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14. ABSTRACT: This work focuses on how arrestin regulates trafficking and signaling of the N-formyl peptide receptor (FPR), a G protein-coupled receptor (GPCR). GPCRs are involved in almost all physiologic processes and numerous pathologic processes. There is an intimate relationship between GPCR trafficking and signaling that controls many cellular processes. However, the protein-protein interactions that control post-endocytic trafficking and signaling of GPCRs are poorly understood. Our previous reports demonstrated that three events take place upon FPR activation in the absence of arrestins: accumulation of FPR in the perinuclear recycling endosome, lack of FPR recycling and apoptosis. All of these phenotypes were rescued by reintroduction of arrestin-2 cDNA. We therefore hypothesized that 1) FPR trafficking and signaling defects were linked and causal and 2) specific regions of arrestin-2 regulate normal FPR trafficking and signaling. To address these hypotheses, we generated mutants of arrestin-2 that were previously described or changed regions of similar amino acids to alanine. We then screened these mutants for the ability to rescue FPR-mediated apoptosis. Subsequently, we examined the role of these arrestin mutants in FPR trafficking. We found that two arrestin-2 mutants demonstrated altered binding to adaptor protein (AP)-2. Furthermore, FPR recycling was inhibited in the presence of either arrestin-2 mutant or the absence of AP-2. We also examined the role of Src kinase in FPR trafficking and signaling and determined that Src kinase has two independent roles in FPR-arrestin-2 regulation: one that controls FPR trafficking and one that mediated FPR signaling. Finally, we found that different SH3-binding domains of arrestin-2 regulate FPR trafficking and signaling independently. One arrestin-2 mutant did not rescue FPR-mediated apoptosis, but did mediate normal FPR trafficking. These results indicate that FPR trafficking and signaling are coordinated processes, but may also be regulated independently. These studies have revealed novel aspects of arrestin-2 that regulate FPR signaling and trafficking.

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INTRODUCTION

This work focuses on how arrestin regulates trafficking and signaling of the *N*-formyl peptide receptor (FPR), a G protein-coupled receptor (GPCR). GPCRs are involved in almost all physiologic processes and numerous pathologic processes. There is an intimate relationship between GPCR trafficking and signaling that controls many cellular processes. However, the protein-protein interactions that control post-endocytic trafficking and signaling of GPCRs are poorly understood.

Our previous reports demonstrated that three events take place upon FPR activation in the absence of arrestins: accumulation of FPR in the perinuclear recycling endosome, lack of FPR recycling and apoptosis. All of these phenotypes were rescued by reintroduction of arrestin-2 cDNA. We therefore hypothesized that 1) FPR trafficking and signaling defects were linked and causal and 2) specific regions of arrestin-2 regulate normal FPR trafficking and signaling.

To address these hypotheses, we generated mutants of arrestin-2 that were previously described or changed regions of similar amino acids to alanine. We then screened these mutants for the ability to rescue FPR-mediated apoptosis. Subsequently, we examined the role of these arrestin mutants in FPR trafficking. We found that two arrestin-2 mutants demonstrated altered binding to adaptor protein (AP)-2. Furthermore, FPR recycling was inhibited in the presence of either arrestin-2 mutant or the absence of AP-2. We also examined the role of Src kinase in FPR trafficking and signaling and determined that Src kinase has two independent roles in FPR-arrestin-2 regulation: one that controls FPR trafficking and one that mediated FPR signaling. Finally, we found that different SH3-binding domains of arrestin-2 regulate FPR trafficking and signaling independently. One arrestin-2 mutant did not rescue FPR-

mediated apoptosis, but did mediate normal FPR trafficking. These results indicate that FPR trafficking and signaling are coordinated processes, but may also be regulated independently.

These studies have revealed novel aspects of arrestin-2 that regulate FPR signaling and trafficking. We hope they will serve as a model for the regulation of other GPCRs. Furthermore, we hope these data are used to create small molecule inhibitors to serve as experimental tools and chemotherapeutics to better understand and treat diseases caused by defects in GPCR trafficking and signaling.

BODY

Task 1. Adaptor Protein-2 interaction with Arrestin regulates *N*-formyl peptide receptor post-endocytic trafficking

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Adaptor Protein-2 interaction with Arrestin regulates *N*-formyl peptide receptor post-endocytic trafficking

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2.1 ABSTRACT

G protein-coupled receptors (GPCRs) are integral to cellular function in nearly all physiologic and numerous pathologic processes. GPCR signaling is an intricate balance between receptor activation, inactivation (desensitization and internalization) and resensitization (recycling and resynthesis). While much is known regarding the first two processes, the latter has not been as thoroughly studied. To better understand the process of GPCR post-endocytic trafficking, we focused on the *N*-formyl peptide receptor (FPR), a chemoattractant receptor found primarily on neutrophils and macrophages. Previous studies have demonstrated that, although FPR internalization occurs in the absence of arrestins, FPR recycling is arrestin-dependent. Furthermore, FPR stimulation in the absence of arrestins leads to receptor accumulation in recycling endosomes and apoptotic signaling. In this study, we determined that the carboxy terminus of arrestin-2 is critical for intracellular receptor trafficking and performed scanning mutagenesis of this region to ascertain the mechanisms involved. Our results reveal that two arrestin-2 mutants (F391A and K397A/M399A/K400A), at sites known to be involved in AP-2 binding, fail to rescue the trafficking and signaling defects observed in the absence of arrestins. Further results demonstrate that AP-2 associates with the receptor-arrestin complex in recycling endosomes and is required for proper post-endocytic trafficking of the FPR, as revealed by siRNA knockdown of AP-2, which inhibits recycling of the FPR to the cell surface. Finally, we observe that AP-1 is associated with the receptor-arrestin complex under recycling-competent conditions, suggesting a transfer of receptors from AP-2- to AP-1-associated vesicles. This is the

first study to demonstrate a requirement for AP-2 in the post-endocytic trafficking of a GPCR and serves as a model for future studies in GPCR trafficking and resensitization.

2.2 INTRODUCTION

G protein-coupled receptors (GPCR) are involved in signaling in virtually every part of the human body including cardiovascular (*1*), immune (*2*) and neuronal systems (*3*). An important feature of GPCR signaling is the cycle of receptor activation, desensitization, internalization, down-regulation/degradation, recycling and resensitization. When these processes are interrupted, they can detrimentally affect cellular migration (*4*), proliferation and cell adhesion (*5*). In the case of v2 vasopressin receptors, constitutive desensitization and internalization leads to nephrogenic diabetes insipidus (*6*).

GPCRs are activated by a myriad of ligands including, but not limited to, peptides, amino acids and their derivatives, proteins, ions, lipids and photons. Ligand-bound GPCRs activate heterotrimeric G protein signaling pathways, resulting in calcium mobilization and changes in cyclic-AMP levels, and are subsequently phosphorylated on intracellular domains by serine/threonine kinases, which reduce receptor affinity for G proteins and increases receptor affinity for arrestins. Arrestin binding sterically blocks receptor-G protein interactions, thereby effectively terminating G protein signaling, while simultaneously providing a scaffold protein that can coordinate the recruitment of internalization machinery, leading to receptor sequestration (*7*). In this model, based primarily on studies of the beta2-adrenergic receptor (β2-AR), the most thoroughly described GPCR, adaptor protein (AP)-2 and clathrin (*8*) bind the carboxy terminus of arrestin, initiating association with clathrin-coated pits and internalization. Arrestin-recruited Src then phosphorylates dynamin, which pinches off the plasma membrane invagination to form an endosome containing receptor, from which arrestin rapidly

dissociates (*9*). Internalized β2-AR in the rab5-containing early endosomal compartment, is then sorted to lysosomes (via a Rab7-containing compartment) for degradation or to the cell surface (via Rab4-positive endosomes) (*10*).

This classic pathway of GPCR internalization and post-endocytic trafficking is, however, not observed with all GPCRs. For example, internalization of the m2-muscarinic acetylcholine receptor is not dependent on either arrestin or clathrin, utilizing instead an ARF6-dependent pathway (*11*). Similarly, the *N*-formyl peptide receptor (FPR) has been demonstrated to internalize in the absence of arrestins using knockout cell lines, in which β2-AR internalization is completely inhibited (*12, 13*). In contrast to GPCRs that follow the rab4-mediated, rapid recycling pathway or the rab7-mediated, lysosomal degradation pathway (*14*), some GPCRs recycle via perinuclear Rab11-containing recycling endosomes. Such receptors include the FPR (*12*), v2 vasopressin receptor (*15*), somatostatin 3 receptor (*16*) and CXC chemokine receptor 2 (*17*). In many cases, such GPCRs have been shown to recycle more slowly and form stable complexes with arrestins, resulting in prolonged endosome-associated arrestin (*12, 18, 19*). Although the stable endosomal association of arrestin with receptors has been shown to be critical in a second prolonged phase of ERK activation in the cytoplasm (*19, 20*), little is known regarding the role of arrestin in regulating intracellular trafficking of GPCRs, particularly via the perinuclear Rab11-containing recycling compartment. Arrestin dissociation has however been implicated as an essential step in the recycling and resensitization of the bradykinin B2 receptor (*21*). In cells lacking both arrestin-2 and arrestin-3, recycling of the FPR is absent resulting in receptor accumulation in perinuclear recycling endosomes, suggesting a critical role for arrestin in efferent

trafficking of the FPR from this compartment (*12*). Thus, arrestin appears to mediate multiple aspects of GPCR trafficking.

Subsequent studies of FPR stimulation in arrestin-deficient cells revealed that, in addition to a recycling defect, cells also underwent apoptosis (*22*). A requirement for receptor internalization was demonstrated using a signaling-competent, internalization-defective mutant of the FPR. Furthermore, apoptosis was prevented by MAPK and other signaling inhibitors. This led to the hypothesis that the accumulation of ligand-activated FPR in recycling endosomes results in aberrant signaling, which initiates apoptotic pathways culminating in caspase activation. Based on the fact that both the trafficking and signaling defects could be rescued by the re-introduction of either arrestin-2 or arrestin-3, we hypothesized that these two apparently distinct defects may be mechanistically linked. Because of the large number of arrestin-interacting proteins, we speculated that the absence of specific interactions might be responsible for the observed defects. Therefore, in this study, we undertook a mapping study to identify the sites within arrestin that when mutated, result in aberrant signaling and trafficking. Our results reveal novel mechanisms in which AP-2 specifically regulates efferent FPR trafficking from recycling endosomes.

2.3 RESULTS

2.3.1 Rescue of FPR-mediated apoptosis by arrestin-2 domains.

We have previously described a requirement for arrestins in preventing apoptosis resulting from activation of multiple GPCRs, including the FPR, IL-8R/CXCR2 and Angiotensin II (Type 1A) receptor (*22*). Arrestins also play a critical role in the proper intracellular trafficking of GPCRs such as the FPR, although FPR internalization does not require arrestin. To better understand the role of arrestin-2 in FPR post-endocytic trafficking, we sought to define the region(s) of arrestin-2 responsible for preventing FPR-mediated apoptosis. To this end, we generated four constructs producing large fragments of arrestin-2 (arr2): amino acids 1-186, 177-418, 319-418 and 1-382. The structure of arrestin-2 is composed of two domains of beta-pleated sheets and a C-terminal "tail" (*23*). Arr2-(1-186) contains the amino terminal domain of beta-pleated sheets of arrestin-2; arr2-(177-418) contains the carboxy terminal domain of beta-pleated sheets and the carboxy terminal "tail" of arrestin-2; arr2-(319-418) contains the "tail" (amino acids 357-418) that acts as a dominant-negative construct inhibiting β2-AR internalization (*24*). Arr2-(1-382) is a truncated form of arrestin-2 that has been previously described as constitutively active with respect to receptor binding, displaying binding to unphosphorylated, liganded receptors as well as phosphorylated, unliganded receptors, receptor forms that wild type arrestin-2 does not bind (*25-28*).

Mouse embryonic fibroblasts (MEF) deficient in both arrestin-2 and -3, but stably expressing the FPR ($arr2^{-/-}/3^{-/-}$ FPR), were used to assess arrestin-2 mutants in the absence of competition from endogenous arrestins. Arrestin-deficient cells transiently transfected with the four GFP-fused arrestin-2 fragments, wild type arrestin-2 and empty

GFP vector were assayed for apoptosis upon FPR stimulation by evaluation of cell rounding. In a previous report, cell rounding was demonstrated to correlate absolutely with classical markers of apoptosis including annexin-V staining, caspase activation and propidium iodide (PI) staining (*22*). As shown in Figure 2.1, unstimulated cells (expressing GFP, wild type or mutant arrestins) did not exhibit significant cell rounding (<20-30%). On the contrary, 70-80% of fMLF-stimulated cells expressing GFP alone exhibited a rounded cell phenotype, whereas cells expressing wild type arrestin-2 were indistinguishable from unstimulated cells, as previously described (*22*). Furthermore, none of the four expressed arrestin-2 domains were capable of preventing FPR-mediated apoptosis, although the arr2-(177-418) showed a small reduction in the number of rounded cells. Of the four arrestin mutants, arr2-(1-382) is the only domain that demonstrated association with the FPR upon stimulation (as determined by confocal fluorescence microscopy, unpublished data). This is consistent with previous data that demonstrated colocalization of arr2-(1-382) with the FPR upon receptor activation (*29*) and binding to the FPR in reconstitution assays (*25, 28*). These results demonstrate that the binding of arrestin-2 alone is insufficient to prevent FPR-mediated apoptosis and furthermore that sequences within the carboxy terminus of arrestin-2 (amino acids 383-418) are essential to prevent apoptotic signaling.

2.3.2 Rescue of FPR-mediated apoptosis by arrestin-2 tail mutants.

The tail of arrestin-2 contains recognition sites for multiple adapter and signaling proteins, including clathrin (*24, 30, 31*), AP-2 (*8, 23*) and ERK, which phosphorylates a serine at position 412 (*32*). Based on the cell rounding results (Figure 2.1), we

hypothesized the amino acids in arrestin-2 responsible for suppressing FPR-mediated apoptosis lie within its carboxy terminus. To test this hypothesis, we generated nine mutants of arrestin-2 using alanine-scanning mutagenesis and previously described mutations (Figure 2.2). Previously described mutations include arr2-F391A, which demonstrates reduced AP-2 binding (*23*), arr2-4A, in which K397, M399 and K400 have individually been shown to mediate AP-2 binding (*8*), arr2-ΔLIELD, which results in reduced clathrin binding (*24, 30, 31*) and S412D/S412A, which mimic the phosphorylated and unphosphorylated states of arrestin-2, respectively (*32*).

GFP-fusions of the nine arrestin-2 tail mutants were tested for their ability to prevent FPR-mediated apoptosis. Following transient transfection, GFP-expressing cells (representing transfected cells) were stimulated with fMLF and scored for apoptosis by propidium iodide staining. Previous results have demonstrated a direct correlation between cell rounding and PI staining in cells undergoing FPR-mediated apoptosis (*22*). Greater than 95% of GFP vector- and arr2- (1-382)-expressing cells were PI positive upon fMLF stimulation (Figure 2.3), consistent with cell rounding results (Figure 2.1). Expression of wild type arrestin-2 and all mutants with the exception of the arr2-F391A and -4A rescued cells from FPR-mediated apoptosis (Figure 2.3). All transfected cells showed minimal PI staining in the absence of ligand stimulation. To confirm that positive PI staining was the result of FPR-mediated apoptosis, empty GFP, wild type arrestin-2, -(1-382), -F391A and -4A were treated with the pan-caspase inhibitor, zVAD-FMK, prior to fMLF stimulation. Transfected cells that underwent apoptosis in response to FPR stimulation failed to do so in the presence of zVAD-FMK,

demonstrating that the PI staining in the presence of the arrestin-2 mutants F391A and 4A represents FPR-mediated, caspase-dependent apoptosis (Figure 2.11).

2.3.3. Arrestin-2 mutants that rescue apoptosis do not inhibit FPR internalization.

Previous results have demonstrated that receptor internalization, which occurs via arrestin-independent mechanisms, is essential for FPR-mediated apoptosis (*22*). Because AP-2 is required for the internalization of certain GPCRs, we hypothesized that overexpression of arrestin-2 mutants might inhibit FPR internalization, thereby preventing FPR-mediated apoptosis. To this end, we measured FPR internalization for each arrestin mutant. We determined that the extent of FPR internalization varied between 65-80%, with no significant difference between the extents (Figure 2.4) or rate (unpublished data) of internalization for any of the arrestin constructs expressed. This indicates that rescue of FPR-mediated apoptosis by arrestin mutants is not due to effects on FPR internalization. In addition, internalization of the ΔLIELD and F391A mutants is consistent with our previous studies showing that FPR internalization is independent of clathrin (*33*) and, similar to other GPCRs whose internalization is arrestin-independent (*34*), is also independent of AP-2.

2.3.4 Arrestin-2 mutants that fail to rescue FPR-mediated apoptosis accumulate in recycling endosomes.

As we have previously observed in arrestin-deficient cells, FPR-mediated apoptosis is associated with defective intracellular trafficking, raising the question as to whether the arr2-F391A and arr2-4A mutants allow normal trafficking of the FPR and to

what extent they remain associated with the receptor as it traffics intracellularly. Although the 6A mutant contains F391 and the 8A mutant contains residues that have also been shown to regulate interactions with AP-2 (*35, 36*), these mutants rescue FPR-mediated apoptosis. Therefore, we decided to focus our subsequent studies on mutants that do not rescue FPR-mediated apoptosis, namely the F391A and 4A mutants. Localization of the FPR (visualized using an Alexa633 labeled *N*-formyl-Leucyl-Leucyl-Phenylalanyl-Leucyl-Tyrosinyl-Lysine ligand (633-6pep)) and arrestin-2 mutants (tagged with monomeric red fluorescent protein, mRFP1 (*37*)) was determined using confocal fluorescence microscopy. Recycling endosomes were visualized using a GFP fusion of Rab11. In unstimulated cells, the Rab11 compartment is located in the perinuclear region and arrestin-2 (either wild type or mutant) is dispersed throughout the cytoplasm. This is consistent for all arrestin-2 mutants (unpublished data). Activation of the FPR with fluorescent ligand (633-6pep) in the absence of arrestin-2 (empty mRFP1 vector-transfected) resulted in accumulation of the FPR in recycling endosomes (Figure 2.5). Expression of wild type arrestin-2, while also leading to localization of the FPR in perinuclear recycling endosomes, resulted in a greater proportion of cytosolic vesicles outside the recycling endosome containing FPR and arrestin, consistent with normal trafficking and recycling of the FPR (*12*).

Cytosolic complexes of FPR-633-6pep and arrestin-2 are believed to represent both internalized afferent endosomes as well as recycling efferent vesicles based on two independent results. First, following a 10 min stimulation of arrestin-deficient cells with 633-6pep, followed by 50 min chase without ligand, the FPR is not seen in cytosolic vesicles, as it is in the presence of arrestins (unpublished data). The presence of the

FPR in vesicles after 50 min of agonist depletion suggests that the vesicle-localized receptor represents recycling receptor, since no such vesicles are seen in the arrestin-deficient cells. Second, FPR internalization experiments performed with 10nM fMLF (the concentration of 633-6pep used in imaging experiments) in the presence of wild type arrestin-2, reveal that the FPR achieves an equilibrium within 10 min wherein only approximately 25-30% of the total receptor is internalized (compared to 1 μM fMLF, where 75-80% of the FPR is internalized). This equilibrium with less total internalized receptor suggests that robust recycling is taking place under these conditions.

Localization results for the mutant arrestins paralleled their apoptotic phenotype. Expression of both the arr2-F391A and -4A mutants (which did not rescue FPR-mediated apoptosis) resulted in accumulation of the FPR with associated mutant arrestin in recycling endosomes. All other mutants (which did rescue the apoptotic phenotype) produced FPR trafficking patterns indistinguishable from wild type arrestin-2 (Figure 2.5 and Fig 2.12). These results provide additional support for the correlation between FPR trafficking defects and the initiation of FPR-mediated apoptosis observed in the complete absence of arrestins as well as in the presence of the arr2-F391A and -4A mutants.

2.3.5 Arrestin-2 mutants that prevent normal FPR trafficking exhibit altered associations with AP-2.

Both the arr2-F391A and -4A mutants fail to rescue FPR-mediated apoptosis and also demonstrate trafficking defects exemplified by accumulation in perinuclear recycling endosomes. The region of arrestin-2 encompassing amino acids 391-400 has

previously been associated with altered binding to AP-2 (*8, 23*). The F391A mutant exhibits decreased binding to AP-2, resulting in inhibition of β2-AR internalization. While the 4A mutant has not previously been described, three of the amino acids within this mutant (K397, M399 and K400) have been individually assessed and shown to exhibit decreased binding to AP-2 as well as decreased β2-AR internalization similar to the F391A mutant (*8*). To better understand the relationship between AP-2 association and FPR trafficking, we used confocal fluorescence microscopy to track the association of AP-2 with the FPR-arrestin complex. Although previous studies of other GPCRs have examined arrestin-2/AP-2 dynamics at early time points upon receptor internalization (*38*), we observed trafficking defects most clearly following one hour of receptor stimulation. Therefore, we used a time course that examines FPR trafficking at both short and long stimulation times.

To assess the interaction between AP-2 and the FPR-arrestin-2 complex, we examined the subcellular localization of the GFP-fused α-subunit of AP-2, in the context of internalized FPR (by tracking the activated 633-6pep-FPR complex) and arrestin (by tracking RFP-fused arrestins) using confocal fluorescence microscopy. In unstimulated cells, all arrestins (wild type and mutants) show a cytosolic distribution consistent with a lack of GPCR stimulation. AP-2 is distributed throughout the cytoplasm as well as in puncta at the plasma membrane. In the arr2$^{-/-}$/3$^{-/-}$ FPR cells, following 60 minutes of stimulation with 633-6pep in the absence of arrestins, the FPR accumulates in a perinuclear region (Figure 2.6) identified as the Rab11 recycling endosome (*cf*. Figure 2.5). This observation and the lack of AP-2 association with the FPR in cytosolic endosomes at 15 and 30 minutes in the absence of arrestins (Figure 2.13) are

consistent with a requirement for arrestin-2 in the recruitment of AP-2. Upon expression of wild type arrestin-2 in the arr2$^{-/-}$/3$^{-/-}$ FPR cells, ligand stimulation results in the accumulation of AP-2 with the 633-6pep-FPR/arrestin-2 complex in the perinuclear region at 60 minutes. However, AP-2 is not observed to associate with cytosolic endosomes containing FPR-arrestin-2 complexes, suggesting that AP-2 is not associated with the complex after exiting the recycling endosome. At 15 minutes, almost no AP-2 was seen associated with FPR-arrestin-2 complexes in recycling endosomes. It is not until 30 minutes that significant levels of AP-2 are concentrated with the FPR and wild type arrestin-2 in recycling endosomes. We interpret these results to suggest that AP-2 does not associate with FPR-arrestin-2 complexes prior to internalization.

Expression of the F391A and 4A arrestin mutants yield substantially different results with respect to AP-2 association. In the presence of the arr2-F391A mutant, AP-2 showed no association with the 633-6pep-FPR/arr2-F391A complex at any of the time points (Figure 2.6 and Figure 2.13). This is consistent with published reports that demonstrate decreased AP-2 binding to arr2-F391A (*8*). However, while we hypothesized that the same would be true of the arr2-4A mutant, this was not the case. Following 60 minutes stimulation, AP-2 showed strong colocalization with the 633-6pep-FPR/arr2-4A complex, as strong as or stronger than that observed with wild type arrestin-2. A similar association of AP-2 with the 633-6pep-FPR/arr2-4A complex was also observed following 15 and 30 minutes stimulation. Our results suggest that the arr2-4A mutant, in contrast to the arr2-F391A mutant, is capable of binding AP-2. The major difference between wild type arrestin-2 and the 4A mutant in this assay is that

there is little, if any, cytosolic 633-6pep-FPR/arr2-4A complex (particularly at 60 min) suggesting an accumulation of complex in recycling endosomes.

To determine the rate of association of AP-2 with FPR/arrestins, we measured AP-2 colocalization with FPR/arrestin-2 complexes over time by visually scoring the fraction of cells in which AP-2 was colocalized with FPR/arrestin-2 perinuclear complexes (Figure 2.14). Results are normalized to the response for wild type arrestin-2 at 60 minutes. At 15 minutes, 20-30% of cells show AP-2 to be clustered with arrestin when either wild type arrestin-2 or arr2-4A is expressed. The percentage of cells showing colocalization increased at the 30 and 60 minute time points for both wild type arrestin-2 (~35 and ~80%, respectively) and arr2-4A (~60 and ~95%, respectively). The arr-2-F391A mutant however showed essentially no AP-2 localization at any of the time points assayed.

To confirm the above results and validate our use of a GFP-tagged α-subunit of AP-2, we examined U937 cells that stably express the FPR (U937 FPR) and both endogenous arrestins. U937 cells are a promonocytic cell line used extensively as a model for FPR function (*39, 40*). Confocal immunofluorescence microscopy of U937 FPR cells transiently expressing Rab11-GFP and stained with antibodies against endogenous AP-2 confirms our results regarding the subcellular localization of AP-2 (Figure 2.15A). In unstimulated cells, Rab11-GFP is localized to the perinuclear region while AP-2 shows puncta at the cell membrane with limited cytosolic staining. Upon stimulation of the FPR with Alexa 546-6pep (546-6pep) for 30 min, ligand-FPR complexes colocalize with endogenous AP-2 in the Rab11 endosome. In addition, ligand-receptor complexes exist outside the perinuclear region, but with little or no AP-2

staining, consistent with the idea that AP-2 only associates significantly with the FPR at recycling endosomes. Alternatively, these vesicles may represent efferent recycling endosomes (see below).

While colocalization of scaffolded proteins helps to define aspects of receptor trafficking and provides sub-micron resolution, it cannot directly measure protein complex formation. To extend our microscopy results (Figure 2.6), we immunoprecipitated FLAG-tagged arrestins and assayed AP-2 complex binding over a time course of FPR-activation (Figure 2.7A). We added additional shorter time points as compared microscopy assays to determine whether FPR/arrestin/AP-2 complexes form prior to their detection by colocalization in punctate structures. Western blotting with antibodies directed against the β-subunit of AP-2 (the subunit that directly binds wild type arrestin-2 (*38*)) did not detect any adaptin following immunoprecipitation with anti-FLAG antibodies using arrestin-deficient cell lysates. Upon expression of FLAG-tagged wild type arrestin-2 in arrestin-deficient cells and immunoprecipitation of arrestin-2, β-adaptin was detected in the immunoprecipitate as early as 5 minutes (quantitated in Figure 2.7B), but not in unstimulated cells. Although there was virtually no detectable binding of AP-2 to the F391A mutant, particularly at the later time points, AP-2 bound to the 4A mutant to a much greater extent than wild type arrestin-2 (~6-fold increase).

2.3.6 FPR displays differential trafficking with AP-1 complexes in the presence of arrestins.

AP-1 has been localized to the TGN and perinuclear recycling endosome of cells and is required for trafficking of proteins from these compartments to the plasma

membrane (*41, 42*). In addition, the beta-subunit of AP-1 (which in AP-2 directly binds arrestin-2) shows significant overall homology to the beta-subunit of AP-2. In addition, amino acids shown to be necessary for arrestin-2 binding to β2-adaptin (E849, Y888 and E902) (*43*) are absolutely conserved in β1-adaptin (*44*). To determine whether AP-1 might also play a role in FPR trafficking, we examined the localization of the FPR, arrestins and AP-1 using confocal fluorescence microscopy. In unstimulated cells, AP-1 was localized primarily in a perinuclear region, but was also present in the cytoplasm. When the FPR was stimulated in the absence of arrestins, receptor accumulated in the perinuclear recycling endosome (see Figure 2.5) and it colocalized extensively with AP-1, with little or no receptor observed outside this compartment (Figure 2.8A). Upon stimulation of cells expressing wild type arrestin-2, the FPR-arrestin-2 complexes were extensively localized with AP-1 in the perinuclear region. However, arrestin was also observed outside this region in cytoplasmic vesicles in association with the FPR and AP-1. This suggests that upon internalization, the FPR traffics to recycling endosomes where it associates with AP-1, which is likely involved in escorting the receptor back to the cell surface. The fact that arrestin appears to mediate these late trafficking events indicates a role for arrestin in receptor trafficking at a much later stage than previously thought. In contrast to wild type arrestin-2, the F391A and 4A mutants both accumulated with the FPR in the AP-1-positive endosome in the perinuclear region with no receptor, arrestin or AP-1 observed outside this cellular compartment, consistent with the lack of recycling. To further assess AP-1 trafficking with FPR/arrestin-2 complexes, we quantitated the number of cells that show AP-1 puncta outside of the perinuclear region (Figure 2.8B). Results demonstrate that only when wild type arrestin-

2 is present is AP-1 observed on vesicles outside the perinuclear region following FPR activation. Finally, pulse-chase experiments (10 min pulse with 633-6pep, 50 min chase) using arr2$^{-/-}$/3$^{-/-}$ FPR cells transiently transfected with γ-GFP show that AP-1 is colocalized with 633-6pep-FPR-wild type arrestin-2 in cytoplasmic vesicles following the 50 min chase, but not in early endosomes immediately following internalization (10 min pulse with no chase, unpublished data).

In order to confirm that the GFP-fused γ-subunit of AP-1 accurately reflects endogenous AP-1 trafficking, we used confocal immunofluorescence microscopy in U937 FPR cells transiently transfected with Rab11-GFP (Figure 2.15B). In unstimulated cells, AP-1 is predominantly colocalized with perinuclear Rab11 with minimal cytosolic staining. Upon stimulation of the FPR with 546-6pep, ligand-receptor complexes colocalize with Rab11 and AP-1 in the perinuclear region. In addition, ligand-receptor complexes found in endosomes outside the perinuclear region also colocalize significantly with AP-1 consistent with efferent trafficking of the FPR.

2.3.7 AP-1 binds arrestin in an FPR activation-dependent manner.

Based on the colocalization of the FPR, arrestin and AP-1 upon receptor activation, we sought to determine whether arrestin also forms a complex with AP-1. Arr2-WT-FLAG was transiently transfected into arr2$^{-/-}$/3$^{-/-}$ FPR cells, and activated prior to immunoprecipitation with anti-FLAG antibodies (Figure 2.8C). AP-1 complex was detected in the immunoprecipitates by blotting for the γ-subunit of AP-1. In the absence of arrestins, AP-1 was not detected in anti-FLAG immunoprecipitates. However, in the presence of arr2-WT-FLAG, the γ-subunit of AP-1 was detected in the anti-FLAG

immunoprecipitates in a stimulation-dependent manner, suggesting an association between the FPR-arrestin complex and AP-1, particularly at times consistent with receptor recycling.

2.3.8 Arrestin mutants and AP-2 regulate recycling of the FPR.

Previous reports have demonstrated that recycling of the FPR is impaired in the absence of arrestins and that this phenotype is rescued by reconstitution with wild type arrestin-2 (*12*). Similar to our previous results, in the current experiments, $arr2^{-/-}/3^{-/-}$ FPR cells recycled ~6% of internalized FPR whereas $arr2^{+/+}/3^{+/+}$ FPR cells recycled ~30% of internalized receptor with similar initial levels of internalization (unpublished data). $Arr2^{-/-}/3^{-/-}$ FPR cells transfected with EGFP alone do not recycle significant amounts of internalized FPR whereas reconstitution with wild type arrestin-2 significantly increases the amount of FPR recycled to ~15% (unpublished data). Expression of either the F391A or 4A mutants yielded no significant recycling of FPR (~5% for the 4A mutant and no recycling for the F391A mutant, unpublished data). These results confirm that the accumulation of the FPR in recycling endosomes in the presence of the F391A or 4A mutants corresponds to a lack of recycling as a result of a block in efferent trafficking from Rab11-containing recycling endosomes.

In order to determine explicitly whether AP-2 and AP-1 regulate FPR recycling, we used siRNAs to knockdown the μ1A and μ2 subunits of AP-1 and AP-2, respectively in U937 FPR cells (Figure 2.9A). Knockdown of μ1A and μ2 subunit expression was >90% in both cases (unpublished data). In addition, expression of the β-subunit of AP-2 and γ-subunit of AP-1 was decreased as well, but not to the same extent (>70% and

>50% respectively, unpublished data). Although the rate of FPR internalization was not affected by knockdown of either AP-2 or AP-1 (control, 1.4+/-0.2 min; AP-1, 1.5+/-0.15 min; AP-2, 1.6+/-0.18 min), the extent of FPR internalization was modestly reduced upon AP-2 knockdown (control, 75+/-2%; AP-1, 82+/-3%; AP-2, 57+/-5%). FPR recycling was measured relative to the amount of internalization for each condition normalized to the extent of recycling in cells electroporated with control siRNA. Whereas knockdown of AP-2 produced a significant decrease of about 40% in FPR recycling ($p<0.05$), knockdown of AP-1 produced no effect (Figure 2.9B). Finally, U937 FPR cells treated with siRNAs were stimulated with 546-6pep for 30min and imaged by confocal fluorescence microscopy. Ligand-receptor complexes were observed in the perinuclear region under all conditions, but when AP-2 levels were reduced, liganded FPR exhibited increased juxtanuclear accumulation with reduced peripheral vesicles being observed (unpublished data). These results confirm an essential role for AP-2 in the recycling of the FPR.

2.3.9 Endogenous AP-2 and AP-1 traffic differentially with the FPR.

In order to determine whether AP-2 and AP-1 specifically colocalize with each other in response to FPR stimulation and trafficking, U937 FPR cells were transiently transfected with GFP subunits of either AP-2 or AP-1 and stained for the complimentary adaptor protein with antibodies to AP-2 and AP-1 subunits (Figure 2.9C). In unstimulated cells, GFP-fused AP-1 and antibody staining of endogenous AP-1 show a perinuclear region and some cytosolic distribution. Similarly, GFP-fused AP-2 and staining of endogenous AP-2 both demonstrate a punctate localization at the plasma

membrane with additional diffuse cytosolic expression. Upon stimulation of the FPR with 546-6pep for 30min, ligand-FPR complexes are present in a perinuclear location colocalized with both AP-2 and AP-1. Under these conditions, AP-2 is only seen with the FPR in the perinuclear location and AP-1 is seen in the perinuclear location as well as in cytosolic endosomes consistent with normal efferent FPR trafficking. These results not only validate the use of GFP-fused AP constructs but also confirm and extend the conclusions drawn from studies in MEF cells.

2.4 DISCUSSION

Receptor trafficking is important to proper receptor and therefore cell function. We have previously suggested a link between FPR-mediated apoptosis and altered FPR trafficking in the absence of arrestins (*12, 22*). In this report, we demonstrate that arrestin mutants that do not rescue FPR-mediated apoptosis accumulate in the Rab11, perinuclear recycling compartment. Furthermore, accumulation of receptor in this compartment was shown to be due to impaired recycling of internalized FPR in the presence of F391A and 4A mutants, resulting from altered AP-2 binding to arrestin-2. Associations between arrestin and AP-2 and AP-1 were also demonstrated by confocal microscopy and co-immunoprecipitations. siRNA-mediated knockdown of AP-2 confirmed the necessity of this interaction in FPR recycling, although an absolute requirement for an interaction with AP-1 in FPR recycling could not be demonstrated. As signaling inhibitors have been shown to inhibit FPR-mediated apoptosis, our results suggest that inhibition of proper FPR trafficking also alters proper spatial control of FPR signaling complexes leading to the initiation of apoptosis within the cell. This is supported by results that show spatial control of GPCRs can induce or limit their potential to create signaling complexes and initiate cellular signaling pathways (*45*).

While the internalization of numerous GPCRs require arrestin, AP-2 and clathrin, the FPR was one of the first GPCRs suggested to internalize through arrestin- and clathrin-independent mechanisms (*33, 46, 47*). Arrestin independence was ultimately definitively demonstrated using arrestin-deficient mouse embryonic fibroblasts, in which the stably expressed FPR exhibited normal internalization (*12*). Consistent with this observation is the fact that none of the arrestin mutants used in this study defective in

AP-2/clathrin binding (F391A and ΔLIELD, respectively) or arrestin dephosphorylation (S412D) inhibited FPR internalization. These results also indicate that a lack of receptor internalization was not responsible for the observed rescue of apoptosis by certain arrestin mutants (ΔLIELD and S412D). Overall, these results support the conclusion that FPR internalization is independent of AP-2, clathrin and the phosphorylation state of arrestin.

Both arrestin mutants used in this study that did not rescue FPR-mediated apoptosis did inhibit FPR trafficking by preventing FPR recycling as a result of altered interactions with AP-2. The F391A arrestin mutant was previously shown to exhibit decreased binding to the β-subunit of AP-2 through *in vitro* binding assays while its overexpression in cells inhibited internalization of the β2-AR (*23*). While the 4A mutant has not been previously described, the mutation and characterization of individual amino acids (K397, M399 and K400) have been described (*8*). These individual mutants showed similar binding properties with the β-subunit of AP-2 and produced similar effects on β2-AR internalization as the F391A mutant. What we found most surprising was the differential interaction these arrestin mutants displayed with the AP-2 complex. While the F391A mutant did not significantly colocalize or co-immunoprecipitate with AP-2 at any time point or at any location within the cell, the 4A mutant strongly colocalized with AP-2 upon FPR activation. In fact, co-immunoprecipitation assays demonstrated increased binding of AP-2 to the 4A mutant compared to wild type arrestin-2. The reason for increased AP-2 binding to an arrestin mutant whose component mutations show decreased binding is unclear, but may be due to conformational changes within the protein that alter the binding properties of this

specific motif or other secondary sites. It is intriguing that in addition to decreased binding of AP-2 to arrestin-2, increased association also inhibits the proper trafficking of the FPR.

A recent report (*35*) suggests that the region of arrestin between amino acids 383-402 forms an α-helix as it binds the β-subunit of AP-2. The 4A mutant may form a more stable α-helix that increases binding to AP-2 through the remaining AP-2 interacting residues such as F391, R393 and R395 or alternatively it may exist in a constitutively active state as previously described for the arrestin-2 3A mutant (I386A/V387A/F388A), which dislodges the tail of arrestin from the body of the protein (*48*). Another constitutively active mutant of arrestin (R169E in the polar core of the protein) has also been shown to exhibit increased binding to AP-2 (*8*). Surprisingly, the 6A mutant, which rescued FPR-mediated apoptosis and bound to activated FPR, lacked many of the residues that have been demonstrated to be involved in the association with AP-2, suggesting the 6A mutant may bind AP-2 at alternate or secondary sites. Similar to our 4A mutant, an AP-2 mutant R879A shows significantly enhanced (10-fold) binding to arrestin-2 (*35*), suggesting that this interaction can be both positively and negatively modulated by mutations in both arrestin and AP-2. The inhibitory effect of increased AP-2 binding by the 4A mutant suggests that appropriate cycling of AP-2 with arrestin (association followed by dissociation) is required for the exit of the FPR from recycling endosomes. This idea is supported by recent evidence demonstrating Src activity as a necessary component for AP-2/arrestin dissociation and internalization of the β2-AR (*38*). When Src activity was inhibited, AP-2 remained bound to arrestin and β2-AR internalization was decreased. While the regulation of β2-AR trafficking by AP-2

clearly occurs at the plasma membrane, arrestin's role in FPR trafficking appears to lie specifically with its recycling and not its internalization leaving AP-2's role in GPCR trafficking an intriguing phenomenon in GPCR function. Other GPCRs likely colocalize with AP-2 in the perinuclear region as well. In a recent report (*49*), the authors found that two purinergic receptors, $P2Y_1$ and $P2Y_{12}$, colocalized with AP-2 in perinuclear "endocytic compartments", though the identity of this compartment was not established.

A significant remaining question is: What regulates the location at which AP-2 mediates its effects on GPCR-arrestin complexes? We have recently shown that the phosphorylation pattern of the FPR is highly complex with respect to its regulation of arrestin binding, internalization and desensitization (*50*). Although the presence of a single phosphorylation site can be sufficient to initiate internalization and arrestin binding, the presence of additional sites can inhibit arrestin binding without affecting receptor internalization. Furthermore, work by our lab has suggested that the pattern of phosphorylation sites within the FPR carboxy terminus regulates arrestin affinity for the FPR, its ability to induce changes in ligand affinity, as well as the intracellular trafficking pattern of internalized receptors (*27-29, 51*). The stability/affinity of the receptor-arrestin interactions also regulates the trafficking of the β2-AR and vasopressin V2 receptors. These effects are likely a result of carboxy terminus phosphorylation patterns, as exchanging the carboxy terminus of the β2-AR for that of the vasopressin V2 receptor produced a trafficking pattern typical of the vasopressin V2 receptor (*52, 53*). A similar conclusion regarding the complexity of receptor phosphorylation has recently been made based on functional studies of the β2-AR following manipulations of GRK and arrestin expression (*54, 55*). The results suggested that GPCR phosphorylation

patterns resulting from the activity of GRK4/5 but not GRK2/3 produce arrestin-mediated signaling functions through ERK1/2. We therefore speculate that the initial phosphorylation of the FPR upon ligand binding may be sufficient to initiate internalization but insufficient to promote arrestin binding. Only upon additional phosphorylation, perhaps following internalization, is the receptor then competent for arrestin binding. Furthermore, it is possible that additional receptor or arrestin phosphorylation/modification may be required to permit the binding of AP-2 to FPR-arrestin complexes within endosomes.

Although AP-1 is predominantly known for mediating vesicle traffic from the Golgi compartment to the plasma membrane, it has also been shown to be involved in the formation of recycling vesicles (*56, 57*). To our knowledge, our report is the first to suggest a role for AP-1 in the post-endocytic processing of GPCRs. Colocalization studies with the FPR and arrestins demonstrate that upon FPR stimulation, receptor and arrestins colocalize with AP-1 in a perinuclear region. However, while the FPR and arrestins-F391A/4A remain with AP-1 in this region, in the presence of wild type arrestin-2, vesicles containing FPR, arrestin and AP-1 are observed outside the perinuclear region. Although AP-1 associates with arrestin in an FPR activation-dependent manner, siRNA studies do not show an effect on FPR recycling. In a recent review (*58*), AP-4 was described as having possible additional functions, besides being localized in the Golgi and trafficking cargo to the cell surface. It may be that AP-4, or another trafficking protein, is capable of serving a redundant function to AP-1 when its levels are decreased.

Based on the results of this study, we propose a new model of FPR internalization and recycling (Figure 2.10). In this model, following ligand binding the FPR internalizes in an arrestin-independent manner. After, or perhaps during internalization, the FPR binds to arrestin, resulting in the presence of FPR/arrestin complexes in early endosomes. At some point during its trafficking to the Rab11, perinuclear recycling endosome (or after its arrival at this location) the FPR-arrestin complex recruits AP-2. Within or as it exits the recycling compartment, the FPR/arrestin complex releases AP-2. AP-1 mediated trafficking subsequently facilitates exit of the FPR/arrestin complex from recycling endosomes and their return to the cell surface. Along the path to the cell surface the complex dissociates and the FPR is dephosphorylated. Finally, the FPR completes its return to the cell surface in a resensitized form ready to continue signaling.

In this report, we have described a novel mechanism for GPCR post-endocytic trafficking and recycling. This is the first report that begins to reveal the protein-protein interactions necessary for the recycling of certain GPCRs, revealing a definitive role for arrestin in the late stages of GPCR trafficking as compared to the β2-AR, where arrestins are involved in the earliest events of GPCR trafficking (*59*). Furthermore, the fact that FPR-mediated apoptosis occurs under conditions where arr2-F391A/-4A are bound to the FPR demonstrates that the FPR-initiated apoptotic signaling is not purely a result of receptor activation in the absence of arrestins and confirms that receptor-arrestin accumulation in recycling endosomes results in aberrant signaling that leads to apoptosis. With this report of novel roles for arrestins and adaptor proteins in the

trafficking and signaling of GPCRs, new avenues for the targeting of GPCR function are presented that may lead to therapeutic interventions for disease processes.

2.5 MATERIALS AND METHODS

2.5.1 Reagents, Plasmids and Mutagenesis. All reagents are from Sigma unless otherwise noted. With the exception of the arr2-F391A and -ΔLIELD mutants, regions of arrestin-2 were mutated by site-directed mutagenesis and cloned into EGFP-N1 vector or mRFP1 vector using standard subcloning procedures and HindIII/ApaI restriction sites. Arr2-F391A and -ΔLIELD constructs were a gift from Jeffrey Benovic. These constructs were amplified using PCR with primers that created HindIII/ApaI restriction sites and subcloned as describe above. Arr2-WT-FLAG was created by cutting Arr2-WT-GFP at the ApaI/NotI restriction sites to remove GFP and inserting a linker that contained the FLAG sequence. Arr2-F391A- and Arr2-4A-FLAG were constructed by digesting with HindIII/ApaI sites and subcloning the ~1300bp fragment into the HindIII/ApaI restriction sites of Arr2-WT-FLAG. All mutants were confirmed by DNA sequencing. Rab11-GFP was a gift from Angela Wandinger-Ness. GFP-fused α-subunit of AP-2 and GFP-fused γ-subunit of AP-1 (*60*) were gifts from Lois Greene.

2.5.2. Cell Culture and Transfection. $Arr2^{-/-}/3^{-/-}$ FPR cells were grown in DMEM with 10% fetal bovine serum, 100 units/mL penicillin and 100units/mL streptomycin at 37°C and 5% CO_2. U937 FPR cells were grown in RPMI with 10% fetal bovine serum, 100 units/mL penicillin and 100units/mL streptomycin at 37°C and 5% CO_2. Transient transfections of mouse embryonic fibroblasts were performed with Lipofectamine 2000 according to manufacturer's instructions. siRNA transfection of U937 FPR cells was performed using siPORT Electroporation Buffer (Ambion) with a Genepulser Xcell (Bio Rad) according to manufacturer instructions. Cells were transfected with 20μg of siRNA twice at 72 hour intervals. Cells were assayed 72 hours after the second transfection.

Cell survival was measured using Trypan Blue and was >95% for all transfections. siRNAs used for depletion of μ1A (AP-1) and μ2 (AP-2) were as previously described (*61*). mRNA gene target sequences for siRNA design were as follows: μ1A—GGCAUCAAGUAUCGGAAGA; μ2—GUGGAUGCCUUUCGGGUCA. A nonfunctional siRNA (Ambion) was used as a control.

2.5.3 Cell Rounding. Arr2$^{-/-}$/3$^{-/-}$ FPR cells transiently transfected with GFP-fused arrestins were plated on 12mm-glass coverslips, incubated overnight and serum-starved for 30 minutes. Cells were incubated in SFM or were stimulated with 10nM fMLF in SFM 5 hours at 37°C. Cells were washed twice with PBS, fixed with 2% paraformaldehyde and mounted using Vectashield. Slides were viewed by phase-contrast microscopy and random fields were evaluated (at least five) until 100-300 GFP-expressing cells were assayed. GFP-expressing cells were counted "positive" for cell death if they rounded or if they were spherical refractile cells with no extensions. Data are expressed as a percentage of rounded/GFP-expressing cells.

2.5.4. Apoptosis. Arr2$^{-/-}$/3$^{-/-}$ FPR cells transiently transfected with GFP-fused arrestins were plated on 12mm-glass coverslips, incubated overnight and serum-starved for 30 minutes. Cells were incubated with SFM or were stimulated with 10nM fMLF in SFM for 5 hours at 37°C. Cells were stained with 100ng/mL propidium iodide in SFM at room temperature for 5-10 minutes, washed twice with PBS, fixed with 2% paraformaldehyde and mounted using Vectashield. Slides were viewed by fluorescence microscopy and random fields were evaluated (at least five) until 100-300 GFP-expressing cells were assayed. GFP-expressing cells were counted "positive" for cell death if they were

stained with propidium iodide. Data are expressed as a percentage of PI-positive/GFP-expressing cells.

2.5.5. FPR Internalization. Arr2$^{-/-}$/3$^{-/-}$ FPR cells transiently transfected with GFP-fused arrestins were harvested by trypsinization. U937 FPR cells were electroporated with siRNAs and harvested by centrifugation. Cells were resuspended in SFM and stimulated for 2, 5, 10, 20 and 30 minutes with 1μM fMLF. Cells were then washed extensively with cold SFM to remove excess unlabelled ligand. Remaining receptors on the cell surface were labeled with 10nM 633-6pep. Cells were then assayed by flow cytometry using a Becton-Dickinson FACSCalibur. Cells (100,000) were gated for live cells using forward and side scatter parameters. GFP-fused arrestin mutant expressing cells were subsequently gated using FL-1 and the mean channel fluorescence (MCF) was determined in FL-4 to monitor cell surface expression of the FPR. Non-specific binding was determined by labeling arr2$^{-/-}$/3$^{-/-}$ cells not expressing the FPR with 633-6pep and assaying as described. Non-specific binding was subtracted before further calculations. MCF from unstimulated cells represented 100% FPR cell surface expression. Cell surface expression from stimulated cells was calculated by dividing the MCF following treatment by the MCF from unstimulated cells. Internalization data were then plotted using GraphPad Prism to calculate maximum internalization extents using a singe exponential decay. Statistical analysis was performed using Student's t-test.

2.5.6. Confocal Fluorescence Microscopy. Arr2$^{-/-}$/3$^{-/-}$ FPR cells or U937 FPR cells were transiently transfected with RFP-fused arrestins and GFP-fused Rab11, α-subunit of AP-2 or γ-subunit of AP-1. Arr2$^{-/-}$/3$^{-/-}$ FPR cells were plated on 25mm-glass coverslips and grown overnight. U937 FPR cells were harvested by centrifugation.

Cells were serum-starved for 30 minutes and stimulated with 10 nM 633- or 546-6pep in SFM for indicated time at 37°C. Coverslips or harvested cells were rinsed with PBS, fixed with 2% paraformaldehyde at room temperature and mounted with Vectashield. For experiments using antibody staining, after fixation cells were incubated in 0.02% Saponin/3% BSA for 15-20 minutes at room temperature. Cells were washed and incubated in 1:100 primary antibody (100/3 for γ1-adaptin antibody or AP-6 for α-adaptin (Affinity Bioreagents)) in 3% NGS for 30 minutes at room temperature. After further washing, cells were incubated in 1:200 secondary antibody (Cy5-conjugated Donkey Anti-Mouse (Jackson Immunoresearch)) in 3% NGS for 30 minutes at room temperature. Cells were then washed and mounted using Vectashield. Fluorescence images were acquired using a Zeiss LSM 510 inverted laser scanning microscope equipped with He-Ne and Kr-AR lasers. To assess the extent of colocalization, cells with perinuclear arrestin clusters were viewed and scored for the presence of any corresponding AP-2 clusters. Data were expressed as mean +/- SEM AP-2 clusters/arrestin clusters in >25 cells/experiment and were normalized to the wild type arrestin response at 60 minutes.

2.5.7. Cell Lysis and Immunoprecipitation. Arr2$^{-/-}$/3$^{-/-}$ FPR cells transiently transfected with FLAG-tagged arrestins were grown to confluence. Cells were serum-starved for 30 min and stimulated for the designated times with 10nM fMLF in SFM at 37°C. Media was removed and 1mL of cold co-IP lysis buffer (1% v/v TX-100, 150mM NaCl, 10mM Tris-HCl pH 7.4 supplemented with protease inhibitor cocktail (Calbiochem)) was added immediately. Lysates were collected, incubated on ice for 30 min, and centrifuged at maximum speed for 30 min at 4°C. An aliquot from each tube

was set aside for determining pre-immunoprecipitation levels of proteins by Western blot. For immunoprecipitations, 25µL of protein A Sepharose (Pierce) was washed three times with co-IP lysis buffer. Beads were rotated for at least one hour at 4°C in 250µL of co-IP lysis buffer with 1:1000 M2 Anti-FLAG antibody. Beads were washed with co-IP lysis buffer and lysates were added and rotated overnight at 4°C. The following day, beads were washed again with co-IP lysis buffer, 40µL of 2X Sample Buffer was added and immunoprecipitated proteins released by boiling for 5 minutes.

2.5.8. Western Blotting. Lysates or immunoprecipitates were resolved with SDS-PAGE. Proteins were transferred to PVDF membranes and blocked for 1 hour with 5% dry milk in TBS-T. Blotting was carried out using 1:1000 dilutions of biotinylated M2 mouse anti-FLAG antibody, M2 mouse Anti-FLAG, mouse anti-β-adaptin antibody (BD Biosciences), mouse 100/3 to γ1-adaptin antibody, rabbit RY/1 to *µ*1A (gift from L. Traub), rabbit R11-29 to *µ*2 ((*62*), gift from J. Bonifacino) and 1:4000 mouse anti-GAPDH antibody (Chemicon) in 5% dry milk in TBS-T overnight at 4°C. Blots were washed with TBS-T and incubated with 1:2500 HRP-streptavidin (in 5% BSA in TBS-T), 1:2500 HRP rabbit anti-mouse antibody or 1:2500 HRP goat anti-rabbit antibody (in 5% Dry Milk in TBS-T) at room temperature for 1 or 3 hours, respectively. Bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Densitometry was performed using Quantity One (BioRad) and ratios expressed as mean ratio +/- SEM adaptin/arrestin immunoprecipitated and normalized to zero time points for each vector expressed.

2.5.9. FPR Recycling. Arr2$^{-/-}$/3$^{-/-}$ FPR cells transiently transfected with GFP-fused arrestins or U937 FPR cells transfected with siRNA were harvested and resuspended in

SFM. An aliquot was removed to measure total cell surface receptor. The remaining cells were stimulated with 1μM fMLF in SFM at 37°C for 1 hour and were then washed extensively to remove excess unlabelled ligand. Half the remaining cells were resuspended in pre-warmed SFM for 30 min at 37°C (20 min for U937 FPR cells) to allow the FPR to recycle. The other half was kept on ice to measure post-internalization cell surface receptor levels. All aliquots were then resuspended in SFM containing 10nM 633-6pep and assayed by flow cytometry using a Becton-Dickinson FACSCalibur. For analysis, assayed cells were gated for live cells using forward and side scatter parameters. These cells were then gated using FL-1 for GFP-fused arrestin mutant expression and the mean channel fluorescence was measured in FL-4 to monitor cell surface expression of the FPR. Non-specific binding was determined by labeling arrestin knockout cell lines (or U937 cells lines) that did not express FPR and was subtracted from all values. To account for differences in total recycling that could be due to differences in the initial extent of internalization, the fraction of recycled FPR (starting from the final internalization time point) was divided by the fraction of internalized FPR. Data are expressed as a percentage of recycled receptor /internalized receptor.

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2.7 ABBREVIATIONS

AP-1—adaptor protein-1 complex
AP-2—adaptor protein-2 complex
Arr2—arrestin-2
Arr2$^{-/-}$/3$^{-/-}$ FPR—arrestin-2$^{-/-}$/-3$^{-/-}$ knockout MEF cells stably expressing the FPR
fMLF—*N*-formyl-Methionyl-Leucyl-Phenylalanine
FPR—*N*-formyl peptide receptor
GPCR—G protein-coupled receptor
MEF—mouse embryonic fibroblast
PI—propidium iodide
SFM—serum-free medium (DMEM or RPMI)
6pep—*N*-formyl-Leucyl-Leucyl-Phenylalanyl-Leucyl-Tyrosinyl-Lysine
U937 FPR—U937 cells stably expressing the FPR

2.8 FIGURE LEGENDS

2.8.1. Figure 2.1. Structural regions of arrestin-2 do not rescue FPR-mediated apoptosis. Arr2$^{-/-}$/3$^{-/-}$ FPR cells were transiently transfected with wild type or structural regions of arrestin-2 fused to GFP. Cells were stimulated for 5 hours in SFM with 10nM fMLF or vehicle at 37°C. GFP-expressing cells were randomly viewed using phase-contrast microscopy and counted for normal morphology or cell rounding. Only wild type arrestin-2 was capable of preventing FPR-mediated apoptosis. Data are expressed as mean +/- SEM rounded cells/GFP-expressing cell from three independent experiments.

2.8.2. Figure 2.2. Sequence of arrestin-2 carboxy terminus and selected mutants. Arrestin-2 mutants within the carboxy terminus were designed based on previously described mutations or by changing regions of qualitatively similar amino acids (e.g. groups of charged residues) to alanine. An additional mutant, ΔLIELD (located just before the site of truncation at amino acid 382), lacking the clathrin-binding motif, was also used.

2.8.3. Figure 2.3. Rescue of FPR-mediated apoptosis by arrestin-2 mutants. Arr2$^{-/-}$/3$^{-/-}$ FPR cells transiently transfected with GFP-fused arrestin mutants were assayed for apoptosis. Cells were stained with PI and 100-300 GFP-expressing cells were viewed and scored for the presence of PI staining. Only in the absence of arrestin (EGFP vector only) or in the presence of arr2-(1-382), arr2-4A and arr2-F391A did the cells continue to undergo FPR-mediated apoptosis. All other mutants prevented apoptosis

like wild type arrestin-2. Data expressed as mean ± SEM PI positive/GFP cell from three independent experiments.

2.8.4. Figure 2.4. Internalization of the FPR in presence of arrestin-2 mutants. Arr2$^{-/-}$/3$^{-/-}$ FPR cells transiently transfected with GFP-fused arrestins were stimulated with 1μM fMLF and aliquoted at multiple time points. Cells were washed free of fMLF, labeled with 633-6pep and analyzed by flow cytometry for residual cell surface receptor. Cells were gated for GFP expression (FL1) to restrict the analysis to transfected cells. Data are expressed as the maximum extent of internalization based on curve fitting using a single exponential decay. Data are expressed as mean ± SEM from three independent experiments

2.8.5. Figure 2.5. Arrestin-2 mutants incapable of rescuing apoptosis accumulate in recycling endosomes. Arr2$^{-/-}$/3$^{-/-}$ FPR cells were transiently transfected with GFP-fused Rab11 and RFP-fused arrestins (wild type, arr2-4A, arr2-F391A and arr2-S412D) and plated on coverslips. Cells were stimulated with the 633-6pep for 1 hour at 37°C and imaged by confocal fluorescence microscopy using a 63X objective. In the absence of arrestin or the presence of arr2-4A or arr2-F391A, the FPR-ligand complex accumulated extensively in the Rab11 compartment. In cells expressing wild type arrestin-2 and arr2-S412D, the receptor is also seen in cytoplasmic vesicles. Representative images are shown from three independent experiments. Scale bars, 10μm. See Figure 2.12 for images of additional mutants.

2.8.6. Figure 2.6. Arrestin-2 mutants differentially colocalize with AP-2. Arr2$^{-/-}$/3$^{-/-}$ FPR cells were transiently transfected with RFP-fused arrestins (wild type, arr2-F391A and arr2-4A) and the GFP-fused α-subunit of AP-2. Cells were subsequently stimulated with 633-6pep at 37°C for the indicated time and imaged by confocal fluorescence microscopy with a 63X objective. In cells expressing wild type arrestin-2 or arr2-4A, following 60 min stimulation, AP-2 is localized with the FPR and arrestin in perinuclear endosomes. On the contrary, in cells expressing arr2-F391A, no AP-2 is associated with internalized receptor. Representative images are shown from three independent experiments. Scale bars, 10µm. See Figure 2.13 for images of additional time points.

2.8.7. Figure 2.7. Immunoprecipitation of AP-2 with arrestin-2 mutants. ***A)*** Arr2$^{-/-}$ /3$^{-/-}$ FPR cells were transiently transfected with FLAG-tagged arrestins. Cells were stimulated for the indicated times with 10nM fMLF and lysed. Lysates were immunoprecipitated with anti-FLAG antibodies, resolved by SDS-PAGE and blotted for FLAG-tagged arrestins and the β-adaptin subunit of AP-2. Representative blots are shown. ***B)*** Quantitation of immunoprecipitated bands by optometric density. Data are expressed as mean +/- SEM ratio of β-adaptin/arrestin intensity and normalized to respective zero time points. Data are from three independent experiments.

2.8.8. Figure 2.8. FPR-arrestin-2 trafficking and binding with AP-1. ***A)*** Arr2$^{-/-}$/3$^{-/-}$ FPR cells were transiently transfected with RFP-fused arrestins (wild type, arr2-F391A and arr2-4A) and the GFP-fused γ-subunit of AP-1. Cells were then stimulated with the 10nM 633-6pep ligand, fixed and viewed by confocal fluorescence microscopy. In all cases, after 60min stimulation, FPR-arrestin complexes were associated with AP-1.

Note that only with the stimulated cells expressing wild type arrestin-2 are cytoplasmic vesicles containing AP-1, FPR and arrestin observed outside the recycling endosomes. Representative images are shown from three independent experiments. Scale bars, 10µm. ***B)*** AP-1 differentially associates with cytosolic arrestin clusters. Association of AP-1 with cytosolic arrestin-containing clusters was determined by viewing cells with cytosolic arrestin clusters and scoring whether or not colocalized AP-1 clusters were also present. Data are expressed as mean +/- SEM for >30 cells from three independent experiments. ***C)*** Arrestin-2 and AP-1 bind in response to FPR activation. $Arr2^{-/-}/3^{-/-}$ FPR cells were transiently transfected with arr2-WT-FLAG or empty vector and immunoprecipitated with anti-FLAG antibodies after a time course of FPR activation. Protein was resolved by SDS-PAGE and blotted as indicated. Representative blots from three independent experiments are shown.

2.8.9. Figure 2.9. FPR recycling upon adaptor protein depletion. ***A)*** U937 FPR cells were electroporated with control, AP-1 or AP-2 siRNA. A fraction of the electroporated cells were harvested before experimentation and lysed for evaluation of knockdown efficiency. Lysates were resolved by SDS-PAGE and blotted with the indicated antibodies. Representative blots are shown. ***B)*** U937 FPR cells were treated with the indicated siRNA and assayed for FPR recycling. FPR recycling was normalized to that of the control siRNA transfected cells. Control cells internalized 75% of the surface FPR and recycled ~50% of the internalized receptor. Data are expressed as mean+/- SEM FPR recycling/internalization and are representative of three independent experiments. ***C)*** U937 FPR cells were transiently transfected with GFP-fused α- or γ-

subunits. Cells were then stimulated with 546-6pep for the indicated times and stained with antibodies to α- or γ- subunits (AP-2 or AP-1, respectively). Cells were imaged by confocal fluorescence microscopy with a 63X objective. Representative images are shown from three independent experiments. Scale bars, 10μm.

2.8.10. Figure 2.10. Model of FPR post-endocytic trafficking. Following binding of ligand to the receptor and initial G protein-mediated signaling, the FPR becomes phosphorylated and internalizes in an arrestin-independent manner. The FPR nevertheless binds to arrestin either during or shortly after internalization. AP-2 is then recruited to the FPR-arrestin complex. This occurs either at some point during its trafficking to the Rab11, perinuclear recycling endosome or commensurate with or immediately after its arrival at this location. The FPR/arrestin complex then releases AP-2, either within recycling endosomes or as the FPR-arrestin complex exits the recycling compartment. Regardless, AP-1-associated trafficking subsequently results in the FPR/arrestin complex exiting the recycling compartment and returning to the cell surface. During this latter process, the FPR-arrestin complex dissociates and the FPR is dephosphorylated. Finally, the efferent vesicles fuse with the plasma membrane completing the recycling of the FPR to the cell surface in a resensitized form ready to begin signaling anew. This figure was generated using ScienceSlides (www.visislides.com).

2.8.11. Figure 2.11. Arrestin-2 mutants that do not rescue FPR-mediated apoptosis are sensitive to caspase inhibitor treatment. $Arr2^{-/-}/3^{-/-}$ FPR cells were

transiently transfected with GFP-fused arrestins and incubated overnight with 10nM zVAD-FMK (pan-caspase inhibitor). Cells were then stimulated with 10 nM fMLF for 5 hours at 37°C, stained with PI and 100-300 GFP-expressing cells (from at least five random fields) were viewed and scored for PI staining. In all cases where FPR-mediated apoptosis occurred, the zVAD-FMK caspase inhibitor blocked apoptosis. Data expressed as mean ± SEM PI positive/GFP cell from three independent experiments.

2.8.12. Figure 2.12. Arrestin-2 mutants capable of rescuing apoptosis do not accumulate in recycling endosomes. Arr2$^{-/-}$/3$^{-/-}$ FPR cells were transiently transfected with GFP-fused Rab11 and RFP-fused arrestins and stimulated with 10nM 633-6pep. Cells were imaged by confocal fluorescence microscopy using a 63X objective. As with cells expressing wild type arrestin-2 or arr2-S412D (*cf*. Figure 2.5) all cells expressing mutants that rescued the apoptotic phenotype showed the presence of cytoplasmic FPR-arrestin complexes in addition to the accumulated FPR-arrestin complexes in the recycling compartment. Representative images are shown and are representative of three experiments. Scale bars, 10µm.

2.8.13. Figure 2.13. Arrestin-2 mutants differentially colocalize with AP-2. Arr2$^{-/-}$ /3$^{-/-}$ FPR cells were transiently transfected with RFP-fused arrestins (wild type, arr2-F391A and arr2-4A) and GFP-fused α-subunit of AP-2. Cells were stimulated with the 633-6pep ligand for intermediate times (15 and 30 min, *cf*. Figure 2.6). Cells were imaged by confocal fluorescence microscopy with a 63X objective. Representative

images are shown and are representative of three independent experiments. Scale bars, 10μm.

2.8.14. Figure 2.14. Quantitation of AP-2 colocalization with FPR/arrestin complexes. Rate of AP-2 association with arrestin was determined by viewing individual cells and ascertaining whether AP-2 clusters were present with perinuclear arrestin clusters. Data were normalized to the WT response at 60 minutes. Cells were counted from three independent experiments and data are expressed as the mean AP-2 clusters/arrestin clusters +/- SEM.

2.8.15. Figure 2.15. Antibody staining of AP-2 and AP-1 in U937 FPR cells. U937 FPR cells were transiently transfected with GFP-fused Rab11. Cells were then stimulated with 546-6pep for the times indicated and stained with antibodies to the α- or γ- subunits (AP-2 or AP-1, respectively). Cells were imaged by confocal fluorescence microscopy with a 63X objective. Representative images are shown from three independent experiments. Scale bars, 10μm.

Task 2. Src Kinase-Arrestin interaction has two independent roles in *N*-formyl peptide receptor signaling and trafficking

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Src Kinase-Arrestin interaction has two independent roles in *N*-formyl peptide receptor signaling and trafficking

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Running Title: Src Kinase Regulates FPR Signaling and Trafficking

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3.1 ABSTRACT

Arrestins were originally described as molecules that bound ligand-activated, phosphorylated G protein-coupled receptors (GPCR) to block further G protein signaling. Subsequently, they were demonstrated to mediate internalization of the β2-adrenergic and other GPCRs. In addition to blocking G protein signaling and mediating GPCR internalization, arrestins recruit a number of signaling proteins including, but not limited to, Src family kinases, ERK1/2, and JNK3. GPCR-arrestin binding can control the spatial and temporal signaling of these signaling complexes. In previous reports, we have shown that *N*-formyl peptide receptor- (FPR) mediated apoptosis, which occurs upon receptor stimulation in the absence of arrestins, is associated with receptor accumulation in the Rab11, perinuclear recycling endosome. Under these conditions, inhibition of Src kinase and ERK1/2 rescued cells from apoptosis. To better understand the role of Src kinase in these processes, we used an arrestin-2 mutant deficient in Src binding (P91G/P121E). Unlike wild type arrestin, this mutant did not inhibit FPR-mediated apoptosis. However, cells expressing this mutant could be rescued by Src kinase inhibition with PP2. Finally, PP2, while inhibiting apoptosis in the presence of arr2-P91G/P121E, did not prevent FPR accumulation in the perinuclear recycling endosome. On the contrary, Src kinase inhibition by PP2 caused wild type arrestin-2 to accumulate in the recycling endosome without initiating FPR-mediated apoptosis. Based on these observations, we conclude that Src kinase has two independent roles with respect to arrestin-2 binding in FPR signaling and trafficking.

3.2 INTRODUCTION

Arrestins were originally described as cytosolic proteins that bound phosphorylated G protein-coupled receptors (GPCR) (*63*). Through binding to phosphorylated GPCRs, arrestins sterically block the binding of heterotrimeric G proteins. This inhibits the GDP-GTP exchange of the Gα subunit and abates G protein signaling cascades. This was believed to be their primary function. Subsequently, arrestins were discovered to mediate GPCR internalization (*64*). Binding sites for adaptor protein (AP)-2 and clathrin were described within the C-terminus of arrestin (*8, 23, 30*). Classical GPCRs such as the beta2-adreneric receptor (β2-AR) (*65*) and the angiotensin II (Type 1A) receptor ($AT1_AR$) (*38*) require arrestin and subsequent AP-2 and clathrin binding for proper internalization. In addition, arrestin-bound Src kinase is also required for proper internalization of the β2-AR (*66*). However, receptors including, but not limited to, the *N*-formyl peptide receptor (FPR) (*12*) and m2-muscarinic acetylcholine receptor (*67*) do not require arrestin for ligand-dependent internalization.

More recently, arrestins have been described as scaffolds for non-G protein GPCR signaling complexes. Signaling proteins including Src kinase (*66*), ERK1/2 (*20*), JNK3 (*68*) bind arrestins and are activated by ligand-bound GPCRs. It is hypothesized that arrestins recruit activated signaling mediators to activated GPCR signaling scaffolds to prevent crosstalk. For example, following β2-AR activation in the absence of arrestin binding, ERK1/2 is phosphorylated and translocated to the nucleus where it activates downstream effectors leading to changes in transcription (*20*). On the other hand, when ERK1/2 is phosphorylated in the context of a GPCR-arrestin scaffold,

activated ERK1/2 remains in the cytosol and activates unknown effector molecules (*55, 69*). Processes affected by the latter form of ERK1/2 activation are, as of yet, unknown.

Arrestin-Src kinase interaction is the best described of the signaling scaffolds mentioned above. This interaction is necessary for such processes as cell migration and degranulation (*5*). In addition, disruption of the interaction between arrestin-2 and Src kinase, by mutation of arrestins, decreased the phosphorylation of ERK1/2 in response to β2-AR activation (*66*). However, this disruption had no affect on ERK1/2 phosphorylation following stimulation of $AT1_AR$. Finally, proper interaction between arrestin-2 and Src kinase is required to phosphorylate tyrosine residues in AP-2 (*38*). Without phosphorylation of AP-2, the $AT1_AR$ does not internalize in response to activation by ligand.

In the absence of arrestins, FPR activation leads to receptor accumulation in the Rab11, perinuclear endosomes (*12*) and apoptosis (*22*). Apoptosis was rescued by reconstitution of cells with wild type arrestin-2 or by pretreating cells with inhibitors of Src family kinases, ERK1/2, JNK3 and p38. In addition, arrestin-2 mutants that have altered binding to AP-2 did not rescue apoptosis and prevented recycling from the Rab11 endosome (Chapter 2).

Whereas arrestins control early signaling and trafficking events for many GPCRs, it has become apparent that arrestins control post-endocytic events of the FPR. As the interaction between arrestin-2 and Src kinase can mediate both GPCR internalization via AP-2 and activation of ERK1/2, we hypothesized that decreased binding of Src kinase to arrestin-2 would lead to FPR accumulation in the recycling endosome and aberrant activation of ERK1/2 thereby initiating apoptosis. To this end, we used a

mutant of arrestin-2 (arr2-P91G/P121E) that has been previously described to exhibit decreased binding to Src kinase in response to β2-AR activation (*66*) to better understand the role of arrestin-2-Src kinase interaction in FPR trafficking and signaling. Our results demonstrate two independent roles for Src kinase in its association with arrestin-2 that independently regulate FPR signaling and trafficking.

3.3 RESULTS

3.3.1. Src kinase binding-deficient arrestin mutant does not rescue FPR-mediated apoptosis.

We have previously shown that FPR-mediated apoptosis is inhibited by signaling inhibitors of p38, p44/42, JNK and Src family kinases (*22*). Arrestins are thought to serve as scaffolds in signaling complexes to control temporal and spatial signaling (*70*). To better understand how arrestins control signaling within the context of FPR activation and apoptosis, we used a mutant of arrestin-2 that is deficient in Src kinase binding (arr2-P91G/P121E) (*66*). This mutant does not support ERK1/2 phosphorylation in response to β2-AR activation and inhibits the internalization of this receptor.

To determine whether arrestin-Src interaction plays a role in FPR signaling, we examined the arr2-P91G/P121E mutant for its ability to rescue FPR-mediated apoptosis (Figure 3.1A). In the absence of ligand, cells expressing any of the GFP vectors were not stained with propidium iodide (PI) demonstrating a dependence on receptor activation. Large fractions of arr-$2^{-/-}$-$3^{-/-}$ FPR cells expressing EGFP vector (no arrestins present) stained with PI (>90%) consistent with previous reports (*22*). In contrast, when arr-$2^{-/-}$/-$3^{-/-}$ FPR cells are activated in the presence of wild type arrestin-2-GFP, very few cells stained with PI (<5%). When arr-$2^{-/-}$/-$3^{-/-}$ FPR cells are activated in the presence of this mutant, a large proportion of cells (>90%) stained with PI. This led us to conclude that arrestin-Src regulated non-G protein GPCR signaling and prevented FPR-mediated apoptosis.

Our previous report (*22*) has described this phenomenon in detail; including annexin V staining, caspase involvement and PI staining and these data confirm that

this process is FPR- and caspase-dependent apoptosis. To confirm that the observed PI staining was apoptosis, arr-2$^{-/-}$/-3$^{-/-}$ FPR cells expressing GFP-fused arrestins were assayed for apoptosis in the presence of zVAD-FMK (pan-caspase inhibitor) (data not shown). Cells expressing EGFP and arr2-P91G/P121E, were not stained with PI in the presence of caspase inhibitor (<5%), but were stained at high levels in the presence of vehicle (>90%). Wild-type arrestin-2 expressing cells did not stain in either case.

3.3.2. Inhibition of Src kinase rescues FPR-mediated apoptosis in the presence of the P91G/P121E arrestin mutant.

Overexpression of arr2-P91G/P121E inhibits ERK1/2 activation in response to β2-AR activation (*66*). However, this mutant did not affect ERK1/2 activation by $AT1_AR$. These results demonstrated that Src association with arrestin was selectively required to activate ERK1/2 following stimulation of the β2-AR. To determine whether Src kinase activity was required to initiate apoptosis in response to FPR stimulation, independent of arrestin-2, we used the Src family kinase inhibitor, PP2, (Figure 3.1B). In the arr-2$^{-/-}$/-3$^{-/-}$ FPR cells expressing EGFP and the arr2-P91G/P121E mutant <5% stained with PI in the presence of PP2, while >90% stained in the presence of vehicle. In the arr-2$^{-/-}$/-3$^{-/-}$ FPR cells expressing arr2-WT only ~5% stained in the presence of either vehicle or PP2.

Because PP2 is a general inhibitor of Src family kinases, we confirmed that our results were due to inhibition of Src kinase activity and not one of the other family members subject to this inhibitor. Accordingly, arr-2$^{-/-}$/-3$^{-/-}$ FPR cells were cotransfected with GFP-fused arrestins and either wild type or kinase dead (K298M) Src kinase (Figure 3.1C). In the absence of FPR stimulation only ~5% of the cells stained with PI,

indicating apoptosis did not occur. In the presence of wild type Src kinase, only arr-2$^{-/-}$/-3$^{-/-}$ FPR cells expressing EGFP or arr2-P91G/P121E (>90%) stained with PI, consistent with the above results (Figure 3.1A). In arr-2$^{-/-}$/-3$^{-/-}$ FPR cells expressing GFP-fused arrestins and kinase dead Src kinase, <5% cells were stained with PI in response to FPR activation , suggesting that Src kinase activity was required to induce the apoptotic phenotype (Figure 3.1B). These data suggest that although expression of an arrestin mutant with decreased binding to Src kinase does not rescue apoptosis. However, inhibition of Src kinase activity with PP2 does rescue apoptosis indicating that Src kinase signaling does play a role in FPR-mediated apoptosis.

3.3.3. Src kinase does not affect FPR internalization.

FPR internalization is required for FPR-mediated apoptosis in the absence of arrestins (*22*). Based on these data, we expected that in the presence of the arr2-P91G/P121E mutant, the FPR would internalize upon activation. Maximum internalization of the FPR in the presence of the arr2-P91G/P121E mutant is similar to FPR internalization in the presence of EGFP and Arr2-WT (Figure 3.2A). In addition, the rate of FPR internalization was unaffected (data not shown). Because PP2 was capable of inhibiting FPR-mediated apoptosis in the absence of arrestins and presence of Arr2-P91G/P121E-GFP, we tested FPR internalization in the presence of GFP-fused arrestins and PP2. Although Src kinase activity was required for FPR-mediated apoptosis in the arr-2$^{-/-}$/-3$^{-/-}$ FPR cells (*22*) or presence of arr2-P91G/P121E-GFP (Figure 3.1B and C) neither PP2 nor vehicle had any effect on the internalization of the FPR (Figure 3.2B). These data suggest that FPR-mediated apoptosis is not being rescued due to inhibition of FPR internalization.

3.3.4. P91G/P121E mutant alters normal FPR trafficking.

FPR-arrestin complex accumulation in the Rab11-positive, perinuclear recycling endosome correlates directly with FPR-mediated apoptosis (Chapter 2). To determine whether the arr2-P91G/P121E mutant accumulated in the recycling endosome, RFP-fused arrestins and GFP-fused Rab11 WT were transiently expressed in arr-$2^{-/-}$/-$3^{-/-}$ FPR cells. The FPR was activated with Alexa 633-*N*-formyl-Leucyl-Leucyl-Phenylalanyl-Leucyl-Tyrosinyl-Lysine (633-6pep) for 1 hour and ligand-FPR-arrestin complexes were tracked using confocal fluorescence microscopy (Figure 3.3A). In all cases, arrestin was distributed throughout the cytosol and Rab11 was localized in a perinuclear location in unstimulated cells (data not shown, Chapter 2). In the presence of mRFP1 alone, the FPR accumulated in the Rab11 compartment with no significant ligand-receptor complexes present outside this area. In the presence of arr2-WT-mRFP, receptor-arrestin complexes were localized to the Rab11 compartment. However, significant amounts of receptor-arrestin complexes were present outside the Rab11 compartment in endocytic vesicles. These phenotypes are consistent with the FPR's ability to traffic normally in the presence of wild type arrestin-2 (Chapter 2). In the presence of Arr2-P91G/P121E-RFP, receptor-arrestin complexes accumulate in the Rab11 compartment and very little receptor-arrestin complex was seen outside this compartment. This is consistent with the inability of the FPR to recycle and has been hypothesized as an underlying cause of FPR-mediated apoptosis (Chapter 2). These results demonstrate that the arr2-P91G/P121E mutant binds activated receptor.

3.3.5. Inhibition of Src kinase alters normal FPR trafficking.

Because arrestins play a critical role in normal FPR post-endocytic trafficking (Chapter 2) and FPR-mediated apoptosis was inhibited by PP2 in the presence of Arr2-P91G/P121E, we hypothesized that PP2 restores normal FPR-arrestin complex trafficking. To test this hypothesis, we used transfected arr-2$^{-/-}$/-3$^{-/-}$ FPR cells stimulated in the presence of PP2 or vehicle (Figure 3.3B). Vehicle did not affect FPR-arrestin trafficking (Figure 3.7A). Use of PP2 demonstrated no effect on FPR trafficking in the absence of arrestins (mRFP1) as receptor accumulated in the Rab11 compartment. Interestingly, PP2 inhibitor did not affect the trafficking of the FPR in the presence of the P91G/P121E mutant as receptor-arrestin complexes were retained in the Rab11 endosome. Furthermore, in the presence of wild type arrestin-2, PP2 lead to accumulation of the receptor-arrestin complex in the Rab11 recycling endosome. Our results suggest Src kinase activity is necessary for normal trafficking of FPR-arrestin complexes.

To confirm that this was due to inhibition of Src kinase specifically, the same assay was performed with cells co-transfected with wild type Src kinase or kinase dead Src kinase. Wild type Src kinase had no effect on the trafficking of any of the receptor-arrestin complexes (Figure 3.7B) similar to vehicle (Figure 3.7A). The use of kinase dead Src kinase yielded the same results as the use of PP2 (Figure 3.3C) in which all ligand-FPR-arrestin complexes accumulated in the Rab11 compartment.

3.3.6. P91G/P121E arrestin mutant alters FPR dynamics with AP complexes.

Our previous results demonstrated a role for AP-2 in FPR post-endocytic trafficking (Chapter 2). In this study, we determined that AP-2 must bind and release arrestin to allow FPR to exit the perinuclear recycling endosome. Previous reports

demonstrate that Src kinase regulates arrestin-AP-2 interaction to control $AT1_AR$ internalization (*38*). To better understand the role of Src kinase in arrestin-AP-2 interaction, we utilized arr-$2^{-/-}$/-$3^{-/-}$ FPR cells transfected with the GFP-fused α-subunit of AP-2 and RFP-fused arrestins to monitor trafficking via confocal fluorescence microscopy (Figure 3.4A). Antibody staining of the α-subunit of AP-2 confirmed that the α-GFP subunit represented endogenous AP-2 (Chapter 2). In unstimulated cells, arrestins showed a cytosolic distribution with AP-2 which was also present in membrane-associated puncta, consistent with previous data (data not shown, Chapter 2). In the absence of arrestins, following a one hour stimulation of the FPR with 633-6pep, AP-2 failed to colocalize with FPR in the perinuclear region. In contrast, as reported in the presence of wild type arrestin (Chapter 2), AP-2 colocalized with FPR and arr2-WT in the perinuclear region. Interestingly, in the presence of the arr2-P91G/P121E mutant, AP-2 colocalized with receptor-arrestin complex in the perinuclear region suggesting arrestin-AP-2 binding occurs despite the fact that arrestin may not bind Src kinase.

We have shown that AP-1 colocalizes with receptor-associated vesicles outside the perinuclear region only in the presence of wild type arrestin-2 (Chapter 2). Because FPR-arr2-P91G/P121E complexes accumulate in the perinuclear recycling endosome, we hypothesized that arr2-P91G/P121E would not colocalize with AP-1 vesicles outside of the recycling endosome consistent with FPR post-endocytic trafficking in the presence of arrestin mutants that do not rescue FPR-mediated apoptosis. To test this hypothesis, we used arr-$2^{-/-}$/-$3^{-/-}$FPR cells transfected with RFP-fused arrestins and the GFP-fused γ-subunit of AP-1 (Figure 3.4B). Antibody staining of the γ-subunit of AP-1

confirmed that the γ-GFP subunit reveals the localization of endogenous AP-1 (Chapter 2). In unstimulated cells, arrestins were cytosolic and AP-1 was localized in a perinuclear region consistent with previous data (data not shown, Chapter 2). When cells were stimulated for 1 hour by 633-6pep in the absence of arrestins (mRFP1), the FPR accumulated in the AP-1-positive endosome with no little ligand outside this compartment. In the presence of Arr2-WT, receptor-arrestin complex was colocalized with AP-1 in a perinuclear region, but receptor-arrestin-AP-1 vesicles were also present throughout the cell. As expected, Arr2- P91G/P121E colocalized with AP-1 and receptor in the perinuclear region and not vesicles outside this area consistent with the inhibition of normal FPR trafficking.

3.3.7. ERK1/2 signaling plays a role in Src- and FPR- mediated apoptosis.

While arr2-P91G/P121E has decreased Src association and inhibits ERK1/2 activation in response to β2-AR activation, ERK1/2 activation in response to $AT1_AR$ activation is not impaired (*66*). In addition, GPCR-arrestin-Src scaffolds regulate localized activation of ERK1/2 and therefore other downstream processes (*70*). Because Src has a clear role in FPR-mediated apoptosis (Figure 3.1) and because ERK1/2 inhibitors can inhibit FPR-mediated apoptosis in the absence of arrestins (*22*), we wanted to understand ERK1/2's role with respect to the arr2-P91G/P121E mutant. To this end, we performed apoptosis assays using arr-$2^{-/-}$/-$3^{-/-}$ FPR cells transfected with GFP-fused arrestins in the presence of ERK1/2 inhibitors (Figure 3.5). In the presence of vehicle, apoptosis in cells transfected with GFP alone and arr2-P91G/P121E-GFP is >90% and with wild type arrestin-2 is <5% consistent with previous data (Figure 3.1B). In the presence of MEK inhibitors U0126 or PD98059, transfected cells did not stain

with PI (<5%) indicating that these inhibitors could stop FPR-mediated apoptosis in the absence of arrestins or presence of the P91G/P121E mutant. These results lead us to hypothesize that Src kinase may be aberrantly activating ERK1/2 in the absence of arrestin binding.

3.4 DISCUSSION

Receptor trafficking and signaling are intimately related within the cell and their proper control is paramount for proper cell function. We have previously demonstrated that these processes are linked by the fact that arrestin mutants that alter FPR signaling also alter its post-endocytic trafficking. Previous results have also suggested a role for Src kinase within these processes. In our present study, we used an arrestin mutant (P91G/P121E) previously shown to be defective in binding to Src kinase (*66*). We demonstrated that this mutant was incapable of rescuing FPR-mediated apoptosis, but that cell death in the presence of this mutant was rescued by inhibition of Src kinase or ERK1/2 activity. Additionally, this mutant and Src kinase inhibition did not affect FPR internalization. Finally, our results indicated that FPR-arr2-P91G/P121E complexes accumulate in the perinuclear recycling endosome with Rab11, AP-2 and AP-1. Although Src kinase inhibition rescued FPR-mediated apoptosis in the presence of arr2-P91G/P121E, it did not restore normal trafficking. Interestingly, Src kinase inhibition caused accumulation of FPR-arr2-WT in the Rab11 endosome without initiating cell death. These results indicate that Src kinase has two independent roles with respect to arrestin binding: one that controls its trafficking and one that controls non-G protein FPR signaling.

The most interesting results from our study involve the roles of Src kinase-arrestin interaction on FPR trafficking and signaling. The P91G/P121E arrestin mutant (decreased binding to Src kinase) did not rescue FPR-mediated apoptosis. However, inhibition of Src kinase and ERK1/2 activity in the presence of this mutant did prevent cell death. These results indicate that proper arrestin-Src kinase binding is necessary

to control non-G protein FPR signaling. Interestingly, Src kinase and ERK1/2 are active in the absence of binding to arrestin-2. Where and how these signaling proteins are being activated by FPR stimulation is unclear at this time. Perhaps there is unknown, non-G protein GPCR activation of cellular signaling that is arrestin-independent. Alternatively, arrestin-2 binding to other proteins not affected by Src kinase binding may activate these pathways. For example, the ubiquitous signaling molecule calmodulin binds arrestins (*71*) and the effects of this binding on GPCR signaling are currently unknown.

Another interesting result from our study involves the role of arrestin-Src kinase interaction in FPR trafficking. Our Src kinase-binding deficient arrestin mutant allowed accumulation of FPR-arrestin complexes in the perinuclear region with Rab11, AP-2 and AP-1. This is not surprising given the fact that FPR-mediated apoptosis and FPR trafficking defects have been linked in previous work (Chapter 2). However, while Src kinase inhibition rescued FPR-mediated apoptosis, it did not restore normal FPR-arrestin trafficking. Furthermore, inhibition of Src kinase in the presence of wild type arrestin-2 caused FPR-arrestin complexes to accumulate in the perinuclear recycling endosome without initiating apoptosis. These results indicate a role for Src kinase in normal FPR post-endocytic trafficking. Previous reports have indicated that phosphorylation of tyrosine residues on AP-2 is necessary to release it from arrestin and mediate internalization of the $AT1_AR$ (*38*). Additionally, our previous results suggest that binding and release of AP-2 from arrestin-2 is necessary to insure proper post-endocytic trafficking of the FPR (Chapter 2). Finally, our results indicate an accumulation of AP-2 with FPR-arrestin in the perinuclear recycling endosome in the

absence of Src kinase activity. Therefore, we hypothesize in the absence of arrestin-2 binding or inhibition of its activity, Src kinase cannot phosphorylate arrestin-bound AP-2 in the perinuclear recycling endosome. This leads to accumulation of FPR-arrestin in this region and is independent of Src kinase's role in apoptotic signaling.

Finally, our previous results have shown that FPR internalization is not dependent on clathrin, AP-2, or arrestin (Chapter 2). In this study, we demonstrate further that FPR internalization is independent of Src kinase. Our data demonstrate that FPR internalization extent and rate was not affected by an arrestin mutant that does not bind Src kinase (P91G/P121E) and use of PP2. While the factors that control FPR internalization remain unclear at this time, possible candidates include G protein-receptor kinases and ADP-ribosylation factor 6. Both of these proteins have shown effects on internalization of the β2-AR (*72*) and m2-muscarinic acetylcholine receptor (*73*), respectively.

Based on our results, we present a model for the two independent roles Src kinase plays in FPR trafficking and signaling (Figure 3.6). In this model, following ligand binding the FPR activates G proteins which, in turn, activate Src kinase-mediated apoptotic signaling pathways. The FPR internalizes in an arrestin-independent manner. After, or perhaps during internalization, the FPR binds arrestin (wild type or P91G/P121E), resulting in the presence of FPR/arrestin complexes in early endosomes. At some point during its trafficking to the Rab11, perinuclear recycling endosome (or after its arrival at this location) the FPR-arrestin complex recruits AP-2. At this point (or earlier), Src kinase is recruited to the FPR-wild type arrestin-AP2 complex and initiates anti-apoptotic signaling pathways to counter G protein-mediated apoptotic signaling. In

addition, Src kinase phosphorylates AP-2 leading to its dissociation from arrestin. This results in FPR-arrestin egress from the perinuclear recycling endosome. Along the path to the cell surface the complex dissociates and the FPR is dephosphorylated. Finally, the FPR completes its return to the cell surface in a resensitized form ready to continue signaling. Alternately, FPR-arr2-P91G/P121E-AP-2 complex accumulates in the perinuclear recycling endosome due to inability to bind Src kinase without subsequent phosphorylation of AP-2. This, in turn, inhibits Src kinase from initiating anti-apoptotic signaling within the context of a GPCR-arrestin signaling scaffold.

This report elucidates two independent roles for Src kinase in FPR trafficking and signaling. To our knowledge, this is the first indication that Src kinase has multiple roles within the same GPCR signaling complex: one in control of its trafficking and one in mediating proper cellular signaling. Both of these roles are properly regulated with binding to arrestin-2. Our results clarify a role for arrestin-2 in the spatial and temporal control of non-G protein GPCR signaling and serve as a model for further study. An interesting question that arises from this work is: do trafficking defects alter cell signaling or vice-versa? We plan to answer this question in the future with further analysis of SH3-binding domains within arrestin-2 to establish a framework for the regulation of these processes. With this report of novel roles for arrestins and Src kinase in the trafficking and signaling of GPCRs, new avenues for the targeting of GPCR function are presented that may lead to therapeutic interventions for disease processes.

3.5 MATERIALS AND METHODS

3.5.1. Materials, Plasmids and Mutagenesis – All materials are from Sigma unless otherwise specified. Arr2-P91G/P121E-GFP was created by using two-step PCR mutagenesis. Briefly, primer pairs 5'-GCCCATATGGGCGACAAAGGGACGCGG-3' and 5'-GCCCAGCTTCTTGATGAGGCGCTCCTGCAGCCGCGTCAGGGGCTTCTTGTCCTCACCGGCCGGCGGGAAAGACTGCACG-3' and primer pairs 5'-GCAGGAGCGCCTCATCAAGAAGCTGGGCGAGCATGCCTACCCTTTCACCTTTGAGATCCCTGAGAACTCCCATGCTCTGTGACTTTGCAGCCG-3' and 5'-GGATCCCGGGCCCATCTGTCGTTGAGCCGCGG-3' were used to create N- and C-terminal fragments that contain each mutation respectively. Fragments were purified and amplified using the outside primers to create an Arr2-P91G/P121E fragment containing HindIII/ApaI restriction sites. Standard subcloning procedures were used to insert this fragment into EGFP or mRFP1 vector. mRFP1, Arr2-WT-RFP, EGFP, Arr2-WT-GFP, Rab11-WT-GFP, γ-subunit (AP-1)-GFP and α-subunit (AP-2)-GFP are previously described (Chapter 2). Murine wild type and kinase dead Src kinase are gifts from Steve Abcouwer (Upstate Cell Signaling Solutions).

3.5.2. Cell Culture and Transfection – Arr-$2^{-/-}$/-$3^{-/-}$ FPR cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, 100 units/mL penicillin and 100units/mL streptomycin at 37°C and 5% CO_2. Transient transfections of arr-$2^{-/-}$/-$3^{-/-}$ FPR cells were performed with Lipofectamine 2000 according to manufacturer's instructions.

3.5.3 Apoptosis – Apoptosis assay was performed as previously described (*22*). Briefly, arr-$2^{-/-}$/-$3^{-/-}$ FPR cells were transiently transfected and plated on 12mm glass coverslips. The next day, cells were serum-starved for 30 minutes and incubated with serum-free medium (SFM) for 5 hours with 10nM formyl-methionyl-leucyl-phenylalnine (fMLF) or vehicle at 37°C. Propidium iodide (PI) was then added to a final concentration of 100pg/μL for 5-10 minutes at room temperature. Coverslips were washed and fixed with 2% paraformaldehyde and mounted using Vectashield. Random fields were viewed by fluorescence microscopy until 100-300 GFP expressing cells were assayed. GFP cells were scored for the presence of PI staining. Data are expressed as mean PI positive/GFP cell.

3.5.4. Internalization – Internalization was performed as previously described (*12*). Briefly, transiently transfected arr-$2^{-/-}$/-$3^{-/-}$ FPR cells were grown to confluence and harvested by trypsinization. Cells were incubated with 1μM fMLF and aliquots were removed to cold SFM at 0, 2, 5, 10, 20 and 30 minutes. Cells were washed extensively with cold SFM to remove excess fMLF. Cells were then resuspended in cold SFM containing 10 nM Alexa633-*N*-formyl-Leucyl-Leucyl-Phenylalanyl-Leucyl-Tyrosinyl-Lysine (633-6pep) and analyzed using a Becton Dickinson FACSCalibur at appropriate wavelengths. Live cells were gated using forward- and side-scatter parameters. Cells expressing the GFP-fused protein of interest were gated using FL-1 and mean channel fluorescence (MCF) was measured in FL-4 to determine amount of cell surface receptor. Non-specific background was determined by labeling arr-$2^{-/-}$/-$3^{-/-}$ cells not expressing the FPR with 10nM 633-6pep and assaying as described. Non-specific binding was subtracted before further analysis. MCF from unstimulated cells

represented 100% FPR cell surface expression. Cell surface expression from stimulated cells was calculated by dividing the MCF following treatment by the MCF from unstimulated cells. Internalization data were then plotted using GraphPad Prism to calculate maximum internalization extents using a one phase exponential decay.

3.5.5. Confocal Fluorescence Microscopy – Microscopy was performed as previously described (*12*). Briefly, arr-2$^{-/-}$/-3$^{-/-}$ FPR cells were transiently transfected with plasmids described. Cells were plated on 25mm coverslips, grown overnight and serum-starved for 30 minutes. Cells were then incubated in SFM containing 10nM 633-6pep for the indicated times. Cells were washed with PBS, fixed with 2% paraformaldehyde and mounted using Vectashield. Fluorescence images were acquired using a Zeiss LSM 510 inverted laser scanning microscope equipped with He-Ne and Kr-AR lasers.

3.6. FOOTNOTES

We thank Charlotte Vines for helpful comments during the preparation of this manuscript. Flow cytometry data and confocal images in this study were generated in the Flow Cytometry and Fluorescence Microscopy Facilities, respectively, at the University of New Mexico Health Sciences Center, which received support from NCRR 1 S10 RR14668, NSF MCB9982161, NCRR P20 RR11830, NCI R24 CA88339, the University of New Mexico Health Sciences Center, and the University of New Mexico Cancer Center. This work was funded by grant BC030217 from the Department of Defense Breast Cancer Research Program to B.M.W. and NIH grants AI36357 and GM68901 to E.R.P.

3.7. ABBREVIATIONS

AP-1—adaptor protein-1 complex
AP-2—adaptor protein-2 complex
Arr2—arrestin-2
Arr2$^{-/-}$/3$^{-/-}$ FPR—arrestin-2$^{-/-}$/-3$^{-/-}$ knockout MEF cells stably expressing the FPR
fMLF—*N*-formyl-Methionyl-Leucyl-Phenylalanine
FPR—*N*-formyl peptide receptor
GPCR—G protein-coupled receptor
MEF—mouse embryonic fibroblast
PI—propidium iodide
SFM—serum-free medium (DMEM)
6pep—*N*-formyl-Leucyl-Leucyl-Phenylalanyl-Leucyl-Tyrosinyl-Lysine

3.8. FIGURE LEGENDS

3.8.1 Figure 3.1. Inhibition of Src kinase, but not expression of Arr2-P91G/P121E, rescues FPR-mediated apoptosis. Arr-$2^{-/-}$/-$3^{-/-}$ FPR cells were transiently transfected with constructs described, stimulated with 10nM fMLF and stained with PI. Random fields were viewed by fluorescence microscopy until 100-300 cells GFP cells were counted. GFP cells were scored for the presence of PI staining. Data are expressed as mean PI positive/GFP cell +/- SEM from three independent experiments. ***A)*** Arr-$2^{-/-}$/-$3^{-/-}$ FPR cells were transiently transfected with GFP-fused arrestins. ***B)*** Arr-$2^{-/-}$/-$3^{-/-}$ FPR cells were transiently transfected with GFP-fused arrestins and incubated with DMSO or PP2 (10nM, 30 min) before and during stimulation. ***C)*** Arr-$2^{-/-}$/-$3^{-/-}$ FPR cells were transiently transfected with GFP-fused arrestins and either wild type Src kinase or kinase dead (K298M) Src kinase.

3.8.2 Figure 3.2. FPR internalization is not affected by arrestins or Src inhibition. ***A)*** Arr-$2^{-/-}$/-$3^{-/-}$ FPR cells were transiently transfected with GFP-fused arrestins and assayed for internalization. Data are expressed as mean maximum internalization +/- SEM from three independent experiments. ***B)*** Arr-$2^{-/-}$/-$3^{-/-}$ FPR cells were transiently transfected with GFP-fused arrestins, pre-incubated with PP2 (10nM, 30 min) and assayed for internalization in the presence of inhibitor. Data are expressed as mean maximum internalization +/- SEM from three independent experiments.

3.8.3. Figure 3.3. FPR-arrestin complexes traffic differentially with Rab11 in response to Src inhibition. Arr-$2^{-/-}$/-$3^{-/-}$ FPR cells were transiently transfected with constructs listed individually below. Cells were stimulated with 10nM 633-6pep for 1 hour and viewed by confocal fluorescence microscopy. Images are representative of three independent experiments. ***A)*** Arr-$2^{-/-}$/-$3^{-/-}$ FPR cells were transfected with RFP-

fused arrestins and GFP-fused Rab11-WT. ***B)*** Arr-$2^{-/-}$/-$3^{-/-}$ FPR cells were transiently transfected with RFP-fused arrestins and GFP-fused Rab11-WT. Cells were incubated with PP2 (10nM, 30min) before and during stimulation ***C)*** Arr-$2^{-/-}$/-$3^{-/-}$ FPR cells were transiently transfected with GFP-fused Rab11-WT, RFP-fused arrestins and kinase dead Src kinase.

3.8.4. Figure 3.4. FPR-arrestin complexes traffic differentially with AP-2 and AP-1. Arr-$2^{-/-}$/-$3^{-/-}$ FPR cells were transiently transfected with constructs described. Cells were stimulated with 10nM 633-6pep for 1 hour and viewed by confocal fluorescence microscopy. Images are representative of three independent experiments. ***A)*** Arr-$2^{-/-}$/-$3^{-/-}$ FPR cells were transfected with RFP-fused arrestins and GFP-fused α-subunit of AP-2. ***B)*** Arr-$2^{-/-}$/-$3^{-/-}$ FPR cells were transiently transfected with RFP-fused arrestins and GFP-fused γ-subunit of AP-1.

3.8.5. Figure 3.5. FPR-mediated apoptosis in the absence of arrestins or presence of P91G/P121E is sensitive to ERK inhibition. Arr-$2^{-/-}$/-$3^{-/-}$ FPR cells were transiently transfected with GFP-fused arrestins and pre-incubated with U0126 (10μM, 30 min), PD98059 (25μM, 30 min) or DMSO. Cells were stimulated with 10nM fMLF and stained with PI. Five random fields were viewed by fluorescence microscopy until 100-300 cells GFP cells were counted. GFP cells were scored for the presence of PI staining. Data are expressed as mean PI positive/GFP cell +/- SEM from three independent experiments.

3.8.6. Figure 3.6. Role of Src kinase in FPR trafficking and signaling. Based on our results, we present a model for the two independent roles Src kinase plays in FPR trafficking and signaling. In this model, following ligand binding the FPR activates G

proteins which, in turn, activate Src kinase-mediated apoptotic signaling pathways. The FPR internalizes in an arrestin-independent manner. After, or perhaps during internalization, the FPR binds arrestin (wild type or P91G/P121E), resulting in the presence of FPR/arrestin complexes in early endosomes. At some point during its trafficking to the Rab11, perinuclear recycling endosome (or after its arrival at this location) the FPR-arrestin complex recruits AP-2. At this point (or earlier), Src kinase is recruited to the FPR-wild type arrestin-AP2 complex and initiates anti-apoptotic signaling pathways to counter G protein-mediated apoptotic signaling. In addition, Src kinase phosphorylates AP-2 leading to its dissociation from arrestin. This results in FPR-arrestin egress from the perinuclear recycling endosome. Along the path to the cell surface the complex dissociates and the FPR is dephosphorylated. Finally, the FPR completes its return to the cell surface in a resensitized form ready to continue signaling. Alternately, FPR-arr2-P91G/P121E-AP-2 complex accumulates in the perinuclear recycling endosome due to inability to bind Src kinase without subsequent phosphorylation of AP-2. This, in turn, inhibits Src kinase from initiating anti-apoptotic signaling within the context of a GPCR-arrestin signaling scaffold. This figure was generated using ScienceSlides (www.visislides.com).

3.8.7. Figure 3.7. Vehicle and Src wild type do not alter FPR-arrestin trafficking phenotypes. Arr-2$^{-/-}$/-3$^{-/-}$ FPR cells were transiently transfected with constructs listed individually below. Cells were stimulated with 10nM 633-6pep for 1 hour and viewed by confocal fluorescence microscopy. Images are representative of three independent experiments. ***A)*** Arr-2$^{-/-}$/-3$^{-/-}$ FPR cells were transiently transfected with RFP-fused arrestins and GFP-fused Rab11-WT. Cells were incubated with PP2 (10nM, 30min)

before and during stimulation ***B)*** Arr-2$^{-/-}$/-3$^{-/-}$ FPR cells were transiently transfected with GFP-fused Rab11-WT, RFP-fused arrestins and kinase dead Src kinase.

Task 3. Arrestin-2 SH3-binding domains differentially regulate *N*-formyl peptide receptor trafficking and signaling

To be submitted to *Traffic*

Arrestin-2 SH3-binding domains differentially regulate *N*-formyl peptide receptor trafficking and signaling

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4.1 ABSTRACT

Arrestin binding to Src kinase is an important event that regulates localized activation of MAPK signaling cascades and cellular proliferation. This interaction is controlled by SH3-binding domains (P-X-X-P) within arrestin at three locations: P88/P91, P121/P124 and P175/P178. When this interaction is disrupted by mutation of prolines within SH3-binding domains, ERK1/2 activation is decreased upon activation of the β2-adrenergic receptor. In a recent report, we demonstrated that arrestin-Src kinase interaction was vital to proper regulation of *N*-formyl peptide receptor- (FPR) mediated signaling, trafficking and apoptosis. We elucidated two roles for arrestin-Src kinase interaction in these processes. First, Src kinase was hypothesized to control trafficking of the FPR by phosphorylating tyrosine resides in the adaptor protein (AP)-2 complex to release AP-2 from arrestin and allow egress of the FPR from the perinuclear recycling endosome. Second, Src kinase activity was also necessary to mediate FPR-mediated apoptosis independent of FPR trafficking. An important question that arose from this work was whether altered trafficking of the FPR lead to aberrant Src kinase-MAPK signaling or vice-versa. In order to understand the mechanisms of FPR trafficking and signaling that lead to FPR-mediated apoptosis, we generated three mutants of arrestin-2 in which prolines within SH3-binding domains were replaced with alanine. Our studies demonstrate two important results. First, arrestin-Src kinase binding is necessary to rescue FPR-mediated apoptosis and one SH3-binding domain (P88-X-X-P91) controls this phenotype. Second, as FPR trafficking was not altered in the presence of any of the arrestin mutants used in this study, we conclude that altered FPR signaling can occur without altered FPR trafficking. This study elucidates

important mechanisms in G protein-coupled receptor (GPCR) signaling and trafficking which will give insight to mechanisms of temporal and spatial control of GPCR signaling complexes.

4.2 INTRODUCTION

Arrestins are cytosolic proteins that have roles in mediating G protein-coupled receptor (GPCR) desensitization, internalization and non-G protein scaffold signaling (*74*). These functions are controlled by arrestin binding to ligand-activated, C-terminal phosphorylated GPCRs, clathrin, adaptor protein (AP)-2 and Src kinase (*75*). Arrestin binding to these cellular components controls both the temporal and spatial signaling of GPCRs (*70*). This, in turn, regulates various cellular functions including proliferation and migration.

Arrestin-Src kinase interaction is vital for mediating activation of extracellular-regulated kinase (ERK) 1/2 (*66*). When cells expressing the β2-adrenergic receptor (β2-AR) were transiently transfected with an arrestin mutant that exhibited decreased binding to Src kinase, ERK1/2 activation was decreased in response to stimulation with isoproterenol. This arrestin mutant also inhibited the internalization of the β2-AR. Interestingly, ERK1/2 activation was not affected when cells expressing the angiotensin II (Type 1A) receptor ($AT1_AR$) were transfected with the same arrestin mutant and stimulated with angiotensin II.

An indirect interaction between Src kinase and arrestin also controls internalization of GPCRs. When arrestin and AP-2 were not allowed to dissociate, internalization of the $AT1_AR$ was inhibited (*38*). The authors went on to demonstrate that phosphorylation of tyrosine resides within AP-2 was necessary for arrestin and AP-2 to dissociate and internalization of the $AT1_AR$ to occur. Given that binding of AP-2 and arrestin are also necessary for proper GPCR internalization (*65*), these results indicate that Src kinase plays an intimate role in arrestin-AP-2 interaction cycling.

Our previous results demonstrate that arrestin-Src kinase interaction is required for rescue of cells from *N*-formyl peptide receptor- (FPR) mediated apoptosis (*22*). In addition, we showed that inhibition of Src kinase with PP2 could rescue FPR-mediated apoptosis, but not the associated trafficking defects (Chapter 3). In fact, inhibition of Src kinase activity led to aberrant FPR trafficking in the presence of wild type arrestin without initiating apoptosis. This result led us to believe that Src kinase plays two independent, arrestin-associated roles in FPR signaling and trafficking. One role is involved with control of FPR post-endocytic trafficking by phosphorylating AP-2 to allow its dissociation from arrestin and receptor egress from the perinuclear recycling endosome. The other role is to regulate proper FPR non-G protein scaffold signaling to prevent FPR-mediated apoptosis.

Our results were complicated by the fact that the arrestin-2 (arr2) mutant deficient in Src kinase binding contained mutations in individual SH3-binding domains (P91G and P121E). Therefore this led us to ask an important question in GPCR trafficking and signaling: Do GPCR signaling defects initiate GPCR trafficking defects or vice-versa? In order to answer this question, we further evaluated the role of SH3-binding domains (P-X-X-P) within arrestin-2 (P88/P91, P121/P124 and P175/P178). Prolines were mutated to alanine within individual SH-3 domains and evaluated for their ability to alter FPR-mediated signaling and trafficking. In addition, arrestin mutants were designed that mimic the original arrestin mutant that has decreased binding to Src kinase to insure that our results were not due to changes in the secondary structure of arrestin-2 due to replacement of prolines with alanine. Our results demonstrate that altered arrestin-Src kinase interaction can alter FPR signaling without changing its

normal trafficking. These results suggest that GPCR signaling may control GPCR trafficking, at least within the context of the FPR.

4.3 RESULTS

4.3.1. Mutations in SH3-binding domains of arrestin have differential effects on FPR-mediated apoptosis.

Previous results have demonstrated that mutation of individual prolines within multiple SH3-binding domains (P91G/P121E) do not rescue FPR-mediated apoptosis and cause receptor-arrestin complexes to accumulate within the Rab11, perinuclear recycling endosome (Chapter 2). To better understand the role of arrestin-Src kinase interaction in FPR signaling and trafficking we generated three mutants of arrestin that mutated prolines in SH3-binding domains (P-X-X-P) to alanine (P88A/P91A, P121A/P124A and P175A/P178A). This method of analysis allows us to assess the individual contributions of arrestin-2 SH-3 binding to Src kinase in FPR signaling and trafficking.

Mouse embryonic fibroblasts (MEF) deficient in both arrestin-2 and -3, but stably expressing the FPR (arr2$^{-/-}$/3$^{-/-}$ FPR), were used to assess arrestin-2 mutants in the absence of competition from endogenous arrestins. Arr2$^{-/-}$/3$^{-/-}$ FPR cells were transiently transfected with empty GFP vector, arr2-WT-GFP and GFP-fused arrestin mutants and assayed for rescue of FPR-mediated apoptosis (Figure 3.1A). Our use of propidium iodide (PI) staining has been validated previously (*22*) as an accurate method of monitoring apoptosis. When GFP expressing cells were unstimulated, <10% of cells were stained with PI consistent with previous results exhibiting that apoptosis is FPR-dependent. When cells expressing empty GFP vector (absence of arrestins) were stimulated with *N*-formyl-Methionyl-Leucyl-Phenylalanine (fMLF), >90% of cells stained with PI. However, cells expressing arr2-WT-GFP stained <10% with PI consistent with

the ability of arrestin-2 to rescue FPR-mediated apoptosis. Arr2$^{-/-}$/3$^{-/-}$ FPR cells expressing GFP-fused mutants demonstrated differential ability to rescue FPR-mediated apoptosis. Arrestin-2 mutants with alterations in the first (P88A/P91A) and third (P175A/P178A) SH3-binding domains rescued FPR-mediated apoptosis (<10% PI staining. However, in cells expressing arr2-P121A/P124A-GFP, >90% of cells stained with PI. This indicates that the second SH3-binding domain of arrestin is responsible for regulating Src kinase binding and preventing FPR-mediated apoptosis.

To confirm that our results were due to activation of Src kinase in the absence of arrestin binding, we transiently transfected arr2$^{-/-}$/3$^{-/-}$ FPR cells with empty GFP vector, arr2-WT-GFP and arr2-P121A/P124A-GFP and inhibited Src kinase activity (with PP2, a Src family kinase inhibitor) during FPR stimulation (Figure 3.1B). In the presence of vehicle, empty GFP and arr2-P121A/P124A-GFP >90% transfected cells stained with PI while <10% of cells transfected with arr2-WT-GFP stained with PI. When cells were incubated with PP2, <10% of GFP cells stained with PI in all conditions. These results indicate that although Src kinase binding to arrestin may be decreased due to mutation of one of arrestin-2's SH3-binding domains, Src kinase activity initiates apoptotic signaling.

4.3.2. Rescue of FPR-mediated apoptosis by SH3-binding arrestin mutants is not due to inhibition of internalization.

In a previous report, a signaling competent, internalization-defective FPR mutant was incapable of initiating FPR-mediated apoptosis (*22*). Because internalization is required to initiate FPR-mediated apoptosis, we hypothesized those SH3-binding mutants that rescue FPR-mediated apoptosis may inhibit FPR internalization. To test

this hypothesis, we transfected arr2$^{-/-}$/3$^{-/-}$ FPR cells with GFP-fused arrestins and measured FPR internalization. FPR internalization extents were 60-70% for all GFP conditions (Figure 3.2) and rates of internalization were also similar (data not shown). Therefore, rescue of FPR-mediated apoptosis by SH3-binding mutants is not due to inhibition of FPR internalization.

4.3.3. SH3-binding mutants allow normal FPR trafficking.

Previous reports reveal FPR trafficking defects associated with arrestin-2 mutants that do not rescue FPR-mediated apoptosis including mutation in AP-2 binding domains (F391A, 4A—Chapter2) and Src-binding domains (P91G/P121E—Chapter 3). These arrestin mutants accumulate in the perinuclear recycling endosome, with little receptor-arrestin complex seen outside this location. Therefore, we hypothesized the arr2-P121A/P124A mutant would accumulate with the FPR in the Rab11, perinuclear recycling endosome. To test this hypothesis, arr2$^{-/-}$/3$^{-/-}$ FPR cells were transfected with RFP-fused arrestins and Rab11-GFP and viewed by confocal fluorescence microscopy (Figure 3.3). In unstimulated cells, Rab11 is localized in a perinuclear location and arrestins are distributed throughout the cytosol consistent with a lack of FPR stimulation. When the FPR is stimulated with Alexa 633-*N*-formyl-Leucyl-Leucyl-Phenylalanyl-Leucyl-Tyrosinyl-Lysine (633-6pep) in the absence of arrestins (empty vector), ligand-FPR complexes accumulate in the Rab11 endosome with little to no ligand outside this location (data not shown). When cells are transfected with mRFP-fused wild type arrestin-2 and stimulated with 633-6pep, ligand-FPR complexes are colocalized with the Rab11 endosome (data not shown). However, ligand-FPR complexes are distributed in vesicles throughout the cytosol. These phenotypes are

consistent with previously published results (Chapter 2, (*12*). Interestingly, cells transfected with SH3-binding mutants are stimulated, all mutants have ligand-receptor complexes in vesicles within the cytosol as well as colocalized with the Rab11 endosome. Our results imply that while the arr2-P121A/P124A mutant does not rescue FPR-mediated apoptosis, it does allow normal trafficking of the FPR.

AP complexes demonstrate differential trafficking patterns with FPR-arrestin signaling complexes (Chapter 2). AP-2 either accumulates with receptor-arrestin complexes in the Rab11 endosome or does not bind these complexes at all. AP-1 does colocalize with all mutants in the Rab11 endosome, but is only in vesicles outside this location with the FPR in the presence of wild type arrestin-2. We hypothesized that while FPR-SH3-binding mutants traffic normally with respect to the Rab11 compartment, they may have altered trafficking with either of the AP complexes.

Arr2$^{-/-}$/3$^{-/-}$ FPR cells were transfected with RFP-fused arrestins and either α-GFP and viewed by confocal fluorescence microscopy (AP-2, Figure 3.4A) or γ-GFP (AP-1, Figure 3.4B). In unstimulated cells, arrestins have a cytosolic distribution, AP-2 has some protein in the cytosol with most in puncta within the cell membrane and AP-1 is localized in a perinuclear region. In cells expressing empty vector and stimulated with 633-6pep, AP-2 did not colocalize with ligand-FPR complexes in the perinuclear region. While AP-1 did colocalize with FPR in the perinuclear region, neither AP-1 nor ligand-FPR was outside this location (data not shown). When cells were stimulated with 633-6pep in the presence of wild type arrestin, both AP-2 and AP-1 colocalized with FPR-arrestin complexes in the perinuclear region. In addition, ligand-FPR-AP-1 complexes were localized outside the perinuclear region consistent with normal FPR trafficking.

These phenotypes are consistent with previously published data (data not shown, Chapter 2). Once again, in the presence of all RFP-fused SH3-binding arrestin mutants, FPR-arrestin trafficking was similar to that of wild type arrestin-2. When stimulated, AP-2 and AP-1 were colocalized with FPR-arrestin complexes in the perinuclear region. However, FPR-arrestin-AP-1 complexes were trafficking outside the perinuclear region.

4.3.4. Individual SH-3 binding point mutations in arrestin differentially regulate FPR-mediated apoptosis.

In previous reports demonstrating that arrestin-Src kinase interaction regulate FPR trafficking and signaling (Chapter 3), we used a mutant containing individual point mutations within different SH3-binding domains. In previous sections, we have demonstrated that the second SH3-binding domain (P121/P124) alters FPR signaling by not rescuing FPR-mediated apoptosis, but demonstrated no effect on FPR trafficking. To remove the possibility that our results are due to mutations within SH3-binding domains that decrease arrestin-Src kinase binding and not alteration of arrestin-2 secondary structure, two arrestin-2 mutants were generated with single point mutations (P91G and P121E). These mutants contain the same mutations as previous SH3-binding mutants described (P91G/P121E). However, they are done individually to more accurately describe the role of SH3-binding domains of arrestin-2 in FPR signaling and trafficking.

Arr2$^{-/-}$/3$^{-/-}$ FPR cells were transfected with GFP-fused arrestin point mutations and assayed for rescue of FPR-mediated apoptosis (Figure 3.5A). In unstimulated GFP-expressing cells, there is <10% PI staining. When the FPR is activated with fMLF, arr2-

WT and arr2-P91G rescue apoptosis (<10% PI staining). However, empty GFP and arr2-P121E cells were >90% PI positive. These results confirm that FPR-mediated apoptosis is due to mutations within the P121/P124 SH3-binding domain and not due to alteration of arrestin-2 secondary structure.

To confirm that Src kinase activity was necessary to initiate FPR-mediated apoptosis in the presence of the arr2-P121E mutant, $arr2^{-/-}/3^{-/-}$ FPR cells expressing GFP-fused arrestins were assayed for apoptosis while incubated with PP2 (Figure 3.5B). In GFP cells incubated with vehicle, FPR activation did not rescue FPR-mediated apoptosis in the absence of arrestins (empty vector) or the presence of arr2-P121E >90% PI staining). Arr2-WT did rescue FPR-mediated apoptosis when incubated with vehicle (<10% PI staining). In the presence of PP2, all GFP cells were rescued from FPR-mediated apoptosis (<10% PI staining).

4.3.5. FPR internalization is not affected by arrestin-2 point mutations.

As previously mentioned, FPR-mediated apoptosis requires FPR internalization. $Arr2^{-/-}/3^{-/-}$ FPR cells were transfected with GFP-fused arrestins and FPR internalization was evaluated (Figure 3.6). In all GFP-expressing cells, FPR internalization extent varies between ~65-70% and rates of FPR internalization were similar (data not shown). These data indicate that rescue of FPR-mediated apoptosis is not due to inhibition of its internalization.

4.3.6. Arrestin-2 SH-3 binding domain point mutations do not alter FPR trafficking.

To assess that normal FPR trafficking allowed by an arrestin mutant (P121A/P124A) was due to mutation of the SH3-binding domain and not due to alterations of arrestin-2 secondary structure, we assayed arr2$^{-/-}$/3$^{-/-}$ FPR cells expressing arrestin-2 point mutants fused to RFP for FPR trafficking with Rab11-GFP by confocal fluorescence microscopy (Figure 3.7). When the FPR was stimulated, both arrestin-2 point mutants colocalize with ligand-FPR in the Rab11 endosome. In addition, FPR-arrestin complexes were localized in vesicles outside the perinuclear region consistent with normal FPR trafficking (Chapters 2 and 3, (*12*).

To ascertain whether FPR and AP complexes traffic normally with arrestin SH3-binding point mutants, we used confocal fluorescence microscopy. Arr2$^{-/-}$/3$^{-/-}$ FPR cells were transfected with RFP-fused arrestins and either α-GPF or γ-GFP (Figures 3.8A and 3.8B, respectively). Upon stimulation of the FPR ligand-FPR-arrestin point mutant complexes colocalize with AP-2 and AP-1 in the perinuclear region. Additionally, FPR-arrestin-AP-1 complexes are localized in vesicles outside the perinuclear region consistent with previous data evaluating normal FPR trafficking (Chapters 2 and 3).

4.4. DISCUSSION

Proper temporal and spatial control of GPCR signaling complexes is vital to proper cellular function. We have previously shown that FPR-mediated apoptosis is linked to receptor-arrestin accumulation in the perinuclear recycling endosome (Chapter 2). Additionally, we have demonstrated that two roles for Src kinase are involved in this process (Chapter 3). However, the Src binding-deficient mutant used in this work contained two point mutations in different SH3-binding domains of arrestin-2. An important question that arose from this work is whether altered FPR trafficking led to aberrant FPR signaling or vice-versa. We hypothesized that individual SH3-binding domains within arrestin-2 may be responsible cause aberrant trafficking and signaling sequentially. Our results indicate this is not the case.

In experiments to assay the effect of mutation of individual SH3-binding domains of arrestin-2 (P88A/P91A, P121A/P124A and P175A/P178A), we found the second SH3-binding domain (P121A/P124A) did not rescue FPR-mediated apoptosis. However, none of the mutants assayed altered FPR internalization of post-endocytic trafficking. FPR-mediated apoptosis allowed by the P121A/P124A mutant was rescuable by inhibition of Src kinase activity. Because use of alanine to replace prolines within the SH3-binding domains could significantly alter arrestin-2 secondary structure, we also generated point mutants of arrestin-2 (P91G and P121E) that were exactly like the original mutant used (P91G/P121E, Chapter 3), but with mutation of only one SH3-binding domain at a time. The P121E mutant did not rescue FPR-mediated apoptosis (although this was rescued by Src kinase inhibition) and neither mutant altered FPR trafficking.

These results suggest that FPR signaling and trafficking may operate independently (within the context of arrestin-Src kinase binding) rather than causally. Clearly, the second SH3-bindng domain (P121/P124) is responsible for regulating FPR signaling complexes. However, it does not alter FPR trafficking. Additionally, the first domain (P88/P91) has no effect on FPR signaling and trafficking when mutated. It is only when both SH3-binding domains are mutated (P91G/P121E, Chapter 3) that defects in both FPR signaling and trafficking occur. This implies that Src kinase binding to either domain properly regulates FPR post-endocytic trafficking while mutation of the second domain (P121/P124) is sufficient to alter FPR signaling. Only when both domains are mutated, are alterations observed. A role for the third domain (P175/P178) is unclear at this time. By itself, this domain is not sufficient to rescue FPR from signaling and trafficking defects. However, its mutation does not alter FPR function in the assays we used. It is possible that this domain regulates some other function of FPR signaling or trafficking such as migration or degranulation.

Our findings elucidate differences in the regulation of FPR trafficking and signaling. Previously, we hypothesized a causal link between receptor-arrestin accumulation in the perinuclear recycling endosome and FPR-mediated apoptosis. However, our findings indicate this is not the case. It is clear that FPR signaling defects can occur independently of trafficking defects. Whether the opposite is true is unknown at this time. This report further elucidates the intimate and intricate dualities of GPCR trafficking and signaling. Other GPCRs, such as the β2-AR (*45*), and receptor tyrosine kinases, such as the epidermal growth factor (*76*) receptor also demonstrate a causal link between receptor internalization and ERK1/2 activation. Is it possible that trafficking

and signaling can be altered independently of one another in these systems? The implication that receptor trafficking and signaling can be modified independently of other processes creates new avenues for the targeting of GPCR function that may lead to novel therapeutic interventions for disease processes.

4.5. MATERIALS AND METHODS

4.5.1. Plasmids

Empty GFP vector, Arr2-WT-GFP, Rab11-WT-GFP, γ-GFP (AP-1), α-GFP (AP-2), empty mRFP1 vector and Arr2-WT-RFP have been previously described (Chapter 2). Arrestin-2 mutants (P88A/P91A, P121A/P124A, P175A/P178A, P91G and P121E) were generated by site-directed mutagenesis using QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) using Arr2-WT-GFP as the staring construct. RFP-fused arrestin-2 mutants were generated standard subcloning procedures and HindIII/ApaI restriction sites.

4.5.2. Cell Culture and Transfection

$Arr2^{-/-}/3^{-/-}$ FPR cells were grown in DMEM with 10% fetal bovine serum, 100 units/mL penicillin and 100units/mL streptomycin at 37°C and 5% CO_2. Transient transfections of $arr2^{-/-}/3^{-/-}$ FPR cells were performed with Lipofectamine 2000 according to manufacturer's instructions.

4.5.3. Apoptosis

Apoptosis of $arr2^{-/-}/3^{-/-}$ FPR cells was performed as previously described (*22*). Briefly, $arr2^{-/-}/3^{-/-}$ FPR cells were transiently transfected with GFP-fused arrestins. Cells were then plated to 12mm glass coverslips and serum-starved for 30min. Cells were then stimulated with 10nM fMLF for 5 hours at 37°C. Propidium iodide (PI) was added to a final concentration of 100ng/mL for 5-10min at room temperature. Cell were washed with PBS, fixed and mounted with Vectashield. Slides were viewed by fluorescence microscopy and random fields were evaluated (at least five) until 100-300 GFP-expressing cells were assayed. GFP-expressing cells were counted "positive" for cell

death if they were stained with propidium iodide. Data are expressed as a percentage of PI-positive/GFP-expressing cells. For assays in which inhibitor was used, cells were treated with 10nM PP2 during serum-starvation and treatment remained throughout FPR stimulation.

4.5.4. Internalization

FPR internalization was assayed as previously described (*12*). Briefly, arr2$^{-/-}$/3$^{-/-}$ FPR cells were transiently transfected with GFP-fused arrestins. Cells were harvested and resuspended in serum-free medium (SFM). The FPR was stimulated with 1μM fMLF and aliquots were removed at 3, 6, 10, 20 and 30min and added to pre-chilled SFM. One aliquot was removed before stimulation to measure total cell surface receptor. Cells were washed extensively with cold SFM to remove excess unlabelled ligand. Remaining cell surface receptor was labeled with 10nM 633-6pep and assayed by flow cytometry using a Becton-Dickinson FACSCalibur. For analysis, cells live cells were gated using forward- and side-scatter parameters. Cells were then gated for arrestin expression using FL-1. Mean channel fluorescence (MCF) was then measured using FL-4. Non-specific binding was determined by labeling arr2$^{-/-}$/3$^{-/-}$ FPR cells not expressing the FPR and this value was subtracted from all internalization values. Unstimulated cells were considered to have 100% cell surface receptor. Cell surface expression of stimulated cells was calculated by dividing its MCF by the MCF of the corresponding unstimulated cells. Internalization data were then plotted using GraphPad Prism to calculate maximum internalization extents using a singe exponential decay.

4.5.5. Confocal Fluorescence Microscopy

Confocal microscopy was performed as previously described (*12*). Briefly, arr2$^{-/-}$/3$^{-/-}$ FPR cells were transiently transfected with RFP-fused arrestins and GFP-fused Rab11, AP-1 or AP-2. Cells were plated to 25mm coverslips and serum-starved for 30min. Cells were stimulated with 10nM 633-6pep for 1 hour at 37°C in SFM. Cells were washed with PBS, fixed with 2% paraformaldehyde and mounted using Vectashield. Fluorescence images were acquired using a Zeiss LSM 510 inverted laser scanning microscope equipped with He-Ne and Kr-AR lasers.

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4.7. ABBREVIATIONS

AP-1—adaptor protein-1 complex
AP-2—adaptor protein-2 complex
Arr2—arrestin-2
Arr2$^{-/-}$/3$^{-/-}$ FPR—arrestin-2$^{-/-}$/-3$^{-/-}$ knockout MEF cells stably expressing the FPR
fMLF—*N*-formyl-Methionyl-Leucyl-Phenylalanine
FPR—*N*-formyl peptide receptor
GPCR—G protein-coupled receptor
MEF—mouse embryonic fibroblast
PI—propidium iodide
SFM—serum-free medium (DMEM or RPMI)
6pep—*N*-formyl-Leucyl-Leucyl-Phenylalanyl-Leucyl-Tyrosinyl-Lysine

4.8. FIGURE LEGENDS

4.8.1. Figure 4.1. Rescue of FPR-mediated apoptosis by arrestin-2 SH3-binding mutants. ***A)*** Arr2$^{-/-}$/3$^{-/-}$ FPR cells transiently transfected with GFP-fused arrestin mutants were assayed for apoptosis. Cells were stained with PI and 100-300 GFP-expressing cells were viewed and scored for the presence of PI staining. Data expressed as mean ± SEM PI positive/GFP cell from three independent experiments. ***B)*** Arr-2$^{-/-}$/-3$^{-/-}$ FPR cells were transiently transfected with GFP-fused arrestins and incubated with DMSO or PP2 (10nM, 30 min) before and during stimulation.

4.8.2. Figure 4.2. Internalization of the FPR in presence of arrestin-2 SH3-binding mutants. Arr2$^{-/-}$/3$^{-/-}$ FPR cells transiently transfected with GFP-fused arrestins were stimulated with 1μM fMLF and aliquoted at multiple time points. Cells were washed free of fMLF, labeled with 633-6pep and analyzed by flow cytometry for residual cell surface receptor. Cells were gated for GFP expression (FL1) to restrict the analysis to transfected cells. Data are expressed as the maximum extent of internalization based on curve fitting using a single exponential decay. Data are expressed as mean ± SEM from three independent experiments.

4.8.3. Figure 4.3. Arrestin-2 SH3-binding mutants traffic normally with respect to Rab11. Arr2$^{-/-}$/3$^{-/-}$ FPR cells were transiently transfected with GFP-fused Rab11 and RFP-fused arrestins and plated on coverslips. Cells were stimulated with the 633-6pep for 1 hour at 37°C and imaged by confocal fluorescence microscopy. Representative images are shown from three independent experiments.

4.8.4. Figure 4.4. Arrestin-2 SH3-binding mutants traffic normally with AP-2 and AP-1. ***A)*** Arr2$^{-/-}$/3$^{-/-}$ FPR cells were transiently transfected with RFP-fused arrestins and

the GFP-fused α-subunit of AP-2. Cells were subsequently stimulated with 633-6pep at 37°C for the indicated time and imaged by confocal fluorescence microscopy. Representative images are shown from three independent experiments. ***B)*** Arr2$^{-/-}$/3$^{-/-}$ FPR cells were transiently transfected with RFP-fused arrestins and the GFP-fused γ-subunit of AP-1. Cells were then stimulated with the 10nM 633-6pep ligand, fixed and viewed by confocal fluorescence microscopy. Representative images are shown from three independent experiments.

4.8.5. Figure 4.5. Rescue of FPR-mediated apoptosis by arrestin-2 SH3-binding point mutants. ***A)*** Arr2$^{-/-}$/3$^{-/-}$ FPR cells transiently transfected with GFP-fused arrestin mutants were assayed for apoptosis. Cells were stained with PI and 100-300 GFP-expressing cells were viewed and scored for the presence of PI staining. Data expressed as mean ± SEM PI positive/GFP cell from three independent experiments. ***B)*** Arr-2$^{-/-}$/-3$^{-/-}$ FPR cells were transiently transfected with GFP-fused arrestins and incubated with DMSO or PP2 (10nM, 30 min) before and during stimulation.

4.8.6. Figure 4.6. Internalization of the FPR in presence of arrestin-2 SH3-binding point mutants. Arr2$^{-/-}$/3$^{-/-}$ FPR cells transiently transfected with GFP-fused arrestins were stimulated with 1μM fMLF and aliquoted at multiple time points. Cells were washed free of fMLF, labeled with 633-6pep and analyzed by flow cytometry for residual cell surface receptor. Cells were gated for GFP expression (FL1) to restrict the analysis to transfected cells. Data are expressed as the maximum extent of internalization based on curve fitting using a single exponential decay. Data are expressed as mean ± SEM from three independent experiments.

4.8.7. Figure 4.7. Arrestin-2 SH3-binding point mutants traffic normally with respect to Rab11. Arr2$^{-/-}$/3$^{-/-}$ FPR cells were transiently transfected with GFP-fused Rab11 and RFP-fused arrestins and plated on coverslips. Cells were stimulated with the 633-6pep for 1 hour at 37°C and imaged by confocal fluorescence microscopy. Representative images are shown from three independent experiments.

4.8.8. Figure 4.8. Arrestin-2 SH3-binding point mutants traffic normally with AP-2 and AP-1. ***A)*** Arr2$^{-/-}$/3$^{-/-}$ FPR cells were transiently transfected with RFP-fused arrestins and the GFP-fused α-subunit of AP-2. Cells were subsequently stimulated with 633-6pep at 37°C for the indicated time and imaged by confocal fluorescence microscopy. Representative images are shown from three independent experiments. ***B)*** Arr2$^{-/-}$/3$^{-/-}$ FPR cells were transiently transfected with RFP-fused arrestins and the GFP-fused -subunit of AP-1. Cells were then stimulated with the 10nM 633-6pep ligand, fixed and viewed by confocal fluorescence microscopy. Representative images are shown from three independent experiments.

KEY RESEARCH ACCOMPLISHMENTS

- Arrestin-2 mutants have been constructed
- We have found mutants that are responsible for FPR-mediated apoptosis
- We have seen that arrestin effect on apoptosis is independent of their effect on FPR internalization
- We have demonstrated that trafficking patterns of arrestin mutants that do not inhibit apoptosis are similar to when no arrestin is present.
- We have demonstrated that trafficking patterns of arrestin mutants that inhibit apoptosis are similar to when wild-type arrestin is present.
- We have realized that arrestin mutants that do not inhibit apoptosis all fail to interact with AP-2 (a regulator of receptor trafficking) and are investigating its role on GPCR/arrestin interactions and apoptosis.
- We have demonstrated that AP-2 has differential interaction with arrestin mutants by both fluorescence microscopy and co-immunoprecipitation.
- We have demonstrated that AP-1 is likely to be involved in FPR/arrestin post-endocytic trafficking.
- We have found that both arrestin mutants involved in Task One show a defect in FPR recycling.
- We have constructed a model of FPR trafficking based on evidence attained in Task One.
- We have shown that a mutant of arrestin deficient in Src binding (PP) does not rescue FPR-mediated apoptosis and this effect is sensitive to Src family kinase inhibitor.
- We have shown that PP mutant trafficking with Rab11 is similar to when no arrestin is present.
- PP mutant appears to bind to AP-2 by microscopy.
- PP mutant trafficking with AP-1 is similar to mutants in Task One.
- Src family kinase inhibitor does not change trafficking pattern of PP mutant/FPR or FPR in the absence of arrestin, but does seem to inhibit trafficking of wild-type arrestin.
- Mutants of arrestin with differing mutations in SH3-binding motifs show varying patterns of FPR-mediated apoptosis.
- These SH3 mutants effect on apoptosis is independent of their effect on internalization.
- All SH3 mutants appear to traffic normally with Rab11, AP-2 and AP-1 despite their differing effects on FPR-mediated apoptosis.

REPORTABLE OUTCOMES

- Presented poster on current research at the American Society for Cell Biology Conference in Washington, DC—December 2004.
- Awarded Edmund J. and Thelma W. Evans Charitable Trust Scholarship
- Awarded Biomedical Sciences Graduate Program Travel Award
- Awarded Office of Graduate Studies Travel Award
- Passed Comprehensive Exam with Distinction
- Presented poster on research from Task One at Era of Hope Conference in Philadelphia, PA—June 2005. "Arrestin Domains that Regualte N-Formyl Peptide Receptor Trafficking and Signaling."
- Awarded Biomedical Sciences Graduate Program Travel Award.
- Manuscript in Preparation – "Arrestin-2 interaction with adaptor proteins regulates *N*-formyl peptide receptor post-endocytic trafficking."
- Defended dissertation and passed with distinction.

CONCLUSION

5.1. SUMMARY

In the preceding studies, we sought to identify 1) whether aberrant signaling causal to FPR-mediated apoptosis in the absence of arrestins was linked to FPR trafficking defects and 2) which region(s) of arrestin-2 were responsible for these phenotypes. Our findings suggest that FPR-mediated apoptosis and FPR-arrestin trafficking defects are linked with respect to decreased binding by certain arrestin-2 binding partners. Additionally, we have done a thorough analysis of arrestin-2 and found that altered binding by certain accessory proteins (AP-2 and Src kinase) are responsible for these phenotypes. Finally, we have discovered that arrestin-2 has a new binding partner (AP-1) whose role in FPR-mediated trafficking and signaling is unknown at this time, but is under further investigation.

5.1.1. Task 1.

Results prior to this work demonstrated that in the absence of arrestins, FPR activation caused receptor accumulation in the Rab11, perinuclear recycling endosome (*12*) and initiated apoptosis (*22*). In addition, FPR internalization was unaffected, but FPR recycling was inhibited. These phenotypes were rescued by reconstitution of arrestin-2 or -3 cDNAs. As arrestin-2 has numerous accessory protein binding partners involved in GPCR trafficking and signaling (*5, 7, 63, 70, 74, 77*), we hypothesized that apoptosis and trafficking alterations were linked and that specific regions of arrestin-2 were responsible for these phenotypes.

We first narrowed our search for arrestin-2 regions responsible for these phenotypes by screening large domains of arrestin-2 for the ability to rescue FPR-mediated apoptosis. While none of the domains assayed rescued apoptosis, only one

region was found to bind the activated FPR, arr2-(1-382). This arrestin-2 mutant is a constitutively active truncation (*24*) that lacks amino acid residues 383-418. The missing amino acids constitute numerous binding sites for accessory proteins. We then used scanning mutagenesis to generate mutants that had been previously described or change qualitatively similar amino acids to alanine (Figure 2.2). When assayed for apoptosis, all mutants except arr2-F391A and -4A rescued FPR-mediated apoptosis. Additionally, in the presence of all mutants (except F391A and 4A), FPR trafficking was unaffected while the F391A and 4A mutants caused FPR accumulation in the perinuclear recycling endosome.

The F391A mutant had been previously described as having decreased AP-2 binding and inhibiting β2-AR endocytosis (*23*). The 4A mutant had not been previously described, but individual mutations (K397A, M399A and K400A) yielded similar AP-2 binding defects and inhibition of β2-AR internalization (*8*). Therefore, we hypothesized that FPR-arrestin trafficking with AP-2 may be altered. In confocal fluorescence microscopy experiments using cells transiently transfected with AP-2-GFP, RFP-fused arrestins and stimulated with 633-6pep, FPR-arrestin trafficking was indeed altered. In cells expressing wild type arrestin-2, FPR-arrestin trafficking was normal and AP-2 was colocalized with receptor-arrestin complexes in the perinuclear region. In cells lacking endogenous arrestins or expressing the F391A mutant, FPR-arrestin accumulated in the perinuclear recycling endosome without AP-2 consistent with an inability to bind AP-2. However, in the presence of the 4A mutant, FPR-arrestin accumulated in the perinuclear region with AP-2. In addition, AP-2 appeared to colocalize as strongly as or more strongly with the 4A mutant than wild-type arrestin-2. This was confirmed using

co-immunoprecipitation of FLAG-tagged arrestins after FPR stimulation and looking for AP-2 binding. Our microscopy results were confirmed: after immunoprecipitation with FLAG antibodies, the F391A mutant did not bind AP-2, wild-type arrestin-2 did bind AP-2 and the 4A mutant bound AP-2 six times as much as wild-type arrestin-2.

As AP-1 had been previously shown to be necessary for formation of recycling endosomes (*42*) and was important to egress from the Golgi to the cell membrane for certain proteins (*41*), we hypothesized it may play a role in efferent FPR trafficking. Confocal fluorescence microscopy experiments in cells transiently transfected with AP-1-GFP, RFP-fused arrestins and stimulated with 633-6pep showed differential AP-1 trafficking with FPR-arrestin complexes. Without stimulation, AP-1 was localized to the perinuclear region. Upon FPR activation, cells lacking arrestins or expressing the F391A or 4A mutants, FPR-arrestin complexes accumulated with AP-1 in the perinuclear region. However, in cells expressing wild type arrestin-2, FPR-arrestin complexes colocalized with AP-1 in the perinuclear region but FPR-arrestin-AP-1 complexes were also seen outside this region consistent with normal FPR trafficking. We hypothesized that arrestin-2 could bind AP-1 upon FPR activation and this was confirmed using co-immunoprecipitation assays.

To confirm that receptor-arrestin accumulation in the perinuclear region was consistent with an inability of the FPR to recycle, we measured FPR recycling by flow cytometry. While expression of wild type arrestin-2 allowed FPR recycling, there was virtually no recycling in the presence of the F391A and 4A mutants. To confirm a role for AP-2 and -1 in normal FPR trafficking, we used siRNA against each in U937 FPR cells (a monocyte cell line that expresses FPR and both endogenous arrestins) and

measured FPR recycling and accumulation in the perinuclear region. Surprisingly, we found that a decrease in AP-2 levels inhibited FPR recycling and caused FPR accumulation in the perinuclear region while decrease of AP-1 levels had no effect.

These findings led to the creation of a model in which after FPR ligand binding and internalization, arrestin-2 binding occurs followed by AP-2 binding to arrestin-2. In the perinuclear recycling endosome, AP-2 must release from arrestin-2 and only then can FPR-arrestin complexes leave the recycling endosome and travel back to the cell surface. Along the latter path, AP-1 interacts with FPR-arrestin, but the physiologic consequences of this binding are unclear at this time. Finally, if AP-2 does not bind or release FPR-arrestin complexes, receptor will be trapped in the recycling endosome and aberrant signaling complexes will initiate apoptosis.

5.1.2. Task 2.

Previous results by our laboratory have demonstrated that FPR-mediated apoptosis in the absence of arrestin can be rescued by inhibition of Src kinase, ERK1/2, JNK3 and p38 MAPKs (*22*). It has also been demonstrated that Src kinase binding to arrestin-2 is necessary for ERK1/2 phosphorylation within the context of β2-AR activation (*66*). Based on these findings, we hypothesized that an arrestin-2 mutant deficient in Src kinase binding (P91G/P121E) could alter FPR-non genomic signaling and initiate apoptosis. In apoptosis assays, expression of the P91G/P121E mutant did not rescue FPR-mediated, but Src kinase inhibition was able to stop apoptosis. These results indicated that although arrestin-2 and Src kinase have decreased binding, Src kinase activity can still initiate FPR-mediated apoptosis. In addition, FPR internalization was not affected by the arrestin-2 mutant or Src kinase inhibition.

To test the P91G/P121E mutant's effect on FPR trafficking, we used confocal fluorescence microscopy. Cells deficient in arrestins were transfected with RFP-fused arrestins and either Rab11-GFP, AP-2-GFP or AP-1-GFP. Upon FPR stimulation in the presence of the P91G/P121E mutant, FPR-arrestin complexes accumulated in the perinuclear recycling endosome with Rab11, AP-2 and AP-1. Because Src kinase inhibition rescued FPR-mediated apoptosis in the presence of the P91G/P121E mutant, we hypothesized that this inhibition may rescue the FPR trafficking defects caused by the mutant. To our surprise, this was not the case. In the presence of PP2, the P91G/P121E mutant accumulated with the FPR and Rab11 in the perinuclear recycling endosome. In addition, PP2 caused wild type arrestin-2 to accumulate with the FPR in the Rab11 endosome, but did not initiate apoptosis. The above results led us to conclude that Src kinase plays two independent roles when it binds arrestin-2: a role in controlling FPR-arrestin signaling cascades and a role in FPR-arrestin complex trafficking.

Finally, we hypothesized that if Src kinase cannot bind the P91G/P121E which results in signaling defects that initiate apoptosis, this might also mean that ERK1/2 signaling was aberrantly affected. To test this hypothesis, we assayed apoptosis in the presence of the P91G/P121E mutant and ERK1/2 inhibitors U0126 and PD98059. Our results demonstrated that in the presence of this arrestin-2 mutant, ERK1/2 inhibition rescues FPR-mediated apoptosis. This indicates that in the absence of Src kinase binding to arrestin-2, not only is Src kinase signaling aberrantly, but ERK1/2 is as well.

Based on these results, we presented a model for the two independent actions of Src kinase with respect to arrestin-2 binding. After FPR activation and internalization,

arrestin-2 binds phosphorylated FPR and AP-2 binds arrestin-2. Upon arrival at the recycling endosome, Src kinase also binds arrestin-2. Src kinase then phosphorylates AP-2 causing its release from arrestin-2. This has been previously demonstrated to be integral to internalization of the $AT1_AR$ (*38*). After AP-2 release, FPR-arrestin can leave the perinuclear recycling endosome and travel back to the cell surface. However, when the P91G/P121E mutant binds FPR, AP-2 binds the arrestin mutant, but Src kinase cannot bind the complex. The FPR complex accumulates in the recycling endosome and Src kinase signals aberrantly independent of the complex and initiates apoptosis. Therefore, we conclude that arrestin-independent Src kinase activity initiates apoptosis, but arrestin-dependent Src kinase activity leads to normal FPR trafficking and signaling.

5.1.3. Task 3.

The results from Chapter 3 demonstrated that Src kinase has two roles in arrestin-mediated FPR trafficking and signaling. However, this led us to ask an important question: do FPR trafficking defects lead to FPR signaling defects or vice-versa? There are three SH-3 binding domains within arrestin-2: P88/P91, P121/P124 and P175/P178. In our previous work, we used an arrestin-2 mutant that had contained point mutations within different SH3-binding domains. To differentiate the effects of different SH3-binding domains on FPR trafficking and signaling we created three mutants that altered prolines within each SH3-binding domain (P88A/P91A, P121A/P124A and P175A/P178A).

FPR-mediated apoptosis was rescued by the above arrestin mutants except P121A/P124A which could only be rescued by Src kinase inhibition. This implicated the second SH3-binding domain as responsible for Src kinase binding that rescued FPR-

mediated apoptosis. However, when these arrestin-2 mutants were assayed by confocal fluorescence microscopy for FPR trafficking defects a surprising result occurred. All mutants trafficked normally with FPR in respect to Rab11, AP-2 and AP-2 colocalization and FPR internalization was not inhibited. These results led us to believe that FPR signaling defects could occur in the absence of FPR trafficking defects.

In the above results, we used mutants that changed prolines within SH3-binding domains to alanine. We feared that alanine substitutions of multiple prolines within the same SH3-binding domain may cause different effects on FPR trafficking and signaling compared to the original mutant used (P91G/P121E). To alleviate this complication, we made point mutations similar to those used in Chapter 3, but in one SH3-binding domain at a time (P91G and P121E). In apoptosis assays, P91G rescued FPR-mediated apoptosis, but the P121E mutant could not rescue the phenotype. However, apoptosis in the presence of the P121E mutant was rescuable by Src kinase inhibition. In trafficking assays using confocal fluorescence microscopy, both mutants trafficked normally with FPR in correlation with Rab11, AP-2 and AP-1. Once again, FPR internalization was not affected. These results indicated that our previous results were not the effect of alanine replacing two prolines in SH3-binding domains of arrestin-2, but were the result of decreased Src kinase binding to the second SH3-binding domain (P121/P124) of arrestin-2.

Our results led us to conclude that FPR signaling defects could occur in the absence of associated trafficking defects. With respect to Src kinase-arrestin-2 binding, the second SH3-binding domain (P121/P124) alters FPR signaling and initiates apoptosis. However, this does not alter FPR trafficking. Additionally, mutation of the

other SH3-binding domains can affect FPR signaling or trafficking. It is only when the first (P88/P91) and the second (P121/P124) SH3-binding domains are mutated that *both* FPR trafficking and signaling are aberrantly affected.

5.2. MODEL AND IMPLICATIONS.

5.2.1. Our new model of FPR trafficking and signaling.

Based on our findings in Chapters 2, 3 and 4 and previously published results, we suggest the following model for the FPR life cycle (Figure 5.1). FPR is expressed on the cell surface and binds its naturally occurring ligand, fMLF. This alters the conformation of the receptor, initiating heterotrimeric G protein and activating G protein-mediated signaling pathways. The C-terminus is then phosphorylated by GRKs. This lowers receptor affinity for G protein binding and increases its affinity for binding by arrestins effectively desensitizing G protein signaling. While arrestin may bind FPR at the membrane or in endocytic vesicles, FPR internalization occurs independently of arrestins, dynamin, clathrin, AP-2, AP-1 and Src kinase and receptor is transferred to a Rab5-positive, early endosome. Before or during transfer to the Rab11, perinuclear recycling endosome AP-2 and Src kinase bind the FPR-arrestin complex and initiate non-G protein, FPR-dependent signaling pathways. In the perinuclear endosome, Src kinase phosphorylates tyrosine residues on AP-2, causing dissociation of AP-2 from the FPR-arrestin complex. This allows the receptor to leave the recycling endosome in AP-1-associated vesicles. During its return to the cell surface, the FPR-arrestin complex dissociates, ligand is uncoupled from receptor and dephosphorylation of the receptor occurs. After these events, the FPR is once again expressed on the cell membrane ready to begin the cycle anew.

5.2.2. FPR internalization and that of other GPCRs.

In the previous chapters, we determined that FPR internalization is independent of arrestins, clathrin, AP-2, AP-1 and Src kinase. Previous reports have further demonstrated that FPR internalization occurs independently of dynamin (*33*). Our unpublished results also suggest that ADP-ribosylation factor 6 and the Rho-Rac-Cdc42 pathway are unnecessary for internalization of the FPR. An important question raised by these results is: what regulates internalization of this receptor? Jokingly, we suggest that only supernatural forces can inhibit FPR internalization. However, in a more serious manner, we suggest some differences in results and assay design from other laboratories that may provide insight to this dilemma.

Protein determinants for β2-AR internalization have been well studied and reviewed. Currently, β2-AR internalization is demonstrated to be dependent upon arrestins (*13*), dynamin (*78*), Src kinase phosphorylation of dynamin (*9*), clathrin (*30*), AP-2 (*65*) and ADP-ribosylation factor 6 (*72*). Many GPCRs have internalization pathways which operate independently of some of these proteins. For example, the m2-muscarinic acetylcholine receptor internalizes in an arrestin-independent, ADP-ribosylation factor 6-dependent manner (*67, 73*). An interesting point common to these studies is the fact that experiments were all performed in HEK293 cells. FPR internalization studies in our laboratory have been performed primarily in MEF or U937 cell lines. It is possible that the cellular contexts between cell lines used in various studies may allow or inhibit GPCR use of various internalization pathways and machinery.

Furthermore, our internalization studies use saturating concentrations of ligand to "flood" the FPR. While this is also true of the aforementioned studies, it is possible that within our cellular context, multiple avenues of receptor internalization exist and pathway divergence is used by the FPR under these high concentrations of ligand. This hypothesis not only explains why we see FPR internalization occurring independently of so many proteins, but is supported by interesting results from our laboratory. Unpublished results indicate that use of lower ligand concentrations during FPR internalization assays has demonstrable effects on FPR internalization. When using 1μM fMLF to initiate FPR internalization, the half-time of internalization is ~90 seconds and the extent of internalization is ~80-90%. However, when 10nM ligand is used in the same assays, the rate of FPR internalization is similar with an extent of 20-25% at 10 minutes that remains constant. This indicates that recycling pathways may be balancing FPR internalization after 10 minutes. Also, by using 10nM fMLF, only a fraction of surface FPR is occupied with receptor at the beginning of the internalization assay leaving open the possibility that under these conditions, alternative avenues of internalization may not be required. Based on these data, we suggest that there are multiple internalization pathways for the FPR, and use of lower ligand concentrations in future experiments may help discovery of the protein(s) that mediate FPR internalization.

5.2.3. AP-2 regulates FPR post-endocytic trafficking and trafficking of other GPCRs.

In our studies, we have shown that AP-2 plays a critical role in FPR post-endocytic trafficking. It was demonstrated that both binding and release of AP-2 from

arrestin-2 is necessary for it to leave the perinuclear recycling endosome and return to the cell surface. Additionally, we hypothesize, based on our evidence from Chapter 3, that Src phosphorylation of AP-2 may also be necessary for normal FPR trafficking. The FPR is not the only GPCR for which AP-2 regulates its trafficking. $AT1_AR$ internalization at the cell membrane may be regulated similarly. One report demonstrated that AP-2 binding to arrestin-2 was necessary for β2-AR internalization (*65*). Furthermore, a different study examined the regulation of $AT1_AR$ internalization by Src kinase (*38*). It was determined that Src kinase phosphorylation of tyrosine residues on AP-2 was necessary for AP-2 release from arrestin-2 and subsequent internalization of $AT1_AR$. Given the functional similarity of the two receptors (*55, 69*), AP-2-dependent internalization for the $AT1_AR$ is likely. Finally, in a recent report (*49*), the authors found that two purinergic receptors, $P2Y_1$ and $P2Y_{12}$, colocalized with AP-2 in perinuclear "endocytic compartments", though the identity of this compartment was not established.

While the trafficking of individual GPCRs is regulated by AP-2 interactions at different places within the cell, it is unclear how AP-2 regulates GPCR-arrestin trafficking. An answer may be found in the differential phosphorylation of GPCRs after ligand binding and activation. Our laboratory has demonstrated that FPR phosphorylation patterns by GRKs are highly complex and differentially regulate receptor desensitization, internalization and arrestin binding (*50*). For example, the presence of one phosphorylation site in the FPR C-terminus is adequate to mediate receptor internalization and arrestin binding while the presence of additional sites of phosphorylation can inhibit arrestin binding without affecting FPR internalization. Furthermore, exchanging the β2-AR C-terminus for the V2-vasopressin C-terminus

produces a V2-vasopressin receptor trafficking phenotype upon β2-AR stimulation (*52, 53*). Finally, differential phosphorylation of $AT1_AR$ significantly affected arrestin-mediated ERK1/2 activation (*55*). In this study, GPCR phosphorylation by GRK4/5 activity, but not GRK2/3 activity, produced ERK1/2 phosphorylation upon receptor activation.

The above studies suggest that different amounts and location FPR phosphorylation within its C-terminus differentially regulates trafficking and signaling. We suggest that minimal receptor phosphorylation mediates FPR internalization. Upon further phosphorylation, perhaps during of after internalization, arrestin binding occurs. Finally, after possibly additional phosphorylation, arrestin binding is sufficient to support binding of AP-2. Due to similar regulation of the FPR and $AT1_AR$ by AP-2 interaction, it is possible to answer the aforementioned hypothesis by swapping the C-termini of the receptors and assaying differences in receptor trafficking. We hypothesize that an FPR/$AT1_AR$ C-terminal chimera will traffic similarly to $AT1_AR$ upon FPR stimulation and vice-versa.

5.2.4. Arrestin and Src kinase regulation of FPR signaling.

Throughout our previous studies (Chapters 2-4), we have demonstrated that proper control of FPR signaling is mediated by arrestins and Src kinase. Additionally, we have demonstrated that Src kinase binding to arrestin is necessary for proper regulation of FPR signaling. Finally, we have demonstrated that the FPR trafficking and signaling defects can be mediated independently of one another. When cells expressing wild type arrestin-2 are subjected to Src kinase inhibition with PP2, FPR-arrestin complexes accumulated in the perinuclear recycling endosome without initiation

of apoptosis. On the contrary, expression of an arrestin mutant deficient in Src kinase binding (P121E) permits normal FPR-arrestin complex trafficking but initiates apoptosis.

Differential GPCR signaling via arrestin and Src kinase is demonstrated by the β2-AR and $AT1_AR$ (*66*). ERK1/2 phosphorylation via β2-AR activation was inhibited by arrestin mutants that did not bind Src kinase (P91G/P121E) or cannot be dephosphorylated (S412D). However, expression of the arrestin mutant that did not bind Src kinase in the context of $AT1_AR$ stimulation did not alter ERK1/2 signaling. Furthermore, different ligands that bind the same GPCR can initiate differential activation of ERK1/2. In the context of CCR7 signaling, CCL19 activates ERK1/2 at early time points and is subsequently dephosphorylated over time whereas stimulation with CCL21 does not activate ERK1/2 at all (personal communication, Charlotte Vines, KUMC).

Given the complexity of arrestin regulation of FPR signaling, it is likely that numerous pathways of MAPK activation and deactivation exist. In addition, these pathways may both converge and diverge to control cellular function. More rigorous examination of these pathways is necessary to understand the effect of MAPK signaling on FPR trafficking and cellular physiology. These experiments are further outlined in Future Study 2.

5.3. FUTURE STUDIES.

5.3.1. Future Study 1. Understanding arrestin-2/AP-1 binding.

An interesting finding from Chapter 2, is the discovery that AP-1 binds arrestin-2 upon FPR activation. While the physiologic effects of this binding are currently unclear, an understanding of where AP-1 binds arrestin-2 may give us clues to its function.

Currently, amino acids Asp385, Phe388, Phe391, Arg393, Arg395, Lys397, Met399 and Lys400 in arrestin-2 have all been demonstrated as binding sites for the β2-subunit of AP-2 (*8, 23*). Additionally, amino acids E849, Y888, and E902 within the β2-subunit of AP-2 are binding sites for arrestin-2 (*43*). Furthermore, the β1-subunit of AP-1 shares significant homology with the β2-subunit of AP-2 and the aforementioned amino acid residues in the β2-subunit are conserved (*44*).

To better understand where AP-1 and arrestin-2 bind each other, GST fusions of the critical regions of the β1-subunit containing the aforementioned amino acid binding sites for arrestin-2 could be used in *in vitro* binding assays with wild-type arrestin-2 to discover whether binding could be interrupted upon amino acid mutation. Furthermore, GST fusions of the β1-subunit could be used in similar assays with arrestin-2 mutations at sites critical for AP-2 binding. These assays will determine two important results: 1) whether it is the β1-subunit that binds arrestin-2 and 2) where the binding sites in the respective proteins are. These results will give us more data to help determine the role of AP-1/arrestin-2 binding in FPR trafficking and signaling. Alternatively, we may find that it is not the β1-subunit that binds arrestin-2 or that, in trafficking assays, other proteins (AP-3 or AP-4) have redundant functions that allow normal FPR trafficking in the absence of AP-1 binding. Regardless, as this protein-protein interaction is novel to GPCR trafficking and signaling, data accrued from these experiments will aid us in elucidating its effects on cellular physiology.

5.3.2. Future Study 2. The effects of aberrant GPCR trafficking on non-G protein signaling cascades.

Our studies have indicated an intimate association between FPR trafficking and signaling defects. While these two defects are often linked, it is possible for each defect to occur independently of the other. In the previous chapters, FPR trafficking defects are well documented, whereas FPR signaling defects are examined by apoptotic signaling and use of signaling protein inhibitors. We hypothesize that specific alterations in MAPK phosphorylation lead to FPR-mediated apoptosis in the absence of arrestins or presence of arrestin mutants.

To test our hypothesis, we suggest using Western blot analysis of MAPK phosphorylation patterns. These experiments would be done over a time course of FPR activation in the absence of arrestins and presence of wild type arrestin-2 or selected arrestin mutants. Phosphorylation patterns for n-terminal c-jun kinase 3, p38 and ERK1/2 should be explored. For example, preliminary results suggests in the absence of arrestins, FPR stimulation leads to early phosphorylation of ERK1/2, but in the presence of wild type arrestin-2 ERK1/2 has both an early and late phase of phosphorylation. In the presence of an arrestin mutant that does not bind Src kinase (P91G/P121E), the ERK1/2 phosphorylation pattern is similar to wild type arrestin-2, but is increased at all time points and is phosphorylated in the absence of ligand stimulation. Additionally, location of activated MAPKs relative the FPR-arrestin complexes should be ascertained by confocal immunofluorescence microscopy. These experiments may reveal a difference between the levels of MAPK activation and the possibility of differential location of the activated signaling complexes. The results of these experiments would lead to an increased understanding of signaling pathways induced by FPR activation and could be applicable to other GPCRs of interest.

5.3.3. Future Study 3. The role of aberrant GPCR trafficking on migration.

It is widely hypothesized, although not directly proven, that inhibition of GPCR trafficking may lead to defects in cellular migration (*79, 80*). One problem has been the inability to stop chemokine/chemoattractant receptor trafficking without undue toxicity towards the cell itself. Our results demonstrate that the FPR accumulates in a perinuclear region and exhibits impaired recycling in the absence of arrestins or when AP-2 levels are decreased with siRNA. These results give us the ability to address the role of GPCR trafficking in cellular migration and we hypothesize that when the FPR traffics abnormally and does not recycle properly, FPR-mediated migration will be decreased.

To test this hypothesis, we would use U937 cells expressing the FPR. Then, siRNAs against both arrestin-2 and -3 or AP-2 could be transfected into cells to decrease the levels of the respective proteins. At this point, any of a variety of chemotaxis and/or migration assays could be used to monitor the effect of decreased arrestins or AP-2 on FPR-mediated cell migration. We could expect, in either case, that decreased migration in response to fMLF would be observed. Additionally, other chemokine/chemoattractant receptors could be tested in these assays. For example, while CXCR4 stimulation does not initiate apoptosis in the absence of arrestins (*22*), CXCR4-mediated migration of cells is arrestin-dependent (*81*). However, in neither case was CXCR4 trafficking under their respective conditions examined.

5.3.4. Future Study 4. Rational design of small molecule inhibitors to interrupt GPCR-arrestin binding.

In previous studies we have found that GPCRs, including the FPR and IL-8R, undergo apoptosis upon activation in the absence of arrestins (*22*). In addition, we have demonstrated that it is not merely the absence of arrestins that mediates this phenotype. On the contrary, FPR-mediated apoptosis can occur in the presence of arrestin mutants that bind FPR (Chapters 2-4). Based on these results, we suggest that small molecules that interrupt GPCR-arrestin binding will lead to apoptosis upon stimulation of the respective GPCR.

To test this hypothesis, we would isolate biotinylated arrestin-2 similar to that described previously (*25*). Next, we would bind biotinylated arrestin-2 to streptavidin beads. To ascertain GPCR C-termini binding, purified FITC labeled, IL-8R or FPR phosphorylated C-termini would be incubated with the arrestin-bound beads. When assayed with flow cytometer, the arrestin-bound beads can be gated using the forward- and side-scatter parameters and bound FITC-GPCR C-termini can be determined by reading the mean channel fluorescence in FL1. Then, the same assay can be used with a library of small molecules to determine which compounds interrupt GPCR-arrestin binding (mean channel fluorescence decreases in FL1). After compound screening, small molecules can be used in cell based assays to determine whether they alter receptor trafficking and/or initiate apoptosis upon ligand stimulation. This study would provide us an opportunity to discover small molecules that may be able to treat breast cancer metastasis as the IL-8R is known to be involved in this process (*82*).

5.3.5. Future Study 5. Rational design of small molecule inhibitors to interrupt AP-2-arrestin binding.

In our work from Chapter 2, we determined that the interaction between AP-2 and arrestin-2 was integral to normal FPR trafficking and signaling. Altered interaction between the two proteins led to FPR-arrestin complex accumulation in the perinuclear recycling endosome and apoptosis. Based on these results, we hypothesize that small molecules that inhibit the association of AP-2 and arrestin-2 may lead to FPR trafficking defects and apoptosis.

To test this hypothesis, we would isolate the GST-tagged β-adaptin subunit of AP-2 and bind it to glutathione beads. To examine binding of arrestin-2 to this subunit, we would incubate the protein-bound beads with purified GFP-fused wild type arrestin-2. Similar, to Future Study 4, arrestin binding would be determined by flow cytometry. Small molecules could then be incubated with the protein-bound beads and arrestin, to screen for compounds that interrupted the binding of AP-2 and arrestin-2. From this point, cell based assays could be employed to determine whether the compound could interrupt AP-2/arrestin binding *in vivo*. For example, co-immunoprecipitation, trafficking and apoptosis assay employed in Chapter 2 could be used to determine the physiologic effect of screened small molecules. In addition to the use of a compound that disrupted binding of AP-2 and arrestin-2 to GPCR researchers in general, the compound could be useful as a chemotherapeutic. For instance, if our hypothesis in Future Study 3 was correct, small molecules screened in this study design could be used to inhibit CXCR4-mediated migration which could be used to treat breast cancer metastasis.

5.4. Concluding Remarks

In this study, we have made great strides in determining the trafficking and signaling patterns of the FPR. These results have led to discovery of novel interactions

of AP-2 and AP-1 with arrestin-2 as well as the roles of Src kinase regulation of FPR trafficking and signaling. These results pertain to many GPCRs and will serve as a model for GPCR trafficking and signaling. We have also proposed numerous future studies that make use of our discoveries to further understand the implications of arrestins in GPCR migration, trafficking and signaling. It is our hope that these data and future studies will lead to the design of novel chemotherapeutics to treat countless patients affected by diseases that involve dysregulation of GPCR trafficking and signaling.

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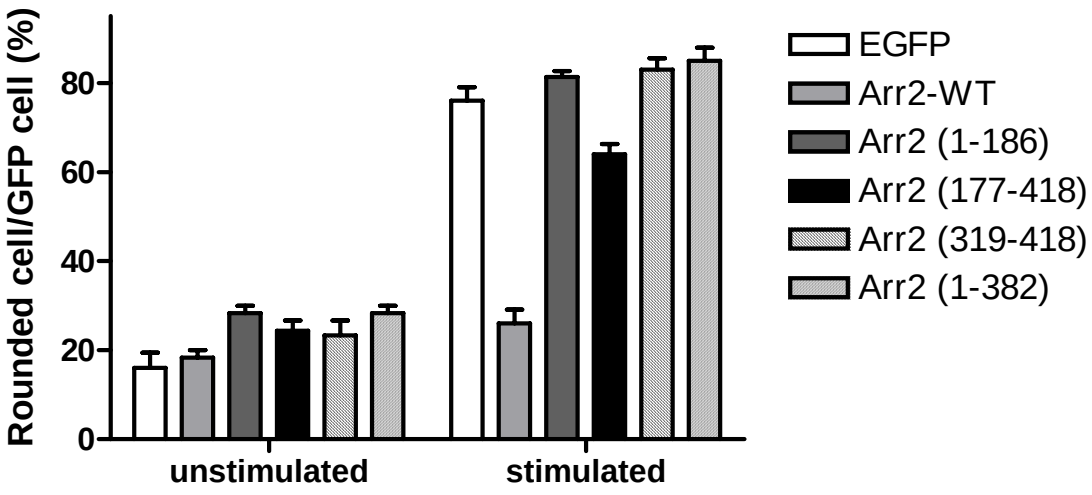


Figure 2.1. Arrestin domains do not rescue FPR-mediated apoptosis.

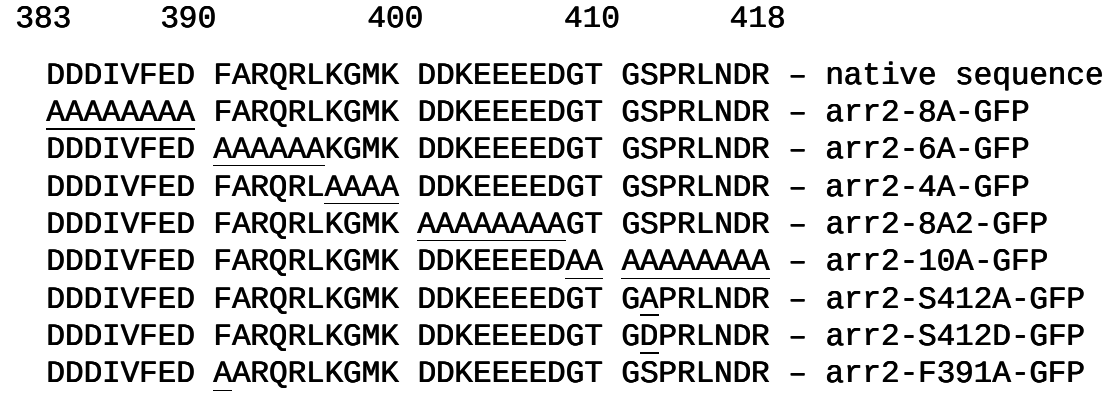


Figure 2.2. Sequence of arrestin-2 carboxy terminus and selected mutants.

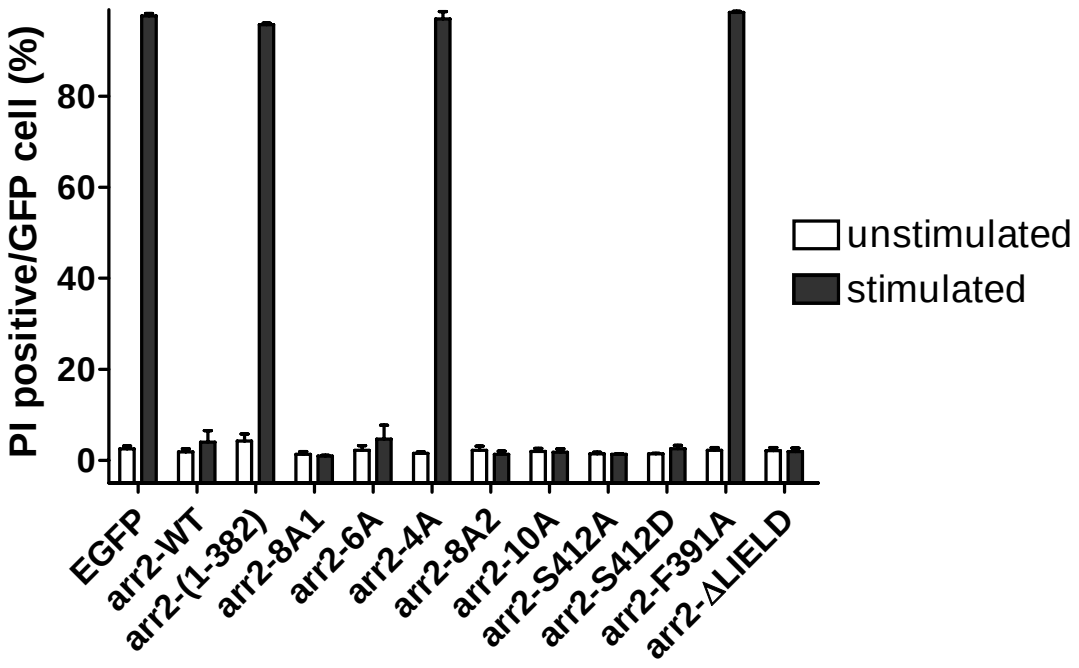


Figure 2.3. Rescue of FPR-mediated apoptosis by arrestin-2 mutants.

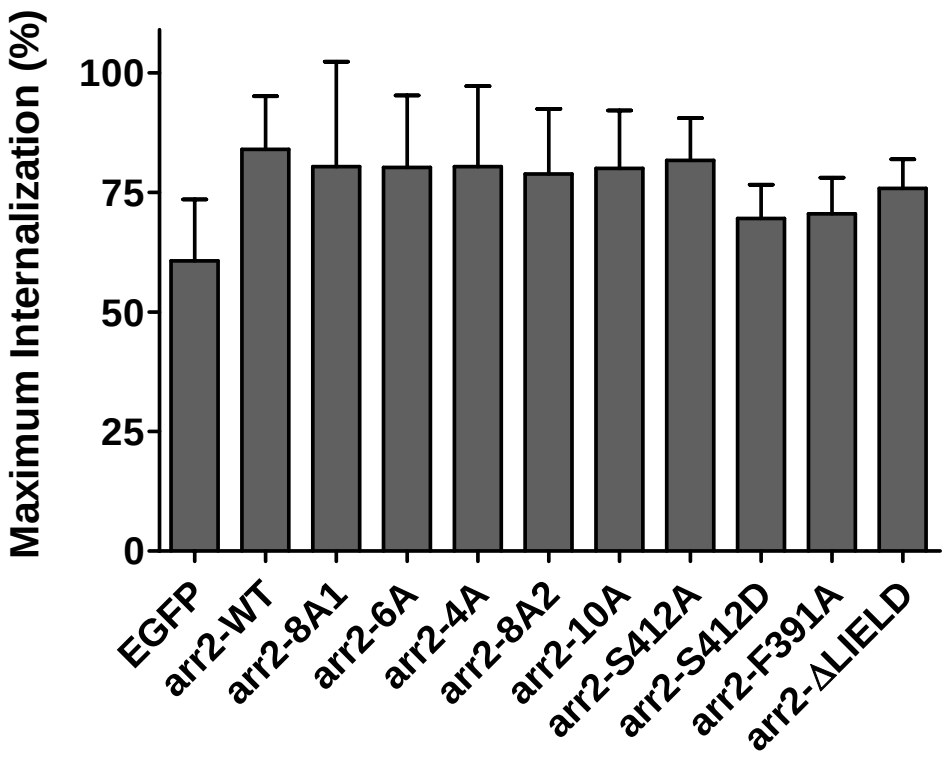


Figure 2.4. Internalization of the FPR in presence of arrestin-2 mutants.

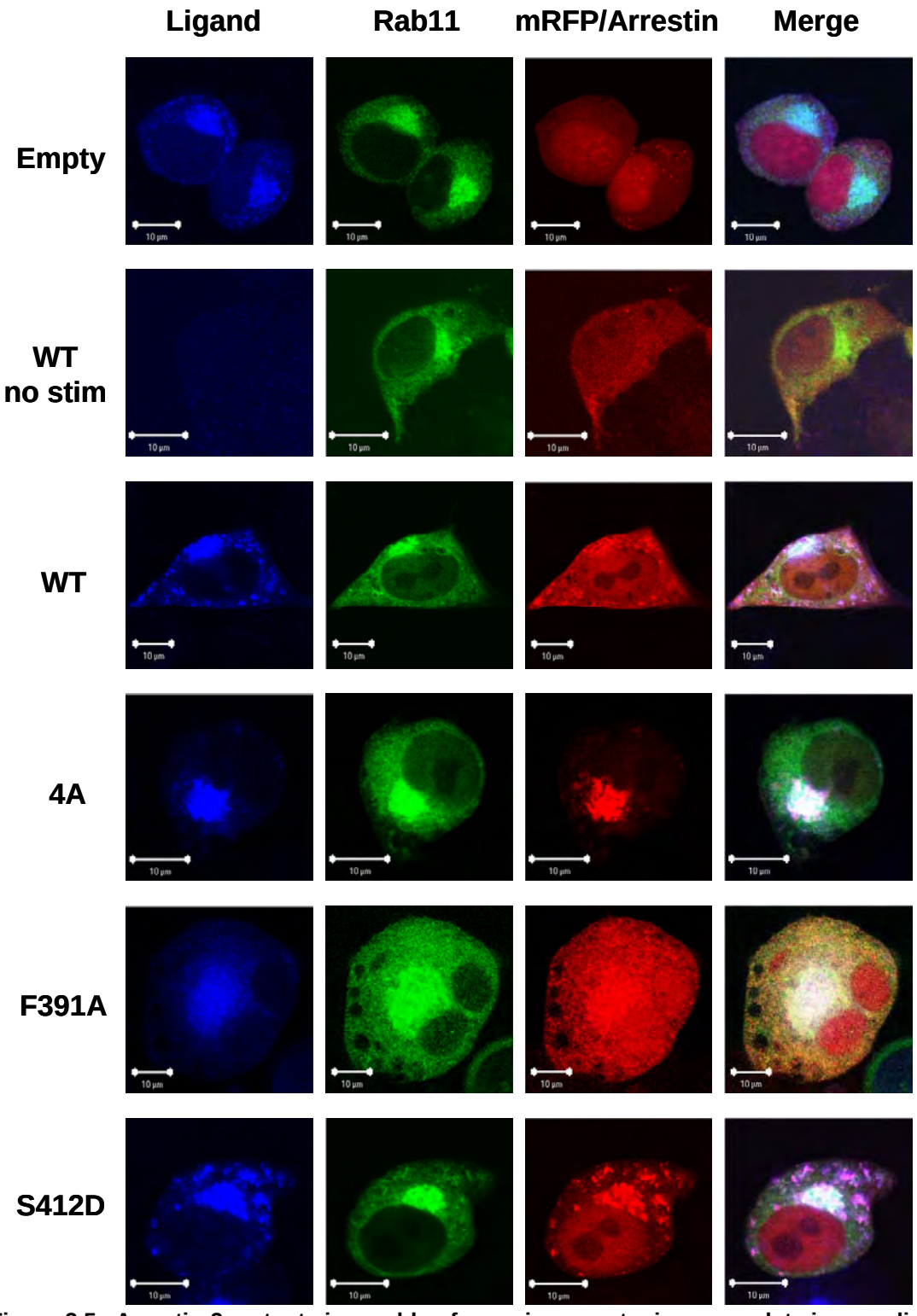


Figure 2.5. Arrestin-2 mutants incapable of rescuing apoptosis accumulate in recycling endosomes.

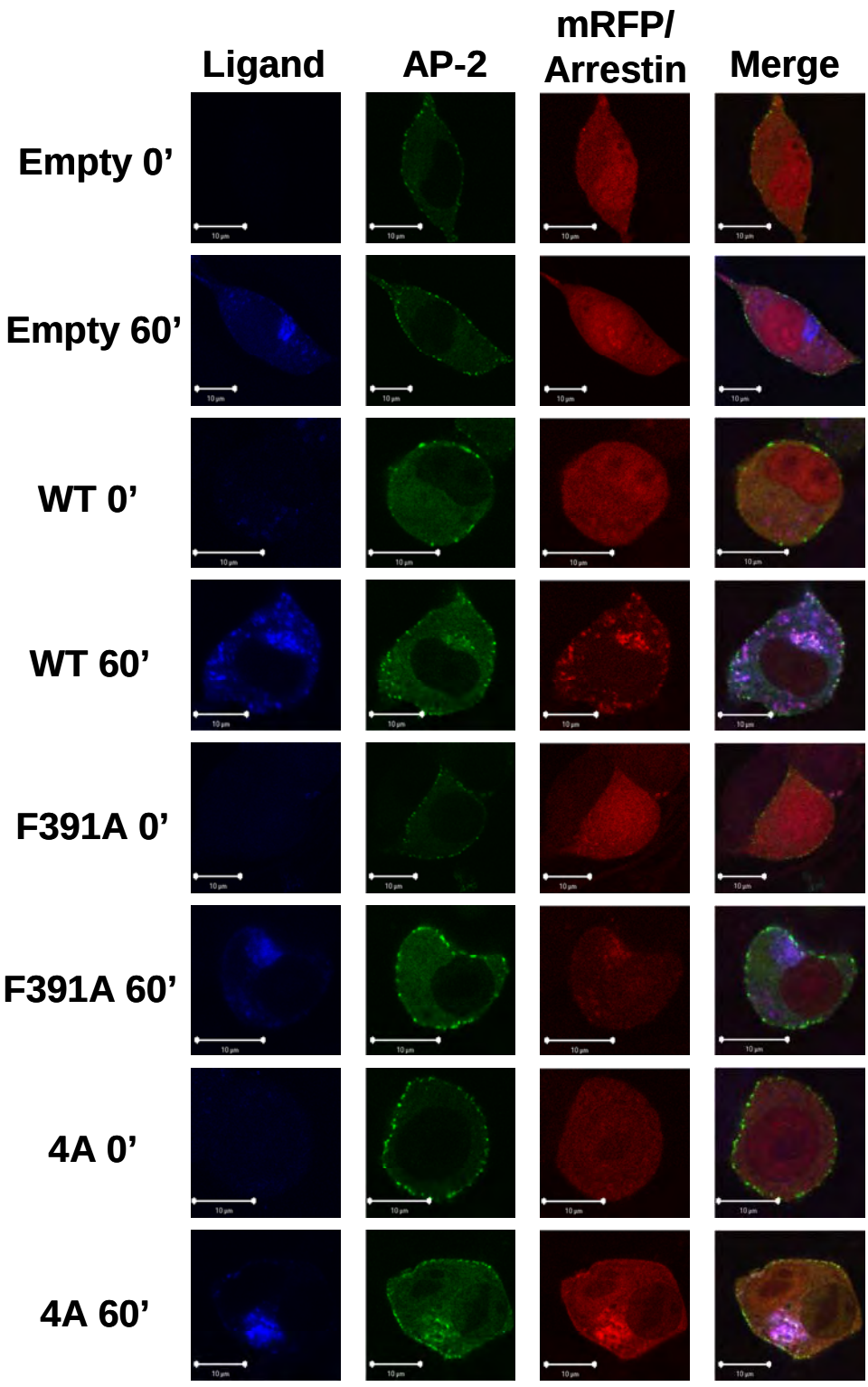


Figure 2.6. Arrestin-2 mutants differentially colocalize with AP-2.

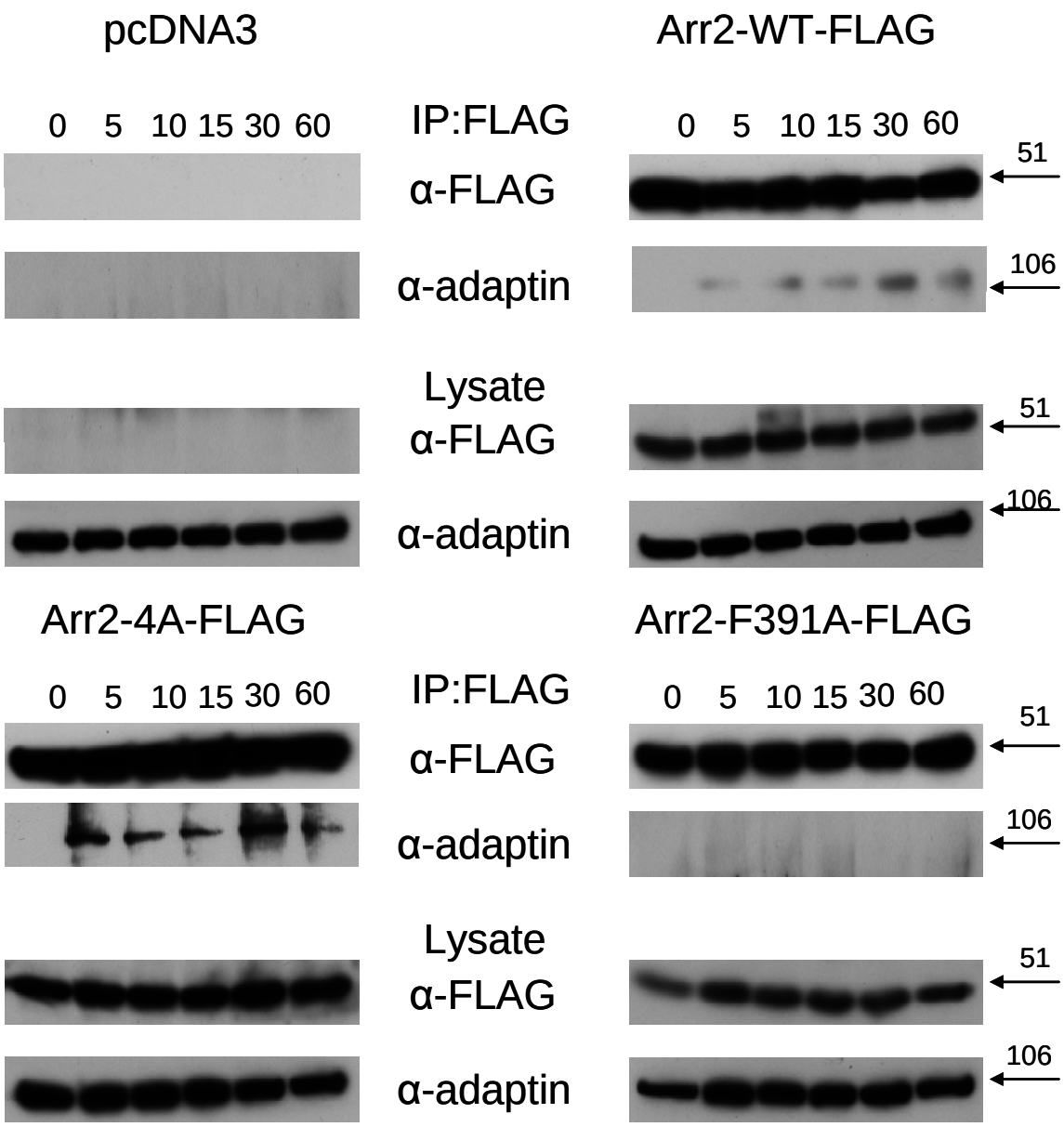


Figure 2.7A. Immunoprecipitation of AP-2 with arrestin-2 mutants.

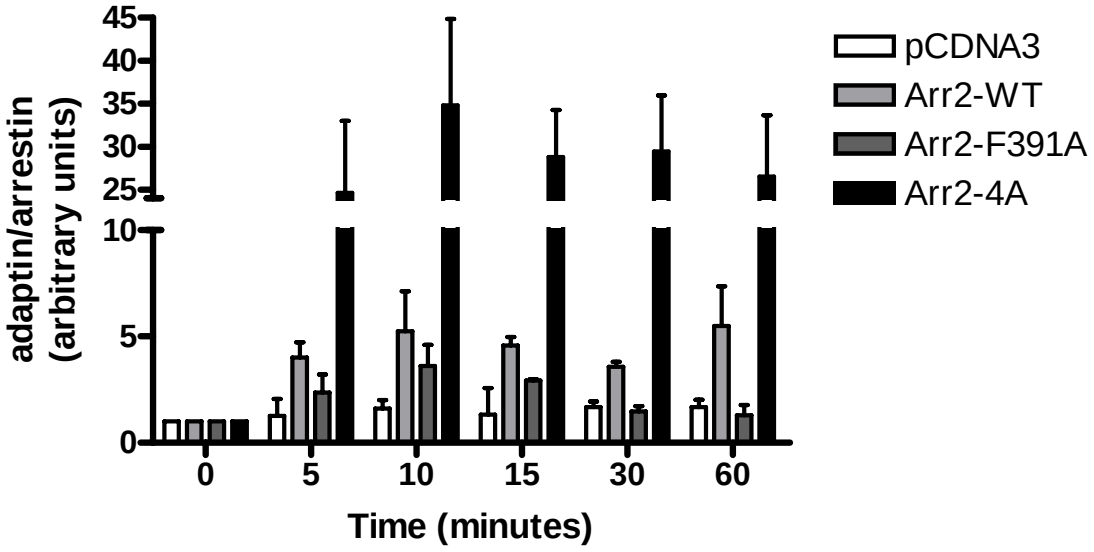


Figure 2.7B. Quantification of AP-2/arrestin-2 interaction.

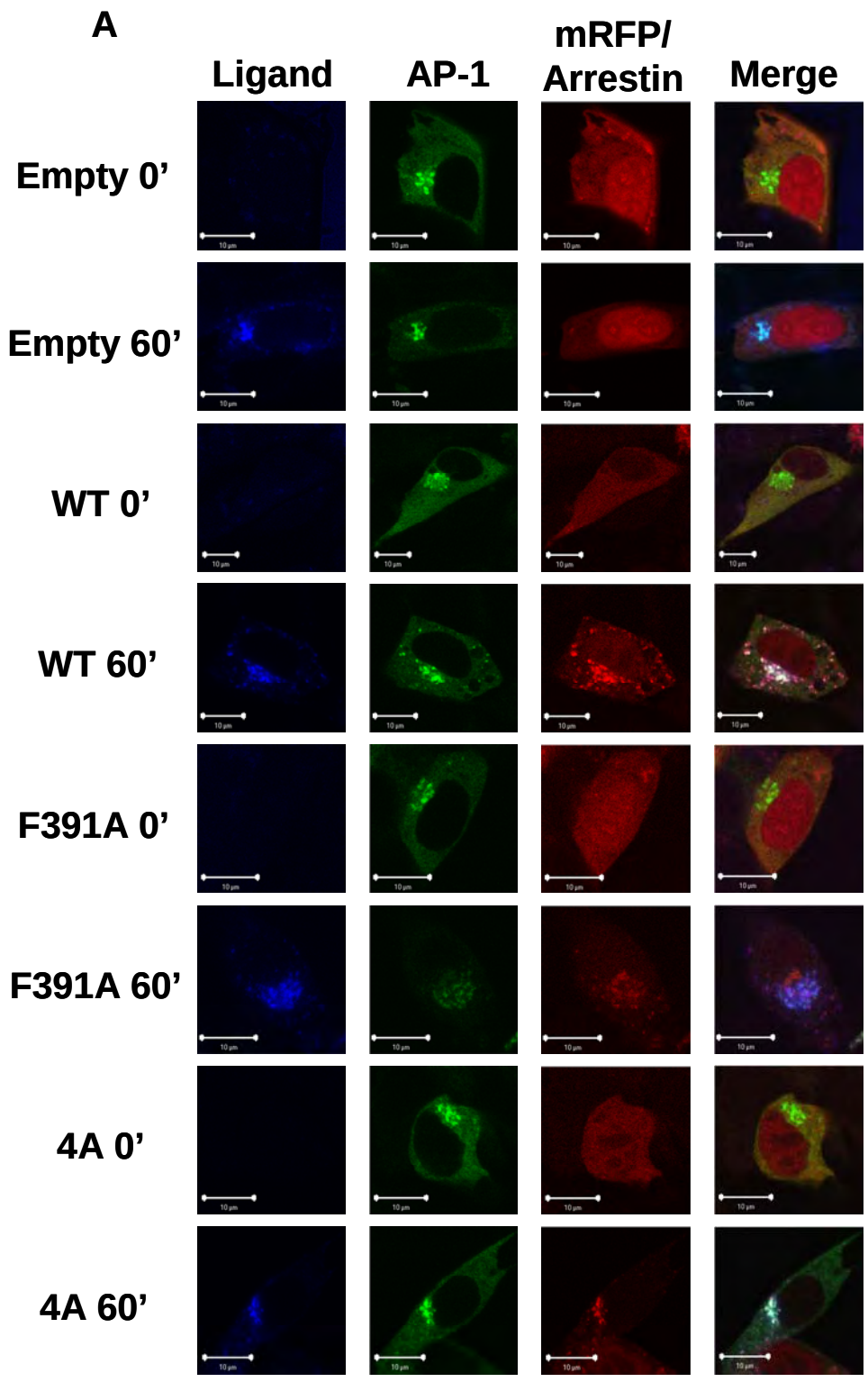
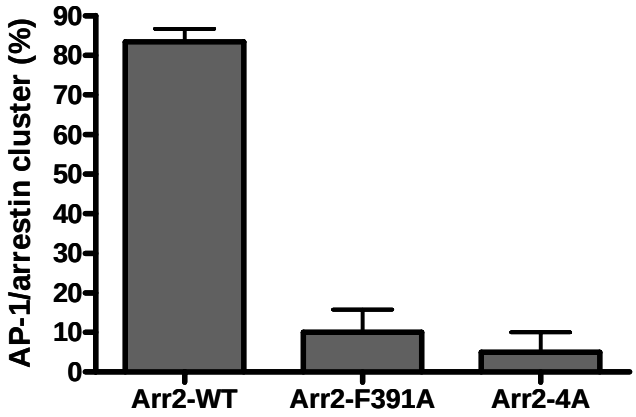


Figure 2.8A. FPR-arrestin-2 trafficking and binding with AP-1.

B



C

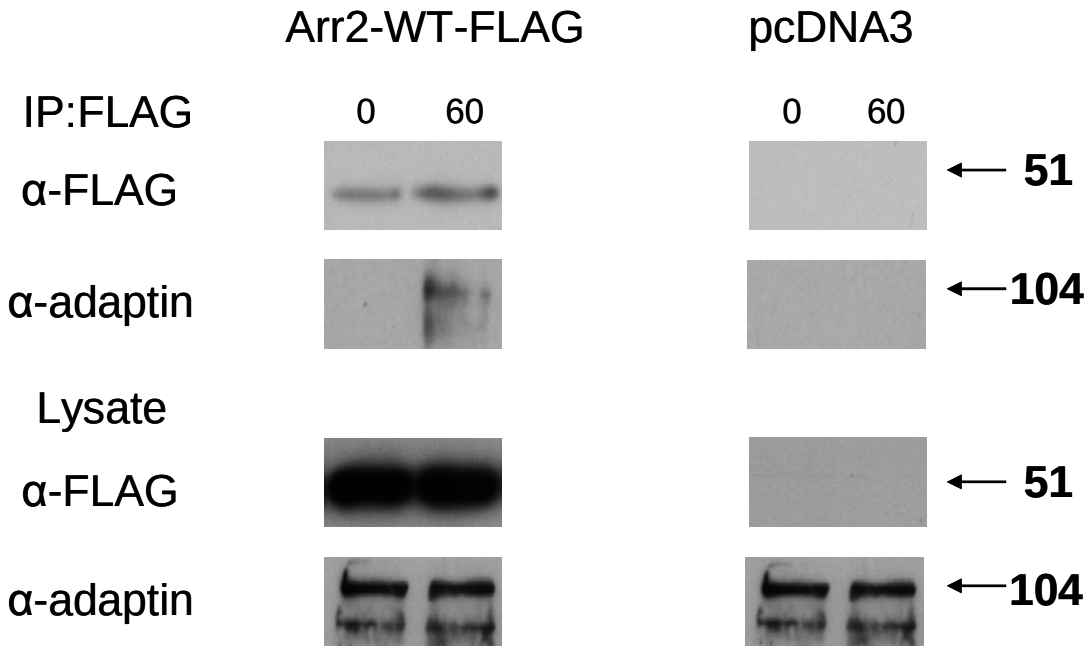
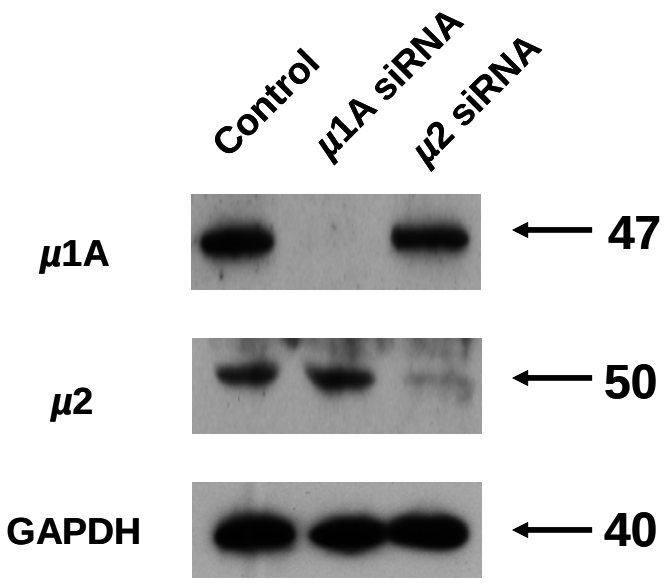


Figure 2.8B and C. Quantification of 2.8A and FPR-dependent AP-1/arrestin binding.

A



B

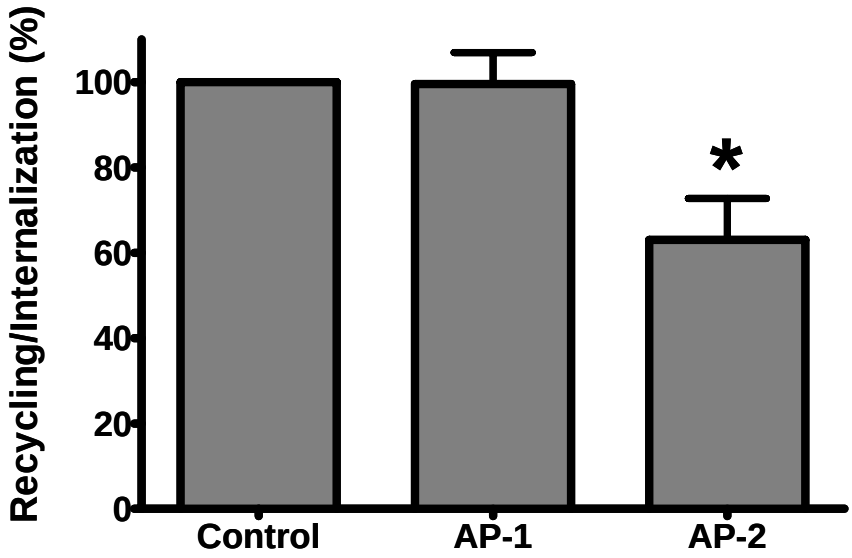


Figure 2.9A and B. FPR recycling upon adaptor protein depletion.

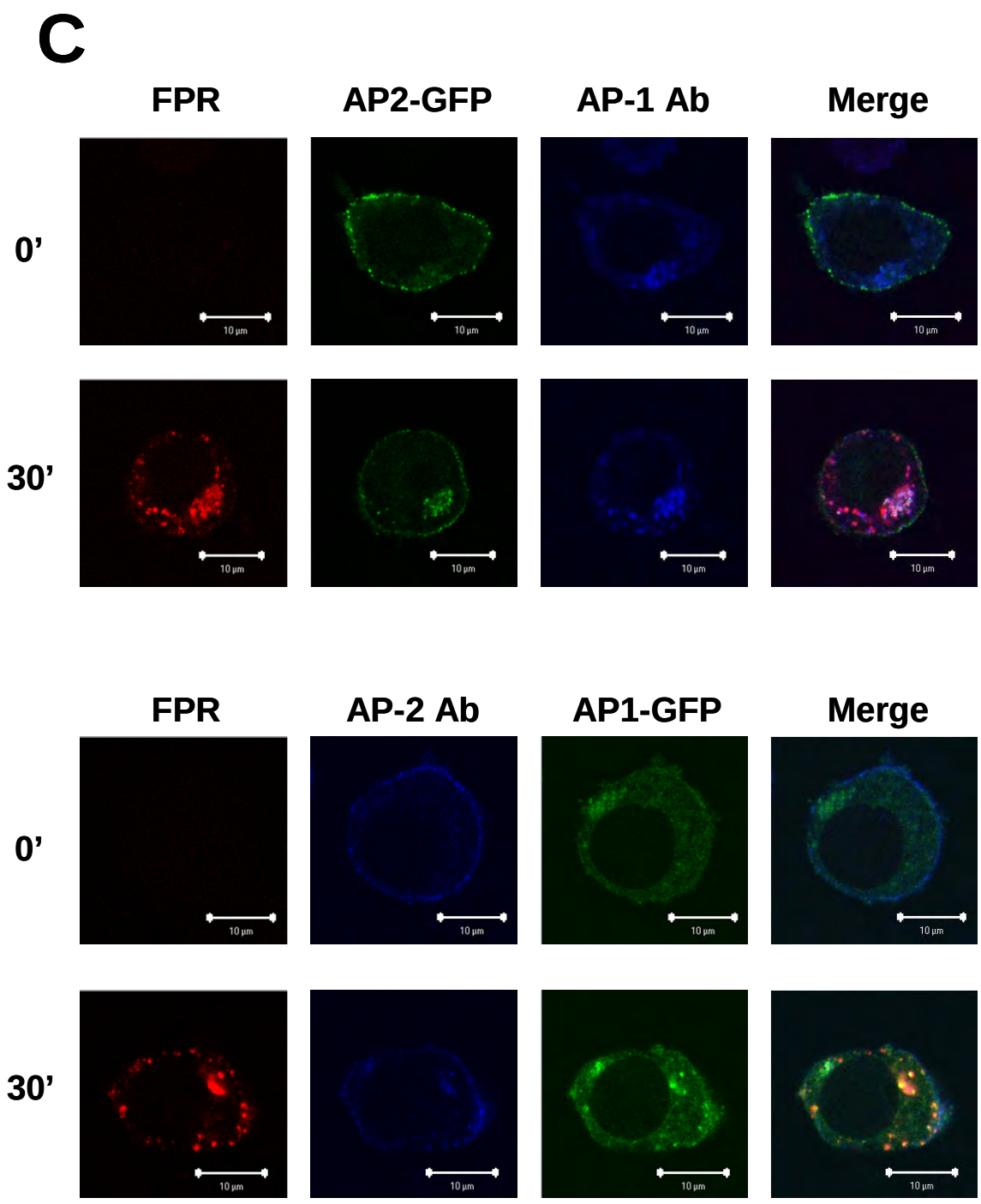


Figure 2.9C. Localization of endogenous AP-2 and AP-1 with FPR.

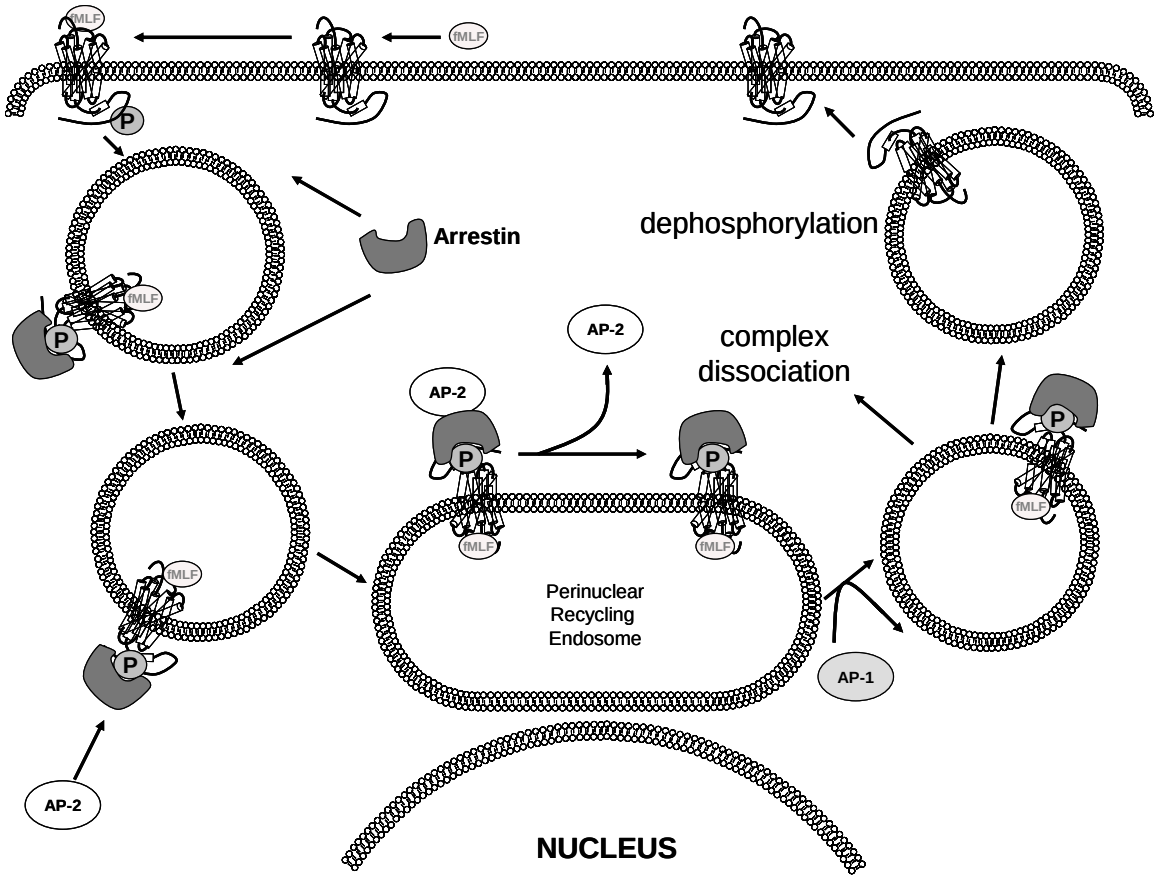


Figure 2.10. Model of FPR post-endocytic trafficking.

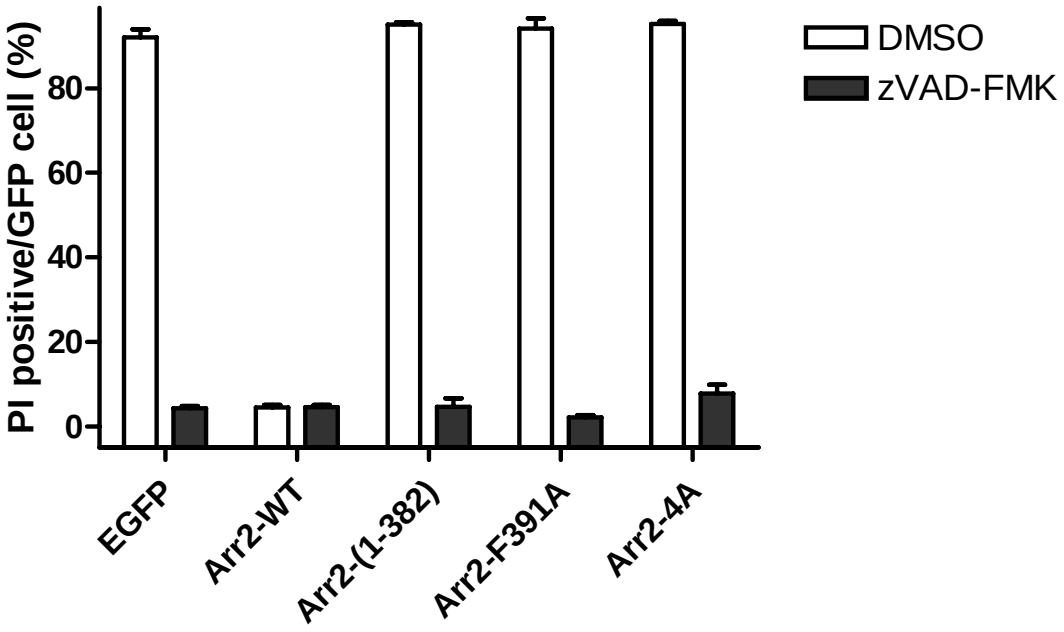


Figure 2.11. Arrestin-2 mutants that do not rescue FPR-mediated apoptosis are sensitive to caspase inhibitor treatment.

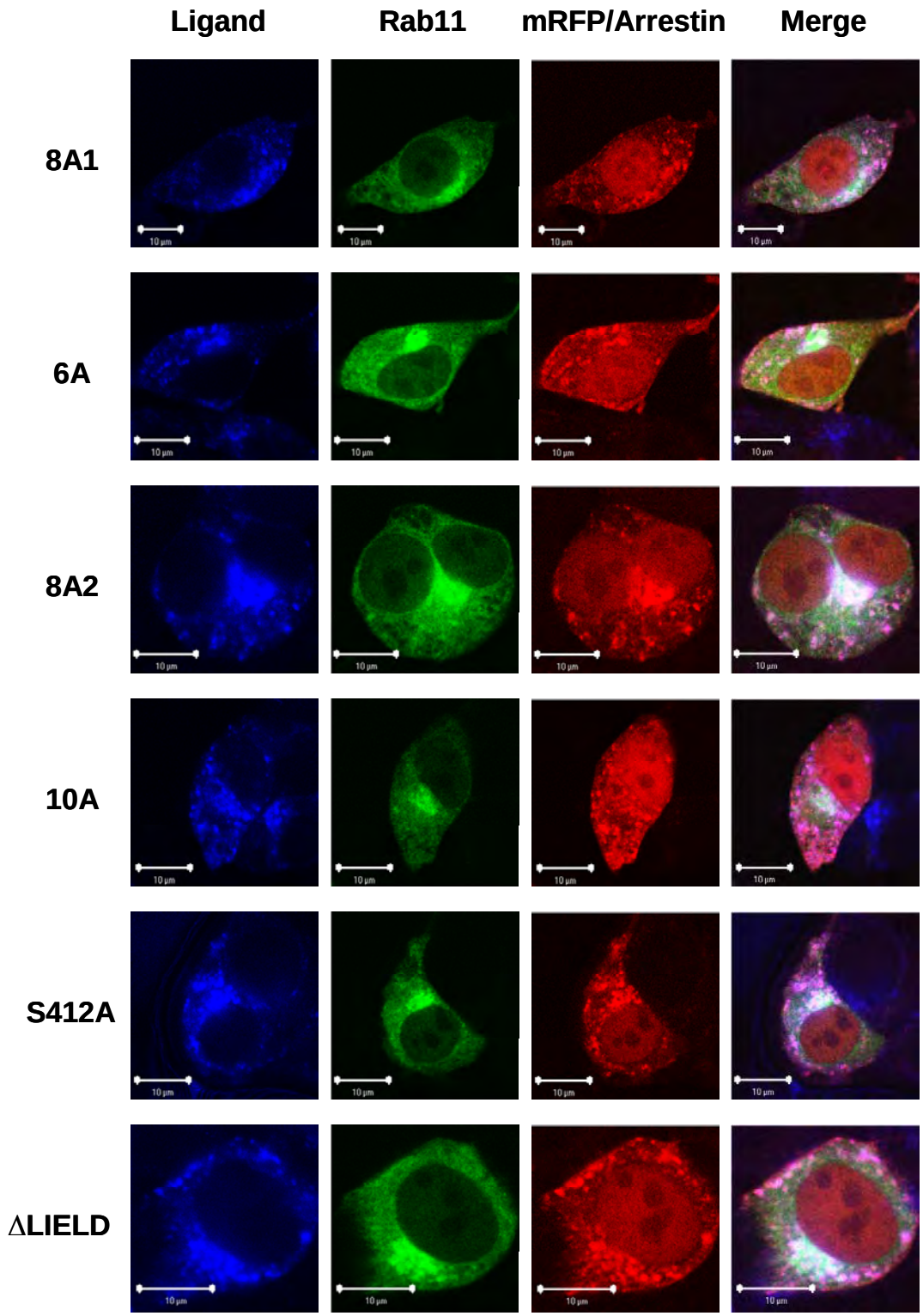


Figure 2.12. Arrestin-2 mutants capable of rescuing apoptosis do not accumulate in recycling endosomes.

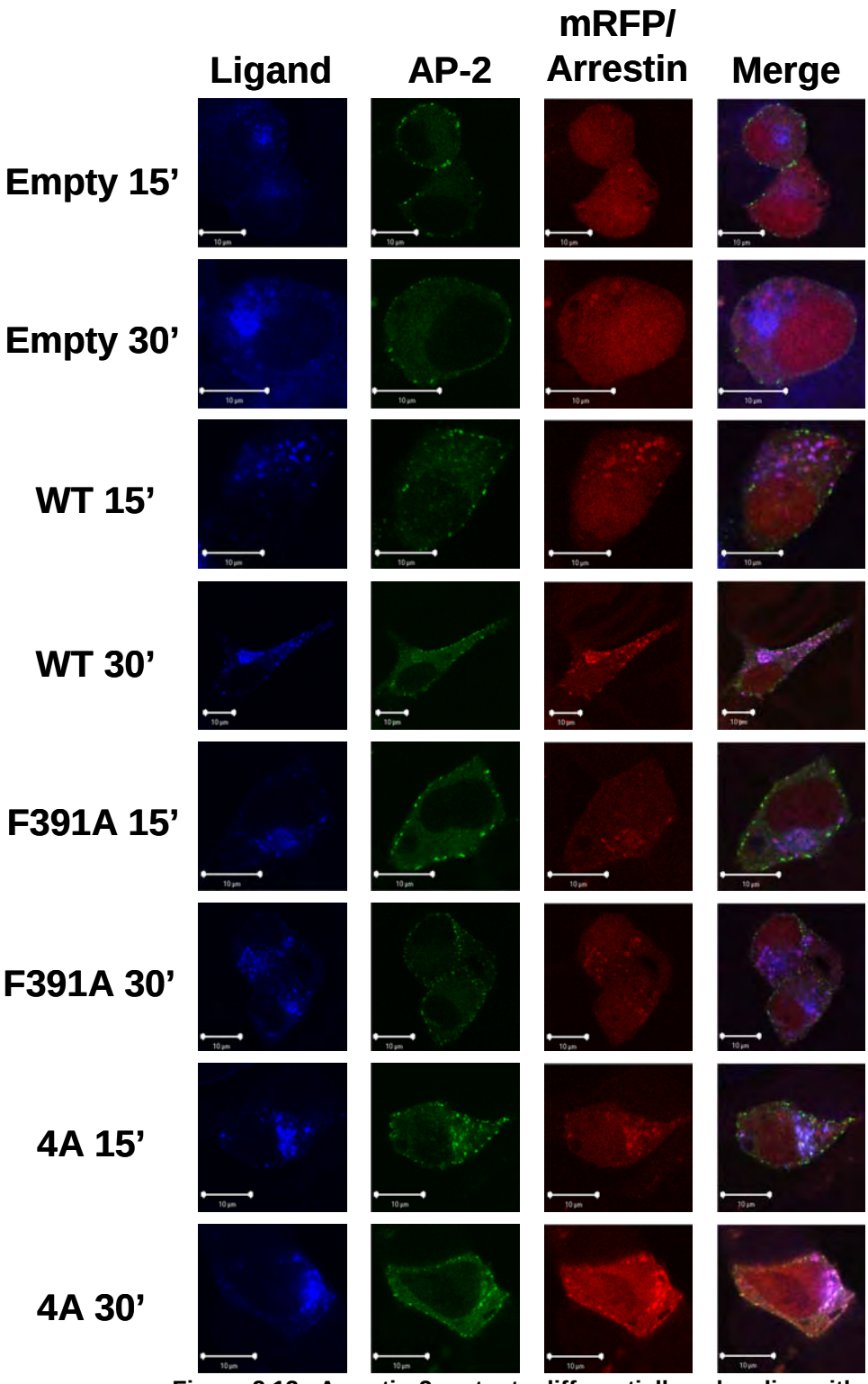


Figure 2.13. Arrestin-2 mutants differentially colocalize with AP-2.

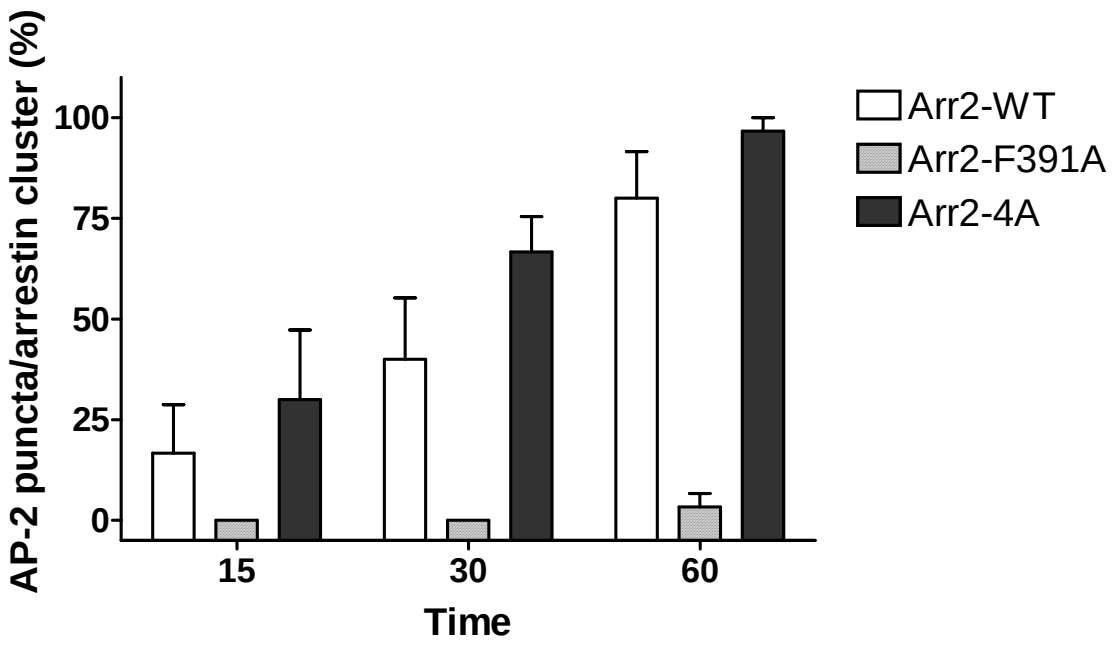


Figure 2.14. Quantitation of AP-2 colocalization with FPR/arrestin complexes.

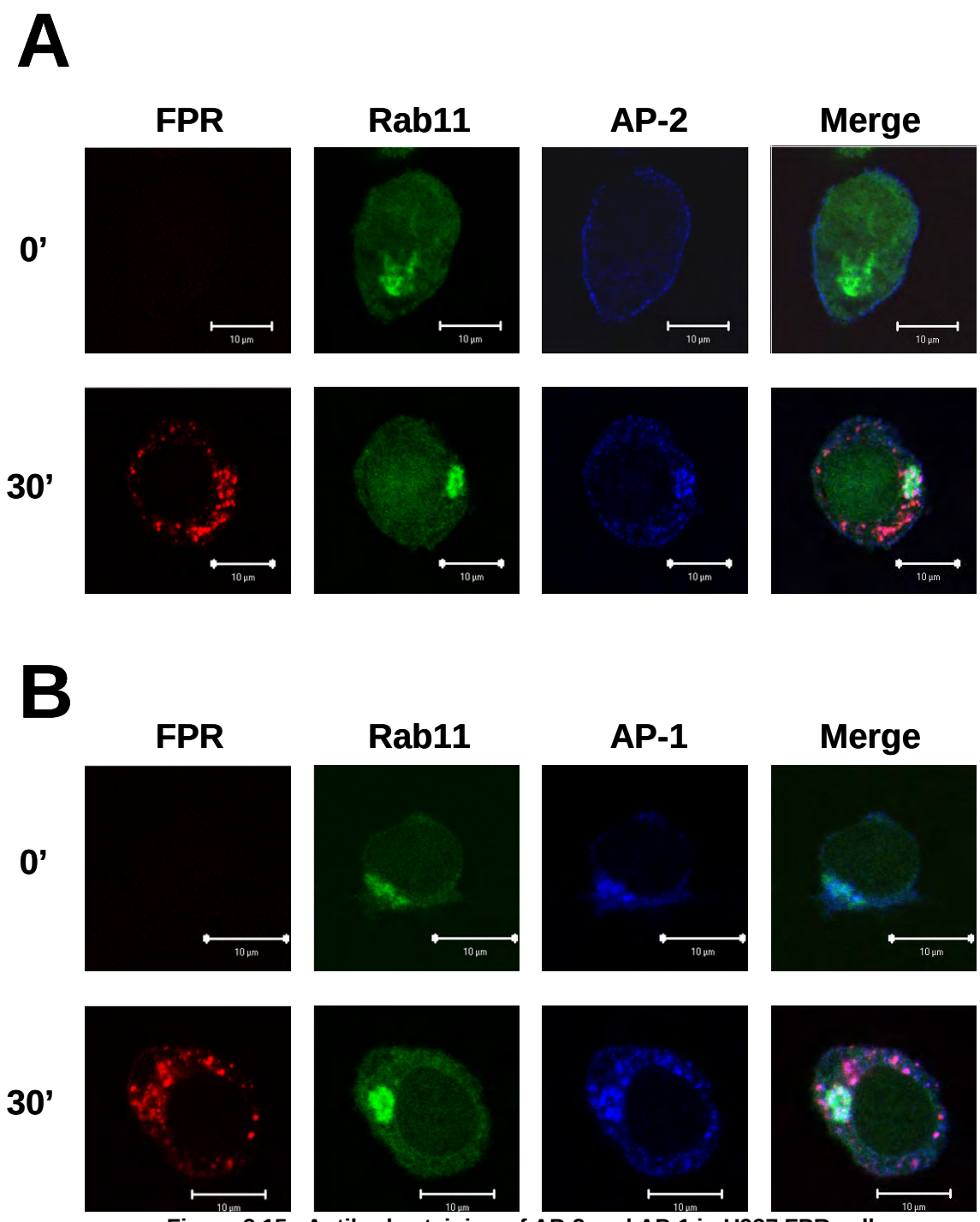


Figure 2.15. Antibody staining of AP-2 and AP-1 in U937 FPR cells.

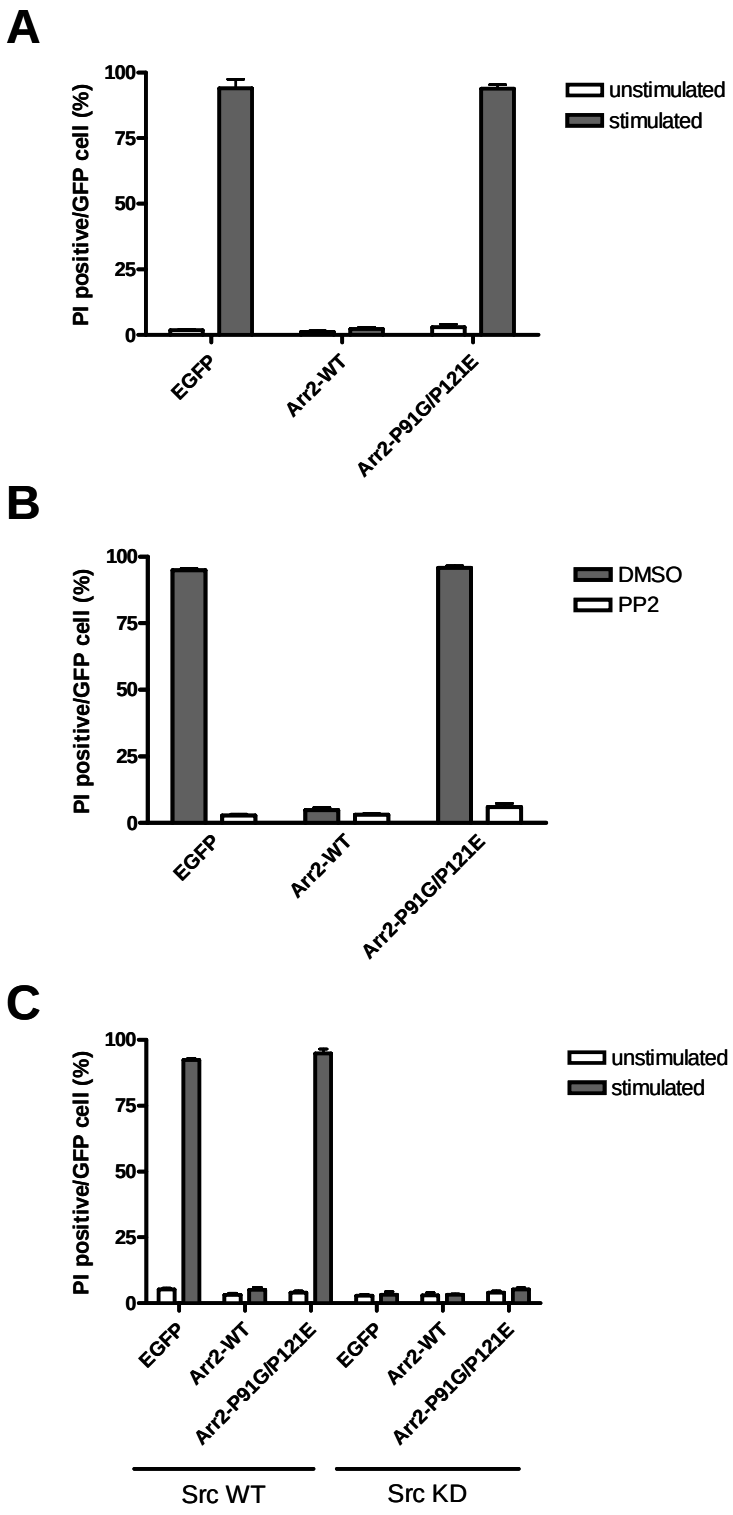


Figure 3.1. Inhibition of Src kinase, but not expression of Arr2-P91G/P121E, rescues FPR-mediated apoptosis.

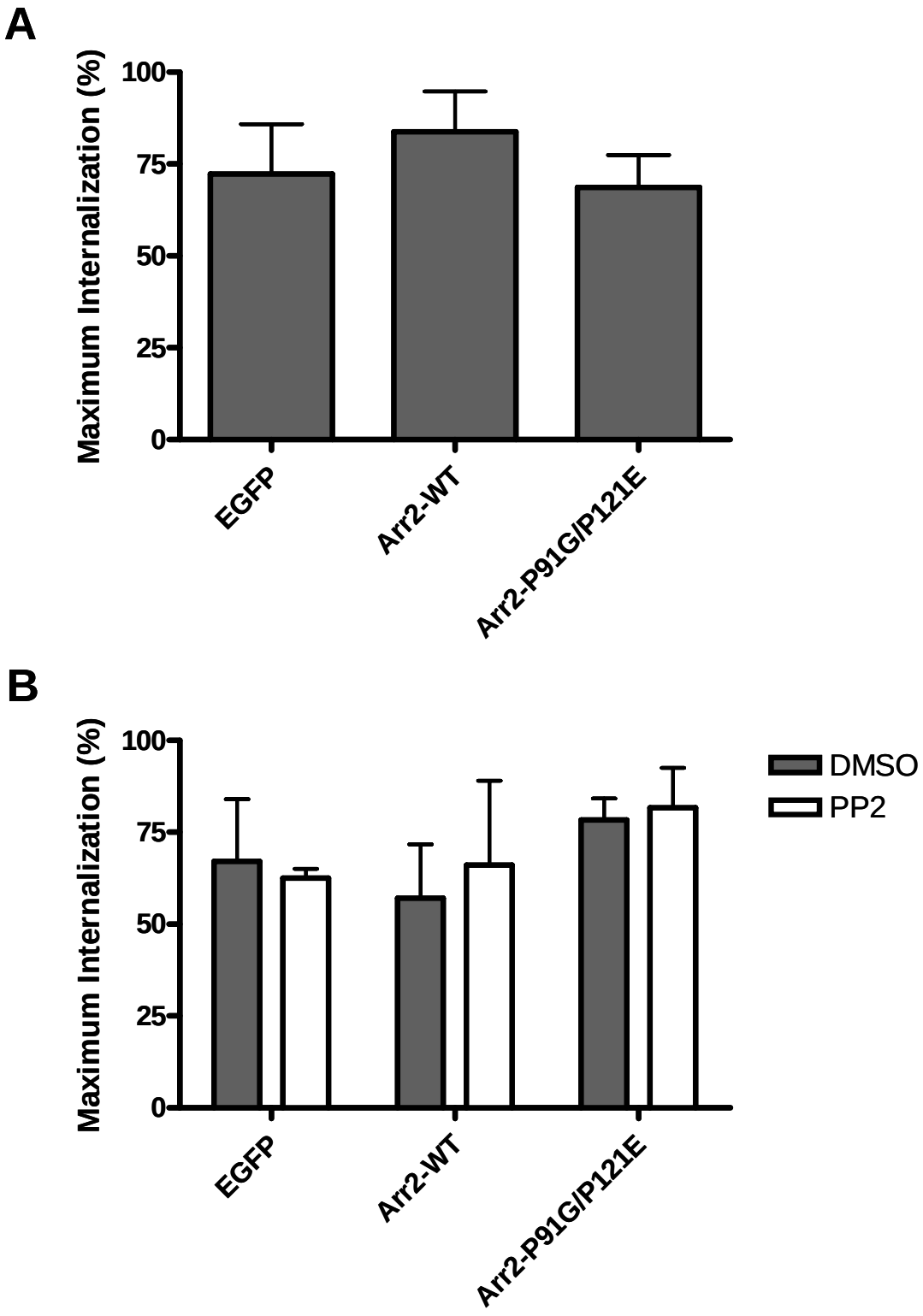


Figure 3.2. FPR internalization is not affected by arrestins or Src inhibition.

A. Control

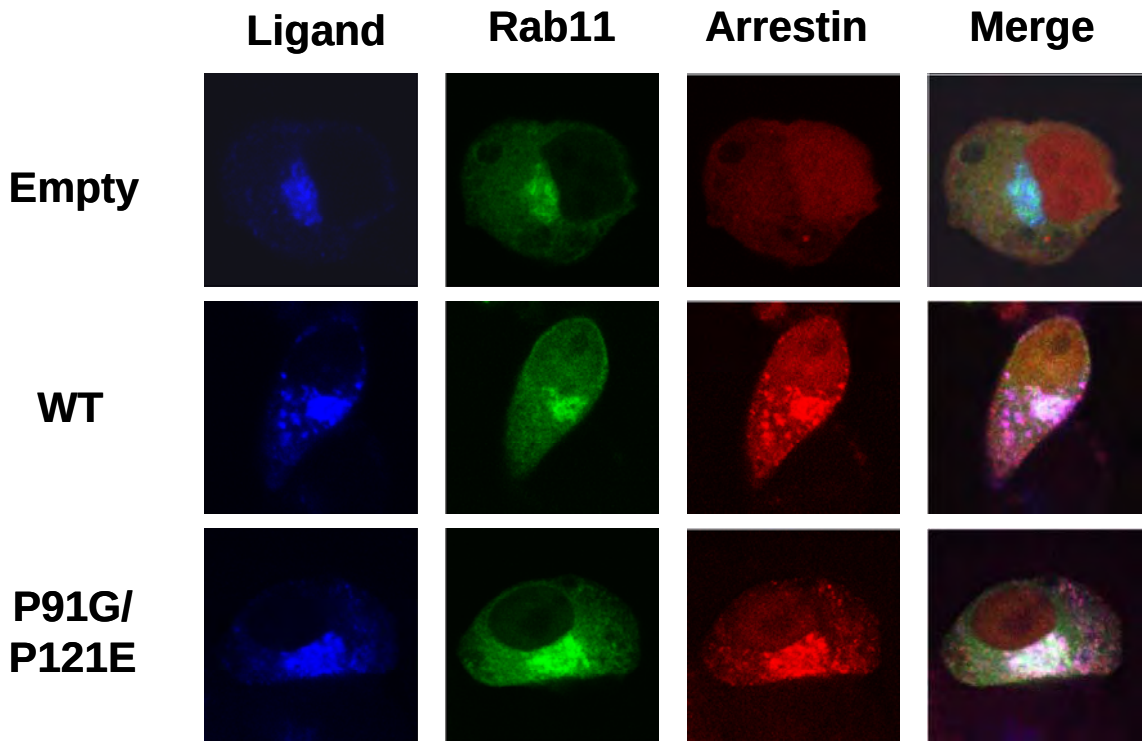


Figure 3.3A. FPR-arrestin complexes traffic differentially with Rab11.

B. PP2

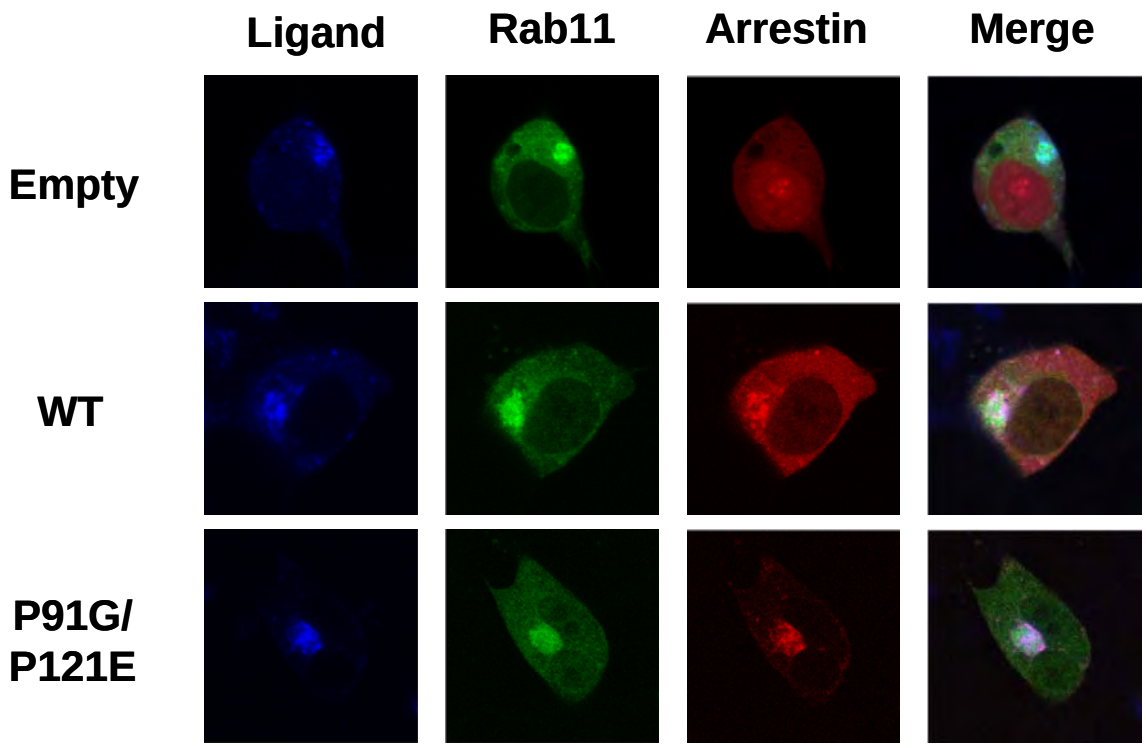


Figure 3.3B. FPR-arrestin complexes traffic differentially with Rab11 in response to Src inhibition.

C. Src KD

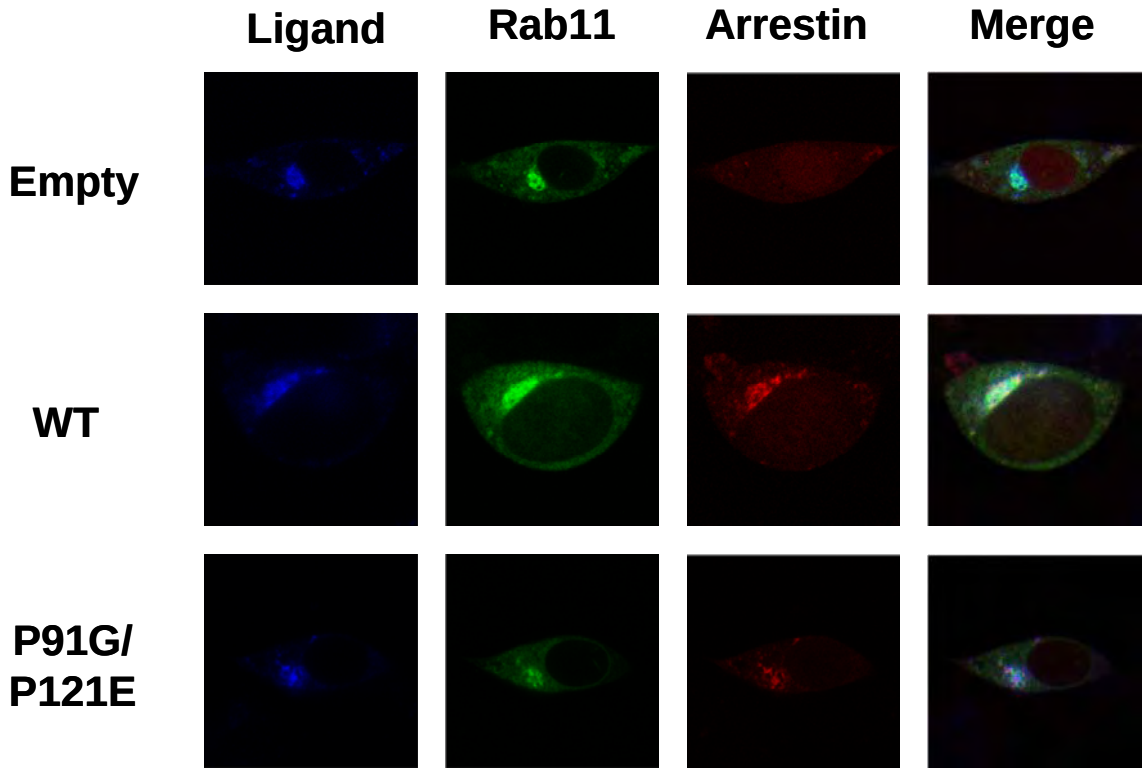


Figure 3.3C. FPR-arrestin complexes traffic differentially with Rab11 in response to Src inhibition.

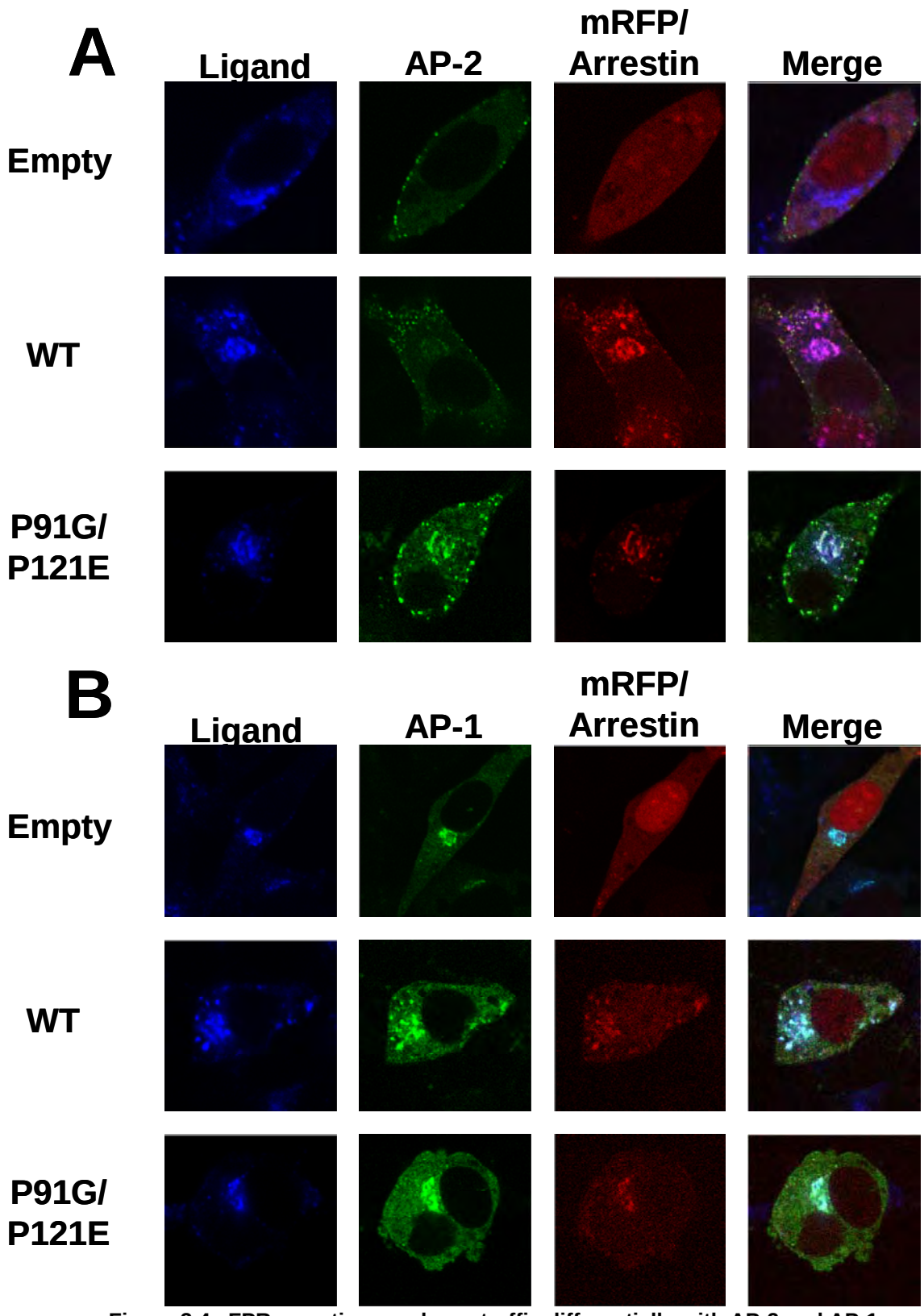


Figure 3.4. FPR-arrestin complexes traffic differentially with AP-2 and AP-1.

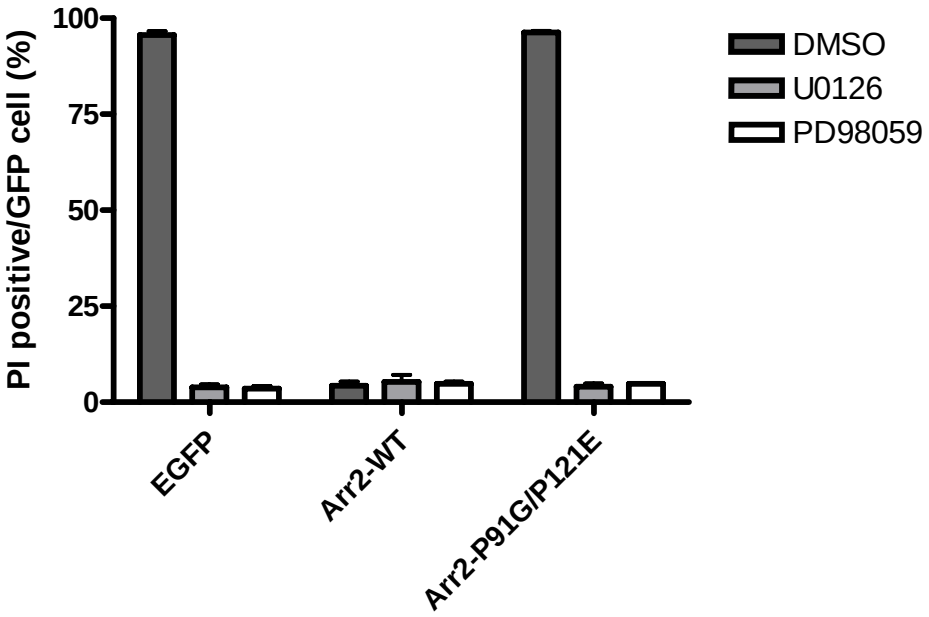


Figure 3.5. FPR-mediated apoptosis in the absence of arrestins or presence of P91G/P121E is sensitive to ERK inhibition.

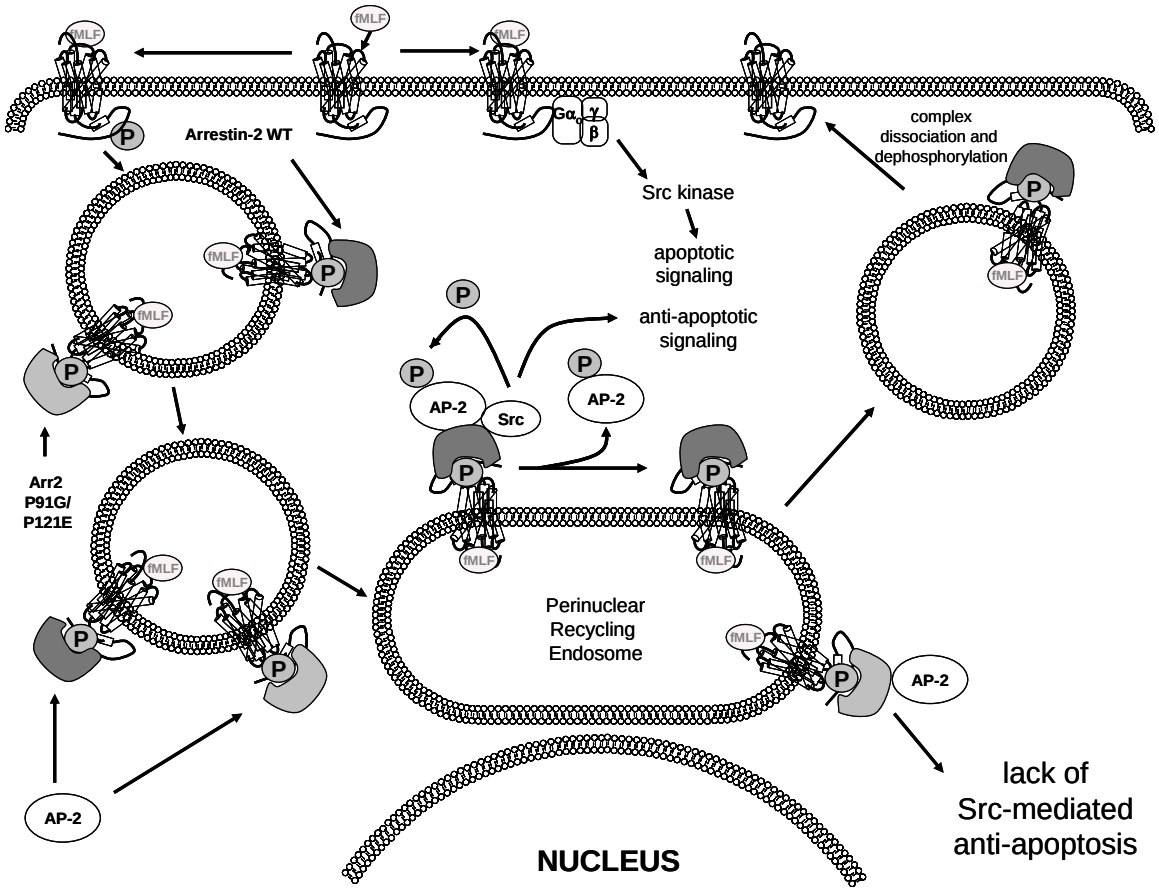


Figure 3.6. Role of Src kinase in FPR trafficking and signaling.

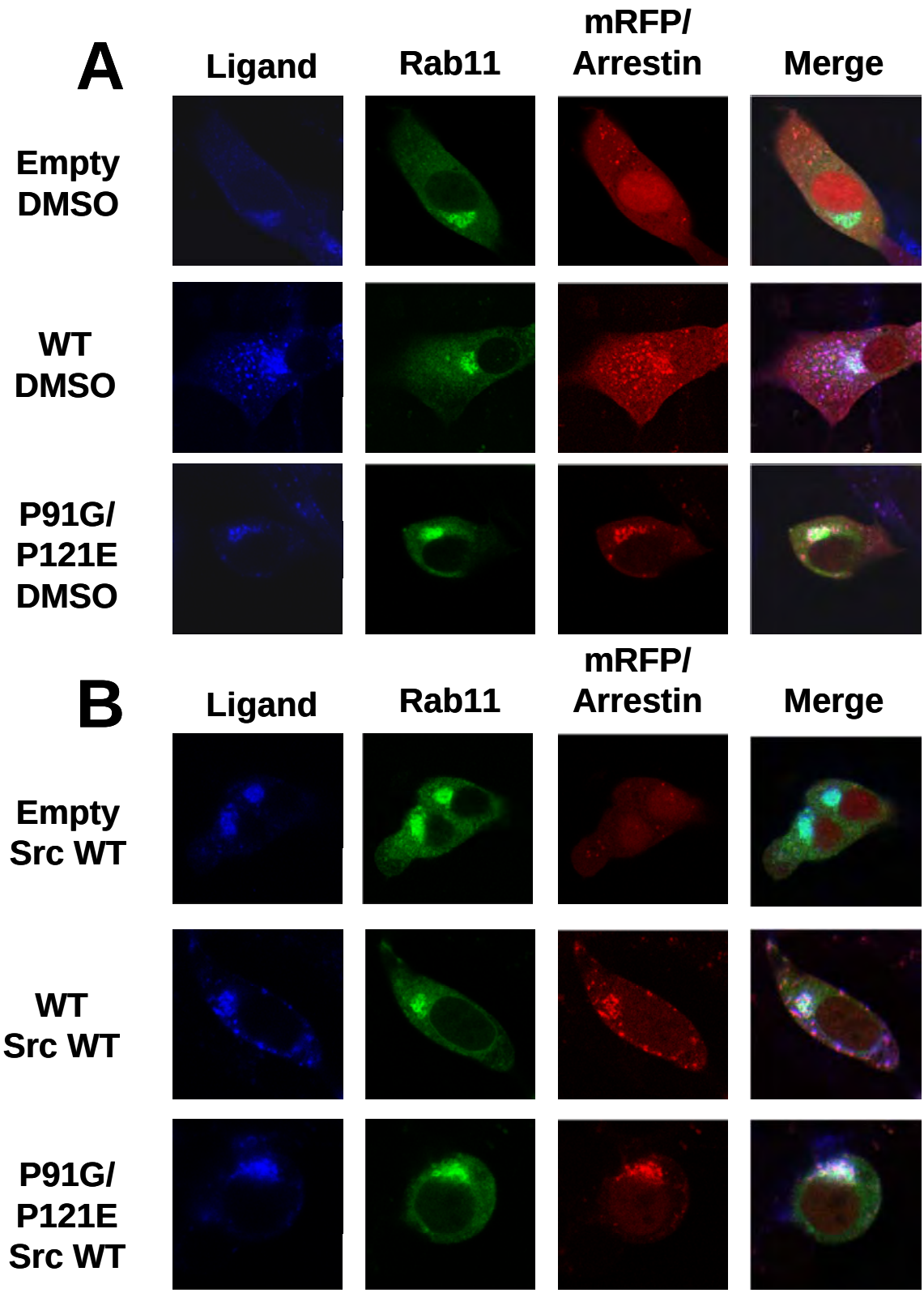


Figure 3.7. Vehicle and Src wild type do not alter FPR-arrestin trafficking phenotypes.

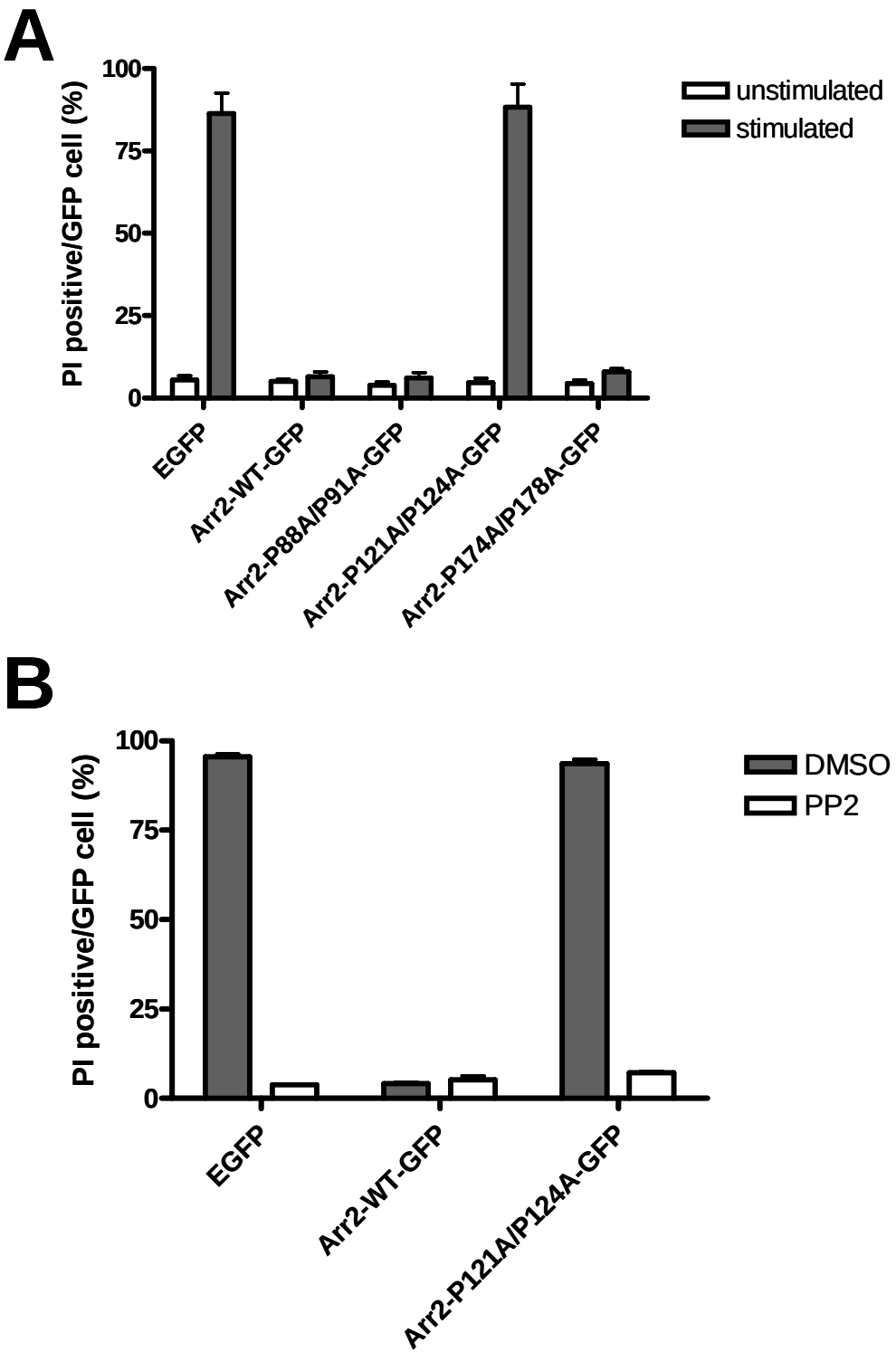


Figure 4.1. Rescue of FPR-mediated apoptosis by arrestin-2 SH3-binding mutants.

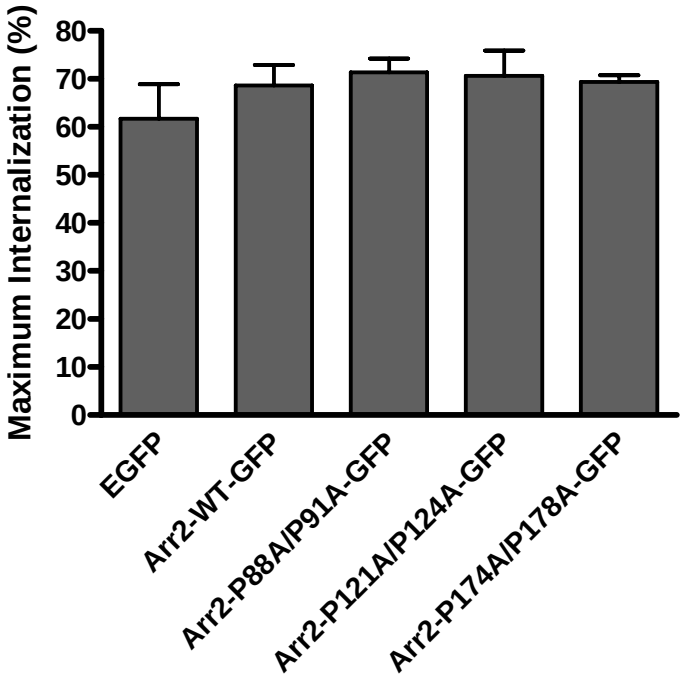


Figure 4.2. Internalization of the FPR in presence of arrestin-2 SH3-binding mutants.

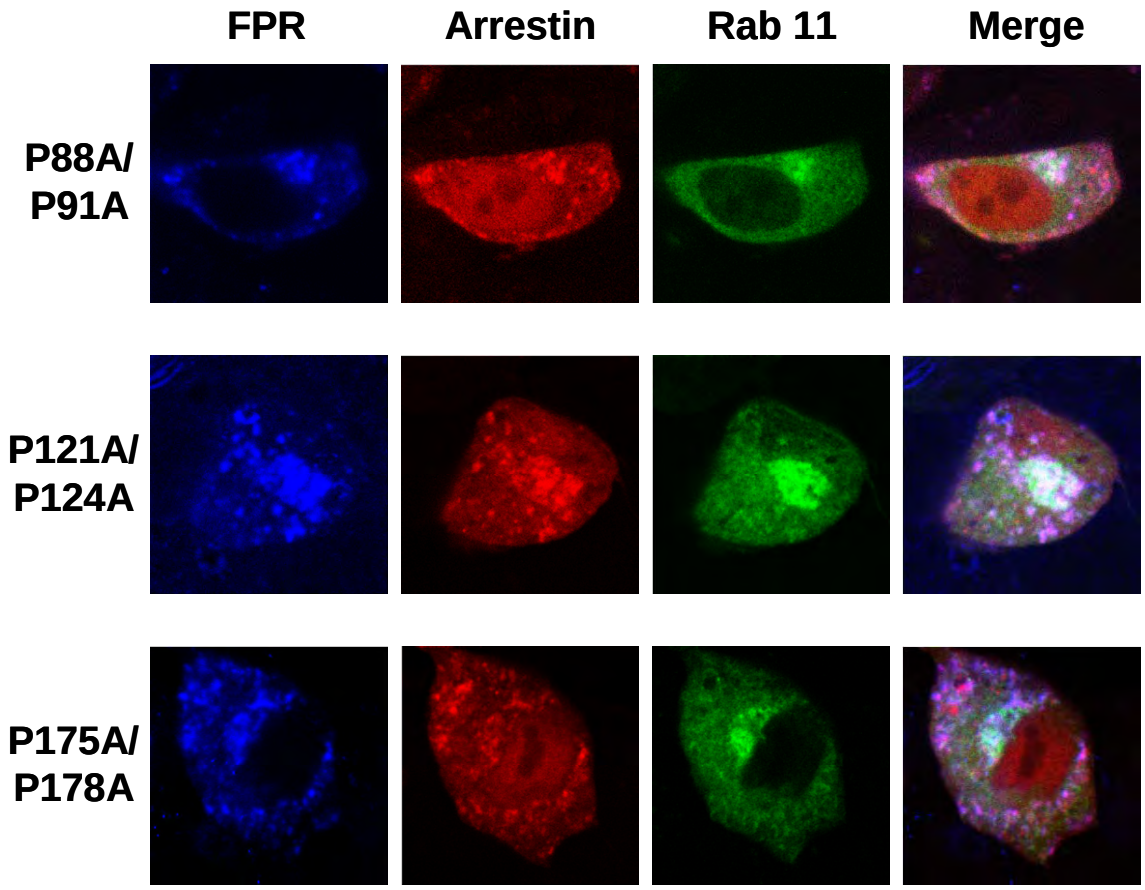


Figure 4.3. Arrestin-2 SH3-binding mutants traffic normally with respect to Rab11.

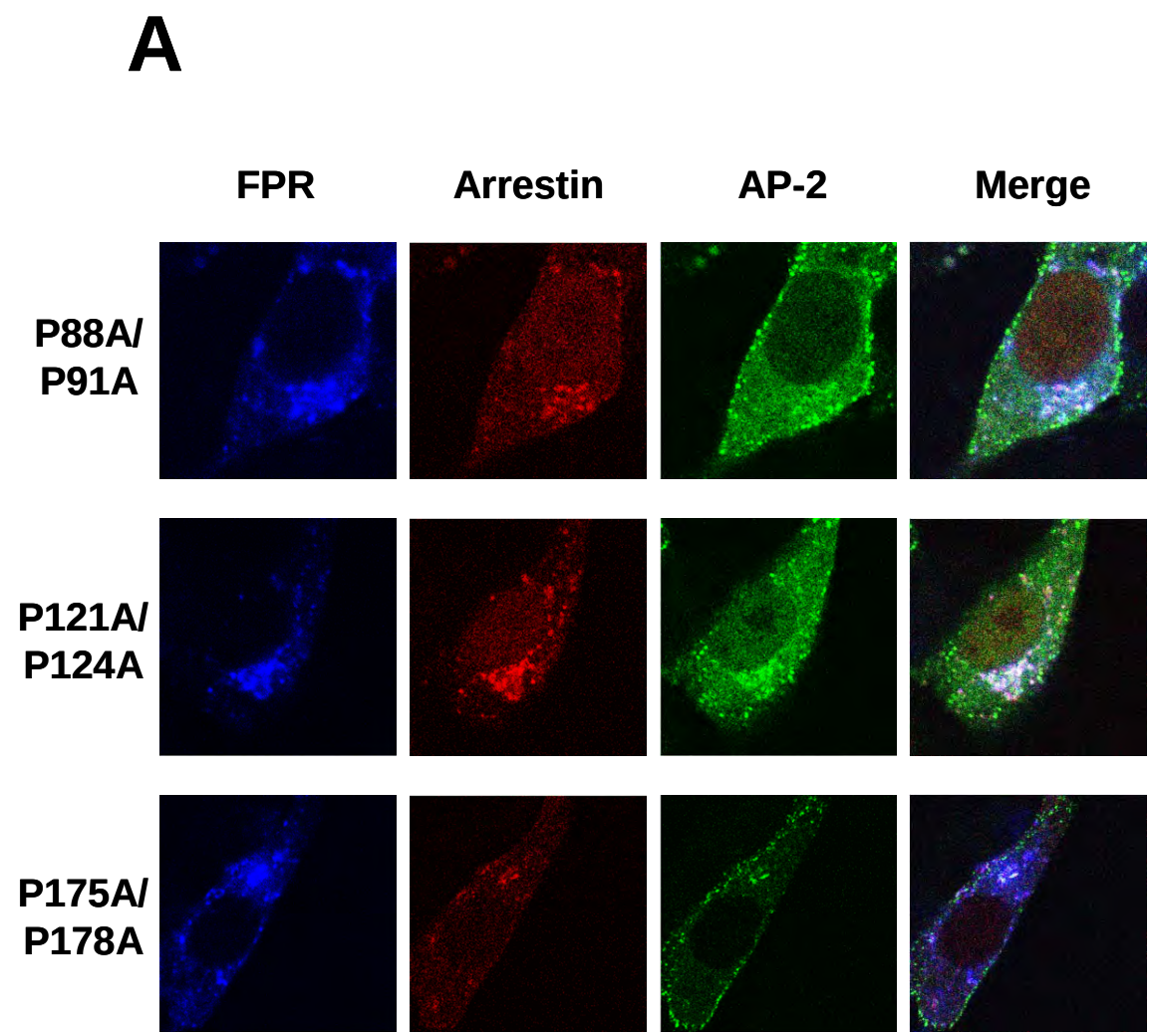


Figure 4.4A. Arrestin-2 SH3-binding mutants traffic normally with AP-2.

B

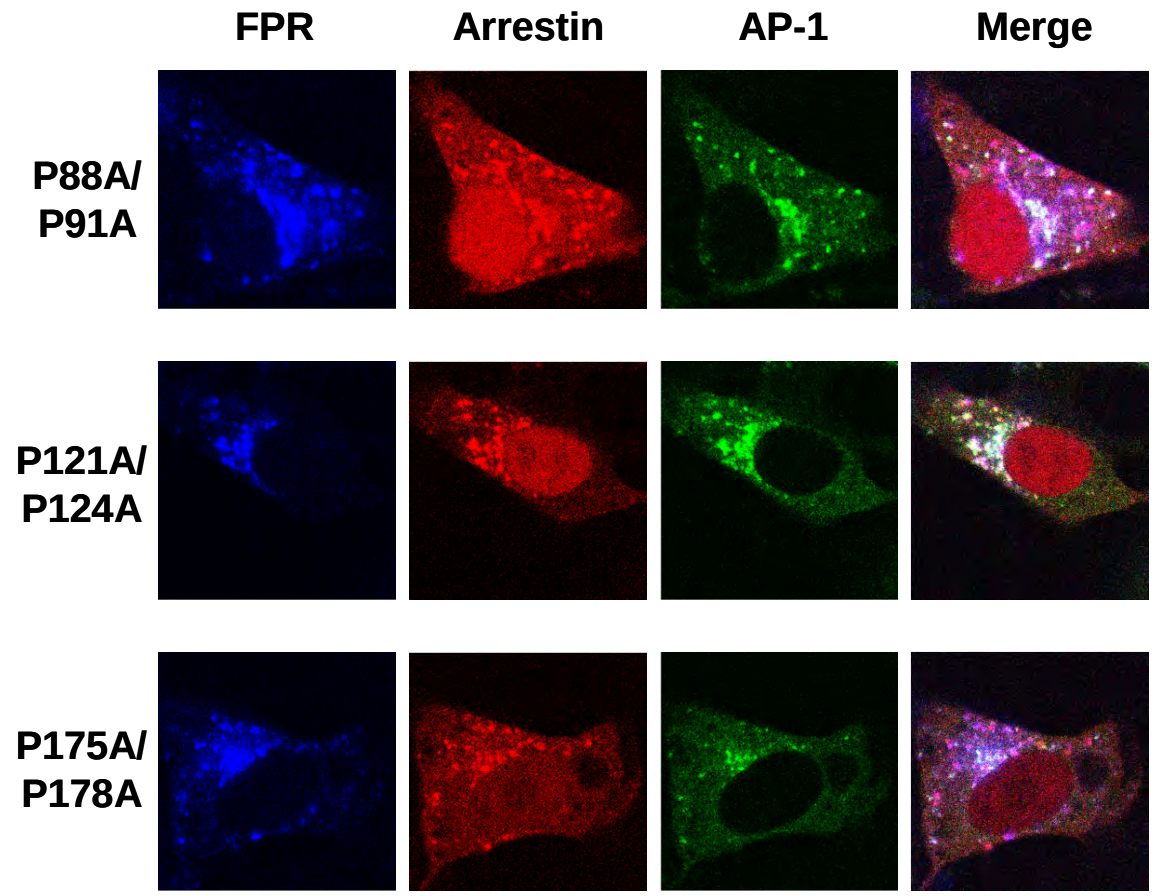


Figure 4.4B. Arrestin-2 SH3-binding mutants traffic normally with AP-1.

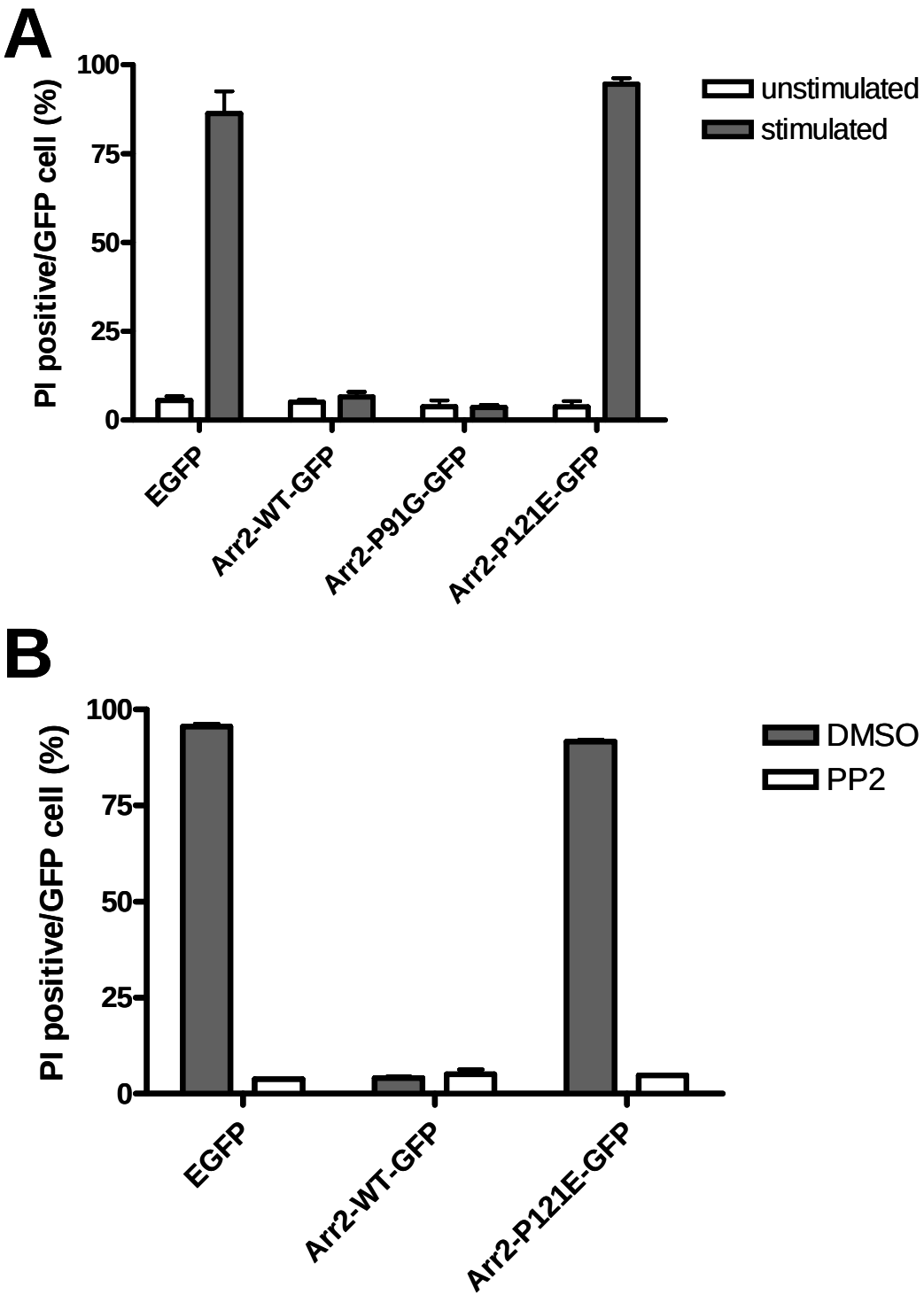


Figure 4.5. Rescue of FPR-mediated apoptosis by arrestin-2 SH3-binding point mutants.

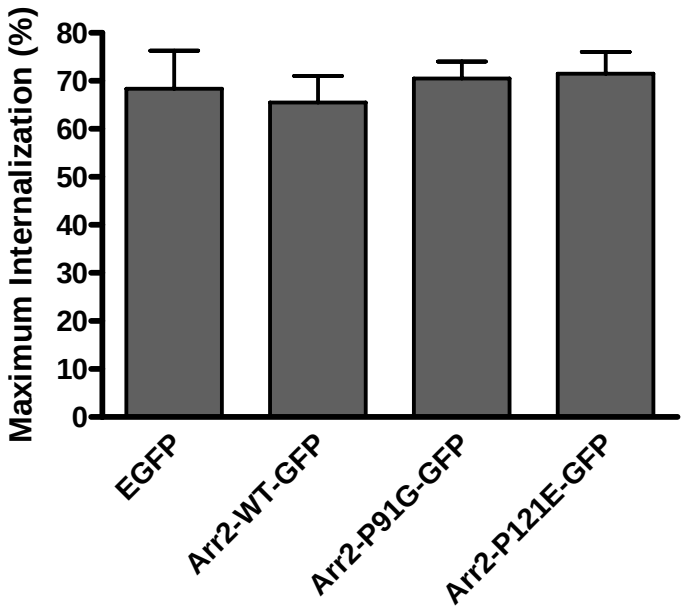


Figure 4.6. Internalization of the FPR in presence of arrestin-2 SH3-binding point mutants.

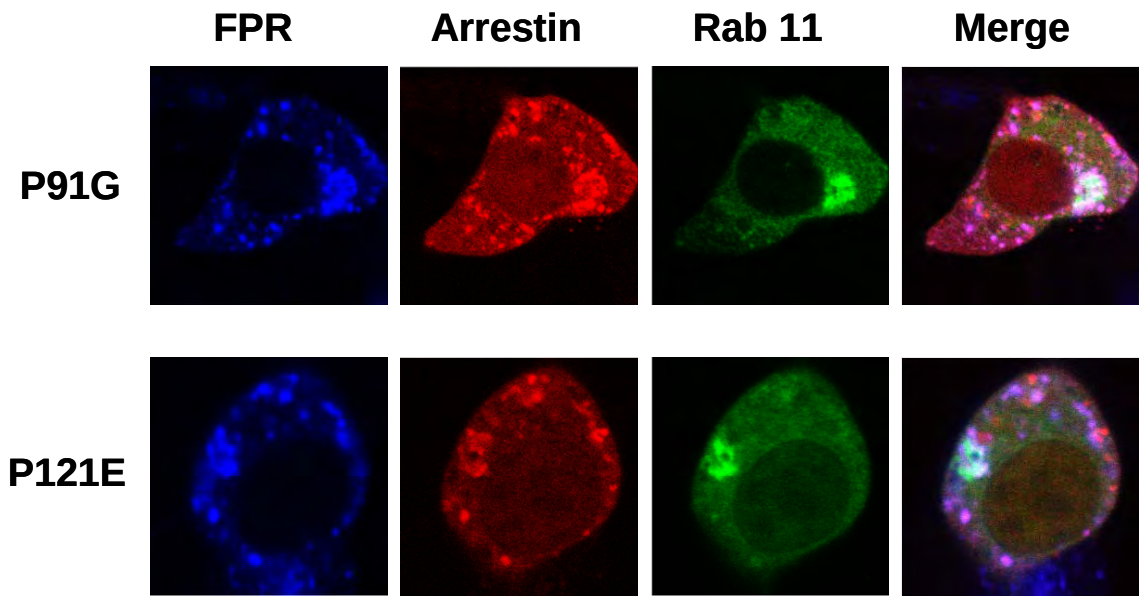


Figure 4.7. Arrestin-2 SH3-binding point mutants traffic normally with respect to Rab11.

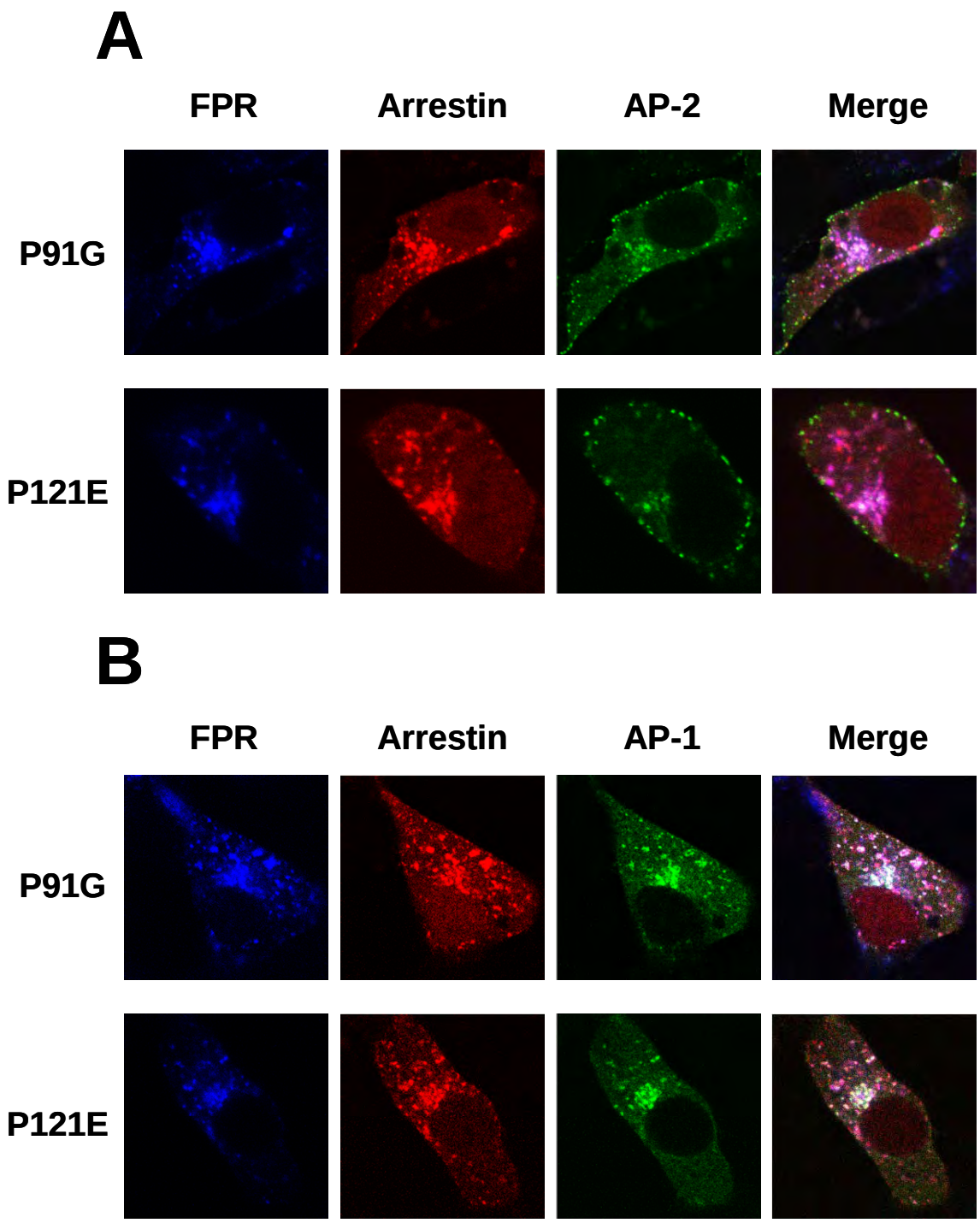


Figure 4.8. Arrestin-2 SH3-binding point mutants traffic normally with AP-2 and AP-1.

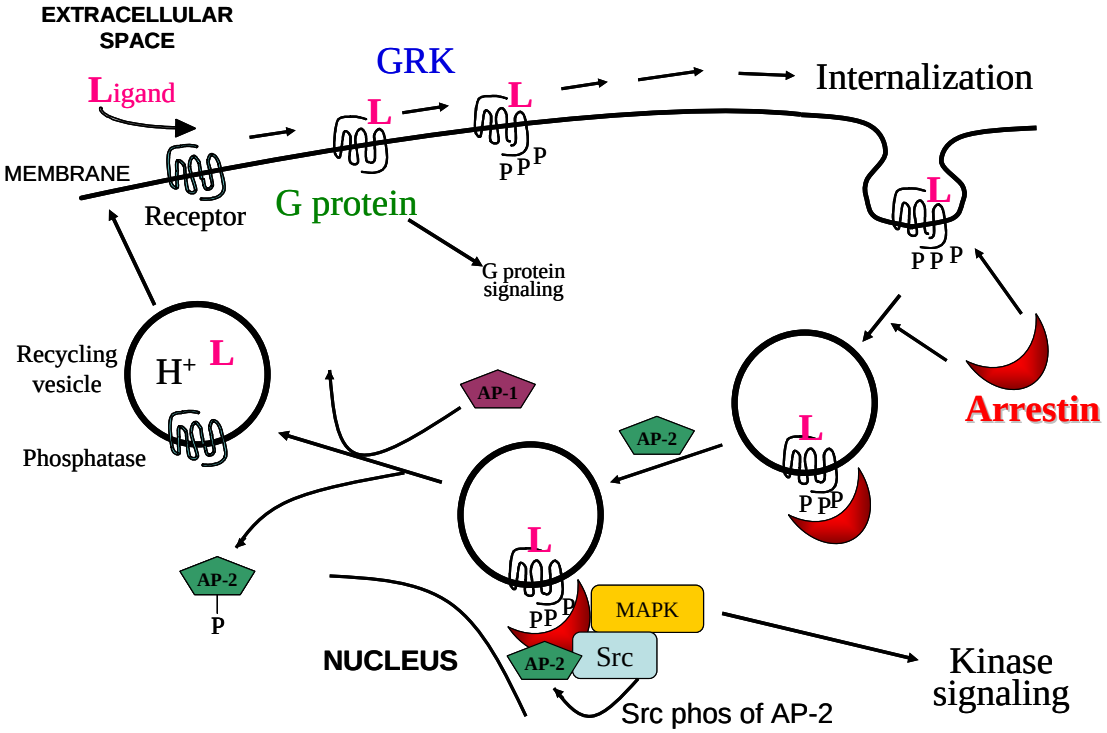


Figure 5.1. New model of FPR trafficking and signaling.