CHARACTERIZATION OF THE ANGIOTENSIN TYPE 1 RECEPTOR AND THE BETA2 ADRENERGIC RECEPTOR PROPERTIES: THE INVOLVEMENT OF ARRESTIN2, RAB1 AND SOME MOLECULAR CHAPERONES IN THE ASSEMBLY AND TRAFFICKING OF GPCRS

by

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Abstract

Current drugs used to treat Congestive Heart Failure target the renin-angiotensin and adrenergic systems. Studies showed increased mortality rates in patients treated with a combination of these medications. Angiotensin-AT1 and β2-Adrenergic receptors were shown to form receptor heteromers. Blockade of one receptor in the complex can affect the signal transmitted by the other suggesting that ligand-based therapy is not as selective as we might think. Modulating receptor trafficking after synthesis might prove to be a valid therapeutic strategy. Unfortunately, little is known about receptor assembly and transport from Endoplasmic Reticulum to Plasma Membrane. The objectives of this study are to identify the proteins that participate in the assembly of AT1R-β2AR heteromers and the regulators of the anterograde trafficking of G-Protein Coupled Receptors. This thesis introduces the role of important targets in those poorly understood processes. The identification of such targets could lead to developing better drugs with fewer adverse effects.

List of Abbreviations and Symbols Used

7TMRs	Seven Transmembrane Receptors
β2-AR	β2- adrenergic receptor
AC	Adenylyl Cyclase
ACE	Angiotensin-Converting Enzyme
ARFs	ADP Ribosylation Factors
Arr2	Arrestin2
AT1R	Angiotensin Type 1 Receptor
BiFC	Bimolecular Fluorescence Complementation
Bip	immunoglobulin binding protein
BRET	Bioluminescence Resonance Energy Transfer
BSA	Bovine Serum Albumin
cAMP	cyclic Adenosine MonoPhosphate
CCVs	Clathrin-coated vesicles
CHF	Congestive Heart Failure
co- IP	co- immunoprecipitation
COP	Coat Protein Complex
CPCs	Coat Protein Complexes
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DN	dominant negative
DNA	Deoxyribonucleic acid
DRiP78	Dopamine Receptor-interacting Protein 78
DTT	1,4-disulfanylbutane-2,3-diol
EDTA	Ethylene Diamine Tetra Acetate
ER	Endoplasmic Reticulum

ERAD	ER-associated degradation
ERGIC	ER-Golgi Intermediate Compartment
ERESs	ER-exit sites
ERQC	ER-Quality Control
FBS	Fetal Bovine Serum
FRET	Fluorescence Resonance Energy Transfer
G protein	Guanine nucleotide binding protein
GABA	γ-aminobutyric acid
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green Fluorescent Protein
GPCRs	G-Protein Coupled Receptors
GRK	G- Protein Kinase
GRP	Glucose-Related Protein
GST	Glutathione-S-Transferase
GTP	Guanosine triphosphate
HA	hemagglutinin
HEK	Human Embryonic Kidney Cells
HERG	human ether-a-go-go related gene
HSP	Heat Shock Protein
IB	Immunoblot
IP3	Inositol triphosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
MAPK	Mitogen-Activated Protein Kinase
MEFs	Mouse Embryonic Fibroblasts
ND	Asparagine at positions 4, 176, 188 to Aspartic Acid
NP40	Nonidet P40

NQ	Asparagine at positions 6, 15, 187 to Glutamine
PBS	Phosphate- Buffered Saline
PCR	Polymerase Chain Reaction
PDI	Protein Disulfide Isomerase
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PM	Plasma Membrane
PS	Penicillin streptomycin
Rab	Ras- related protein in brain
RAS	Renin Angiotensin System
RIPA	Radioimmunoprecipitation assay buffer
Rluc	Renilla luciferase
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
shRNA	short hairpin RNA
siRNA	small interfering RNA
SNS	Sympathetic Nervous System
TBS	Tris- Buffered Saline
TEMED	Tetramethylethylenediamine
V1	venus 1 (N-terminal of venus - amino acids 1 to 157)
V2	venus 2 (C-terminal of venus - amino acids 158 to 238)
WT	wild type
YFP	Yellow Fluorescent Protein

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CHAPTER 1: INTRODUCTION

1.1 Congestive Heart Failure

Heart failure is a condition in which the heart has lost the ability to pump adequate blood to the tissues. This leads to malfunction in all other tissues and organs since the levels of blood circulating and hence oxygen and nutrients supply are significantly reduced. Heart failure is an example of many other cardiovascular diseases such as stroke, arrhythmia, hypertension and atherosclerosis that account for the death of many Canadians every year. According to 2010 data from statistics Canada, cardiovascular diseases are the second leading cause of death and account for 22.4% of all deaths in the country (1). One of the most serious cardiovascular diseases is congestive heart failure (CHF) since it is progressive. It is estimated that there are 400,000 Canadians living with CHF (2). Congestive heart failure (CHF) is the result of damage in the heart muscle that leads to a decrease in heart contractility and reduced cardiac output. Hypertrophy, increased in heart size, is another characteristic of CHF. This remodelling of the cardiac muscle is the cause for progressive weakening in the pumping function of the heart (3). The two major systems involved in CHF are the renin-angiotensin and the adrenergic nervous systems since they are activated to compensate for reduced cardiac output in CHF. However, it was noticed that the activation of these systems makes CHF even worse and increases its damaging effects (4). It is known that these systems are controlled by two groups of receptors; the angiotensin receptors and the adrenergic receptors. This has led to the development of drugs that inhibit the renin-angiotensin system and the adrenergic nervous system to treat CHF and the most common examples are angiotensin converting enzyme (ACE) inhibitors, angiotensin II

type I receptor (AT1R) blockers and beta-adrenergic receptor (βAR) blockers. This group of drugs has shown positive long-term effects on morbidity and mortality in CHF patients by improving their clinical signs and symptoms (5). Nonetheless, these drugs were not able to give a dramatic solution for the heart failure problem which still represents a major concern being one of the leading reasons for hospitalization and morbidity. Therefore, combination therapies are now being administered for the patients to increase their survival rates. In such treatments, two or even three of the common classes of drugs (ACE inhibitors, β- blockers and AT1R blockers) are being given to patients simultaneously. It was observed that this type of therapy leads to the accumulation of huge amounts of active angiotensin II in the circulation despite the administration of ACE inhibitors (6, 7) and therefore this new approach of therapy has to be further studied. Unfortunately, it was recently found that although each drug taken alone has beneficial effects, the combination of all three drugs has adverse effects in a subgroup of patients, leading to an increase in their mortality rate (5, 8). The reason for this observation is not determined yet.

1.2 Renin-Angiotensin System (RAS): Angiotensin Type I Receptor (AT1R)

The renin-angiotensin system plays a crucial role in the regulation of blood pressure and fluid homeostasis by the activation of angiotensin II which is considered the main active component in the system (9). This is initiated when angiotensinogen which is released from the liver is converted into angiotensin I (ANG I) by the action of renin, secreted from the kidney. ANG I is then converted to the active form, angiotensin II by angiotensin converting enzyme (ACE) **(Figure 1.1)** (9). This is the classical activation

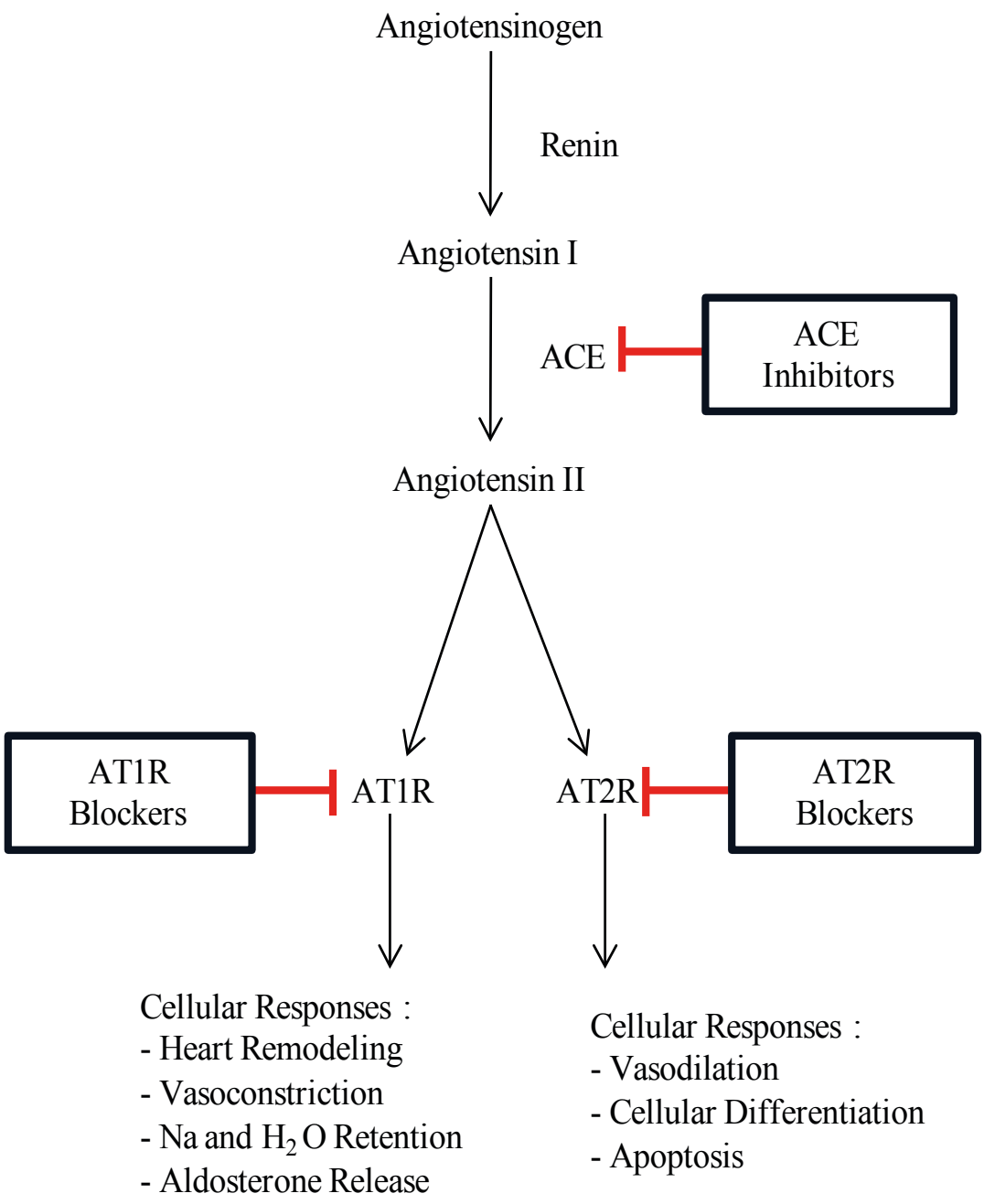


Figure 1.1: Renin Angiotensin System
Angiotensinogen is converted to ANG I via renin, ANG I is converted to ANG II which can activate angiotensin receptors to induce signal transduction.

mechanism; however, another alternative cascade that involves ACE-2 can also occur. ACE-2 is a homolog of ACE that converts either angiotensin I to ANG (1-9) or angiotensin II to ANG (1-7) (10, 11). Angiotensin II mediates a variety of physiological processes by binding to and activating the angiotensin receptors type I and II (AT1R and AT2R) which are expressed in the heart, brain, kidney and blood vessels (12, 13). This group of receptors belongs to the G Protein-Coupled Receptor Family (GPCRs) and they can trigger distinct cellular signals upon ligand binding. In fact, they show opposite effects and this is illustrated clearly when considering their function on the smooth muscle cells since AT1R is a vasoconstrictor while AT2R activation leads to vascular relaxation (9, 14, 15). Furthermore, AT1R and AT2R have the ability to elicit other biological responses. AT2R mediates apoptosis and cellular differentiation (16-18) while AT1R mediates aldosterone release from the adrenal gland, sympathetic activation, sodium and water retention and cellular growth (19, 20). In addition to the aforementioned effects, AT1R plays a key role in cardiovascular and heart remodelling. The current proposed mechanism links this hypertrophic effect of AT1R with its ability to promote growth signals in an ANGII-dependent mechanism since AT1R stimulation leads to the release of several secondary growth factors such as transforming growth factor-beta (TGF-β) and endothelin-1 (ET-1) (21, 22). AT1R signals via Gq/G11 or Gαi. Activation of Gq/G11 stimulates phospholipase C (PLC) to produce Inositol triphosphate (IP3) while activation of Gαi inhibits adenylyl cyclase and reduces cyclic AMP (cAMP) production. Studies have demonstrated that vascular muscle relaxation occurs in response to tyrosine kinase activation (23) as well as Rho/Rho kinase activation (24). The studies

suggest a dual role for AT1R signalling in the regulation of myosin-light chain (MLC) phosphorylation by Ca^{2+}-dependent and Ca^{2+}-independent pathways.

1.3 Sympathetic Nervous System (SNS): β2-Adrenergic Receptor (β2AR)

The sympathetic nervous system represents the other important effector in maintaining homeostasis and regulating cardiac physiology. In the SNS, L-tyrosin is converted to norepinephrine which is converted to epinephrine via a cascade of enzymatic reactions **(Figure 1.2)**. Both epinephrine and norepinephrine can bind to and activate the different subtypes of adrenergic receptors, another subfamily in the GPCRs superfamily. There are three subfamilies of the adrenergic receptors; α1-ARs (has three members: α1A, α1B and α1D), α2-ARs (has three members: α2A, α2B and α2C) and β-ARs (has three members: β1, β2 and β3) (25, 26). All adrenergic receptors are activated by the same group of catecholamines but they stimulate distinct cellular signalling pathways. α1-ARs and β-ARs are expressed in the heart, however, β-ARs are the main receptors that have crucial effects on cardiac function (25). The activation of β1- and β2-ARs leads to increased cardiac output and contractility but β3-AR function is still unclear and needs further characterization (27). Few studies examined β3-ARs signalling and showed that it seems to be coupling to Gαi leading to blocking AC activity and reducing cardiac contractility (28, 29). Both β1-AR and β2-AR signal via Gαs to activate adenylyl cyclase and produce cAMP due to ligand stimulation. This also triggers the activation of protein kinase A (PKA). The ability of β-ARs to regulate heart function is associated with this step of their signalling pathway since PKA activation induce L-type calcium channel phosphorylation and phospholamban phosphorylation and therefore control Ca^{2+} levels in

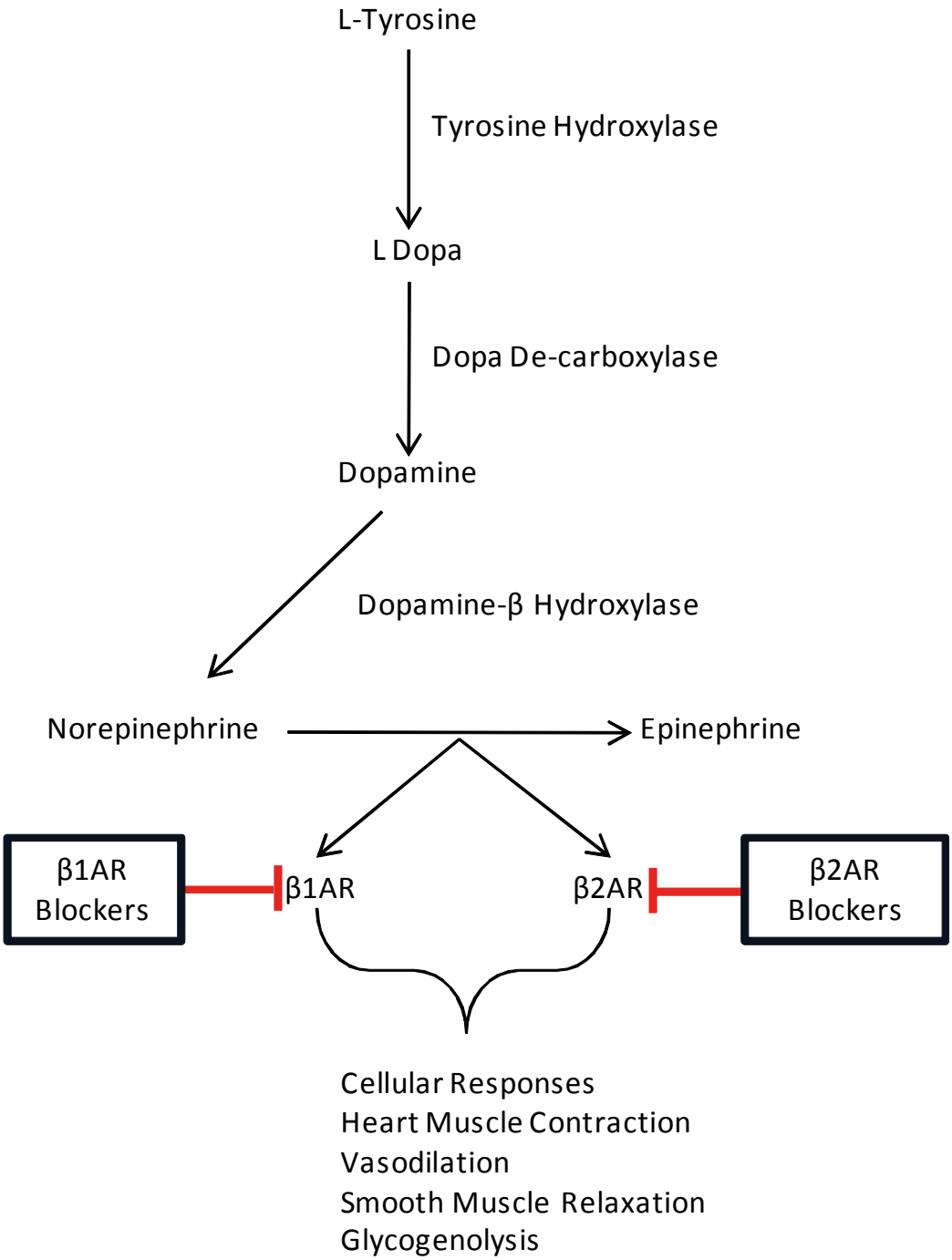


Figure 1.2: Sympathetic Nervous System

L-tyrosine is converted to Dopamine which is then hydrolysed to Norepinephrine. Both Catecholamines (epinephrine and norepinephrine) can activate β-adrenergic receptors to activate corresponding pathways.

the heart (25-27). Both β1-AR and β2-AR were shown to signal through Gαi as well. For the β2-AR, the signalling pathway to be activated is determined by the ligand used to stimulate the receptor. Studies on the rat cardiomyocyte showed that fenoterol binding enhances signalling via Gαs while terbutaline and salbutamol induce signalling through both Gαs and Gαi (30, 31). Interestingly, β2-AR signalling via Gαi did not lead only to AC inhibition, it was also shown to induce mitogen-activated protein kinase (MAPK) in a PKA-dependent mechanism (32). Furthermore, Gαi activation could also mediate the release of cytosolic phospholipase A2 (cPLA2). Signalling via Gαi/cPLA2 leads to increased Ca^{2+} levels and hence increases contractility. What determines which pathway is activated is still uncertain, however some evidences suggest that it depends on the cAMP pathways; meaning that those alternative mechanisms are only mediated when the main cAMP pathway is defective (33). Heart failure is characterized by rapid internalization and downregulation of β-adrenergic receptors, especially β1-ARs (34, 35). It is not clear though whether this should be considered as a beneficial or detrimental event (27). Studies show that overexpressing β1-AR results in cardiac hypertrophy and fibrosis (36, 37). On the other hand, β2-AR overexpression increases heart contractility and enhances cardiac function until a certain limit where expressing higher levels could lead to cardiomyopathy (38). A possible reason for the difference between the two receptor subtypes is the different signalling pathways they mediate. Another possibility is the different polymorphisms of the adrenergic receptors. Interestingly a β1-AR polymorphism showed better contractility levels due to functional enhancement of the receptor (39) while a β2-AR polymorphism showed lower AC activity and increased mortality rates in patients (40, 41). Studies also show that Gαs levels are downregulated

while Gαi and its mRNA levels are doubled during heart failure and that the levels of catecholamines are also increased and that is another cause for the opposite effects of receptor activation since β1-AR induces apoptosis while β2-AR has an anti-apoptotic effect.

1.4 G-Protein Coupled Receptors (GPCRs)

G-Protein Coupled Receptors (GPCRs) is a superfamily of signalling proteins that are encoded by 3-5% of human genes. All the members of the family share a similar structure of seven transmembrane α helical domains spanning the cellular membranes, an extracellular N- terminal domain and an intracellular C- terminal domain; therefore they are also called Seven Transmembrane Receptors (7TMRs) especially that a subgroup of this family signals through a pathway other than G-proteins (42). GPCRs have a different variable range of ligands including; light, odorants, hormones, neurotransmitters, chemokines, amino acids and Ca^{2+} ions and activating GPCRs leads to the release of several downstream effectors including adenylyl cyclases, GPCR kinases and phospholipases. Some receptors are also targets for more than one ligand. Therefore each receptor has the ability to signal through different pathways because different ligands induce different signalling pathways. GPCRs are usually classified based on the arrangement of the terminal ends into classes A, B or C (42).

Family A:

This family represents the largest group of G- protein coupled receptors since over 80% of GPCRs belong to it. An important member is the Rhodopsin receptor, a GPCR that has been studied thoroughly and the first to be crystallized; therefore the family is commonly called rhodopsin-like receptors or Family 1 (42). There are a number

of conserved residues in the sequences of the receptors in this group such as the DRY box and the Proline residues in loops 6 and 7 which are responsible for G protein activation and the structure and function of the helices respectively. Another characteristic feature of the receptors in this family is the disulfide bridge between the first and second transmembrane domains. In addition, most of the members in the group has an active palmitoylation site at the entrance of their c-tail to the intracellular domain (43). Examples for the receptors in this group are odorant, protease, dopamine, chemokine and muscarinic cholinergic receptors. In addition, the β2-adrenergic receptor which is the first receptor of interest in the project belongs to this family.

Family B:

This group is a relatively small group compared with Family A. It is also known as Family 2. This group is distinguished by a large N-terminal end with multiple cysteine residues forming disulfide bridges. The long end acts as a binding domain for ligands; specifically hormones. Common examples include glucagon, calcitonin, the parathyroid hormone receptor and the angiotensin type 1 receptor, the second receptor of interest in this study (42).

Family C:

Family C (or Family 3) contains the $GABA_B$, metabotropic and the mGLU receptors to mention a few. These receptors need to be in dimers to be able to function properly. The crystal structure of the metabotropic glutamate receptors showed a large extracellular domain at the N-terminus that was described as a Venus fly trap. Another important property of family C is the short highly conserved third loop, but other than this, little is known about the rest of the transmembrane domains (43).

1.5 GPCR Dimerization

GPCRs were initially believed to function as monomers; however, the concept of receptors dimerization has replaced this classical view of monomeric entities. Recent evidences have shown that dimerization is a very basic and important requirement for some receptors and can play a key role in their function (42-45). For example, studies have shown that blocking one receptor in a dimer pair can affect the signal transduction activated by the other. This observation could present a possible explanation or at least part of the problem of the increased mortality rates that are caused by taking drugs targeting GPCRs. The model in this study is the heterodimer AT1R-β2AR. These receptors were shown to form homo as well as heterodimers. They were also shown to exhibit this cross-inhibition effect where blocking AT1R affect the signal activated by β2-AR and vice versa. In addition, it was also interesting to study those two receptors because of the opposite effects they have on the heart muscle given that one acts as a vasoconstrictor (AT1R) while the other is a vasodilator (β2AR).

1.5.A Oligomerization; Disease and Drug Therapy

G protein- coupled receptors represent important targets for drugs because they are involved in most physiological processes and more than 30% of the currently available drugs are synthesized to act directly or indirectly on GPCRs. There are at least 800 members that have been identified as GPCRs, however only a small number of them has been used as targets in drugs synthesis. Some GPCRs are ubiquitous and can be expressed in different organs and tissues; therefore targeting a receptor in a certain tissue could lead to complex side effects due to the activation/ inactivation of the receptor in other tissues. Heteromerization and protein-protein interactions are important

mechanisms to regulate GPCRs function (46). Heteromerization can be tissue-specific or disease-specific. Tissue-specific heteromers occur in certain tissues and a good example for it would be AT1R-β2AR which has been shown to control heart rate and cardiac contractility (47), while disease-specific heteromers occur in certain pathological events. There are a couple of examples for disease-specific heteromers including AT1R-bradykinin B2 receptor which occurs in AngII-induced hyper-responsiveness in pre-eclampsia, prostaglandin EP1-β2AR which occurs in asthma and AT1R-APJ (apelin receptor) which occurs in atherosclerosis (48-50). Some of the previous studies looked at heteromerization in vivo by studying pre-eclampsia patients or ApoE-KO models. Heteromerization changes ligand-binding pockets which can either increase or decrease ligand affinity by changing the extent of G-protein coupling, the G-protein that the receptor usually binds to and the molecules recruited upon activation.

GPCRs are linked to a wide range of human diseases which is understandable given their wide distribution all over the different organs of the body. An important example would be heart diseases. About 200 members of GPCRs are targeted by the currently available heart drugs (cardiac GPCRs); however, most of these drugs act on the angiotensin and the adrenergic receptors. The available drugs; although improving the quality of life for patients, still have their limitations since they show major side effects on some patients. To produce better drugs with fewer side effects, further characterization of GPCRs, their dimerization, their assembly into signalling complexes as well as their trafficking from the endoplasmic reticulum to the cell surface is required to establish a complete understanding of the key factors in the processes.

1.6 GPCRs Transport from ER to PM

The different members in the G Protein-Coupled Receptors family are expressed in different tissues of the body and most of them can be found in more than one system. The level of their expression at the plasma membrane is highly regulated by three important processes in the receptor's life; export, internalization and recycling/degradation (51). GPCRs are first synthesized and folded in the ER. In contrast to what was thought in the past (that ligand binding recruits signalling molecules to the receptors at the plasma membrane), there are now many evidences that the signalling complex is also assembled in the ER after the early steps of biosynthesis (52-57). Following this, the receptor is transported via secretory vesicles to the ER-Golgi intermediate complex (ERGIC) then to the Golgi apparatus and eventually to the plasma membrane (58). When the receptor is expressed at the cell surface, endogenous ligands can bind to the extracellular domain of the receptor inducing its endocytosis. The receptor is then phosphorylated by kinases including G protein receptor kinase (GRK), protein kinase A (PKA) and protein kinase C (PKC). The receptor is then internalized upon arrestin recruitment to be recycled back to the plasma membrane or degraded. The process of internalization has been investigated thoroughly and so many studies have characterized the endocytic pathways and the events following the internalization of the receptors. In contrast, very little is known about the anterograde trafficking of GPCRs and the events leading to their transport from the ER through Golgi to the plasma membrane. This pathway has just recently become the focus of some research and new evidences are starting to emerge about the molecular mechanisms governing the process (59-61).

1.7 Protein Synthesis:

Protein synthesis starts with transcription (transfer of genetic information from DNA to mRNA), followed by translation (transfer of genetic information from mRNA to the primary amino acids sequence) and finally protein folding (conversion of the amino acids chains to the biologically active form). The key operators of protein synthesis are the ribosomes. Some ribosomes are located in the cytoplasm and those are responsible for the synthesis of cytosolic, peroxisomal and nuclear proteins. On the other hand, plasma membrane proteins and secretory polypeptides are synthesized via ribosomes that are located at the rough endoplasmic reticulum membranes. GPCRs are plasma membrane proteins; therefore, the rest of this section will focus on describing the synthesis of the proteins that are destined to the cell surface. Protein synthesis is tightly controlled since it is very crucial to conserve the amino acids sequence in the native form; however, protein synthesis is still subjected to errors that could occur due to an array of reasons such as cellular stress and genetic mutations. This could result in the development of some serious conditions such as cystic fibrosis and Alzheimers (8). Therefore, the folding process is controlled by many scaffold proteins, folding enzymes and molecular chaperones to ensure conserving the amino acids sequence of the native polypeptide. Those proteins interact with the polypeptides once they arrive at the ER and help them to fold properly. Examples of this group of regulators include Bip, calnexin, calreticulin, ERp57, HSP70, PDI and GRP94. The interactions that occur between the newly synthesized polypeptides and those ER-proteins slow the folding process, prevent aggregation and stabilize the intermediate forms of proteins.

The outcome of the folding process determines the fate of the protein; whether it is going to be secreted and transported to its appropriate cellular location or sent to the proteosome for degradation. Proteins that are not properly folded are retained in the ER. This accumulation causes ER stress and an unfolded protein response is activated. This response slows protein synthesis and facilitates the degradation of misfolded proteins. The degradation process has been linked to the folding process because in order to maintain the capacity of the cell to synthesize and fold proteins, efficient degradation machinery must be occurring simultaneously. Therefore, ER quality control mechanisms and ER-associated degradation (ERAD) pathways have evolved to monitor the formation of polypeptides (62, 63). The ERAD pathway is composed of five main steps; Recognition, Targeting, Retrotranslocation, Ubiquitination and Proteasomal Degradation. Bip is believed to be responsible for recognition of substrates to be degraded. It was shown to interact with ERAD substrates and that misfolded proteins that can escape the degradation machinery are not recognized by Bip (Kar2p in yeast) (64, 65). The targeting step is the process where the misfolded protein leaves the ER and actually enters the ERAD pathway. The molecule that seems to be interacting with the aberrant protein at this level is EDEM (Mnl1p/Htm1p in yeast) which is a lectin chaperone (66). The next step after targeting is translocation back to the cytosol. Both Derlin-1 and PDI were shown to be involved in this step. The following step in ERAD is Ubiquitination. Ubiquitin acts as a signal for degradation following endocytosis as well as for misfolded proteins undergoing proteasomal degradation; however, the length of the ubiquitin molecule determines which process would be performed on the labelled protein (67). Ubiquitination is processed by three enzymes that sequentially act on the proteins, E1

(ubiquitin-activating enzyme) which activates a ubiquitin molecule in an ATP-dependent step, E2 (ubiquitin-conjugating enzyme) which receives the active ubiquitin and transfers it to E3 (ubiquitin ligase) which would recognize the misfolded protein and label it with the ubiquitin molecule. The actual degradation process takes place in the cytosolic 26S proteasome where misfolded proteins are broken down into smaller polypeptides and eventually individual amino acids that can be later used to synthesize new proteins (62, 68). The degradation of some GPCRs such as δ opioid receptor, thyrotropin receptor, and rhodopsin mutants was shown to be controlled by the ERAD machinery.

1.8 Molecular Chaperones

Molecular chaperones are located in compartments where the synthesis and transport of proteins take place such as the cytoplasm, the mitochondria and of course the endoplasmic reticulum. Generally, their main role is to facilitate the maturation of newly synthesized polypeptides, ensure their proper folding, facilitate their maturation and regulate the post-translational modifications that they have to undergo such as glycosylation and formation of disulfide bridges (64, 69). Chaperones were shown to play an important role in the transfer of some proteins to the Golgi and the plasma membrane. In addition, chaperone proteins were also shown to be involved in the activation of specific transcription factors, regulation of Ca^{2+} homeostasis and oxidative stress (8).

Bip, Calnexin, Calreticulin, ERp57, HSP70, PDI and GRP94 were selected to be investigated in this study because they were shown to interact with newly synthesized proteins, they present important components of the quality control mechanism, and their specific functions at distinct steps of the protein folding process allow predicting the

process of GPCRs folding. **Figure 1.3** summarizes the role of some chaperones in the folding of newly synthesized proteins.

Bip (GRP78): Bip is considered to be the most important molecular chaperone in the ER given its numerous functions that vary from regulating protein folding and oligomerization to balancing calcium levels in the ER (70). In addition, it can also associate with other chaperones such as calnexin, calreticulin and PDIs and facilitate their functions. The importance of Bip in the ERQC was illustrated in Bip-mutant knock-in mice. Bip is capable of binding the proteins directly at their hydrophobic residues and therefore is known to target misfolded proteins with exposed hydrophobic patches (63). Bip acts with the help of many co-factors including the Hsp40 members (ERdj1-5), BAP, GRP94 and Torsin A (62). Since it belongs to the heat-shock proteins, Bip contains two important domains; the ATPase domain at the N-terminal end and the domain responsible for the binding of peptides which is at the C-terminal end (63).

The Lectin Chaperones (Calnexin and Calreticulin): Maintaining calcium concentration in the ER is an important requirement for regulating some of the essential processes that take place in this compartment such as chaperone-chaperone interactions and chaperone-substrate interactions. As their names indicate, both calnexin and calreticlulin associate with calcium ions to regulate the ratio of free:bound calcium. The lectin chaperones are parts of the quality control cycle. They interact with polypeptides in a calcium-dependent fashion. Polypeptides are glycosylated in the ER by the addition of two acetylglucosamines and nine mannoses with three glucose molecules to asparagine residues. The glycoprotein is then cleaved by glucosidases I and II (71). Calnexin and calreticulin bind the monoglycosylated protein to facilitate its correct folding by

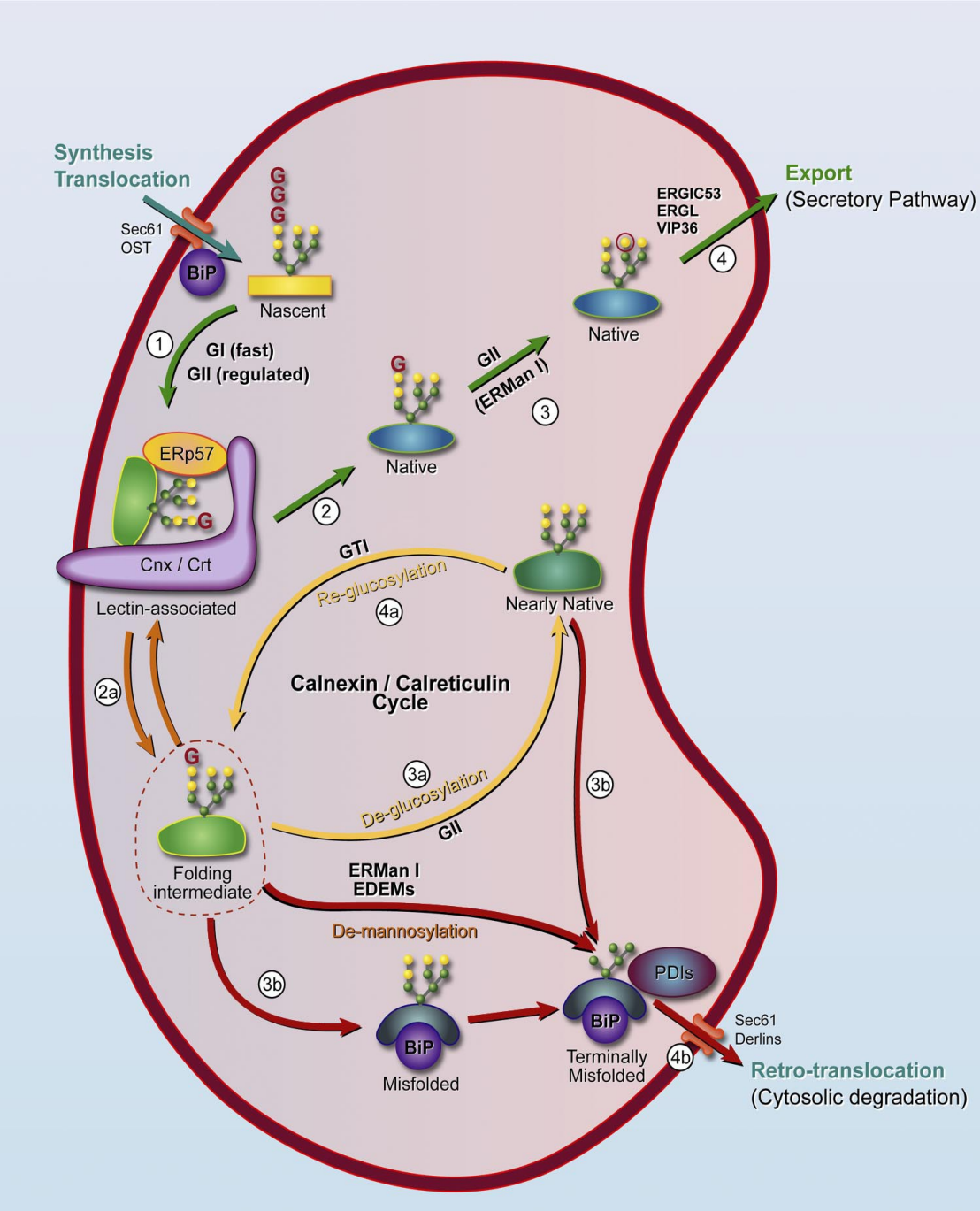


Figure 1.3: Molecular Chaperones
Some of the molecular chaperones involved in regulating the folding and trafficking of newly synthesized polupeptides. (Hebert and Molinari; Physiol. Rev. 2007. Am Physiol Soc, with permission)

preventing aggregation as well as premature degradation. The last glucose is cleaved and the chaperones dissociate. The protein can be reglycosylated if it is not completely folded to undergo a second round of this quality control cycle. Glycosylation was thought to be a pre-requisite for the binding of calnexin and calreticulin to all their substrates (72, 73), however, some evidences are emerging about the ability of calnexin and calreticulin to bind non-glycosylated proteins. In addition, a recent study showed the ability of a lectin-deficient calreticulin to bind polypeptides and fulfill its function as a chaperone (74). The crystal structure of calnexin shows two distinguishable domains, a single carbohydrate-binding domain and a Proline-rich domain (P domain) (62). The three-dimensional structure of calreticulin has not been identified yet but studies show that it is composed of a highly conserved amino-terminal domain, a Proline-rich domain for Ca^{+2} binding and an acidic carboxy-terminal domain (75).

PDI family (PDI and ERp57): The members of the PDI family are mainly responsible for the formation of disulfide bonds which is considered to be a very crucial step for the maturation of newly synthesized proteins. They catalyze the oxidation/reduction reaction of the S-S bridges and the isomerization of such bonds. They are characterized by thioredoxin-like domains (TLDs) that encompass the active site motif (usually two cysteine residues spaced by any two amino acids-CXXC). The different members vary in the number of TLDs as well as the active motifs. Many of these members (including human PDI and ERp57) act as molecular chaperones in addition to their enzymatic activity (62). They do so by preventing the formation of aggregates but the mechanism of their association with the protein is still unknown. There are 19 known homologues up to now which could indicate their specificity to

different substrates (63). Despite their large homology, PDI and ERp57 have their differences. ERp57 is known to interact with calnexin and catreticulin (76). Although both enzymes assist in the formation of disulfide bridges, ERp57 limits this catalysis to glycoproteins while PDI acts on non-glycosylated proteins (77, 78).

GRP94: The ER levels of GRP94 are very high. The main role of GRP94 is to assist the proteins in their maturation; however, a study showed that GRP94 participates in the T-cell immunity. The three functional domains in GRP94 are the regulatory domain at the NH_2 terminus, a substrate binding domain in the center and a dimerization domain at the COOH terminus (62).

1.8.A Molecular Chaperones and GPCRs:

Some recent studies have started to link molecular chaperones to GPCRs showing chaperones specificity in their interactions with distinct forms of receptors and their involvement in multiple functions. Yet, very few studies have addressed the roles of chaperones in GPCRs maturation. Examples include NinaA and RAN-binding protein 2 which were shown to help in the proper folding of Drosophila and vertebrates' rhodopsin, DRiP78 and calnexin which were proven to be important in the export of dopamine D1 receptor from the ER and its expression at the cell surface and calreticulin which was shown to enhance the maturation of B2 bradykinin receptor (79-81). In addition, some chaperones were shown to associate with immature hormone receptors in a different pattern than their association with the wild type completely folded forms. In addition, this study which looked at a group of multiple chaperones has also noted differences among the loss-of-function mutants themselves (82). Further studies are required to completely understand the function of molecular chaperones in the biosynthesis of GPCRs. In

addition, a closer look on the different oligomeric complexes and how chaperones affect them can also lead to interesting findings distinguishing monomers, homodimers and heterodimers.

1.9 Assembly

It was assumed in the past that GPCRs are transported to the plasma membrane as individual units after synthesis in the endoplasmic reticulum. When the receptor is expressed, it represents a target for the different endogenous and exogenous ligands that would bind and activate it. The classical old understanding of how GPCRs signalling complexes form suggested that upon ligand stimulation, trimeric G- proteins are recruited to the receptor, the Gαβγ complex dissociates into Gα and Gβγ, the later subunit then activates ion channels and enzymes which in turn would produce second messengers and initiate cellular responses (83). It was not clear whether the dissociation of the trimeric G protein into Gα and Gβγ occurs physically and the two subunits actually detach or is just a molecular rearrangement that the proteins undergo due to the activation and studies supporting the opposite ideas were presented (84, 85). However, this model had its own pitfalls because it does not really go with the specificity that each receptor has and their ability to select distinct transduction pathways to signal through. The last decade revealed new concepts and advanced techniques that changed this picture and allowed scientists to have a closer look at these complicated though highly organized systems. In addition, the idea of the existence of other functional forms of GPCRs (dimers and higher oligomers) has led to a strong debate about how these conformations assemble, get expressed and signal. Furthermore, the discovery of new state-of the art technologies to look at protein-protein interactions in living cells has also caused a huge impact on understanding these

systems. The emergence of fluorescence energy transfer techniques enabled researchers to determine interactions between two, three or even four proteins. The current understanding of the process suggests that the signalling complex is formed during the early biosynthesis events before the plasma membrane expression and activation of the receptor. This also suggests that the complex is being transported to the plasma membrane as a complete signalling unit. This was first proven for Saccharomyces cerevisiae and Drosophila (86, 87). These studies showed interactions among signalling proteins and how they might be regulated. The yeast study suggested that a group of scaffold proteins stabilize the subunits of the signalling complex for the yeast mating pheromone response in a specific orientation that would enable their sequential interactions. The Drosophila study was on visual transduction and showed the role of a supermolecular signalling complex (Signalplex) that contains a protein of 5 PDZ units that would allow the binding of the components. Showing similar results in mammalian systems was hard until the development of fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET). These methods allow studying interactions between the different components of the signalling complex in living cells. Therefore, research focused on studying the interactions between the components of the signalling complex. Evidences were provided about interactions between human receptors and their G-protein subunits (Gα, Gβ and Gγ). It was shown that the interactions between Galpha and GPCRs are specific; for example, the prostacyclin receptor showed an association with Gαs but not Gαi; in contrast, the α2-Adrenergic receptor was shown to interact with Gαi but not Gαs (57). This study has also demonstrated the ability of some GPCRs such as muscarinic M4 receptors,

Dopamine D2 receptors, α2-AR and the adenosineA1 receptor to interact with Gβ1 and Gγ2. Another study looked at the interaction between β2-AR and the trimeric G proteins and showed associations with Gβ, Gγ and Gαs subunits where BRET was used to detect the interaction between the receptor and the G-protein subunits (88). In addition, these studies showed that such associations occur in a basal level, and then tend to increase rapidly upon agonist stimulation before decreasing slowly which was an indication of receptor desensitization. German studies showed that Gαi does not really dissociate from the Gβγ subunits upon activation of α2- AR. These studies suggest that ligand binding leads to a conformational rearrangement of the heterotrimeric G protein subunits that elucidate further signal initiation (84, 89).

In addition to specifically interacting with their G- proteins, evidences show that the GPCRs have the ability to associate with their cognate effectors in the early steps of formation of the signalling complex before being expressed at the plasma membrane (90). This concept has been applied on different GPCRs such as the dopamine D2 and D4 receptors and the β2-adrenergic receptor. Different members of the inwardly rectifying potassium channels (GIRK or Kir3) were shown to associate with the dopamine receptors in HEK293 cells and COS-7 cells. In a study on rat brains, β2-AR was shown to associate with the class C L-type calcium channel in addition to G-proteins (Gαs and Gβγ), adenyl cyclase and PKA basally without the need for agonist stimulation (91). It is not well understood yet how this assembly is regulated and what target the formation of the signalling complex. Although some suggestions that G-proteins are the determinant factor in this equation were made, using dominant negative forms with some of these examples (dopamine receptor-Kir3 channel) did not affect the association. After showing

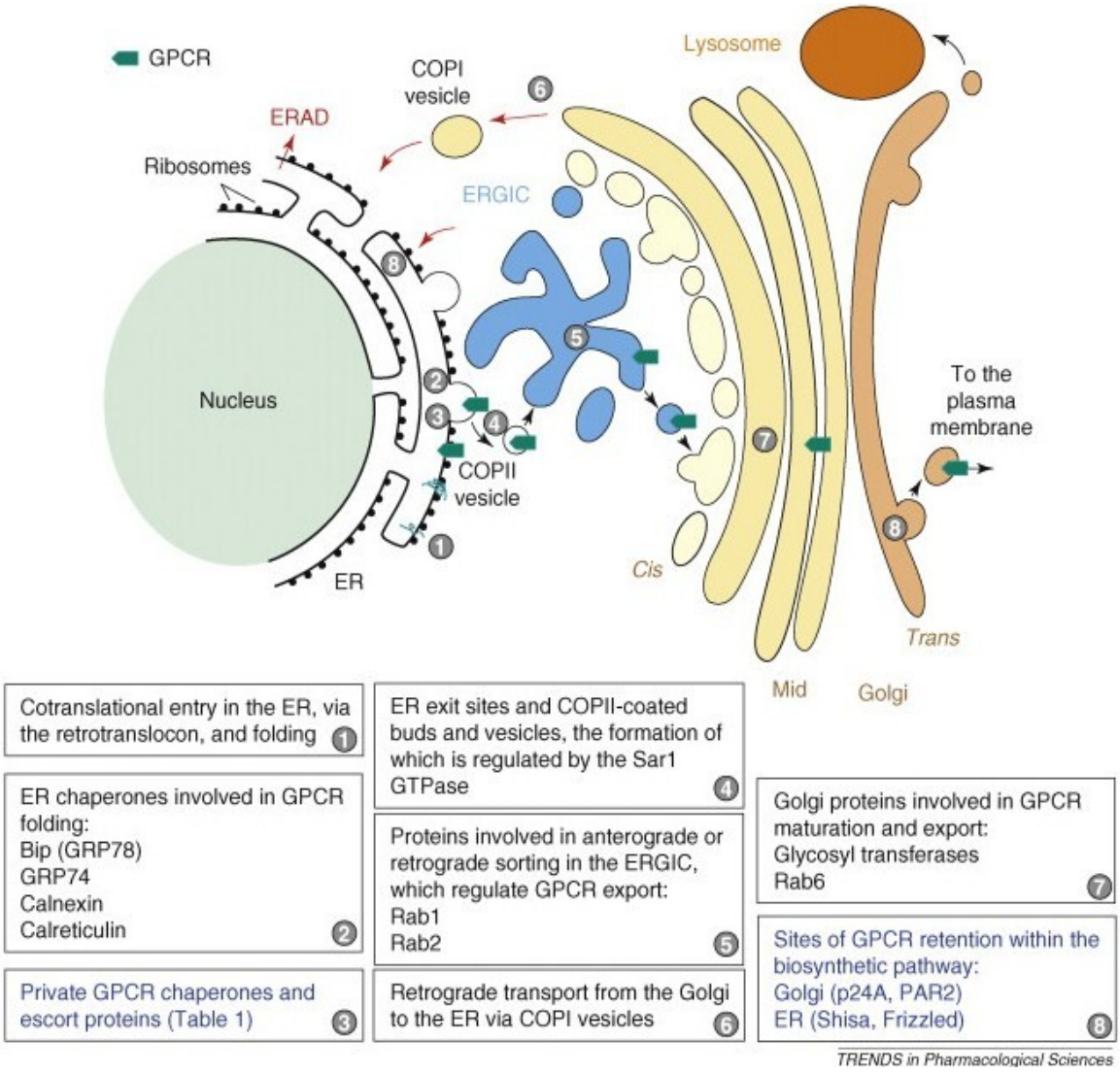
the interactions between GPCRs and their specific G- protein subunits and effector molecules, studies started investigating the interactions between the heterotrimeric G-proteins and the effector molecules. Previous work by Dr. Dupré on the β2-AR showed that there is a basal association between Gβγ subunits with both adenylyl cyclase and Kir3.1 channels (54).

1.10 Anterograde Pathway

Completely folded proteins have to be transported between the distinct subcellular organelles in order to reach their destination. The process of protein trafficking from the endoplasmic reticulum to the plasma membrane is known as the anterograde pathway while the opposite direction is known as the retrograde pathway. The two processes are highly regulated and have been fairly investigated; however, the exocytic pathway is not as detailed as the endocytic pathway. A specialized group of vesicles and tubules is responsible for the regulation of the processes. This group is referred to as Coat Protein Complexes (CPCs) and encounters COPII (coat protein II) and COPI (coat protein I) (92). COPII is known to mediate the anterograde pathway while COPI is responsible for cargo trafficking in the retrograde pathway (**Figure 1.4a**). A number of proteins were shown to associate with the coat proteins and/or to be required for the trafficking machinery, however, their functions have not been fully understood yet and there is still a possibility for more regulators to exist.

Proteins start their anterograde pathway in the endoplasmic reticulum, more specifically at the endoplasmic reticulum exit sites (ERESs), also known as translational ER sites. These domains are widely distributed on the ER membranes and were found to contain high levels of COPII proteins (92). In fact, studies demonstrated that the

(a)



(b)

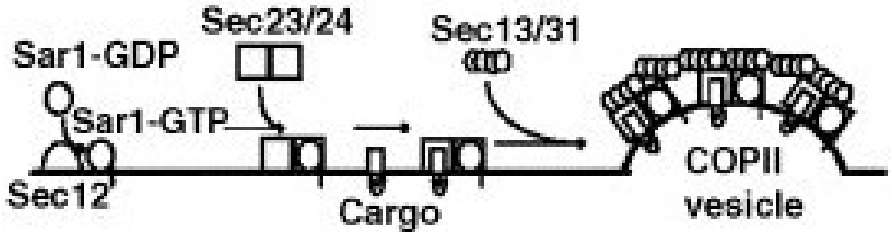


Figure 1.4: The Anterograde Trafficking Pathway

a) Anterograde pathway of GPCRs and some of the regulators involved. b) Formation of COPII vesicles via Sec23-Sec24 and Sec13-Sec32 complexes. (Achour *et al.*; Trends in Pharmacological Science. 2008, with permission, Dupré and Hébert: Cellular Signalling. 2006, with permission)

formation of ERESs and COPII vesicles is determined by the cargo protein being transported (93, 94). There are currently some proteins that were identified to have an essential role in ER exit; those are Sar1 GTPase, Sec23-Sec24 and Sec13-Sec31 **(Figure 1.4b)**. In addition, there is also the effector molecule that activates Sar1; the guanine nucleotide-exchange factor (GEF) known as Sec12. GEF converts the GDP-bound Sar1 to the active GTP-bound Sar1 which is sequentially translocated from the cytosol to the ER and embedded in the ER membranes via its N-terminal helix. Here comes the role of Sec23-Sec24 since they would also be recruited to the ER when Sar1 is activated to select the cargo proteins to be packaged and exported. Sec23 and Sec24 were shown to form a heterodimer that would be assembled with GTP-Sar1 (95). Sec23 and Sec24 show very similar structures, however, they each have their own specific function. Sec23 is the subunit that binds Sar1 and acts as a GTPase activating protein to hydrolyze GTP, while Sec24 is responsible for cargo binding to the complex (92). This recognition of cargo by the Sar1-Sec23-Sec24 complex is required for the formation of the cargo-containing pre-budding vesicles. Cargo proteins have also shown a requirement for a transmembrane receptor to link them to the COPII machinery. This receptor binds COPII directly when the cargo is secretory but only has to be exposed to COPII when a plasma membrane protein is being transported. The next step after the formation of a Sar1-Sec23-Sec24-cargo complex is the actual formation of COPII vesicles. This is initiated via Sec13-Sec31 complex; studies show that this complex can self assemble to form a heterotetramer of two Sec13 subunits and two Sec31 subunits that is similar in size to COPII vesicles (96). How cargo proteins are transferred to those vesicles is explained by the association that was observed between Sec13-Sec31 complex and Sec23-Sec24

complex (97). Both Sec23 and the cargo-containing subunit Sec24 were shown to interact with Sec13-Sec31 (98). This suggests that Sec13-Sec31 is translocated to the ER-membranes, then it binds the complex, and when it self-assembles, it would form the vesicle. It was suggested that the fission of this vesicle occurs via recruitment of more GTP-Sar1 but how this is regulated remains to be determined. The trafficking of these cargo-containing vesicles to Golgi occurs in two steps; the short range vesicular transport between ERESs and ERGIC and the long range vesicular transport from ERGIC to Golgi (The Stable Compartment Model). In the first step, COPII vesicles are transported along the cytoskeleton network. Ras GTPases play an important role in this step since Rab proteins manage the recruitment of motor proteins that link COPII vesicles to the microtubules in a Sar1-dependent mechanism (99). A very specialized sorting machinery is developed in the ERGIC to direct proteins to their right destinations, either back to the ER (retrograde pathway) or to Golgi (anterograde pathway). This is achieved by having different ADP Ribosylation Factors (ARFs) isoforms whose combinations would dictate the transport (100). The long range transport is still not quite understood. The possible factors regulating it are spectrin/ankyrin, dynactin/dynein and ZW10. The available model suggests that ZW10 mediates the connection between the ERGIC membranes and the microtubule cytoskeleton. In addition, ZW10 binds dynactin which facilitates the physical association between the microtubules and spectrin/ankyrin. This seems to provide a route for vesicles to traffic to the Golgi compartment (100). These anterograde carriers and vesicles are directed and fused into their destined compartment by a group of tethering proteins and SNARE complexes. In yeast, the proteins that were identified are syntaxin 5, Sec22b, membrin and Bet1 and isoforms were later discovered in mammalian

cells. These proteins form a complex that regulates tethering and fusion of COPII vesicles from the ER to ERGIC, while another complex composed of syntaxin 5, Bet1,GOS 28 and Ykt6 was shown to be responsible in the later ERGIC-Golgi transport (101, 102).

1.10.A Rab GTPases

Rab GTPases belong to the Ras superfamily. GTPases were shown to be responsible for regulating different cellular processes such as growth, cellular differentiation and cell movement, in addition they are known for their specificity in vesicular transport, especially Rab proteins which mainly mediate vesicle targeting. Sixty members of the Rab family have been identified to date (103). They are all localized in the cytosol in an inactive form (GDP-bound) and in complex with the Rab guanine nucleotide dissociation inhibitor (GDI). This complex is responsible for distributing Rab proteins to their right compartment, therefore, each Rab protein is associated with one or more organelles and they are very specific (60, 103, 104). It has been shown recently that Rab proteins can regulate the anterograde trafficking between specific organelles. For example; Rab1 was shown to regulate the trafficking between the ER and Golgi. Blocking Rab1 inhibits the transport of GPCRs like the adrenergic receptors (α1A, α1B, β1 and β2-AR) and angiotensin II type 1 receptor (AT1R) (105-107). Rab6 controls the anterograde transport of some receptors (β2-AR and AT1R) in the trans-Golgi network while Rab8 is known to mediate vesicular trafficking of receptors from Golgi to plasma membrane (108). Some of the previous studies have also examined the effect of these Rab GTPases on signalling and showed that inhibiting the function of GTPases such as Rab6 and Rab8 reduced ERK activation following stimulation of some GPCRs. Research has been focusing lately on studying the mechanism by which these proteins function but

there are still many questions to be answered. In view of these recent evidences and the anterograde pathway described above, the question would be is Rab1 a specific equivalent to Sar1 in GPCRs trafficking or a co-pilot for Sar1 in the process.

1.10.B β-Arrestins

Arrestins are scaffold proteins distributed in the cytosol. They are divided into two subgroups; visual (arrestin1 in retinal rod cells and arrestin4 in cone cells) and non visual (arrestin2 and arrestin3 also known as β-arrestin1 and β-arrestin2). The visual arrestins are specific to the retinal tissue and are responsible for regulating the signalling of photoreceptors. Non visual arrestins on the other hand are ubiquitous proteins and are involved in the trafficking and signalling of GPCRs (59). Arrestin2 and 3 act as mediator proteins in the internalization process of GPCRs through clathrin-coated vesicles (CCVs). They do so by associating with GRK-phosphorylated GPCRs, clathrin and AP-2 complexes to bring GPCRs together with CCVs. Most GPCRs are internalized by CCVs, however, there are some receptors that were shown to internalize through caveolae such as β2AR, endothelin, adenosine-A1 and M2-muscarinic receptors (59, 60). Although arrestins are thought of as signal terminating factors considering their main role in the desensitization/ internalization processes, evidence of their involvement in other cell functions such as promoting signal transduction has been shown. A role for arrestins in initiating signalling pathways such as ERK activation has been proposed (109). The idea of non visual arrestins being involved in GPCRs internalization was first adopted because of the observation that it directly binds clathrin (59). Further studies gave evidence that an arrestin-dependent internalization mechanism is a predominant pathway for many GPCRs. Arretsin2 is a ubiquitous scaffolding protein and therefore is considered to be a

multifunctional protein with several new cellular functions. A recent description for a direct interaction between arrestin2 and Rab1 using proteomic analysis was revealed (110). Furthermore, arrestin was shown to regulate the cell surface expression of some GPCRs. For example, the protease-activated receptor 2 (PAR2) showed a significant reduction in the number of receptors going to the plasma membrane when expressed in an arrestin2-KO microfibroblasts cell line compared with the wild type cells (111). Another example is the effect of arrestin on granule release by CXCR1. A study have demonstrated that arrestin is required for the formation of the complex targeted for exocytosis (arrestin-Hck/Fgr complex) and that expressing dominant negative forms of arrestin blocked this pathway (109). This role that arrestin is playing in the exocytic pathway does not seem to be limited to GPCRs. Arrestin was shown to be important for the translocation of a guanine nucleotide exchange factor (Ral-GDS) from ER to PM. This GEF activates Ral, a Ras GTPase that is involved in multiple cellular functions such as differentiation, cell migration and gene transcription (112). Our work would present further investigation of Rab1/arrestin2 interaction and study the role of arrestin in the transport of GPCRs from the ER to PM, suggesting the possibility that a similar mechanism with common proteins is regulating the trafficking to the cell surface.

1.11 The Project:

The main objectives of this project can be divided into two aspects:

- Study the anterograde pathway of the receptors of interest and identify the key molecules involved in the limiting step of the process (ER exit) ;
- Study the assembly of the AT1R-β2AR heterodimer and the signalling complexes it is capable to form with different trimeric G proteins and effectors.

1.11.A New Ideas:

While the process of GPCRs internalization has been well studied, many links remain missing in understanding the transport of receptors from the endoplasmic reticulum to the plasma membrane. Members of the Ras superfamily are showing key roles in this pathway and some of them are showing direct interactions with certain GPCRs (113-116). However, most of the work currently available for this mechanism explains transport beyond ER export and focuses on the later stages. Therefore, this project studies earlier steps in the trafficking, mainly ER export of GPCRs which is considered to be the rate limiting step for receptor expression. This was achieved by selecting some proteins that are beginning to show potential roles in this process and investigating how they are involved in β2-AR trafficking from the ER to PM. The two targets that were studied are Rab1 and arrestin2. Rab1 is a GTPase that was shown to be involved in β2-AR expression without a description of how it can regulate the exocytic pathway. Arrestin2 is a scaffolding protein with a major role in the internalization machinery. It has recently shown an association with Rab1. In addition, it was shown to be important for the expression of some plasma membrane proteins.

The assembly of GPCRs signalling complex is another poorly understood process and the ability of this group of receptors to form higher oligomers has even made it more complex to study. There is currently a body of evidence suggesting that the signalling complexes of GPCRs, which are typically formed from receptors, G proteins and effectors, are assembled in the early steps of synthesis before the transport of the receptor to the PM (56, 57, 88, 90, 108, 117, 118), but this was only described for the monomeric forms of the receptors. Therefore this project explores the different forms of homo and

heterodimeric pairs of the β2-AR and AT1R in terms of their maturation and assembly and whether the molecular chaperones that were shown to control receptor monomers could have an effect on the higher oligomers.

CHAPTER 2: MATERIALS AND METHODS

2.1 Constructs

The following constructs were kindly provided by Dr. Terrence E. Hébert (McGill University) and cloned as described earlier; AT1R WT- venus1, AT1R WT- venus2, c-myc Rab1 WT, c-myc Rab1 S25N (119); β2-AR WT- venus1, β2AR WT- venus2, β2-AR-GFP_{10}, β2-AR-Rluc, β2-AR S355-356A, HA-arrestin2 WT, arrestin2 V53D, arrestin2 219-418, arrestin2 shRNA, CD4-Rluc, ACII-Rluc, Gαs-Rluc, Gαi-Rluc, Gβ-Rluc and Gγ-Rluc. Chaperone constructs (Bip WT and T37G), ERp57 and calnexin were obtained from Dr. William Green (University of Chicago). AT1R and β2-AR F(X)-$_6$LL mutants (AT1R A(X)-$_6$AA and β2-AR A(X)-$_6$AA) were obtained from Dr. Guangyu Wu (Lousiana State University, Health Sciences Center). LacZ shRNA construct was kindly provided by Dr. Kishore Pasumarthi (Dalhousie University). AT1R N4, 176, 188D-GFP was obtained from Dr. Gaétan Guillemette (Université de Sherbrooke). AT1R N4, 176, 188D venus1 and AT1R N4, 176, 188D venus2 were cloned by amplifying AT1RN4D-GFP by PCR using AT1R (N4D) FWD primer (5'-AAGCTGCTAGCATTCTCGACTCTTCTACTGAAGATGGT-3') and AT1R RVS primer (5'-GCCACCTTCGAACTCAACCTCAAAACATGGTGCAGGCTT-3'). The PCR fragment was subcloned into pcDNA3.1 vectors containing either zip-venus1 or venus2 using NheI-ClaI/BstBI. β2-AR N4, 15, 176Q was synthesized in PUC57. This construct was then amplified by PCR using β2-AR (NQ) FWD primer (5'-ATGTGCGGCCGCACCATGGGGCAACCCGGGCAGGGC-3') and β2-AR (NQ) RVS primer (5'-GCCACCATCGATCAGCAGTGAGTCATTTGT-3') and subcloned into

pcDNA3.1 vectors containing either zip-venus1 or venus2 using NotI-ClaI. All constructs were confirmed by bidirectional sequencing.

2.2 Reagents

Dulbecco's Modified Eagle's Medium high glucose and all chemicals were obtained from Sigma-Aldrich (Oakville, ON, Canada), unless noted. Fetal bovine serum and Lipofectamine 2000 transfection reagent were from Invitrogen (Etobicoke, ON, Canada). Polyclonal HA, polyclonal Rab1, monoclonal β-arrestin-1/2(A-1), polyclonal β-arrestin-1 (K-16), polyclonal GFP, c-myc, CD4 and secondary HRP antibodies (anti-mouse, anti-rabbit and anti-goat) were purchased from Santa Cruz (Santa Cruz, CA, USA). Monoclonal GFP antibody was purchased from Sigma-Aldrich (Oakville, ON, Canada). Chaperone antibodies (Bip, ERP57, calnexin, calreticulin, HSP70, PDI and GRP94 antibodies), Coelenterazine H, Coelenterazine 400a and Covance monoclonal anti-HA raw ascites were from Cedarlane Labs (Hornby, ON, Canada). Phorbol 12-myristate 13-acetate (PMA) and H89 were purchased from Enzo Life Sciences (Burlington, Ontario, Canada). EZ-link-Biotin, streptavidin beads, complete EDTA-free protease inhibitor cocktail tablets were purchased from Roche Molecular Biochemicals. Calreticulin and ERp57 inhibitory RNAs were obtained from Applied BioSystems (Carlsbad, California, USA). HSP70 inhibitory RNA was purchased from Santa Cruz (Santa Cruz, CA, USA).

2.3 Cell Lines

Two cell lines were used in this project; Human Embryonic Kidney Cells (HEK293A) (purchased from ATCC) and Mouse Embryonic Fibroblasts (MEFs) (kind gift from Dr. Robert J. Lefkowitz, Duke University).

HEK293A cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 2% penicillin streptomycin (PS). They were passed in 100 mm cell culture plates when they were 80-90% confluent. All experiments were performed on 75-80% confluent plates with cells of a passage of 25 or less.

MEFs were maintained in DMEM supplemented with 15% heat inactivated FBS, they were subcultured in 100 mm cell culture plates when they were 80-90% confluent and 0.05% trypsin-EDTA was used to detach them. Experiments were performed on 75-80% confluent plates with cells of a passage of 15 or less.

Co-immunoprecipitations and cell lyses were performed in 100 mm plates while BRET and cell surface assays were performed in 6-well plates.

2.4 Transfections

Cells were transfected using Lipofectamine 2000 transfecting reagent as described by the manufacturer. For transfecting cells in 100 mm plates, 4 μg of DNA was diluted in 250 μl DMEM, 10 μl of Lipofectamine 2000 was diluted in 250 μl DMEM and then DNA was mixed with Lipofecatmine 2000 and incubated for 20 minutes before the total mix (500 μl) was added to the cells. The same method was used for transfecting cells in 6-well plates with 1 μg of DNA in 100 μl DMEM and 6 μl of Lipofectamine in 100 μl

DMEM. Transfection efficiency was tested and 40% was achieved. Experiments were carried out 48 hours after transfection.

2.5 Cell Lysis and Co-Immunoprecipitations

Cells were harvested after 48 hours of transfection and were washed with PBS. 0.8 ml of RIPA (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, complete EDTA-free protease inhibitors and DNase I) was used for cell lysis. Samples were pre-cleared by incubation with 35 µl protein- A sepharose beads for 30 minutes at 4°C. The lysate was then clarified by centrifugation at 13000 RPM for 10 minutes at 4°C. Samples were then incubated with the appropriate primary antibody for 30 minutes at 4°C. The protein-antibody complex was then precipitated by adding 50 µl protein- A sepharose beads and leaving it overnight to precipitate the desired protein. Samples were then washed vigorously with RIPA and proteins were eluted with 50 µl SDS sample buffer that contains β-mercaptoethanol. For cell lysis, 200 µl of RIPA was used to lyse the cells and proteins were eluted directly after the centrifugation step with 200 µl SDS sample buffer. Samples were run on SDS-PAGE and western blots were then performed with the appropriate antibodies. **Figure 2.1** illustrates the steps of co-IPs.

2.6 GST and His Pull-Downs

β2-AR c-tail constructs were cloned into pGEX-4T1 vector, then transformed using BL21(DE3)pLysS and left to grow overnight in 20 ml TB medium. In the next day, 10 ml of the grown medium was diluted into 90 ml TB medium and left in a 37°C shaker for 3 hours then 1 mM IPTG was added to medium and incubated for another 3 hours.

IPTG induces protein synthesis by inducing the expression of Ptac promoter (the promoter for GST-fusion protein transcription). Medium with growing bacteria was centrifuged, the pellet was lysed with lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole, lysozyme, protease inhibitors) and left to dissolve at 4°C. Another centrifugation was performed at 12000 RPM for 20 mins and the supernatant was harvested and incubated with glutathione beads for an hour. A series of centrifugations and washes of the beads was performed using wash buffer (50mM NaH_2PO_4, 300mM NaCl and 20mM imidazole). His pull-downs were performed in the same way except that Ni-NTA-sepharose beads were used to bind proteins. Proteins quantification was performed by running samples on 10% SDS gel and then staining the gel with Coomassie Blue Dye. For detecting protein-protein interactions, pure His-Rab1 was obtained by elution buffer (50mM NaH_2PO_4, 300mM NaCl and 250mM imidazole) then incubated with GST-β2AR fusion protein overnight. Samples were then run on SDS-gel and western blot analysis was performed. In histidine pull-downs, HEK293 cells were transfected with arrestin2, cells were lysed as previously described and lysate was incubated with His-Rab1 fusion protein. **Figure 2.2** shows the fusion protein produced upon adding IPTG and how it can be purified using glutathione coated beads.

2.7 Biotin- Labelling Cell Surface Assay

HEK293 cells were co-transfected with the indicated constructs for each experiment and 48 hours after transfection, cells were washed with PBS and incubated with EZ-link Sulfo-NHS-LC-Biotin for 30 min. The cells were then washed with 100 mM glycine in PBS and lysed in 0.25 ml of RIPA buffer. The lysate was solubilized as described earlier in the immunoprecipitation steps. The pre-cleared lysate was then

incubated with 80 µl of streptavidin agarose resin overnight. The samples were then washed rapidly with RIPA buffer and biotin-labelled proteins were eluted with 80 µl DTT in SDS sample buffer then separated on SDS-PAGE. Western blots were performed using antibodies against the surface protein to be tested. **Figure 2.3** demonstrates the protocol of the biotin-labelling cell surface assay.

2.8 ELISA- like Cell-Surface Expression Assay

HEK293 cells were co-transfected with HA-β2-AR and/or HA-arrestin2 (WT or mutated V53D, or 219-418) or Arrestin2 shRNA target set. 1 µg of each cDNA was transfected into each well of a 6-well plate, and total DNA/dish was kept constant by adding pcDNA vector as required. 48 hours after transfection, cells were washed with PBS and fixed with 3.7% formaldehyde in TBS for 5 minutes. After 3 washes with TBS, cells were incubated for 45 minutes in TBS + 1% BSA, and then for 1 hour in TBS + 1% BSA + relevant primary antibody. Cells were gently washed twice with TBS, blocked again in TBS +1% BSA for 15 min, and then incubated with TBS + 1% BSA + the relevant fluorescent secondary antibody (ALEXA 488) for 1 hour. Cells were washed again twice with TBS and suspended in 100 µl TBS. The assay was then read on a plate reader (Perkin Elmer Envision) using 525/35 nm band pass filters.

2.9 Western Analysis

Most of the experiments; co-IPs, cell lysis, biotin-labelling cell surface assay and GST pull-downs, were followed by western blots for the analysis of the samples. 30-35 µl of the samples from the different assays which is equivalent to about 25 µg of proteins was diluted in β-mercaptoethanol containing laemmli loading buffer (or DTT containing

buffer for biotin-labelling cell surface assay) and then applied to 10% SDS-PAGE (30% acrylamide mix, 1.5M tris-HCl, 20% SDS, 10% ammonium persulfate and TEMED). Separated proteins were then transferred to nitrocellulose membranes and membranes were then blocked in 5% milk in PBS for 1 hour. Membranes were then blotted overnight by incubation with the appropriate antibody at 4°C. In the next day, membranes were washed at least three times with 1X PBS+0.05% tween 20, and then incubated with a Horseradish Peroxidase secondary antibody for 1 hour. Membranes were finally washed again with 1X PBS+0.05% tween 20 three times before being developed using a Kodak chemiluminescence system.

2.10 Bimolecular Fluorescence Complementation (BiFC)

BiFC is used to study protein-protein interactions and can be performed using specific proteins that have the ability to fluoresce. The most commonly used is YFP (yellow fluorescent protein). The idea is to cut a fluorescent protein into two non-fluorescent fragments and use each half to label the two proteins to be studied. The protein can't fluoresce unless the two halves (N fragment and C fragment) are reconstituted. The fluorescent protein used for this study is called Venus and is a variant of YFP. Venus 1 is the fragment that has the N-terminal (the first 157 amino acids of the fluorescent protein) while Venus 2 has the C-terminal (amino acids 158 to 238). In BiFC experiments, HEK293 cells were co-transfected with Venus 1 and Venus 2 tagged receptors (1 µg of each cDNA was transfected into each well of a 6-well plate, and total DNA/dish was kept constant by adding pcDNA vector as required). Twenty-four hours after transfection cells were harvested and washed once with phosphate-buffered saline (PBS). The cells were then suspended in PBS and distributed into 96-well microplates

(white Optiplate; Perkin-Elmer Life and Analytical Sciences). Cells were examined with a fluorescence microscope using 525/35 nm band pass filters optimized for detection of the BiFC signal of the co-expressed intact fluorescent protein and images were collected. The ratio of the fluorescence level due to BiFC and that due to the intact fluorescent protein was calculated for each well.

2.11 Fluorescence Microscopy

Twenty four hours post-transfection, HEK293 cells were harvested and seeded on laminin-coated cover slips for 4 hours at 37 °C. The cells were then fixed for 20 min. in PBS, pH 7.4, containing 3% (w/v) paraformaldehyde. The cover slips were washed with PBS, drained, and mounted onto glass slides using a drop of 0.4% 1,4-diazabicyclo{2.2.2}octane/glycerol medium. Cover slips were fixed to the slides with nail polish. Fluorescence microscopy was performed with an Olympus IX81 equipped with a Photometrics coolSNAP HQ2 camera and excite series 120Q light source. YFP (venus) was excited at 488 nm, and image acquisition was done at fluorescence emission 525 nm.

2.12 Bioluminescence Resonance Energy Transfer (BRET)

Cells were co-transfected with the indicated proteins. The different G-protein subunits (Gαs, Gαi, Gβ1 and Gγ2) were tagged with Renilla luciferase (Rluc) and used as the energy donor. The receptor dimers were used as energy acceptor, receptor A was tagged with venus1 and receptor B was tagged with venus2. The β2-AR homodimer was used as a positive control (β2-AR-GFP_{10} and β2-AR-Rluc) while AT1R/β2AR-venus1 and AT1R/β2AR-venus2 with $PRlucN_3$ vector were used as negative controls. 48 hours

after transfection, cells were harvested, washed twice with PBS then suspended with 100µl of PBS. 90µl of each sample was distributed into 96-well microplates (white Optiplate; PerkinElmer Life Sciences). Experiments were conducted using the BRET^1 technology using coelenterazine H at a final concentration of 5 µM or BRET^2 technology using coelenterazine 400a at a final concentration of 5 µM. Signals were collected on a Packard Fusion instrument (Perkin-Elmer Life and Analytical Sciences) using 410/80-nm (luciferase) and 515/30-nm (GFP) band pass filters for GFP constructs. Whether or not BRET occurred was determined by calculating the ratio of the light passed by the 515/30 filter (luciferase) to that passed by the 410/80 filter (GFP). This ratio is referred to as the BRET ratio. To avoid possible variations in the BRET signal resulting from fluctuation in the relative expression levels of the energy donor and acceptor, we designed transfection conditions to maintain constant GFP/Rluc expression ratios in each experimental set. BRET background was determined under conditions where resonance energy transfer between Rluc and GFP either could not or did not occur. This was accomplished by expressing Rluc or Rluc-tagged proteins either alone or together with GFP or GFP-tagged proteins, none of which interact physiologically. The background was the same regardless of which of the aforementioned individual proteins or combinations of proteins were expressed. **Figure 2.4** illustrates how both BiFC and BRET were utilized to study the interaction between three proteins, the two receptors and a G-protein. The two receptors were tagged with complementary non-fluorescent fragment of venus, while the G proteins were tagged with Rluc.

2.13 Statistical analysis

Comparison was performed using two-tailed student's t test. All measurements are represented as mean ± SEM. *, **, *** indicate p values less than 0.05, 0.01, 0.001 respectively and all considered to be significant.

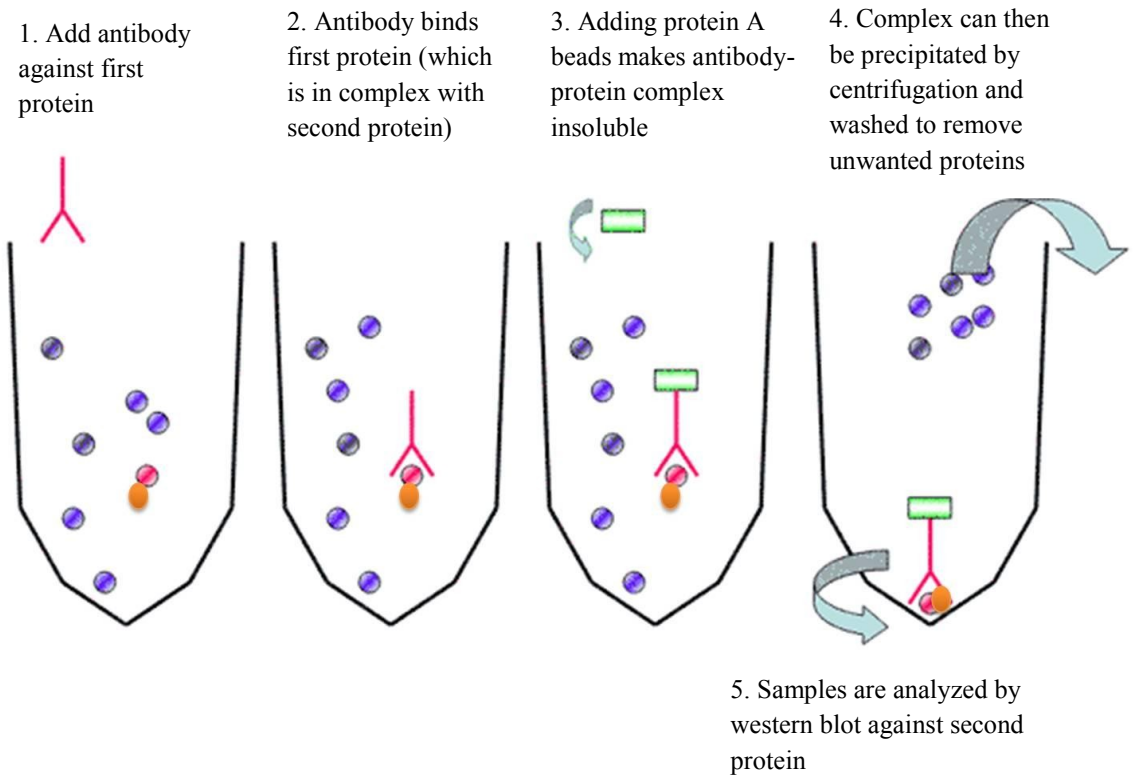


Figure 2.1: Co-immunoprecipitations
A schematic presentation for the procedures in co-IPs and the concept of the assay.

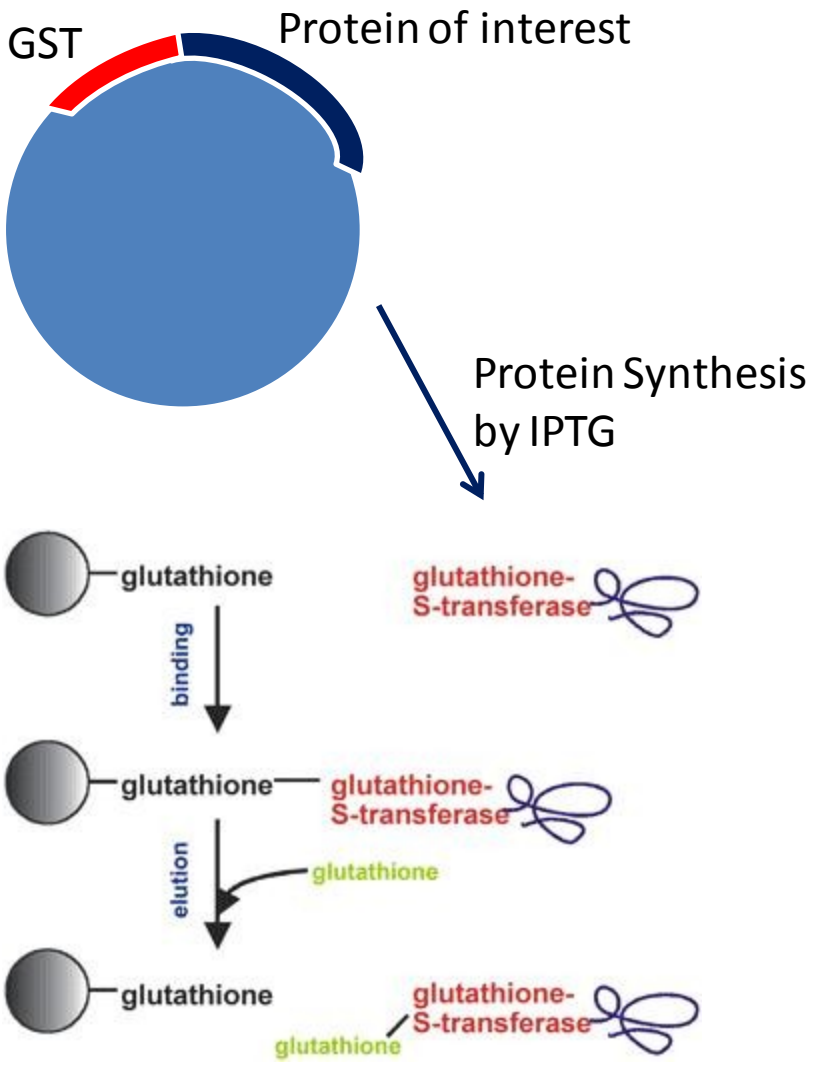
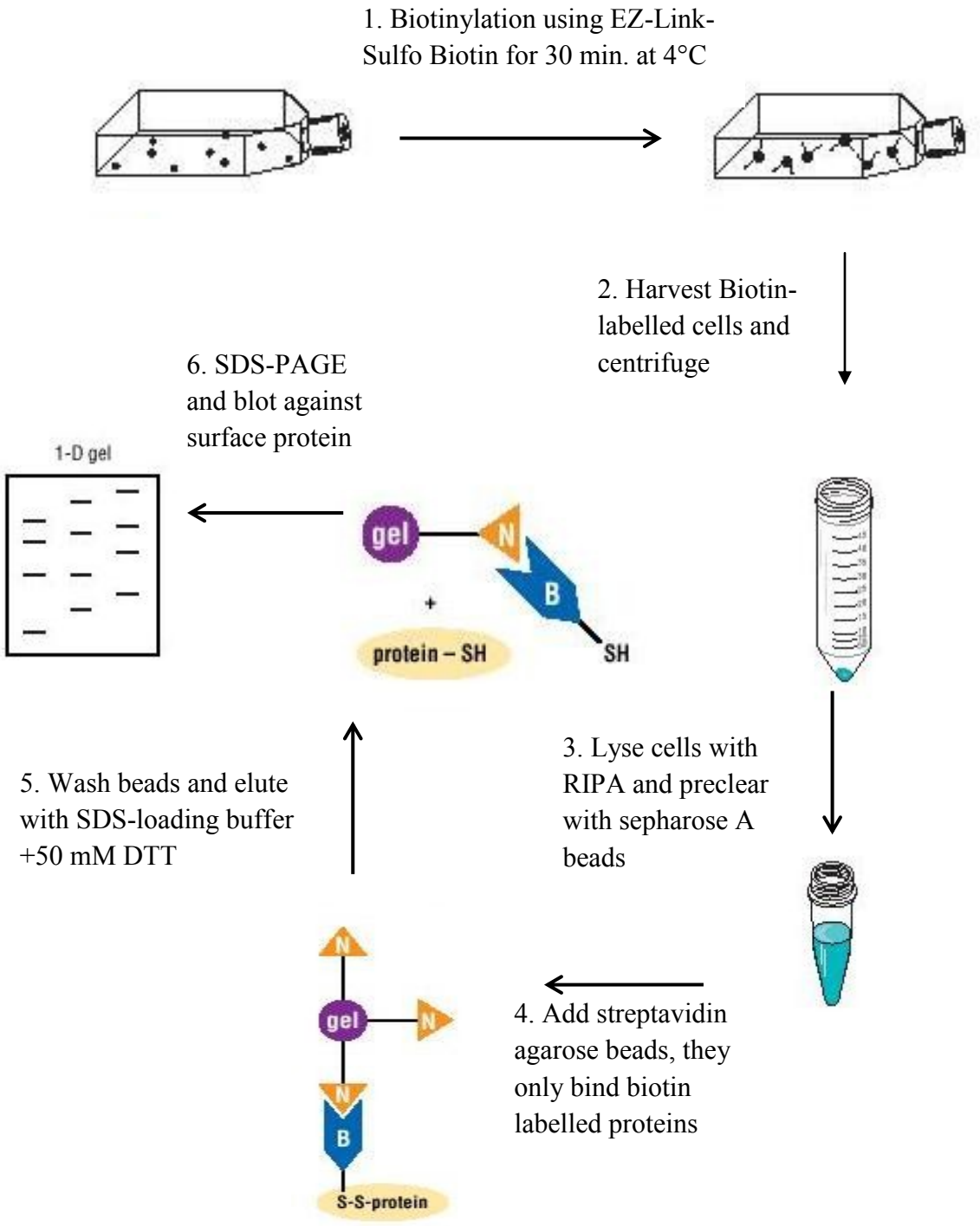


Figure 2.2: GST Pull-downs

A schematic presentation illustrating the constructs used in GST pull-downs. The GST sequence is cloned into an expression vector alongside the gene sequence encoding the protein of interest and inducing protein synthesis results in a fusion protein that can be purified by glutathione-coated beads.



2.3: Biotin labelling cell surface assay:

A schematic presentation for the procedures in biotin labelling assay and the concept of the experiment.

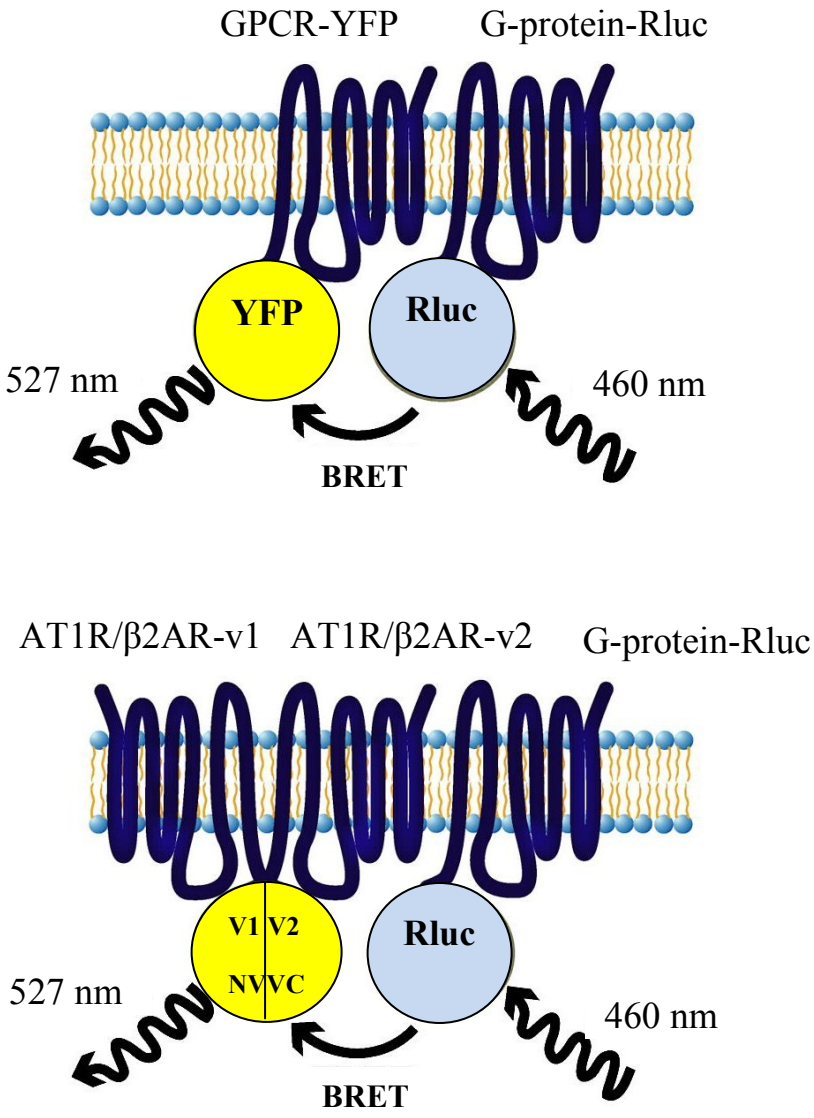


Figure 2.4: BiFC-BRET

Each receptor is tagged with a non-fluorescent fragment of venus, G- Proteins are tagged with Rluc. When Rluc is excited with energy of 460 nm wavelength, energy would be transferred to the reconstituted venus protein; emission energy of Rluc can excite venus which would in turn emit energy that can be measured with a plate reader at 527 nm. This figure was modified from (Vidi,P.A.*et al*.; 2008).

CHAPTER 3: RESULTS

3.1 The Trafficking of β2-AR from the Endoplasmic Reticulum to the Plasma Membrane

The association between Rab1 and β2-AR

Previous studies showed important roles for Rab GTPases in vesicles transport between specific cellular compartments. An important example is Rab1 because of its involvement in the early ER-exit step (105, 107, 119). Although it was previously demonstrated that Rab1 regulates the export of β2-AR from the ER, no mechanism has been explained about how this is happening (117). For example, is Rab1 capable of interacting directly or does it simply regulate receptor trafficking by controlling the vesicles in which the receptors are present. Therefore our first step was testing whether there is an association between the receptor and Rab1. GST pull-downs were performed with a purified Rab1 (WT or GDP-bound S25N) and a GST-tagged construct of the β2-AR c-tail where the two proteins were incubated overnight then the samples were resolved on SDS-PAGE. Western blots were performed using anti-Rab1 antibody. The results showed an association between the wild type form of Rab1 and the β2-AR c-tail while there was no interaction detected with the GDP-bound form (Rab1 S25N) (**Figure 3.1**). To further characterize this association and identify a possible binding site, deletion mutants of the β2-AR c-tail were designed by removing specific intracellular domains of the receptor. **Figure 3.2a** shows the GST fusion constructs and illustrates the amino acid composition of the different mutants. β2-AR T3 mutant stops at the entrance of the c-tail

and contains amino acids 322 to 331, β2-AR T2.5 ends at the palmitoylation site and contains amino acids 322 to 342 , and β2-AR T2 removes half of the c-tail and contains amino acids 322 to 369. A western blot showing the expression of the different β2AR constructs is presented in **Figure 3.2b**. **Figure 3.2c** shows the results of performing GST pull-downs on the mutated constructs with purified Rab1 WT. There was a significant reduction in association between Rab1 and β2AR T3 (where almost all of the c-tail was removed) compared to the WT construct, however, an association was still observed with the other two mutants. In fact, the bands were much stronger with the β2-AR T2.5 and T2 and a possible explanation for this strong association would be that these alterations in the structure induce a conformational change to make it more susceptible to protein binding. These results suggest that the binding site for Rab1 on the c-tail of β2-adrenergic receptor is located in the domain between the T3 cut site and the palmitoylation site. This domain contains the F(X)-$_6$LL motif (where F is Phenylalanine, X indicates any amino acid residue and L is Leucine). This motif is conserved in almost all GPCRs and was shown to be important for their expression at the plasma membrane. Studies on multiple GPCRs showed that mutations in this site lead to a reduction in the amount of receptors trafficking to the cell surface (58, 120). This is suggesting that Rab1 could control receptor trafficking through this motif. To confirm the hypothesis that Rab1 binds the β2-AR c-tail at this motif, another β2-AR construct was designed where the Phenylalanine residue was replaced with an Alanine and the dileucine residues with two Valine residues. This mutant was also tagged with GST to perform GST pull-downs with purified Rab1 WT. A complete loss of the association was observed with this construct compared with β2-AR WT and β2-AR T3 **(Figure 3.3)**.

The association between Rab1 and arrestin2

Arrestins are now identified as multifunctional proteins and therefore are found to bind a wide array of cellular proteins. A global proteomic analysis was performed on β-arrestins 1 and 2 (known as arrestin2 and 3 respectively) and showed that 173 proteins are capable of interacting with arrestin2 and 266 proteins can bind arrestin3 (110). Usually, if an association was observed between two proteins where one of them is known to regulate a specific function, a hypothesis would arise about the involvement of the other protein in the same function. For example, linking β-arrestins with the internalization machinery and their role in the endocytosis pathway first arose from observing its association with clathrin and AP2 (121-123). The proteomic analysis has also suggested the involvement of β-arrestins in the anti-apoptotic response as well as cellular organization and movement because it interacted with proteins that play important roles in those functions. Arrestin2 showed an ability to associate with Rab1; therefore it could have a role in the regulation of trafficking of GPCRs to the plasma membrane. The first step was to regenerate the association between the two proteins using a different approach than mass spectrometry so co-immunoprecipitations and Histidine pull-downs were used. For co-IPs, HEK293 cells were transfected with c-myc Rab1 and HA-arrestin2, then harvested after 48 hours to perform co-IPs. An antibody against c-myc was used for precipitation and western blots were performed with poly HA antibody. **Figure 3.4a** shows a strong association between the two proteins when a wild type form of Rab1 is used and this association is significantly reduced with the dominant negative Rab GTPase (Rab1S25N). To confirm this result, Histidine pull-downs were performed where HEK293 cells were transfected with HA-arrestin2, then lysed and

incubated with purified Rab1 (WT and S25N). Similar results to the co-IPs were obtained (**Figure 3.4b**). In an attempt to search for the binding domain for Rab1 on arrestin2, co-IPs were performed with a deletion mutant (arrestin2 219- 418) that contains the last 199 amino acids of the protein. For this co-IP, an antibody that targets the c-terminal end of arrestin was used. The results in **Figure 3.4c** indicate that there was still as association with this mutant although it was weaker than that observed with arrestin WT and arrestin V53D, a dominant negative form that blocks the function of arrestin2. However, this suggests that Rab1 binds arrestin2 at the c-terminal portion of the protein.

Characterization of β2- adrenergic receptor binding to arrestin2

Arrestin2 is known for its association with GRK-phosphorylated receptors upon their activation to initiate their internalization process. However, recent studies on some GPCRs proved the ability of arrestin to bind receptors despite their activation and phosphorylation states. These studies have investigated the functions of arrestins in the signalling pathways of some GPCRs and showed results where arrestins terminate the signals of PAR1 (124) and initiate the desensitization of dopamine D1 (125). They have used phosphorylation-deficient mutants of the receptors and examined their interaction with arrestins. In addition, a study has proposed a phosphorylation-independent mechanism for the internalization of Leukotriene B4 Receptors regulated by β-arrestins (126).

Since the hypothesis implied a possible role for arrestin2 in regulating the trafficking of β2-AR, it was important to study whether arrestin2 binding to β2-AR could occur basally and if it required receptor phosphorylation. To determine this, HEK cells

were transfected with β2-AR and HA- arrestin2, co-IPs were performed against the receptor and immunoblots were performed using an anti-HA antibody. The first lane in **Figure 3.5a** shows the result of this co-IP and demonstrates an association that can occur with arrestin without activating the receptor. This observation eliminates the requirement of β2-AR stimulation for arrestin2 to bind to it. This result isn't that surprising, given that other receptors like the angiotensin type 1 receptor (AT1R) was shown to interact basally with arrestin (127). In order to determine whether phosphorylation is necessary, the cells were treated with PMA or H89 overnight, 24 hours post-tranfection. PMA (phorbol 12-myristate 13-acetate) is a potent phosphorylation activator that specifically activates PKC signalling, and H89 is a potent cell permeable phosphorylation inhibitor. The same co-IPs were then performed without β2-AR stimulation. DMSO was used as the control treatment since it was the solvent used for PMA and H89. As observed in lanes 2, 3 and 4 in **Figure 3.5a**, the binding between β2-AR and arrestin2 was not significantly altered by any of the treatments suggesting a phosphorylation- independent association. The same co-IPs were also performed with the dominant negative form of arrestin2 and similar conclusions were drawn (**Figure 3.5b**). H89 is not a very specific inhibitor and no GRK-specific inhibitor has been synthesized to date, therefore a mutant β2-AR construct was used where the Serine residues in positions 355 and 356 were altered to Alanine. These sites were shown to be important for GRK-mediated desensitization and therefore are thought to be the sites responsible for GRK phosphorylation (128). A previous study showed that using this β2-AR mutant resulted in a significant reduction in the internalization and the phosphorylation of β2-AR. The results presented here show that the association was still occurring between this construct and arrestin2 (WT and V53D)

and this suggests that either GRK phosphorylation is not important for arrestin2 recruitment, but phosphorylation by other kinases is required, or that arrestin can associate with the receptor in the non-phosphorylated state (**Figure 3.5c**).

The effects of arrestin2 on β2-AR expression at the plasma membrane

Arrestin was shown to be involved in the cell surface expression of some proteins. For example, it was shown to regulate cell surface expression of PAR2, the granule release by CXCR1 stimulation and the translocation of Ral-GDS from the cytosol to the plasma membrane (109, 111, 112). Arrestin was also shown to associate with Rab1 which was shown to be important for the trafficking of some GPCRs (105). Among these receptors was the β2-adrenergic receptor. Therefore the effects of arrestin2 on the cell surface expression of β2-AR were investigated in the present report. To do so, an ELISA-like cell surface assay was developed to allow measuring the amount of receptor going to the plasma membrane. HEK293 cells were transfected with HA-β2AR and arrestin2 (WT, V53D and 219-418). After 48 hours, cells were fixed, blocked with 1% BSA in TBS and then labelled with mono-HA antibody. After an incubation period for 1 hour, cells were washed and labelled with a conjugated fluorescent anti- mouse antibody (ALEXA 488). The fluorescence of the cells was then measured using a plate reader at 527 nm. Since the receptor was tagged on the N-terminal end with a hemagglutinin molecule, using an antibody against HA ensures picking up the signal only from the receptors that are expressed at the plasma membrane and therefore, the fluorescence counts reflect the amount of receptor at the cell surface. The fluorescence levels that were obtained from transfecting the wild type forms of arrestin2 were normalized to 100% and were used as a reference to compare the expression levels from using mutated arrestin

constructs (**Figure 3.6a**) or shRNA against arrestin (**Figure 3.6b**). The results showed a significant reduction in the plasma membrane expression of β2-AR when arrestin2 expression was altered. When a dominant negative form of arrestin2 was used (arrestin2 V53D), a reduction of 55% ± 12 was observed. This construct is a functionally inactive form of arrestin. When using a deletion mutant of arrestin2 that contains the 199 amino acids closest to the c-terminal end of the protein (arretsin2 219- 418), a significant reduction in receptor levels by 42% ± 8 was also observed. To confirm this effect that arrestin2 seems to be having, arrestin2 shRNA was also used to silence the expression of the gene. Transfecting the cells with an individual shRNA has lead to 30% ± 8 reduction in β2-AR levels and transfections with a set of 5 shRNAs resulted in 26% ± 8 reduction. In order to confirm that arrestin2 shRNA was specifically blocking arrestin2, LacZ shRNA was used as a control with the wild type form of arrestin2 and similar levels of fluorescence were detected (**Figure 3.6b**). The results indicate that arrestin2 can play an important role in β2AR expression. However, another approach was necessary at this point. Therefore, the expression of β2AR was studied in a cell line of arrestin2 knockout mouse embryonic fibroblasts. First a biotin-labelling assay was performed with a wild type cell line versus the arrestin2 knockout cells and a significant reduction in the amount of receptors expressed at the plasma membrane was detected (**Figure 3.7a**). Another way to show this effect was transfecting the cells with β2-AR-GFP and preparing fluorescent images to visualize the expression of the receptor. The wild type cells were expressing most of the receptors at the plasma membrane (**Figure 3.7b**) while with the arrrestin2 knockout cells, a great proportion of receptors was retained inside the cells (**Figure 3.7c)**.

This confirms the importance of arrestin2 for the expression of β2-AR at the plasma membrane.

Arrestin2/Rab1 complex regulation of β2-AR exit from ER

The previous results have shown the involvement of arrestin2 in the expression of β2-AR at the plasma membrane and suggested that its activity might contribute in the ER-exit process of the receptor. Xiao *et al.* showed an interaction of arrestin2 with Rab1, and Rab1 was previously shown to control the trafficking of β2-AR. Therefore, the next hypothesis was that a complex (Arrestin2/Rab1) regulates the trafficking. To further investigate this, a sequestration assay was developed to identify the mechanism by which the two proteins perform their actions. In this assay, HEK293 cells were transfected with β2-AR and arrestin2 (WT or V53D) with increasing amounts of transfected Rab1 (WT or S25N). 48 hours post-transfection, cells were harvested and washed with PBS, then co-immunoprecipitation was performed against β2-AR and immunoblots were performed against HA-arrestin2. **Figure 3.8a** shows that increasing concentrations of Rab1WT in the presence of arrestin2 WT didn't affect the association between arrestin2 and β2-AR. **Figure 3.8b** shows that using arrestin2 V53D with increasing levels of Rab1 WT didn't affect the association as well. **Figure 3.8c** shows that when arrestin2 V53D is expressed, the association between β2-AR and Rab1 is higher than when arrestin2 WT is expressed. A tentative explanation for those results could be that the wild type form of arrestin2 facilitates the trafficking, so the receptor can detach from Rab1 and then further move along the exocytic pathway. While when arrestin2 V53D is expressed, the receptor is retained in the ER and the interaction between Rab1 and β2-AR is prolonged. **Figures 3.9a** and **3.9b** show that increasing the concentration of Rab1 S25N does not affect the

association between β2-AR and the wild type form of arrestin2 as well as the dominant negative form. The quantitative analysis of this (**Figure 3.9c**) shows that there is no difference in the association of β2-AR with arrestin2 WT versus arrestin2 V53D. This is probably because the dominant negative form of Rab1 binds both forms of arrestin2 equally (**Figure 3.4c**).

Effect of GPCRs, arrestin2 and phosphorylation on CD4 expression

The previous results have clearly showed that arrestin2 is involved in the expression of β2-AR at the plasma membrane; therefore it was interesting to investigate whether this phenomenon is specific to GPCRs or would be true for other plasma membrane proteins that traffic from the ER to the surface of the cell. It was important to select a plasma membrane protein that does not interact with β2-AR to ensure that any results obtained won't be the effect of such an association. Therefore CD4, a glycoprotein that acts as a co-receptor for T cell receptors, was selected. The first step was to ensure that CD4 does not interact with β2-AR. To do so, BRET assay was used where HEK cells were transfected with CD4-Rluc and β2AR-GFP_{10}. The BRET ratio obtained indicated that there was no interaction because it was similar to the ratio obtained with the negative control that was used which was ACII-Rluc with HERG-GFP_{10} (**Figure 3.10a**). The positive control used was the β2-AR homodimer (β2AR-Rluc with β2AR-GFP_{10}) because the two proteins to be tested are plasma membrane proteins. BRET was also performed with AT1R and CD4 and no interaction was detected as seen with β2-AR. This was important to confirm since the effect of both receptors on CD4 expression will be investigated. CD4 levels at the plasma membrane were measured using a biotin-labelling cell surface assay. HEK293 cells were transfected with CD4, GPCR (WT and ER-

retained mutants (β2AR-A(X)-$_6$AA) or (AT1R-A(X)-$_6$AA)) and arrestin2 (WT or DN (V53D)). Cells were then lysed with RIPA and incubated with biotin (a probe that does not have the ability to penetrate the cell membrane unless permeabilized so it only recognizes and labels the proteins on the cell surface). Streptavidin agarose beads which have a very high affinity for biotin were then added to the samples and incubated overnight. Western analysis was then performed using an antibody against CD4. The results in **Figure 3.10b** show the detected levels of CD4 on the cell surface when expressing the wild type forms of the receptor and arrestin2 (lane 3). However when arrestin2 V53D was used, a significant reduction in CD4 reaching the plasma membrane was observed (lane 4). More interestingly, when mutant forms of the GPCRs (AT1R and β2-AR) in the F(X)-$_6$LL motif were expressed (lanes 1 and 2), CD4 expression was significantly reduced as well. The histogram to the right presents a quantitative analysis of the western blot (**Figure 3.10c**). This analysis was done using Image J software. The previous results indicate that GPCRs have a role in regulating CD4 expression at the plasma membrane. The effect of phosphorylation on CD4 expression was examined next. HEK293 cells were treated with H89 and a biotin-labelling assay was used to measure CD4 levels at the plasma membrane. Inhibiting phosphorylation has led to a significant reduction in CD4 expression at the plasma membrane and this can be due to affecting the transport or removing the protein from the cell surface (**Figure 3.11**). Although it is not quite understood yet, current evidences are proposing the existence of kinase signalling cascades, other than PKA, PKC and PKD, that regulate cargo export in the early steps of transport from the ER to Golgi and link this control to Sar1 GTPase activity and COPII

recruitment. Given the fact that H89 is still not completely characterized in terms of its specificity, we expect that it could be inhibiting the kinases that affect ER export.

Overall, this part of the study on the trafficking of β2-AR from ER to PM demonstrates that Rab1 can interact directly with GPCRs at the F(X)-$_6$LL conserved motif to regulate their export from the ER to Golgi and Plasma membrane. It also shows that aresstin2 interact with GPCRs basally and is important for the expression of GPCRs at the plasma membrane as well, just like Rab1. Therefore; both Rab1 and arrestin2 might be parts of the complex responsible for the anterograde trafficking of GPCRs. In addition, our results show that GPCRs themselves can regulate the expression of other plasma membrane proteins that do not associate with receptors through an arrestin-dependent mechanism.

3.2 The Assembly of the Heterodimeric Complex AT1R-β2-AR

G-protein coupled receptors have historically been studied as monomeric entities. However, their ability to exist in higher dimeric arrangements has been well documented (42-44) and more studies are starting to focus on the dimerization property of GPCRs to understand how they form, how they signal and what effects these oligomers could have on drug use and development. This second part of the project was designed to study the heterodimer AT1R-β2-AR and characterize some of its properties.

Molecular chaperones interactions with AT1R-β2AR homo- and heterodimers

Most of the previous studies that have been investigating GPCRs examined the receptor as a monomer and therefore very little is known about receptor dimers. This part

of the project was directed toward studying oligomers of GPCRs. More specifically, we were interested in tracking the differences between homodimers and heterodimers in their immature and completely folded states. We have selected some molecular chaperones (BiP, Calnexin, Calreticulin, ERp57, HSP70, PDI and GRP94) to start our investigation because they were shown to interact with GPCRs, including AT1R or β2-AR. Molecular chaperones are important scaffolding proteins that have many important key roles in polypeptides synthesis, modifications and folding. Therefore, the first set of experiments in this part involved co-immunoprecipitations of chaperones with receptor pairs to see if there were any differences between homo- and heterodimers. In order to express the receptors in the dimer form, a venus molecule which is a yellow fluorescent protein variant was used to tag the receptors. This venus protein was cleaved into two parts; the N-terminal end which was called venus1 (or v1) and the C-terminal end which was called venus2 (or v2). Each receptor was tagged with one of the two parts (**Figure 3.12a**), when the receptors dimerize, the two ends of venus reconstitute forming the whole molecule that could be detected by a specific antibody against GFP. Since the plan was to detect the interactions between the different receptor pairs and endogenous chaperones, the function of the chaperone antibodies was tested by lysing HEK293 cells, running the samples on SDS-PAGE and blotting them against the different chaperones. **Figure 3.12b** shows that the antibodies were selectively recognizing their corresponding chaperones and that endogenous levels are adequate for detection. **Figure 3.12c** shows the specificity of this monoclonal GFP antibody to the reconstituted tag since it does not give any signal when receptors tagged with only one part of venus are transfected.

The expression of the different wild type dimers was visualized by preparing fluorescent images as shown in **Figure 3.13** and the receptors were expressed at the plasma membrane. Then the interaction between the chaperones and the wild type forms of the receptors was tested. HEK293 cells were transfected with AT1R-v1 and AT1R-v2 (to generate an AT1R homodimer) or β2AR-v1 and β2AR-v2 (to generate a β2-AR homodimer) or β2AR-v1 and AT1R-v2 (to generate an AT1R-β2AR heterodimer). Cells were harvested and washed after 48 hours of transfection and immunoprecipitations were performed with antibodies raised against the different molecular chaperones identified in **Figure 3.14**. As illustrated from the blots in the figure, there were indeed some differences in the interaction pattern and strength observed. BiP and HSP70 showed an association with the homodimers (AT1R/AT1R and β2AR/β2AR) but not with the heterodimer. ERp57 interacted with the AT1R containing pairs; the heterodimer and the AT1R homodimer. In addition, some chaperones (Calreticulin, PDI and GRP94) were interacting with all the dimers while calnexin did not interact with any.

Effects of glycosylation on chaperones associations

Some of the chaperones investigated (mainly calnexin and calreticulin) were suggested to require glycosylation to interact with their targets; therefore mutant receptor constructs were designed where the three glycosylation sites of the receptors were altered. For the β2-AR, the three N- glycosylation sites (Asn6, Asn15 and Asn187) were substituted with Glutamine while for the AT1R, the three N- glycosylation sites at positions 4, 176 and 188 were mutated to Aspartic acid. These glycosylation deficient constructs were then cloned into a pcDNA with venus1 or venus2 and the dimers they form were visualized with fluorescent images. **Figure 3.15** shows that receptors are

retained inside the cells which was expected since they are immature proteins that would be recognized as misfolded cargo. Co-IPs were then performed to examine their interaction with the chaperones. Results in **Figure 3.16** showed very similar interactions between the homodimers and the heterodimer in this glycosylation- deficient group. However, when the results were compared with the ones obtained from **Figure 3.14**, a difference in the chaperones associating with the β2-AR homodimer was noted. The interaction with BiP was lost in the β2-AR NQ construct and an interaction with ERp57 occured.

The next set of combinations used contained dimers of mixed constructs (WT with glycosylation deficient). These dimers were also shown to be expressed inside the cell as illustrated in **Figure 3.17**. The results for the co-IPs of this group of dimers with the chaperones are illustrated in **Figure 3.18**. Interestingly, a heterodimer with one glycosylation deficient receptor in the pair showed interactions with BiP and HSP70 and some traces with calnexin (**Figure 3.18 a** and **b**). Those chaperones did not show any interaction with the wild type heterodimer or when both receptors lacked their glycosylation sites. In addition, it was also observed that the interactions with these chaperones were stronger when the AT1R was the mutant receptor. The β2-AR homodimer (with a wild type receptor paired to a mutant receptor) resulted in a pattern that looked more like the glycosylation-deficient homodimer than the wild type dimer with the strong interaction with ERp57 and losing BiP interaction (**Figure 3.18d**). On the other hand, chaperones interactions with AT1R homodimer in which one receptor was in the wild type form and the other in the mutated form were different from both the wild

type homodimer and the mutant homodimer since there was no association with HSP70 **(Figure 3.18c)**.

Effect of mutation in the conserved F(X)-$_6$LL motif on chaperone interactions

We and others have demonstrated the importance of the F(X)-$_6$LL motif in the ER export of GPCRs and showed that introducing mutations to the Phenylalanine and Leucine residues leads to trapping the receptors in the endoplasmic reticulum (58, 120). Since the chaperones of interest are found in the ER and are known to facilitate the folding of newly synthesized as well as misfolded polypeptides, it was interesting to study how such ER-retained receptors would associate with the chaperones. **Figures 3.18e** and **f** show that receptor pairs that contain the AT1Rm1 (AT1R A(X)-$_6$AA) showed interactions with calnexin for the first time among the other dimers that were tested. In addition, associations with BiP and HSP70 look stronger with this mutant in the AT1R homodimer condition. These results might be suggesting that dimerization leads to masking or revealing the interaction sites for those chaperones. Another possible explanation could be related to the function of the chaperones themselves and the fact that they associate with certain mutations to correct them and complete the folding process.

Effects of chaperones on homo and heterodimer formation

Several chaperones are known to facilitate the assembly of the signalling complex of GPCRs in the early steps of biosynthesis. Since the formation of receptor dimers occur at the ER, it was interesting to study how those dimers form and what chaperones are required for their assembly. BiFC (Bimolecular fluorescence complementation) was used

to study this effect. HEK293 cells were transfected with venus1 and 2-tagged receptors along transfections with chaperones (WT, DN or shRNA), and a plate reader was used to measure the signal with emission filters adjusted to wavelength of 527 nm to meet YFP conditions. Therefore, the fluorescence signal measured is an indication for dimer formation. The results show that ERp57 is important for the formation of the AT1R homodimer and the heterodimer (AT1R-β2AR) (**Figure 3.19**) since using siRNA against ERp57 led to a significant reduction in the signal measured. However ERp57 did not affect the assembly of the β2-AR homodimer. HSP70 and Bip on the other hand did not affect the formation of any receptor dimers.

Effects of chaperones on G-protein coupling to different receptor pairs

In the last set of experiments, the effects of chaperones on the coupling of G-protein subunits to the receptors were investigated. BiFC/BRET[1] was performed where G- protein subunits with Rluc tags were used as energy donors and venus-tagged receptors as energy acceptors. The interaction between the G proteins (Gαs, Gαi, Gβ and Gγ) and all receptor dimers in their wild type form (AT1R homodimer, β2-AR homodimer and AT1R/β2-AR) was examined in the presence of wild type forms, dominant negative forms or siRNA of the chaperones BiP, calreticulin and ERp57. The β2-AR homodimer was used as a positive control (β2AR-Rluc and β2AR-EGFP) and the different receptor pairs with the appropriate venus tags with an empty pRlucN3 vector as negative controls. All the selected chaperones did not have significant effects on the interaction of any of the different G- protein subunits with the heterodimer (**Figure 3.20**), the AT1R homodimer (**Figure 3.21**) and the β2AR homodimer (**Figure 3.22**). These

results indicate that this group of chaperones is not required for the formation of this part of the signalling complex.

Overall, our results on the effects of chaperones on GPCRs assembly suggest that there are indeed differences among receptor oligomers that occur early during biosynthesis and folding. Our data also illustrate that the immature receptor in a pair composed of wild type-misfolded receptors dictates its association with chaperones. In addition, the results indicate that ERp57 is important for the formation of AT1R-containing dimers (AT1R homodimer and AT1R-β2AR heterodimer), but not for the β2-AR homodimer formation. The effect of chaperones on the assembly of the signalling complexes was also investigated but the chaperones examined did not seem to be important for the formation of receptor- G proteins complex.

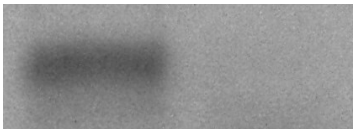


Figure 3.1: Rab1 interacts with the c-tail of β2-AR
GST pull-downs of wild type β2-AR c-tail with purified Rab1 (WT or S25N) incubated for 24 hours and run on 10% SDS-PAGE, and then blotted with an antibody against Rab1. Results are representative of 3 independent experiments.

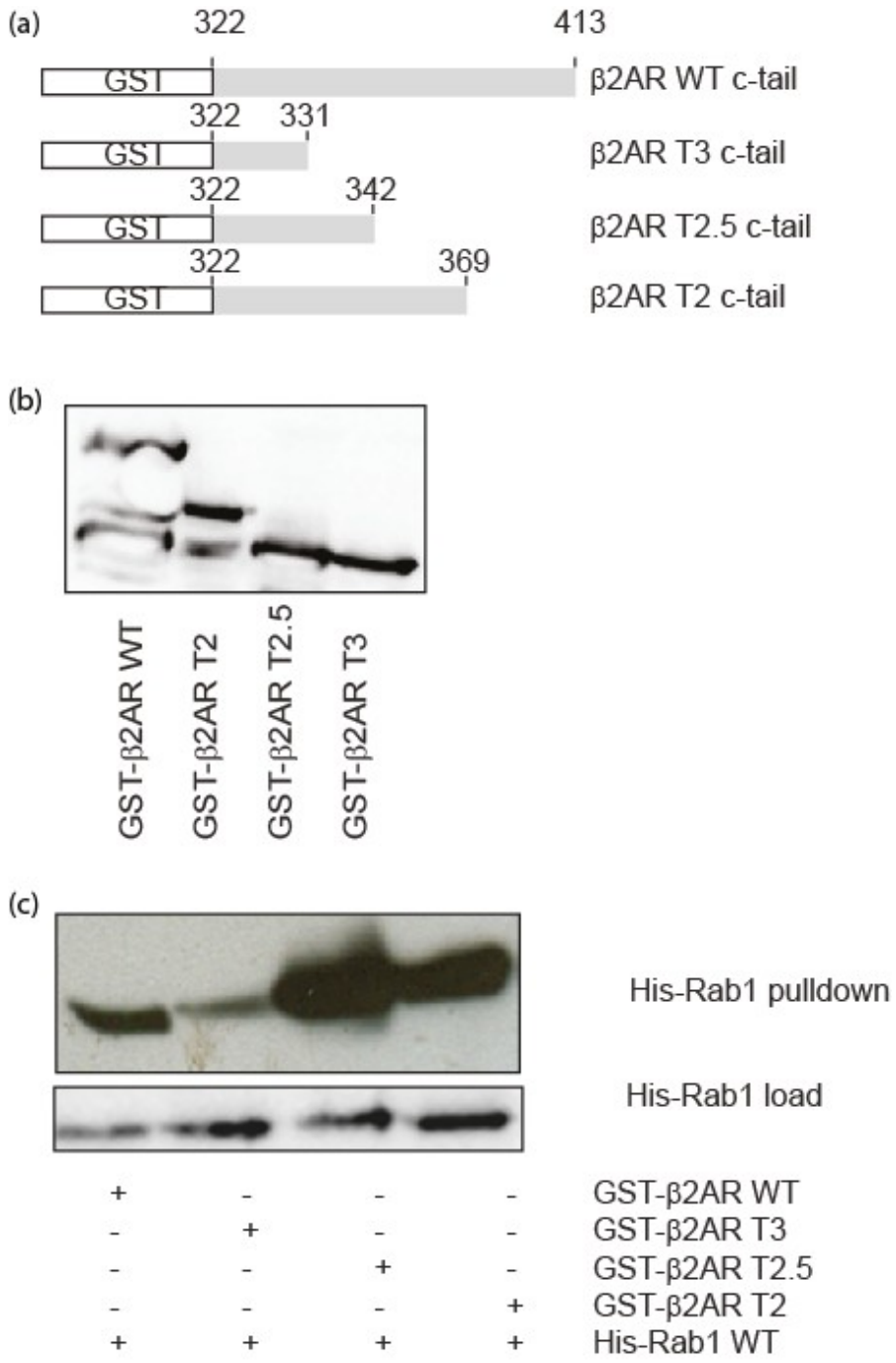


Figure 3.2: Characterization of β2-AR domain responsible for Rab1 interaction

a) Schematic representation of the different β2-AR mutant constructions used and their amino acid sequence; β2-AR T2, β2-AR T2.5 and β2-AR T3. b) A western blot showing the expression levels of the different β2-AR constructs used. c) GST pull-downs of β2-AR wt or truncated constructs with purified Rab1 WT, western blot was performed against Rab1 antibody. Rab1 load is illustrated as a positive control. Results are representative of 3 independent experiments.

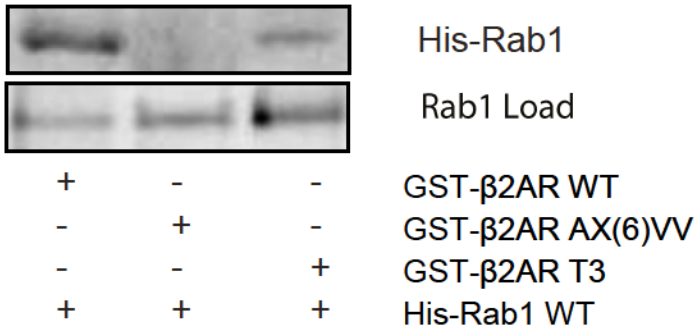


Figure 3.3: Rab1 interacts with β2-AR at the conserved F(X)-$_6$LL motif
GST pull-downs of β2-AR (WT, A(X)-$_6$VV or T3) and purified Rab1 WT, western blot was blotted with Rab1 antibody. Rab1 load is illustrated as a positive control. Results are representative of 3 independent experiments.

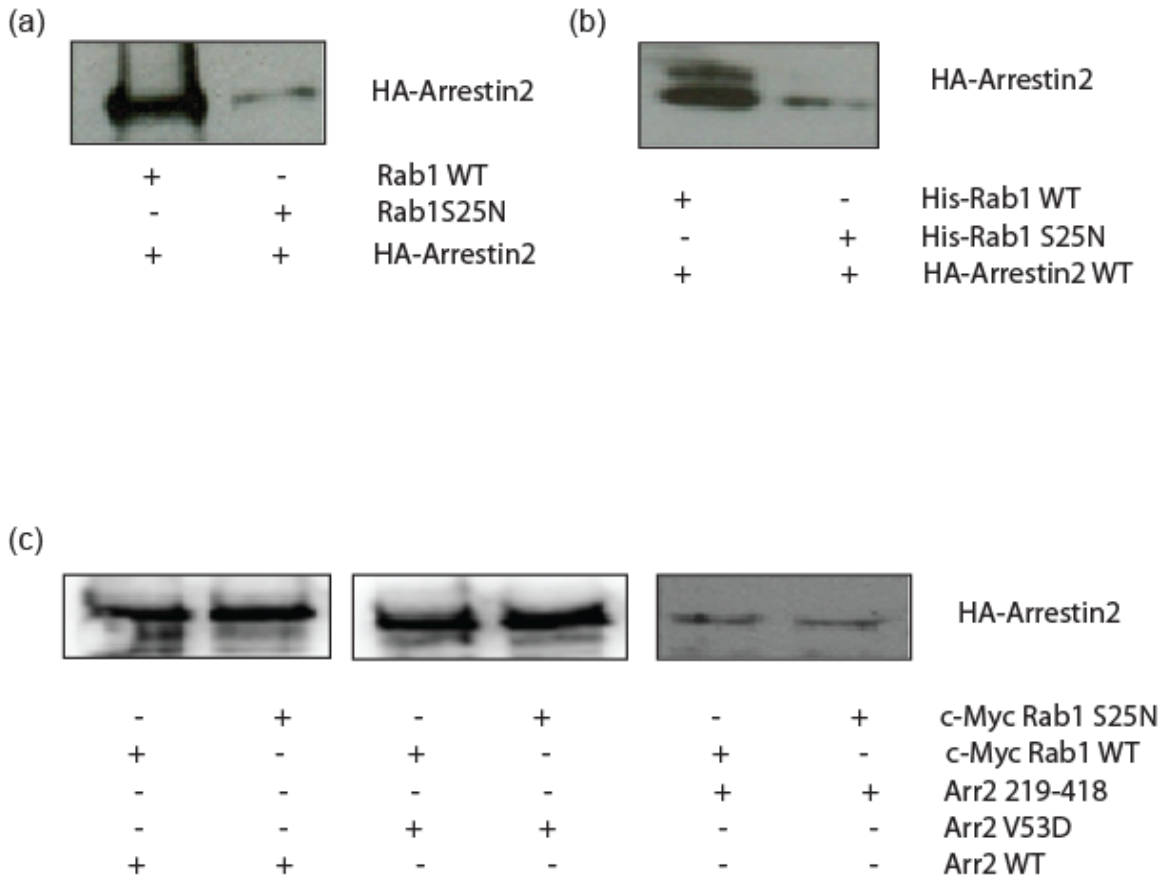


Figure 3.4: Rab1 interacts with arrestin2 at the last 199 amino acids

a) HEK293 cells were co-transfected with HA-arrestin2 and c-myc-Rab1, co-IPs were performed using an antibody against c-myc and immunobloting was performed using poly HA antibody. b) Histidine pull-downs were performed to confirm the results. HA-arrestin was transfected in HEK293 cells, cells were then lysed and incubated with His tagged Rab1 and an immunoblot was performed using poly HA antibody. c) HEK293 cells were transfected with the indicated forms of arrestin2 and Rab1, co-IPs were performed with c-myc antibody and immunoblots were performed with arrestin2 antibody. Results are representative of 3 independent experiments.

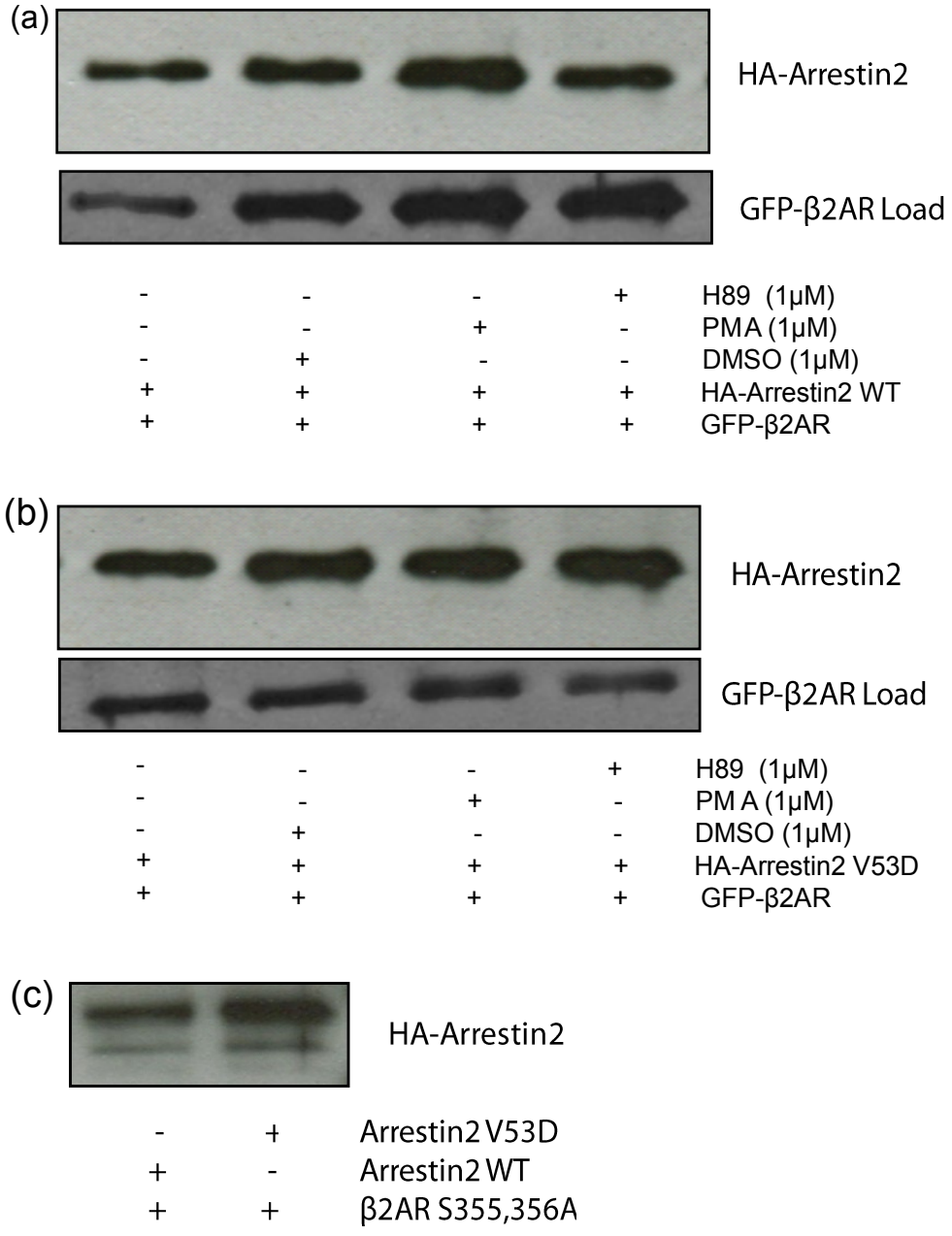


Figure 3.5: Arrestin2 interacts with β2-AR basally despite phosphorylation state
HEK293 cells were co-transfected with GFP-β2AR and arrestin2. 24 hours post transfection, they were treated with 1 μM DMSO, PMA or H89 overnight. Co-IPs were performed with mono GFP and immunoblots were performed against poly HA. a) Arrestin2 WT. b) Arrestin2 V53D. c) No treatments were performed in this case, a receptor that should not undergo GRK phosphorylation was used instead. Results are representative of 4 independent experiments.

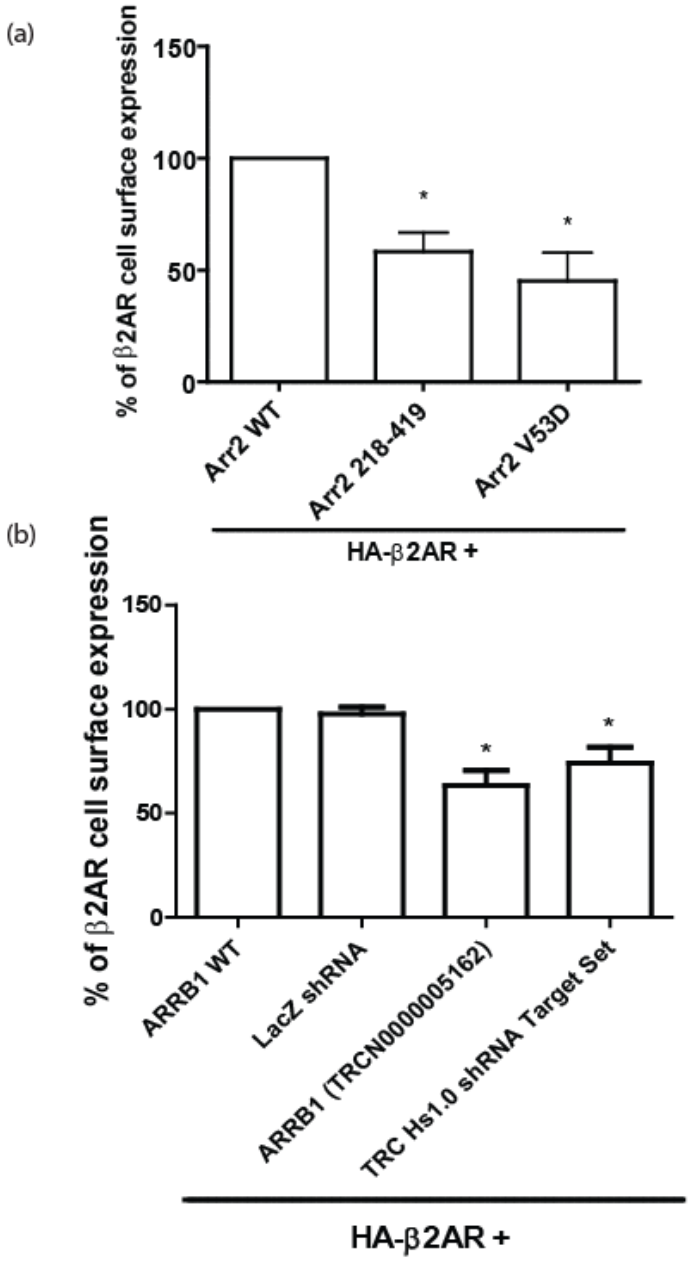


Figure 3.6: The effect of Arrestin2 on β2-AR expression at the plasma membrane
HEK293 cells were transfected with the indicated constructs; cell- surface assays were performed where β2-AR was labelled with an antibody against HA followed by an Alexa- Fluor 488 secondary antibody. The fluorescence measured indicated the levels of expression of the β2-AR at the plasma membrane. The assay was then read on a plate reader (Perkin Elmer Envision) at 527 nm. * indicates that $p < 0.05$ using two-tailed student's t test. a) Arrestin2 V53D, 219-418. b) Individual arrestin2 shRNA (TRCN0000005162) and a group of multiple arrestin2 shRNA. Results are representative of 5 independent experiments.

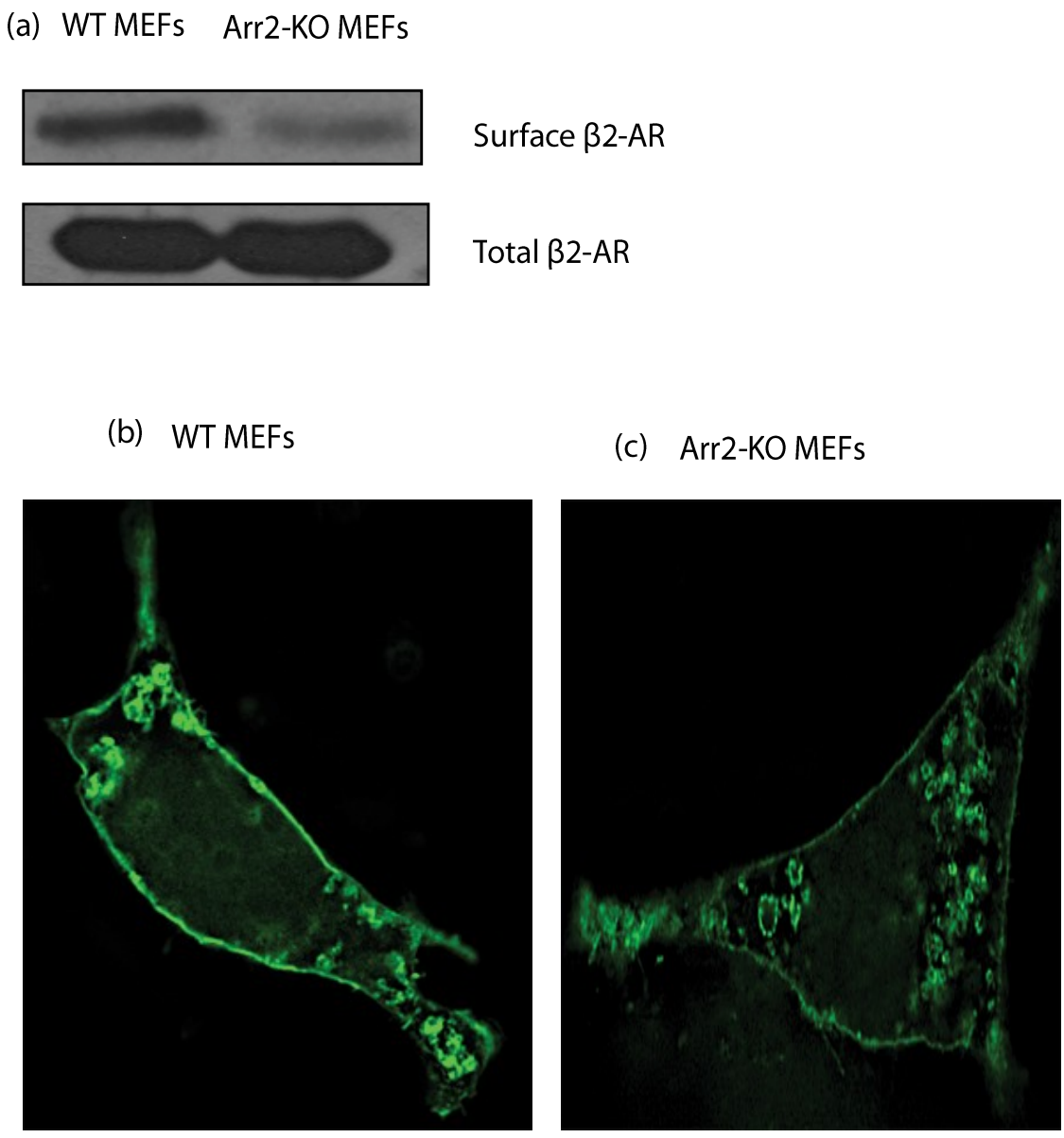


Figure 3.7: The expression of β2-AR in MEFs

a) Biotin-labelling cell surface assay of mouse embryonic fibroblasts cells. Cells were incubated with biotin, lysed, and then samples were incubated with streptavidin agarose resin. Immunoblots were performed using a β2-AR antibody. Lysate was loaded as a control to show total levels of endogenous β2-AR. b) Expression levels of β2AR-GFP in wild type MEFs and c) arrestin2 knockout MEFs. Results are representative of 3 independent experiments.

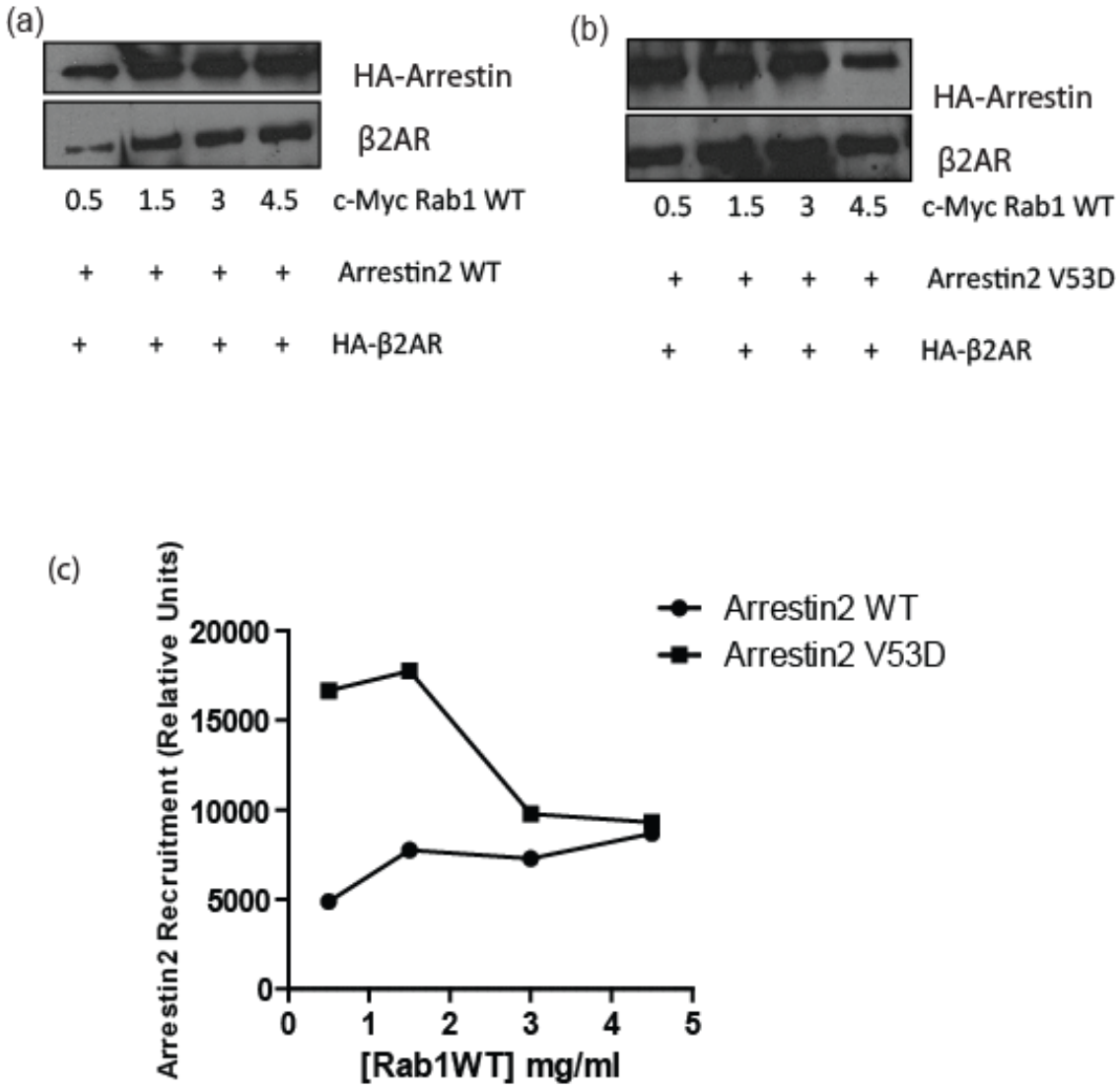


Figure 3.8: The effect of arrestin2 on Rab1 association with β2-AR

Cells were harvested with PBS after 48 hours of transfection, and lysed with RIPA. Co-IPs were performed against β2-AR. Immunoblots were performed using anti-HA antibody (upper panels) or anti-β2-AR antibody (lower panels) as a control. a) Cells were co-transfected with β2-AR and HA-arrestin2 WT and increased concentrations of Rab1 WT. b) Cells were co-transfected with β2-AR and HA-arrestin2 V53D and increased concentrations of Rab1 WT. c) A quantitative histogram for immunoblots (a) and (b). Results are representative of 3 independent experiments.

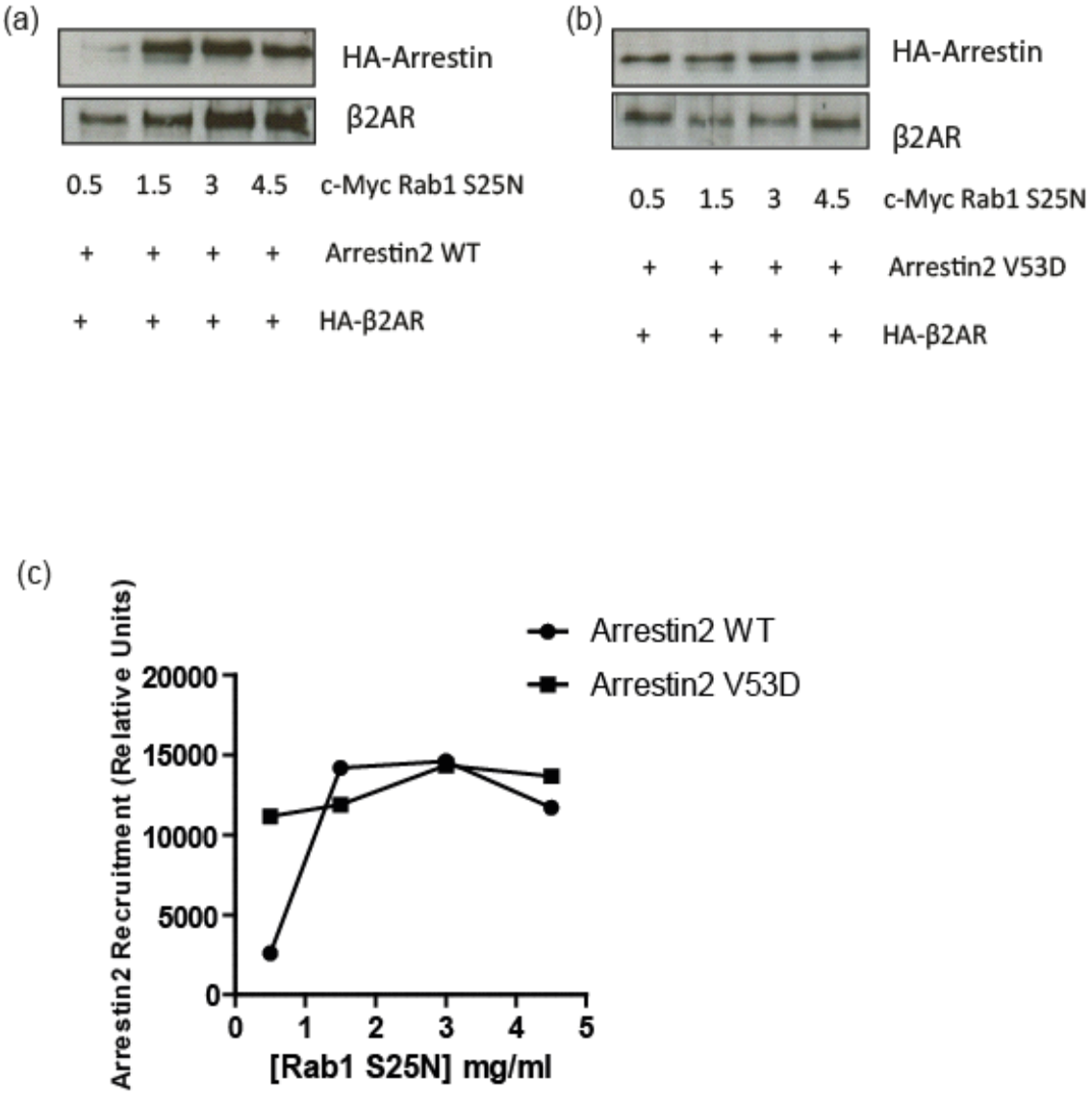


Figure 3.9: Rab1 S25N can interact with arrestin2 WT and V53D

Cells were harvested with PBS after 48 hours of transfection, and lysed with RIPA. Samples were immunoprecipitated against β2-AR and were run on SDS gels. Immunoblots were performed using anti-HA antibody (upper panels) or anti-β2-AR antibody (lower panels) as a control. a) Cells were co-transfected with β2-AR and HA-arrestin2 WT and increased concentrations of Rab1 S25N. b) Cells were co-transfected with β2-AR and HA-arrestin2 V53D and increased concentrations of Rab1 S25N. c) A quantitative histogram for immunoblots (a) and (b). Results are representative of 3 independent experiments.

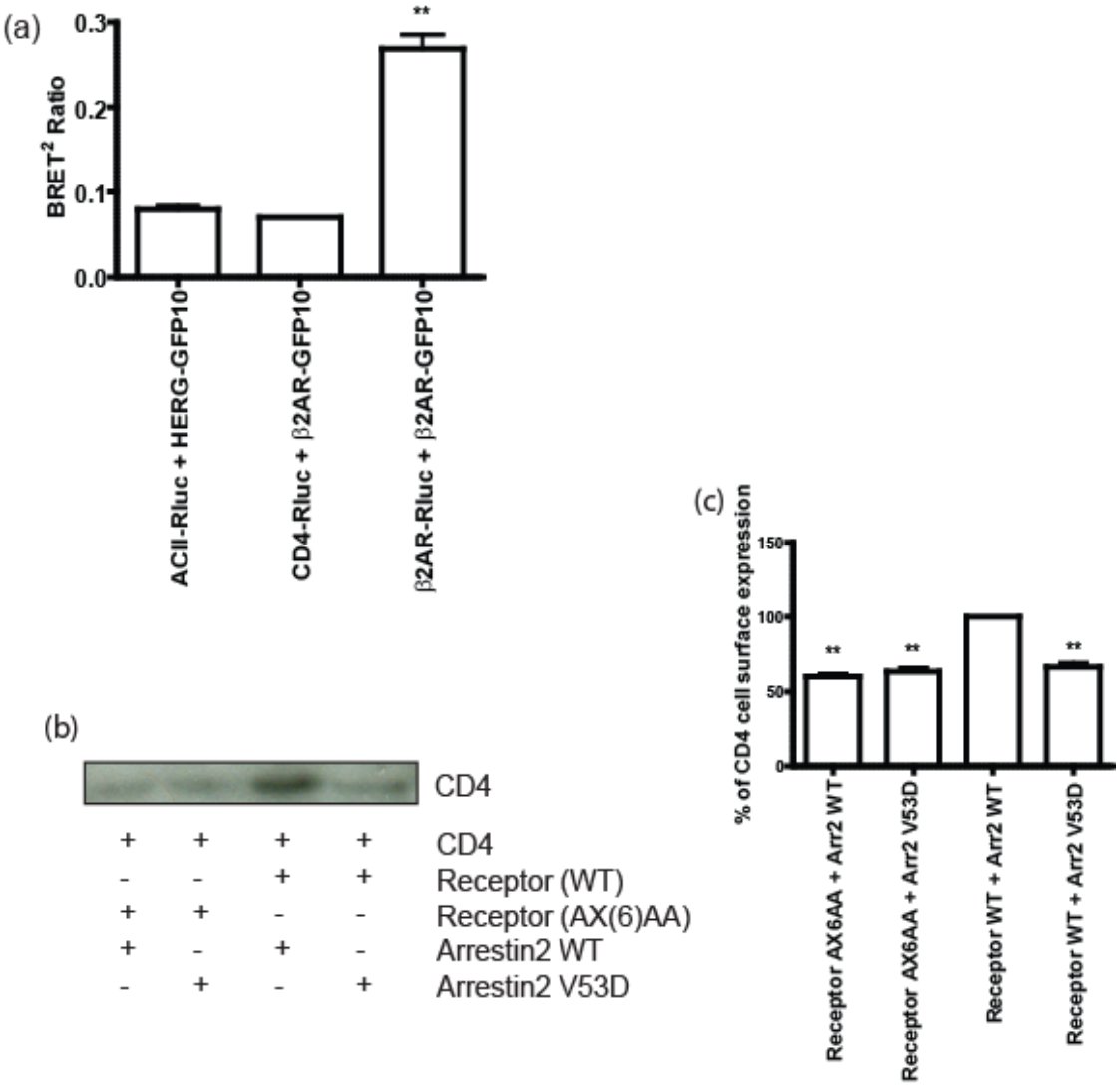


Figure 3.10: Regulation of CD4 expression at plasma membrane by GPCRs

a) BRET2 results from co-transfecting HEK 293A cells with CD4-Rluc and β2-AR-GFP_{10}. The first column is the negative control and the third column is the positive control. ** indicates that $p < 0.01$ using two-tailed paired student's t test. b) Biotin labelling assay for CD4 expression. The first two lanes show CD4 levels when the ER-retained form of a GPCR (A(X)-$_6$AA) is expressed compared to lane 3 where the receptor is in the WT form. The last lane shows the PM expression of CD4 when the wild type form of the receptor is used with arrestin2 V53D. c) A quantitative presentation for the immunoblot. ** indicates that $p < 0.01$ using two-tailed paired student's t test. Results are representative of 3 independent experiments.

Figure 3.11: The effect of phosphorylation on CD4 expression at the plasma membrane

Cells were transfected with the indicated constructs. Cells were then incubated with biotin, lysed, and then samples were incubated with streptavidin agarose resin. Immunoblots were performed using anti-CD4 antibody. The first two lanes show the effect of arrestin2 V53D on CD4 expression at the PM. The last two lanes show the effect of the phosphorylation inhibitor H89 on CD4 expression in the presence of arrestin2 (WT or V53D). Results are representative of 3 independent experiments.

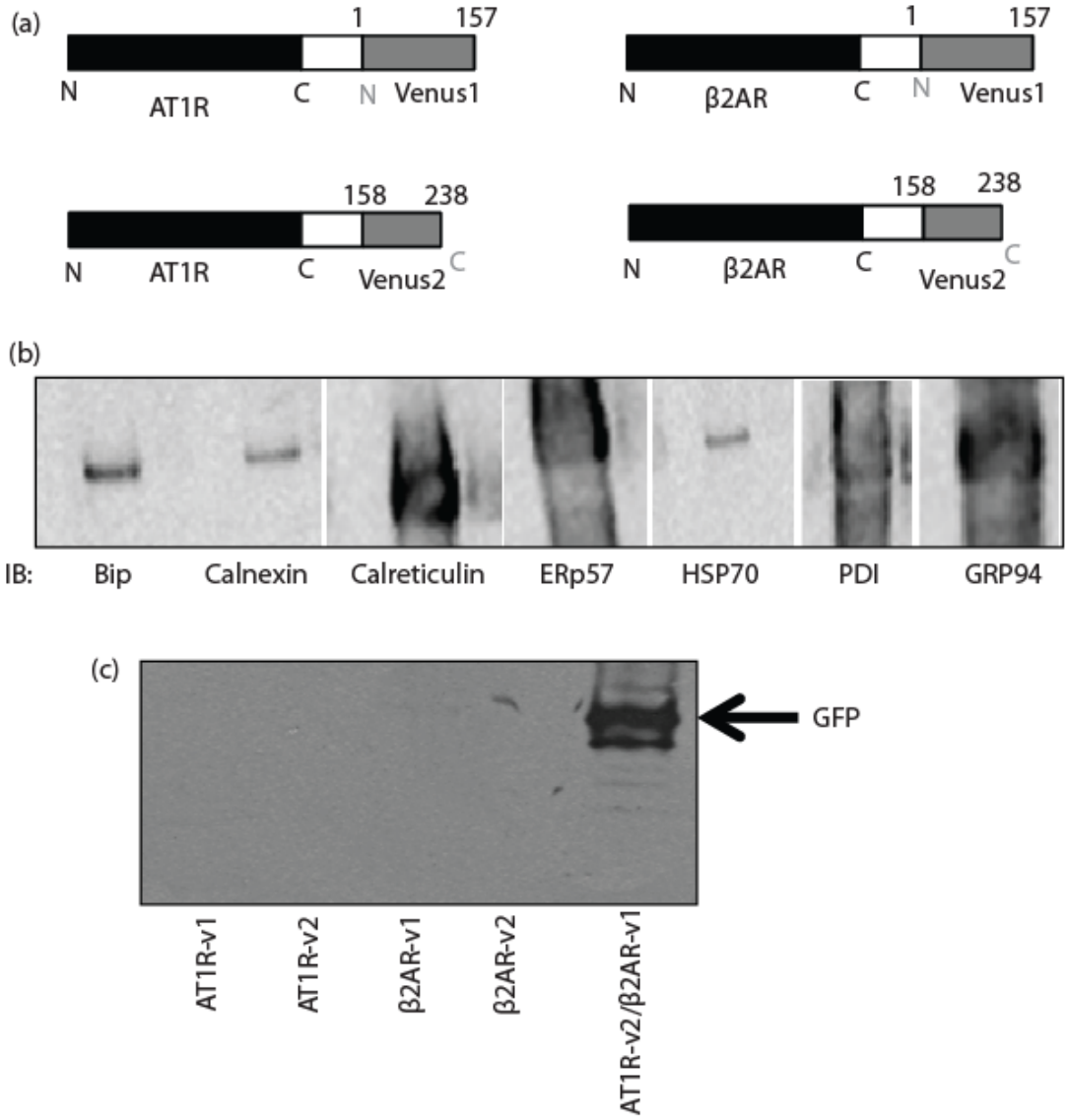


Figure 3.12: The expression of the receptor dimer pairs and the chaperones tested
a) Schematic presentation of the constructs used in the experiments. Venus1 contains the N- terminal end of the yellow fluorescent protein variant (venus) and represents the first 157 amino acids while venus2 contains the C- terminal end of the protein and represents amino acids 158 to 238. b) HEK293 cells were harvested, washed and lysed with 200µl of RIPA then precleared with 50µl protein A-sepharose beads. Proteins were then dissolved in 50 µl β-mercaptoethanol containing SDS loading buffer. Samples were then run on SDS-PAGE and Western analysis was performed using indicated chaperone antibodies. c) Specificity of monoclonal GFP antibody to dimers was tested in HEK293 cells. The cells were transfected with indicated receptors. Lysate was prepared as previously explained. Western analysis was performed using mono-GFP. Results are representative of 3 independent experiments.

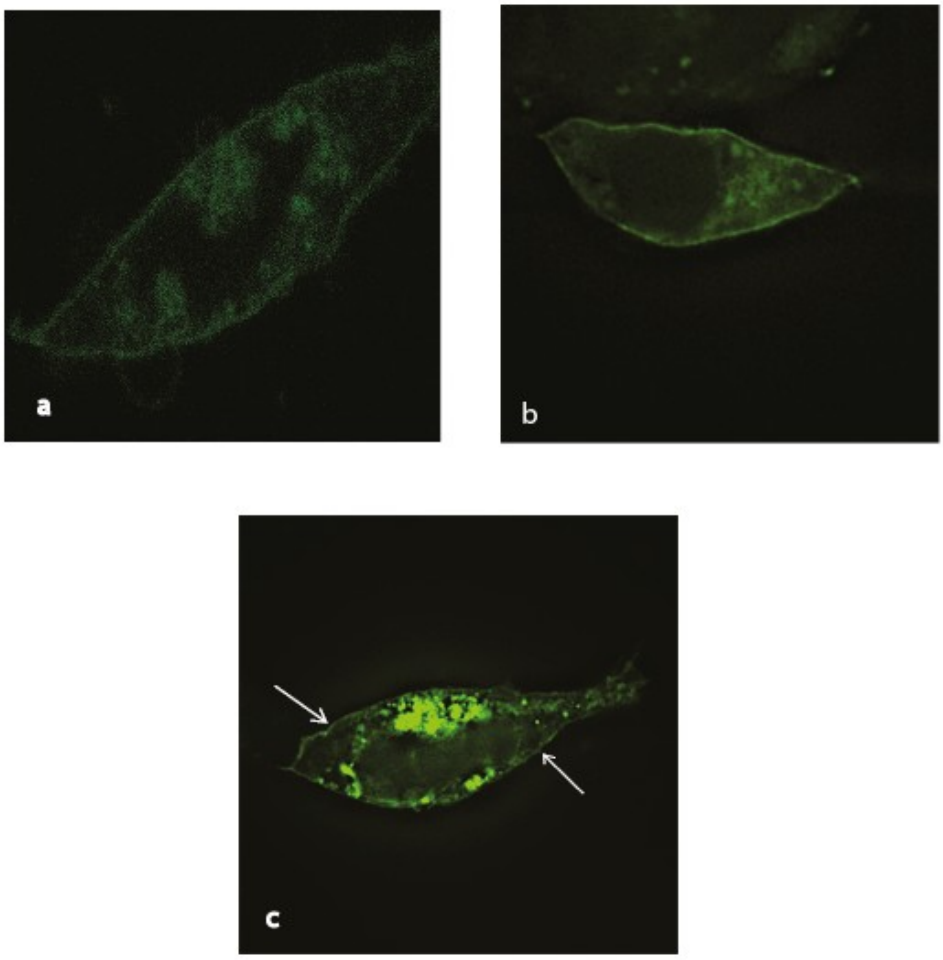


Figure 3.13: The expression of wild type forms of homo- and heterodimers
Fluorescent images of HEK293 cells transfected with a) AT1R-v1 + AT1R-v2, b) β2AR-v1 + β2AR-v2, c) AT1R-v2 + β2AR-v1. Results are representative of 4 independent experiments.

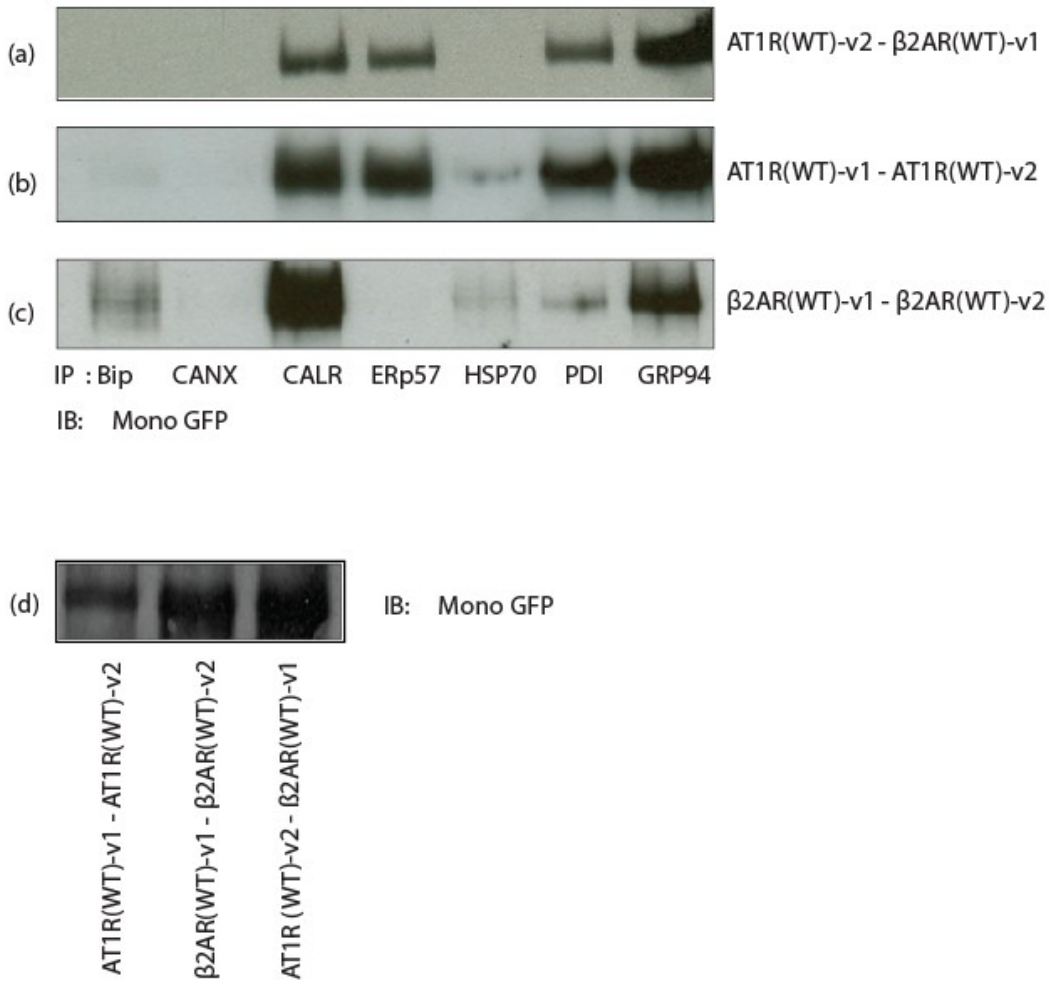


Figure 3.14: Molecular chaperones association with wild type receptors dimers
HEK293 cells were transfected with AT1R (WT)-v1/v2 and β2AR (WT)-v1/v2. After 48 hours, cells were harvested, washed, lysed with RIPA and precleared with protein A-sepharose beads. This lysate was distributed into eight different microcentrifuge tubes and co-immunoprecipitations were performed using the indicated chaperone antibody. a) AT1R/β2AR (WT) Heterodimer, b) AT1R (WT) Homodimer, c) β2AR (WT) Homodimer. The eighth sample was loaded as a control to show the expression level of the wild type receptor dimers as shown in d. CANX: Calnexin, CALR: Calreticulin. Results are representative of 4 independent experiments.

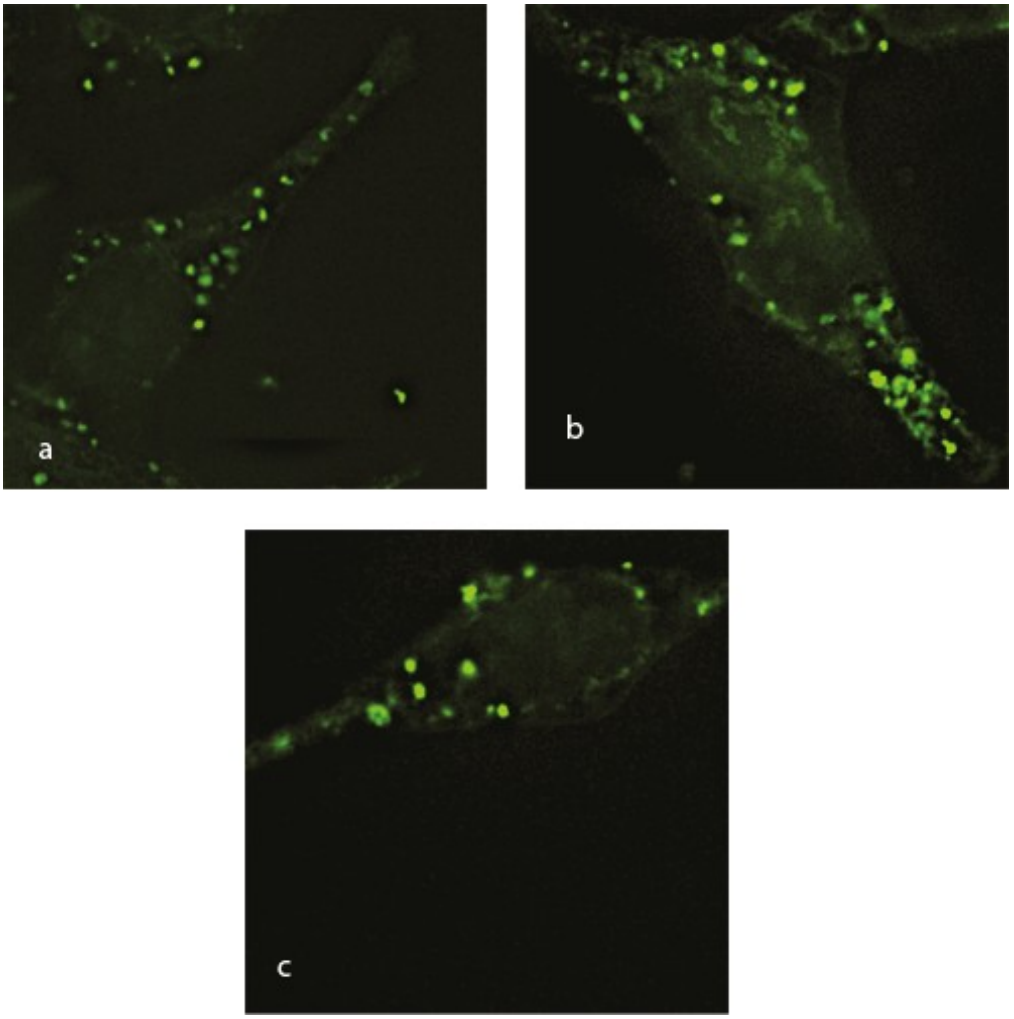


Figure 3.15: The expression of glycosylation-deficient forms of homo- and heterodimers

Fluorescent images of HEK293 cells transfected with a) AT1R (ND)-v1 + AT1R (ND)-v2, b) β2AR (NQ)-v1 + β2AR (NQ)-v2, c) AT1R (ND)-v2 + β2AR(NQ)-v1. Results are representative of 4 independent experiments.

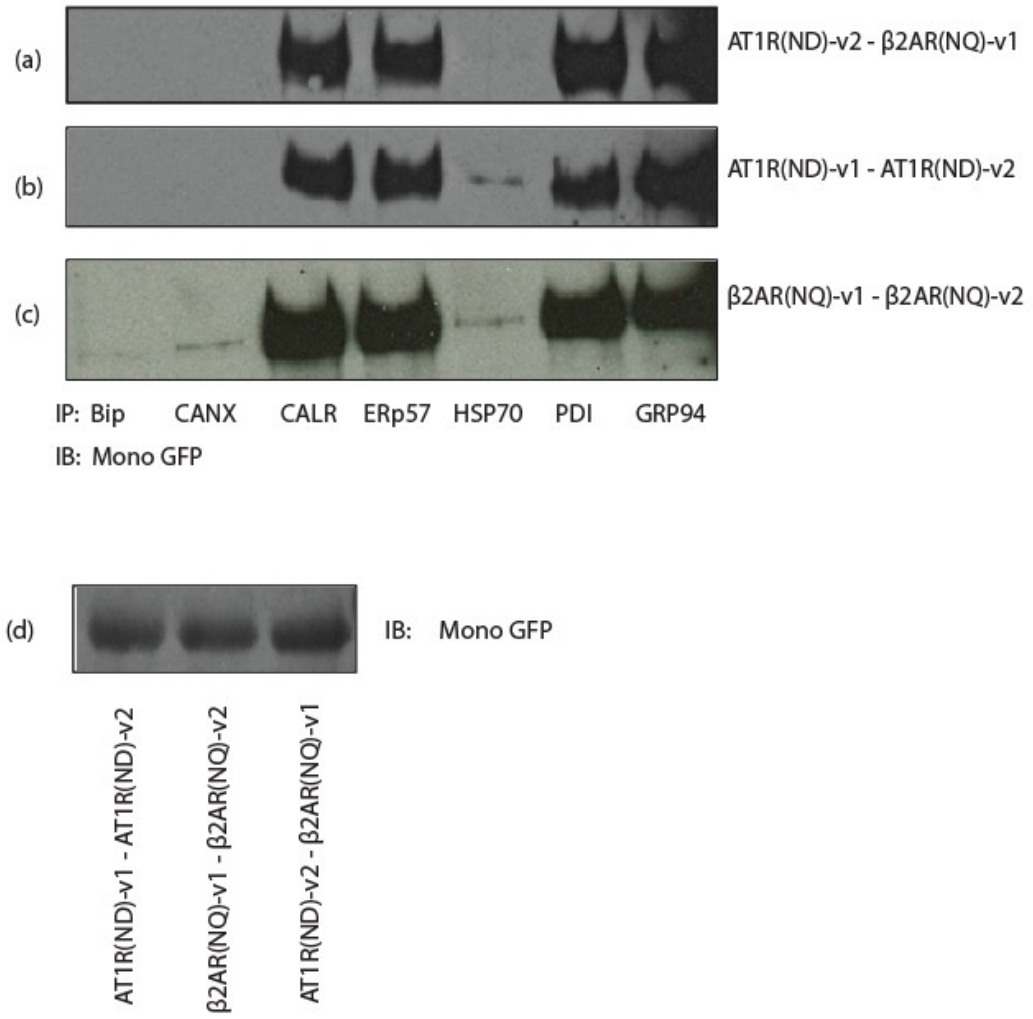


Figure 3.16: Molecular chaperones association with immature receptors dimers
HEK293 cells were transfected with AT1R (N4, 176, 188D)-v1/v2 and β2AR (N4, 15, 176Q)-v1/v2. After 48 hours, cells were harvested, washed, lysed with RIPA and precleared with protein A-sepharose beads. This lysate was distributed into eight different microcentrifuge tubes and co-immunoprecipitations were performed using the indicated chaperone antibody. a) AT1R(ND)/β2AR(NQ) heterodimer, b) AT1R (ND) homodimer, c) β2AR(NQ) homodimer. The eighth sample was loaded as a control to show the expression level of the immature receptor dimers as shown in d. CANX: Calnexin, CALR: Calreticulin. Results are representative of 4 independent experiments.

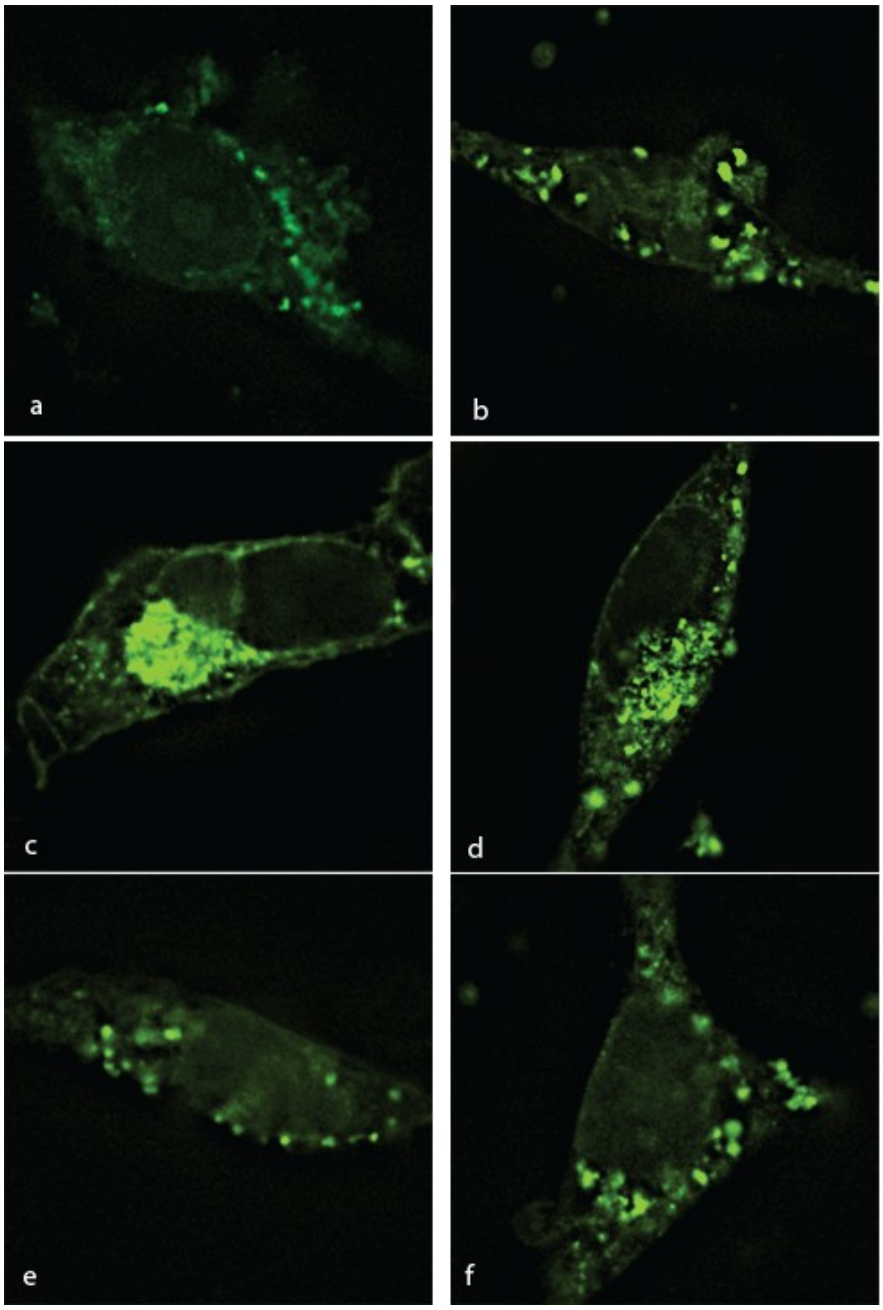


Figure 3.17: The expression of dimers composed of a wild type receptor with a mutant receptor

Fluorescent images of HEK293 cells transfected with a) AT1Rv2 + β2AR (NQ)-v1, b) AT1R (ND)-v2 + β2AR-v1, c) AT1R-v1 + AT1R (ND)-v2, d) β2ARv1 + β2AR (NQ)-v2, e) AT1R(m1)-v1 + AT1Rm1-v2, f) AT1R-v1+ AT1R(m1)-v2. Results are representative of 4 independent experiments.

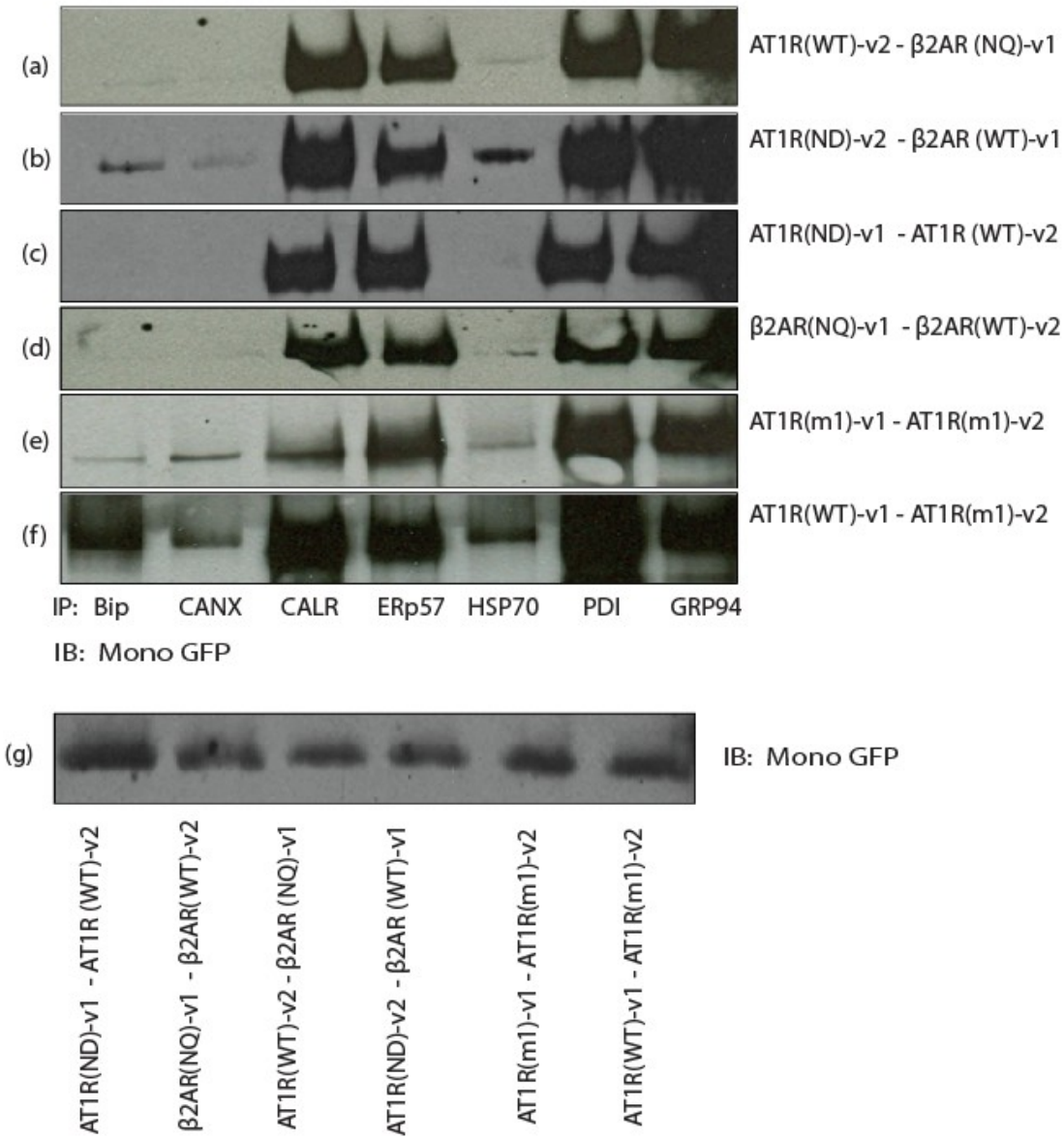


Figure 3.18: The effect of co- expressing immature receptors with wild type receptors on chaperones association

HEK293 cells were transfected with AT1R (WT, ND or m1)-v1/v2 and β2AR (WT or NQ)-v1/v2. After 48 hours, cells were harvested, washed, lysed with RIPA and precleared with protein A-sepharose beads. The lysate was distributed into eight different microcentrifuge tubes and co-immunoprecipitations were performed using the indicated chaperone antibody. The eighth sample was loaded as a control to show the expression level of the different receptor dimers as shown in g. CANX: Calnexin, CALR: Calreticulin. Results are representative of 4 independent experiments.

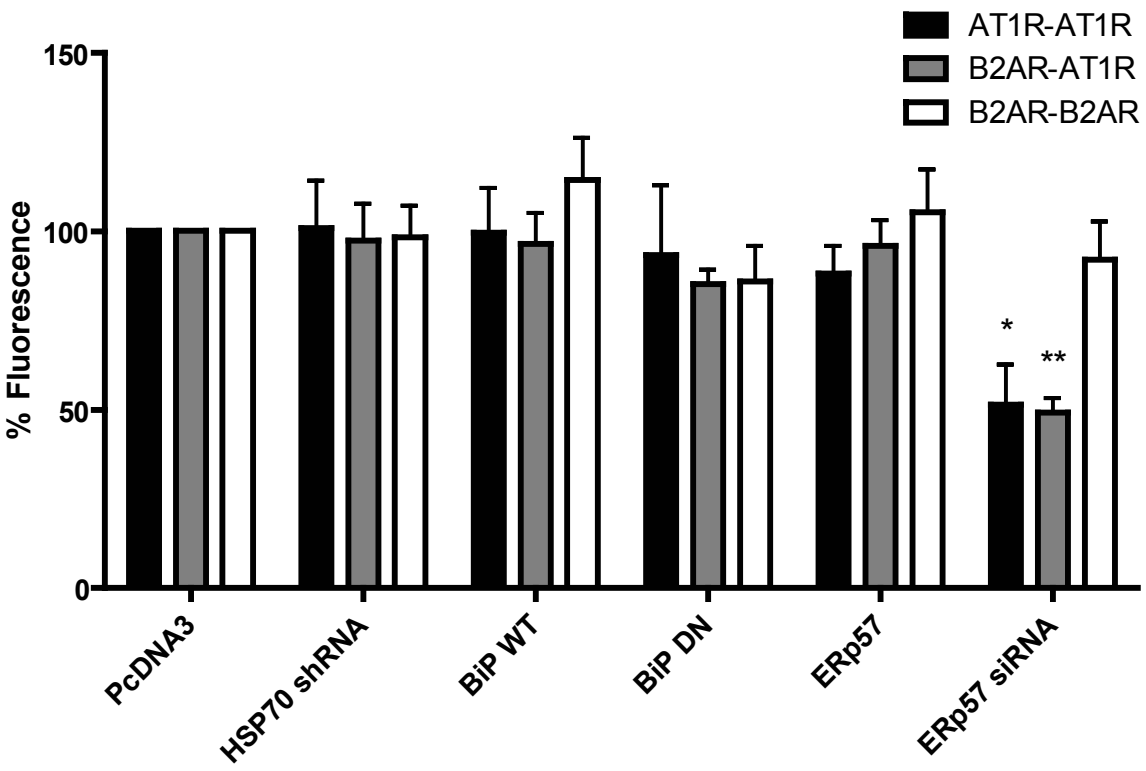


Figure 3.19 Chaperones effect on the formation of β2-AR/AT1R homo- and heterodimers

HEK293 cells were transfected with WT AT1R-v1/v2 and β2AR-v1/v2 and indicated chaperones. After 48 hours, cells were harvested, washed with PBS1X and resuspended in 100 µl PBS. Fluorescence was then measured using an envision plate reader at 527 nm. Results are expressed as means ± SEM. Statistical analysis was performed using two-tailed paired Student's t test. Results are representative of 4 independent experiments.

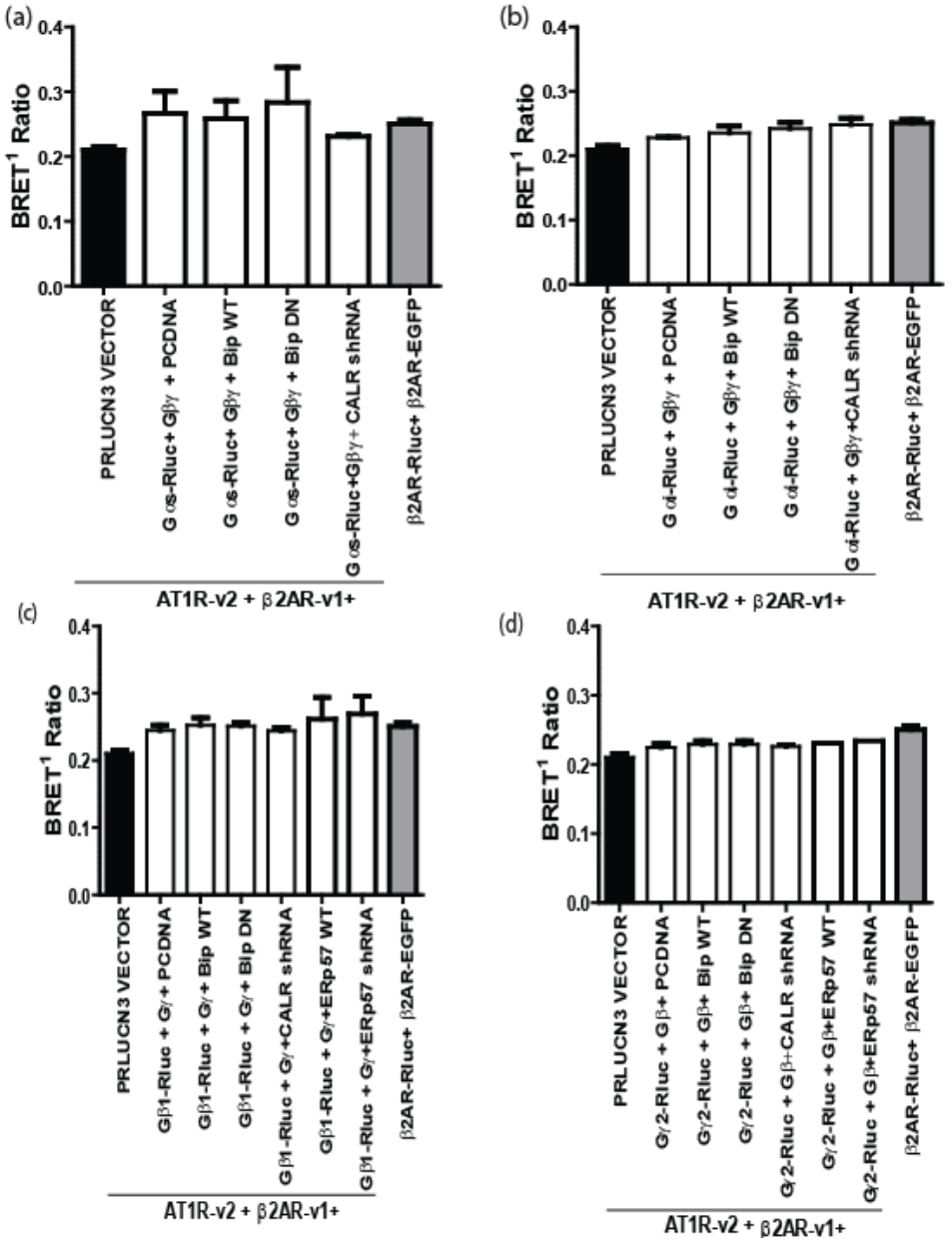


Figure 3.20: The effect of some chaperones on G- proteins coupling to AT1R-β2AR Heterodimer

HEK293 cells were transfected with indicated constructs. 48 hours post transfection, cells were harvested, washed three times with PBS then suspended in 100 µl PBS. 90 µl of the samples was distributed into 96 well plates and 10 µl of 5 µM coelenterazine H was added and mixed then the fluorescence energy emitted by Rluc and YFP (venus) was measured at their corresponding emission wavelengths (460 and 528 nm, respectively). The BRET ratio was then calculated and plotted with the ratios for a negative control and a positive control. Results are expressed as means ± SEM of at least 3 experiments. Statistical analysis was performed using two-tailed paired Student's t test.

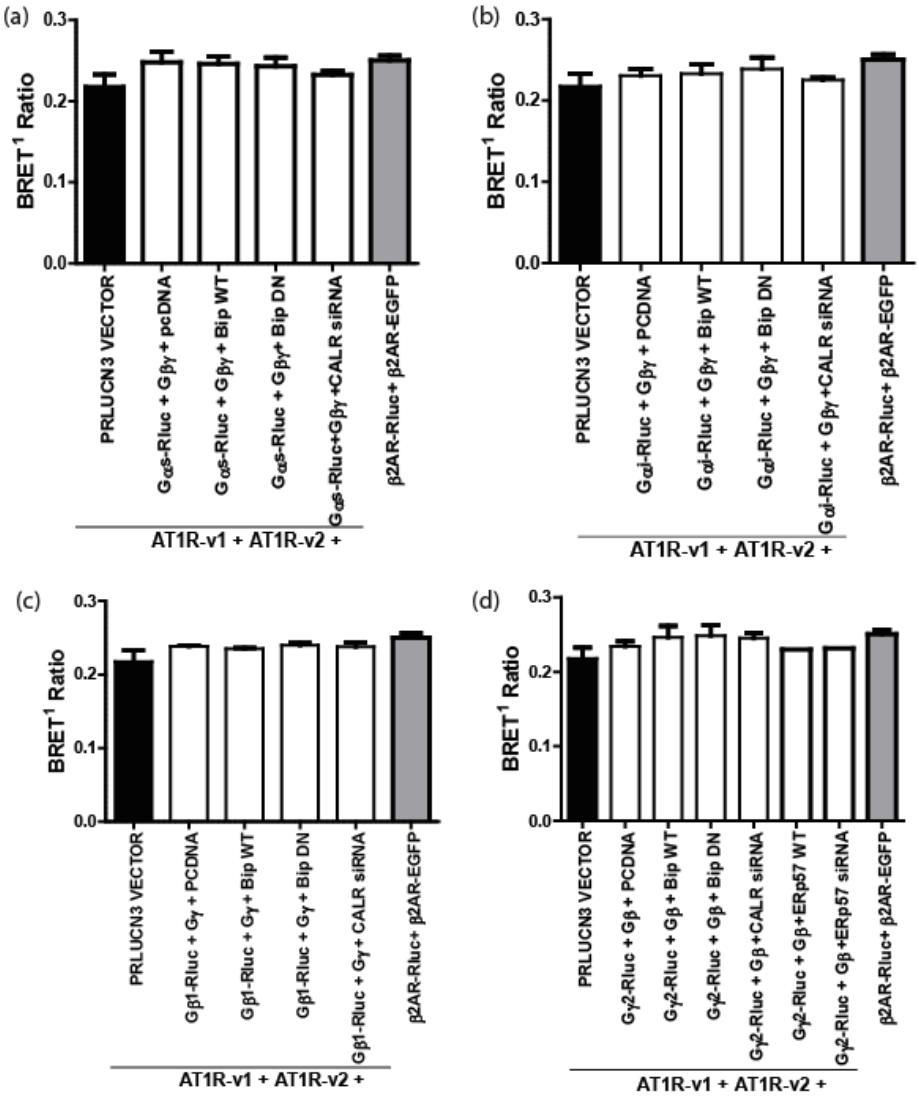


Figure 3.21: The effect of some chaperones on G- proteins coupling to AT1R homodimer

HEK293 cells were transfected with AT1R-v1, AT1R-v2 and Rluc G-proteins (Gαs, Gαi, Gβ1 and Gγ2). 48 hours post transfection, cells were harvested, washed three times with PBS then suspended in 100 µl PBS. 90 µl of the samples was distributed into 96 well plates and 10 µl of 5 µM coelenterazine H was added and mixed then the fluorescence energy emitted by Rluc and YFP (venus) was measured at their corresponding emission wavelengths (460 and 528 nm, respectively). The BRET ratio was then calculated and plotted with the ratios for a negative control and a positive control. Results are expressed as means ± SEM of at least 3 experiments. Statistical analysis was performed using two-tailed paired Student's t test.

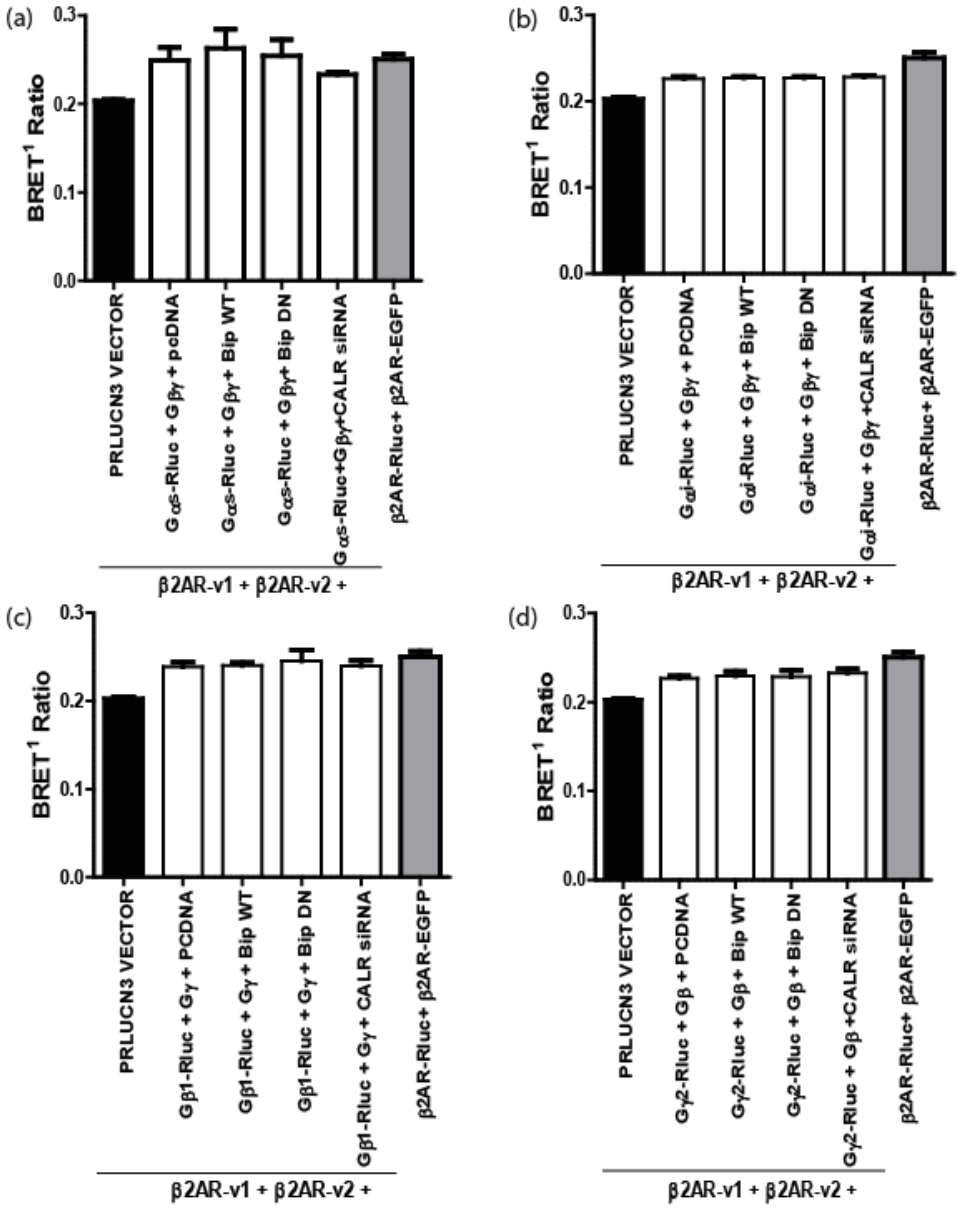


Figure 3.22: Effect of some chaperones on G- proteins coupling to β2AR homodimer
HEK293 cells were transfected with β2AR-v1, β2AR-v2 and Rluc G-proteins (Gαs, Gαi, Gβ1 and Gγ2). 48 hours post transfection, cells were harvested, washed three times with PBS then suspended in 100 µl PBS. 90 µl of the samples was distributed into 96 well plates and 10 µl of 5 µM coelenterazine H was added and mixed then the fluorescence energy emitted by Rluc and YFP (venus) was measured at their corresponding emission wavelengths (460 and 528 nm, respectively). The BRET ratio was then calculated and plotted with the ratios for a negative control and a positive control. Results are expressed as means ± SEM of at least 3 experiments. Statistical analysis was performed using two-tailed paired Student's t test.

CHAPTER 4: DISCUSSION

4.1 General overview

G-protein coupled receptors family is a very important group of proteins given their wide distribution all over the body and hence their involvement in the different physiological functions that control body homeostasis. It is therefore very crucial to understand the different phases of their life cycle and the molecules that are involved and responsible for regulating the processes that each receptor undergoes. Lots of research has been dedicated to GPCRs, however, many questions regarding some of their properties have not been fully answered yet. For example, the trafficking of GPCRs from the endoplasmic reticulum to the plasma membrane is not described as detailed as the endocytic pathway. In addition, the steps followed or the regulators needed for the assembly of receptor dimers and higher oligomers are not fully understood. This project focuses on some of these aspects in an attempt to identify the molecules that are involved in the regulation of these processes. This is important for Pharmacology since such molecules would represent interesting targets for new therapeutics. Therefore, the first aspect studied was the trafficking of the receptors from their synthesis site in the endoplasmic reticulum to their expression site at the plasma membrane. Studying the exocytic pathway of the β2- adrenergic receptor enabled the identification of two scaffolding proteins (Rab1 and arrestin2) that are important for the expression of β2-AR at the plasma membrane. The second part of the project focused on an earlier step in GPCRs life which is the formation of homo- and heterodimers. The dimer model used was the heterodimer angiotensin type I receptor- β2- adrenergic receptor and a role for the molecular chaperone ERp57 in the formation of this heterodimer was indicated. In

addition differences in the effects of chaperones on the formation of dimers were observed between the homo- and heterodimeric complexes.

4.2 Rab1 and Arrestin2 are Involved in the Plasma Membrane Expression of the β2-Adrenergic Receptor

There is currently a quite clear picture of how a receptor gets activated upon the binding of an appropriate ligand, desensitized and then internalized via the endocytic machineries such as the clathrin- coated vesicles and caveolae- dependent pathways (60, 129). Depending on the internalization mechanism, the receptor's fate is determined and it either gets degraded in the proteasome or recycled back to the plasma membrane. On the other hand, the exocytic pathway and how GPCRs get transported from the ER to the PM remains unresolved. Studies are currently trying to investigate this process in an attempt to understand the details of the pathway and whether it is a similar process that is controlled by the same factors for all receptors. This hypothesis is driven by the fact that studying the anterograde and the retrograde pathways of proteins resulted in the discovery of many similar regulators playing equivalent roles in the opposite pathways. For example, clathrin which is known to initiate the formation of the internalized vesicles is equivalent to the Sec13-Sec31 complex that assembles to coat cargo in the ER (92, 96, 98). Another example would be the role that dynamin plays in the cession of the clathrin-coated vesicles from the plasma membrane which is similar to the Sar1-GTPase role in separating the COPII vesicles from the ER membranes (92). Some GTPases and especially the members of the Rab proteins family have shown specificity in vesicular transport between the distinct cellular compartments (103, 105, 107). For example, Rab6 was shown to regulate transport in the trans-Golgi network and from Golgi to ER (130-

132), Rab8 was shown to be involved in cargo transport between Golgi and the plasma membrane (115, 133) and Rab11 has been related to vesicles transport from Golgi to recycling endosomes (134). Another member of the Rab family that was shown to be regulating cargo trafficking is Rab1. Studies have shown that Rab1 is involved in the exocytic pathway of β2- AR (107, 117, 135); however, the mechanism by which this occurs remains unknown. Interestingly, recent studies have shown that some Rab proteins can interact directly with GPCRs to regulate their trafficking. Studies on the thromboxane A2 receptor and the β2-Adrenergic Receptor showed that Rab11 has the ability to regulate the recycling of those GPCRs through binding directly to the c- tail of the receptors (113, 114). Other studies on a different GPCR, the angiotensin II type 1 receptor, looked at a group of Rab proteins, namely Rab5, Rab7 and Rab11 and found that these proteins are required for proper trafficking of AT1R (116). Those previous observations trigger the question of a direct interaction between Rab1 and β2-AR. The results obtained from GST pull-downs show a direct interaction between Rab1 and the c-tail of the β2- AR. Furthermore, the exact interaction site of Rab1 appeared to be the conserved F(X)-$_6$LL motif. As mentioned earlier, this motif has exhibited functional importance in ER export of multiple GPCRs (58, 120). Therefore, these results suggest that this c-terminal motif could be acting as a sorting signal that allows recognition of the receptor to be transported from the ERGIC to Golgi. In addition, the association between Rab1 and this motif confirms that Rab1 role in the exocytic pathway arises in the early steps of ER export and transport from ER to Golgi. A previous report showed contradicting results to ours where Rab1 did not interact with the β2-AR c-tail (115). Although the other group was using GST pull-downs, as was the case in the results

presented here, the β2-AR constructs that were used in the other study are designed in different ways which could be a possible reason for the opposite results obtained. The F(X)-$_6$LL motif was shown to be important for the export of GPCRs such as angiotensin II type 1 receptor and $\alpha 2_B$-adrenergic receptor. Previous studies showed that receptors with a mutated motif are retained in the ER and this leads to their inability to induce signal transduction (136). Interestingly, the β2-adrenegric receptor was shown to be expressed at the plasma membrane when the dileucine residues in this motif were substituted with alanine (137) which could mean that the phenylalanine residue is the essential factor in the motif for ER export or that the entire motif needs to be available for ER export to occur.

Polypeptides are transported between the different intracellular compartments and membranes in vesicular arrangements. These vesicles facilitate the anterograde trafficking as well as the retrograde movement of the proteins. Rab GTPases are considered important regulators for this vesicular transport. In fact, each Rab protein is associated with a distinct organelle and therefore seems to be responsible for vesicles transport between specific compartments. A relevant example of a process regulated through such vesicle arrangements would be GPCRs endocytosis. Upon ligand binding, GPCRs are phosphorylated via a group of specific kinases and this leads to the recruitment of β-arrestins. This would target clathrin and dynamin recruitment to form internalized vesicles from the plasma membrane. Arrestin molecules used to be linked to the internalization pathway; however, recent evidences in the last decade presented arrestins as multifunctional proteins due to their involvement in many cellular functions such as the induction of signal transduction, regulation of granule release and regulation

of chemotaxis and apoptosis. Given the wide range of their physiological function, it was expected that arrestins could interact with many cellular molecules and this was indeed represented in a global proteomic analysis that was performed on arrestin2 and arrestin3 using tandem mass spectrometery (110). This analysis showed the ability of arrestin molecules to interact with more than 200 cellular molecules and hence implied for multiple roles of arrestin in the processes regulated by its interacting partners. For instance, arretsin2 showed an interaction with actin, tubulin and filamin, suggesting that it is involved in regulating cellular movement. Another group of proteins were mortalin, APLP and BIP and therefore, apoptosis was added to the list of processes regulated by arrestin. Interestingly, Rab1 was one of the positive hits in the analysis and showed an interaction with both arrestin2 and arrestin3. In order to confirm the results, co-immunoprecipitations and Histidine pull-downs were performed between the two proteins (Rab1 and arrestin2). A strong association was detected between the wild type form of Rab1 with arrestin2. In addition, the interaction domain of Rab1 appears to be at the c-terminal end of arrestin2 in the region harboring the last 199 amino acids of the sequence. As previously indicated, Rab1 was shown to localize at the ER and control vesicles transport from the endoplasmic reticulum to the plasma membrane, and given the wide range of functions controlled by arrestin, the question is could GPCRs exocytosis be one of these processes. Arrestin2 usually associates with receptors upon their phosphorylation by a group of kinases, like GRKs, PKA and PKC to induce endocytosis (129). In order to regulate export of GPCRs, arrestin would probably have the ability to interact with unphosphorylated receptors as well. In fact, two reports that were released around the same time showed that phosphorylation is not a requirement for arrestin2-

dependent internalization. Arrestin2 was shown to associate with the D1 Dopamine receptor (125) and the Protease Activated Receptor1 (PAR1) (124) when they are in an unphosphorylated state; i.e. when the receptors are mutated at their serine/ threonine phosphorylation sites. The results represented here add the β2-adrenergic receptor to this group since it appears to associate with arrestin2 when phosphorylation is inhibited by H89 and when a receptor mutated in some of its GRK phosphorylation sites is used. In addition, this association is also occurring basally which was the case for PAR1. This represents the first clue for a role of arrestin2 distinct from β2-AR internalization. To further investigate that, an ELISA-like assay was applied and illustrated this role of arrestin2 by showing its involvement in the expression of β2-AR at the plasma membrane. A significant reduction in the number of receptors expressed at the cell surface was reported when arrestin2 function was corrupted by dominant negative constructs and shRNA. In addition, switching to a mouse embryonic fibroblast cell line and using the arrestin2 knockout cells clearly showed that compared to the wild type cell line, a great number of β2- adrenergic receptors was retained inside the cells. This was illustrated by two different approaches; fluorescence microscopy and a biotin labelling assay against the receptor. This is the second report for arrestin's role in GPCRs expression at the cell surface, however, the previous data did not illustrate at what point of the anterograde pathway arrestin would be required. In the data presented here, arrestin2-Rab1 association is taken in consideration to understand this function. The role of arrestin2 in receptor function might also come from its pleiotropic effects on cell physiology. The fact that arrestin2 interacts with Rab1 suggests that the role of arrestin2 in regulating receptor expression arises at the early stages of ER export and the transport

from ER to Golgi through the ERGIC since those are the steps where Rab1 seems to be involved. The stable compartment model of anterograde membrane traffic through the ERGIC suggests multiple roles for Rab1 in the pathway including: recruitment of COPII components, initiating vesicles budding and fusion as well as cargo sorting (99). Despite the various evidences available to support the idea, Rab1 function is not completely established and the mechanisms are not described. This could be due to limitation of using dominant negative forms of Rab1 and could suggest a need for knock-down models. The variability in the functions might also suggest the existence of Rab1 isoforms that has not been identified yet. The Rab1-arrestin2 association and the fact that both can regulate receptor expression suggested a mutual sequestration mechanism of action for the two proteins. However, the results showed that Rab1 does not seem to be regulating β2AR export from the ER via sequestering the interaction between arrestin2 and the receptor; alternatively, a possible mechanism is that Rab1 seems to be binding the receptor first and when arrestin2 is recruited, the receptor can detach from Rab1 to bind arrestin which in turn would facilitate its trafficking, however, if arrestin2 is not functional, the receptor is retained in the ER, and would likely be in complex with Rab1. Further studies are required to fully understand the role of arrestin2 and Rab1 in the anterograde trafficking. Moreover, a wide interaction screen is needed to identify the other molecules that act as activators or switches for those targets because Rab1 and arrestin2 are probably just parts of the higher complex that is running this system.

G protein- coupled receptors are not the only membrane proteins that traffic from the endoplasmic reticulum to the plasma membrane. There is a large number of other plasma membrane proteins that are synthesized at the rough endoplasmic reticulum

membranes and then get transported to the plasma membrane. The trafficking of those membrane proteins has also become the focus of many studies yet not all the details have been discovered. Arrestin2 was shown to regulate the expression of some GPCRs, therefore we hypothesized that it could be required for the expression of other plasma membrane proteins. In addition, we also proposed a role for the GPCRs themselves in regulating this transport. CD4 was selected to be the plasma membrane protein to study because it does not interact with β2-AR or AT1R (the two GPCRs that we were working on). Our results show that GPCRs seem to be regulating the PM expression of CD4 in an arrestin-dependent mechanism since having a mutant arrestin2 or a mutant receptor led to significant blockade of plasma membrane expression of CD4. Phosphorylation was another important factor to investigate when studying the expression of a plasma membrane protein. A previous report showed that blocking phosphorylation could affect COPII recruitment, an early step in vesicle formation and budding to initiate cargo exit from ER (138). The inhibitor used here (H89) is a non-specific inhibitor and therefore, it would be difficult to determine the kinases required for ER exit, however, it was demonstrated in the other study that H89 inhibits COPII activation by preventing Sar1 recruitment. Rab1 is another GTPase that was shown to be recruited to the ER and activated to initiate the formation of COPII vesicles. Further work is required to study the effect of inhibiting phosphorylation on Rab1 activity as well because the available evidences suggest that H89 is acting on specific kinases that seems to be involved in the activation of small GTPases.

Overall, this part of the project suggests that both Rab1 and arrestin2 control ER export and plasma membrane expression by interacting with cargo proteins. Interestingly,

we showed that arrestin2, a protein capable of binding Rab1, can also contribute to ER export of GPCRs. Both arrestin2 and Rab1 can interact with GPCRs to control receptor expression at the plasma membrane. In addition, arrestin2 seems to be important for the expression of other membrane proteins. Our results show that GPCRs can contribute to the regulation of the export of proteins not usually associated with them.

4.3 Chaperones Contribute to G protein Coupled Receptor Oligomerization, but do not Participate in the Assembly of the G Protein with the Receptor Signalling Complex

The members of the G- protein coupled receptor family are synthesized at the endoplasmic reticulum, and then transported from ER exit sites to Golgi before they reach the plasma membrane. During this process, receptors have to undergo a series of folding and post- translational modifications to become properly folded and ready for expression. These modifications include proteolytic cleavage of hydrophobic signal sequence, disulfide bonds formation, N and O glycosylation as well as quaternary structure maturation (139). Molecular chaperones are important regulators in these tightly controlled processes. Although chaperones are known to facilitate folding of polypeptides, finalize their correct assembly and prevent their aggregation, it is still not quite clear how they are functioning for GPCRs. In addition, several studies have showed recently that GPCRs signalling complexes are preassembled in early stages of receptors biosynthesis and that chaperones seem to have an important role in the process but this has not been fully characterized yet in terms of which chaperones are involved and how they participate in the process. GPCRs can exist as homo- and hetero- oligomeric complexes and studies showed that this is occurring before the receptor get transported and expressed at the plasma membrane. Interestingly, some chaperones were shown to

associate with receptors of the GPCRs family. A previous study reported an interaction between calnexin and Dopamine receptors (D1 and D2) (79). In addition, they have shown that calnexin is important for the expression of the receptors at the plasma membrane and that having the receptor in a non-glycosylated form would reduce this interaction. On the other hand, another study reported a role for calreticulin in the maturation of the B2 bradykinin receptor by showing a strong association with immature forms (81). Furthermore, the study illustrated that calreticulin is important for the dimerization of B2 bradykinin receptor with the angiotensin type 1 receptor. These two examples represent good evidence that the diverse functions of chaperones are receptor-specific, as those studies were focusing on two similar lectin chaperones (calnexin and calreticulin).

The receptors investigated for our study are the β2- adrenergic receptor and the angiotensin II type 1 receptor. β2-AR is a prototypic GPCR that has been the focus of lots of studies and was characterized in many aspects such as its signalling and the cellular responses that result from its activation as well as its endocytosis and how it gets internalized. However, little is known about how it is folded and transported to the plasma membrane. In addition, the β2-AR is an important cardiac receptor, despite being expressed in many other tissues and systems including the eye, the gastrointestinal tract, the muscular system and even the brain and it is in fact one of the first targeted receptors in the treatment of cardiovascular diseases. Therefore, it is very important to further characterize the expression of β2-AR. There are two main reasons for choosing AT1R, it was shown to form a heterodimer with the β2-AR and it is the other important target in designing drugs for heart failure (47). It is noteworthy that these receptors have opposite

effects since AT1R is a vasoconstrictor while β2AR is a vasodilator (21, 25, 27). Interestingly, a recent report about higher mortality rates in a sub group of patients administered a combination of ACE inhibitors, AT1R and β2-AR blockers was revealed (5). Taken together, these two observations suggest that a possible explanation for the higher mortality rate could be the effects that these drugs have on the AT1R-β2AR heterodimer especially with the study that illustrated that blocking one of the receptors in the heterodimeric complex could have effects on the signalling initiated by the other receptor.

GPCRs can be expressed in different arrangements, and therefore ligand- based therapy does not seem to be the ideal treatment anymore, at least not until a detailed characterization of these oligomeric complexes is performed (44, 140). Meanwhile, a more specific approach can be developed to target receptors in the early stages of their synthesis before they get transported and expressed at the plasma membrane. In order to do that, a better understanding of how the receptors are assembled into their signalling complexes and what molecules regulate their anterograde trafficking from the ER to the PM needs to be achieved. The focus of this part of the project was switched to the formation of the heterodimeric and the homodimeric forms of the receptors. The experiments were designed to investigate whether the chaperones favor specific conformations by comparing homodimers with heterodimers and immature receptors with wild type completely folded ones. Furthermore, the effects of some chaperones on the formation of the signalling complexes, specifically the coupling of trimeric G protein subunits with the different receptor pairs were also studied.

One of the challenges in studying heterodimeric complexes is to distinguish between them and homodimeric pairs. However, new techniques have been developed to at least partially overcome this problem. Bimolecular Fluorescence Complementation (BiFC) is the basic approach used in most of the experiments in this part of the project. This technique facilitates the study of interactions occurring between three proteins which are basically represented by the two receptors and the chaperone under study in this case (141, 142). In addition, the way venus (a yellow fluorescent protein variant) was used enabled us to specifically blot against the dimer or visualize it in the fluorescence images (143). First, the expression of the different receptor pairs included in the study was illustrated by images of the HEK cells expressing the fluorescent receptor pairs. The results confirm that for a receptor to be expressed at the plasma membrane, it has to be completely modified and folded since glycosylation-deficient receptors and receptors with mutations at the F(X)-$_6$LL motif were retained inside the cell. This was also shown for the glycoprotein hormone receptor lutropin/choriogonadotropin (LHR) where two loss-of-fuction misfolded mutants were retained intracellulary compared to the wild type form of the receptor which was expressed at the plasma membrane (82).

The next set of results demonstrates that there are indeed some differences in the interaction pattern between those different receptor pairs and chaperone proteins. Not much is known about what chaperones can associate with β2-AR since DRiP78 is the only chaperone that was shown to interact with this receptor so far (53). On the other hand, few studies looked at the AT1R and showed that it interacts with calnexin, HSP70 as well as DRiP78 (144, 145). The results from our work show that Bip can associate with the β2-AR homodimer but not the AT1R homodimer or the heterodimer AT1R-

β2AR, that HSP70 interacts with the two homodimers but not the heterodimer and that ERp57 only associates with AT1R containing dimers. The difference observed with HSP70 was interesting because it could be indicating that it is a homodimer- specific chaperone. In addition, this also suggests that the different dimeric complexes start acting in distinct fashions during the early steps of their synthesis and formation. A previous report showed that AT1R can associate with calnexin and HSP70 (144), but this group was not selecting the forms of receptor detected while the results presented here show that AT1R homodimer does not interact with calnexin. One could conclude that some chaperones can be dimer-specific, meaning that it can select among monomeric versus dimeric complexes. Another report that could support this hypothesis was with the dopamine D1 receptor. Interestingly, this group was also studying calnexin and they saw that only the lower molecular weight species which was reflecting the monomer form of D1 receptor was co-precipitated with calnexin while the oligomeric higher molecular weight species was not (79). These results can suggest that calnexin acts as a retention protein by prolonging the binding to monomeric receptors and allowing the ER export of higher oligomers. Furthermore, this observation provides evidence that in addition to being different in the signalling pathways that they activate, dimeric complexes seem to be processed in a different way as well. There is a good number of examples that show the difference in signalling among these different arrangements. For instance, a heterodimer could couple to G- protein subunits other than those recruited by the homodimers and hence activate effectors other than those activated by homodimers. This was suggested for the mu/delta opioid heterodimer because it did not show sensitivity to pertussis toxin while the homodimers of these receptors are sensitive to it (146). This was

also illustrated for the chemokine receptors CCR5-CCR2 as well where the heterodimer was shown to couple to Gq/11, a subunit that does not interact with CCR5 homodimer or CCR2 homodimer (147).

Molecular chaperones have the ability to promote the folding and maturation of polypeptides (chaperoning function) (8, 64), but at the same time, they also can act as ER retaining proteins of misfolded polypeptides (quality control function) (63, 65, 139, 148). However, the previous studies did not really discriminate between monomers and dimers. Therefore, the next set of experiments involved using a presumably immature form of the dimers. Both receptors of interest contain three potential N-glycosylation sites; β2-AR sites at residues 6, 15 and 187 and AT1R at residues 4, 176 and 188 (149-151). Hence, constructs were designed to introduce mutations at these sites given the importance of glycosylation in the recognition of completely folded proteins and confocal images were obtained to verify their intracellular retention. The chaperones association with the homo- and heterodimeric complexes was then detected. The results did not show any difference with the AT1R homodimer or the heterodimer, however, some interesting outcomes were observed with the β2-AR homodimer. Two chaperones that did not interact with the wild type form (calnexin and ERp57) showed an interaction with the receptor lacking the glycosylation sites. In addition, the interaction with PDI was much stronger when the mutated receptor was used. The previous results suggest a role for calnexin, ERp57 and PDI in the quality control mechanism for the β2-AR homodimer and could be indicating that this GPCR undergoes a series of calnexin/calreticulin cycles with the help of ERp57 for the formation of disulfide bridges till it becomes completely folded and ready to pursue its trafficking itinerary. This is probably the case for the other dimers (AT1R

homodimer and AT1R-β2AR heterodimer) except that they are either controlled by a different mechanism that needs to be identified or that it is the same mechanism but the complexes that they form with the chaperones are not stable enough for detection. A surprising result was the loss of interaction between Bip and the β2-AR homodimer since Bip is known to bind misfolded proteins to prevent their aggregation. The fact that Bip and calnexin did not interact with the other dimeric receptors (AT1R homodimer and the heterodimer) was also surprising. This is because it is contradicting the results obtained from studies on the glycoprotein hormone receptors (TSHR, LHR and FSHR) where Bip, calnexin and calreticulin were interacting with the immature forms (82, 152). However, this could be interpreted by assuming that Bip's role in dimers folding is at a later phase. Another tentative explanation is that GRP94 can be mimicking Bip function in this system. This can be possible because first GRP94 is a co-chaperone for Bip and more importantly it was demonstrated before that it has the ability to associate with proteins in a more advanced stage of folding than Bip (139). In addition, studies on the light and heavy chains of immunoglobulins showed that they can bind both Bip and GRP94 with an average dissociation time of few minutes for Bip and fifty minutes for GRP94 (153). Given the complexity of the dimeric complexes compared with individual receptors, one would expect the need of this higher chaperone system to completely fold the receptors. The fact that GRP94 was interacting with the wild type forms as well does not really negate this hypothesis, contrarily, it might be confirming the complexity of these higher oligomeric receptors illustrated in a prolonged binding with GRP94. A similar conclusion was made about PDI interaction in the hormone receptors paper. The immature proteins were interacting with calnexin and calreticulin but not with ERp57, which is a co-

chaperone for these lectins. The receptors were interacting with PDI instead and given that both ERp57 and PDI are members of the thiol oxidoreductase family and both catalyze disulfide bonds formation, a conclusion was made indicating that PDI acts as a co-chaperone for calnexin and calreticulin (82).

The last group of receptor dimers that was studied for the chaperones interaction represented the pairs where one of the receptors was in the wild type form and the other was an immature receptor and a combination of homo- and hetero- dimeric complexes was used. When those interactions were compared with the results obtained in **Figure 3.13** (wild type receptors) and **3.15** (immature receptors), a tendency toward following the pattern of the glycosylation deficient receptor was concluded, suggesting that the less mature form dictates the chaperones interactions. In addition, the less mature form in a dimeric pair controls the expression of it and leads to the retention of receptors inside the cell. This could be interpreted as a protective effect because blocking the transport of such receptors to the plasma membrane prevents the possible signalling responses that they would initiate which would most likely be harmful or at least abnormal. An interesting result was the interaction of all the chaperones under study with the AT1R (m1) – containing pairs. As mentioned earlier, this construct has a mutation at the site responsible for ER exit of GPCRs (F(X)-$_6$LL). Since we believe that the chaperones are acting at different stages of the pathway, this result indicates that this ER-retained form of receptor is blocked all the way in the anterograde trafficking pathway. Again, this is probably a protective mode that the cell undergoes in the case of the existence of not-fully folded receptors and it represents a part of the quality control mechanism (8, 62, 63, 65, 139).

A general observation was a different binding profile for calnexin and calreticulin which are usually put side by side in most studies. At some point, there was a debate about glycosylation being a requirement for these chaperones to bind a polypeptide. This was resolved as there are many evidences showing interactions between these lectins and non-glycosylated proteins (8, 154, 155). More interestingly, some studies have shown that lectin chaperones have the ability to interact with proteins by recognizing the polypeptides part of the glycoproteins (156-159). The results presented here confirm that two mechanisms are also available for the interactions between dimeric GPCRs and lectin chaperones; glycan-dependent and glycan-independent. Although they are usually classified in the same group, some differences were detected among the two chaperones in their distribution on human oocyte (8). In addition, studies on mice that lack calnexin or calreticulin indicated that they don't have the ability to compensate for one another and therefore suggested distinct functions of the two proteins (160, 161). It is also noteworthy that despite their important role in the quality control cycle, the main functions of these lectin chaperones are different than protein folding; calreticulin is essential for calcium homeostasis and cell adhesion while calnexin is mainly essential for recognizing misfolded proteins rather than facilitating their synthesis (8). Furthermore, both calnexin and calreticulin were shown to be expressed at the cell surface but their function has not been clarified yet, however studies have been showing a potential link between them and some autoimmune diseases where autoantibodies against lectin chaperones are detected (148, 162). These conclusions suggest that these chaperone are complex systems and that their functions are beyond what was thought to be.

In addition to their main role in facilitating the folding of polypeptides, and as another proof of their involvement in proteins processing, some molecular chaperones were shown to assist in the formation of protein complexes. For instance, Phosducin-like protein (PhLP) and the cytosolic chaperonin complex were shown to mediate the assembly of Gβ subunit with Gγ subunit (163, 164). Another chaperone that was shown to be involved in this assembly was DRiP78 (53). How the chaperones regulate GPCRs assembly requires further studies and therefore, the effect of knocking down some selected chaperones on the formation of the homo- and heterodimeric complexes of the AT1R and the β2-AR was investigated here. Interestingly, a significant reduction in the formation of AT1R homodimer and the heterodimer AT1R-β2AR was observed when ERp57 inhibitory RNA was co-expressed compared with the case where a wild type form of ERp57 was used. No effect was detected on the formation of β2-AR homodimer. This provides a possible explanation for ERp57 interaction with the AT1R – containing dimers only and suggests a crucial role for ERp57 in the formation of specific dimers. ERp57 is an ER- protein and therefore, the previous results suggest that the formation of GPCRs oligomers occurs in the early stages of biosynthesis in the endoplasmic reticulum. Using a dominant negative construct of Bip had no effects on the formation of the dimers. Similar results were obtained with HSP70 shRNA and no significant difference was detected in the homo- as well as heterodimeric formation. This is another illustration of how this group of proteins acts in a specific way and that even if chaperones belong to the same family, they can have distinct functions. It is noteworthy that over-expressing wild type forms of Bip and ERp57 did not enhance the formation of dimers which could

be an indicator of the availability of the required amount of chaperones for protein assembly.

GPCRs were shown to preassemble with the components of their signalling complex before being expressed at the plasma membrane. This was illustrated by showing interactions between the different components of the GPCRs signalling complexes in living cells without the need for ligand activation (55-57, 117, 135, 164). The β2-AR is known to signal through Gαs and Gαi mediated pathways while the AT1R signalling occurs via Gαi and Gαq- dependent pathways. However, the signalling via the heterodimer AT1R/β2AR has not been characterized yet. Given the role of chaperones in the early steps, their effects were tested on the coupling of the different trimeric G proteins to the Homo- and Heterodimeric complexes of β2AR and AT1R. The method used was BRET and the G proteins that were studied are Gβ1, Gγ2, Gαs and Gαi with all the different receptor dimer pairs. Unfortunately, no significant difference was detected with any of the receptors combinations. This might suggest that another group of chaperones are involved in the preassembly of dimers confirming the complexity and the differences in these oligomers compared with the monomeric receptors. Therefore, an advanced search for scaffolding proteins would be necessary. Also Gαq was not tested because no Rluc-tagged construct is currently available so more studies are needed on the effect of chaperones on the signalling complex of Gαq and the dimers.

CHAPTER 5: CONCLUSIONS

This thesis describes the influence of several factors on the assembly of receptor dimers and GPCR trafficking to the plasma membrane. Understanding the functions regulating receptor expression at the plasma membrane is a new field of research that has already provided some new therapeutic drugs, such as the pharmacological chaperones. This study intends to provide more insights in the mechanisms required for GPCR export from the ER. The results in this study demonstrate that the exocytic pathway for β2-adrenergic receptor is regulated by proteins that are either part of or similar to those regulating the endocytic pathway of GPCRs. Rab1 seems to be directing ER export of the β2-AR via a direct interaction with the c-tail portion of the receptor, specifically with the F(X)-$_6$LL motif. The results have also shown that arrestin2 is playing an important role in the expression of receptors at the plasma membrane as well. The findings of the project propose that Rab1 and arrestin2 are parts of a higher complex that might be involved in regulating the anterograde pathway.

The results also demonstrate that the ER-molecular chaperones can associate with dimeric arrangements of AT1R and β2AR with slight changes between homodimers versus heterodimers. This would suggest the specificity of chaperones towards different receptor complexes. The functions of some chaperones were determined, for example, Bip, calnexin and HSP70 mainly interact with immature dimers therefore could be part of the ERAD machinery or could facilitate the folding while ERp57 is responsible for the formation of certain dimers. Although the chaperones tested did not have an effect on G-protein coupling, this suggests that other chaperones might be involved in the tight regulation of G protein coupled receptor signalling complex assembly. More studies are required to identify such chaperones and characterize their functions.

Figure 5.1 summarizes the most important findings of the study and highlights the roles of Rab1, arrestin2 and ERp57 in the anterograde pathway and the assembly of GPCRs.

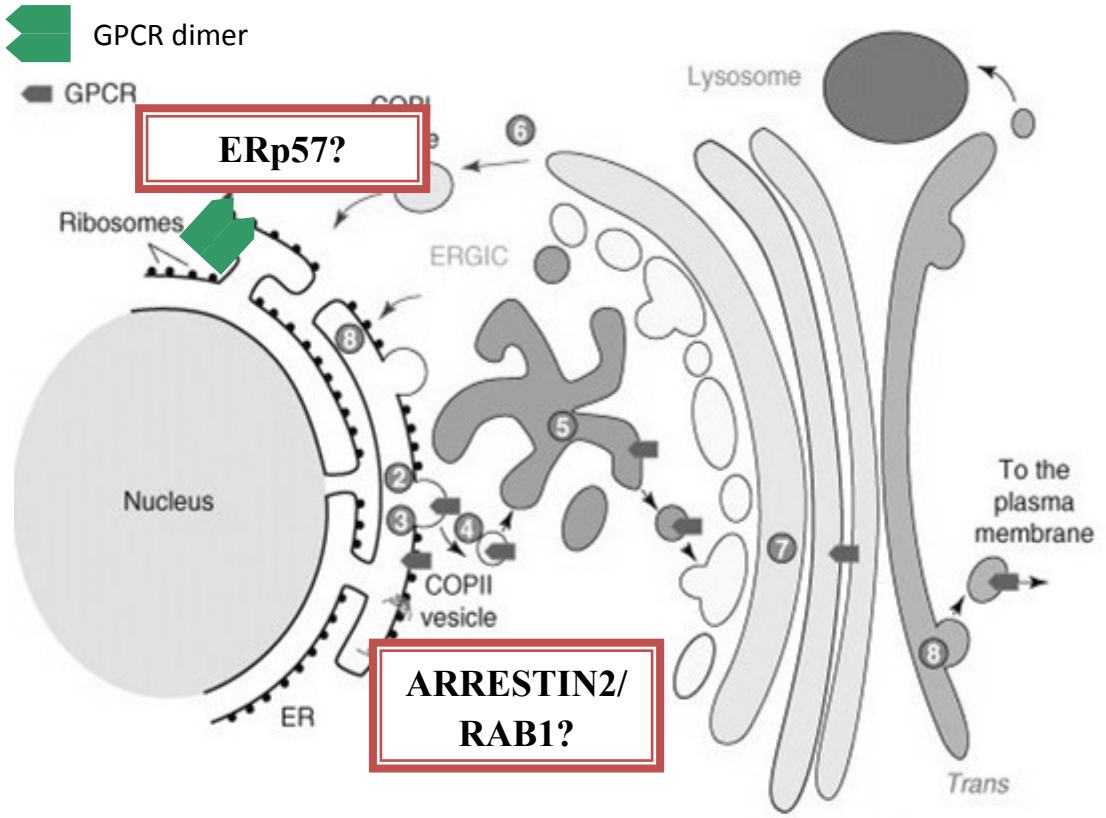


Figure 5.1: Potential roles for arrestin2, Rab1 and ERp57 in GPCRs trafficking and assembly

The results from this study highlighted the roles of arrestin2 and Rab1 in the early steps of GPCRs trafficking from ER to PM as well as ERp57 importance in the formation of some homo- and heterodimers.

References

1. Leading causes of death [homepage on the Internet]. Canada: Statistics Canada; 2010 [updated April 16, 2010; cited July 1, 2010]. Available from: http://www40.statcan.gc.ca/l01/cst01/hlth36a-eng.htm.

2. Arnold JM, Liu P, Demers C, Dorian P, Giannetti N, Haddad H, et al. Canadian cardiovascular society consensus conference recommendations on heart failure 2006: Diagnosis and management. Can J Cardiol. 2006 Jan;22(1):23-45.

3. Houser SR, Piacentino V,3rd, Weisser J. Abnormalities of calcium cycling in the hypertrophied and failing heart. J Mol Cell Cardiol. 2000 Sep;32(9):1595-607.

4. Rockman HA, Koch WJ, Lefkowitz RJ. Seven-transmembrane-spanning receptors and heart function. Nature. 2002 Jan 10;415(6868):206-12.

5. Cohn JN, Tognoni G, Valsartan Heart Failure Trial Investigators. A randomized trial of the angiotensin-receptor blocker valsartan in chronic heart failure. N Engl J Med. 2001 Dec 6;345(23):1667-75.

6. Jorde UP, Ennezat PV, Lisker J, Suryadevara V, Infeld J, Cukon S, et al. Maximally recommended doses of angiotensin-converting enzyme (ACE) inhibitors do not completely prevent ACE-mediated formation of angiotensin II in chronic heart failure. Circulation. 2000 Feb 29;101(8):844-6.

7. Kawamura M, Imanashi M, Matsushima Y, Ito K, Hiramori K. Circulating angiotensin II levels under repeated administration of lisinopril in normal subjects. Clin Exp Pharmacol Physiol. 1992 Aug;19(8):547-53.

8. Bedard K, Szabo E, Michalak M, Opas M. Cellular functions of endoplasmic reticulum chaperones calreticulin, calnexin, and ERp57. Int Rev Cytol. 2005;245:91-121.

9. Oliveira L, Costa-Neto CM, Nakaie CR, Schreier S, Shimuta SI, Paiva AC. The angiotensin II AT1 receptor structure-activity correlations in the light of rhodopsin structure. Physiol Rev. 2007 Apr;87(2):565-92.

10. Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, et al. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. Circ Res. 2000 Sep 1;87(5):E1-9.

11. Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G, Turner AJ. A human homolog of angiotensin-converting enzyme. cloning and functional expression as a captopril-insensitive carboxypeptidase. J Biol Chem. 2000 Oct 27;275(43):33238-43.

12. Sechi LA, Griffin CA, Grady EF, Kalinyak JE, Schambelan M. Characterization of angiotensin II receptor subtypes in rat heart. Circ Res. 1992 Dec;71(6):1482-9.

13. Shanmugam S, Monnot C, Corvol P, Gasc JM. Distribution of type 1 angiotensin II receptor subtype messenger RNAs in the rat fetus. Hypertension. 1994 Jan;23(1):137-41.

14. Ishii K, Takekoshi K, Shibuya S, Kawakami Y, Isobe K, Nakai T. Angiotensin subtype-2 receptor (AT2) negatively regulates subtype-1 receptor (AT1) in signal transduction pathways in cultured porcine adrenal medullary chromaffin cells. J Hypertens. 2001 Nov;19(11):1991-9.

15. Carey RM. Cardiovascular and renal regulation by the angiotensin type 2 receptor: The AT2 receptor comes of age. Hypertension. 2005 May;45(5):840-4.

16. Gendron L, Payet MD, Gallo-Payet N. The angiotensin type 2 receptor of angiotensin II and neuronal differentiation: From observations to mechanisms. J Mol Endocrinol. 2003 Dec;31(3):359-72.

17. Horiuchi M. Functional aspects of angiotensin type 2 receptor. Adv Exp Med Biol. 1996;396:217-24.

18. Stoll M, Unger T. Angiotensin and its AT2 receptor: New insights into an old system. Regul Pept. 2001 Jun 15;99(2-3):175-82.

19. Fitzsimons JT. Angiotensin, thirst, and sodium appetite. Physiol Rev. 1998 Jul;78(3):583-686.

20. Spat A, Hunyady L. Control of aldosterone secretion: A model for convergence in cellular signaling pathways. Physiol Rev. 2004 Apr;84(2):489-539.

21. Campbell SE, Katwa LC. Angiotensin II stimulated expression of transforming growth factor-beta1 in cardiac fibroblasts and myofibroblasts. J Mol Cell Cardiol. 1997 Jul;29(7):1947-58.

22. Gray MO, Long CS, Kalinyak JE, Li HT, Karliner JS. Angiotensin II stimulates cardiac myocyte hypertrophy via paracrine release of TGF-beta 1 and endothelin-1 from fibroblasts. Cardiovasc Res. 1998 Nov;40(2):352-63.

23. Eguchi S, Inagami T. Signal transduction of angiotensin II type 1 receptor through receptor tyrosine kinase. Regul Pept. 2000 Jul 28;91(1-3):13-20.

24. Gohla A, Schultz G, Offermanns S. Role for G(12)/G(13) in agonist-induced vascular smooth muscle cell contraction. Circ Res. 2000 Aug 4;87(3):221-7.

25. Barki-Harrington L, Perrino C, Rockman HA. Network integration of the adrenergic system in cardiac hypertrophy. Cardiovasc Res. 2004 Aug 15;63(3):391-402.

26. Brodde OE, Bruck H, Leineweber K. Cardiac adrenoceptors: Physiological and pathophysiological relevance. J Pharmacol Sci. 2006;100(5):323-37.

27. Lohse MJ, Engelhardt S, Eschenhagen T. What is the role of beta-adrenergic signaling in heart failure? Circ Res. 2003 Nov 14;93(10):896-906.

28. Gauthier C, Langin D, Balligand JL. Beta3-adrenoceptors in the cardiovascular system. Trends Pharmacol Sci. 2000 Nov;21(11):426-31.

29. Varghese P, Harrison RW, Lofthouse RA, Georgakopoulos D, Berkowitz DE, Hare JM. Beta(3)-adrenoceptor deficiency blocks nitric oxide-dependent inhibition of myocardial contractility. J Clin Invest. 2000 Sep;106(5):697-703.

30. Xiao RP, Zhang SJ, Chakir K, Avdonin P, Zhu W, Bond RA, et al. Enhanced G(i) signaling selectively negates beta2-adrenergic receptor (AR)--but not beta1-AR-mediated positive inotropic effect in myocytes from failing rat hearts. Circulation. 2003 Sep 30;108(13):1633-9.

31. Ponicke K, Groner F, Heinroth-Hoffmann I, Brodde OE. Agonist-specific activation of the beta2-adrenoceptor/Gs-protein and beta2-adrenoceptor/Gi-protein pathway in adult rat ventricular cardiomyocytes. Br J Pharmacol. 2006 Apr;147(7):714-9.

32. Daaka Y, Luttrell LM, Lefkowitz RJ. Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. Nature. 1997 Nov 6;390(6655):88-91.

33. Pavoine C, Behforouz N, Gauthier C, Le Gouvello S, Roudot-Thoraval F, Martin CR, et al. beta2-adrenergic signaling in human heart: Shift from the cyclic AMP to the arachidonic acid pathway. Mol Pharmacol. 2003 Nov;64(5):1117-25.

34. Engelhardt S, Bohm M, Erdmann E, Lohse MJ. Analysis of beta-adrenergic receptor mRNA levels in human ventricular biopsy specimens by quantitative polymerase chain reactions: Progressive reduction of beta 1-adrenergic receptor mRNA in heart failure. J Am Coll Cardiol. 1996 Jan;27(1):146-54.

35. Bristow MR. Why does the myocardium fail? insights from basic science. Lancet. 1998 Aug;352 Suppl 1:SI8-14.

36. Engelhardt S, Hein L, Wiesmann F, Lohse MJ. Progressive hypertrophy and heart failure in beta1-adrenergic receptor transgenic mice. Proc Natl Acad Sci U S A. 1999 Jun 8;96(12):7059-64.

37. Bisognano JD, Weinberger HD, Bohlmeyer TJ, Pende A, Raynolds MV, Sastravaha A, et al. Myocardial-directed overexpression of the human beta(1)-adrenergic receptor in transgenic mice. J Mol Cell Cardiol. 2000 May;32(5):817-30.

38. Milano CA, Allen LF, Rockman HA, Dolber PC, McMinn TR, Chien KR, et al. Enhanced myocardial function in transgenic mice overexpressing the beta 2-adrenergic receptor. Science. 1994 Apr 22;264(5158):582-6.

39. Mialet Perez J, Rathz DA, Petrashevskaya NN, Hahn HS, Wagoner LE, Schwartz A, et al. Beta 1-adrenergic receptor polymorphisms confer differential function and predisposition to heart failure. Nat Med. 2003 Oct;9(10):1300-5.

40. Green SA, Cole G, Jacinto M, Innis M, Liggett SB. A polymorphism of the human beta 2-adrenergic receptor within the fourth transmembrane domain alters ligand binding and functional properties of the receptor. J Biol Chem. 1993 Nov 5;268(31):23116-21.

41. Liggett SB, Wagoner LE, Craft LL, Hornung RW, Hoit BD, McIntosh TC, et al. The Ile164 beta2-adrenergic receptor polymorphism adversely affects the outcome of congestive heart failure. J Clin Invest. 1998 Oct 15;102(8):1534-9.

42. Gurevich VV, Gurevich EV. GPCR monomers and oligomers: It takes all kinds. Trends Neurosci. 2008 Feb;31(2):74-81.

43. Nature Reviews Drug Discovery GPCR Questionnaire Participants. The state of GPCR research in 2004. Nat Rev Drug Discov. 2004 Jul;3(7):575, 577-626.

44. Gurevich VV, Gurevich EV. How and why do GPCRs dimerize? Trends Pharmacol Sci. 2008 May;29(5):234-40.

45. Szidonya L, Cserzo M, Hunyady L. Dimerization and oligomerization of G-protein-coupled receptors: Debated structures with established and emerging functions. J Endocrinol. 2008 Mar;196(3):435-53.

46. Rozenfeld R, Devi LA. Receptor heteromerization and drug discovery. Trends Pharmacol Sci. 2010 Mar;31(3):124-30.

47. Barki-Harrington L, Luttrell LM, Rockman HA. Dual inhibition of beta-adrenergic and angiotensin II receptors by a single antagonist: A functional role for receptor-receptor interaction in vivo. Circulation. 2003 Sep 30;108(13):1611-8.

48. AbdAlla S, Lother H, el Massiery A, Quitterer U. Increased AT(1) receptor heterodimers in preeclampsia mediate enhanced angiotensin II responsiveness. Nat Med. 2001 Sep;7(9):1003-9.

49. McGraw DW, Mihlbachler KA, Schwarb MR, Rahman FF, Small KM, Almoosa KF, et al. Airway smooth muscle prostaglandin-EP1 receptors directly modulate beta2-adrenergic receptors within a unique heterodimeric complex. J Clin Invest. 2006 May;116(5):1400-9.

50. Chun HJ, Ali ZA, Kojima Y, Kundu RK, Sheikh AY, Agrawal R, et al. Apelin signaling antagonizes ang II effects in mouse models of atherosclerosis. J Clin Invest. 2008 Oct;118(10):3343-54.

51. Duvernay MT, Filipeanu CM, Wu G. The regulatory mechanisms of export trafficking of G protein-coupled receptors. Cell Signal. 2005 Dec;17(12):1457-65.

52. David M, Richer M, Mamarbachi AM, Villeneuve LR, Dupre DJ, Hebert TE. Interactions between GABA-B1 receptors and kir 3 inwardly rectifying potassium channels. Cell Signal. 2006 Dec;18(12):2172-81.

53. Dupre DJ, Robitaille M, Richer M, Ethier N, Mamarbachi AM, Hebert TE. Dopamine receptor-interacting protein 78 acts as a molecular chaperone for ggamma subunits before assembly with gbeta. J Biol Chem. 2007 May 4;282(18):13703-15.

54. Rebois RV, Robitaille M, Gales C, Dupre DJ, Baragli A, Trieu P, et al. Heterotrimeric G proteins form stable complexes with adenylyl cyclase and Kir3.1 channels in living cells. J Cell Sci. 2006 Jul 1;119(Pt 13):2807-18.

55. Petaja-Repo UE, Hogue M, Laperriere A, Walker P, Bouvier M. Export from the endoplasmic reticulum represents the limiting step in the maturation and cell surface expression of the human delta opioid receptor. J Biol Chem. 2000 May 5;275(18):13727-36.

56. Gales C, Van Durm JJ, Schaak S, Pontier S, Percherancier Y, Audet M, et al. Probing the activation-promoted structural rearrangements in preassembled receptor-G protein complexes. Nat Struct Mol Biol. 2006 Sep;13(9):778-86.

57. Nobles M, Benians A, Tinker A. Heterotrimeric G proteins precouple with G protein-coupled receptors in living cells. Proc Natl Acad Sci U S A. 2005 Dec 20;102(51):18706-11.

58. Dong C, Filipeanu CM, Duvernay MT, Wu G. Regulation of G protein-coupled receptor export trafficking. Biochim Biophys Acta. 2007 Apr;1768(4):853-70.

59. Marchese A, Chen C, Kim YM, Benovic JL. The ins and outs of G protein-coupled receptor trafficking. Trends Biochem Sci. 2003 Jul;28(7):369-76.

60. Gaborik Z, Hunyady L. Intracellular trafficking of hormone receptors. Trends Endocrinol Metab. 2004 Aug;15(6):286-93.

61. Achour L, Labbe-Jullie C, Scott MG, Marullo S. An escort for GPCRs: Implications for regulation of receptor density at the cell surface. Trends Pharmacol Sci. 2008 Oct;29(10):528-35.

62. Hebert DN, Molinari M. In and out of the ER: Protein folding, quality control, degradation, and related human diseases. Physiol Rev. 2007 Oct;87(4):1377-408.

63. Vembar SS, Brodsky JL. One step at a time: Endoplasmic reticulum-associated degradation. Nat Rev Mol Cell Biol. 2008 Dec;9(12):944-57.

64. Fewell SW, Travers KJ, Weissman JS, Brodsky JL. The action of molecular chaperones in the early secretory pathway. Annu Rev Genet. 2001;35:149-91.

65. Nishikawa S, Brodsky JL, Nakatsukasa K. Roles of molecular chaperones in endoplasmic reticulum (ER) quality control and ER-associated degradation (ERAD). J Biochem. 2005 May;137(5):551-5.

66. Hosokawa N, Wada I, Hasegawa K, Yorihuzi T, Tremblay LO, Herscovics A, et al. A novel ER alpha-mannosidase-like protein accelerates ER-associated degradation. EMBO Rep. 2001 May;2(5):415-22.

67. Marchese A, Paing MM, Temple BR, Trejo J. G protein-coupled receptor sorting to endosomes and lysosomes. Annu Rev Pharmacol Toxicol. 2008;48:601-29.

68. Yano M, Kanesaki Y, Koumoto Y, Inoue M, Kido H. Chaperone activities of the 26S and 20S proteasome. Curr Protein Pept Sci. 2005 Apr;6(2):197-203.

69. Trombetta ES, Parodi AJ. Quality control and protein folding in the secretory pathway. Annu Rev Cell Dev Biol. 2003;19:649-76.

70. Hendershot LM. The ER function BiP is a master regulator of ER function. Mt Sinai J Med. 2004 Oct;71(5):289-97.

71. Helenius A, Aebi M. Roles of N-linked glycans in the endoplasmic reticulum. Annu Rev Biochem. 2004;73:1019-49.

72. Hammond C, Braakman I, Helenius A. Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. Proc Natl Acad Sci U S A. 1994 Feb 1;91(3):913-7.

73. Parodi AJ. Role of N-oligosaccharide endoplasmic reticulum processing reactions in glycoprotein folding and degradation. Biochem J. 2000 May 15;348 Pt 1:1-13.

74. Ireland BS, Brockmeier U, Howe CM, Elliott T, Williams DB. Lectin-deficient calreticulin retains full functionality as a chaperone for class I histocompatibility molecules. Mol Biol Cell. 2008 Jun;19(6):2413-23.

75. Bansal S, Ponnan P, Raj HG, Weintraub ST, Chopra M, Kumari R, et al. Autoacetylation of purified calreticulin transacetylase utilizing acetoxycoumarin as the acetyl group donor. Appl Biochem Biotechnol. 2009 May;157(2):285-98.

76. Okazaki Y, Ohno H, Takase K, Ochiai T, Saito T. Cell surface expression of calnexin, a molecular chaperone in the endoplasmic reticulum. J Biol Chem. 2000 Nov 17;275(46):35751-8.

77. Elliott JG, Oliver JD, High S. The thiol-dependent reductase ERp57 interacts specifically with N-glycosylated integral membrane proteins. J Biol Chem. 1997 May 23;272(21):13849-55.

78. Zapun A, Darby NJ, Tessier DC, Michalak M, Bergeron JJ, Thomas DY. Enhanced catalysis of ribonuclease B folding by the interaction of calnexin or calreticulin with ERp57. J Biol Chem. 1998 Mar 13;273(11):6009-12.

79. Free RB, Hazelwood LA, Cabrera DM, Spalding HN, Namkung Y, Rankin ML, et al. D1 and D2 dopamine receptor expression is regulated by direct interaction with the chaperone protein calnexin. J Biol Chem. 2007 Jul 20;282(29):21285-300.

80. Bermak JC, Li M, Bullock C, Zhou QY. Regulation of transport of the dopamine D1 receptor by a new membrane-associated ER protein. Nat Cell Biol. 2001 May;3(5):492-8.

81. Abd Alla J, Reeck K, Langer A, Streichert T, Quitterer U. Calreticulin enhances B2 bradykinin receptor maturation and heterodimerization. Biochem Biophys Res Commun. 2009 Sep 11;387(1):186-90.

82. Mizrachi D, Segaloff DL. Intracellularly located misfolded glycoprotein hormone receptors associate with different chaperone proteins than their cognate wild-type receptors. Mol Endocrinol. 2004 Jul;18(7):1768-77.

83. Tolkovsky AM, Levitzki A. Mode of coupling between the beta-adrenergic receptor and adenylate cyclase in turkey erythrocytes. Biochemistry. 1978 Sep 5;17(18):3795.

84. Bunemann M, Frank M, Lohse MJ. Gi protein activation in intact cells involves subunit rearrangement rather than dissociation. Proc Natl Acad Sci U S A. 2003 Dec 23;100(26):16077-82.

85. Digby GJ, Lober RM, Sethi PR, Lambert NA. Some G protein heterotrimers physically dissociate in living cells. Proc Natl Acad Sci U S A. 2006 Nov 21;103(47):17789-94.

86. Dohlman HG, Thorner JW. Regulation of G protein-initiated signal transduction in yeast: Paradigms and principles. Annu Rev Biochem. 2001;70:703-54.

87. Montell C. Visual transduction in drosophila. Annu Rev Cell Dev Biol. 1999;15:231-68.

88. Gales C, Rebois RV, Hogue M, Trieu P, Breit A, Hebert TE, et al. Real-time monitoring of receptor and G-protein interactions in living cells. Nat Methods. 2005 Mar;2(3):177-84.

89. Frank M, Thumer L, Lohse MJ, Bunemann M. G protein activation without subunit dissociation depends on a G{alpha}(i)-specific region. J Biol Chem. 2005 Jul 1;280(26):24584-90.

90. Lavine N, Ethier N, Oak JN, Pei L, Liu F, Trieu P, et al. G protein-coupled receptors form stable complexes with inwardly rectifying potassium channels and adenylyl cyclase. J Biol Chem. 2002 Nov 29;277(48):46010-9.

91. Davare MA, Avdonin V, Hall DD, Peden EM, Burette A, Weinberg RJ, et al. A beta2 adrenergic receptor signaling complex assembled with the Ca2+ channel Cav1.2. Science. 2001 Jul 6;293(5527):98-101.

92. Gurkan C, Stagg SM, Lapointe P, Balch WE. The COPII cage: Unifying principles of vesicle coat assembly. Nat Rev Mol Cell Biol. 2006 Oct;7(10):727-38.

93. Aridor M, Bannykh SI, Rowe T, Balch WE. Cargo can modulate COPII vesicle formation from the endoplasmic reticulum. J Biol Chem. 1999 Feb 12;274(7):4389-99.

94. Forster R, Weiss M, Zimmermann T, Reynaud EG, Verissimo F, Stephens DJ, et al. Secretory cargo regulates the turnover of COPII subunits at single ER exit sites. Curr Biol. 2006 Jan 24;16(2):173-9.

95. Bi X, Corpina RA, Goldberg J. Structure of the Sec23/24-Sar1 pre-budding complex of the COPII vesicle coat. Nature. 2002 Sep 19;419(6904):271-7.

96. Antonny B, Gounon P, Schekman R, Orci L. Self-assembly of minimal COPII cages. EMBO Rep. 2003 Apr;4(4):419-24.

97. Lee MC, Miller EA, Goldberg J, Orci L, Schekman R. Bi-directional protein transport between the ER and golgi. Annu Rev Cell Dev Biol. 2004;20:87-123.

98. Stagg SM, Gurkan C, Fowler DM, LaPointe P, Foss TR, Potter CS, et al. Structure of the Sec13/31 COPII coat cage. Nature. 2006 Jan 12;439(7073):234-8.

99. Appenzeller-Herzog C, Hauri HP. The ER-golgi intermediate compartment (ERGIC): In search of its identity and function. J Cell Sci. 2006 Jun 1;119(Pt 11):2173-83.

100. Volpicelli-Daley LA, Li Y, Zhang CJ, Kahn RA. Isoform-selective effects of the depletion of ADP-ribosylation factors 1-5 on membrane traffic. Mol Biol Cell. 2005 Oct;16(10):4495-508.

101. Hay JC, Klumperman J, Oorschot V, Steegmaier M, Kuo CS, Scheller RH. Localization, dynamics, and protein interactions reveal distinct roles for ER and golgi SNAREs. J Cell Biol. 1998 Jun 29;141(7):1489-502.

102. Zhang T, Hong W. Ykt6 forms a SNARE complex with syntaxin 5, GS28, and Bet1 and participates in a late stage in endoplasmic reticulum-golgi transport. J Biol Chem. 2001 Jul 20;276(29):27480-7.

103. Schwartz SL, Cao C, Pylypenko O, Rak A, Wandinger-Ness A. Rab GTPases at a glance. J Cell Sci. 2007 Nov 15;120(Pt 22):3905-10.

104. Beraud-Dufour S, Balch W. A journey through the exocytic pathway. J Cell Sci. 2002 May 1;115(Pt 9):1779-80.

105. Wu G, Zhao G, He Y. Distinct pathways for the trafficking of angiotensin II and adrenergic receptors from the endoplasmic reticulum to the cell surface: Rab1-independent transport of a G protein-coupled receptor. J Biol Chem. 2003 Nov 21;278(47):47062-9.

106. Filipeanu CM, Zhou F, Claycomb WC, Wu G. Regulation of the cell surface expression and function of angiotensin II type 1 receptor by Rab1-mediated endoplasmic reticulum-to-golgi transport in cardiac myocytes. J Biol Chem. 2004 Sep 24;279(39):41077-84.

107. Filipeanu CM, Zhou F, Fugetta EK, Wu G. Differential regulation of the cell-surface targeting and function of beta- and alpha1-adrenergic receptors by Rab1 GTPase in cardiac myocytes. Mol Pharmacol. 2006 May;69(5):1571-8.

108. Dupre DJ, Hebert TE. Biosynthesis and trafficking of seven transmembrane receptor signalling complexes. Cell Signal. 2006 Oct;18(10):1549-59.

109. Barlic J, Andrews JD, Kelvin AA, Bosinger SE, DeVries ME, Xu L, et al. Regulation of tyrosine kinase activation and granule release through beta-arrestin by CXCRI. Nat Immunol. 2000 Sep;1(3):227-33.

110. Xiao K, McClatchy DB, Shukla AK, Zhao Y, Chen M, Shenoy SK, et al. Functional specialization of beta-arrestin interactions revealed by proteomic analysis. Proc Natl Acad Sci U S A. 2007 Jul 17;104(29):12011-6.

111. Kumar P, Lau CS, Mathur M, Wang P, DeFea KA. Differential effects of beta-arrestins on the internalization, desensitization and ERK1/2 activation downstream of protease activated receptor-2. Am J Physiol Cell Physiol. 2007 Jul;293(1):C346-57.

112. Bhattacharya M, Anborgh PH, Babwah AV, Dale LB, Dobransky T, Benovic JL, et al. Beta-arrestins regulate a ral-GDS ral effector pathway that mediates cytoskeletal reorganization. Nat Cell Biol. 2002 Aug;4(8):547-55.

113. Parent A, Hamelin E, Germain P, Parent JL. Rab11 regulates the recycling of the beta2-adrenergic receptor through a direct interaction. Biochem J. 2009 Feb 15;418(1):163-72.

114. Hamelin E, Theriault C, Laroche G, Parent JL. The intracellular trafficking of the G protein-coupled receptor TPbeta depends on a direct interaction with Rab11. J Biol Chem. 2005 Oct 28;280(43):36195-205.

115. Dong C, Yang L, Zhang X, Gu H, Lam ML, Claycomb WC, et al. Rab8 interacts with distinct motifs in {alpha}2B- and {beta}2-adrenergic receptors and differentially modulates their transport. J Biol Chem. 2010 Apr 27.

116. Dale LB, Seachrist JL, Babwah AV, Ferguson SS. Regulation of angiotensin II type 1A receptor intracellular retention, degradation, and recycling by Rab5, Rab7, and Rab11 GTPases. J Biol Chem. 2004 Mar 26;279(13):13110-8.

117. Dupre DJ, Robitaille M, Ethier N, Villeneuve LR, Mamarbachi AM, Hebert TE. Seven transmembrane receptor core signaling complexes are assembled prior to plasma membrane trafficking. J Biol Chem. 2006 Nov 10;281(45):34561-73.

118. Rebois RV, Hebert TE. Protein complexes involved in heptahelical receptor-mediated signal transduction. Receptors Channels. 2003;9(3):169-94.

119. Zhang X, Wang G, Dupre DJ, Feng Y, Robitaille M, Lazartigues E, et al. Rab1 GTPase and dimerization in the cell surface expression of angiotensin II type 2 receptor. J Pharmacol Exp Ther. 2009 Jul;330(1):109-17.

120. Duvernay MT, Dong C, Zhang X, Zhou F, Nichols CD, Wu G. Anterograde trafficking of G protein-coupled receptors: Function of the C-terminal F(X)6LL motif in export from the endoplasmic reticulum. Mol Pharmacol. 2009 Apr;75(4):751-61.

121. Kim YM, Benovic JL. Differential roles of arrestin-2 interaction with clathrin and adaptor protein 2 in G protein-coupled receptor trafficking. J Biol Chem. 2002 Aug 23;277(34):30760-8.

122. Laporte SA, Oakley RH, Holt JA, Barak LS, Caron MG. The interaction of beta-arrestin with the AP-2 adaptor is required for the clustering of beta 2-adrenergic receptor into clathrin-coated pits. J Biol Chem. 2000 Jul 28;275(30):23120-6.

123. Laporte SA, Oakley RH, Zhang J, Holt JA, Ferguson SS, Caron MG, et al. The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis. Proc Natl Acad Sci U S A. 1999 Mar 30;96(7):3712-7.

124. Chen CH, Paing MM, Trejo J. Termination of protease-activated receptor-1 signaling by beta-arrestins is independent of receptor phosphorylation. J Biol Chem. 2004 Mar 12;279(11):10020-31.

125. Kim OJ, Gardner BR, Williams DB, Marinec PS, Cabrera DM, Peters JD, et al. The role of phosphorylation in D1 dopamine receptor desensitization: Evidence for a novel mechanism of arrestin association. J Biol Chem. 2004 Feb 27;279(9):7999-8010.

126. Jala VR, Shao WH, Haribabu B. Phosphorylation-independent beta-arrestin translocation and internalization of leukotriene B4 receptors. J Biol Chem. 2005 Feb 11;280(6):4880-7.

127. Lee C, Bhatt S, Shukla A, Desnoyer RW, Yadav SP, Kim M, et al. Site-specific cleavage of G protein-coupled receptor-engaged beta-arrestin. influence of the AT1 receptor conformation on scissile site selection. J Biol Chem. 2008 Aug 1;283(31):21612-20.

128. Seibold A, Williams B, Huang ZF, Friedman J, Moore RH, Knoll BJ, et al. Localization of the sites mediating desensitization of the beta(2)-adrenergic receptor by the GRK pathway. Mol Pharmacol. 2000 Nov;58(5):1162-73.

129. Ferguson SS, Zhang J, Barak LS, Caron MG. Molecular mechanisms of G protein-coupled receptor desensitization and resensitization. Life Sci. 1998;62(17-18):1561-5.

130. Jiang S, Storrie B. Cisternal rab proteins regulate golgi apparatus redistribution in response to hypotonic stress. Mol Biol Cell. 2005 May;16(5):2586-96.

131. Martinez O, Schmidt A, Salamero J, Hoflack B, Roa M, Goud B. The small GTP-binding protein rab6 functions in intra-golgi transport. J Cell Biol. 1994 Dec;127(6 Pt 1):1575-88.

132. Del Nery E, Miserey-Lenkei S, Falguieres T, Nizak C, Johannes L, Perez F, et al. Rab6A and Rab6A' GTPases play non-overlapping roles in membrane trafficking. Traffic. 2006 Apr;7(4):394-407.

133. Chen W, Wandinger-Ness A. Expression and functional analyses of Rab8 and Rab11a in exocytic transport from trans-golgi network. Methods Enzymol. 2001;329:165-75.

134. Ullrich O, Reinsch S, Urbe S, Zerial M, Parton RG. Rab11 regulates recycling through the pericentriolar recycling endosome. J Cell Biol. 1996 Nov;135(4):913-24.

135. Dupre DJ, Baragli A, Rebois RV, Ethier N, Hebert TE. Signalling complexes associated with adenylyl cyclase II are assembled during their biosynthesis. Cell Signal. 2007 Mar;19(3):481-9.

136. Duvernay MT, Zhou F, Wu G. A conserved motif for the transport of G protein-coupled receptors from the endoplasmic reticulum to the cell surface. J Biol Chem. 2004 Jul 16;279(29):30741-50.

137. Gabilondo AM, Hegler J, Krasel C, Boivin-Jahns V, Hein L, Lohse MJ. A dileucine motif in the C terminus of the beta2-adrenergic receptor is involved in receptor internalization. Proc Natl Acad Sci U S A. 1997 Nov 11;94(23):12285-90.

138. Aridor M, Balch WE. Kinase signaling initiates coat complex II (COPII) recruitment and export from the mammalian endoplasmic reticulum. J Biol Chem. 2000 Nov 17;275(46):35673-6.

139. Kim PS, Arvan P. Endocrinopathies in the family of endoplasmic reticulum (ER) storage diseases: Disorders of protein trafficking and the role of ER molecular chaperones. Endocr Rev. 1998 Apr;19(2):173-202.

140. Terrillon S, Bouvier M. Roles of G-protein-coupled receptor dimerization. EMBO Rep. 2004 Jan;5(1):30-4.

141. Kerppola TK. Visualization of molecular interactions by fluorescence complementation. Nat Rev Mol Cell Biol. 2006 Jun;7(6):449-56.

142. Kerppola TK. Bimolecular fluorescence complementation (BiFC) analysis as a probe of protein interactions in living cells. Annu Rev Biophys. 2008;37:465-87.

143. Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K, Miyawaki A. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nat Biotechnol. 2002 Jan;20(1):87-90.

144. Lanctot PM, Leclerc PC, Escher E, Guillemette G, Leduc R. Role of N-glycan-dependent quality control in the cell-surface expression of the AT1 receptor. Biochem Biophys Res Commun. 2006 Feb 10;340(2):395-402.

145. Leclerc PC, Auger-Messier M, Lanctot PM, Escher E, Leduc R, Guillemette G. A polyaromatic caveolin-binding-like motif in the cytoplasmic tail of the type 1 receptor for angiotensin II plays an important role in receptor trafficking and signaling. Endocrinology. 2002 Dec;143(12):4702-10.

146. George SR, Fan T, Xie Z, Tse R, Tam V, Varghese G, et al. Oligomerization of mu- and delta-opioid receptors. generation of novel functional properties. J Biol Chem. 2000 Aug 25;275(34):26128-35.

147. Mellado M, Rodriguez-Frade JM, Vila-Coro AJ, Fernandez S, Martin de Ana A, Jones DR, et al. Chemokine receptor homo- or heterodimerization activates distinct signaling pathways. EMBO J. 2001 May 15;20(10):2497-507.

148. Schrag JD, Bergeron JJ, Li Y, Borisova S, Hahn M, Thomas DY, et al. The structure of calnexin, an ER chaperone involved in quality control of protein folding. Mol Cell. 2001 Sep;8(3):633-44.

149. Mialet-Perez J, Green SA, Miller WE, Liggett SB. A primate-dominant third glycosylation site of the beta2-adrenergic receptor routes receptors to degradation during agonist regulation. J Biol Chem. 2004 Sep 10;279(37):38603-7.

150. Jayadev S, Smith RD, Jagadeesh G, Baukal AJ, Hunyady L, Catt KJ. N-linked glycosylation is required for optimal AT1a angiotensin receptor expression in COS-7 cells. Endocrinology. 1999 May;140(5):2010-7.

151. Lanctot PM, Leclerc PC, Escher E, Leduc R, Guillemette G. Role of N-glycosylation in the expression and functional properties of human AT1 receptor. Biochemistry. 1999 Jul 6;38(27):8621-7.

152. Siffroi-Fernandez S, Giraud A, Lanet J, Franc JL. Association of the thyrotropin receptor with calnexin, calreticulin and BiP. efects on the maturation of the receptor. Eur J Biochem. 2002 Oct;269(20):4930-7.

153. Melnick J, Dul JL, Argon Y. Sequential interaction of the chaperones BiP and GRP94 with immunoglobulin chains in the endoplasmic reticulum. Nature. 1994 Aug 4;370(6488):373-5.

154. Swanton E, High S, Woodman P. Role of calnexin in the glycan-independent quality control of proteolipid protein. EMBO J. 2003 Jun 16;22(12):2948-58.

155. Danilczyk UG, Williams DB. The lectin chaperone calnexin utilizes polypeptide-based interactions to associate with many of its substrates in vivo. J Biol Chem. 2001 Jul 6;276(27):25532-40.

156. Arunachalam B, Cresswell P. Molecular requirements for the interaction of class II major histocompatibility complex molecules and invariant chain with calnexin. J Biol Chem. 1995 Feb 10;270(6):2784-90.

157. Baksh S, Burns K, Andrin C, Michalak M. Interaction of calreticulin with protein disulfide isomerase. J Biol Chem. 1995 Dec 29;270(52):31338-44.

158. van Leeuwen JE, Kearse KP. Calnexin associates exclusively with individual CD3 delta and T cell antigen receptor (TCR) alpha proteins containing incompletely trimmed glycans that are not assembled into multisubunit TCR complexes. J Biol Chem. 1996 Apr 19;271(16):9660-5.

159. Zhang Q, Tector M, Salter RD. Calnexin recognizes carbohydrate and protein determinants of class I major histocompatibility complex molecules. J Biol Chem. 1995 Feb 24;270(8):3944-8.

160. Denzel A, Molinari M, Trigueros C, Martin JE, Velmurgan S, Brown S, et al. Early postnatal death and motor disorders in mice congenitally deficient in calnexin expression. Mol Cell Biol. 2002 Nov;22(21):7398-404.

161. Mesaeli N, Nakamura K, Zvaritch E, Dickie P, Dziak E, Krause KH, et al. Calreticulin is essential for cardiac development. J Cell Biol. 1999 Mar 8;144(5):857-68.

162. Eggleton P. Stress protein-polypeptide complexes acting as autoimmune triggers. Clin Exp Immunol. 2003 Oct;134(1):6-8.

163. Lukov GL, Baker CM, Ludtke PJ, Hu T, Carter MD, Hackett RA, et al. Mechanism of assembly of G protein betagamma subunits by protein kinase CK2-phosphorylated phosducin-like protein and the cytosolic chaperonin complex. J Biol Chem. 2006 Aug 4;281(31):22261-74.

164. Robitaille M, Dupre DJ, Hebert TE. The role of molecular chaperones in the assembly of heterotrimeric G proteins. Med Sci (Paris). 2009 Oct;25(10):821-5.

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