Investigating G protein βγ subunit interactions with nuclear proteins

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December 2014

A thesis submitted in partial fulfillment of the requirements of the degree of Master's of Science

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Abstract

Heterotrimeric G proteins, composed of functional α and βγ subunits, canonically act at the cell surface to transmit extracellular signals received by G protein-coupled receptors (GPCRs) to modulate internal signalling pathways. In this capacity, Gβγ subunits are generally found at the cell surface. However, they have also been localized to intracellular compartments including the nucleus where they interact with a number of transcription factors. To characterize these broader functions of Gβγ in the nucleus, we designed a screen to capture Gβγ interacting proteins in the nucleus. This thesis describes optimization of the tandem affinity purification protocol for use with nuclear lysates, and the results that were obtained by using this protocol with cytosolic and nuclear lysates. In addition to the standard TAP protocol, we describe two additional methods, a split and a single TAP method, in addition to Flag affinity purification. In the nucleus of HEK 293 cells, Gβγ was found to interact with a number of transcription factors and RNA/DNA binding proteins, supporting previous evidence of function in the nucleus. In the cytosol of HEK 293 cells, Gβγ interacted with some novel proteins, including mitochondrial ribosomes, which may indicate a novel localization and function for Gβγ. In comparing the three affinity purification methods used, we show an altered composition of interacting proteins, indicating the importance of considering the affinity purification technique used. By activating endogenously expressed muscarinic receptors in HEK 293 cells with carbachol, the pattern of nuclear Gβγ-interacting proteins was altered, suggesting that such signalling networks respond to surface GPCRs. This work opens the door to further understanding nuclear G protein signalling, and provides the tools to further study specific Gβγ signalling events in the nucleus.

Résumé

Les protéines G hétérotrimériques sont composées de trois sous-unités : α et le dimère βγ. Elles sont localisées à la surface des cellules et transmettent les signaux reçus par les récepteurs couplés aux protéines G (RCPG) aux différents effecteurs intracellulaires. Cette fonction des protéines G fut relativement bien étudiée durant les dernières décennies. Toutefois, de récents travaux ont permis de découvrir que les protéines G sont aussi localisées à l'intérieur du noyau des cellules et qu'elles peuvent interagir avec certains facteurs de transcription. Afin de déterminer les principales fonctions de ces protéines G nucléaires, nous avons établi un criblage des différentes protéines qui interagissent avec elles. Cette thèse décrit les étapes d'optimisation de deux protocoles utilisant la purification par affinité en tandem (unimoléculaire et bimoléculaire), permettant de déterminer les protéines qui interagissent avec βγ localisé dans le noyau, et en décrit les résultats. De plus, ces techniques furent comparées à une autre technique de purification de protéines couramment utilisée, la purification par affinité au peptide Flag. Dans les noyaux des cellules HEK 293, βγ interagit avec un grand nombre de facteurs de transcription et de protéines liant l'ADN et l'ARN. Au niveau du cytoplasme, nous démontrons de nouvelles interactions entre βγ et certaines protéines incluant des ribosomes mitochondriales. En comparant les trois protocoles, nous démontrons que nous pouvons modifier la composition des protéines interagissant avec βγ. Nous avons également modifié la composition des protéines interagissant avec βγ en traitant les cellules avec du carbachol, agoniste de certains RCPG endogènes des cellules HEK 293, démontrant ainsi que la stimulation d'un récepteur à la surface des cellules peut modifier la signalisation des protéines G nucléaires. Cette thèse ouvre la voie à une meilleure compréhension de la signalisation de βγ et procure les outils permettant l'étude de la signalisation du dimère βγ nucléaire.

Acknowledgements

The completion of this thesis would have not have been possible without the support and encouragement from a number of people, namely those in the Hébert Lab. I would first like to thank Dr. Hébert for the opportunity to undertake my MSc. thesis in his laboratory. Here I have been able to develop my analytical and critical thinking skills, as well as my scientific writing and presentation skills. Terry has always been there to answer my questions and appease my concerns. He has been highly supportive and has enabled me to accomplish as much as I possibly could in a MSc. thesis. One of the reasons I chose McGill was for the atmosphere of excellence and high standard of academic achievement, and in Terry's lab I have been able to excel.

First, thanks to Dr. Sarah Gora who taught me my first protocols at McGill. She was patient and understanding in my learning process, and her sense of humour and care made all the difference in my transition to McGill. To Darlaine Pétrin, who answered many of my emails in my first months of optimization, and provided advice that was invaluable in the optimization process. To Dr. Mélanie Robitaille, a former Hébert lab graduate, who has answered countless emails and offered up tips and tricks for mass spectrometry. She also ran all my mass spec samples in the Angers Lab at the University of Toronto, so without her this project really would not have been possible. Thanks also go out to Dr. Stéphane Angers for his collaboration. I would also like to thank Dr. Dan Bernard for the use of his speed vac equipment. Special thanks to Dr. Wolfgang Reinstch for help with confocal microscopy and image analysis.

Thank you to both of my students, Cole Krensky and Sanchari Banerjee. Thank you Cole for help with the immense amount of tissue culture I undertook in the preparation of samples. Thank you Sanchari for your work ethic and for the enormous amount of work accomplished in the three months of summer.

Thank you to my thesis advisory committee, Dr. Bruce Allen, Dr. Paul Clarke and Dr. Jason Tanny. Thanks Bruce, for your sense of humour and questions that made me think about my thesis from a broader perspective. Thanks Paul for your

mentorship and advice, and for teaching me. Thanks Jason for your questions and feedback. Special thanks also to all the professors at McGill I have had the opportunity to interact with, who have taught me so much and have made my experience at McGill the best it could be.

I would like to thank members of the Hébert lab for welcoming me into their ranks, and providing much needed stress relief and perspective outside of the laboratory. To Dr. Dominic Devost for insightful conversations and advice. I appreciate the time taken to discuss questions; it is impossible not to learn more than you had anticipated when speaking with you, and for the numerous things I have learnt from you I am tremendously grateful. “Tanks” to Dr. Nicolas Audet for your advice, fighting techniques, and the levity brought to the workplace. Nico has also been crucial in the optimization process through teaching me to trust my instincts and to fully understand any technique used. Thanks to Dr. Dany Fillion for helpful discussions, conversation and for keeping perspective. To Dr. Richard Wargachuk for his advice, the humour brought to the workplace, for camaraderie during late work nights, and the pumpkin caper. To Phan Trieu, for answering my many questions, and always knowing how and where to find laboratory reagents. Finally, but not at all least, thank you to the other graduate students in the Hébert Lab. To Shahriar Khan and Rory Sleno for being great friends over the past two years! They made all the difference in my move to Montréal, and aided in my transition. Special thanks to Rory for heated discussions, where if I didn’t learn something at the time, I definitely did in the long run. Special thanks to Gayane Machkalyan for your conversation and friendship. Your critical thinking, knowledge, advice and attitude were highly welcomed and appreciated during the time she joined our laboratory. Thanks also to the new graduate students, Ryan Martin and Jennifer Sung, in addition to the many undergraduate students and volunteers who passed through the Hébert lab. All the members of the Hébert lab have made it a truly enjoyable experience to undertake my MSc. here, and I am grateful to everyone for creating such an engaging and congenial environment to work in.

This work was supported by grants from the Canadian Institutes of Health Research (CIHR; MOP-79354 to Terry Hébert). The manuscript authors within

were supported by a postdoctoral fellowship from the McGill-CIHR Drug Development Training Program (DDTP) awarded to Nicolas Audet and a graduate studentship from the National Science and Engineering Research Council of Canada (NSERC) awarded to Rhiannon Campden.

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14-3-3 – protein eluted in the 14th fraction of bovine brain homogenate and were found on positions 3.3 of subsequent electrophoresis, hence 14-3-3

3T3 L1 – 3-day transfer, inoculum 3 x 10^5 cells L1 cells

α_{2A}-AR – α_{2A}-adrenergic receptor

AC – adenylyl cyclase

AcTEV™ – active tobacco etch protease

AEBP1 – adipocyte enhancer binding protein 1

AEBSF – 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride

AF2 – activation function 2

AGS – Activator of G protein signalling

AKAP – A kinase anchoring protein

ALK4 – Activin receptor type 1B

ALK5 – TGFβ type I receptor

Ang II – angiotensin II

AP1 – activator protein 1

Arf – ADP ribosylation factor

Arl – ADP ribosylation factor like

ATP – adenosine triphosphate

AT_1R – angiotensin receptor 1

βAR – β-adrenergic receptor

BRET – bioluminescence resonance energy transfer

BSA – bovine serum albumin

$CaCl_2$ – calcium chloride

CaM – calmodulin

cAMP – cyclic adenosine monophosphate

CAND1 – cullin-associated and neddylation-dissociated 1

Ca_v – voltage-dependent calcium channel

$Ca_v\beta$ – voltage-dependent calcium channel β subunit

$Ca_v\alpha1$ – voltage-dependent calcium channel α1 subunit

CBD – calcium binding domain

CCT – chaperonin-containing TCP1 protein

Chang – Chang liver cell line

ChIP – chromatin immunoprecipitation

CHO – Chinese hamster ovary cells

cFos – cFBJ murine osteosarcoma viral oncogene homolog B

c-H-Ras – carcinoma Harvey rat sarcoma viral oncogene homolog

cJun – Jun proto-oncogene

COPI – coat protein I

COS1 – CV-1 (simian) in Origin, and carrying the SV40 genetic material 1

COS7 – CV-1 (simian) in Origin, and carrying the SV40 genetic material 7

COX2 – cyclooxygenase 2

CSN – COP9 signalosome subunit

DCN1 – defective in Cul neddylation 1 protein

DNA – deoxyribonucleic nucleic acid

DMEM – Dulbecco's modified eagle media

DRiP78 – dopamine receptor interacting protein 78

DTT – dithiothreitol

Ect2 – Epithelial cell transforming 2 cells

EDTA – ethylenediaminetetraacetic acid

EGF – epithelial growth factor

EGTA – ethylene glycol tetraacetic acid

ER – endoplasmic reticulum

ERK – extracellular signal-regulated kinase

ETB – endothelin B receptor

FBS – fetal bovine serum

FASTA – FAST-All

FLAG – FLAG octopeptide epitope

FRET – fluorescence resonance energy transfer

FSHβ – Follicle-stimulating hormone β

FosB – FBJ murine osteosarcoma viral oncogene homolog B

Gα – G protein α subunit

Gβ – G protein β subunit

Gβγ – G protein βγ subunits

Gγ – G protein γ subunit

GAP – GTPase-accelerating protein

GAP43 – growth associated protein 43

GDP – guanosine diphosphate

GEF – guanine nucleotide exchange factor

GEM – GTP Binding Protein Overexpressed In Skeletal Muscle

GH3 – glycoside hydrolase family 3

GNRH1 – Gonadotropin releasing hormone 1

GOA-1 – G protein α subunit Go

GPA-1 – G protein α subunit family

GPB-1 – G protein β subunit family

GPC-1 – G protein γ subunit family

GPCR – G protein coupled receptor

GR – glucocorticoid receptor

GRE – GR response element

GRK2 – G protein-coupled receptor kinase 2

GRK2ct – G protein-coupled receptor kinase 2 C-terminal domain

GST – glutathione S-transferase

GTP – Guanosine triphosphate

GTPγS – Guanosine triphosphate analogue γS

GTPase – guanosine triphosphate hydrolase

HA – Human influenza hemagglutinin epitope

HCT116 – human colorectal carcinoma cell line

HDAC – histone deacetylase

HEK 293 – Human embryonic kidney 293 cells

HeLa – Henrietta Lacks cell

Hep3B – Hepatocellular carcinoma 3B cells

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HepG2 – Hepatocellular carcinoma G2 cell

HP1α – heterochromatin protein 1 α

H-Ras – Harvey rat sarcoma viral oncogene homolog

H-Ras4B – Harvey rat sarcoma viral oncogene homolog 4B

Huh7 – Human hepatotoma 7 cells

IgG – Immunoglobulin G

JNK – c-Jun N terminal kinase

JunB – Jun B proto-oncogene

Kir – inwardly rectifying potassium channel

K-Ras4B – Kirsten rat sarcoma viral oncogene homolog 4B

LCMS – Liquid chromatography mass spectrometry

LPA – lysophosphatidic acid

LPA1R – lysophosphatidic acid 1 receptor

mACh – muscarinic acetylcholine receptor

MAPK – mitogen-activated protein kinase

MBP – maltose binding protein

MEF2C – myocyte enhancer factor 2C

MEK1/2 – mitogen-activated protein kinase kinase 1/2

Mfn1 – mitofusin 1

mRNA – messenger ribonucleic acid

MS – mass spectrometry

NDPK – nucleotide disphosphate kinase

NEDD8 – neddylin 8

NES – nuclear export signal

Neuro2A – Neuroblast 2A cells

Net1 – neuroepithelial cell transforming 1

NFκB – Nuclear factor kappa-light-chain-enhancer of activated B cells

NHDF – normal human dermal fibroblasts

NIH 3T3 – NIH 3-day transfer, inoculum 3 x 10^5 cells

NLS – nuclear localization signal

NOS – nitric oxide synthase

NPC – nuclear pore complex

NTF2 – nuclear transport factor 2

NTP – nucleotide triphosphate

$P34^{cdc2}$ – cyclin dependent kinase 1

PAFR – platelet-activating factor receptor

PBS – phosphate buffered saline

PdBu – phorbol 12,13-dibutyrate

PGE2 – prostaglandin E2

PI3K – phosphoinositide 3 kinase

PhLP – phosducin-like protein

PKA – protein kinase A

PKB – protein kinase B

PKC – protein kinase C

PKD – protein kinase D

PLC – phospholipase C

PMA – phorbol 12-myristate 13-acetate

PMSF – phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride

ProtA – Protein A of Staphylococcus aureus

POLR2A – Polymerase (RNA) II (DNA Directed) Polypeptide A

POLR2D – Polymerase (RNA) II (DNA Directed) Polypeptide D

POLR2E – Polymerase (RNA) II (DNA Directed) Polypeptide E

POLR2H – Polymerase (RNA) II (DNA Directed) Polypeptide H

PTX – pertussis toxin

R7BP – Regulator of G protein signalling 7 binding protein

RACK1 – receptor for activated C kinase 1

Rab – Member of ras oncogene family

Rad – GTP binding protein RAD

Raf1 – Proto-oncogene serine/threonine-protein kinase 1

Ran – Ras related nuclear protein

RanBP1/2 – Ran binding protein 1/2

RanGAP – Ran GTPase activating protein 1

Rap1 – member of Ras oncogene family 1a

Ras – Ras sarcoma viral oncogene homolog

RCC1 – regulator of chromosome condensation 1

Rce1 – Ras converting CAAX endopeptidase 1

Rem – RAS (RAD And GEM)-Like GTP-Binding

Rem2 – RAS (RAD And GEM)-Like GTP-Binding 2

RGK – Rad/Rem/Rem2/Gem/Kir

RGS – Regulator of G protein signalling

RhoA – Ras homolog gene family member A

Ric8 – Resistance to inhibitors of cholinesterase 8

Rin1 – Ras and Rab interactor 1

RNA – ribonucleic acid

RNAPII – RNA polymerase II

RT-qPCR – reverse transcriptase quantitative polymerase chain reaction

RTK – receptor tyrosine kinase

SBP – streptavidin binding domain

SCC – Squamous cell carcinoma cells

SII – Sar(1),Ile(4),Ile(8)]Ang II

siRNA – small interfering RNA

Swiss 3T3 – Swiss 3-day transfer, inoculum 3 x 10^5 cells

TAP – tandem affinity purification

TCP1 – T complex 1

TEV – tobacco etch virus

TFE3 – Transcription Factor Binding To IGHM Enhancer 3

TRHR – thryrotropin releasing hormone receptor

U – Units

VDCC – voltage-dependent calcium channels

VEGF – vascular endothelial growth factor

VIP – vasoactive intestinal peptide

WD40 – Tryptophan asparagine 40

XPO1 – exportin 1

Y2H – yeast-two-hybrid

1 Introduction

This introduction includes a modified version of a review entitled, “Nuclear G protein signalling: New tricks for old dogs”, to be published in *J. Cardiovasc. Pharmacol*.[1]. I wrote 60% of the manuscript and prepared the figures. Nicolas Audet wrote the other portion of the manuscript and Terry Hébert edited the work.

1.1 G protein-coupled receptor and G protein signalling

G protein-coupled receptors (GPCRs) are ubiquitously expressed proteins that receive extracellular signals and transmit them inside the cell. There are 800 genes that code for GPCRs, indicating their diversity and importance[2,3]. Heterotrimeric G proteins, which are responsible for transducing signals received by GPCRs into cellular responses, therefore mediate a vast range of events involved in many different cell processes.

The majority of GPCRs signal through G proteins in response to hormones and neurotransmitters. In the canonical model of GPCR signalling, receptors are localized to cell membranes were they are coupled with the G protein heterotrimer. GPCRs receive extracellular signals and transmit them to G proteins, which then signal to other downstream effectors. Conformational changes in the GPCR activate coupled G proteins. This mechanism involves the GPCR acting as a guanine nucleotide exchange factor (GEF) on the Gα subunit. The Gα subunit then releases GDP and binds GTP, allowing activation of the G protein[4]. This action allows the heterotrimer to activate downstream effectors. Both G proteins (Gα and Gβγ) can activate different downstream effectors including adenylyl cyclase[5], inwardly rectifying K^+ channels[6,7], phospholipase C β (PLCβ)[8-10], phosphoinositide 3 kinase (PI3K)[11], and voltage-dependent N-type Ca^{2+} channels[12,13]. It was originally thought that GPCR activation resulted in separation of heterotrimeric G protein subunits, where Gα would modulate one pathway, and Gβγ another. However, using fluorescent resonance energy transfer (FRET), it was revealed that Gαi, when coupled to Gβ1γ2, remains a heterotrimer but undergoes a

conformational change following GPCR activation to modulate effectors[14]. Whether or not the heterotrimer dissociates into the Gα and Gβγ subunits or undergoes conformational changes to carry out functional roles following receptor activation is still under discussion. Under different contexts evidence for both mechanisms has been found[15,16].

GPCRs contain seven transmembrane domains, an extracellular N-terminus and an intracellular C-terminus, which is involved in coupling to G proteins. Ligand binding to the extracellular portion of the GPCR leads to movement in the transmembrane domains which are translated to the intracellular side[17]. Different ligands can induce different changes in GPCRs, and likewise different ligands can induce different movements within one GPCR and thus activate different signalling pathways[17,18]. This phenomenon is called biased signalling. One well-studied example of biased signalling comes from the angiotensin receptor 1 (AT_1R). Both the endogenous ligand, angiotensin II (Ang II), and the biased agonist SII can recruit β-arrestin to the GPCR in a G protein-independent manner. However, SII does not activate the canonical pathway mediated by Gαq. Both agonists can activate Gαi and Gαq, however the different ligands lead to different downstream signalling pathways. In the case of Ang II, Gαq controls activation of Gαi-dependent effectors, such as ERK1/2 and phospholipase C. Activation of AT_1R by SII leads to direct adenylyl cyclase regulation by Gαq- and Gαi-independent mechanisms[19]. In addition, AT_1R activation by SII increases the recruitment of β arrestin, whereas other angiotensin analogs do not[20]. This example outlines the complexity of GPCR signalling at the level of ligand and G proteins coupled to the GPCR.

The large variety of downstream Gβ and Gγ subunits adds another layer of complexity to GPCR signalling. The formation of unique Gβγ dimers within a particular cell context may govern how receptor-based signalling complexes are assembled, thus orchestrating the signalling pathways activated in a given cell. In mammals, there are five Gβ subunits and twelve Gγ subunits. Gβ subunits 1-4 share between 79 and 90% sequence similarities, while the Gβ5 subunit is the most divergent and shares only approximately 52% similarity with the others. Gγ

subunits are much more divergent than Gβ subunits, and share between 26 and 76% similarity. This may suggest that Gγ subunits are responsible for the diversity of signalling partners shared by Gβγ (reviewed by Khan *et al.*)[21].

Gβγ can interact with multiple effectors, including, but not limited to, adenylyl cyclase[22], G protein coupled receptor kinase (GRK)[23], phospholipase C[24], phosphoinositide 3-kinase[25], and inwardly rectifying potassium channels (GIRK)[26]. Analysis of the wide range of Gβγ binding partners reveals that there is no consensus binding sequence for interaction with Gβγ. The limited number of crystal structures of Gβγ with the interactors GRK2, Gα, phosducin, and a peptide sequence (SIGKAFKILGYPDYD) show that these proteins all interact with the same surface of Gβ created by the β propeller structure of WD40 repeats. Further analysis revealed that although all these proteins bind to the same surface, they use unique residues on Gβ to mediate their binding[27]. This evidence suggests that Gβ is the main mediator of Gβγ effector modulation. The question of the specific role of individual Gγ subunits remains uncertain at present.

Although not the focus of this manuscript, another important factor is the diversity in Gα subunits. There are four major families of G protein α: Gαs, Gαi, Gαq, and Gα12, which contain the sixteen subtypes of Gα. Coupling of GPCRs to different Gα subunits, as outlined above, can lead to different signalling outputs[19,28]. Understanding G protein composition, signalling and processing is therefore an important consideration.

1.2 G protein βγ structure and processing

The G protein heterotrimer is composed of a Gα subunit and a Gβγ subunit. The Gα subunit contains a helical domain and a GTPase domain, which provides binding sites for Gβγ (**Figure 1**). The GTPase domain adopts different structures, depending on whether it is bound to GTP or GDP.

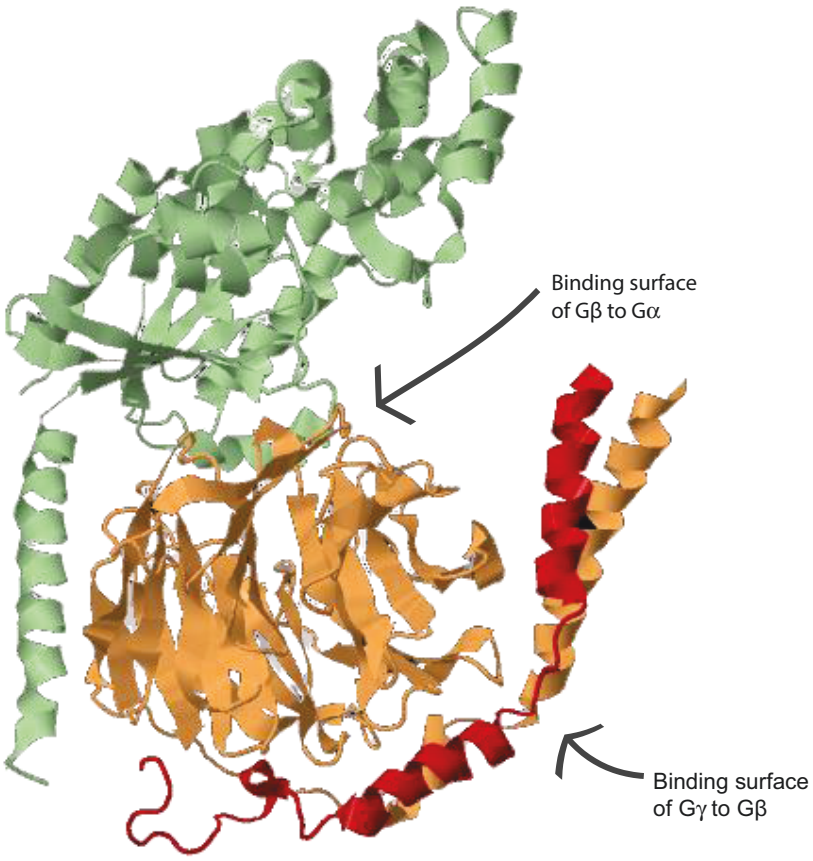


Figure 1: *Structure of the Gα and Gβγ heterotrimer bound to GDP.* Gα subunit is in green, Gβ subunit in orange, and Gγ subunit in red. The structural image was generated using the RSCB Protein Data Bank and structural data by Lambright *et al*.[29]. GDP is not visualized in this image, although structural information was taken from the Gα and Gβγ heterotrimer bound to GDP. Gβ binds to Gα at the GTPase domain on Gα. Gγ binds to Gβ at the N-terminal domain of Gβ.

The Gβγ subunit is folded in a seven-bladed propeller structure of seven WD40 repeats, which is approximately 40 amino acids long and ends in a tryptophan and asparagine (hence WD40). WD40 motifs are conserved in other proteins and usually mediate protein-protein interactions. The N-terminus of Gβ has a α-helical confirmation and is where the N-terminus of the Gγ subunit binds. The C-terminus of Gγ folds around to bind blades 5 and 6 of Gβ[30].

Although in some cases the heterotrimer can dissociate into the Gβγ and Gα subunits, Gβγ dimers will only dissociate under denaturing conditions[14-16,31]. Similarly, in the absence of Gβ, Gγ subunits are not processed properly, and show reduced protein levels[32]. In the absence of Gγ, Gβ forms misfolded protein aggregates[33]. In addition, proper targeting to the cell membrane requires the dimerization of Gβγ and proper processing of the dimer[32]. The presence of both Gβ and Gγ are required for the proper folding and expression of Gβγ.

Cell membrane localization of Gβγ and Gα subunits require post-translational modification. The Gα subunit can be both myristoylated and palmityolated. Myristoylation is the covalent (and therefore irreversible) attachment of a 14-carbon myristate moiety to an N-terminal glycine on Gα via an amide bond. Palymitoylation is the reversible attachment of a 16 carbon palmitate on a cysteine via a thioester bond[34]. Gγ subunits can be isoprenylated, which refers to the addition of either a 15-carbon farnesyl or a 20-carbon geranylgeranyl isoprenoid group linked to a cysteine on the Gγ subunit. Gγ isoforms 1,8 and 11 are farnesylated, while the other Gγ subunits are geranyl geranylated[30]. Addition of a prenyl group occurs at the CAAX motif, where C is a cysteine, A is an aliphatic amino acid, and X is a variable group. Following prenylation, the cysteine of the CAAX motif is methylated, and then the –AAX is cleaved by CAAX prenyl protease (Rce1). Of particular note, Gγ5 is processed differently from the other Gγ subunits. Instead of an aliphatic amino acid at position A2, Gγ5 has the aromatic amino acid, phenylalamine, rendering the protein resistant to Rce1 proteolysis[35].

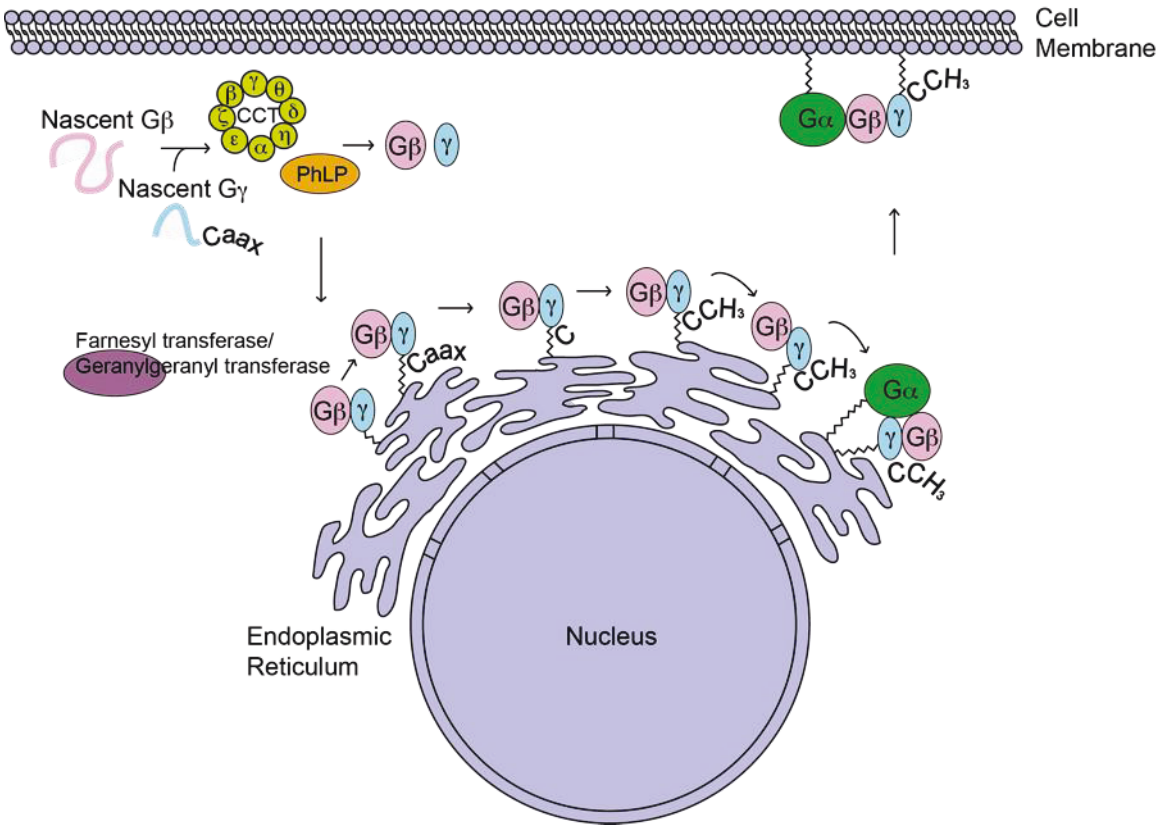


Figure 2: *Gβ and Gγ are folded separately with the chaperone proteins PhLP and CCTs*. PhLP promotes the proper folding of Gβγ into a dimer. Following dimerization, Gγ undergoes lipid modification and further processing such as carboxymethylation and protein cleavage. Next, the dimer is bound to Gα and the heterotrimer is recruited to the plasma membrane.

Gβγ subunits are synthesized in the ER and processed in the cytosol[21,36]. Separately, Gβ and Gγ bind to chaperone proteins to facilitate their proper folding, and are then brought together to form the Gβγ dimer (**Figure 2**). Chaperonin containing TCP-1 (CCT) proteins bind to Gβ and promote adoption of its seven bladed propeller structure[29,36]. Dopamine receptor-interacting protein 78 (DRiP78) may facilitate proper folding of Gγ[21]. The phosducin-like protein (PhLP) is required for the assembly of Gβγ and promotes disassembly of Gβ and CCT[21]. Following the binding of Gβγ, Gγ undergoes lipid modification [34].

After Gβγ is post-translationally modified, Gβγ associates with Gα and is transported to the cell membrane. Although the specific requirements for Gα trafficking to the cell membrane appear to be cell and protein context-dependent, Gβγ may be important as a targeting step for Gα direction to the cell membrane[34,37]. The relative contribution of N- myrisotoylation, palmityoylation and binding to Gβγ in membrane targeting of the Gα subunit is not completely understood. Although Gβγ may not be required for Gα localization to the cell membrane, Gα is required for Gβγ targeting[34]. Under different conditions, and with different Gα subunits, N-myristoylation has been shown to be required for palmitoylation and membrane localization, and in some cases N-myristoylation is dispensible and rather Gβγ is required for further palmitoylation and membrane localization[37]. However, when Gα is not palmitoylated, Gβγ fails to localize efficiently to the cell membrane[34]. Localization at the cell membrane, whether it occurs through myristoylation of Gα, Gβγ binding, or both, allows palmitoylation to occur which anchors the heterotrimeric G protein[37].

Although most Gγ subunits are prenylated, there may be a population that escapes prenylation, is alternately prenylated, or is processed in a different manner. These examples come from studies by Cook *et al.* who found multiple alternately processed Gγ variants including seven Gγ2 variants, the highest expressed Gγ isoform in the brain[3]. It is likely that these variants are found in all other isoforms, but were only isolated in the study due to the high expression of Gγ2. Of particular note were the variants of Gγ2 and Gγ7 which lacked a prenyl group[3]. Since prenylation is required for Gβγ insertion into the plasma membrane,

this evidence would suggest that there are Gβγ isoforms that are purely cytosolic[34]. Additionally, Gγ2 was found to be farnesylated in this study, whereas previous reports show that Gγ2 is geranylgeranylated[34]. Alternate processing may lead to physiologically different roles of Gγ subunits[3]. This is highlighted by studies on the A1 adenosine receptor, which was shown to prefer Gγ subunits that were geranylgeranylated[38]. Although there is limited data to date, the above evidence suggests that alternately processed Gγ subunits have different physiological roles, that could be affected by receptor recognition, fidelity of receptor signalling, cellular localization and turnover of G proteins[3].

Another factor in Gβγ signalling is isoform specificity. The diversity in Gβ and Gγ subunits is a product of evolution, indicating a divergence in roles for individual subunits[21]. Evidence from yeast-two-hybrid (Y2H) and immunoprecipitation studies show that not all Gβ and Gγ subunits are able to bind to each other with the same affinities[39,40].

Gβ1 and Gβ2 have the highest affinity for Gγ subunits as determined using by Y2H experiments, while Gβ3-5 have variable affinities[39]. By immunoprecipitation, it appears that while Gβ1 and Gβ4 have the highest affinities for Gγ subunits, Gβ2 is more specific for individual Gγ subunits (having little affinity for Gγ 1,8,11 and 13) and Gβ3 and Gβ5 have the lowest or non-existent affinities for Gγ[40].The low affinity of Gβ5 for Gγ is of particular interest, since in a cellular context it binds to RGS protein family 7[41]. As discussed above, sequence analysis shows that Gβ5 shares the least similarity with all other Gβ isoforms, and may indicate an unique role[21]. Unique Gβγ pairs add an additional layer of complexity to the specificity of cellular signalling as in cell specific contexts, different GPCRs can couple to particular Gβγ and Gα subunits[42,16,21].

1.3 G protein activation independent of GPCRs

The localization of G proteins to distinct subcellular compartments, such as the Golgi apparatus[43] and nucleus[21,44], suggest that they function either independent of GPCRs, or perhaps are not yet linked to a GPCR, and suggests alternate

mechanisms of activation of G proteins in these locations[45]. Further, lipophilic antagonists (tetracaine, bupivacaine, lidocaine) of β-adrenergic receptor (βAR) were able to directly activate GTP hydrolysis by isolated G proteins bound within liposomes. The more lipid soluble the molecule, the more activation of the G protein was achieved[46]. These results indicate that in some cases G proteins might act independently of GPCRs.

The Activators of G protein Signalling (AGS) family of proteins can activate G proteins, thus fulfilling the GEF function of the GPCR. AGS proteins act in three ways: 1) by directly binding to the Gα subunit, 2) by modulating the Gα and Gβγ interaction via binding to Gα, and 3) by modulating the Gα and Gβγ interaction by binding to Gβγ[45]. AGS2/3 proteins can activate the pheromone response pathway in a Gα-GTP independent mechanism[47]. There is evidence to suggest that AGS proteins may compete with GPCRs for a limited pool of G protein partners[45]. G proteins can also be activated by a number of other proteins including GTPase activator protein 43 (GAP43), which facilitates the binding of GTP to Gαo and amplifies GPCR signalling cascades[48], and Ric8, which binds Gαq and Gαi/o and functions as a guanine exchange factor[49,50]. GTPase accelerating proteins (GAPs) bind to G proteins and stimulate their innate GTPase function, causing the hydrolysis of GTP to GDP, thus inactivating the protein. Guanine nucleotide exchange factors (GEFs) are required for the exchange of GDP for GTP on the G protein, thus aiding in their activation[51].

Another protein that can activate G proteins independent from GPCRs is nucleotide disposphate kinase (NDPK). NDPKs are enzymes that catalyze the transfer of the γ phosphate group from an NTP onto a phospho-histidine kinase[52]. The family I of NDPKs, including NDPK-B, are expressed mainly in the cytosol, but have also been reported at cell membranes, in the cell nucleus and mitochondria[52,53]. Functions of NDPKs include DNA binding, transcriptional modulation, DNA cleavage, and histidine kinase function. The two main isoforms share 88% identity, however only NDPK-B has been reported to be associated with G protein activation[52,54-57]. In this context, NDPK-B catalyzes the transfer of a high-energy phosphate from the γ position of ATP[52]. This high-energy phosphate

is transferred from ATP to histidine 116 on NDPK-B, and then transferred to histidine 266 on Gβ. From here, the phosphate group is transferred to GDP bound by Gα, leading to receptor-independent G protein activation[55,57]. Evidence for the activation of Gαs, Gαt and Gαi through this mechanism has been reported[54].

Although the above examples present a mechanism for G protein activation independent of the GPCR, and in alternate cell locations from the cell membrane, the majority of G proteins are not thought to be activated by this method[54]. In support of this, NDPK-B depletion, or mutation of His266 on Gβ did not affect cAMP production by stimulation of βARs with isoproterenol in cardiac myocytes. These changes did, however, lead to reduced receptor-independent Gαs activation in cardiomyocytes, affecting cAMP production and contractility in this context. It is important to note that NDPK-B content at the cell membrane in patients with congestive heart failure is increased by 300%[58]. Thus, it is likely that only in this pathological cell context does independent activation of G proteins affect cell function.

Despite the evidence for G protein activation independent of GPCRs under particular conditions, there is no evidence yet to suggest this occurs in the nucleus. Indeed, there are a number of GPCRs localized to the cell nucleus [59-61], suggesting that G proteins may function in concert with GPCRs in this context. However, to date, the mechanism of G protein activation in the nucleus has not been fully characterized.

These examples indicate that signalling pathways bypassing GPCRs, which directly activate heterotrimeric G proteins, are possible. These pathways may be involved in the activation of G proteins, in particular Gβγ subunits, in subcellular locations and under particular contexts, where, as of yet, no GPCRs have been found. This is exemplified in the Golgi apparatus where G protein activation leads to Golgi vesiculation[62]. This is mediated through the activation of PLCβ3 by Gβ1γ2, leading to the activation of protein kinase D (PKD)[63,64]. Sequestration of endogenous Gβγ by GRK2 c-terminal domain (GRK2ct) inhibited the transport of cargo directed to the plasma membrane[65]. Although movement of Gβγ to the Golgi apparatus following cell membrane bound GPCR activation has been

demonstrated, whether GPCRs are present and can be activated at the Golgi is suspected but not convincingly demonstrated[21].

1.4 Expression patterns of Gβγ

Gβγ subunits are not ubiquitously expressed in all cell types, which further limits the possible Gβγ dimers that can form in particular cell types. *In situ* hybridization studies on rat brains indicated that all Gβ subunits showed widespread expression within the brain, with highest concentrations in the cortex, striatum, hippocampus, hypothalamus, thalamus and dorsal raphe. The expression patterns of Gβ2, 3, and 5 were very similar, while Gβ4 had distinct localization to deep layers of the parietal and occipital cortex. Indeed, across all tissues, Gβ expression is nearly ubiquitous[66]. Gγ subunits, on the other hand, showed more distinctive localization. Gγ1, Gγ9 (cone-specific isoform) and Gγ11 were undetectable in the brain, while Gγ2, 3, 4, 7 and 10 were expressed exclusively in neurons[67]. The highest expression levels of Gγ7, 3 2 were found in the brain, while Gγ5 was found at lower levels in the brain, heart and spleen, and at higher levels in the kidney, liver and lung. Gγ2 and 3 were only found in the brain[68]. This indicates the differentiation of Gγ subunit expression, and may indicate that individual Gγ subunits are involved in unique, tissue-specific signalling pathways.

Inside the cell, Gβγ localization is not restricted to the plasma membrane. Gβγ has been reported in endosomes, in the mitochondria, in the endoplasmic reticulum, in the Golgi apparatus and in the nucleus (reviewed by Khan *et al.*)[21]. While it is true that GPCRs are internalized as a mechanism of desensitization of a primary signalling pathway, relocation of such signalling complexes may also allow modulation of additional signalling pathways. Just as endocytosis functions to control cell surface signalling, cell signalling can also control endocytic pathways[69].

Gβ1γ2 was found to interact with Rab11a in endosomes following activation of the lysophosphatidic acid (LPA) receptor. This interaction leads to the

recruitment and activation of PI3Kγ and protein kinase B (PKB) signalling pathways. Stimulation of the LPA receptor leads to cell proliferation and protection against apoptosis in human embryonic kidney cells (HEK 293)[70]. Gβ2[71], Gγ1[71], Gα12[72] and Gαi1[73] have all been localized to the mitochondria. However, overexpression of Gα subunits in mitochondria was able to disrupt the interaction of Gβ2 with mitofusin 1 (Mfn1), indicating that Gβγ in this context may be independent of Gα. In this context, Gβ2 interacts with Mfn1 through interaction its WD40 repeats in HeLa cells to regulate mitochondrial fusion. Gβ2 was found to restrict the movement of Mfn1 along the mitochondrial surface[71]. The above examples present novel Gβγ localization, where Gβγ acts potentially independently of Gα and presumably, at the mitochondria, independently of a GPCR. However, further research needs to be done to confirm this.

In contrast to the above examples, in the endoplasmic reticulum, Gβγ subunits interact with diverse components of GPCR signalling[21]. Further examples, discussed below, also suggest independent roles of Gβγ subunits in the nucleus. The emerging picture is that Gβγ subunits function with GPCR signalling components in some situations, and in others they act alone. This again highlights the need to think about G proteins in terms of distinct “pools”; for example, those that are found at the cell membrane, compared with those that exist in the nucleus. Further complexity may arise from different subunits occupying different pools in different cellular contexts.

1.5 Heterotrimeric G protein signalling mediated by nuclear GPCRs

The standard model of GPCR signalling holds that receptors are trafficked to the plasma membrane where they become functional and responsive to agonist stimulation. These receptors can be internalized following prolonged or repetitive agonist stimulation, which leads to desensitization of their primary signalling pathways. However, internalizing receptors can also activate signalling pathways distinct from those activated by cell surface receptors[74-76]. An increasing number of GPCRs have also been demonstrated to be targeted to the endomembrane locations, as have their associated signalling cascades[77,78]. Thus, the signalling

landscape inside the cell may be as complex as that assembled to detect and interpret signals from outside the cell. It is clear that different organelles must communicate in real-time on a variety of distinct time-scales using a number of intracrine signalling pathways. That said, cell surface- and endomembrane-localized GPCRs both signal via heterotrimeric G proteins, which can either remain associated with their cognate GPCRs during such signalling events or be released to operate in distinct locations of their own.

In the past decade, an increasing number of cell surface GPCRs have been detected on the nuclear membrane (reviewed in Boivin et al. 2008, Vaniotis et al. 2011, Tadevosyan et al. 2012)[76,79,80]. Direct activation of these receptors by intracellular ligands can be followed by activation of nuclear resident pool of heterotrimeric G protein that can in turn lead to increases in second messenger levels inside the nucleus, protein kinase activation and for some, transcriptional regulation. Even if it is not clear whether these receptors couple with the same G proteins and effectors in the nucleus membrane as at the cell membrane, there are many known examples of nuclear GPCR signalling through G proteins.

A large number of signalling proteins, normally associated with receptor-mediated events at the cell surface have been detected in the nucleus and/or on the nuclear membrane. In addition to heterotrimeric G proteins[81-84] (see Willard *et al.*, Dupré *et al.*, Dupré *et al*., and **Table 1**)[13,85,86], effector molecules such adenylyl cyclases[87,88], phospholipase A_2[89], phospholipase Cβ[90] and phospholipase D[91], and regulatory proteins such as β-arrestin1[92,93], G protein-coupled receptor kinases[94], A kinase anchoring proteins (AKAPs), PKA[95] and RGS proteins (reviewed in Burchett *et al*. 2003)[96], among others, have been detected at these sites as well (**Figure 3**). Further, these intracrine signalling loops do not seem to be restricted to GPCRs and may include a number of other classes of “surface” receptors such as ALK4/ALK5, TGF-β superfamily receptors responsive to activin A[97], VEGF receptors[98], (reviewed in Re and Cook 2007)[99] and EGF receptors[100-102].

Table 1: *Evidence for G proteins in the nucleus including specific localization, cell type, and evidence type.* Table was constructed based on the literature cited below.

Protein class	Protein	Localization	Cell type	Evidence	Reference
Gαi	Gαi	Nucleus, Kinetochores	Swiss 3T3	Western blot, cell fractionation, fluorescent microscopy	Crouch *et al.* 1991 Crouch *et al.* 1997 Crouch *et al.* 2000
	Gαo	Mitotic spindle		Immunohistochemistry, immunoelectron microscopy	Wu *et al.* 1994
Gαs	Gαs	Nuclear membrane	S49 lymphoma cells	Cell fractionation, western blot, immunoelectron microscopy	Saffitz *et al.* 1994
Gαq	Gαq	Nuclear membrane	HEK 293 Neurons Cardiac myocytes	Nuclear isolation	O'Malley *et al*. 2003 Kumar *et al.* 2008 Wu *et al.* 2014
	Gα16	Nucleus	COS7 HEK 293 Cardiomyocytes	Yeast two-hybrid, GST-pull-down, fluorescent microscopy	Sato *et al.* 2011
Gβ	Gβ1	Nucleus	HEK 293 HCT116	Fluorescent resonance energy transfer, nuclear isolation, western blot, chromatin immunoprecipitation, co-immunoprecipitation	Robitaille *et al.* 2010 Kino *et al. 2005* Spiegelberg and Hamm 2005
	Gβ2		HCT116	Chromatin immunoprecipitation	Kino *et al.* 2005
	Gβ5	Nucleus	Mouse primary neurons	Immunofluorescence, nuclear isolation, western blot	Panicker *et al.* 2010

Gγ	Gγ2	Nucleus	HEK 293 HCT116	Fluorescent resonance energy transfer, nuclear isolation, western blot, chromatin immunoprecipitation	Robitaille *et al.* 2010 Kino *et al.* 2005
	Gγ5	Nucleus	3T3-L1 cells	Electrophoretic gel mobility shift assay, western blot, cell fractionation, co-immunoprecipitation	Park *et al.* 1999
Ras	H-Ras	Nucleus	NIH 3T3 L-cells Fibroblasts	Western blot, cell fractionation, immunofluorescence	Contente *et al.* 2011
RGK	Rem2	Nucleus	COS1 HeLa C2C12	Immunofluorescence, nuclear localization signal	Beguin *et al.* 2005 Mahalakshmi *et al.* 2007
	Rem	Nucleus	COS1 HeLa A549 H9c2(1-2)	Immunofluorescence, nuclear localization signal, co-immunoprecipitation	Mahalakshmi *et al.* 2007a
	Rad	Nucleus	COS1 HeLa A549 H9c2(1-2)	Immunofluorescence, nuclear localization signal, co-immunoprecipitation	Mahalakshmi *et al.* 2007a

	Kir/Gem	Nucleus	COS1 HeLa	Immunofluorescence, nuclear localization signal, co-immunoprecipitation	Mahalakshmi *et al.* 2007b
Rab	Rab1A	Nucleus	SCC cell lines SCC tissue	Immunohistochemistry, cell fractionation, western blot	Mitra *et al.* 2003
	Rab1B	Nucleus	SCC cell lines SCC tissue	Immunohistochemistry, cell fractionation, western blot	Mitra *et al.* 2003
	Rab 24	Perinuclear, Nuclear inclusions	HEK 293	Immunofluorescence, cell fractionation, western blot	Maltese *et al.* 2002
Rho	RhoA	Nucleus	HEK 293, HeLa		Dubash *et al.* 2011
Sar1/Arf	Arf1	Nuclear vesicles	*Xenopus* oocytes	Nuclear vesicle fusion assay, light and electron microscopy	Boman *et al.* 1992
	Arl4	Nucleus/nucleoli	COS7 Sertoli (TM4) Neuro 2A	Western blot, cell fractionation, co-immunoprecipitation, fluorescent microscopy, nuclear localization signal	Jacobs *et al.* 1999 Lin *et al.* 2000
	Arl5	Nucleus/nucleoli	COS7 Hep3B HuH7	Fluorescent microscopy, western blot, cell fractionation, nuclear localization signal	Lin *et al.* 2002
	Arl6	Nucleus		Fluorescent microscopy	Jacobs *et al.* 1999
	Arl7	Nucleus		Fluorescent microscopy	Jacobs *et al.* 1999
Ran	Ran	Nuclear membrane	Eukaryotic cells		

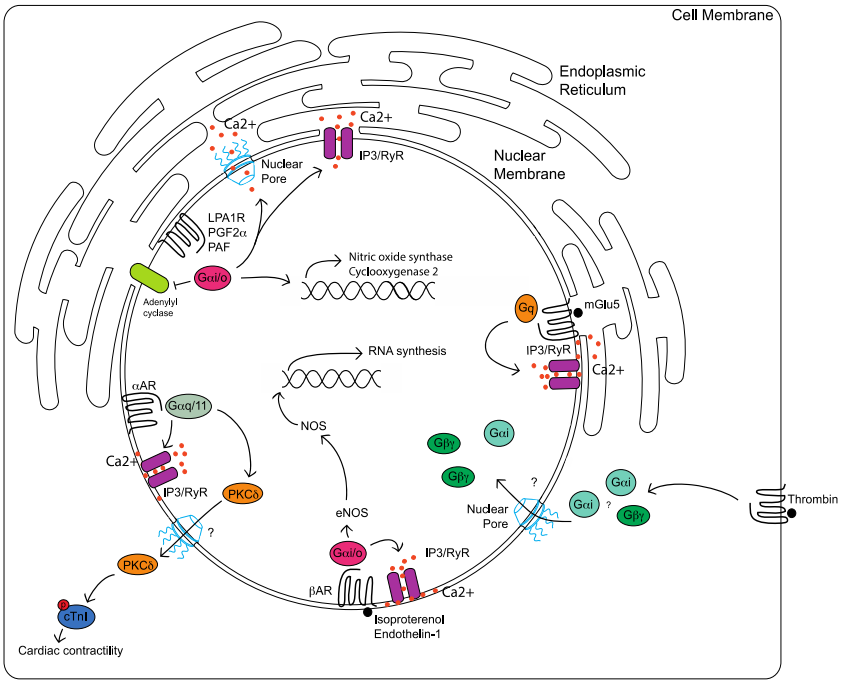


Figure 3: *G protein-modulated pathways in the nucleus include calcium and NOS signalling*. These pathways can lead to regulation of transcription and other events within the nucleus, but could potentially act to regulate cytoplasmic events as well. Within the nucleus, stimulation of nuclear lysophosphatidic acid receptors (LPA1R), prostaglandin F2 α receptor (PGF2α), and platelet activating factor receptor (PAF) lead to activation of Gαi /o leading to increased calcium in the nucleus, inhibition of adenylyl cyclase and increased nitric oxide synthase (NOS) and cyclooxygenase 2 (COX2) gene synthesis. Activation of nuclear α adrenergic receptor (αAR) activates Gαq/ll leading to increased calcium signalling and activation of protein kinase C delta (PKCδ). PCKδ is then transported out of the cell nucleus and leads to an increase in cardiac contractility via phosphorylation of cardiac troponin I (cTnI). Nuclear β-adrenergic receptor (βAR) activation causes a calcium influx via Gs (C. Merlen, T.E. Hébert and B.G. Allen, unpublished results) and also leads to increased gene expressed via Gi and NOS. Activation of the muscarinic glutamate receptor 5 (mGlu5) also leads to an increase in calcium in the nucleus via Gq. Thrombin receptor activation at the cell membrane leads to an increased of Gα and Gβγ in the nucleus, which may enter via the nuclear pore.

1.6 *Examples of heterotrimeric G protein signalling in the nucleus.*

Activation of platelet-activating factor receptor (PAFR), detected on isolated nuclei from porcine cerebral microvascular endothelial cells and CHO cells, led to a decrease in nuclear cAMP production and an increase in intra-nuclear calcium and an increase in expression of pro-inflammatory genes (nitric oxide synthase (NOS) and cyclooxygenase 2 (COX-2)) (**Figure 3**). In this context, these events were sensitive to pertussis toxin, in contrast to cell surface PAFR signalling dependent on Gq, indicating the involvement of Gi/o proteins[103]. NOS transcription could also be modulated by activation of PTX-sensitive G proteins and nuclear calcium signalling following activation of nuclear lysophosphatidic receptor type-1 (LPA1R)[104] and nuclear PGE2 receptor[105,106] (**Figure 3**).

Gq-coupled receptors are also found on the nuclear membrane. It has been demonstrated that stimulation of metabotropic glutamate receptor mGlu5 on isolated nuclei of both HEK 293 cells and neurons leads to an increase in calcium signalling through Gq[107,108] (**Figure 3**). Nuclear α_{1A}-adrenergic receptor also signals through Gq/11 to activate protein kinase C δ (PKCδ) in cardiomyocytes[109] (**Figure 3**). PKCδ activation is followed by its translocation into the cytoplasm where it phosphorylates cardiac troponin I and induces cardiomyocyte contractility. This was the first example found of a cytoplasmic target for nuclear GPCR signalling and raises many questions about the orientation of nuclear signalling events and the directionality of signalling downstream of these receptors[110].

Other nuclear GPCRs in the heart can also signal into the nucleus via heterotrimeric G proteins. Using UV-caged ligands (endothelin-1 and isoproterenol) that can be released inside living cells and activate intracellular receptors, it was shown that endothelin B (ETB) and β-adrenergic receptors (βARs) can regulate calcium and nitric oxide signalling in the nuclei of intact adult cardiomyocytes via Gq and Gi, respectively[111,112] (**Figure 3**). Activation of nuclear βARs in the heart also increased RNA synthesis through a PTX-sensitive G protein[84,113] (**Figure 3**).

In addition to the examples above, the GPCRs apelin[59], AT1 angiotensin[59,114] and bradykinin 2 receptors[115], are localized to the nuclear membrane. However,

direct signalling through G proteins for apelin and bradykinin receptors still needs to be demonstrated.

1.7 Nuclear signalling by heterotrimeric G protein mediated by surface GPCRs

Independently of nuclear-localized GPCRs *per se*, the involvement of GPCRs and G proteins in transcription has been well-documented[21,116]. Using a proteomic approach with tandem affinity purification (TAP) followed by mass spectroscopy, we showed that when TAP-tagged Gβγ subunits were purified from isolated nuclei, most of the endogenous Gα subunit subtypes were also detected in HEK 293 cells under basal conditions[117] (**Table 2**, see subsequent chapters). It is also possible that some of the G proteins can reversibly shuttle into and out of the nucleus in response to stimulation. For example, treatment of Swiss 3T3 cells with thrombin for 20 hours led to an increase of Gαi and Gβ in the nucleus, with a corresponding decrease of Gαi in the cytosol with pertussis toxin sensitive Gαi (**Figure 3**). Gαi was found to be involved in cell division[118-120]. This may mean that the effects of G proteins on transcription, DNA replication or repair may be altered depending on their localization or cell cycle status. For example, Gαi can activate all three classes of MAPKs following activation of GPCRs at the cell membrane. Depending on the cell context this can lead to the activation of different signalling cascades involving JNK, ERK and p38 leading to gene expression through the activation or inhibition of transcription factors that are trafficked into the nucleus[118]. Another example of extracellular signals leading to translocation of heterotrimer G protein subunits into the nucleus involves modulation of glucocorticoid receptor (GR) activity.

Table 2: *Heterotrimeric G proteins in the nucleus of HEK 293 cells pulled down with TAP Gβ1 or Gβ1γ7 in the cytosol and nucleus.* Proteins identified as Gβγ interactors with TAP tagged Gβ1 and identified by mass spectrometry.

Protein Class	Protein
Gα	Gαs
	Gαq
	Gαi1
	Gαi2
	Gαi3
	Gα11
	Gα12
	Gα13
Gβ	Gβ1
	Gβ2
	Gβ4
Gγ	Gγ7

In GH3 cells, dexamethasone, which activates GR, and subsequent somatostatin treatment to activate Gβγ through activation of cognate receptors, led to an increased interaction between Gβγ subunits and GR, which resulted in movement of this complex into the nucleus (**Figure 4**). Gβ1 and Gβ2 were found to interact with GR in HCT116 colorectal carcinoma cells and GH3 rat pituitary carcinoma cells. This interaction was dependent on the ability of Gβ to bind Gγ but intriguingly, not Gαi. Gβγ was able to inhibit GR function, which required binding of Gα to the AF-2 domain of GR. This effect was enhanced when a nuclear localization signal was added to Gα and conversely decreased when a nuclear export signal was added. This complex associates with DNA, however, chromatin immunoprecipitation (ChIP) revealed that Gβ2 was precipitated with GR response elements (GRE) only in the presence of GR[121]. What has become clear in recent years is that many transcription factors and DNA modification are direct targets for G protein action in the nucleus (**Figure 4**). G proteins found in the nucleus can modulate transcription, or have interaction with transcription factors and transcriptional regulators. These include Arl5[122], Gα16[123], Gβ (Gβ1, 2)[121,123-126] and Gγ (Gγ5)[127].

1.8 G protein regulation of nuclear events

G proteins in the nucleus have been documented to both inhibit and activate transcription[124,128]. They can either interact directly with transcription factors, or act indirectly by inhibiting or activating regulators of transcription factors[125,128]. This points to further complexity of regulation of transcription and some new mechanisms behind how cells integrate external and internal signals. This has become a rich new area of study with a number of interesting implications. As of yet, the interactions of Gβγ in the nucleus do not form a cohesive story of Gβγ signalling in this location. As further data begins to emerge, it is likely that the understanding of Gβγ signalling in the nucleus will correspondingly increase as well, and a clearer picture of the function of Gβγ in the nucleus will develop.

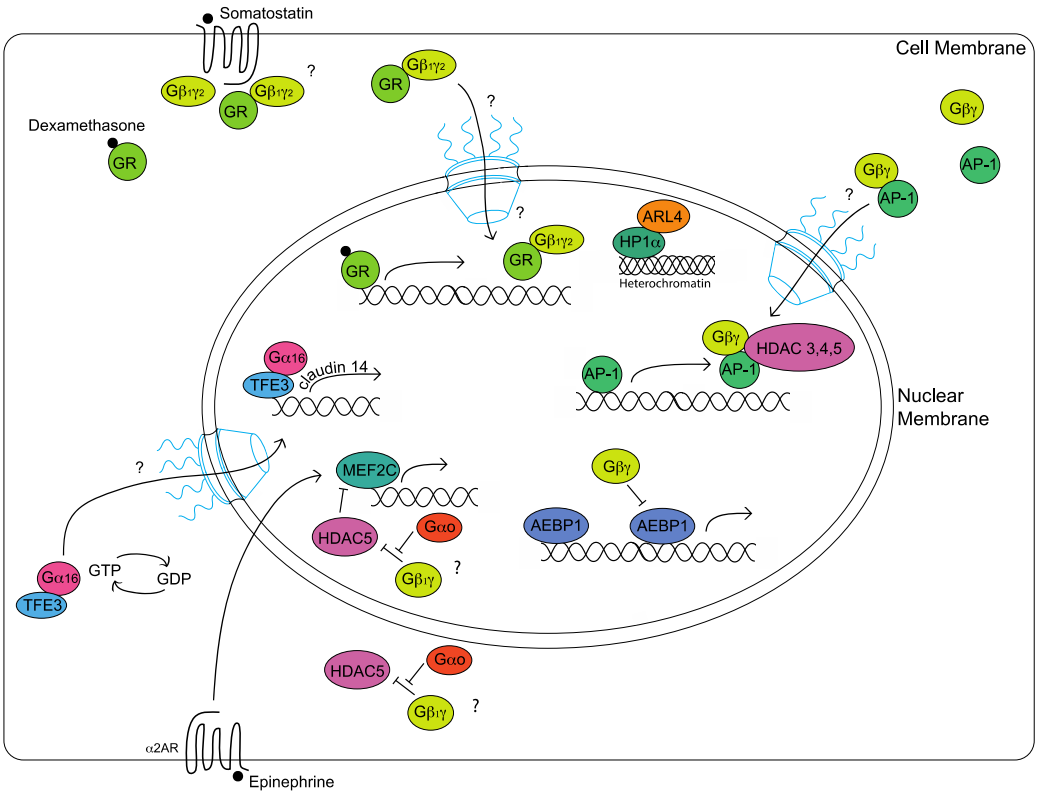


Figure 4: *Heterotrimeric G proteins are involved in the regulation of transcription factors within the nucleus*. In many cases regulation occurs through signalling pathways that originate at the cell surface, as outlined in greater detail within the text. Following glucocorticoid receptor (GR) stimulation, Gβ1γ2 inhibits GR mediated transcription. It is unknown whether Gβ1γ2 binds GR in the cytosol or the nucleus. Gβγ can also inhibit activator protein 1 (AP-1) transcription factor activity by recruiting histone deacetylase 3, 4 or 5 (HDAC 3, 4, 5). This recruitment occurs through the binding of Gβγ to AP-1. Gβγ can similarly inhibit AEBP1, however in this case transcriptional inhibition is inhibited. Through inhibition of HDAC, Gβ1γ can relieve the inhibition of gene transcription from MEF2C sensitive genes. Gαo can block this, and it is unknown whether this function occurs in the nucleus or the cytosol. Gα16 can bind transcription factor binding to IGHM enhancer 3 (TFE3) either in the cytosol or nucleus, leading to increased transcription of claudin 14. Arf-like protein 4 (Arl4) interacts with HP1α in the nucleus, leading to the hypothesis that it is involved in the regulation of heterochromatin.

1.8.1 *HDAC isoforms*

The control of gene expression in cardiac cells is tightly regulated and involves many transcription factors and chromatin modulators[129]. One way that gene expression is modulated in cardiac cells is by acetylation and deacetylation. Acetylation refers to the addition of acetyl groups onto histone N-terminal lysines. This removes the positive charge on the histones, leading to less compaction. Histone deacetylase (HDAC) enzymes are histone-modifying enzymes that remove this acetyl group, leading to increased DNA compaction and therefore decreased gene expression. Both histone acetyltransferase (HATs) and HDACs have been implicated in cardiac hypertrophy, which involves aberrant gene expression in cardiac myocytes[129]. In the heart, HDAC5 binds to the muscle differentiation factor (MEF2C) sensitive promoters and leads to inhibition of transcription[130-132]. HDAC5 interacts with MEF2C-sensitive promoters and causes decreased histone acetylation leading to inhibition of transcription[130]. Spiegelberg and Hamm found that HA-tagged Gβ1 was purified with HDAC (**Figure 4**). Gαo can inhibit this interaction, indicating that HDAC5 binds to free Gβγ. When the α_{2A}-AR was activated, the inhibitory effect of Gαo on Gβγ was reduced as it was released[125]. Thus, Gβγ consequently reduces the inhibitory effect of HDAC5 on MEF2C by binding directly to HDAC5. It is not clear where the HDAC5/Gβγ interactions occur in the cell. Although free Gβγ can interact with a transcriptional repressor alone[125], in other contexts, Gβγ binds to Gαi and is expressed in equimolar concentrations in the nucleus, suggesting that these proteins can function as a heterotrimer under certain conditions as well[119]. A more recent study has also shown the Gβ2 interacts with MEF2, HDAC5 and histone proteins in neonatal rat ventricular cardiomyocytes and knockdown was associated with changes in expression for a large number of genes[126].

1.8.2 *AP-1*

Gβγ subunits have also been shown to interact with activator protein 1 (AP-1) transcription factors (**Figure 4**). This transcription factor is composed of two proteins from the Jun (c-Jun, Jun and JunD) and Fos (c-Fos, FosB, Fra1 and Fra2) subfamilies; the dimer combination determines the gene regulatory specificity[133]. In human embryonic kidney cells (HEK 293), over-expression of Gβγ leads to a 50% reduction of luciferase reporter under control of the AP-1 sensitive promoter, while reducing Gβγ levels with siRNA lead to an increase in luciferase levels. In this context, Gβγ functions to repress AP-1 action. This result was also observed in the gonadotrope cell line LβT2, where gonadotropic-releasing hormone 1 (GNRH1) stimulates follicle stimulating hormone β (FSHβ) gene promoter via AP-1. Gβγ was able to inhibit the activation of FSHβ by combinations of the overexpressed AP-1 proteins FosB/cJun, FosB/JunB, and cFos/JunB. Gβ was co-immunoprecipitated with cFos or FosB, but not with Jun, and the interaction was dependent on the leucine zipper domain of Fos that is involved in AP-1 formation. Although no interaction of Gγ was found with Fos, the over-expression of Gγ in tandem with Gβ increased the effect of Gβ on AP-1. This interaction is believed to occur within the nucleus, as Gβγ localized to the nucleus under basal conditions. When the interaction between Gβ and Fos was made irreversible, the proteins accumulated in the nucleus, suggesting that the interaction between AP-1 and Gβγ leads to movement of the complex into the nucleus. Gβγ was again able to recruit the transcriptional co-repressor HDAC3, 4 and 5. A ChIP assay showed that without Gβγ there was limited HDAC associated with the FSHβ promoter. However, when Gβγ is expressed, there was increased association of HDAC with the promoter[124]. In this example, Gβγ recruits a transcriptional regulator to repress AP-1 sensitive genes and highlights the fact that individual transcriptional complexes may contain unique subsets of Gβγ subunits leading to gene- and cell-specific outcomes.

1.8.3 *AEBP1*

In 3T3-L1 cells, Gγ5 and Gβ are localized to the nucleus. Co-immunoprecipitation revealed binding of Gγ5 to adipocyte enhancer binding protein 1 (AEBP1) (**Figure 4**). AEBP1 is expressed in pre-adipocytes and may function as a transcriptional repressor in the maturation of adipocytes[134].The interaction of AEBP1 and Gγ5 leads to the inhibition of AEBP1-sensitive gene transcription that is concentration dependent. Electrophoretic mobility shift assays were used to show that Gγ5 prevents the binding of AEBP1 to AE-1 sequences, but not other transcription factors, highlighting Gγ5 specificity for AEBP1. Therefore, in this context, Gγ acts to repress a repressor, although how exactly this occurs has yet to be elucidated[128]. Evidence of G proteins binding to transcription factors brings up two important points: 1) to date, no direct DNA binding ability of G proteins has been found, and 2) G proteins generally act to modify the activity of transcription factors either by recruiting repressors/activators, or as chaperones to traffic transcription factors in or out of the nucleus.

1.8.4 Gβ5 subunits

Gβ5 is structurally distinct from the other Gβ isoforms, sharing approximately 50% sequence similarity with Gβ1-4 (reviewed in Khan *et al.*)[21]. That said, Gβ5 binds the RGS family 7 proteins, RGS 6, 7, 9-1, 9-2 and 11. The dimer formed between Gβ5 and RGS7 binds the RGS 7 binding protein (R7BP) and is anchored to the cell membrane through the addition of a palmitate group of R7BP[135]. Loss of this palmitate group results in accumulation of the Gβ5/RGS7 complex in the nucleus in neuronal cell lines[136]. Interestingly, the palmitoylation of R7BP is highly dynamic and turnover occurs at a high rate. In neuronal cells, signalling through Gαi/o controls palmitoylation. When Gαi/o is activated, it inhibits the depalmitoylation of R7BP and leads to expression at the cell membrane[82,137]. When Gαi/o is inactivated by PTX, there is nuclear accumulation of R7BP[138]. Shuttling of RGS7-Gβ5 can also be inhibited in the absence of R7BP[82,137]. The shuttling of RGS7-

Gβ5 to the nucleus by R7BP may represent a new mechanism of transmitting neurotransmitter signals from the cell surface into the nucleus, however the function of Gβ5 in the nucleus is yet be determined[135].

1.8.5 *TFE3*

The effects of heterotrimeric G protein subunits on transcriptional activity are not restricted to free Gβγ subunits but may also depend on Gα. Within the Gαq family, Gα16 was found to bind to the activator of G protein signalling (AGS) protein transcription factor binding to IGHM enhancer 3 (TFE3) in COS-7 and HEK 293 cells (**Figure 4**). TFE3 is ubiquitously expressed and involved in the regulation of genes in tandem with Smad proteins[139]. The interaction is dependent on guanine nucleotide binding; constitutively active Gα16(Q212L) or inactive Gα16(G211A) mutants were not co-immunoprecipitated with TFE3. The interaction of TFE3 and Gα16 leads to accumulation of the dimer in the nucleus, and causes increased expression of TFE3-sensitive genes such as claudin 14. When the inactive or constitutively active Gα16 mutant was expressed with TFE3, the effect on claudin 14 was abolished[123].

1.8.6 *Arl5*

Small G proteins may also be important in the regulation of nuclear function (**Table 1**). The ADP ribosylation-like (ARL) proteins ARL4, 5, 6 and 7 have all been found in the nucleus[122,140]. Endogenous ARL4 is localized to the nucleus of Sertoli and neuroblastoma cells. The localization of ARL4 appears to be dependent on myristoylation, as an ARL4 mutant lacking the C-terminal tail (which is myristoylated) was not localized to the nucleus[122]. Endogenous ARL5 was found in the nucleus of human hepatoma Hep3B and Huh7 cells[141]. Transiently expressed ARL4 mutants that bind only GDP or GTP were both found in the nucleus, indicating the nuclear localization of ARL4 is not dependent on the GTPase activity of ARL4. However, GDP-bound ARL4 and ARL5 were enriched in the nucleoli[122]. A yeast two-hybrid assay using ARL5 bound to GTP was used to

identify the transcription factor human heterochromatin protein 1 (HP1α) as an interactor, which was confirmed by GST-pulldown[122]. HP1α is involved in epigenetic gene repression[142]. The inactive ARL4 mutant did not bind HP1α, nor did any other ARL isoform[122]. Therefore, through its interaction with HP1α, ARL5 may be involved in heterochromatin and transcriptional repression (**Figure 4**).

1.9 Other G proteins in the nucleus

1.9.1 *Ras*

H-Ras is also localized to the cell nucleus and levels fluctuate based on cell cycle stage[143]. Ras proteins are involved in gene expression, apoptosis, cell cycle regulation, endocytosis and extracellular signal transduction[144]. Although its role in the nucleus may not be direct, changes in nuclear levels suggests its importance in the nucleus during the cell cycle. Both oncogenic Ras and wild type H-Ras are localized to the nucleus. Wild type H-Ras is found in the nuclei of primary fibroblasts, NIH 3T3 cells, and intestinal enteroendocrine cells (L cells). Serum starvation followed by stimulation of NIH 3T3 cells indicated that H-Ras shuttles in and out of the nucleus during the cell cycle, and showed strongest nuclear expression directly following starvation. Nuclear localization was decreased over 16 hours post stimulation with serum. When the same experiment was done with the oncogenic NIH 3T3 cell line transformed with c-H-Ras, H-ras did not localize in the nucleus following starvation, but increased in the nucleus over eight hours following serum treatment. This indicates that the localization of c-H-Ras is altered as compared to H-Ras[143] and may contribute to its oncogenic phenotype[145]. Oncogenic forms of H-Ras are found in the nuclei of mouse liver tumours where they bind nuclear transport factor 2 (NTF2), an interaction that is thought to occur because of aberrant nuclear accumulation of H-Ras in this context[145]. Interestingly, this protein also binds NF□B and prevents the phosphorylation of p27 in the nucleus[145]. Another study showed that a splice variant of H-Ras, termed p19, was found in the nucleus of COS1 and HeLa cells. In addition to its nuclear localization, the splice variant could not bind effectors of the dominant splice form such as Raf1

and Rin1, but did bind RACK1[146]. There are a number of other small G proteins whose mutant forms or splice variants are localized to the nucleus. These include Rab24[147], and Rap1A and B[148]. These mutant proteins have different subcellular distributions than their wild type forms, can associate with different proteins, and serve different cellular functions.

The importance of careful analysis of the evidence of G proteins in the nucleus is highlighted by the example of K-Ras4B. Expression of H-Ras4B in the nucleus of normal human dermal fibroblasts (NHDF), human liver carcinoma cells (HEPG2), Chang, and HEK 293 cells was reported using fluorescent microscopy and cell fractionation followed by western blot[149]. The reactivity of the α-K-Ras4B antibody used was blocked by pre-incubation with peptide sequences specific for K-Ras4B, but not H-Ras or K-Ras4A. The expression of K-Ras4B was reported to be specific to the nucleolus, and immunoprecipitated with nucleolin[149]. Further evidence demonstrated that nucleolin/K-Ras4B complexes regulated transcriptional processes to control gene expression[149]. However, when a K-Ras4B double KO mouse was developed and the nuclear expression of K-Ras4B analysed using the same antibody, nuclear expression of K-Ras4B was still noted in the K-Ras4B-/- mouse fibroblasts[150]. This demonstrated a cross-reactivity of this antibody, and highlights the importance of using multiple techniques when detecting nuclear localization of G proteins.

1.9.2 *RGK proteins regulate voltage-dependent calcium channel signalling*

Another subfamily of Ras small GTPases has been found in the nucleus of cells, the RGK proteins (Kir/Gem, Rad, Rem and Rem2)[151]. One of their most important physiological functions is regulation of voltage-dependent calcium channels (VDCC)[152]. In addition to a plasma membrane targeting signal located in the C-terminus, these small GTPase possess three different nuclear localisation signals[151,152] and can interact with multiple proteins to regulate their subcellular localization[153]. Shuttling of RGK proteins between cytoplasm and nucleus is controlled by phosphorylation of two serine residues that drive interactions with 14-

3-3 proteins and calmodulin (CaM) (see [154] for review). Depending on the RGK protein, CaM and 14-3-3 differentially regulate their localization to the nucleus[153,155]. In the absence of 14-3-3 and CaM, RGK proteins interact with importin α5 leading to a direct accumulation of RGK in the nucleus[153,156].

The roles of RGK proteins in the nucleus are not well understood yet. Mutant Rem2 and Kir/Gem proteins that cannot bind 14-3-3 or CaM show nuclear localization in different cell types including HeLa, Neuro2A and HEK 293. Both Rem2 and Kir/Gem bind the $Ca_V\beta$ subunit of the VDCC and down regulate its activity, a function that is independent of binding to CaM and 14-3-3[152,155,157]. The expression of wild type Rem2 or mutant proteins blocked cell surface transport of $Ca_V\alpha$ by $Ca_V\beta$. Disruption of the interaction between $Ca_V\alpha1$ and $Ca_V\beta$ subunits decreases the trafficking of VDCC to the membrane[155]. It has been proposed that nuclear RGK-dependent sequestration of $Ca_V\beta$ subunits leads to a reduction in VDCC currents as a result of reduced trafficking to the cell surface, although this hypothesis is still controversial[151-153,155]. The cellular function of nuclear accumulation of the RGK/$Ca_V\beta$ subunit complex may also be involved in regulating gene expression. It has been demonstrated that $Ca_V\beta$ subunits can regulate transcription when localized to the nucleus[71,158,159]. Due to their nuclear localization with $Ca_V\beta$, RGK may be involved in this process. Another possibility is that the presence of RGK proteins in cell nuclei is related to the regulation of its GTPase activity[160]. However, at this point the biological function of nuclear localization of RGK proteins remains obscure.

1.10 *Monomeric G proteins in the nucleus: Getting to the site of action*

1.10.1 *Nucleocytoplasmic Shuttling*

The double nuclear envelope ensures tight control over what enters and exits the nucleus. Proteins are imported and exported through the central channel of the nuclear pore through interactions with the monomeric G protein Ran and nuclear import and export proteins, called karyopherins[161,162] (**Figure 5**). Proteins that are found at the inner nuclear membrane can also enter the nucleus through the

peripheral channel of the nuclear pore, by vesicle-mediated transport, and by diffusion from the outer nuclear membrane[163] (**Figure 5**). Proteins transported through the central channel of the nuclear pore, bind karyopherin proteins via a nuclear localization signal (NLS) for import, or a nuclear export signal (NES) for export[164].

1.10.2 *Ran shuttling system*

One of the best-described pathways of nucleocytoplasmic shuttling involves Ran, a monomeric GTP binding protein, which binds karyopherins and aids in the transport of proteins in and out of the nucleus. Karyopherins bind cargo proteins with a NLS or NES and transport them through the nuclear pore via interactions with NPC components, nucleoporins. In the nucleus, export karyopherins, with bound cargo, bind Ran-GTP and are chaperoned out of the nucleus through the nuclear pore. Following hydrolysis of GTP to GDP via Ran binding protein (RanBP1/2), Ran-GDP bound exportins and their cargo proteins are released[165]. Ran-GDP is transported back into the nucleus through its association with NTF2[166-168]. Once back in the nucleus, Ran-GDP is converted to Ran-GTP by the guanine nucleotide exchange factor, regulator of chromatin condensation (RCC1), causing release of cargo proteins (**Figure 5**)[165]. This process has been reviewed in more detail elsewhere[164-167].

Although there is some evidence identifying particular pathways that G proteins use to gain entry into the nucleus, much is still unknown. To enter the nucleus, most proteins use the Ran system of entry through the nuclear pore. In order to be transported through the nuclear pore they require a NLS. Interestingly, many GPCRs possess a canonical or non-canonical NLS, including the bradykinin receptor[169], the α_1-adrenergic receptor[170], and the gonadotropin hormone releasing receptor[171].

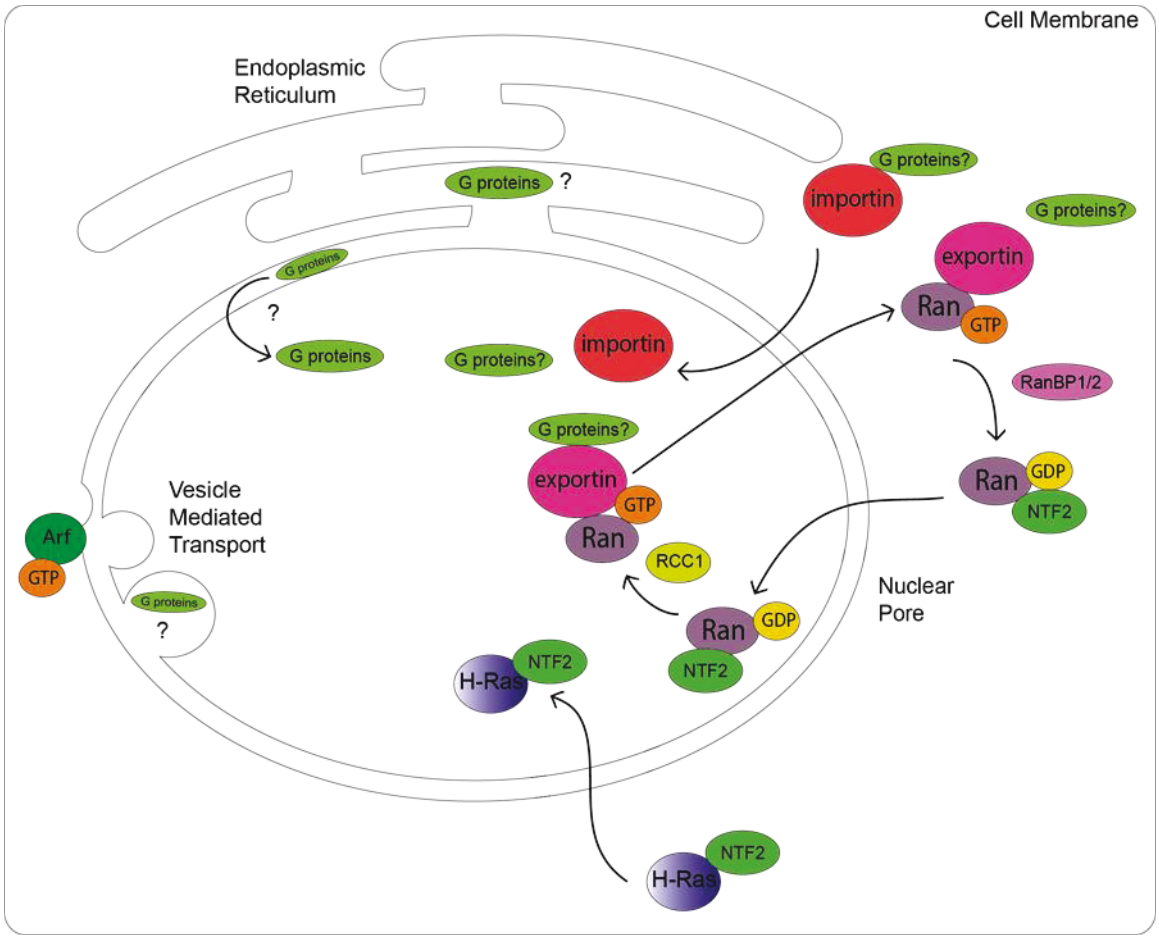


Figure 5: *Import and export pathways of G proteins in and out of the nucleus include nuclear pores and vesicle mediated transport.* How G proteins, aside from Ran, enter and exits the nucleus has not yet been determined, however interactions with karyopherins and other import/export machinery suggest routes of entry and exit. Ran shuttles other proteins in and out of the nucleus with importins and exportins. It is imported back into the nuclear via nuclear transport factor 2 (NTF2). NTF2 can also bind oncogenic H-Ras to import it into the nucleus. G proteins may be entering via the central or peripheral channel of the nuclear pore, or by vesicle mediated transport. The small G protein Arf may inhibit vesicle fusion.

To date, the G proteins Arl 4-7[140], Rem, Kir/Gem and Rad have identified NLS[151,153]. It has been proposed that these proteins either function like Ran, to shuttle proteins in and out of the nucleus, or they use this system to enter the nucleus and have functions, yet unverified, within the nucleus and nucleoli (**Figure 5**). ARL4 and 5 were found to interact with importin α following a yeast two-hybrid screen and confirmed by GST-pull down assays[122,141]. Rem associates with importin α5, Rad binds importins β, α3[151] and α5 and Kir/Gem binds importin α5[156]. It is also possible that the other proteins, such as Gβ and Gα that localize to the nucleus either have as yet unidentified NLS variants, or they bind to proteins with an NLS and enter the nucleus through the nuclear pore. For example, RhoA binds Net1 and Ect2 which both have an identified NLS[172].

Another likely possibility is that GPCR/Gαβγ complexes are trafficked to the nucleus during nuclear envelope assembly directly from the ER. Free G proteins could also enter the nucleus is through the same route of entry used by Ran (**Figure 5**). Ran binds NTF2, which targets Ran to the NPC, and allows its transport to the nuclear side[168] (**Figure 5**). Oncogenic H-Ras can bind NTF2, and may use this pathway to gain entry into the nucleus[145]. Although few of the G proteins found in the nucleus have identified routes of entry, it is likely that many of them use the Ran/NPC system, since some G proteins have been identified as binding to importins[122,141,151,156]. Mass spectrometry data from our own laboratory indicates that the Gβγ complex may interact with karyopherins (see subsequent chapters).

1.10.3 *Vesicle-mediated transport and nuclear envelope formation*

Vesicle-mediated transport may be another route for protein entry into the nucleus[173]. There is evidence that both Ran and the ADP-ribosylation factor (Arf), are involved in vesicle-mediated transport[174,175] (**Figure 5**). Ran is implicated in the formation of the nuclear pore, the nuclear envelope and vesicle-mediated transport, while Arf is involved in vesicle fusion with the nuclear envelope. Vesicle-mediated transport mediated by Ran is related to its role in nuclear envelope formation[176]. Arf is involved in vesicle formation for transport of proteins between

the rough endoplasmic reticulum and the Golgi apparatus[177]. There is evidence to suggest that Arf is involved in a similar process of vesicle fusion at the cell nucleus. At the nucleus, Arf inhibits vesicle fusion in the presence of the hydrolysis-resistant GTP analogue, GTPγS[175,178]. This inhibition is dependent on the myristoylation of Arf[174,178]. Brefeldin A, a compound that blocks coat protein I (COPI)-mediated vesicle transport (see Pinot *et. al.* for review)[179], was also able to prevent binding of endogenous Arf to nuclear membranes, indicating the involvement of Arf in vesicle fusion that is similar to that seen at the Golgi. This further suggests that Arf associates with the nuclear envelope in a way similar to vesicle transport between the endoplasmic reticulum and the Golgi apparatus[177,178].

Rab24 in the Rab family of small G proteins does not have a defined function like most of the other Rab proteins. A mutation at position 123 leads to accumulation of the Rab24 in nuclear inclusion bodies, which disrupt the nuclear envelope over time in HEK 293 and NIH 3T3 cells. This data lead Maltese *et al.* to suggest the Rab24 is normally involved in nuclear protein trafficking or in the assembly and maintenance of the nuclear envelope[147].

To date, Ran is the only protein that has a confirmed function in nucleocytoplasmic shuttling. However, many other G proteins use these systems to gain entry into the nucleus.

1.10.4 *Prenylation as a means to localize Gβγ subunits or G protein heterotrimers*

Another factor in the localization of G proteins to the nucleus is their post-translational modifications. Out of the G protein subtypes found in the nucleus, Gγ is prenylated[180,181], Gα can by myristoylated or palmitoylated[37], H-Ras is farnesylated[182], and RhoA is prenylated[172]. Disrupting these post-translational modifications can disrupt G protein signalling and localization[183,184]. Gγ5 and Gγ2 have both been localized to the nucleus in 3T3-LI, and HCT116 cells respectively. Gγ7, in contrast, is mainly localized to the cell membrane[128]. This difference in Gγ subunit expression patterns is not observed in NIH 3T3 cells, suggesting that

nuclear localization may also be cell type-specific leading to unique physiological outputs. Following prenylation of Gγ5, there is no protein cleavage as with other Gγ subunits, leading to the hypothesis that distinct post-translational processing events lead to alternative targeting of Gγ5 under specific physiological conditions[35,128]. In HCT116 cells, Kino *et al.* concluded that the nonprenylated Gγ2 preferentially localized to the nucleus compared with the prenylated form. Further, this group reported that blocking prenylation using lovastatin resulted in a shift in Gβ2γ2 from the nucleus to the cytoplasm[185]. This is in contrast to other data from studies on Gγ2[128], H-Ras[143,145], and RhoA[172]. H-Ras is found in the nuclei of primary fibroblasts, NIH 3T3 cells and L cells. When cells were treated with a farnesyltransferase inhibitor that blocked addition of a farnesyl group, H-Ras levels in the nucleus were decreased in transformed oncogenic NIH 3T3 cells, suggesting that Ras requires membrane anchoring for proper localization[143,145,146]. In addition, treatment with HEK 293 cells with lovatostatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, prevented nuclear accumulation of RhoA[172].

1.11 G proteins in DNA repair, replication and mitosis

G proteins have been implicated in both DNA replication and mitosis. Ran is involved with nuclear envelope formation, spindle formation[176,186], and DNA synthesis[187], Gαi in the regulation of cell division following S phase[120,188,189], and H-Ras moves in and out of the nucleus dependent on cell cycle phase[143]. The involvement of Ran in nuclear envelope formation, spindle formation and DNA synthesis have been described elsewhere[175,176,186].

As indicated above, Gαi has been implicated in cell division by evidence that it binds to kinetochores during mitosis. Cells can be stimulated to undergo mitosis by serum treatment, leading to DNA synthesis and mitosis with a corresponding increase of Gαi and Gβ in the nucleus through activation of receptors such as GPCRs and receptor tyrosine kinase receptors (RTKs)[120]. These receptors include the GPCRs for thrombin, bombesin, and lysophosphatidic

acid, and the epidermal growth factor RTK[120,188,189]. In *C. elegans,* heterotrimeric G proteins are required for embryonic development, as the Gα subunits, GOA-1 and GPA-16, Gβ GPB-1 and Gγ GPC-2 control spindle orientation and positioning events during early embryonic stages[190]. Mutant *gpb-1* embryos have a highly disorganized tissue distribution and fail to hatch. GPB-1 and GPC-2 function as negative regulators of the activity of GOA-1 and GPA-16, as spindle-positioning defects in *gpb-1* depleted embryos are rescued by co-depletion of these two Gα subunits[190-192].

Mitosis and cell proliferation can also be stimulated by activation of protein kinase C with the phorbol ester phorbol dibutyrate (PdBu), and inhibited by pertussis toxin, which inactivates Gαi/o. This suggests a role for nuclear Gαi in cell division. Immunofluorescence indicated that movement of Gαi into the nucleus corresponded with a decrease at the cell membrane, leading to the conclusion that Gαi partially translocates from the cell membrane to the nucleus[120,188]. The analysis also established that Gαi associates with chromatin during metaphase, anaphase and the beginning of telophase, and more specifically, that Gαi binds to kinetochores. Although cell division could be blocked with pertussis toxin, the association of Gαi with kinetochores could not, suggesting that the GTPase activity of Gαi is important for cells to undergo mitosis, but not for its association with chromatin. Further analysis of cell cycle progression revealed that the cyclin $p34^{cdc2}$, which is involved with cyclin B in the progression of mitosis, has depleted kinase activity following treatment of pertussis toxin. This indicates that Gαi likely binds to kinetochores in the nucleus and contributes to the activity of cyclins that help the cell progress from G2 to M phases of the cell cycle. Some G proteins may move in and out of the nucleus depending on cell cycle, however the only clear evidence of a G protein involved with the progression of cell cycle is from Gαi[189].

Finally, a monomeric GTPase known to regulate the actin cytoskeleton in the formation of stress fibres, RhoA, has been detected in small amounts in the HEK 293 and HeLa cell nuclei. It was discovered that a pool of RhoA is independent of the cytosolic pool, and can be activated with the Rho-A specific GEF, Net1 in isolated nuclei by ionizing radiation. The nuclear localization of this

Ras family member depends on its prenylation and by the expression level of Rho GDP-dissociation inhibitor 1, which may retain RhoA in the cytosol. Therefore, the nuclear pool of RhoA and Net1 appear to be important in transmitting DNA damage signals following ionization radiation[172,193].

1.12 Summary

The connections between nuclear GPCRs and nuclear G proteins (which may function as independent proteins regulated by mechanisms distinct from receptors *per se*) or between cell surface and nuclear receptors remain to be disentangled. It is clear that G proteins may impact nuclear function downstream of both cell surface or nuclear-localized GPCRs. We still need to understand how the two arms of nuclear signalling (receptor-dependent and –independent) might be intertwined with each other and with cell surface signalling. Interestingly, stimulation of endogenous cell surface muscarinic receptors, but not βAR in HEK 293 cells, results in accumulation of Gβγ in the nucleus (Sarah Gora, Shahriar Khan and Terry Hébert, unpublished data). Further, we have noted that stimulation with treatment with phorbol myristate acetate (PMA) alters the pattern of Gβγ occupation of several candidate promoter regions in a ChIP experiment using TAP-tagged Gβ (Sarah Gora, Shahriar Khan and Terry Hébert, unpublished data). This suggests that there is crosstalk between signalling events in the different subcellular locations and highlights again the need to understand nuclear G protein- and GPCR-controlled gene expression networks before proceeding further.

2 Thesis Objectives and Rationale

In my thesis, I endeavour to characterize the binding partners of Gβγ in the nucleus using affinity purification followed by protein identification through mass spectrometry (MS). In addition, my project contributes to the long-term goal of understanding the signalling contribution made by Gβγ in the nucleus. The specific aims of this thesis are: 1) to identify endogenous binding partners of Gβγ in the nucleus under control conditions and following stimulation with carbachol, and 2) to determine whether Gβγ localization is affected by post-translational processing of Gγ.

The aims of the study have been achieved through the following approaches:

1) Tandem affinity purification (TAP) and FLAG-affinity purification were used on isolated nuclei to identify Gβγ interactors in the nucleus. In this approach I used four different constructs: TAP-Gβ1, TAP-Gβ1γ7, TAP empty vector, and Flag-Gβ1. TAP was used to identify interactors with Gβ1 and Gβ1γ7 in the nucleus and cytosol. Due to the large size of the TAP tag, I also used Flag-Gβ1 to provide an alternative method of protein identification. The Flag protocol also offers the benefit of using a single tag, therefore it is more likely to preserve weak interactions.

2) Confocal imaging and nuclear isolation followed by western blot were used to help understand the precise location of Gβγ within the cell, and whether this localization is affected by altered processing of Gβγ. The prenylation mutant Gγ C68S was used to assess the localization of Gβγ when Gγ was not prenylated.

3) Pharmacological approaches were used to block nuclear export, and activate or inactive specific GPCR signalling pathways to determine Gβγ localization under different conditions. We also determined whether the nuclear binding partners of Gβγ were altered under different conditions such as serum deprivation and in the absence of Gα proteins.

3 Optimizing tandem affinity purification for use with nuclear lysates

This chapter outlines the tandem affinity purification protocol developed for use with nuclear lysates and is adapted from the manuscript "Tandem affinity purification to identify cytosolic and nuclear Gβγ interacting proteins" written by Rhiannon Campden and Terry Hébert[194]. I optimized the protocol and wrote 90% of the manuscript. I also generated 60% of the results that are presented and optimized the protocol used. Darlaine Pétrin generated the other portion of the results and Mélanie Robitaille ran all the MS samples. The other contributing authors were involved in the optimization of the protocol. Terry Hébert edited the manuscript.

3.1 Summary

Protein affinity purification is a common method to identify and confirm protein interactions. When used in conjugation with mass spectrometry it can be used to identify novel protein interactions with a given bait protein. The tandem affinity purification (TAP) technique identifies partner proteins bound to tagged protein bait. Combined with protocols to enrich the nuclear fraction of whole cell lysate through sucrose cushions, TAP allows for purification of interacting proteins found specifically in the nucleus. It has become clear in recent years that the Gβγ subunits of heterotrimeric proteins serve broad roles in the regulation of cellular activity and interact with many proteins in different subcellular locations including the nucleus. This manuscript details the steps of the TAP technique on cytosolic and nuclear lysates to identify candidate proteins, through mass spectrometry, that bind to Gβ1 subunits.

3.2 Introduction

The TAP protocol was first developed by Rigaut *et al*. for use in yeast, but has since been used for identifying protein interactions in numerous mammalian cell

types[195]. The technique was developed to identify multimeric protein complexes under mild, physiological conditions in the context of heterologous expression. Toward this aim, high affinity protein tags are attached to a bait protein, and stable cell lines can be created[196]. As initially developed, TAP used the binding domains *Staphylococcus aureus* Protein A (ProtA) and calmodulin binding domains (CBD), separated by a tobacco etch virus (TEV) protease cleavage site, which resulted in efficient recovery of bait proteins. The tag could be attached to either the N- or C-termini of the bait protein[197]. The bait protein and bound protein interactors are selected through affinity for the IgG ProtA and calmodulin beads and tandem mass spectrometry was initially used to identify proteins in eluates[196]. The technique has since been modified for use in mammalian cell lines such as human embryonic kidney cells (HEK 293)[197,198].

In addition to the ProtA and CBD tags there are also small peptide tags such as GST (glutathione-S transferase) and MBP (maltose binding protein), small peptide tags such as CBD (choline binding domain), methionine, FLAG, and streptavidin binding domains (SBD), and combination tags such as choline-binding histidine and nanoscale gold particle tags[199-211]. The choice of these tags depends on the protein being isolated, the conditions required for binding and elution of the bait protein, whether or not the tags will disrupt protein binding, and the conditions required for affinity purification[207]. These tags are used with corresponding beads including streptavidin Sepharose beads and magnetic gold particle beads, broadening the repertoire for TAP[199-202]. An overview of these tags and their corresponding affinities has been reported elsewhere[202,207].

The TAP protocol can also be adapted to engineer half of a TAP tag onto two different proteins, allowing for identification of protein complexes that require two separate proteins, known as bimolecular-TAP[202]. In addition, the TAP protocol can be used for identification of proteins from specific cellular compartments and organelles[212]. Prior to application of the TAP protocol, specific cell fractions of total lysates from cells expressing bait proteins can be isolated by differential centrifugation. Previous work has described the use of TAP with cytoplasmic, nuclear, and chromosomal fractions[212].

The TAP protocol has previously been used to identify protein interactions with the G protein dimer βγ in whole cell lysates from HEK 293 cells[198]. One of the important advantages of TAP is that eluates can be directly applied for mass spectrometry experiments without having to run 1- or 2D gels to isolate bands of interest. We have also adapted this technique for direct injection of TAP-tagged GPCR eluates, allowing us to understand their signalling networks from the perspective of receptors or G proteins (see below)[195]. Recent evidence shows that Gβγ interacts with a number of different proteins in the nucleus (reviewed by *Khan et al.*)[21]. Functional expression of GPCRs, G proteins, and their associated signalling machinery have been detected on the nuclear membranes or in nuclei in various cell types (reviewed elsewhere)[21,79,80,120]. Several lines of evidence have shown functional interactions of Gβγ in the nucleus with transcription factors. $G\beta_1\gamma_2$ was found to interact with histone deacetylase 5 (HDAC5) to prevent its repression of muscle differentiation factor (MEF2C)-sensitive genes[125]. We showed that Gβγ decreased phorbol 12-myristate 13-acetate (PMA)-stimulated activating protein (AP-1) reporter gene activity in different cell lines[124]. These effects were dependent on Gβγ interactions with members of AP-1 transcription factor complex and HDACs, acting specifically in the nucleus. $G\gamma_5$ can also prevent the repression of adipocyte enhancer binding protein (AEBP1)-sensitive genes by binding to AEBP1 and preventing its interaction with DNA[128]. $G\gamma_5$ and AEBP1 are both localized to the nucleus; however the authors did not show whether this interaction occurs specifically in the nucleus.

The extent of such nuclear G protein networks remains to be understood. Functions of distinct Gβγ isoforms in the nucleus remain largely unknown. As an initial screen to better understand nuclear Gβγ signalling networks in the nucleus, we have adapted the TAP protocol for use with nuclear lysates using TAP-tagged $G\beta_1$ subunits. This technique involves isolation of nuclei from HEK 293 cells through a sucrose cushion, lysing nuclei, binding tagged $G\beta_1$ lysates to and eluting them from streptavidin Sepharose beads, followed by binding to and elution from calmodulin Sepharose beads. Samples can then be processed for western blotting or mass spectrometry. Problems encountered in focusing on the nuclear fraction

include isolating sufficient protein, contaminating DNA in samples, and defining appropriate buffers for compatibility with mass spectrometry.

Another aspect to consider in defining the function of Gβγ is the number of possible Gβγ isoforms. There are 5 Gβ and 12 Gγ subunits that comprise different dimers expressed in different cell types[21]. For this reason, we have also adopted a split-tag approach, placing half of the TAP tag onto individual Gβ and Gγ subunits. Following tandem affinity purification, this allows identification of proteins that bind to specific Gβγ dimer pairs. These constructs can be used on nuclear isolates as well as on whole cell lysates to identify isoform-specific Gβγ interactions.

The following method describes the use of the TAP tag technique on nuclear lysates followed by liquid chromatography/mass spectroscopy (LC/MS) for protein identification. The technique presents particular challenges in the steps of nuclear isolation, tandem affinity purification, and mass spectrometry, which will be discussed in detail in the text below.

3.3 Materials

Prepare all solutions using ultrapure Milli-Q water. Particular steps need to be taken to limit contaminants in samples for MS. Contamination can occur from various sources, from skin to plasticware. Any glassware used must be washed thoroughly in Milli-Q water. Keratin contamination in dust is largely from human origin[213]. To reduce this, wear gloves, lab coats, and tie back long hair at all times. Other precautions include ensuring a clean environment, such as working in a safety cabinet or at minimum, carefully cleaning your workspace. Choose plastic containers and pipette tips that are compatible with MS, and avoid using plastic ware to store acids and bases used in the protocol. All plastic ware used must be new. Further contamination can result from autoclaving solutions[213]. If solutions must be sterilized, use 0.22μm filters.

3.3.1 *Cell Culture*

1. Tissue culture dishes (T175).
2. Tissue culture hood.
3. Incubator: 37°C, 5% CO_2.
4. Dulbecco's modified Eagle's medium (DMEM) high glucose supplemented with fetal bovine calf serum (FBS) (10% final concentration) and penicillin/streptomycin (P/S) (100 U/mL final concentration).
5. Puromycin (see Note 1).
6. Hygromycin.
7. Lipofectamine 2000.
8. TAP-tagged vector pIRESpuro-GLUE with HA-$G\beta_1$ inserted: human Gβ was cloned by PCR using EcoRI and BamHI into the pIRESpuro-GLUE-N1 vector, described elsewhere[195,203]. Split-TAP tagging of Gβ1, Gβ2 with the streptavidin binding domain plus an HA tag, and Gγ2 or Gγ7 with calmodulin binding domain plus a Flag tag allows purification of complexes specifically associated with unique Gβγ combinations.
9. TAP-tagged vector pIRESpuro-GLUE-N1[195,203].
10. Human embryonic kidney 293 cells (HEK 293T).

3.3.2 *Isolation of Nuclei*

1. PBS: Prepare a 10× stock of PBS: 1.37M NaCl, 27mM KCl, 100mM Na_2HPO_4, 18mM KH_2PO_4. Place a 1L graduated cylinder or glass beaker on a stir plate; place a stir bar inside with approximately 500mL of water. Weigh out 80g NaCl, 2g KCl, 14.4g Na_2PO_4, 2.4g KH_2PO_4. Cover beaker or cylinder with Parafilm to prevent contamination while mixing. Mix until dissolved. Add distilled water up to 1L. Store at room temperature.
2. EDTA: Prepare 0.5M EDTA pH 8.0. Add 18.61g of disodium EDTA $2H_2O$ to 80 mL of water in a beaker or graduated cylinder on a stir plate. While stirring, add

~2g of NaOH pellets (see Note 2). Adjust to 100mL with H_2O. Store at room temperature.

3. EGTA: Prepare 0.2M EGTA pH 7.5. Add 7.61g of EGTA to 80mL of sterile water on a stir plate. Adjust pH to 7.5 using NaOH pellets. Adjust to 100mL with H_2O. Store at room temperature.

4. PBS plus EGTA and EDTA. For 200mL, add 20mL of 10× PBS to 170mL of sterile water. Add 800µL of 0.5M EDTA pH 8.0 for a final concentration of 2mM. Add 2mL of EGTA pH 7.5 for a final concentration of 2mM. Adjust volume to 200mL. This solution should be made fresh, but can be stored at 4 °C for a few days.

5. 2M $MgCl_2$: For 50mL, add 20.33g to 20mL water. Mix until dissolved and then adjust volume to 50mL. Store at room temperature.

6. 1M HEPES-NaOH pH 8.0: For 50mL, add 11.92g of HEPES to 30mL of water. Mix until dissolved. Check pH and add NaOH pellets until pH 8.0 is reached. Adjust volume to 50mL with water. Store at room temperature.

7. 1M DTT: For 10mL, add 1.5g of DTT to 8mL of water. Adjust to 10mL. Aliquot and freeze at −20 °C.

8. 100mM PMSF: For 10mL, add 174mg of PMSF to 9mL of isopropanol. Adjust to 10mL. Aliquot and freeze at −20 °C (see Note 3).

9. 200mM Na_3VO_4: For 10mL add 0.368g to 9mL of water. Check pH and adjust to 10.0 using 1M NaOH or 1M HCl. Boil for 10 min or until solution clears. Cool on ice and adjust pH again. Boil the solution again and recheck pH after cooling. Repeat, adjusting pH as necessary, until pH stabilizes at 10.0. Aliquot and store at −20 °C.

10. Lysis Buffer: 320mM sucrose, 10mM HEPES-NaOH, 5mM $MgCl_2$, 1mM DTT, 1mM PMSF, 1mM Na_3VO_4, 1% Triton X-100. For a 70mL solution, weigh out 7.667g of sucrose and add to 30mL of water. Mix using a stir bar. Add 700µL 1M HEPES-NaOH, 175µL 2M $MgCl_2$, 70µL 1M DTT, 175µL PMSF, 700µL Na_3VO_4, 700µL 100% Triton X-100. Adjust volume to 70mL. Prepare fresh, just prior to use (see Note 4).

11. High-sucrose buffer: 1.8M sucrose, 10mM HEPES-NaOH, 5mM $MgCl_2$, 1mM DTT, 1mM PMSF, 1mM Na_3VO_4, 1% Triton X-100. Weigh out 43.127g of sucrose. Prepare as stated above, heat solution to dissolve sucrose. Prepare fresh, just prior to use.
12. Re-suspension buffer: 320mM sucrose, 10mM HEPES-NaOH, 5mM $MgCl_2$, 1mM DTT, 1mM PMSF, 1mM Na_3VO_4, 1% Triton X-100, 1× Protease Inhibitor. For a 20mL solution, weigh out 1.919 g of sucrose and add to 10mL of water. Prepare as stated above. Prepare fresh, just prior to use.

3.3.3 *Nuclear Lysis*

1. 1M HEPES-KOH pH 8.0: For 50mL, add 11.92g of HEPES to 30mL of water. Mix until dissolved. Check pH and add KOH pellets until pH 8.0 is reached. Adjust volume to 50mL with water. Store at room temperature.
2. 1M KCl: For 50mL, add 3.73g of KCl to 40mL water. Mix until dissolved. Adjust volume to 50mL. Store at room temperature.
3. TAP lysis buffer: 1mM $MgCl_2$, 10% glycerol, 50mM HEPES-KOH pH 8.0, 100mM KCl, 0.1% NP-40, 0.2mM EDTA, 2mM DTT, 1x Protease Inhibitor. For 50mL combine 25µL 2 M $MgCl_2$, 5mL glycerol, 2.5mL 1M HEPES-KOH, 5mL 1 M KCl, 71.43µL 70% NP-40, 50µL 0.2M EDTA, 100µL 1M DTT, 50µL 1,000× Protease Inhibitor to 10mL of water. Adjust to 50mL. Prepare fresh.
4. Dounce tissue homogenizer, 2mL capacity.
5. Benzonase® Nuclease (Sigma-Aldrich, Saint Louis, MO, USA).

3.3.4 *Tandem Affinity Purification*

1. Streptavidin Sepharose™ High Performance beads (GE Healthcare).
2. Calmodulin Sepharose™ 4B beads (GE Healthcare).
3. 5M NaCl: For50 mL, add 14.61g of NaCl to 25mL of water. Mix until dissolved. Adjust volume to 50mL. Store at room temperature.
4. AcTEV™ (Tobacco Etch Virus) Protease (Invitrogen Life Technologies,

Carlsbad, CA, USA).

5. TEV buffer: 150mM NaCl, 10mM HEPES-KOH pH 8.0, 0.5mM EDTA, 0.1% NP-40, and 1mM DTT. For 50mL combine 1.5mL 5M NaCl, 500µL HEPES-KOH, 125µL 0.2M EDTA, 71.43µL 70% NP-40, 50µL 1M DTT to 10mL water. Adjust to 50mL. Prepare fresh.

6. 1M MgOAc: For 25mL add 5.381g of MgOAc to 15mL of water. Adjust to 25mL. Store at room temperature.

7. 1M imidazole: For 10mL add 0.6808g of imidazole to 5mL of water. Adjust to 10mL. Can be stored at 4 °C protected from light for up 1 year.

8. 2M $CaCl_2$: For 50mL, add 7.351g of $CaCl_2$ to 30mL of water. Adjust to 50mL. Store at room temperature.

9. Calmodulin binding buffer: 10mM HEPES-KOH pH 8.0, 150mM NaCl, 1mM MgOAc, 1mM imidazole, 0.1% NP-40, 2mM $CaCl_2$, 10mM β-mercaptoethanol. For 50mL add 500µL 1M HEPES-KOH, 1.5mL 5M NaCl, 50µL 1M MgOAc 50µL 1M imidazole, 71.43µL 70% NP-40, 100µL 2M $CaCl_2$, 39µL 14M β-mercaptoethanol to 30mL water. Adjust to 50mL. Prepare fresh.

10. 1M ammonium bicarbonate pH 8.0: For 25mL add 98.8mg of ammonium bicarbonate to 20mL of water. Adjust to 25mL with water (see Note 5). Prepare fresh.

11. Calmodulin rinsing buffer: 50mM ammonium bicarbonate, 75mM NaCl, 1mM MgOAc, 1mM imidazole, 2mM $CaCl_2$. For 25mL add 1,250µL 1M ammonium bicarbonate, 375µL 5M NaCl, 25µL 1M MgOAc, 25µL 1M imidazole, 50µL 2M $CaCl_2$ to 15mL water. Adjust to 25mL. Prepare fresh.

12. 0.5M ammonium hydroxide: For 25mL, add 0.86mL of 14.534M stock solution to 6.25mL of water. Adjust to 25mL with water. Prepare fresh.

13. Monoclonal antibody, mouse anti-HA.11, clone 16B12 (Covance, Montreal, QC, CA).

14. Nucleoporin p62 antibody, mouse, clone 53 (BD Transduction Laboratories™).

15. $G\beta_{1-4}$ antibody (T-20), rabbit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

3.4 Methods

All steps must be carried out on ice or at 4 °C unless otherwise indicated (see Note 6). Centrifugation steps must be performed at 4 °C. Samples (of approximately 50μL) are taken at various stages of the protocol to ensure the bait protein binds and elutes from the beads. This is critical to determining whether the protocol has worked.

3.4.1 *Tissue Culture*

1. Grow 10 T175 flasks of HEK 293 cells to 40% confluency in Dulbecco's MEM supplemented with FBS and P/S (see Note 7).
2. Transfect cells with TAP tag plasmid expressing $G\beta_1$ or the empty vector using Lipofectamine 2000 according to the manufacturer's instructions (see Note 8).
3. Harvest after 2 days as outlined below.

3.4.2 *Isolation of Nuclei*

To ensure the isolation of intact nuclei, cells are lysed using a Triton X-100 based buffer and nuclei are pelleted through a 1.8M sucrose cushion. This results in intact nuclei that can be viewed under the microscope. To confirm the purity of the nuclear fraction, we used antibodies against nucleoporin p62, a nucleoporin protein found exclusively in the nucleus, and against β-tubulin, found in the cytosol (**Figure 6**)[124]. Endogenous Gβ was detected using anti-$G\beta_{1-4}$ antibody; tagged Gβ was detected with anti-HA antibody. To increase protein yield from nuclear isolations, they were done in 50 mL conical tubes.

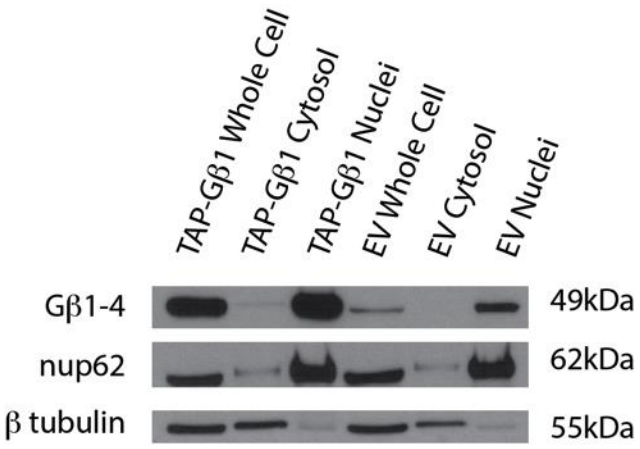


Figure 6: Western blot showing n*uclear isolation of TAP-tagged Gβ1 and empty vector (EV) transfected in HEK 293 cells*. TAP-Gβ and endogenous Gβ was detected using a pan Gβ1-4 antibody. The proteins nucleoporin 62, which is found in the nucleus, and β-tubulin, which is found in the cytosol, were detected using the antibodies Nup62 and β-tubulin antibodies respectively. This figure shows that both TAP tagged Gβ and endogenous Gβ are found in both the cytosolic and nuclear fractions of HEK 293 cells.

Due to increased protein expression in the cytosol compared with the nucleus, the cytosolic fraction was used as a positive control to confirm the success of purification, ensuring expression of the bait protein and proper binding and elution in all steps of the protocol. Endogenous Gβγ subunits are also localized to the nucleus (**Figure 7**). The optimization of the protocol for use with nuclear lysates will more clearly reveal new interactions for Gβγ that occur specifically in the nucleus.

1. Remove flasks from incubator and place on ice (see Note 9).
2. Wash cells 2× with 10mL PBS plus EGTA, EDTA (see Note 10).
3. Add another 10mL of PBS plus EGTA, EDTA and tap the side of the flask to remove cells. Pipette up and down to remove cells from the surface. Remove cells from flasks and add to new 15mL conical tubes on ice.
4. Centrifuge at 300×g for 5 min.
5. Discard supernatant.
6. Add 20mL of lysis buffer to each tube and incubate on ice for 6 min (see Note 11).
7. While cells are incubating, add 15mL of high sucrose buffer to new 50mL conical tubes.
8. After 6 min, slowly place the lysed cells on top of the sucrose cushion being very careful not to disturb the barrier (see Notes 12 and 13).
9. Centrifuge at 4,600 × g for 30 min at 4 °C (see Note 14).
10. Remove the top 18mL cytosolic layer to a new 50mL tube, being sure to collect only top layer, not the high sucrose cushion. Remove a sample for western blot. Proceed to tandem affinity purification step for cytosolic fraction or store at −80 °C (see Notes 15 and 16).
11. Slowly remove the rest of the cytosol followed by the high sucrose cushion. This can be discarded (see Note 17).
12. Re-suspend the nuclear pellet in 1mL of re-suspension buffer. Place the 1mL in a new clean Eppendorf tube. Centrifuge at 500 × g for 5 min. Remove a sample for western blot.

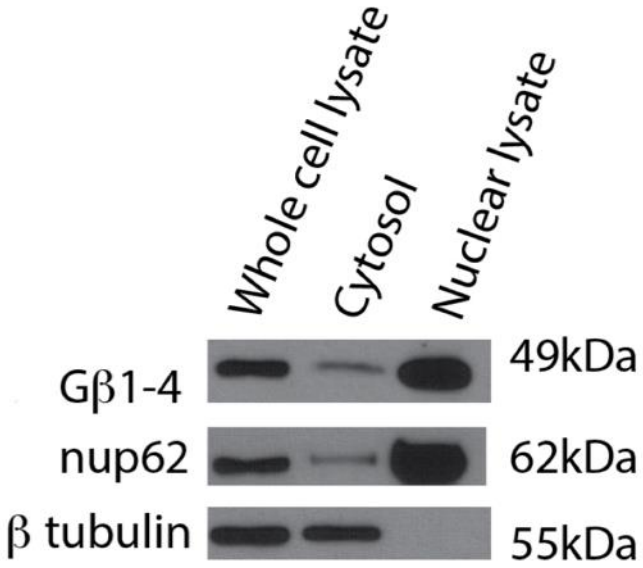


Figure 7: *Western blot of nuclear isolation of HEK 293 cells*. This figure indicates thepresence of Gβ in the whole cell, cytocolic and nuclear fractions. Gβ was detected using a pan Gβ1-4 antibody. The proteins nucleoporin 62, which is found in the nucleus, and β-tubulin, which is found in the cytosol, were detected using the antibodies Nup62 and β-tubulin antibodies respectively. This figure shows that endogenous Gβ is found in both the cytosolic and nuclear fractions of HEK 293 cells.

13. Remove the re-suspension buffer. If not immediately lysing the nuclei, re-suspend in re-suspension buffer and store at 4°C. Remove a sample for western blot.

3.4.3 *Nuclear Lysis*

1. Re-suspend all nuclei in 2mL Tap lysis buffer with phosphatase and protease inhibitors (see Note 18).
2. Transfer to Dounce homogenizer (tight fit) and subject to ten passes. Transfer to new 2mL tube (see Note 19).
3. Add 2µL of benzonase (250 U/µL) and leave on a shaker overnight (see Note 20).
4. Centrifuge lysate at 17.6g for 10 min to remove insoluble fraction (see Note 21).

3.4.4 *Tandem Affinity Purification*

The general workflow for TAP is shown in **Figure 8**. In our hands, the standard TAP protocol works for whole cell lysates (**Table 3**), pulling down a number of known Gβγ-interacting proteins. Further, we noted Gβγ-interacting proteins identified from screens in whole cell lysates with either TAP-Gβ1 or the different split-TAP pairs include proteins with known functions in the nucleus (**Table 4**) further supporting a broader role for Gβγ in the nucleus. However, the standard Gβγ TAP protocol, as described by Ahmed et al., did not work with nuclear lysates[198]. Therefore, we have made changes that are outlined below. With this adjusted protocol (see **Figure 8**, **Figure 9**), we were able to identify known interactors with $G\beta_1$ in both the cytosol and the nucleus (**Table 3**, **Table 4**, **Table 5** and **Table 6**).

1. Wash 2× 100µL of streptavidin Sepharose beads 3× with 1mL of TAP lysis buffer. Spin down beads at 200×g for 1 min.

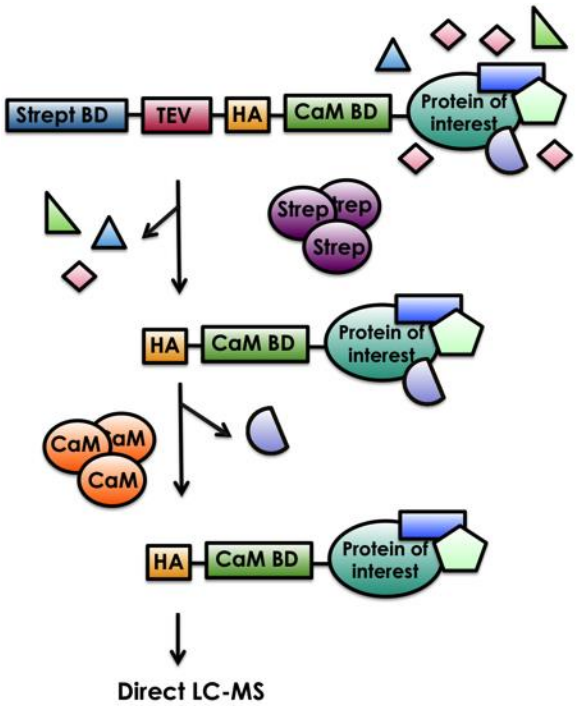


Figure 8: *Schema depicting the tandem affinity purification protocol*. A calmodulin binding domain and streptavidin binding domain are fused to the protein of interest together or in the case of split-TAP alone. These tags allow binding to streptavidin and calmodulin beads. The tobacco etch virus (TEV) site allows for release of the bait protein from the streptavidin beads. The HA tag allows for identification of the fusion protein. In some split TAP constructs, Flag tags were used instead.

Table 3: *Proteins pulled down with $G\beta_1$ from whole cell lysates.* Proteins identified as Gβγ interactors with TAP tagged $G\beta_x\gamma_x$ and identified by mass spectrometry.

Bait Protein (TAP or split-TAP)	Protein ID	Description	Unique Peptides
Gβ1γ2, Gβ1γ7, Gβ2γ7, Gβ2γ2	GNA11	Guanine nucleotide binding protein (G Protein), alpha 11 (Gq Class)	11, 2, 5, 4
Gβ1γ2, Gβ1γ7, Gβ2γ7, Gβ2γ2	GNA13	Guanine nucleotide binding protein (G protein), alpha 13	11, 4, 5, 7
Gβ1γ2, Gβ2γ7, Gβ2γ2	GNAI2	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2	3, 1, 2
Gβ1γ2, Gβ1γ7, Gβ2γ7, Gβ2γ2	GNAI3	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3	7, 3, 6, 3
Gβ1γ2, Gβ2γ2	GNAI1	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1	3, 1
Gβ1γ2, Gβ1γ7, Gβ2γ7, Gβ2γ2	GNAQ	Guanine nucleotide binding protein (G protein), q polypeptide	5, 2, 3, 6
Gβ1γ2	GNAS	Guanine nucleotide binding protein (G Protein), alpha stimulating activity polypeptide 1	5
Gβ1γ2	GNAZ	Guanine nucleotide binding protein (G protein), alpha z polypeptide	3
Gβ2γ2	GNAO1	Guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O	1
Gβ1γ2, Gβ1γ7, Gβ2γ7	GNB1	Guanine nucleotide binding protein (G protein), beta polypeptide 1	25, 18, 8
Gβ1γ2	GNB2	Guanine nucleotide binding protein (G protein), beta polypeptide 2	3
Gβ1γ7	GNB3	Guanine nucleotide binding protein (G protein), beta polypeptide 3	1

Gβ1γ2, Gβ2γ7	GNG12	Guanine nucleotide binding protein (G protein), gamma 12	4, 1
Gβ1γ2, Gβ2γ2	GNG2	Guanine nucleotide binding protein (G protein), gamma 2	7, 2
Gβ1γ2	GNG5	Guanine nucleotide binding protein (G protein), gamma 5	3
Gβ1γ2, Gβ1γ7, Gβ2γ7, Gβ2γ2	GNG7	Guanine nucleotide binding protein (G protein), gamma 7	1, 3, 2, 1
Gβ1γ2, Gβ1γ7, Gβ2γ7, Gβ2γ2	CCT2	Chaperonin containing TCP1, subunit 2 (beta)	11, 8, 4, 6
Gβ1γ2, Gβ1γ7, Gβ2γ7, Gβ2γ2	CCT3	Chaperonin containing TCP1, subunit 3 (gamma)	9, 9, 4, 4
Gβ1γ2, Gβ1γ7, Gβ2γ2	CCT4	Chaperonin containing TCP1, subunit 4 (delta)	10, 5, 2
Gβ1γ2, Gβ1γ7, Gβ2γ7, Gβ2γ2	CCT5	Chaperonin containing TCP1, subunit 5 (epsilon)	6, 6, 2, 4
Gβ1γ2, Gβ1γ7, Gβ2γ7, Gβ2γ2	CCT6A	Chaperonin containing TCP1, subunit 6A (zeta 1)	8, 9, 2, 4
Gβ1γ2, Gβ1γ7, Gβ2γ2	CCT7	Chaperonin containing TCP1, subunit 7 (eta)	10, 4, 2
Gβ1γ2, Gβ1γ7, Gβ2γ7, Gβ2γ2	CCT8	Chaperonin containing TCP1, subunit 8 (theta)	14, 7, 5, 6
Gβ1γ2, Gβ1γ7, Gβ2γ2	TCP1	T-complex 1	10, 6, 2
Gβ1γ2	KCTD2	Potassium channel tetramerization domain containing 2	9
Gβ1γ2	KCTD12	Potassium channel tetramerization domain containing 12	11
Gβ1γ2	KCTD16	Potassium channel tetramerization domain containing 16	1
Gβ1γ2	KCTD17	Potassium channel tetramerization domain containing 17	5
Gβ1γ2, Gβ1γ7, Gβ2γ7, Gβ2γ2	KCTD5	Potassium channel tetramerization domain containing 5	14, 2, 3, 3
Gβ1γ2, Gβ1γ7, Gβ2γ7, Gβ2γ2	RADIL	Ras association and DIL domains	24, 5, 10, 6
Gβ1γ2, Gβ1γ7, Gβ2γ7, Gβ2γ2	PDCL	Phosducin-like	4, 1, 7, 5

Gβ1γ2	RAP1A	Member of RAS oncogene Family	4
Gβ1γ2, Gβ2γ7, Gβ2γ2	RAP1B	Member of RAS oncogene Family	1, 1, 1

Split TAP combinations are listed as $G\beta_x\gamma_y$, single TAP constructs as $G\beta_x$ or $G\gamma_y$.

Table 4: *Nuclear proteins pulled down from various Gβ and Gγ combinations.* Proteins identified as Gβγ interactors with TAP tagged $G\beta_x\gamma_x$ and identified by mass spectrometry.

Bait Protein (TAP or split-TAP)	Protein ID	Description	Unique Peptides
Gβ1γ2	IPO7	Importin 7	2
Gβ1γ2, Gβ1, Gγ2	KPNB1	Karyopherin β1	1, 5, 1
Gγ2	EXP1	Exportin 1	1
Gγ2	EXP5	Exportin 5	1
Gβ2γ7	NPM1	Nucleophosmin 1	2
Gβ1γ2, Gγ2, Gβ2γ2, Gβ2γ7	PRKDC	Protein Kinase, DNA activated, catalytic polypeptide isoform 1	8, 50, 2, 6
Gβ1γ2	NCOA5	Nuclear receptor coactivator 5	1
Gβ2γ7, Gβ2γ2	NFKB	NF-κB	1, 1
Gγ2	HNRNH1	Heterogenous nuclear ribonucleoprotein H1	1

Split TAP combinations are listed as $G\beta_x\gamma_y$, single TAP constructs as $G\beta_x$ or $G\gamma_y$.

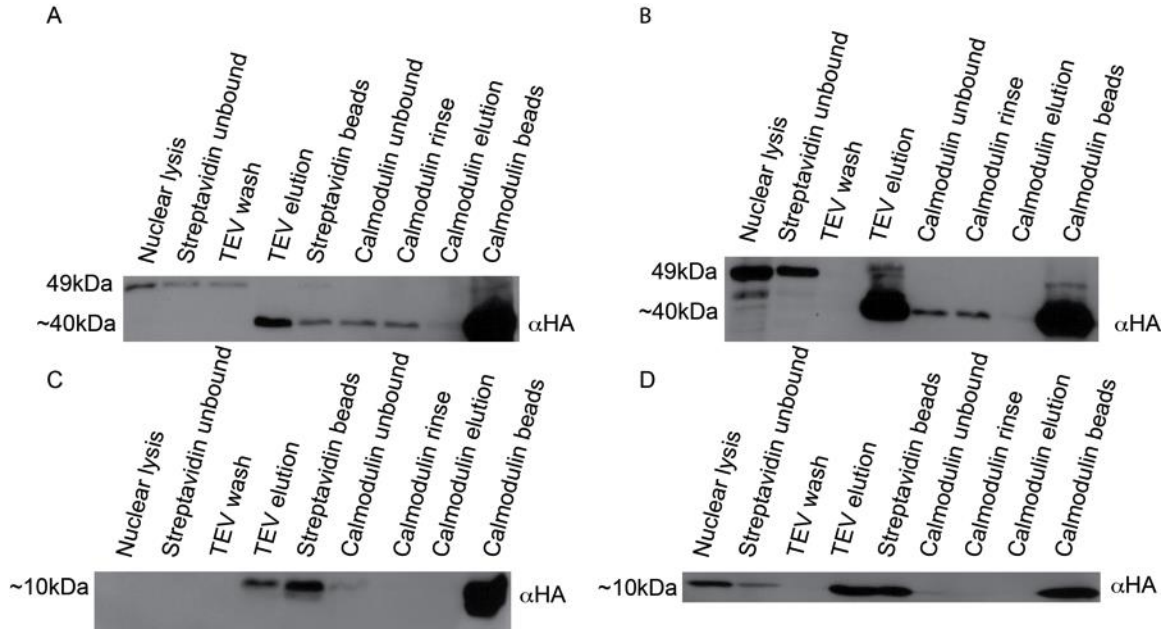


Figure 9: *TAP of Gβ1 from nuclear and cytosolic fraction.* Panel (a) shows the cytosolic fraction of HEK 293 cells transfected with the TAP-Gβ1. An HA antibody wasused to detect TAP- Gβ1 in all panels (which contains an HA epitope). Panel (b) shows the nuclear fraction of HEK 293 cells transfected with TAP-Gβ1. Panel (c) shows the cytosolic fraction of HEK 293 cells transfected with empty vector. Panel (d) shows the nuclear fraction of HEK 293 cells transfected with empty vector. Gβ1 is bound and eluted from the streptavidin and calmodulin beads and runs at 49 kDa. A 30μL sample was taken from each step of the TAP protocol and run on a gel. To determine how much sample was left on the beads following elution, the Calmodulin and Streptavidin beads were run on the gel.The HA tag from empty vector transfected cells runs at ~10 kDa and can be seen on the gel.

Table 5: *Proteins pulled down from $G\beta_1$ following nuclear isolation.* Proteins identified as Gβγ interactors with TAP tagged $G\beta_x\gamma_x$ and identified by mass spectrometry.

Bait Protein	Protein ID	Description	Unique Peptides
Gβ1γ7	HNRNPM	Heterogeneous nuclear ribonucleoprotein M	27
Gβ1γ7, Gβ1	GNB1	Guanine nucleotide binding protein (G Protein), beta polypeptide 1	26, 12
Gβ1γ7	GNB2	Guanine nucleotide binding protein (G protein), beta polypeptide 2	25
Gβ1γ7, Gβ1	GNA13	Guanine nucleotide binding protein (G protein), alpha 13	7, 2
Gβ1γ7, Gβ1	GNAS	Guanine nucleotide binding protein (G Protein), alpha stimulating activity polypeptide 1	6, 2
Gβ1γ7	GNA11	Guanine nucleotide binding protein (G protein), alpha 11 (Gq class)	5
Gβ1γ7	CKAP4	Cytoskeleton-associated protein 4	5
Gβ1γ7	RADIL	Ras association and DIL domains	4
Gβ1γ7	PHB2	Prohibitin 2	4
Gβ1γ7	GNAI3	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3	4
Gβ1	HNRNPM	Heterogeneous nuclear ribonucleoprotein M	4
Gβ1	DNAJC25-GNG10	DNAJC25-GNG10 read through transcript	4
Gβ1	ACTBL2	Actin, beta-like 2	4
Gβ1γ7, Gβ1	GNG7	Guanine nucleotide binding protein (G protein), gamma 7	3, 1

Gβ1	FBLN5	Fibulin 5	3
Gβ1γ7	KCTD12	Potassium channel tetramerization domain containing 12	3
Gβ1γ7	HNRNPF	Heterogeneous nuclear ribonucleoprotein F	3
Gβ1γ7	MATR3	Matrin 3	3
Gβ1γ7	HNRNPU	Heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)	2
Gβ1γ7	MYBBP1A	MYB binding protein (P160) 1a	2
Gβ1γ7	RBM12	RNA binding motif protein 12	2
Gβ1γ7	ATAD3B	ATPase family, AAA domain containing 3B	2
Gβ1	HIST1H1C	Histone cluster 1, H1c	2
Gβ1	ANXA5	Annexin A5	2
Gβ1	AKR7A2	Aldo-keto reductase family 7, member A2 (aflatoxin aldehyde reductase)	2
Gβ1	GNAI2	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2	2
Gβ1	FAM82B	Regulator of microtubule dynamics 1	2
Gβ1γ7	RFC1	Replication factor C (activator 1) 1, 145kDa	1
Gβ1γ7, Gβ1	HNRNPD	Heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37kDa)	1, 1
Gβ1γ7	BSG	Basigin	1

Gβ1γ7	ANXA6	Annexin A6	1
Gβ1γ7	PTPLAD1	Protein tyrosine phosphatase-like A domain containing 1	1
Gβ1γ7	RFC2	Replication factor C (activator 1) 2, 40kDa	1
Gβ1γ7	TESC	Tescalcin	1
Gβ1γ7	HIST1H2AI	Histone cluster 1, H2ai	1
Gβ1γ7	RPS27A	Ribosomal protein S27a	1
Gβ1γ7	HNRNPA3	Heterogeneous nuclear ribonucleoprotein A3	1
Gβ1γ7	PHB	Prohibitin	1
Gβ1γ7	KCTD5	Potassium Channel Tetramerization Domain Containing 5	1
Gβ1	GNG12	Guanine nucleotide binding protein (G protein), gamma 12	1
Gβ1	SPA17	Sperm autoantigenic protein 17	1
Gβ1	GNAQ	Guanine nucleotide binding protein (G protein), q polypeptide	1
Gβ1	MTERFD2	MTERF domain containing 2	1

Split TAP combinations are listed as $G\beta_x\gamma_y$, single TAP constructs as $G\beta_x$ or $G\gamma_y$.

Table 6: *Proteins pulled down from $G\beta_1$ following cytosolic isolation.* Proteins identified as Gβγ interactors with TAP tagged $G\beta_1\gamma_x$ and identified by mass spectrometry.

Bait Protein	Protein ID	Description	Unique Peptides
Gβ1, Gβ1γ7	GNAI3	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3	88, 33
Gβ1, Gβ1γ7	GNAI2	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2	77, 23
Gβ1, Gβ1γ7	GNAI1	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1	67, 24
Gβ1	CAPNS1	Calpain, small subunit 1	35
Gβ1γ7, Gβ1, Gβ1	GNB1	Guanine nucleotide binding protein (G protein), beta polypeptide 1	34, 20, 4
Gβ1	MYLK2	Myosin light chain kinase 2	32
Gβ1, Gβ1γ7	GNAS	Guanine nucleotide Binding Protein (G Protein), Alpha Stimulating Activity Polypeptide 1	30, 17
Gβ1γ7, Gβ1	GNA13	Guanine nucleotide binding protein (G protein), alpha 13	28, 28
Gβ1	GNAQ	Guanine nucleotide binding protein (G protein), q polypeptide	28
Gβ1, Gβ1γ7	GNA11	Guanine nucleotide binding protein (G protein), alpha 11 (Gq class)	28, 14
Gβ1γ7	DSP	Desmoplakin	26

Gβ1, Gβ1γ7, Gβ1	TUBB4B	Tubulin, beta 4B class IVb	26, 8, 4
Gβ1, Gβ1	PDCL	Phosducin-like	20, 5
Gβ1γ7	GNAO1	Guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O	20
Gβ1γ7, Gβ1	KCTD2	Potassium channel tetramerization domain containing 2	19, 7
Gβ1γ7, Gβ1	KCTD12	Potassium channel tetramerization domain containing 12	17, 9
Gβ1γ7	GNAQ	Guanine nucleotide binding protein (G protein), q polypeptide	15
Gβ1γ7, Gβ1	KCTD5	Potassium channel tetramerization domain containing 5	15, 5
Gβ1γ7	HNRNPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1	13
Gβ1, Gβ1γ7	GNG7	Guanine nucleotide binding protein (G protein), gamma 7	10, 2
Gβ1γ7	TUBB	Tubulin, beta class I	9
Gβ1γ7	SERPINB3	Serpin peptidase inhibitor, clade B (ovalbumin), member 3	8
Gβ1γ7	JUP	Junction plakoglobin	8
Gβ1γ7	ALB	Albumin	8
Gβ1, Gβ1γ7	GNAZ	Guanine nucleotide binding protein (G protein), alpha z polypeptide	7, 2
Gβ1	CPNE1	Copine I	7
Gβ1	TP53I11	Tumour protein p53 inducible protein 11	7

Gβ1	MYL6	Myosin, light chain 6, alkali, smooth muscle and non-muscle	7
Gβ1	HPCAL1	Hippocalcin-like 1	6
Gβ1	GNG11	Guanine nucleotide binding protein (G protein), gamma 11	6
Gβ1	IPO5	Importin 5	5
Gβ1	PDCD6	Programmed cell death 6	5
Gβ1	CCT3	Chaperonin containing TCP1, subunit 3 (gamma)	5
Gβ1γ7	DSG1	Desmoglein 1	5
Gβ1γ7	TGM3	Transglutaminase 3	5
Gβ1	ATAD3A	ATPase family, AAA domain containing 3A	4
Gβ1	PHB2	Prohibitin 2	4
Gβ1	CAND1	Cullin-associated and neddylation-dissociated 1	4
Gβ1	CPNE3	Copine III	4
Gβ1γ7	HSPA1L	Heat shock 70kDa protein 1-like	4
Gβ1γ7	FUS	Fused in sarcoma	4
Gβ1γ7	AMOT	Angiomotin	4
Gβ1γ7	CAP14	Catabolite activator gene protein	4

Gβ1γ7, Gβ1	SLC25A5	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	4, 2
Gβ1γ7	CCT5	Chaperonin containing TCP1, subunit 5 (epsilon)	4
Gβ1	PPP3R1	Protein phosphatase 3, regulatory subunit B, alpha	3
Gβ1	PTPLAD1	Protein tyrosine phosphatase-like A domain containing 1	3
Gβ1	CKAP4	Cytoskeleton-associated protein 4	3
Gβ1	ERLIN2	ER lipid raft associated 2	3
Gβ1	IQGAP1	IQ motif containing GTPase activating protein 1	3
Gβ1	CHP1	Calcineurin-like EF-hand protein 1	3
Gβ1	ST13	Suppression of tumorigenicity 13 (colon carcinoma) (Hsp70 interacting protein)	3
Gβ1	MAPK1	Mitogen-activated protein kinase 1	3
Gβ1	VPS35	Vacuolar protein sorting 35 homolog (*S. cerevisiae*)	3
Gβ1 Gβ1γ7	CCT4	Chaperonin containing TCP1, subunit 4 (delta)	3, 2
Gβ1	ANXA1	Annexin A1	3
Gβ1	ACTBL2	Actin, beta-like 2	3
Gβ1	MYL12A	Myosin, light chain 12A, regulatory, non-sarcomeric	3
Gβ1	FBLN5	Fibulin 5	3

Gβ1	CCT5	Chaperonin containing TCP1, subunit 5 (epsilon)	3
Gβ1γ7	HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1	3
Gβ1γ7	LGALS7	Lectin, galactoside-binding, soluble, 7	3
Gβ1γ7	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	3
Gβ1γ7	ANAX2	Transient receptor potential cation channel, subfamily V, member 5	3
Gβ1γ7	SERPINB12	Serpin peptidase inhibitor, clade B (ovalbumin), member 12	3
Gβ1γ7	IDE	Insulin-degrading enzyme	3
Gβ1	ATP6V1D	ATPase, H+ transporting, lysosomal 34kDa, V1 subunit D	2
Gβ1, Gβ1γ7	PHB	Prohibitin	2, 1
Gβ1	HADHA	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, alpha subunit	2
Gβ1	CAPN2	Calpain 2, (m/II) large subunit	2
Gβ1	TLN1	Talin 1	2
Gβ1	CUL3	Cullin 3	2
Gβ1	MYO6	Myosin VI	2
Gβ1	TMEM109	Transmembrane protein 109	2

Gβ1	YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	2
Gβ1	S100A4	S100 calcium binding protein A4	2
Gβ1	MYO1B	Myosin IB	2
Gβ1	SPTAN1	Spectrin, alpha, non-erythrocytic 1	2
Gβ1, Gβ1γ7	CCT6A	Chaperonin containing TCP1, subunit 6A (zeta 1)	2, 2
Gβ1, Gβ1, Gβ1γ7	GNG12	Guanine nucleotide binding protein (G protein), gamma 12	3, 2, 2
Gβ1γ7	HSPB1	Heat shock 27kDa protein 1	2
Gβ1γ7	DSC1	Desmocollin 1	2
Gβ1γ7	KCTD17	Potassium channel tetramerization domain containing 17	2
Gβ1γ7	RPS27A	Ribosomal protein S27a	2
Gβ1γ7	TXN	Thioredoxin	2
Gβ1γ7	PLEC	Plectin	2
Gβ1	DOLPP1	Dolichyldiphosphatase 1	1
Gβ1	COX4I1	Cytochrome c oxidase subunit IV isoform 1	1
Gβ1	LMNB1	Lamin B1	1
Gβ1	SNX1	Sorting nexin 1	1

Gβ1	GNG4	Guanine nucleotide binding protein (G protein), gamma 4	1
Gβ1	VPS26A	Vacuolar protein sorting 26 homolog A (S. pombe)	1
Gβ1	HADHB	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit	1
Gβ1	G6PD	Glucose-6-phosphate dehydrogenase	1
Gβ1	TCP1	T-complex 1	1
Gβ1	RPLP2	Ribosomal protein, large, P2	1
Gβ1	CSTB	Cystatin B (stefin B)	1
Gβ1γ7	SBSN	Suprabasin	1
Gβ1γ7	COX7A2	Cytochrome c oxidase subunit VIIa polypeptide 2 (liver)	1
Gβ1γ7	CTSA	Cathepsin A	1
Gβ1γ7	HNRNPAB	Heterogeneous nuclear ribonucleoprotein A/B	1
Gβ1γ7	HNRNPA3	Heterogeneous nuclear ribonucleoprotein A3	1
Gβ1γ7	CCT7	Chaperonin containing TCP1, subunit 7 (eta)	1
Gβ1γ7	PABPC1	Poly(A) binding protein, cytoplasmic 1	1
Gβ1γ7	CDC42BPA	CDC42 binding protein kinase alpha (DMPK-like)	1
Gβ1γ7	RPS20	Ribosomal protein S20	1

Gβ1γ7	CDSN	Corneodesmosin	1

Split TAP combinations are listed as $G\beta_x\gamma_y$, single TAP constructs as $G\beta_x$ or $G\gamma_y$.

2. Re-suspend in 400μL of TAP lysis buffer in 2mL tube.3. Combine the supernatant from nuclear lysis into a 15 mL tube and add washed beads. Add second set of beads to 50 mL of cytosolic fraction. Incubate on shaker overnight (see Note 22).

4. Spin down beads at 200×g for 1 min. Remove supernatant and save sample for western blot.

5. Wash beads (from cytosol and nuclei) 3× with TAP lysis buffer.

6. Wash beads 2× with TEV buffer. Save a sample of TEV wash for western blot.

7. Add 250μL of TEV buffer to beads. Add 10 U of TEV and incubate overnight on shaker.

8. Spin down beads and remove supernatant to 2 mL tube. Add 300 μL of calmodulin binding buffer to streptavidin beads. Mix by inverting tube. Spin down and collect supernatant. Repeat 2×, adding each wash to the streptavidin elution. Save a sample for western blot.

9. Add 1/250 volume (5μL) of 2M $CaCl_2$ to tube for a final concentration of 1mM.

10. Spin down to remove residual streptavidin beads that may have transferred over.

11. Remove supernatant to new tube.

12. Wash calmodulin Sepharose beads 3× with calmodulin binding buffer.

13. Resuspend beads in 400μL of calmodulin binding buffer and transfer to streptavidin elution.

14. Incubate overnight on shaker.

15. Spin down beads and remove supernatant. Save this sample for western blot.

16. Wash beads 3× with 1mL calmodulin binding buffer. Save a sample of the wash for western blot.

17. Wash beads 3× with calmodulin rinsing buffer. Save a sample for western blot.

18. Add 150μL 0.5M ammonium hydroxide. Leave on bench for 5 min.

19. Spin down beads and remove supernatant to new tube. Save 30μL for silver stain gel and a sample for western blot.

20. Repeat 2× and combine supernatant.

21. Lyophilize supernatant for approximately 2h (see Note 23).

22. Add 600 µL of water to the pellet to wash.
23. Lyophilize for another 2h.
24. Send lyophilized sample for MS, run samples on a polyacrylamide gel followed by silver stain and western blot to detect bait protein.

3.4.5 *Silver Stain*

Silver stain allows detection of protein at concentrations in the nanogram range. If protein can be detected on a silver-stained gel, there should be sufficient protein eluted from the beads to be sent for MS. The protocol of choice should be determined based on whether the gel is used for identifying protein, or whether protein will be cut out of the gel and sent for MS[214].

1. Load 30µL from first elution.
2. Run gel at 120 V for 60 min.
3. Stain gel with silver stain [215] (see Note 24).
4. Send sample for LC/MS [195].
5. Tandem mass spectra were searched against a FASTA file containing the human NCBI sequences as previously described[203]. The resulting peptide identifications were compared to control data and data from the "Crapome" database[215]. Protein identifications found in less than "60 Crapome" control experiments were determined to be positive hits.

Protein identifications from cytosolic fractions indicate that $G\beta_1$ can bind to known Gβ interactors such as the Gα subunits and Gγ subunits in both the cytosolic and nuclear fractions (**Table 4**, **Table 5**, and **Table 6**). Therefore our $G\beta_1$ construct is functional as it pulls down previously characterized $G\beta_1$ protein interactors. In both our whole cell and nuclear isolation screens, we have been able to identify importin and exportin proteins (**Table 3** and **Table 5**). This may suggest a possible mechanism for import of Gβγ into the nucleus. Interesting interactors with $G\beta_1$ found through our screens include heterogeneous nuclear ribonuclear proteins,

RNA binding motif protein, and replication factors (**Table 5**). Although these interactors remain to be validated, this data, along with accumulated evidence of Gβγ subunit interactions with transcription factors, point to a broad role for Gβγ in regulating transcription in the nucleus [21,124,125,128]. Further research in our lab will focus on replicating and validating these results, allowing characterization of Gβγ signalling networks important in gene transcription.

3.5 Notes

1. Puromycin and hygromycin are used to select for TAP plasmids expressing tagged bait proteins in stable cell lines. Other appropriate antibiotics to select for the TAP plasmid can be used.
2. EDTA/EGTA will not dissolve until pH of 8.0 is reached. There is a small window of pH in which EDTA/EGTA will dissolve; therefore, add NaOH pellet very slowly while stirring and check pH frequently.
3. PMSF is rapidly inactivated in water. Therefore it should always be added to solutions just before use.
4. Depending on the number of T175 flasks, you will need to calculate the amount of lysis buffer, high sucrose buffer, and resuspension buffer required.
5. Ammonium bicarbonate must be made fresh for each experiment as the pH changes rapidly over time. Check pH using pH strips to avoid contamination. Do not adjust the pH when making ammonium bicarbonate; the solution should be at pH 8.0 when made if the concentration is correct.
6. If storing samples at any point, flash freeze them in liquid nitrogen and store at −80 °C. This will ensure that protein stability is maintained.
7. The binding capacity of streptavidin Sepharose beads (GE Healthcare) is 0.6 mg of biotinylated BSA/100μL of medium. However, due to the number of wash steps involved in this protocol, we found that the optimal starting amount of protein is 100 mg. This translates into 10 or 20 T175 flasks of HEK 293 cells, respectively, for cytosolic or nuclear preparations.
8. Our lab has found that transfecting at much lower concentrations than

recommended by the manufacturer is appropriate for HEK 293 cells. This should be optimized based on the cell line used and whether you will be using stable or transiently transfected cell lines. In our lab, we have generated stable cell lines for use with the TAP protocol to ensure levels of protein expression closer to that of the endogenous protein.

9. Cell detachment and lysis must be done on ice, large dishes or containers can be filled with ice, and tissue culture flasks placed on top during the process.

10. EGTA and EDTA chelate calcium and magnesium, which are required by integrins for surface adhesion[216]. Therefore, the use of EGTA and EDTA facilitates cell detachment. If cells are hard to detach, trypsin can be used in combination with EDTA.

11. The 6 min incubation time on ice is very important; if cells are lysed for longer, the amount of intact nuclei isolated will be decreased.

12. Slowly pipette the lysed solution down the side of the conical tube. This will prevent the two layers from mixing.

13. The 1.8M sucrose cushion allows for enrichment of nuclei from HEK 293 cells, by preventing the cytosol and cell membrane from passing through the cushion during centrifugation. Intact nuclei pass through the cushion and are found as a pellet at the bottom of the tube.

14. This centrifuge speed is optimal to pellet the nuclei of HEK 293 cells. If using another cell type, this may differ.

15. There will be more protein isolated from the cytosol than the nuclei. Because the cytosol is diluted compared with the nuclei, combine the cytosol in a 50mL conical tube and add the streptavidin beads to that.

16. Protein that will be used for further steps of the TAP protocol must be stored at −80 °C to ensure it will bind to the beads when thawed.

17. It is very important to remove the cytosolic fraction before removing the high sucrose cushion. This ensures a clean nuclear pellet with low levels of cytosolic contamination.

18. Phosphatase and protease inhibitors that are appropriate for your cells can be added. If using an inhibitor cocktail that contains 4-(2-aminoethyl) benzenesulfonyl

fluoride hydrochloride (AEBSF), PMSF can be omitted as they have similar specificities.

19. To ensure that proper lysis of nuclei has occurred, place 10μL of nuclear lysis on a glass slide and look at under light microscope with 10× magnification. You should not see any intact nuclei.

20. This amount will need to be optimized depending on the cell type used. You can test out differing concentrations of benzonase by determining the amount of DNA left (measure absorbance at 260nm). It is very important to digest the DNA in the sample as it will be very highly concentrated and will affect the ability of nuclear proteins to bind to beads. Take a sample for western blot analysis following digestion with benzonase. Benzonase is effective at temperatures from 0 to 42°C, Mg^{2+} concentrations of 1 to 10mM, at pH 6.0–10.0, 0–100mM DTT, and 0–150mM monovalent cation (Na^{+}, K^{+}) concentrations. Because the buffer and temperatures used in this protocol are not optimal for benzonase activity, lysis should be left overnight using higher concentrations of benzonase as per manufacturer's instructions.

21. This centrifugation step allows separation of DNA bound proteins from soluble nuclear proteins. If this step is removed, the beads will clump together with the non-soluble fraction, preventing proper elution of protein from the beads in the following step.

22. The cytosolic sample does not require DNA digestion with benzonase. While nuclear sample is incubating with benzonase overnight, the cytosolic sample can remain incubating with streptavidin beads on the shaker or flash frozen in liquid nitrogen and stored at −80°C for later use.

23. For further details on preparing proteins for MS analysis see Ahmed *et al.*[203].

24. If a band is seen following silver stain, there will be enough protein in the sample for detection by mass spectrometry. Otherwise, the protocol should be done again with more starting material.

4 An unbiased screen for nuclear and cytosolic Gβγ interactors

The chapter below describes results of the tandem affinity purification technique, described in detail above, to identify nuclear and cytosolic Gβγ interactors. A manuscript entitled “Analysis of nuclear and cytosolic Gβγ interactions” is in preparation[217]. I contributed all of the lab work except the mass spectrometry, which was done by Mélanie Robitaille. I wrote this draft of the manuscript with edits from Terry Hébert.

4.1 Summary

G proteins are involved in the transduction of extracellular signals received by GPCRs to intracellular targets. Although canonical signalling pathways localize the Gβγ subunits to the cell membrane, a number of lines of evidence show the functional presence of Gβγ subunits to intracellular compartments including the nucleus. Tandem affinity purification can be used to identify protein interacting partners; therefore we used this technique to identify Gβγ binding partners in the nucleus and cytosol of HEK 293 cells. By modifying the protocol to target specific Gβγ dimer subunit pairs, we were able to alter the composition of proteins identified. We were also able to modulate the composition of identified proteins by treating cells with 1mM carbachol. Our results demonstrate the importance of Gβγ subunit composition, identifies novel nuclear and cytosolic interacting proteins, and demonstrates that G protein signalling in the nucleus can be modified by activation of endogenously expressed cell surface receptors.

4.2 Introduction

GPCR signalling involves activation of the receptor, canonically localized to the cell surface, leading to activation of heterotrimeric G proteins and activation of downstream effectors. GPCRs are involved in a number of different signalling pathways, including those that modulate transcription[218,219]. In addition, GPCR

signalling can be modulated based on association with G proteins of particular composition, effector molecules, physiological context, receptor isoforms, homo- and heterodimerization, polymorphisms, ligands, cell type expression, and subcellular localization (Reviewed by Maurice *et al.*)[28]. Recently, there have been a number of reports of GPCRs found at the cell nucleus. These include the bradykinin receptor[169], β-adrenergic receptor[113], endothelin receptor[112] and angiotensin receptor[60]. Additionally, many downstream effector molecules have been localized to the cell nucleus including adenylyl cylcases[87,88], phospholipase A_2 [89], phospholipase β[90], phospholipase D[91], β arrestin-1[92,93], G protein coupled receptor kinases (GRKs)[94], A kinase anchoring proteins (AKAPs), protein kinase A (PKA)[95], and regulators of G protein signalling (RGS) proteins[96]. Thus, a picture is developing that we have only scratched the surface of GPCR signalling in the nucleus.

Both small G proteins and heterotrimeric G proteins have been localized to the nucleus in a number of different cell types (reviewed in Chapter 1). Within the nucleus, heterotrimeric G proteins have function with calcium signalling[108,220], nitrous oxide signalling[108], and transcription[44,123,128,185]. Our research has shown that many of the Gα proteins are found in the nucleus[194]. This indicates that nuclear G proteins may regulate many of the same functions that cytosolic G proteins do, although in many instances it is yet to be determined whether these events are controlled by G proteins that move into the nucleus, or an existing pool of nuclear G proteins. This study aims to understand Gβγ signalling in the nucleus by developing a starting point for further investigation.

Another aspect of heterotrimeric G protein signalling involves the specific combinations of Gβ and Gγ. In mammals, there are five Gβ subunits and twelve Gγ subunits. All the Gβ subunits except Gβ5 share high sequence similarity, however Gγ subunits are less conserved (reviewed in Khan *et al.*)[21]. To date, our own data from tandem affinity purification (TAP) followed by liquid chromatography mass spectrometry (LC/MS), has shown that the β1, 2, and 4 subunits and the γ7 subunit are endogenously found in nuclei from human embryonic kidney 293 (HEK 293) cells[194].

Although many binding partners for Gβγ and Gα subunits, much is still unknown about what these proteins are doing in the nucleus. To this end we used TAP and other affinity-based purification methods to specifically identify Gβγ interacting partners in the nucleus[194]. As a means to identify Gβγ combination-specific interacting proteins, we also developed a "split TAP" procedure where the individual TAP protein tags are engineered into a Gβ and a Gγ subunit[202]. This allows us to determine whether different Gβγ combinations have distinct sets of interacting proteins.

There have been reports of cell surface signalling events that trigger movement of G proteins into the nucleus. For example, following treatment of HEK 293 cells with thrombin for 20 hours, levels of Gαi were increased in the nucleus with a corresponding decrease in cytosolic Gαi[120]. Following activation of the somatostatin receptor, Gβγ is able to inhibit the glucocorticoid receptor, an effect that was increased following addition of a nuclear localization signal on Gβ2[185]. Therefore, activation of cell surface receptors can lead to the movement of G proteins into the nucleus where they interact with a distinct set of proteins from their cytosolic counterparts. To understand this phenomenon, we used carbachol to activate cell surface muscarinic receptors expressed endogenously in HEK 293 cells. Carbachol is a stable analog of acetylcholine and binds to mACh receptors.

We have determined that Gβγ subunits interact with a diverse number of proteins in the nucleus. The population of proteins that interact with Gβγ can be altered by activating cell surface receptors or by changing the Gβγ isoforms used as bait. This work opens the door for further understanding of Gβγ involvement in nuclear signalling pathways, as well as validation of these proteins as *bona fide* Gβγ binding partners.

4.3 *Methods*

4.3.1 *Transfection*

Stable HEK 293 cell lines were made using the lipofectamine 2000® reagent. Human Gβ1 and Gγ7 were inserted into the TAP-tag pIRESpuro-GLUE-N1 vector backbone using EcoRI and BamHI for G β1, and ClaI and BamHI for Gγ7. All enzymes were purchased from New England Biolabs. The plasmid expressing Flag-Gβ1 was obtained from Missouri S&T cDNA Resource Centre (www.cdna.org).

4.3.2 *Tissue Culture*

HEK 293 cells were grown in Gibco® Dulbecco's modified Eagle's medium (DMEM) (Thermo Scientific, 12491-015) in 5% fetal bovine serum (FBS) (Wisent, St. Bruno, QC; 081-105) in T175 flasks (Thermo Scientific, 130191) until 90% confluent. Cells were cultured in an atmosphere of 5% CO_2 at 37°C and were passaged on average of each 3-4 days. Cells were grown to confluency and then serum-deprived overnight in DMEM. In some cases, cells were treated with 1mM carbamoylcholine chloride (Sigma-Aldrich, St Louis, MO, C4382) for 5 minutes followed by a 45 minute wash out period.

4.3.3 *Nuclear Isolation*

Starting material was determined based on protein concentration. A minimum of 25mg of protein in the cytosolic and nuclear samples was required for purification. Cells were washed with 10mL 1x phosphate buffered saline (PBS) and removed in 10mL of 1xPBS from T175 culture flasks by tapping gently on the side. Cells were then spun down at 1200rpm (Sorvall Mach1.6R) for ten minutes in 15mL conical tubes (Thermo Scientific, 339650). After removing the PBS, cells were re-suspended in 10mL lysis buffer (320mM sucrose, 10mM HEPES NaOH, 5mM $MgCl_2$, 1mM DTT, 1mM PMSF, 1mM Na_3VO_4, 1% Triton-X) and incubated on ice for six minutes. After six minutes, the lysis solution was layered on top of 10mL

high sucrose buffer (1.8M sucrose, 10mM HEPES NaOH, 5mM $MgCl_2$, 1mM DTT, 1mM PMSF, 1mM Na_3VO_4, 1% Triton-X) in 50mL conical tubes (Thermo Scientific, 339652). Cells were centrifuged at 4600rpm (Eppendorf 5415R) for twenty minutes to pellet nuclei. Following centrifugation, the cytosolic layer was removed to new 50mL tubes and frozen at -80°C. The sucrose cushion was then removed, and the pellet nuclei re-suspended in re-suspension buffer (320mM sucrose, 10mM HEPES NaOH, 5mM $MgCl_2$, 1mM DTT, 1mM PMSF, 1mM Na_3VO_4, 1% Triton-X). Nuclei were spun down at 300x g for 5min (Eppendorf 5415R) in 1.5mL tubes (Fisherbrand, 05-408-129). Nuclei were then re-suspended in 200µL TAP lysis buffer (1mM $MgCl_2$, 10% glycerol, 50mM HEPES-KOH pH8.0, 100mM KCl, 0.1% NP-40, 0.2mM EDTA, 2mM DTT, 1x Protease Inhibitor) and pooled together. TAP lysis buffer was added to 5mL. Nuclear lysate was homogenized with a dounce homogenizer (Kontes, size 19) with ten strokes. 5µL of 25KU/µL benzonase (Sigma-Aldrich, St. Louis, MO, E1014) was added to the nuclear lysate and left on a shaker at 4°C overnight. The following day the solution was frozen at -80°C for protein purification.

4.3.4 *Western blotting*

Lysates were added to loading buffer (62.5mM Tris pH 6.8, 16.3% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.025% bromophenol blue). Samples were heated for 10 minutes at 65°C followed by sonication (Misonix sonicator 3000) for 5 seconds at setting “0.5”. Samples were run on a 10% polyacrylamide gel for 15 minutes at 120V, followed by one and a half hours at 160V. Gels were transferred to Immun-Blot® polyvinylidene fluoride (PVDF) membranes (BioRad; 162-0177) for one hour at 100V and blotted in 5% milk for one hour. Antibodies were re-suspended in 5% milk and left on membranes for one hour, followed by three five minute washes. Anti-Flag (Sigma-Aldrich, St Louis, MO; F7425) and anti-HA (Roche, Indianapolis, IN; 12CA5) were used at a dilution of 1:1000. Anti-β-tubulin (Life Technologies, 32-2600) and anti-nup62 (BD Transduction Laboratories™, Mississauga, ON; 610497) were used at a dilution of 1:4000. All HRP-conjugated

secondary antibodies (mouse and rabbit; Sigma-Aldrich, St Louis, MO) were used at a dilution of 1:20,000.

4.3.5 *Tandem Affinity Purification*

100µL of streptavidin Sepharose™ beads (GE Healthcare, 17-5113-01) were washed three times with 1mL of TAP lysis buffer (320mM sucrose, 10mM HEPES NaOH, 5mM $MgCl_2$, 1mM DTT, 1mM PMSF, 1mM Na_3VO_4, 1% Triton-X) and spun down at 1600 rpm for 1 min (Eppendorf 5415R). Beads were then added to thawed cytosolic and nuclear lysates and left on shaker overnight at 4°C. The following day, beads were spun down at 1600 rpm for 10 minutes (Sorvall Mach1.6R). The supernatant was removed, and the beads washed three times in TAP lysis buffer. Beads were then washed three times in Tobacco Etch Virus (TEV) buffer (150mM NaCl, 10mM HEPES-KOH pH8.0, 0.5mM EDTA, 0.1% NP-40, 1mM DTT) and finally re-suspended in 250µL of TEV buffer. 3µL of 10U/µL AcTEV protease (Invitrogen, 1332340) was added to the beads and left on shaker overnight at 4°C. The following day, beads were spun down at 1600 rpm for 1 minute, and the supernatant removed to new 1.5mL tubes. The beads were then washed three times in calmodulin binding buffer (10mM HEPES-KOH pH8.0, 150mM NaCl, 1mM MgOAc, 1mM imidazole, 0.1% NP-40, 2mM $CaCl_2$, 10mM β-mercaptoethanol), and each wash was added to a new tube. 100µL of calmodulin Sepharose™ beads (GE Healthcare, 17-0529-01) were washed three times with 1mL of calmodulin binding buffer and re-suspended in 300µL of calmodulin binding buffer. Beads were added to the new tube, and 5µL of 1M calcium chloride was added. Beads were then left on shaker overnight at 4°C. The next day, beads were spun down at 1600 rpm for 1 min (Eppendorf 5415R) and the supernatant removed. Beads were washed three times in 1mL of calmodulin binding buffer, three times in calmodulin rinsing buffer (50mM ammonium bicarbonate, 75mM NaCl, 1mM MgOAc, 1mM imidazole, 2mM $CaCl_2$), and three times in 50mM ammonium bicarbonate (pH 8-8.5). Bound protein was eluted from calmodulin beads using 0.5M ammonium hydroxide at pH 11. Beads were re-suspended in 150µL of

ammonium hydroxide and added to a Micro Bio-Spin™ Chromatography Column (BioRad; 732-6204). Sample was left 10 minutes and then eluted through the column. This was repeated eight times for maximal protein elution. The resulting sample was lyophilized using a speed-vac system for 2 hours at “high” setting. The sample was re-suspended in 300µL of double distilled water and lyophilized again.

4.3.6 *Flag Purification*

Flag beads (Sigma-Aldrich, St. Louis, MO, A2220) were washed three times in 1mL of TAP lysis buffer. Beads were added to cytosolic and nuclear lysates and incubated overnight on a shaker at 4°C. Beads were spun down at 1600 rpm for 10 minutes (Sorvall Mach1.6R) and washed three times with TAP lysis buffer. Beads were then washed three times in Flag rinsing buffer (50mM ammonium bicarbonate pH 8-8.5, 75mM KCl), and three times in 50mM ammonium bicarbonate. Beads were eluted through a Micro Bio-Spin™ column using 0.5M ammonium hydroxide pH 11 five times. The resulting sample was sent for MS (see below).

4.3.7 *Processing of mass spectrometric data*

The resulting samples were sent for MS at the laboratory of Dr. Stéphane Angers. Proteins were digested with sequencing-grade trypsin (Promega), and the resulting peptide mixture was analyzed by liquid chromatography- tandem mass spectrometry using a LTQ-XL Linear Ion Trap Mass spectrometer (Thermo Scientific). The acquired tandem mass spectra were searched against a FASTA file containing the human NCBI sequences using a normalized implementation of SEQUEST running on the Sorcerer platform (Sage-N Research). The resulting peptide identifications returned by SEQUEST were filtered and assembled into protein identifications using peptide and protein prophets (Institute of Systems Biology, Seattle) and the Mascot database (RefSeqV45). Only proteins found in 2 out of 3 separate runs were analyzed and discussed below, unless otherwise indicated (**Figure 10**). Comparisons of proteins were made using BioVenn[221].

Biological functions and classes of proteins were sorted using Panther[222,223]. Protein interactions and pathway analysis was performed with String[224]. Proteins identified under untreated conditions are coloured yellow, in treated conditions blue, and in both conditions green. Heat maps were generated using R (R Core Team. R: A language and environment for statistical computing (R foundation for statistical computing. Vienna, Austria. 2014, http://www.R-project.org).

4.4 Results

Based on previous evidence showing the involvement of Gβγ subunits in transcriptional regulation, we used affinity purification (AP) to capture Gβγ interactors in the nucleus on a broader scale. We used two different methods, Flag AP or tandem affinity purification (TAP) to both preserve weak interactors and identify distinct Gβγ interactors[198,203,225]. TAP involves sequential elution from streptavidin Sepharose™ beads followed by calmodulin Sepharose™ beads[202]. In addition, we modified the TAP method so the calmodulin and streptavidin binding domains were fused to two different proteins (**Figure 11**). This allows us to specifically identify interactors for unique Gβγ combinations. **Figure 10** demonstrates the overall work flow that was used to identify Gβγ interacting proteins. In our initial experiments with TAP-Gβ1, Gγ7 was the only Gγ subunit pulled down with Gβ1 in the nucleus; therefore, split-TAP experiments were performed initially using Gβ1γ7 (see **Appendix** for all proteins identified by MS, not excluding those found only once).

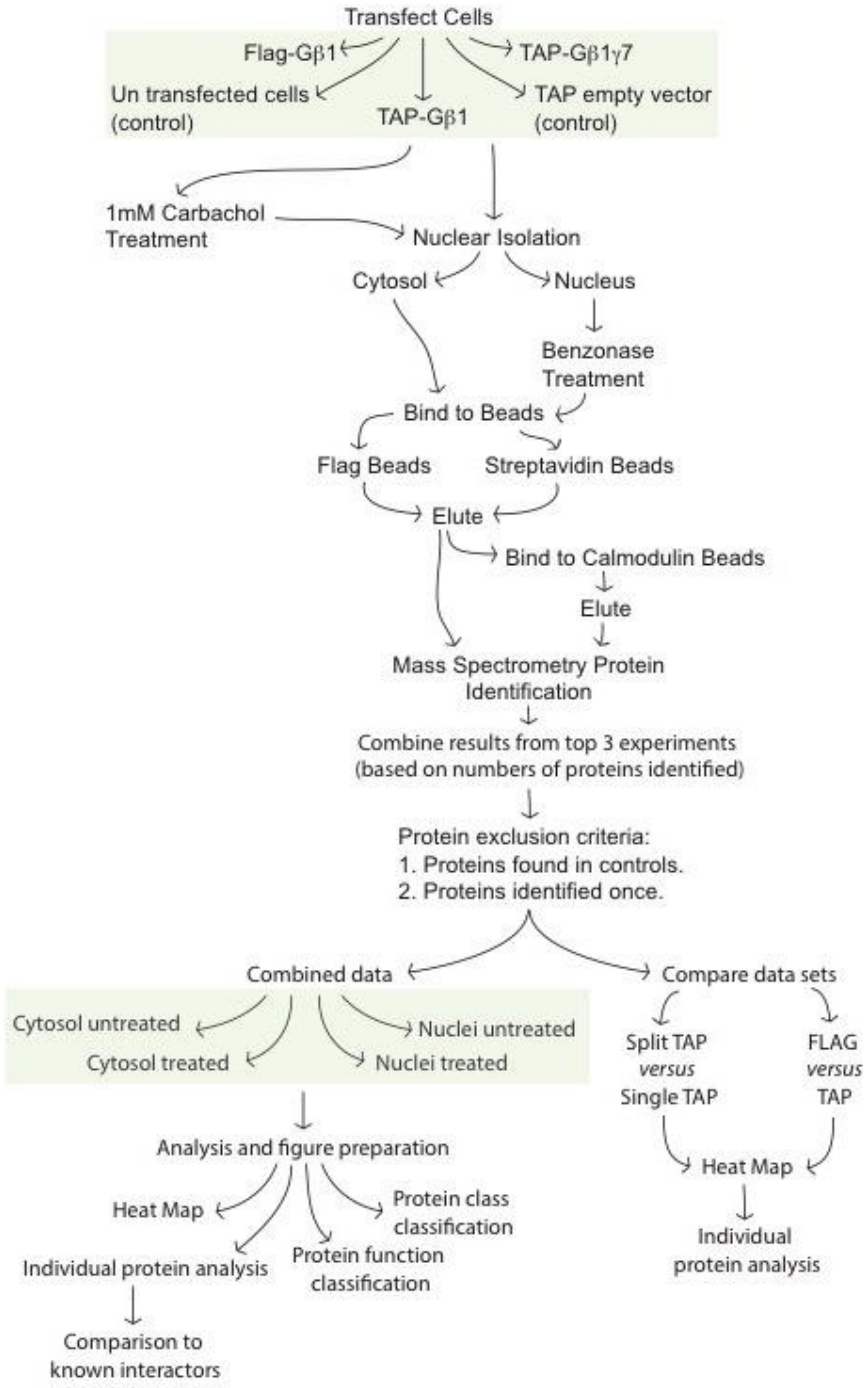

Transfect Cells
Flag-Gβ1
TAP-Gβ1γ7
Un transfected cells (control)
TAP-Gβ1
TAP empty vector (control)
1mM Carbachol Treatment
Nuclear Isolation
Cytosol
Nucleus
Benzonase Treatment
Bind to Beads
Flag Beads
Streptavidin Beads
Elute
Bind to Calmodulin Beads
Elute
Mass Spectrometry Protein Identification
Combine results from top 3 experiments (based on numbers of proteins identified)
Protein exclusion criteria:
1. Proteins found in controls.
2. Proteins identified once.
Combined data
Compare data sets
Cytosol untreated
Cytosol treated
Nuclei untreated
Nuclei treated
Split TAP versus Single TAP
FLAG versus TAP
Analysis and figure preparation
Heat Map
Protein class classification
Individual protein analysis
Protein function classification
Heat Map
Individual protein analysis
Comparison to known interactors

Figure 10: *Schematic describing the workflow of identification and analysis of Gβγ interacting proteins.* HEK 293 cells are transfected with single TAP Gβ1, split TAP Gβ1, Flag Gβ1, empty TAP vector, or un-transfected. Some cells are treated with 1mM carbachol. Following nuclear isolation, nuclei are lysed for protein recovery. Protein is bound to either Streptavidin and Calmodulin beads, or Flag beads. Eluted protein samples were identified by Mass Spectrometry. The top three experiments are taken for further analysis, and only proteins found in two out of three experiments are used. Data from all Gβ and Gβγ constructs are combined together to compare treated and untreated conditions, cytosolic and nuclear fractions. Individual construct data are also compared to understand the effects of protein tags and modulating Gβγ signalling. Proteins were analyzed using heat maps, pie charts, protein functional classification, and protein class sorting.

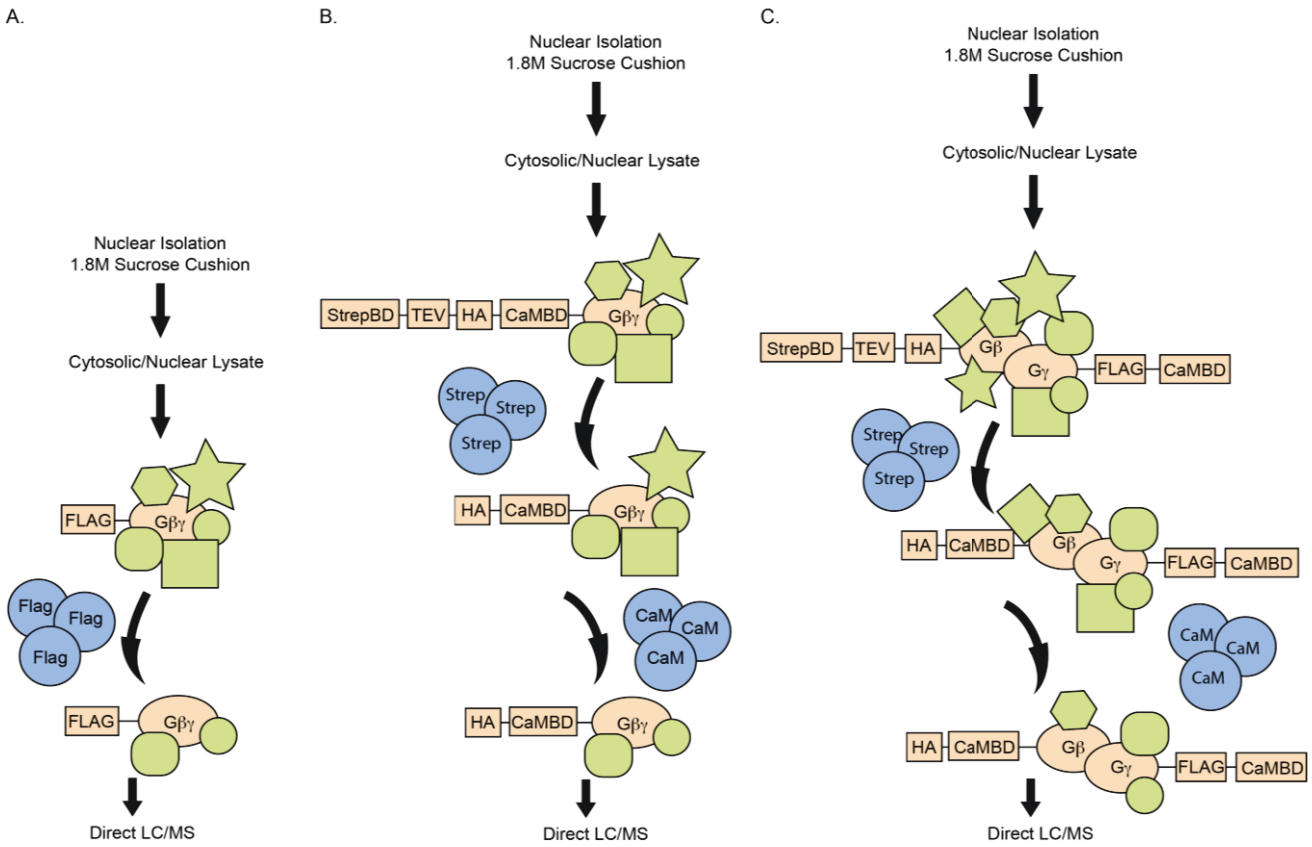


Figure 11: *Schematic of Flag affinity purification, single tandem affinity purification and split tandem affinity purification*. A) Flag affinity purification, B) single tandem affinity purification, and C) split tandem affinity purification.

Using these three purification protocols, we were able to capture numerous Gβγ-interacting proteins. As Gβγ subunits have known and well-documented functions in the cytosol, cytosolic lysates were used as a positive control to identify Gβγ interactors. Members of all four families of Gα proteins were pulled down, in addition to the chaperonin proteins that are required for proper folding of Gβ prior to binding to Gγ[21] (**Table 7** and **Table 8**). **Figure 12** shows the presence of the bait protein, Gβ1, in elutions from TAP and Flag protocols.

4.4.1 *Gβ1 interactors*

Using criteria outlined in the methods, we identified 123 potential interactions with Gβ1 in the cytosol and 73 in the nucleus. **Figure 13** shows global changes in the Gβ1 interacting proteome in the cytosol and nucleus with and without carbachol treatment by plotting the total peptide counts obtained for each protein by mass spectrometry. Values were averaged over three experiments and combined between tag conditions to get a general picture of protein levels bound to Gβγ in the cytosbol and nucleus in treated and untreated conditions. It is important to mention that although these values are not the best indication of protein levels bound to Gβγ due to the variation between experiments and protocols, they do allow an interpretation of the data, and enable us to focus on protein targets for further quantitative analysis of changes. To further understand the function of Gβγ, we analyzed the molecular function and protein classes of these potential interactors in both cytosol and nucleus (**Table 9** and **Table 10**). These changes are visualised in **Figure 14** and **Figure 15**. Overlap of proteins between the cytosolic and nuclear fractions is likely due to shuttling of proteins in and out of the nucleus (such as in the case of heterogenous ribonucleoproteins (HNRNPs)), or due to incomplete fractionation of the cytosol and nucleus. This data supports known interactions of Gβγ with DnaJ[226], t-complex polypeptide (TCP)[227], phosducin-like proteins[228], ras-related protein (Rap1) and Radil[203].

Table 7: *Proteins pulled down with Flag-Gβ1, TAP-Gβ1 and TAP-Gβ1γ7*. Proteins identified as Gβγ interactors with TAP tagged $G\beta_x\gamma_x$ and identified by mass spectrometry. Proteins pulled down in two out of three experiments.

Flag Gβ1 *Cytosol*	Flag Gβ1 *Nuclei*	TAP Gβ1 *Cytosol*	TAP Gβ1 *Nuclei*	TAP Gβ1γ7 *Cytosol*	TAP Gβ1γ7 *Nuclei*
GNA11	GNA11	GNA11	GNA11	GNA11	GNA11
GNA12	GNA12		GNA12	GNA12	GNA12
GNA13	GNA13	GNA13	GNA13	GNA13	GNA13
GNAI1	GNAI1	GNAI1	GNAI1	GNAI1	GNAI1
GNAI2	GNAI2	GNAI2	GNAI2	GNAI2	GNA2
GNAI3	GNAI3	GNAI3	GNAI3	GNAI3	GNAI3
		GNAL			
				GNAO1	
GNAQ	GNAQ	GNAQ	GNAQ	GNAQ	GNAQ
GNAS	GNAS		GNAS	GNAS	GNAS
GNAZ				GNAZ	
GNB1	GNB1	GNB1	GNB1	GNB1	GNB1
GNB2				GNB2	GNB2
GNB2L1	GNB2L1				
		GNB4			GNB4
GNG10					
	GNG11				
GNG12	GNG12	GNG12		GNG12	GNG12
					GNG2
GNG4				GNG4	
GNG5	GNG5				
GNG7			GNG7	GNG7	GNG7

Table 8: *Chaperonin proteins pulled down with Flag-Gβ1, TAP Gβ1 and TAP-Gβ1γ7*. Proteins identified as Gβγ interactors with TAP tagged $G\beta_x\gamma_x$ and identified by mass spectrometry. Proteins pulled down in two out of three experiments.

Flag-Gβ1	TAP-Gβ1	TAP-Gβ1γ7
CCT2		CCT2
CCT3	CCT3	CCT3
CCT4	CCT4	CCT4
CCT5	CCT5	CCT5
CCT6A	CCT6A	CCT6A
CCT7	CCT7	CCT7
CCT8		CCT8

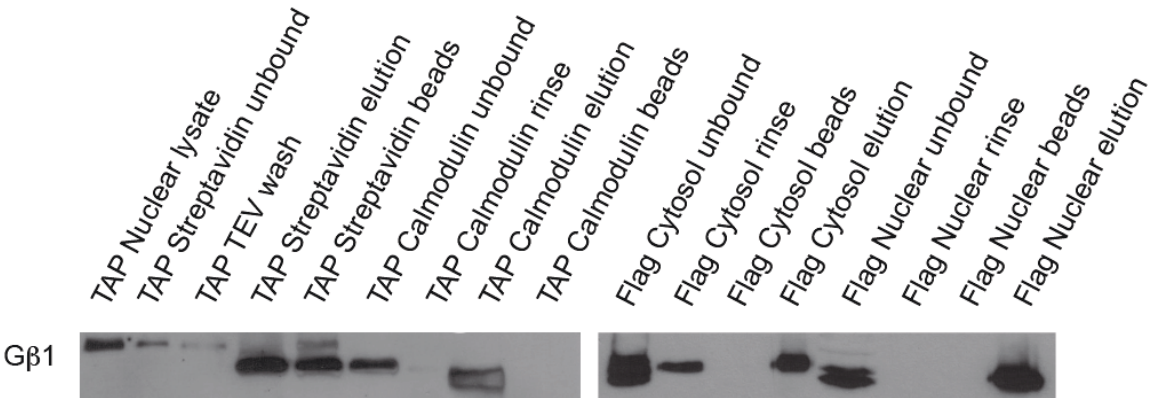


Figure 12: *TAP and Flag affinity purification*. In the TAP protocol, Gβ1 is tagged with streptavidin binding domains and calmodulin binding domains (left). In the Flag protocol Gβ1 is tagged with Flag (right). 30μL samples were taken from each step of the TAP or Flag protocol and run on a gel. TAP data is representative of 12 independent experiments. Flag data is representative of six independent experiments.

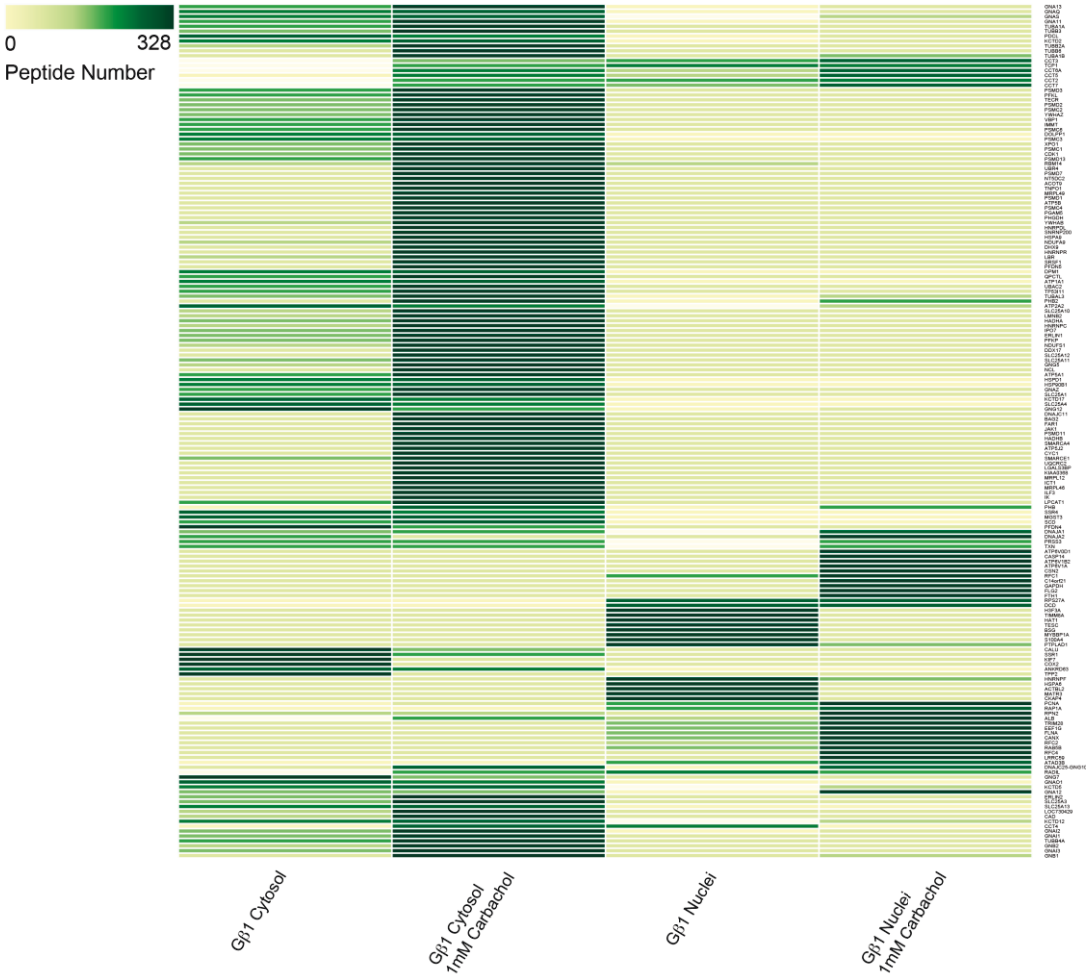


Figure 13: *Heat map of Gβγ binding proteins from Flag-tagged Gβ1, single TAP-tagged Gβ1 and split TAP-tagged Gβ1 with and without 1mM carbachol treatment.* The heat map represents the total peptide contains obtained for each protein identified by mass spectrometry. Values were averaged over three experiments and combined between tag conditions (Gβ1 Cytosol, Gβ1 Cytosol 1mM carbachol, Gβ1 Nuclei, Gβ1 Nuclei 1mM carbachol) and a heat map was generated using the statistical software R. Light green indicates low levels of total peptide identified, dark green indicates high levels of peptides identified. Data is representative of three experiments.

Table 9: *Cytosolic fraction proteins pulled down in using the Flag AP and TAP protocols*. Proteins identified as Gβγ interactors with TAP tagged $G\beta_x\gamma_x$ and identified by mass spectrometry and then grouped by molecular function and protein class using Panther online software[223].

Molecular Function	Number of proteins	Protein Class	Number of proteins
Antioxidant activity	1	Calcium-binding protein	10
Binding	53	Chaperone	16
Catalytic Activity	71	Cytoskeletal protein	10
Enzyme regulator activity	8	Defense/immunity protein	1
Nucleic acid binding transcription factor activity	3	Enzyme modulator protein	23
Protein binding transcription factor activity	1	Hydrolase	21
Receptor activity	7	Isomerase	1
Structural molecule activity	16	Kinase	5
Translation regulator activity	2	Ligase	1
Transporter activity	14	Lyase	1
		Membrane traffic protein	1
		Nucleic acid binding	19
		Oxidoreductase	11
		Phosphatase	2
		Protease	5
		Receptor	7
		Signalling molecule	2
		Structural protein	1
		Transcription factor	3
		Transfer/carrier protein	9
		Transferase	12
		Transporter	14

Table 10: *Nuclear fraction proteins pulled down in using the Flag AP and TAP protocols*. Proteins identified as Gβγ interactors with TAP tagged $G\beta_x\gamma_x$ and identified by mass spectrometry and then grouped by molecular function and protein class using Panther online software[223].

Molecular Function	Number of proteins	Protein Class	Number of proteins
Binding	34	Calcium binding protein	3
Catalytic Activity	33	Chaperone	12
Enzyme Regulator Activity	3	Cytoskeletal protein	5
Nucleic Acid Binding Transcription Factor Activity	2	Enzyme modulator protein	16
Receptor Activity	2	Hydrolase	11
Structural Molecule Activity	7	Isomerase	1
Translation Regulator Activity	1	Nucleic Acid Binding Protein	14
Transporter Activity	5	Oxidoreductase	3
		Phosphatase	1
		Protease	2
		Receptor	2
		Signalling Molecule	2
		Storage Protein	2
		Transcription Factor	2
		Transfer/carrier Protein	1
		Transferase	5
		Transmembrane receptor regulatory/adaptor protein	1
		Transporter	5

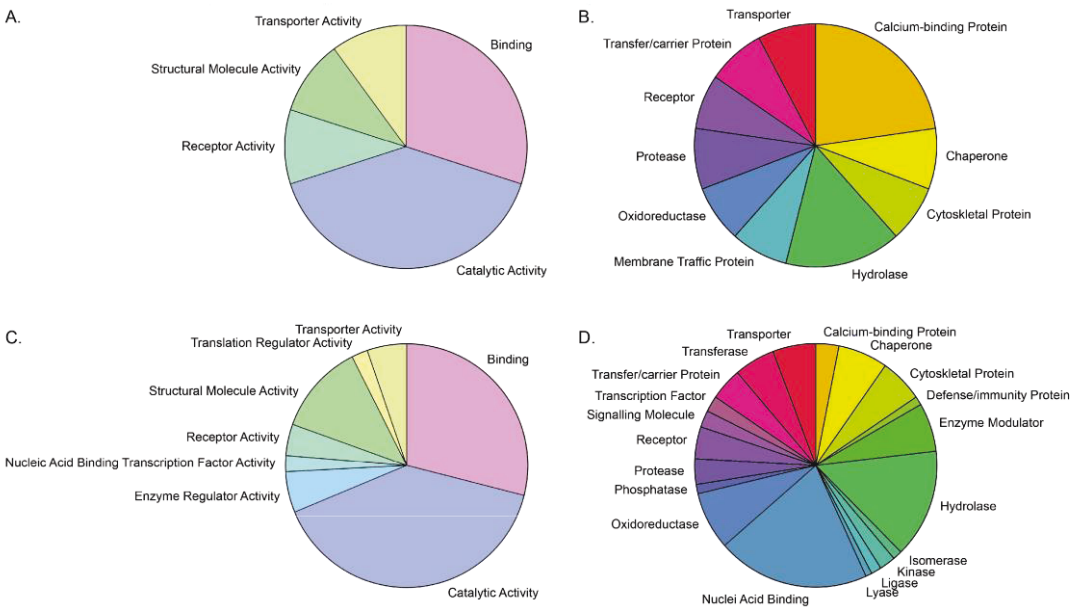


Figure 14: *Analysis of cytosolic proteins identified in untreated and treated (1mM carbachol) conditions organized by molecular function and protein class*. A) Cytosolic untreated proteins arranged by molecular function, B) Cytosolic untreated proteins arranged by protein class, C) Cytosolic treated proteins arranged by molecular function, D) Cytosolic treated proteins arranged by protein class. Proteins identified as Gβγ interactors with TAP tagged $G\beta_x\gamma_x$ and identified by mass spectrometry and grouped by molecular function and protein class using Panther online software[223].

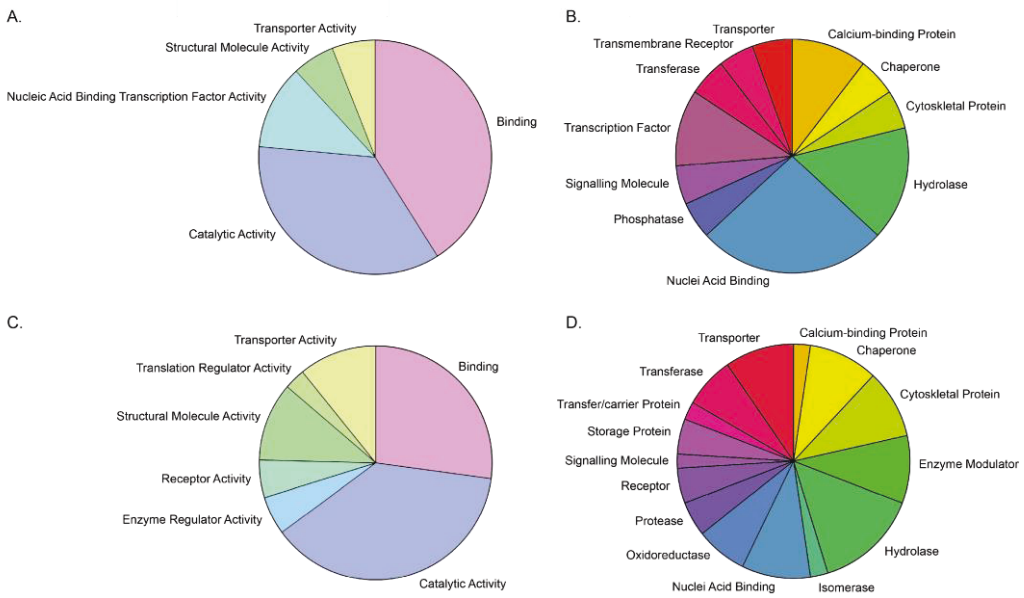


Figure 15: *Analysis of nuclear proteins identified in untreated and treated (1mM carbachol) conditions organized by molecular function and protein class*. A) Nuclear untreated proteins arranged by molecular function, B) Nuclear untreated proteins arranged by protein class, C) Nuclear treated proteins arranged by molecular function, D) Nuclear treated proteins arranged by protein class. Proteins identified as Gβγ interactors with TAP tagged $G\beta_x\gamma_x$ and identified by mass spectrometry and grouped by molecular function and protein class using Panther online software[223].

The four largest classes of proteins identified in the cytosolic and nuclear isolates were enzyme modulator proteins, chaperone proteins, hydrolases, and nucleic acid binding proteins (**Table 9** and **Table 10**).

The largest class of proteins identified was the enzyme modulator protein class (23 proteins identified in the cytosol, 16 in the nucleus), which includes Gα and Gβγ proteins. The chaperone class (16 proteins identified in the cytosol, 12 in the nucleus) includes chaperonin proteins (CCT) and other proteins involved in the proper folding of Gβ and Gγ including phosducin-like protein (PhLP) [21]. In addition, Von Hippel-Lindau Binding Protein 1 (VBP1, a prefoldin protein) was found to interact with Gβ1 in the cytosol. Although there is no reported interaction of Gβ1 with VBP1 (also known as PFDN3), as a prefoldin, VBP1 is known to chaperone proteins to complexes involved in protein assembly. Prefoldins are involved in the targeting of unfolded proteins to CCTs[229]. In addition to VBP1, PFDN4 and PFDN5 were pulled down with Gβ1 in the cytosol, suggesting the involvement of prefoldins in Gβ folding. Within this class, the 14-3-3 proteins ζ/δ and α/β (YWHAZ and YWHAB) were also identified as interactors of Gβ1 in the cytosol. These proteins can also interact with the small G proteins Rnd3, Rnd1, Rnd2, and Rap1A (also pulled down in our screen) and are thought to be involved in G protein inhibition [230]. This interaction may suggest the ability of 14-3-3 to modulate heterotrimeric G proteins in a similar manner. With a potential link to Rap1A and Radil, we pulled down kinesin super family member 7 (KIF7). Rap1A, Radil and KIF7 were pulled down from both cytosolic and nuclear lysates (from all data: Kif7 in the cytosol, Radil and Rap1a in the cytosol and nucleus).

Within the hydrolase class, numerous ATPases were pulled down with Gβ in both the cytosol and nucleus, including P type, V type (vacuolar) and F type (mitochondrial) ATPases[231,232]. In the cytosol, ATP5A1 (F type), ATP1A1 (P type), ATP5B (F type), ATP5J2 (F type) and ATP2A2 (P type) were identified. In the nucleus, ATP6V1B2 (V type), ATP6V0D1 (V type), ATP6V1A (V type), and ATP2A2 (P type) were identified. F type ATPases use a trans-membrane gradient of cations to generate ATP[232]. V and P type ATPases work in an opposite manner,

using the energy from ATP hydrolysis to pump cations back across a membrane (reviewed in Stewart *et al.* and Palmgren and Nissen)[231,232].

The proteins ATP5A1, ATP5B and ATP5J2 belong to F type ATPases that are expressed in the mitochondria. In addition to these proteins, SLC25A13[233,234], SLC25A1[234], SLC25A11[235], SLCA12[236], SLC25A4[237], SLC25A3[238], SLC25A10[239], CYC1[240], NDUFS1, NDUFA9[241], ACOT9[242], ICT1[243], UQCRC2[244], MRPS27, MRPL49, MRPL12[245], PGAM5[246], IMMT[247], HADHA, HADHB[248], HSPD1[249], and HSPA9[250] were also pulled down with Gβ1 and are found in the mitochondria, suggesting that Gβγ may be involved in regulatory processes within the mitochondria (**Table 11**). Interestingly, expression of Gβ2 on the surface of mitochondria, and interaction with the mitochondrial protein mitofusin 1 (Mfn1) has been reported, corroborating Gβγ's localization there[71].

Gβ1 was also found to interact with PSMC1, 2, 3, and 4, members of the 26S proteasome. In addition, RPN2, a regulatory subunit of non-ATPase members of the 26S proteasome was pulled down with Gβ1[251]. Gβ1 also pulled down a number of proteins that have been identified as interactors with the cullin ubiquitin E3 ligase system (**Table 12**)[252]. Although ubiquitin-associated domain containing 2 (UBAC2), a protein which binds ubiquitin and involved in various cell processes[253], was pulled down with Gβ1, Gβ1 did not capture any known E3 ligases [254]. Based on this evidence, it is unclear whether Gβγ is ubiquitinated and targeted for degradation, or whether Gβγ is involved in regulating protein ubiquitination by acting as a targeting protein.

Among nucleic acid binding proteins in the cytosol, Gβ1 pulled down the RNA helicases DEAH (Asp-Glu-Ala-His) box helicase 9 (DHX9), DEAD (Asp-Glu-Ala-Asp) box helicase 17 DDX17 and small nuclear ribonucleoprotein 200kDa (SNRNP200), the splicing factor SRSF1, and three heterogenous ribonucleoproteins (HNRNPR, HNRPDL, HNRNPC). Gβ1 also pulled down SMARCE1 and SMARCA4, SWI/SNF related, matrix-associated, actin-dependent regulators of chromatin. These proteins have helicase activity, and are thought to regulate gene expression by modulating chromatin structure[255].

Table 11: *Mitochondrial proteins pulled down with Flag-Gβ1 in two or more experiments.* Proteins identified as Gβγ interactors with TAP tagged $G\beta_x\gamma_x$ and identified by mass spectrometry. Mitochondrial proteins identified through literature searches and online databases.

Protein	Name	Function
ACOT9	Acyl-coA thioesterase 9	Acyl-coA thioesterase, unknown function
ATP5A1	ATP synthase, H+ transporting, α subunit 1	Subunit of mitochondrial ATP synthase, which catalyzes ATP synthesis using an H+ gradient
ATP5B	ATP synthase, H+ transporting β polypeptide	Subunit of mitochondrial ATP synthase, which catalyzes ATP synthesis using an H+ gradient
ATP5J2	ATP synthase, H+ transporting subunit F2	Subunit of mitochondrial ATP synthase, which catalyzes ATP synthesis using an H+ gradient
CYC1	Cytochrome C1	Subunit of cytochrome bc1 complex, involved in mitochondrial respiration
HADHA	Hydroxylacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase α subunit	Subunit of the mitochondrial trifunctional protein that catalyzes the last three steps of β oxidation of long chain fatty acids
HADHB	Hydroxylacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase β subunit	Subunit of the mitochondrial trifunctional protein that catalyzes the last three steps of β oxidation of long chain fatty acids
HSPA9	Heat shock 70kDa protein 9	Involved in cell proliferation, stress response, and maintenance of the mitochondria
HSPD1	Heat shock 60kDa protein 1	Required for the folding of nascent proteins transported into the mitochondria
ICT1	Immature colon carcinoma protein 1	Component of peptidyl-tRNA hydrolase of the mitochondrial large ribosomal subunit
IMMT	Inner membrane protein, mitochondrial	
MRPL12	Mitochondrial ribosome protein L12	Involved in protein synthesis in the mitochondria
MRPL49	Mitochondrial ribosome protein L49	Involved in protein synthesis in the mitochondria
MRPS27	Mitochondrial ribosome protein S27	Involved in protein synthesis in the mitochondria
NDUFA9	NADH dehydrogenase 1 α sub complex 9	First enzyme complex involved in the electron transport chain
NDUFS1	NADH dehydrogenase Fe-S protein 1	Transfers electrons from NADH to the respiratory chain
PGAM5	Phosphoglycerate mutase family member 5	Dephosphorylates and activates MAP3K5 kinase
SLC25A1	Solute carrier family 25, member 1	Translocates small metabolites across the mitochondrial membrane
SLC25A10	Solute carrier family 25, member 10	Exchanges dicarboxylates such as malate and succinate for phosphate, sulfate and other small molecules
SLC25A11	Solute carrier family 25, member 11	Transports 2-oxoglutarate across the mitochondrial membrane

SLC25A13	Solute carrier family 25, member 13	Catalyzes the exchange of aspartate for glutamate and a proton across the mitochondrial membrane
SLC25A3	Solute carrier family 25, member 3	Translocates phosphate into the mitochondria
SLC25A4	Solute carrier family 25, member 4	Translocates ADP into the mitochondria, and ATP to the cytoplasm
UQCRC2	Ubiquinol-cytochrome c reductase core protein II	Required for the cytochrome c reductase complex, involved in mitochondrial respiration

Table 12: *E3 ligase interactors pulled down with Gβ1 in the cytosol, nucleus, treated and untreated conditions*. E3 ligase interactors include proteins that were pulled down with Cullin 1, 2, 3, 4A, 5, neddylin 8 (NEDD8), cullin-associated and neddylation-dissociated 1 (CAND1), COP9 signalosome subunit 6 (CSN6), CSN5 and defective in Cul neddylation 1 protein (DCN1)[252]. E3 ligase interactors were compared to Gβγ interactors in each condition. Overlapping interactors were organized by condition and fraction in which they were found.

Gβ1 Cytosol	Gβ1 Cytosol 1mM carbachol	Gβ1 Nuclei	Gβ1 Nuclei 1mM carbachol
		ACTBL2	
	ALB		ALB
		ATAD3B	ATAD3B
ATP1A1	ATP1A1		ATP6V1A
ATP2A2	ATP2A2		ATP2A2
ATP5A1	ATP5A1		
	ATP5B		
	BAG2		
		BSG	
	CAD		
			CANX
			CASP14
CCT2	CCT2	CCT2	CCT2
CCT3	CCT3	CCT3	CCT3
CCT5	CCT5	CCT5	CCT5
CCT6A	CCT6A	CCT6A	CCT6A
CCT7	CCT7	CCT7	CCT7
		CKAP4	
			DCD
	DDX17		
	DHX9		
	DNAJA1		DNAJA1
DNAJA2			DNAJA2

			EEF1G
	FAR1		
			FLG2
			FLNA
			GAPDH
	HADHA		
		HAT1	
	HNRNPC		
		HNRNPF	HNRNPF
	HNRNPR		
	HNRPDL		
HSP90B1	HSP90B1		
	HSPA9	HSPA6	
	HSPD1		
	IL3		
KCTD12	KCTD12	KCTD12	KCTD12
KCTD17	KCTD17		
KCTD2	KCTD2	KCTD2	KCTD2
KCTD5	KCTD5	KCTD5	KCTD5
	LGALS3BP		
			LRRC59
		MATR3	
	MRPL12		
	MRPL49		
	MRPS27		
		MYBB1A	
	NCL		
	NDUFS1	PTPLAD1	PTPLAD1
	NT5DC2		
	PCNA	PCNA	PCNA
PFKP	PFKP		
	PGAM5		
	PHB		PHB
	PHGDH		
	PSMC1		
	PSMC2		
	PSMC4		
	PSMC6		
	PSMD1		
	PSMD2		
		RFC2	RFC2
RPN2	RPN2		RPN2
SLC25A3	SLC25A3		

SLC25A4			
	SMARCA4		
	SMARCE1		
	SNRNP200		
SSR1	SSR4		
SSR4		PTPLAD1	
TCP1	TCP1	TCP1	TCP1
			TRIM28
TUBA1A	TUBA1A		
	TUBB2A		
	TUBB3		
	TUBB6		
TXN	TXN		TXN
	YWHAB		
	YWHAZ		

The transcriptional regulator, interleukin enhancer binding factor (ILF3), a protein involved in RNA stabilization, was also associated with Gβ1, suggesting a general role for Gβ1 in transcriptional regulation, RNA stability and processing. To support this, Gβ1 also interacted with a number of mitochondrial ribosome proteins (MRPS27, MRPL49 and MRPL12). Gβγ association with mitochondrial ribosomal proteins may indicate a similar role for Gβγ in transcriptional regulation in this organelle as well. Gβ1 also interacted with 59 different ribosomal and ribosomal associated proteins found in the cytosol (see **Appendix**). However, these interactions were only seen once, and were therefore excluded from further analysis. It is possible that these interactions might occur during the translation of Gβ1. However this does not explain its association with mitochondrial ribosomal proteins, which may suggest a potential regulatory role. In addition, the proteins HNRPDL, HNRNPC, NCL, HNRNPR and RBM14 all interact with the ribosome.

Nucleic acid binding proteins bound to Gβ1 from nuclear lysates also included proteins that bind DNA. These proteins included replication factors 1,2 and 4, which are AAA+ ATPases (RFC1, RFC2, and RFC4), histone H3F3A and proliferating cell nuclear antigen (PCNA), involved in DNA replication and repair. RFC proteins make up replication factor C, a five subunit DNA polymerase accessory protein. This DNA-dependent ATPase is required for eukaryotic DNA replication and repair. These proteins are involved in disrupting the PCNA-DNA interface. This results in ATP hydrolysis by RFC and conformational change which decreases their affinity for PCNA, thus triggering the closing of the replication clamp around the DNA and expulsion of the clamp loader[256] (for a review of DNA replication see Méchali)[257]. Further, Gβ1 isolated from the nucleus also interacted with proteins associated with the ribosome, including the ribosomal protein S27a (RPS27A), RBM14, and eukaryotic translation elongation factor (EEF1G). Gβ1 also pulled down matrin 3 (MATR3) involved in stabilizing RNA. The large number of RNA and DNA binding proteins captured by Gβ1 in both the cytosol and nucleus suggests that Gβ1 interacts with proteins involved in RNA processing and stability, in addition to transcription and transcriptional regulation. This does not, however,

prove Gβγ involvement in these processes, but rather points to novel functions that need to be verified.

Finally, Gβ1 pulled down a number of proteins that can shuttle in and out of the nucleus, suggesting a mechanism for Gβγ entry and exit. In the cytosol and nucleus, Gβ1 interacted with exportin 1 (XPO1), importin 7 (IPO7), transportin 1 (TNPO1) and the heterogenous ribonucleoproteins (HNRNPF, HNRPDL, HNRNPR) that can shuttle in and out of the nucleus. Gβ1 was also associated with different heterogenous ribonucleoproteins, karyopherins, splicing factors, which are known to shuttle in and out of the nucleus, and nucleoporins, key components of the nuclear pore through which proteins enter and exit the nucleus. However, these interactions were only seen once, and were therefore excluded from our analysis (**Appendix**).

4.4.2 Split versus Single TAP

To determine whether there were unique protein interactions when a specific Gβγ dimer was used as bait, we compared proteins isolated by TAP-Gβ1 (single TAP) or TAP-Gβ1γ7 (split TAP). **Figure 16** and **Figure 17** show a comparison of proteins identified by each method in the cytosol or nucleus, identified through their molecular function or protein class. A comparison of total peptide numbers and total proteins identified did not reveal a significant difference between split and single TAP procedures (**Figure 18**). Therefore, the TAP method used did not affect the total number of proteins isolated, only the composition of the interacting proteins (**Figure 16** and **Figure 17**). It is evident from sorting according to molecular function of protein classes identified that the diversity of proteins is greater using the split-TAP method. Although true for both nuclear and cytosolic fractions, this is most evident when looking at the composition of nuclear proteins.

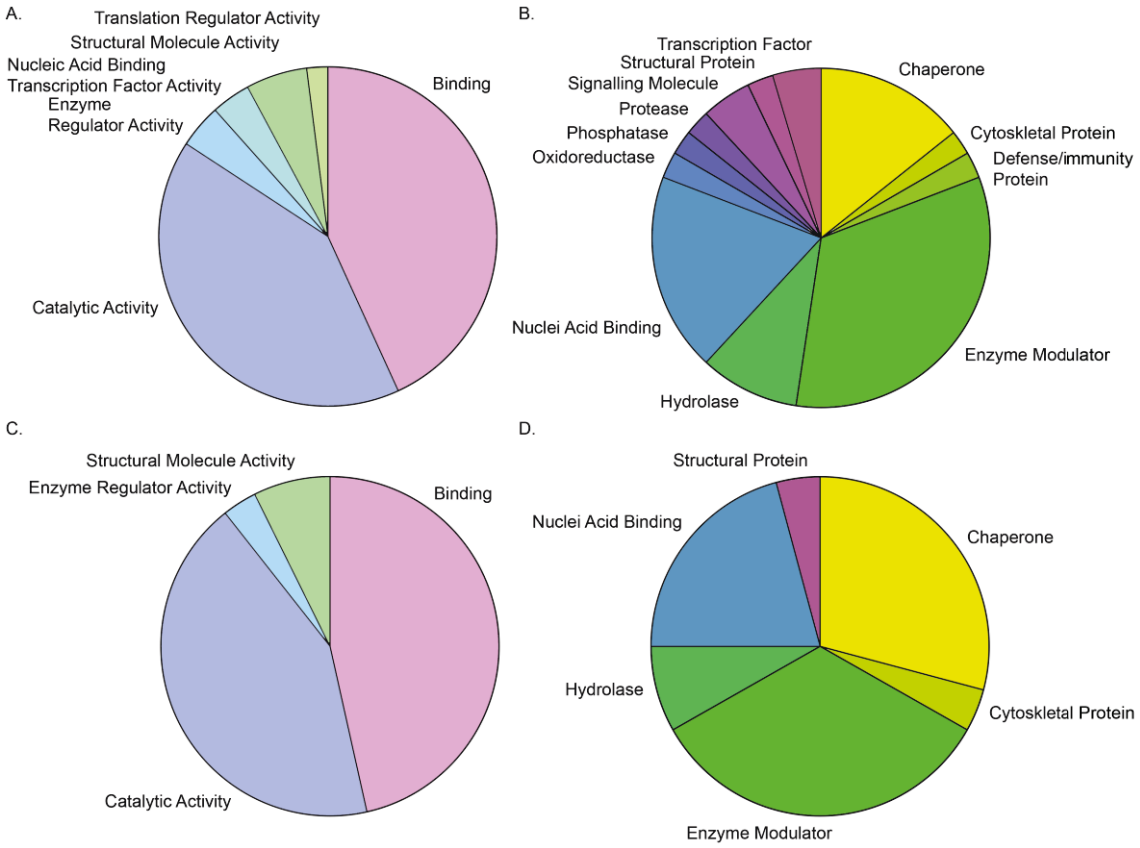


Figure 16: *Analysis of cytosolic proteins identified by Single-TAP and Split-TAP organized by molecular function and protein class*. A) Cytosolic Split-TAP proteins arranged by molecular function, B) Cytosolic Split-TAP proteins arranged by protein class, C) Cytosolic Single-TAP proteins arranged by molecular function, D) Cytosolic Single-TAP proteins arranged by protein class. Proteins identified as Gβγ interactors with TAP tagged $G\beta_x\gamma_x$ and identified by mass spectrometry and grouped by molecular function and protein class using Panther online software[223].

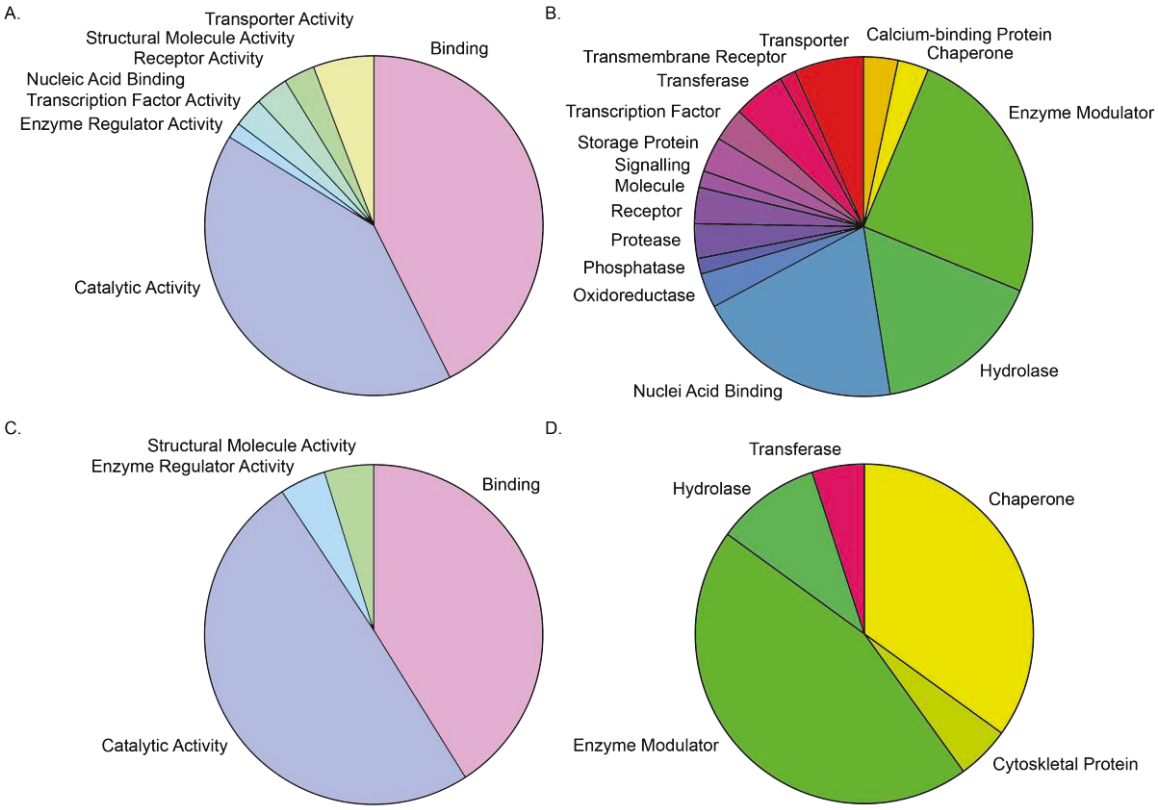


Figure 17: *Analysis of nuclear proteins identified by Single-TAP and Split-TAP organized by molecular function and protein class.* A) Nuclear Split-TAP proteins arranged by molecular function, B) Nuclear Split-TAP proteins arranged by protein class, C) Nuclear Single-TAP proteins arranged by molecular function, D) Nuclear Single-TAP proteins arranged by protein class. Proteins identified as Gβγ interactors with TAP tagged $G\beta_x\gamma_x$ and identified by mass spectrometry and grouped by molecular function and protein class using Panther online software[223].

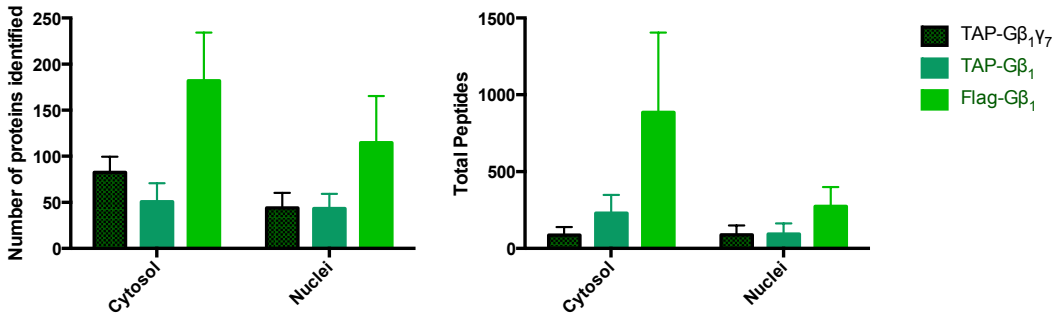


Figure 18: *Comparison of proteins identified by Flag, Single-TAP or Split-TAP.* The left panel indicates the total number of proteins identified using each protocol. The right panel indicates the total number of peptide fragments detected by mass spectrometry using each protocol. All treatment conditions and fractions combined, averaged across cytosol and nucleus.

There were 11 new classes of proteins identified in the nuclear fraction: transporter, transmembrane receptor, transcription factor, storage protein, signalling molecule, receptor, protease, phosphatase, oxidoreductase, nucleic acid binding, and calcium binding protein. There was a decrease in chaperone proteins pulled down with Gβ1γ7 (CCTs, PDCL, TCP) compared with Gβ1 alone. This indicates that by enriching for fully folded Gβγ dimers, we may decrease the chaperonin proteins required for Gβγ folding that are captured. In this way, the split-TAP method may enrich for the signalling function of Gβγ, rather than the processing of the protein. When specifically selecting for proteins bound to Gβ1γ7 we pulled down the ATPases ATP6V1B2, ATP6V0D1, and ATPV1A, in addition to the ATP-dependent chromatin remodelling complex SMARCE1 following treatment of cells with 1mM carbachol. Split-TAP Gβ1γ7 pulled down 12 RNA/DNA binding proteins and two transcription factors, compared with zero for both fractions using single-TAP Gβ1. Three of these include the replication factor C (RFC) proteins 1,2 and 4. The absence of these proteins in any of the single TAP pull downs may indicate the requirement for functional Gβ1γ7 in binding to RFC. Interestingly, MYBB1A, MATR3, H3F3A, CKAP4 and RBM14 comprise all of the proteins pulled down with Gβ1γ7 that play roles in DNA/RNA binding from the nuclear fraction in the overall analysis of carbachol-treated conditions (discussed in more detail below). In comparison, TAP-Gβ1 alone did not capture any DNA/RNA binding proteins under untreated conditions. This comparison shows the advantage of using the split-TAP method to enrich for particular Gβγ dimers, in addition to suggesting that different Gβγ dimers may be involved in distinct cell processes. This also suggests that Gγ7 is required for Gβγ function with the identified transcription factors in this context.

Finally, Gβ1γ7 captured a number of novel interactors from the nuclear fraction including the immunoglobulin superfamily member basigin (BSG), the intracellular iron storage protein ferritin (FTH1), casein 2 (CSN2), the calcium binding protein S100A4, the serine protease PRSS3, cystic-aspartic acid protease (CASP14) involved in apoptosis, tescalcin (TESC) required for cell pH regulation, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the redox protein

thioredoxin (TXN). The interaction between Gβ1γ7 and GAPDH could potentially be involved in RNA/DNA binding, since GAPDH has been found to bind tRNA and AU-rich RNA[258]. However, this requires further study. The split Gβ1γ7 indicate the importance of considering specific Gβγ dimer. In addition, our data suggests that Gβ1γ7 is specifically involved in transcriptional regulation.

4.4.3 *Flag versus TAP*

The Flag tag is a small eight amino acid tag[259], in comparison to calmodulin binding domain[260] and streptavidin binding domain[261], which are 148 and eight amino acids in length respectively. The size of the calmodulin-binding domain may result in steric hindrance of protein interactions with Gβγ. This may have resulted in the difference in protein interactions seen when using the Flag purification in comparison to the TAP protocol. Another difference between these two protocols is the number of wash steps, which may have reduced the number of interacting proteins identified using the split and single TAP methods. There were also some proteins that were only found in TAP elutions.

Proteins only found using the Flag tag include a number of ATPases (ATP1A1, ATP2A2, ATP5A1, ATP5B, ATP5JC), mitochondrial ribosomal proteins (MRP12, MRP49, MRPS27), solute carrier family members (SLC25A3, SLC25A4, SLC25A13, SLC25A1, SLC25A11, SLC25A10, SLC25A12), NADH dehydrogenase proteins (NDUFS1), elongation factors (EEF1G), ER lipid raft associated proteins (ERLIN1, 2), 26S proteasome subunits (PSMC1, 2,3,4,6, PSMD1, 2,3,7,11,13), importins and exportin proteins, and proliferating cell nuclear antigen (PCNA). These proteins may have been prevented from binding to TAP-Gβγ due to the presence of bulkier tags.

A possible constraint to protein binding of TAP-tagged Gβγ could be localization. Although we have shown that the tags do not affect Gβγ localization to the nucleus (**Figure 19**), we do not know whether these tags affect Gβγ localization to mitochondria.

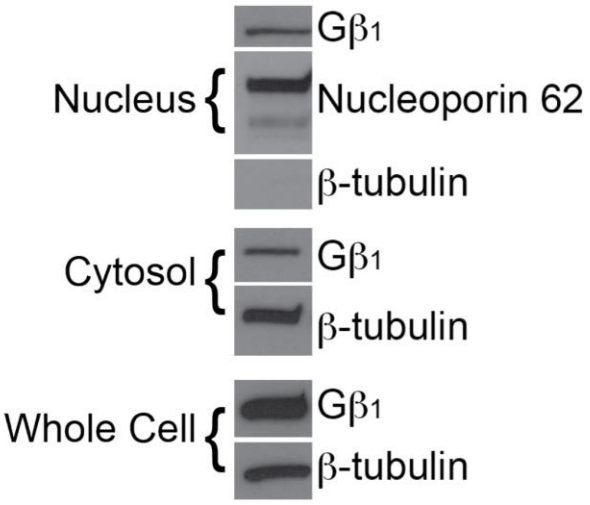


Figure 19: *Localization of Flag-tagged Gβ1 in the nucleus, cytosol and whole cell*. Nucleoporin 62 used as a marker of the nucleus, β tubulin used as a marker of the cytosol.

Only one (HSPA9) of the mitochondrial proteins found associated with Flag-tagged Gβγ were associated with streptavidin or calmodulin tagged Gβγ (**Appendix**). This protein is primarily associated with the mitochondria, but is also localized to the endoplasmic reticulum, cytoplasmic vesicles, and plasma membrane where it could interact with TAP-tagged $G\beta\gamma^{250,262}$. Therefore, it is possible that the calmodulin and streptavidin tags exclude Gβγ from the mitochondria, where they can interact with a number of mitochondrial proteins (**Table 11**).

4.4.4 Carbachol treatment modulates Gβγ interactions

To further understand the protein interactions of Gβγ in the nucleus and cytosol, we treated cells with carbachol, an agonist for the endogenously expressed

muscarinic acetylcholine receptor. In our hands, we observed the expression of M3 muscarinic receptors in HEK 293 cells by both western blot and qPCR (Shahriar Khan, unpublished data). The presence of M3 receptors in HEK 293 cells allows us to activate an endogenous cell surface GPCR to determine whether this changes the composition of proteins that interact with Gβγ in the cytosol and nucleus. Cells were treated for 5 minutes followed by a 45-minute wash out period and prepared for MS (using conditions determined by Shahriar Khan to facilitate Gβγ interactions with nuclear proteins, unpublished data). Treatment with carbachol shifted the composition of proteins that interacted with Gβγ (**Figure 13**). In addition, treatment with carbachol altered the numbers of peptides captured by Gβ1 in both cytosol and nucleus (**Figure 14 and Figure 15).** This indicates that carbachol alters pathways that Gβγ regulates in the nucleus and cytosol. To understand how carbachol affects Gβγ signalling in the nucleus, we analyzed Gβγ interacting proteins using String pathway analysis (**Figure 20**, **Figure 21** and **Figure 22**).

Treatment with carbachol did not alter the interaction of Gβ1 with other G proteins or chaperonin proteins suggesting that it modulated function rather than assembly of Gβγ.

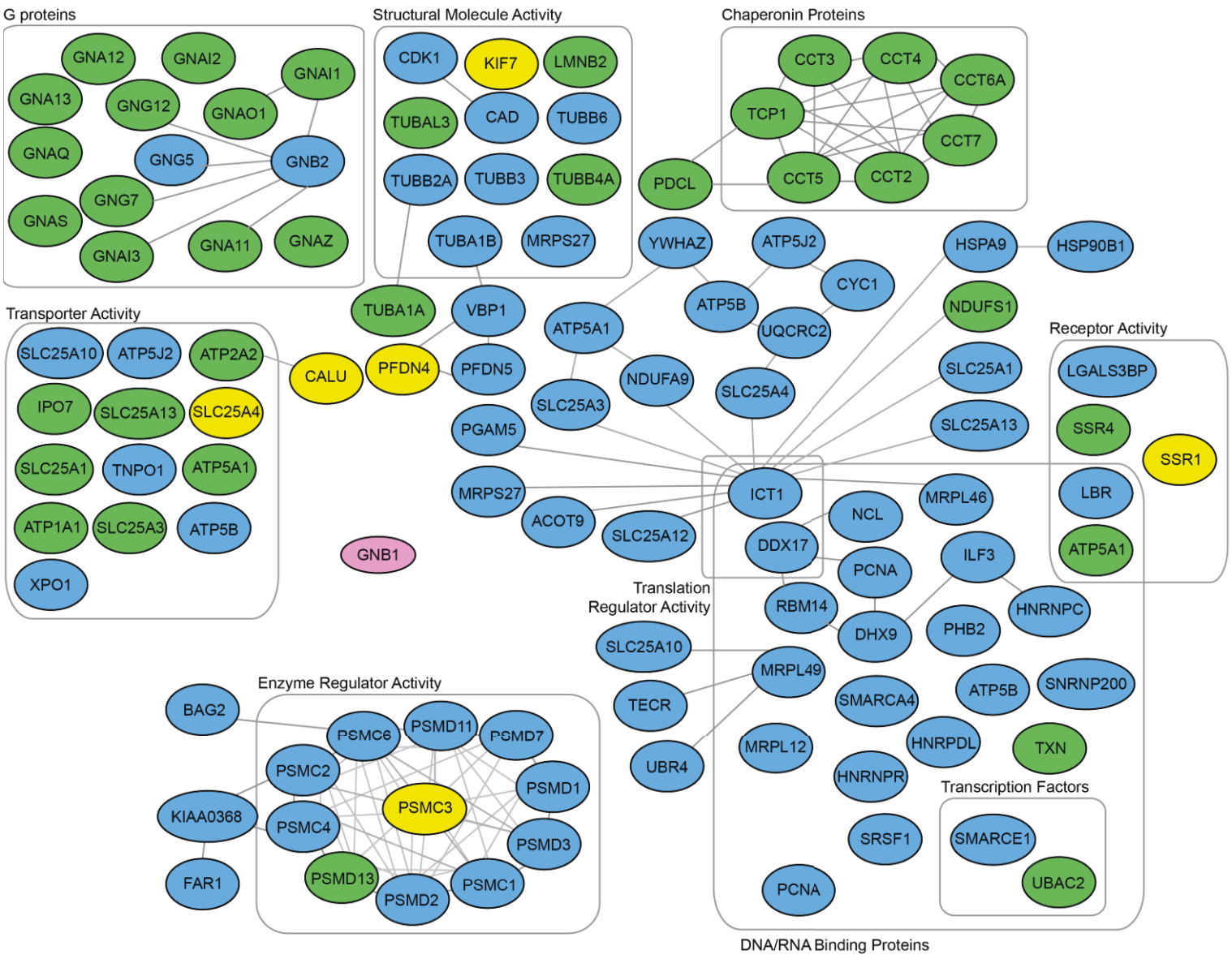

G proteins
GNA12
GNAI2
GNAI1
GNA13
GNG12
GNAO1
GNAQ
GNG5
GNB2
GNG7
GNAS
GNAI3
GNA11
GNAZ
Structural Molecule Activity
CDK1
KIF7
LMNB2
TUBAL3
CAD
TUBB6
TUBB2A
TUBB3
TUBB4A
TUBA1B
MRPS27
Chaperonin Proteins
CCT3
CCT4
CCT6A
TCP1
CCT7
CCT5
CCT2
PDCL
Transporter Activity
SLC25A10
ATP5J2
ATP2A2
IPO7
SLC25A13
SLC25A4
SLC25A1
TNPO1
ATP5A1
ATP1A1
SLC25A3
ATP5B
XPO1
CALU
TUBA1A
VBP1
PFDN4
PFDN5
PGAM5
GNB1
YWHAZ
ATP5J2
CYC1
ATP5B
UQCRC2
ATP5A1
NDUFA9
SLC25A3
SLC25A4
HSPA9
HSP90B1
NDUFS1
SLC25A1
SLC25A13
Receptor Activity
LGALS3BP
SSR4
SSR1
LBR
ATP5A1
MRPS27
ACOT9
SLC25A12
ICT1
MRPL46
NCL
DDX17
PCNA
ILF3
Translation
Regulator Activity
RBM14
DHX9
HNRNPC
PHB2
SLC25A10
MRPL49
TECR
SMARCA4
ATP5B
SNRNP200
UBR4
MRPL12
HNRNPDL
TXN
HNRNPR
Transcription Factors
SRSF1
SMARCE1
UBAC2
PCNA
DNA/RNA Binding Proteins
Enzyme Regulator Activity
BAG2
PSMC6
PSMD11
PSMD7
PSMC2
PSMD1
KIAA0368
PSMC3
PSMC4
PSMD3
FAR1
PSMD13
PSMC1
PSMD2

Figure 20: *Network of cytosolic proteins identified by Flag-Gβ1, Single-TAP Gβ1, and Split TAP-Gβ1 in two or more experiments*. Proteins organized by molecular function based on Panther groupings. Protein interactions obtained from String. Proteins coloured in yellow indicated proteins identified only in untreated conditions, proteins in blue indicate proteins identified only in treated (1mM carbachol) conditions, proteins in green indicate proteins found in both conditions. Proteins identified as Gβγ interactors with TAP tagged $G\beta_x\gamma_x$ and identified by mass spectrometry.

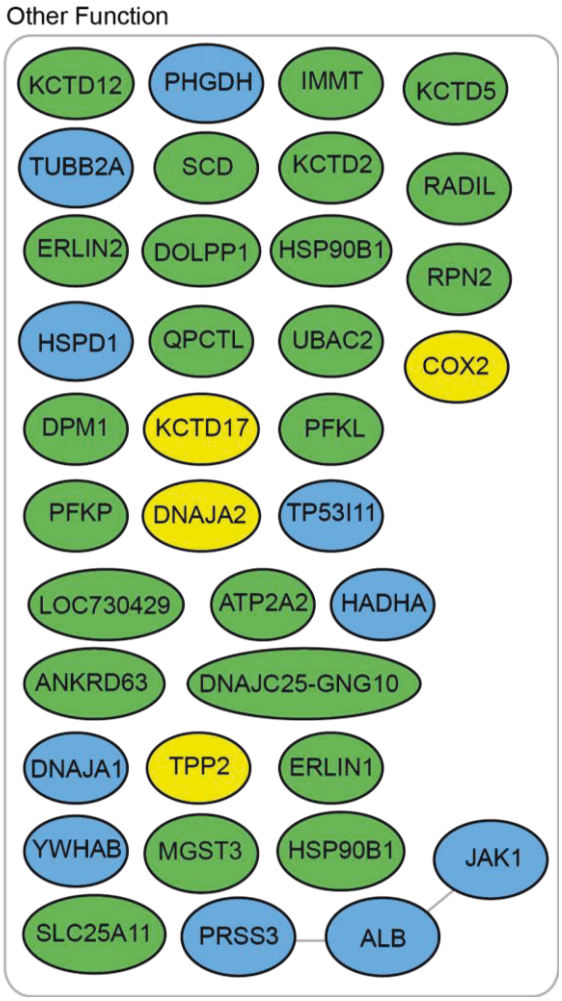


Figure 21: *Other cytosolic proteins identified by Flag-Gβ1, Single-TAP Gβ1, and Split TAP-Gβ1 in two or more experiments that did not fit into Panther classifications*. Protein interactions obtained from String. Proteins coloured in yellow indicated proteins identified only in untreated conditions, proteins in blue indicate proteins identified only in treated (1mM carbachol) conditions, proteins in green indicate proteins found in both conditions. Proteins identified as Gβγ interactors with TAP tagged $G\beta_x\gamma_x$ and identified by mass spectrometry.

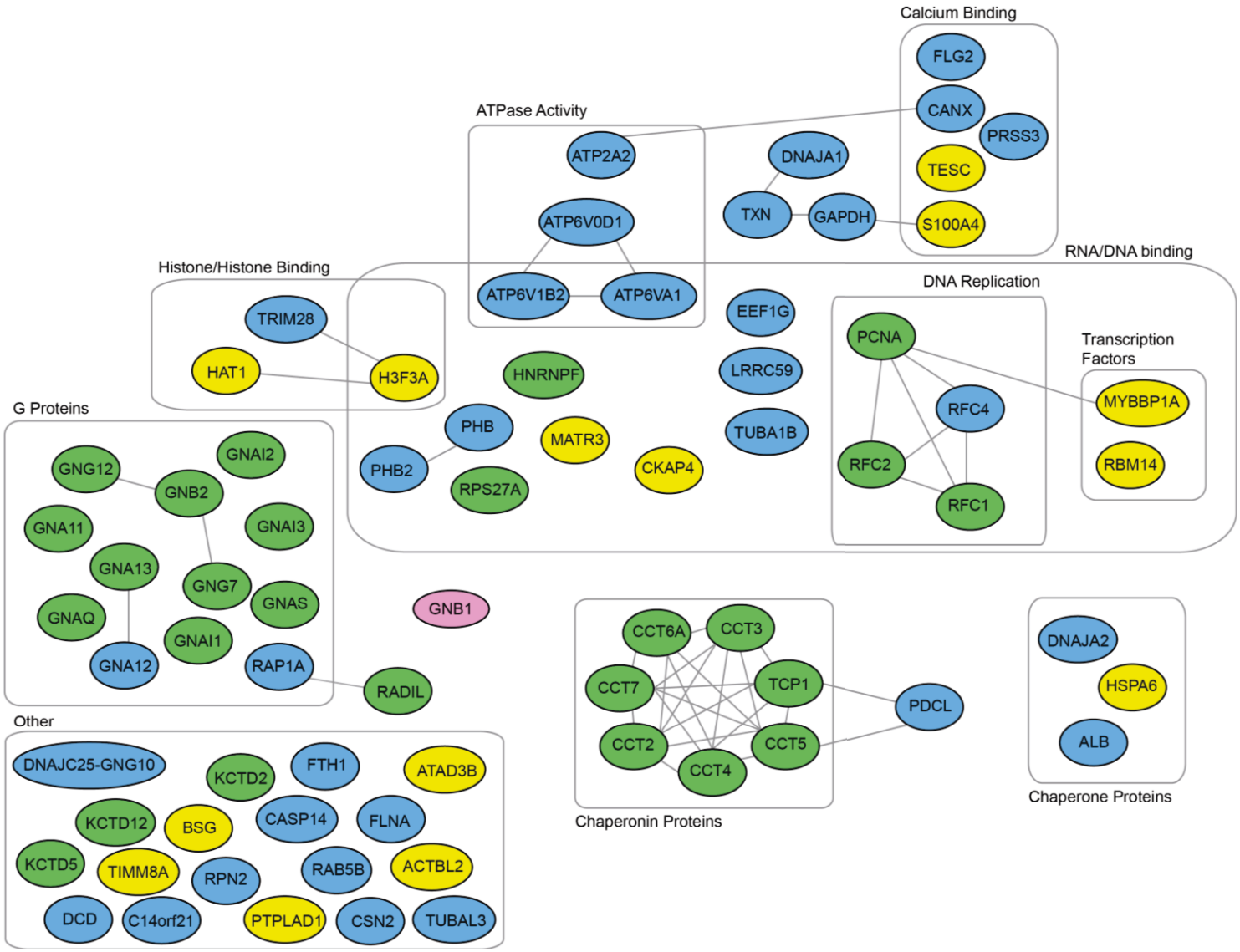

Calcium Binding
FLG2
CANX
PRSS3
TESC
S100A4
ATPase Activity
ATP2A2
ATP6V0D1
ATP6V1B2
ATP6VA1
DNAJA1
TXN
GAPDH
RNA/DNA binding
Histone/Histone Binding
TRIM28
HAT1
H3F3A
HNRNPF
PHB
PHB2
MATR3
RPS27A
CKAP4
EEF1G
LRRC59
TUBA1B
DNA Replication
PCNA
RFC4
RFC2
RFC1
Transcription Factors
MYBBP1A
RBM14
G Proteins
GNG12
GNAI2
GNB2
GNA11
GNAI3
GNA13
GNG7
GNAS
GNAQ
GNAI1
GNA12
RAP1A
RADIL
GNB1
Other
DNAJC25-GNG10
KCTD2
FTH1
ATAD3B
KCTD12
BSG
CASP14
FLNA
KCTD5
TIMM8A
RPN2
RAB5B
ACTBL2
DCD
C14orf21
PTPLAD1
CSN2
TUBAL3
CCT6A
CCT3
CCT7
TCP1
CCT2
CCT5
CCT4
PDCL
Chaperonin Proteins
DNAJA2
HSPA6
ALB
Chaperone Proteins

Figure 22: *Network of nuclear proteins identified by Flag-Gβ1, Single-TAP Gβ1, and Split TAP-Gβ1 in two or more experiments*. Proteins organized by molecular function based on Panther groupings. Protein interactions obtained from String. Proteins coloured in yellow indicated proteins identified only in untreated conditions, proteins in blue indicate proteins identified only in treated (1mM carbachol) conditions, proteins in green indicate proteins found in both conditions. Proteins identified as Gβγ interactors with TAP tagged $G\beta_x\gamma_x$ and identified by mass spectrometry.

In both cytosol and nucleus under treated and untreated conditions, Gβ1 bound to G proteins, enzyme regulator proteins, and chaperone proteins.

In the cytosol, carbachol treatment lead to an increased association of Gβ1 with proteins associated with RNA/DNA, including transcription factors (ILF, SMARCE1), ribosomal proteins (MRPL46, MRPL49, MRPL12), heterogenous ribonucleoproteins (HNRNPDL, HNRNPR, HNRNPC) and other RNA binding proteins (SNRNP200, SRSF1, RBM14, DHX9, DDX17). Our data suggests that carbachol treatment increases the association of Gβγ with proteins involved with the stability and processing of RNA in the cytosol. It is also interesting to note that carbachol stimulation leads to association of Gβ1 with immature colon carcinoma transcript 1 (ICT1), a protein with codon nonspecific and aminoacyl-tRNA hydrolase activity that is active in mitochondria[243]. ICT1 interacts with a number of other mitochondrial proteins pulled down in our screen, suggesting a role for Gβγ in mitochondrial translation following carbachol stimulation. In addition, carbachol treatment led to the association of Gβγ with the 26S proteasome regulatory enzymes PSMC 1, 2, 4, 6 and PSMD 1, 2, 3, 7, 11, and 13.

The association of Gβγ with these proteins led us to compare Gβγ association with proteins identified in screens of ubiquitin ligase-binding proteins[252] (**Table 12**). Under untreated conditions, Gβγ was associated with 23 proteins in the cytosol and 22 proteins in the nucleus that have been found associated with cullin E3 ligases. When treated with carbachol, the number of proteins increased to 60 in the cytosol and 31 in the nucleus. It is worth mentioning that overall, the number of proteins found under treated conditions increased relative to untreated (untreated cytosol 92, untreated nuclei 59, treated cytosol 161, treated nuclei 93).

In the nucleus, carbachol treatment increases Gβγ association with proteins with ATPase activity, some of which can bind RNA/DNA (ATP2A2, ATP6V0D1, ATP6V1B2, ATP6VA1). However, following carbachol treatment, proteins isolated from the nucleus appear to have more links with DNA binding proteins than with RNA binding proteins or those involved in transcriptional regulation. Before stimulation, Gβ1 interacted with histone acetylase 1 (HAT1), histone 3.3 (H3F3A), MTR3, cytoskeletal associated protein 4 (CKAP4), and the transcription factors

Myb-binding protein 1A (MYBBP1A) and RBM14. These proteins are all involved in transcriptional regulation, and suggest that carbachol treatment shifts Gβγ function in the nucleus away from transcriptional regulation *per se*. This trend is more striking when proteins pulled down only once are included. It is interesting that the trend towards transcriptional regulation following carbachol treatment occurs in the cytosol but not the nucleus. Carbachol treatment may be differentially regulating Gβγ signalling in the nucleus compared with the cytosol or helping to sequester transcriptional regulators. This may suggest further independence of Gβγ subunits in the nucleus from cytosolic Gβγ.

4.5 Discussion

In this study, we identified numerous candidate interactors for Gβγ subunits in the nucleus, in addition to identifying a number of novel interactors in the cytosol and other organelles. These interactors came from proteins found in the soluble fraction of HEK 293 cells. Our results support the involvement of Gβγ in transcriptional regulation, which has been reported elsewhere[124,127]. Further, association of Gβγ with mitochondrial proteins may identify a broader role for Gβγ in regulation of mitochondrial energy production, beyond its previously identified functions[71]. In addition, we show that GPCR stimulation alters the profile of Gβγ-associated proteins. Cytosolic and nuclear proteins are regulated differently, based on distinct changes in Gβγ binding partners caused by carbachol.

It is unlikely that carbachol, as a charged molecular, enters the cell, but is rather activating mACh receptors expressed on the outside of the cell. Therefore this suggests that by activating cell surface GPCR signalling pathways we can alter G protein signalling within the cell and nucleus. For communication to originate from the outside of the cell and to trigger changes within the nucleus, this suggests either the movement of proteins into nucleus following cell surface activation, or lines of communication that cross the nuclear membrane, such as through receptors on the internal and external nuclear membrane. This second suggestion is more complex, given the double membrane structure of the nucleus, as it would

require the activation of two receptors on the nuclear membrane in addition to the availability of a signalling molecule that could transmit a signal from one to the other. To support the first hypothesis, our lab has detected fluctutations in the concentration of Gβγ in and out of the nucleus, however in our hands this movement was not dependent on a range of GPCR agonist stimulations (**Figures 33, 34 amd 36**, discussed further in Chapter 5). Therefore, at this point, it is unknown whether there is a protein that passes information following cell surface GPCR activation to nuclear G proteins to change their signalling.

Our data offers a new way of understanding the function of nuclear G proteins. We were also able to capture a distinct set of Gβγ binding partners by using a specific Gβγ dimer combination. Using Gβ1γ7 as bait, we were able to identify a number of transcription factors and DNA/RNA binding proteins that associated with this dimer combination. Interestingly, there were other Gβ and Gγ proteins pulled down from nuclear lysates with Gβ1γ7 (**Table 7**). This may suggest that some protein complexes require more than one Gβγ dimer, and that there may be a requirement for more than one type of Gβγ dimer. In this model Gβγ may bind to multiple surfaces to link proteins into larger macromolecular units. Additionally, it could also mean that the Gβγ itself may be composed of more than one β and one γ, resulting in mutlimeric Gβγ complexes.

We also found that Gβγ interacted with a number of E3 ligase associated proteins. An analysis of the effect of GPCR activation on ubiquitination found an increase in protein ubiquitination following activation[263]. Although these results do not specifically include the mACh receptor, it is possible that this is what is occurring here. The interaction of Gβγ with these associated proteins may suggest that Gβγ is involved in the regulation of this process. The problem of whether Gβγ has a regulatory role with ubiquitination, or is being ubiquitinated itself, is mirrored in Gβγ's interaction with ribosomal proteins. In this case, however, a nascent Gβ subunit that has not yet bound Gγ, would not be able to pull down ribosomal proteins as is seen in the split-TAP condition (**Appendix**).

The interaction of Gβγ with Rap1A, Radil and a kinesin super family member protein draws a parallel with results suggesting that Gβ2 and Gγ2 binds

Radil and Rap1a to promote cell matrix adhesiveness[203]. Rap1A is Ras-related small G protein that functions as a molecular switch between GTP-bound active and GDP-bound inactive states, facilitated by guanine nucleotide exchange factors (GEFs) and GTPase activator proteins (GAPs)[203,264]. It integrates extracellular and intracellular signalling in cell adhesion and integrin activation. In addition, the kinesin super family (KIF) protein KIF14, was found to bind to Radil, and inhibit the interaction of Rap1a and Radil[264]. In our screen, we pulled down kinesin super family member 7 (KIF7), which primarily acts as a repressor of hedgehog signalling in myocyte differentiation[265]. KIF7 can interact with GLI transcription factors and is able to suppress GLI-dependent transcription through the regulation of their processing and protein levels[265]. This may suggest a role for Gβγ in an alternate regulatory role of GLI transcription factors in tandem with KIF7. This interaction may involve Rap1a and Radil.

The interaction of KIF7 with Gβγ suggests that its involvement in regulating transcription may occur through regulating the levels and abundance of transcription factors in the nucleus, in addition to affecting their activity in the nucleus (as is true in other examples)[44,125,128]. This may indicate that cytosolic and nuclear Gβγ play different roles in regulating transcription, the cytosolic counterpart involved in signalling pathways originating from the cell surface, while nuclear Gβγ could directly involved with the transcriptional partners themselves. If this is true, this study identifies Gβγ as a broad signalling regulator and integrator, much in the same way that GPCRs integrate extracellular signals to control cell function depending on where they are localized.

This study also highlights the importance of careful consideration of protein tags when designing fusion constructs. Here, it appears that the larger tag found on TAP tagged Gβγ excludes a number of protein interactions, similar to what we found when purifying the β_2AR using TAP and Flag purification[195]. Tags that are under 10 amino acids in length, such as the Flag tag we used, may be advantageous to larger tags such as the calmodulin tag. That said there is obvious benefit to using the split-TAP method in this case, indicated by the changes in protein interactions observed when studying specific Gβγ pairs. To address this

problem, further split-TAP constructs should be designed using smaller tags to allow for the localization of the tagged protein in endogenous situations, and to prevent the exclusion of protein interactions due to steric hindrance. However, an intrinsic problem of altering proteins via the addition of epitope tags may in itself change the composition of bound proteins through the formation of a novel binding site between the tag and bait proteins. This highlights the requirement of validation of all protein interactors using endogenous, untagged proteins. Although *in situ* this is limited by the availability of good antibodies, it is nevertheless an important step that must be taken. Further methods may include attempting to reconstitute protein interactions outside of the cell and to detect the resulting protein aggregates.

5 General Discussion and Future Directions

This thesis provides evidence for G protein function in the nucleus in HEK 293 cells. The results presented in Chapters 3 and 4 detail putative $G\beta_x\gamma_x$ binding partners in both the cytosol and nucleus that need to be further validated. The emerging picture from my results is that Gβγ has a significant functional role in the nucleus as well as the cytosol in transcription and potentially translation. Additionally, interactions with mitochondrial and endoplasmic reticulum proteins provide insight into Gβγ interactions in other locations within the cell. These results highlight the diverse signalling interactions of Gβγ subunits in different subcellular compartments. In addition, it highlights a novel notion in GPCR signalling, that there may be distinct pools of Gβγ subunits found in different locations throughout the cell. Whether localization of these pools is dependent on particular Gβγ dimer combinations, processing of Gγ, or other activation of surface signalling pathways is yet to be determined.

A consideration regarding data analysis was the exclusion of proteins found in less than two out of three experiments. This allowed us to focus on proteins consistently isolated with Gβγ, however it may have removed some interesting interactors and categories of interactors. For example, this criteria removes importin and exportin proteins that were pulled down with Gβγ. It also removes cytosolic ribosomal proteins, and a number of RNA polymerase II (RNAPII)-associated proteins. This might indicate that these are non-specific interactors, however, Gβγ also pulled down mitochondrial ribosome proteins, suggesting its ability to couple with ribosomal proteins. Although there are some differences between mitochondrial and cytosolic ribosomes[266], these differences are minor, and since Gβγ was able to pull down both types, it is unlikely that any sequence differences affected binding to Gβγ subunits. Further validation of these interactions will help to determine whether this simply represents an artefact of the protocol, or whether Gβγ is interacting with these proteins.

In addition to studying the proteins interactions of Gβγ subunits in the nucleus, we also approached Gβγ signalling in the nucleus by investigating

combination-specific interactions. This both answered questions raised following our initial MS analysis, and created areas for further study.

5.1 Interaction of Gβ with RNA polymerase II

Based on our analysis, a number of proteins bound to Gβγ in the nucleus are involved at some level in transcriptional regulation. This, and additional evidence from the lab (Shahriar Khan, Sarah Gora unpublished data), led us to investigate the interaction of RNA polymerase II with Gβγ.

To assess the interaction of Gβ with ribonucleic acid (RNA) polymerase II (RNAPII) I compared Gβ and Gβγ protein interactors from TAP and Flag screens with known interactors of RNAPII from the literature[267-272] (**Table 13**). I found that Gβ and Gβγ interacted with four of the main RNAPII subunits (POLR2A, POLR2D, POLR2E, POLR2H; each pulled down once) in addition to many RNAPII-interacting proteins (**Appendix**). In addition, when TAP purifications were blotted for RNAPII, RNAPII subunits were associated with TAP-Gβ1 streptavidin bead elutions (**Figure 23**). RNAPII binding proteins found bound to Gβγ include core RNAPII subunits, ribosome proteins, splicing factors, chaperone proteins, tubulin, 26S proteasome proteins, replication factors, DNA helicases, karyopherins, and transcription factors. No RNAPII was detected in calmodulin elutions, presumably because many protein interactions were lost due to multiple washes. This corroborates data from our lab suggesting that treatment of HEK 293 cells with carbachol leads to an increased association of Gβγ with RNAPII (Shahriar Khan, unpublished data). Based on this data, carbachol stimulation for five minutes followed by a 45-minute wash out period resulted in significant association of Gβγ and RNAPII. To quantify this interaction, Shahriar Khan used co-immunoprecipitaton followed by western blotting and densitometric analysis.

Table 13: *Overlapping interactors from screen with RNAPII screens*. Proteins pulled down with Gβ1 two out of three times[267,269-271]. RNAPII interactors were compared to Gβγ interactors in each condition. Overlapping interactors were organized by condition and fraction in which they were found.

Cytosol		*Nucleus*	
Untreated	1mM carbachol	Untreated	1mM carbachol
		ACTBL2	
	ALB		ALB
	BAG2		
			CASP14
CCT2	CCT2	CCT2	CCT2
CCT3	CCT3	CCT3	CCT3
CCT4	CCT4	CCT4	CCT4
CCT5	CCT5	CCT5	CCT5
CCT6A	CCT6A	CCT6A	CCT6A
CCT7	CCT7	CCT7	CCT7
	DNAJA1		DNAJA1
	HNRNPC		
	HNRPDL		
		HSPA6	
	HSPA9		
	HSPD1		
		MATR3	
		MYBBP1A	
PFDN4			
	PFDN5		
	PHGDH		
	PSMC1		
	PSMC2		
PSMC3			
	PSMC4		
	PSMD1		
	PSMD11		
PSMD13	PSMD13		
	PSMD2		
	PSMD3		
		RFC2	RFC2
			RFC4
	SMARCA4		
	SMARCE1		
	SRSF1		

TCP1	TCP1	TCP1	TCP1
TUBA1A	TUBA1A		
	TUBA1B		TUBA1B
	TUBB2A		
	TUBB3		
	TUBB6		
	VBP1		
	XPO1		
	YWHAZ		

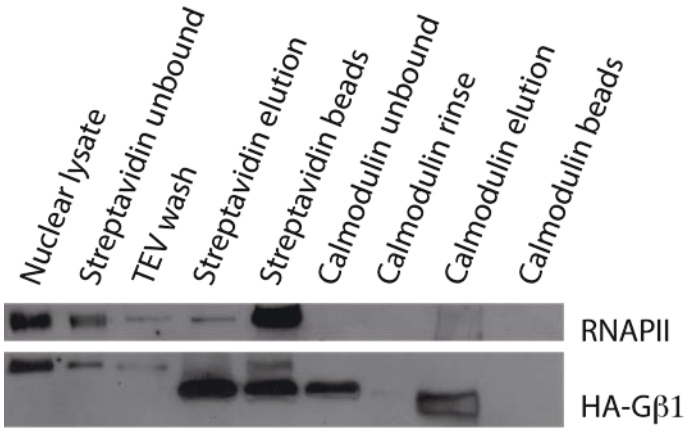


Figure 23: *Western blot of tandem affinity purification samples blotted with RNAPII*. HA-Gβ1 stably expressed in HEK 293 cells was bound and eluted from streptavidin Sepharose™ and calmodulin Sepharose™ beads in the TAP protocol. A 10% sample from each step of the TAP (see Chapter 2 for method) was run on a 10% polyacrylamide gel at 120V for 20 minutes, followed by one hour at 160V. Proteins were transferred to a polyvinyl difluoride (PVDF) membrane at 100V for one hour. The membrane was blotted with skim milk (5% skim milk, 1.37M NaCl, 27mM KCl, 100mM KH_2HPO_4, 18mM KH_2PO_4). RNAPII (1:1000, Millipore, Darmstadt, Germany; CTD4H8), HA (1:1000, Roche Diagnostics; 3F10), and α-mouse (1:20,000, Sigma-Aldrich, St. Louis, MO; A9917) were each blotted for one hour. Membranes were oxidized using Western Lightning Plus ECL (PerkinElmer Inc., Waltham, MA; NEL103001 EA) and autoradiography film was exposed. Data represents a single preliminary experiment.

I followed up on these experiments by treating Flag-Gβ1 or TAP-Gβ1γ7-transfected HEK 293 cells with carbachol, isolating the nuclei, and sending the samples for MS to determine what proteins Gβγ interacted with under these conditions. Following 1 mM carbachol treatment, I found an increased number of RNAPII-interacting proteins were associated with Gβ and Gβγ in the cytosol and nucleus (**Table 13**; 5 additional unique proteins in the nuclear fraction, 26 additional unique proteins in the cytosolic fraction). Carbachol treatment did not disrupt the processing of Gβγ, as it still bound CCT and TCP1 proteins. It is likely that these proteins are also involved in the processing of RNAPII[273]. Since chaperonin proteins are involved in the proper folding of both Gβγ and RNAPII, Gβγ interactions are likely separate from the potential regulatory role Gβγ plays with RNAPII. In the nucleus, treatment with carbachol decreased the association of Gβγ with ribosomal proteins, transcription factors, and splicing factors, when comparing all proteins pulled down by Flag-Gβ1 or TAP-Gβ1γ7. The increased association with RNAPII, but decreased association with RNAPII-associated proteins in this context, may indicate that Gβγ is bound to different proteins at different stages of transcription. Different factors are associated with RNAPII when it is found in the pre-initiation complex, in the abortive initiation and paused RNAPII stage, the productive transcription initiation stage, the transcription elongation stage, or the transcription termination stage[274]. Based on the data presented here, we cannot yet determine at which stage Gβγ is associated with RNAPII binding proteins. Since we only identified four core RNAPII proteins interacting with Gβγ in one experiment, and pending further validation, at this stage we cannot yet determine whether Gβγ interacts with core RNAPII proteins or RNAPII interacting proteins. There are many different types of proteins that are involved in transcriptional regulation, that include but are not limited to transcription factors. Histone and nucleosome remodelling proteins function to change the DNA architecture allowing more or less transcription. In addition, factors that can positively or negatively modulate core promoter elements can influence transcription[274]. Therefore the effect of Gβγ subunits on transcriptional regulation may be due to interactions with proteins generally involved with DNA that allow

transcription to occur, rather than with transcription factors *per se*. Further investigation into RNAPII and Gβγ will reveal which proteins Gβγ binds to directly, and at what stage of transcription this association occurs. Understanding the interaction of Gβγ with RNAPII will help further understand Gβγ function in the nucleus, and the general role of GPCR signalling in transcriptional regulation by G proteins.

5.2 Movement of Gβγ into the nucleus

I also investigated how Gβγ subunits enter and exit the nucleus. Based on our MS results, Gβγ interacts with a number of importin and exportin proteins, which would theoretically enable its entry and/or exit into the nucleus (**Table 14**). In addition, Gβγ also interacts with a number of proteins such as heterogenous ribonucleoproteins (hnRNP) and spliceosome components, which can shuttle in and out of the nucleus (**Table 14**). These interactions suggest that Gβγ enters the nucleus through the nuclear pore in association with one or more chaperones. To further investigate the movement of Gβγ subunits into the nucleus we compared the localization of prenylated and unprenylated Gγ subunits, blocked nuclear export using leptomycin B, and activated cell surface receptors to determine whether Gβγ nuclear localization could be altered.

Table 14: *Proteins pulled down with Gβ1 and Gβ1γ7 that can shuttle in and out of the nucleus*. Proteins identified as Gβγ interactors with TAP tagged $G\beta_x\gamma_x$ and identified by mass spectrometry. Shuttling proteins identified through literature searches and online databases.

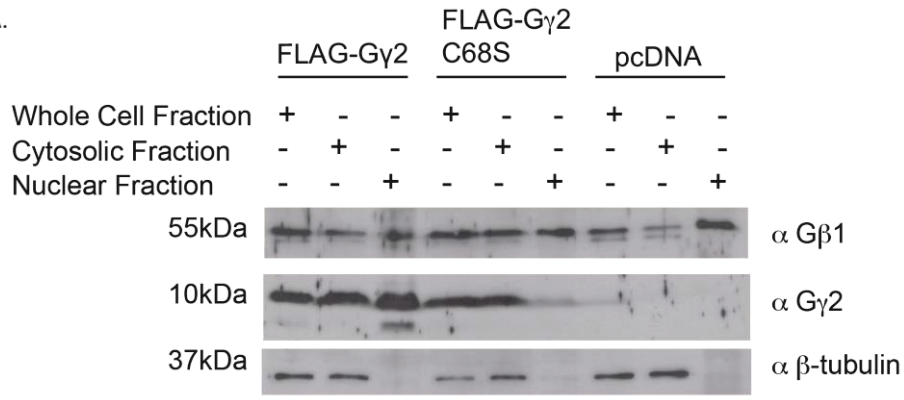
Split TAP				Single TAP				Flag			
Cytosol	Cytosol 1mM carbachol	Nuclei	Nuclei 1mM carbachol	Cytosol	Cytosol 1mM carbachol	Nuclei	Nuclei 1mM carbachol	Cytosol	Cytosol 1mM carbachol	Nuclei	Nuclei 1mM carbachol
HNRNPC	HNRNPC				HNRNPC	HNRNPC	HNRNPC				
	HNRNPF	HNRNPF	HNRNPF	HNRNPF		HNRNPF					
	HNRNPR				HNRNPR						
	HNRPDL					HNRPDL					
					HNRNPUL2		HNRNPUL2				
								IPO7	IPO7		
									IPO8		
								IPO9			
										KPNA2	
					SNRNP200						
					SNRNP70						
	SNRPC										
									SNRPB		
					SNRPD2						
	SNRPD3				SNRPD3						
					SNRPE						
					SNRPG						
					SNRPN						
								XPO1	XPO1		
									XPO7		

5.2.1 *The role of prenylation in Gβγ nuclear localization*

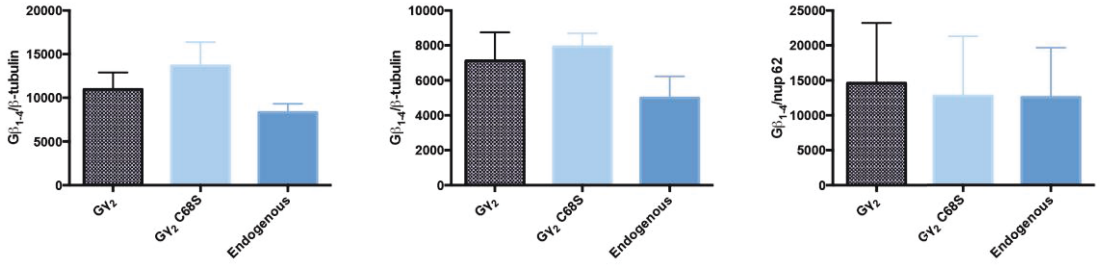
Gγ subunits are modified with a lipid moiety, allowing insertion into the plasma membranes[34,181]. Although the majority of Gγ subunits are prenylated, there is a small pool that is unprenylated, in additional to pools of Gγ subunits that are alternately modified from the standard geranylgeranyl and farnesyl modifications[3]. Therefore, in order to assess whether prenylation was involved in targeting of Gβγ to the nucleus, a Gγ2 prenylation mutant (C68S) was used. This mutant contains a serine in place of the cysteine in the CAAX motif of Gγ2, preventing prenylation while preserving association with Gβ subunits[275]. Gγ2, and Gγ2C68S were transfected into HEK 293 cells and nuclei were isolated. Levels of Gγ2 and Gγ2C68S in the whole cell fractions were not statistically different (normalized to β tubulin), however, significantly more wild type Gγ2 was found in the nucleus compared with Gγ2C68S (Paired T test, $p<0.05$) (**Figure 24**). Although transfected Gγ2 levels were altered in the nucleus compared with the whole cell and cytosol, Gβ1-4 levels remained the same. This likely means that the amount of Gγ2 transfected was insufficient to alter the majority of Gβ subunits found in the cell.

To further investigate the precise localization of Gγ within the cell, confocal microscopy was used. Upon analysis, we found that confocal analysis supported evidence from western blot (**Figure 24**, **Figure 25** and **Figure 26**). However, transfected Gγ2 did not alter the localization of Gβ1-4. Based on these results we conclude that normal prenylation of Gγ is important for its localization to the nucleus. This indicates that the nuclear localization of Gβγ must be a regulated process that requires the proper processing of Gβγ. In contrast, other studies suggest that in the absence of a prenyl moiety on Gγ2, Gβγ translocated into the nucleus but this may have been due to other effects of the statins used to block prenylation[183].

A.


FLAG-Gγ2
FLAG-Gγ2 C68S
pcDNA
Whole Cell Fraction + - - + - - + - -
Cytosolic Fraction - + - - + - - + -
Nuclear Fraction - - + - - + - - +
55kDa
α Gβ1
10kDa
α Gγ2
37kDa
α β-tubulin

B.


Gβ1-4/β-tubulin
Gγ2
Gγ2 C68S
Endogenous
Gβ1-4/nup 62

C.

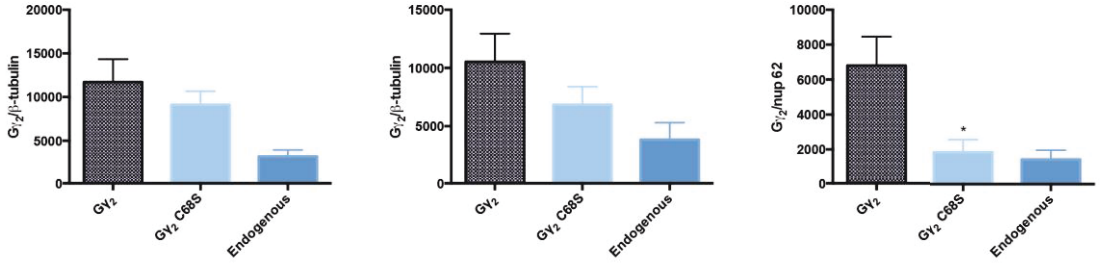
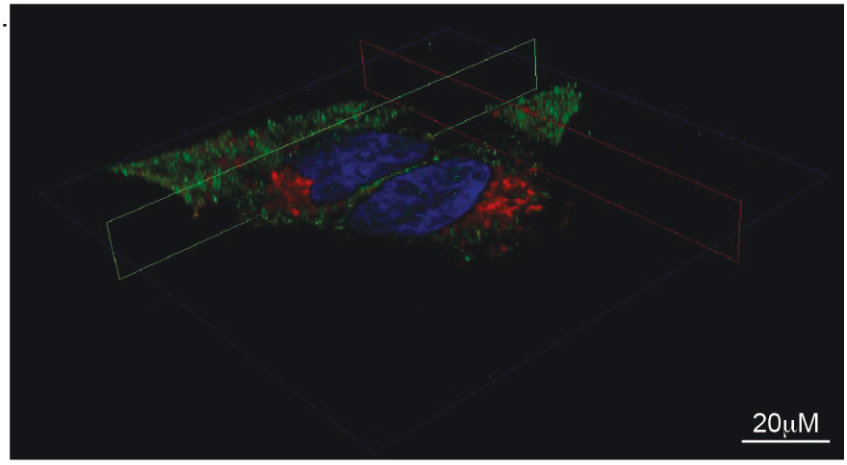
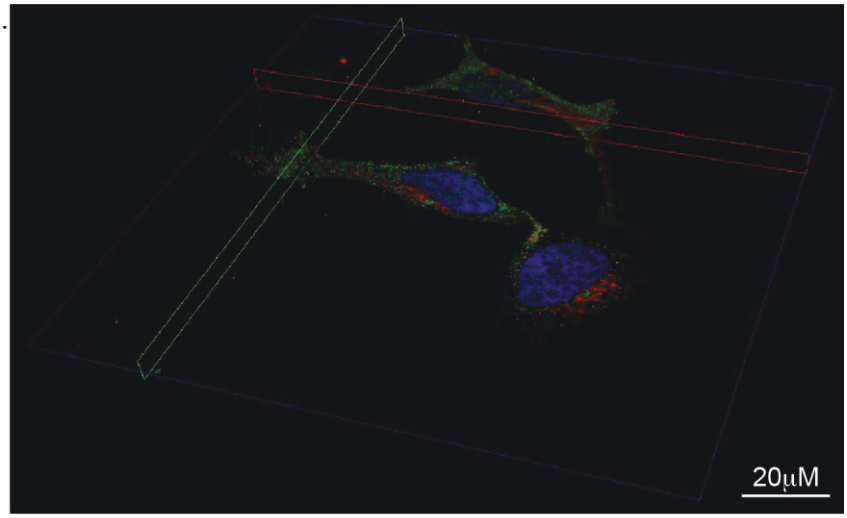

Gγ2/β-tubulin
Gγ2
Gγ2 C68S
Endogenous
Gγ2/nup 62
*

Figure 24: *Western blot and quantification of Gγ2 or Gγ2 C68S prenylation mutant transfected into HEK 293 cells*. A) Western blot showing expression of Gγ2 and Gγ2 C68S in whole cell, cytosolic and nuclear fractions of HEK 293 cells. B) Quantification of Gβ1 in each fraction (from left to right, whole cell, cytosol and nuclear fractions) and C) Quantification of Gγ2 in each fraction (from left to right, whole cell, cytosol and nuclear fractions). 1.6µg of Gγ2 or Gγ2C68S plasmids (Gγ or Gγ2 C68S were inserted into the pIRES-Hyg3-CBP-FLAG vector using EcoRI and BamHI) were transiently transfected into $1.2x10^6$ HEK 293 cells using 5µL of lipofectamine 2000®, according to manufacturers instructions. Two days later, cells were lysed using cell lysis solution (320mM sucrose, 10mM HEPES NaOH, 5mM $MgCl_2$, 1mM DTT, 1mM PMSF, 1mM Na_3VO_4, 1% Triton-X100) for six minutes. The cell lysis was layered over a sucrose cushion (1.8M sucrose, 10mM HEPES NaOH, 5mM $MgCl_2$, 1mM DTT, 1mM PMSF, 1mM Na_3VO_4, 1% Triton-X100) and centrifuged at maximum speed for twenty minutes to isolate nuclei. Whole cell, cytosolic and nuclear fractions were run on a gel and blotted for protein expression. Samples were run on a 15% polyacrylamide gel at 120V for 20 minutes, followed by one hour at 160V. Proteins were transferred to a polyvinyl difluoride (PVDF) membrane at 100V for one hour. The membrane was blotted with 5% skim milk (5% skim milk, 1.37M NaCl, 27mM KCl, 100mM KH_2HPO_4, 18mM KH_2PO_4), followed by 1:1000 Gβ (1:1000, Gβ T20 Santa Cruz Biotechnology, Dallas, Texas; SC378) or Gγ2 (1:500, Gγ2 A16 Santa Cruz Biotechnology, Dallas Texas; SC374) for one hour, and α-mouse (1:20,000, Sigma-Aldrich, St. Louis, MO; A9917) or α rabbit (1:20,000, Sigma-Aldrich, St. Louis, MO; A0545) for one hour. Membranes were oxidized using Western Lightning Plus ECL (PerkinElmer Inc., Waltham, MA; NEL103001 EA) and autoradiography film was exposed. ImageJ was used to quantify band intensities, and the resulting values were graphed using GraphPad Prism. One-way T test with Bonferroni correction ($p<0.05$) was used to determine significance. Graphs represent 3-5 independent experiments.

A.



B.



C.

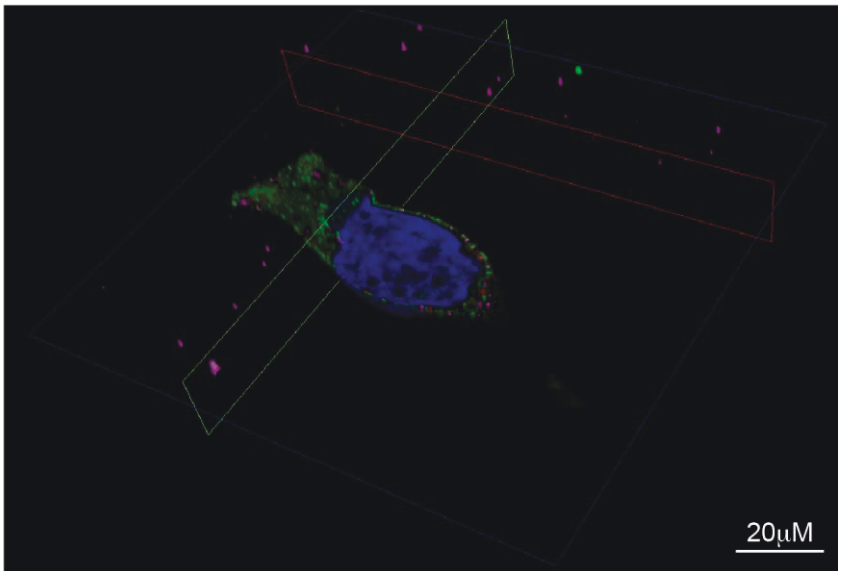


Figure 25: *Confocal images of HEK 293 cells transfected with Gγ2 or Gγ2 C68S prenylation mutant*. A) Untransfected cells, B) Gγ2-transfected cells, and C) Gγ2 C68S-transfected cells were quantified using IMARIS (Bitplane, USA). The nucleus is stained blue, the whole cell red, Gβ1-4 green and Gγ2 in pink. 250,000 cells per well were plated on glass 22x22 glass slides (Fisher Scientific, Ottawa, ON; 12-543-B) in a six well plate and treated with 0.1mg/mL Poly-L-ornithine Hydrobromide (Sigma-Aldrich, St. Louis, MO; P3655) two days before use. $1.2x10^6$ HEK 293 cells were transfected with 1.6µg Gγ or Gγ2C68S (Gγ or Gγ2 C68S were inserted into the pIRES-Hyg3-CBP-FLAG vector using EcoRI and BamHI) using 5µL of lipofectamine 2000® according to manufacturers instructions. Cells were fixed on glass slides using 2% paraformaldehyde and permeabilized with 0.1% Triton X100. Cells were blotted in 1% bovine serum albumin. Primary antibodies (Gγ2 A16 Santa Cruz Biotechnology, Dallas Texas; SC374 and Gβ T20 Santa Cruz Biotechnology, Dallas, Texas; SC378) were used at a dilution of 1:200, Secondary antibody (α mouse Alexa Fluor® 488, Life Technologies; A11029 and α rabbit Alexa Fluor® 555, Life Technologies; A21428) at 1:500. Whole cell and nuclei were stained with Wheat Germ Agglutinin, Alexa Fluor® 647 (Life Technologies, W32466) and Hoechst 33342 (Life Technologies, H3570), respectively. Cover slips were secured to slides using PermaFluor™ (Thermo Scientific, TA-006-FM). Slides were imaged using Leica Confocal Scanning Microscope TCS SP8. A 63x/1.4NA objective lens airy 1, zoom 1.42. An average of 20 Z stacks were taken depending on cell size. Images were produced using 3D rendering by Leica Application Suite X software. Slices were taken to demonstrate protein expression within the cell. Data are representative of three independent experiments.

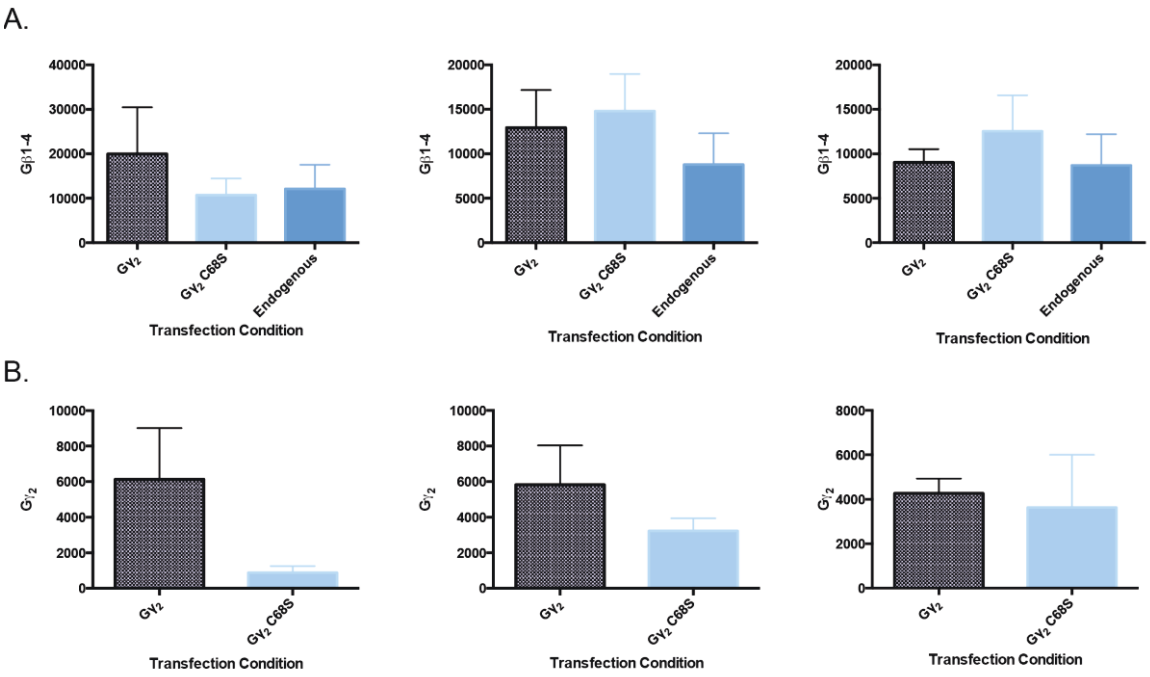


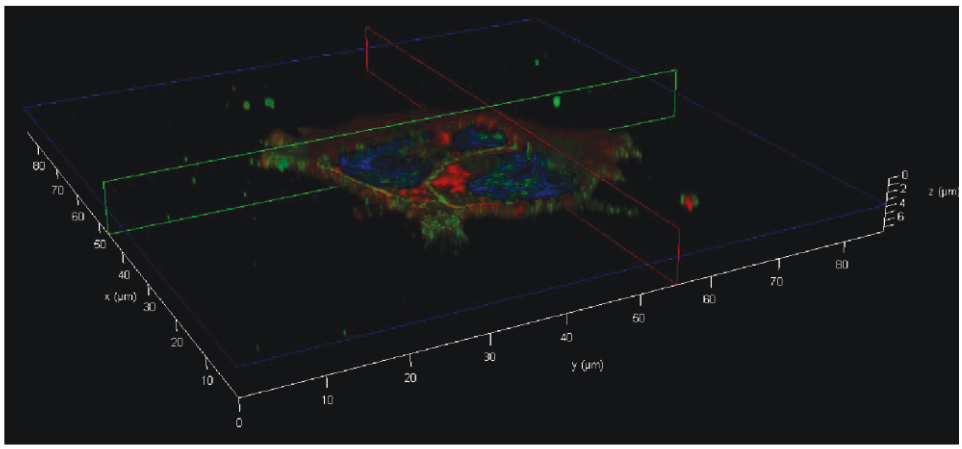
Figure 26: *Quantification of confocal images of HEK 293 cells transfected with Gγ2 or Gγ2 C68S prenylation mutant*. A) Quantification of Gβ1-4 levels. B) Quantification of Gγ2 levels. Confocal images were analyzed using IMARIS (BitPlane, USA). Images were rendered in 3D and volume calculations were made for whole cell and nuclear compartments using Wheat Germ Agglutinin and Hoechst staining to define the whole cell and nuclear volumes respectively. The cytosolic volume was determined by subtracting the nuclear volume from the whole cell volume. Intensity of staining for Gβ was determined by calculating the number of voxels within each volume. The concentration was calculated by dividing the voxels by the total volume in each compartment. This value was averaged across all images. Background levels were calculated from cells stained only with secondary antibody. These values were subtracted from concentration values after averaging, and the resulting concentrations were graphed in GraphPad Prism. Data analysed by unpaired T tests and Mann Whitney U tests were variance unequal (Bonferroni correction). Results include data from three independent experiments.

Western blotting, which uses a larger sample of cells, perhaps ensures that sampling errors do not bias results. Confocal images reveal what crude lysis followed by western blot cannot: that transfection efficiency is lower than 100%. This is particularly important considering the technique of confocal microscopy. The benefit of this technique is the ability to visualize protein expression in high detail within the cell. However, in order to do this, there is a limitation on how many cells can be imaged. Therefore, the cells imaged may not fully represent the full population even when selected at random. To illustrate this point, **Figure 27** shows three different cells, all transfected with Gγ2C68S. It is evident from the images that some cells have been transfected with more Gγ2 C68S than others, and that in some cases there appears to be more Gγ2 in the nucleus than in other cells. Therefore it does appear that Gγ2 C68S increases the concentration of Gβγ in the nucleus, however analysis of the images did not reveal a difference between conditions (**Figure 26**). In these initial experiments, transient transfection was used, however following these experiments, and to avoid the problem discussed above, cells were stably transfected.

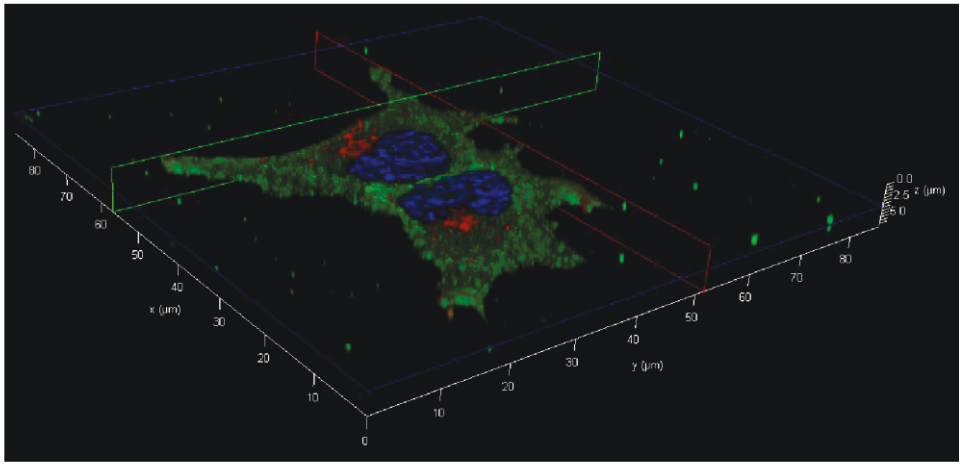
If prenylation excluded Gβγ from the nucleus, one would expect that there would be an identifiable population of unprenylated Gβγ present in the cell, especially given the high levels of Gβ endogenously expressed within cell nuclei (

Figure 6) Studies examining prenylation of all Gγ subunits expressed in bovine brain extracts found that the majority of Gγ subunits were prenylated as expected (either farnesylated or geranylgeranylated). Unprenylated forms of Gγ2 and Gγ7 were detected at <1% and 1-5% abundance respectively. In addition, there is variation in the types of posttranslational modifications that occur on Gγ subunits, including arginylation, cysteinylation and versions that escape proteolysis of the –AAX motif following prenylation. Some Gγ2 subunits were found to escape carboxymethylation. Unmethylated Gγ2 subunits made up 30-50% of the detected Gγ2 subunits[3]. Based on these results, it is much more likely that methylation, rather than prenylation, is involved in the nuclear localization of Gβγ. Further analysis of the processing of nuclear Gγ subunits, including the methylation status, may help to resolve this issue.

A.



B.



C.

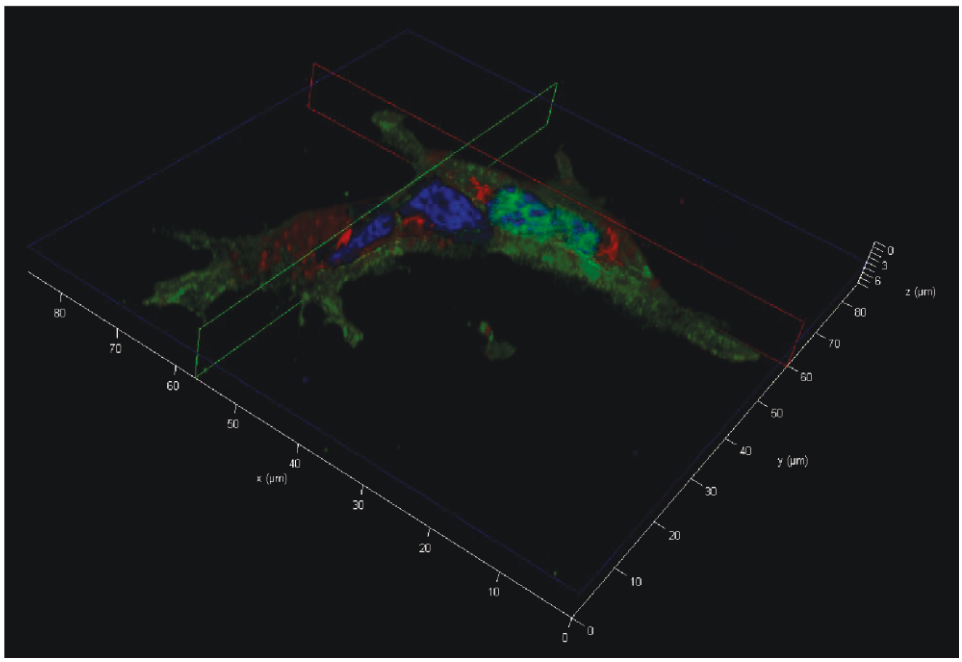


Figure 27: *Confocal images of varying Gβ concentrations in Gγ2 C68S transfected cells*. A) Gβ1γ2C68S-transfected cells. B) Gγ2C68S-transfected cells. C) Gγ2C68S-transfected cells. The nucleus is stained blue, the whole cell red, and Gβ1-4 green. 250,000 cells per well were plated on 22x22 glass slides (Fisher Scientific, Ottawa, ON; 12-543-B) in six well plates and treated with 0.1mg/mL Poly-L-ornithine Hydrobromide (Sigma-Aldrich, St. Louis, MO; P3655) two days before use. Cells were treated with 2μM leptomycin B (Cell Signalling Technology, Inc., Whitby, ON; 9676) for 30 minutes. Cells were fixed on glass slides using 2% paraformaldehyde and permeabilized with 0.1% Triton X. Cells were blotted in 1% bovine serum albumin. Primary antibody (Gβ T20 Santa Cruz Biotechnology, Dallas, Texas; SC378) was used at a concentration of 1:200, secondary antibody (α mouse Alexa Fluor® 488, Life Technologies; A11029 and α rabbit Alexa Fluor® 555, Life Technologies; A21428) at 1:500. Whole cell and nucleus were stained with Wheat Germ Agglutinin, Alexa Fluor® 647 (Life Technologies, W32466) and Hoechst 33342 (Life Technologies, H3570). Cover slips were secured to slides using PermaFluor™ (Thermo Scientific, TA-006-FM). Slides were imaged using Leica Confocal Scanning Microscope TCS SP8. A 63x/1.4NA objective lens airy 1, zoom 1.42. An average of 20 Z stacks were taken depending on cell size. Images were produced using 3D rendering by Leica Application Suite X software. Slices were taken to demonstrate protein expression within the cell. Data are representative of three independent experiments.

5.2.2 *The effect of leptomycin B on nuclear localization of Gβ1-4*

To understand the mechanism of Gβγ trafficking to and from the nucleus, the nuclear export blocker leptomycin B was used. Leptomycin B binds to exportin 1 (XPO1) and subsequently prevents it from binding to proteins with a nuclear export signal. Therefore, in the presence of leptomycin B, proteins that require XPO1 for transport will be sequestered in the nucleus[276]. XPO1 is one of the primary export pathways for proteins leaving the nucleus and was a likely candidate for nuclear export of Gβ1-4[161]. In addition, MS data identified XPO1 as a potential Gβγ interactor (**Table 14**). Therefore, we hypothesized that by blocking this particular route of exit from the nuclear pore, we would increase the concentration of Gβγ within the nucleus.

Initial experiments were conducted to determine the concentration of leptomycin B required to block protein export from the nucleus in HEK 293 cells (**Figure 28**), using mitogen-activated protein kinase kinase (MEK1/2) as a positive control based on manufacturer's instructions for leptomycin B. Based on these results, 2μM leptomycin B was used for further experiments. However, leptomycin B treatment of HEK 293 cells followed by nuclear isolation did not reveal changes in nuclear Gβ1-4 levels (**Figure 29**). The same result was seen using confocal microscopy (**Figure 30** and **Figure 31**). Differences between MEK1/2 were significantly different (one-way ANOVA, Bonferroni correction, $p<0.05$) in all fractions compared across time points. This was not true for Gβ1-4. Regardless of this, the data indicate that MEK1/2 increases in expression over time, while Gβ1-4 decreases. This suggests that either Gβ1-4 is not using this pathway, or that there is no net movement of total Gβ1-4 out of the nucleus. Studies of individual Gβγ pairs would be significantly more informative in this regard as the antibody we used would not detect exchange of different Gβ subunits.

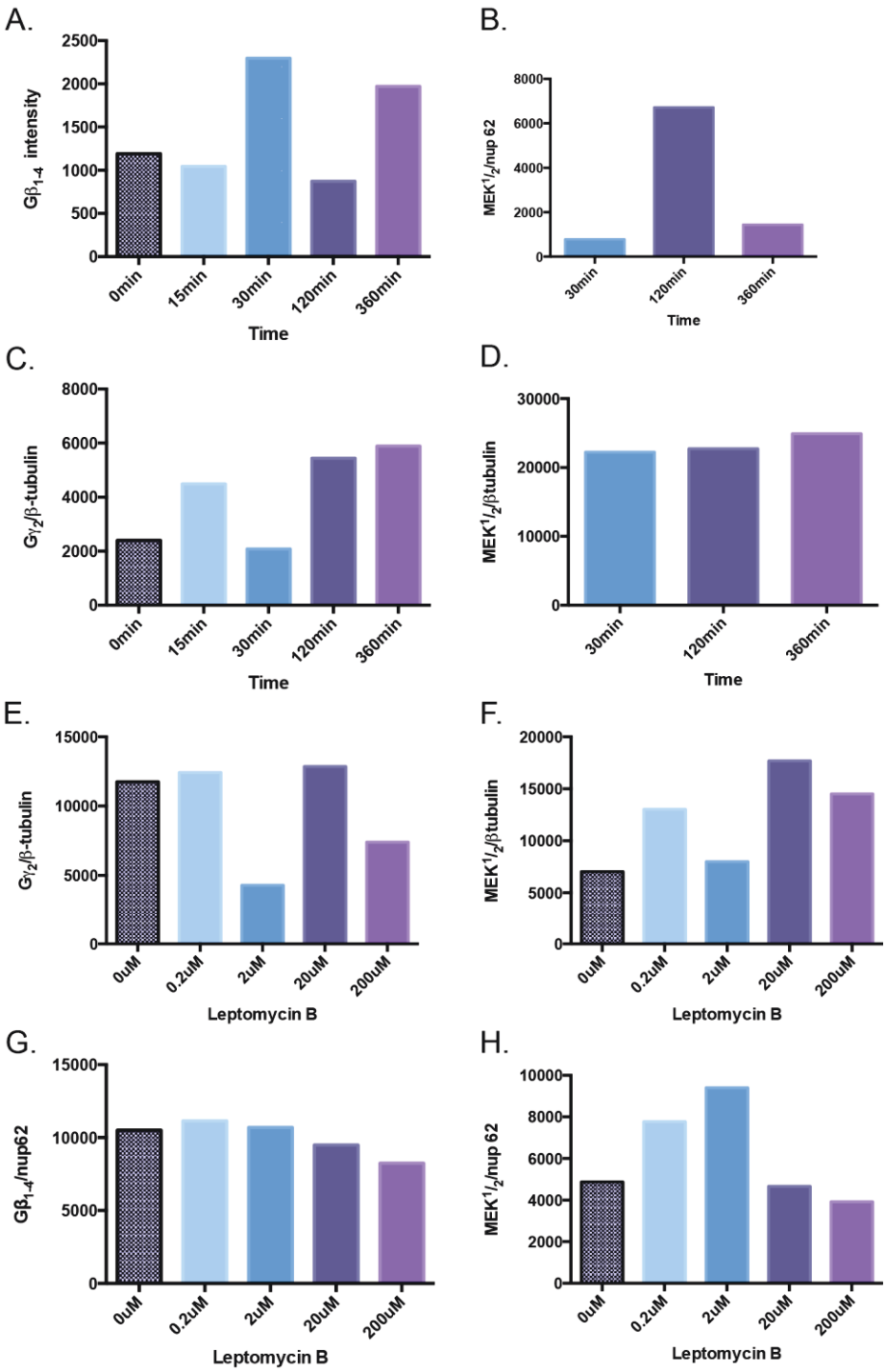

A.
Gβ1-4 intensity
2500
2000
1500
1000
500
0
0min
15min
30min
120min
360min
Time
B.
MEK1/2/nup 62
8000
6000
4000
2000
0
30min
120min
360min
Time
C.
Gγ2/β-tubulin
8000
6000
4000
2000
0
0min
15min
30min
120min
360min
Time
D.
MEK1/2/βtubulin
30000
20000
10000
0
30min
120min
360min
Time
E.
Gγ2/β-tubulin
15000
10000
5000
0
0uM
0.2uM
2uM
20uM
200uM
Leptomycin B
F.
MEK1/2/βtubulin
20000
15000
10000
5000
0
0uM
0.2uM
2uM
20uM
200uM
Leptomycin B
G.
Gβ1-4/nup62
15000
10000
5000
0
0uM
0.2uM
2uM
20uM
200uM
Leptomycin B
H.
MEK1/2/nup 62
10000
8000
6000
4000
2000
0
0uM
0.2uM
2uM
20uM
200uM
Leptomycin B

Figure 28: *Quantification of nuclear and cytosolic Gβ following HEK 293 cell treatment with leptomycin B*. Cells were treated with 0.2, 2, 20, or 200μM leptomycin B (Cell Signalling Technology, Inc., Whitby, ON; 9676) for 2 hours. Further experiments were conducted with treatment of cells with leptomycin B for 15, 30, 120 and 360 minutes. Cells were lysed using cell lysis solution (320mM sucrose, 10mM HEPES NaOH, 5mM $MgCl_2$, 1mM DTT, 1mM PMSF, 1mM Na_3VO_4, 1% Triton-X) for six minutes. The cell lysis was layered over top of a sucrose cushion (1.8M sucrose, 10mM HEPES NaOH, 5mM $MgCl_2$, 1mM DTT, 1mM PMSF, 1mM Na_3VO_4, 1% Triton-X) and centrifuged at max speed for twenty minutes to isolate the nuclei. The whole cell, cytosolic and nuclear fractions were run on a gel and blotted for protein expression. Samples were run on a 15% polyacrylamide gel at 120V for 20 minutes, followed by one hour at 160V. Proteins were transferred to a polyvinyl difluoride (PVDF) membrane at 100V for one hour. The membrane was blotted with 5% skim milk (5% skim milk, 1.37M NaCl, 27mM KCl, 100mM KH_2HPO_4, 18mM KH_2PO_4), followed by 1:1000 Gβ (1:1000, Gβ T20 Santa Cruz Biotechnology, Dallas, Texas; SC378) or Mek1/2 (1:1000, New England Biolabs, UK; 9122) for one hour, and α-mouse (1:20,000, Sigma-Aldrich, St. Louis, MO; A9917) and α rabbit (1:20,000, Sigma-Aldrich, St. Louis, MO; A0545) for one hour. Membranes were oxidized using Western Lightning Plus ECL (PerkinElmer Inc., Waltham, MA; NEL103001 EA) and autoradiography film was exposed. ImageJ was used to quantify band intensities. Gβ and Mek1/2 values were normalized to nucleoporin 62 in the nucleus and β tubulin in the cytosolic and whole cell fractions. The resulting values were graphed using GraphPad Prism. Graphs represent data from one experiment.

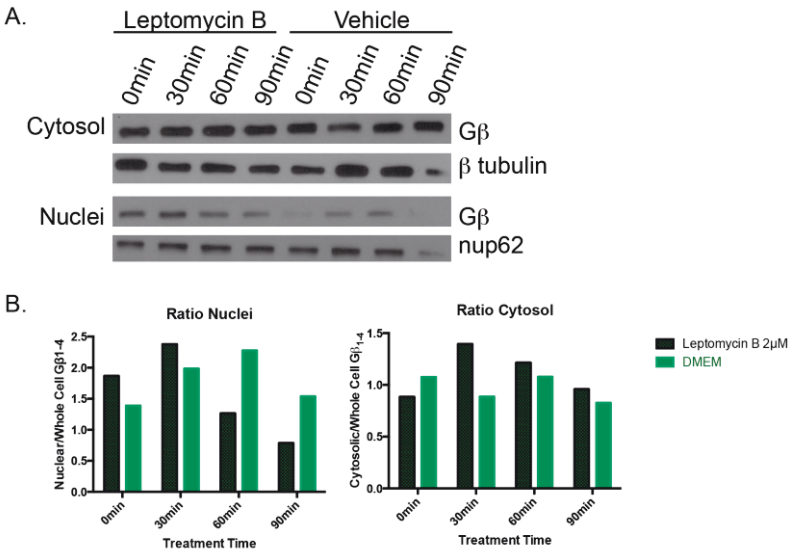


Figure 29: *Western blot and quantification of nuclear and cytosolic Gβ following HEK 293 treatment with 2μM leptomycin B*. Cells were treated with 2μM leptomycin B for 30 minutes. Samples were run on a 10% polyacrylamide gel at 120V for 20 minutes, followed by one hour at 160V. Proteins were transferred to a polyvinyl difluoride (PVDF) membrane at 100V for one hour. The membrane was blotted with skim milk (5% skim milk, 1.37M NaCl, 27mM KCl, 100mM KH_2HPO_4, 18mM KH_2PO_4), followed by Gβ1-4 (1:1000, Gβ T20 Santa Cruz Biotechnology, Dallas, Texas; SC378) for one hour, and α-mouse (1:20,000, Sigma-Aldrich, St. Louis, MO; A9917) for one hour. Membranes were oxidized using Western Lightning Plus ECL (PerkinElmer Inc., Waltham, MA; NEL103001 EA) and autoradiography film was exposed. Protein bands were quantified by densitometry using ImageJ. Resulting values were normalized to control bands and displayed using GraphPad Prism. Graphs represent data from one experiment.

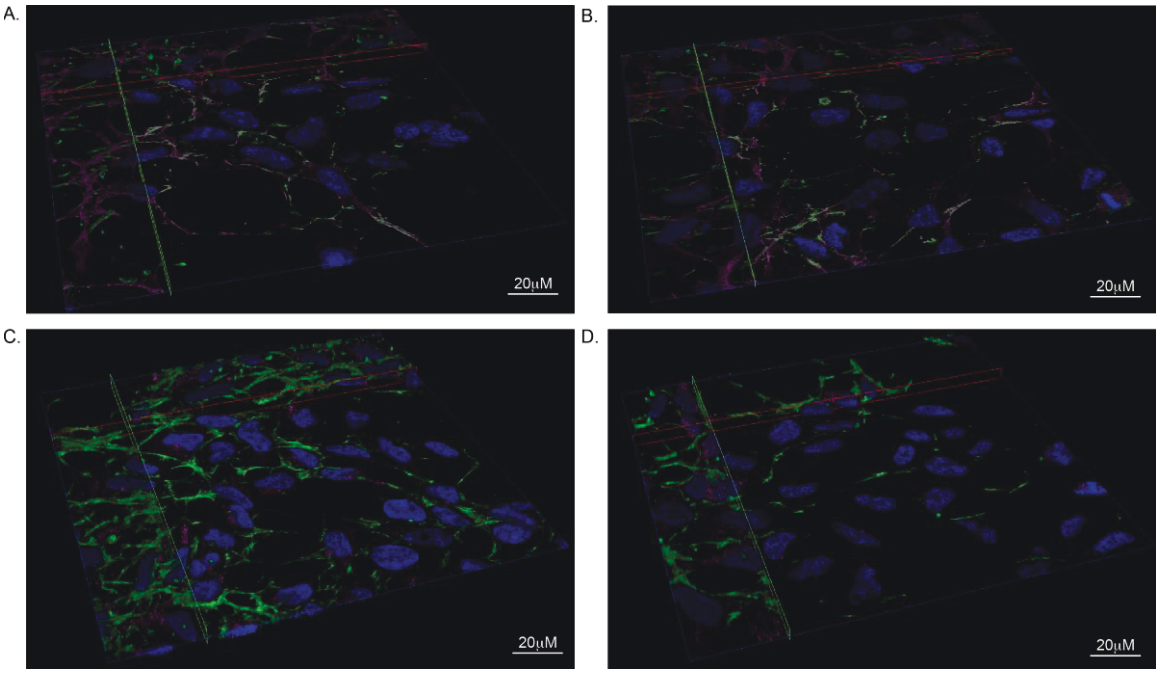

A.
20μM
B.
20μM
C.
20μM
D.
20μM

Figure 30: *Confocal images of Gβ in HEK 293 cells following treatment with 2μM leptomycin B*. A) Cells stained with Mek1/2, no leptomycin B, B) Cells stained with Mek1/2, 30 minutes leptomycin B, C) Cells stained with Gβ1-4, no leptomycin B and D) Cells stained with Gβ1-4, 30 minutes leptomycin B. The nucleus is stained blue, the whole cell green, Gβ1-4 or Mek1/2 purple. 250,000 cells per well were plated on 22x22 glass slides (Fisher Scientific, Ottawa, ON; 12-543-B) in a six well plate and treated with 0.1mg/mL Poly-L-ornithine Hydrobromide (Sigma-Aldrich, St. Louis, MO; P3655) in a 6-well plate two days prior to use. Cells were treated with 2μM Leptomycin B for 30 minutes. Cells were fixed on glass slides using 2% paraformaldehyde and permeabilized with 0.1% Triton X. Cells were blotted in 1% bovine serum albumin. Primary antibodies Gβ (Gβ T20 Santa Cruz Biotechnology, Dallas, Texas; SC378) and Mek1/2 (1:1000, New England Biolabs, UK; 9122) were used at a concentration of 1:200, Secondary antibody (α rabbit Alexa Fluor® 555, Life Technologies; A21428, α mouse Alexa Fluor® 555, Life Technologies; A-21422) at 1:500. Whole cell and nucleus were stained with phalloidin Alexa Fluor® 488 (1:5000, Life Technologies; A12379) and Hoechst 33342 (1:1000, Life Technologies, H3570). Cover slips were secured to slides using PermaFluor™ (Thermo Scientific, TA-006-FM). Slides were imaged using Leica Confocal Scanning Microscope TCS SP8. A 63x/1.4NA objective lens airy 1 was used. An average of 20 Z stacks were taken depending on cell size. Images were produced by Leica Application Suite X software. Data are representative of one experiment.

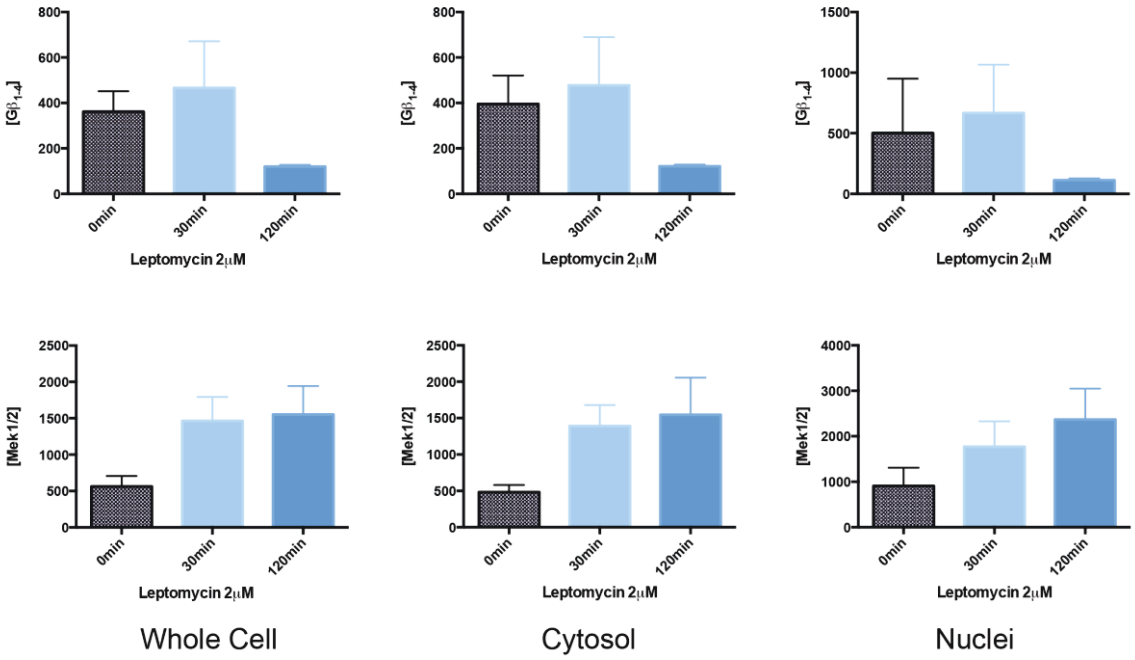


Figure 31: *Quantification of confocal microscopy images of Gβ and MEK1/2 in HEK 293 cells following treatment with 2μM Leptomycin B*. Three images were taken for each condition. Confocal images were analyzed using IMARIS BitPlane software. Images were rendered in 3D and volume calculations were made for whole cell and nuclear compartments using wheat germ agglutinin and Hoechst staining to define the whole cell and nucleus respectively. The cytosolic volume was determined by subtracting the nuclear volume from the whole cell volume. Intensity of staining for Gβ was determined by calculating the number of voxels within each volume. The concentration was calculated by dividing the voxels by the total volume in each compartment. This value was averaged across all images. Background levels were calculated from cells stained only with secondary antibody. These values were subtracted from concentration values after averaging, and the resulting concentrations were graphed in GraphPad Prism. Graphs represent one experiment. One-way repeated measures ANOVA was used to analyze MEK1/2 data.

5.2.3 *The effect of cell surface signalling on Gβγ nuclear localization*

To determine whether cell surface GPCR signalling pathways could alter the localization of Gβγ in the cell, HEK 293 cells were treated with the GPCR agonists isoproterenol, epinephrine, norepinephrine, angiotensin II, and carbachol. Isoproterenol is a synthetic isopropyl analogue of epinephrine and is a β adrenergic receptor agonist with relative selectivity for β_2-adrenergic receptor. Angiotensin II is the endogenous agonist for angiotensin receptors. Epinephrine is the endogenous agonists for α- and β-adrenergic receptors, while norepinephrine preferentially activates α-adrenergic receptors. Finally, carbachol is a synthetic compound that activates muscarinic acetylcholine receptors. RT-qPCR and western blot data from our lab indicates that the M3 receptor is expressed endogenously in HEK 293 cells (Shahriar Khan, unpublished data). Analysis of mRNA expression suggests the presence of the angiotensin, α- and β- adrenergic receptors in the cells used (Phan Trieu, unpublished data). The idea of using a spectrum of GPCR agonists was to determine whether there were GPCR signalling pathways that could modulate Gβγ entry to or egress from the nucleus. Based on initial experiments using confocal microscopy, epinephrine caused an influx of Gβ1-4 into the nucleus after 15 minutes of stimulation (**Figure 32** and **Figure 33**). However, further follow up western blot experiments were unable to confirm these results (**Figure 34**). Due to the unavailability of good antibodies for individual Gβ subunits, it is possible that we were unable to detect any movement of the total pool of Gβ. Therefore, we followed up with epinephrine and carbachol, in line with MS experiments, in cells stably expressing Flag-Gβ1. Treatment with either of these compounds did not cause a change in concentration of Flag-Gβ1 in the nuclei of HEK 293 cells when compared to controls (**Figure 35** and **Figure 36**). What is evident however is that there appears to be movement of Gβ1 in and out of the nucleus that is independent of drug treatment. This prompted us to look into the effect of serum starvation of Flag-Gβ1 nuclear localization.

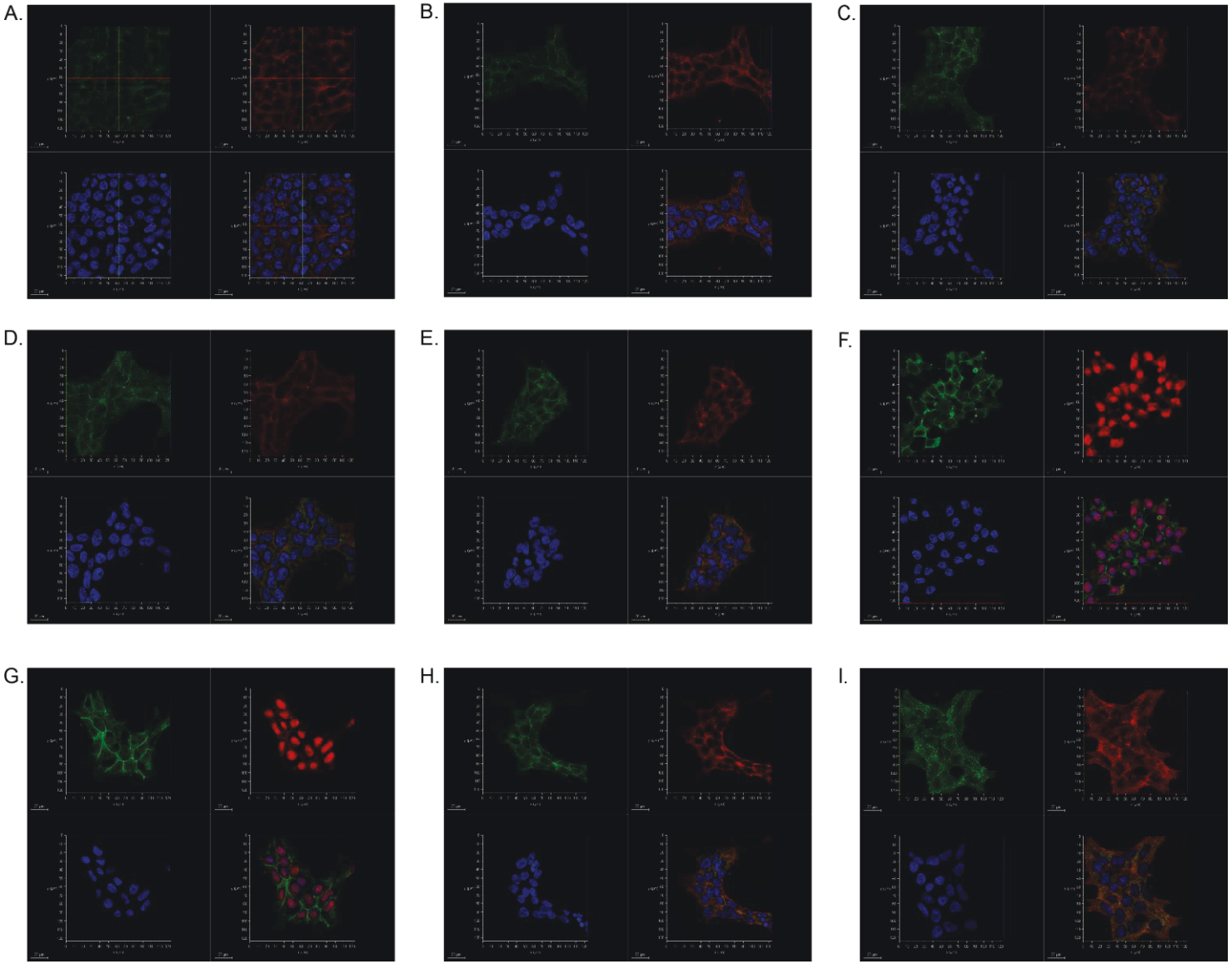

A.
B.
C.
D.
E.
F.
G.
H.
I.

Figure 32: *Representative images from confocal microscopy of Gβ in HEK 293 cells following treatment with GPCR ligands*. Agonists (final concentrations- 1μM angiotensin (Sigma-Aldrich, St. Louis, MA; A9525), 10μM norepinephrine (Sigma-Aldrich, St. Louis, MA; 7257), 10μM epinephrine (Sigma-Aldrich, St. Louis, MA; E4250), and 10μM isoproterenol (I6504) were re-suspended in Dulbecco's modified eagle media (DMEM). A) Angiotensin control, B) Angiotensin 15 minutes, C) Epinephrine control, D) Epinephrine 5 minutes, E) Epinephrine 30 minutes, F) Isoproterenol control, G) Isoproterenol 15 minutes, H) Norepinephrine control, and I) Norepinephrine 15 minutes. The nucleus is stained blue, the whole cell red, and Gβ1-4 green. 250,000 cells per well were plated on 22x22 glass slides (Fisher Scientific, Ottawa, ON; 12-543-B) in a six well plate and treated with 0.1mg/mL Poly-L-ornithine Hydrobromide (Sigma-Aldrich, St. Louis, MO; P3655) in a 6-well plate two days prior to use. Each well was treated with the respective agonist for 0, 5, 15, 30, 120 or 180 minutes. Cells were fixed on glass slides using 2% paraformaldehyde and permeabilized with 0.1% Triton X. Cells were blotted in 1% bovine serum albumin. Primary antibody Gβ (Gβ T20 Santa Cruz Biotechnology, Dallas, Texas; SC378) was used at a dilution of 1:200; secondary antibody (α mouse Alexa Fluor® 488, Life Technologies; A11029) at 1:500. Whole cell and nucleus were stained with HCS CellMask™ Deep Red Stain (1:5000, Life Technologies Inc.; H32721) and Hoechst 33342 (1:1000, Life Technologies, H3570). Cover slips were secured to slides using PermaFluor™ (Thermo Scientific, TA-006-FM). Slides were imaged using Leica Confocal Scanning Microscope TCS SP8. A 63x/1.4NA objective lens airy 1 was used. An average of 20 Z stacks were taken depending on cell size. Images were produced using 3D rendering by Leica Application Suite X software. Data are representative of one to three independent experiments.

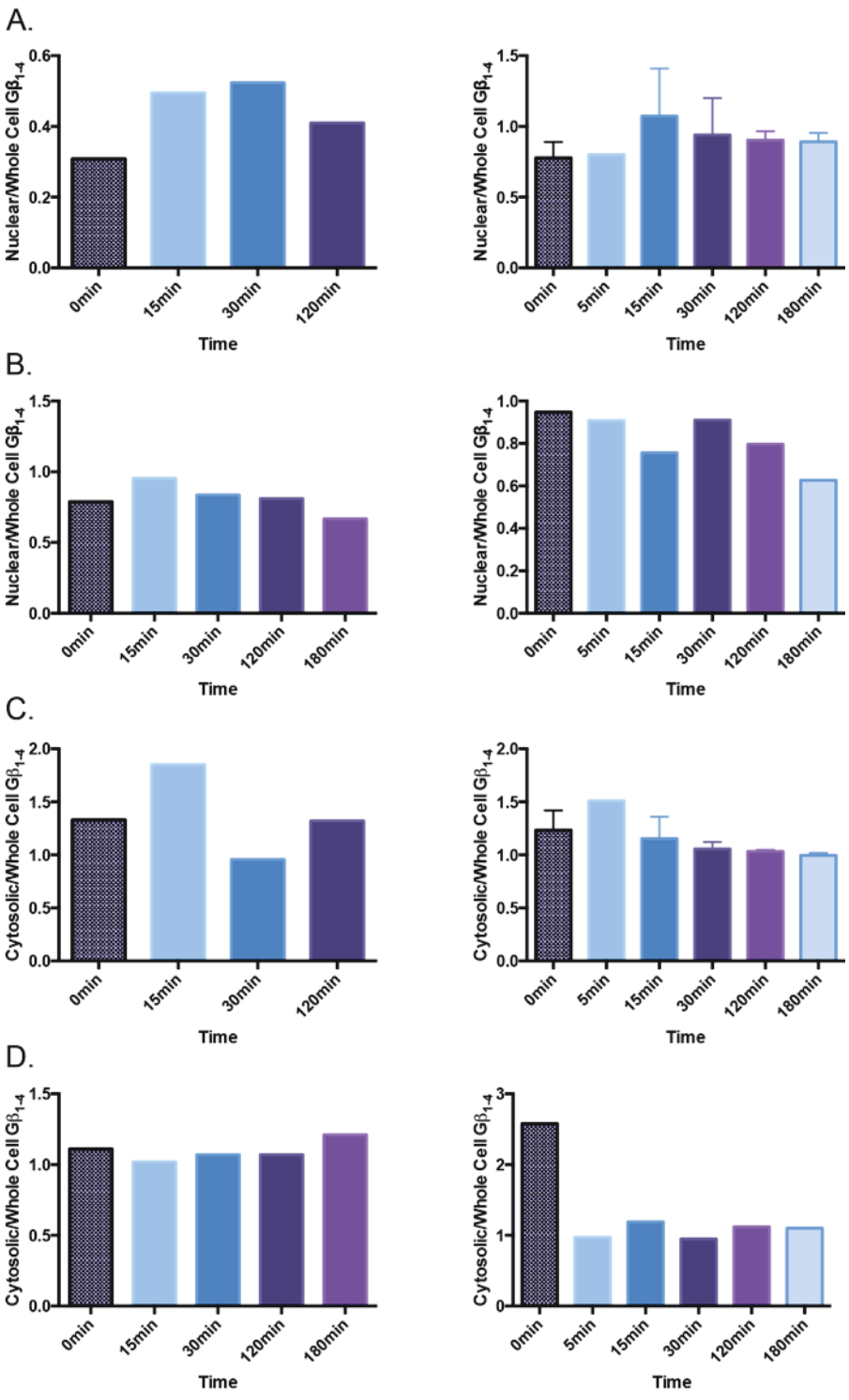
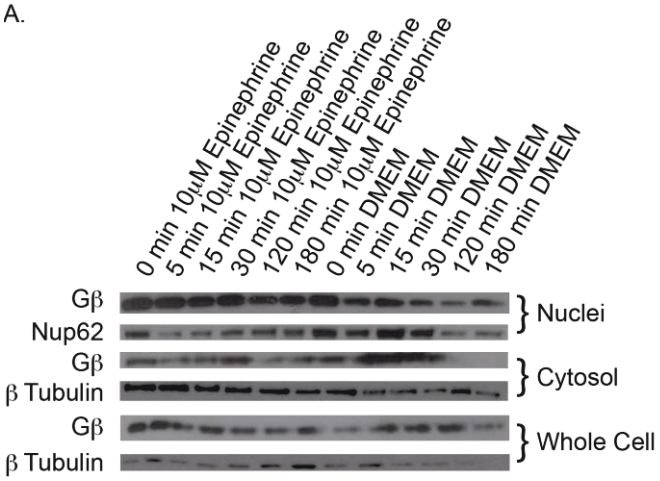

A.
Nuclear/Whole Cell Gβ1-4
0.6
0.4
0.2
0.0
0min
15min
30min
120min
Time
Nuclear/Whole Cell Gβ1-4
1.5
1.0
0.5
0.0
0min
5min
15min
30min
120min
180min
Time
B.
Nuclear/Whole Cell Gβ1-4
1.5
1.0
0.5
0.0
0min
15min
30min
120min
180min
Time
Nuclear/Whole Cell Gβ1-4
1.0
0.8
0.6
0.4
0.2
0.0
0min
5min
15min
30min
120min
180min
Time
C.
Cytosolic/Whole Cell Gβ1-4
2.0
1.5
1.0
0.5
0.0
0min
15min
30min
120min
Time
Cytosolic/Whole Cell Gβ1-4
2.0
1.5
1.0
0.5
0.0
0min
5min
15min
30min
120min
180min
Time
D.
Cytosolic/Whole Cell Gβ1-4
1.5
1.0
0.5
0.0
0min
15min
30min
120min
180min
Time
Cytosolic/Whole Cell Gβ1-4
3
2
1
0
0min
5min
15min
30min
120min
180min
Time

Figure 33: *Quantification of confocal microscopy of Gβ in in HEK 293 cells following receptor activation*. A) Cells treated with 1μM angiotensin II. Left panel shows ratio of Gβ1-4 found in the cytosol, right panel shows ratio of Gβ1-4 found in the nucleus. B) Cells treated with 10μM epinephrine. Left panel shows ratio of Gβ1-4 found in the cytosol, right panel shows ratio of Gβ1-4 found in the nucleus. C) Cells treated with 10μM isoproterenol. Left panel shows ratio of Gβ1-4 found in the cytosol, right panel shows ratio of Gβ1-4 found in the nucleus. D) Cells treated with 10μM angiotensin II. Left panel shows ratio of Gβ1-4 found in the cytosol, right panel shows ratio of Gβ1-4 found in the nucleus. Confocal images were analyzed using IMARIS (BitPlane, USA). Images were rendered in 3D and volume calculations were made for whole cell and nuclear compartments using CellMask™ and Hoechst staining to define the whole cell and nucleus respectively. The cytosolic volume was determined by subtracting the nuclear volume from the whole cell volume. Intensity of staining for Gβ was determined by calculating the number of voxels within each volume. The concentration was calculated by dividing the voxels by the total volume in each compartment. This value was averaged across all images. Background levels were calculated from cells stained only with secondary antibody. These values were subtracted from concentration values after averaging. The resulting concentrations were taken as a ratio of the whole cell concentration and were graphed in GraphPad Prism. The experiment was run one to three times depending on drug treatment. Graphs represent one to three independent experiments.

A.


0 min 10μM Epinephrine
5 min 10μM Epinephrine
15 min 10μM Epinephrine
30 min 10μM Epinephrine
120 min 10μM Epinephrine
180 min 10μM Epinephrine
0 min DMEM
5 min DMEM
15 min DMEM
30 min DMEM
120 min DMEM
180 min DMEM
Gβ
Nup62
Nuclei
Gβ
β Tubulin
Cytosol
Gβ
β Tubulin
Whole Cell

B.

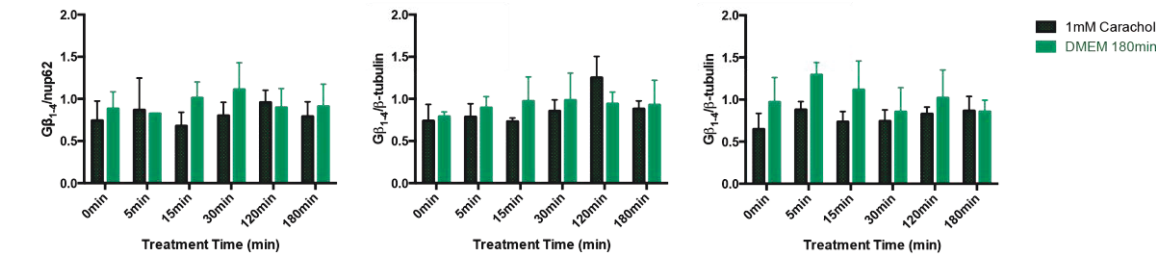

Gβ1-4/nup62
Gβ1-4/β-tubulin
Gβ1-4/β-tubulin
0.0
0.5
1.0
1.5
2.0
0min
5min
15min
30min
120min
180min
Treatment Time (min)
1mM Carachol
DMEM 180min

Figure 34: *Western blot and quantification of HEK 293 cells treated with epinephrine*. Cells were treated with 10μM epinephrine (Sigma-Aldrich, St. Louis, MO; E4250) for 0, 5, 15, 30, 120 or 180 minutes. Epinephrine was re-suspended in Dulbecco's modified eagle media (DMEM). Cells were washed in twice in PBS (1.37M NaCl, 27mM KCl, 100mM KH_2HPO_4, 18mM KH_2PO_4), spun down, and re-suspended in cell lysis buffer (320mM sucrose, 10mM HEPES NaOH, 5mM $MgCl_2$, 1mM DTT, 1mM PMSF, 1mM Na_3VO_4, 1% Triton-X) for six minutes. The cell lysis was layered over top of a sucrose cushion (1.8M sucrose, 10mM HEPES NaOH, 5mM $MgCl_2$, 1mM DTT, 1mM PMSF, 1mM Na_3VO_4, 1% Triton-X) and centrifuged at max speed for twenty minutes to isolate the nuclei. The whole cell, cytosolic and nuclear fractions were run on a gel and blotted for protein expression. Samples were run on a 15% polyacrylamide gel at 120V for 20 minutes, followed by one hour at 160V. Proteins were transferred to a polyvinyl difluoride (PVDF) membrane at 100V for one hour. The membrane was blotted with 5% skim milk (5% skim milk, 1.37M NaCl, 27mM KCl, 100mM KH_2HPO_4, 18mM KH_2PO_4), followed by Gβ (1:1000, Gβ T20 Santa Cruz Biotechnology, Dallas, Texas; SC378), β tubulin (1:4000, Life Technologies; 32-2600) and nucleoporin 62 (1:6000, BD Transduction Laboratories™, 610497) for one hour, and α-mouse (1:20,000, Sigma-Aldrich, St. Louis, MO; A9917) for one hour. Membranes were oxidized using Western Lightning Plus ECL (PerkinElmer Inc., Waltham, MA; NEL103001 EA) and autoradiography film was exposed. ImageJ was used to quantify band intensities. Gβ1 was normalized to β tubulin or nucleoporin 62 and the resulting values were graphed using GraphPad Prism. Graphs represent three independent experiments.

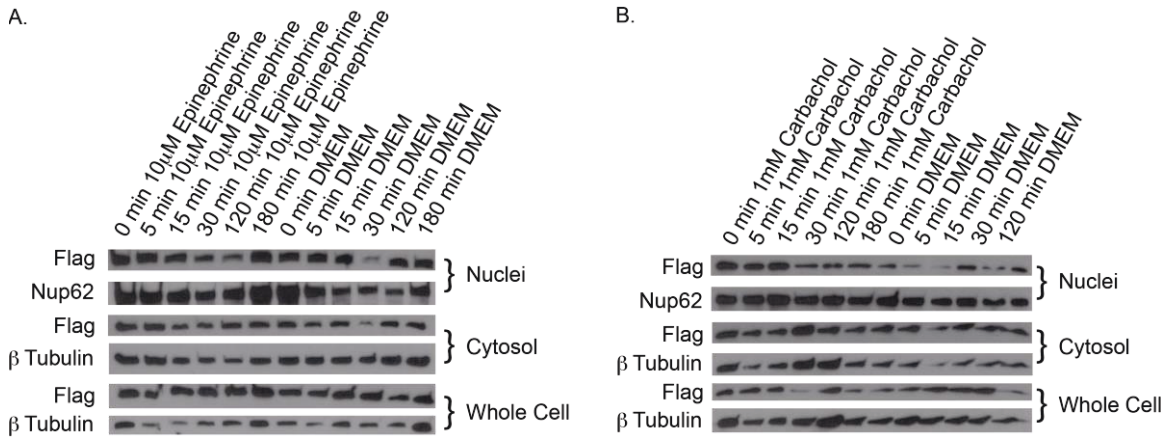


Figure 35: *Subcellular localization of Flag-tagged Gβ1 in stable HEK 293 cells*. Cells were treated with A) 10μM epinephrine (Sigma-Aldrich, St. Louis, MO; E4250) or B) 1mM carbachol (Sigma-Aldrich, St. Louis, MO; C4382) for 0, 5, 15, 30, 120 or 180 minutes and separated into whole cell, cytosolic and nuclear fractions. Epinephrine was re-suspended in Dulbecco's modified eagle media (DMEM). Cells were washed in twice in PBS (1.37M NaCl, 27mM KCl, 100mM KH_2HPO_4, 18mM KH_2PO_4), spun down, and re-suspended in solution A (320mM sucrose, 10mM HEPES NaOH, 5mM $MgCl_2$, 1mM DTT, 1mM PMSF, 1mM Na_3VO_4, 1% Triton-X) for six minutes. The cell lysis was layered over top of a sucrose cushion (1.8M sucrose, 10mM HEPES NaOH, 5mM $MgCl_2$, 1mM DTT, 1mM PMSF, 1mM Na_3VO_4, 1% Triton-X) and centrifuged at max speed for twenty minutes to isolate the nuclei. The whole cell, cytosolic and nuclear fractions were run on a gel and blotted for protein expression. Samples were run on a 15% polyacrylamide gel at 120V for 20 minutes, followed by one hour at 160V. Proteins were transferred to a polyvinyl difluoride (PVDF) membrane at 100V for one hour. The membrane was blotted with 5% skim milk (5% skim milk, 1.37M NaCl, 27mM KCl, 100mM KH_2HPO_4, 18mM KH_2PO_4), followed by Flag (1:1000, Sigma-Aldrich, St. Louis, MO, F1804), β tubulin (1:4000, Life Technologies; 32-2600) and nucleoporin 62 (1:6000, BD Transduction Laboratories™, 610497) for one hour, and α-mouse (1:20,000, Sigma-Aldrich, St. Louis, MO; A9917) for one hour. Membranes were oxidized using Western Lightning Plus ECL (PerkinElmer Inc., Waltham, MA; NEL103001 EA) and

autoradiography film was exposed. Data are representative of three independent experiments.

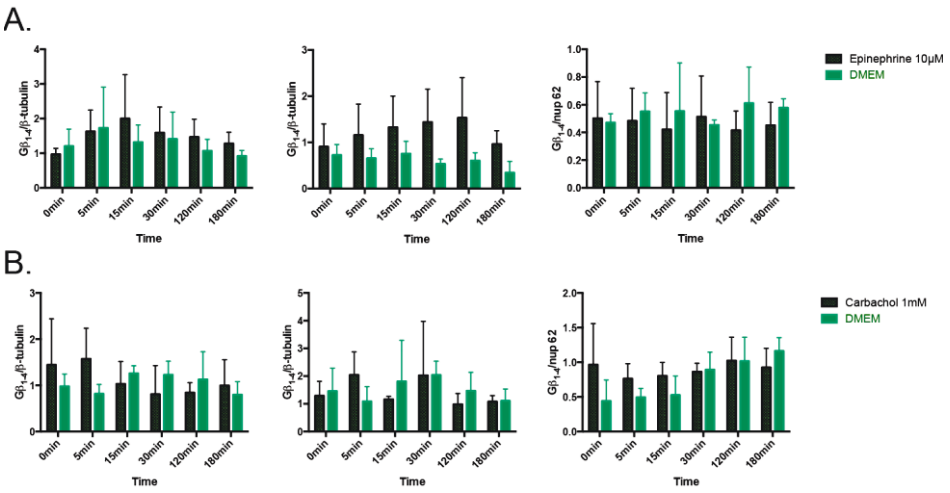


Figure 36: *Localization of Flag-tagged Gβ1 in stable HEK 293 cells*. Cells were treated with A) 10μM epinephrine or B) 1mM carbachol for 0, 5, 15, 30, 120 and 180 minutes and separated into whole cell, cytosolic and nuclear fractions. ImageJ was used to quantify band intensities. Flag-tagged Gβ1 was normalized to β tubulin or nucleoporin 62 and the resulting values were graphed using GraphPad Prism. Graphs represent three independent experiments.

5.2.4 *Effect of serum on nuclear Gβ1-4 localization*

Previous studies indicated that growth factors lead to the movement of Gαi into the nucleus of Swiss 3T3 cells[120,188]. When Swiss 3T3 cells were treated with thrombin, it was noted that both Gαi and Gβ migrated into the nucleus[188]. Whether or not the proteins remained associated when they moved into the nucleus was not determined. In our hands, serum deprivation followed by serum treatment of HEK 293 cells did not lead to an accumulation of Gβ in the nucleus following serum treatment (**Figure 37**). Instead, the same pattern was seen as in previous experiments. Gβ nuclear levels appear to fluctuate, but are not dependent on treatment condition. The mechanisms underlying Gβγ cycling into and out of the nucleus remain to be determined.

5.3 Conclusions

Based on the above data, Gβγ is found in the nucleus, and interacts with a number of nuclear proteins including those involved in RNA/DNA binding and transcriptional regulation. Additionally, Gβγ may be involved in binding to RNAPII through association with interacting proteins. The question of how Gβγ gains access to the nucleus is still unclear. Gβγ can bind to a number of different karyopherins (**Table 13**) that are characterized for their ability to shuttle proteins in and out of the nucleus, in addition to a number of proteins that can shuttle in and out of the nucleus. However, stimulation with the GPCR agonists angiotensin II, isoproterenol, epinephrine, norepinephrine and carbachol were unable to affect net levels of Gβγ in the nucleus as measured by both confocal microscopy and western blotting. Surprisingly, although carbachol is able to alter the composition of Gβγ interacting proteins in the nucleus, this is not accompanied by a corresponding increase or decrease of total Gβγ in the nucleus. We have been unable to detect the presence of adrenergic receptors in the nucleus of HEK 293 cells (Nicolas Audet, unpublished data), but have not searched for other nuclear GPCRs in these cells.

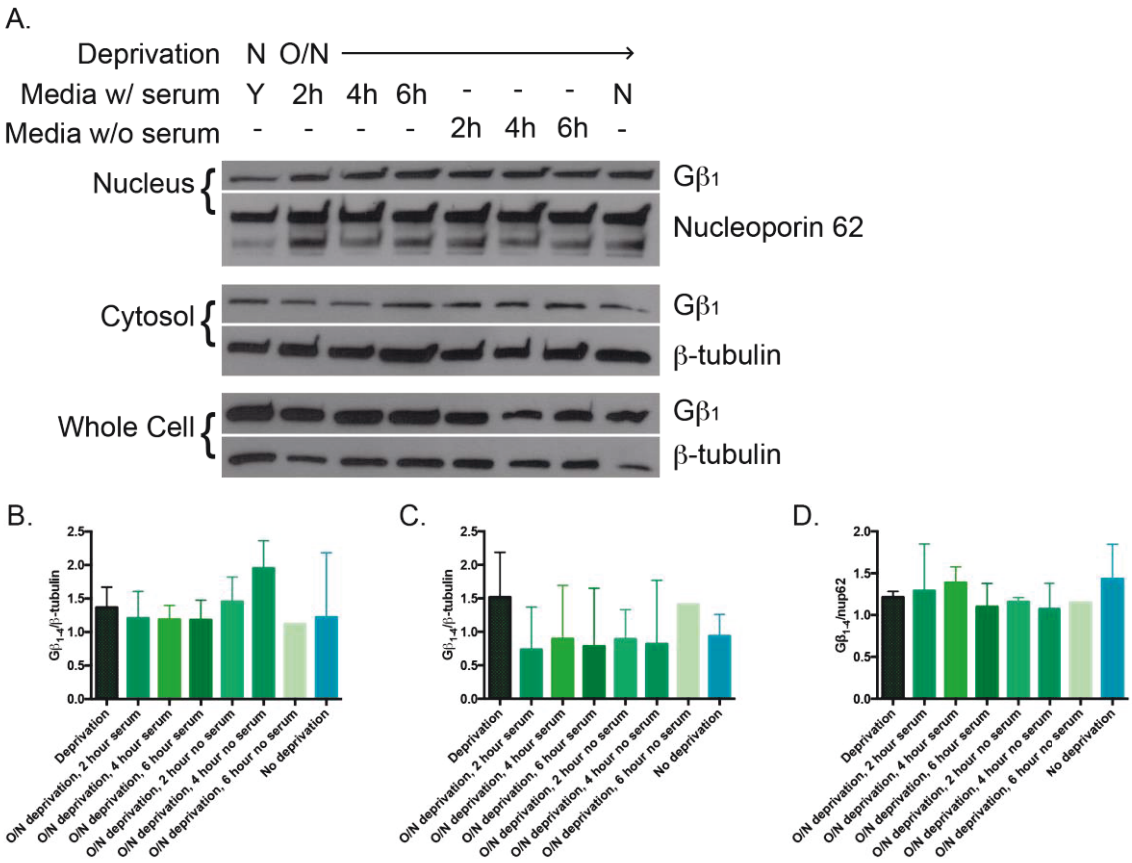

A.
Deprivation N O/N
Media w/ serum Y 2h 4h 6h - - - N
Media w/o serum - - - - 2h 4h 6h -
Nucleus
Gβ1
Nucleoporin 62
Cytosol
Gβ1
β-tubulin
Whole Cell
Gβ1
β-tubulin
B.
C.
D.
Deprivation
O/N deprivation, 2 hour serum
O/N deprivation, 4 hour serum
O/N deprivation, 6 hour serum
O/N deprivation, 2 hour no serum
O/N deprivation, 4 hour no serum
O/N deprivation, 6 hour no serum
No deprivation

Figure 37: *Western blot and quantification of HEK 293 cells deprived or treated with serum*. Cells were deprived overnight (O/N) and then treated with media containing 5% fetal bovine serum for 2, 4, or 6 hours. A) Western blot of serum treated cells, B) quantification of whole cell fraction, C) quantification of cytosolic fraction, and D) quantification of nuclear fraction. Controls were treated with media without serum. Cells were washed in twice in PBS (1.37M NaCl, 27mM KCl, 100mM KH_2HPO_4, 18mM KH_2PO_4), spun down, and re-suspended in solution A (320mM sucrose, 10mM HEPES NaOH, 5mM $MgCl_2$, 1mM DTT, 1mM PMSF, 1mM Na_3VO_4, 1% Triton-X) for six minutes. The cell lysis was layered over top of a sucrose cushion (1.8M sucrose, 10mM HEPES NaOH, 5mM $MgCl_2$, 1mM DTT, 1mM PMSF, 1mM Na_3VO_4, 1% Triton-X100) and centrifuged at max speed for twenty minutes to isolate the nuclei. The whole cell, cytosolic and nuclear fractions were run on a gel and blotted for protein expression. Samples were run on a 15% polyacrylamide gel at 120V for 20 minutes, followed by one hour at 160V. Proteins were transferred to a polyvinyl difluoride (PVDF) membrane at 100V for one hour. The membrane was blotted with 5% skim milk (5% skim milk, 1.37M NaCl, 27mM KCl, 100mM KH_2HPO_4, 18mM KH_2PO_4), followed by Gβ1 (1:1000, Gβ T20 Santa Cruz Biotechnology, Dallas, Texas; SC378), β-tubulin (1:4000, Life Technologies; 32-2600) and nucleoporin 62 (1:6000, BD Transduction Laboratories™, 610497) for one hour, and α-mouse (1:20,000, Sigma-Aldrich, St. Louis, MO; A9917) for one hour. Membranes were oxidized using Western Lightning Plus ECL (PerkinElmer Inc., Waltham, MA; NEL103001 EA) and autoradiography film was exposed. ImageJ was used to quantify band intensities. Flag-tagged Gβ1 was normalized to β tubulin or nucleoporin 62 and the resulting values were graphed using GraphPad Prism. Graphs represent 1-2 independent experiments.

Our data suggests that cell surface receptors can control nuclear Gβγ signalling and interactions but the question of whether cell membrane and cytosolic pools of G proteins are separate from nuclear pools of G proteins remains. In our hands, we observed members of all four families of G proteins in the nucleus, in addition to a number of different Gβ and Gγ subunits. Does the localization of these proteins separate them from the cytosolic counterparts? Whether there is direct movement of Gβγ from the cytosol into the nucleus is unknown. It does appear that there are fluctuating levels of Gβγ in the nucleus, however as of yet, we have not been able to affect this movement.

To further investigate Gβγ signalling within the nucleus, the question of GPCR expression in the nucleus and their association with nuclear G proteins in the nucleus must be addressed. Cell-permeable ligands that bypass cell surface receptors may help us to better understand intracellular signalling pathways, including those in the nucleus[112,277].

Future study of Gβγ signalling in the nucleus should be oriented toward validating protein interactions we detected that are involved in transcriptional regulation and DNA/RNA binding. This is line with unpublished data from our lab that suggests that Gβγ interacts with RNAPII (Shahriar Khan, unpublished data). In addition to nuclear interactions, novel interactions of Gβγ in the mitochondria have also been identified. This opens up a relatively un-studied area regarding localization of Gβγ, and points to candidates for Gβγ interaction, as well as pointing to novel functions of Gβγ in energy production in the mitochondria.

In addition to highlighting novel Gβγ targets for validation in both the cytosol and the nucleus, I have optimized a TAP protocol for use with nuclear lysates. By comparing protein interactions isolated by Flag and tandem affinity purification, my research indicates that multiple purification schemes are desirable and necessary. The protein targets of Gβγ, potential nuclear signalling pathways regulated by Gβγ and nuclear TAP consideration highlighted by this project will further the understanding of Gβγ signalling in the nucleus and help to elucidate the role of Gβγ in transcriptional regulation.

Works Cited

1 Campden, R., Audet, N. & Hébert, T. E. Nuclear G protein signalling: New tricks for old dogs. *The Journal of Cardiovascular Pharmacology* (2015).

2 Consortium, I. H. G. S. Initial sequencing and analysis of the human genome. *Nature* **409**, 860-921 (2001).

3 Cook, L. A. *et al.* Proteomic analysis of bovine brain G protein γ subunit processing heterogeneity. *Molecular and Cellular Proteomics* **5**, 671-685, (2006).

4 Downes, G. B. & Gautam, N. The G protein subunit gene families. *Genomics* **62**, 544-552 (1999).

5 Wittpoth, C., Scholich, K., Yigzaw, Y., Stringfield, T. M. & Patel, T. B. Regions on adenylyl cyclase that are necessary for inhibition of activity by βγ and Giα subunits of heterotrimeric G proteins. *Proceedings of the National Academy of Sciences* **96**, 9551-9556 (1999).

6 Ivanina, T. *et al.* Gαi1 and Gαi3 differentially interact with, and regulate, the G protein-activated K+ channel. *The Journal of Biological Chemistry* **279**, 17260-17268, (2004).

7 Wada, Y. *et al.* A region of sulfonylurea receptor critical for a modulation of ATP-sensitive K+ channels by G-protein βγ-subunits. *The EMBO Journal* **19**, 4915-4925 (2000).

8 Rebecchi, M. J. & Pentyala, S. N. Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiological Reviews* **4**, 1291-1335 (2000).

9 Park, D., Jhon, D.-Y., Lee, C.-W., Lee, K.-H. & Rhee, S. G. Activation of phopsholipase C isozymes by G protein βγ subunits. *The Journal of Biological Chemistry* **268**, 4573-4576 (1993).

10 Hubbard, K. B. & Hepler, J. R. Cell signalling diversity of the Gqα family of heterotrimeric G proteins. *Cell Signalling* **18**, 135-150, (2006).

11 Czupalla, C. *et al.* Identification and characterization of the autophosphorylation sites of phosphoinositide 3-kinase isoforms β and γ. *The Journal of Biological Chemistry* **278**, 11536-11545, (2003).

12 Catterall, W. A. Structure and regulation of volate-gated Ca^{2+} channels. *Annual Review of Cell and Developmental Biology* **16**, 521-555 (2000).

13 Dupre, D. J., Robitaille, M., Rebois, R. V. & Hebert, T. E. The role of Gβγ subunits in the organization, assembly, and function of GPCR signalling complexes. *Annual Review of Pharmacology and Toxicology* **49**, 31-56, (2009).

14 Bunemann, M., Frank, M. & Lohse, M. J. Gi protein activation in intact cells involves subunit rearrangement rather than dissociation. *Proceedings of the National Academy of Sciences* **100**, 16077-16082, (2003).

15 Digby, G. J., Lober, R. M., Sethi, P. R. & Lambert, N. A. Some G protein heterotrimers physically dissociate in living cells. *Proceedings of the National Academy of Sciences* **103**, 17789-17794, (2006).

16 Gales, C. *et al.* Probing the activation-promoted structural rearrangements in preassembled receptor-G protein complexes. *Nature Structural & Molecular Biology* **13**, 778-786, (2006).
17 Gether, U. & Kobilka, B. K. G Protein-coupled Receptors: II. Mechanism of agonist activation. *Journal of Biological Chemistry* **273**, 17979-17982, (1998).
18 Akam, E. C., Challiss, R. A. J. & Nahorski, S. R. Gq/11 and Gi/o activation profiles in CHO cells expressing muscarinic acetylcholine receptors: dependence on agonist as well as receptor-subytpe. *British Journal of Pharmachology* **132**, 950-958 (2001).
19 Sauliere, A. *et al.* Deciphering biased-agonism complexity reveals a new active AT1 receptor entity. *Nature Chemical Biology* **8**, 622-630, (2012).
20 Zimmerman, B. *et al.* Differential β-arrestin-dependent conformational signalling and cellular responses revealed by angiotensin analogs. *Science Signalling* **5**, ra33, (2012).
21 Khan, S. M. *et al.* The expanding roles of Gβγ subunits in G protein-coupled receptor signalling and drug action. *Pharmacological Reviews* **65**, 545-577, (2013).
22 Tang, W.-J. & Gilman, A. G. Type-specific regulation of adenylyl cyclase by G protein βγ subunits. *Science* **254**, 1500-1503 (1991).
23 Koch, W. J., Hawes, E. H., Inglese, J., Luttrell, L. M. & Lefkowitz, R. J. Cellular expression of the carboxyl terminus of a G protein coupled receptor kinase attentuates Gβγ-mediated signalling. *The Journal of Biological Chemistry* **269**, 6193-6197 (1994).
24 Park, D., Jhon, D.-Y., Lee, C.-W. & Rhee, S. G. Activation of phospholipase C isozymes by G protein βγ subunits. *The Journal of Biological Chemistry* **268**, 4573-4576 (1993).
25 Thomason, P. A., James, S. R., Casey, P. J. & Downes, C. P. A G-protein βγ-subunit-responsibe phosphoinositide 3-kinase activity in human platelet cytosol. *The Journal of Biological Chemistry* **269**, 16525-16528 (1994).
26 Kofuji, P., Davidson, N. & Lester, H. A. Evidence that neuronal G-protein-gated inwardly rectifying K+ channels are activated by Gβγ subunits and function as heteromultimers. *Proceedings of the National Academy of Sciences* **92**, 6542-6546 (1995).
27 Lin, Y. & Smrcka, A. V. Understanding molecular recognition by G protein βγ subunits on the path to pharmacological targeting. *Molecular Pharmacology* **80**, 551-557, (2011).
28 Maurice, P., Benleulmi-Chaachoua, A. & Jockers, R. Differential assembly of GPCR signalling complexes determines signalling specificity. *Subcellular Biochemistry* **63**, 225-240, (2012).
29 Lambright, D. G. *et al.* The 2.0 A cyrstal structure of a heterotrimeric G protein. *Nature* **379**, 311-319 (1996).
30 Oldham, W. M. & Hamm, H. E. Heterotrimeric G protein activation by G-protein-coupled receptors. *Nature Reviews Molecular Cell Biology* **9**, 60-71, (2008).

31 Fung, B. K.-K. Characterization of transducin from bovine retinal rod outer segments. *The Journal of Biological Chemistry* **258**, 10495-10502 (1983).
32 Knol, J. C., Engel, R., Blaauw, M., Visser, A. J. & van Haastert, P. J. The phosducin-like protein PhLP1 is essential for Gβγ dimer formation in Dictyostelium discoideum. *Molecular and Cellular Biology* **25**, 8393-8400, (2005).
33 Pronin, A. N. & Gautam, N. Proper processing of a G protein γ subunit depends on complex formation with a β subunit. *FEBS Letters* **328**, 89-93 (1993).
34 Takida, S. & Wedegaertner, P. B. Heterotrimer formation, together with isoprenylation, is required for plasma membrane targeting of Gβγ. *The Journal of Biological Chemistry* **278**, 17284-17290, (2003).
35 Kilpatrick, E. L. & Hildebrandt, J. D. Sequence dependence and differential expression of Gγ5 subunit isoforms of the heterotrimeric G proteins variably processed after prenylation in mammalian cells. *The Journal of Biological Chemistry* **282**, 14038-14047, (2007).
36 Hewavitharana, T. & Wedegaertner, P. B. Non-canonical signalling and localizations of heterotrimeric G proteins. *Cellular Signalling* **24**, 25-34, (2012).
37 Chen, C. A. & Manning, D. R. Regulation of G proteins by covalent modification. *Oncogene* **20**, 1643-1652 (2001).
38 Yasuda, H., Lindorfer, M. A., Woodfork, K. A., Fletcher, J. E. & Garrison, J. C. Role of the Prenyl Group on the G Protein Subunit in Coupling Trimeric G Proteins to A1 Adenosine Receptors. *Journal of Biological Chemistry* **271**, 18588-18595, (1996).
39 Kang, Y., Kalyanaraman, V. & Gautam, N. Differential ability to form the G Protein βγ complex among members of the β and γ subunit families. *Journal of Biological Chemistry* **271**, 7141-7146, (1996).
40 Dingus, J., Wells, C. A., Cleator, J. H., Robinson, K. & Hildebrandt, J. D. G protein βγ dimer formation: Gβ and Gγ differentially determine efficiency of in vitro dimer formation. *Biochemistry* **2005**, 11882-11890 (2005).
41 Howlett, A. C., Gray, A. J., Hunter, J. M. & Willardson, B. M. Role of molecular chaperones in G protein β5/regulator of G protein signalling dimer assembly and G protein βγ dimer specificity. *The Journal of Biological Chemistry* **284**, 16386-16399, (2009).
42 Johansen, P. W., Lund, H. W. & Gordeladze, J. O. Specific combinatinos of G protein subunits discriminate hormone signalling in rat pituitary (GH3) cells in culture. *Cellular Signalling* **13**, 251-256 (2001).
43 Stow, J. L. *et al.* The heterotrimeric G protein, Gαi3, on golgi membranes regulates the secretion of a heparan sulfate proteoglycan in LLC-PK1 epithelial cells. *The Journal of Cell Biology* **114**, 1113-1134 (1991).
44 Robitaille, M. *et al.* Gβγ is a negative regulator of AP-1 mediated transcription. *Cell Signalling* **22**, 1254-1266, (2010).
45 Cismowski, M. J. Non-receptor activators of heterotrimeric G-protein signalling (AGS proteins). *Seminars in Cell and Developmental Biology* **17**, 334-344,(2006).

46 Hagelüken, A. *et al.* Liphilic β-adrenoceptor antagonists and local anesthetics are effective direct activators of G-proteins. *Biochemical Pharmacology* **47**, 1789-1795 (1994).
47 Takesono, A. *et al.* Receptor-independent Activators of Heterotrimeric G-protein Signalling Pathways. *Journal of Biological Chemistry* **274**, 33202-33205, (1999).
48 Strittmatter, S. M. GAP-43 as a modulator of G protein transduction in the growth cone. *Perspectives on Developmental Neurobiology* **1**, 13-19 (1992).
49 Nishimura, A. *et al.* Ric-8A potentiates Gq-mediated signal transduction by acting downstream of G protein-coupled receptor in intact cells. *Genes to Cells* **11**, 487-498, (2006).
50 Miller, K. G., Emerson, M. D., McManus, J. R. & Rand, J. B. RIC-8 (Synembryn): A novel conserved protein that is required for Gqα signalling in the C. elegans nervous system. *Neuron* **27**, 289-299 (2000).
51 Siderovski, D. P. & Willard, F. S. The GAPs, GEFs and GDIs of heterotrimeric G-protein α subunits. *International Journal of Biological Sciences* **1**, 51-66 (2005).
52 Boissan, M. *et al.* The mammalian Nm23/NDPK family: from metastasis control to cilia movement. *Molecular and Cellular Biochemistry* **329**, 51-62, 2009).
53 Bosnar, M. H. *et al.* Subcellular localization of A and B Nm23/NDPK subunits. *Experimental cell research* **298**, 275-284, (2004).
54 Wieland, T. Interaction of nucleoside diphosphate kinase B with heterotrimeric G protein βγ dimers: consequences on G protein activation and stability. *Naunyn-Schmiedeberg's Archives of Pharmacology* **374**, 373-383, (2007).
55 Hippe, H. J. & Wieland, T. High energy phosphate transfer by NDPK B/G βγ complexes--an alternative signalling pathway involved in the regulation of basal cAMP production. *Journal of Bioenergetics and Biomembranes* **38**, 197-203, (2006).
56 Cuello, F. *et al.* Activation of heterotrimeric G proteins by a high energy phosphate transfer via nucleoside diphosphate kinase (NDPK) B and Gβ subunits. Complex formation of NDPK B with Gβ γ dimers and phosphorylation of His-266 IN Gβ. *The Journal of Biological Chemistry* **278**, 7220-7226, (2003).
57 Hippe, H. J. *et al.* The interaction of nucleoside diphosphate kinase B with Gβγ dimers controls heterotrimeric G protein function. *Proceedings of the National Academy of Sciences* **106**, 16269-16274, (2009).
58 Hippe, H. J. *et al.* Regulation of cardiac cAMP synthesis and contractility by nucleoside diphosphate kinase B/G protein β γ dimer complexes. *Circulation Research* **100**, 1191-1199, (2007).
59 Lee, D. K. *et al.* Agonist-independent nuclear localization of the Apelin, angiotensin AT1, and bradykinin B2 receptors. *The Journal of Biological Chemistry* **279**, 7901-7908, (2004).

60 Tadevosyan, A. *et al.* Nuclear-delimited angiotensin receptor-mediated signalling regulates cardiomyocyte gene expression. *The Journal of Biological Chemistry* **285**, 22338-22349, (2010).
61 Lu, D., Yang, H., Shaw, G. & Raizada, M. K. Angiotensin II-induced nuclear targeting of the angiotensin type 1 (AT1) receptor in brain neurons. *Endocrinology* **139**, 365-375 (1998).
62 Jamora, C., Takizawa, P. A., Denesvre, C., Faulkner, D. J. & Malhotra, V. Regulation of gogli structure through hetertrimeric G proteins. *Cell* **91**, 617-626 (1997).
63 Jamora, C. *et al.* Gβγ-Mediated Regulation of Golgi Organization Is through the Direct Activation of Protein Kinase D. *Cell* **98**, 59-68, (1999).
64 Diaz Anel, A. M. Phospholipase C β3 is a key component in the Gβγ/PKCη/PKD-mediated regulation of trans-Golgi network to plasma membrane transport. *Biochemical Journal* **406**, 157-165, (2007).
65 Irannejad, R. & Wedegaertner, P. B. Regulation of constitutive cargo transport from the trans-Golgi network to plasma membrane by Golgi-localized G protein βγ subunits. *The Journal of Biological Chemistry* **285**, 32393-32404, (2010).
66 Ray, K., Hansen, C. A. & Robishaw, J. D. Gβγ-mediated signalling in the heart: implications of β and γ subunit heterogeneity. *The Journal of Chinese Medicine* **6**, 115-121 (1996).
67 Betty, M., Harnish, S. W., Rhodes, K. J. & Cockett, M. I. Distribution of heterotrimeric G protein β and γ subunits in the rat brain. *Neuroscience* **85**, 475-486 (1998).
68 Cali, J. J., Balcueva, E. A., Rybalkin, I. & Robishaw, J. D. Selective tissue distribution of G protein γ subunits, including a new form of the γ subunits identified by cDNA cloning. *The Journal of Biological Chemistry* **267**, 24023-24027 (1992).
69 Von Zastrow, M. & Sorkin, A. Signalling on the endocytic pathway. *Current Opinion in Cell Biology* **19** (2007).
70 Garcia-Ragalado, A. *et al.* G Protein-coupled Receptor-promoted Trafficking of Gβ1γ2 Leads to AKT Activation at Endosomes via a Mechanism Mediated by Gβ1γ2-Rab11a Interaction. *Molecular Biology of the Cell* **19**, 4188-4200, (2008).
71 Zhang, J. *et al.* G-protein β2 subunit interacts with mitofusin 1 to regulate mitochondrial fusion. *Nature Communications* **1**, 101, (2010).
72 Andreeva, A. V., Kutuzov, M. A. & Voyno-Yasenetskaya, T. A. Gα12 is targeted to the mitochondria and affects mitochondrial morphology and motility. *The FASEB Journal* **22**, 2821-2831, (2008).
73 Lyssand, J. S. & Bajjalieh, S. M. The heterotrimeric [corrected] G protein subunit Gαi is present on mitochondria. *FEBS Letters* **581**, 5765-5768, (2007).
74 Sorkin, A. & Von Zastrow, M. Signal transduction and endocytosis: close encounters of many kinds. *Nature Reviews Molecular Cell Biology* **3**, 600-614, (2002).

75 Calebiro, D., Nikolaev, V. O., Persani, L. & Lohse, M. J. Signalling by internalized G-protein-coupled receptors. *Trends in Pharmacological Sciences* **31**, 221-228, (2010).
76 Boivin, B., Vaniotis, G., Allen, B. G. & Hebert, T. E. G protein-coupled receptors in and on the cell nucleus: a new signalling paradigm? *Journal of Receptors and Signal Transduction* **28**, 15-28, (2008).
77 Sallese, M., Pulvirenti, T. & Luini, A. The physiology of membran transport and endomembrane-based signalling. *The EMBO Journal* **25**, 2663-2673, (2006).
78 Sallese, M., Giannotta, M. & Luini, A. Coordination of the secretory compartments via inter-organelle signalling. *Seminars in Cell & Developmental Biology* **20**, 801-809, (2009).
79 Vaniotis, G., Allen, B. G. & Hébert, T. E. Nuclear GPCRs in cardiomyocytes: an insider's view of β-adrenergic receptor signalling. *American Journal of Heart and Circulatory Physiology* **301**, 1754-1764, (2011).
80 Tadevosyan, A., Vaniotis, G., Allen, B. G., Hebert, T. E. & Nattel, S. G protein-coupled receptor signalling in the cardiac nuclear membrane: evidence and possible roles in physiological and pathophysiological function. *The Journal of Physiology* **590**, 1313-1330, (2012).
81 Gobeil, F. *et al.* G-protein-coupled receptors signalling at the cell nucleus: an emerging paradigm. *Canadian Journal of Physiology and Pharmacology* **84**, 287-297, (2006).
82 Zhang, J. H. *et al.* Nuclear localization of G protein β 5 and regulator of G protein signalling 7 in neurons and brain. *The Journal of Biological Chemistry* **276**, 10284-10289, (2001).
83 Boivin, B., Villeneuve, L. R., Farhat, N., Chevalier, D. & Allen, B. G. Sub-cellular distribution of endothelin signalling pathway components in ventricular myocytes and heart: lack of preformed caveolar signalosomes. *Journal of Molecular and Cellular Cardiology* **38**, 665-676, (2005).
84 Boivin, B. *et al.* Functional β-adrenergic receptor signalling on nuclear membranes in adult rat and mouse ventricular cardiomyocytes. *Cardiovascular Research* **71**, 69-78, (2006).
85 Willard, F. S. & Crouch, M. F. Nuclear and cytoskeletal translocation and localization of heterotrimeric G-proteins. *Immunology and Cell Biology* **78**, 387-394 (2000).
86 Dupre, D. J. *et al.* Seven transmembrane receptor core signalling complexes are assembled prior to plasma membrane trafficking. *The Journal of Biological Chemistry* **281**, 34561-34573, (2006).
87 Yamamoto, S., Kawamura, K. & James, T. N. Intracellular Distribution of Adenylate Cyclase in Human Cardiocytes Determined by Electron Microscopic Cytochemistry. *Microscopy Research and Technique* **40**, 479-487 (1998).
88 Schulze, W. & Buchwalow, I. B. Adenylyl cyclase in the heart: an enzymocytochemical and immunocytochemical approach. *Microscopy Research and Technique* **40**, 473-478 (1998).

89 Schievella, A. R., Regier, M. K., Smith, W. L. & Lin, L. L. Calcium-mediated Translocation of Cytosolic Phospholipase A2 to the Nuclear Envelope and Endoplasmic Reticulum. *Journal of Biological Chemistry* **270**, 30749-30754, (1995).
90 Kim, C. G., Park, D. & Rhee, S. G. The Role of Carboxyl-terminal Basic Amino Acids in Gqα -dependent Activation, Particulate Association, and Nuclear Localization of Phospholipase C-β 1. *Journal of Biological Chemistry* **271**, 21187-21192, (1996).
91 Freyberg, Z. *et al.* Intracellular Localization of Phospholipase D1 in Mammalian Cells. *Molecular Biology of the Cell* **12**, 943-955 (2001).
92 Scott, M. G. *et al.* Differential nucleocytoplasmic shuttling of β-arrestins. Characterization of a leucine-rich nuclear export signal in β-arrestin2. *The Journal of Biological Chemistry* **277**, 37693-37701, (2002).
93 Wang, P., Wu, Y., Ge, X., Ma, L. & Pei, G. Subcellular localization of β-arrestins is determined by their intact N domain and the nuclear export signal at the C terminus. *The Journal of Biological Chemistry* **278**, 11648-11653, (2003).
94 Johnson, L. R., Scott, M. G. & Pitcher, J. A. G protein-coupled receptor kinase 5 contains a DNA-binding nuclear localization sequence. *Molecular and Cellular Biology* **24**, 10169-10179, (2004).
95 Sastri, M., Barraclough, D. M., Carmichael, P. T. & Taylor, S. S. A-kinase-interacting protein localizes protein kinase A in the nucleus. *Proceedings of the National Academy of Sciences* **102**, 349-354, (2005).
96 Burchett, S. A. In through the out door: nuclear localization of the regulators of G protein signalling. *Journal of Neurochemistry* **87**, 551-559, (2003).
97 Gressner, O. A. *et al.* Intracrine signalling of activin A in hepatocytes upregulates connective tissue growth factor (CTGF/CCN2) expression. *Liver International* **28**, 1207-1216, (2008).
98 Lee, T.-H. *et al.* Vascular Endothelial Growth Factor Mediates Intracrine Survival in Human Breast Carcinoma Cells through Internally Expressed VEGFR1/FLT1. *PloS one* **4**, e186, (2007).
99 Re, R. N. & Cook, J. L. Mechanisms of disease: Intracrine physiology in the cardiovascular system. *Nature Clinical Practice Cardiovascular Medicine* **4**, 549-557, (2007).
100 Du, Y., Hsu, J. L., Wang, Y. N. & Hung, M. C. in *Receptor Tyrosine Kinase: Structure, Functions and Role in Human Disease* (eds D. L. Wheeler & Y. arden) (Springer, 2014).
101 Wang, Y. N., Hsu, J. & Hung, M. C. in *Vesicle Trafficking in Cancer* (eds Y. Yarden & G. arcic) 159-176 (Springer, 2013).
102 Huo, L., Hsu, J. & Hung, M. C. in *Nuclear Signalling Pathways and Targeting Transcription in Cancer* (ed R. Kumar) 189-229 (Springer, 2014).

103 Marrache, A. M. *et al.* Proinflammatory gene induction by platelet-activating factor mediated via its cognate nuclear receptor. *The Journal of Immunologyl.* **169**, 6474-6481 (2002).
104 Gobeil, F., Jr. *et al.* Modulation of pro-inflammatory gene expression by nuclear lysophosphatidic acid receptor type-1. *The Journal of Bioliological Chemistry.* **278**, 38875-38883 (2003).
105 Gobeil, F., Jr. *et al.* Regulation of eNOS expression in brain endothelial cells by perinuclear EP(3) receptors. *Circ.Res.* **90**, 682-689 (2002).
106 Zhu, T. *et al.* Intracrine signalling through lipid mediators and their cognate nuclear G-protein-coupled receptors: a paradigm based on PGE2, PAF, and LPA1 receptors. *Canadian Journal of Physiology and Pharmacology* **84**, 377-391, (2006).
107 O'Malley, K. L., Jong, Y. J., Gonchar, Y., Burkhalter, A. & Romano, C. Activation of metabotropic glutamate receptor mGlu5 on nuclear membranes mediates intranuclear Ca2+ changes in heterologous cell types and neurons. *The Journal of Biological Chemistry* **278**, 28210-28219, (2003).
108 Kumar, V., Jong, Y. J. & O'Malley, K. L. Activated nuclear metabotropic glutamate receptor mGlu5 couples to nuclear Gq/11 proteins to generate inositol 1,4,5-trisphosphate-mediated nuclear Ca2+ release. *The Journal of Biological Chemistry* **283**, 14072-14083, (2008).
109 Wu, S. C. *et al.* Nuclear localization of a1A-adrenergic receptors is required for signalling in cardiac myocytes: an "inside-out" α1-AR signalling pathway. *Journal of the American Heart Association.* **3**, e000145 (2014).
110 Vaniotis, G., Allen, B. G. & Hebert, T. E. Nuclear GPCRs in cardiomyocytes: an insider's view of β-adrenergic receptor signalling. *American Journal of Physiology Heart and Circulatory Physiology.* **301**, H1754-H1764 (2011).
111 Merlen, C. *et al.* Intracrine endothelin signalling evokes IP3-dependent increases in nucleoplasmic Ca(2)(+) in adult cardiac myocytes. *Journal of Molecular Cell Cardiology.* **62**, 189-202 (2013).
112 Vaniotis, G. *et al.* Regulation of cardiac nitric oxide signalling by nuclear β-adrenergic and endothelin receptors. *Journal of Molecular and Cellular Cardiology* **62**, 58-68, (2013).
113 Vaniotis, G. *et al.* Nuclear β-adrenergic receptors modulate gene expression in adult rat heart. *Cell Signalling.* **23**, 89-98 (2011).
114 Tadevosyan, A. *et al.* Nuclear-delimited angiotensin receptor-mediated signalling regulates cardiomyocyte gene expression. *The Journal of Biological Chemistry.* **285**, 22338-22349 (2010).
115 Lee, D. K. *et al.* Agonist-independent nuclear localization of the Apelin, angiotensin AT1, and bradykinin B2 receptors. *The Journal of Biological Chemistry.* **279**, 7901-7908 (2004).
116 Ho, M. K., Su, Y., Yeung, W. W. & Wong, Y. H. Regulation of transcription factors by heterotrimeric G proteins. *Current Moleclar Pharmacology.* **2**, 19-31 (2009).

117 Campden, R. *et al.* in *Nuclear G-Protein Coupled Receptors* (eds B. G. Allen & T. E. Hebert) (Springer, 2014).
118 Crouch, M. F., Osborne, G. W. & Willard, F. S. The GTP-binding protein G(iα) translocates to kinetochores and regulates the M-G(1) cell cycle transition of Swiss 3T3 cells. *Cell Signalling.* **12**, 153-163 (2000).
119 Crouch, M. F. & Simson, L. The G-protein G(i) regulates mitosis but not DNA synthesis in growth factor-activated fibroblasts: a role for the nuclear translocation of G(i). *The FASEB Journal.* **11**, 189-198 (1997).
120 Crouch, M. F. Growth factor-induced cell division is paralleled by translocatino of Giα to the nucleus. *The FASEB Journal* **5**, 200-206 (1991).
121 Kino, T. *et al.* G protein β interacts with the glucocorticoid receptor and suppresses its transcriptional activity in the nucleus. *The Journal of Cell Biology.* **69**, 885-896 (2005).
122 Lin, C. Y., Li, C. C., Huang, P. H. & Lee, F. J. A developmentally regulated ARF-like 5 protein (ARL5), localized to nuclei and nucleoli, interacts with heterochromatin protein 1. *The Journal of Cell Science.* **115**, 4433-4445 (2002).
123 Sato, M. *et al.* Identification of transcription factor E3 (TFE3) as a receptor-independent activator of Gα16: gene regulation by nuclear Gα subunit and its activator. *The Journal of Biological Chemistry* **286**, 17766-17776, (2011).
124 Robitaille, M. *et al.* Gβγ is a negative regulator of AP-1 mediated transcription. *Cell Signalling.* **22**, 1254-1266 (2010).
125 Spiegelberg, B. D. & Hamm, H. E. G βγ binds histone deacetylase 5 (HDAC5) and inhibits its transcriptional co-repression activity. *The Journal of Biological Chemistry* **280**, 41769-74176, (2005).
126 Bhatnagar, A. *et al.* Interaction of G-protein βγ complex with chromatin modulates GPCR-dependent gene regulation. *PLoS.One.* **8**, e52689 (2013).
127 Park, J. G., Muise, A., He, G. P., Kim, S. W. & Ro, H. S. Transcriptional regulation by the γ5 subunit of a heterotrimeric G protein during adipogenesis. *The EMBO Journal.* **18**, 4004-4012 (1999).
128 Park, J.-G., Muise, A., He, G.-P., Kim, S.-W. & Ro, H.-W. Transcriptional regulation by the γ5 subunit of a heterotrimeric G protein during adipogenesis. *The EMBO Journal* **18**, 4004-4012 (1999).
129 Mathiyalagan, P., Keating, S. T., Du, X. J. & El-Osta, A. Chromatin modifications remodel cardiac gene expression. *Cardiovascular Research* **103**, 7-16, (2014).
130 McKinsey, T. A., Zhang, C.-L., Lu, J. & Olson, E. N. Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. *Nature* **408**, 106-111 (2000).
131 Haberland, A. *et al.* Structural appearance of linker histone H1/siRNA complexes. *Molecular Biology Reports.* **36**, 1083-1093 (2009).

132 Kee, H. J. & Kook, H. Roles and targets of class I and IIa histone deacetylases in cardiac hypertrophy. *Journal of Biomedicine and Biotechnology* **2011**, 1-10, (2011).
133 Shaulian, E. AP-1--The Jun proteins: Oncogenes or tumor suppressors in disguise? *Cellular Signalling* **22**, 894-899, (2010).
134 Sorisky, A., Gagnon, A. & Abaiian, K. Aortic carboxypeptidase-like protein (ACLP): what is a protein with a name like that doing in adipose tissue? *Hormone and Metabolic Research* **34**, 764-766 (2002).
135 Drenan, R. M. *et al.* Palmitoylation regulates plasma membrane-nuclear shuttling of R7BP, a novel membrane anchor for the RGS7 family. *The Journal of Cell Biology* **169**, 623-633, (2005).
136 Grabowska, D. *et al.* Postnatal induction and localization of R7BP, a membrane-anchoring protein for regulator of G protein signalling 7 family-Gβ5 complexes in brain. *Neuroscience* **151**, 969-982 (2008).
137 Panicker, L. M. *et al.* Nuclear localization of the G protein β 5/R7-regulator of G protein signalling protein complex is dependent on R7 binding protein. *Journal of Neurochemistry.* **113**, 1101-1112 (2010).
138 Jia, L., Linder, M. E. & Blumer, K. J. Gi/o signalling and the palmitoyltransferase DHHC2 regulate palmitate cycling and shuttling of RGS7 family-binding protein. *The Journal of Biological Chemistry.* **286**, 13695-13703 (2011).
139 Shimano, H. SREBP-1c and TFE3, energy transcription factors that regulate hepatic insulin signalling. *The Journal of Molecular Medicine* **85**, 437-444, (2007).
140 Jacobs, S. *et al.* ADP-ribosylation factor (ARF)-like 4, 6, and 7 represent a subgroup of the ARF family characterization by rapid nucleotide exchange and a nuclear localization signal. *FEBS Letters.* **456**, 384-388 (1999).
141 Lin, C. Y. *et al.* ARL4, an ARF-like protein that is developmentally regulated and localized to nuclei and nucleoli. *The Journal of Biological Chemistry.* **275**, 37815-37823 (2000).
142 Lehming, N., Le, S. A., Schuller, J. & Ptashne, M. Chromatin components as part of a putative transcriptional repressing complex. *Proceeding of the National Academy of Sciences U.S.A* **95**, 7322-7326 (1998).
143 Contente, S., Yeh, T. J. & Friedman, R. M. H-ras localizes to cell nuclei and varies with the cell cycle. *Genes and Cancer* **2**, 166-172, (2011).
144 Karnoub, A. E. & Weinberg, R. A. Ras oncogenes: split personalities. *Nature Reviews Molecular Cell Biology* **9**, 517-531, (2008).
145 Wurzer, G., Mosgoeller, W., Chabicovsky, M., Cerni, C. & Wesierska-Gadek, J. Nuclear Ras: Unexpected Subcellular Distribution of Oncogenic Forms. *Journal of Cellular Biochemistry* **36**, 1-11 (2001).
146 Guil, S. *et al.* Alternative splicing of the human proto-oncogene c-H-ras renders a new Ras family protein that trafficks to cytoplasm and nucleus. *Cancer Research.* **63**, 5178-5187 (2003).
147 Maltese, W. A., Soule, G., Gunning, W., Calomeni, E. & Alexander, B. Mutant Rab24 GTPase is targeted to nuclear inclusions. *BMC Cell Biology* **3**, 1-17 (2002).

148 Mitra, R. S. *et al.* Rap1A and rap1B ras-family proteins are prominently expressed in the nucleus of squamous carcinomas: nuclear translocation of GTP-bound active form. *Oncogene* **22**, 6243-6256, (2003).
149 Birchenall-Roberts, M. C. *et al.* K-Ras4B proteins are expressed in the nucleolus: Interaction with nucleolin. *Biochemical and Biophysical Research Communications.* **348**, 540-549 (2006).
150 Fuentes-Calvo, I., Blazquez-Medela, A. M., Santos, E., Lopez-Novoa, J. M. & Martinez-Salgado, C. Analysis of k-ras nuclear expression in fibroblasts and mesangial cells. *PLoS.One.* **5**, e8703 (2010).
151 Mahalakshmi, R. N. *et al.* Nuclear localization of endogenous RGK proteins and modulation of cell shape remodeling by regulated nuclear transport. *Traffic.* **8**, 1164-1178 (2007).
152 Correll, R. N., Pang, C., Niedowicz, D. M., Finlin, B. S. & Andres, D. A. The RGK family of GTP-binding proteins: regulators of voltage-dependent calcium channels and cytoskeleton remodeling. *Cell Signalling.* **20**, 292-300 (2008).
153 Beguin, P. *et al.* Nuclear sequestration of β-subunits by Rad and Rem is controlled by 14-3-3 and calmodulin and reveals a novel mechanism for Ca2+ channel regulation. *Journal of Molecular Biology.* **355**, 34-46 (2006).
154 Charnet, P. *et al.* in *Modulation of Presynaptic Calcium Channels* (eds S. Mochida & G. Stephens) 131-149 (Springer, 2013).
155 Beguin, P. *et al.* Regulation of Ca2+ channel expression at the cell surface by the small G-protein kir/Gem. *Nature* **411**, 701-706 (2001).
156 Mahalakshmi, R. N. *et al.* Nuclear transport of Kir/Gem requires specific signals and importin α5 and is regulated by calmodulin and predicted serine phosphorylations. *Traffic.* **8**, 1150-1163 (2007).
157 Beguin, P. *et al.* Roles of 14-3-3 and calmodulin binding in subcellular localization and function of the small G-protein Rem2. *Biochemical Journal* **390**, 67-75, (2005).
158 Hibino, H. *et al.* Direct interaction with a nuclear protein and regulation of gene silencing by a variant of the Ca2+-channel β 4 subunit. *Proceeding of the National Academy of Sciences U.S.A.* **100**, 307-312 (2003).
159 Tadmouri, A. *et al.* Cacnb4 directly couples electrical activity to gene expression, a process defective in juvenile epilepsy. *The EMBO Journal.* **31**, 3730-3744 (2012).
160 Yang, T., Xu, X., Kernan, T., Wu, V. & Colecraft, H. M. Rem, a member of the RGK GTPases, inhibits recombinant CaV1.2 channels using multiple mechanisms that require distinct conformations of the GTPase. *Journal of Physiology.* **588**, 1665-1681 (2010).
161 Mosammaparast, N. & Pemberton, L. F. Karyopherins: from nuclear-transport mediators to nuclear-function regulators. *Trends in Cell Biology.* **14**, 547-556, (2004).
162 Christophe, D., Christophe-Hobertus, C. & Pichon, B. Nuclear targeting of proteins: how many different signals? *Cell Signalling.* **12**, 337-341 (2000).
163 Katta, S. S., Smoyer, C. J. & Jaspersen, S. L. Destination: inner nuclear membrane. *Trends in Cell Biology.* **24**, 221-229 (2014).

164 Lange, A. *et al.* Classical nuclear localization signals: definition, function, and interaction with importin α. *The Journal of Biological Chemistry* **282**, 5101-5105, (2007).
165 Pemberton, L. F. & Paschal, B. M. Mechanisms of receptor-mediated nuclear import and nuclear export. *Traffic.* **6**, 187-198, (2005).
166 Kuersten, S., Ohno, M. & Mattaj, I. W. Nucleocytoplasmic transport: Ran, β and beyond. *Trends in Immunology.* **11**, 497-503 (2001).
167 Freitas, N. & Cunha, C. Mechanisms and signals for the nuclear import of proteins. *Current Genomics.* **10**, 550-557 (2009).
168 Ribbeck, K., Lipowsky, G., Kent, H. M., Stewart, M. & Gorlich, D. NTF2 mediates nuclear import of Ran. *The EMBO Journal.* **17**, 6587-6598 (1998).
169 Takano, M. & Matsuyama, S. Intracellular and nuclear bradykinin B2 receptors. *European Journal of Pharmacology* **732**, 169-172, (2014).
170 Wright, C. D., Wu, S. C., Dahl, E. F., Sazama, A. J. & O'Connell, T. D. Nuclear localization drives α-1 adrenergic receptor oligodimerization and signalling in cardiac myocytes. *Cellular Signalling* **24**, 794-802, (2012).
171 Re, M. *et al.* The human gonadotropin releasing hormone type I receptor is a functional intracellular GPCR expressed on the nuclear membrane. *PloS one* **5**, e11489, (2010).
172 Dubash, A. D. *et al.* The small GTPase RhoA localizes to the nucleus and is activated by Net1 and DNA damage signals. *PloS one* **6**, e17380, (2011).
173 Zuleger, N., Korfali, N. & Schirmer, E. C. Inner nuclear membrane protein transport is mediated by multiple mechanisms. *Biochemical Society Transactions* **36**, 1373-1377, (2008).
174 Boman, A. L., Taylor, T. C., Melançon, P. & Wilson, K. L. A role for ADP-ribosylation factor in nuclear vesicle dynamics. *Nature* **358**, 512-514 (1992).
175 Hetzer, M., Bilbao-Cortés, D., Walther, T. C., Gruss, O. J. & Mattaj, I. W. GTP Hydrolysis by Ran Is Required for Nuclear Envelope Assembly. *Molecular Cell* **5**, 1013-1024 (2000).
176 Hetzer, M., Gruss, O. J. & Mattaj, I. W. The Ran GTPase as a marker of chromsome position in spindle formation and nuclear envelope assembly. *Nature* **4**, E177-E184 (2002).
177 D'Souza-Schorey, C. & Chavrier, P. ARF proteins: roles in membrane traffic and beyond. *Nature Reviews Molecular Cell Biology* **7**, 347-358, (2006).
178 Boman, A. L., Delannoy, M. R. & Wilson, K. F. GTP Hydrolysis is required for vesicle fusion during nuclear envelope assembly in vitro. *The Journal of Cell Biology* **116**, 281-294 (1992).
179 Pinot, M., Goud, B. & Manneville, J. B. Physical aspects of COPI vesicle formation. *Molecular Membrane Biology* **27**, 428-442, (2010).
180 Higgins, J. B. & Casey, P. J. In vitro processing of recombinant G protein γ subunits. *The Journal of Biological Chemistry* **269**, 9067-9073 (1994).

181 Zhang, F. L. & Casey, P. J. Protein prenylation: molecular mechanisms and functional consequences. *Annual Review of Biochemistry* **65**, 241-269 (1996).
182 Garcia, A. M., Rowell, C., Ackermann, K., Kowalczyk, J. J. & Lewis, M. D. Peptidomimetic Inhibitors of Ras Farnesylation and Functionin Whole Cells. *The Journal of Biological Chemistry* **268**, 18415-18418 (1993).
183 Kino, T., Kozasa, T. & Chrousos, G. P. Statin-induced blockade of prenylation alters nucleocytoplasmic shuttling of GTP-binding proteins γ2 and β2 and enhances their suppressive effect on glucocorticoid receptor transcriptional activity. *European Journal of Clinical Investigation* **35**, 508-513 (2005).
184 Mulligan, T., Blaser, H., Raz, E. & Farber, S. A. Prenylation-deficient G protein γ subunits disrupt GPCR signalling in the zebrafish. *Cellular Signalling* **22**, 221-233, (2010).
185 Kino, T. *et al.* G protein β interacts with the glucocorticoid receptor and suppresses its transcriptional activity in the nucleus. *The Journal of Cell Biology* **169**, 885-896, (2005).
186 Melchior, F. Ran GTPase cycle: One mechanism — two functions. *Current Biology* **11**, 257-260 (2001).
187 Ren, M., Drivas, G., D'Eustachio, P. & Rush, M. G. Ran/TC4: A Small Nuclear GTP-binding Protein That Regulates DNA Synthesis. *The Journal of Cell Biology* **120**, 313-323 (1993).
188 Crouch, M. F. & Simson, L. The G-protein Gi regulates mitosis but not DNA synthesis in growth factor-activated fibroblasts: a role for the nuclear translocation of Gi. *The FASEB Journal* **11**, 189-198 (1997).
189 Crouch, M. F., Osborne, G. W. & Willard, F. S. The GTP-binding protein Giα translocates to kinetochores and regulates the M-G1 cell cycle transition of Swiss 3T3 cells. *Cellular Signalling* **12**, 153-163 (2000).
190 Zwaal, R. R. *et al.* G proteins are required for spatial orientation of early cell cleavages in C. elegans embryos. *Cell* **86**, 619-629 (1996).
191 Gotta, M. & Ahringer, J. Distinct roles for Gα and Gβγ in regulating spindle position and orientation in Caenorhabditis elegans embryos. *Nature Cell Biology* **3** (2001).
192 Tsou, M. F., Hayashi, A. & Rose, L. S. LET-99 opposes Gα/GPR signalling to generate asymmetry for spindle positioning in response to PAR and MES-1/SRC-1 signalling. *Development* **130**, 5717-5730, (2003).
193 Guilluy, C., Dubash, A. D. & Garcia-Mata, R. Analysis of RhoA and Rho GEF activity in whole cells and the cell nucleus. *Nature Protocols* **6**, 2050-2060, (2011).
194 Campden, R. *et al. Tandem affinity purification to identify cytosolic and nuclear Gβγ-interacting proteins*. Vol. 1234 (2015).
195 Roy, S. J. *et al.* Novel, gel-free proteomics approach identifies RNF5 and JAMP as modulators of GPCR stability. *Molecular Endocrinology* **27**, 1245-1266, (2013).

196 Rigaut, G. *et al.* A generic protein purification method for protein complex characterization and proteome exploration. *Nature Biotechnology* **17**, 1030-1032 (1999).
197 Puig, O. *et al.* The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods* **24**, 218-229, (2001).
198 Ahmed, S. M., Daulat, A. M. & Angers, S. Tandem affinity purification and identification of heterotrimeric g protein-associated proteins. *Methods in Molecular Biology* **756**, 357-370, (2011).
199 Gloeckner, C. J., Boldt, K. & Ueffing, M. Strep/FLAG tandem affinity purification (SF-TAP) to study protein interactions. *Current Protocols in Protein Science* **Chapter 19**, (2009).
200 Stamsas, G. A., Havarstein, L. S. & Straume, D. CHiC, a new tandem affinity tag for the protein purification toolbox. *Journal of Microbiological Methods* **92**, 59-63, (2013).
201 Okada, Y. *et al.* New protein purification system using gold-magnetic beads and a novel peptide tag, "the methionine tag". *Bioconjugate Chemistry* **22**, 887-893, (2011).
202 Maine, G. N., Gluck, N., Zaidi, I. W. & Burstein, E. Bimolecular affinity purification (BAP): tandem affinity purification using two protein baits. *Cold Spring Harbor Protocols* **4**, 1-7, (2009).
203 Ahmed, S. M., Daulat, A. M., Meunier, A. & Angers, S. G protein βγ subunits regulate cell adhesion through Rap1a and its effector Radil. *The Journal of Biological Chemistry* **285**, 6538-6551, (2010).
204 Chong, S. *et al.* Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. *Gene* **192**, 271-281, (1997).
205 Kim, J. S. & Raines, R. T. Ribonuclease S-peptide as a carrier in fusion proteins. *Protein Science : A Publication of the Protein Society* **2**, 348-356, (1993).
206 Korndörfer, I. P. & Skerra, A. Improved affinity of engineered streptavidin for the Strep-tag II peptide is due to a fixed open conformation of the lid-like loop at the binding site. *Protein Science : A Publication of the Protein Society* **11**, 883-893, (2002).
207 Li, Y. Commonly used tag combinations for tandem affinity purification. *Biotechnology and applied biochemistry* **55**, 73-83, (2010).
208 Raines, R. T., McCormick, M., Van, T. R. & Mierendorf, R. C. The S· tag fusion system for protein purification. *Methods in Enzymology* **326**, 362-376 (2000).
209 Stirling, D. A., Petrie, A., Pulford, D. J., Paterson, D. T. & Stark, M. J. Protein A-calmodulin fusions: a novel approach for investigating calmodulin function in yeast. *Molecular Microbiology* **6**, 703-713, (1992).
210 Stofko-Hahn, R. E., Carr, D. W. & Scott, J. D. A single step purification for recombinant proteins. Characterization of a microtubule associated protein (MAP 2) fragment which associates with the type II cAMP-dependent protein kinase. *FEBS letters* **302**, 274-278, (1992).

211 Uhlén, M., Guss, B., Nilsson, B., Götz, F. & Lindberg, M. Expression of the gene encoding protein A in Staphylococcus aureus and coagulase-negative staphylococci. *Journal of Bacteriology* **159**, 713-719 (1984).
212 Lavallee-Adam, M. *et al.* Discovery of cell compartment specific protein-protein interactions using affinity purification combined with tandem mass spectrometry. *Journal of Proteome Research* **12**, 272-281, (2013).
213 Keller, B. O., Sui, J., Young, A. B. & Whittal, R. M. Interferences and contaminants encountered in modern mass spectrometry. *Analytica Chimica Acta* **627**, 71-81, (2008).
214 Chevallet, M., Luche, S. & Rabilloud, T. Silver staining of proteins in polyacrylamide gels. *Nature Protocols* **1**, 1852-1858, (2006).
215 Mellacheruvu, D. *et al.* The CRAPome: a contaminant repository for affinity purification-mass spectrometry data. *Nature Methods* **10**, 730-736 (2013).
216 Doaga, I. O., Savopol, T., Neagu, M., Neagu, A. & Kovacs, E. The kinetics of cell adhesion to solid scaffolds: an experimental and theoretical approach. *Journal of Biological Physics* **34**, 495-509, (2008).
217 Campden, R., Robitaille, M., Angers, S. & Hébert, T. E. Analysis of nuclear and cytosolic Gβγ interactions (2015).
218 Chang, C. W. *et al.* Acute β-adrenergic activation triggers nuclear import of histone deacetylase 5 and delays G(q)-induced transcriptional activation. *The Journal of Biological Chemistry* **288**, 192-204, (2013).
219 Ma, L. & Pei, G. β-arrestin signalling and regulation of transcription. *Journal of cell science* **120**, 213-218, (2007).
220 Boivin, B., Chevalier, D., Villeneuve, L. R., Rousseau, E. & Allen, B. G. Functional endothelin receptors are present on nuclei in cardiac ventricular myocytes. *The Journal of Biological Chemistry* **278**, 29153-29163, (2003).
221 Hulsen, T., de Vlieg, J. & Alkema, W. BioVenn - a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams. *BMC genomics* **9**, 488, (2008).
222 Mi, H., Muruganujan, A., Casagrande, J. T. & Thomas, P. D. Large-scale gene function analysis with the PANTHER classification system. *Nature Protocols* **8**, 1551-1566, (2013).
223 Mi, H., Muruganujan, A. & Thomas, P. D. PANTHER in 2013: modeling the evolution of gene function, and other gene attributes, in the context of phylogenetic trees. *Nucleic Acids Research* **41**, D377-386, (2013).
224 Franceschini, A. *et al.* STRING v9.1: protein-protein interaction networks, with increased coverage and integration. *Nucleic Acids Research* **41**, D808-815, (2013).
225 Zhang, M. *et al.* Progesterone receptor membrane component 1 is a functional part of the GLP-1 receptor complex in pancreatic β cells. *Molecular & Cellular Proteomics* **13**, 3049-3062 (2014).
226 Magga, J. M., Jarvis, S. E., Arnot, M. I., Zamponi, G. W. & Braun, J. E. A. Cysteine string protein regulates G protein modulation of N-type calcium channels. *Neuron* **28**, 195-204 (2000).

227 Dupre, D. J. *et al.* Dopamine receptor-interacting protein 78 acts as a molecular chaperone for Gγ subunits before assembly with Gβ. *The Journal of Biological Chemistry* **282**, 13703-13715, (2007).
228 Willardson, B. M. & Howlett, A. C. Function of phosducin-like proteins in G protein signalling and chaperone-assisted protein folding. *Cell Signalling* **19**, 2417-2427, (2007).
229 Vainberg, I. E. *et al.* Prefoldin, a chaperone that delivers unfolded proteins to cytosolic chaperones. *Cell* **93**, 863-873 (1998).
230 Riou, P. *et al.* 14-3-3 proteins interact with a hybrid prenyl-phosphorylation motif to inhibit G proteins. *Cell* **153**, 640-653, (2013).
231 Palmgren, M. G. & Nissen, P. P-type ATPases. *Annual Review of Biophysics* **40**, 243-266, (2011).
232 Stewart, A. G., Sobti, M., Harvey, R. P. & Stock, D. Rotary ATPases: models, machine elements and technical specifications. *Bioarchitecture* **3**, 2-12, (2013).
233 Kobayashi, K. *et al.* The gene mutated in adult-onset type II citrullinaemia encodes a putative mitochondrial carrier protein. *Nature Genetics* **22**, 159-163 (1999).
234 Kaplan, R. S., Mayor, J. A. & Wood, D. O. The mitochondrial tricarboxylate transport protein. *The Journal of Biological Chemistry* **268**, 13682-13690 (1993).
235 Kabe, Y. *et al.* Porphyrin accumulation in mitochondria is mediated by 2-oxoglutarate carrier. *The Journal of Biological Chemistry* **281**, 31729-31735, (2006).
236 Palmieri, L. *et al.* Citrin and aralar1 are Ca2+-stimulated aspartate/glutamate transporters in mitochondria. *The EMBO Journal* **20**, 5060-5069 (2001).
237 Wang, W. *et al.* Identification of rare DNA variants in mitochondrial disorders with improved array-based sequencing. *Nucleic Acids Research* **39**, 44-58, (2011).
238 Dolce, V., Fiermonte, G., Messina, A. & Palmieri, F. Nucleotide sequence of a human heart cDNA encoding the mitochondrial phosphate carrier. *Journal of DNA Sequencing and Mapping* **2**, 133-135 (1991).
239 Fiermonte, G. *et al.* Organization and sequence of the gene for the human mitochondrial dicarboxylate carrier: evolution of the carrier family. *Biochemical Journal* **344**, 953-960 (1999).
240 Duncan, A. M. V., Ozawa, T., Suzuki, H. & Rozen, R. Assignment of the gene for the cytochrome C1 subunit of the mitochondrial cytochrome bc1 complex (CYC1) to human chromosome 8q24.3. *Genomics* **19**, 400-401 (1994).
241 Murray, J. *et al.* The subunit composition of the human NADH dehydrogenase obtained by rapid one-step immunopurification. *The Journal of Biological Chemistry* **278**, 13619-13622, (2003).
242 Poupon, V. *et al.* Molecular Cloning and Characterization of MT-ACT48, a Novel Mitochondrial Acyl-CoA Thioesterase. *Journal of Biological Chemistry* **274**, 19188-19194, (1999).

243 Richter, R. *et al.* A functional peptidyl-tRNA hydrolase, ICT1, has been recruited into the human mitochondrial ribosome. *The EMBO Journal* **29**, 1116-1125, (2010).
244 Duncan, A. M. V., Ozawa, T., Suzuki, H. & Rozen, R. Assignmnet of the gene for the core protein II (UQCR2) subunit of the mitochondrial cytochrome bc1 complex to human chromosome 16p12. *Genomics* **18**, 455-456 (1993).
245 Koc, E. C. *et al.* The large subunit of the mammalian mitochondrial ribosome. Analysis of the complement of ribosomal proteins present. *The Journal of Biological Chemistry* **276**, 43958-43969, (2001).
246 Lo, S.-C. & Hannink, M. PGAM5 tethers a ternary complex containing Keap1 and Nrf2 to mitochondria. *Experimental Cell Research* **314**, 1789-1803 (2008).
247 Gieffers, C., Korioth, F., Heimann, P., Ungermann, C. & Frey, J. Mitofilin is a transmembrane protein of the inner mitochondrial membrane expressed as two isoforms. *Experimental Cell Research* **232**, 395-399 (1997).
248 Aoyama, T., Wakui, K., Orii, K. E., Hashimoto, T. & Fukushima, Y. Fluorescence in situ hybridization mapping of the α and β subunits (HADHA and HADHB) of human mitochondrial fatty acid β-oxidation multienzyme complex to 2p23 and their evolution. *Cytogenetics and Cell Genetics* **79**, 221–224 (1997).
249 Hansen, J. J. *et al.* Genomic structure of the human mitochondrial chaperonin genes: HSP60 and HSP10 are localised head to head on chromosome 2 separated by a bidirectional promoter. *Human Genetics* **112**, 71-77, (2003).
250 Kang, P.-J. *et al.* Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins. *Nature* **348**, 137-143 (1990).
251 Hartmann-Petersen, R., Tanaka, K. & Hendil, K. B. Quaternary structure of the ATPase complex of human 26S proteasomes determined by chemical cross-linking. *Archives of Biochemistry and Biophysics* **386**, 89-94, (2001).
252 Bennett, E. J., Rush, J., Gygi, S. P. & Harper, J. W. Dynamics of cullin-RING ubiquitin ligase network revealed by systematic quantitative proteomics. *Cell* **143**, 951-965, (2010).
253 Su, V. & Lau, A. F. Ubiquitin-like and ubiquitin-associated domain proteins: significance in proteasomal degradation. *Cellular and Molecular Life Sciences* **66**, 2819-2833, (2009).
254 Komander, D. The emerging complexity of protein ubiquitination. *Biochemical Society Transactions* **37**, 937-953, (2009).
255 Peterson, C. L. & Tamkun, J. W. The SWI-SNF complex: a chromatin remodelling machine? *Trends in Biochemical Sciences* **20**, 143-146 (1995).
256 Tainer, J. A., McCammon, A. & Ivanov, I. Recognition of the ring-opened state of proliferating cell nuclear antigen by replication factor C promotes

eukaryotic clamp-loading. *Journal of the American Chemical Society* **132**, 7372-7378 (2010).
257 Méchali, M. Eukaryotic DNA replication origins: many choices for appropriate answers. *Nature Reviews Molecular Cell Biology* **11**, 728-738, (2010).
258 Baxi, M. D. & Vishwanatha, J. K. Uracil DNA-glycosylase/glyceraldehyde-3-phosphate dehydrogenase is an Ap4A binding protein. *Biochemistry* **34**, 9700-9707 (1995).
259 Einhauer, A. & Jungbauer, A. The FLAG peptide, a versatile fusion tag for the purification of recombinant proteins. *Journal of Biochemical and Biophysical Methods* **49**, 455-465 (2001).
260 Melkko, S. & Dario, N. *Calmodulin as an Affinity Purifcation Tag*. Vol. 205 69-77 (Humana Press Inc. , 2003).
261 Schmidt, T. G. & Skerra, A. The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins. *Nature Protocols* **2**, 1528-1535, (2007).
262 Wittrup, A. *et al.* magnetic nanoparticle-based isolation of endocytic vesicles reveals a role of the heat shock protein GRP75 in macromolecular delivery. *Proceeding of the National Academy of Sciences U.S.A* **107**, 13342-13347 (2010).
263 Wojcikiewicz, R. J. Regulated ubiquitination of proteins in GPCR-initiated signalling pathways. *Trends in Pharmacological Sciences* **25**, 35-41, (2004).
264 Ahmed, S. M. *et al.* KIF14 negatively regulates Rap1a-Radil signalling during breast cancer progression. *The Journal of Cell Biology* **199**, 951-967, (2012).
265 Tay, S. Y., Ingham, P. W. & Roy, S. A homologue of the Drosophila kinesin-like protein Costal2 regulates Hedgehog signal transduction in the vertebrate embryo. *Development* **132**, 625-634, (2005).
266 Barreto, F. S. & Burton, R. S. Evidence for compensatory evolution of ribosomal proteins in response to rapid divergence of mitochondrial rRNA. *Molecular biology and evolution* **30**, 310-314, (2013).
267 Ip, J. Y. *et al.* Global impact of RNA polymerase II elongation inhibition on alternative splicing regulation. *Genome research* **21**, 390-401, (2011).
268 Cloutier, P. *et al.* High-resolution mapping of the protein interaction network for the human transcription machinery and affinity purification of RNA polymerase II-associated complexes. *Methods* **48**, 381-386, (2009).
269 Forget, D. *et al.* The protein interaction network of the human transcription machinery reveals a role for the conserved GTPase RPAP4/GPN1 and microtubule assembly in nuclear import and biogenesis of RNA polymerase II. *Molecular & Cellular Proteomics* **9**, 2827-2839, (2010).
270 Forget, D. *et al.* Nuclear import of RNA polymerase II is coupled with nucleocytoplasmic shuttling of the RNA polymerase II-associated protein 2. *Nucleic Acids Research* **41**, 6881-6891, (2013).

271 Jeronimo, C. *et al.* Systematic analysis of the protein interaction network for the human transcription machinery reveals the identity of the 7SK capping enzyme. *Molecular Cell* **27**, 262-274, (2007).
272 Moller, A. *et al.* Proteomic analysis of mitotic RNA polymerase II reveals novel interactors and association with proteins dysfunctional in disease. *Molecular & Cellular Proteomics* **11**, M111.011767, (2012).
273 Fislova, T., Thomas, B., Graef, K. M. & Fodor, E. Association of the influenza virus RNA polymerase subunit PB2 with the host chaperonin CCT. *Journal of Virology* **84**, 8691-8699, (2010).
274 Shandilya, J. & Roberts, S. G. E. The transcription cycle in eukaryotes: from productive initiation to RNA polymerase II recycling. *Biochimica et Biophysica Acta*, (2012).
275 Akgoz, M., Kalyanaraman, V. & Gautam, N. G protein βγ complex translocation from plasma membrane to Golgi complex is influenced by receptor γ subunit interaction. *Cell Signalling* **18**, 1758-1768, (2006).
276 Fornerod, M., Ohno, M., Yoshida, M. & Mattaj, I. W. CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90**, 1051-1060 (1997).
277 Merlen, C., Villeneuve, L. R. & Allen, B. G. Using caged ligands to study intracrine endothelin signalling in intact cardiac myocytes. *Methods in Molecular Biology* **1234**, 31-41, (2015).

Appendix

Table 15: *Proteins identified in the cytosol by Flag-Gβ1, untreated conditions.* Proteins identified as Gβγ interactors with Flag tagged Gβ1 and identified by mass spectrometry and grouped by molecular function and protein class using Panther online software[223].

Protein	Molecular Function	Molecular Class	Average Total Peptides	Average Unique Peptides	Coverage	n
TUBB4A	Structural Constituent Of Cytoskeleton	Tubulin	131	2	41.35	2
TUBB3	Structural Constituent Of Cytoskeleton	Tubulin	47	1	25.8	1
GNAI3	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	89	8	30.8	2
NAV2			33	1	0.5	1
TUBA1A	Structural Constituent Of Cytoskeleton	Tubulin	61	2	36.4	2
GNAI2	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	65	16	31.8	2
GNAQ	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	48	20	38.4	2
GNAI1	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	52	6	28	2
PDCL	Catalytic Activity Protein Binding Small GTPase Regulator Activity	G-Protein Modulator Chaperone	52	13	26.1	2
GNB1	GTPase Activity Protein Binding	Hydrolase Heterotrimeric G-Protein	21	4	12.1	1

GNAS	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	36	13	16.1	2
KCTD12	Protein Binding	Enzyme Modulator	26	13	25.25	2
GNA11	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	34	9	35.9	2
GNA13	Pyrophosphatase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	34	12	24.65	2
CCT3		Chaperonin	35	20	23.05	2
SLC25A13	Amino Acid Transmembrane Transporter Activity Transmembrane Transporter Activity Calcium Ion Binding Calmodulin Binding	Amino Acid Transporter Mitochondrial Carrier Protein Transfer/Carrier Protein Calmodulin	18	16	14.7	2
TCP1		Chaperonin	28	20	23.75	2
CCT7		Chaperonin	29	16	23.55	2
KCTD2	Protein Binding	Enzyme Modulator	15	12	32.3	2
ATP2A2	Hydrolase Activity Ion Channel Activity Cation Transmembrane Transporter Activity	Cation Transporter Ion Channel Hydrolase	11	10	6.95	2
CCT2		Chaperonin	27	19	27.65	2
SLC25A4	Amino Acid Transmembrane Transporter Activity Transmembrane Transporter Activity Calcium Ion Binding Calmodulin Binding	Amino Acid Transporter Mitochondrial Carrier Protein Transfer/Carrier Protein Calmodulin	13	2	14.8	2
CCT5		Chaperonin	17	14	14.75	2
KCTD17	Protein Binding	Enzyme Modulator	12	5	18.85	2

GNA12	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	13	3	6.55	2
KCTD5	Protein Binding	Enzyme Modulator	9	4	15	2
ATP5A1	Hydrolase Activity Receptor Activity Anion Channel Activity Ligand-Gated Ion Channel Activity Cation Transmembrane Transporter Activity Proton-Transporting ATP Synthase Activity, Rotational Mechanism Single-Stranded DNA Binding	ATP Synthase Anion Channel Ligand-Gated Ion Channel Ligand-Gated Ion Channel DNA Binding Protein Hydrolase	8	8	9.35	2
HSP90B1		Hsp90 Family Chaperone	11	8	7.5	2
CCT6A		Chaperonin	20	17	24.5	2
ERLIN2			13	8	17.85	2
SLC25A3	Amino Acid Transmembrane Transporter Activity Transmembrane Transporter Activity Calcium Ion Binding Calmodulin Binding	Amino Acid Transporter Mitochondrial Carrier Protein Transfer/Carrier Protein Calmodulin	10	6	9.1	2
UBAC2	Transcription Cofactor Activity Sequence-Specific DNA Binding Transcription Factor Activity Aspartic-Type Endopeptidase Activity Sequence-Specific DNA Binding Transcription Factor Activity Protein Binding	Transcription Cofactor Aspartic Protease Aspartic Protease	7	7	18.1	2
CCT4		Chaperonin	14	10	13.35	2
ATP1A1	Hydrolase Activity Ion Channel Activity Cation Transmembrane Transporter Activity	Cation Transporter Ion Channel Hydrolase	7	6	3.8	2
LOC730429			4	4	2.5	1

TPP2	Serine-Type Peptidase Activity	Serine Protease Serine Protease	9	8	4.8	2
SLC25A1	Amino Acid Transmembrane Transporter Activity Transmembrane Transporter Activity Calcium Ion Binding Calmodulin Binding	Amino Acid Transporter Mitochondrial Carrier Protein Transfer/Carrier Protein Calmodulin	9	8	14.1	2
DPM1	Transferase Activity, Transferring Glycosyl Groups	Glycosyltransferase	7	7	16.7	2
TUBAL3	Structural Constituent Of Cytoskeleton	Tubulin	6	2	5.4	2
ANKRD63	Protein Kinase Activity	Non-Receptor Serine/Threonine Protein Kinase	7	7	11.35	2
ERLIN1			6	4	11.05	2
GNAZ	GTPase Activity Pyrophosphatase Activity Adenylate Cyclase Activity Nucleotide Binding Receptor Binding Enzyme Regulator Activity	Heterotrimeric G-Protein	7	7	16.35	2
IPO7	GTPase Activity Transmembrane Transporter Activity Protein Binding	Transporter Small GTPase	6	6	3.4	2
NDUFS7	Oxidoreductase Activity	Dehydrogenase Reductase	3	2	10.8	1
TECR	Oxidoreductase Activity	Dehydrogenase	3	2	7.1	1
QPCTL	Transferase Activity	Transferase	6	4	10.4	2
GNG12	GTPase Activity Protein Binding	Heterotrimeric G-Protein	5	2	22.2	2
DNAJC25-GNG10			6	2	9.8	2
KIF7	Microtubule Motor Activity Structural Constituent Of Cytoskeleton	Microtubule Binding Motor Protein	6	2	1.4	2
RADIL			3	3	1.95	2

COX2	Oxidoreductase Activity	Oxidoreductase	4	4	15.9	2
SSR1	Calcium Ion Binding G-Protein Coupled Receptor Activity	Membrane Traffic Protein Calcium-Binding Protein G-Protein Coupled Receptor	4	4	8.55	2
PFKL	Carbohydrate Kinase Activity	Carbohydrate Kinase	4	3	2.85	2
PFKP	Carbohydrate Kinase Activity	Carbohydrate Kinase	5	4	3.35	2
STOM	Structural Constituent Of Cytoskeleton	Carbohydrate Kinase Cytoskeletal Protein	2	2	7.3	1
PSMD13	Catalytic Activity Protein Binding Enzyme Regulator Activity	Enzyme Modulator	4	4	9	2
NIPSNAP1			2	2	10.2	1
IMMT			3	3	2.3	2
AGK	Carbohydrate Kinase Activity	Carbohydrate Kinase	2	2	5.2	1
CALU	Calcium Ion Binding Calmodulin Binding	Calmodulin	5	3	8.45	2
SLC25A11	Amino Acid Transmembrane Transporter Activity Transmembrane Transporter Activity Calcium Ion Binding Calmodulin Binding	Amino Acid Transporter Mitochondrial Carrier Protein Transfer/Carrier Protein Calmodulin	3	3	5.4	2
MGST3	Oxidoreductase Activity Transferase Activity Peroxidase Activity	Transferase	3	3	13.2	2
SCD	Oxidoreductase Activity	Oxidoreductase	2	2	3.6	2
NDUFS1		Dehydrogenase Reductase	1	1	2.2	1
PFDN4			2	2	9	2
PSMC3	Hydrolase Activity	Hydrolase	4	4	7.3	2
SLC25A24	Amino Acid Transmembrane Transporter Activity Transmembrane Transporter Activity	Amino Acid Transporter Mitochondrial Carrier Protein Transfer/Carrier Protein Calmodulin	1	1	2.8	1

	Calcium Ion Binding Calmodulin Binding					
SSR4	G-Protein Coupled Receptor Activity	G-Protein Coupled Receptor	4	4	15.65	2
IPO9	GTPase Activity Transmembrane Transporter Activity Protein Binding	Transporter Small GTPase	1	1	1.1	1
LPCAT1	Transferase Activity, Transferring Acyl Groups Calcium Ion Binding Calmodulin Binding Calcium-Dependent Phospholipid Binding	Acyltransferase Annexin Calmodulin	1	1	2.8	1
HSD17B12	Oxidoreductase Activity	Dehydrogenase Reductase	1	1	4.5	1
MRPL43	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	1	1	5.1	1
GNG5	GTPase Activity Protein Binding	Heterotrimeric G-Protein	1	1	39.7	1
DNAJA2		Chaperone	3	3	5.45	2
RPN2	Transferase Activity, Transferring Glycosyl Groups	Glycosyltransferase	3	2	1.9	2
YWHAB		Chaperone	1	1	4.1	1
GNG4	GTPase Activity Protein Binding	Heterotrimeric G-Protein	1	1	21.3	1
GNB2	GTPase Activity Protein Binding	Hydrolase Heterotrimeric G-Protein	19	5	18.5	1
HSPD1		Chaperonin	9	6	18.8	1
TP53I11			5	2	13.2	1
HADHA	Oxidoreductase Activity Hydro-Lyase Activity Racemase And Epimerase Activity	Dehydrogenase Hydratase Epimerase/Racemase	4	4	6.9	1
PSMD3	Catalytic Activity Protein Binding Enzyme Activator Activity	Enzyme Modulator	4	4	11.2	1

CDK1	Non-Membrane Spanning Protein Tyrosine Kinase Activity	Non-Receptor Serine/Threonine Protein Kinase Non-Receptor Tyrosine Protein Kinase Non-Receptor Serine/Threonine Protein Kinase Non-Receptor Tyrosine Protein Kinase	4	2	7.1	1
VBP1		Chaperone	3	3	22.3	1
PSMC2	Hydrolase Activity	Hydrolase	3	3	7.9	1
EEF1G	Oxidoreductase Activity Transferase Activity Racemase And Epimerase Activity Structural Constituent Of Cytoskeleton Translation Elongation Factor Activity Translation Elongation Factor Activity Receptor Binding Translation Elongation Factor Activity	Transferase Signalling Molecule Reductase Translation Elongation Factor Epimerase/Racemase Cytoskeletal Protein	3	3	8.2	1
PHB2			3	3	12.7	1
PSMC6	Hydrolase Activity	Hydrolase	3	3	9.2	1
UBR5	Ligase Activity	Ubiquitin-Protein Ligase	3	3	1.5	1
PSMD2	Catalytic Activity Protein Binding Enzyme Regulator Activity	Enzyme Modulator	3	2	2.8	1
PRKAR1A	Kinase Activity Protein Binding Kinase Regulator Activity	Kinase Modulator	3	2	7.3	1
SLC25A10	Amino Acid Transmembrane Transporter Activity Transmembrane Transporter Activity Calcium Ion Binding Calmodulin Binding	Amino Acid Transporter Mitochondrial Carrier Protein Transfer/Carrier Protein Calmodulin	3	1	8.4	1

PSMC1	Hydrolase Activity	Hydrolase	2	2	8.2	1
XPO1		Receptor	2	2	2.1	1
DNAJA1		Chaperone	2	2	9.6	1
GNG7	GTPase Activity Protein Binding	Heterotrimeric G-Protein	2	1	23.5	1
LBR	Oxidoreductase Activity Receptor Activity	Receptor Reductase	1	1	3.1	1
NDUFS2	Oxidoreductase Activity	Dehydrogenase Reductase	1	1	1.9	1
PCNA	Catalytic Activity Nucleic Acid Binding DNA Polymerase Processivity Factor Activity	DNA Polymerase Processivity Factor	1	1	5	1
NDUFA9	Oxidoreductase Activity	Dehydrogenase Reductase	1	1	3.4	1
CAD	Transferase Activity Ligase Activity	Transferase Ligase	1	1	0.7	1
HSPA6		Hsp70 Family Chaperone	8	1	8.1	1
AMOT			7	4	11	1
LOC645870			7	3	44.2	1
CTNNA1	Structural Constituent Of Cytoskeleton Actin Binding	Non-Motor Actin Binding Protein Cell Adhesion Molecule	6	3	7.5	1
MYO1D	Motor Activity Structural Constituent Of Cytoskeleton Protein Binding Enzyme Regulator Activity	G-Protein Modulator Actin Binding Motor Protein Cell Junction Protein	5	5	7.6	1
H2AFY	DNA Binding	Histone	4	4	18.3	1
MYO1B	Motor Activity Structural Constituent Of Cytoskeleton Protein Binding Enzyme Regulator Activity	G-Protein Modulator Actin Binding Motor Protein Cell Junction Protein	4	4	4.7	1
FBL	Methyltransferase Activity RNA Binding	Methyltransferase Ribonucleoprotein	3	2	13.4	1

AP2B1		Membrane Traffic Protein	3	2	4.1	1
DSP	Structural Constituent Of Cytoskeleton Actin Binding	Non-Motor Actin Binding Protein	3	3	2.1	1
NMT1	Transferase Activity	Transferase	3	3	11.3	1
SF3A1	Catalytic Activity mRNA Binding	mRNA Splicing Factor	3	3	6.2	1
YWHAQ		Chaperone	2	1	11.8	1
MDN1		Chaperone	2	2	0.6	1
MYO1C	Motor Activity Structural Constituent Of Cytoskeleton Protein Binding Enzyme Regulator Activity	G-Protein Modulator Actin Binding Motor Protein Cell Junction Protein	2	2	2.4	1
TFCP2	Sequence-Specific DNA Binding Transcription Factor Activity Sequence-Specific DNA Binding Transcription Factor Activity	Transcription Factor	2	2	7	1
MYEF2	RNA Binding	Ribonucleoprotein	2	2	5.8	1
GTF3C4			2	2	4.3	1
SKIV2L2	DNA Helicase Activity RNA Helicase Activity Hydrolase Activity mRNA Binding	DNA Helicase RNA Helicase mRNA Splicing Factor Helicase Hydrolase	2	2	2.2	1
CKB	Amino Acid Kinase Activity	Amino Acid Kinase Amino Acid Kinase	2	1	4.5	1
NOP16			2	1	8.4	1
RPA3			2	1	19	1
CBX1	Sequence-Specific DNA Binding Transcription Factor Activity Sequence-Specific DNA Binding Transcription Factor Activity Chromatin Binding	Transcription Factor Chromatin/Chromatin-Binding Protein	1	1	8.6	1
U2SURP	mRNA Binding	mRNA Processing Factor	1	1	1.5	1

RBM3	Catalytic Activity Structural Constituent Of Ribosome Poly(A) RNA Binding	mRNA Polyadenylation Factor mRNA Splicing Factor Ribonucleoprotein Ribosomal Protein	1	1	20.4	1
NIPBL	Nucleic Acid Binding Chromatin Binding	Chromatin/Chromatin-Binding Protein	1	1	0.5	1
SUPT6H	Sequence-Specific DNA Binding Transcription Factor Activity Sequence-Specific DNA Binding Transcription Factor Activity Chromatin Binding	Transcription Factor Chromatin/Chromatin-Binding Protein	1	1	0.8	1
ADD1	Structural Constituent Of Cytoskeleton Actin Binding	Non-Motor Actin Binding Protein	1	1	2.4	1
TAGLN2	Structural Constituent Of Cytoskeleton Actin Binding	Non-Motor Actin Binding Protein	1	1	9	1
POLR2D	Sequence-Specific DNA Binding Transcription Factor Activity Sequence-Specific DNA Binding Transcription Factor Activity	Transcription Factor	1	1	11.3	1
CTNND1	Structural Constituent Of Cytoskeleton Intermediate Filament Binding	Intermediate Filament Binding Protein Cell Junction Protein Cell Adhesion Molecule	1	1	2.7	1
DDX46	RNA Helicase Activity Translation Initiation Factor Activity Translation Initiation Factor Activity Translation Initiation Factor Activity	RNA Helicase Helicase	1	1	1.3	1
ZC3H18			1	1	1.9	1
NOL9			1	1	2.7	1
POLR2E	DNA-Directed RNA Polymerase Activity Nucleotidyltransferase Activity Nucleic Acid Binding	Nucleotidyltransferase DNA-Directed RNA Polymerase	1	1	8.1	1
EEF2	GTPase Activity Translation Initiation Factor Activity	Translation Elongation Factor Translation Initiation Factor	1	1	1.5	1

	Translation Elongation Factor Activity Translation Initiation Factor Activity Translation Elongation Factor Activity Protein Binding Translation Initiation Factor Activity Translation Elongation Factor Activity	Hydrolase G-Protein				
MORF4			1	1	7.7	1
WDR18			1	1	3.9	1
BRIX1	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	1	1	2	1
NAA38	Nuclease Activity mRNA Binding	mRNA Splicing Factor Nuclease	1	1	25	1
RRP9			1	1	5.9	1
IMPDH2	Oxidoreductase Activity	Dehydrogenase Reductase	1	1	3.1	1
PCBP2	Catalytic Activity mRNA Binding Protein Binding	mRNA Splicing Factor Ribonucleoprotein Enzyme Modulator	1	1	3.6	1
ZCCHC8	Nucleic Acid Binding	Nucleic Acid Binding	1	1	2.7	1
RBM17	mRNA Binding	mRNA Processing Factor	1	1	3	1

Table 16: *Proteins identified in the cytosol by Flag-Gβ1, 1mM carbachol treated conditions.* Cells treated with 1mM carbachol for 5 minutes followed by a 45 minute wash out period. Proteins identified as Gβγ interactors with TAP tagged Gβ1 and identified by mass spectrometry and grouped by molecular function and protein class using Panther online software[223].

Protein	Molecular Function	Molecular Class	Average Peptides	Average Unique Peptides	Average Coverage	n
TUBB4A	Structural Constituent Of Cytoskeleton	Tubulin	244	5	62.5	3

TUBB3	Structural Constituent Of Cytoskeleton	Tubulin	117	2	36.5	2
GNAI3	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	296	33	46.8	3
TUBA1A	Structural Constituent Of Cytoskeleton	Tubulin	132	3	41.8	3
GNAI2	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	222	23	42.2	3
GNAQ	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	65	24	36.6	3
GNAI1	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	223	16	45.1	3
PDCL	Catalytic Activity Protein Binding Small GTPase Regulator Activity	G-Protein Modulator Chaperone	45	19	24.6	3
GNB1	GTPase Activity Protein Binding	Hydrolase Heterotrimeric G-Protein	328	32	42.9	3
GNAS	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	55	24	16.6	3
KCTD12	Protein Binding	Enzyme Modulator	40	22	30.4	3
GNA11	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	56	20	35.7	3
GNA13	Pyrophosphatase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	76	26	34.1	3
CCT3		Chaperonin	65	40	28.8	3

SLC25A13	Amino Acid Transmembrane Transporter Activity Transmembrane Transporter Activity Calcium Ion Binding Calmodulin Binding	Amino Acid Transporter Mitochondrial Carrier Protein Transfer/Carrier Protein Calmodulin	24	23	14.5	3
TCP1		Chaperonin	59	39	30.1	3
CCT7		Chaperonin	44	27	25.7	3
KCTD2	Protein Binding	Enzyme Modulator	25	15	32.3	3
ATP2A2	Hydrolase Activity Ion Channel Activity Cation Transmembrane Transporter Activity	Cation Transporter Ion Channel Hydrolase	10	10	5.1	3
CCT2		Chaperonin	75	41	38.1	3
SLC25A4	Amino Acid Transmembrane Transporter Activity Transmembrane Transporter Activity Calcium Ion Binding Calmodulin Binding	Amino Acid Transporter Mitochondrial Carrier Protein Transfer/Carrier Protein Calmodulin	13	1	17.4	1
CCT5		Chaperonin	68	37	26.7	3
KCTD17	Protein Binding	Enzyme Modulator	1	1	4	1
GNA12	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	13	3	6.6	2
KCTD5	Protein Binding	Enzyme Modulator	12	9	18.5	3
ATP5A1	Hydrolase Activity Receptor Activity Anion Channel Activity Ligand-Gated Ion Channel Activity Cation Transmembrane Transporter Activity Proton-Transporting ATP Synthase Activity, Rotational Mechanism Single-Stranded DNA Binding	ATP Synthase Anion Channel Ligand-Gated Ion Channel Ligand-Gated Ion Channel DNA Binding Protein Hydrolase	14	13	10.2	3
HSP90B1		Hsp90 Family Chaperone	12	10	5.9	3

CCT6A		Chaperonin	64	28	27.3	3
ERLIN2			33	18	22.8	3
SLC25A3	Amino Acid Transmembrane Transporter Activity Transmembrane Transporter Activity Calcium Ion Binding Calmodulin Binding	Amino Acid Transporter Mitochondrial Carrier Protein Transfer/Carrier Protein Calmodulin	31	17	19.8	3
UBAC2	Transcription Cofactor Activity Sequence-Specific DNA Binding Transcription Factor Activity Aspartic-Type Endopeptidase Activity Sequence-Specific DNA Binding Transcription Factor Activity Protein Binding	Transcription Cofactor Aspartic Protease	12	11	13.5	3
CCT4		Chaperonin	37	22	19.7	3
ATP1A1	Hydrolase Activity Ion Channel Activity Cation Transmembrane Transporter Activity	Cation Transporter Ion Channel Hydrolase	8	8	4.1	3
LOC730429			41	37	6.4	3
SLC25A1	Amino Acid Transmembrane Transporter Activity Transmembrane Transporter Activity Calcium Ion Binding Calmodulin Binding	Amino Acid Transporter Mitochondrial Carrier Protein Transfer/Carrier Protein Calmodulin	17	12	14.0	3
DPM1	Transferase Activity, Transferring Glycosyl Groups	Glycosyltransferase	10	10	15.4	3
TUBAL3	Structural Constituent Of Cytoskeleton	Tubulin	13	3	5.7	3
ANKRD63	Protein Kinase Activity	Non-Receptor Serine/Threonine Protein Kinase	5	5	8.2	2
ERLIN1			14	4	11.2	3
GNAZ	GTPase Activity Pyrophosphatase Activity Adenylate Cyclase Activity	Heterotrimeric G-Protein	18	14	18.7	3

IPO7	Nucleotide Binding Receptor Binding Enzyme Regulator Activity GTPase Activity Transmembrane Transporter Activity Protein Binding	Transporter Small GTPase	15	13	5.8	3
TECR	Oxidoreductase Activity	Dehydrogenase	7	7	8.3	3
QPCTL	Transferase Activity	Transferase	10	5	11.4	2
GNG12	GTPase Activity Protein Binding	Heterotrimeric G-Protein	6	3	22.2	3
DNAJC25-GNG10			10	5	17.7	3
RADIL			11	10	4.3	3
SSR1	Calcium Ion Binding G-Protein Coupled Receptor Activity	Membrane Traffic Protein Calcium-Binding Protein G-Protein Coupled Receptor	2	2	9.1	1
PFKL	Carbohydrate Kinase Activity	Carbohydrate Kinase	7	5	4.4	3
PFKP	Carbohydrate Kinase Activity	Carbohydrate Kinase	14	14	8.2	3
STOM	Structural Constituent Of Cytoskeleton	Cytoskeletal Protein	1	1	9.8	1
PSMD13	Catalytic Activity Protein Binding Enzyme Regulator Activity	Enzyme Modulator	9	7	11.5	3
NIPSNAP1			2	1	3.2	1
IMMT			6	5	4.3	2
AGK	Carbohydrate Kinase Activity	Carbohydrate Kinase	1	1	5.9	1
CALU	Calmodulin Binding	Calmodulin	2	2	8.6	1
SLC25A11	Amino Acid Transmembrane Transporter Activity Transmembrane Transporter Activity Calcium Ion Binding Calmodulin Binding	Amino Acid Transporter Mitochondrial Carrier Protein Transfer/Carrier Protein Calmodulin	13	13	18.9	3
MGST3	Oxidoreductase Activity Transferase Activity Peroxidase Activity	Transferase	3	3	13.5	2

SCD	Oxidoreductase Activity	Oxidoreductase	3	3	3.6	3
NDUFS1		Dehydrogenase Reductase	12	12	9.4	3
PFDN4			1	1	9	1
PSMC3	Hydrolase Activity	Hydrolase	5	5	18	1
SSR4	G-Protein Coupled Receptor Activity	G-Protein Coupled Receptor	3	3	11.9	2
LPCAT1	Transferase Activity, Transferring Acyl Groups Calcium Ion Binding Binding calcium-Dependent Phospholipid Binding	Acyltransferase Annexin Calmodulin	2	2	2.8	2
MRPL43	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	1	1	5.1	1
GNG5	GTPase Activity Protein Binding	Heterotrimeric G-Protein	20	4	34.6	2
DNAJA2		Chaperone	2	2	8.5	1
RPN2	Transferase Activity, Transferring Glycosyl Groups	Glycosyltransferase	3	3	2	3
YWHAB		Chaperone	4	2	11.8	2
GNG4	GTPase Activity Protein Binding	Heterotrimeric G-Protein	2	2	41.3	1
GNB2	GTPase Activity Protein Binding	Hydrolase Heterotrimeric G-Protein	261	4	25.5	3
HSPD1		Chaperonin	13	9	7.3	3
TP53I11			11	5	9.2	3
HADHA	Oxidoreductase Activity Hydro-Lyase Activity Racemase And Epimerase Activity	Dehydrogenase Hydratase Epimerase/Racemase	16	15	10.4	3
PSMD3	Catalytic Activity Protein Binding Enzyme Activator Activity	Enzyme Modulator	7	7	7.9	2
CDK1	Non-Membrane Spanning Protein Tyrosine Kinase Activity	Non-Receptor Serine/Threonine Protein Kinase	10	9	12.1	3

VBP1		Non-Receptor Tyrosine Protein Kinase Chaperone	6	6	22.3	2
PSMC2	Hydrolase Activity	Hydrolase	8	7	10.8	2
EEF1G	Oxidoreductase Activity Hydro-Lyase Activity Racemase And Epimerase Activity	Transferase Signalling Molecule Reductase Translation Elongation Factor Epimerase/Racemase	2	2	5.3	1
PHB2		Cytoskeletal Protein	10	10	14.0	3
PSMC6	Hydrolase Activity	Hydrolase	6	6	6.6	3
PSMD2	Catalytic Activity Protein Binding Enzyme Regulator Activity	Enzyme Modulator	8	8	4.2	3
PRKAR1A	Kinase Activity Protein Binding Kinase Regulator Activity	Kinase Modulator	1	1	2.9	1
SLC25A10	Amino Acid Transmembrane Transporter Activity Transmembrane Transporter Activity Calcium Ion Binding Calmodulin Binding	Amino Acid Transporter Mitochondrial Carrier Protein Transfer/Carrier Protein Calmodulin	16	10	14.4	3
PSMC1	Hydrolase Activity	Hydrolase	5	4	7.3	2
XPO1	Receptor Activity	Receptor	6	6	5	2
DNAJA1		Chaperone	4	4	8.1	2
GNG7	GTPase Activity Protein Binding	Heterotrimeric G-Protein	5	3	23.5	3
LBR	Oxidoreductase Activity Receptor Activity	Receptor Reductase	9	7	6.3	3

PCNA	Catalytic Activity Nucleic Acid Binding DNA Polymerase Processivity Factor Activity	DNA Polymerase Processivity Factor	2	2	5	2
NDUFA9	Oxidoreductase Activity	Dehydrogenase Reductase	7	6	8.4	3
CAD	Transferase Activity Ligase Activity	Transferase Ligase	43	41	9.4	3
TUBB2A	Structural Constituent Of Cytoskeleton	Tubulin	164	2	53.5	2
TUBA1B	Structural Constituent Of Cytoskeleton	Tubulin	136	28	42.8	3
TUBB6	Structural Constituent Of Cytoskeleton	Tubulin	127	12	41.4	3
SLC25A12	Amino Acid Transmembrane Transporter Activity Transmembrane Transporter Activity Calcium Ion Binding Calmodulin Binding	Amino Acid Transporter Mitochondrial Carrier Protein Transfer/Carrier Protein Calmodulin	14	5	8.8	3
EMD			5	5	18.5	1
ATP5B	Hydrolase Activity Receptor Activity Anion Channel Activity Ligand-Gated Ion Channel Activity Cation Transmembrane Transporter Activity Proton-Transporting ATP Synthase Activity, Rotational Mechanism Single-Stranded DNA Binding	ATP Synthase Anion Channel Ligand-Gated Ion Channel Ligand-Gated Ion Channel DNA Binding Protein Hydrolase	6	6	7.8	2
SLC16A1	Transmembrane Transporter Activity	Transporter	4	3	6.6	1
PSMD7	Catalytic Activity Protein Binding Enzyme Regulator Activity	Enzyme Modulator	5	3	6.2	2
NDUFS3			3	3	16.3	1
PSMD1	Catalytic Activity Protein Binding Enzyme Regulator Activity	Enzyme Modulator	6	6	3.8	2

PFDN5			8	6	22.3	3
PSMC4	Hydrolase Activity	Hydrolase	6	5	6.0	3
PGAM5			6	4	8.0	2
UBR4			5	5	0.7	2
YWHAZ		Chaperone	7	3	10.5	3
PHGDH	Oxidoreductase Activity	Dehydrogenase	6	6	5.1	3
BAG2			4	4	13.3	2
DNAJC11		Chaperone	4	4	4.7	2
FAR1			4	4	5.1	2
JAK1	Non-Membrane Spanning Protein Tyrosine Kinase Activity	Non-Receptor Tyrosine Protein Kinase	4	4	2.9	2
TOMM22	Receptor Activity	Transfer/Carrier Protein Receptor	2	2	16.2	1
PSMD11	Catalytic Activity Protein Binding Enzyme Regulator Activity	Enzyme Modulator	4	4	5.9	2
NT5DC2	Nucleotide Phosphatase Activity	Nucleotide Phosphatase	5	5	7.5	2
HADHB	Nucleotide Phosphatase Activity Acetyltransferase Activity	Acetyltransferase	4	4	5.2	2
CANX	Calcium Ion Binding	Chaperone Calcium-Binding Protein	2	2	5.6	1
ALB		Transfer/Carrier Protein	4	4	4.0	2
KDELR2	Receptor Activity	Receptor Membrane Trafficking Regulatory Protein	2	2	12.7	1
ACOT9	Hydrolase Activity, Acting On Ester Bonds	Esterase	5	5	7.3	2
UQCR10	Oxidoreductase Activity	Reductase	2	2	38.1	1
OXA1L	Transmembrane Transporter Activity	Transporter	2	2	3.6	1

LGALS3BP	Oxidoreductase Activity Serine-Type Peptidase Activity Receptor Activity	Receptor Serine Protease Oxidase Serine Protease	2	2	2.7	2
DHRS7B	Oxidoreductase Activity	Dehydrogenase Reductase	1	1	4.3	1
CYC1			2	2	3.7	2
IPO8	GTPase Activity Transmembrane Transporter Activity Protein Binding	Transporter Small GTPase	1	1	1.2	1
ATP5J2	Catalytic Activity Cation Transmembrane Transporter Activity Hydrogen Ion Transmembrane Transporter Activity	ATP Synthase	3	3	13.1	3
UQCRC2	Oxidoreductase Activity Metallopeptidase Activity Hydrolase Activity, Acting On Ester Bonds	Metalloprotease Reductase Esterase Metalloprotease	2	2	3.5	2
CSE1L	GTPase Activity Transmembrane Transporter Activity Protein Binding	Transporter Small GTPase	1	1	1.8	1
PHB			3	3	5.9	2
COX5A	Oxidoreductase Activity	Oxidase	1	1	6	1
TXN	Oxidoreductase Activity	Oxidoreductase	3	3	12.4	3
MRPS18B			1	1	8.5	1
COX4I1			1	1	6.5	1
KIAA0368			2	2	0.7	2
USMG5			1	1	25.9	1
ABCE1			1	1	2	1
MRPL12	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	2	2	6.1	2
TNPO1	Transmembrane Transporter Activity	Transporter Transfer/Carrier Protein	5	5	3.0	3

PEX14	Receptor Activity	Receptor	1	1	2.4	1
DOLPP1	Hydrolase Activity, Acting On Ester Bonds	Phosphatase Esterase Phosphatase	1	1	5.5	1
MRPL17	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	1	1	4.6	1
PNPLA6	Hydrolase Activity, Acting On Ester Bonds	Esterase	1	1	0.9	1
CLGN	Calcium Ion Binding	Chaperone Calcium-Binding Protein	1	1	2.6	1
NDUFA4	Oxidoreductase Activity	Oxidoreductase	1	1	12.3	1
MRPL49	Calcium Ion Binding	Ribosomal Protein	5	5	19.9	2
TRIM28			3	2	3.8	1
ATAD3B	Hydrolase Activity	Hydrolase	2	2	3.4	1
CHCHD3			2	2	8.4	1
NDUFS8			2	2	10	1
SLC25A18	Amino Acid Transmembrane Transporter Activity Transmembrane Transporter Activity Calcium Ion Binding Calmodulin Binding	Amino Acid Transporter Mitochondrial Carrier Protein Transfer/Carrier Protein Calmodulin	2	1	7.9	1
MRPS27	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	3	3	3.9	2
HIST1H1D	DNA Binding	Histone	2	2	9	1
MRPL4	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	2	2	13.2	1
GBAS			2	2	9.4	1
PFKM	Carbohydrate Kinase Activity	Carbohydrate Kinase	2	2	3.2	1
MRPS5	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	1	1	3.7	1
XPO7		Transfer/Carrier Protein	1	1	1.5	1
ICT1	Translation Factor Activity, Nucleic Acid Binding Translation Factor Activity, Nucleic	Translation Release Factor	2	2	5.8	2

	Acid Binding Translation Release Factor Activity					
FASTKD5			1	1	1.8	1
PFDN6			1	1	9.3	1
MTCH1		Mitochondrial Carrier Protein Transfer/Carrier Protein	1	1	3	1
PTPMT1	Phosphoprotein Phosphatase Activity	Protein Phosphatase Protein Phosphatase Kinase Inhibitor	1	1	6	1
RPS27	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	1	1	15.5	1
RAF1	Non-Membrane Spanning Protein Tyrosine Kinase Activity	Non-Receptor Serine/Threonine Protein Kinase Non-Receptor Tyrosine Protein Kinase	1	1	1.5	1
MRPL46			2	2	7	2
MRPS35	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	1	1	3.1	1
SNRPB	Catalytic Activity mRNA Binding	mRNA Splicing Factor	4	3	13.4	1
DAP3	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	4	3	12.3	1
STOML2	Structural Constituent Of Cytoskeleton	Cytoskeletal Protein	2	2	8.4	1
FLNA	Structural Constituent Of Cytoskeleton Actin Binding	Non-Motor Actin Binding Protein	2	2	0.9	1
RAP1A	GTPase Activity Protein Binding	Small GTPase	2	1	6.5	1
MMS19	Transcription Cofactor Activity Sequence-Specific DNA Binding Transcription Factor Activity Sequence-Specific DNA Binding Transcription Factor Activity Protein Binding	Transcription Cofactor Nucleic Acid Binding	2	2	2.4	1

DGKH	Kinase Activity	Kinase	2	2	1.4	1
TMEM109			1	1	4.9	1
HSPB1	Structural Molecule Activity	Structural Protein Chaperone	1	1	4.9	1
STIP1		Chaperone	1	1	2.4	1
GCDH	Oxidoreductase Activity Transferase Activity	Transferase Dehydrogenase Oxidase	1	1	5.3	1

Table 17: *Proteins identified in the nucleus by Flag-Gβ1, untreated conditions.* Proteins identified as Gβγ interactors with TAP tagged Gβ1 and identified by mass spectrometry and grouped by molecular function and protein class using Panther online software[223].

Protein	Molecular Function	Protein Class	Average Peptides	Average Unique Peptides	Average Coverage	n
TUBB4A			19	1	24.3	1
GNAI3	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	46	15	28.4	2
GNAI2	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	38	8	26.6	2
GNAQ	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	3	3	12	1
GNAI1	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	34	4	18.65	2
PDCL	Catalytic Activity Protein Binding Small GTPase Regulator Activity	G-Protein Modulator Chaperone	2	2	7	1

GNB1	GTPase Activity Protein Binding	Hydrolase Heterotrimeric G-Protein	14	5	7.95	2
GNAS	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	17	8	16.35	2
GNA13	Pyrophosphatase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	11	5	10.85	2
CCT3		Chaperonin	85	28	31.4	2
TCP1		Chaperonin	78	30	33.6	2
CCT7		Chaperonin	52	21	28.8	2
CCT2		Chaperonin	77	34	46.55	2
CCT5		Chaperonin	57	24	23.7	2
GNA12			3	1	5.5	1
CCT6A		Chaperonin	56	28	36.25	2
CCT4		Chaperonin	36	16	20.7	2
TUBAL3	Structural Constituent Of Cytoskeleton	Tubulin	1	1	3.8	1
ANKRD63			1	1	3.2	1
GNG12	GTPase Activity Protein Binding	Heterotrimeric G-Protein	1	1	22.2	1
DNAJC25-GNG10			2	1	9.8	1
RADIL			2	2	2.6	1
DNAJA2		Chaperone	2	1	2.4	1
EEF1G	Oxidoreductase Activity Transferase Activity Racemase And Epimerase Activity Structural Constituent Of Cytoskeleton Translation Elongation Factor Activity Translation Elongation Factor Activity Receptor Binding Translation Elongation Factor Activity	Transferase Signalling Molecule Reductase Translation Elongation Factor Epimerase/Racemase Cytoskeletal Protein	2	2	5.9	1

PHB2			2	2	8.4	1
DNAJA1			1	1	6.3	1
PCNA	Catalytic Activity Nucleic Acid Binding DNA Polymerase Processivity Factor Activity	DNA Polymerase Processivity Factor	7	5	12.45	2
AMOT			3	3	6.4	1
H2AFY	DNA Binding	Histone	5	4	15.9	1
FBL	Methyltransferase Activity RNA Binding	Methyltransferase Ribonucleoprotein	2	2	9.3	1
SF3A1	Catalytic Activity mRNA Binding	mRNA Splicing Factor	1	1	1.6	1
NOP16			2	1	8.4	1
PHGDH			2	2	4.3	1
CANX	Calcium Ion Binding	Chaperone Calcium-Binding Protein	2	2	5.6	1
ALB		Transfer/Carrier Protein	3	3	5.6	1
TRIM28			2	2	2.8	1
ATAD3B	Hydrolase Activity	Hydrolase	6	6	4.7	2
FLNA			2	2	0.9	1
RAP1A	GTPase Activity Protein Binding	Small GTPase	3	2	12	1
BANF1			3	2	29.2	1
SERBP1			1	1	3.9	1
RPL19	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	1	1	8.7	1
KPNA2			1	1	2.8	1
REST			4	2	2.4	1
KTN1			3	3	2.7	1
ACACA	Ligase Activity	Ligase	3	2	0.9	1
PC	Ligase Activity	Ligase	3	2	3.1	1

GNG11	GTPase Activity Protein Binding	Heterotrimeric G-Protein	3	2	24.7	1
PCCB			1	1	3.2	1
VAPB			1	1	4.9	1
RAB5B			1	1	5.1	1
RRP1			1	1	2.8	1
LOC731751			8	7	2	1
CPSF1	Damaged DNA Binding Poly(A) RNA Binding	Damaged DNA-Binding Protein mRNA Polyadenylation Factor	1	1	1.2	1

Table 18: *Proteins identified in the nucleus by Flag-Gβ1, 1mM carbachol treated conditions.* Cells treated with 1mM carbachol for 5 minutes followed by a 45 minute wash out period. Proteins identified as Gβγ interactors with TAP tagged Gβ1 and identified by mass spectrometry and grouped by molecular function and protein class using Panther online software[223].

Protein	Molecular Function	Protein Class	Average Peptides	Average Unique Peptides	Average Coverage	n
GNAI3	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	46	15	32.1	3
NAV2			4	1	0.5	1
TUBA1A			20	1	33.5	1
GNAI2	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	45	26	31.3	3
GNAQ	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	12	11	12.5	3

GNAI1	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	31	8	21.8	3
PDCL	Catalytic Activity Protein Binding Small GTPase Regulator Activity	G-Protein Modulator Chaperone	8	8	13.1	3
GNB1	GTPase Activity Protein Binding	Hydrolase Heterotrimeric G-Protein	46	13	15.2	3
GNAS	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	29	18	22.6	3
KCTD12	Protein Binding	Enzyme Modulator	9	9	9.9	3
GNA11	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	10	4	10.3	3
GNA13			19	10	14	3
CCT3		Chaperonin	71	37	28.1	3
TCP1		Chaperonin	58	35	26.0	3
CCT7		Chaperonin	52	28	25.4	3
ATP2A2			5	5	2.0	3
CCT2		Chaperonin	82	43	38.7	3
CCT5		Chaperonin	75	42	29.8	3
GNA12			16	9	10.1	3
KCTD5	Protein Binding	Enzyme Modulator	1	1	4.3	1
HSP90B1			2	1	2.9	1
CCT6A		Chaperonin	72	35	26.5	3
ERLIN2			2	2	7.1	1
CCT4		Chaperonin	32	19	16.2	3
TUBAL3	Structural Constituent Of Cytoskeleton	Tubulin	5	2	5.4	2
ANKRD63			1	1	3.2	1

QPCTL	Transferase Activity	Transferase	1	1	2.9	1
GNG12	GTPase Activity Protein Binding	Heterotrimeric G-Protein	2	1	22.2	1
DNAJC25-GNG10			8	4	13.7	3
RADIL			2	1	3.7	1
SSR1			1	1	5.2	1
SSR4	G-Protein Coupled Receptor Activity	G-Protein Coupled Receptor	1	1	6.4	1
GNG5	GTPase Activity Protein Binding	Heterotrimeric G-Protein	1	1	13.2	1
DNAJA2		Chaperone	4	4	6.0	2
RPN2	Transferase Activity, Transferring Glycosyl Groups	Glycosyltransferase	7	7	4.2	3
TP53I11			1	1	5.3	1
EEF1G			6	6	6.9	2
PHB2			6	6	7.6	3
DNAJA1			4	4	9.6	2
PCNA	Catalytic Activity Nucleic Acid Binding DNA Polymerase Processivity Factor Activity	DNA Polymerase Processivity Factor	11	8	14.2	3
CAD	Transferase Activity Ligase Activity	Transferase Ligase	2	2	1.4	1
TUBA1B	Structural Constituent Of Cytoskeleton	Tubulin	46	23	35.5	2
PGAM5			2	2	6.6	1
CANX	Calcium Ion Binding	Chaperone Calcium-Binding Protein	7	7	5.7	3
ALB		Transfer/Carrier Protein	7	7	5.0	3
PHB			2	2	4.4	2
TXN			2	2	12.4	2
TRIM28			6	6	6	2

ATAD3B			7	7	10.5	1
FLNA			5	5	1.2	2
RAP1A	GTPase Activity Protein Binding	Small GTPase	4	4	12.3	2
GNG11	GTPase Activity Protein Binding	Heterotrimeric G-Protein	4	2	27.4	1
VAPB			2	2	10.7	1
RAB5B			4	4	6.8	3
RRP1			1	1	3.7	1
LRRC59			4	4	8.7	2
ACTR3	Structural Constituent Of Cytoskeleton	Actin And Actin Related Protein	2	2	7.2	1
RBM5			1	1	1.8	1
SRSF8			1	1	2.8	1
STT3B	Transferase Activity, Transferring Glycosyl Groups	Glycosyltransferase	2	2	3	1
RPN1	Transferase Activity, Transferring Glycosyl Groups	Glycosyltransferase	2	2	3.8	1
COPG2		Vesicle Coat Protein	1	1	1.4	1
API5			1	1	1.6	1
DNAJA3		Chaperone	1	1	3.3	1
UBE2V2			1	1	6.9	1
VDAC1			1	1	4.6	1
RAN			1	1	5.1	1

Table 19: *Proteins identified in the cytosol by TAP-Gβ1, untreated conditions.* Proteins identified as Gβγ interactors with TAP tagged Gβ1 and identified by mass spectrometry and grouped by molecular function and protein class using Panther online software[223].

Protein	Molecular Function	Protein Class	Average Peptides	Average Unique Peptides	Average Coverage	n

CCT3		Chaperonin	19	17	13.3	3
PDCL	Catalytic Activity Protein Binding Small GTPase Regulator Activity	G-Protein Modulator Chaperone	36	14	20.3	3
GNB1	GTPase Activity Protein Binding	Hydrolase Heterotrimeric G-Protein	7	5	7.7	2
CCT4		Chaperonin	21	13	12.1	3
ANXA1			3	3	10.1	1
ACTBL2	Structural Constituent Of Cytoskeleton	Actin And Actin Related Protein	3	1	11.2	1
CCT5		Chaperonin	20	14	9.1	3
YWHAZ		Chaperone	2	2	11.8	1
S100A4	Calcium Ion Binding Growth Factor Activity Calmodulin Binding	Growth Factor Calmodulin	2	2	18.8	1
CCT6A		Chaperonin	21	14	13.7	3
GNG12	GTPase Activity Protein Binding	Heterotrimeric G-Protein	4	2	22.2	2
DCD			1	1	10	1
TCP1		Chaperonin	22	17	14.5	3
CSTB	Cysteine-Type Peptidase Activity Protein Binding Cysteine-Type Endopeptidase Inhibitor Activity	Cysteine Protease Inhibitor	1	1	12.2	1
CCT7		Chaperonin	12	9	11.0	2
HSPA6		Hsp70 Family Chaperone	5	1	5.9	1
GNAQ	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	4	4	14.8	1
ATAD3B	Hydrolase Activity	Hydrolase	3	3	5.2	1
KCTD12	Protein Binding	Enzyme Modulator	2	2	7.1	1

GNA11	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	2	1	6.7	1
DNAJC25-GNG10			1	1	9.8	1
HNRNPF	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	1	1	4.1	1
GNAL	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	1	1	2.9	1
H3F3A	DNA Binding	Histone	1	1	5.1	1
KIF11	Microtubule Motor Activity Structural Constituent Of Cytoskeleton	Microtubule Binding Motor Protein	5	2	4.2	1
PRSS3	Serine-Type Peptidase Activity	Serine Protease	2	1	8.1	1

Table 20: *Proteins identified in the cytosol by TAP-Gβ1, 1mM carbachol treated conditions.* Cells treated with 1mM carbachol for 5 minutes followed by a 45 minute wash out period. Proteins identified as Gβγ interactors with TAP tagged Gβ1 and identified by mass spectrometry and grouped by molecular function and protein class using Panther online software[223].

Protein	Molecular Function	Protein Class	Average Peptides	Average Unique Peptides	Average Coverage	n
CCT3		Chaperonin	19	17	13.4	3
PDCL	Catalytic Activity Protein Binding Small GTPase Regulator Activity	G-Protein Modulator Chaperone	25	15	21.1	3
GNB1	GTPase Activity Protein Binding	Hydrolase Heterotrimeric G-Protein	9	6	8.8	2
CCT4		Chaperonin	9	8	11.8	2

CCT5		Chaperonin	12	11	8.2	3
CCT6A		Chaperonin	26	19	15.2	3
GNG12	GTPase Activity Protein Binding	Heterotrimeric G-Protein	1	1	22.2	1
TCP1		Chaperonin	21	17	13.6	3
CCT7		Chaperonin	22	15	12.5	3
GNAQ	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	20	17	19.4	3
GNA11	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	14	7	14	3
DNAJC25-GNG10			3	2	9.8	2
NCL	Catalytic Activity Structural Constituent Of Ribosome Poly(A) RNA Binding	mRNA Polyadenylation Factor mRNA Splicing Factor Ribonucleoprotein Ribosomal Protein	17	12	9.85	2
HNRNPC	mRNA Binding	mRNA Processing Factor Ribonucleoprotein	6	5	10.5	2
HNRNPR			3	3	5.4	1
SRSF1			8	6	13.1	2
GNAI2	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	4	3	7.15	2
GNAI3	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	8	5	8.9	3
DHX9	RNA Helicase Activity Nucleic Acid Binding	RNA Helicase Helicase	6	6	3.45	2

LMNB2	Structural Constituent Of Cytoskeleton	Structural Protein Intermediate Filament	3	3	2.5	2
SRSF3			1	1	8.5	1
SNRNP200	DNA Helicase Activity RNA Helicase Activity Hydrolase Activity mRNA Binding	DNA Helicase RNA Helicase mRNA Splicing Factor Helicase Hydrolase	7	7	1.8	2
TOP1			7	7	10.6	1
SRSF2	Catalytic Activity mRNA Binding	mRNA Splicing Factor	7	2	11.3	1
EFTUD2	GTPase Activity Translation Initiation Factor Activity	Ribonucleoprotein Translation Elongation Factor Translation Initiation Factor Hydrolase G-Protein	4	4	4.3	1
SRSF5	Catalytic Activity mRNA Binding	mRNA Splicing Factor	4	3	11.8	1
SNRNP70			4	3	10.3	1
SRSF7			4	2	9.7	1
PRPF8	Catalytic Activity mRNA Binding	mRNA Splicing Factor	3	3	1.8	1
EIF3A			3	3	2.9	1
SNRPD2	mRNA Binding	mRNA Processing Factor	3	2	23.7	1
SNRPD3			3	2	15.1	1
GNG10	GTPase Activity Protein Binding	Heterotrimeric G-Protein	3	2	41.2	1
GNA13	Pyrophosphatase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	5	3	6.6	2
SNRPN	Catalytic Activity mRNA Binding	mRNA Splicing Factor	2	2	9.6	1
SNRPE	Catalytic Activity mRNA Binding	mRNA Splicing Factor	2	2	25	1

CSNK2A1			2	2	8.7	1
H1FX	DNA Binding	Histone	2	2	10.3	1
DDX23	RNA Helicase Activity Translation Initiation Factor Activity	RNA Helicase Helicase	2	2	2.7	1
RPS6	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	2	1	6	1
PRPF6	Catalytic Activity mRNA Binding	mRNA Splicing Factor	1	1	1.3	1
SNRPG	Catalytic Activity mRNA Binding	mRNA Splicing Factor	1	1	15.8	1
HNRNPUL2	RNA Binding	RNA Binding Protein	1	1	1.9	1
PES1	Structural Constituent Of Ribosome RNA Binding	Ribosomal Protein	1	1	1.7	1
KLK15	Serine-Type Peptidase Activity	Serine Protease	1	1	2.7	1
CKAP4	Hydrolase Activity Nucleic Acid Binding Chromatin Binding	Chromatin/Chromatin-Binding Protein Hydrolase	1	1	3	1
GNAS	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	3	1	6.9	1
SRSF8			2	1	2.8	1

Table 21: *Proteins identified in the nucleus by TAP-Gβ1, untreated conditions.* Proteins identified as Gβγ interactors with TAP tagged Gβ1 and identified by mass spectrometry and grouped by molecular function and protein class using Panther online software[223].

Protein	Molecular Function	Protein Class	Average Peptides	Average Unique Peptides	Average Coverage	n
CCT3		Chaperonin	7	7	7.8	2

PDCL	Catalytic Activity Protein Binding Small GTPase Regulator Activity	G-Protein Modulator Chaperone	1	1	4	1
GNB1	GTPase Activity Protein Binding	Hydrolase Heterotrimeric G-Protein	22	7	7.5	3
CCT4		Chaperonin	9	7	9.1	2
ACTBL2	Structural Constituent Of Cytoskeleton	Actin And Actin Related Protein	6	2	10.1	2
CCT5		Chaperonin	3	3	8.1	1
S100A4	Calcium Ion Binding Growth Factor Activity Calmodulin Binding	Growth Factor Calmodulin	2	2	18.8	1
CCT6A		Chaperonin	11	10	13.8	2
GNG12	GTPase Activity Protein Binding	Heterotrimeric G-Protein	1	1	22.2	1
DCD			1	1	10	1
TCP1		Chaperonin	8	8	8.1	2
CCT7		Chaperonin	9	8	9.55	2
HSPA6		Hsp70 Family Chaperone	4	1	5.9	1
GNAQ	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	7	4	7.3	3
ATAD3B	Hydrolase Activity	Hydrolase	2	2	3.4	1
KCTD12	Protein Binding	Enzyme Modulator	10	7	27.1	1
GNA11	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	7	5	19.2	1
DNAJC25-GNG10			4	1	9.8	1

HNRNPF	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	4	1	8.2	1
KIF11	Microtubule Motor Activity Structural Constituent Of Cytoskeleton	Microtubule Binding Motor Protein	1	1	1.9	1
HNRNPC	mRNA Binding	mRNA Processing Factor Ribonucleoprotein	1	1	3.6	1
GNAI2	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	12	4	12.4	2
GNAI3	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	16	8	17.5	2
DHX9	RNA Helicase Activity Nucleic Acid Binding	RNA Helicase Helicase	1	1	1	1
GNA13	Pyrophosphatase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	14	10	14.9	2
CKAP4	Hydrolase Activity Nucleic Acid Binding Chromatin Binding	Chromatin/Chromatin-Binding Protein Hydrolase	12	11	24.9	1
GNAS	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	13	6	7.6	2
VCL	Structural Constituent Of Cytoskeleton Actin Binding	Non-Motor Actin Binding Protein Cell Adhesion Molecule	2	2	2.3	1
PTPLAD1			3	2	4.9	2
KCTD5	Protein Binding	Enzyme Modulator	4	2	7.1	2
FECH			1	1	3.5	1
AKR7A2	Oxidoreductase Activity Transporter Activity	Reductase	2	2	5.6	1
FAM82B			2	2	6.7	1
MTERFD2			1	1	2.4	1

GNG7	GTPase Activity Protein Binding	Heterotrimeric G-Protein	1	1	23.5	1
PRKDC	Protein Kinase Activity Nucleotide Kinase Activity Nucleic Acid Binding	Nucleotide Kinase Non-Receptor Serine/Threonine Protein Kinase Nucleic Acid Binding Nucleotide Kinase Non-Receptor Serine/Threonine Protein Kinase	20	20	5.3	1
TUBA1B	Structural Constituent Of Cytoskeleton	Tubulin	8	6	19.1	1
RADIL			7	5	8.2	1
GNA12	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	5	1	5.5	1
NONO	Catalytic Activity mRNA Binding	mRNA Splicing Factor	4	4	12.1	1
HNRPDL	Catalytic Activity Structural Constituent Of Ribosome Poly(A) RNA Binding	mRNA Polyadenylation Factor mRNA Splicing Factor Ribonucleoprotein Ribosomal Protein	4	4	10.5	1
RAP1A	GTPase Activity Protein Binding	Small GTPase	3	3	20.7	1
RAP1B	GTPase Activity Protein Binding	Small GTPase	3	1	20.7	1
BSG		Transmembrane Receptor Regulatory/Adaptor Protein	2	2	7.3	1
RBMX	Catalytic Activity Structural Constituent Of Ribosome Poly(A) RNA Binding	mRNA Polyadenylation Factor mRNA Splicing Factor Ribonucleoprotein Ribosomal Protein	2	2	10.7	1
RPN2	Transferase Activity, Transferring Glycosyl Groups	Glycosyltransferase	2	2	6.3	1

MATR3	DNA Binding RNA Binding	DNA Binding Protein RNA Binding Protein	2	2	2.8	1
ATP6V0A1	Hydrolase Activity Cation Transmembrane Transporter Activity Hydrogen Ion Transmembrane Transporter Activity	ATP Synthase Hydrolase	2	2	2.4	1
RALY	mRNA Binding	mRNA Processing Factor Ribonucleoprotein	1	1	2.9	1

Table 22: *Proteins identified in the nucleus by TAP-Gβ1, 1mM carbachol treated conditions.* Cells treated with 1mM carbachol for 5 minutes followed by a 45 minute wash out period. Proteins identified as Gβγ interactors with TAP tagged Gβ1 and identified by mass spectrometry and grouped by molecular function and protein class using Panther online software[223].

Protein	Molecular Function	Protein Class	Average Peptides	Average Unique Peptides	Average Coverage	n
CCT3		Chaperonin	32	25	18.3	3
PDCL	Catalytic Activity Protein Binding Small GTPase Regulator Activity	G-Protein Modulator Chaperone	7	7	14.3	2
GNB1	GTPase Activity Protein Binding	Hydrolase Heterotrimeric G-Protein	21	9	7.7	3
CCT4		Chaperonin	7	7	8	2
CCT5		Chaperonin	18	15	10.9	3
CCT6A		Chaperonin	27	18	11.9	3
GNG12	GTPase Activity Protein Binding	Heterotrimeric G-Protein	1	1	22.2	1
TCP1		Chaperonin	31	21	16.7	3
CCT7		Chaperonin	20	15	13.1	3
GNAQ	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity	Heterotrimeric G-Protein	7	7	10.2	2

	Phospholipase Activity Adenylate Cyclase Activity Receptor Binding					
ATAD3B	Hydrolase Activity	Hydrolase	8	7	4.4	3
GNA11	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	8	4	7.7	3
DNAJC25-GNG10			5	3	9.8	3
HNRNPC	mRNA Binding	mRNA Processing Factor Ribonucleoprotein	1	1	3.3	1
GNAI2	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	9	5	12.0	2
GNAI3	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	17	12	15.7	3
GNA13	Pyrophosphatase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	3	1	6.9	1
HNRNPUL2	RNA Binding	RNA Binding Protein	2	2	3.2	1
GNAS	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	16	9	11	3
KCTD5	Protein Binding	Enzyme Modulator	2	2	4.3	2
RADIL			4	4	3.3	2
RAP1A	GTPase Activity Protein Binding	Small GTPase	3	3	9.0	2
RPN2	Transferase Activity, Transferring Glycosyl Groups	Glycosyltransferase	2	2	1.9	2
RPN1	Transferase Activity, Transferring Glycosyl Groups	Glycosyltransferase	4	4	9.1	1

DDOST	Transferase Activity, Transferring Glycosyl Groups	Glycosyltransferase	3	3	6.8	1
OR6K3			2	1	2.5	1
TP53I11			2	2	13.2	1

Table 23: *Proteins identified in the cytosol by TAP-Gβ1γ7, untreated conditions.* Proteins identified as Gβγ interactors with TAP tagged Gβ1 and identified by mass spectrometry and grouped by molecular function and protein class using Panther online software[223].

Protein	Molecular Function	Protein Class	Average Peptides	Average Unique Peptides	Average Coverage	n
GNAI3	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	105	19	28.3	3
TUBB2A	Structural Constituent Of Cytoskeleton	Tubulin	17	1	29.4	1
GNAI1	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	63	8	19.5	3
GNAI2	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	68	13	24.4	3
GNB1			76	17	16.5	3
GNAQ	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	44	21	29.3	3
GNAS	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	43	12	11.5	3
GNA13	Pyrophosphatase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	57	26	29.7	3

KCTD12	Protein Binding	Enzyme Modulator	34	18	20.5	3
GNG7	GTPase Activity Protein Binding	Heterotrimeric G-Protein	24	6	28.9	3
GNA11	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	33	14	24.7	3
KCTD2	Protein Binding	Enzyme Modulator	47	16	31.8	3
RPS25	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	7	4	24	1
IRS4			6	5	6	1
KCTD5	Protein Binding	Enzyme Modulator	27	9	21.8	3
SLC25A4			5	1	14.8	1
RPS2	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	4	2	7.8	1
GNG12	GTPase Activity Protein Binding	Heterotrimeric G-Protein	8	3	22.2	3
S100A9	Calcium Ion Binding Receptor Binding Calmodulin Binding	Signalling Molecule Calmodulin	2	2	26.3	1
SUPT6H			2	2	2.6	1
TFG			2	2	6.8	1
DOLPP1			4	3	9.05	2
RPL17			2	2	14.7	1
RBM14			2	2	4.9	1
RPS15A	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	2	1	17.7	1
CCT7		Chaperonin	3	2	2.4	2
LMNB2			3	3	2.75	2
TXN			3	2	12.4	2
RPL37A			1	1	19.6	1

ITPR3	Receptor Activity Ligand-Gated Ion Channel Activity	Ligand-Gated Ion Channel	1	1	0.5	1
RPL27A			1	1	7.4	1
CCT3		Chaperonin	4	3	4.05	2
GNAZ			4	3	3.9	3
ENAH			1	1	1.8	1
WDR6			1	1	0.8	1
GNB2	GTPase Activity Protein Binding	Hydrolase Heterotrimeric G-Protein	22	1	14.7	1
GNAO1			29	4	13.6	2
GNA12			6	1	5.5	1
CCT5		Chaperonin	8	5	7.3	2
KCTD17	Protein Binding	Enzyme Modulator	6	3	10.1	2
HNRNPC	mRNA Binding	mRNA Processing Factor Ribonucleoprotein	3	3	12.1	1
PDCL	Catalytic Activity Protein Binding Small GTPase Regulator Activity	G-Protein Modulator Chaperone	3	3	10.3	1
CCT4		Chaperonin	4	4	4.9	2
PRSS3			2	2	8.1	1
ATAD3B			2	2	2.9	1
PRKDC			2	1	0.5	1
CCT6A		Chaperonin	4	4	3.4	2
TCP1		Chaperonin	2	2	4	1
H3F3A			2	1	5.1	1
GNG4			2	1	21.3	1
PTPLAD1			1	1	3.9	1
DPF2			1	1	4.1	1
DNAJA1			1	1	3.3	1
RANBP6			1	1	1.3	1

TRA2A	Catalytic Activity mRNA Binding	mRNA Splicing Factor	1	1	5	1
C17orf85			1	1	3.8	1
SMARCE1	Sequence-Specific DNA Binding Transcription Factor Activity Sequence-Specific DNA Binding Transcription Factor Activity Chromatin Binding Receptor Binding	HMG Box Transcription Factor Signalling Molecule Chromatin/Chromatin-Binding Protein	1	1	3.2	1
SERPINB3			8	7	24.1	1
JUP			8	7	9.1	1
DSG1	Calcium Ion Binding	Cell Junction Protein Cadherin	5	4	5.3	1
TGM3			5	4	7.1	1
CASP14			4	3	10.3	1
KRT80	Structural Constituent Of Cytoskeleton	Structural Protein Intermediate Filament	4	1	5	1
LGALS7			3	3	28.7	1
GAPDH	Oxidoreductase Activity	Dehydrogenase	3	2	8.7	1
ANXA2			3	2	9.1	1
CALML5			3	2	21.2	1
SERPINB12			3	3	8.4	1
IDE			3	3	2.9	1
HSPB1	Structural Molecule Activity	Structural Protein Chaperone	2	2	9.8	1
DSC1			2	2	3.9	1
RPS27A	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	2	2	16	1
SBSN			1	1	3.1	1
COX7A2			1	1	8.7	1
CTSA	Serine-Type Peptidase Activity	Serine Protease	1	1	2.6	1
CDC42BPA			1	1	0.4	1

CDSN		1	1	3.4	1

Table 24: *Proteins identified in the cytosol by TAP-Gβ1γ7, 1mM carbachol treated conditions.* Cells treated with 1mM carbachol for 5 minutes followed by a 45 minute wash out period. Proteins identified as Gβγ interactors with TAP tagged Gβ1 and identified by mass spectrometry and grouped by molecular function and protein class using Panther online software[223].

Protein	Molecular Function	Protein Class	Average Peptides	Average Unique Peptides	Average Coverage	n
GNAI3	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	135	22	27.6	3
GNAI1	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	65	8	30.7	2
GNAI2	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	75	12	24.3	3
GNB1	GTPase Activity Protein Binding	Hydrolase Heterotrimeric G-Protein	141	17	18.8	3
GNAQ	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	47	24	29.6	3
GNAS	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	31	12	24	2
GNA13	Pyrophosphatase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	51	15	18.7	3
KCTD12	Protein Binding	Enzyme Modulator	24	14	26.2	2

GNG7	GTPase Activity Protein Binding	Heterotrimeric G-Protein	8	4	24.5	3
GNA11	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	34	9	31.2	2
KCTD2	Protein Binding	Enzyme Modulator	44	15	26.5	3
KCTD5	Protein Binding	Enzyme Modulator	28	7	19.2	3
RPS2	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	2	2	8.5	1
GNG12	GTPase Activity Protein Binding	Heterotrimeric G-Protein	6	3	22.2	3
SUPT6H			5	4	3.5	1
DOLPP1			4	2	5.5	2
RPL17			1	1	5.4	1
RBM14			9	5	5.6	2
LMNB2			12	9	8.9	2
RPL27A			2	1	7.4	1
GNAO1			20	3	14.3	2
GNA12			5	1	5.5	1
CCT5		Chaperonin	2	2	3.9	1
KCTD17	Protein Binding	Enzyme Modulator	12	4	10.1	3
HNRNPC	mRNA Binding	mRNA Processing Factor Ribonucleoprotein	12	11	12.3	3
PRSS3			4	2	6.7	2
ATAD3B			7	5	7.7	1
PTPLAD1			2	1	5.8	1
DPF2			2	2	7.9	1
C17orf85			1	1	3.8	1
SMARCE1	Sequence-Specific DNA Binding Transcription Factor Activity	HMG Box Transcription Factor	4	2	3.2	2

	Sequence-Specific DNA Binding Transcription Factor Activity Chromatin Binding Receptor Binding	Signalling Molecule Chromatin/Chromatin-Binding Protein				
RPS27A	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	2	2	16	1
HNRPDL	Catalytic Activity Structural Constituent Of Ribosome Poly(A) RNA Binding	mRNA Polyadenylation Factor mRNA Splicing Factor Ribonucleoprotein Ribosomal Protein	7	5	5.7	3
HNRNPR			6	6	5.3	2
ILF3	Kinase Activity Hydrolase Activity Deaminize Activity DNA Binding RNA Binding Protein Binding Kinase Activator Activity	DNA Binding Protein RNA Binding Protein Deaminase Kinase Activator Defense/Immunity Protein	2	2	1.4	2
HSPA9			7	7	7	2
MRE11A			1	1	1.7	1
MCM7			7	7	16.8	1
DDX17			12	6	7.2	2
SF3B3	Catalytic Activity Damaged DNA Binding Poly(A) RNA Binding	Damaged DNA-Binding Protein mRNA Polyadenylation Factor mRNA Splicing Factor	3	3	3.3	1
DHX9			3	3	3.1	1
LMNA			2	2	3.5	1
NCL			2	2	2.7	1
SMARCA4	DNA Helicase Activity Nucleic Acid Binding	DNA Helicase Helicase	4	4	1.5	2

PRPF19	Catalytic Activity mRNA Binding	mRNA Splicing Factor	2	2	4.2	1
SRSF6			2	2	5.2	1
H1FX	DNA Binding	Histone	2	2	10.3	1
SMARCC1	Transcription Cofactor Activity Sequence-Specific DNA Binding Transcription Factor Activity Sequence-Specific DNA Binding Transcription Factor Activity Chromatin Binding Protein Binding	Transcription Cofactor Chromatin/Chromatin-Binding Protein	2	1	1.8	1
RUVBL1			2	2	5	1
DDX21			2	2	2.6	1
GATA6			1	1	2.4	1
SNRPC			1	1	7.5	1
SNRPD3			1	1	7.9	1
OBSL1	Protein Kinase Activity Structural Constituent Of Cytoskeleton Protein Binding Small GTPase Regulator Activity Guanyl-Nucleotide Exchange Factor Activity	Non-Receptor Serine/Threonine Protein Kinase Non-Receptor Serine/Threonine Protein Kinase Guanyl-Nucleotide Exchange Factor Actin Family Cytoskeletal Protein Cell Adhesion Molecule	1	1	0.6	1
RRP1B			1	1	2.5	1
IK	Cytokine Activity	Cytokine	2	2	1.6	2
YWHAB			2	2	4.1	2
DDX3X	RNA Helicase Activity Translation Initiation Factor Activity Translation Initiation Factor Activity Translation Initiation Factor Activity	RNA Helicase Helicase	7	4	8.2	1

SMARCC2	Transcription Cofactor Activity Sequence-Specific DNA Binding Transcription Factor Activity Sequence-Specific DNA Binding Transcription Factor Activity Chromatin Binding Protein Binding	Transcription Cofactor Chromatin/Chromatin-Binding Protein	3	2	2.6	1
PPIL1	Isomerase Activity	Isomerase	1	1	8.4	1
POLR2H			1	1	7.3	1
RPS6	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	1	1	6	1
NUP153			1	1	1	1
SFPQ			1	1	2.3	1
NONO	Catalytic Activity mRNA Binding	mRNA Splicing Factor	1	1	1.9	1
HNRNPF			1	1	4.1	1
CYC1			1	1	3.7	1
FIP1L1			1	1	2.7	1
SRSF2			1	1	7.7	1

Table 25: *Proteins identified in the nucleus by TAP-Gβ1γ7, untreated conditions.* Proteins identified as Gβγ interactors with TAP tagged Gβ1 and identified by mass spectrometry and grouped by molecular function and protein class using Panther online software[223].

Protein	Molecular Function	Protein Class	Average Peptides	Average Unique Peptides	Average Coverage	n
GNAI3	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	7	3	8.45	2
GNB1	GTPase Activity Protein Binding	Hydrolase Heterotrimeric G-Protein	30	6	9.15	2
GNAQ	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity	Heterotrimeric G-Protein	2	2	7.2	1

	Phospholipase Activity Adenylate Cyclase Activity Receptor Binding					
GNAS	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	6	3	13.2	1
GNA13	Pyrophosphatase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	10	8	13.1	2
KCTD12	Protein Binding	Enzyme Modulator	9	7	12.45	2
GNG7	GTPase Activity Protein Binding	Heterotrimeric G-Protein	8	5	27.93333 333	3
GNA11	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	5	5	15.6	1
KCTD2	Protein Binding	Enzyme Modulator	2	2	12.5	1
KCTD5	Protein Binding	Enzyme Modulator	3	3	11.55	2
GNG12	GTPase Activity Protein Binding	Heterotrimeric G-Protein	3	1	22.2	1
RBM14	Sequence-Specific DNA Binding Transcription Factor Activity Catalytic Activity DNA Replication Origin Binding Single-Stranded DNA Binding Sequence-Specific DNA Binding Transcription Factor Activity Poly(A) RNA Binding	Transcription Factor DNA Binding Protein mRNA Polyadenylation Factor mRNA Splicing Factor Ribonucleoprotein	2	2	4.9	1
GNB2	GTPase Activity Protein Binding	Hydrolase Heterotrimeric G-Protein	25	1	12.6	1
ATAD3B	Hydrolase Activity	Hydrolase	2	2	2.9	1
H3F3A	DNA Binding	Histone	2	1	5.1	1
PTPLAD1			1	1	3.9	1

RPS27A	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	1	1	10.3	1
HNRNPF	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	3	1	10.6	1
TESC	Phosphoprotein Phosphatase Activity Phosphoprotein Phosphatase Activity Calcium Ion Binding Calmodulin Binding	Protein Phosphatase Protein Phosphatase Calmodulin	3	3	10.05	2
HAT1	Acetyltransferase Activity	Acetyltransferase	1	1	3.1	1
CKAP4	Hydrolase Activity Nucleic Acid Binding Chromatin Binding	Chromatin/Chromatin-Binding Protein Hydrolase	7	7	8.65	2
RADIL			4	4	4.5	1
MATR3	DNA Binding RNA Binding	DNA Binding Protein RNA Binding Protein	3	3	5.8	1
MYBBP1A	Sequence-Specific DNA Binding Transcription Factor Activity DNA-Directed DNA Polymerase Activity Sequence-Specific DNA Binding Transcription Factor Activity	Transcription Factor DNA-Directed DNA Polymerase	3	3	1.15	2
RFC1	Double-Stranded DNA Binding	DNA Binding Protein	1	1	1.1	1
BSG			1	1	2.6	1
RFC2			1	1	2.8	1
HSPA6		Hsp70 Family Chaperone	4	1	5.9	1
S100A4	Calcium Ion Binding Growth Factor Activity Calmodulin Binding	Growth Factor Calmodulin	2	2	18.8	1
TIMM8A	Cation Transmembrane Transporter Activity	Cation Transporter	1	1	11.3	1

Table 26: *Proteins identified in the nucleus by TAP-Gβ1γ7, 1mM carbachol treated conditions.* Cells treated with 1mM carbachol for 5 minutes followed by a 45 minute wash out period. Proteins identified as Gβγ interactors with TAP tagged

Gβ1 and identified by mass spectrometry and grouped by molecular function and protein class using Panther online software[223].

Protein	Molecular Function	Protein Class	Average Peptides	Average Unique Peptides	Average Coverage	n
GNAI3	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	50	13	19.5	3
GNAI1	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	25	2	13.6	2
GNAI2	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	32	11	24.8	2
GNB1	GTPase Activity Protein Binding	Hydrolase Heterotrimeric G-Protein	85	17	18.0	3
GNAQ	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	12	6	14.5	2
GNAS	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	9	3	11.9	1
GNA13	Pyrophosphatase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	21	7	14.5	2
KCTD12	Protein Binding	Enzyme Modulator	30	18	22.5	3
GNG7	GTPase Activity Protein Binding	Heterotrimeric G-Protein	8	3	23.5	3
GNA11	Pyrophosphatase Activity Phosphoric Diester Hydrolase Activity Phospholipase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	16	9	12.7	3

KCTD2	Protein Binding	Enzyme Modulator	11	7	14.7	3
KCTD5	Protein Binding	Enzyme Modulator	16	9	18.0	3
GNG12	GTPase Activity Protein Binding	Heterotrimeric G-Protein	4	2	22.2	2
TXN	Oxidoreductase Activity	Oxidoreductase	1	1	12.4	1
GNB2	GTPase Activity Protein Binding	Hydrolase Heterotrimeric G-Protein	18	1	12.6	1
GNA12	Pyrophosphatase Activity Adenylate Cyclase Activity Receptor Binding	Heterotrimeric G-Protein	18	7	11.4	2
PRSS3	Serine-Type Peptidase Activity	Serine Protease	4	4	8.1	2
PTPLAD1			1	1	3.9	1
DNAJA1			1	1	3.3	1
CASP14			2	2	9.9	1
GAPDH	Oxidoreductase Activity	Dehydrogenase	1	1	4.2	1
RPS27A	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	1	1	10.3	1
HNRNPF	Structural Constituent Of Ribosome Nucleic Acid Binding	Ribosomal Protein	2	1	8.2	1
RADIL			5	5	3.4	2
RFC1	Double-Stranded DNA Binding	DNA Binding Protein	2	2	2.2	1
RFC2			7	5	9.6	2
LRRC59			5	5	20.8	1
RFC4	DNA-Directed DNA Polymerase Activity Nucleotidyltransferase Activity Nucleic Acid Binding	Nucleotidyltransferase DNA-Directed DNA Polymerase	4	4	6.0	2
ATP6V0D1	Hydrolase Activity Cation Transmembrane Transporter Activity Hydrogen Ion Transmembrane Transporter Activity	ATP Synthase Hydrolase	2	2	6.6	1
ATP6V1B2	Hydrolase Activity Receptor Activity	ATP Synthase Anion Channel	2	2	4.1	1

	Anion Channel Activity Ligand-Gated Ion Channel Activity Cation Transmembrane Transporter Activity Proton-Transporting ATP Synthase Activity, Rotational Mechanism Single-Stranded DNA Binding	Ligand-Gated Ion Channel Ligand-Gated Ion Channel DNA Binding Protein Hydrolase				
ATP6V1A	Hydrolase Activity Receptor Activity Anion Channel Activity Ligand-Gated Ion Channel Activity Cation Transmembrane Transporter Activity Proton-Transporting ATP Synthase Activity, Rotational Mechanism Single-Stranded DNA Binding	ATP Synthase Anion Channel Ligand-Gated Ion Channel Ligand-Gated Ion Channel DNA Binding Protein Hydrolase	2	2	3.9	1
RAP1A	GTPase Activity Protein Binding	Small GTPase	2	2	11.4	1
C14orf21			1	1	2.4	1
DCD			1	1	10	1
FLG2			1	1	0.5	1
FTH1	Storage Protein		1	1	3.8	1
CSN2			2	1	8.5	1