

Regulation of by *N*-Formyl Peptide Receptor Signaling and Trafficking by Arrestins Initiates or Prevents Apoptosis

Comprehensive Exam Proposal**

Brant M. Wagener*
MD/PhD Student

University of New Mexico
School of Medicine

Albuquerque, NM 87131

*Correspondence:

Phone Number: 272-1496

E-mail: bwagener@salud.unm.edu

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1.1 ABSTRACT

G protein-coupled receptors (GPCRs) comprise the largest family of cell surface receptors in the human genome and are the therapeutic target of ~60% of pharmaceuticals currently prescribed by physicians. Different members of this superfamily are found in essentially all tissues and are involved in numerous physiologic and pathologic processes.

While GPCRs interact with many proteins, one group that is beginning to stand out is arrestins. Arrestins are involved with trafficking, desensitization and regulation of scaffold signaling of GPCRs. Many reports have described GPCR trafficking, signaling and survival defects in cells lacking arrestins.

Two recent reports have demonstrated N-formyl peptide receptor (FPR)-mediated apoptosis and receptor recycling defects in cells lacking arrestin-2 and -3. Both of these defects were rescued by transient transfection of arrestin-2 or arrestin-3 cDNAs. In preliminary data, we reintroduced important structural regions of arrestin-2 and limited the region likely responsible for FPR-mediated apoptosis to the tail of the protein (amino acids 383-418). Also, inhibitors of the Src kinase/mitogen-activated protein kinase (MAPK) signaling cascade resulted in inhibition of FPR-mediated apoptosis. Finally, FPR mutants defective in internalization upon ligand binding could not initiate apoptosis indicating that FPR internalization is a necessary component of FPR-mediated apoptosis in the absence of arrestins.

Our proposal focuses on these defects in murine embryonic fibroblasts (MEF) lacking both arrestin-2 and arrestin-3 that stably and transiently express the FPR. We have generated ten arrestin mutants, focusing on its tail. Using these mutants and cell biology and biochemical approaches, we hope to elucidate arrestin-dependent mechanisms of FPR signaling and trafficking that lead to apoptosis.

1.2 HYPOTHESIS

Specific regions of arrestin are responsible for FPR trafficking and signaling which control its apoptotic phenotype.

Based on results from previous reports and preliminary data, we hypothesize that the tail of arrestin (amino acids 383-419) controls FPR-mediated apoptosis by regulating FPR signaling and trafficking. We further hypothesize that arrestin controls the temporal and spatial signaling of the Src kinase/MAPK signaling complex, which is dysregulated in the absence of arrestin resulting in apoptosis. The goal of this proposal is to outline experiments that will further understand the mechanisms of FPR-mediated apoptosis.

1.3 SPECIFIC AIMS

AIM 1: Characterize regions in the tail of arrestin that regulate FPR-mediated apoptosis.

Preliminary data have narrowed the likely region of arrestin responsible for FPR-mediated apoptosis to the tail (amino acids 383-418). We will use alanine scanning mutagenesis and previously described mutations in the tail of arrestin to determine regions responsible for apoptosis. MEFs lacking both arrestins will be transiently transfected with these arrestin mutants fused to GFP. Transfected cells will then be assayed by previously described techniques to determine regions of arrestin that do not rescue the apoptotic phenotype.

AIM 2: Determine the role of arrestin-mediated Src/MAPK signaling and interactions in FPR-mediated apoptosis.

Previous lab reports have described MAPK signaling through Src kinase may contribute to the FPR-mediated apoptotic phenotype. This report used inhibitors of Src kinase and MAPK signaling components and found FPR-mediated apoptosis was inhibited. We aim to demonstrate that Src kinase

and its downstream effectors are involved in FPR-mediated apoptosis in the absence of arrestins and determine how their temporal/spatial dysregulation contributes to this phenotype.

AIM 3: Determine the contributions of FPR signaling and trafficking defects to the apoptotic phenotype.

Previous reports and preliminary data demonstrate that there are both trafficking and signaling defects associated with FPR-mediated apoptosis in the absence of arrestins or presence of arrestin mutants. This includes accumulation of the FPR in a Rab11-positive recycling endosome, impaired recycling of the receptor and Src kinase/MAPK signaling-dependent apoptosis. We hypothesize that the trafficking defects of the receptor are causing the accumulation of FPR-mediated signaling complexes, which in the absence of arrestin, are aberrant, uncontrolled and leading to the initiation of apoptosis. Experiments in this aim will be designed to test this hypothesis.

2.1 BACKGROUND AND SIGNIFICANCE

2.1.1 GPCR Overview

GPCRs are the largest family (600-1000) of cell surface receptors in the human body [1, 2]. They are found in virtually every tissue in the body and regulate a variety of physiologic responses. In addition, they are gaining notoriety for their involvement in a variety of disease states including immune dysfunction [3], heart disease [4] and cancer metastasis [5].

Recent reviews have extensively described the signaling and trafficking of GPCRs [6-8]. To summarize, these receptors bind ligand extracellularly, changing their conformation to an activated state that binds heterotrimeric G proteins intracellularly and mobilizes a variety of secondary messengers. These second messengers include, but are not limited to, calcium and cyclic-adenosine monophosphate. These secondary messengers have a multitude of effects depending on cell type and the intracellular milieu.

After activation of G protein signaling cascades, GPCRs are phosphorylated at serine and threonine residues in the cytoplasmic, C-terminal tail of the receptor and/or intracellular loops by receptor kinases. This phosphorylation lowers the receptor's affinity for G proteins and increases its affinity for arrestins. Arrestin binding to phosphorylated receptor sterically blocks the association of G proteins with activated receptor, thereby effectively stopping the G protein signaling cascade. This process is known as desensitization.

After or during desensitization, the receptor/arrestin complex is bound by proteins of the internalization machinery including, but not limited to, adaptor protein-2 (AP-2) [9], clathrin [10-12] and dynamin [13]. Scaffolds, which induce signaling through the MAPK signaling cascades (extracellular signal-regulated kinase (ERK) 1/2 and c-jun n-terminal kinase (JNK) 3) are formed [14-16]. The internalization machinery aids the internalization of the receptor/arrestin complex where it generally has one of two fates: degradation or resensitization and recycling [17]. These details are further outlined in Appendix I.

2.1.2 N-Formyl Peptide Receptor

The FPR is a chemoattractant GPCR found commonly on macrophages and neutrophils [18]. It is one of the best described chemoattractant GPCRs and its functions in the immune system include regulation of adhesion, chemotaxis, superoxide production and degranulation [18]. The FPR induces ERK1/2 phosphorylation upon activation with ligand [19]. It can also be found expressed in neuromuscular, vascular and endocrine tissues [20], fibroblasts [21] and hepatic cells [22] although its role in these tissues is not well understood. It has recently been discovered that FPR activation induces trafficking and signaling defects as well as apoptosis in the absence of one of its activated state binding partners, arrestin [23, 24].

2.1.3 *Arrestin*

Arrestin was long thought to only play a role in the desensitization of G protein signaling of activated GPCRs [6]. In the mid-1990s, reports began to describe a role for arrestin in trafficking of GPCRs and the formation of signaling scaffolds that could regulate the temporal and spatial signaling of kinase cascades [14-16]. Since then, numerous reports have described the importance of arrestin in internalization of activated GPCRs. In addition, it has been demonstrated that arrestin interacts with members of the internalization apparatus and signaling molecules including AP-2, clathrin, Src kinase, ERK1/2 and JNK3 in response to activation of different GPCRs.

For instance, AP-2 and clathrin binding to arrestin are necessary for ligand-induced β_2 -adrenergic receptor (β_2 AR) internalization (although AP-2 is not as critical) [9]. Binding of Src kinase to arrestin is necessary for β_2 AR-mediated ERK1/2 activation [25] and is necessary for AP-2/clathrin interactions with arrestin for Angiotensin II Type I receptor (AT1R) internalization [26]. ERK1/2 phosphorylates serine-412 in the tail of arrestin and lack of dephosphorylation of this serine leaves the β_2 AR unable to internalize [27, 28]. However, this dephosphorylation is not required for receptor binding or desensitization. Finally, JNK3 has been shown to bind arrestin-3 upon activation of the AT1R [29].

2.1.4 *GPCR Trafficking*

Upon activation of GPCRs, a number of proteins can be recruited to the plasma membrane to aid internalization of activated GPCRs from the cell surface [30, 31]. While it was believed earlier that many or all GPCRs were dependent on the same families of molecules to aid internalization, more recent reports have described a variety of determinants for GPCR internalization [32]. For example, GPCRs can move into clathrin-coated pits or undergo clathrin-independent internalization. Many GPCRs are dependent upon dynamin [33], AP-2 [34], arrestin [35] or ARF6 [36]. Others are dependent upon none, some or all of these factors. At this time, it remains unclear which proteins are utilized for FPR endocytosis.

After removal from the cell surface, endosomes formed during internalization need to be directed to different locations for further processing. These cellular functions are controlled in part by the Rab GTPases, including Rab4, 5, 7 and 11 [37]. These GTPases cycle between a GTP-bound form that is an active protein and a GDP-bound state that is primarily inactive. GTP-GDP cycling is regulated by GTP hydrolysis to GDP and release of GDP to bind GTP. These reactions are aided by accessory proteins that regulate the rate of GTP binding or GTP hydrolysis. Understanding the Rab GTPase pathway allows investigators to understand where GPCRs are located at specific times of GPCR processing.

Generally, endosomes derived from the plasma membrane contain Rab5. Rab5-positive endosomes are generally indicative of early endosomes. Some GPCRs are dependent upon Rab5 for internalization [38], while there are some that do not require Rab5 for internalization, but do colocalize with it at later time points (see 3.3.2). After internalizing via a Rab5-positive endosome, GPCRs follow three main pathways. They can translocate to 1) a Rab4-positive endosome indicative of an early recycling pathway, 2) a Rab7-positive endosome that will lead to degradation of the cargo by the lysosome or 3) a Rab11-positive endosome indicative of a late recycling pathway [37]. This model is summarized in Appendix II.

2.1.5 *GPCRs and Apoptosis*

Apoptosis is programmed cell death that can be beneficial to organisms in many ways [39]. It is a process used during fetal development and to remove excess tissue from organs after completion of certain tasks (i.e. removal of extra epithelial cells and ducts in breast tissue after breast-feeding is discontinued or endometrial breakdown during the menstrual cycle). In addition, apoptosis has also been described in pathologic processes, including reperfusion injuries resulting from cerebral or myocardial ischemia [40-42].

Classic apoptosis is an organized process that has some defining steps [39]. To summarize, a signal is seen by the cell that initiates apoptotic signaling in the cell. These signals can range from death ligands (i.e. Fas-ligand and tumor necrosis factor) to withdrawal of growth factors or hormones to noxious stimuli, including radiation, toxins or free radicals. These signals initiate release of cytochrome c from the intermembranous space of mitochondria. Cytochrome c activates members of the caspase cascade of proteins (the “executioners” of the cell) and this activation is regulated by pro-apoptotic and anti-apoptotic members of the Bcl-2 family. These activated caspases, classically caspase-9 and caspase-3, activate a variety of proteases and endonucleases that degrade critical components of the cell. Finally, the cell forms apoptotic “bodies” and is recognized by phagocytes for removal from the tissue.

The first GPCR discovered to initiate apoptosis when activated was rhodopsin [43]. When chronically activated by light, rhodopsin forms a stable complex with arrestin (arrestin-1 or rod-arrestin), and rod cells undergo degeneration and apoptosis. This is considered a possible mechanism for macular degeneration. In contrast, other GPCRs have been described that initiate apoptosis in the absence of arrestins, including the FPR [23] and IL-8R [44]. Stimulation of the FPR in the absence of arrestins has been linked to trafficking defects [24] and possible overactivation of the Src-kinase-MAPK signaling cascades since use of inhibitors of this pathway rescue the apoptotic phenotype. IL-8R activation in the absence of arrestins has demonstrated increased signaling through ERK1/2 and JNK3 and normal p38 activation by Western analysis. These MAPKs may be integral players in FPR and/or IL-8R-mediated apoptosis.

2.1.6 *Model of FPR-mediated apoptosis in the absence of arrestins.*

First, the FPR was demonstrated to internalize in the absence of arrestins [24]. Two reports from our laboratory demonstrated that activated FPR has trafficking defects, does not recycle to the cell surface and initiates apoptosis in the absence of arrestins [23, 24]. Reconstituting wild-type arrestin-2, -3 or both to arrestin-deficient cells expressing the FPR rescued the trafficking and apoptotic phenotypes. In addition, inhibitors of Src family kinases and MAPKs (p38, ERK1/2 and JNK3) rescued the apoptotic phenotype.

We propose that in the absence of arrestins, activated FPR internalizes from the cell surface and merges with a Rab5-positive endosome. The receptor eventually transfers to a Rab11-positive endosome indicative of the recycling compartment. Normally, in the presence of arrestins, the receptor/arrestin complex would be dissociated and the FPR would travel back to the cell surface. However, in the absence of arrestins, the receptor accumulates in the Rab11-positive endosome in a perinuclear location. We hypothesize in this Rab11-positive signaling compartment, FPR continuously activates signaling complexes (MAPKs) leading to the initiation of apoptosis. This model is summarized in Appendix III. The aims presented hereafter are designed to determine regions of arrestin are responsible for these defects and the mechanisms by which they initiate apoptosis.

2.2 SIGNIFICANCE

These studies will help us to better understand the role arrestin and other proteins play in GPCR trafficking and signaling that lead to apoptosis. In addition, these studies can be used as a model to better understand GPCR-mediated apoptosis, signaling and trafficking in a variety of disease states including cancer. Finally, understanding the role of arrestins in GPCR-mediated apoptosis, trafficking and signaling will allow the design of novel chemotherapeutics to target cancer cells abnormally expressing GPCRs including, but not limited to, CXCR4 and IL-8R [5, 45].

3 PRELIMINARY DATA

3.1 Specific Aim 1.

3.1.1 Structural domains of arrestin-2 do not rescue FPR-mediated apoptosis.

Because a previous report has demonstrated that FPR-mediated apoptosis is arrestin-dependent, we wanted to determine which regions of arrestin-2 were responsible. Therefore, four

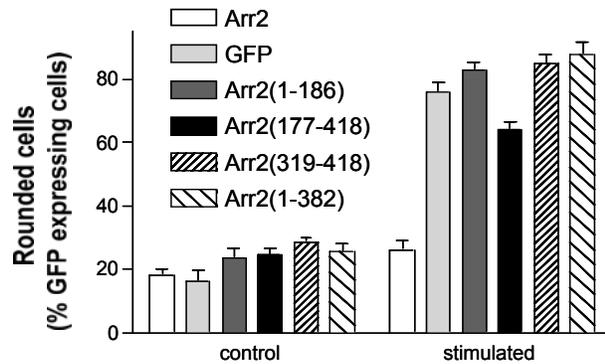


Figure 1. Structural regions of arrestin-2 do not rescue FPR-mediated apoptosis. KOFPRs were transiently transfected with important structural regions of arrestin fused to GFP and assayed for cell rounding. GFP expressing cells were randomly viewed and counted for normal architecture or rounding. Data are expressed as rounded cells/GFP expressing cell (%). Three hundred cells were counted per experiment and these results are the product of three independent experiments.

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383      390      400      410      418
DDDIVFED FARQLKGMK DDKEEEDGT GSPRLNDR - native sequence
DDDAAAA FARQLKGMK DDKEEEDGT GSPRLNDR - arr2-3A-GFP
AAAAAAA FARQLKGMK DDKEEEDGT GSPRLNDR - arr2-8A-GFP
DDDIVFED AAAAAAGMK DDKEEEDGT GSPRLNDR - arr2-6A-GFP
DDDIVFED FARQLAAAA DDKEEEDGT GSPRLNDR - arr2-4A-GFP
DDDIVFED FARQLKGMK AAAAAAAGT GSPRLNDR - arr2-8A'-GFP
DDDIVFED FARQLKGMK DDKEEEDGT GAPRLNDR - arr2-5A-GFP
DDDIVFED FARQLKGMK DDKEEEDAA AAAAAAA - arr2-10A-GFP
DDDIVFED FARQLKGMK DDKEEEDGT GDPRLNDR - arr2-S412D-GFP
DDDIVFED AARQLKGMK DDKEEEDGT GSPRLNDR - arr2-F391A-GFP

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-There is a clathrin sequence just before this region (LIELD) that has also been deleted - arr2-ΔLIELD-GFP.

Figure 2. Map of arrestin-2 and mutants generated by PCR mutagenesis. Arrestin mutants were designed based on previously known mutations or by changing qualitatively similar amino acids to alanine. Mutants were generated with PCR mutagenesis and subcloned into pEGFP-N1 or mRFP1.

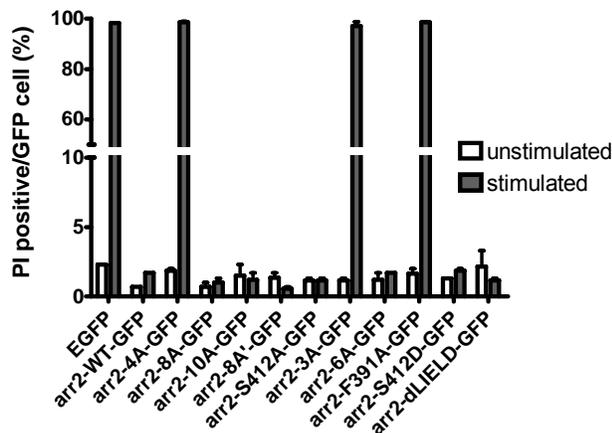


Figure 3. Regions of arrestin that are responsible for FPR-mediated apoptosis. Transiently transfected KOFPRs were plated on glass coverslips and stimulated with 10nM 633-6pep for five hours. Cells were stained with 100pg/μL PI, washed, fixed, mounted and assayed for percentage PI positive cells/GFP expressing cells. Data expressed as mean ±SEM and are representative of two independent experiments.

important structural domains of arrestin-2 (1-186, 177-418, 383-418 and 1-382) were amplified using PCR and subcloned into pEGFP-N1 vector using HindIII/ApaI restriction sites. MEFs lacking arrestin-2 and -3 stably transfected with the FPR (KOFPR) were transiently transfected with one of the above mutants and plated on glass coverslips. Cells were then stimulated with fNle-Leu-Phe-Nle-Tyr-Lys-Alexa633 (633-6pep) for five hours, fixed, mounted and viewed by confocal fluorescence microscopy.

Figure 1 shows the results of these experiments. As can be seen, none of the transiently transfected cell lines underwent cell rounding when exposed only to serum-free medium (SFM). However, when transfected KOFPRs are stimulated with agonist, cells round in the presence of all arrestin constructs except for arr-2 WT, which rescues the FPR-mediated apoptotic phenotype consistent with previous reports. Of the regions tested, 1-382 has previously been shown to be able to bind activated receptor [46]. This result demonstrates that although none of the structural regions rescued the FPR-mediated phenotype, the 1-382 mutant is important because it can bind receptor. By binding receptor, but not rescuing apoptosis, this mutant indicates that the region of arrestin likely responsible for regulating FPR-mediated apoptosis is in the tail of arrestin (amino acids 383-419).

3.1.2 Design of arrestin-2 mutants.

Mutants of arrestin-2 were designed to narrow regions in the tail that regulate FPR-mediated apoptosis (Figure 2). Some of these mutants were made based on previously known mutations of arrestin that prevent interaction with AP-2 [9], ERK1/2 [27], and clathrin [10-12] or were known to be constitutively active (arr2-3A) [47] Arr2-3A has trafficking defects that lead to impaired recycling [48]. The remainder of the mutants were designed by mutating qualitatively similar amino acids in regions of arrestin to alanine. These mutants were created by PCR mutagenesis and cloned into HindIII/ApaI sites into pEGFP-N1 vector or pmRFP1. All mutants were confirmed by DNA sequencing.

3.1.3 FPR-mediated apoptosis is controlled by specific regions in the tail of arrestin.

Our lab has demonstrated the importance of arrestin in preventing GPCR-mediated apoptosis. Preliminary data has localized the region of arrestin responsible for this phenotype to amino acids 383-418. We have further narrowed the regions that may be responsible by analyzing transiently transfected KOFPRs for uptake of propidium iodide after stimulation (5hr) with 633-6pep or SFM alone. Three hundred arrestin mutant-expressing KOFPRs (green) were randomly viewed via confocal fluorescence microscopy. Those cells were noted to be propidium iodide positive or negative. Results are shown in Figure 3 and are represented as the percentage of PI positive cells per GFP positive cell. Empty GFP vector or arrestin-2-WT-GFP were used as controls. In addition, KOFPRs and wild-type littermates stably expressing the FPR (WTFPR) were assayed as well and are consistent with previously published results (data not shown) [23].

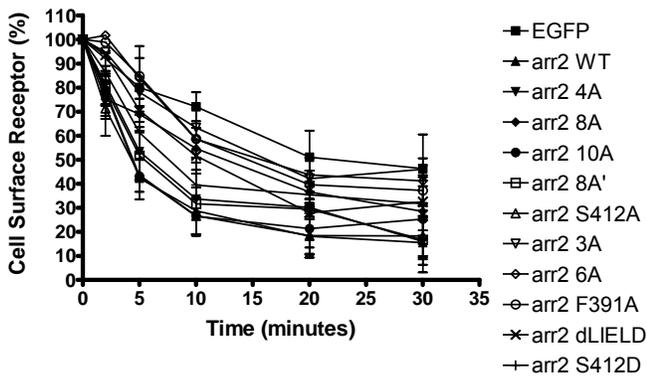


Figure 4. Internalization of the FPR in presence of arrestin-2 mutants. Transiently transfected KFs were stimulated with 1 μ M fMLF, aliquoted at time points shown, washed 3 times with SFM and labeled with 633-6pep for analysis by flow cytometry. Data are expressed as mean \pm SEM and are representative of three independent experiments.

3.1.4 Internalization of arrestin-2 mutants.

To determine whether FPR internalization was required to generate apoptosis we assayed the internalization of the FPR in the presence of GFP-fused arrestin-2 mutants generated above. KOFPRs transiently transfected with GFP-fused arrestin-2 mutants were assayed for FPR internalization as described in 4.4.4. The FPR internalizes in the presence of arrestin-2 mutants as well or better than (EGFP) empty vector (Figure 3). At this time, we conclude that any arrestin-2 mutant that will inhibit FPR-mediated apoptosis is doing so for some other reason than preventing the FPR from leaving the cell surface. Untransfected KOFPRs and WTFPRs were also assayed and consistent with previous published results (data not shown) [24].

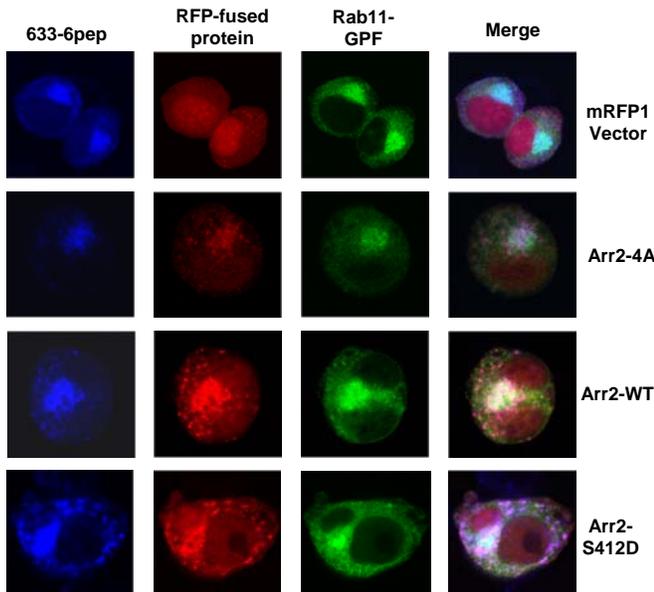


Figure 5. Arrestin mutants incapable of rescuing apoptosis accumulate in a Rab11-positive, perinuclear location. Transiently transfected KOFPRs were plated on glass coverslips, stimulated with 633-6pep for 1 hour, fixed, mounted and viewed by confocal fluorescence microscopy. Representative images are shown and are indicative of one experiment.

3.1.5 Arrestin mutants that do not rescue apoptosis accumulate in a Rab11-positive, perinuclear location.

Previous results have demonstrated that in stimulated KOFPRs, the FPR accumulates in a perinuclear location [24]. This result was further described as being a Rab11-positive endosome indicative of a recycling compartment. The report also noted that the result corresponded with an inability of the receptor to recycle. We sought to understand the relationship between arrestin-2 mutants that do not rescue apoptosis and their trafficking with respect to Rab11. Experiments were conducted in which KOFPRs were transiently transfected with Rab11-WT-GFP and various RFP-fused arrestin mutants. Transfected cells were then plated on glass coverslips, stimulated for 1 hour with 633-6pep, fixed and mounted. Cells were imaged by confocal fluorescence microscopy. As can be seen in Figure 5, in KOFPRs transfected with mRFP

vector (empty), the FPR accumulates in the Rab11-positive endosome in a perinuclear location. In cells transfected with wild-type arrestin, the receptor/arrestin complex is found colocalized with Rab11, but significant amounts are also found in other, more peripheral areas of the cell. This is indicative of the receptor's ability to traffic normally. Also, the arrestin-2-4A mutant (which does not rescue apoptosis) behaves similarly to arrestin-deficient cells with respect to FPR trafficking while the arrestin-2-S412D mutant (which does rescue apoptosis) behaves like cells expressing wild-type arrestin. Although not shown, the remainder of the arrestin mutants behave in a manner consistent with their ability to rescue FPR-mediated apoptosis. Therefore, normal trafficking of the FPR appears to predict arrestin's ability to rescue apoptosis.

3.2 Specific Aim 2.

3.2.1 Arrestin mutants deficient in ability to bind Src kinase do not rescue apoptosis.

Our hypothesis that FPR-mediated apoptosis involves Src kinase and MAPK signaling led to the construction of an arrestin mutant previously reported to be deficient in binding Src kinase [25]. To generate this mutant we changed two prolines in arrestin found in SH3-binding domains (P-X-X-P), P91G and P121E (hereafter referred to as "2Pro"). We first assayed FPR-mediated apoptosis in the presence of this mutant as described in 3.1.3 and found it did not rescue apoptosis (data not shown). Next, we assayed internalization and trafficking of the receptor/arrestin complex in the presence of the double proline mutant as described in 3.1.4 and 3.1.5. The 2Pro mutant did internalize and it accumulated in a Rab11-positive, perinuclear location as described above (data not shown). Although this data may seem to conflict with previous reports (that Src inhibitors inhibit apoptosis whereas the 2Pro presumably does not bind and activate Src and does not rescue apoptosis), we have designed experiments below to resolve this apparent conflict.

3.3 Specific Aim 3.

3.3.1 MAPK activity does not distinguish the receptor/arrestin complex and its colocalization with Rab11 in a perinuclear location.

Inhibitors of the MAPK cascade can inhibit FPR-mediated apoptosis. While the inhibition of aberrant cascades may provide clues to understanding this mechanism, we sought to understand what, if any, effect the loss of MAPK signaling would have on the receptor/arrestin complex and its trafficking pattern. This answer will help us understand whether the trafficking defect initiates the signaling defect leading to apoptosis or vice-versa.

To answer this question, we used an inhibitor of MAP kinase kinase (MEK), PD98059, which inhibits downstream activation of ERK1/2. KOFPRs transiently transfected with Rab11-GFP and mRFP1 vector or arrestin-2-WT-RFP were assayed by fluorescence microscopy. In Figure 6, transiently transfected KOFPRs were exposed to PD98059, stimulated with fluorescent ligand and imaged by confocal fluorescence microscopy. As can be seen, the FPR is highly colocalized with Rab11 in the presence of empty

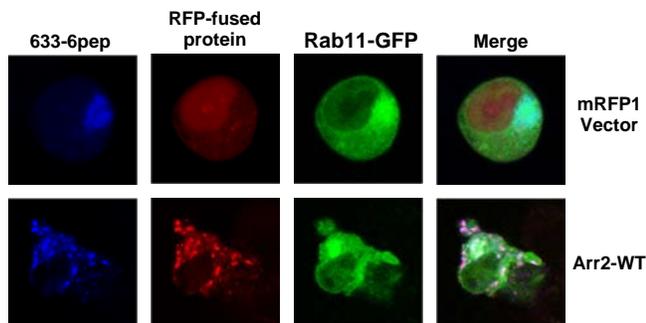


Figure 6. The MEK inhibitor, PD98059, does not cause dissociation of the receptor/arrestin complex with mutants of arrestin that do not rescue FPR-mediated apoptosis. Transiently transfected cells were plated on glass coverslips and preincubated with 10 μ M PD98059 for 30 minutes. Cells were then stimulated with 10nM 633-6pep in the continuous presence of inhibitor. Cells were washed twice with PBS, fixed with 2% paraformaldehyde, mounted and imaged by confocal fluorescence microscopy. Representative pictures are shown and are indicative of one experiment.

mRFP vector and inhibitor. However, in the presence of arr2-WT-mRFP, the receptor/arrestin complex appears to traffic normally despite inhibition of ERK1/2 activity. We interpret this data to mean that despite PD98059's ability to inhibit FPR-mediated apoptosis in the absence of arrestins it does not

release the receptor/arrestin complex from the Rab11 compartment. This indicates that accumulation of the receptor/arrestin complex in the Rab11 compartment may be an early step in the initiation of FPR-mediated apoptosis and precedes aberrant signaling complex formation.

3.3.2 Rab5 WT and EEA1 colocalize with internalized FPR, but dominant negative Rab5 does not.

Many GPCRs including the β_2 AR colocalize with Rab5 and EEA1, both markers of early, clathrin-derived endosomes. In addition, the β_2 AR is dependent upon Rab5 for proper internalization [38]. However, many receptors are dependent upon Rab5 for proper trafficking independent of internalization [49]. We suspected that although Rab5 was not necessary for internalization of the FPR, it may be necessary to mediate its proper trafficking.

Therefore, we transiently transfected U937 cells stably transfected with FPR with Rab5 WT, Rab5 S34N (dominant negative) or early endosomal antigen 1 (EEA1) fused to GFP and exposed them to labeled ligand for 2, 5 and 8 minutes to monitor trafficking of the FPR with respect to these proteins. In panel 7A, the FPR can be seen to colocalize with Rab5 at all time points, but more strongly at five and eight minutes. In addition, the FPR also colocalizes with transfected EEA1-GFP at all time points

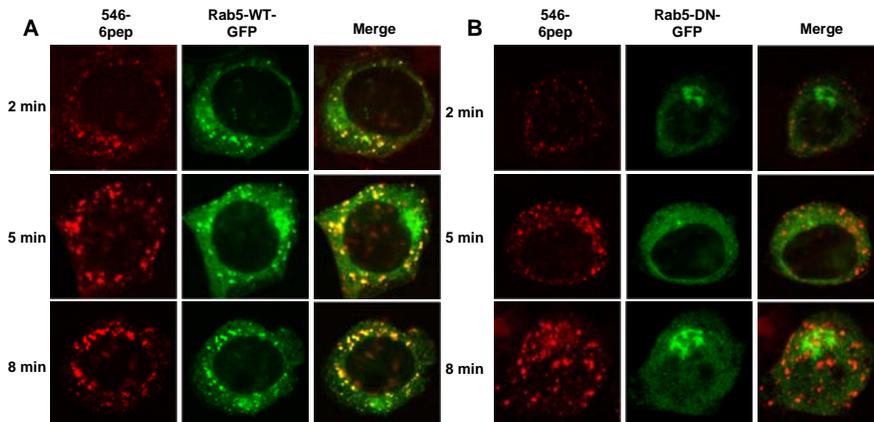


Figure 7. Internalization of the FPR and its colocalization with Rab5-WT-GFP and Rab5-DN-GFP. Transfected cells were harvested in SFM, stimulated with 546-6pep for described time points and aliquoted to cold 2% paraformaldehyde for fixation. Cells were then rinsed three times with cold PBS, mounted on glass coverslips and viewed by confocal fluorescence microscopy. Panel A was transfected with Rab5-WT-GFP, panel B with dominant negative Rab5 (S34N) and panel C with EEA1-GFP. Representative pictures are shown and are indicative of two independent experiments.

and more strongly at later time points (data not shown). Finally, in panel 7B, dominant-negative Rab5 does not appear to colocalize with receptor at any of the time points. The fact that dominant-negative Rab5 does not form vesicles is consistent with previous reports concerning the β_2 AR [38]. **We plan to demonstrate that when the FPR cannot transfer to a Rab11 endosome, there is no accumulation of FPR signaling complexes and no apoptosis.**

4 RESEARCH DESIGN AND METHODS

4.1 Specific Aim 1: Characterize regions in the tail of arrestin that regulate FPR-mediated apoptosis.

4.1.1 Research Rationale

Preliminary data reconstituted important domains of arrestin into KOFPRs, none of which were capable of rescuing FPR-mediated apoptosis. One of these mutants is a truncated arrestin (1-382) lacking the C-terminal 37 amino acids. As described previously, many of arrestin's binding partners interact with the tail of arrestin. Based on this finding, we hypothesized that regions of arrestin responsible for rescuing FPR-mediated apoptosis would likely be found in amino acids 383-418. Novel or known arrestin binding partners may not be able to carry out their normal functions when these regions are absent.

4.1.2 Research Design

While there are a few known interactions between the C-terminus of arrestin and other proteins (AP-2, clathrin and ERK1/2), there is little else known about its function or binding partners. In addition, preliminary data suggested that amino acids 383-418 of arrestin were responsible for FPR-mediated

apoptosis. Therefore, we constructed previously known and new mutations in the tail of arrestin (see 3.1.2, Figure 2).

These arrestin mutants were assayed for their effect on FPR-mediated apoptosis. All mutants designed rescued FPR-mediated apoptosis except for the arr2-4A, arr2-3A and arr2-F391A mutants (see 3.1.4, Figure 3). Apoptosis will be confirmed by using caspase inhibitors and CaspaTag to confirm caspase activation and apoptosis consistent with previous reports.

To understand the cellular mechanism resulting in apoptosis in the presence of these arrestin mutants, we sought to determine whether these mutants were capable of internalizing the FPR. Previous data has demonstrated that when the FPR does not internalize, KOFPRs do not undergo apoptosis. Therefore, FPR internalization was assayed using KOFPRs transiently transfected with arrestin mutants fused to GFP. Data from the experiments demonstrate the FPR internalizes in the presence of all mutants (see 3.1.3, Figure 4).

After determining mutants that were capable of rescuing FPR-mediated apoptosis, we wanted to understand mutant arrestins affected FPR trafficking. A previous report demonstrated that in the absence of arrestins, the FPR accumulates in a Rab11-positive, perinuclear location [24]. In wild-type cells, the FPR is seen with Rab11, but is also dispersed throughout the cell, presumably undergoing normal trafficking. Fluorescence micrographs demonstrate that all mutants that rescued the apoptotic phenotype resemble receptor/arrestin trafficking seen in WTFPRs. However, KOFPRs expressing arrestin mutants that did not rescue apoptosis showed trafficking defects, consistent with FPR trafficking in cells lacking arrestins, but with mutant arrestins associated with the receptor (see 3.1.5, Figure 5). **This result demonstrates that FPR-mediated apoptosis is not simply the lack of arrestin at the recycling endosome.**

Therefore, we are now interested in determining the function of arrestin mutants that do not rescue apoptosis. One of these mutants, arr2-F391A mutant, has been previously shown not to bind AP-2 [9], a member of the internalization machinery. To understand the role of AP-2 in FPR-mediated apoptosis, we will monitor its trafficking and binding to the receptor/arrestin complex. We will monitor AP-2 trafficking by confocal fluorescence microscopy of GFP-fused alpha subunit of AP-2 and co-immunoprecipitation to detect binding of AP-2 to the receptor/arrestin complex. In addition, we can monitor this interaction by using known mutants of AP-2 that do not bind arrestin [34] and monitoring FPR-mediated apoptosis, binding and trafficking of AP-2 with the receptor/arrestin complex.

Finally, we plan to overexpress the 319-418 mutant in WTFPRs and monitor apoptosis. If our hypothesis that the tail of arrestin binds accessory proteins and a lack of binding initiates apoptosis, we hypothesize that overexpression of this mutant should cause apoptosis in wild-type MEFs. We would attribute this result to the mutant stoichiometrically binding important accessory proteins that would normally bind endogenous arrestins, therefore making them unavailable, with apoptosis ensuing as is seen in KOFPRs.

4.1.3 *Expected Outcomes, Possible Pitfalls and Alternative Methods*

We anticipate that are that AP-2 will not bind arrestin-F391A as has been previously published [34]. In addition, we expect that AP-2 will not bind receptor and will not colocalize with the receptor/arrestin complex upon FPR activation. However, we do expect that AP-2 will bind and colocalize with wild-type arrestin and the receptor throughout the cell. We will interpret this to mean that AP-2 binding is an essential component of FPR trafficking (particularly receptor recycling) that controls the apoptotic phenotype. In the absence of arrestin binding to AP-2, FPR trafficking is not properly controlled, thereby initiating aberrant signaling and apoptosis.

Possible problems are presented by co-immunoprecipitation. The problems presented are mainly technical as the investigator has not used the assay previously. Potential problems include lack of stability of the protein complex and dissociation of its components during the lysis, binding or rinsing steps. However, crosslinking of proteins may help solve these problems. Also, there are collaborators that have extensive experience with the assay and all foreseen issues appear solvable.

Alternative possibilities to this hypothesis are that AP-2 will colocalize with the receptor/arrestin complex, will bind receptor directly, but will not bind arrestin. In this case we would conclude that AP-2 participates fully in this ternary complex, and is necessary to inhibit FPR-mediated apoptosis. In addition, there are three other members of the adaptor protein family (AP-1, AP-3 and AP-4) [50]. If AP-2 is not responsible for binding arrestin and preventing FPR-mediated apoptosis, it is possible that one of these family members is responsible. We will run similar assays with these family members to determine the role of arrestin-2-F391A in FPR-mediated apoptosis.

4.2 Specific Aim 2: *Determine the role of arrestin-mediated Src/MAPK signaling and interactions in FPR-mediated apoptosis.*

4.2.1 Research Rationale

Previous inhibitor studies from our laboratory have demonstrated the involvement of Src kinase and the MAPK (ERK1/2, JNK3 and p38) cascades in FPR-mediated apoptosis [23]. Based on these results, we hypothesize that FPR-mediated apoptosis is mediated by MAP kinase signaling through Src kinase.

4.2.2 Research Design

To understand the role of arrestin and Src in FPR-mediated apoptosis, we will use the 2Pro mutant of arrestin that mutates prolines in two of three SH3-binding domains (P91G and P121E). This mutant has been previously demonstrated to be deficient in binding Src kinase and stimulating ERK1/2 activity upon β_2 AR activation [25]. We have made and characterized effects of this mutant which are described in 3.2.1.

Preliminary data have shown that the 2Pro mutant does not rescue apoptosis. This is interesting as we would expect that lack of arrestin binding to Src would not activate Src kinase. However, reports describing impaired β_2 AR-mediated ERK1/2 activation in the presence of this arrestin mutant also demonstrated that AT1R-mediated ERK1/2 activation was not impaired [25]. This indicates that this arrestin mutant's function may be GPCR-specific. To understand the role of Src kinase in this process, we will use PP2 (an inhibitor of Src family kinases) and a kinase dead mutant (K298M) of Src kinase.

In addition, we will monitor activation of the MAPK cascades (ERK1/2, p38m JNK3) in the presence of the 2Pro mutant. We expect that their activation will be altered because IL-8R-mediated activation of ERK1/2 and JNK3 in the absence of arrestins was increased and prolonged [44]. Further, we will use PP2 and kinase-dead Src kinase overexpression to demonstrate that this alternate MAPK signaling is due to aberrant Src kinase activation.

Finally, because the 2Pro mutant has previously shown deficiency in binding arrestin, we want to understand the trafficking of Src kinase and the MAPKs in relation to the receptor/arrestin complex. This is important as expression of this mutant did not rescue apoptosis. This will be done using confocal fluorescence microscopy and combinations of GFP-fused kinases (or appropriate antibodies), receptor and arrestin.

4.2.3 Expected Outcomes, Possible Pitfalls and Alternative Methods

We expect dominant-negative forms of Src kinase and inhibitors of Src kinase will rescue FPR-mediated apoptosis. Further, we expect that MAPK cascades will show altered activation which will be reversed by PP2 and kinase-dead Src kinase. Finally, we expect there to be changes in trafficking by Src kinase or the MAPK members in relation to the receptor/arrestin complex.

Alternative possibilities are that inhibitors of the Src kinase will rescue FPR-mediated apoptosis, but that kinase-dead Src kinase will not. PP2 is a non-specific inhibitor of many Src family kinases. We would then hypothesize that altered signaling is occurring not through Src kinase specifically, but through one of the other family members. If this is the case we will assay Fyn and Yes (two other family members expressed in fibroblasts) as described to ascertain their role in FPR-mediated

apoptosis. Finally, the MAPKs could traffic with the receptor/arrestin complex (however this does not necessarily mean direct binding of the proteins). If this is the case, we will co-IP members of the signaling complex (receptor, arrestin, Src kinase, MAPK members) to understand changes in binding or complex formation that may be different when wild-type arrestin 2 is present.

4.3 Specific Aim 3: *Determine the contributions of FPR signaling and trafficking defects to the apoptotic phenotype.*

4.3.1 *Research Rationale*

Based on previous reports and our own preliminary data, we hypothesize that there are both trafficking and signaling defects involved in FPR-mediated apoptosis in the absence of arrestins. Furthermore, we hypothesize that the receptor trafficking defect precedes and causes the signaling defect leading to the initiation of apoptosis. In the absence of arrestins, the FPR accumulates in a Rab11-positive, perinuclear region, scaffold signaling complexes cannot be dissociated and MAPK signaling is altered and initiates apoptosis. Finally, we hypothesize that when the FPR cannot transfer to the recycling endosome (Rab11) or leaves the endosome in some other manner, FPR signaling complexes cannot accumulate and initiate apoptosis.

4.3.2 *Research Design*

To test this hypothesis, we will use KOFPRs and WTFPRs (or KOFPRs transfected with arr2-WT or blank vector). We will first demonstrate that use of MAPK inhibitors (PD98059, SB202190, U0126 and SP600125) does not dissociate the receptor/arrestin complex from the Rab11-positive endosome using confocal fluorescence microscopy. We expect that none of the inhibitors will dissociate the complex meaning that the trafficking defect does precede the signaling defect.

Preliminary data demonstrates that trafficking of the FPR is altered in the presence of Rab5 S34N (see 3.3.3, Figure 7). We believe that this altered trafficking may not allow the receptor to reach the recycling endosome and therefore will not initiate apoptosis. This will be tested by apoptosis methods described in KOFPRs. We will also demonstrate that in the presence of Rab5 S34N, the receptor does not traffic to the Rab11-positive endosome. This will demonstrate that when the FPR cannot transfer to a Rab11 endosome, there is no accumulation of FPR signaling complexes and no initiation of apoptosis.

In addition, other dominant-negative or constitutively-active trafficking proteins may alter the apoptotic phenotype. For instance, we will test whether overexpression of constitutively-active Rab11 will assist the receptor returning to the cell surface. This would result in dissociation of the receptor/arrestin complex in the Rab11-positive endosome and presumably rescue FPR-mediated apoptosis.

4.3.3 *Expected Outcomes, Possible Pitfalls and Alternative Methods*

We expect that use of MAPK inhibitors will not stop accumulation of the receptor/arrestin complex in a Rab11-positive, perinuclear location. This will help us understand whether trafficking precedes and causes the signaling defect or vice-versa.

In addition, we expect that overexpression of Rab5 S34N will prevent transfer of the receptor/arrestin complex to a Rab11-positive endosome. This should prevent accumulation of the receptor/arrestin complex and rescue apoptosis. Also, we expect that use of other dominant-negative or constitutively-active proteins should rescue apoptosis (for example, the constitutively-active Rab11 should stop accumulation of the receptor/arrestin complex in the recycling endosome and rescue apoptosis).

Possible pitfalls include Rab5 S34N overexpression not preventing accumulation of the receptor/arrestin complex in Rab11-positive endosomes and initiation of apoptosis. If this is the case, there are a variety of dominant-negative and constitutively active proteins involved with internalization of GPCRs that can be assayed including, but not limited to, ARF6 and dynamin. In addition, different

dominant-negative and constitutively active proteins may not behave as we expect. This data will only help us to understand their role in GPCR trafficking and FPR-mediated apoptosis.

4.4 Methods

4.4.1 *Mutagenesis.* Regions of arrestin were mutated by PCR-mutagenesis and cloned into vectors fused to GFP (EGFP-N1 vector) or RFP (mRFP vector) using standard subcloning procedures and HindIII/ApaI restriction sites. All mutants will be confirmed by DNA sequencing.

4.4.2 *Cell culture.* Mouse embryonic fibroblasts are grown in DMEM with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100units/mL streptomycin at 37°C and 5% CO₂. U937 cells are grown in RPMI with 10% FBS, 100units/mL penicillin and 100units/mL streptomycin at 37°C and 5% CO₂.

4.4.3 *Transfection.* All mouse embryonic fibroblast transfections will be performed with Lipofectamine 2000 according to manufacturer's instructions. All U937 transfections will be performed using previously described electroporation procedures [48].

4.4.4 *Internalization Assay.* Internalization assays will be run according to previously published protocols [24, 48]. Briefly, transiently transfected cells will be harvested, resuspended in SFM and stimulated for various times with 1 μ M fMLF. Cells will then be washed extensively with cold SFM and labeled with 633-6pep. Cells are then assayed by flow cytometry using a Becton-Dickinson 4-color FacsCalibur at appropriate wavelengths. Assayed cells are gated for live cells using forward and side scatter parameters. These cells are then gated using FL-1 for GFP-fused arrestin mutant expression and the mean channel fluorescence is measured in FL-4 to monitor cell surface expression of the FPR. Zero time points are considered 100% expression and cell surface expression at non-zero time points is the MCF divided by the MCF at zero time. All values have had the non-specific binding subtracted before calculation. Non-specific binding is determined by labeling arrestin-deficient cells not expressing the FPR with 633-6pep and assaying as described. Statistical analysis will be performed using Student's t-test.

4.4.5 *Apoptosis Assay.* This assay will be carried out using previously published protocols [23]. Briefly, transiently transfected cells will be plated on glass coverslips and stimulated with 10nM 633-6pep or fMLF in SFM or SFM with no stimulus for 5 hours. Cells will then be stained with 100pg/ μ L propidium iodide in SFM. Cells will then be washed twice with PBS, fixed with 2% paraformaldehyde and mounted using Vectashield. Slides will be viewed by fluorescence microscopy and random fields will be counted until 300 GFP expressing cells have been viewed. GFP expressing cells will be counted "positive" for death if they are stained with propidium iodide. Data will be expressed as a percentage of PI positive/GFP expressing cells. Statistical analysis will be performed using Student's t-test.

4.4.6 *Immunofluorescence Microscopy.* These studies will be carried out according to previously published protocols [24, 48]. Briefly, transiently transfected KOFPRs are plated on glass coverslips and incubated overnight. Cells are then stimulated with fluorescently labeled ligand (Alexa 488-, 546- or 633-6pep) for 1 hour, rinsed with SFM and fixed with 2% paraformaldehyde. Cells can then be permeabilized and stained with labeled or unlabeled primary antibodies of interest and labeled secondary antibodies as necessary. Coverslips are then mounted with Vectashield and viewed using a Zeiss LSM 510 META Inverted Fluorescence microscope.

4.4.7 *Western Blotting.* This work will be carried out as described [51]. Briefly, transiently transfected cells are stimulated with 100nM fMLF in SFM for time periods ranging from 0 to 30 minutes. Cells are then lysed and lysates are resolved with SDS-PAGE. Blotting will be carried out using appropriate primary antibodies and HRP-conjugated secondary antibodies. Generally, these will typically be phospho- or whole cell antibodies to ERK1/2, p38 and JNK3.

4.4.8 *Immunoprecipitation Assay.* Assay will be carried out as described [52]. Briefly, transiently transfected cells will be grown to confluence and stimulated with 100nM fMLF in SFM. Cells will be washed once with PBS and then scraped off in PBS. Cells will be pelleted and snap-frozen in liquid nitrogen. Pellets will be thawed on ice and resuspended in 200 μ L of co-IP buffer (20mM HEPES pH

7.4, 100mM NaCl, 0.5mM EDTA, and protease inhibitor cocktail). Samples containing 750µg protein will be pre-cleared with 2µg IgG and 10µL of protein A/G Plus agarose for 30' at 4°C. Agarose will be pelleted and supernatant transferred to a new tube with 2µg primary Ab, incubated at 4°C with rotation for 2-3hrs. Protein A/G Plus will be added (25µL) and incubated at 4°C overnight with mixing. Immunocomplexes will be pelleted and washed 3X with co-IP buffer. Immunocomplexes will be eluted with 50µL 2X sample buffer and resolved by SDS-PAGE. Western blots for AP-2 will be carried out as described above.

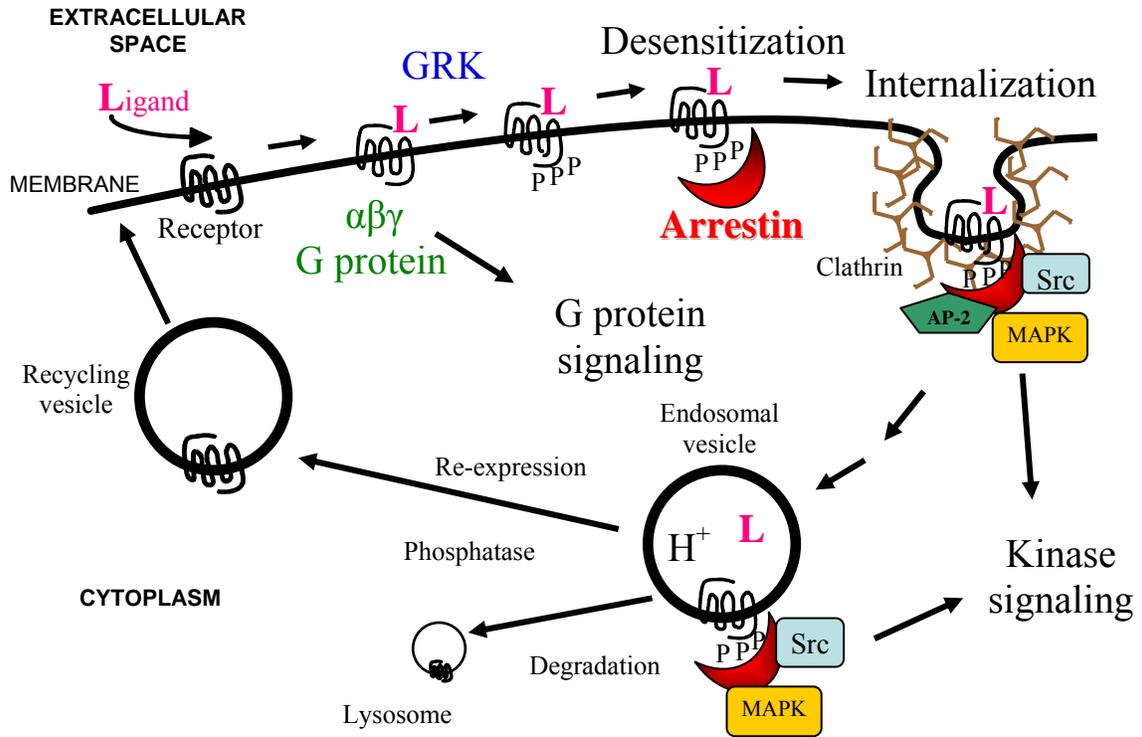
5 TIMELINE

Although these research aims are aggressive, we believe that within one year, this work can be completed. This is based on the fact that the primary investigator is competent in most of the techniques described and others in the ERP laboratory and surrounding, collaborating laboratories have the experience necessary to aid the PI in these studies should problems arise.

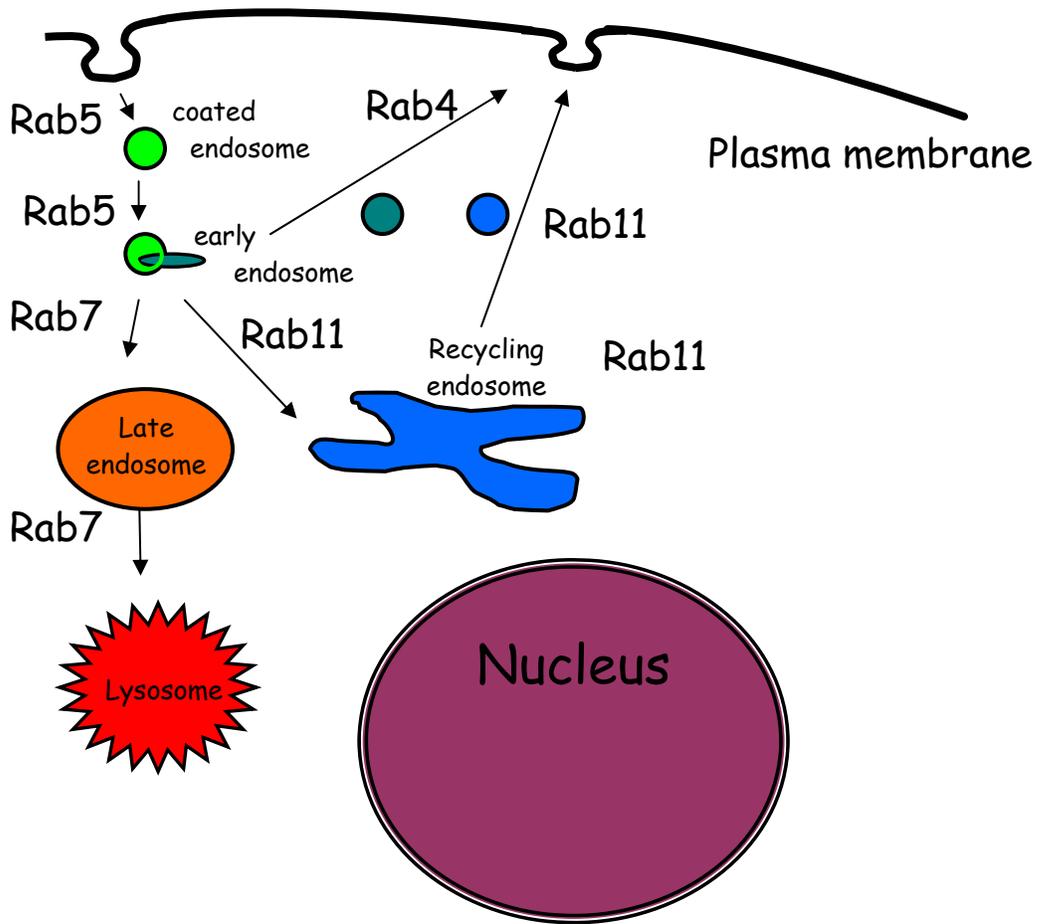
AIM1-----4MO
AIM2-----8MO
AIM3-----12MO

6 APPENDIX

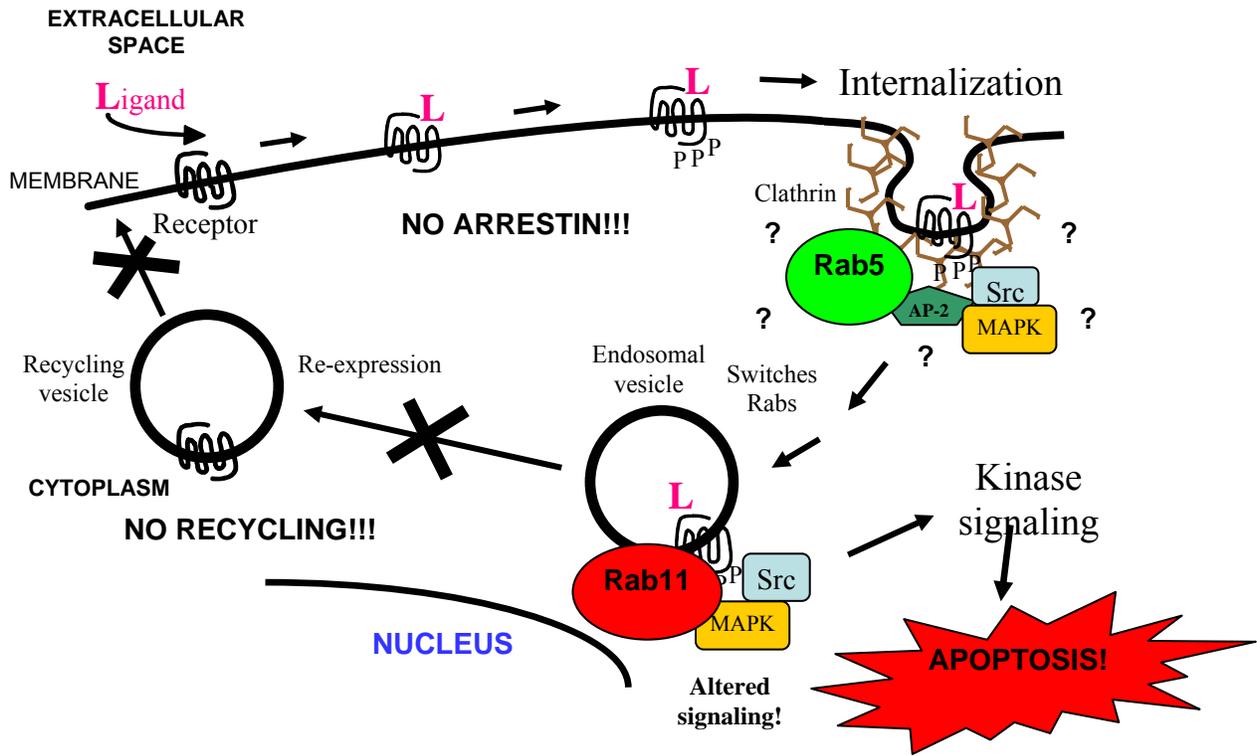
6.1 Appendix I. Life cycle of a GPCR.



6.2 Appendix II. GPCR Trafficking and Rabs.



6.3 Appendix III. Model of FPR-mediated apoptosis in the absence of arrestins.



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