these subunits may be responsible for imparting specifity of the functions they serve.

2.2 Abstract

Gβγ subunits from heterotrimeric G proteins perform a vast array of functions in cells with respect to signalling, often independently as well as in concert with Gα subunits. However, the eponymous term "Gβγ" does not do justice to the fact that 5 Gβ and 12 Gγ isoforms have evolved in mammals to serve much broader roles beyond their canonical roles in cellular signalling. Here, we explore the phylogenetic diversity of Gβγ subunits with a view toward understanding these expanded roles in different cellular organelles. We suggest that the particular content of distinct Gβγ subunits regulates cellular activity and that the granularity of Gβ and Gγ action is only beginning to be understood. Given the therapeutic potential of targeting Gβγ action, this larger view serves as a prelude to more specific development of drugs aimed at individual isoforms.

2.3 Introduction

The presence of diverse, yet sequence-similar Gβ and Gγ subunits may be the result of an evolutionary process reflecting the emergence of distinct functions. Assuming a broader role for Gβγ beyond their roles in cellular signalling *per* se, different receptor complexes may also have used the diversification of Gβ and Gγ subunits, or vice-versa, resulting in Gβ and Gγ sequence diversity and varying cellular function. G protein-coupled receptor (GPCR) complexes are organized sets of signalling-specific proteins (reviewed in [336-338]). Unique Gβγ pairs, involved in the specificity of cellular signalling may also be involved in assembly of particular GPCR complexes. Given the potential involvement of Gβγ subunits in GPCR signalling complex formation (discussed below, [6]), it is possible that the pool of Gβγ dimers in a particular cell may drive and/or dictate which receptor complexes can form in that cell. Here, we will discuss the evolutionary expansion of Gβγ function and the implications of Gβγ subunit diversity.

With the exception of $G\beta_5$, Gβ subunits share high amino acid sequence conservation (Table 1). $G\beta_{1\text{-}4}$ share between 79-90% sequence similarity, whereas $G\beta_5$ is approximately 52% similar to the other Gβ subunits. Compared to Gβ subunits, Gγ subunits are more diverse and their protein sequences are between 26-76% similar (Table 2). Given their evolutionary divergence, the question of the different roles these homologous Gβ subunits play in signal transduction becomes important. It is now generally recognized that we cannot consider an eponymous Gβγ subunit. Thus, evolution has played a large and largely unappreciated role in a plethora of Gβγ functions.

2.4 Methods

2.4.1 *Gβ and Gγ protein sequence alignment* – Amino acid sequences for human Gβ and Gγ subunits were obtained from the NCBI Protein database. Sequence alignments of the different Gβ subunits were performed using EMBL-EBI's ClustalW2 via a slow pairwise alignment. All known subtypes of human Gβ and Gγ subunits were included in the alignment, including the long and short-length forms of $G\beta_5$.

2.4.2 *Phylogenetic analysis of Gβ and Gγ subunits across various species* – In order to construct the trees, amino acid sequences for known Gβ homologs were collected from the NCBI Pubmed and Ensembl databases. Redundancy of amino acid sequences was eliminated in order to conserve only one copy of each distinct amino acid sequence. Multiple sequence alignments were produced for each family using T-coffee [339] and quality of the alignments were ascertained using T-coffee's alignment scoring mechanism. Adequate parameters for inference of the phylogeny were obtained by submitting the produced alignments to the ProtTest evolutionary model selection software [340]. For Gβ subunits, Maximum Likelihood-based phylogeny was then inferred using PhyML [341] using the parameters suggested by ProtTest based on the Akaike Information Criterion framework (AIC) score, namely, the LG model along with γ correction. For the Gγ phylogenetic analysis, the parameter used for this Maximum-likelihood phylogeny tree was the JTT evolutionary model along with γ correction. Tree topology optimization was accomplished through the sub-tree pruning and regrafting (SPR) technique and a total of 5 random starting trees were used in each inference. Robustness of the obtained tree was evaluated by running 1000 bootstrap iterations of the inference process. In

order to preserve the integrity of the tree, redundant Gβ and Gγ sequences from different species that aligned at the same node are represented as groups, linked to the tree by dashed lines. Clusters, clades and nodes are identified by their different color backgrounds and text colour. A distance bar scale is shown under each tree.

2.4.3 *Structural mapping of Gβγ subunits* – Modelling of conserved and nonconserved regions of $G\beta_{1\text{-}5}$ and $G\gamma_{1\text{-}13}$, based on the published structures of $G\beta_1\gamma_1$ and $G\beta_1\gamma_2$. SWISS MODEL was used to generate structures. The ALIGN feature (CLUSTAL) in Pymol was used to align the different subunits. PROTSKIN [342] was used to colour the level of conservation (red – conserved, blue – nonconserved). Views from three different vantage points are presented with either the Gγ or Gβ modelled using PROTSKIN.

2.5 Results and Discussion

We performed a phylogenetic analysis of Gβ and Gγ subunit protein sequences from species including invertebrates in which Gβγ function has been characterized, plant species and a wide variety of mammalian species. Upon analysis of the Gβ phylogeny tree in Figure 2.1, it can be seen that Gβ subunits from various species cluster into 5 groups – that is, 5 clusters around each mammalian Gβ subunit. On an intra-subunit level, it is evident that Gβ diverged from a common ancestor into two superfamilies very early on in their evolution giving rise to one superfamily consisting of the $G\beta_{1\text{-}4}$ subtypes and another consisting of $G\beta_5$ subtypes. This observation is not surprising, given that, as mentioned earlier, mammalian $G\beta_5$ is least similar to the other Gβ subtypes.

However, the phylogeny tree for Gγ subunits in Figure 2.2 paints a more complex picture. Gγ subunits diverged from each other into 5 classes, which can be grouped as follows: Class I: $G\gamma_7$, $G\gamma_{12}$; Class II: $G\gamma_2$, $G\gamma_3$, $G\gamma_4$, $G\gamma_8$; Class III: $G\gamma_5$, $G\gamma_{10}$; Class IV: $G\gamma_1$, $G\gamma_9$, $G\gamma_{11}$; Class V: $G\gamma_{13}$. Interestingly, $G\gamma_7$ and $G\gamma_{12}$ diverged from the rest of the group early in the evolutionary process, with $G\gamma_{12}$ representing a more ancestral Gγ subunit than $G\gamma_7$. It is also interesting to note that these two subunits are most similar to each other, as they exhibit 76% sequence similarity (Table 2).

2.5.1 Gβ and Gγ subunits in lower eukaryotes

We did not include many fungal or *Dictyostelium* Gβγ subunits in our comparative analysis of protein sequences, with the exception of the budding yeast *S. cerevisiae*. In *S. cerevisiae,* Gβγ has been shown to play a role in the pheromone response pathway [343, 344] [345]. Gβ subunits in *S. cerevisiae* reflect a different evolutionary pattern with respect to invertebrates: both GPB1 and GPB2 are homologous with vertebrate $G\beta_{1\text{-}4}$ counterparts, with GPB1 being most similar to vertebrate $G\beta_1$ and GPB2 being most similar to $G\beta_3$ (Figure 2.1). It is interesting to note that the Gγ subunit from *S. cerevisiae* displays a tight co-divergence pattern with eukaryotic $G\gamma_5/G\gamma_{10}$ class counterparts, suggesting a basic, yet absolute functional requirement for this class of Gγ subunits in cellular processes. Interestingly, a non-canonical Gβ subunit, Vps15, has also been identified in *S. cerevisiae*, and is coupled to a PI-3K pathway that does not seem to involve a Gγ subunit [346]. The fission yeast, *S. pombe*, expresses a single Gβγ pair (git5 [347] and git11 [348], respectively) that is also involved in pheromone signalling. Another Gγ-independent Gβ subunit, Gnr1 likely negatively regulates pheromone signalling in *S. pombe* [349].

Most filamentous fungi and *Dictyostelium* also express single Gβ and Gγ subunits (reviewed in [350]). Within the filamentous fungi, the sequence of Gβ subunits is fairly similar (between 70-90%, see [351]), while their Gγ subunits show more sequence diversity (between 40-90%, see [351]). Functional Gβ (GNB-1) and Gγ (GNG-1) subunits have been identified in *N. crassa*, where they play a role in regulating female fertility and asexual development [352]. Interestingly, Gβγ in *N. crassa* stabilizes Gα subunits, suggesting that the heterotrimer is the

functional unit. Although GNG-1 is highly similar to a number of other fungal Gγ isoforms, it is only 40% similar to Gγ in *S. cerevisiae* and 9% similar to Gγ in *S. pombe* [352], already suggesting a potential divergence of function. However, all of these Gγ subunits possess a conserved CAAX box, which allows farnesylation and thus facilitates membrane association, suggesting that their activity consistently depends on membrane anchoring.

2.5.2 Invertebrate Gβγ

From our analysis, invertebrate Gβ isoforms from the nematode *C. elegans* and the fruit fly *D. melanogaster* share a common pattern of subunit evolution. Intriguingly, Figure 2.1 demonstrates that each of these species contains a Gβ subtype highly divergent from the rest of the Gβ subunits analyzed (GPB-1 and $G\beta_{13}$F from *C. elegans* and *D. melanogaster*, respectively) and one Gβ that is homologous to vertebrate $G\beta_5$ (GPB-2 and $G\beta_5$ from *C. elegans* and *D. melanogaster*, respectively). *D. melanogaster* contains one additional Gβ (Gβ76C) that is homologous to vertebrate $G\beta_{1\text{-}4}$. With regard to invertebrate Gγ subtypes, a similar divergence pattern as with Gβ subtypes is also observed for *C. elegans* and *D. melanogaster*: GPC-1 and $G\gamma_1$ are most similar to the vertebrate $G\gamma_{1/9/11}$ superfamily, whereas GPC-2 and Gg30 are most similar to vertebrate $G\gamma_{13}$ subunits, respectively.

In *C. elegans,* Gβ and Gγ are required for embryonic development, as GPB-1 and GPC-2 control spindle orientation and positioning events during early embryonic stages (Zwaal et al, 1996, Gotta and Ahringer, 2001, Tsou et al., 2003). Accordingly, *gpb-1* mutant embryos fail to

hatch and have a highly disorganized tissue distribution. In this system, GPB-1 and GPC-2 are likely to function as negative regulators of the activity of two Gα subunits, GOA-1 and GPA-16, as the spindle positioning defect observed in *gpb-1* depleted embryos can be suppressed by co-depletion of these two Gα subunits [353]. Regarding GPB-2, this vertebrate Gβ5 homolog has been shown to bind Gγ-like (GGL) domain containing RGS proteins, much like its vertebrate counterparts, and is believed to regulate the GTPase activity of Gα subunits [354-356]. GPC-2 shows ubiquitous expression in *C. elegans* and is most related to the vertebrate $G\gamma_{13}$, which is least similar to all the other Gγ subunits in humans (see Table 2). On the other hand, *C. elegans* GPC-1 is only expressed in sensory neurons and has been shown to be involved in chemosensation [357]. Figure 2.2 shows that this subunit is more closely related to vertebrate $G\gamma_1$ and $G\gamma_9$. Interestingly, these two vertebrate subunits have been shown to be expressed in the rods and cones of the human eye [358, 359], suggesting that GPC-1 and the two vertebrate homologs are specifically required in the nervous system.

In *D. melanogaster*, G protein subunits are encoded by 3 Gβ and 2 Gγ genes. Gβγ dimers are mainly involved in control of asymmetric cell division in neuroblasts and sensory organs, gastrulation, heart function [360-363] and the visual system [364, 365]. One study showed that free Gβγ subunits are involved in wing expansion accompanied by epithelial-mesenchymal transition [366]. Interestingly, the sea squirt, *Ciona intestinalis* and *Drosophila* display a very similar evolutionary pattern in that *C. intestinalis* also expresses 3 Gβ subunits that are either divergent from the rest of the Gβ subunits of the species analyzed ($G\beta_1$), similar to vertebrate $G\beta_5$ or similar to vertebrate $G\beta_{1\text{-}4}$ ($G\beta_{2\text{-like-}1}$). However, the *C. intestinalis* Gγ homologs cluster with the vertebrate $G\gamma_{5/10}$ superfamily. *C. intestinalis* has the smallest genome of manipulable

chordates, making it an excellent candidate to study evolutionary and developmental biology, and in particular, given its similarity to the $G\gamma_{5/10}$ superfamily, would also make an excellent model to understand G protein subunit diversity as well [367].

2.5.3 Plant Gβγ

We analyzed the sequence of Gβ subunits in three different plant species: AGB1 in *Arabidopsis thaliana* (thale cress), NGB1 in *Nicotiana bethamiana* (similar to tobacco plant), and RGB1 in *Oryza sativa* (rice). While *Arabidopsis thaliana* and *Oryza sativa* Gβ subunits are similar to each other and diverge from a common ancestor in the $G\beta_5$ cluster, Gβ from *Nicotiana bethamiana* is more similar to vertebrate $G\beta_2$ counterparts (Figure 2.1). This finding is intriguing, as this suggests that perhaps during evolution, plants have been able to retain certain classes of Gβ subtypes according to particular cellular requirements. *Arabidposis thaliana* AGB1 has been shown to be involved in the negative regulation of auxin-induced cell division, gene transcription regulation, and pathogen resistance pathways [368, 369].

Oryza sativa expresses two different types of Gγ (RGG1 and RGG2) [370] but unfortunately had to be removed from our phylogeny analysis as their sequence divergence proved too disruptive to the inference process. Gγ subtypes from *Nicotiana bethamiana* are yet to be characterized and were also not included in the present analysis. However, phylogenetic analysis of three *Arabidopsis thaliana* Gγ subunits, including the recently characterized third Gγ subunit [371], reveals that all three subunits, AGG1, AGG2 and AGG3, share a common

ancestor with the vertebrate $G\gamma_{1/9/11}$ class. Since this common ancestor was the most diverged ancestral Gγ from the initial Gγ subunit, this suggests that Gγ subunits have evolved to become highly specialized in *Arabidopsis thaliana*. Despite their tight co-divergence, AGG1 and AGG2 have been shown to exhibit functional selectivity within this species, playing different roles in pathogen resistance, germination, lateral root development and gravitropism [372].

2.5.4 Fish and mammalian Gβγ

Throughout evolution, fish and mammals have acquired a larger and more diverse set of Gβ and Gγ subunits. Whether these sets of Gβγ subunits are redundant, or serve specific cellular roles remains unknown. We analyzed the Gβ sequences from two different types of fish, *Danio rerio* (zebra fish) and *Gadus morhua* (cod), and observed that all Gβ subunits (with the exception of $G\beta_4$) from both these species seemed to have evolved from the same common ancestor that yielded Gβ in mammalian species (Figure 2.1). The same can be concluded regarding Gγ subunits in both of these fish species, however, it is interesting to note that $G\gamma_1$ and $G\gamma_{11}$ were found to be redundant for our analysis. Whether these subunits are functionally redundant remains to be determined.

Genomic analysis of humans and mice Gγ genes revealed a general, but not absolute conservation, with differences appearing primarily at the 5'-ends of these genes [373, 374]. Certain Gγ genes were found to be less than 10 kb in length, whereas others were greater than 100 kb in length, which is remarkable given that Gγ protein sequences typically contain 65-75

amino acids [373]. Our phylogenetic analysis of Gβ and Gγ from various species indicates a modest level of sequence conservation between both fish and mammalian species.

Mammalian Gβ and Gγ subunits display a subunit specific clustering pattern as described earlier. The roles that these specific G protein subunits play in cellular signalling is also described in more detail below. Our analysis reveals that regardless of differences in species types, mammalian Gβ and Gγ subunits display tight conservation of protein sequence within each subtype of Gβ or Gγ, with each subtype in each species coming from a particular common ancestral Gβ and Gγ. The observation that different Gβ and Gγ subunit subtypes are similar/conserved across species has been used as sound reasoning to use certain mammalian species such as mice (*Mus musculus*) and rats (*Rattus norvegicus*) as experimental models to study G protein function, and has yielded great insight into the roles that these Gβ and Gγ subunits play. However, the crucial question that remains to be answered is: what does the evolutionary diversity of Gβ and Gγ subunits imply for broader G protein function? While it is quite possible that some of these subunits may serve redundant roles, it is highly probable that these differences in Gβ and Gγ protein sequences within a certain species impart essential structural differences to these subunits, conferring them specificity and selectivity in their function.

2.5.5 Structural features of Gβγ subunits

The number of Gβ and Gγ genes is strikingly higher in mammals compared to *C. elegans* and other simpler organisms. As discussed above, humans express five distinct Gβ subunits

along with their variants (β_1, β_2, β_3, β_{3S}, β_4, β_5, β_{5L}) and twelve Gγ proteins $G\gamma_{1\text{-}5,7\text{-}13}$ [375] while *C. elegans* possesses only two genes for each subunit [376, 377]. Thus over evolution a number of new and distinct functions for Gβγ may have come into play through gene duplication and subsequent selection and they may not be limited to the open reading frames of the various genes. However, our current understanding of the basis of mammalian Gβγ diversity is rudimentary and has been mostly focused on canonical signalling functions. Almost nothing is known about how this diversity affects either the "organizing" or the transcriptional regulatory functions of different Gβγ subunits discussed below.

A number of crystal structures have been generated for Gβγ subunits, alone and in complexes with known effectors (reviewed in [378]. The β-propeller structure of the WD repeats in Gβ and its association with Gγ [379] and Gα [380, 381] have become iconic. All of these have used either $G\beta_1\gamma_1$ or $G\beta_1\gamma_2$ and have addressed how subunit diversity might impact function. The Gγ subunits are where this diversity is most obvious, a somewhat curious notion given that they are among the smallest proteins involved in G protein signalling. Molecular modelling of the human Gβ and Gγ subunits give some indication as to why this is important (Figure 2.3). Here we can see that the three non-conserved regions of Gγ subunits, the N- and C-termini, as well as the central hinge, all face outward, away from the Gβ subunit (which is generally much more conserved) where they can interact differentially with a number of different and possibly unique effectors. Thus the Gγ subunits have evolved to provide a great deal of the structural diversity or "granularity" necessary for serving the diverse roles of Gβγ in cellular function.

2.6 Conclusions

Overall, our analyses reveal a clearer understanding of Gβ and Gγ subunit phylogeny and diversity, and our structural analysis of mammalian Gβγ provides clues regarding the importance of these diverse subunits with respect to function. Our data suggests that different classes of Gβ and Gγ may exist, however whether such phylogenetic classifications translate to functional selectivity remains to be identified. Moreover, it remains to be determined whether the granularity imparted by Gγ subunits in the overall structure of Gβγ translates to specificity of signalling downstream of GPCR activation. Taken together, our data sheds light on the importance of regarding Gβγ dimers as dimers of specific subunit composition, and not just the eponymous term "Gβγ" as referred to in the majority of studies.

2.7 Acknowledgements

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2.8 Author Contributions

Shahriar M. Khan, Sarah Gora, Rory Sleno, Peter Zylbergold, Jean-Philippe Laverdure, Jean-Claude Labbé, Gregory J. Miller and Terence E. Hébert wrote the entire review manuscript. Shahriar M. Khan, Jean-Philippe Laverdure, Jean-Claude Labbé, Gregory J. Miller and Terence E. Hébert performed the bioinformatics analysis.

2.9 Figures Legends for Chapter 2

Figure 2.1 ***Phylogenetic relationships of Gβ subunits across various species*****.**

In order to construct the trees, amino acid sequences for known Gβ homologs were collected from the NCBI Pubmed and Ensembl databases, and the phylogenetic analysis was perfomed as described in the Methods section. The 5 Gβ subunit subtypes from different species form clusters, and these are depicted by the following color scheme: $G\beta_1$ (red), $G\beta_2$ (dark blue), $G\beta_3$ (orange), $G\beta_4$ (green) and $G\beta_5$ (light blue).

Figure 2.2 ***Phylogenetic relationship of Gγ subunits across various species***.

The phylogenetic tree for Gγ subunits was constructed by a similar method as previously described in Figure 1..As in Figure 1, redundant sequences are grouped and linked to the tree by dashed lines, and a distance bar scale is shown under the tree. Gγ subunits group into five main clusters, and consist of: Cluster 1 (pink) – $G\gamma_2$, $G\gamma_3$, $G\gamma_4$ and $G\gamma_8$; Cluster 2 (green) – $G\gamma_1$, $G\gamma_9$ and $G\gamma_{11}$; Cluster 3 (red) – $G\gamma_5$ and $G\gamma_{10}$; Cluster 4 (blue) – $G\gamma_{13}$; Cluster 5 – $G\gamma_7$ and $G\gamma_{12}$. $G\gamma_7$ and $G\gamma_{12}$ appear to be the least divergent Gγ subunit, being most similar to the initial ancestral Gγ subunit.

Figure 2.3 ***Structural mapping of Gβγ subunits.***

Modelling of conserved and nonconserved regions of $G\beta_{1\text{-}5}$ and $G\gamma_{1\text{-}13}$, based on the published structures of $G\beta_1\gamma_1$ and $G\beta_1\gamma_2$. Views from three different vantage points are presented with either the Gγ (left) or Gβ (right) coloured using PROTSKIN. Nonconserved N-termini, hinge and C-termini of Gγ subunits are located on the external face of the Gβγ subunit. Gβ subunits show greater conservation (especially on the face which contacts Gα).

2.10 Figures for Chapter 2

Table 1 - Percentage sequence similarities of human Gβ subunits

	Gβ1	Gβ2	Gβ3	Gβ4	Gβ5-5	Gβ-L
Gβ1	100%	-	-	-	-	-
Gβ2	90%	100%	-	-	-	-
Gβ3	83%	80%	100%	-	-	-
Gβ4	90%	90%	79%	100%	-	-
Gβ5-S	52%	51%	52%	52%	100%	-
Gβ5-L	52%	51%	52%	52%	100%	100%

Table 2.1 Sequence similarities of human Gβ subunits.

Assessment of human G β sequence conservation. Values indicated in each cell represent percentage sequence similarity between Gβ subtypes.

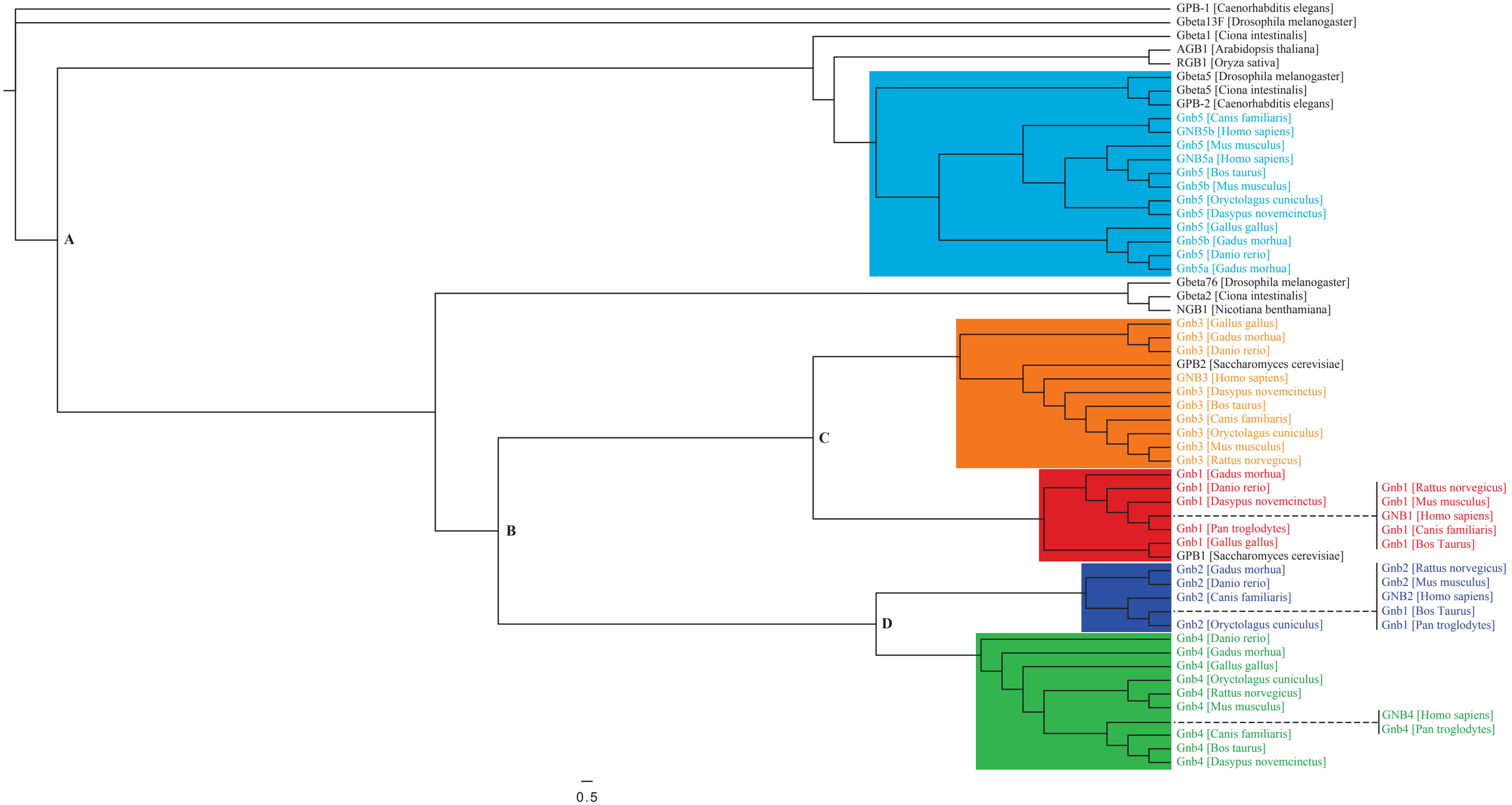
Table 2 - Percentage sequence similarities of human Gγ subunits

	Gγ1	Gγ2	Gγ3	Gγ4	Gγ5	Gγ7	Gγ8	Gγ9	Gγ10	Gγ11	Gγ12	Gγ13
Gγ1	100%	-	-	-	-	-	-	-	-	-	-	-
Gγ2	32%	100%	-	-	-	-	-	-	-	-	-	-
Gγ3	29%	76%	100%	-	-	-	-	-	-	-	-	-
Gγ4	31%	77%	69%	100%	-	-	-	-	-	-	-	-
Gγ5	25%	45%	45%	42%	100%	-	-	-	-	-	-	-
Gγ7	30%	66%	58%	55%	51%	100%	-	-	-	-	-	-
Gγ8	31%	70%	55%	60%	42%	51%	100%	-	-	-	-	-
Gγ9	63%	34%	31%	28%	26%	38%	30%	100%	-	-	-	-
Gγ10	29%	48%	45%	45%	52%	52%	45%	33%	100%	-	-	-
Gγ11	76%	29%	28%	27%	27%	35%	28%	63%	30%	100%	-	-
Gγ12	31%	56%	54%	50%	45%	76%	47%	36%	44%	36%	100%	-
Gγ13	28%	28%	25%	26%	23%	28%	26%	32%	23%	31%	25%	100%

Table 2.2 Sequence similarities of human Gγ subunits.

Assessment of human G γ sequence conservation. Values indicated in each cell represent percentage sequence similarity between Gγ subtypes.

Figure 2.1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Gnb1 [Danio rerio]
Gnb1 [Dasypus novemcinctus]
Gnb1 [Pan troglodytes]
Gnb1 [Gallus gallus]
GPB1 [Saccharomyces cerevisiae]
Gnb2 [Gadus morhua]
Gnb2 [Danio rerio]
Gnb2 [Canis familiaris]
Gnb2 [Oryctolagus cuniculus]
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Gnb4 [Gadus morhua]
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Gnb1 [Mus musculus]
GNB1 [Homo sapiens]
Gnb1 [Canis familiaris]
Gnb1 [Bos Taurus]
Gnb2 [Rattus norvegicus]
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B
C
D
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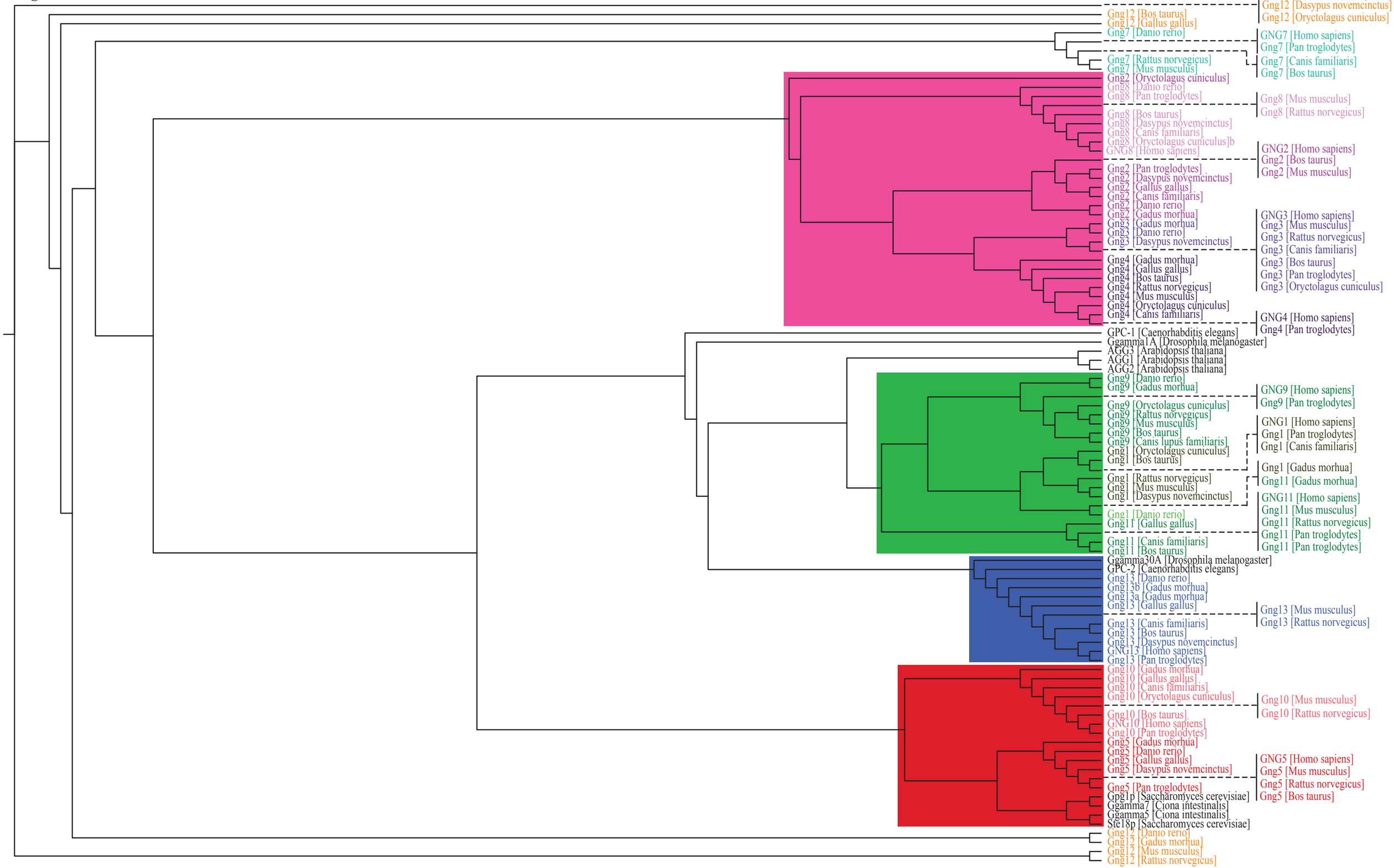
Figure 2.2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Gng7 [Rattus norvegicus]
Gng7 [Mus musculus]
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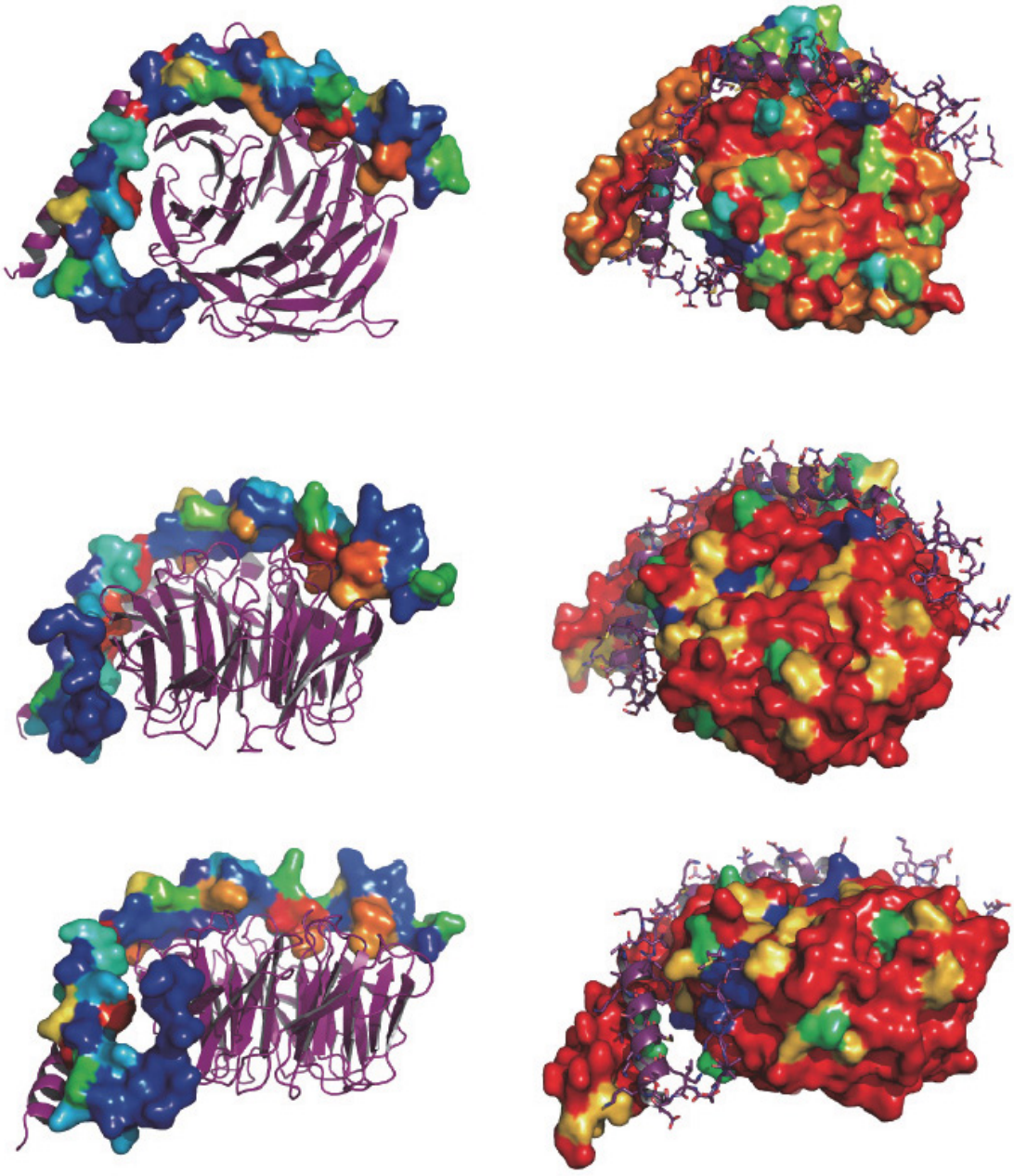
2.0

Figure 2.3



CHAPTER 3: $G\beta_4\gamma_1$ as a modulator of M3 muscarinic receptor signalling and novel roles of $G\beta_1$ subunits in the modulation of cellular signalling.

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3.1 Preface

In the previous Chapter, we demonstrated that Gβ and Gγ subunits may have evolutionarily diverged as classes and as such, functions may have also evolved whereby subunits within certain classes perform similar roles in signalling. Moreover, we describe that Gγ subunits impart levels of granularity to the overall structure of Gβγ, suggesting that they may be more important for imparting specificity to the signalling pathway components Gβγ dimers modulate. Next, with the aim of trying to understand whether these observations of differential divergence of Gβ and Gγ subunits translate into functions for modulation of signalling, in collaboration with Integrated DNA Technologies, we designed a RNAi screen to study the roles of specific Gβ and Gγ subunits in signalling downstream of endogenously expressed M3-mAChR in HEK 293 cells. Here, we demonstrate that $G\beta_4\gamma_1$ is the preferential and predominant Gβγ dimer that acts to regulate Ca^{2+} release upon activation of M3- mAChR with carbachol, with limited redundant functions observed for $G\beta_4\gamma_2$, $G\beta_4\gamma_4$ and $G\beta_4\gamma_7$. In addition, we demonstrate that knockdown of $G\beta_1$ subunits results in dysregulation of M3- mAChR signalling as measured by Ca^{2+} release, ERK1/2 phosphorylation and expression of signalling pathway components. Moreover, this manuscript showed for the first time that $G\beta_1$ subunits are capable of binding the promoters of over 700 genes, including that of $G\beta_4$, pointing to novel non-canonical roles of $G\beta_1\gamma$ in GPCR signalling systems.

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3.2 Abstract

Much is known about how Gβγ subunits regulate effectors in response to G protein-coupled receptor stimulation. However, there is still a lot we do not know about how specific combinations of Gβ and Gγ are wired into different signalling pathways. Here, using an siRNA screen for different Gβ and Gγ subunits, we examined an endogenous M3 muscarinic receptor signalling pathway in HEK 293 cells. We observed that $G\beta_4$ subunits were critical for calcium signalling and a downstream surrogate measured as ERK1/2 MAP kinase activity. A number of Gγ subunits could partner with $G\beta_4$ but the best coupling was seen via $G\beta_4\gamma_1$. Intriguingly, knocking down $G\beta_1$ actually increased signalling through the M3-mAChR most likely via an increase in $G\beta_4$ levels. We noted that $G\beta_1$ occupies the promoter of $G\beta_4$ and may participate in maturation of its mRNA. This highlights a new role for Gβγ signalling beyond the canonical roles these dimers play in cellular signalling.

3.3 Introduction

Much is known regarding the roles that "eponymous" Gβγ subunits play in signal transduction (reviewed in [6, 66, 335]). Although a number of studies have also examined the specific roles of individual Gβ and Gγ subunits, we still do not have a clear view of their individual functions. Functions for individual Gβ and Gγ subunits have been attributed using antisense approaches and the roles they play in receptor signalling pathways as well as embryonic development have been characterized in animal knockout models [83, 84, 86, 382-393]. $G\beta_{1\text{-}4}$ subunits share 78-88% identity over their approximately 340 amino acid sequences (reviewed in [335], [394]). $G\beta_5$ is structurally distinct from the other Gβ subunits (see below), sharing approximately 50% sequence identity with the other Gβ subunits. Gγ subunits are considerably more structurally diverse than the Gβ subunits sharing between 27 and 76% sequence homology. Sequence homology among related family members is much higher. For example, $G\gamma_1$, $G\gamma_{11}$ and $G\gamma_{13}$ share 62-73% homology [335, 394].

If all Gβ subunits formed dimers randomly with all Gγ subunits there would be 60 possible combinations. Most can form pairs *in vitro* although exceptions have been reported [65]. Some of this is due to specialized function or cellular distribution. For example, $G\gamma_1$ expression is restricted to retinal rod cells [395]. $G\beta_1$ can interact with all Gγ subunits while $G\beta_2$ is more restricted in its Gγ partners [396, 397] with a region of Gγ that defines specificity for the interaction with $G\beta_1$ or $G\beta_2$ subunits localized to a 14-amino acid segment [398]. Specific Gβγ interactions are certainly restricted by differential expression in particular cell types – with

the extreme examples of the visual system and vasculature [399] as lower and upper limits for combinatorial diversity. We still do not understand the functional consequences of this diversity.

The combinatorial association of the different G protein subunits could provide the level of selectivity that is needed to generate the broad range of signals transmitted by G proteins. It has been difficult to demonstrate that subunit diversity plays an important role in signalling specificity. Biochemical approaches have revealed modest differences among the various subunit combinations (reviewed in [6, 67, 335]). However, genetic approaches have been more successful where specific roles for $G\beta_1$, $G\beta_5$, $G\gamma_3$ and $G\gamma_7$ have been demonstrated in distinct tissues [84, 382, 383, 388-391]. For most receptors though, the G protein subunit combination required to generate specific signalling events *in vivo* is still unknown. Here, we use a systematic Gβγ RNAi approach to understand coupling of endogenous muscarinic receptors in HEK 293 cells to the G_q/PLCβ/calcium signalling pathway.

3.4 Materials and Methods

3.4.1 Reagents – Custom qPCR assays and DsiRNAs against Gγ subunits were obtained from Integrated DNA Technologies (Coralville, IA, USA) and sequences for DsiRNA duplexes and qPCR assays are listed in Supplemental Tables 1 and 2, respectively. Carbachol, TRI Reagent RNA isolation reagent, Bovine serum albumin, β-glycerophosphate, sodium fluoride (NaF), ethylenediaminetetraacetic acid (EDTA), sodium orthovanadate (Na_3VO_4), Triton X-100, microcystin, dithrothreitol (DTT), leupeptin, phenylmethylsulfonyl fluoride (PMSF), 70% NP-40 (Tergitol), sodium deoxycholate and anti-mouse Protein G Agarose beads, goat anti-rabbit IgG (whole molecule) conjugated to peroxidase secondary antibody and goat anti-mouse IgG (Fab specific) conjugated to peroxidase secondary antibody were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Lipofectamine 2000, zeocin, coelenterazine h, blasticidine were purchased from Invitrogen (Burlington, ON, Canada). Enhanced chemiluminescence (ECL) Plus reagent and white opaque-bottom 96 well microplates were purchased from Perkin Elmer (Woodbridge, ON, Canada). Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L glucose, L-glutamine with or without phenol red, Penicillin-Streptomycin solution, Tris base buffer, fetal bovine serum and geneticin (G418) were purchased from Wisent (St. Bruno, QC, Canada). Puromycin was purchased from Invivogen (San Diego, CA, USA). Moloney murine leukemia virus reverse transcriptase (MMLV-RT) enzyme and recombinant RNasin ribonuclease inhibitor were purchased from Promega (Madison, WI, USA). SsoAdvanced SYBR Green supermix was purchased from BioRad (Hercules, CA, USA). Formaldehyde, siGENOME SMARTpool siRNA for Gβ subunits were purchased from Dharmacon Incorporated (Lafayette, CO, USA). Sodium chloride was purchased from Fisher Scientific (Ottawa, ON, Canada). Ethylene glycol bis (2-aminooethyl ether) N,N,N',N' tetraacetic acid (EGTA) was purchased

from BioShop (Burlington, ON, Canada). Anti-PLCβ_1 and anti-PLCβ_3 antibodies were a kind gift from Dr. Alan Smrcka (University of Rochester Medical Center, Rochester, NY, USA). Anti-phosphoERK1/2 antibody was purchased from Cell Signalling Technology (Danvers, MA, USA). Anti-ERK1/2-total, anti-G$\alpha_{q/11}$, anti-GRK2, anti-GRK3, anti-GRK5 and anti-GRK6 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-mouse monoclonal anti-HA antibody was purchased from Roche Applied Science (Laval, QC, Canada). Anti-β tubulin antibody was purchased from Invitrogen (Burlington, ON, Canada).

3.4.2 *Cell Culture and Transfection* – A stable HEK 293F cell line expressing HA-tagged Tandem Affinity Purification-Gβ_1 (TAP-Gβ_1) was generated by transfection with pIRESpuro-GLUE-HA-TAP-Gβ_1, as previously described [400]. Native HEK 293F cells were used for ERK1/2 MAPK assays. A previously characterized stable Flp-In T-Rex HEK 293 cell line expressing apo-aequorin (Aequorin-HEK 293) was a generous gift from Jonathan Javitch [401]. All cell lines 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. Aequorin-HEK 293 stable lines were maintained with 700 μg/ml G418, 15 μg/l blasticidine and 10 μg/ml zeocin selection while TAP-Gβ_1 HEK 293 stable lines were maintained with 10 ug/ml puromycin selection. For transient transfections for aequorin assays, cells were reverse-transfected with 10 nM of DsiRNAs targeting Gγ subunits or 50 nM of siRNAs targeting Gβ subunits for a duration of 72 hours in 150,000 – 175,000 cells using Lipofectamine 2000 according to the manufacturer's protocol.

3.4.3 *Reverse Transcription and qPCR* – Total RNA was isolated from transfected HEK 293F cells with TRI reagent using a modified protocol from Ambion (Burlington, ON, Canada).

Reverse transcription was performed on 2-8 μg of RNA using a Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) reaction assay as per the manufacturer's protocol. Custom Primetime qPCR 5' Nuclease assays from Integrated DNA Technologies were used for validation of knockdown of Gγ subunits and for all subsequent analyses of Gγ expression. For validation of ChIP-on-CHIP data, qPCR assays were performed using the Bio-Rad SsoAdvanced SYBR Green Supermix platform. All qPCR reactions were run using a Corbett Rotorgene 6000 qPCR instrument. mRNA expression data were normalized to the levels of the housekeeping genes actinB (ActB). Sequences of qPCR primers are depicted in Supplemental Table 3.

3.4.4 *Aequorin Assay* – The luminescence-based aequorin assay, which measures changes in intracellular Ca^{2+} levels, was conducted as previously described with minor modifications [401]. Briefly, 72 hours after transfection in 12-well plates of Aequorin-HEK 293 stable cells, cells were washed twice with phosphate-buffered saline and resuspended in DMEM without phenol red supplemented with 0.1% bovine serum albumin. Samples were then loaded with 5μM coelenterazine h and incubated on a rotator for 3 hours at room temperature, protected from light. Cells were subsequently loaded on white opaque-bottom 96 well microplates. Dose-response curves were obtained by injecting 50 μl of 2X preparations of carbachol to obtain appropriate final concentrations. Luminescence measurements were collected for a total integration time of 20 seconds using a Bio-Tek Synergy 2 Multi-Mode Microplate Reader. For data analysis, integrated luminescence counts were normalized to values obtained for calcium release using the highest concentration of carbachol in control condition samples.

3.4.5 *Western blotting* – An immunoblot-based assay to assess levels of ERK1/2 phophorylation was performed as previously described [402, 403]. Briefly, HEK 293 cells, reverse transfected in 12-well plates, were first serum-starved for 5 hours at 37°C. Cells were subsequently treated with 1 mM carbachol for a duration of 5 minutes (as determined by a carbachol treatment time-course experiment, Supplemental Figure 3.1) and placed on ice immediately after agonist treatment. Samples were subsequently washed twice with cold 1X PBS, and then lysed in MAPK lysis buffer (50 mM Tris pH 7.5, 20 mM β-glycerophosphate, 20 mM NaF, 5 mM EDTA, 10 mM EGTA, 1 mM Na_3VO_4, 1% Triton-X, 1 μM microcystin, 5 mM DTT, 5 μg/ml leupeptin, 0.5 mM PMSF). Samples were then sonicated in a sonicator bath three times for 5 minutes each at 4° C, and frozen immediately at -20° C. Lysates were quantified using a standard Bradford assay, and 50 μg of total lysates were subjected to SDS-PAGE on 10% acrylamide gels and subsequently western blotted. Proteins were visualized using anti-phospho ERK1/2 and anti-ERK1/2 total primary antibodies used in conjunction with peroxidase-conjugated secondary antibodies and a chemiluminescence detection system. For detection of $PLC\beta_1$, $PLC\beta_3$, $G\beta_4$, $G\alpha_{q/11}$, GRK2/3/5/6 under $G\beta_1$ knockdown conditions, aequorin-HEK 293 cells were transfected with 50 nM $G\beta_1$ siRNA for 72 hours in 12-well plates. Cells were then lysed in 1X 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) with rotation for 1 hour at 4° C. Lysates were again quantified by Bradford assay, and 50 μg of total lysates were subjected to SDS-PAGE on 8% acrylamide gels and protein expression was assessed by western blot. Proteins were visualized using anti-$PLC\beta_1$, anti- $PLC\beta_3$, anti-$G\alpha_{q/11}$ or anti-β tubulin antibodies used in conjunction with peroxidase-conjugated secondary antibodies and a chemiluminescence detection system. Band intensities were quantified using ImageJ.

3.4.6 *Chromatin Immunoprecipitation (ChIP)* – ChIP was performed according to a protocol from Millipore, provided with the EZ ChIP Kit. Briefly, proteins were cross-linked to DNA using fresh 37% formaldehyde (1% final concentration) and the reaction was quenched using 1X glycine solution provided with kit and then cells were lysed with SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0). The cell lysate was then sonicated for 20 min (30 sec on/off cycle) to shear DNA. Each immunoprecipitate sample was then pre-cleared by incubating samples with with anti-mouse Protein G Agarose beads (Sigma). Immunoprecipitation (IP) of cross-linked protein/DNA was achieved by incubating overnight with mouse monoclonal anti-HA at 4°C. Anti-mouse protein G Agarose beads were then added to precipitate the protein/DNA complexes. The protein/DNA complexes were then eluted using Elution Buffer (provided in the kit) and the protein/DNA cross-links were reversed by Proteinase K to free DNA. The DNA was then analyzed by real-time quantitative PCR. For each qPCR reaction, 20 ng DNA from IP fractions was amplified and the protocol was performed as described above. The input fraction was 1% of the chromatin used in IP fractions. Primers corresponded to regions within the $G\beta_1$ and $G\beta_4$ promoters. Primer sequences are listed in Supplemental Table 3. Data were analyzed to calculate site occupancy, in which $2^{-\Delta\Delta Ct}$ represents IP fold enrichment in occupancy above background (no antibody, noAb). ΔCt values were calculated relative to the input fraction: ΔCt = Ct(IP or noAb) – Ct(Input – Dilution Factor) and $2^{-\Delta\Delta Ct} = 2^{-[\Delta Ct(IP)-\Delta Ct(noAb)]}$.

3.4.7 *ChIP on chip experiments* – 100 ng of purified nucleic acid from ChIP reactions was amplified following the Genomeplex complete WGA kit (Sigma, St. Louis, MO, USA). 2 µg of product of bound DNA and Input samples were labeled with cyanine-5 (Cy5) and cyanine-3 (Cy3) UTP (VWR, Ville Mont-Royal, QC, Canada) respectively, using the BioPrime® Array

CGH Genomic Labeling System (Invitrogen, Burlington, ON, Canada). Dye incorporation was verified using a Nanodrop spectrophotometer. 3.5ug labeled amplified DNA (labelled with Cy5 and Cy3) was combined and hybridized to Agilent Custom Sureprint CGH Microarray 2x400K two-arrays in Agilent hybridization chambers for 40 hours at 65°C using solution provided in Agilent's Gene Expression Hybridization kit, according to manufacturer's recommendations. The arrays were then washed according to manufacturer's recommendations. The chips were scanned using an Agilent SureScan Microarray scanner. Numerical data was produced using Agilent Feature Extraction software, FE 10.7 Grid: 026112_D_F_20100808. We partitioned the regions tiled by probes into 1000bp regions and identified those with high probe intensities.

3.4.8 *Proteomic analysis of Gβ interactions* – The HA-TAP-Gβ_1, construct has already been previously described [184]). The TAP-tagged pIRESpuro-GLUE-N1 vector backbone was engineered to express either HA-Gβ_1 or Flag-Gγ_7 for use in split-TAP experiments. Gβ_1 was inserted using EcoRI and BamHI, Gγ7 with ClaI and BamHI. Briefly, cells transiently transfected with these constructs were treated or not with 1mM carbamoylcholine chloride (Sigma-Aldrich, St Louis, MO, USA) for 5 minutes followed by a 45 minute wash out period. Total or nuclear extracts of cells expressing TAP-Gβ_1 were purified by tandem affinity purification and characterized as described [184]. Raw MS files were created and analyzed for protein identification according to [404] using the Mascot database (RefSeqV45).

3.4.9 *Statistical analysis* – Statistical tests were performed using GraphPad Prism 6.0c software. Unpaired t-tests were used in Figure 3.2, 3.7 and 3.8 using data normalized to siRNA

control values represented as 100%. For dose response curves generated from aequorin assays, one-way analysis of variance followed by Dunnett's test (i.e. compared to respective siRNA control values) was used on pooled normalized values for Ca^{2+} release at the three highest doses of carbachol used for Figures 3.3, 3.4, and 3.5. For MAPK activation assays, one-way analysis of variance followed by Dunnett's correction was used on fold change over basal values normalized to siRNA control conditions in Figure 3.5. All results are expressed as mean ± S.E.M. Data presented are from pooled experiments whose sample sizes (n) and p values are indicated in respective figure legends. A two-tailed p value of $p<0.05$ was considered to be significant.

3.5 Results

3.5.1 Determination of muscarinic acetylcholine receptor subtypes in HEK 293 cells

A microarray analysis of HEK 293 cells was first performed to identify candidate endogenous GPCRs to assess the effect Gβ/Gγ knockdown mediated changes to downstream effector controlled second messenger signalling (data not shown). Of several receptors where expression was detected at the mRNA level, muscarinic acetylcholine receptors (mAChR) were selected for further study based on their relatively high expression levels. Further, it had previously been reported that M3-mAChRs were endogenously expressed in HEK 293 cells [405-407] whereas M1-mAChRs were not [408]. Aequorin-based assays of calcium signalling were used to confirm that M3-mAChR is the specific muscarinic receptor subtype expressed in HEK 293 cells. Pre-treatment of HEK 293 cells stably expressing the calcium-sensing aequorin biosensor (Aequorin-HEK 293 cells) with increasing concentrations of either atropine (a pan-muscarinic receptor antagonist), pirenzepine (an M1-mAChR selective antagonist), or darifenacin (an M3-mAChR selective antagonist) followed by treatment with 10 mM carbachol (a pan-muscarinic receptor agonist) yielded IC_{50} values of 0.4594 nM ($LogIC_{50}$ = -9.34 ± 0.089), 217.2 nM ($LogIC_{50}$ = -6.66 ± 0.059) and 51.97 nM ($LogIC_{50}$ = -7.28 ± 0.039), respectively (Figure 3.1, Table 3.1). Upon comparison with previously reported IC_{50} values of these antagonists for these receptors [409] and expected IC_{50} values as calculated by the Cheng-Prusoff equation (Table 1), it was determined that M3-mAChR was the predominant receptor subtype in our HEK 293 cells. In addition, this observation was confirmed by RT-PCR using total RNA isolated from these cells (data not shown). With regard to the expression of Gβ and Gγ subunits, microarray analysis revealed that all 5 subtypes of Gβ subunits and 8 Gγ subunits

with the exception of $G\gamma_3$, $G\gamma_8$, $G\gamma_9$ and $G\gamma_{13}$ were expressed in our cells (data not shown). These data were confirmed by proteomic analysis of $G\beta_1$- or $G\gamma_2$-interacting proteins (DP, RC and TEH, unpublished data).

3.5.2 An RNAi screen for Gβ and Gγ subunits

We previously conducted a structural and molecular mapping analysis of human Gβ and Gγ subunits [335] where it was noted that Gγ subunits were poorly conserved around their N-termini, hinge region and C-termini on the face external to their Gβ binding regions. In contrast, Gβ subunits displayed fewer regions of low conservation (with the exception of Gβ5). This suggests that Gγ subunits may impart structural and functional diversity to Gβγ dimers in effector modulation. Thus, an RNA interference (RNAi) screen was devised to knockdown Gβ and Gγ subunits in HEK 293 cells to assess specific roles in muscarinic receptor signalling. DsiRNAs against $G\gamma_3$, $G\gamma_8$ and $G\gamma_{13}$ were not included in our screen because cell lines with sufficient mRNA expression could not be identified to validate knockdown efficiency and because these subunits were not expressed in HEK 293 cells. Moreover, DsiRNAs against $G\gamma_{10}$ could not be used due to off-target effects noted in initial experiments. Several (between 9 and 11) different DsiRNAs for the remaining Gγ subunits, used at three different concentrations, were screened in cell types with high detectable expression of their respective Gγ targets in order to determine knockdown efficiencies (Supplemental Figure 3.2a, b). DsiRNAs with the highest knockdown efficiencies when used at concentrations of 10 nM were selected from this initial screen for further use in functional assays. As depicted in Figure 3.2, significant knockdown was obtained for Gγ subunits expressed in these HEK 293 cells. With regard to Gβ subunits, $G\beta_1$ and

$G\beta_4$ were chosen as candidates for our RNAi screen. The rationale behind this choice was that even though $G\beta_{1-4}$ are highly similar, $G\beta_1$ and $G\beta_3$ subunits and $G\beta_2$ and $G\beta_4$ subunits may have evolved as two separate respective groups [335]. $G\beta_1$ and $G\beta_4$ protein knockdown experiments were performed as previously described, yielding similar efficiencies [59].

3.5.3 Identification of specific Gβ and Gγ subunits that modulate M3-mAChR mediated Ca^{2+} release

In order to assess the effect of knocking down Gβ and Gγ subunits individually on GPCR-stimulated generation of second messengers, we applied our RNAi screen in Aequorin-HEK 293 cells to measure changes in intracellular calcium levels following M3-mAChR stimulation. Knockdown of $G\gamma_1$, $G\gamma_2$, $G\gamma_4$ and $G\gamma_7$ (Figure 3.3A-D) resulted in significant decreases in overall carbachol-stimulated calcium release, where as knockdown of $G\gamma_5$, $G\gamma_{11}$ and $G\gamma_{12}$ did not (Figure 3.3E-G). This suggests that the former set of Gγ subunits may be involved in the modulation of PLCβ activity downstream of M3-mAChR. Indeed, knock down of $G\gamma_1$, $G\gamma_2$, $G\gamma_4$ and $G\gamma_7$ all together results in the highest reduction in Ca^{2+} release, by 61% (Figure 3.3I). It should be noted that knockdown of $G\gamma_1$ (Figure 3.3A) had the greatest effect on intracellular calcium release compared to $G\gamma_2$, $G\gamma_4$ and $G\gamma_7$ (Figure 3.3B-D). This implies that even though multiple Gγ subunits may be involved in regulating signalling events downstream of M3-mAChR activation, there is Gγ subunit selectivity in this signalling cascade. As a control, knockdown of $G\gamma_9$, a Gγ subunit that is not expressed in HEK 293 cells, caused no change in calcium release compared to control (Figure 3.3H). This suggests that our DsiRNAs in our RNAi

screen are specific to their targets, and strengthens the notion that our observed signalling phenotypes are not a result of off-target effects.

Similar to the effects of silencing expression of $G\gamma_1$, $G\gamma_2$, $G\gamma_4$, and $G\gamma_7$ subunits individually, knockdown of $G\beta_4$ revealed a 40% decrease in calcium release upon carbachol stimulation (Figure 3.4A), suggesting that $G\beta_4$ is required to activate PLCβ upon receptor activation. In order to evaluate the effect of dual Gβ and Gγ knockdown, we eliminated the Gγ subunits from our RNAi screen that were not implicated in Ca^{2+} release (i.e., $G\gamma_5$, $G\gamma_9$, $G\gamma_{11}$ and $G\gamma_{12}$ were excluded). Next, $G\beta_4$ was knocked down in combination with $G\gamma_1$, $G\gamma_2$, $G\gamma_4$, or $G\gamma_7$, allowing us to identify specific dimers that may be implicated in signalling downstream of M3-mAChR activation. Knockdown of $G\beta_4\gamma_2$, $G\beta_4\gamma_4$, and $G\beta_4\gamma_7$ all caused decreases in Ca^{2+} release upon carbachol treatment (40%, 47%, 48%, respectively; Figure 3.4C-E). Interestingly, knockdown of $G\beta_4\gamma_1$ resulted in the highest loss of Ca^{2+} release (68.46%; Figure 3.4B) compared to the other dimers, an effect greater than the decrease in Ca^{2+} release when $G\beta_4$ and $G\gamma_1$ were knocked down individually. Intriguingly, dual knockdown of $G\beta_4$ and $G\gamma_2$ resulted in no difference in Ca^{2+} release when compared to knockdown of $G\beta_4$ alone (Figure 3.4C vs 3.4A). In addition, combinatorial knockdown of $G\gamma_1$, $G\gamma_2$, $G\gamma_4$ and $G\gamma_7$ together with $G\beta_4$ knockdown yielded a suppression of calcium release that was higher than knockdown of $G\beta_4$ alone (70 vs. 61%, respectively; Figure 3.4F). Taken together, our results suggest that $G\beta_4\gamma_1$ is a predominant and preferential Gβγ dimer modulating PLCβ activity downstream of M3-mAChR activation. It is possible that compensatory changes in M3AChR levels might have occurred in response to Gβ or Gγ knockdown. However, we detected no changes in the EC_{50} values for receptor activation when comparing control and siRNA conditions (Supplemental Table 3.4) suggesting that the

response of the receptor was preserved in each case. We also detected no changes in the number of receptors as determined by ligand binding in preliminary experiments comparing control and $G\gamma_2$ siRNAs (data not shown). Thus we conclude the effects are likely explained by the loss of specific receptor/effector coupling events.

3.5.4 Unexpected effects of $G\beta_1$ knockdown on M3-mAChR mediated signalling activity

As previously mentioned, $G\beta_1$ was included in our RNAi screen as it diverged from $G\beta_4$ over the course of evolution. Interestingly, unlike the signalling phenotypes observed for $G\beta_4$ or $G\gamma$ knockdown, knockdown of $G\beta_1$ resulted in a three-fold increase in calcium release compared to control (Figure 3.5A). With respect to dual knockdown of $G\beta_1$ with the $G\gamma$ subunits in our screen, increases in overall calcium release were blunted when $G\beta_1\gamma_7$, $G\beta_1\gamma_2$, $G\beta_1\gamma_4$ and $G\beta_1\gamma_1$ were knocked down compared to $G\beta_1$ knockdown alone (Figure 3.5B-E). Interestingly, combined knockdown of $G\beta_1$, $G\gamma_1$, $G\gamma_2$, $G\gamma_4$, and $G\gamma_7$ ablated the increased Ca^{2+} release completely compared to control (Figure 3.5F). This suggests that the mechanisms leading to increased signalling output due to $G\beta_1$ knockdown also require an intact $G\beta\gamma$ complex.

3.5.5 Effect of Gβ and Gγ knockdown on M3-mAChR mediated phosphorylation of ERK1/2

Stimulation of M3-mAChRs with carbachol leads to PKC-dependent activation of the MAPK cascade, as detected by phosphorylation of ERK1/2 [410, 411]. To provide a second

readout on the effect of knockdown of specific Gβγ dimers identified as relevant for calcium signalling, we paired our RNAi screen with measures of MAPK activation. Transfected Aequorin-HEK 293 cells were treated with 1 mM carbachol (~EC_{90}) for 5 minutes (as determined in Supplemental Figure 3.1) and level the phosphorylation of ERK1/2 due to M3-mAChRs activation was assessed using immunoblotting. Similar to the approach taken for the RNAi screen coupled to calcium-sensing aequorin assays, Gβ and Gγ subunits were first knocked down individually to assess effects on pERK1/2 levels. As for calcium, knockdown of $G\gamma_1$ and $G\gamma_4$ resulted in decreased levels of carbachol-stimulated M3-mAChR activated phosphorylation of ERK1/2 (Figure 3.6A, B). Similar to what was observed in our aequorin assays, knockdown of $G\gamma_5$, $G\gamma_9$, $G\gamma_{11}$ and $G\gamma_{12}$ did not alter pERK1/2 levels upon receptor activation (Figure 3.6A, B). In contrast to results obtained in calcium assays, knockdown of $G\gamma_2$ and $G\gamma_7$ alone did not affect M3-mAChR mediated phosphorylation of ERK1/2, and in fact knockdown of $G\gamma_2$ resulted in a trend for increased levels of pERK1/2 (Figure 3.6A, B). This suggests that Gγ subunits can propagate distinct signalling events downstream of PLCβ activation. $G\beta_1$ and $G\beta_4$ were knocked down individually to determine which Gβ subunits were implicated in ERK1/2 phosphorylation downstream of M3-mAChR activation. Analogous to what was observed for the aequorin assays, knockdown of $G\beta_4$ showed a trend for decreased levels of pERK1/2 (Figure 3.6C, D) while knockdown of $G\beta_1$ caused a 2-fold increase in phosphorylation of ERK1/2 when normalized to total ERK levels (Figure 3.6C, D). Intriguingly, knockdown of $G\beta_1$ caused a downregulation of total ERK1/2 protein levels (Figure 3.6C). This strengthens the notion that that the signalling phenotypes observed as a result of silencing $G\beta_1$ expression result form changes to global signalosome components, again suggesting that $G\beta_1$ plays roles beyond canonical GPCR signalling.

We next assessed the effect of dual Gβ and Gγ knockdown on carbachol-stimulated phosphorylation of ERK1/2. Knockdown of $G\beta_4$ with $G\gamma_1$, $G\gamma_2$, $G\gamma_4$ and $G\gamma_7$ all resulted in reduced pERK1/2 levels compared to control (Figure 3.6E,F). However, simultaneous knockdown of $G\beta_4$ with $G\gamma_2$ and $G\beta_4$ with $G\gamma_7$ caused a decrease in pERK1/2 levels, unlike knockdown of $G\gamma_2$ and $G\gamma_7$ alone. This implies that decrease in pERK1/2 levels are primarily due to loss of $G\beta_4$ and not as a result of loss of $G\beta_4\gamma_2$ and $G\beta_4\gamma_7$ dimers, which are likely redundant. Similar to what was observed with our calcium assays, simultaneous knockdown of $G\beta_1$ with Gγ subunits all reduced the effect of knockdown of $G\beta_1$ alone. That is, knockdown of $G\gamma_1$, $G\gamma_7$, and $G\gamma_4$ individually with $G\beta_1$ led a reduction in $G\beta_1$ knockdown-induced increases in pERK1/2 in that rank order respectively (Figure 3.6E,F). Interestingly, knockdown of $G\beta_1$ and $G\gamma_2$ reduced the increase observed with $G\beta_1$ knockdown alone to levels similar to control (Figure 3.6E,F), again highlighting the need for functional Gβγ subunits.

3.5.6 Effect of $G\beta_1$ knockdown on M3-mAChR signallosome components

M3-mAChRs have previously been described to interact directly with PLCβ3 [412]. Of the four isoforms of PLCβ known to exist, $PLC\beta_1$, $PLC\beta_3$ and $PLC\beta_4$ are expressed in HEK 293 cells [405]. Of these three isoforms, only $PLC\beta_1$ and $PLC\beta_3$ are directly modulated by Gβγ [413]. It has previously been described that simulatenous knockdown of $G\beta_1$ and $G\beta_2$ in HeLa cells resulted in increased protein expression of adenylyl cyclase subtypes VI and III [414]. Furthermore, with regards to M3-mAChR desensitization following receptor activation,

knockdown of GRK2, GRK3 and GRK6, but not GRK5, resulted in an increase in carbachol-stimulated M3-mAChR mediated calcium release [406]. Results from our RNAi screen and effector assays suggested the possibility that the signalling phenotypes as a result of knockdown of $G\beta_1$ may be due changes in expression of effectors within the M3-mAChR signallosome. Indeed, knockdown of $G\beta_1$ resulted in decreased total ERK1/2 levels (Figure 3.6C). In order to understand how knockdown of $G\beta_1$ leads to increased Ca^{2+} release upon M3-mAChR activation, we first assessed whether of $G\beta_1$ regulated PLCβ3 protein expression. $G\beta_1$ was knocked down and PLCβ3 expression was analyzed using western blot. Loss of $G\beta_1$ did not alter PLCβ3 (Figure 3.7A,G) or $PLC\beta_1$ levels (Figure 3.7A,H). This eliminates the possibility of compensation by other PLC isoforms to cause increased signalling via the M3-mAChR under these RNAi conditions. Interestingly, as in previous studies [59, 414], knockdown of $G\beta_1$ resulted in 1.4±0.2 fold increase in expression of $G\beta_4$ (Figure 3.7A,I). We also sought to determine whether the increased second messenger signalling observed under $G\beta_1$ knockdown conditions were due to changes in levels of Gα subunits involved in signal transduction. Knockdown of $G\beta_1$ did not result in significant changes in levels of $G\alpha_{q/11}$ (Figure 3.7B,J). Furthermore, knockdown of $G\beta_1$ did not result in significant changes in expression of GRK2, GRK3, GRK5, but led to a significant increase in GRK6 expression (Figure 3.7C-F, K-N, respectively).

3.5.7 Transcriptional effects of $G\beta_1$

Our data suggests $G\beta_1$ might serve a non-canonical function distinct from modulation of effector activity in receptor signalling *per se*. Many Gβγ subunits have been found to play a wide

variety of functions beyond their known canonical signalling roles; Gβγ non-canonical functions include regulation of anterograde trafficking from the trans-Golgi apparatus, as well as affects on transcriptional regulators [335]. Given that the effects of $G\beta_1$ knockdown cannot be attributed to loss of agonist-induced GPCR signalling activity, we sought to provide clues regarding the mechanism by which the observed phenotypes occur. We have previously demonstrated that $G\beta_1$ interacts with cFos of the AP-1 transcription factor complex, acting as a negative regulator of AP-1 mediated transcription, suggesting further transcriptional roles of Gβγ in the nucleus [400]. We performed a ChIP-on-chip experiment to analyze whether $G\beta_1$ binds promoters. Human promoter microarrays were used to to assess $G\beta_1$ promoter occupancy whereby promoter regions were defined as 1000 basepairs directly upstream of a given gene's transcription start site. Upon analysis of the ~21,000 best defined human genes on RefSeq at the time, we found that $G\beta_1$ occupied the promoters of more than 700 genes (data not shown). Interestingly, an examination of specific promoter regions where $G\beta_1$ binds revealed that it occupied the promoter of $G\beta_4$ at 4 distinct putative binding regions (Figure 3.8A). To validate this finding, ChIP-qPCR experiments were designed to amplify regions using specific primers for the $G\beta_4$ promoter to which $G\beta_1$ was predicted to bind (regions amplified defined as red regions #1-4; Figure 3.8A). Of the four regions of the $G\beta_4$ promoter that were amplified, regions #3 and #4 displayed a 1.28 fold and 1.42 fold increase in $G\beta_1$ binding over control, respectively (Figure 3.8B). It has previously been described that knockdown of $G\beta_1$ leads to increased protein levels of $G\beta_4$ [59, 414]. However, it is not known how $G\beta_1$ influences this change in expression. In order to assess whether $G\beta_4$ transcript levels were influenced by $G\beta_1$, we quantified its expression upon $G\beta_1$ knockdown. Intriguingly, knockdown of $G\beta_1$ (Figure 3.8D) resulted in no change in Gβ4 mRNA levels as measured by qPCR (Figure 3.8C).

To further examine the putative role of $G\beta_1$ as a modulator of $G\beta_4$ protein expression, we used tandem affinity purification and LC/MS to identify candidate mechanisms by which $G\beta_1$ might regulate $G\beta_4$ levels. In particular, our analysis identified both cytosolic and nuclear $G\beta_1$ interactors using tagged versions of $G\beta_1$ and $G\gamma_7$ under conditions of vehicle or 1 mM carbachol treatment. Our analysis revealed a number of novel interactors in both cellular compartments investigated [184]. We noted interactors involved in shuttling mRNA in and out of the nucleus. Upon subtraction of non-specific interactors from data sets obtained from negative controls, we identified one group of interactors of particular interest – the heterologous ribonuclear (hnRNP) family of proteins. $G\beta_1$ was found to interact with hnRNP C, hnRNP R and hnRNP D-like (Supplemental Table 3.5). In particular, interaction of $G\beta_1$ with these proteins was detected in at least 2 of 3 repeated LC/MS experimental runs following carbachol treatment especially in cytosol-enriched samples. Given that hnRNPs are known to be involved in co-transcriptional processing of nascent mRNA transcripts, mRNA transport, nuclear trafficking and nuclear retention, our data suggests that interaction of the aforementioned hnRNPs with $G\beta_1$ may suggest a novel role for $G\beta_1$ in mRNA processing [415, 416] which could explain the effect on $G\beta_4$ protein levels independently of transcript levels. This would be an interesting area for further study.

3.6 Discussion

Here, using an endogenous GPCR signalling system coupled with an RNAi screen for Gβ and Gγ subunits, we provide insight into how effectors downstream of a GPCR are modulated by Gβγ dimers of specific subunit composition. Previous studies have characterized GPCR signalling modulation by specific Gβ and Gγ subunits [384, 386, 392, 414, 417-421] [reviewed in 335], however, our approach involved a more comprehensive RNAi screen that attributes multiple roles in functional modulation of a second messenger system by specific Gβγ subunits in HEK 293 cells. Overall, we draw two main conclusions from our studies. The first is that $G\beta_4\gamma_1$ comprises the key specific Gβγ dimer that modulates calcium signalling downstream of M3-mAChRs in HEK 293 cells. This may be different in other cells. The second is that $G\beta_1$ subunits play roles beyond their known canonical signalling function. This is potentially via interactions with other protein partners, our data suggests it occupies the promoters of various genes and may function in co-transcriptional modulation of $G\beta_4$ synthesis.

The results from our calcium biosensor assays and MAPK assays reveal that specific combinations of Gβ and Gγ subunits, in particular $G\beta_4\gamma_1$, $G\beta_4\gamma_4$ and $G\beta_4\gamma_7$, are involved in the modulation of effectors signalling downstream of M3-mAChR activation. Based on results from both assays, it is evident that loss of $G\beta_4\gamma_1$ had profound effects on both calcium release and phosphorylation of ERK1/2 compared to loss of $G\beta_4\gamma_4$ and $G\beta_4\gamma_7$. The extent to which these effectors is modulated by these dimers is in the order of $G\beta_4\gamma_1 >> G\beta_4\gamma_4 > G\beta_4\gamma_7$. This suggests that the $G\beta_4\gamma_1$ is a predominant and preferred Gβγ dimer associated with M3-mAChR signalling complexes, but also that there is at least some redundancy. Indeed, evidence of such redundancy

has previously been reported with respect to the functional coupling of galanin receptors to voltage-gated calcium channels by G proteins consisting of $G\alpha_{o1}\beta_2\gamma_2$ and $G\alpha_{o1}\beta_3\gamma_4$ [418]. However, it must be noted that this redundancy is Gβγ dimer-specific, and limited. Moreover, with regard to modulation of PLCβ, our analysis provides evidence that the extent to which a Gβγ dimer preferentially modulates PLCβ3 activity is determined by which specific Gγ subtype is present. That is, knockdown of $G\beta_4\gamma_1$ produced an even greater decrease in Ca^{2+} release compared to $G\beta_4$ alone than $G\beta_4\gamma_4$ or $G\beta_4\gamma_7$ knockdown compared to $G\beta_4$ alone (Figure 3.4A-E). However, modulation of ERK1/2 phosphorylation was observed to be more dependent on the interacting Gβ subunit alone as no significant differences were observed between $G\beta_4\gamma_1$, $G\beta_4\gamma_4$ and $G\beta_4\gamma_7$ knockdown conditions (Figure 3.6F). In other words, regardless of which Gγ subunit was implicated, knockdown of that particular subunit with $G\beta_4$ produced similar decreases in pERK1/2 levels. This suggests an increased layer of selectivity and definition of the roles played by Gβ and Gγ subunits individually although they are obligate dimers. In addition, the differences observed in the extents to which these dimers modulated PLCβ and MAPK cascade activity may be explained by the proximity to which these effector cascades are to Gβγ activation. That is, the effects of knockdown produced more striking effects in our calcium assays as PLCβ3 is more proximal to receptor activation compared to MAPK activation which is more distal.

We further provide insight into a novel non-canonical role for $G\beta_1$ in GPCR signalling. Our results depicting loss of signalling due to knockdown of Gβ or Gγ expression are expected as these observations can be explained by loss of required signalling partners that propagate signalling upon GPCR activation. However, $G\beta_1$ knockdown led to a paradoxical increase in

downstream signalling (Figure 3.5A, 3.6D). Such increases were lost when specific Gγ subunits were knocked down simultaneously (Figure 3.5B-F, 3.6F) – likely through effects on $G\beta_4$ containing dimers. The lack of increase in Ca^{2+} observed when $G\gamma_1$, $G\gamma_2$, $G\gamma_4$ and $G\gamma_7$ were all knocked down in combination with $G\beta_1$ in turn suggests that regardless of the independent effects of $G\beta_1$ on the cellular environment, partner Gγ subunits are absolutely required for $G\beta_1$-mediated effects on signalling and their loss can still override such mechanisms (Figure 3.5F). We tested whether the phenotypes observed under conditions of loss of $G\beta_1$ resulted as a consequence of (1) dysregulation of signalling control at the level of effector expression, in which increased levels of effector would be observed or (2) at the level of receptor desensitization upon agonist-treatment, in which decreases of GRK proteins would be observed. Our analysis of the expression of signallosome components of the M3-mAChR reveal that $G\beta_1$ knockdown did not alter levels of $PLC\beta^3$ and $PLC\beta_1$ (Figure 3.7A, G, H), nor did it decrease protein expression of GRK2, GRK3, GRK5 (Figure 3.7C-E, K-M), all of which have been implicated in controlling M3-mAChR desensitization [406]. Intriguingly, knockdown of $G\beta_1$ resulted in increased GRK6 expression (Figure 3.7F, N), further suggesting that increases in signalling observed under these conditions are not due to decreased receptor desensitization *per se*. The effect of $G\beta_1$ knockdown is likely due to the observed effects on $G\beta_4$ levels, as $G\beta_4$ is critical to effector coupling that we measured.

Our studies uncover mechanisms by which $G\gamma_2$ may also regulate components of M3-mAChR complexes through dimerization with other Gβ subunits. Our aequorin assays reveal that knockdown of $G\gamma_2$ alone results in decreased calcium release upon M3-mAChR activation (Figure 3.3B), whereas dual knockdown of $G\gamma_2$ with $G\beta_4$ produced no change in Ca^{2+} release

compared to knockdown of $G\beta_4$ alone (Figure 3.4A, C). Had $G\gamma_2$ been in a dimer with $G\beta_4$, we would expect a further decrease in Ca^{2+} release, as observed with dual $G\beta_4$ and $G\gamma_1$ knockdown (Figure 3.4B). Furthermore, dual knockdown of $G\gamma_2$ with $G\beta_1$ supressed the increase in Ca^{2+} release observed when $G\beta_1$ is knocked down. Dual knockdown of $G\beta_1\gamma_2$ would cause a further increase in carbachol stimulated Ca^{2+} release, yet we observed a decrease of that increased Ca^{2+} release. These findings suggest that $G\gamma_2$ dimerizes with another Gβ subunit – that is, as either $G\beta_2$ or $G\beta_3$ expressed in HEK 293 cells – that in turn opposes the actions of $G\beta_1$ in its control of the M3-mAChR system components.

Previous studies have described Gβγ mediated effects in the nucleus and its roles in the regulation of transcriptional activity [239, 240, 246, 400, 422, 423]. Here, we describe for the first time that $G\beta_1$ is able to interact with proteins directly involved in co-transcriptional events related to mRNA processing in the nucleus. In addition, we also demonstrate that $G\beta_1$ can occupy the promoters of various genes using ChIP on chip (data not shown) and ChIP-qPCR (Figure 3.8). With regard to the observation of no change in total $G\beta_4$ mRNA levels, we speculate that $G\beta_1$ does not alter control of $G\beta_4$ mRNA transcription, suggesting that there are other factors in this transcriptional complex that remain to be identified or that $G\beta_1$ is perhaps a co-transcriptional modulator. One potential mechanism may involve heterologous ribonuclear proteins family (hnRNPs). hnRNPs are RNA binding proteins that have been found to have roles in various aspects of RNA processing and expression, ranging from RNA capping, mediating RNA stability, nuclear export, splicing and modulation of mRNA translation [415]. Indeed, our results suggest that hnRNP C1/C2 is an interacting partner of $G\beta_1$, having been detected in our LC/MS experiments especially in response to receptor activation by carbachol (Supplemental

Table 3.4). hnRNP C1/C2, core constituents of the 40S ribonucleoprotein particles, contain a nuclear retention sequence and thus have been suggested to play roles in nuclear retention of pre-mRNA and mRNA stability [424-426]. Furthermore, these proteins are multimeric RNA binding proteins that bind intronic regions and are crucial for the packaging of co-transcriptional packaging of most RNAs [427, 428]. Thus in the context of increased $G\beta_4$ protein expression under $G\beta_1$ knockdown conditions (Figure 3.7A,I), loss of $G\beta_1$ change interactions with hnRNP C1/C2, altering the processing of its $G\beta_4$ pre-mRNA, nuclear retention and eventual export to the cytosol. Our data also suggest interactions between $G\beta_1$ and hnRNP R as well as hnRNP D. These proteins have been shown to play roles in mRNA splicing as well as mRNA shuttling between the cytoplasm and the nucleus [429, 430], furthering the potential involvement of $G\beta_1$ in mRNA processing.

Taken together, our results suggest that limited but redundant $G\beta\gamma$ dimers are capable of modulating signalling downstream of M3-mAChRs. While the mechanisms leading to decreases in overall second messenger signalling (due to $G\beta_4$, $G\gamma_1$, $G\gamma_2$, $G\gamma_4$, or $G\gamma_7$ knockdown) may be the result of an effector coupled to a receptor losing its cognate signalling modulator, it is likely that the phenotypes obtained where overall increases in second messenger signalling are observed due to $G\beta_1$ knockdown to disruption of non-canonical signalling events. It is likely that other $G\beta$ and $G\gamma$ subunits also subserve a mixture of canonical and noncanonical signalling events. There is still a great deal we do not know.

3.7 Conclusions

- $G\beta_4\gamma_1$ is the key specific Gβγ dimer that modulates signalling activity downstream of M3-mAChR activation.
- Our data broadens the current understanding of the specificity of Gβγ dimers in GPCR signalling and re-affirms the notion that the functions of these dimers are not entirely redundant.
- Our data reveals, for the first time, a novel non-canonical role for $G\beta_1$ whereby this subunit occupies the promoters of various genes. The function of this role remains to be elucidated, although our data provide clues for roles of $G\beta_1$ as co-transcriptional regulator of mRNA export.

3.8 Acknowledgements

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oligonucleotides for sale similar to some of the compounds described in the manuscript. IDT is however not a publicly traded company and the authors do not personally own any shares or equity in IDT.

3.9 Figure Legends for Chapter 3

Figure 3.1 ***HEK 293 cells endogenously express only the M3 subtype of muscarinic acetylcholine receptors.***

Naïve HEK 293 cells stably overexpressing the calcium sensing aequorin biosensor (aequorin-HEK 293) were loaded with 5 μM coelenterazine for 3 hours, pretreated with increasing amounts of indicated antagonist for 5 minutes and then treated with 10 mM carbachol. Calcium release readings were obtained as a measure of luminescence emitted and values obtained were normalized as a percentage of the lowest concentration of antagonist used. Data is represented as mean ± S.E.M. of two independent experiments for the atropine curve and of three independent experiments for the pirenzepine and darifenacin curves.

Figure 3.2 ***Validation of knockdown of individual Gγ isoforms in HEK 293 cells.***

HEK 293 cells were reverse transfected with 10 nM of DsiRNAs against the indicated targets in panels A-G for a duration of 72 hours. Total RNA was then isolated, reverse transcribed and indicated transcripts were then amplified quantitatively by qPCR. Data is depicted as mean ± S.E.M. and is representative of at least three independent pooled experiments. Means were compared using Student's t-test; ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Figure 3.3 ***The effect of individual and combinational Gγ knockdown on carbachol stimulated, M3-mAChR mediated calcium release.***

Aequorin-HEK 293 cells were reverse transfected for 72 hours with 10 nM of DsiRNA against the indicated Gγ subunits endogenously (A-G), DsiRNA against $G\gamma_9$ not expressed in HEK 293

cells (H), or combinational knockdown of $G\gamma_{1/2/4/7}$ (I). Cells were harvested, loaded with 5 μM coelenterazine for 3 hours and treated with increasing concentrations of carbachol. Calcium release was recorded as a measure of luminescence emitted, and resulting values were normalized to calcium release upon vehicle treatment. Data is depicted as mean ± S.E.M and is representative of three independent pooled experiments. Calcium release at individual doses of carbachol were analysed using one-way ANOVA followed by a Dunnett's multiple comparisons post-hoc test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 3.4 ***Knockdown of Gβ$_4$ (alone or in combination with Gγ) results in a decrease in Ca^{2+} release upon M3-mAChR activation.***

$G\beta_4$ was knocked down using 50 nM of siRNA either alone (A) or in combination with 10 nM DsiRNAs against $G\gamma_1$, $G\gamma_2$, $G\gamma_4$ or $G\gamma_7$ (individually: B-E; in combination: F) for a duration of 72 hours in Aequorin-HEK 293 cells. The efficiency of knockdown of $G\beta_4$ was assessed using western blot (*Inset*, A). Calcium release was measured as previously described in Figure 3. Data is depicted as mean ± S.E.M and is representative of six independent pooled experiments. Calcium release at individual doses of carbachol were analysed using one-way ANOVA followed by a Dunnett's multiple comparisons post-hoc test.

Figure 3.5 ***Gβ$_1$ knockdown results in overall increases in Ca^{2+} release.***

Similar to Figure 4, $G\beta_1$ was knocked down using 50 nM of siRNA either alone (A) or in combination with 10 nM DsiRNAs against $G\gamma_1$, $G\gamma_2$, $G\gamma_4$ or $G\gamma_7$ (individually: B-E; in combination: F) for a duration of 72 hours in aequorin-HEK 293 cells. The efficiency of knockdown of $G\beta_1$ was assessed using western blot (*Inset*, A). Calcium release was measured as

previously described in Figure 3. Data is depicted as mean ± S.E.M and is representative of six independent pooled experiments. Calcium release at individual doses of carbachol were analysed using one-way ANOVA followed by a Dunnett's multiple comparisons post-hoc test. * $p<0.05$.

Figure 3.6 ***Effect of Gβ and Gγ knockdown on carbachol stimulated, M3-mAChR mediated phosphorylation of ERK1/2.***

Aequorin-HEK 293 cells were reverse transfected with DsiRNAs against Gγ subunits (A; individually), Gβ subunits (C; individually), or Gβ and Gγ subunits (E; combinational) for 72 hours. Cells were lysed in MAPK lysis buffer, and phospho-ERK1/2 and total ERK1/2 protein levels were assessed by western blot. Quantifications of the blots are depicted in B, D, and F for individual Gγ knockdown analysis, individual Gβ knockdown analysis and combinational Gβ and Gγ analysis respectively. Resulting bands were quantified using densitometry and values were normalized to control conditions and vehicle treated conditions. Data is represented as fold change over basal ± S.E.M and is representative of at least three independent pooled experiments. Fold change over basal values were statistically analyzed using one-way ANOVA followed by a Dunnett's multiple comparisons post-hoc test. * $p<0.05$.

Figure 3.7 ***Knockdown of $G\beta_1$ does not change protein expression levels of components of the M3-mAChR signalling complex.***

Aequorin-HEK 293 cells transfected with 50 nM of siRNA $G\beta_1$ were lysed in 1X RIPA buffer and protein expression of $PLC\beta_1$, $PLC\beta_3$, $G\beta_4$, $G\alpha_{q/11}$, GRK2, GRK3, GRK5 and GRK6 (A-F) was subsequently analyzed by western blot. Resulting bands were quantifed by densitometry, normalized to β-tubulin bands (loading control) and subsequently normalized to siRNA control

conditions for PLCβ_1 (G), PLCβ_3 (H), Gβ_4 (I) G$\alpha_{q/11}$ (J), GRK2 (K), GRK3 (L), GRK5 (M), and GRK6 (N). Data is represented as mean ± S.E.M for three independent pooled experiments. Fold change over control values were statistically analyzed using Student's t-test.

Figure 3.8 ***Gβ$_1$ occupies and sits on the promoter of Gβ$_4$.***

(A) Gene track data representation of the regions of the Gβ_4 gene and promoter region where Gβ_1 was discovered to bind according to ChIP-on-CHIP data. (B) – Validation of promoter occupancy by Gβ_1 using ChIP to pull down Gβ_4 promoter regions depicted in (A), and amplified using qPCR using region specific primers. Data is represented as fold enrichment over no antibody control (for ChIP) ± S.E.M. (C and D) Assessment of Gβ_4 (C) and Gβ_1 (D) mRNA levels upon Gβ_1 knockdown in aequorin-HEK 293 cells using qPCR. Data is depicted as mean ± S.E.M. and is representative of at least three independent pooled experiments. Means were statistically analyzed using Student's t-test. *** $p<0.001$.

Table 3.1 ***Observed and expected IC$_{50}$ values for antagonists used to characterize mAChR subtype expression.***

Calcium release was measured under conditions of pre-treatment with increasing concentrations of atropine, pirenzepine or darifenacin followed by 10 mM carbachol treatment as described in Figure 1. Observed IC50 values were obtained from curve-fitting data reported in GraphPad Prism 6. * indicates as described in [409]; ** indicates values as determined by the Cheng-Prusoff equation. Data is represented as mean ± S.E.M. of two independent experiments for atropine and three independent experiments for pirenzepine and darifenacin.

Supplemental Figure 3.1 ***Establishment of carbachol treatment conditions to obtain maximal phosphorylation of ERK1/2.***

(A) Aequorin-HEK 293 cells were treated with 1 mM carbachol or vehicle for the indicated time periods, cells were lysed in MAPK lysis buffer, and phospho-ERK1/2 levels were assessed using Western blot. (B) Bands resulting from the Western blot analysis were quantified using densitometry, and pERK1/2 levels were normalized to total ERK1/2 levels in each respective time sample, and resulting normalized ratios were calculated as a fold change over a 0 minutes treatment control. 5 minutes of 1 mM carbachol treatment was chosen as a optimal treatment condition because resulting fold change over control pERK1/2:totalERK1/2 ratios were maximal at this condition.

Supplemental Figure 3.2a ***Establishment of optimal knockdown conditions for individual $G\gamma_1$, $G\gamma_2$, $G\gamma_4$, $G\gamma_5$ subunits.***

To identify the best DsiRNAs and establish optimal transfection conditions, 9 to 11 different DsiRNAs were tested to obtain the highest level of knockdown for each Gγ subunit target. Cell lines in which each respective Gγ target found to be highly expressed are indicated above each graph. DsiRNAs were transfected for that particular target at three concentrations – 0.1, 1 and 10 nM. The 5' nuclease qPCR assays used to assess percentage values of the mRNA of targets upon knockdown are also indicated per graph. Red arrows indicate the particular DsiRNA and concentration chosen for subsequent use and assays.

Supplemental Figure 3.2b ***Establishment of optimal knockdown conditions for individual $G\gamma_7$, $G\gamma_9$, $G\gamma_{11}$, $G\gamma_{12}$ subunits.***

To identify the best DsiRNAs and establish optimal transfection conditions, 10 different DsiRNAs were tested to obtain the highest level of knockdown for each Gγ subunit target. Optimization of DsiRNAs were performed identically to as described in Supplemental Figure 2a. Red arrows indicate the particular DsiRNA and concentration chosen for subsequent use and assays.

Supplemental Table 3.1 ***Sequences of DsiRNAs chosen for use in cellular assays.***

DsiRNAs used for RNAi in conjunction with cell based assays. 10 nM of each listed DsiRNA was used for knockdown experiments. Sequences were obtained from proprietary design algorithms from Integrated DNA Technologies (Coralville, IA, USA).

Supplemental Table 3.2 ***Sequences of primers and probes used for qPCR assays.***

Sequences listed were used at concentrations of 500 nM for each reverse and forward primer, and 250 nM for each respective qPCR probe. Sequences were obtained using proprietary design algorithms from Integrated DNA Technologies (Coralville, IA, USA).

Supplemental Table 3.3 ***Sequences of primers used for ChIP-qPCR experiments.***

Primers used to amplify promoter regions of the Gβ4 gene to which $G\beta_1$ was found to occupy/bind.

Supplemental Table 3.4 ***Dose response analysis of calcium signalling following Gβ or Gγ knockdown.***

LogEC50 values were obtained from dose response curves generated from calcium sensing aequorin assays upon knockdown of Gβ or Gγ subunits either individually or in combination. None of the LogEC50 values obtained from the different Gβ and Gγ knockdown curves were statistically different from their respective controls. Statistical significance of these values was assessed using one-way ANOVA followed by a Dunnett's post-hoc correction ($p<0.05$).

Supplemental Table 3.5 ***Members of the heterologous nuclear ribonucleoprotein (hnRNP) family interacting with $G\beta_1$ or $G\beta_1\gamma_7$ using a proteomics approach.***

TAP-tagged $G\beta_1$ or split-TAP tagged $G\beta_1$ and $G\gamma_7$ were used to identify novel interactors using LC/MS in both cytosolic and nuclear fractions in carbachol treated or untreated HEK 293 cells. Average number of peptides identified, average unique peptides and average coverage for each identified interacting hnRNP is depicted. At least three independent experiments were run for each TAP screen.

3.10 Figures for Chapter 3

Table 3.1 – ***Observed IC50 values for Muscarinic Acetylcholine receptor antagonists in HEK 293 cells***

	Atropine	Pirenzepine	Darifenacin
Observed LogIC50 (M)	-9.34 ± 0.089	-6.66 ± 0.059	-7.28 ± 0.039
Observed IC50	0.4594 nM	217.2 nM	51.97 nM
Expected IC50 (for M3-mAChR)	0.44 nM*	253.9 nM**	71.137 nM**

Figure 3.1

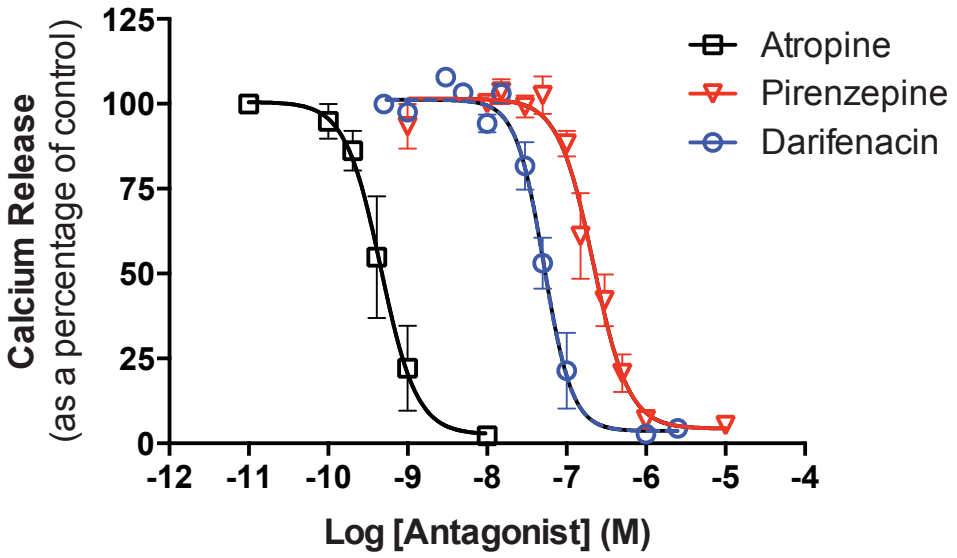
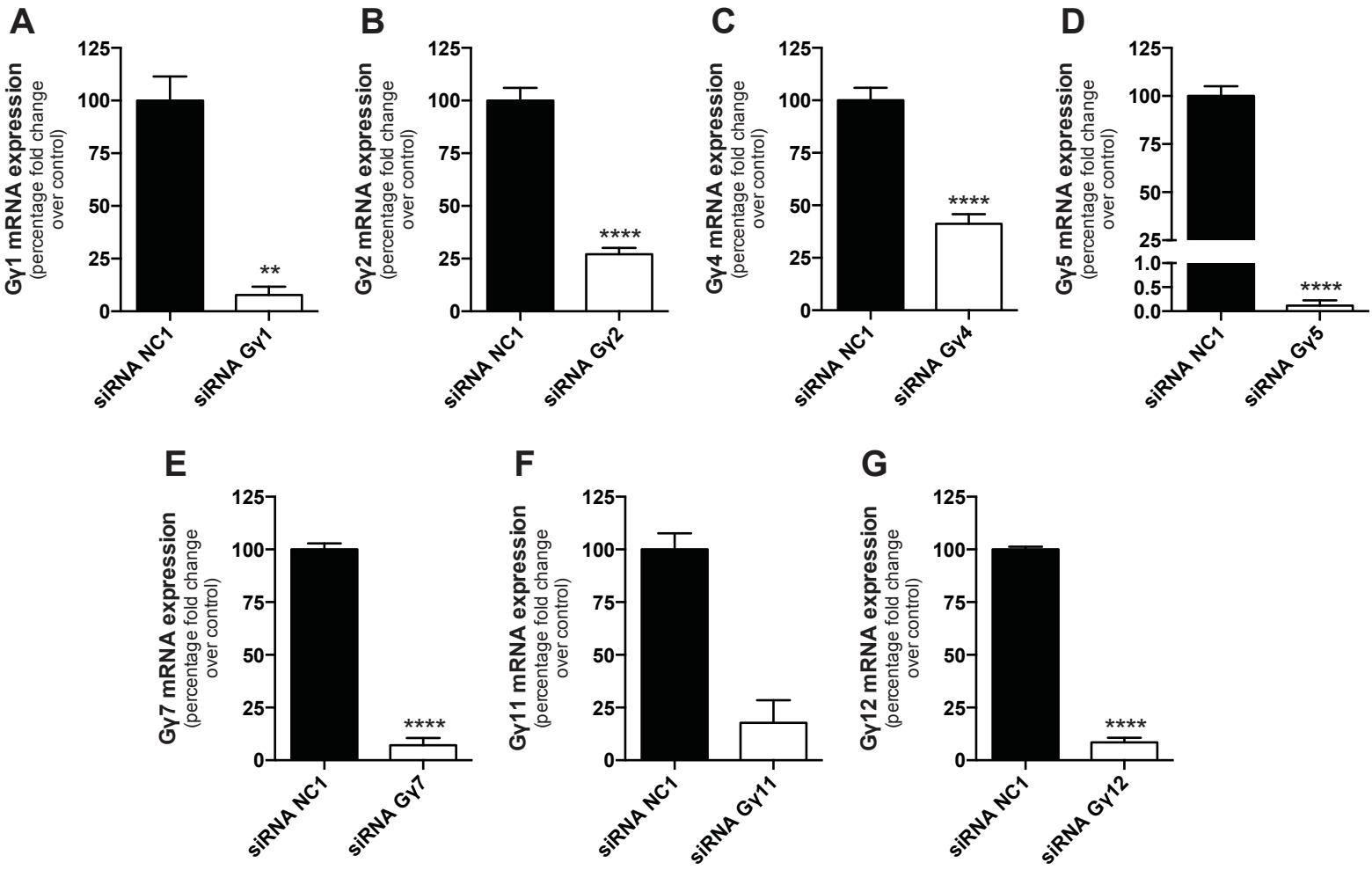

Calcium Release
(as a percentage of control)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
-4
Log [Antagonist] (M)
Atropine
Pirenzepine
Darifenacin

Figure 3.2


A
Gγ1 mRNA expression
(percentage fold change over control)
125
100
75
50
25
0
**
siRNA NC1
siRNA Gγ1
B
Gγ2 mRNA expression
(percentage fold change over control)
125
100
75
50
25
0
****
siRNA NC1
siRNA Gγ2
C
Gγ4 mRNA expression
(percentage fold change over control)
125
100
75
50
25
0
****
siRNA NC1
siRNA Gγ4
D
Gγ5 mRNA expression
(percentage fold change over control)
125
100
75
50
25
1.0
0.5
0.0
****
siRNA NC1
siRNA Gγ5
E
Gγ7 mRNA expression
(percentage fold change over control)
125
100
75
50
25
0
****
siRNA NC1
siRNA Gγ7
F
Gγ11 mRNA expression
(percentage fold change over control)
125
100
75
50
25
0
siRNA NC1
siRNA Gγ11
G
Gγ12 mRNA expression
(percentage fold change over control)
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100
75
50
25
0
****
siRNA NC1
siRNA Gγ12

Figure 3.3

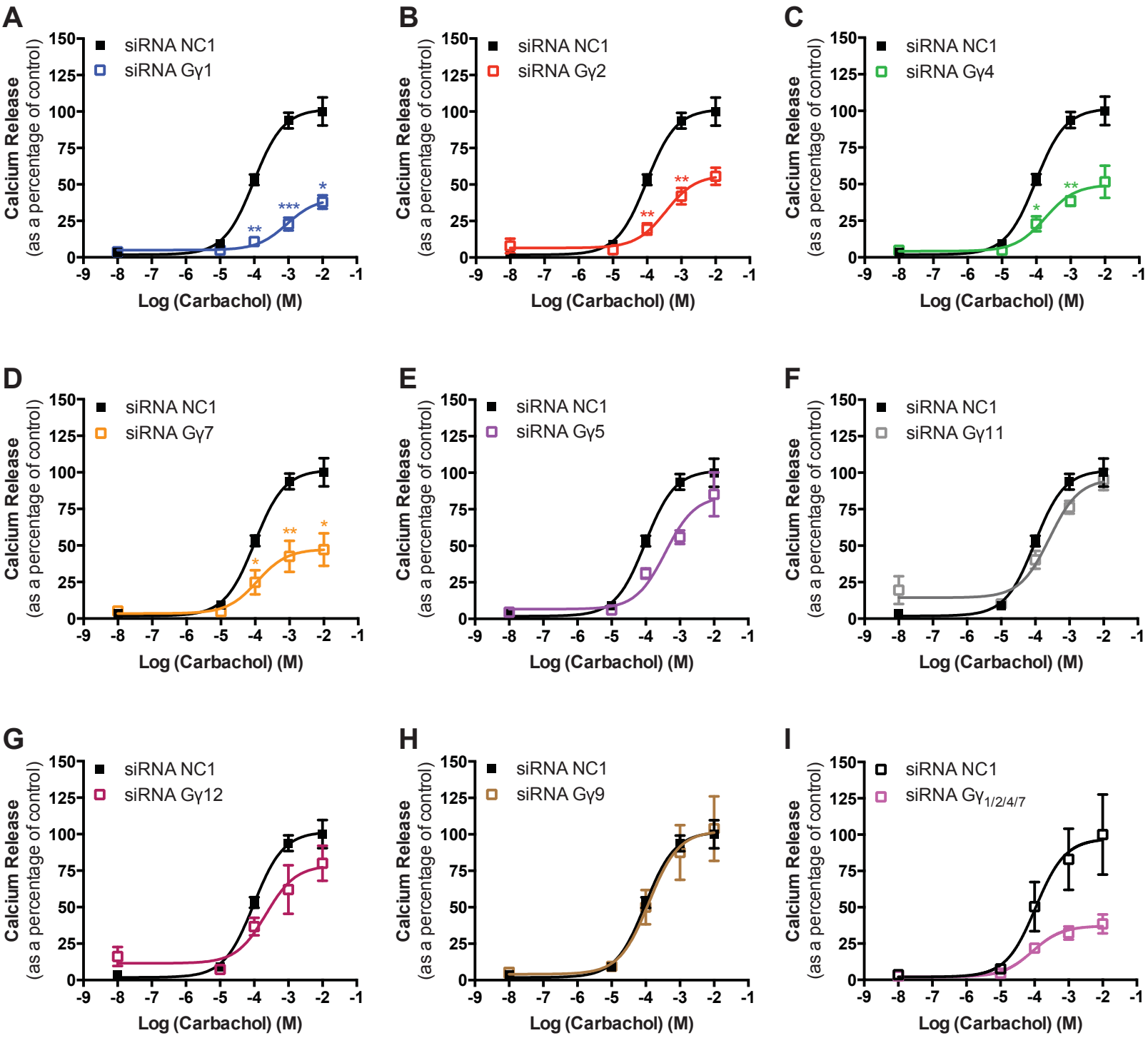

A
siRNA NC1
siRNA Gγ1
B
siRNA NC1
siRNA Gγ2
C
siRNA NC1
siRNA Gγ4
D
siRNA NC1
siRNA Gγ7
E
siRNA NC1
siRNA Gγ5
F
siRNA NC1
siRNA Gγ11
G
siRNA NC1
siRNA Gγ12
H
siRNA NC1
siRNA Gγ9
I
siRNA NC1
siRNA Gγ1/2/4/7
Calcium Release
(as a percentage of control)
Log (Carbachol) (M)

Figure 3.4

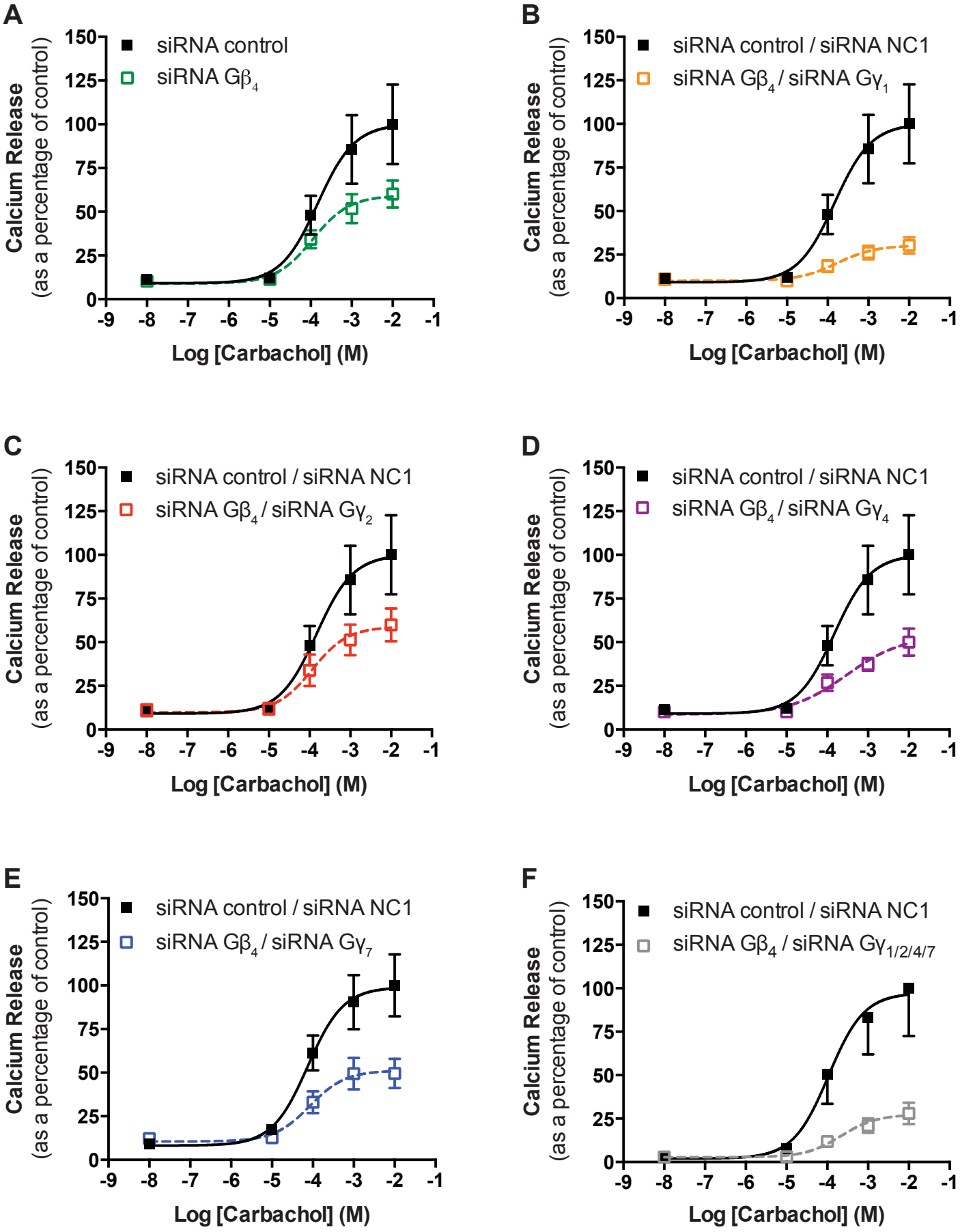

A
siRNA control
siRNA Gβ4
B
siRNA control / siRNA NC1
siRNA Gβ4 / siRNA Gγ1
C
siRNA control / siRNA NC1
siRNA Gβ4 / siRNA Gγ2
D
siRNA control / siRNA NC1
siRNA Gβ4 / siRNA Gγ4
E
siRNA control / siRNA NC1
siRNA Gβ4 / siRNA Gγ7
F
siRNA control / siRNA NC1
siRNA Gβ4 / siRNA Gγ1/2/4/7
Calcium Release
(as a percentage of control)
Log [Carbachol] (M)
150
125
100
75
50
25
0
-9
-8
-7
-6
-5
-4
-3
-2
-1

Figure 3.5

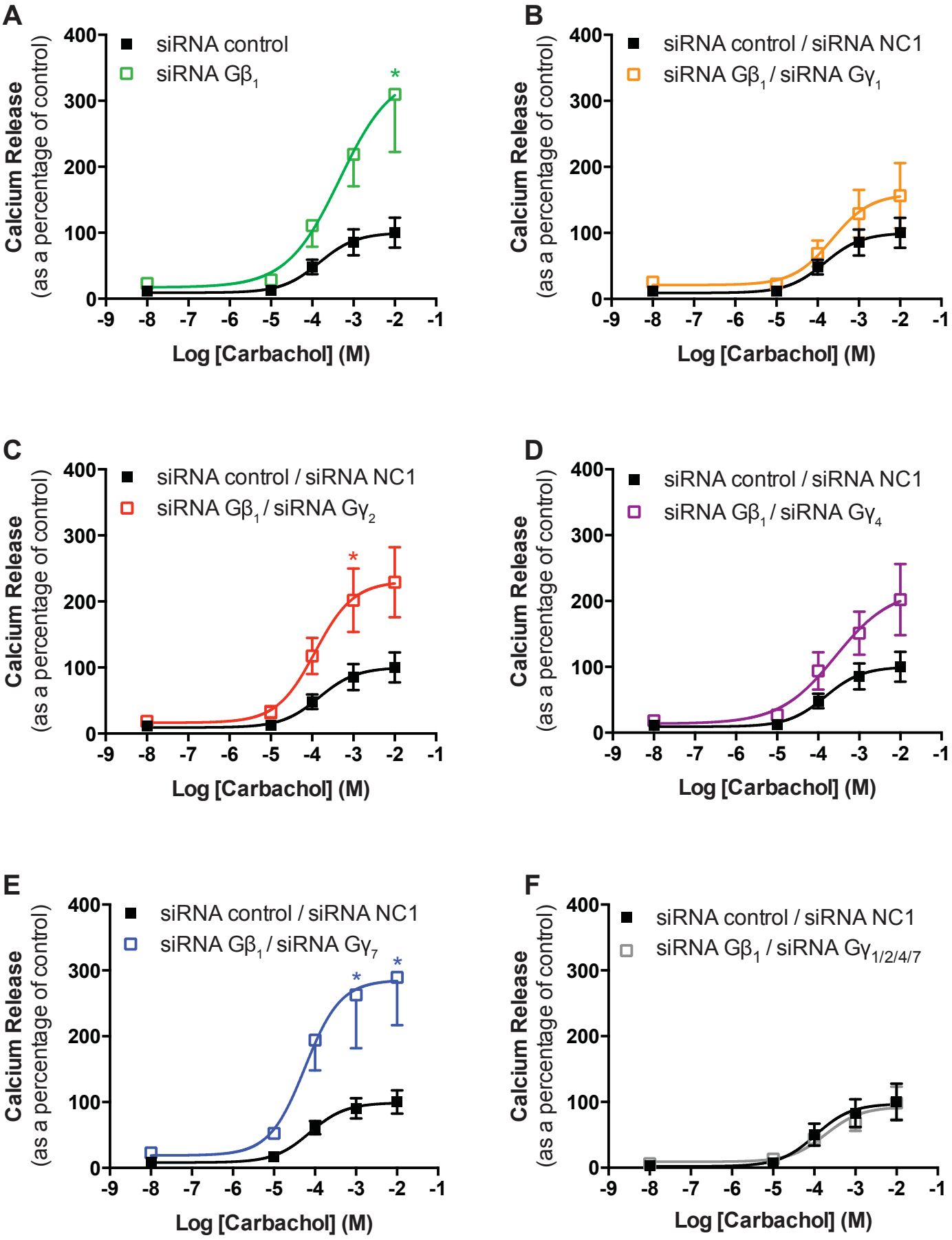

A
siRNA control
siRNA Gβ1
Calcium Release
(as a percentage of control)
Log [Carbachol] (M)
B
siRNA control / siRNA NC1
siRNA Gβ1 / siRNA Gγ1
C
siRNA control / siRNA NC1
siRNA Gβ1 / siRNA Gγ2
D
siRNA control / siRNA NC1
siRNA Gβ1 / siRNA Gγ4
E
siRNA control / siRNA NC1
siRNA Gβ1 / siRNA Gγ7
F
siRNA control / siRNA NC1
siRNA Gβ1 / siRNA Gγ1/2/4/7

Figure 3.6

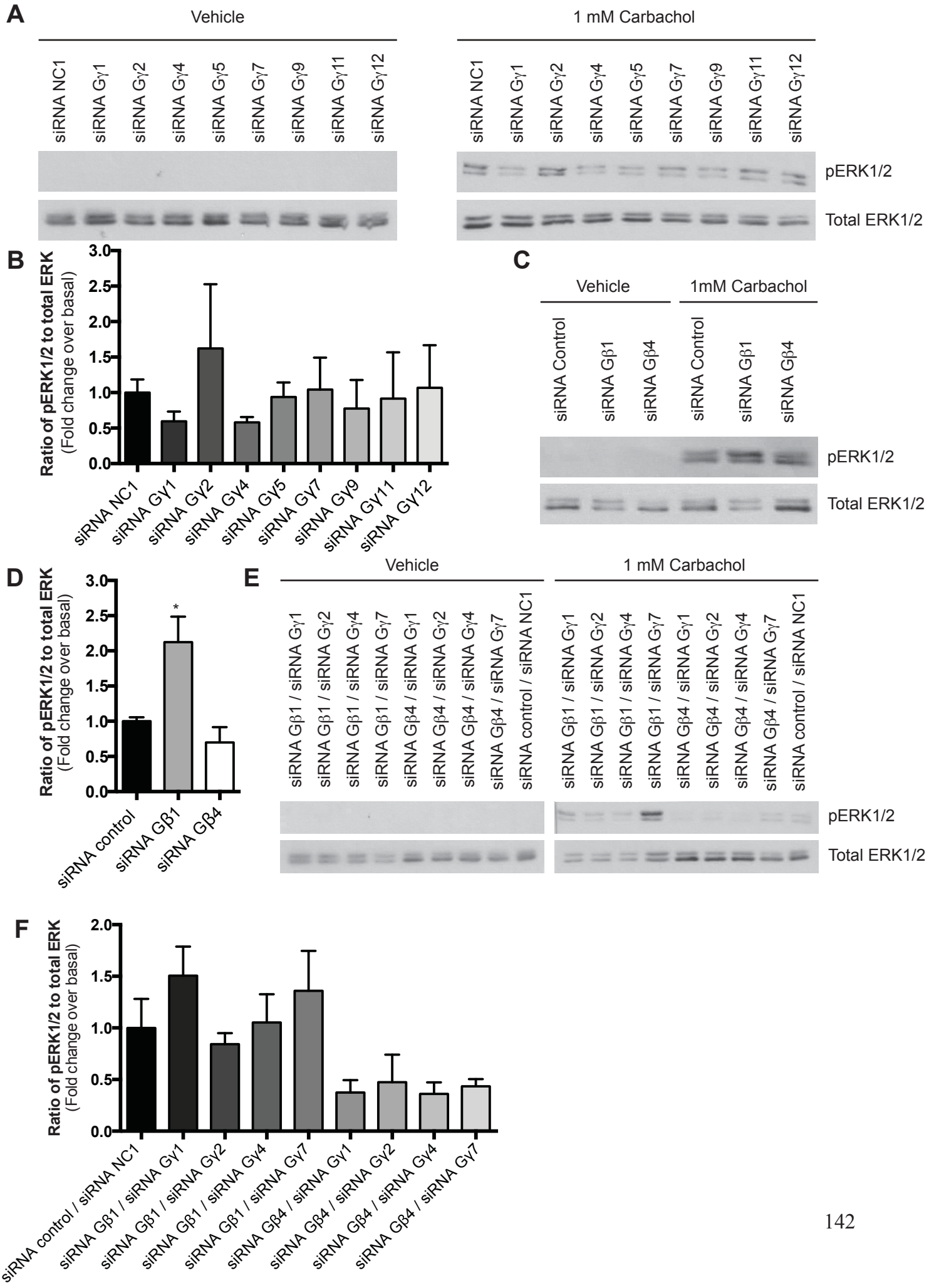

A
Vehicle
1 mM Carbachol
siRNA NC1
siRNA Gγ1
siRNA Gγ2
siRNA Gγ4
siRNA Gγ5
siRNA Gγ7
siRNA Gγ9
siRNA Gγ11
siRNA Gγ12
pERK1/2
Total ERK1/2
B
Ratio of pERK1/2 to total ERK
(Fold change over basal)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
C
Vehicle
1mM Carbachol
siRNA Control
siRNA Gβ1
siRNA Gβ4
D
*
siRNA control
siRNA Gβ1
siRNA Gβ4
E
siRNA Gβ1 / siRNA Gγ1
siRNA Gβ1 / siRNA Gγ2
siRNA Gβ1 / siRNA Gγ4
siRNA Gβ1 / siRNA Gγ7
siRNA Gβ4 / siRNA Gγ1
siRNA Gβ4 / siRNA Gγ2
siRNA Gβ4 / siRNA Gγ4
siRNA Gβ4 / siRNA Gγ7
siRNA control / siRNA NC1
F
siRNA control / siRNA NC1
siRNA Gβ1 / siRNA Gγ1
siRNA Gβ1 / siRNA Gγ2
siRNA Gβ1 / siRNA Gγ4
siRNA Gβ1 / siRNA Gγ7
siRNA Gβ4 / siRNA Gγ1
siRNA Gβ4 / siRNA Gγ2
siRNA Gβ4 / siRNA Gγ4
siRNA Gβ4 / siRNA Gγ7

Figure 7

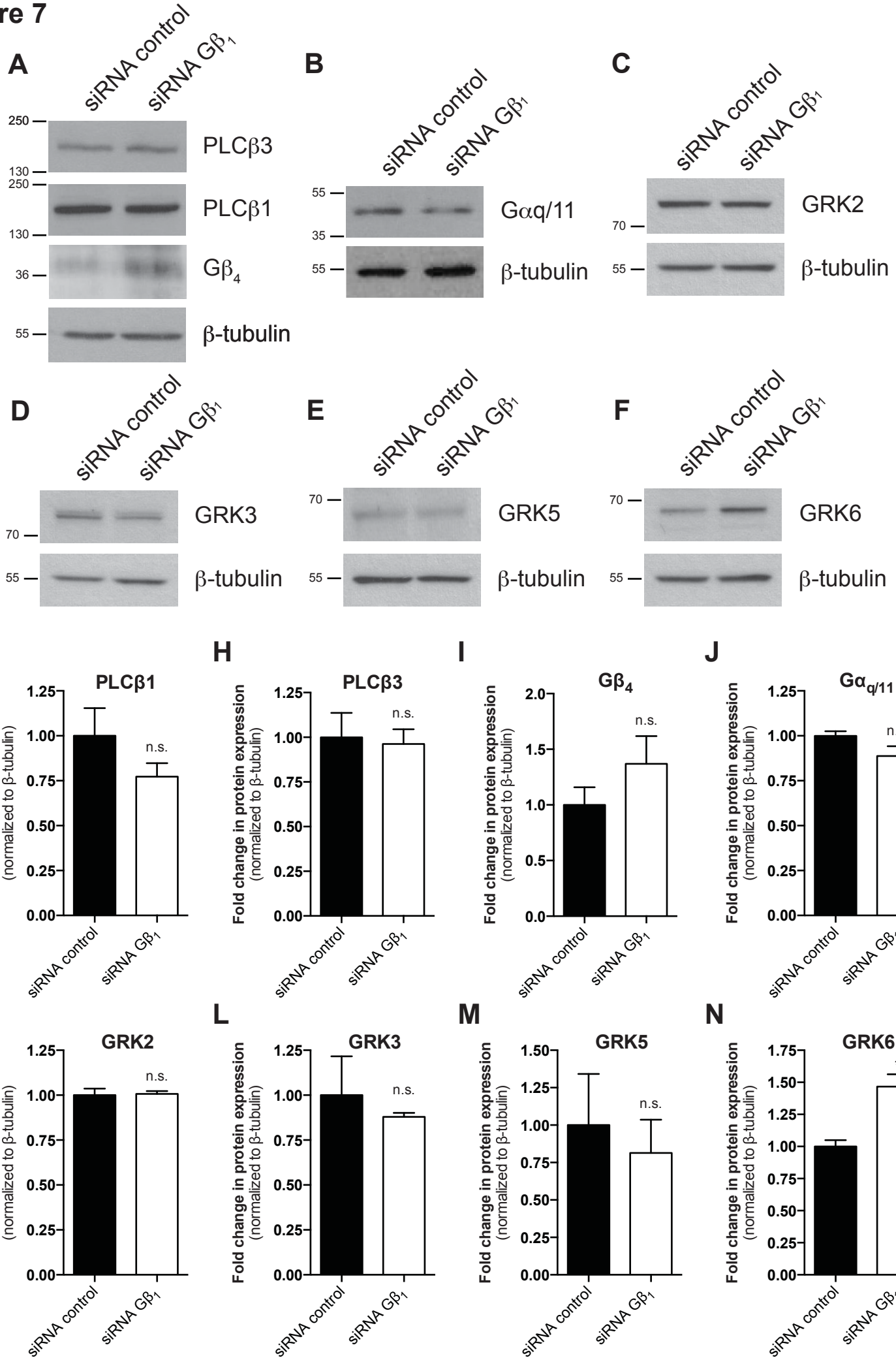
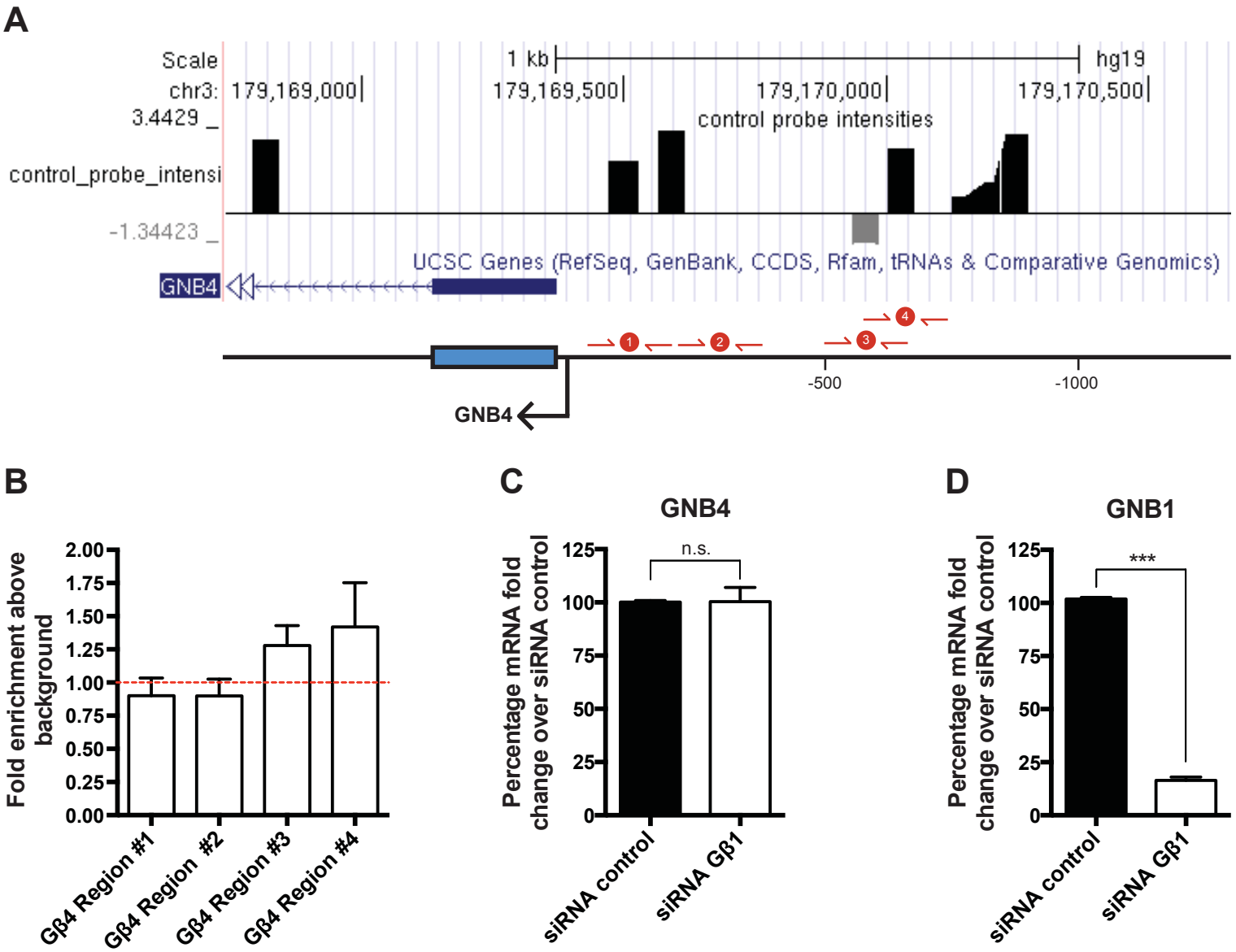
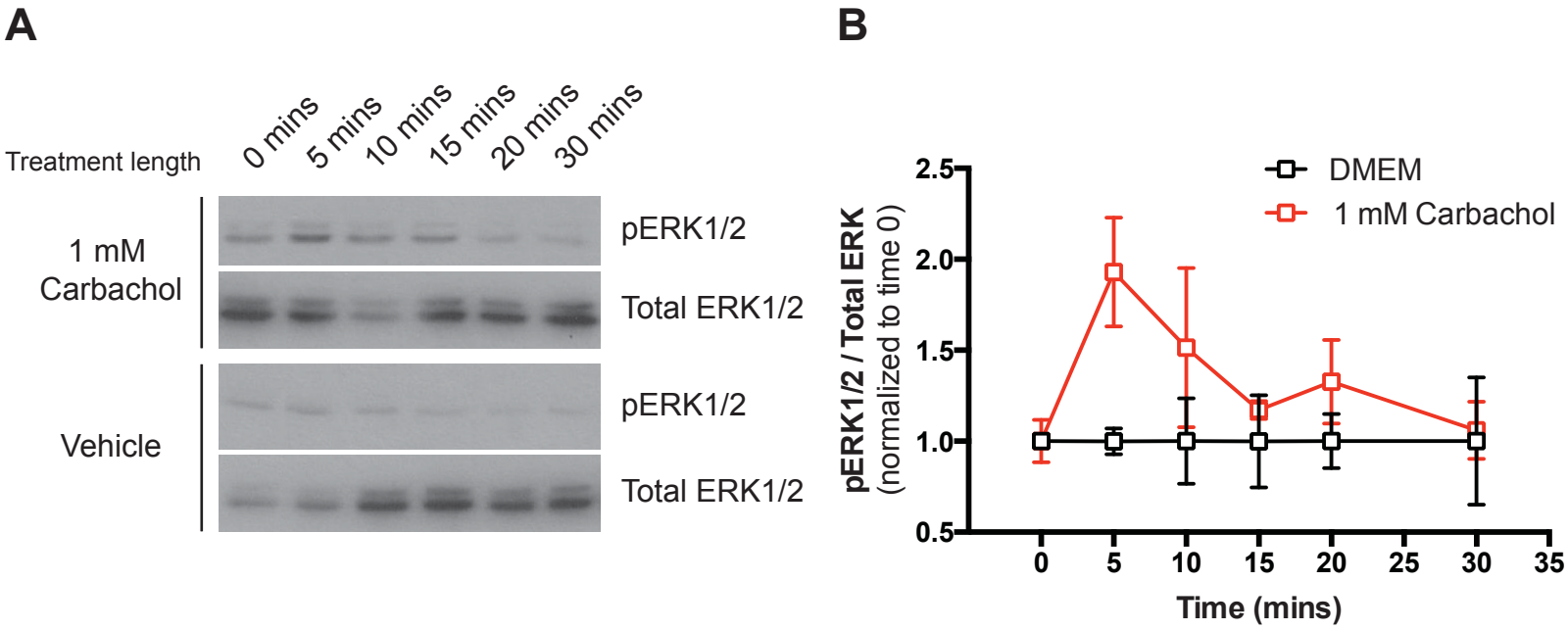

A
siRNA control
siRNA Gβ1
250
130
250
130
36
55
PLCβ3
PLCβ1
Gβ4
β-tubulin
B
siRNA control
siRNA Gβ1
55
35
55
Gαq/11
β-tubulin
C
siRNA control
siRNA Gβ1
70
55
GRK2
β-tubulin
D
siRNA control
siRNA Gβ1
70
55
GRK3
β-tubulin
E
siRNA control
siRNA Gβ1
70
55
GRK5
β-tubulin
F
siRNA control
siRNA Gβ1
70
55
GRK6
β-tubulin
G
PLCβ1
H
PLCβ3
I
Gβ4
J
Gαq/11
Fold change in protein expression (normalized to β-tubulin)
n.s.
siRNA control
siRNA Gβ1
K
GRK2
L
GRK3
M
GRK5
N
GRK6
*

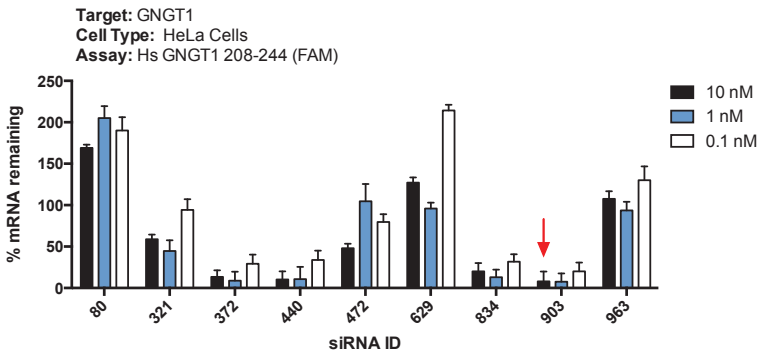
Figure 3.8

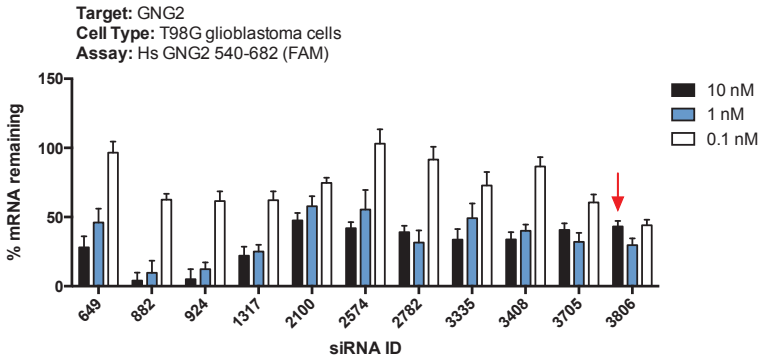

A
Scale
chr3:
3.4429 _
control_probe_intensi
-1.34423 _
1 kb
hg19
179,169,000
179,169,500
179,170,000
179,170,500
control probe intensities
UCSC Genes (RefSeq, GenBank, CCDS, Rfam, tRNAs & Comparative Genomics)
GNB4
1
2
3
4
-500
-1000
GNB4
B
Fold enrichment above background
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Gβ4 Region #1
Gβ4 Region #2
Gβ4 Region #3
Gβ4 Region #4
C
GNB4
Percentage mRNA fold change over siRNA control
125
100
75
50
25
0
n.s.
siRNA control
siRNA Gβ1
D
GNB1
Percentage mRNA fold change over siRNA control
125
100
75
50
25
0
***
siRNA control
siRNA Gβ1

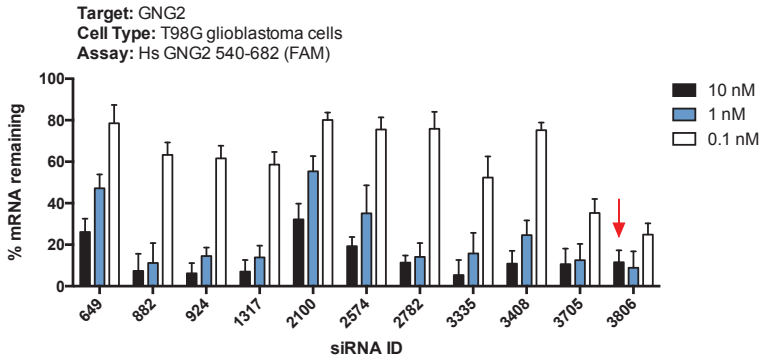
Supplemental Figure 3.1

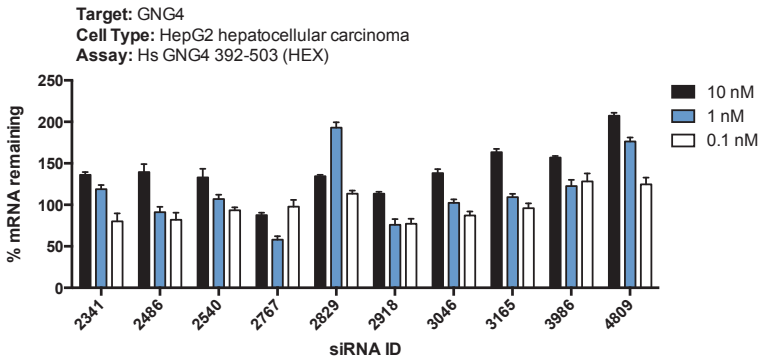


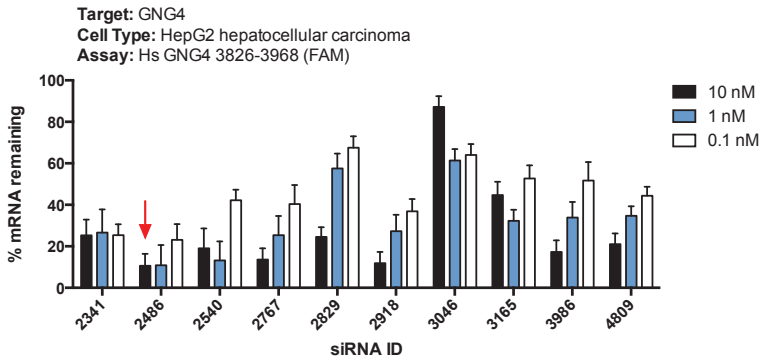
Supplemental Figure 3.2a
Establishment of RNAi screen

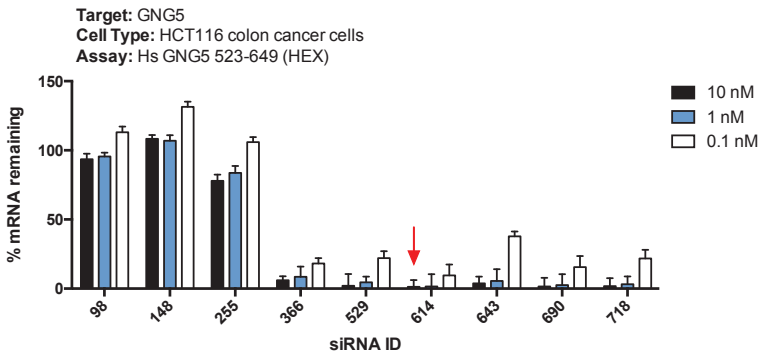


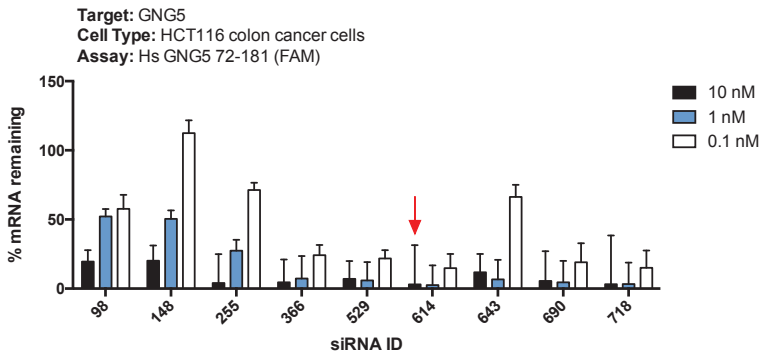


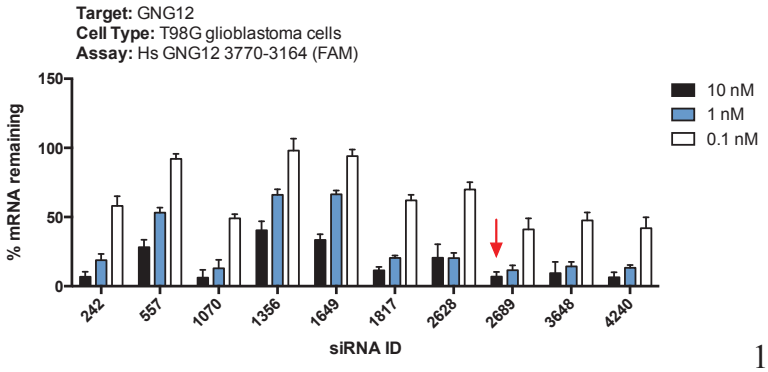


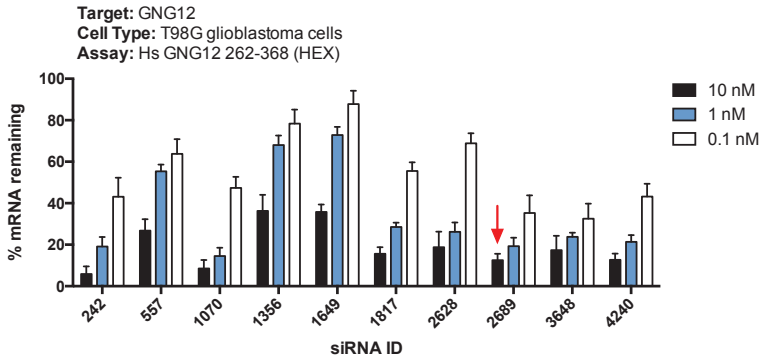




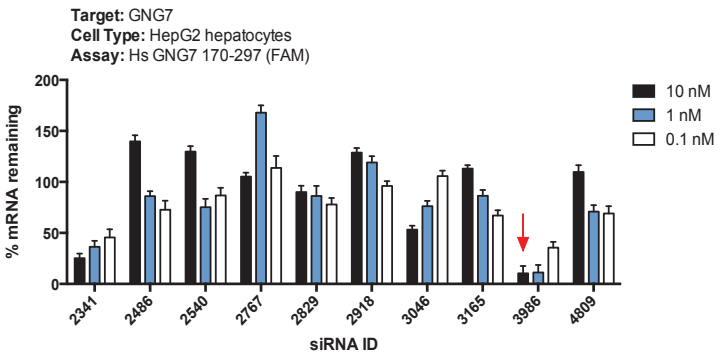


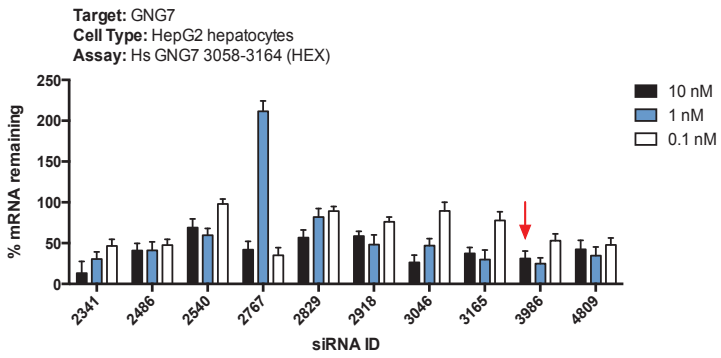


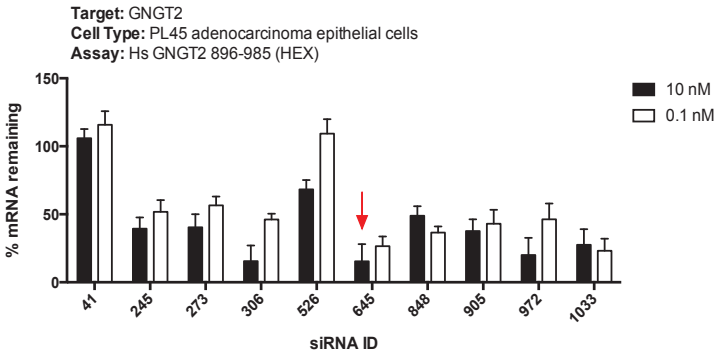


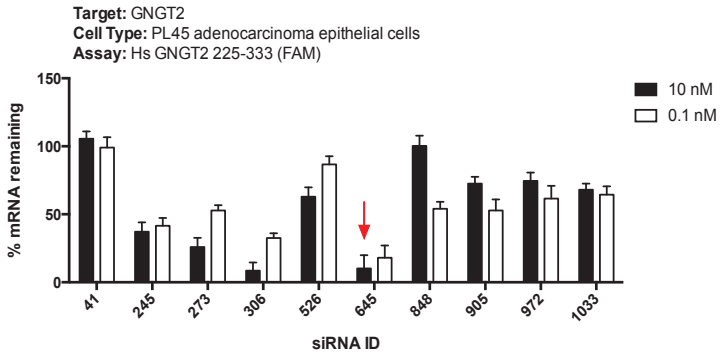


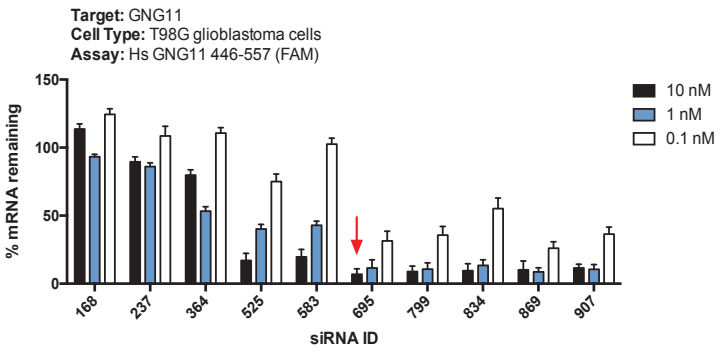
Supplemental Figure 3.2b
Establishment of RNAi screen

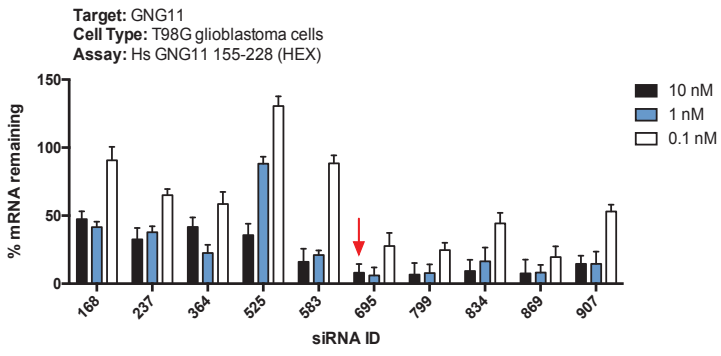


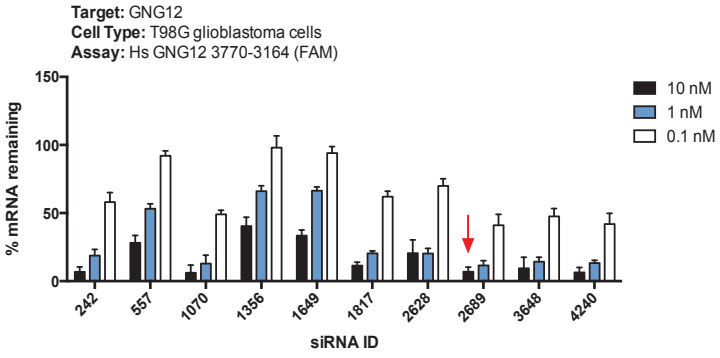


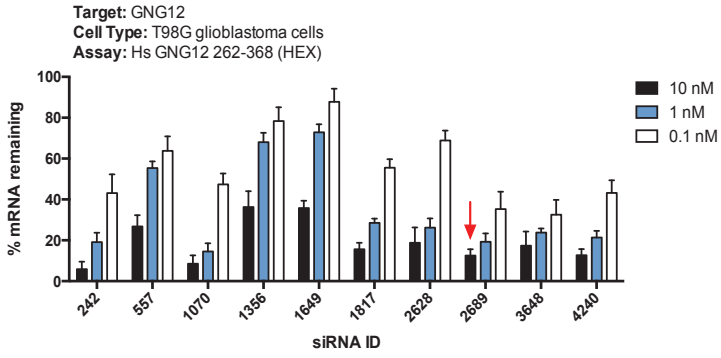












Supplemental Table 3.1 DsiRNA sequences used for RNAi assays

Target	DsiRNA sequence
NC1 27mer (negative control)	5'- CGUUAAUCGCGUAUAAUACGCGUat
	3'- CAGCAAUUAGCGCAUAUUAUGCGCAUA
NC5 27mer (negative control)	5′- CAUAUUGCGCGUAUAGUCGCGUUag
	3′- UGGUAUAACGCGCAUAUCAGCGCAAUC
GNGT1-903	5'- UCAUGCAUACUAUUACUACUAAGca
	3'- AGAGUACGUAUGAUAAUGAUGAUUCGU
GNG2-3806	5'- ACUACAGAAAUGUUAAAGCAGGAaa
	3'- CUUGAUGUCUUUACAAUUUCGUCCUUU
GNG4-2486	5'- GCUAGACAUCGAUGAAUUAGCUCtt
	3'- UCCGAUCUGUAGCUACUUAAUCGAGAA
GNG5-614	5'- ACCACUCCUUAUGAUCCAGUGAAta
	3'- UUUGGUGAGGAAUACUAGGUCACUUAU
GNG7-3986	5'- CGCAUCCAUCUCCAAGUUAACAGcc
	3'- GUGCGUAGGUAGAGGUUCAAUUGUCGG
GNGT2-645	5'- UGAGUAAAGAUCCCUGAACAAAUgc
	3'- UUACUCAUUUCUAGGGACUUGUUUACG
GNG11-695	5'- ACUGCAUCCUAAGUGGAAGAACUag
	3'- UUUGACGUAGGAUUCACCUUCUUGAUC
GNG12-2689	5'- CAGAUACAAUAAAGGCUAUGAGUat
	3'- CAGUCUAUGUUAUUUCCGAUACUCAUA

Supplemental Table 3.2 Sequences of primers and probes used for qPCR assays

qPCR Assay	Forward Primer (5'-3')	Reverse Primer (5'-3')	Probe (5'-3')
Hs GNG4 3826-3968	CCACTCTGTCAGTGGAAGATGAA	CTAAGGCATGATGTCCCTGCT	/56-FAM/CCACAATGC/ZEN/AAGCAGGCTTTATTCC/3IABkFQ/
Hs GNG5 523-649	AATCCCTTCAGACCCCAGA	GCTTTTGTACAGGCTTCAAATGTAG	/56-FAM/CCCAAACCA/ZEN/CTCCTTATGATCCAGTGAA/3IABkFQ/
Hs GNG7 170-297	CAAGCTGTGATTCCTGGGA	TTCTATGCGTAGCTGTTCCAC	/56-FAM/ACATTGTCT/ZEN/GCCATCAGCTCTGGG/3IABkFQ/
Hs GNG11 155-228	ACTTAACCCTCATCTAGCACCC	GACCTGAGAACTCTGCCTTT	/56-FAM/CCTCCTGGG/ZEN/TTGCAGGGACTTG/3IABkFQ/
Hs GNG12 3770-3857	CTGGATCATTTCCCAGTCTGTC	GCTCTCAGCCATCATCTACT	/56-FAM/TTGGACAAG/ZEN/GCGCTTCATCTCTCT/3IABkFQ/
Hs GNG8 34-121	CGCAAGACGGTGGAACA	CGCAGAAAGCCAGGAGTT	/56-FAM/AGGTGAACA/ZEN/TCGACCGCATGAAGG/3IABkFQ/
Hs GNG13 32-134	GCCGTTGTCATTGTCCCT	TTCTTCATCTGTGGCACGTC	/56-FAM/CTGTCACCT/ZEN/TTTCAAGCCCCAGG/3IABkFQ/
Hs GNG2 3004-3168	TGGAATTACAATTTCTAGGGAGCA	AGAGGCATAAGTAATCGAAAGAC	/56-FAM/TGGCTTCTT/ZEN/GGTATTTGTACTTATTCAGGG/3IABkFQ/
Hs GNG3 615-691	CCTGACCATGTGAGGTTATCTG	CACAAAGGTGGTAGGAAATGGG	/5-HEX/AAGGCCCAC/ZEN/CCTCACCTATCTGT/3IABkFQ/
Hs GNGT1 208-244	CCAGCTCAAGAAAGAAGTGACA	GATGCCCTTTACCAGTGGAT	/56-FAM/AGTTTCCAA/ZEN/ATGTTGTGAAGAAGTAAGAGATTACGT/3IABkFQ/
Hs GNGT2 225-333	CAAACACTATCTTACCTGAGTCCA	TCCTTCTCGCTGAGATCCT	/56-FAM/TAGACAGAC/ZEN/CTGATCCTGCCACAGA/3IABkFQ/
Hs HPRT 517-591	GACTTTGCTTTCCTTGGTCAGGCA	GGCTTATATCCAACACTTCGTGGG	/56-FAM/ATGGTCAAG/ZEN/GTCGCAAGCTTGCTGGT/3IABkFQ/
Hs SFRS9 569-712	TGTGCAGAAGGATGGAGT	CTGGTGCTTCTCTCAGGATA	/5-HEX/TGGAATATGC/ZEN/CCTGCGTAAACTGGA/3IABkFQ/

Supplemental Table 3.3 Sequences of primers used for ChIP-qPCR experiments

Target	Forward Primer (5'-3')	Reverse Primer (5'-3')
GNB4 Promoter #1	5'-CGCGCAGCTTCTGCTTCCTATTT-3'	5'-AACGAAACTGCCCGGGAATGAATG-3'
GNB4 Promoter #2	5'-CGCCAGTTTGCTTGTTAGCAGGAT-3'	5'-GTTGTGCAACTCTGGGCTTCAGTT-3'
GNB4 Promoter #3	5'-CCCGCTCTTCCATCACTAGTTGTA-3'	5'-GGCCATTTATTTGGAGGCAGGCTT-3'
GNB4 Promoter #4	5'-AGATATCCCTGCCAAGTCGCTCTT-3'	5'-CGGTACTGACTTCAGAGGAAGCAA-3'

Supplemental Table 3.4 LogEC50 values obtained from calcium release assays following Gβ and Gγ knockdown

Condition	LogEC50 ± S.E.M
siRNA control	-3.83 ± 0.1269
siRNA Gβ1	-3.488 ± 0.09694
siRNA Gβ4	-3.906 ± 0.1345
siRNA Gβ1 / siRNA Gγ1	-3.666 ± 0.1159
siRNA Gβ1 / siRNA Gγ2	-3.891 ± 0.1038
siRNA Gβ1 / siRNA Gγ4	-3.819 ± 0.1408
siRNA Gβ1 / siRNA Gγ7	-4.026 ± 0.2511
siRNA Gβ4 / siRNA Gγ1	-3.67 ± 0.198
siRNA Gβ4 / siRNA Gγ2	-3.85 ± 0.1652
siRNA Gβ4 / siRNA Gγ4	-3.621 ± 0.2488
siRNA Gβ4 / siRNA Gγ7	-3.846 ± 0.2614
siRNA NC5	-4.012 ± 0.06598
siRNA Gγ1	-3.099 ± 0.3314
siRNA Gγ2	-3.476 ± 0.05613
siRNA Gγ4	-3.733 ± 0.352
siRNA Gγ5	-3.458 ± 0.1784
siRNA Gγ7	-3.886 ± 0.095
siRNA Gγ9	-3.885 ± 0.04832
siRNA Gγ11	-3.595 ± 0.09544
siRNA Gγ12	-3.296 ± 0.5588
siRNA control / siRNA NC1	-4.194 ± 0.2356
siRNA control / siRNA Gγ1/2/4/7	-4.305 ± 0.2705
siRNA Gβ1 / siRNA Gγ1/2/4/7	-4.03 ± 0.3703
siRNA Gβ4 / siRNA Gγ1/2/4/7	-3.802 ± 0.3339

Supplemental Table 3.5 Identification of hnRNPs as Gβ and Gβγ interacting partners using LC/MS

Treatment	Type of Isolation	Bait Protein (TAP/split-TAP)	Protein ID	Description	Average Peptides	Average Unique Peptides	Average Coverage	Number of times detected
1mM Carbachol	Cytosolic	TAP-Gβ1	HNRNPC	Heterogeneous nuclear ribonucleoprotein C (C1/C2)	6	5	10.5	2
			HNRNPR	Heterogeneous nuclear ribonucleoprotein R	3	3	5.4	1
Untreated	Nuclear	TAP-Gβ1	HNRNPC	Heterogeneous nuclear ribonucleoprotein C (C1/C2)	1	1	3.6	1
			HNRPDL	Heterogeneous nuclear ribonucleoprotein D-like	4	4	10.5	1
1mM Carbachol	Nuclear	TAP-Gβ1	HNRNPC	Heterogeneous nuclear ribonucleoprotein C (C1/C2)	1	1	3.3	1
Untreated	Cytosolic	TAP-Gβ1γ7	HNRNPC	Heterogeneous nuclear ribonucleoprotein C (C1/C2)	3	3	12.1	1
1mM Carbachol	Cytosolic	TAP-Gβ1γ7	HNRNPC	Heterogeneous nuclear ribonucleoprotein C (C1/C2)	12	11	12.3	3
			HNRNPR	Heterogeneous nuclear ribonucleoprotein R	6	6	5.3	2
			HNRPDL	Heterogeneous nuclear ribonucleoprotein D-like	7	5	5.7	3

CHAPTER 4: Characterization of a novel interaction between Gβγ subunits and RNA Polymerase II

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4.1 Preface

In Chapter 3, I presented data that suggests that $G\beta_1$ participates in non-canonical functions as a possible regulator of transcription. We discussed potential roles for this subunit as a potential regulator of mRNA processing, in attempt to explain our observations of $G\beta_1$ displaying promoter occupancy properties and interactions with different members of the hnRNP family. While $G\beta_1$ may indeed play roles in mRNA processing, the connections between the two aforementioned observations are difficult to make, especially given the fact that $G\beta_1$ was found to bind over 700 promoters. Instead, a pilot analysis in our lab was undertaken by Dr. Sarah Gora (former post-doctoral fellow, Hébert Lab) to assess whether $G\beta\gamma$ dimers bind DNA by interacting with proteins that are known to bind promoters. One candidate interactor that was assessed was RNA polymerase II – indeed, it was shown that direct activation of PKC by 100 nM of PMA results in an interaction between $G\beta_1\gamma$ and Rpb1 in HEK293 cells stably expressing $G\beta_1$. The goal of the following study was to assess whether endogenous $G\beta\gamma$ interacts with Rpb1 under conditions of endogenous GPCR stimulation. Here, we demonstrate that $G\beta_1\gamma$ interacts with RNAPII upon agonist-stimulation in two different cell types, and plays a role in RNAPII pause release, and ultimately, the regulation of gene expression. Furthermore, we describe roles for $G\beta_2\gamma$ as transducers of AT1R signalling, using similar approaches taken in Chapter 3. Taken together, this chapter demonstrates a clear, more direct role for $G\beta\gamma$ subunits in gene expression regulating, furthering our understanding of non-canonical roles that these dimers play in cellular signalling.

4.2 Abstract

Gβγ subunits are involved in an array of distinct signalling processes in various compartments of the cell, and of particular interest, Gβγ subunits modulate the activities of a variety of proteins in the nucleus. We previously performed ChIP-on-CHIP experiments that have revealed that Gβγ dimers occupy the promoters of more than 700 genes. Our *in silico* analyses have shown that neither Gβ and Gγ subunits are able to bind DNA on their own, leading to the hypothesis that Gβγ dimers occupy promoters in conjunction with other proteins – transcription factors or proteins involved in the process of transcription. Since Gβγ dimers occupied so many promoters, we assessed whether Gβγ could interact with RNA polymerase II (RNAPII). Here, we demonstrate that Gβγ dimers interact with RNAP II in a GPCR-dependent, agonist-induced, pathway-specific manner. In particular, we show that this interaction is induced both by endogenous M3 muscarinic acetylcholine receptors in human embryonic kidney cells and angiotensin II type I receptors in primary rat neonatal cardiac fibroblasts. Subcellular fractionation studies reveal that upon GPCR activation, Gβγ subunits translocate to the nucleus and interact with hRpb1, the largest subunit of RNA polymerase II. *In vitro* protein interaction assays confirm that Gβγ interacts with GST-tagged hRpb6, hRpb8 and hRpb9. Various inhibitors and cell lines with CRISPR/Cas9-mediated knockout of different G proteins potentially downstream of receptor activation were used to decipher the mechanisms underlying how Gβγ interacts with Rpb1. In addition, using siRNA-mediated knockdown of Gβ subtypes, we explored the functional roles of this interaction in terms of gene expression during a fibrotic response using qPCR arrays in cardiac fibroblasts. Taken together, our studies reveal a novel

interaction between Gβγ subunits and RNA polymerase II, further shedding light on the complex roles Gβγ dimers play in GPCR signalling.

4.3 Introduction

Heterotrimeric G proteins, specific combinations of Gα, Gβ and Gγ subunits, act as signal transducers that relay extracellular stimuli sensed by G protein-coupled receptors and to the activation of distinct intracellular signalling pathways (reviewed in [335]), in which Gβ and Gγ subunits form obligate dimers. Various biochemical and genetic studies have revealed that Gβγ dimers possess functions beyond their original role as negative regulators of Gα subunit activity [431]. Indeed, Gβγ subunits have been shown to modulate a wide variety of canonical effectors including adenylyl cyclases, phospholipases and inwardly rectifying potassium channels [6, 66, 335]. However, Gβγ subunits have been found to be regulators a variety of non-canonical functions in distinct intracellular locations – number of studies have implicated roles for Gβγ in the nucleus (reviewed in [186, 335]).

With respect to transcriptional activity downstream of GPCR signalling, G proteins are known to modulate various transcription factors and cofactors such as STAT and NFκB that activates various biological responses such as proliferation, differentiation and hypertrophy [5, 184, 246]. Gene expression regulatory pathways downstream of Gα subunit activation have been extensively described [432], however, the understanding of how Gβγ and their complex signalling networks regulate gene expression remains rudimentary. Nevertheless, the specific roles of Gβγ subunits involved in gene expression regulation have only begun to be described in great detail. Indeed, $G\beta_1\gamma_2$ has been shown to interact with histone deacetylase 5 (HDAC5) resulting in the release of MEF2 and subsequent stimulation of transcriptional activity under conditions of α_{2A}-adrenergic receptor activation [240].

With respect to interactions of Gβγ with specific transcription factors, we have previously shown that Gβγ interacts with cFos to decrease AP-1 mediated transcription – Gβγ/Fos colocalized in the nucleus, Gβγ did not prevent Jun/Fos dimerization or interaction with DNA, and recruited HDACs to repress transcription [183]. In addition, we have previously reported that Gβγ is capable of binding promoters; in particular, $G\beta_1$ was shown to occupy the promoter of its another Gβ isoform – $G\beta_4$ [433]. We next sought to examine whether these dimers interact with a protein complex that binds transcription regulatory and promoter regions ubiquitously. Here, we describe a novel interactor for the Gβγ dimer – RNA polymerase II. Using a cell-context and pathway-specific approach, we describe the spatiotemporal modulation and function of this interaction downstream of activation of endogenous GPCRs in both HEK 293 cells and rat neonatal cardiac fibroblasts. Our findings indicate: (1) a specificity in function for Gβγ dimers whereby different specific Gβ containing Gβγ dimers interact with the Rpb1 of RNA polymerase II under basal and GPCR agonist-stimulated conditions, (2) an interaction that is regulated by a series of kinases and phosphatases, and (3) roles for specific Gβγ subunits as basal repressors of transcription.

4.4 Materials and Methods

4.4.1 *Reagents* – Carbachol, angiotensin II, isoproterenol, 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, 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 and polybrene were purchased from Sigma-Aldrich Corp. (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 and dithiothreitol (DTT) 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, low glucose DMEM supplemented with 1.0 g/L glucose, L-glutamine and phenol red, 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 was 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). Anti-$G\beta_{1\text{-}4}$ (T-20) antibody, anti-RNA Polymerase I Rpa194 (N-16) antibody, anti-ERK1/2 antibody and anti-Gαq antibody was 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-GST antibody was purchased from Rockland Immunochemicals (Limerick, PA, USA). Rat Fibrosis PCR arrays were purchased from SABiosciences (Qiagen; Toronto, ON, Canada). The $G_{q/11}$-specific inhibitor FR900359 [434] was purchased from the Institute of Pharmaceutical Biology (University of Bonn, Germany).

4.4.2 *cDNA constructs* – FLAG-$G\beta_1$, FLAG-$G\beta_4$ and FLAG-$G\beta_5$ were obtained from UMR cDNA Resource (www.cdna.org). GST-tagged hRbpb3, hRbpb4, hRbpb6, hRbpb7, hRbpb8, hRbpb9, and hRbpb10β contained in pGEX-2T plasmids were generous gifts from Dr. Jeffrey Parvin (Ohio State University, Columbus, Ohio; described in [435]).

4.4.3 *Tissue culture, transfection and treatments* –HEK 293, HEK 293T cells and CRISPR-Cas9 mediated $\Delta G\alpha_{q/11/12/13}$ knockout HEK 293 cells (quadKO cells) [436], a generous gift from Dr. Asuka Inoue (Tohuku University, Sendai, Japan), were grown at 37°C in 5% CO_2 in high glucose 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-$G\beta_{1/4/5}$ using Lipofectamine 2000 as per manufacturer's recommendations and as previously described

[183]. Primary rat neonatal cardiac fibroblasts (RNCFs) were isolated as previously demonstrated with minor modifications [223]. Briefly, hearts from 1-3 one-day old rat pups were cut into 2-3 pieces and trypsinized overnight at 4°C with gentle agitation. The next morning, trypsin was neutralized by the addition of fibroblast growth medium (DMEM supplemented with 7% FBS (v/v) and 1% P/S (v/v)) and cells were subsequently treated with collagenase five times for 1-3 mins in a 37°C water bath. Cells were pelleted, resuspended in HBSS and filtered through a 40 μm filter and pelleted again at $400g^{-1}$ for 5 mins at 4°C. The resulting cell pellet was resuspended in a total of 40 mL of fibroblast growth medium and plated in 100mM plates and grown at 37°C in 5% CO_2 for 1h. After one hour of plating, media was removed from the plates to minimize cardiomyocyte attachment, cells were washed once with fibroblast media, and then grown for 48 hours at 37°C in 5% CO_2. Two days post plating, cells were trypsinized and seeded in 100mM plates at a density of $5x10^5$ cells per plate (for immunoprecipitation (IP) experiments) in fibroblast growth medium for 48h. RNCFs were then starved in low glucose DMEM overnight for 10-12h prior to subsequent inhibitor treatments for interaction pathway/mechanism assessment. For treatment of HEK 293 cells, quadKO cells or RNCF, cells were starved in DMEM (with no FBS and no P/S) overnight for between 10-12 hours and subsequently treated with pathway inhibitors, 1mM carbachol or 1 μM Ang II for the treatment lengths indicated in the various assays listed below.

4.4.4 *RT-qPCR* – Reverse transcription of RNA isolated from rat neonatal cardiac fibroblasts was performed using a protocol previously described [433]. Briefly, cells plated in 100mM dishes 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 on $G\beta_1$ and $G\beta_2$ transcripts was performed with Evagreen Dye qPCR master-mixes using a Corbette Rotorgene 6000 thermocycler. 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 3.

4.4.5 *Purification of GST-tagged protein, G proteins and in vitro interaction assays* – GST-tagged hRPB subunits were purified as previously described with minor modifications [437]. Briefly, pGEX-2T plasmids containing sequences for GST-hRPB subunits were transformed into Bl21-DE3 *E. coli* cells, plated on LB/Ampicillin-agar plates overnight and inoculated overnight in a volume of 35-40 mL LB/ampicillin overnight at 37°C with shaking at 225 rpm. The next day, 10mL of this starter culture was transferred to a total culture volume of 100 mL, grown until $O.D._{600}$ reached between 0.6 and 1.0. Expression of GST-tagged proteins was induced with IPTG at a final concentration of 0.5 mM and then cultures were grown overnight at 16°C in a shaker at 180 rpm. Cells were then harvested by centrifugation and lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 120mM NaCl, 0.3 mg/mL lysozyme, 5 mM $MgCl_2$, 0.5% TritonX-100, 0.5 mM PMSF, 1X protease inhibitor cocktail), and subsequently frozen at -80°C to facilitate further lysis. Lysates were thawed on ice, sonicated using a Misonix Sonicator 3000 (8 bursts of 15 seconds pulses with 45 seconds of cooling time between each pulse), and then cleared by ultracentrifugation at 39240 g^{-1} for 45 minutes. Cleared lysates were then incubated with washed glutathione sepharose beads (GST-beads) overnight to enrich for GST-tagged proteins, washed twice in lysis buffer and once with 100 mM Tris pH 8.0 the next day,

and eluted in elution buffer (100 mM Tris pH 8.0, 20 mM reduced glutathione, 1mM DTT, 0.5 mM PMSF, 1X protease inhibitor cocktail) overnight. Eluates were then collected, quantified using a Bradford assay and subsequently aliquoted and frozen at -80°C. For *in vitro* interaction assays, 100-250 μg of lysates from HEK 293 cells or RNCFs were first precleared with GST-beads for 2 hours at 4°C with rotation. 50 μL of GST-beads were added to the pre-cleared lysates, to which 100 μg of purified GST-hRPB subunit proteins were subsequently added and the mixture was incubated overnight at 4°C with end-over mixing. The next day, the GST-beads were washed three times with cold MLB and proteins were eluted off in 100 μl of elution buffer. Efficiency of Gβ pull down by GST-tagged hRPB subunit fusion proteins was then assessed by SDS-PAGE. Briefly, Sf9 cells were simultaneously infected with baculovirus constructs expressing β_1, γ_2 and His6-tagged α_{i1}, and βγ subunits were purified as previously described [129, 438, 439]. βγ subunits were then concentrated using 0.3-ml macro-prep ceramic hydroxyapatite columns (Bio-Rad) and eluted into βγ vehicle (20 mM Hepes pH 8.0, 100 mM NaCl, 1 mM DTT, 1% octyl glucoside; and 200 mM potassium phosphate pH 8.0). Purified TAP-tagged hRpb2 protein was a generous gift from Dr. Benoit Coulombe (Institutes de Recherches Clinique de Montréal (IRCM), Montréal, QC, Canada).

4.4.5 *Nuclear isolation* – Nuclei from HEK 293 cells were isolated as previously described [184]. Briefly, cells seeded in T175 flasks 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 minutes 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^{-1} for 5 minutes and subsequently lysed in 1X RIPA buffer.

4.4.6 *Immunoprecipitation and Western blotting* – Immunoprecipitation (IP) assays of Gβ and Rpb1 were performed as previously described, with minor alterations [183]. Treated HEK293 cells and RNCFs lysed in 1X 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 first quantified with Bradford assay, upon which 500 µg of lysates were precleared with 15 µl of anti-rabbit IgG-agarose beads. Precleared lysates were then incubated with 1 µg anti-$G\beta_{1\text{-}4}$ or 2 µg of anti-Rpb1 overnight at 4°C with end-over mixing. The next day, 40 µl of washed 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 1X RIPA. Proteins were then eluted off the beads by the addition of 4X Laemmli buffer followed by denaturation at 65°C. Protein immunoprecipitation and co-IP was then assessed by western blot as previously described [433]. Resulting western blot images were then quantified using ImageJ 1.48v and analyzed in GraphPad Prism 6.0c software. Lysates prepared from hearts of TAC and sham mice were a kind gift from Dr. Benoit Boivin (Montreal Heart Institute, Montreal, Canada) and lysates of aged rat hearts were a generous gift from Dr. Bruce Allen (Montreal Heart Institute, Montreal, Canada).

4.4.7 *Rat Fibrosis qPCR arrays* – Fibrosis qPCR arrays were performed as per the manufacturer's protocols (Qiagen, Toronto, ON, Canada). Briefly, 0.5 µg of isolated total RNA

(A_{260}:A_{230} ratios greater than 1.7, A_{260}:A_{280} ratios between 1.8 and 2.0) from siRNA transfected and vehicle/AngII treated rat neonatal cardiac fibroblasts were 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 RT^2 First Strand Kits with protocols according to the manufacturer's instructions. Resulting cDNA mixes were then mixed with RT2 SYBR Green mastermixes and subsequently dispensed in wells of a 96 well plate containing pre-loaded lyophilized primers provided by the manufacturer. qPCR reactions were then run on a 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. A list of all the genes whose expressions were detected can be found at https://www.qiagen.com/ca/shop/pcr/primer-sets/rt2-profiler-pcr-arrays?catno=PARN-120Z#geneglobe. mRNA expression data were normalized to levels of two housekeeping genes contained on each plate – Ldha1 and Hprt.

4.4.8 *Statistical Analysis* – Statistical tests were perfomed using GraphPad Prism 6.0c software. For analysis on quantifications of immunoprecipitation experiments, one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's correction was used on raw quantifications of western blot bands and comparisons were made to vehicle-vehicle conditions. For assessment of Ca^{2+} release using Fura-2 AM-based assays, one way ANOVA followed by Dunnett's correction was used on areas under curves derived from Ca^{2+} release – time graphs and comparisons were made back to either siRNA control conditions or vehicle/vehicle conditions. For fibrosis qPCR arrays, repeated measures one way ANOVA followed by Bonferroni's post-

hoc analysis was used to determine differences in gene expression with all comparisons made to respective siRNA control or no treatment conditions within siRNA conditions. For validation of $G\beta_1$ and $G\beta_2$ knockdown in RNCFs, fold changes over siRNA control were compared to siRNA control using Student's t-tests. Comparisons that resulted with p values that were $p<0.05$ were considered significant. All results are expressed as mean ± S.E.M and data are represented as pooled experiments whose sample sizes are indicated in figure legends.

4.5 Results

4.5.1 Initial characterization of the interaction between RNA polymerase II and Gβ$_{1-4}$

Our initial observation of the interaction between RNA polymerase II and Gβγ was in HEK 293 cells overexpressing TAP-Gβ$_1$ upon activation of protein kinase C (PKC) with 100nM PMA (Supplemental Figure 4.1A). We sought to first elucidate whether activation of a GPCR induces endogenous Gβ to interact with Rpb1, the largest subunit of RNAPII. It has been previously demonstrated that HEK 293 cells endogenously express M3-muscarinic acetylcholine receptors (M3-mAChR) [433]. In order to characterize the kinetics of the interaction upon M3-mAChR stimulation, HEK 293 cells were treated with 1mM carbachol for different intervals between 0-300 minutes of treatment upon which Gβ$_{1-4}$ was immunoprecipitated and levels of Rpb1 co-immunoprecipitated were assessed. It was observed that the amount of Rpb1 interacting with Gβ$_{1-4}$ increases two-fold between 0-60 minutes, upon which the interaction decreases over the next two hours down to basal levels (Figure 4.1A, B). Under the same conditions, we observed no basal or carbachol-dependent interaction of Gβγ with the A194 subunit of RNA polymerase I (Supplemental Figure 4.1B). Using the time-point at which the initial interaction was observed (45 mins 1mM carbachol stimulation), a reverse co-IP experiment revealed that immunoprecipitation of Rpb1 also co-immunoprecipitates Gβ in an agonist-dependent, time-dependent manner (Figure 4.1C). Additionally, we observed no carbachol-dependent or basal interaction of Rpb1 with Gα$_{q/11}$ or ERK1/2 (Supplemental Figure 4.1C, D). In order to validate this interaction using a different approach, GST-pull down assays were used to assess the interaction between different purified subunits of RNAPII or GST-tagged RNAPII and purified

Gβγ or Gβγ from cellular lysates. In addition to our observation of $G\beta_{1\text{-}4}$ interacting with Rpb1, we observe that GST-tagged hRpb6, hRpb8 and hRpb9 pull down Gβ from HEK 293 cell lysates, but GST-alone does not (Figure 4.1D).

Upon confirmation of this interaction *in vitro*, the specificity of Gβ subunits interacting with RNAPII was determined. We have previously described that $G\beta_4\gamma_1$ is the primary specific Gβγ dimer that mediates signalling downstream of M3-mAChR and that $G\beta_1$ subunits play a role in the control of expression of components involved in this GPCR signalling cascade. In attempt to further characterize specificities of these Gβ subunits, FLAG-$G\beta_1$, FLAG-$G\beta_4$ or FLAG-$G\beta_5$ overexpressed in HEK 293 cells were immunoprecipitated and the amounts of Rpb1 co-immunoprecipitated were assessed. Intriguingly, activation of M3-mAChR with carbachol leads to an overall increase in FLAG-$G\beta_1$ interacting with Rpb1, but a decrease in FLAG-$G\beta_4$ interacting with Rpb1, suggesting a regulatory interplay between basal and GPCR-activated states of Gβγ interaction with RNAPII (Figure 4.1E). No net change in the amount of FLAG-$G\beta_5$ interacting with Rpb1 was observed (Figure 4.1E).

Next, the localization of this interaction was assessed. RNAPII has been described as a strictly nuclear protein, and although it has previously been shown that Gβγ is present in the nucleus [184], the mechanisms that cause the entry of Gβγ into the nucleus is unknown. Using a nuclear isolation technique in conjunction with endogenous GPCR stimulation, we demonstrate that M3-mAChR activation causes a net increase in the amount of Gβ in the nucleus and a net decrease in cytosol at 45 mins post stimulation (Supplemental Figure 4.1E). Nuclear import was then inhibited using the importin-β inhibitor importazole to determine whether Gβ is indeed

being imported into the nucleus. Again, using nuclear isolation techniques coupled with M3-mAChR activation, we demonstrate that blocking nuclear import blocks the M3-mAChR dependent movement of Gβ into the nucleus (Supplemental Figure 4.1F). In order to determine whether Gβ nuclear import is essential for the interaction to occur with Rpb1, immunoprecipitation of Gβ from isolated nuclei and isolated cytosol was performed and results reveal that the interaction only occurs in the nuclear fraction and not in the cytosol (Figure 4.1F,G). In addition, the increase in interaction is blocked by importazole, suggesting that upon M3-mAChR stimulation, nuclear import of Gβ is absolutely required for its interaction with RNAPII.

4.5.2 Pathways involved in mediating the Gβγ-RNAPII interaction in HEK 293 cells

Since endogenous M3-mAChRs primarily couple to $G\alpha_{q/11}$ in HEK 293 cells [406], signalling players downstream of $G_{q/11}$ activation were inhibited to assess the overall mechanism of inducing the Gβγ-RNA Pol II interaction and the involvement of specific signalling players. FR900359-mediated inhibition of Gαq/11 revealed a loss in the carbachol-induced interaction (Figure 4.2A). In addition, CRISPR-Cas9 mediated quadruple knockout of $G\alpha_{q/11/12/13}$ in HEK 293 cells also prevents the carbachol-mediated increase in interaction (Figure 4.2B). Inhibition of PLCβ using U71322 also blocked the carbachol-induced interaction, however, basal levels of the interaction were increased without receptor stimulation (Figure 4.2C). Intriguingly, chelation of calcium using BAPTA-AM increased basal levels of the interaction, and did not block the carbachol-induced interaction (Figure 4.2D), suggesting an integral role for calcium in

modulating and regulating this interaction. Similar effects were observed upon inhibition of kinases activated downstream of $G_{q/11}$ coupled GPCRs – inhibition of PKC with Gö6983 and CamKII with KN-93 both increased basal levels and did not block the carbachol-induced interaction between Gβ and Rpb1 (Figure 4.2E, F). Indeed, inhibition of PP2B with cyclosporinA blocks the carbachol-mediated increase in interaction between Gβ and Rpb1, suggesting roles for this phosphatase in mediating the interaction upon M3-mAChR activation (Figure 4.2H). The role of PP1α could not be assessed as its inhibitor, CalyculinA, proved to be toxic for HEK 293 cells (data not shown).

4.5.3 Characterization of the interaction in rat neonatal cardiac fibroblasts

We next sought to investigate this interaction in a more physiologically relevant model. In the progression of cardiac hypertrophy and remodeling of the heart, pathologic crosstalk between cardiomyocytes and cardiac fibroblasts has been implicated [440]. Aortic banding is a well established model to study left ventricular hypertrophy, with ascending and transverse aortic constriction (AAC and TAC mice, respectively) being the most popular techniques used to study pressure overload hypertrophy [441]. We first sought to determine the state of the interaction in mice subjected to transverse aortic constriction (TAC) to study the state of the interaction in a hypertrophied heart. Compared to sham surgery mice, we observed an increased interaction between Gβγ and RNAPII in the TAC animals (Supplemental Figure 4.3A). Furthermore, we detected the presence of this interaction in aged rat hearts (Supplemental Figure 4.3B). These observations indicate the presence of the interaction not only in diseased hearts, but also

maintenance of the interaction with in adult rat hearts. Various signalling systems are involved in the development of cardiac hypertrophy and fibrosis [273, 302, 442-445], so we explored the role of the AT1R on the interaction between Gβγ and RNAPII. First, cultured rat neonatal cardiac fibroblasts treated with Ang II were used to determine whether the interaction was also induced in these cells. A timecourse experiment demonstrated that Ang II induced an increase in interaction between Gβγ and Rpb1 75 minutes post stimulation of AT1R (Figure 4.3A,B). Using a similar approach to validating the interaction in HEK 293 cells, GST-pull down assays were used to assess the interaction between Gβγ in lysates of rat neonatal cardiac fibroblasts with purified GST-hRpb6, GST-hRpb8, GST-hRpb9 or GST-alone. These assays showed that Gβγ was pulled down with all three GST tagged RNA polymerase II subunits (hRpb6, 8, 9) but not GST-alone (Figure 4.3C), confirming the interaction *in vitro*.

Cardiac fibroblasts express both angiotensin II type I and type II receptors [446]. In order to distinguish which receptor induced the interaction in fibroblasts, an AT1R specific antagonist, losartan, was used. Pretreatment of cells with losartan prior to Ang II treatment completely blocked the agonist-induced interaction, suggesting that AT1R and not AT2R was responsible for the increased interaction in cardiac fibroblasts (Figure 4.3D). Next, we determined whether nuclear localization of Gβγ is necessary for the interaction to occur. Using importazole as previously described, we observed a blockage of the interaction when nuclear import via importin-β is inhibited, again suggesting that Gβγ must translocate to the nucleus for the interaction to occur (Figure 4.3E). Intriguingly, inhibition of importin-β under basal conditions showed an increase in the interaction between Gβγ and Rpb1 (Figure 4.3E). We also sought to determine the specificity of Gβ subunits interacting with Rpb1. Immunoprecipitation of $G\beta_1$

revealed an increase in the amount of Rpb1 co-immunoprecipitated in response to Ang II treatment, whereas immunoprecipitation of $G\beta_2$ revealed a higher basal interaction with Rpb1 that was reduced in response to Ang II treatment (Supplemental Figure 4.3C). Compared to what was observed downstream of M3-mAChR signalling in HEK 293 cells, we observed a similar interplay of Gβ subunits associating or dissociating from Rpb1 in response to GPCR activation. Given that $G\beta_2$ is more similar to $G\beta_4$ [335], and that we previously described $G\beta_4$ containing Gβγ dimers to be important as transducers of signalling responses and $G\beta_1$ as regulators of players involved in GPCR signalling [433], we next determined which Gβ subunit was necessary to initiate signalling cascades proximally downstream of AT1R activation. Upon validation of $G\beta_1$ and $G\beta_2$ knockdown in cardiac fibroblasts (Supplemental Figure 4.2D,E), the effects of knockdown of these specific Gβ subunits on AT1R-mediated calcium release was assessed. Using Fura2-AM to measure Ca^{2+}, knockdown of $G\beta_1$ did not alter Ca^{2+} release downstream of AT1R stimulation with Ang II (8.04 ± 7.04% decrease, $p>0.05$; Figure 4.3F). However, knockdown of $G\beta_2$ resulted in a significant 31.6 ± 8.97% decrease in Ca^{2+} release (Figure 4.3G), suggesting that $G\beta_2$-containing Gβγ dimers mediates signalling downstream of AT1R activation. Knockdown of $G\beta_2$ also resulted in a reduced Ang II-mediated induction Gβγ/Rbp1 interaction, reversing the trend and resulting in lower overall interaction (Figure 4.3H, I). Intriguingly, we observed an inhibition of the Ang II-induced interaction upon knockdown of $G\beta_1$ despite not being required for initation of downstream AT1R signalling (Figure, 4.3F, H, I). Furthermore, we observed that under basal conditions, Ang II treatment resulted in an increase in Gβγ interaction with Ser5-phosphorylated form of Rpb1, and that this increase was inhibited with $G\beta_1$ knockdown, suggesting $G\beta_1$ increases its interaction with paused RNAPII (Supplemental Figure 4.3F). Taken together, our results suggest distinct roles for specific Gβ subunits – it appears that

$G\beta_2$ is required for signalling downstream of AT1R activation and acts as a basal interactor of Rpb1, whereas $G\beta_1$ is required for interaction with Rpb1 under conditions of GPCR agonist stimulation.

4.5.4 Deciphering pathways that modulate the Gβγ-RNAPII interaction in rat neonatal cardiac fibroblasts

Next, signalling pathways that modulate the interaction between Gβγ and RNAPII in rat neonatal cardiac fibroblasts were determined. It has previously been demonstrated that AT1R couples to both $G_{q/11}$ and $G_{i/o}$ G-proteins [282, 447, 448]. FR900359-mediated inhibition of AT1R resulted in an increased basal interaction and a partial reduction of the Ang II-induced response (Figure 4.4A, Supplemental Figure 4.4C). Intriguingly, PTX-mediated inhibition of Gαi/o resulted in enhanced basal Gβγ-Rpb1 interactions, and a maintenance of the Ang II-induced increases in the interaction (Figure 4.4B, Supplemental Figure 4.4D). Dual inhibition of both $G\alpha_{q/11}$- and $G\alpha_{i/o}$-coupled AT1R resulted in a phenotype similar to $G\alpha_{i/o}$ inhibition alone, possibly because PTX pre-treatment preceded FR900359 pre-treatment in this case. With respect to AT1R-stimulated Ca^{2+} release under conditions of PTX and FR900359 pretreatment, we observe that PTX diplayed a trend for increased Ca^{2+} release while FR900359 pre-treatment results in a significant reduction in Ca^{2+} release (Figure 4.4D).

Proximal to the activation of the G protein, we next inhibited the activity of PLCβ, and effector activated downstream of both $G_{q/11}$ and $G_{i/o}$. U71322-mediated inhibition of PLCβ

showed a small increase in the basal interaction between Gβγ and RNAPII, but reduced agonist-induced interaction, suggesting a role for PLCβ in mediating the interaction (Figure 4.4E). Unlike the effects seen in HEK 293 cells, chelation of Ca^{2+} using BAPTA-AM abrogated the agonist-induced interaction in RNCFs (Figure 4.4F), suggesting a central role for Ca^{2+} as well. Interestingly, inhibition of inhibition of CaMKII with KN-93 or PKC with Go6983 both blocked the Ang II-dependent increase in interaction (Figure 4.4G, H). Conversely, inhibition of calcineurin with cyclosporin A increased the basal interaction and further amplified the Ang II-dependent increase in interaction (Figure 4.4I). Inhibition of PP1α with calyculinA was attempted but not pursued it proved toxic for RNCFs (data not shown).

Gβγ has previously been demonstrated to be involved in the autophosphorylation of and nuclear import of ERK1/2 in cardiomyocytes [188]. Although we demonstrate that ERK1/2 does not interact with Rpb1 in HEK 293 cells (Supplemental Figure 4.1C), we next assessed its involvement in inducing the Gβγ/Rpb1 interaction by inhibiting the activity of the Raf-MEK1-ERK1/2 pathway via the inhibition of MEK1 with U0126. Interestingly, inhibition of MEK1 led to an increased basal interaction but a loss of the Ang II-induced interaction (Figure 4.4J). Taken together with our observations of the induced interaction in HEK 293 cells, these findings suggest that the pathways responsible for regulating the interaction between Gβγ and RNAPII are both cell type-, and pathway-specific.

4.5.5 Analysis of effects of the interaction on gene expression in RNCFs

Cdk7 and Cdk9 are known to phosphorylate heptad repeats contained within the C-terminal domain of Rpb1 at serine positions 5 and 2, respectively (Ser5p-Rpb and Ser2p-Rpb1). In order to assess the significance of these differentially phosphorylated subtypes of Rpb1 and the types of Rpb1 that interacts with Gβγ, we first assessed the effect of both Cdk9 and Cdk7 inhibition on our agonist-induced interaction in either HEK 293 cells or RNCFs. Using DRB at concentrations known to affect the addition of both phosphorylation marks on Rbp1 (50 μM), we observed that inhibition of both these enzymes resulted in the loss of Ang II-induced interaction between Gβγ and Rpb1 in RNCFs, and carbachol-induced interaction in HEK 293 cells (Figure 4.5A-B). Calcium signalling has been previously described to be involved in the PP2B and PP1α-mediated disruption of the 7SK snRNP-HEXIM-P-TEFb complex, which leads to pause release and eventual transcription [449]. Therefore, it would appear that this interaction is dependent upon recruitment of RNAPII to promoter regions and the subsequent control of proximal promoter pausing and release. In order to test whether Gβγ interacts with paused RNA Pol II, we performed co-immunoprecipitation assays of Ser5p-Rpb1 with Gβ under conditions of $G\beta_1$ and $G\beta_2$ knockdown.We observed a net increase in interaction of Gβγ with Ser5p-Rpb1 upon Ang II treatment, whereas knockdown of $G\beta_1$ resulted in a loss of this increase and $G\beta_2$ knockdown did not affect Ang II-induced interaction (Supplemental Figure 4.3F). This suggests that $G\beta_1$ interacts with Ser5p-Rpb1 following Ang II treatment.

To assess the significance of this net increase in association of Gβγ with Rpb1 in response to Ang II, we next assessed the effect of knockdown of $G\beta_1$ and $G\beta_2$ on both basal and

Ang II-modulated gene expression in rat neonatal cardiac fibroblasts. Using a qPCR array based gene expression profiler, we assessed the expression of 84 genes known to be regulated in fibrosis. Of these 84 genes whose expression levels were measurable with our arrays, 11 genes were beyond our chosen limit of detection (i.e. Ct > 35) and were thus excluded from subsequent analysis. Of the remaining 73 genes analyzed, with respect to transcripts known to be upregulated upon Ang II stimulation of AT1R [312, 314, 442, 443, 450], we observed significant increases (fold change > 2.0, $p<0.05$) in the expression of Ctgf, End1, Itga1, Itgb3, Pdgfa, Tgfb3 and Timp1 (Figure 4.5C, D, Supplemental Table 1). Furthermore, Ang II treatment yielded trends for greater than two-fold increases in expression of Acta2, Ccl12, Itga2, Itga3, Itgb1, Itgb8, Lox, Smad3, Thbs1 and Timp 4 (Supplemental Table 1). Ang II-induced increases in mRNA expression of these particular genes was used to validate our qPCR array.

Upon observation that $G\beta_2$ is necessary for the initiation of Ang II-induced, AT1R mediated downstream signalling cascades (as measured by Ca^{2+} release assays, Figure 4.3F), we next assessed the effect of $G\beta_2$ knockdown on Ang II-regulated gene expression. Our analysis revealed that $G\beta_2$ acts as both an inhibitor and promoter of gene expression under basal conditions. Knockdown of $G\beta_2$ resulted in a basal upregulation of Bmp7, Bcl2, Itga3 and Ccl12 while also causing downregulation of Ila1 and Tnfa ($p<0.05$, Figure 4.5E-J, Table 1). Moreover, with respect to the changes in expression of Bmp7, Bcl2 and Il1a, changes to basal levels of these genes were maintained with Ang II treatment, i.e., Ang II did not result in gene expression changes significantly different from basal changes due to $G\beta_2$ knockdown. However, it is interesting to note that knockdown of $G\beta_2$ did not only significantly decrease basal expression of Tnfa, but also blocked the trend for the Ang II-mediated increase expression observed under

control conditions (Figure 4.5H). Such was not the case for Itga3 and Ccl12. We observed opposite results with respect to Itga3 gene expression – knockdown of $G\beta_2$ resulted in a significant upregulation in basal expression and also a trend for increased expression with concurrent Ang II treatment higher than siRNA control and Ang II treatment levels (Figure 4.5I). With regards to expression of Ccl12, Ang II treatment with concurrent $G\beta_2$ knockdown caused a 4.43-fold increase over conditions of $G\beta_2$ knockdown and Ang II treatment (Supplemental Table 2) over significant basal downregulation of Ccl12, almost a doubling of the fold change response under conditions of no $G\beta_2$ knockdown (2.53 fold change, Supplemental Table 2). Such varying responses suggest that $G\beta_2$-containing $G\beta\gamma$ dimers act not only as positive modulators of gene expression under basal conditions and in response to Ang II, but also as repressors of gene expression in some cases.

Our data suggests that $G\beta_1$/RNAPII interactions increase under conditions of M3-mAChR stimulation with carbachol in HEK 293 cells and AT1R stimulation with Ang II in rat neonatal cardiac fibroblasts. In order to extract clues regarding this agonist-induced interaction, we assessed changes in basal and Ang II-regulated gene expression under conditions of $G\beta_1$ knockdown. Unlike $G\beta_2$, knockdown of $G\beta_1$ did not result in significant downregulation of any of the 73 genes assessed in our arrays, with the possible exception of Ccl12 (0.181 fold change compared to siRNA control, Supplemental Table 4.2). Conversely, $G\beta_1$ knockdown resulted in a significant upregulation of 18 different genes that included Akt1, Bcl2, Bmp7, Cav1, Cxcr4, Egf, Itga2, Itga3, Itgb3, Itgb6, Jun, Mmp8, Plau, Smad4, Smad6, Smad7, Sp1 and Tgfbr2 (Figure 4.6A-E, Table 4.2). Interestingly, concurrent knockdown of $G\beta_1$ and activation of AT1R with did not significantly change the observed upregulation of expression for all of these genes except for

Itga2 (Figure 4.6F), although trends for increase in expression beyond the increased basal expression for Itgb3 and Cav1 were observed (Figure 4.6G, H). It is worth noting that in the case of these genes, $G\beta_2$ knockdown did not affect basal or Ang II-induced gene expression, suggesting that regulation of expression is under control of $G\beta_1$-containing dimers, and not those containing $G\beta_2$. Our results suggest that $G\beta_1$ acts as a repressor of gene expression at the level of transcription and that $G\beta_2$ regulates signalling pathways that converge on gene expression. Taken together, considering the interaction of $G\beta\gamma$ with RNAPII under conditions of AT1R activation, it would appear that $G\beta_1$-containing $G\beta\gamma$ dimers are involved in regulation of initiation and pause release of RNAPII-mediated transcription, with $G\beta_1\gamma$ dimers acting as repressors of pause release.

4.6 Discussion

Here, we demonstrate for the first time, a novel interaction between Gβγ and RNA polymerase II that occurs under basal and GPCR -stimulated conditions in both transformed cell lines (HEK 293 cells) and in primary rat neonatal cardiac fibroblasts. Although a majority of previous studies have focused on elucidating the significance of Gβ and Gγ subunit specificity for signalling proximal to GPCR activation (i.e., the regulation of effector activity downstream of receptor stimulation) (reviewed in [335]), our findings provide further insight regarding non-canonical roles of specific Gβ-containing Gβγ dimers for more distal signalling processes in the nucleus, and in particular, the regulation of gene expression. The observation of the interaction between Gβγ and RNA polymerase II is a significant addition to the expanding list of Gβγ interactors (reviewed in [335]), and our results suggest that the regulation of this interaction is dependent on cellular context and is also signalling pathway-specific.

Our analysis of this interaction revealed that endogenous Gβγ interacts with Rpb1 upon activation of endogenous M3-mAChR and AT1R in HEK 293 cells and rat neonatal cardiac fibroblasts, respectively. Although these two receptors are both coupled to G_q, the wiring of signalling pathways that lead to the interaction were different both in terms of kinetics (45 minutes vs 75 minutes for maximal interaction, Figures 4.1B, 4.3B) and signalling pathways involved. Distinct signalling pathways regulated the interaction downstream of M3-mAChR and AT1R in the two different cell types. *In vitro* immunoprecipiation studies of other specific subunits of RNA Polymerase II revealed that in addition to the Gβγ-Rpb1 interaction observed *in cellulo*, Gβγ from HEK 293 cell and RNCF lysates also interacted with purified GST-hRpb6, -

hRpb8 and h-Rpb9, validating the occurrence measured via co-immunoprecipitation in both cells (Figures 4.1D, 4.3C). Previously published structures of RNA polymerase II suggest that Rpb1, Rpb6, Rpb8 and Rpb9 are arranged in such a way that forms a common interface on RNAPII to which we hypothesize Gβγ binds [451, 452]. While it remains to be identified exactly which structural interface of Gβγ binds RNAPII, it must first be determined whether Gβγ is a direct interactor of RNAPII. Preliminary analysis of *in vitro* immunoprecipitation experiements with purified proteins suggests that purified $G\beta_1\gamma_2$ interacts with purified GST-hRpb8 (data not shown). Furthermore, we demonstrate that this interaction is specific to RNAPII, and not RNAPI (Supplemental Figure 4.1B). Although this study focuses on the induction of this interaction upon activation of two different Gq-coupled GPCRs in two different cell types, we believe that such a phenomenon may be common to all GPCR signalling pathways that converge on regulation of gene expression. Indeed, in a separate assessment of this interaction, we observed that stimulation of endogenous β_2-adrenergic receptors with isoproterenol also induces the interaction to occur, albeit with different kinetics and presumably via activation of different G proteins (data not shown).

In addition, our results show that import of Gβγ to the nucleus is dependent on importin-β that in turn facilitates Gβγ interacting with RNAPII (Figures 4.1F, 4.3E). These observations align with previous TAP-tagged mass spectrometry studies that demonstrate that Gβγ dimers interact with different proteins that regulate import/export, various nucleoporins and heterologous ribonuclearproteins [184]. We demonstrated that inhibition of importin-β with importazole results in blockade of Gβγ nuclear import (Supplemental Figures 4.1E, 4.F) and

abrogration the Gβγ-Rpb1 interaction; what remains to be identified are the specifics of Gβγ nuclear import and export upon both basal conditions and under conditions of GPCR activation.

Assessment of the signalling pathways responsible for inducing the Gβγ-RNAPII interaction has yielded three main conclusions: (1) different GPCR signalling systems show different kinetics in the induction of this interaction, (2) different signalling pathways downstream of GPCR activation act to both induce or regulate this interaction and (3) different Gq-coupled GPCRs induce this interaction via involvement of different kinases and phosphatases known to be activated upon receptor stimulation in different cell models. Indeed, our results suggest that cell context is important when regarding the mechanism of action by which this interaction occurs. Our group, in addition to others, have previously demonstrated that M3-mAChRs are the primary muscarinic acetylcholine receptors subtypes expressed in HEK 293 cells [406, 433]. From our analysis of the pathways that induce the interaction in these cells, we observes that the interaction occurs maximally 45-60 minutes after M3-mAChR stimulation and depends critically on a G_q-PLCβ-Ca^{2+}-calcineurin pathway downstream of M3-mAChR activation, whereby PKC and CamKII both negatively regulate this interaction under basal conditions (summarized in Supplemental Figure 4.6).

With respect to the interaction in RNCFs, although both AT1R and AT2R receptor subtypes have been found to be expressed in rat neonatal cardiac fibroblasts [453, 454], our experiments using losartan to block AT1R showed a loss of the Ang II-induced interaction (Figure 4.3D). This suggests that the observed induction of the Gβγ-RNAPII interaction is AT1R specific in cardiac fibroblasts and is not induced by AT2R activation. Assessment of the roles of

$G\alpha_q$ vs. $G\alpha_i$ in inducing the interaction reveals a regulatory role for G_i-coupled AT1R whereby inhibiting these receptors acted to potentiate the interaction observed as a result of activating G_q-coupled AT1Rs (Figure 4.4B-D). One potential mechanism of regulation is the role of AT2R vs AT1R. AT2R has previously been shown to counter act AT1R mediated effects in cardiac contexts [455, 456] by altering the conformation of AT1R and thus altering its signalling [457]. Furthermore, AT2R has been found to couple to G_i [458, 459]. Taken together, our results suggest that $G\alpha_{i/o}$ coupled AT2R may act to control of the state of the $G\alpha_{q/11}$-coupled AT1R, either by altering its conformation at the cell surface or its activity, resulting in a dysregulation of AT1R-mediated signalling and thus a dysregulated Gβγ-RNAPII interaction. Therefore, the observed pathway required for induction of the interaction in RNCFs is a G_q-PLCβ-Ca^{2+}-CamKII/PKC/MEK dependent pathway downstream of AT1R activation, whereby calcineurin acts as a basal negative regulator (summarized in Supplemental Figure 4.7).

Although such opposing signalling modes of PKC and calcineurin is contrary to previous reports that suggest cosignalling between these two kinases and phosphatases downstream of toll-like receptor 4 leading to suppression of fibrotic markers [460], our results shed light on the complexity of signalling downstream of different receptors in response to different extracellular stimuli that lead to opposing responses. The involvement of Ca^{2+}, PKC and ERK1/2 in inducing the Gβγ-Rpb1 interaction is supported by previous reports that demonstrate that Ang II-induced fibrosis requires these entities [461, 462]. Although Gβγ has been described as an interactor of ERK1/2 and is involved in its auto-phosphorylation, dimerization and subsequent nuclear entry [188], we observe that neither Gα1 nor ERK1/2 are co-interactors of the Gβγ-RNAPII interaction (Supplemental Figures 4.1C, D). The requirement of these proteins involved in inducing the

Gβγ-RNAPII interaction is corroborated by the fact that these signalling molecules been described to be activated upon Ang II treatment in RNCFs [463]. Irrespective of the different pathways taken to induce this novel Gβγ-RNAPII interaction, it appears that both these pathways converge on the activity of Cdk9 and Cdk7 as inhibition of both of these kinases with DRB results in the loss of both the carbachol-induced interaction in HEK 293 cells and Ang II-induced interaction in cardiac fibroblasts.

With respect to $G\beta_2$-containing Gβγ dimers, we did not observe dramatic changes in gene expression, as only 4 genes were observed to be upregulated and 3 downregulated (Table 4.1), with the rest of the genes analyzed following expression patterns similar to control conditions. Assessment of the roles of specific Gβγ that control second messenger release downstream of AT1R activation demonstrates that $G\beta_2$ knockdown in RNCFs resulted in a ~30% decrease in AT1R-mediated Ca^{2+} release, while $G\beta_1$ knockdown did not significantly alter Ca^{2+} release (Figure 4.3F). We hypothesize that the direct role of $G\beta_2$ in gene expression regulation in response to Ang II is minimal, and that $G\beta_2$ is likely more important for proximal AT1R mediated signal transduction; evidence supporting this notion previous studies that have also shown $G\beta_2\gamma$ coupling to AT1R [238]. Such a role for $G\beta_2$ further corroborates our hypothesis that $G\beta_2$-containing Gβγ dimers are more important for signal transduction in AT1R signalling, whereas $G\beta_1$ containing Gβγ dimers are more important for direct regulation of RNAPII. The roles of specific Gγ subunits in mediating proximal signal transduction must also be considered as previously performed [433], and will be the subject of future studies.

Roles for Gβγ in the regulation of gene expression has primarily been described in the context of modulation and control of signalling pathways upon GPCR activation that ultimately converge on such regulations in the nucleus (reviewed in [5]), with examples being the Gβγ-PI3K-Pax8 dependent transcription of sodium-iodide transporter and the modulation of interleukin-2 mRNA levels in CD4+ T-helper cells [173, 234]. Other studies have described direct roles for Gβγ in gene expression regulation that include the relief of transcriptional repression exhibited by its interactions with AEBP1 [239] and HDAC5 (in the context of MEF2A transcriptional activity repression) [240]. While these studies have described Gβγ-mediated regulation of signalling pathways or proteins known to regulate gene expression, our results suggest a more direct role for Gβγ in gene expression regulation. Our qPCR array suggests that $G\beta_1$-containing Gβγ dimers serve as repressors of gene expression. Taken together with the fact that $G\beta_1$ increases its association with Rpb1 with AngII stimulation (Supplemental Figure 4.2C), DRB inhibits the Ang II-mediated net increase of Gβγ binding to Rpb1 (Figure 4.5B), and that knockdown of Gβ1 results in a loss of Gβγ interacting with Ser5p-Rpb1 in particular upon AT1R activation (Supplemental Figure 4.3F), it would appear as though AngII treatment results in a phenomena that induces $G\beta_1$ to interact with paused Pol II that prevents release into elongating RNA Pol II. Such a mechanism corroborates well with the fact that knockdown of $G\beta_1$ results in upregulation of 18 genes out of the 73 that are implicated in fibrosis (Table 4.2), with a particular example including basal upregulation of Itga2 and maintenance of Ang II-mediated increase in gene expression under $G\beta_1$ knockdown conditions (Figure 4.6F), which suggest loss of pause regulation at this gene. Intriguingly, $G\beta_1$ knockdown resulted in upregulation of Jun (Figure 4.6C), a component of the AP-1 transcription factor alongside cFos. Given that Gβγ has been previously shown to interact with cFos/Jun [183], such observations

suggests regulatory mechanisms whereby Gβγ act to regulate the expression of their own interactors. Indeed, we have previously described such a phenomena whereby knockdown of $G\beta_1$ in HEK 293 cells results in upregulation of ERK1/2 protein expression [433]. Overall, it would appear that the function of $G\beta_1\gamma$ in the fibrotic response is inhibitory.

Activation of TGF-β receptors, endothelin-1 receptors, AT1R signalling have been extensively described to be important mediators of pro-fibrotic responses in cardiac fibroblasts. Ang II has been found to be an important driving factor in fibrotic responses [445] in which Ang II signalling acts to induce the activities of both TGF-β and ET-1 signalling pathways [442]; examples of such Ang II-dependent induction include the upegulation of TGF-β1 expression [312, 443] and ET-1 expression [464]. Furthermore, Ang II treatment is known to induce gene expression directly via its own signalling pathways, for example the expression of pro-fibrotic genes such as CTGF [442, 450], or indirectly through activation of TGF-β and ET-1 signalling that causes expression of genes like collagen I [314, 442]. Considering how Ang II signalling is so pivotal to regulation of fibrotic responses in cardiac fibroblasts, deciphering the precise mechanisms of how its own signalling pathways induce gene expression becomes important. Our demonstration of the $G\beta_1\gamma$-RNAPII interaction provides for a mechanism by which this interaction may directly regulate the expression of known pro-fibrotic genes such as Tgfbr2, members of the Smad family and integrins in AT1R signalling in fibroblasts.

4.7 Conclusions

Overall, our characterization of this novel interaction between Gβγ and RNA polymerase II suggests a new level of regulatory function for Gβγ in gene expression. Our studies highlight specificities of interaction for different Gβ subtypes containing Gβγ dimers that is maintained upon stimulation of different Gq-coupled receptors and suggests divergent functions for different kinases and phosphatases that are activated downstream of GPCR activation in regulating this interaction. Since $G\beta_1\gamma$ dimers possess negative regulatory roles for the expression of fibrotic genes in cardiac fibroblasts, pharmacological inhibition of these specific dimers in the context of cardiac fibrosis may pose as an avenue for potential therapeutic intervention.

4.8 Acknowledgements

The authors thank the aforementioned investigators for their kind gifts of reagents, cell lines, and purified proteins. We would also like to thank Dr. Nicolas Audet (McGill University) for isolation of cardiac fibroblasts used in the initial characterization of the interaction as well as his assistance with the statistical analysis. This work was supported by grants from the Canadian Institutes of Health Research (CIHR, MOP-119290) and from the Natural Sciences and Engineering Research Council of Canada (NSERC). SMK was supported by a scholarship from the CIHR Drug Discovery Training Program and Graduate Excellence Fellowships from The Department of Pharmacology and Therapeutics, McGill University. RM was supported by a scholarship from the CIHR Drug Discovery Training Program and a CIHR Frederick Banting and Charles Best Canada Graduate Scholarship.

4.9 Figure Legends for Chapter 4

Figure 4.1 ***Characterization of the interaction between Gβγ and Rpb1 in HEK 293 cells***

(A) Time-course analysis of the induction of the Gβγ-Rpb1 interaction – HEK 293 cells treated for the indicated times with 1mM carbachol were subjected to immunoprecipitation (IP) of Gβ from total lysates and the amount of Rpb1 co-immunoprecipitated (co-IP) was assessed by western blot for each time point. Data is representative of 3 independent experiments. (B) Quantification of Gβγ-Rpb1 time-course IP. Densitometry analysis yielding values reflecting bands intensity that corresponding to amount of Rpb1 co-immunoprecipitated in each timepoint was normalized to the band intensity of the amount of Gβ immunoprecipitated to yield ratios of Rpb1 pulled down with Gβ. Resulting ratios were then normalized to the 0 mins treatment time point. Data is representive as mean ± S.E.M; ** indicates $p<0.01$, * indicates $p<0.05$. (C) Reverse-IP analysis of Rpb1 interacting with Gβ using two different antibodies against Rpb1. Western blots are representative of at least two independent experiments. (D) *In vitro* assessment of interaction of GST-hRpb subunits with Gβ expressed in HEK 293 cell lysates. Data is representative of 3 independent experiments. (E) Assessment of specific FLAG-tagged Gβ subunits that interact with Rpb1 under conditions of M3-mAChR stimulation with carbachol. Data is representative of 3 independent experiments. (F) Subcelluar fractionation-based assessment of the Gβγ-Rpb1 demonstrating that the interaction occurs in the nuclear fraction and that nuclear import of Gβγ is necessary to facilitate the interaction. (G) Densitometry-based quantification of the carbachol-induced interaction and the effect of nuclear import inhibition on interaction induction. Data is representative of 3 independent experiments for black bars, and 2 independent experiments for white bars (nuclear import inhibition conditions).

Figure 4.2 ***Analysis of the mechanism through which the carbachol induced Gβγ interaction occurs in HEK 293 cells***

(A-G) HEK 293 cells starved for 10-12 hours in DMEM without FBS were pre-treated with the indicated inhibitors against different proteins for the indicated times. Cells were then treated with carbachol for 45 minutes and analysis of effector inhibition on of the amount of Rpb1 co-immunoprepitated with Gβ was assessed by western blot. Data is representative of atl east 3 independent experiments. Corresponding quantifications of all experiments can be found in Supplemental Figure 4.2.

Figure 4.3 ***Characterization of Gβγ-Rpb1 in rat neonatal cardiac fibroblasts***

(A) Ang II-stimulated interaction induction timecourse – assessment of the amount of Rpb1 co-immunoprecipitated with Gβ upon treatment of 1 μM Ang II treatment at the indicated timepoints in RNCFs. (B) Quantification of Ang II interaction timecourse; data representative of two independent experiments. (C) *In vitro* demonstration of the interaction between Gβ from RNCF lysates with GST-tagged hRpb subunits. Data is representative of 3 independent experiments. (D) Effect of AT1R antagonist (Losartan) pre-treatment on the Ang II interaction to demonstrate angiotensin receptor subtype specificity for interaction in RNCF. Data is representative of 3 independent experiments. (E) Assessment of the necessity of Gβγ import into the nucleus for interaction to occur upon AT1R stimulation with Ang II. Data representative of 4 independent experiments. (F) Raw traces of calcium release upon AT1R stimulation with Ang II under conditions of $G\beta_1$ and $G\beta_2$ knockdown. Data points are representative of mean ± S.E.M. of fluorescence ratios of 340/516 emission readings to 360/516 emissions recordings normalized to basal ratios, and of three independent experiments. (G) Area under the curve analysis of curves

obtained in (F), * indicates $p<0.05$. (H) siRNA knockdown mediated assessment of specific Gβ subunits that interact with Rpb1 upon AT1R stimulation. RNCFs transfected with siRNA control, $G\beta_1$ or $G\beta_2$ for 72 hours, starved overnight and treated with Ang II for 75 minutes were assessed for interaction induction by IP and western blots. (I) Quantification of knockdown experiments in (H); data is representative of mean ± S.E.M. of six independent experiments.

Figure 4.4 ***Characterization of the mechanism through which Gβγ interacts with Rpb1 in rat neonatal cardiac fibroblasts***

(A-C, E-J) Assessment of the effect of inhibition of signalling molecules and effectors implicated in AT1R signalling on the induction of the Gβγ-Rpb1 interact in RNCFs. Concentrations of inhibitors and lengths of pre-treatment are indicated in each subfigure. 75 minutes of 1 μM Ang II treatment was used in all experiments shown to induce the interaction. Data shown is representative of between 3 and 6 independent co-immunoprecipitation and western blot experiments. Corresponding quantification analyses of inhibitor co-IP experiments are depicted in Supplemental Figure 4.4. (D) Analysis of the effect of $G\alpha_{q/11}$ inhibition with Ubo, $G\alpha_i/o$ inhibition with PTX and co-inhibition of both $G\alpha_{q/11}$ and $G\alpha_{i/o}$ on AT1R-stimulated Ca^{2+} release in RNCFs. Data shown is representative of areas under curves derived from Fura-2AM based Ca^{2+} release assays over time, is representative of atleast 3 independent experiments. ** indicates $p<0.01$; *** indicates $p<0.001$.

Figure 4.5 ***Functional analysis of Gβγ-Rpb1 interaction in rat neonatal cardiac fibroblasts***

(A, B) Effect of Cdk7 and Cdk9 inhibition with DRB on both carbachol- and Ang II-induced Gβγ-Rpb1 interaction in HEK 293 cells and RNCFs, respectively – Length of inhibitor pre-

treatment is indicated on each respective subfigure, and assessment of interaction was performed via co-immunoprecipitation experiments coupled to western blot analysis. Data is representative of 3 independent experiments; quantifications of blots are depicted in Supplemental Figure 4.2G and Supplemental Figure 4.4L for (A) and (B), respectively. (C, D) Validation of fibrosis qPCR array – Graphs depict two different genes that are known to be upregulated upon AngII treatment in RNCFs. (E-J) Representative basal and Ang II-induced fold change expression patterns of genes affected by $G\beta_2$ knockdown. Examples depicted in (E) and (F) are genes that are upregulated upon knockdown of $G\beta_2$. (G) and (H) represent genes that are downregulated basally upon knockdown of $G\beta_2$. (I, J) Examples of genes that display upregulation (I) and downregulation (J) of expression at basal levels, but whose expression induction is not affected by Ang II treatment. Data represented in all graphs are as fold change over siRNA control/DMEM; bars in graphs represent mean fold change over control ± S.E.M and are representative of at least 3 separate independent experiments; * indicates $p<0.05$, ** indicates $p<0.01$.

Figure 4.6 ***Effect of $G\beta_1$ knockdown on AngII induced gene expression in rat neonatal cardiac fibroblasts***

(A-E) Representative examples of genes whose expressions are basally upregulated upon $G\beta_1$ knockdown and whose upregulation is not affected by Ang II treatment. (F-H) Gene expression profiles of genes basally upregulated by $G\beta_1$ knockdown and also dysregulated with Ang II treatment. Data represented in all graphs are as fold change over siRNA control/DMEM; bars in graphs represent mean fold change over control ± S.E.M; * indicates $p<0.05$, ** indicates $p<0.01$.

Table 4.1 ***Effect of $G\beta_2$ knockdown on gene expression***

Table depicts a list of genes affected by $G\beta_2$ knockdown; the 7 genes either upregulated or downregulated under both basal conditions and Ang II-treated conditions, and extent of statistical significance are depicted. Representative fold changes are depicted in Supplemental Table 2.

Table 4.2 ***Effect of $G\beta_1$ knockdown on gene expression***

Depiction of a complete list of the 18 genes basally upregulated upon knockdown of $G\beta_1$ in RNCFs, and the effects of knockdown on Ang II-induced gene responses. Statistical significance of changes are listed alongside changes where necessary.

Supplemental Figure 4.1 ***Supporting data for the induction of the Gβγ-Rpb1 interaction in HEK 293 cells***

(A) Initial observation of interaction between Gβγ and RNAPII. HEK 293 cells stably expressing TAP-tagged $G\beta_1$ were treated with vehicle, 100 nM PMA (PKC activator) or not treated and immunoprecipitation of both Rpb1 and Gβ were performed to assess whether Gβ or Rpb1 are co-immunoprecipitated, respectively. Data is representative of one independent experiment. (B) Assessment of interaction between Gβ and Rpa194, the largest subunit of RNA polymerase I. Data represents analysis of a timecourse experiment blot performed as in Figure 4.1A. These results indicate that Gβγ does not interact with Rpa194. (C,D) Immunoprecipitation experiments demonstrating that carbachol treatment does not induce interaction of Rpb1 with Gαq nor 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) Quantitative analysis demonstrating decreases in Gβ content

in the cytosol and accompanying increases in the nucleus upon carbachol treatment in HEK 293 cells. Cells treated with carbachol for increasing amounts of time were subcellularly fractionated to yield cytosolic and nuclear fractions. Amounts of Gβ in each fraction were then assessed by western blot, upon which intensities from Gβ bands on blots were quantified using ImageJ. Data shown is representative of fold changes over 0 minutes treatment control, and is indicative of a single experiment. (F) Effect nuclear import inhibition with importazole on trafficking of Gβ to the nucleus. Cells pre-treated with 40 μM importazole and treated with carbachol for the indicated times were analyzed for Gβ distribution in the cytosol and nucleus as described in (E).

Supplemental Figure 4.2 ***Quantitative analysis of the effect of inhibition of signalling molecules downstream of M3-mAChR activation***

(A-G) The relative quantities of Rpb1 co-immunoprecipitated with Gβ under different conditions depicted in Figure 4.2 and Figure 4.5A were quantified using Image J and were normalized to amounts pulled down in DMSO/DMEM control conditions (A, C-G) or control parental HEK 293 conditions. Data is represented as fold change over respective controls and error bars represent S.E.M. * indicates $p<0.05$.

Supplemental Figure 4.3 ***Assessment of interaction in animal models and supporting evidence of the Gβγ-Rpb1 interaction in rat neonatal cardiac fibroblasts***

(A) Induction of the Gβγ-Rpb1 interaction in transverse aortic constriction (TAC) mice, but not in sham surgery mice. Mice were subjected to TAC or sham surgery for either 3, 7, 14, 30 or 60 days upon which hearts were surgically removed and lysed. Lysates were subsequently assessed for the presence of the Gβγ-Rpb1 interaction as previously mentioned via immunoprecipitation

and western blot. Blots are representative of a single experiment. (B) Observation of Gβγ-Rpb1 in aged rat hearts. Hearts were surgically removed from aged rats and subsequently lysed. Detection of the interaction was performed as described in (A), with no-antibody controls. (C) Assessment of specific Gβ subunits that interact with Rpb1 upon Ang II treatment in RNCFs. $G\beta_1$ and $G\beta_2$ were immunoprecipitated from RNCF lysates treated with 1 μM Ang II for 75 minutes and the amount of Rpb1 pulled down with either Gβ was assessed. For $G\beta_1$ specific IP, we observe no basal interaction but a net increase in the interaction, whereas for $G\beta_2$ specific IPs, we observe a basal interaction which is lost with Ang II treatment, suggesting interplay of specific Gβ subunits interacting with Rpb1 as seen in Figure 4.1E. (D, E) Validation of $G\beta_1$ and $G\beta_2$ mRNA (D) and protein (E) knockdown in RNCFs. Data in (D) are represented as fold change over control and is representative of 4 independent experiments; *** indicates $p<0.001$ and **** indicates $p<0.0001$. (F) Assessment of the effect of knockdown of $G\beta_1$ and $G\beta_2$ on the ability of Gβ to interact with Ser5-phosphorylated Rpb1 under conditions of Ang II treatment. RNCFs in which $G\beta_1$ or $G\beta_2$ were knocked down were subject to immunoprecipitation of Gβ and amounts of Ser5p-Rpb1 co-immunoprecipitated were assessed by western blot.

Supplemental Figure 4.4 ***Quantitative analysis of the effect of inhibition of signalling molecules downstream of AT1R activation in rat neonatal cardiac fibroblasts***

The relative quantities of Rpb1 co-immunoprecipitated with Gβ under different conditions in conjunction with 1 μM Ang II treatment depicted in Figure 4.4 and Figure 4.5B were quantified using Image J and were normalized to amounts pulled down in DMSO/DMEM control conditions. Data is represented as fold change over respective controls and error bars represent S.E.M. * indicates $p<0.05$, ** indicates $p<0.01$.

Supplemental Figure 4.5 ***Cartoon scheme depicting the pathways necessary to induce the interaction in HEK 293 cells downstream of M3-mAChR activation***

Proteins that have been found to be inducers of the Gβγ-Rpb1 interaction are depicted in blue, whereas proteins that act to regulate the interaction under basal conditions are depicted in orange. Inhibitors against the proteins studied in our assays are depicted in red text.

Supplemental Figure 4.6 ***Cartoon scheme depicting the pathways necessary to induce the interaction in AT1R cells downstream of AT1R activation***

Proteins that have been found to be inducers of the Gβγ-Rpb1 interaction are depicted in blue, whereas proteins that act to regulate the interaction under basal conditions are depicted in orange. Inhibitors against the proteins studied in our assays are depicted in red text.

Supplemental Table 4.1 ***List of genes upregulated in rat neonatal cardiac fibroblasts with AngII treatment***

Fold changes over siRNA control/DMEM of genes that are known to be upregulated with activation of AT1R are listed here, along with p values obtained for the observed changes. * indicates $p<0.05$, ** indicates $p<0.01$.

Supplemental Table 4.2 ***Complete table of fibrosis qPCR array results***

Table portrays complete list of observed changes on gene expression under conditions of siRNA control, siRNA $G\beta_1$ or siRNA $G\beta_2$ with vehicle or Ang II treatment. Fold changes over siRNA control/DMEM conditions are listed in rows next to each gene. Boxes highlighted in green

indicate trends for upregulation, boxes highlighted in yellow indicate significant upregulations compared to respective control, boxes in red indicate trends for downregulation, while boxes in blue indicate genes significantly downregulated compared to respective control. Data is represented as fold change over control calculated from 3 independent samples for each condition run on each replicate's own PCR array plate.

Supplemental Table 4.3 ***List of primers used for validation of $G\beta_1$ and $G\beta_2$ knockdown in rat neonatal cardiac fibroblasts***

Sequences listed were used at concentrations of 300 nM for each reverse and forward primer for each qPCR reaction. Primer sequences were designed using Integrate DNA Technology's PrimerQuest Tool (https://www.idtdna.com/Primerquest/Home/Index) and validated by analysis of standard curve qPCR assays performed in-house.

4.10 Figures for Chapter 4

Figure 4.1

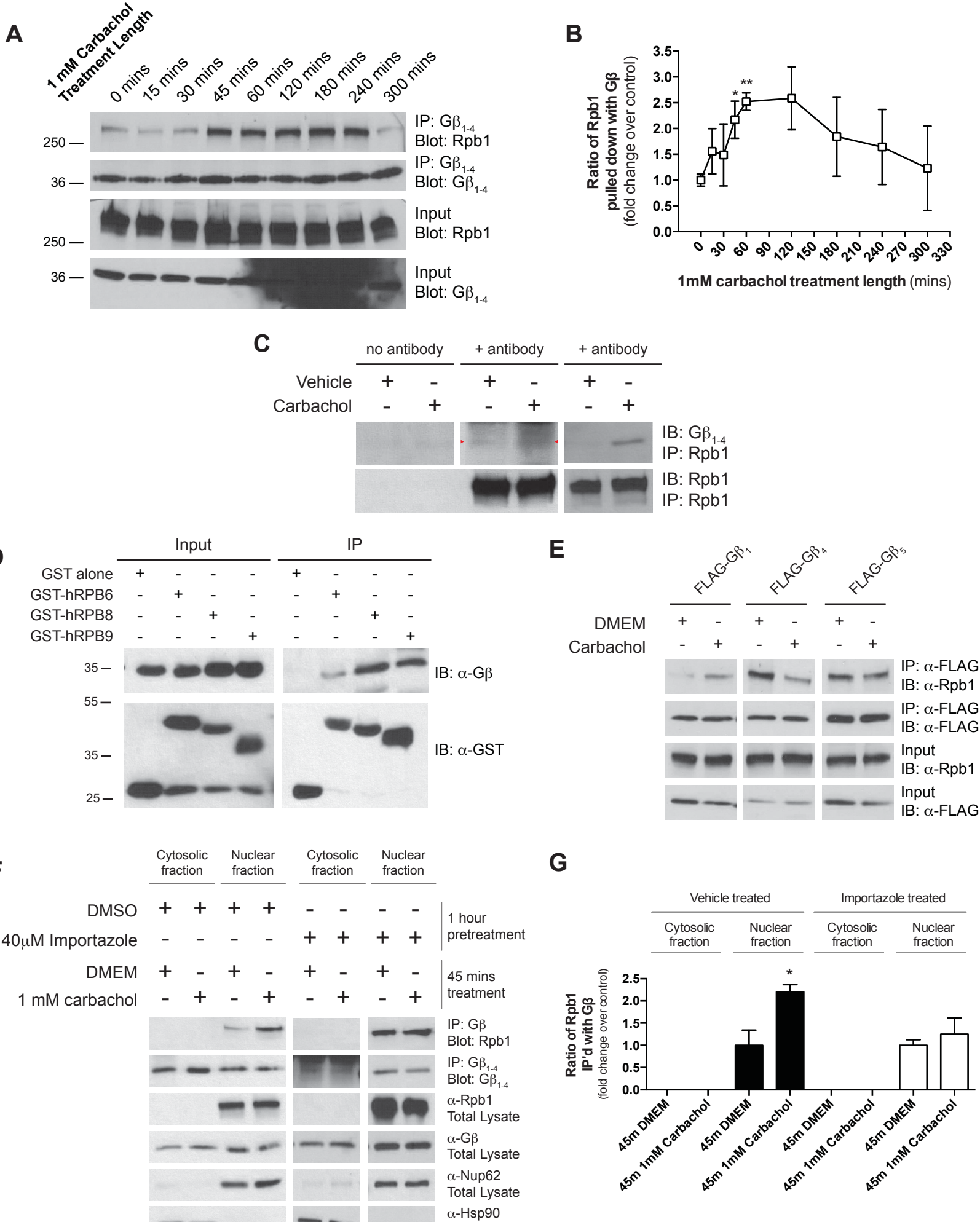

A
1 mM Carbachol Treatment Length
0 mins
15 mins
30 mins
45 mins
60 mins
120 mins
180 mins
240 mins
300 mins
250
36
250
36
IP: Gβ1-4
Blot: Rpb1
IP: Gβ1-4
Blot: Gβ1-4
Input
Blot: Rpb1
Input
Blot: Gβ1-4
B
Ratio of Rpb1 pulled down with Gβ
(fold change over control)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
**
0
30
60
90
120
150
180
210
240
270
300
330
1mM carbachol treatment length (mins)
C
no antibody
+ antibody
+ antibody
Vehicle
Carbachol
IB: Gβ1-4
IP: Rpb1
IB: Rpb1
IP: Rpb1
D
Input
IP
GST alone
GST-hRPB6
GST-hRPB8
GST-hRPB9
35
55
35
25
IB: α-Gβ
IB: α-GST
E
FLAG-Gβ1
FLAG-Gβ4
FLAG-Gβ5
DMEM
Carbachol
IP: α-FLAG
IB: α-Rpb1
IP: α-FLAG
IB: α-FLAG
Input
IB: α-Rpb1
Input
IB: α-FLAG
F
Cytosolic fraction
Nuclear fraction
Cytosolic fraction
Nuclear fraction
DMSO
40μM Importazole
1 hour pretreatment
DMEM
1 mM carbachol
45 mins treatment
IP: Gβ
Blot: Rpb1
IP: Gβ1-4
Blot: Gβ1-4
α-Rpb1
Total Lysate
α-Gβ
Total Lysate
α-Nup62
Total Lysate
α-Hsp90
Total Lysate
G
Vehicle treated
Importazole treated
Cytosolic fraction
Nuclear fraction
Cytosolic fraction
Nuclear fraction
Ratio of Rpb1 IP'd with Gβ
(fold change over control)
2.5
2.0
1.5
1.0
0.5
0.0
*
45m DMEM
45m 1mM Carbachol
45m DMEM
45m 1mM Carbachol
45m DMEM
45m 1mM Carbachol
45m DMEM
45m 1mM Carbachol

Figure 4.2

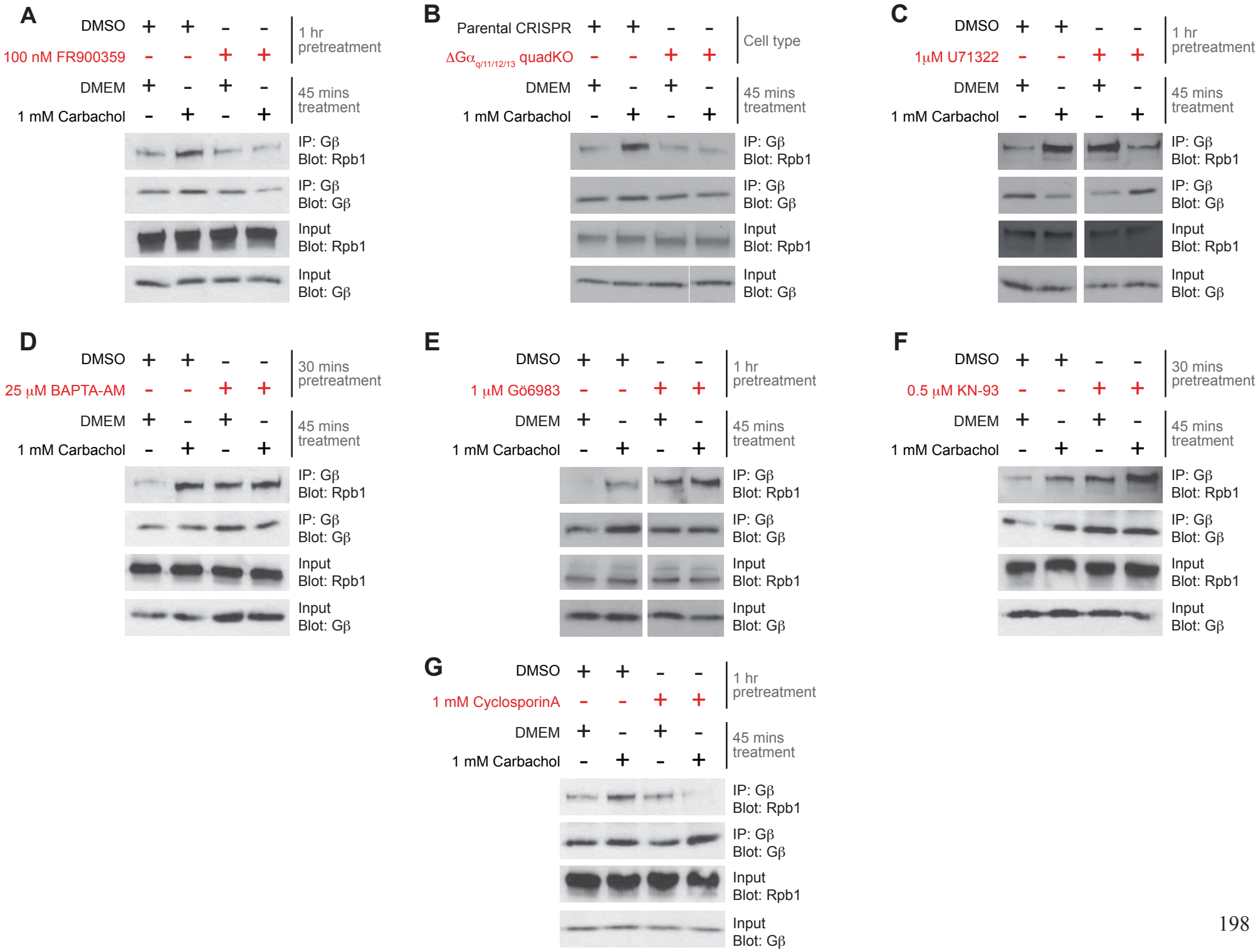

A
DMSO + + - -
100 nM FR900359 - - + +
1 hr pretreatment
DMEM + - + -
1 mM Carbachol - + - +
45 mins treatment
IP: Gβ Blot: Rpb1
IP: Gβ Blot: Gβ
Input Blot: Rpb1
Input Blot: Gβ
B
Parental CRISPR + + - -
ΔGα q/11/12/13 quadKO - - + +
Cell type
DMEM + - + -
1 mM Carbachol - + - +
45 mins treatment
IP: Gβ Blot: Rpb1
IP: Gβ Blot: Gβ
Input Blot: Rpb1
Input Blot: Gβ
C
DMSO + + - -
1μM U71322 - - + +
1 hr pretreatment
DMEM + - + -
1 mM Carbachol - + - +
45 mins treatment
IP: Gβ Blot: Rpb1
IP: Gβ Blot: Gβ
Input Blot: Rpb1
Input Blot: Gβ
D
DMSO + + - -
25 μM BAPTA-AM - - + +
30 mins pretreatment
DMEM + - + -
1 mM Carbachol - + - +
45 mins treatment
IP: Gβ Blot: Rpb1
IP: Gβ Blot: Gβ
Input Blot: Rpb1
Input Blot: Gβ
E
DMSO + + - -
1 μM Gö6983 - - + +
1 hr pretreatment
DMEM + - + -
1 mM Carbachol - + - +
45 mins treatment
IP: Gβ Blot: Rpb1
IP: Gβ Blot: Gβ
Input Blot: Rpb1
Input Blot: Gβ
F
DMSO + + - -
0.5 μM KN-93 - - + +
30 mins pretreatment
DMEM + - + -
1 mM Carbachol - + - +
45 mins treatment
IP: Gβ Blot: Rpb1
IP: Gβ Blot: Gβ
Input Blot: Rpb1
Input Blot: Gβ
G
DMSO + + - -
1 mM CyclosporinA - - + +
1 hr pretreatment
DMEM + - + -
1 mM Carbachol - + - +
45 mins treatment
IP: Gβ Blot: Rpb1
IP: Gβ Blot: Gβ
Input Blot: Rpb1
Input Blot: Gβ

Figure 4.3

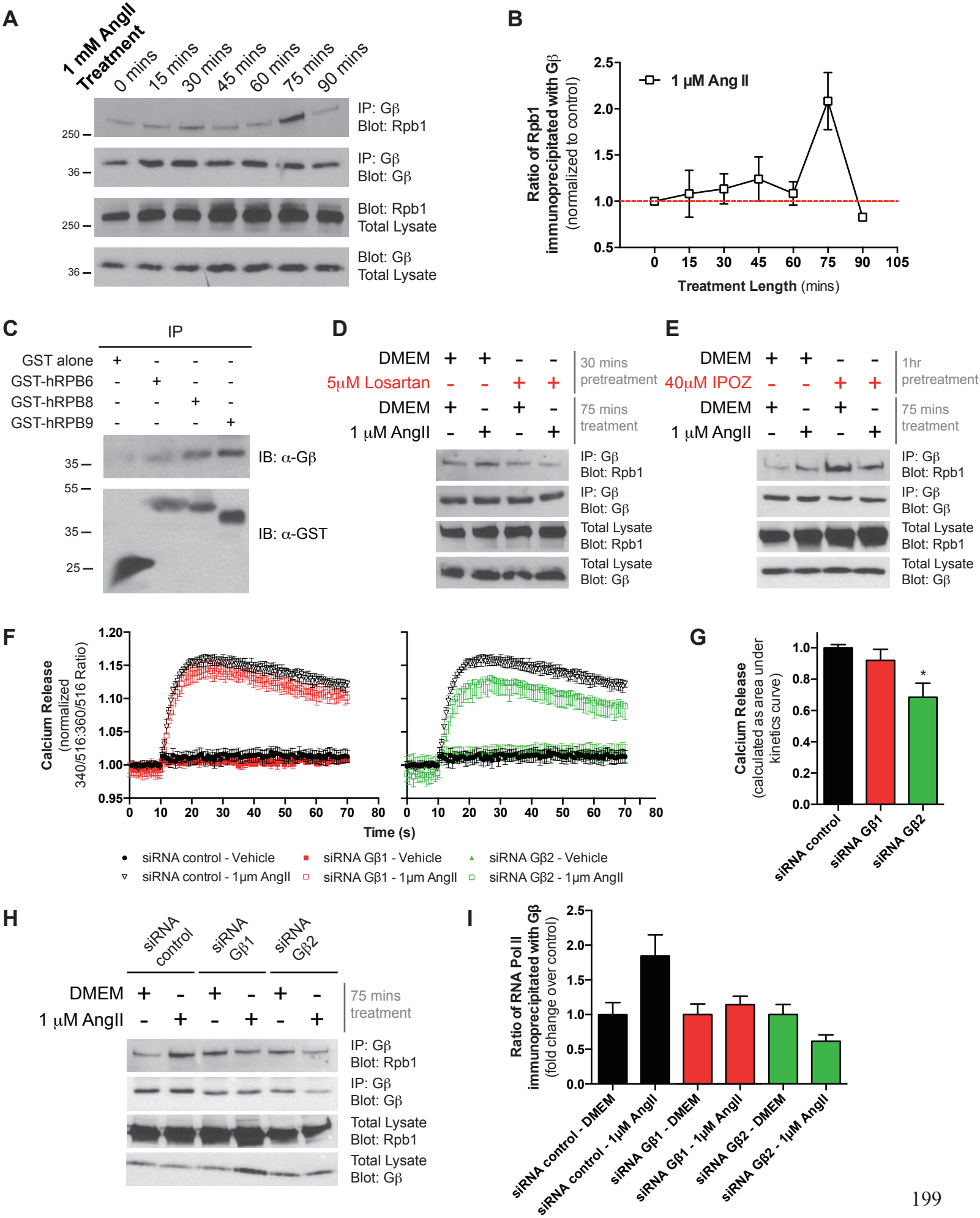

A
1 mM AngII Treatment
0 mins
15 mins
30 mins
45 mins
60 mins
75 mins
90 mins
250
36
250
36
IP: Gβ
Blot: Rpb1
IP: Gβ
Blot: Gβ
Blot: Rpb1
Total Lysate
Blot: Gβ
Total Lysate
B
Ratio of Rpb1 immunoprecipitated with Gβ
(normalized to control)
1 μM Ang II
Treatment Length (mins)
C
IP
GST alone
GST-hRPB6
GST-hRPB8
GST-hRPB9
35
55
35
25
IB: α-Gβ
IB: α-GST
D
DMEM
5μM Losartan
DMEM
1 μM AngII
30 mins pretreatment
75 mins treatment
IP: Gβ
Blot: Rpb1
IP: Gβ
Blot: Gβ
Total Lysate
Blot: Rpb1
Total Lysate
Blot: Gβ
E
DMEM
40μM IPOZ
DMEM
1 μM AngII
1hr pretreatment
75 mins treatment
IP: Gβ
Blot: Rpb1
IP: Gβ
Blot: Gβ
Total Lysate
Blot: Rpb1
Total Lysate
Blot: Gβ
F
Calcium Release
(normalized
340/516:360/516 Ratio)
Time (s)
siRNA control - Vehicle
siRNA control - 1μm AngII
siRNA Gβ1 - Vehicle
siRNA Gβ1 - 1μm AngII
siRNA Gβ2 - Vehicle
siRNA Gβ2 - 1μm AngII
G
Calcium Release
(calculated as area under kinetics curve)
siRNA control
siRNA Gβ1
siRNA Gβ2
H
siRNA control
siRNA Gβ1
siRNA Gβ2
DMEM
1 μM AngII
75 mins treatment
IP: Gβ
Blot: Rpb1
IP: Gβ
Blot: Gβ
Total Lysate
Blot: Rpb1
Total Lysate
Blot: Gβ
I
Ratio of RNA Pol II immunoprecipitated with Gβ
(fold change over control)
siRNA control - DMEM
siRNA control - 1μM AngII
siRNA Gβ1 - DMEM
siRNA Gβ1 - 1μM AngII
siRNA Gβ2 - DMEM
siRNA Gβ2 - 1μM AngII

Figure 4.4

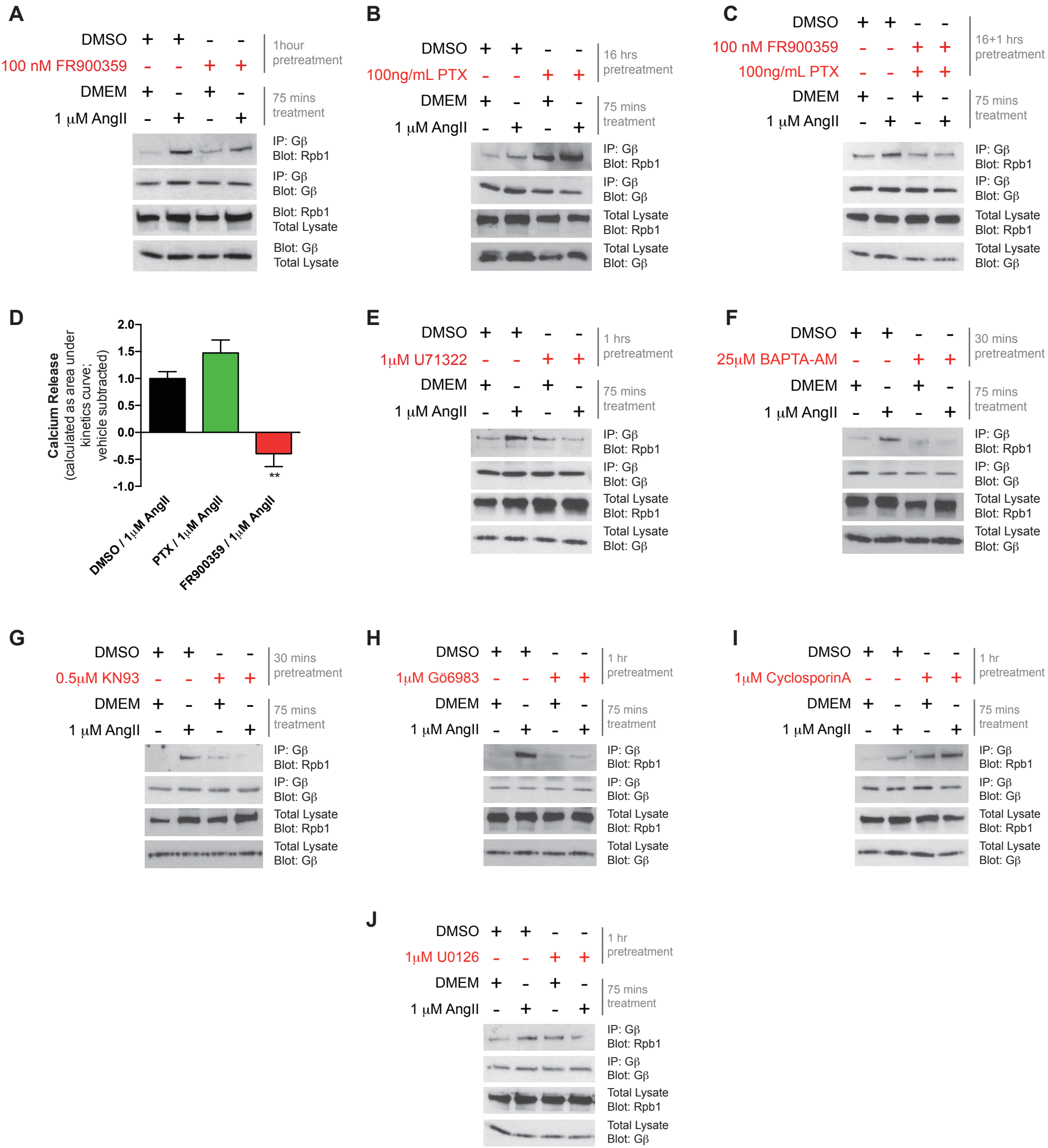
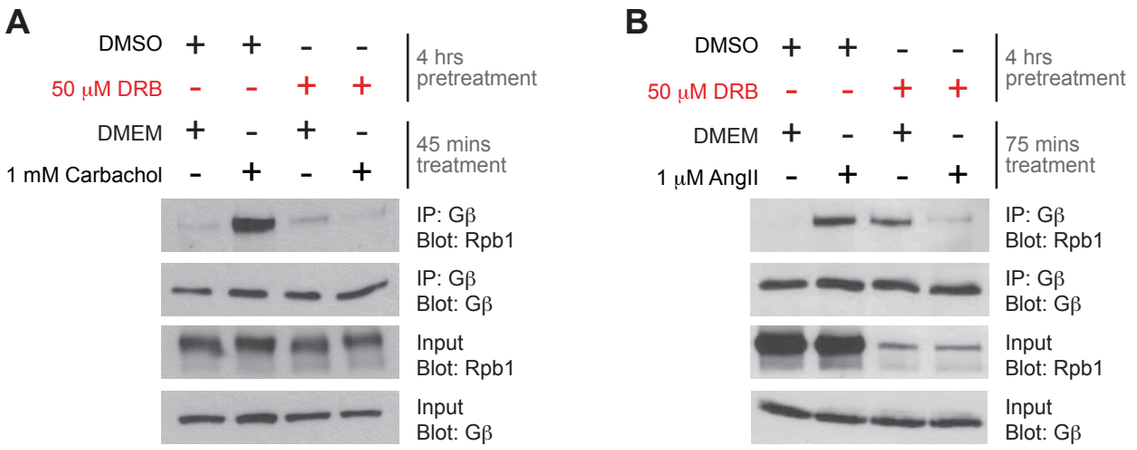

A
DMSO + + - -
100 nM FR900359 - - + +
1hour pretreatment
DMEM + - + -
1 μM AngII - + - +
75 mins treatment
IP: Gβ Blot: Rpb1
IP: Gβ Blot: Gβ
Blot: Rpb1 Total Lysate
Blot: Gβ Total Lysate
B
DMSO + + - -
100ng/mL PTX - - + +
16 hrs pretreatment
DMEM + - + -
1 μM AngII - + - +
75 mins treatment
IP: Gβ Blot: Rpb1
IP: Gβ Blot: Gβ
Total Lysate Blot: Rpb1
Total Lysate Blot: Gβ
C
DMSO + + - -
100 nM FR900359 - - + +
100ng/mL PTX - - + +
16+1 hrs pretreatment
DMEM + - + -
1 μM AngII - + - +
75 mins treatment
IP: Gβ Blot: Rpb1
IP: Gβ Blot: Gβ
Total Lysate Blot: Rpb1
Total Lysate Blot: Gβ
D
Calcium Release
(calculated as area under kinetics curve; vehicle subtracted)
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
**
DMSO / 1μM AngII
PTX / 1μM AngII
FR900359 / 1μM AngII
E
DMSO + + - -
1μM U71322 - - + +
1 hrs pretreatment
DMEM + - + -
1 μM AngII - + - +
75 mins treatment
IP: Gβ Blot: Rpb1
IP: Gβ Blot: Gβ
Total Lysate Blot: Rpb1
Total Lysate Blot: Gβ
F
DMSO + + - -
25μM BAPTA-AM - - + +
30 mins pretreatment
DMEM + - + -
1 μM AngII - + - +
75 mins treatment
IP: Gβ Blot: Rpb1
IP: Gβ Blot: Gβ
Total Lysate Blot: Rpb1
Total Lysate Blot: Gβ
G
DMSO + + - -
0.5μM KN93 - - + +
30 mins pretreatment
DMEM + - + -
1 μM AngII - + - +
75 mins treatment
IP: Gβ Blot: Rpb1
IP: Gβ Blot: Gβ
Total Lysate Blot: Rpb1
Total Lysate Blot: Gβ
H
DMSO + + - -
1μM Gö6983 - - + +
1 hr pretreatment
DMEM + - + -
1 μM AngII - + - +
75 mins treatment
IP: Gβ Blot: Rpb1
IP: Gβ Blot: Gβ
Total Lysate Blot: Rpb1
Total Lysate Blot: Gβ
I
DMSO + + - -
1μM CyclosporinA - - + +
1 hr pretreatment
DMEM + - + -
1 μM AngII - + - +
75 mins treatment
IP: Gβ Blot: Rpb1
IP: Gβ Blot: Gβ
Total Lysate Blot: Rpb1
Total Lysate Blot: Gβ
J
DMSO + + - -
1μM U0126 - - + +
1 hr pretreatment
DMEM + - + -
1 μM AngII - + - +
75 mins treatment
IP: Gβ Blot: Rpb1
IP: Gβ Blot: Gβ
Total Lysate Blot: Rpb1
Total Lysate Blot: Gβ

Figure 4.5


A
DMSO + + - -
50 μM DRB - - + +
4 hrs pretreatment
DMEM + - + -
1 mM Carbachol - + - +
45 mins treatment
IP: Gβ Blot: Rpb1
IP: Gβ Blot: Gβ
Input Blot: Rpb1
Input Blot: Gβ
B
DMSO + + - -
50 μM DRB - - + +
4 hrs pretreatment
DMEM + - + -
1 μM AngII - + - +
75 mins treatment
IP: Gβ Blot: Rpb1
IP: Gβ Blot: Gβ
Input Blot: Rpb1
Input Blot: Gβ

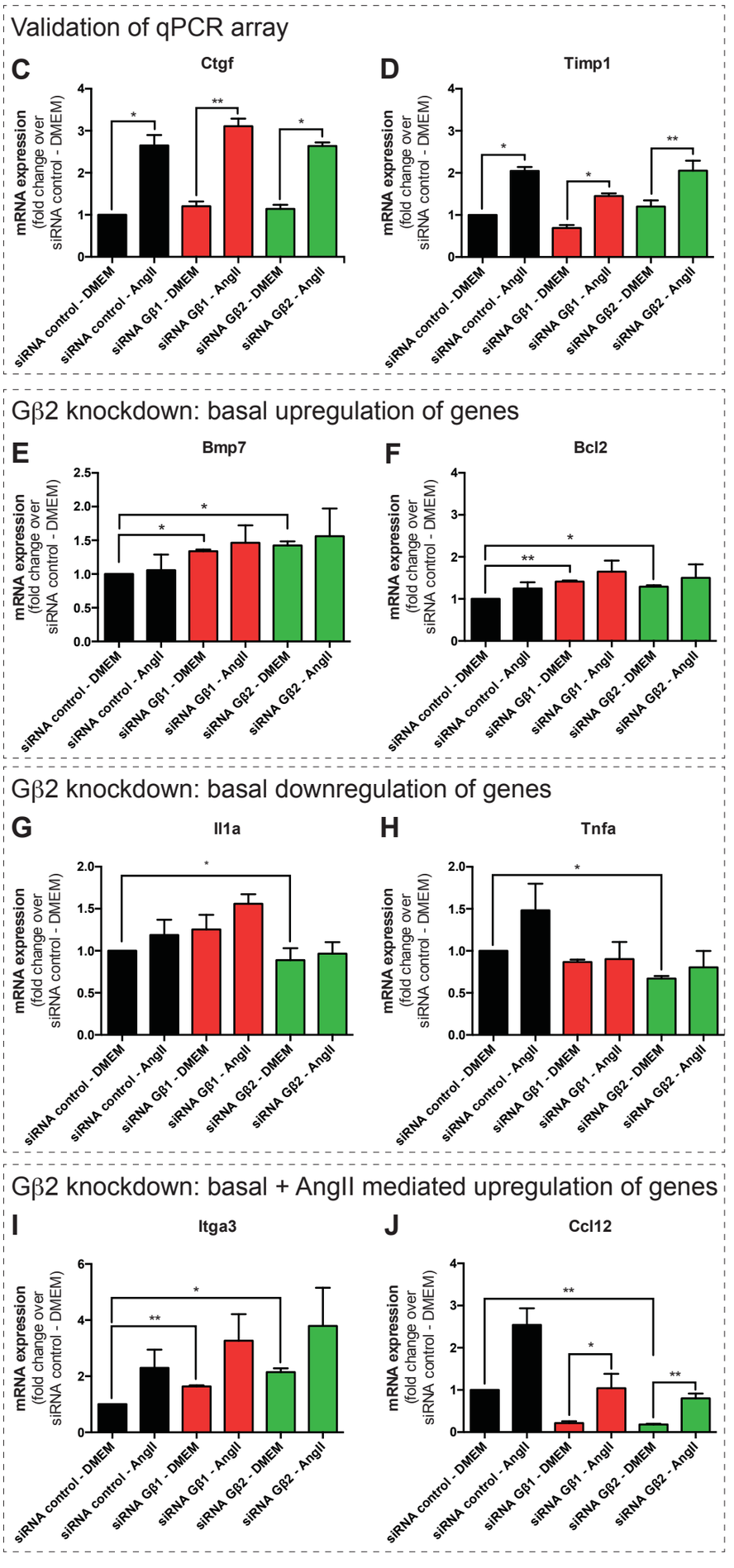

Validation of qPCR array
C
Ctgf
D
Timp1
mRNA expression (fold change over siRNA control - DMEM)
siRNA control - DMEM
siRNA control - AngII
siRNA Gβ1 - DMEM
siRNA Gβ1 - AngII
siRNA Gβ2 - DMEM
siRNA Gβ2 - AngII
Gβ2 knockdown: basal upregulation of genes
E
Bmp7
F
Bcl2
Gβ2 knockdown: basal downregulation of genes
G
Il1a
H
Tnfa
Gβ2 knockdown: basal + AngII mediated upregulation of genes
I
Itga3
J
Ccl12

Figure 4.6

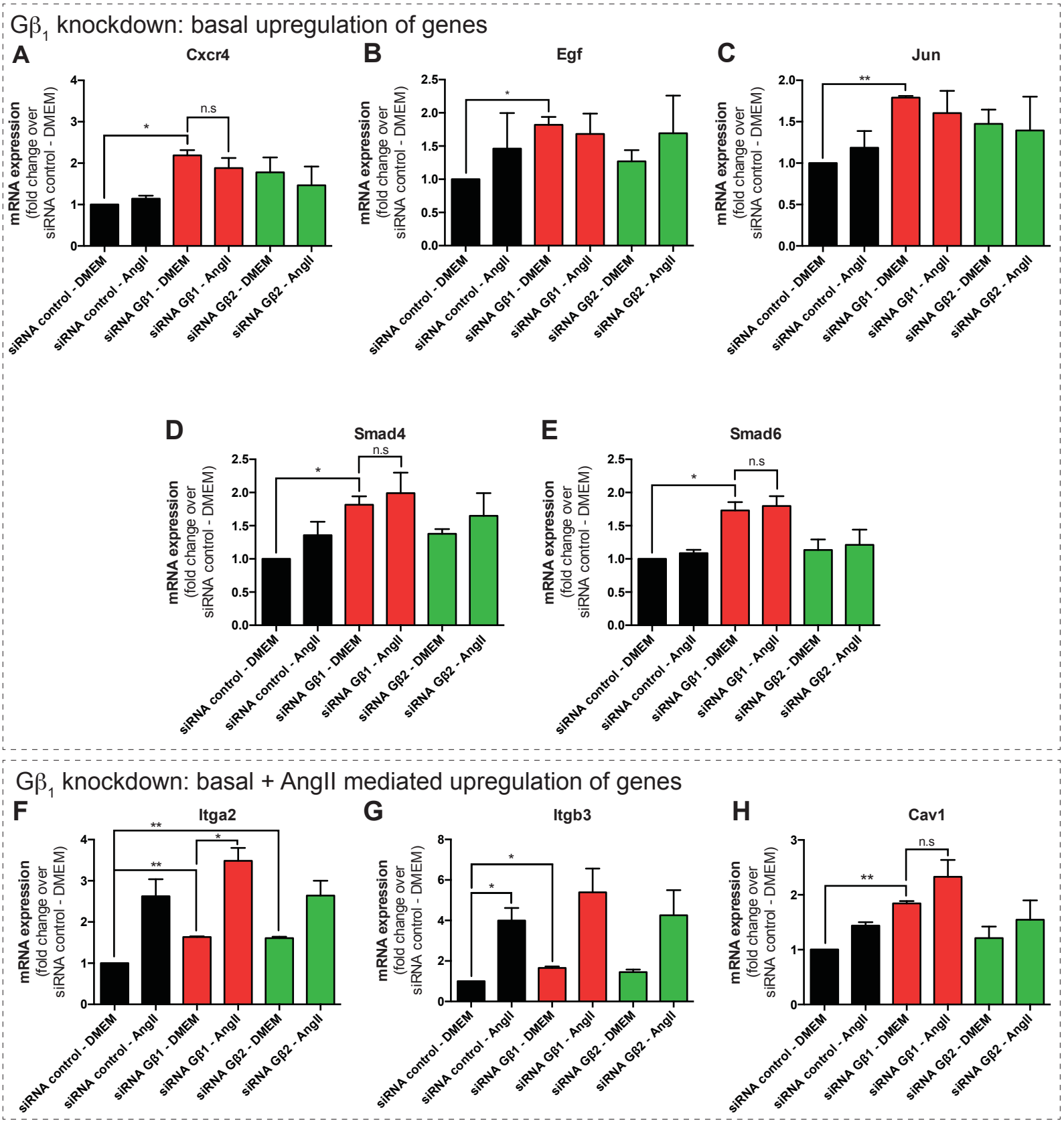

Gβ1 knockdown: basal upregulation of genes
A
Cxcr4
B
Egf
C
Jun
D
Smad4
E
Smad6
mRNA expression
(fold change over
siRNA control - DMEM)
n.s
siRNA control - DMEM
siRNA control - AngII
siRNA Gβ1 - DMEM
siRNA Gβ1 - AngII
siRNA Gβ2 - DMEM
siRNA Gβ2 - AngII
Gβ1 knockdown: basal + AngII mediated upregulation of genes
F
Itga2
G
Itgb3
H
Cav1

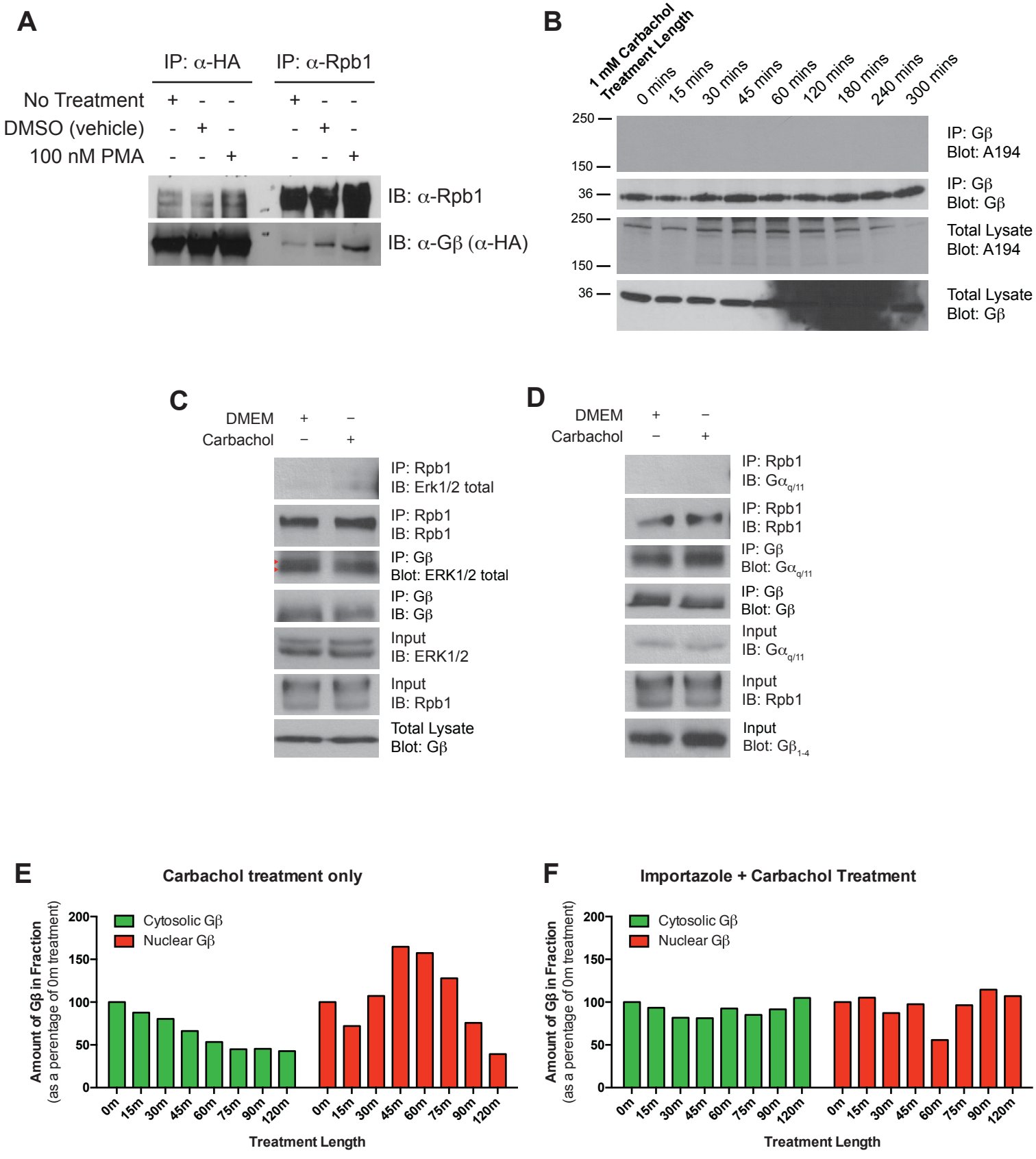
Table 4.1

Effect of Gβ2 knockdown on gene expression		
Gene	***Effect on basal expression***	***Effect on AngII induced expression***
Bcl2	Upregulated (p<0.05)	No change compared to basal
Bmp7	Upregulated (p<0.05)	No change compared to basal
Ccl12	Downregulated (p<0.05)	Upregulated (p<0.05)
Il1a	Downregulated (p<0.05)	No change compared to basal
Itga2	Upregulated (p<0.05)	No change compared to basal
Itga3	Upregulated (p<0.05)	Upregulated (n.s)
Tnf	Downregulated (p<0.05)	No change compared to basal

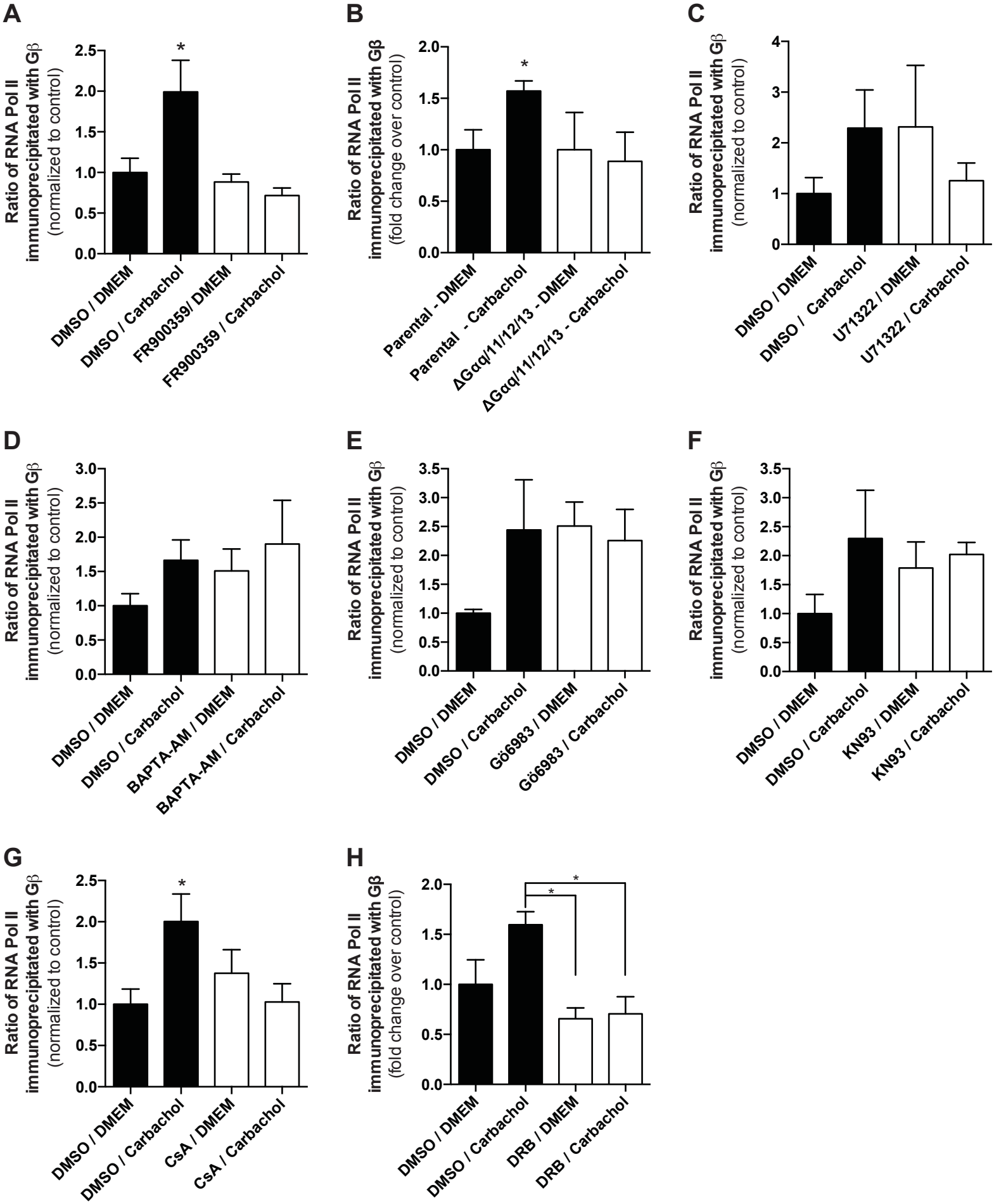
Table 4.2

Effect of Gβ1 knockdown on gene expression		
Gene	***Effect on basal expression***	***Effect on AngII induced expression***
Akt1	Upregulated (p<0.05)	No change compared to basal
Bcl2	Upregulated (p<0.05)	No change compared to basal
Bmp7	Upregulated (p<0.05)	No change compared to basal
Cav1	Upregulated (p<0.05)	Upregulated (n.s)
Cxcr4	Upregulated (p<0.05)	No change compared to basal
Egf	Upregulated (p<0.05)	No change compared to basal
Itga2	Upregulated (p<0.05)	Upregulated (p<0.05)
Itga3	Upregulated (p<0.05)	No change compared to basal
Itgb3	Upregulated (p<0.05)	Upregulated (n.s)
Itgb6	Upregulated (p<0.05)	No change compared to basal
Jun	Upregulated (p<0.05)	No change compared to basal
Mmp8	Upregulated (p<0.05)	No change compared to basal
Plau	Upregulated (p<0.05)	No change compared to basal
Smad4	Upregulated (p<0.05)	No change compared to basal
Smad6	Upregulated (p<0.05)	No change compared to basal
Smad7	Upregulated (p<0.05)	No change compared to basal
Sp1	Upregulated (p<0.05)	No change compared to basal
Tgfbr2	Upregulated (p<0.05)	No change compared to basal

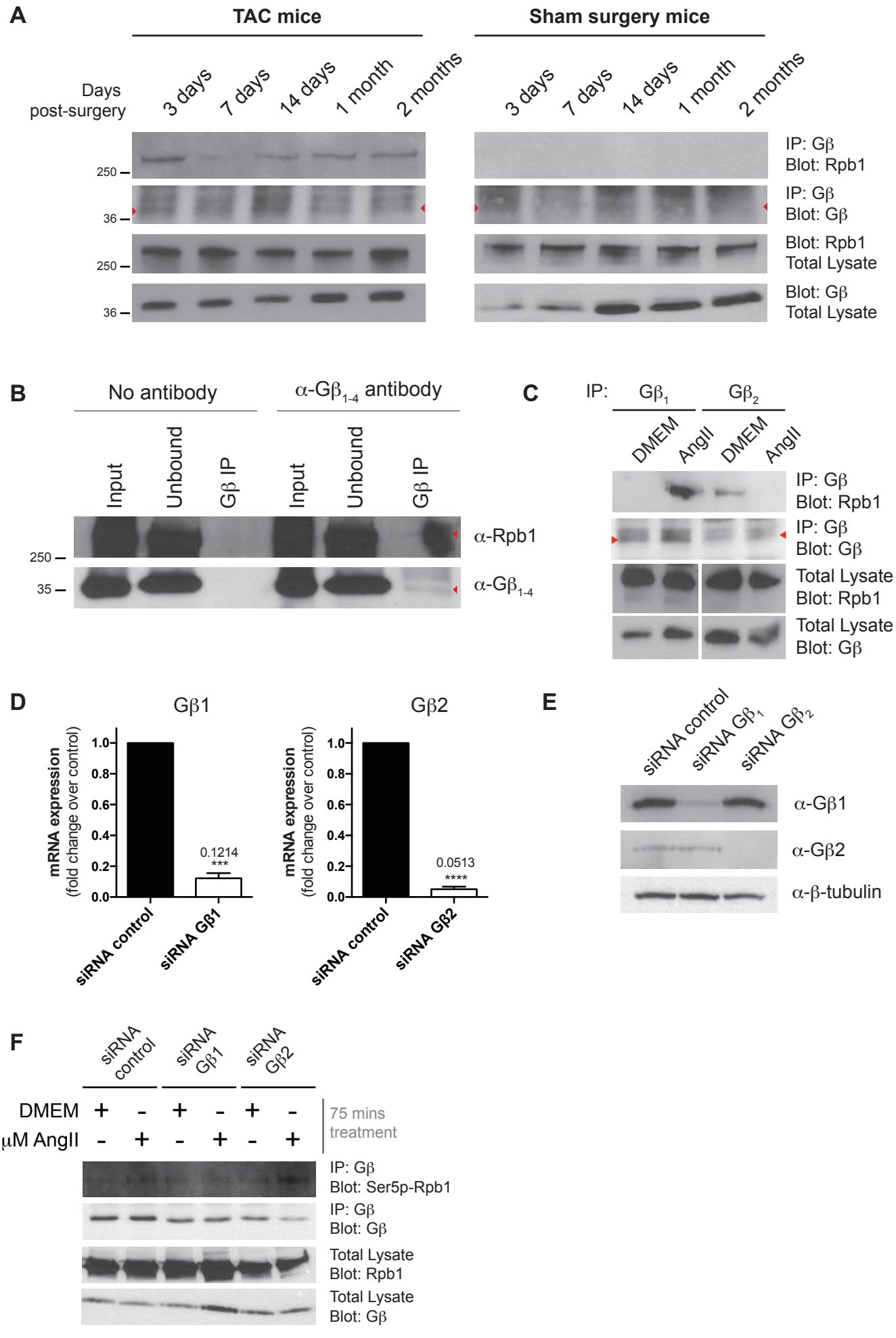
Supplemental Figure 4.1



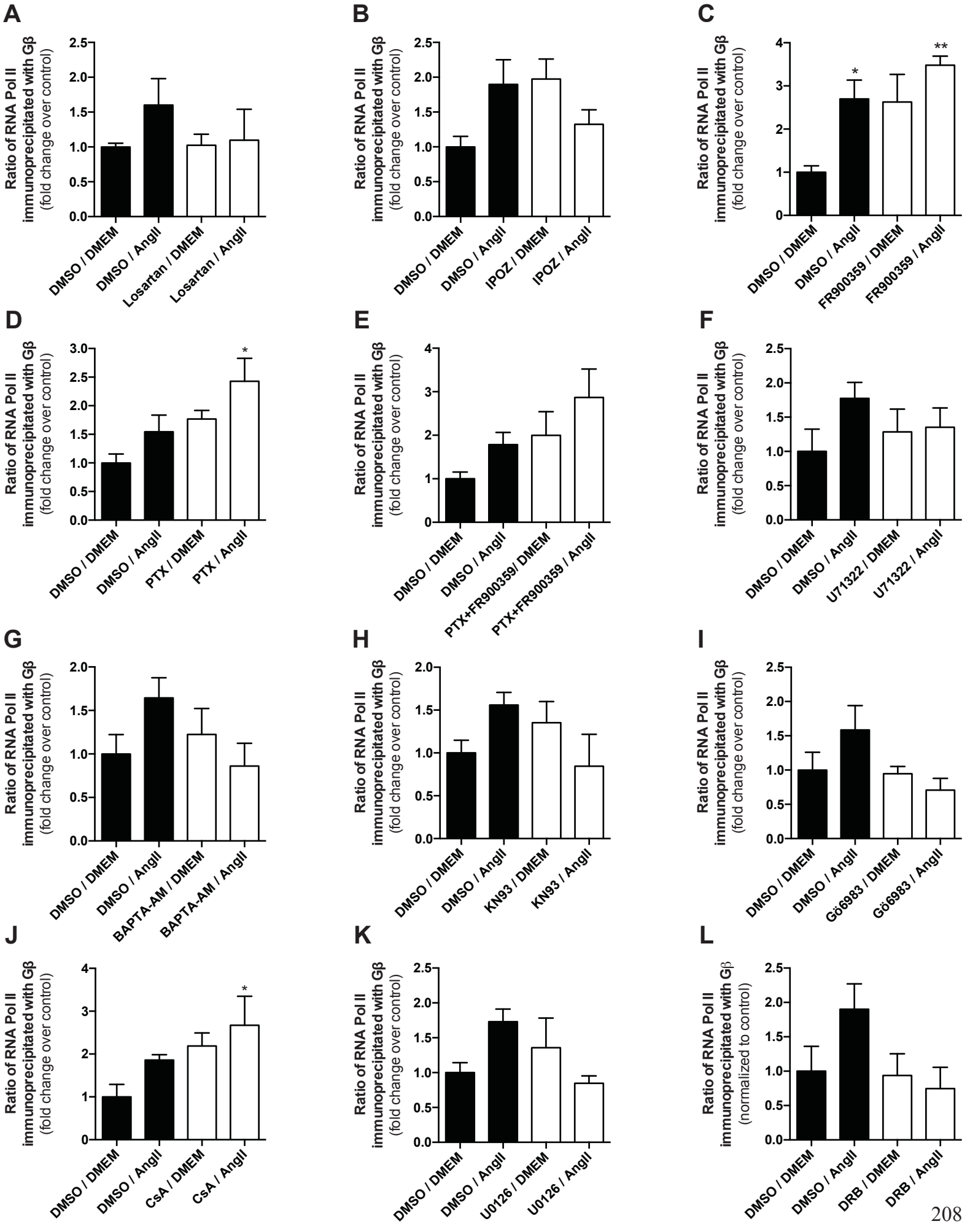
Supplemental Figure 4.2



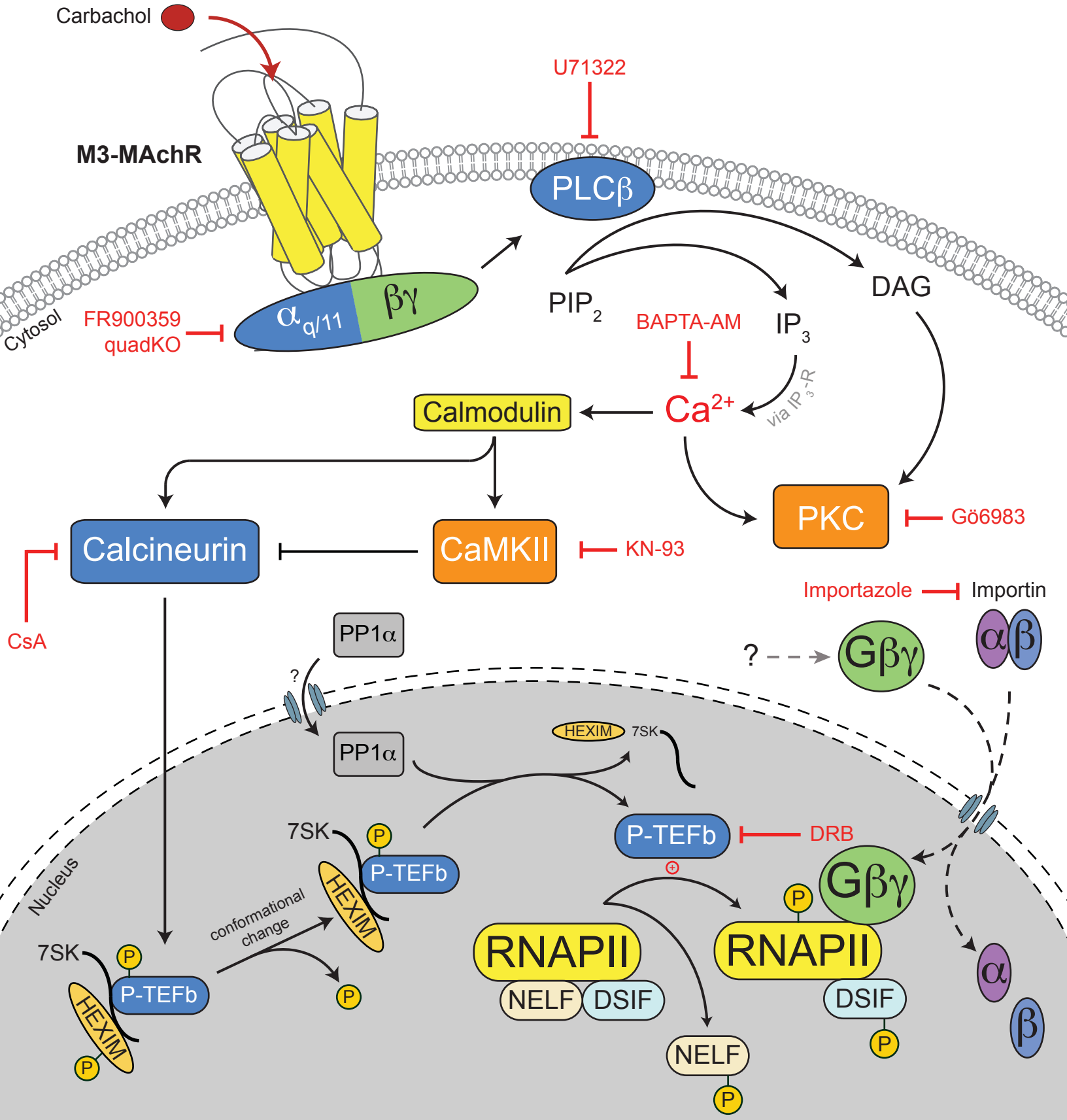
Supplemental Figure 4.3



Supplemental Figure 4.4



Supplemental Figure 4.5

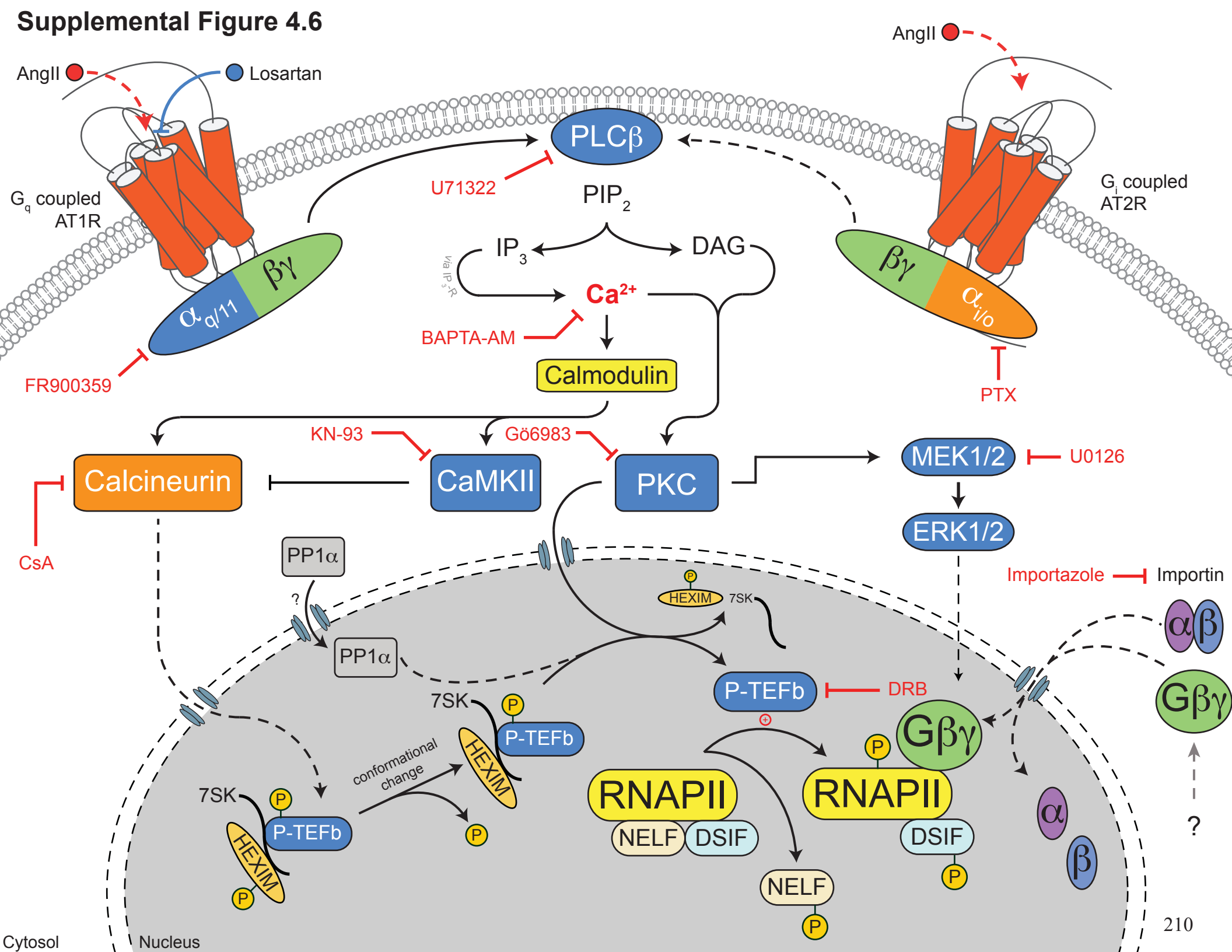


CsA = CyclosporinA
CalyA = CalyculinA
quadKO = $G\alpha_{q/11/12/13}$ knockout HEK293 cells
DRB = 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole

= interaction activating protein

= interaction regulating protein

Supplemental Figure 4.6



Supplemental Table 4.1

Effect of AngII treatment on gene expression				
Gene ID	***Gene description***	***Fold change increase***	***p value***	***Significance***
Ctgf	*Connective tissue growth factor*	2.651	0.0261	*
Edn1	*Endothelin-1*	2.193	0.0027	**
Itga1	*Integrin α subunit 1*	2.113	0.0288	*
Itgb3	*Integrin β subunit 3*	3.995	0.035	*
Pdgfa	*Platelet derived growth factor α*	1.702	0.032	*
Tgfb3	*Transforming growth factor β subunit 3*	4.994	0.0049	**
Timp1	*Timp metallopeptidase inhibitor 1*	2.049	0.0117	*
Acta2	*Smooth muscle actin α 2*	2.681	0.0708	*n.s.*
Ccl12	*Chemokine (C-C motif) ligand 12*	2.543	0.0752	*n.s.*
Eng	*Endoglin*	2.078	0.3262	*n.s.*
Grem1	*Gremlin 1*	2.541	0.1425	*n.s.*
Il10	*Interleukin 10*	10.626	0.1733	*n.s.*
Itga2	*Integrin α subunit 2*	2.624	0.0724	*n.s.*
Itga3	*Integrin α subunit 3*	2.297	0.3027	*n.s.*
Itgb1	*Integrin β subunit 1*	2.413	0.1071	*n.s.*
Itgb8	*Integrin β subunit 8*	2.198	0.1936	*n.s.*
Lox	*Lysyl oxidase*	2.718	0.0767	*n.s.*
Ltbp1	*Latent transforming growth factor β binding protein 1*	2.383	0.0692	*n.s.*
Mmp8	*Matrix metallopeptidase 8*	3.135	0.9111	*n.s.*
Smad3	*SMAD family member 3*	2.025	0.4762	*n.s.*
Thbs1	*Thrombospondin 1*	2.674	0.1536	*n.s.*
Timp4	*Timp metallopeptidase inhibitor 4*	2.173	> 0.9999	*n.s.*

Supplemental Table 4.2-1

	siRNA control DMEM	siRNA control AngII	siRNA Gβ1 DMEM	siRNA Gβ1 AngII	siRNA Gβ2 DMEM	siRNA Gβ2 AngII
	Average Fold Change					
Acta2	1.000	2.681	0.917	**2.793**	1.115	**2.800**
Agt	1.000	1.891	0.964	1.076	1.560	2.183
Akt1	1.000	1.482	**1.442**	1.875	1.322	1.766
Bcl2	1.000	1.248	**1.410**	1.648	**1.293**	1.502
Bmp7	1.000	1.057	**1.339**	1.463	**1.423**	1.562
Cav1	1.000	1.402	**1.854**	2.502	1.331	1.749
Ccl11	1.000	0.953	0.888	0.664	0.934	0.463
Ccl12	1.000	2.543	0.214	**1.042**	**0.181**	**0.800**
Ccl3	1.000	1.137	0.907	0.982	0.798	0.806
Cebpb	1.000	1.972	1.236	1.867	1.019	1.805
Col1a2	1.000	1.352	1.153	**1.612**	0.986	**1.378**
Col3a1	1.000	0.773	1.465	**1.229**	1.217	1.092
Ctgf	1.000	**2.651**	1.205	**3.108**	1.140	**2.640**
Cxcr4	1.000	1.141	**2.189**	1.882	1.779	1.467
Dcn	1.000	**0.685**	1.365	**0.700**	1.487	1.070
Edn1	1.000	**2.193**	1.632	2.928	1.085	2.059
Egf	1.000	1.461	**1.820**	1.682	1.269	1.692
Eng	1.000	2.078	1.667	2.842	1.480	2.554
Faslg	1.000	1.541	1.417	1.638	1.789	1.610
Grem1	1.000	2.541	1.136	3.277	1.098	2.789
Hgf	1.000	1.340	1.574	1.793	1.522	1.817
Il10	1.000	10.626	4.550	15.309	1.835	9.196
Il1a	1.000	1.274	1.158	1.606	**0.749**	0.871
Il1b	1.000	1.186	1.012	1.422	0.635	0.540
Ilk	1.000	1.436	1.141	1.670	1.151	1.541
Itga1	1.000	**2.113**	1.132	2.385	1.031	2.353
Itga2	1.000	2.624	**1.636**	**3.484**	**1.608**	2.642
Itga3	1.000	2.297	**1.639**	3.266	**2.146**	3.793
Itgav	1.000	1.887	1.260	2.144	1.152	2.014
Itgb1	1.000	2.413	0.947	2.485	0.956	2.196
Itgb3	1.000	**3.995**	**1.654**	5.389	1.453	4.256
Itgb5	1.000	1.864	1.112	2.055	1.202	2.338
Itgb6	1.000	1.600	**1.808**	2.086	1.481	2.573
Itgb8	1.000	2.198	1.664	3.115	1.574	3.340
Jun	1.000	1.185	**1.791**	1.605	1.474	1.394
Lox	1.000	2.718	1.045	2.346	0.980	2.398
Ltbp1	1.000	2.383	1.416	2.246	1.221	2.345
Mmp14	1.000	1.569	1.535	1.826	1.190	1.432
Mmp2	1.000	1.192	1.787	1.597	1.327	1.293

Legend	
	Trend for upregulation (>2 fold change)
	Significant upregulation (p<0.05)
	Trend for downregulation (<0.75 fold change)
	Significant downregulation (p<0.05)

Supplemental Table 4.2-2

	siRNA control DMEM	siRNA control AngII	siRNA Gβ1 - DMEM	siRNA Gβ1 - AngII	siRNA Gβ2 - DMEM	siRNA Gβ2 - AngII
	Average Fold Change					
Mmp8	1.000	3.135	**6.943**	10.749	3.535	5.548
Mmp9	1.000	0.907	1.140	0.715	1.261	0.868
Myc	1.000	1.605	1.640	1.891	1.211	1.388
Nfkb1	1.000	1.437	1.433	1.621	1.240	1.317
Pdgfa	1.000	**1.702**	1.520	2.309	1.292	1.830
Pdgfb	1.000	1.175	1.628	1.380	1.458	1.158
Plat	1.000	1.580	1.423	2.111	1.291	1.863
Plau	1.000	1.569	**1.469**	1.935	1.166	1.626
Plg	1.000	1.115	0.652	0.812	1.041	0.823
Serpine1	1.000	**21.210**	0.860	**10.459**	0.601	**9.806**
Serpinh1	1.000	1.851	1.243	2.238	1.053	1.833
Smad2	1.000	1.709	1.399	2.255	1.420	1.993
Smad3	1.000	2.025	1.687	2.737	1.348	2.436
Smad4	1.000	1.357	**1.814**	1.990	1.377	1.650
Smad6	1.000	1.085	**1.729**	1.798	1.135	1.211
Smad7	1.000	1.841	**1.744**	2.550	1.163	2.226
Snai1	1.000	1.017	1.975	1.529	1.273	1.181
Sp1	1.000	1.587	**1.842**	2.484	1.692	2.140
Stat1	1.000	1.639	1.118	1.657	1.020	1.638
Stat6	1.000	1.608	1.603	2.135	1.345	1.580
Tgfb1	1.000	1.490	1.429	1.577	1.123	1.269
Tgfb2	1.000	1.856	1.183	2.045	1.304	2.006
Tgfb3	1.000	**4.994**	1.388	6.966	1.107	5.002
Tgfbr1	1.000	1.591	1.499	1.956	1.221	1.652
Tgfbr2	1.000	1.369	**1.705**	2.074	1.393	1.667
Tgif1	1.000	1.334	1.188	1.370	1.009	1.167
Thbs1	1.000	2.674	1.220	2.338	1.141	2.224
Thbs2	1.000	1.224	0.837	0.700	0.843	0.798
Timp1	1.000	**2.049**	0.692	**1.451**	1.200	**2.055**
Timp2	1.000	1.230	1.354	1.605	1.408	1.659
Timp3	1.000	1.371	1.144	1.360	1.152	1.455
Timp4	1.000	2.173	1.617	1.804	1.892	1.381
Tnf	1.000	1.482	0.867	0.902	**0.670**	0.805
Vegfa	1.000	1.471	1.493	1.758	1.182	1.332

Legend	
	Trend for upregulation (>2 fold change)
	Significant upregulation (p<0.05)
	Trend for downregulation (<0.75 fold change)
	Significant downregulation (p<0.05)

Supplemental Table 4.3

Primers used for qPCR assays		
Target	**Forward Primer (5'-3')**	**Reverse Primer (3'-5')**
U6 snRNA	TGGAACGATACAGAGAAGATTAG	GAATTTGCGTGTCATCCTTG
Gnb1	CTCATGACCTACTCCCATGA	TCAGCTTTGAGTGCATCC
Gnb2	CAGCTACACCACTAACAAGG	CTCTCGGGTCTTGAGACTAT

CHAPTER 5: General discussion

5.1 Contributions to scientific understanding

The overall goal of my thesis was to further our understanding of Gβγ dimers taking a top-down, holistic approach. In the field of G protein signalling, Gβγ biology has long been studied and discussed as a singular entity, $G\beta_1\gamma_2$, despite being well recognized that there are theoretically close to 60 possible combinations of these dimers. In an attempt to understand the notion that combinations of specific Gβγ dimers can no longer be ignored when studying their roles in and outside of GPCR biology, my thesis acts to provide a better understanding of three broad, yet connected themes: (1) Gβ and Gγ subunit evolutionary divergence patterns, (2) roles for specific Gβ and Gγ subunits in canonical signalling paradigms, and (3) novel non-canonical, nuclear roles for specific Gβγ dimers and their modulation of transcriptional events.

In Chapter 2 (Khan et al, 2014), I describe Gβ and Gγ subunits from an evolutionary perspective and discuss potential clues as to how and why different species express varying numbers of subtypes of these individual subunits. Our phylogenetic analysis of Gβ and Gγ subunits from both lower order and higher order organisms reveals intricate patterns of subunit divergence both within and between different subtypes of these subunits. Of particular interest, we demonstrate that with respect to Gβ subunits, a core divergence of an ancestral Gβ subunit gave rise to the two classes/types of Gβ subunits – $G\beta_{1\text{-}4}$ and $G\beta_5$. Regarding Gγ subunit divergence, its phylogenetic trees revealed more intricate patterns of divergence and this work led to suggestions of the existence of different classes of Gγ subunits that may share similar functions. Finally, our structural mapping analysis of human Gβγ dimers suggested that

specificities achieved by different combinations of specific Gβ and Gγ subunits that dimerize may primarily be imparted by Gγ subunits.

Chapter 3 (Khan et al, 2015) focuses on the determination of the aforementioned specificities of Gβγ in the modulation of effectors downstream of GPCR signalling. Using an siRNA based RNAi screen, we demonstrate that $G\beta_4\gamma_1$ is the Gβγ dimer of specific subunit composition that modulates the activation of PLCβ and activation of MAPK signalling pathways upon stimulation of M3-mAChR in HEK 293 cells. In addition, we demonstrated that knockdown of $G\beta_4\gamma_2$, $G\beta_4\gamma_4$ and $G\beta_4\gamma_7$ pairs also diminished Ca^{2+} release upon receptor activation, but not to the same extent of decreased second messenger release as knockdown of $G\beta_4\gamma^1$. This suggested a certain amount of redundancy in Gβγ function, albeit not as grossly redundant as once generally accepted. Furthermore, our studies of the effects of $G\beta_1$ in signalling downstream of M3-mAChRs revealed novel non-canonical roles for $G\beta_1$ containing Gβγ dimers – knockdown of $G\beta_1$ led to increased calcium release upon receptor activation compared to control conditions and increased levels of ERK1/2 phosphorylation. Moreover, we demonstrated that knockdown of $G\beta_1$ led to reduced expression of ERK1/2, increased expression of GRK6, and increased $G\beta_4$ expression (confirmation of findings previously published by other groups). Such findings eluded to $G\beta_1$'s potential roles as a regulator of gene expression. Indeed, in this chapter, we also demonstrate that $G\beta_1$ occupies the promoters of over 700 genes, and validated our ChIP-on-ChIP assays by demonstrating that $G\beta_1$ occupies the $G\beta_4$ promoter. Finally, using data derived from a proteomics screen, we discuss potential mechanisms by which $G\beta_1$ acts a regulator of gene expression.

In Chapter 4 (Khan et al, *to be submitted*), I describe the discovery, characterization and putative functions of a novel interaction between Gβγ and RNAPII. We demonstrate that Gβγ interacts with Rpb1 in under both basal conditions and GPCR agonist-stimulated conditions in HEK 293 cells and rat neonatal cardiac fibroblasts, although with different kinetics of interaction induction. Using *in vitro* interaction assays, we demonstrate that Gβγ also interacts with hRpb6, hRpb8 and hRpb9. Our studies reveal that Gβγ must translocate to the nucleus via an importin-β dependent mechanism to interact with Rpb1 in the nucleus. Furthermore, we demonstrate an interplay of specific Gβ containing Gβγ dimers that act to associate and dissociate with Rpb1 in response to agonist stimulation; we provide evidence that $G\beta_1\gamma$ associates with Rpb1 while $G\beta_{2/4}\gamma$ dissociates from Rpb1 under conditions of M3-mAChR activation in HEK 293 cells and AT1R activation in rat neonatal cardiac fibroblasts. With respect to the mechanism of interaction induction, a pathway involving M3-mAChR/G_q/PLCβ/Ca^{2+}/calcineurin is required to induce the increase in interaction in HEK 293 cells, whereas AT1R/G_q/PLCβ/Ca^{2+}/PKC/CamKII/MEK pathway is necessary in rat neonatal cardiac fibroblasts. Finally, with respect to functional roles served by Gβγ downstream of AT1R activation, we demonstrate that $G\beta_2\gamma$ is necessary for the initiation of signalling upon receptor activation whereas $G\beta_1\gamma$ acts as a repressor of gene expression under both basal and AngII induced conditions. Indeed, our analysis suggests that the $G\beta_1$ knockdown mediated upregulation of gene expression is due to the loss of its interaction with Ser5-phosphorylated Rpb1, thus leading to the conclusion that it serves a function in the pause of RNAPII.

5.2 Novel understanding gained from phylogenetic analysis of Gβ and Gγ subunits

The significance of Gβγ subunit diversity and its implications in GPCR biology remains an under-characterized aspect of G protein signalling. It is still not understood exactly why humans express 5 different Gβ subtypes and 12 different Gγ, let alone exactly what the functions that all the multiple possible combinations of these subunits are responsible for performing. Our phylogenetic analysis was performed to try and answer this question from an evolutionary perspective by assessing divergence patterns of human Gβ and Gγ subunits in comparison to other mammalian species and lower order organisms.

As I mentioned in the Introduction, Gβγ subunits were once thought solely to be negative regulators of Gα subunit activity. While this is understood to no longer be the case and it is well appreciated that Gβγ dimers perform various functions of their own, it remains a question why Gβγ forms a heterotrimer with Gα subunits to begin with. Clues as to why this is the case may come from studying phylogenetic expression patterns of Gα, GPCRs and Gβγ. Indeed, in a study that aimed to elucidate Gα subunit diversity across species, Gα subunit phylogeny as well as absolute counts of 7-transmembrane domain receptors, Gβ and Gγ subunits expressed across eukaryotic species that was assessed [465]. What is interesting from this analysis is that with the exception of *Trichomonas vaginalis*, every species that expressed Gα subunits also expressed 7-transmembrane domain receptors as well as Gβ and Gγ subunits [465]. Such a finding suggests an intricate pattern of Gα, GPCR and Gβγ co-divergence that suggests that signalling cooperativity between these three entities have been a feature of cellular signalling across both protozoan and metazoan species throughout evolution. Whether these subunits have indeed co-

diverged to facilitate one another's functions could possibly be the subject of future investigations of GPCR-G protein phylogenies. More specifically and of particular interest to this thesis, the study of Gβ and Gγ subunit co-divergence may reveal clues as to which Gβ and Gγ subunits evolved with each other and pattern of specific Gβγ dimers that formed, which in turn would provide us more insight about their evolving functions over time.

From a more global perspective, one might also question how Gα, Gβγ and 7-TMR co-divergences feed into the specificities and complexities attained in the evolution of signalling. While such questions may remain largely unanswered, it has been suggested that the complexities of signalling pathways evolve in such a way that stringent selection criteria lead to lower pathway sizes [466]. As a result, it may be speculated that the evolution of complex signalling pathways has been the result of non-adaptive selection cues applied on ancestral organisms that have been retained due to the beneficial nature of the mutations these pressures conferred on the organism [466]. Suggestions of this nature are supported when considering the origins of genome complexity. It has been proposed that increases in genome complexity observed in prokaryotes to eukaryotes are results of processes such as the retention of duplicate genes and increased abundance of spliceosomal introns, which in turn are due to passive emergence of such processes in response to reductions in population size that accompany increases in organism size [467]. While the reason why mammals express 5 Gβ and 12 Gγ subtypes may be that the passive mutational events that led to the emergence of these different isoforms and were retained due to the beneficial value they added to signalling systems, the functional significance of the retention of these divergent subtypes becomes a topic of interest.

One curiosity that is piqued when analyzing our phylogenetic analysis is whether functions have been retained throughout the course of evolution. Assessment of how Gβ and Gγ subunits in species such as *C. elegans* (worms) compare to their human counterparts reveals that that worms express a Gβ subunit similar to $G\beta_5$ (GPB2) and another that closest to the first ancestral Gβ subunit in our analysis (GPB1). This gives rise to questions of what functions were evolved in higher order organisms that required the divergence into 5 different subtypes we see in humans, and what functions have been conserved. In an attempt to understand the extent of divergence between Gβ subunits in humans and *C. elegans*, an ongoing research project in our lab is studying whether GPB1 from *C. elegans* can rescue $G\beta_1$ and $G\beta_4$ knockdown phenotypes I presented in Chapter 3. The rationale for this study is that if structural functions of Gβ subunits have been maintained throughout evolution, despite the species divergence we observe, overexpression of GPB1 should reverse the changes to Ca^{2+} release observed under conditions of $G\beta_1$ and $G\beta_4$ knockdown and M3-mAChR activation. Preliminary results indicate that overexpression of GPB1 rescues the loss of Ca^{2+} release observed under conditions of $G\beta_4$ knockdown and M3-mAChR activation (unpublished data, Charles Harkness, PHAR 599 project), suggesting a certain degree of structural conservation throughout evolution. Furthermore, others have demonstrated that in *C. elegans*, GPB1 and GPC2 are involved in spindle formation and orientation during assymetric mitotic cell division of one-cell stage embryos, a process that is regulated by force generators such as Gα subunits, LIN-5 and GPR-1/2 [468-471]. GPB-1 has been found to act as a negative regulator of these force generators in C. elegans }[471], and work in our lab demonstrates that GPB1 knockouts (*gpb-1* mutants) results in an increased distance between pronuclear meeting to nuclear envelope breakdown and distances involved in the mitotic rocking phase, resulting in the loss of inhibitory effects on the

aforementioned force generators (Fouad El-Shehabi, Connie Yu, unpublished data). Interestingly, in attempt to assess the roles of mammalian Gγ overexpression in these asymmetric mitotic events, it was observed that overexpression $G\gamma_{13}$ significantly increases the distance between pronuclear meeting to nuclear envelope breakdown to levels comparable to *gpb-1* mutants, whereas $G\gamma_{11}$ overexpression significantly increases distances during the rocking phases of mitosis (Fouad El-Shehabi, Connie Yu, unpublished data). This would suggest that $G\gamma_{11}$ and $G\gamma_{13}$ act in dominant negative capacities when over expressed in C. elegans, meaning that these subunits have acquired structural functions not present in C. elegans. What remains to be identified is the effect of overexpression of mammalian Gβ subunits in worms to see if they can rescue the functions lost in *gpb-1* mutants.

5.3 The need for more emphasis on specificity of Gβγ in signalling

One of the major focuses in my project was to demonstrate that no two specific Gβγ subunits are identical functionally and that specificity of Gβγ function is key. Our evolutionary divergence and structural mapping analysis of Gβ and Gγ subunit stands as one line of evidence that suggests that these subunits may have evolved for different functions. Specificity of Gβγ in GPCR signalling is not a novel concept; numerous studies have attempted to understand the role of specific Gβγ combinations and their function using various gene silencing methods that include shRNA and siRNA mediated knockdown, antisense oligonucleotide approaches and gene knockout approaches. The fact that Gβ and Gγ subunit display specific and preferential dimerization patterns imply that levels of specificity are conferred by these dimers on the

"personality" of the signalling pathways they regulate and the phenotypes they exhibit [127, 335, 472, 473]. While a great deal of emphasis has been put on describing specificities of selective Gα coupling to GPCRs and the specificities of effectors activated downstream of Gα activation [5, 474], there is far less known of the specificities and selectivity of Gβγ signalling downstream of GPCR activation.

As mentioned in Chapter 3, knockdown approaches have resulted in modest differences in signalling phenotypes between different Gβγ combinations, while knockout studies have provided insights regarding tissue specific roles. However, previously described RNAi and knockout approaches have not been systematic, describing knockdown of only a subset of either Gβ and Gγ subtypes [385, 417, 420, 475, 476], or knockout of a single subtype [84, 382, 383, 388-390]. Our Gγ-specific RNAi-screen approach can be regarded as one of the first subtype-wide knockdown analyses to assess the functional roles of individual endogenous Gγ subunits expressed in a particular cell type. Our initial hypothesis was that the Gγ subtypes in "classes" of Gγ subunits that our phylogenetic analysis identified performed similar roles and were redundant. By applying the lessons learned from these two analyses in contexts of cellular signalling, our RNAi screen revealed a role for $G\beta_4\gamma_1$ as the specific Gβγ dimer needed for propagating signalling of endogenous M3-mAChR in HEK 293 cells, although loss of Ca^{2+} release was observed when $G\beta_4\gamma_2$, $G\beta_4\gamma_4$, and $G\beta_4\gamma_7$ were knocked down (Figure 3.4). This refuted our classification-based hypothesis for function for classes of Gγ subunits, but revealed that specific Gγ subunits from different phylogenetic arms serve similar roles. It must be noted that the Gβ and Gγ knockdown effects seen in our study may be due to their respective involvements in parallel signalling pathways and not necessarily in a dimer. Elucidation of such

discrepancies should be the subject of future studies. We also describe that $G\beta_2$ containing $G\beta\gamma$ dimers are necessary for similar roles downstream of AT1R in RNCFs (Figure 4.3G). While our results make it tempting to speculate that $G\beta_2$ and $G\beta_4$ containing $G\beta\gamma$ dimers selectively couple to G_q-coupled receptors, previously have demonstrated that $G\beta_2$ acts to modulate purinergic receptor-mediated and C5a receptor-mediated calcium release [417], the latter of which is primary coupled to G_i [477]. What is critical to notice is the lack of a role for $G\beta_1$ in these canonical signalling paradigms. Therefore, our findings in addition to those mentioned here, in themselves, are testaments to the notion that the eponymous $G\beta_1\gamma_2$ dimer can no longer be the benchmark dimer that most studies use to assess the functions of $G\beta\gamma$ in both canonical and non-canonical signalling.

The concept of specificity of $G\beta\gamma$ mediated signalling raises the question of which component of these dimers is more important for determining the functions they serve. Such questions are partially answered in Chapter 3, where it would seem that both dimer partners are important, although the argument can be made that the $G\gamma$ subunit is more important as other subtypes subunits cannot compensate for the largest loss of Ca^{2+} signalling observed under conditions of $G\gamma_1$ knockdown with and without $G\beta_4$ knockdown (Figures 3.3A, 3.4A, B). To assess the contributive capacity of $G\beta$ subunits in compensating for the knockdown of one subtype, our analysis would have had to include $G\beta_2$ and $G\beta_3$ knockdown to assess their roles in M3-mAChR related signalling. Thus, for future considerations, a knockdown matrix that includes all $G\beta$ and $G\gamma$ subunits expressed in a particular cell type should be the subject of experiments that aim to assess specificities and redundancies of these subunits.

Our identification and characterization of a novel Gβγ-RNAPII interaction provides evidence supporting our previously suggested dichotomy of $G\beta_1$-containing Gβγ dimers ($G\beta_1\gamma$) and $G\beta_{2/4}$ containing Gβγ dimers ($G\beta_{2/4}\gamma$) [433], whereby the former appears to possess functions for gene expression regulation downstream of G_q-coupled GPCRs and the latter is more important for proximal signal transduction regulation. Experiments in both HEK 293 cells and RNCFs suggests an interplay of Gβ-specific Gβγ dimers interacting with Rpb1 such that $G\beta_{2/4}\gamma$ dimers interact with Rpb1 in basal condtions (Figure 4.1E, Supplemental Figure 4.2C), whereby $G\beta_1\gamma$ dimers interact with Rpb1 under agonist stimulated conditions. When considered alongside with the fact that the $G\beta_1\gamma$-Rpb1 acts as a transcriptional pause break, it can be speculated that perhaps the basal $G\beta_{2/4}$-Rpb1 may act in a capacity that promotes basal expression of genes affected by this basal interaction. Such a dichotomy of roles for $G\beta_1\gamma$ and $G\beta_{2/4}\gamma$ align well with our phylogenetic analysis [335] that suggest $G\beta_1$ and $G\beta_3$ are more similar to one another and share a common ancestral type of Gβ, whereas similar conclusions can be made for $G\beta_2$ and $G\beta_4$. Our current study provides functional corroboration for such phylogenetic divergences that may help provide insight regarding why mammalians have evolved to express different subtypes of Gβ and Gγ subunits, and also validates our previous findings that suggest $G\beta_4\gamma$ dimers are important for M3-mAChR proximal signalling while $G\beta_1\gamma$ dimers regulate expression of different proteins and occupy the promoters of over 700 genes in HEK 293 cells [433].

Indeed, it is high time for a better global understanding of the specificity of Gβγ dimer signalling and recognition that Gβγ specificity is as important as the specificities of signalling portrayed by different Gα subunits subtypes. The knowledge gained from the study of broad Gβγ function versus specific Gβγ function may reveal implications for their signalling not only for

specific and undiscovered signalling pathways, but also their roles in disease phenotypes. As mentioned in the Introduction, the use of broad-spectrum Gβγ inhibitors have been described to curb the proliferation in prostrate cancer [258] and as analgesics in μ–OR mediated pain [328]. These studies demonstrate the value of Gβγ inhibition in disease, albeit with global Gβγ inhibitors. In order to realize the potential of targeting these dimers as therapeutics of disease, crystallographic identification of specific Gβγ combinations' structures in addition to the currently published structures of $G\beta_1\gamma_2$ [41, 325], in conjunction with medicinal chemistry approaches to develop targeted inhibitors is necessary. Another possible way to develop inhibitors of the Gβγ-RNAPII interaction would be to develop specific RNA aptamers, a class of nucleic acid based ligands similar to antibodies that can be engineered to bind their targets with high affinity [478]. It is of my opinion that the development of inhibitors for specific combinations of Gβγ known to modulate signalling in implicated disease pathways has the potential to become a new class of drugs altogether.

5.4 Gβγ non-canonical signalling as negative feedback mechanisms?

As described in the Introduction, canonical effectors modulated by Gβγ include different isoforms of adenylyl cyclases, PLCβ subtypes, Kir3 channels, PI3K isoforms and components of the MAPK signalling pathway. Specificity of effector regulation is bidirectional phenomena whereby Gβγ acts to modulate specific isoforms of ACs, PLCβs and PI3Ks [38, 82, 106, 109-112, 124, 125, 127, 166-168], and whereby effectors interact with specific Gβγ subunits, examples of which I describe in this thesis by attributing roles for $G\beta_4\gamma_1$ and $G\beta_2$-containing Gβγ

dimers in the activation of PLCβ mediated calcium release upon M3-mAChR and AT1R activation, respectively. Moreover, the work presented in this thesis would suggest that regulation of effectors is not exclusive to regulation of activity, but also to the regulation of effector expression as evidenced by the roles of $G\beta_1$ in regulating ERK1/2 protein levels as well as the gene expression of various other components of signalling (Figure 3.6C). While we have learnt a great deal regarding the mechanisms whereby these effectors interact with and are regulated by Gβγ in terms of activity, the regulation of the expression of these proteins by Gβγ is a novel feature of GPCR signalling. This somewhere serves as a bridge between canonical roles of Gβγ and non-canonical functions such as effector expression regulation. What can then be speculated is whether regulatory feedback mechanisms exist whereby the activities of Gβγ subunits involved in specific signalling pathways act to control expression levels of pathway components upon pathway activation. Results presented in Chapter 3 allude to the existence of such mechanisms; $G\beta_1$ knockdown results in increased M3-mAChR signalling, trends for increased $G\beta_4$ and GRK6 expression and decreased ERK1/2 expression (Figure 3.7N, 3.6C). The presence of such regulatory mechanisms would not be entirely suprising, given that such mechanisms have been described for other receptor superfamilies such as the TGFβ receptor family (reviewed in [479]) and RTK family (reviewed in [480]). Confirmation of these regulatory feedback loops would require assessment of levels of all major signalling components involved in a given GPCR's signalling phenotype, via the assessment of Gβ and Gγ knockdown. Such a grandiose feat was beyond the scope of my project. Nevertheless, these results provide a solid basis for Gβγ mediated feedback regulation and present themselves as topics for future experimentation.

5.5 Insights regarding Gβγ intracellular translocation

With respect to emerging non-canonical functions of Gβγ, it is evident now that Gβγ functions extend far beyond the regulation of effectors proximal to GPCR signalling. As discussed in the Introduction, novel functions for Gβγ have been localized to perinuclear regions [204-206], ER [58, 96, 194], Golgi apparatus [195-197, 201], and mitochondria [60, 207, 208]. One question that remains is what the mechanisms are by which Gβγ dimers translocate within the cell. Regulation of proteins involved in vesicular formation such as SNARE [481] and PKD [197, 198, 200] and the fact that Gγ is anchored to membranes by prenylation may suggest that Gβγ subunits move in cells by virtue of vesicular transport. Another possibility exists whereby there may be different pools of Gβγ – pools of soluble dimers that translocate upon receipt of stimuli, and pools of organelle-specific resident-Gβγ that do not translocate, but rather perform functions when stimuli reach the site of residence (as in the case of plasma membrane bound Gβγ in trimeric form with Gα).

Translocation of Gβγ to the nucleus is a novel paradigm that has been previously been described to be GPCR activation-dependent [184, 238], a notion that is supported by our subcellular fractionation studies presented in Chapter 4 (Supplemental Figure 4.1E, F). As novel roles for Gβγ in the nucleus emerge, the question of how it gets into and out of the nucleus becomes increasingly interesting. In Chapter 4, I provide evidence showing that Gβγ translocates to the nucleus upon M3-mAChR activation, a signalling event that is blocked by the inhibition of importin-β with importazole. This observation tied in with the fact that our lab has demonstrated that Gβγ interacts with various members of processes that control nuclear import and export

(Rhiannon Campden, MSc thesis, unpublished data; [184]) leads to the speculation that Gβγ entry and exit to the nucleus is a regulated process. Gβγ dimers, however, do not possess any known nuclear export sequences (NES) or nuclear localization sequences (Sarah Gora, unpublished data). This makes it difficult to argue that Gβγ do indeed get imported and exported from the nucleus since karyopherin-β (importin/exportin family) mediated translocation require such sequences [482, 483]. Whether pools of specific Gβγ dimers exist as nuclear resident proteins, or a nuclear shuttling of Gβγ is necessary to facilitate their nuclear functions remain to be elucidated. However, it may be speculated that Gβγ shuttle to and from the nucleus as cargo on proteins that are known to shuttle. In the case of Gβγ mediated ERK1/2 dimerization and subsequent nuclear import [188], it is very well possible that Gβγ enters the nucleus as cargo of ERK1/2. Corroborating evidence for such an interaction-mediated Gβγ nuclear import includes demonstrations of Gβγ interactions with AP-1 transcription factors [183], a protein complex that has been described to translocate in response to receptor mediated signalling events [484].

5.6 Gβγ as regulators of gene expression

Activation of GPCR signalling pathways and effectors modulated by G proteins have previously been shown to converge on the regulation of gene expression in the nucleus (reviewed in [5]). Of more interest to this thesis and as discussed in the Introduction, Gβγ dimers indirectly regulate gene expression by activating signalling pathways that in turn promote or repress gene expression, and directly influence gene expression via their interactions with HDACs [240] or transcription factors such as MEF2a and AP-1 [183, 240]. In Chapter 5, I describe a novel

interaction between RNAPII and Gβγ, attributing an even more direct role for these dimers in the regulation of transcriptional activities. It is now evident that Gβγ plays roles in three different capacities pertaining to gene expression regulation, and thus, these findings shed light and concretely expand on an emerging aspect of Gβγ biology – regulation and modulation of transcription.

In higher eukaryotes, RNAPII is responsible for the transcription of protein coding mRNA, small non-coding and non-polyadenylated small nuclear RNAs (snRNAs) and and micro RNAs [485, 486]. After the initation of transcription, RNAPII pauses at regions 25-50 base pairs away from the start site due to its interactions with negative elongation factors such as NELF and DSIF that regulate RNAPII processivity [487, 488]. The C-terminal domain of Rpb1 in RNAPII contains heptad repeats of $Y_1S_2P_3T_4S_5P_6S_7$ which known to be regulated by phosphorylation by kinases such as P-TEFb (Cdk9/Cyclin T1) and Cdk7 [489]. More specifically, Cdk7 acts to phosphorylated these heptad repeats at Serine position 5, whereas P-TEFb acts to phosphorylate serines at position 2 [490, 491]. It is believed that these phosphorylation events are concomitant [489], although reports have indicated that Cdk7 activity is required as a priming step for the activity of P-TEFb [492], which in turn facilitates the release of pausing and transition to elongating RNAPII leading to the production of full length mRNA[449]. Therefore, Ser5p-Rpb1 can be thought of as marks that indicate paused RNAPII, and Ser2/5-Rpb1 can be regarded as elongating RNAPII. In Chapter 4, I describe that treatment of RNCFs with Ang II results in a net association of Gβγ with Rpb1 (Figure 4.3A), specifically with Ser5-phosphorylated Rpb1 (Figure 4.3F), whereby $G\beta_1$ acts to increase its interaction with both total Rpb1 (Figure 4.3C). Morever, I describe that knockdown of $G\beta_1$ results in a loss of the Ang II-induced increase of total Gβγ

interacting with both Rpb1 and Ser5p-Rpb1, suggesting that $G\beta_1\gamma$ increases its association with Ser5-phosphorylated Rpb1. Tying these observations in with the aforementioned fact that Ser5p-Rpb1 represents a paused population of RNAPII, and that our gene expression qPCR arrays reveal significant basal upregulation of 18 genes when $G\beta_1$ is knocked down, it can be speculated that $G\beta_1\gamma$ interacts with paused RNAPII to regulate pause release (Supplemental Figure 4.6). These array results are in line with previous demonstrations of $G\beta_1\gamma$'s roles as repressors of transcription [183].

Given that our results shows $G\beta_1\gamma$ is a regulator of pause release, one question that arises is how does it interact with RNAPII and with which other proteins. In Chapter 5, we provide evidence showing that $G\beta\gamma$ acts to interact with the interface on RNAPII created by Rpb1, Rpb6, Rpb8 and Rpb9, and describe preliminary results suggesting that $G\beta_1\gamma_2$ directly interacts with hRpb8. In order to appreciate how this interaction occurs, a co-crystal structure of $G\beta\gamma$ with the RNAPII holoenzyme complex would be of utmost benefit. Furthermore, structures of this type would allow for the development of inhibitors of this interaction, which could then be used to assess the effects of directly inhibiting the interaction on gene expression. $G\beta$ subunits have been structurally described to contain 7 consecutive repeats of 4 stranded antiparallel sheets that coincide with 7 repeated WD40 repeats [493]. Interestingly, proteins that contain WD40 repeats orchestrate diverse protein-protein interactions including proteins involved in scaffolding and the regulation of assembly of multi-protein complexes [494]. Taking this into consideration with the fact that $G\beta\gamma$ dimers do not possess any enzymatic activity, one reason as to why $G\beta\gamma$ interacts with Rpb1 may be to act as a scaffold to facilitate the binding of other Rpb1 regulatory proteins. To assess this notion, assessment of interactions with known Rpb1 proteins is necessary in future

experiments. Furthermore, our results provide the first line of evidence of the aforementioned Gβγ mediated negative feedback loops in GPCR signalling whereby speculations can be made that the $G\beta_1\gamma$ interaction with Ser5p-Rpb1 acts in a capacity to prevent further transcription of signalling components contained in a particular GPCR signalling pathway. In order to assess whether this is the case, whole genome RNA sequencing experiments with and without $G\beta_1$ knockdown would need to be performed to realize all of the genes that are regulated by $G\beta_1\gamma$.

The interaction between $G\beta_2\gamma$ and Rpb1 is currently more difficult to explain with the results in hand. As mentioned in Chapter 5, we believe that $G\beta_2\gamma$ plays a more prominent role in the propagation of signalling that leads to $G\beta_1\gamma$ increasing its interaction with RNAPII, however basal interaction and subsequent net decrease of $G\beta_2\gamma$ interaction with Rpb1 under conditions of agonist stimulation (Supplemental Figure 4.3C) cannot go ignored. The observation that knockdown of $G\beta_2$ results in both upregulation and downregulation of fibrotic genes makes it even more difficult to understand what the function of its basal interaction with RNAPII is. However, if one were to think along the same lines of the previously proposed notion of feedback regulation, it can be though that $G\beta_2\gamma$ basally interacts with RNAPII to positively regulate the expression of signalling pathway components. Unfortunately, such a process of thought at this point is purely speculative and he true nature of this basal interaction remains to be revealed, with further experiments required to concretely identify a role for this basal interaction. Our data suggests that $G\beta_5$ also interacts with RNAPII (Figure 4.1E), but the characterization of this interaction was again beyond the scope of my project. Studying this interaction would be an interesting area of future experiments that may reveal further differences between $G\beta_{1\text{-}4}$ and $G\beta_5$ as suggested by our phylogenetic analysis. Furthermore, the involvement

of different Gγ subtypes in the Gβγ-RNAPII interaction has been largely ignored throughout Chapter 4. Reasons for this include the fact that a majority of our analysis has involved assessment of interaction between endogenous proteins and antibodies that detect Gγ subtypes are few and far between. Nevertheless, RNAi screens similar to the one presented in Chapter 3 that would knockdown different Gγ subunits to assess their roles in mediated the interaction may be utilized to realize further specificities of the Gβγ-RNAPII interaction.

We demonstrate the Gβγ-RNAPII interaction in cell types whose origins are from two different species – HEK 293 cells derived from humans and primary RNCFs. The observation that this interaction occurs in mammalian species that are known to share around 90% genomic similarity (humans vs. rats, [495]) may lead one to wonder whether the interaction described in Chapter 4 between mammalian Gβγ and RNAPII is a feature acquired in higher organisms or whether such interactions and regulations have been maintained throughout evolution. Work in our lab has demonstrated using bimolecular fluorescence complementation that GPB-1 from *C. elegans* forms dimers with human Gγ subunits, and that GPC-2 is capable of similar dimerization with human Gβ subunits (unpublished data, Charles Harkness, PHAR 599 research project). A way to demonstrate that structural features that allow for interaction with Rpb1 have been retained from lower order species would be to first assess whether tagged versions GPB-1 from *C. elegans* can interact with and pull down Rpb1 in immunoprecipitation experiments. Another method of demonstrating the evolutionary conservation of the Gβγ-RNAPII interaction would be to assess the presence of the interaction from samples taken from lower order species. These two simple approaches to identify the potential conservation of this interaction throughout evolution

would be a great addition to our understanding of Gβ and Gγ subunit divergence and functional conservation.

5.7 Roles for Gβγ in disease

In Chapter 4, I provided preliminary evidence of the Gβγ-RNAPII interaction in whole heart lysates from TAC mice and aged rat hearts. Apart from demonstrating that this interaction occurs not only in hearts obtained from neonatal rats but also in aged rat hearts, we intriguingly observed a time-dependent patterns of interaction upregulation and downregulation in hearts from TAC mice – this provides for a interesting avenue of study to assess why such a phenomenon occurs and what the effects of the interaction induction is on the progression of phenotypes observed in TAC hearts. In a similar sense, it would also be interesting to assess the levels of interaction between RNAPII and the previously mentioned $G\beta_3$ mutation C825T in both sham mice and TAC mice. Demonstrations of interaction regulation in disease models of the type could impact not only our understanding of how hypertrophy is induced in these mice, but would add valuable proof to the existence of our interaction in animal models of disease.

As stated in the introduction, cardiac fibrosis is pathophysiology that is characterized by increased depositions of proteins in the extracellular matrix of the myocardium that results in remodelling of cardiac tissue [496], with the main drivers of these mechanisms in the heart being cardiac fibroblasts [497]. Activation of fibroblasts by stimulation of signalling pathways via activation of receptors such as TGF-β receptors, β_2-ARs and pertinent to this thesis, AT1Rs,

results in their transitition to activated myofibroblasts [496]. Here, I have described potential roles for Gβγ in regulating the expression of genes known to be upregulated or downregulated in fibrotic responses upon activation of AT1R with Ang II. As previously mentioned, our analysis reveals roles for $G\beta_1\gamma$ and $G\beta_2\gamma$ in regulating expression levels of various genes coding for transcription factors, cytokines, cellular adhesion proteins and other pro-fibrotic genes. One interesting observation is the upregulation of proteins that belong to different components of the TGF-β superfamily signalling. With knockdown of $G\beta_1$, we observe significant basal upregulations of Bmp7, Cav1, Smad4, Smad6, Smad7 and Tgfbr2 (Table 4.2), with no changes observed when compared to Ang II treated conditions. AT1R activation is well known to induce the expression of TGF-β1 in cardiac fibroblasts [444], and while the roles of AT1R and TGF-β signalling pathways have been found to not act independently of another and are interlinked [444], our results are the first demonstrations of a direct mechanism for Gβγ in controlling the regulation of AT1R activated signalling pathways that lead to fibrotic gene expression. Our findings hint at a finer layer of complexity relating AT1R signalling and TGF-β signalling in fibroblasts, and provide for a very interesting topic for future studies.

Beyond this novel role in cardiac fibrosis inducing mechanisms, Gβγ dimers are implicated a many diseases, one of which is cancer. Although many GPCRs such as thyroid stimulating hormone receptor, AT1R, M1- and M3-mAChRs and follicle stimulating hormone receptors have been implicated in the initiation and progression of cancer [498], roles for Gβγ in cancer remain largely unexplained. Since an overarching theme of my thesis has been the roles of Gβγ as regulators of gene expression, roles for Gβγ in the development, progression, and regulation of signalling activities in cancer becomes an interesting topic. As previously

mentioned in the Introduction, mutations occurring in $G\gamma_2$ and $G\beta_1$ have recently been described in malignant melanomas and erythroleukaemias, respectively [265, 266] [269], providing evidence for roles of $G\beta_1$ and $G\gamma_2$ as tumor suppresors. One caveat of these studies was that the effect these mutated subunits on signalling pathways was not completely described, although the study regarding $G\gamma_2$ demonstrated a link to FAK activity, and thus a role in tumor migration and invasion.

In essence, cancer is a disease of dysregulated gene expression and signalling [253]. In light of our demonstration of the novel Gβγ-RNAPII interaction, and the fact that $G\beta_1\gamma$ plays roles as a regulator of pause release and thus transcription, the next most logical and impactful course of action would be to assess these interactions in cancer cell types and tumors. By taking advantage of the knowledge of known mutations that lead dysregulated signalling phenotypes – mutations at mutants at positions Lys57, Lys89 and Iso80 of $G\beta_1$ [269] – the first step to take would be to assess whether these mutated $G\beta_1$ subunits display differential interaction capabilities with RNAPII. Since we have demonstrated that $G\beta_1\gamma$ increases and $G\beta_2\gamma$ decreases its interaction with RNAPII in an agonist-dependent manner, it would then be interesting to see whether such interplay and the signalling mechanisms necessary to induce the interplay are maintained in cancer cells or dysregulated altogether.

5.8 Conclusions

With a certain humility, it must be acknowledged that the work presented here is only a fraction of the entire story regarding Gβγ function. Many questions remain unanswered; what are the roles of endogenous $G\beta_3$ containing Gβγ dimers in cellular signalling? How does Gβγ enter the nucleus? What other specific genes do Gβγ regulate through interaction with RNA polymerase II? Are any other proteins recruited to the Gβγ-RNAPII interaction, and if so, why? Attempts to answer any of these questions could be considered the subject of entire projects and was unfortunately beyond of the scope of my PhD project.

As evidenced within this thesis, no two Gβγ dimers of specific subunit composition are completely alike. Although these dimers appear to be redundant to very limited degrees, it should now be appreciated that these specific dimers do indeed possess specific functions. Our analysis of Gβγ function would appear to suggest that in the context of Gq-coupled GPCRs, functions for $G\beta_2$ and $G\beta_4$-containing dimers are functionally similar, while those containing $G\beta_1$ are not; such a model fits our phylogenetic analysis and signalling phenotype analysis. However, the data presented herein is insufficient to truly come to such a conclusion that can be broadly applied to all different types of Gβγ dimers. Further work is necessary to attribute and confirm the presence of such classifications in other GPCR signalling systems.

The novel interaction between Gβγ and RNAPII described herein has the potential to be a ground breaking finding. Observed to be induced under the activation of two different types of Gq-GPCRs in two different cellular models, and the fact that we have also preliminarily

observed this interaction to be induced upon activation of Gs-GPCRs, it can be speculated that this interaction is a general feature of GPCR signalling. Further work is definitely needed to determine whether such a suggestion is true. However, if this interaction is indeed modulated by more members of the GPCR superfamily, this novel finding will change the scope of the field entirely and add another layer of complexity to an already complex playing field. Nevertheless, the demonstration of this interaction to be a GPCR-wide phenomenon will have profound impact on our understanding of GPCR signalling systems, and may possibly aid the development of therapeutics aimed at modulating these systems.

All in all, the work presented in this thesis advances our understanding of Gβγ dimers in three respects: (1) their phylogenetic divergence, (2) their specificity in signalling and (3) their interaction with RNA polymerase II as a regulator of gene expression. It can easily be realized that Gβγ dimers are responsible for a plethora of roles that range from modulation of signal transduction to regulation of non-canonical functions in multiple intracellular compartments. From their functions in effector regulation in the cytosol to newly described roles in the nucleus, it can be appreciated that Gβγ dimers play critical roles in GPCR signalling and beyond. It is an exciting time in the field as we are at the cusp of fully realizing the vast arsenal of functions these dimers possess, a realization that will only grow in magnitude and impact as we learn more about these dimers.

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Appendix