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Protein Interactions Mediating Endocytic Recycling of G Protein-Coupled Receptors

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Protein Interactions Mediating Endocytic Recycling of G Protein-Coupled Receptors

by

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DISSERTATION

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B. Lauffer provided the experimentation and manuscript preparation of the greater majority of the following dissertation, which constitutes a substantial and comparable contribution to that of other dissertations in the greater Pharmacology and Cell Biology fields. Collaborative contributions from others are specified on the title pages of the relevant chapters.

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Abstract

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G protein-coupled receptors (GPCRs) comprise the largest family of transmembrane signaling receptors known and account for roughly half of all pharmaceutical targets. Receptor regulation is known to influence cellular signaling strength and specificity, but the mechanisms conferring cellular-level regulation are largely unclear. An important process impacting cellular sensitivity is the post-endocytic sorting of receptors between a downregulating lysosomal fate and a resensitizing recycling pathway to the cell surface. GPCR recycling, in particular, is itself a regulated process that requires recognition of cytoplasmic receptor motifs. The first motif recognized as a recycling sequence is a protein interaction ligand found at the C-terminus of the β2 adrenoceptor. This ligand has shown affinity for multiple proteins, several of which have been proposed to mediate recycling of the receptor. Using a combination of mutational, protein engineering, and RNA interference techniques to probe the recycling activity of three candidate interaction types in the HEK293 cell culture model, we have distinguished these interactions as necessary, sufficient, or dispensable with respect to mediating receptor recycling. In particular, we have identified a dichotomy between the sufficiency of the connectivity in the NHERF/ERM/Actin protein complex to promote recycling of engineered receptors

and the necessity of a novel and functionally distinct interaction with Sortin Nexin 27 that is the only interaction detectably required for recycling of the β2 adrenoceptor. In addition, a competing interaction with the N-Ethylmaleimide Sensitive Factor was found to be relatively dispensable in this regard despite showing the capacity for physiologic fine-tuning of receptor trafficking and signaling. These results identify a new function of sorting nexin 27 in promoting PDZ-dependent recycling of an integral membrane protein, expand our basic understanding of trafficking mechanisms that regulate a prototypic signaling receptor, while additionally providing information critical to understanding the physiologic consequences of receptor regulation.

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CHAPTER 1: INTRODUCTION

1.1 Overview

This dissertation compiles three projects that all investigate the role of different protein interactions, between a subset of a vastly important class of signaling receptors, and several other types of cellular protein, all which have been implicated in a process of receptor transport between various membrane structures in the cell. This introduction presents a brief description of the concept of a biological receptor, how molecular receptors were classified, and the utility of studying the intricate details of receptors for medicine and biological engineering. It will then go on to introduce the molecular mechanisms of signal transduction related to the receptors of interest, their regulation, and the molecules and cellular transport events relevant to these processes. The following three chapters are arranged according to the receptor-interacting proteins that were focused on, and all include separately published or yet-to-be published content. Additional, presently-unpublished supplemental data pertaining to the NSF-β2AR relationship (Chapter 2), engineered receptor interactions (Chapter 3), and the SNX27-β2AR relationship (Chapter 4) are included in Appendix I, II, and III, respectively. All of these chapters and appendices will be summarized, synthesized, and extrapolated into models and speculations for future research in Chapter 5.

1.2 An Explosion of Receptors

The use of exogenous substances from outside the body to evoke a non-nutritive, physiological response predates recorded history and even the human species. Not only have organisms blindly evolved chemical warfare (toxins) and chemical symbioses with other organisms, but the selective seeking of certain substances for a specific therapeutic gain, in particular, is utilized by many species and organized into a vast enterprise by humans. The ability for various substances to evoke different physiological responses led the pioneers of modern pharmacology to hunt deeper into body tissue for biological entities responsible for relaying the action of a drug or toxin. This led to the realization that many drugs mimic or block the effects of endogenous substances. It also became clear that the molecular structure of a ligand, when purified or synthesized, determined the physiologic response observed. Thus, the *receptors* for drugs and toxins were inferred to be fairly discrete molecules themselves, before they were physically identified.

Many receptors were purified from tissue and characterized as proteinaceous material in cell membranes (Hubbard 1954; Ehrenpreis 1960; Lefkowitz, Haber et al. 1972; Bidlack and Abood 1980). As molecular biology techniques progressed, so did the identification of genes that encoded the receptor proteins and new receptors were identified by homology or functional expression (Patrick, Ballivet et al. 1983; Dixon, Kobilka et al. 1986; Evans, Keith et al. 1992). Pharmacological studies had begun to suggest that multiple receptors could engage similar drugs with variable binding properties, and the advent of receptor cloning confirmed that multiple genes with high

sequence identity would often account for the varied interactions with the same class of drug (Lord, Waterfield et al. 1977; Frielle, Caron et al. 1989; Loh and Smith 1990). Primary and secondary structural analysis also indicated that even distantly-related receptors with different ligand preference could also share certain topological features; the fastest growing group of receptors all spanning the membrane seven times, resembling the molecular receptor for light (rhodopsin) characterized much earlier and recently confirmed structurally (Henderson and Unwin 1975; Kubo, Fukuda et al. 1986; Kobilka, Dixon et al. 1987; Palczewski, Kumasaka et al. 2000; Cherezov, Rosenbaum et al. 2007; Rasmussen, Choi et al. 2007). These varying levels of sequence identity allowed for cloning of new receptor genes of variable similarities to one another, and the numbers of the 7-transmembrane receptor genes (7TMs), in particular, eventually began multiplying past the point to where receptor ligands were known for these 'orphan receptors' (Marchese, George et al. 1999). The genomic era confirmed that this superfamily, allowing biological perception of diverse ligands as small as a photon and as large as a protein hormone, contained hundreds of genes in the human genome (2001; Venter, Adams et al. 2001).

While the number of potential molecular drug binding sites, and thus receptors, are theoretically astronomical, the 7TM superfamily of gene products account for between a third and a half of the drug target molecules in common use (Kroeze, Sheffler et al. 2003). It is clear these receptors evolved to relay signals from the outside of the cell to the inside, and the hijacking of this natural, biochemical communication system by drugs give them a practical advantage as a drug target. It is hard to get an exact number of functional genes in this superfamily, due to the partial uncertainty in predicting gene and

pseudogene sequences. Much of the uncertainty lies in the major proportion of 7TM receptors that serve as sensors for odorants, for many of the odorant receptor genes found in other mammalian lineages are no longer needed (functional) in humans (Menashe, Aloni et al. 2006). Still, discounting this group, there have been estimates between 241 and 369 7TM receptor genes in humans (the possible odorant receptor genes bring the number to over 800 total) that can produce an even larger array of receptor proteins. Only around 10% of these genes are in use as the major class of drug targets already, and since many endogenous ligands and/or physiologic roles of these receptors remain a mystery, it appears as though the potential for therapeutic intervention is largely untapped (Kroeze, Sheffler et al. 2003; Vassilatis, Hohmann et al. 2003; Lagerstrom and Schioth 2008).

The biological responses invoked through the 7TM receptors are as varied as the ligands. Cellular level processes such as motility, electrical activity, metabolism, and gene expression shifts are all influenced by receptor signaling (Kang, Shi et al. 2005; Mathie 2007; Jin, Xu et al. 2008). Higher-level physiological responses such as changes in blood pressure, development of body patterning, and changes in mood are also a small sampling of the types of processes regulated by 7TM function (Wicking, Smyth et al. 1999; Catapano and Manji 2007; Lyssand, DeFino et al. 2008). Exploitation of these systems pharmacologically has been the driving force for studying 7TMs, but genetic rewiring of the receptor signaling system within the cell is an exciting frontier with applications much broader than, but including medicine. Receptors activated solely by a synthetic ligand (RASSLs) have already been engineered as tools for studying spatiotemporally-controlled signal exposure in living tissue and organisms (Conklin, Hsiao et al. 2008). And signal transduction networks downstream of receptors are

becoming ‘rewirable’ (Bashor, Helman et al. 2008). These types of developments hint at the possibility of not just ‘hijacking’ natural biology for benefit, but engineering it for purposes it never evolved for. Almost needless to say, improved understanding of receptor biology will allow increased control over it, and expand its breadth and efficiency of uses. And though harder to put a value to, the unraveling of mechanisms in nature increasingly force reflection on one’s place in it.

1.3 The Nuts and Bolts of GPCR Signal Generation

The search for molecular 'transducers' of the signals of some of the earliest known hormones like epinephrine/adrenaline and glucagon unveiled not just receptors but the intracellular biochemical cascades that ultimately relayed the receptor signal to the rest of the cell. Early work highlighted the role of phosphorylation and dephosphorylation reactions that could be retained in detergent extracts of liver. Eventually it became clear that both hormone and GTP were required for activating an adenylate cyclase protein enzyme and the production of what would be called a 'second messenger' pool of cyclic, adenosine monophosphate (cAMP) that would impact a wide range of intracellular enzymatic processes (reviewed by (Freissmuth, Casey et al. 1989)). Purification of extract components, and the identification of mutant cell lines defective in the signaling cascade all allowed for the deduction of at least 3 proteins involved in generating cAMP: the receptor, adenylate cyclase, and an intermediary, transducer GTPase. These G proteins, as they came to be known, could modulate receptor affinities for agonists (but not antagonists), and again because of similarities to the visual system and the identification of multiple G proteins, it became clear that these components were used by a wide variety of systems (Freissmuth, Casey et al. 1989). Thus we now know that most 7TM receptors activate G protein-linked signaling cascades by catalyzing the guanine nucleotide exchange of particular G proteins. This became a paradigm of how 7TM receptors relay their signal (though important exceptions exist), which has earned 7TMs the second name of G Protein-Coupled Receptors (GPCRs). There are some 7TMRs (such as Smoothened and Frizzled –related proteins) that display alternate

mechanisms of signaling, or have not been clearly established to activate G proteins physiologically. Conversely, there is increasing evidence that so-called 'classical' 7TMRs (such as adrenergic catecholamine and angiotensin peptide receptors) can signal via additional protein interactions distinct from G proteins. GPCR will be the primary denotation used henceforth however, because this mode of signal transduction is highly relevant to most 7TMRs and to the particular receptors examined in the following studies.

G proteins that are directly activated by GPCRs are found as heterotrimers in the inactive state, which are composed of α, β, and γ subunits. The α form is the site of guanine nucleotide binding. The GTP-bound form of the trimer is the active form, and is typically thought to dissociate from the beta-gamma subcomplex to regulate effectors such as adenylyl cyclase. The beta-gamma subcomplex, when dissociated from that alpha:GTP subunit, can also interact with effectors and play an active role in signaling. Inactivation is catalyzed by an intrinsic GTPase present in the alpha subunit structure and, depending on the G protein and cell type studied, the activity of this intrinsic GTPase can be increased substantially by binding of RGS proteins. Once GTP hydrolysis occurs, the alpha:GDP subunit becomes inactive and is thought to rapidly re-associate with the beta-gamma subcomplex. The critical function played by the GPCR is to promote GDP dissociation from the alpha subunit of the inactive heterotrimer, allowing GTP (which is present in considerable molar excess in the cytoplasm) to bind to the transiently 'empty' alpha subunit and promote its activating conformational change (reviewed well by (Oldham and Hamm 2008)). As alluded to above, all subunits come in variant forms, and receptors tend to have preferences for certain G proteins, which start to explain the variation between responses mediated through different receptors. The α

subunits, in particular, can not only stimulate cAMP formation (Gαs group), but some actually inhibit cyclase activity (Gαi), while a third group (Gαq) uses a separate second messenger of calcium ions. Other less common groups exist as well (Simon, Strathmann et al. 1991). The Gαs and Gαi/o groups have an interesting though unfortunate (for the people afflicted) link to pathology; they are chemically modified by the actions of cholera and pertussis toxins, respectively (Simon, Strathmann et al. 1991). This leads to the constitutive activation of Gαs and the inactivation of Gαi, which can be exploited for experimental purposes.

1.4 Desensitization and Resensitization of GPCRs

Drugs (and endogenous agonists) will often lose their potency or efficacy following continuous or repeated administration. This observation is likely as old as the use of opium and other drugs, and can result from many compensatory biological mechanisms, the simplest probably being the enhanced metabolism (upregulated enzyme) of the drug to inactive forms over time. However at the cellular level, Gs-induced cAMP production will often diminish even in cases where active drug is not depleted (DeRubertis and Craven 1976; Lauzon, Kulshrestha et al. 1976). As with many insights to GPCR biochemistry, the first mechanistic hints came from studies of retinal rhodopsin, which was observed to lose its ability to activate transducin (its cognate trimeric G protein) when phosphorylated (McDowell and Kuhn 1977). The regulatory similarities between rhodopsin and other receptors were not immediately obvious in this regard (as with the structural similarities discussed above), but once realized they demonstrated the remarkable versatility of similar mechanisms in controlling perception of the extracellular world.

Paradoxically, the activation of GPCRs makes them available for inactivation (for review see (Lefkowitz 2007)). As previously mentioned, phosphorylation of the receptors turned out to be a widespread mechanism of decreasing GPCR responsiveness (Stadel, Nambi et al. 1982). Identification of a relevant kinase for the β2 adrenergic receptor and for rhodopsin again revealed a subfamily of genes all involved in mediating the specific inactivation of previously activated GPCRs (Benovic, DeBlasi et al. 1989; Lorenz, Inglese et al. 1991). These came to be known as the GPCR kinases (GRKs), and

while they are not the only kinases known to phosphorylate receptors, they are unique in their ability to promote 'homologous' desensitization. This pharmacological term refers to specific reduction in responses mediated by the previously activated receptors, without affecting responses mediated by other receptors that were not previously activated (Benovic, Bouvier et al. 1988; Lohse, Lefkowitz et al. 1989). GRKs are thought to produce homologous desensitization by selectively phosphorylating activated receptors. GPCR signaling can also stimulate other kinases that phosphorylate receptors without regard to their state of activation. An early example comes from studies of beta-2 adrenergic receptor-induced cAMP production. This accumulation of cAMP activates the cyclic AMP-dependent protein kinase (protein kinase A or PKA), which can phosphorylate the adrenergic receptor irrespective of whether or not it is bound by ligand. This phosphorylation also inhibits the ability of receptors to interact with G proteins, providing feedback onto many types of activated and inactivate receptors that are PKA substrates. And since any pathway that stimulates cAMP can activate this kinase, this allows for ‘cross-talk’ between separate signaling ligands and receptors, producing what is called heterologous desensitization (Clark, Kunkel et al. 1988; Clark, Friedman et al. 1989).

The enzymatic phosphorylation of receptors helped explain the rapidity of desensitization, though ironically the ability to desensitize reconstituted receptors was weakened as GRK preparations were made more pure. Though looking again to the retinal system, a type of cofactor was suspected. A rod cell-specific protein was implicated in aiding rhodopsin kinase to attenuate light detection, termed visual arrestin (Kuhn and Wilden 1987). Though specific to rod cells, this protein was able to augment

the desensitization of reconstituted adrenergic receptors, albeit at high molar ratios (Benovic, Kuhn et al. 1987). The deduction of other gene products similar to visual arrestin resulted in the identification of more ubiquitous arrestins, notably arrestins 2 and 3 (also called beta-arrestins-1 and –2) with enhanced ability to desensitize β2 adrenergic receptors. These proteins were shown to bind phosphorylated receptors preferentially, and sterically hinder receptor interactions with G proteins (Lefkowitz 2007). They were later appreciated to augment this regulation at the cAMP level by providing a scaffold for a phosphodiesterase that breaks down cAMP (Perry, Baillie et al. 2002).

In many other ways, the scaffolding functions of arrestins have changed our understanding of GPCR signaling and regulation. The most recent surprise has come from the ability of arrestin proteins to physically interact with enzymes utilized traditionally for other signaling pathways, many in the MAP kinase category (Lefkowitz and Shenoy 2005). This indicates that the signal transduced by a receptor has at least two temporally and mechanistically distinct forms that can contribute to the wide range of biological responses influenced by a receptor. And provocatively, different drugs may evoke bias for what balance and form of the two is achieved (Lefkowitz 2007, Shukla 2008, Drake 2008). Of special interest to the following body of work, however, is the role of arrestins in promoting regulated endocytosis of receptors.

It had long been known that β2 adrenergic receptors detected by a hydrophilic radioligand applied to the extracellular milieu would disappear or ‘sequester’ from the cell surface within minutes after activation (Staehelin and Simons 1982). This was verified to occur by regulated endocytosis, and the major vehicles of regulated endocytosis were identified as clathrin-coated pits (Kurz and Perkins 1992; von Zastrow

and Kobilka 1992). Ligand-induced activation of receptors is not required for forming coated pits; instead, endocytosis of β2 adrenergic receptors was found to be regulated by the lateral movement and concentration of receptors into clathrin-coated pits (von Zastrow and Kobilka 1994). It was then found that arrestins 2 and 3 can bind directly to clathrin heavy chain, a major coat protein, via a conserved 'clathrin box' present in the carboxyl-terminal region of the protein that is not conserved in visual arrestin (Goodman, Krupnick et al. 1996). Arrestins can also link indirectly to clathrin via their ability to bind AP-2, a major clathrin-associated 'adaptor' protein (Laporte, Oakley et al. 1999). This has led to the general model that coated pit formation and endocytic scission occur essentially continuously, and that non-visual or beta-arrestins act as regulated adaptor proteins to specifically recruit receptors into coated pits when activated by ligand and phosphorylated by GRKs. It has become clear more recently that there are other regulators of receptor endocytosis (N'Diaye, Hanyaloglu et al. 2008) and that receptors can in fact regulate the dynamics of the coated pit itself (Puthenveedu and von Zastrow 2006). Nevertheless, it still appears that regulated concentration of receptors in coated pits, as deduced before any of the intervening biochemistry was defined, is the major mechanism by which the ligand-dependent entry of β2 adrenergic receptors into the endocytic pathway is regulated.

At first glance it would seem that this reduction in cell surface receptor number would reduce net cellular responsiveness to adrenergic ligands, as catecholamines are impermeable to the plasma membrane. However, internalized receptors can undergo variable processing in endosomes and rapid subsequent trafficking, thereby influencing surface levels of functional receptors either positively or negatively within minutes of

internalization (Morishima , Thompson et al. 1980; Ferguson 2001)). Sequestration of the beta-2 adrenergic receptor is thought to *promote* rapid recovery of adrenergic responsiveness after functional desensitization mediated by receptor phosphorylation. This process resulting in a net increase in functional receptors present in the plasma membrane, and increased adrenergic responsiveness, was called 'resensitization' to distinguish it from functional 'desensitization' of cellular signaling produced by initial phosphorylation of ligand-activated plasma membranes occurring in the plasma membrane (Pippig, Andexinger et al. 1995). The processes of receptor sequestration and dephosphorylation are proposed to be intimately linked, either because the phosphatase that acts on the β2 adrenergic receptor is associated with endosomes or because its activity is increased by exposure of receptors to the low pH of endosomes (Pitcher, Payne et al. 1995). It remains less clear as to how subcellular location influences the receptor and/or arrestin signaling complexes, though clearly there are more arrestin interaction partners than can coexist at any one point in time. The use of receptor transport to allow multiple types of signaling events seems as likely as important as its effect on regulating signaling dynamics over time (von Zastrow and Sorkin 2007).

These intricacies of GPCR signal transduction and regulation indicate a very concerted effort by the cell to mount a precise response, and base this response on a ‘memory’ of previous activity. While many features of this system are widely used, it should be noted that substantial variation exists in the extent to which each of these features, such as phosphorylation, arrestin complexation, and endocytosis, are used by a particular receptor-ligand complex. These differences likely contribute to the ability of different drug-receptor mixes and different subtypes of receptor to mount different

biological responses. Insight into the mechanisms governing regulation variability should aid in revealing the full impact of these processes on the varying levels of biological response, as the identification of mechanistic features allows for more specific experimental pertubation that reduces complexity in its interpretation. Of special interest is the variability in GPCR processing in the endocytic pathway, as these differences influence opposite effects on signaling sensitivity.

1.5 Membrane Trafficking in the Endocytic Pathway and GPCR Cargo

The plasma membrane houses many proteins and lipids that allow the cell to control its interaction with the external world. Not only does this heterogeneous barrier require turnover for upkeep, but this turnover can aid in the secretion and uptake of soluble substances that also keep the entire cell functioning. Thus, membrane is constantly moving to and from the plasma membrane. In most cases in eukaryotic cells, this is achieved by many small membrane vesicles fusing with and pinching off from the plasma membrane through the processes of exocytosis and endocytosis, respectively. Despite the seeming difficulty in maintaining a balance of these processes, membrane traffic to and from the plasma membrane has evolved regulatory components that allow spatio-temporally controlled delivery of certain 'cargo' (Derby and Gleeson 2007).

While membrane transport events such as synaptic transmission involve the use of vesicles distinct from the constitutive, 'housekeeping' vesicle transport, we have seen that regulated endocytosis of GPCRs simply uses arrestins to tap into already existing endocytic machinery. Clatherin-coated pits represent one of several mechanisms by which plasma membrane is internalized, and there is a complex biochemistry involved in 'maturation' and subsequent dynamin-dependent endocytic scission that forms nascent endocytic vesicles (Schmid, McNiven et al. 1998). These vesicles come together and acquire new machinery (presumably by fusing with pre-existing endosomes) that identifies them as early endosomes. From here, soluble components are retained and mature with certain membrane components until they encounter lysosomal machinery (by

fusion with vesicles delivering newly formed machinery and pre-existing lysosomes), which further acidifies the compartment and provides enzymes for the breakdown of retained cargo (Gruenberg and Maxfield 1995).

The progression of soluble cargo to lysosomes belies the fate of most membrane. Due to the extension of endosomal membrane tubules with high surface-area-to-volume ratios, soluble cargo can be retained while repeated tubulation can remove most of the membrane entering the organelle. This 'iterative fractionation' of endosomes has been demonstrated to be a highly effective way of sorting 'passively-diffusing' membrane receptors from their soluble cargo, exemplified in studies of the transferrin and low-density lipoprotein nutrient uptake systems (Dunn, McGraw et al. 1989; Gruenberg and Maxfield 1995). Not only does this preferential return of membrane to the cell surface help maintain homeostasis, it helps to 'recycle' many membrane proteins that can be reutilized after they drop off their cargo in the endosomes, as opposed to meeting a lysosomal destruction fate after a single use. Inherent in this model of 'geometric sorting' is the idea that membrane-bound cargo destined for the lysosomes must utilize a retention mechanism to avoid recycling, whereas recycling occurs by 'default'. One mechanism for degrading membrane proteins utilizes a covalently-linked protein tag called ubiquitin that is recognized by specialized endosomal degradation machinery collectively called the ESCRT complexes (endosomal complexes required for transport) (Katzmann, Babst et al. 2001). These complexes are proposed to send membrane cargo to the lysosome by pinching them into separate vesicles in the endosomal lumen that then behave more like soluble cargo (Raiborg, Rusten et al. 2003).

As mentioned in the last section, GPCRs exhibit quite a bit of variability in their post-endocytic sorting. It had long been known that agonist-induced sequestration of some receptors like the β2 adrenergic receptor can be recovered, whereas others such as δ-opioid receptors down-regulate, or disappear from cells during hour time frames of agonist administration (Pollet and Levey 1980; Staehelin and Simons 1982; Law, Hom et al. 1983). Most receptor types will exhibit some level of down-regulation if examined for long enough (Shenoy, McDonald et al. 2001). However, during more acute agonist administration, this difference in post-endocytic sorting is quite striking, and it can be observed to happen after distinct subtypes of receptor are endocytosed into the same endosomes (Tsao and von Zastrow 2000). This suggested that various receptors carry recognition information in their varying sequence that target them to different sorting fates. According to the geometric sorting and ESCRT models, only the degrading receptors should need such recognition. However, GPCRs are not constitutively endocytosed like nutrient receptors; their endocytosis is a response to stimulus. And as just mentioned, many exhibit some level of down-regulation. It is tempting to speculate that the default sorting for GPCRs is actually lysosomal then, as the pheromone receptor in yeast, considered the closest extant receptor to the original GPCR dowregulates quite efficiently (Jenness and Spatrick 1986). Yet clearly, passively getting to the lysosomes is counter-intuitive, and in fact ubiquitin and a protein GASP have been implicated in the receptor-proximal, degradative recognition of certain GPCRs (Whistler, Enquist et al. 2002; Hanyaloglu and von Zastrow 2008). The more surprising GPCR invention is the use of a recycling sequence to apparently gain tighter control over receptor numbers over time, and possibly allow regulation of sorting based on the signaling conditions in the cell

(Yudowski, Puthenveedu et al. 2006; Hanyaloglu and von Zastrow 2008). The list of receptors with distinct recycling sequences is growing, and curiously they point to a large number of distinct, recycling recognition machinery (Hanyaloglu and von Zastrow 2008). Not surprisingly, the β2 adrenergic receptor was the first GPCR to have a recognized recycling sequence, and distinct models of its sorting recognition exists (Cao, Deacon et al. 1999; Cong, Perry et al. 2001). We will examine these models in the next section, before we learn more in the following chapters.

1.6 The β2 Adrenergic Receptor as a Model Recycler

As suspected, the β2 adrenergic receptor recycling sequence was found in the receptor's intracellular, carboxyl terminus. Heterologous expression systems useful for this type of study often employ the use of epitope or other identifier tags attached to one of the protein termini, however this can often alter the properties of the protein. In this case the disruption of the receptor C-terminus with an epitope tag helped identify the existence of a critical recycling sequence, and it was subsequently found that even the addition of a single alanine residue to the receptor's C-terminus was sufficient to disrupt recycling behavior (Cao, Deacon et al. 1999). The C-termini of many integral membrane proteins bind to PDZ domains, the largest family of modular protein-interaction domains in mammals, so-named for the first proteins identified to contain this module (Post Synaptic Density-95 kDa protein, Discs Large, Zona Occludens-1) (Kennedy 1995). Further the C-terminus of the β2 adrenergic receptor contains a consensus PDZ domain-interacting sequence and was shown previously to bind PDZ proteins (Hall, Premont et al. 1998).

Affinity chromatography using a β2 adrenergic receptor-derived tail sequence identified a protein in a bovine kidney extract that reacted with an antibody raised against a rabbit-derived protein called NHERF (Na+/H+ Exchange Regulatory Factors), so-named previously for its ability to confer cAMP-dependent regulation on the NHE3 sodium-proton exchanger (Weinman, Steplock et al. 1993; Weinman, Steplock et al. 1995). An independent search for proteins that interact with the actin-binding protein ezrin identified EBP50 (for Ezrin-binding phosphoprotein of 50 kDa) in human placenta

and bovine brain. Sequence analysis revealed two PDZ domains in EBP50, and both of these domains were found to be present also in rabbit NHERF (Reczek, Berryman et al. 1997)). NHERF/EBP50 proved to be the first of several proteins that are now typically dubbed the NHERFs (Na+/H+ Exchange Regulatory Factors) (Donowitz, Cha et al. 2005; Thelin, Hodson et al. 2005). This family arises from 4 genes, each of which is also known by other names derived from independent study of their biochemical functions. Two members of this PDZ protein family, NHERF1 (the initially identified NHERF/EBP50 protein) and NHERF2 (a close homologue also called E3KARP), share the ability to bind to ERM proteins, revealing a protein network connecting the actin cytoskeleton to proteins embedded in the plasma membrane (see Chapter 2, Fig, 1A for a schematic representation) (Reczek, Berryman et al. 1997). And since ezrin has co-expressed paralogs (ezrin, radixin, and moesin making up the ERM family of actin-binding proteins), and NHERF2 (E3KARP) has similar biochemistry to EBP50, this redundant network has the capacity to be quite robust (Bretscher, Edwards et al. 2002). However, clearly interference with the PDZ interaction identified a bottleneck at which recycling was disrupted. And since the NHERF network was ultimately linked to the actin cytoskeleton, this implicated the cytoskeleton as another such bottleneck that was not known to be linked to endosomal sorting. Analysis of β2 adrenergic receptor recycling during cytoskeletal disruption with the actin toxin Latrunculin B suggested the hypothesis that a NHERF-ERM-actin network is involved in recycling of the β2 adrenergic receptor (Cao, Deacon et al. 1999).

Despite the plausibility of the NHERF model for β2 adrenergic receptor recycling, the identification of a competing protein interaction formed another. In a yeast two-

hybrid screen, the β2 adrenergic receptor 'tail' was found to interact with a protein otherwise known to facilitate the fusion of membrane vesicles. This ATP-dependent fusion protein, called NSF (N-ethylmaleimide Sensitive Factor), bound to the PDZ ligand region of the distal β2 tail despite not containing a recognizable PDZ domain (Cong, Perry et al. 2001). Indeed, this study demonstrated that the NSF and NHERF interactions were competitive for the β2 tail, though single amino acid substitutions could be made to preferentially disrupt one interaction over the other (Hall, Ostedgaard et al. 1998; Cong, Perry et al. 2001). While this was used to argue for an NSF-mediated recycling mechanism, a later study found multiple PDZ-interacting sequences to be sufficient in mediating recycling of the non-recycling δ-opioid receptor when fused to the receptor C-terminus (Gage, Matveeva et al. 2004). This receptor-fusion approach had been used previously to establish the sufficiency of the C-terminal 4 amino acids of the β2 adrenergic receptor, a DSLL sequence, to override lysosomal sorting of the δ-opioid receptor (Gage, Kim et al. 2001). The follow-up study using this approach cast doubt on the necessity of the NSF-interaction in mediating recycling, as a non-NSF-binding, DSAL, variant sequence was similarly efficient at driving recycling of the δ-opioid receptor (Gage, Matveeva et al. 2004). This particular variant sequence made for an intriguing argument, because several rodent species possessed a natural variation at this position in their β2 adrenergic receptor sequences (to produce a DSPL C-terminus). This sequence was demonstrated as being required for recycling in mouse cardiomyocytes, in addition to producing an interesting signaling response that will be introduced in Chapter 2 (Xiang and Kobilka 2003). The ability of the rodent PDZ ligand to engage NSF will also be discussed there and in Appendix I.

Because all these models were based on the ability of various mutant receptors to recycle after ligand-induced endocytosis, the interacting proteins that actually mediate β2 adrenergic receptor recycling were not identified. Thus, precise understanding of the recycling recognition machinery was left unclear. Since then, a number of other PDZ ligands in GPCRs have been shown to be necessary for receptor recycling, and the potential significance of the NSF interaction with receptors has remained unclear. And even in the growing subset of PDZ-mediated recycling sequences, little overlap for the same PDZ proteins have been suspected (Hanyaloglu and von Zastrow 2008). However, many PDZ proteins are linked to actin-dense structures, which may present a transferable feature of the NHERF recycling model (Bretscher, Chambers et al. 2000; Kim and Sheng 2004; Mueller and Strange 2004). Addressing these major gaps in our understanding is a major goal of the present work. It is hoped that progress made toward improved mechanistic understanding of β2 adrenergic receptor recycling will provide lessons with broad pharmacological and cell biological significance, and possibly open new doors for understanding fundamental membrane trafficking events and signal transduction mechanisms alike.

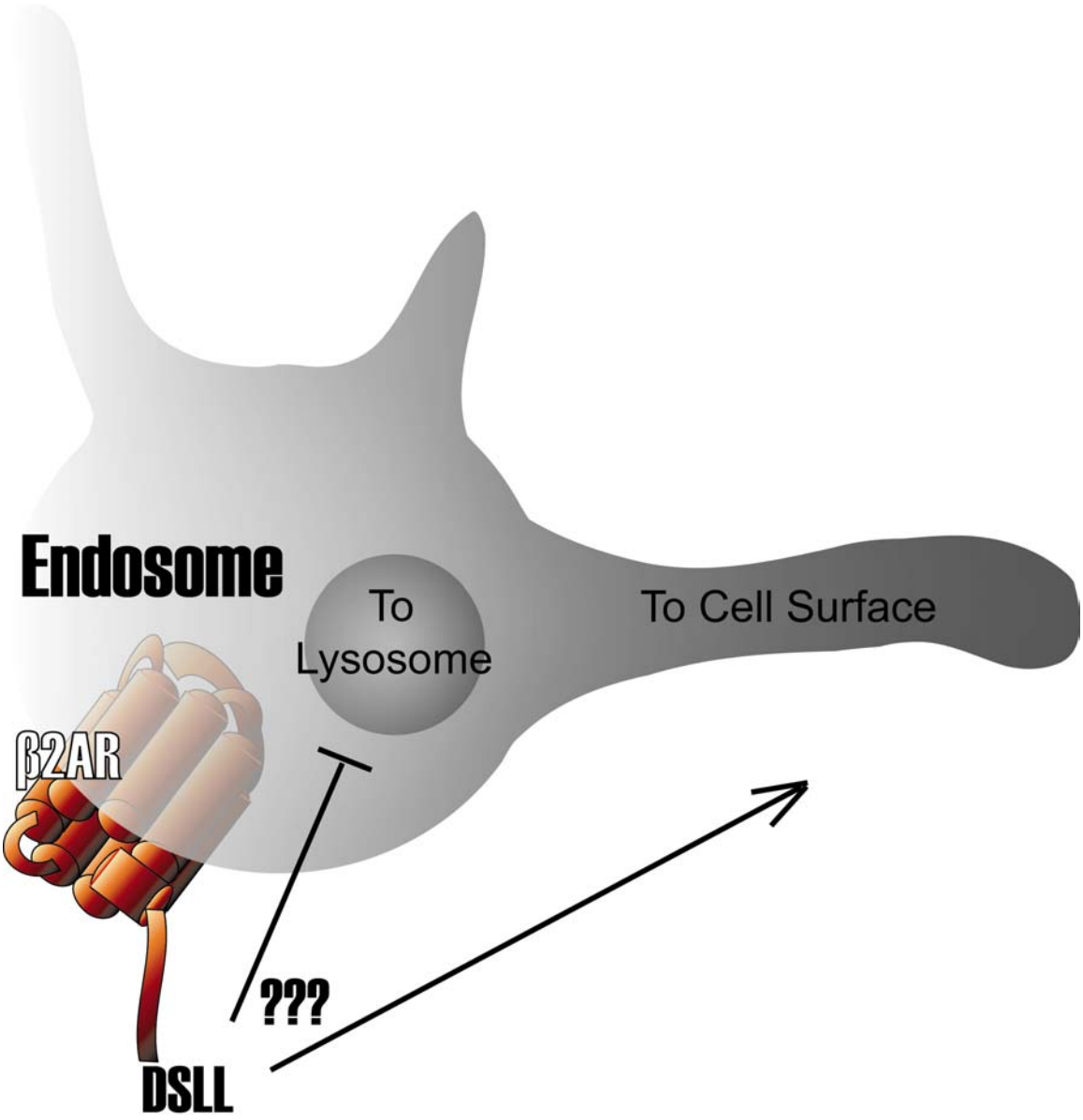

Figure 1. Unknown Features of β2AR recycling.

The PDZ ligand at the β2AR C-terminus is required for recycling of receptors to the cell surface, but the relevant interacting proteins and their connection to endosomal sorting into recycling tubules vs. intralumenal endosome vesicles have not been determined.

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CHAPTER 2:

N-Ethylmaleimide-Sensitive Factor Regulates β2 Adrenoceptor Trafficking And Signaling In Cardiomyocytes

B.E.L. Lauffer contributed Figure 1 and assisted with Figures 2 and 3 in addition to providing earlier drafts of the following chapter, for which omitted figures are provided in Appendix I. The remaining work was contributed by Yang Xiang in the laboratory of Brian Kobilka (Department of Molecular and Cellular Physiology, Stanford Medical Center, Palo Alto, California) and subsequently Yongyu Wang in the laboratory of Yang Xiang (Department of Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, Urbana, Illinois)

2.1 Summary

Recycling of G protein-coupled receptors determines the functional resensitization of receptors and is implicated in switching β_2 adrenoceptor (β_2AR) G protein specificity in cardiomyocytes. The human β_2AR carboxyl end binds to the *N*-ethylmaleimide-sensitive factor (NSF), an ATPase integral to membrane trafficking machinery. It is interesting that the human β_2AR (hβ_2AR) carboxyl end pulled down NSF from mouse heart lysates, whereas the murine one did not. Despite this difference, both β_2ARs exhibited substantial agonist-induced internalization, recycling, and G_i coupling in cardiomyocytes. The hβ_2AR, however, displayed faster rates of agonist-induced internalization and recycling compared with the murine β_2AR (mβ_2AR) and a more profound G_i component in its contraction response. Replacing the mβ_2AR proline (-1) with a leucine generated a gain-of-function mutation, mβ_2AR-P417L, with a rescued ability to bind NSF, faster internalization and recycling than the mβ_2AR, and a significant enhancement in G_i signaling, which mimics the hβ_2AR. Selective disruption of the mβ_2AR-P417L binding to NSF inhibited the receptor coupling to G_i. Mean-while, inhibiting NSF with *N*-ethylmaleimide blocked the mβ_2AR recycling after agonist-induced endocytosis. Expressing the NSF-E329Q mutant lacking ATPase activity inhibited the mβ_2AR coupling to G_i in cardiomyocytes. Our results revealed a dual regulation on hβ_2AR trafficking and signaling by NSF through direct binding to cargo receptor and its ATPase activity and uncovered an unprecedented role for the receptor binding to NSF in regulating G protein specificity that has diverged between mouse and human β_2ARs.

2.2 Introduction

β-Adrenoceptors play a pivotal role in regulating cardiomyocyte contraction through distinct signaling pathways. The β_1AR couples to G_s protein(s), which increases cAMP/protein kinase A activity and the contraction rate, whereas the activated β_2AR sequentially couples to both G_s and G_i in neonatal cardiomyocytes, creating a biphasic change in contraction. β_2AR G_i coupling seems to be dependent on receptor trafficking, which includes both endocytosis and recycling. Inhibiting either process blocks receptor coupling to G_i in cardiomyocytes (Xiang et al., 2002; Xiang and Kobilka, 2003).

Many G protein-coupled receptors (GPCRs) undergo endocytosis in response to activation, yet their subsequent sorting in endosomes is variable, creating variable regulation of their activity during prolonged or repeated stimulation. Some receptors are targeted to lysosomes to down-regulate cellular responses mediated by the receptor, whereas many GPCRs possess the ability to efficiently return to the cell surface. This recycling of receptors underlies the resensitization of corresponding cellular responses (von Zastrow, 2003). Many GPCRs depend on sequences residing in their intracellular domains for recycling. A well-defined class of recycling sequences are PSD-95/Discs-large/ZO-1 (PDZ) domain binding motifs (also called PDZ ligands) that are usually located at the carboxyl-terminal end of different GPCR tails (Bockaert et al., 2004; Gage et al., 2005). The β_2AR has a type I PDZ ligand at its carboxyl-terminal end that is necessary for recycling and sufficient to reroute the δ-opioid receptor from a degradative to a recycling pathway (Cao et al., 1999; Gage et al., 2001). In cultured neonatal mouse cardiomyocytes, this sequence is also required for the temporal switch from G_s to G_i-

mediated signal transduction observed in the contraction-rate response to the agonist isoproterenol (Xiang and Kobilka, 2003). Several lines of evidence now indicate that membrane trafficking of this receptor dictates not only cellular resensitization but also signal transduction specificity. Despite progress in understanding the β_2AR recycling process, numerous questions concerning the core mechanism and physiological variations remain.

Although the recycling sequence at the β_2AR C terminus has been shown to bind PDZ domains in NHERF family proteins (NHERF-1/EBP50 and NHERF-2/E3KARP) (Hall et al., 1998; Cao et al., 1999), it also binds at least one protein with no identifiable PDZ domain: the *N*-ethylmaleimide sensitive factor (NSF) (Cong et al., 2001). NSF has been identified as an ATPase that binds SNAP receptor (SNARE) complexes in an ATP-dependent fashion to separate them during ATP hydrolysis; this and a wealth of other evidence has demonstrated its general role in vesicle fusion between various membrane compartments (Morgan and Burgoyne, 2004; Whiteheart and Matveeva, 2004). Moreover, NSF has been shown to bind to β-arrestin, an adaptor protein involved in GPCR desensitization and endocytosis upon agonist stimulation. β-Arrestin preferentially interacts with the ATP-bound form of NSF, and this NSF binding facilitates clathrin coat-mediated GPCR internalization (McDonald et al., 1999). In heterologous HEK293 cells, selective ablation of NSF binding to the β_2AR was inferred to inhibit recycling of receptors, whereas imparting NSF binding on the δ-opioid receptor slightly enhanced its ability to recycle (Cong et al., 2001; Gage et al., 2005). Although there is also evidence to show that PDZ interactions promote receptor recycling (Cao et al., 1999) and are

functionally important for G_i coupling in cardiomyocytes (Xiang and Kobilka, 2003), it is not clear how NSF may affect β_2AR trafficking and signaling in these cells.

Here, we used neonatal mouse cardiomyocytes as a model system to address these questions. It is interesting that the NSF binding sites on the β_2AR were not conserved among mammalian species, providing a naturally occurring divergence in NSF binding to exploit. The -1 position of the β_2AR carboxyl terminus is proline in mβ_2AR and leucine in hβ_2AR. Because of this single amino acid difference, mβ_2AR binding to NSF was not detectable. Nevertheless, despite the lack of detectable binding of the mβ_2AR carboxyl terminus to NSF in biochemical assays, we found that inhibition of NSF activity with *N*-ethylmaleimide (NEM) inhibited murine β_2AR (mβ_2AR) recycling despite this poor affinity. In addition, both human and murine β_2ARs sufficiently recycled after endocytosis and coupled to G_i pathways in cardiomyocytes. The different affinities for NSF seemed to have a minimum role on receptor trafficking and signaling. In contrast, inactivation of NSF ATPase activity with a point mutation was sufficient to block both human and murine β_2AR recycling and coupling to G_i in cardiomyocytes, indicating that NSF is required for proper trafficking and signaling of β_2ARs in cardiomyocytes independent of a high-affinity interaction with the receptor. This study strengthens the relationship between β_2AR recycling and signaling specificity and demonstrates an unprecedented role for NSF in regulating physiologically relevant signal transduction.

2.3 Materials and Methods

cDNA Constructs and Mutagenesis. Constructs containing the cloned human and murine β_2AR in pcDNA3 (Invitrogen, Carlsbad, CA) with a FLAG epitope attached at the N terminus were used for these studies and have been described before (Cao et al., 1999; Swaminath et al., 2004). Constructs encoding for GST-β_2AR and GST-β_2AR-alanine proteins (encompassing amino acids 328 to 413 of the human β_2AR, and the latter with an additional alanine added to the C terminus) have also been reported (Cao et al., 1999). A comparable murine GST-β_2AR construct was created by insertion of a polymerase chain reaction product of the region encoding amino acids 328 to 418 of the FLAG-mβ_2AR construct using primers containing EcoRI and HindIII appendages and performing the appropriate digestion and ligation into pGEX-KG (Pfizer, New York, NY). The human NSF coding sequence was similarly ligated into pEGFP-N1 (Clontech, Mountain View, CA) after SacI digestion of the vector and a polymerase chain reaction product containing a 3'-SacI appendage and including the 5'-SacI restriction site from the source vector, NSF in pBluescriptR (American Type Culture Collection, Manassas, VA). The P417L mutation was introduced into the FLAG-mβ_2AR and GST-mβ_2AR constructs via the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), as was the E329Q NSF mutation into the pEGFP-N1 construct. Plasmid amplification was done in DH5α *Escherichia coli,* and all sequences were verified by dideoxynucleotide sequencing (University of California San Francisco Biomolecular Resource Center, San Francisco, CA).

Cell Culture and Transfection. Spontaneously beating neonatal cardiomyocytes were prepared from hearts of 1-day-old β_1/β_2AR-KO mouse pups as before (Devic et al., 2001). The myocyte-enriched cells remaining in suspension after preplating were plated in 35-mm dishes for contraction-rate studies and in 12-well plates for immunological assays (with coverslips for immunofluorescent microscopy). Recombinant adenovirus encoding FLAG-mβ_2AR has been described previously (Xiang et al., 2002), and the FLAG-mβ_2AR/P417L, FLAG-hβ_2AR, GFP-NSF, and GFP-NSF-E329Q adenoviral vectors were generated with the same pAdEasy system (Qbiogene Inc., Irvine, CA). Neonatal myocytes were infected with viruses at a multiplicity of infection of 100 after being cultured for 24 h. The receptor expression levels were determined by ligand binding assays as described previously (Xiang et al., 2002). They were expressed at equivalent levels in cardiac myocytes (FLAG-mβ_2AR, 147.3 ± 22 fmol/mg; FLAG-mβ_2AR/P417L, 171.6 ± 9.1 fmol/mg; and FLAG-hβ_2AR, 160.3 ± 21.8 fmol/mg membrane).

GST Pulldown Assays. The various GST-β_2AR fusion proteins were produced in BL21 *E. coli* and bound to glutathione-Sepharose agarose beads (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Beads containing 10 μg of the full-length fusion protein (assessed by densitometry of Coomassie-stained protein resolved by SDS-polyacrylamide gel electrophoresis) were incubated for 4 h at 4°C in 0.5 ml of clarified extracts from frozen mouse hearts with atria removed (Pel-Freez Biologicals, Rogers, AR), prepared to ~10 mg/ml. Beads were washed four times in 1 ml of extract buffer [0.1% (v/v) Triton X-100, 150 mM NaCl, 25 mM KCl, and 10 mM Tris, pH 7.4, complete Roche protease inhibitor cocktail], and protein was eluted in lithium dodecyl sulfate sample buffer (Invitrogen) with dithiothreitol added to 20 mM. Samples were divided in two for SDS-

polyacrylamide gel electrophoresis, transfer to nitrocellulose, and Western blotting using rabbit anti-EBP50 antibodies (courtesy of Dr. Anthony Bretscher, Cornell University, Ithaca, NY) or the mouse 2E5 anti-NSF antibody (courtesy of Dr. Sidney W. Whiteheart, University of Kentucky, Lexington, KY).

Immunofluorescence Microscopy. Myocyte images were obtained using a similar setup on a Zeiss Axioplan 2 microscope (Carl Zeiss Inc., Thornwood, NY). Fluorescent measurements of the myocyte receptor trafficking were made by a ratiometric normalization of fluorescent intensities measured using Metamorph software (Molecular Devices, Sunnyvale, CA). Epitope-tagged receptors were detected using M1 anti-FLAG antibody (Sigma, St. Louis, MO). Selective detection of surface relative to total pools of receptor and its use to estimate receptor recycling have been described previously (Tanowitz and von Zastrow, 2003). The recycling estimates were conducted without the EDTA strip. The primary antibody used in these experiments was M1 conjugated to Alexa Fluor 488 (Invitrogen) using standard procedures as described previously (Tanowitz and von Zastrow, 2003). Secondary staining was performed using a commercial goat antimouse IgG Alexa Fluor 594 conjugate (Invitrogen). Experiments were performed at least in triplicate, and representative results are shown.

Immunofluorescence Spectroscopy. Surface receptor levels were determined as before (Swaminath et al., 2004) in the indicated cell type expressing the indicated FLAG-β_2AR. Media were refreshed 1 h before 10 μM isoproterenol (Sigma) stimulation for 10 or 30 min. Periods of agonist washout after 30-min isoproterenol stimulations were also performed for an additional 30 or 60 min as indicated.

Myocyte Contraction Rate Assay. Measurement of spontaneous contraction rates from myocytes expressing either the endogenous or the indicated FLAG-β_2AR were carried out with and without the use of PTX as described previously (Devic et al., 2001). In some assays, NEM was applied 30 min before the addition of isoproterenol. Tat peptide, Tat-β_2-DSAL consisting of Tat linked to GRQGFSSDSAL of β_2AR, and Tat-β_2-ASLL consisting of Tat linked to GRQGFSSASLL of β_2AR through a cysteine bridge were synthesized in the Stanford Core facility and EZ-Biolab (Indianapolis, IN). Neonatal myocytes were preincubated at 37°C with 10 μM peptide for 25 min before isoproterenol (10 μM; Sigma) exposure.

Statistical Analysis. Curve-fitting and statistical analyses were performed using Prism (GraphPad Software, Inc., San Diego, CA).

2.4 Results

NSF Had Higher Binding Affinity to Human β_2AR than Murine β_2AR. To understand the molecular mechanism of the NSF effect on β_2AR signaling in cardiomyocytes, the interaction between β_2AR and NSF from heart lysate was examined. NSF, a hexameric ATPase involved in membrane fusion, can bind to the carboxyl terminus of the hβ_2AR. The protein-binding region on this receptor involves a four-residue stretch at the distal C terminus of the receptor (Cong et al., 2001). In addition, NHERF-1/EBP50, a cytoskeleton-associated protein, can also bind to the same stretch of residues on the carboxyl terminus of the hβ_2AR. It is interesting that several rodent β_2ARs, including the mβ_2AR, are identical with the hβ_2AR in the carboxyl-binding region except at one residue (leucine -1 of the hβ_2AR, DSLL) that is required for binding to NSF but not to NHERF-1/EBP50 (Cong et al., 2001). Rather, the mβ_2AR has a proline at the -1 position (DSPL). To determine whether both the human and murine β_2ARs have a similar capacity to bind NSF, GST-fusion proteins, including various β_2AR carboxyl-terminal tail sequences, were prepared. Protein binding was evaluated with a pull-down assay using the GST-fusion proteins coupled to glutathione-agarose beads.

GST fusion proteins were incubated with tissue lysate prepared from mouse hearts. NSF only bound to the cytoplasmic tail of the hβ_2AR but not the mβ_2AR (Fig. 1A). In contrast, NHERF-1/EBP50 bound to the cytoplasmic tail of both the hβ_2AR and mβ_2AR under these conditions (Fig. 1B). As a negative control for nonspecific binding, an addition of a single alanine residue to the hβ_2AR carboxyl terminus (GSThβ_2AR-Ala) was tested as well. As reported previously, this mutant failed to exhibit the PDZ domain-mediated and NSF protein binding (Fig. 1, A and B; Cao et al., 1999). In addition, we

attempted to "rescue" an NSF interaction with the mβ$_2$AR tail by substitution of the mβ$_2$AR proline 417 with a leucine residue (mβ$_2$AR-P417L). The mβ$_2$AR-P417L pulled down similar amounts of NSF from lysates compared with the hβ$_2$AR, indicating that the mβ$_2$AR-P417L cytoplasmic tail fully rescued binding to NSF (Fig. 1A). Likewise, this mutant mβ$_2$AR-P417L pulled down NHERF-1/EBP50 from mouse heart lysates (Fig. 1B).

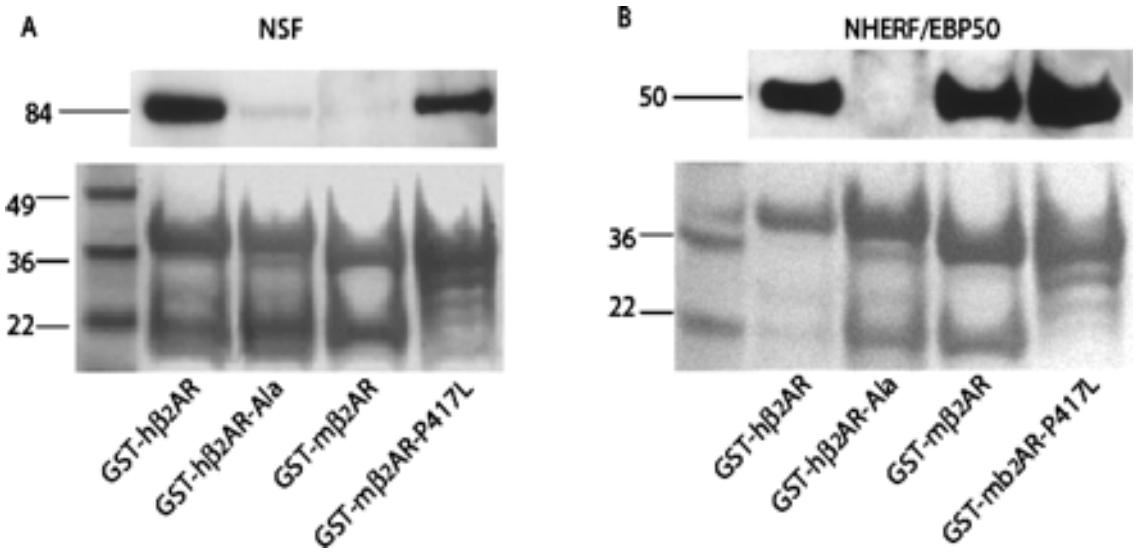

Figure 1. The binding of mβ2AR and hβ2AR to NSF and NHERF-1/EBP50.

The binding of mβ$_2$AR and hβ$_2$AR to NSF and NHERF-1/EBP50 from mouse heart extracts. GST pull-downs were performed as described under *Materials and Methods*. Western detection of NHERF-1/EBP50 pulled down by the indicated GST-β$_2$AR fusion protein from mouse heart extract, and the corresponding detection of NSF is shown in A, whereas the detection of NHERF-1/EBP50 from this extract is shown in B. Ponceau-stained GST fusion proteins are shown under each blot. Images are representative of three or more experiments.

The Binding of NSF and the NSF ATPase Activity Had Distinct Effects on β$_2$AR Trafficking in Cardiomyocytes. To examine whether NSF plays any role in β2AR trafficking in cardiomyocytes, we analyzed the localization of flag-tagged β$_2$ARs

in cardiac myocytes. The mβ_2AR, mβ_2AR-P417L, and hβ_2AR were transiently expressed in cardiac myocytes using recombinant adenovirus. Immunofluorescence studies showed that all three receptors had a cell-surface staining during a nonstimulated state (Fig. 2A). Upon isoproterenol stimulation, all three receptors had reduced cell-surface staining together with increased punctate intracellular staining, suggesting a significant internalization of the receptors in cardiac myocytes. These observations were confirmed quantitatively using an ELISA-based method for assaying surface receptor levels (Swaminath et al., 2004) in a large number of cells and a ratiometric method for analysis of fluorescence micrographs (Tanowitz and von Zastrow, 2003) (Fig. 2B; data not shown). It is interesting that when we measured the short-term decrease in cell-surface receptors after agonist stimulation in cardiac myocytes, we found that the mβ_2AR-P417L had a faster rate ($t_{1/2}$ = 2.63 ± 0.05 min) of cell surface-receptor decrease than the mβ_2AR ($t_{1/2}$ = 10.93 ± 0.01 min; Fig. 3). Because the receptor level change in the short time points after agonist stimulation is primarily determined by agonist-induced endocytosis, these data suggested a faster rate of endocytosis for the mβ_2AR-P417L than for the mβ_2AR in cardiac myocytes. The hβ_2AR had a similar rate ($t_{1/2}$ = 3.4 ± 0.03 min) of cell surface-receptor decrease compared with the mβ_2AR-P417L (Fig. 3). However, after 30 min of agonist stimulation, we observed a similar amount of surface receptor decreases with the mβ_2AR (30.54 ± 2.90%), the mβ_2AR-P417L (28.43 ± 1.69%), and the hβ_2AR (24.94 ± 2.74%). The observed decrease of receptor density at 30 min of stimulation should have been a composite of receptor endocytosis and recycling. The equivalent decreases of receptors at cell surface are usually due to much slower recycling process than endocytosis in cells.

When isoproterenol was removed, both the hβ_2AR and mβ_2AR-P417L recovered cell-surface staining almost completely after a 60-min incubation (Fig. 2). In contrast, the mβ_2AR did not show a fully recovered cell-surface staining pattern, and some residual intracellular staining was observed in these cells (Fig. 2), even though the majority of the internalized receptors seemed to return to the surface within 60 min. When we examined the cell surface-receptor density at different time points with the fluorescent ELISA assay, the mβ_2AR exhibited a lower recovery of cell surface-receptors after recycling for 60 min than the mβ_2AR-P417L mutant and the hβ_2AR (Fig. 2B; *, $p < 0.05$). A significant difference in surface recovery was also observed using ratiometric image measurements 60 min after agonist washout (data not shown). These data indicate that the mβ_2AR, although capable of undergoing agonist-induced internalization and recycling in cardiomyocytes, differs in rates of recycling compared with the hβ_2AR and mβ_2AR-P417L.

It has been well-established that NSF ATPase activity plays an important role in membrane cargo trafficking. We then tested whether NSF activity was necessary for the endocytic recycling of the receptor by using NEM to inhibit NSF activity in myocytes. In the presence of NEM, endocytosis of the receptor was preserved; however, a return of the receptor to the cell surface after removal of agonist for 60 min was not (Fig. 4A). This observation was confirmed with measurements of surface receptor levels by a fluorescent ELISA assay. The cell surface receptor levels dropped after agonist addition and only recovered with agonist withdrawal in the absence of NEM (Fig. 4B). These data suggested that NEM treatment can block the receptor from recycling after endocytosis.

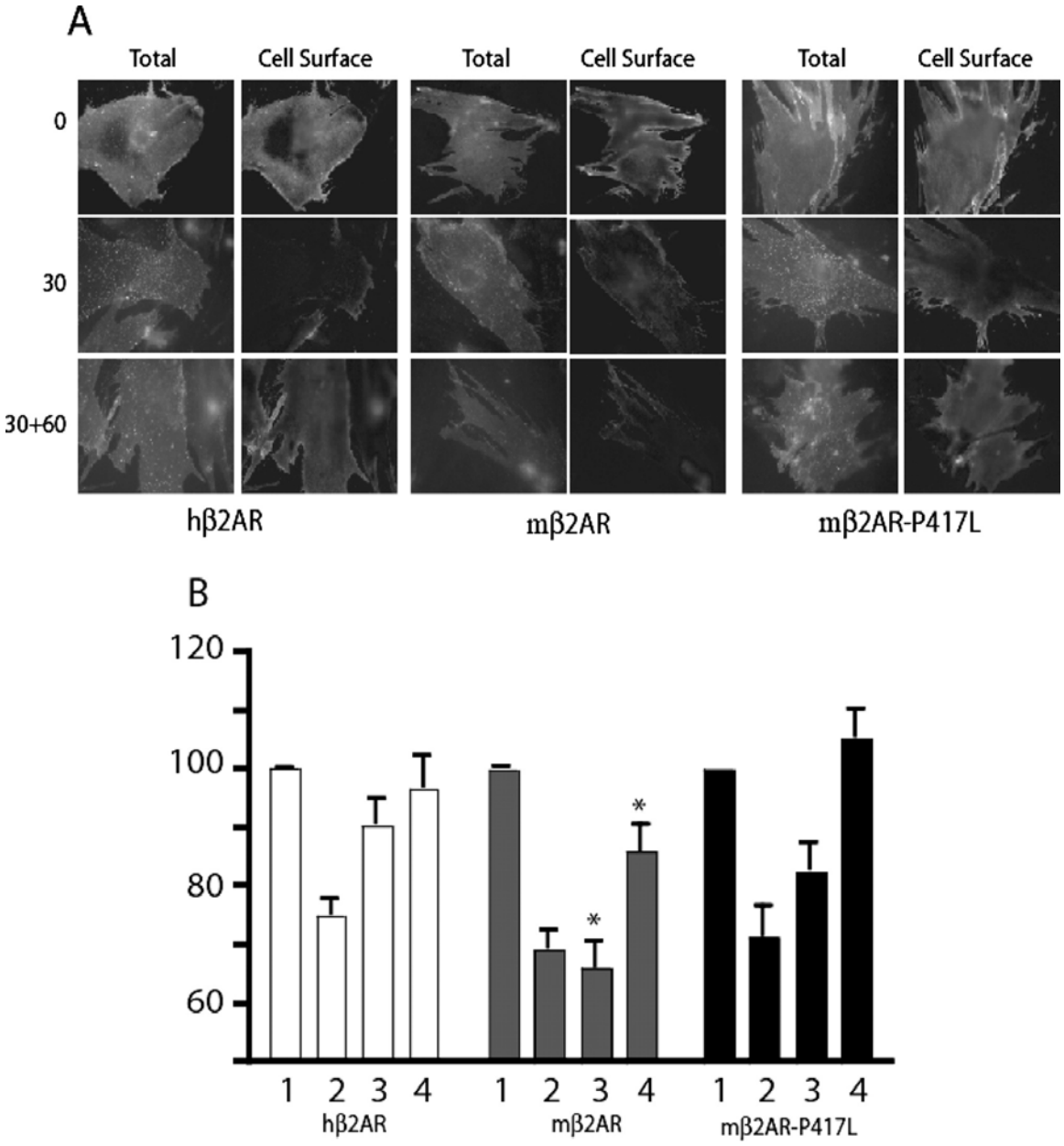

Figure 2. NSF binding enhances recycling of FLAG-β2ARs in neonatal cardiac myocytes from β1/β2AR-KO mice.

A, human and murine β_2ARs internalize and recycle in cardiac myocytes. Cardiac myocytes expressing a FLAG-tagged hβ_2AR, mβ_2AR, or mβ_2AR-P417L were stained with M1 primary antibody conjugated to the Alexa-488 fluorophore to observe a starting "total" receptor population. After no treatment (0), 30-min 10 μM isoproterenol treatment (30), or 30-min isoproterenol treatment followed by a surface antibody strip and 60 min of agonist removal (30 + 60), cells were stained under nonpermeable conditions with a goat

anti-mouse-IgG secondary antibody conjugated to the Alexa-594 fluorophore to observe the relative complement of "surface" receptor. Images are representative of three experiments. B, NSF binding β_2ARs recycle faster. Surface levels of the three β_2ARs were quantified by fluorescence spectroscopy measurements of M1-Alexa 488 associated with the cell surface receptors after the indicated periods of drug administration and removal (1, control; 2, 30 min of isoproterenol stimulation; 3, 30 min of isoproterenol followed by 30 min of drug removal; and 4, 30 min of isoproterenol followed by 60 min of drug removal). Surface levels are normalized as a percentage of untreated cell surface fluorescence, and error bars reflect standard deviations over three experiments. *, $p < 0.05$, significantly different between mβ_2AR and hβ_2AR or mβ_2AR-P417L by *t* test.

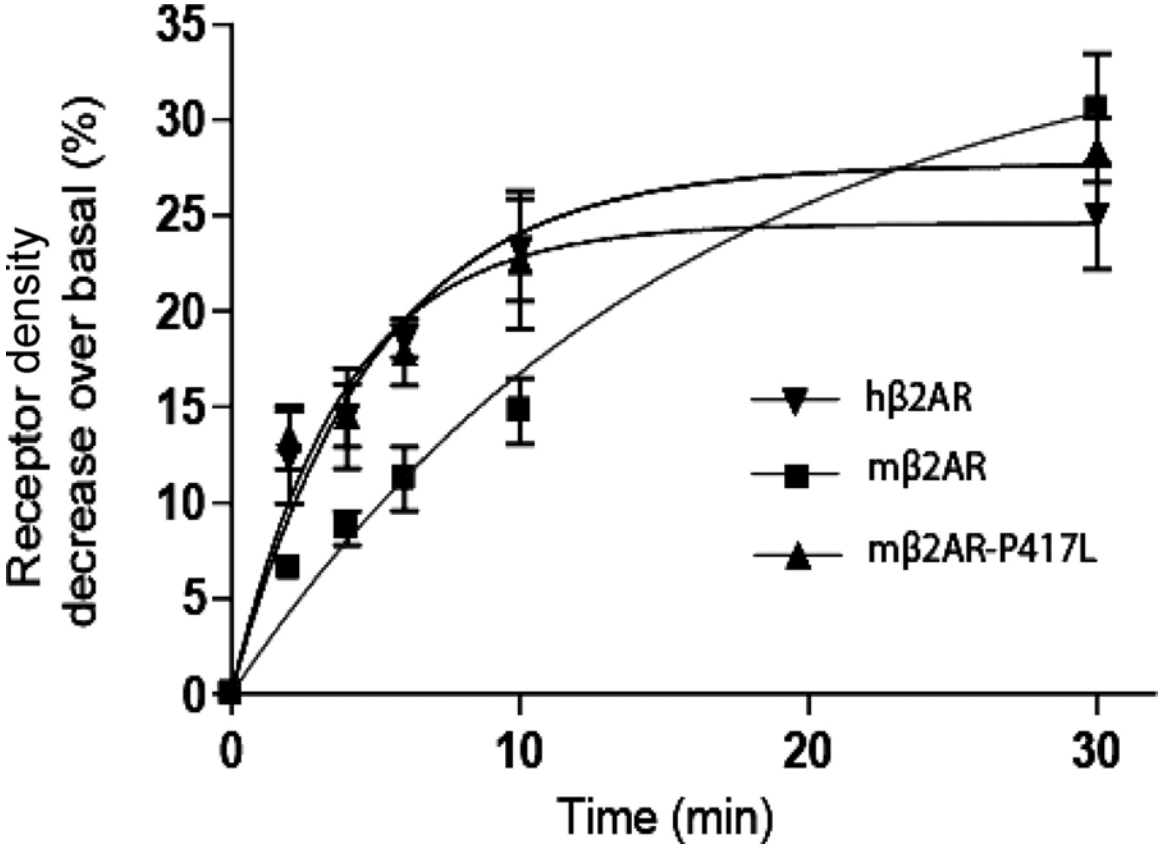

Figure 3. NSF binding enhances the internalization kinetics of FLAG-β2ARs expressed in neonatal cardiac myocytes from β1/β2AR KO mice.

Surface levels of the three β_2ARs were quantified by fluorescent measurement of M1-Alexa 488 associated with the cell surface receptors after the indicated periods of 10 μM isoproterenol administration. Data were

normalized as a percentage decrease of untreated cell surface fluorescence, and error bars reflect standard deviations over three experiments. The data represent the mean ± S.E. of experiments from at least three different myocyte preparations.

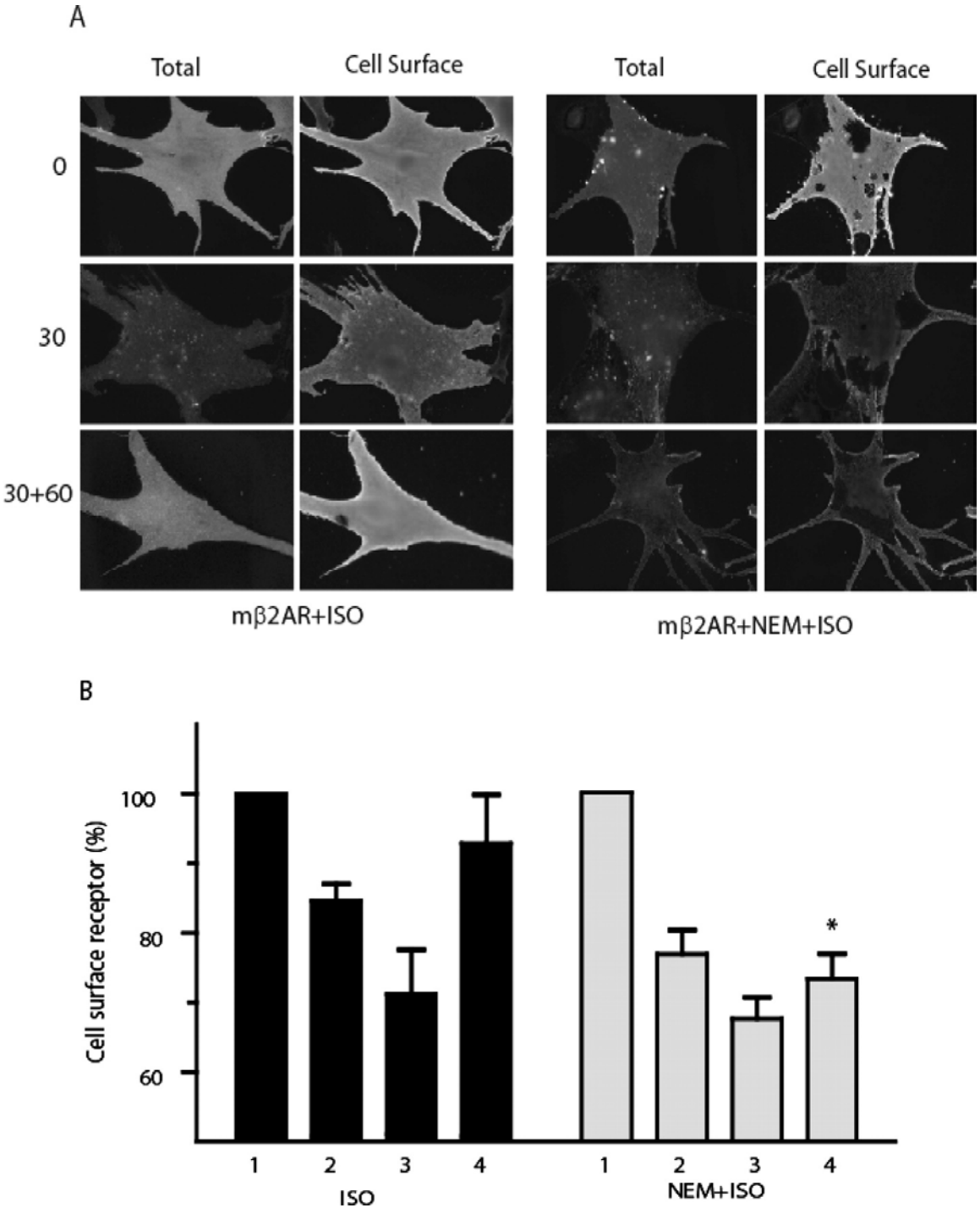

Figure 4. Inhibiting NSF with NEM blocks the FLAG-β2AR recycling after agonist-induced endocytosis in cardiomyocytes.

A, murine β_2ARs internalize and recycle in cardiac myocytes. Cardiac myocytes expressing a FLAG-tagged mβ_2AR were treated as described under *Materials and Methods* and Fig. 3. Images are representative of three experiments. Inhibiting NSF with NEM blocks the FLAG-β_2AR recycling after agonist-induced endocytosis in cardiomyocytes. B, surface levels of the mβ_2ARs were quantified by fluorescent measurement of M1-Alexa 488 associated with the cell surface receptors after the indicated periods of drug administration and removal (1, control; 2, 30 min of isoproterenol stimulation; 3, 30 min of isoproterenol followed by 30 min of drug removal; and 4, 30 min of isoproterenol followed by 60 min of drug removal). Surface levels were normalized as a percentage of untreated cell surface fluorescence, and error bars reflect standard deviations over three experiments. *, $p < 0.05$, significantly different between cells with and without NEM treatment by *t* test.

Dominant-Negative NSF Lacking ATPase Activity Inhibited Endogenous mβ_2AR Coupling to G_i Pathway in Cardiomyocytes. Our finding that NEM inhibits β_2AR recycling in cardiac myocytes suggested that NSF function is required for this process. To further probe whether NSF enzymatic activity can affect the receptor signaling independent from the direct NSF-receptor interaction, we examined the signaling mediated by the endogenous mβ_2AR when overexpressing an inactivated NSF, the E329Q mutant (Whiteheart et al., 1994). This mutation abolishes ATPase activity and has been shown to block AMPA receptor trafficking (Whiteheart et al., 1994; Whiteheart and Matveeva, 2004). When the endogenous mβ_2AR in the β_1AR-KO myocyte was stimulated by isoproterenol, the activated receptor induced a biphasic contraction-rate response with an initial increase mediated by G_s coupling followed by a sustained G_i-dependent decrease to reduce the contraction rate below basal level (Fig. 5A; Xiang et al.,

2002). When wild-type NSF was expressed in β_1AR-KO cardiac myocytes, we did not observe any significant change in the endogenous mβ_2AR-mediated contraction-rate response (Fig. 5A). In contrast, when the NSF-E329Q mutant was overexpressed in cardiomyocytes, the contraction rate mediated by the mβ_2AR was significantly higher than the control and did not display a decrease lower than the basal level (Fig. 5B). This response profile was similar to that observed with an inhibition of G_i by PTX (Fig. 5C). Indeed, additional treatment of PTX did not generate any further increases in contraction rates (Fig. 5D). Therefore, the NSF-E329Q behaved as a dominant-negative to block the receptor coupling to G_i in cardiomyocytes. In addition, when myocytes are pretreated with NEM to inhibit the NSF ATPase activity, we also observed effects similar to those by NSF-E329Q mutant on mβ_2AR signaling mediated contraction-rate response (data not shown).

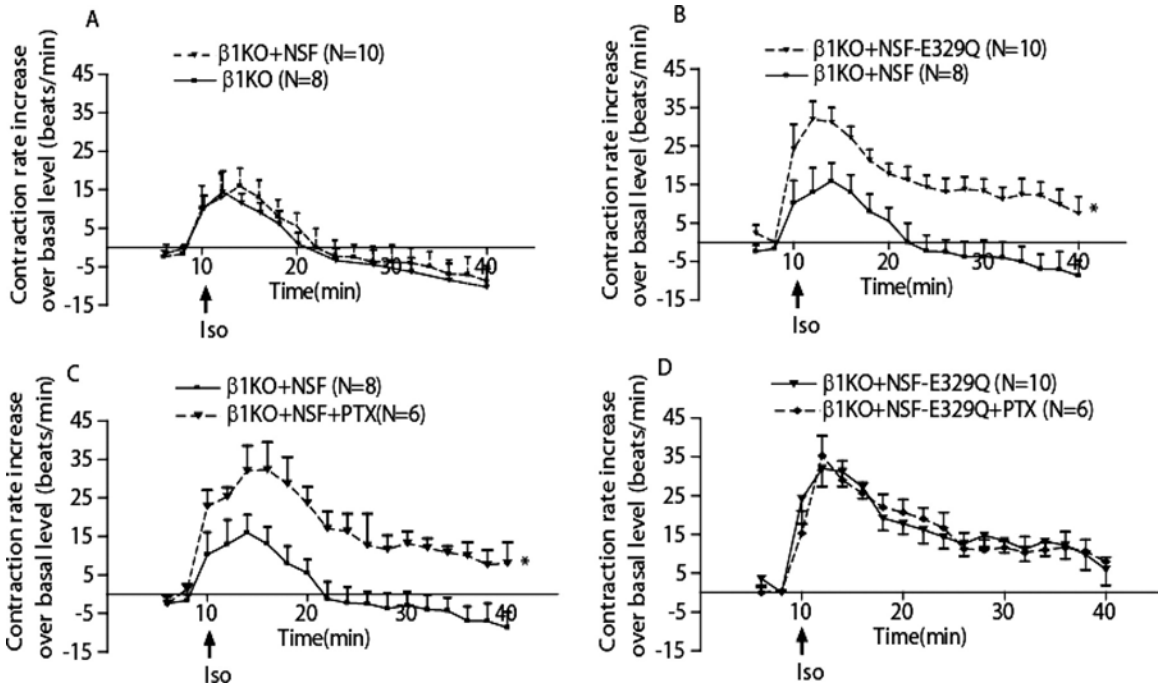

Figure 5. Dominant-negative NSF-E329Q mutant inhibits the mβ2AR coupling to Gi protein.

Spontaneously beating cardiac myocytes from β_1AR KO mice were transfected with a wild-type NSF (A and C) or NSF-E329Q (B and D) mutant adenovirus as indicated. The cells were administered 10 μM isoproterenol with inhibition of G_i by PTX. Overexpressing the NSF E329Q mutant enhanced the contraction-rate increase induced by isoproterenol stimulation. Additional PTX treatment did not further enhance the contraction-rate increase induced by the mβ_2AR. The data represent the mean ± S.E. of experiments from at least three different myocyte preparations. *, $p < 0.05$, time course significantly different by two-way ANOVA.

The Divergent C Termini of the Human and Murine β_2AR Had Different Effects on Contraction Rate Responses in Neonatal Cardiomyocytes. Our previous studies have shown that the localization and trafficking of the mβ_2AR is important for the receptor's G protein signaling specificity and subsequent regulation of the myocyte contraction rate. In the course of this study, we found that the divergent PDZ ligand of the human and murine β_2AR affected the receptor trafficking rates after agonist stimulation in cardiac myocytes. Thus, we wanted to examine whether differences in NSF binding and/or altered trafficking rates could modulate the receptor signaling in cardiac myocytes. When the mβ_2AR was expressed in β_1/β_2AR-KO myocytes and stimulated by isoproterenol, the activated receptor induced a biphasic contraction-rate response with an initial increase followed by a sustained decrease to reduce the contraction rate lower than basal level (Fig. 6A; Xiang et al., 2002). This contraction-rate change is equivalent to that induced by the endogenous mβ_2AR in β_1AR-KO myocytes (Fig. 6A). The mβ_2AR-P417L induced a similar contraction-rate response profile and initial increase compared with the mβ_2AR in β_1/β_2AR-KO myocytes (Figs. 6C and 7D). However, the contraction rate decreased faster, and the contraction rate was lower than that induced by the mβ_2AR during late stimulation in cardiac myocytes (Figs. 6C and 7E). In addition, when

stimulating the hβ_2AR expressed in β_1/β_2AR-KO myocytes with isoproterenol, the activated receptor also induced a biphasic, contraction-rate change with an initial increase followed by a sustained decrease (Fig. 6B). Although it is interesting that the initial contraction-rate increase was smaller than that induced by the activated mβ_2AR and mβ_2AR-P417L (Figs. 6B and 7D), it is more surprising that the late decrease in contraction rate induced by the exogenous hβ_2AR was greater than that induced by the mβ_2AR and mβ_2AR-P417L (Figs. 6B and 7E).

The profound contraction-rate decrease induced by the mβ_2AR-P417L and the hβ_2AR suggests that these receptors may have enhanced coupling to G_i and/or reduced coupling to G_s compared with the mβ_2AR. We therefore examined the G_i signaling induced by the activated receptors in cardiac myocytes. PTX was used to block G_i signaling in cardiac myocytes expressing the different β_2ARs before isoproterenol stimulation. Upon inhibiting G_i with PTX, the isoproterenol-stimulated mβ_2AR induced a slightly greater but not significant contraction-rate increase in myocytes compared with the control and prevented the late G_i-dependent contraction rate decrease (Fig. 7A; Xiang et al., 2002). PTX treatment also inhibited the contraction rate decrease mediated by the mβ_2AR-P417L or the hβ_2AR during the late phase of stimulation (Fig. 7, B and C). These data suggest that compared with the activated mβ_2AR, the mβ_2AR-P417L had an enhanced G_i coupling upon isoproterenol stimulation, and the activated hβ_2AR coupled to G_i more efficiently in neonatal cardiac myocytes (Fig. 7E). This indicates that the divergent receptor C termini can induce different changes in contraction-rate responses that correlate with subtle changes in receptor transportation rates.

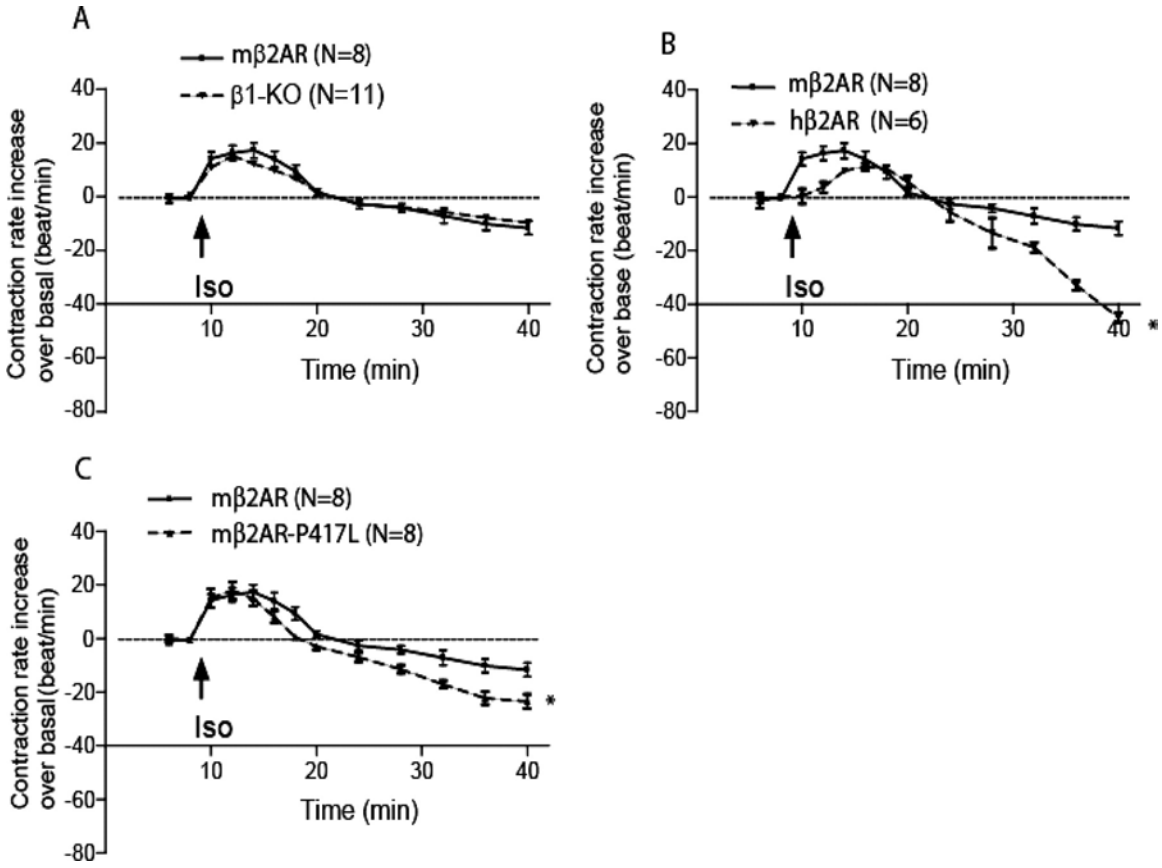

Figure 6. Differences in β2AR contraction-rate responses to isoproterenol in neonatal cardiac myocytes from β1/β2AR KO mice.

The hβ_2AR and mβ_2AR-P417L exhibit different contraction rate profiles than the mβ_2AR at comparable expression levels. Spontaneously beating, cardiac myocytes from β_1/β_2AR KO mice were infected with a FLAG-tagged mβ_2AR (A), hβ_2AR (B), or mβ_2AR-P417L (C) recombinant adenovirus as indicated and infused with 10 μM isoproterenol. Contraction rates were measured and normalized as the change over baseline. The data represent the mean ± S.E. of experiments from at least three different myocyte preparations. *, $p < 0.05$, time course significantly different by two-way ANOVA.

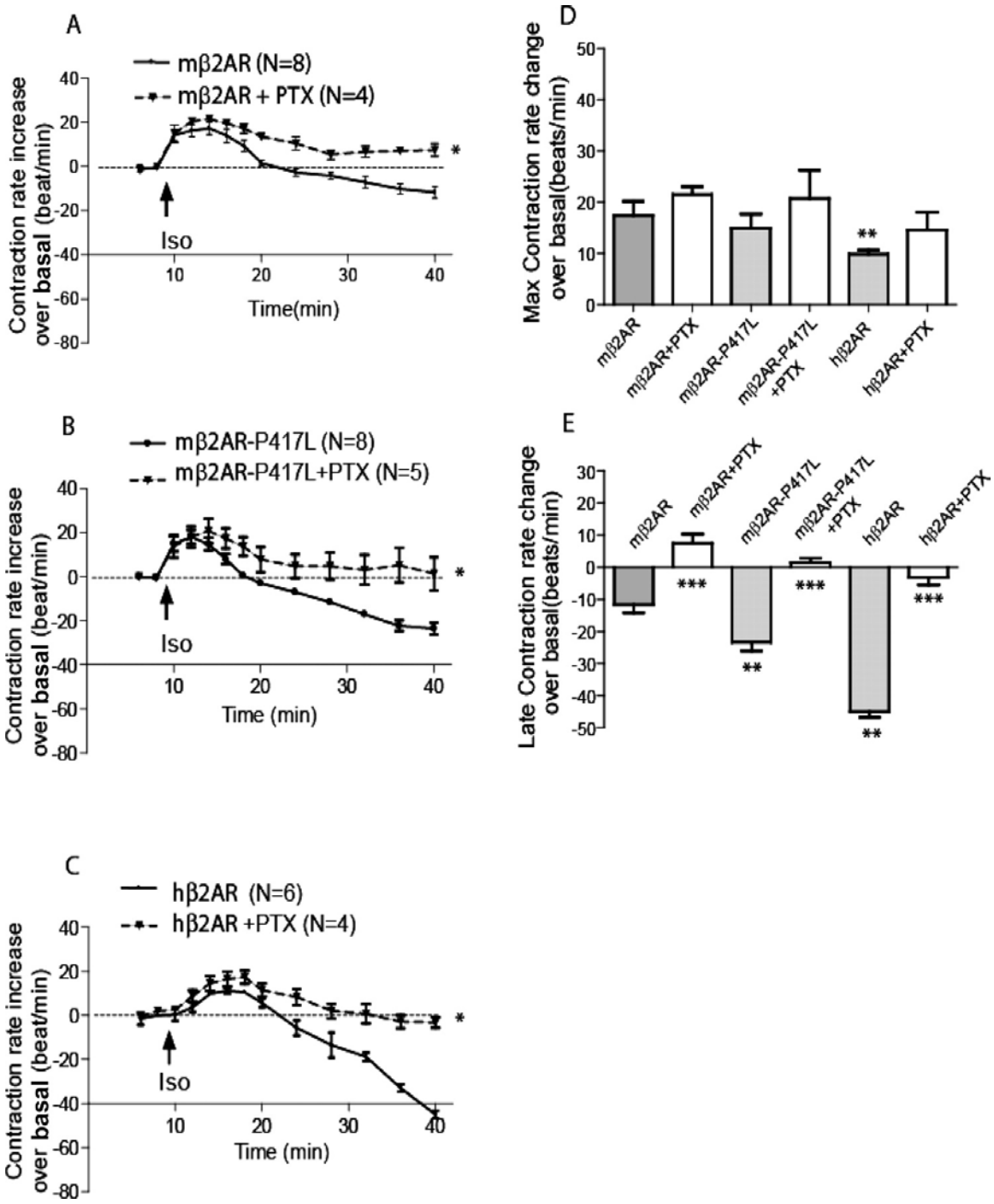

Figure 7. NSF binding enhances the Gi signaling components of β2ARs.

Spontaneously beating cardiac myocytes from β_1/β_2AR KO mice were transfected with a FLAG-tagged mβ_2AR (A), mβ_2AR-P417L (B), or hβ_2AR (C) adenovirus as indicated. The cells were administered 10 μM isoproterenol with inhibition of G_i with PTX. PTX treatment did not affect initial response usually

mediated by receptor/G_s coupling (D) but significantly enhanced the contraction rate during the late stimulation induced by the G_i coupling to the activated hβ_2AR, mβ_2AR, or mβ_2AR-P417L (E). The data represent the mean ± S.E. of experiments from at least three different myocyte preparations. *, $p < 0.05$, time course significantly different by two-way ANOVA. **, $p < 0.05$, unpaired *t* test significantly different on initial maximum contraction rate increases or late contraction-rate decreases mediated by different β_2ARs. ***, $p < 0.05$, unpaired *t* test significantly different on late contraction rate decreases after PTX treatment.

The Binding of NSF and PDZ Had Distinct Effects on β2AR Activation-Induced Contraction Rates in Cardiomyocyte. To further probe the effect of the β_2AR binding to NSF and PDZ on receptor signaling in cardiomyocytes, we took advantage of the different binding affinities between receptor and proteins by using peptides to selectively disrupt the interactions. We expressed either mβ_2AR or mβ_2AR-P417L (the hβ_2AR mimic) in β_1/β_2AR-KO cardiomyocyte for the contraction rate assay. Membrane-permeable peptides containing ASLL sequence and DSAL sequence were used to selectively disrupt NSF and NHERF/EBP50 binding, respectively. When mβ_2AR-expressing myocytes were treated with NSF (ASLL) peptide, the activated receptor induced a slightly bigger but not significant initial increase than the cells without pretreatment (Fig. 8, A and C). The increase was sustained during stimulation and lacked a late decrease mediated by receptor/G_i coupling in control cells (Fig. 8, A and D). When mβ_2AR-expressing myocytes were treated with PDZ (DSAL) peptide, the activated receptor induced a significantly greater initial increase than the control (Fig. 8, B and C), and the increase was sustained and lacked a late G_i-dependent decrease (Fig. 8, B and D). In contrast, pretreatment with NSF (ASLL) peptide did not affect the activated mβ_2AR-P417L-induced initial increase (Fig. 8, E and G). However, the increase was sustained

and did not display a late G_i-induced decrease of contraction rate (Fig. 8, E and H). When myocytes expressing mβ_2AR-P417L were treated with PDZ (DSAL) peptide, the activated receptor induced a significantly greater initial increase in contraction rate than the control (Fig. 8, F and G), and the increase was sustained and did not show a late G_i-induced decrease (Fig. 8, F and H). Together, these data showed that although disrupting the binding to PDZ protein (such as NHERF/EBP50) affects the receptor coupling to both G_s and G_i, disrupting the binding to NSF selectively affects the receptor coupling to G_i in cardiomyocytes.

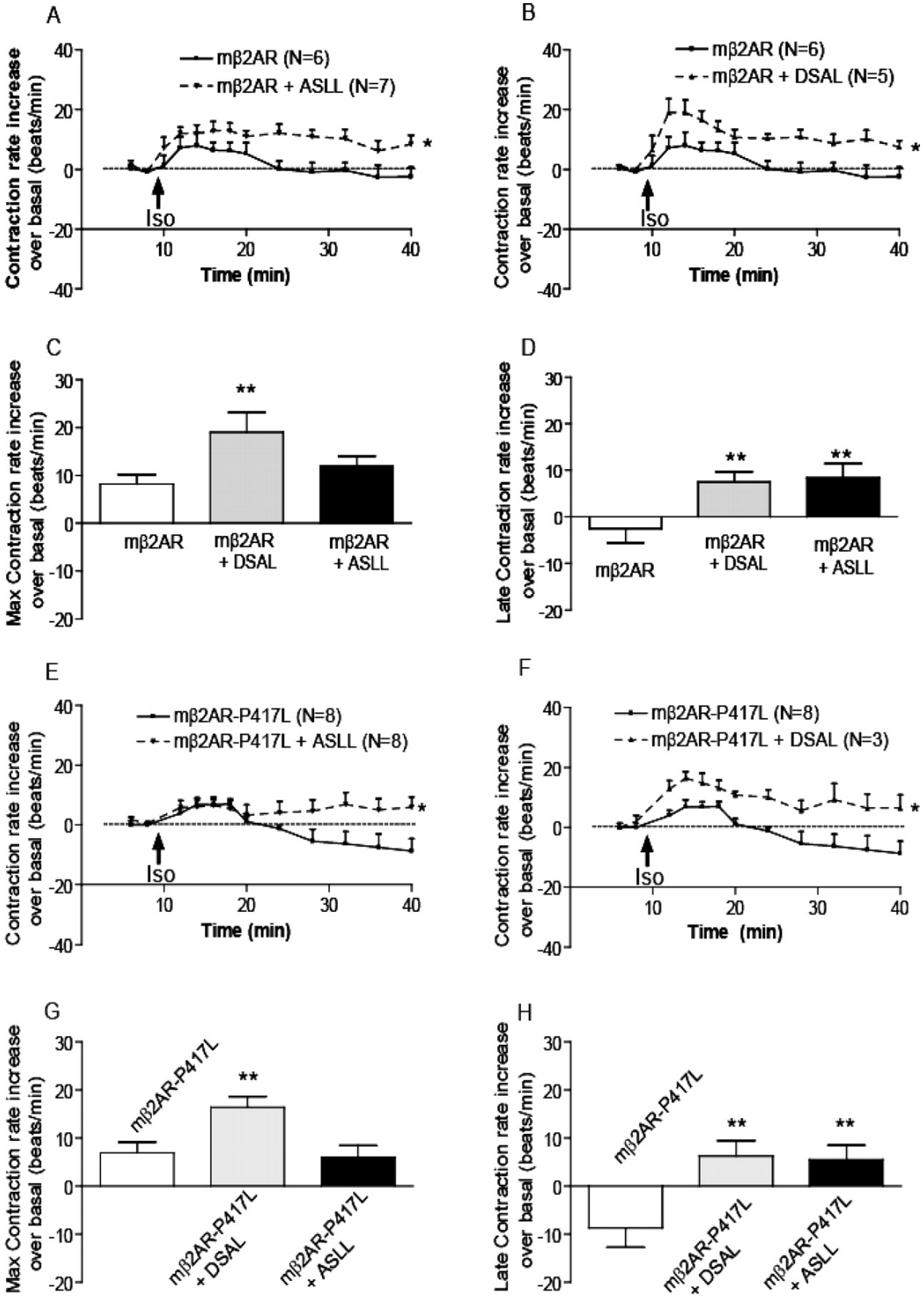
A
mβ2AR (N=6)
mβ2AR + ASLL (N=7)
Contraction rate increase over basal (beats/min)
Iso
Time (min)
B
mβ2AR (N=6)
mβ2AR + DSAL (N=5)
Contraction rate increase over basal (beats/min)
Iso
Time (min)
C
Max Contraction rate increase over basal (beats/min)
mβ2AR
mβ2AR + DSAL
mβ2AR + ASLL
D
Late Contraction rate increase over basal (beats/min)
mβ2AR
mβ2AR + DSAL
mβ2AR + ASLL
E
mβ2AR-P417L (N=8)
mβ2AR-P417L + ASLL (N=8)
Contraction rate increase over basal (beats/min)
Iso
Time (min)
F
mβ2AR-P417L (N=8)
mβ2AR-P417L + DSAL (N=3)
Contraction rate increase over basal (beats/min)
Iso
Time (min)
G
Max Contraction rate increase over basal (beats/min)
mβ2AR-P417L
mβ2AR-P417L + DSAL
mβ2AR-P417L + ASLL
H
Late Contraction rate increase over basal (beats/min)
mβ2AR-P417L
mβ2AR-P417L + DSAL
mβ2AR-P417L + ASLL

Figure 8. Selective disruptions of NSF and NHERF-1/EBP50 binding have distinct effects on β2AR signaling.

Spontaneously beating cardiac myocytes from β_1/β_2AR KO mice were transfected with a FLAG-tagged mβ_2AR (A-D) or mβ_2AR-P417L (E-H) adenovirus as indicated. The cells were administered 10 μM isoproterenol with pretreatment of membrane-permeable NSF peptide ASLL and PDZ peptide DSAL to disrupt the receptor binding to NSF and PDZ protein, respectively. NSF peptide ASLL significantly affected the receptor-mediated contraction response during the late stimulation, which are usually mediated by receptor/G_i coupling (D and H). In contrast, PDZ peptide affected both initial contraction rate increase mediated by receptor/G_s coupling (C and G) and the late contraction-rate response mediated by receptor/G_i coupling (D and H). The data represent the mean ± S.E. of experiments from at least three different myocyte preparations. *, $p < 0.05$, time course significantly different by two-way ANOVA. **, $p < 0.05$, unpaired *t* test significantly different on initial maximum contraction-rate increases or late contraction-rate decreases after treatment with peptides.

2.5 Discussion

In the present study, several approaches were used to test whether NSF regulates β_2AR trafficking and physiological signaling. This idea was extended from the studies of β_2AR-selective interactions with NSF and PDZ proteins. A distal portion of the cytoplasmic C terminus of the hβ_2AR selectively binds to several PDZ domain-containing proteins, such as the cytoskeleton-associated protein NHERF/EBP50, which is implicated in receptor recycling (Cao et al., 1999). However, a subsequent study confirmed the importance of the PDZ ligand for receptor recycling to the cell surface but identified a distinct non-PDZ interaction of this sequence with NSF that was required for proper endocytic recycling (Cong et al., 2001). Although the reported difference could result from the difference between the derived HEK293 cell lines, we have tried to address the functional roles of these receptor-protein interactions in primary cultured cardiomyocytes—a native environment that may have a more precise regulation of receptor function. We have shown previously that the carboxyl-terminal sequence of mβ_2AR was also required for efficient plasma membrane recycling and for receptor coupling to G_i in cardiomyocytes (Xiang and Kobilka, 2003). In this study, we showed that the binding to NSF enhanced both internalization and recycling rates of β_2AR and increased the receptor coupling to G_i signaling in cardiomyocytes (Figs. 2, 3, and 8). We further distinguished the effects of NSF and PDZ binding on β_2AR signaling in myocytes. Although the binding to NSF increases receptor/G_i coupling, the binding to PDZ proteins affects receptor coupling to both G_s and G_i proteins (Fig. 8).

It is interesting that, at the receptor's distal carboxyl terminus, the mβ_2AR (DSPL) differs from the hβ_2AR (DSLL) at the -1 position, at which the hβ_2AR has a leucine critical for binding to NSF. Because the mβ_2AR has a proline residue at the same relative position of the receptor cytoplasmic tail, we predicted that the receptor could not bind to NSF. Our experiments confirmed a very low affinity binding of NSF to the mβ_2AR cytoplasmic tail (Fig. 1). We used a gain-of-function approach by replacing the proline with a leucine to generate a mutant mβ_2AR-P417L. This mutant has a distal terminus identical with that of the hβ_2AR (hβ_2AR mimic) and displayed recovered binding to NSF (Fig. 1). The direct NSF-binding seemed to increase the rates of both agonist-induced endocytosis and recycling of the mβ_2AR-P417L in cardiomyocytes (Fig. 2 and 3). We cannot exclude the possible contribution by the small increase in binding affinities of the mutant mβ_2AR-P417L for PDZ proteins or new binding partners. However, our beating assay data supported that the increased trafficking rates are probably caused by the fact that the mutant mβ_2AR-P417L gained binding to NSF (Fig. 8).

Consistent with the trafficking data, the mβ_2AR-P417L and the hβ_2AR also displayed a more profound coupling to G_i than the mβ_2AR in cardiomyocytes (Fig. 7). We have established previously that activated mβ_2AR undergo sequential coupling to G_s and G_i to modulate cardiomyocyte contraction rate, and the recycling of mβ_2AR is necessary for coupling to G_i (Xiang and Kobilka, 2003). By using membrane-permeable peptides to selectively inhibit the receptor binding to NSF or PDZ proteins, we will be able to distinguish the subtle effects of a specific binding on receptor signaling. Although disruption of PDZ binding affects receptor coupling to both G_s and G_i, disruption of NSF binding selectively inhibits receptor coupling to G_i (Fig. 8). It is interesting that despite

that the mβ_2AR does not bind to NSF well, the NSF peptide ASLL affected the receptor signaling (Fig. 8A). This may result from a low basal interaction between the mβ_2AR and NSF. On the other hand, NSF peptide ASLL is capable of binding to PDZ proteins (Cong et al., 2001); thus, it may compete against DSPL on the mβ_2AR, which is not a perfect PDZ ligand because of the structure of proline. In contrast, the binding between DSLL of the mβ_2AR-P417L and PDZ proteins is less likely to be affected by the NSF peptide (Fig. 8E). Thus, the effect on the mβ_2AR-P417L signaling by the NSF peptides suggested that the binding to NSF affects the receptor/G_i coupling, which is consistent with its role in the modulation of receptor recycling.

NSF was identified as an ATPase, binding to SNARE complexes required for membrane fusion, thus playing critical roles in protein trafficking of many membrane receptors (Whiteheart and Matveeva, 2004). In agreement, we showed that NSF ATPase activity was essential for mβ_2AR trafficking and signaling in cardiomyocytes (Fig. 4 and 5). It is interesting that NSF can bind to β-arrestin, an adaptor-like protein linking most GPCRs to clathrin-coated vesicles for endocytosis (McDonald et al., 1999). NSF binding to β-arrestin, like binding to classic SNARE substrates, is an ATP-dependent event (McDonald et al., 1999). Thus, NSF could play a role together with β-arrestin in recruiting the cargo receptors into clathrin-coated vesicles for budding. This process can be fine-tuned if NSF directly binds membrane cargo receptors, including hβ_2AR (Heydorn et al., 2004). In addition, the binding of NSF to the hβ_2AR is enhanced in the ATP-bound form (Gage et al., 2005), and the NSF ATPase activity dissociates the AMPA receptor from PDZ proteins allowing endocytosis (Osten et al., 1998; Hanley et al., 2002). Therefore, NSF can facilitate receptor recruitment into clathrin-coated vesicle by

both direct binding to the cargo receptor and its ATPase activity. In the case of mβ_2AR, the activated receptor recruits β-arrestin; this brings NSF to the receptor. NSF ATPase activity helps to dissociate the receptor from PDZ proteins to enter clathrin-coated vesicles, and later NSF regulates the vesicle fusion to endosome. In comparison, hβ_2AR can directly bind to the NSF. When NSF is recruited to the receptor/arrestin complexes, it can compete against the receptor binding to PDZ proteins. This competition can lead to an increase in internalization rates (Fig. 3). During the receptor recycling, NSF binding can bridge the cargo receptor to SNARE complexes, which facilitate the docking of recycling vesicles to plasma membrane, hence enhancing the recycling rate of hβ_2AR and mβ_2AR-P417L but not mβ_2AR. In this study, we only measured the cell surface receptor level during endocytosis and recycling. Any additional role of NSF in receptor trafficking among endosomal compartments remains to be addressed.

It is noteworthy that the subtle effects of NSF-hβ_2AR binding on trafficking and signaling is not conserved throughout mammals; such an effect can be overlooked easily in an experimental procedure. Although NSF is a common factor involved in membrane receptor trafficking, the context of the NSF-receptor complex can further complicate the type and degree of receptor regulation. These regulations will probably include the binding of the receptor to PDZ-domain containing proteins and cytoskeleton-associated proteins and additional binding of NSF to other trafficking proteins such as SNARE complexes and arrestin. Further studies using NSF mutants with selective ablation of binding to the β_2AR or other proteins such as arrestin will help to dissect any roles of individual protein-protein interactions on the β_2AR trafficking and signaling in cardiomyocytes or physiological settings.

Indeed, an effect of NSF binding to the hβ_2AR is likely to be complicated by competitive binding of PDZ proteins on the same sequence at the carboxyl-terminal end (Cong et al., 2001). A PDZ binding can have multiple effects on membrane receptor distribution and trafficking. One effect of PDZ binding is to stabilize and restrict the receptors at distinct subcellular domains. This is supported by the recent evidence that overexpressing NHERF-1/EBP50 reduced the agonist-induced internalization of two GPCRs, the parathyroid hormone receptor type-1 and thromboxane A(2)β receptor in HEK293 cells (Rochdi and Parent, 2003; Sneddon et al., 2003). By binding to cytoskeleton and/or scaffold proteins, the receptors can associate with signaling components and form complexes to either facilitate or restrict signal transduction. Consistent with the notion, our previous and current studies support that disrupting the PDZ binding to the β_2ARs enhances the receptor coupling to G_s in cardiomyocytes (Xiang and Kobilka, 2003). In contrast, the PDZ protein GRIP/ABP binding has been shown to play a role in the stabilization of an intracellular pool of AMPA receptors that have been internalized with stimulation, thus inhibiting their recycling to the synaptic membrane (Braithwaite et al., 2002). Therefore, depending on the receptors and their binding partners, the PDZ domain-containing proteins can stabilize the receptor complexes at either the cell surface or intracellular compartments to fine-tune the receptor function in a given cell type. The third effect of PDZ binding seems to promote receptor trafficking to another subcellular location. Both PICK1 and NHERF-1/EBP50 have been shown to be critical for AMPA receptor and β_2AR recycling back to the cell surface (Cao et al., 1999; Xiang and Kobilka, 2003; Lu and Ziff, 2005). In cardiomyocytes, selective disruption of PDZ binding with point mutations or with membrane-permeable peptide

blocks receptor recycling and also inhibits receptor coupling to G_i ((Xiang and Kobilka, 2003) and Fig. 8). This PDZ-promoted trafficking may simply be a result of PDZ sequestration of receptors away from a competing trafficking fate, which could generalize PDZ interactions as hindrances to trafficking. The function of PDZ binding on receptor endocytosis and recycling could be further complicated by agonist-dependent phosphorylation of the receptor C-terminal end by G-protein receptor kinases and subsequent receptor dephosphorylation by pH-sensitive phosphatases (Sibley et al., 1986; Pitcher et al., 1995, 1998; Cao et al., 1999). The significance of this interplay in cardiomyocytes remains to be seen.

When the hβ_2AR was expressed in murine cardiomyocytes, the receptor displayed sequential coupling to G_s and G_i to regulate the myocyte contraction rate (Fig. 6). This result reinforced the notion from our previous studies that the recycling of the β_2AR is part of a mechanism necessary for the receptor to switch from G_s to G_i (Xiang et al., 2002, 2005; Xiang and Kobilka, 2003). Both the human and murine β_2ARs displayed a dual coupling to both G_s and G_i proteins in cardiac myocytes. Our studies revealed a species-dependent difference between human and murine β_2ARs. The hβ_2AR seemed to have a lower efficiency in coupling to the G_s pathway and a significantly higher efficiency in coupling to G_i than the mβ_2AR when regulating the myocyte contraction rate (Figs. 6 and 7). Our results suggest that the profound G_i coupling is in part due to the increased binding to NSF. The mechanism of the low G_s coupling efficiency is not clear, although the higher receptor endocytosis rate could be an indication of enhanced desensitization. Another clue lies in the differences between receptor species. Despite the fact that the mβ_2AR-P417L had recovered the ability to bind NSF, much of the signaling

properties of this mutant mβ_2AR still resembled those of the mβ_2AR rather than the hβ_2AR (Fig. 6). The differences of other structural domains on the hβ_2AR and mβ_2AR must thus account for the differences observed between the mβ_2AR-P417L and the hβ_2AR in cardiomyocytes. The notable regions include both the third loop and the proximal region of the carboxyl tail, which can directly influence G protein coupling. Another species-dependent difference is that a unique sugar-modification site located on the second extracellular domain of the hβ_2AR, but not rodent β_2ARs, promotes receptor degradation upon long-term agonist stimulation (Mialet-Perez et al., 2004). When overexpressed in mice, the hβ_2AR seems to enhance the cardiac contraction in animal hearts without developing heart failure (Milano et al., 1995). The β_2AR/G_s signaling is proapoptotic (Zhu et al., 2001), whereas the β_2AR/G_i signaling plays an antiapoptotic role in both mouse hearts and cultured mouse cardiac myocytes (Zhu et al., 2001; Patterson et al., 2004). Thus, the more preferential coupling of the hβ_2AR to G_i over G_s observed in our experiments could explain the lack of pathologic changes observed with overexpression of the hβ_2AR in the hearts of mice. Further studies characterizing the differences between the hβ_2AR and mβ_2AR are needed to advance our understanding of adrenergic physiology in vivo.

In conclusion, the present results indicate that NSF ATP-ase activity is necessary for agonist-dependent β_2AR trafficking in cardiomyocytes, whereas NSF binding enhances the receptor transportation rates. Both the direct binding to NSF and its ATPase activity are important for the receptor coupling to G_i. Our data also showed different affinities of NSF binding to β_2ARs from different species, and the direct binding to NSF contributes to the differences of receptor signaling in cardiomyocytes. Our data further

revealed distinct effects of NSF and PDZ binding on β_2AR signaling. In contrast to the selective effect on G_i coupling by the receptor binding to NSF, the receptor binding to PDZ proteins affects the receptor coupling to both G_s and G_i proteins. The present results add to the growing appreciation of diversified cellular factors as part of comprehensive mechanisms to fine-tune GPCR signaling and membrane trafficking in native mammalian cardiomyocytes.

2.6 Acknowledgements and Footnotes

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ABBREVIATIONS: β_2AR, β_2-adrenergic receptor; β_1AR, β_1-adrenergic receptor; GPCR, G protein-coupled receptor; KO, knockout; NSF, *N*-ethylmaleimide-sensitive factor; PDZ, PSD-95/Discs-large/ZO-1; NHERF/EBP50, Na^+/H^+ exchanger regulatory

factor/ezrin/radixin/moesin-binding phosphoprotein of 50 kDa; GST, glutathione; PTX, pertussis toxin; NEM, *N*-ethylmaleimide; ELISA, enzyme-linked immunosorbent assay; HEK, human embryonic kidney; ANOVA, analysis of variance; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor.

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CHAPTER 3:

Engineered Protein Connectivity to Actin Mimics PDZ-Dependent Recycling of GPCRs but Not its Regulation by Hrs

B. E. L. Lauffer contributed work towards all Figures and text in the following Chapter. Gabriel Vargas and Stanford Chen conceived and validated the receptor-fusion, experimental strategy employed. Eric Lontauk and Cindy Yang performed further preliminary experimentation. Cristina Melero-Heras and Tanja Kortemme provided an alternative experimental strategy that is discussed in Appendix II.

3.1 Summary

Many G protein-coupled receptors (GPCRs) recycle after agonist-induced endocytosis by a sequence-dependent mechanism, which is distinct from default membrane flow and remains poorly understood. Efficient recycling of the beta-2 adrenergic receptor (β2AR) requires a carboxyl-terminal PDZ (PSD-95 / Discs Large / ZO-1) protein–binding determinant (PDZbd), an intact actin cytoskeleton, and is regulated by the endosomal protein Hrs (hepatocyte growth factor-regulated substrate). The PDZbd is thought to link receptors to actin through a series of protein interaction modules present in NHERF/EBP50 (Na+/H+ exchanger 3 regulatory factor / ezrin-binding phosphoprotein of 50 kDa) -family and ERM (ezrin / radixin / moesin) -family proteins. It is not known, however, if such actin connectivity is sufficient to recapitulate the natural features of sequence-dependent recycling. We addressed this question using a receptor fusion approach based on the sufficiency of the PDZbd to promote recycling when fused to a distinct GPCR, the delta opioid receptor, which normally recycles inefficiently in HEK293 cells. Modular domains mediating actin connectivity promoted receptor recycling with similarly high efficiency as the PDZbd itself, and recycling promoted by all of the domains was actin-dependent. Regulation of receptor recycling by Hrs, however, was conferred only by the PDZbd and not by downstream interaction modules. These results suggest that actin connectivity is sufficient to mimic the core recycling activity of a GPCR-linked PDZbd but not its cellular regulation.

3.2 Introduction

G protein-coupled receptors (GPCRs) comprise the largest family of transmembrane signaling receptors expressed in animals, and transduce a wide variety of physiological and pharmacological information. While these receptors share a common 7-transmembrane-spanning topology, structural differences between individual GPCR family members confer diverse functional and regulatory properties (1-4). A fundamental mechanism of GPCR regulation involves agonist-induced endocytosis of receptors via clathrin-coated pits (4). Regulated endocytosis can have multiple functional consequences, which are determined in part by the specificity with which internalized receptors traffic via divergent downstream membrane pathways (5-7).

Trafficking of internalized GPCRs to lysosomes, a major pathway traversed by the δ-opioid receptor (δOR), contributes to proteolytic down-regulation of receptor number and produces a prolonged attenuation of subsequent cellular responsiveness to agonist (8,9). Trafficking of internalized GPCRs via a rapid recycling pathway, a major route traversed by the β2 adrenergic receptor (β2AR), restores the complement of functional receptors present on the cell surface and promotes rapid recovery of cellular signaling responsiveness (6,10,11). When co-expressed in the same cells, the δOR and β2AR are efficiently sorted between these divergent downstream membrane pathways, highlighting the occurrence of specific molecular sorting of GPCRs after endocytosis (12).

Recycling of various integral membrane proteins can occur by 'default', essentially by bulk membrane flow in the absence of lysosomal sorting determinants (13).

There is increasing evidence that various GPCRs, such as the β2AR, require distinct cytoplasmic determinants in order to recycle efficiently (14). In addition to requiring a cytoplasmic sorting determinant, sequence-dependent recycling of the β2AR differs from default recycling in its dependence on an intact actin cytoskeleton and its regulation by the conserved endosomal sorting protein Hrs (hepatocyte growth factor receptor substrate) (11,14). Compared to the present knowledge regarding protein complexes that mediate sorting of GPCRs to lysosomes (15,16), however, relatively little is known about the biochemical basis of sequence-directed recycling or its regulation.

The β2AR-derived 'recycling sequence' conforms to a canonical PDZ (PSD-95/Discs Large/ZO-1) protein-binding determinant (henceforth called PDZbd), and PDZ-mediated protein association(s) with this sequence appear to be primarily responsible for its endocytic sorting activity (17-20). Fusion of this sequence to the cytoplasmic tail of the δOR effectively re-routes endocytic trafficking of engineered receptors from lysosomal to recycling pathways, establishing the sufficiency of the PDZbd to function as a transplantable sorting determinant (18). The β2AR-derived PDZbd binds with relatively high specificity to the NHERF/EBP50 family of PDZ proteins (21,22). A well-established biochemical function of NHERF/EBP50–family proteins is to associate integral membrane proteins with actin-associated cytoskeletal elements. This is achieved through a series of protein-interaction modules linking NHERF/EBP50–family proteins to ERM (ezrin-radixin-moesin) –family proteins and, in turn, to actin filaments (23-26). Such indirect actin connectivity is known to mediate other effects on plasma membrane organization and function (23), however, and NHERF/EBP50–family proteins can bind to additional proteins potentially important for endocytic trafficking of receptors (23,25).

Thus it remains unclear if actin connectivity is itself sufficient to promote sequence-directed recycling of GPCRs and, if so, if such connectivity recapitulates the normal cellular regulation of sequence-dependent recycling. In the present study we took advantage of the modular nature of protein connectivity proposed to mediate β2AR recycling (24,26), and extended the opioid receptor fusion strategy used successfully for identifying diverse recycling sequences in GPCRs (27-29), to address these fundamental questions.

Here we show that the recycling activity of the β2AR-derived PDZbd can be effectively bypassed by linking receptors to ERM family proteins in the absence of the PDZbd itself. Further, we establish that the protein connectivity network can be further simplified by fusing receptors to an interaction module that binds directly to actin filaments. We found that bypassing the PDZ-mediated interaction using either domain is sufficient to mimic the ability of the PDZbd to promote efficient, actin-dependent recycling of receptors. Hrs-dependent regulation, however, which is characteristic of sequence-dependent recycling of wild type receptors, was recapitulated only by the fused PDZbd and not by the proposed downstream interaction modules. These results support a relatively simple architecture of protein connectivity that is sufficient to mimic the core recycling activity of the β2AR-derived PDZbd, but not its characteristic cellular regulation. Given that an increasing number of GPCRs have been shown to bind PDZ proteins that typically link directly or indirectly to cytoskeletal elements (17,27,30-33), the present results also suggest that actin connectivity may represent a common biochemical principle underlying sequence-dependent recycling of various GPCRs.

3.3 Experimental Procedures

DNA Constructs- All receptor constructs studied were created from a FLAG-tagged version of the murine δOR cloned into pcDNA3.0 (Invitrogen), described previously (12). A carboxyl-terminal fusion of the distal 10 residues derived from the β2AR tail, which contains the PDZbd, was also described previously (18) and is called δOR-PDZbd in the present study. The ERM protein-binding domain (Ebd) was isolated from EBP50 (also called human NHERF1, accession # O14745, generously provided by A. Bretscher) by PCR-mediated amplification of the sequence encoding the carboxyl-terminal 39 residues. The actin-binding domain (Abd) was generated by chemical synthesis of a sequence encoding the 34-residue modular domain defined in a carboxyl-terminal portion human ezrin (26). In both cases, appropriate stop codon and linker sequences were added to facilitate ligation to the Srf1 / Xba1 sites present in the sequence encoding the distal carboxyl-terminal cytoplasmic domain of the FLAG-δOR construct. The δOR-Abdt[6] construct was generated by adding a stop codon at the –6 position in the δOR-ABD construct, using oligonucleotide site-directed mutagenesis (QuickChange, Stratagene), a mutation that disrupts actin binding to the fused Abd (26). FLAG- δOR-GFP was constructed using PCR amplification of the FLAG- δOR coding sequence together with AgeI/HindIII appendages, followed by ligation in frame into pEGFP-N1 (Clontech). The GFP-Hrs construct was created using PCR amplification of a Myc-tagged construct described previously (34) and generously provided by Harald Stenmark (Norwegian Radium Hospital). EcoRI/XbaI appendages were added to facilitate ligation into pEGFP-

C2 (Clontech), effectively replacing the N-terminal Myc tag with EGFP. All constructs were verified by dideoxynucleotide sequencing (Elim Biopharmaceuticals, Inc.).

Cell Culture and Transfections- Human embryonic kidney 293 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (University of California San Francisco Cell Culture Facility). Cells plated in 6-well plates were transfected at ~50% confluency using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Stably transfected cells were selected in 500 μg/mL Geneticin (Life Technologies, Inc.), and cell clones expressing FLAG-tagged receptor constructs were chosen at closely similar levels based on average surface immunofluorescence/cell measured through flow cytometry, and found to have at least 75% of cells expressing surface immunoreactivity (the minor proportion of cells lacking substantial immunoreactivity was excluded in subsequent analyses). Receptor expression was quantified by whole-cell radioligand binding using [^{3}H]-diprenorphine (Amersham) as described previously (18). Cell clones selected for further study expressed receptors in the range of 0.2 – 1 pmol / mg cell protein. For immunofluorescence studies of receptor trafficking in transiently-transfected cells, cells were transfected as above, plated onto coverslips in a 12-well plate 24 hr post transfection, and experiments were conducted 48-72 hr post transfection. In experiments requiring coexpression of GFP-Hrs, cell clones expressing the indicated receptor construct were transiently transfected at ~50% confluency, as above, except using the GFP-Hrs construct. Cells were split into 12 well plates 24 hours after transfection, and used for the preparation of flow cytometry samples 24 hours thereafter.

Fluorescence Microscopy- FLAG-tagged receptors present in the plasma membrane of living cells were labeled with anti-FLAG monoclonal antibody (M1, Sigma) as described previously (18). Endocytosis of labeled receptors was promoted by adding 10 μM [D-Ala2, D-Leu5]-Enkephalin (DADLE, Research Biochemicals) to the culture medium and incubating cells at 37 °C for 25 minutes. Recycling was assessed by carrying out a medium change after DADLE incubation, and incubating cells at 37 °C for an additional 45 minutes in the presence of 10 μM of the opioid antagonist naloxone (Research Biochemicals, used to block residual agonist activity in the culture medium). Following the indicated incubations, specimens were fixed (4% formaldehyde in PBS) and permeabilized (0.1% Triton-X100 in PBS), then antibody-labeled receptors were visualized by incubation with goat anti-mouse IgG conjugates (Invitrogen) linked to AlexaFluor594 (wide field microscopy), AlexaFluor555 (scanning confocal microscopy), or AlexaFluor488 (transferrin colocalization). For visualizing internalized transferrin receptors, 10 μg/ml Texas Red-labeled diferric transferrin (Invitrogen) was added to serum-free culture medium together with DADLE. Wide field fluorescence images were collected using a Nikon Diaphot epifluorescence microscope with mercury arc lamp illumination and 60X/NA1.4 objective. Images were captured using a cooled CCD camera (Princeton Instruments) interfaced to a PC running MetaMorph acquisition and analysis software (Molecular Devices). Confocal images were acquired using a Zeiss LSM510 laser scanning microscope with 63x/NA1.3 objective, using instrument settings verified to produce negligible bleedthrough between channels, and an estimated section

thickness of 1 μm. Micrographs shown are representative optical sections imaged through the center of the cell.

Fluorescence Flow Cytometry- FLAG-tagged receptors present in the plasma membrane were labeled as described previously (18), except that M1 anti-FLAG antibody was conjugated with AlexaFluor647 (Invitrogen) rather than AlexaFluor488 to reduce background autofluorescence in the subsequent analysis. The mean fluorescence of 5,000-20,000 cells/sample was determined using a FACSCalibur instrument and CellQuest Software (BD Biosciences). The mean fluorescence calculated from triplicate samples was normalized to reflect the relative change in surface receptor pools and these levels were averaged from at least 3 experiments per cell clone analyzed. The percentage of receptor recycling occurring after agonist washout was then calculated from mean surface receptor fluorescence values (F) as follows: % recycling = ($F_{washout}$ - $F_{agonist\text{-}treated}$)/($F_{untreated}$ - $F_{agonist\text{-}treated}$) x 100. For analysis of recycling under conditions of cytoskeletal disruption, cytochalasin D (Sigma) was administered in culture medium at a 1:500 dilution from a 1 mg/mL stock in DMSO, which was added 20 minutes prior to incubation with the indicated opioid ligands. Vehicle control was accomplished by adding 1:500 DMSO alone. To evaluate the Hrs-sensitivity of GPCR recycling, 10,000-20,000 cells expressing the indicated receptor construct together with GFP-Hrs (generated as described above) were analyzed. Labeled receptors (AlexaFluor647) and Hrs (EGFP) were quantified simultaneously using separate laser excitations and detection channels in which negligible bleedthrough was verified using singly labeled specimens. This strategy produced a population of cells with uniform levels of receptor expression

(based on stable transfection) and variable levels of Hrs over-expression (based on transient transfection). In the analysis, this range of GFP-Hrs expression was divided into two cell populations based on EGFP fluorescence intensity (regions R2 and R3, see Fig. 6A). These two internally-controlled groups were then analyzed for AlexaFluor647-labeled receptor fluorescence in triplicates, averaged within each group and drug treatment, and normalized to calculate independent recycling percentages as described above. The average percentage recycling calculated from region R3 was subtracted from that calculated from region R2 and averaged to assess dose-dependent inhibition of recycling by GFP-Hrs expression. Data shown represent mean determinations from 4-5 independent experiments per receptor-expressing cell clone. Graphing and statistical analysis was carried out using Prism (GraphPad, Inc.) software. Error bars represent the standard error of the mean determinations across the experiments.

Cosedimentation of Receptors with Purified F-Actin- Cytoplasmic actin purified from Acanthamoeba castellani, as previously described (35), was generously provided by R. D. Mullins and group. Monomeric actin was maintained at 4˚C at a concentration of 50 μM in 0.5 mM TCEP (0.1 mM $CaCl_2$, 0.2 mM ATP, 2 mM Tris pH 8.0) before dilution to 4 μM into KMEH buffer (10 mM HEPES, 50 mM KCl, 1 mM EGTA, and 1 mM $MgCl_2$ pH 7.0) supplemented with 0.04 mg/mL Bovine Serum Albumin. Incubation in this buffer at 25°C for 1 hour allowed polymerization into filaments, which were subsequently stabilized by addition of 4μg/mL phalloidin (Sigma). Extracts from HEK293 cells transiently transfected with the indicated FLAG-tagged receptor construct (or mock-transfected without added plasmid DNA) were prepared in KMEH

supplemented with 1% Triton-X 100, 20 mM dithiothreitol, and protease inhibitors (Complete EDTA-free cocktail, Roche Diagnostics). Extracts were clarified by microcentrifugation for 10 minutes at 20,000 x g followed by ultracentrifugation in a TLA 100 rotor (BD Biosciences) at 48,000 RPM for an additional 30 minutes. Total protein concentration in the supernatant was determined by a Bradford assay and adjusted as needed with KMEH lysis buffer to achieve equal concentrations (2 – 5 μg / mL depending on the individual experiment) of total protein. Extracts (50 μL) were then mixed with 50 μL F-actin mixture and left on ice for 10 minutes before sedimenting in a TLA 100 for 30 minutes at 48K RPM. 20 μL of supernatant was subsequently removed per sample for immunoblot analysis, and the pellet was cleared of remaining supernatant and washed in 100 μL KMEH lysis buffer before solubilizing by boiling for 5 min in 30 μL NuPAGE (Invitrogen) LDS sample buffer (500 mM Tris base, 8% lithium dodecyl sulfate, 40% glycerol, 2 mM EDTA) containing 0.25 M 2-mercaptoethanol, separated on 4–12% NuPAGE LDS gels (Invitrogen), transferred to nitrocellulose (BioRad), and blotted for FLAG-tagged receptors using 2.5 μg/mL M1 anti-FLAG antibody (Sigma) followed by secondary antibody incubation using sheep anti-mouse-HRP conjugate (1:3000 dilution, Amersham Biosciences). Detection was carried out using enzyme-linked chemiluminescence (SuperSignal, Pierce) and immunoreactive signals were analyzed using a FluorChem 8000 imaging system (Alpha Innotech Corporation).

Statistical analysis- Internalization and recycling percentages were calculated for each individual experiment and collected as replicates in a Prism spreadsheet for statistical and graphing analysis (Graphpad, Inc.). Mean percentages were compared between receptors

by ANOVA and analyzed post-hoc for pair-wise differences using the Bonferroni Multiple Comparisons test with a significance level of 0.05. Mean recycling was compared between DMSO and cytochalasin D treated cells, per receptor expressed, by Student's t-test at a significance level of 0.05, whereas comparison of recycling means between cells expressing low and high amounts of GFP-Hrs in the same sample were compared for each receptor using a paired t-test with a significance level of 0.05.

3.4 Results

The ERM protein-binding domain conserved in NHERF-/EBP50–family proteins is sufficient to promote efficient recycling when fused to the δOR. The β2AR-derived PDZbd is sufficient, when fused to the carboxyl-terminal cytoplasmic domain of the δOR (δOR-PDZbd mutant receptor), to re-route endocytic trafficking of this distinct GPCR from its usual lysosomal fate to the rapid recycling pathway (18). To test the hypothesis that downstream protein connectivity to actin is sufficient to mediate this recycling activity, we applied the same receptor fusion approach to modular domains proposed to function downstream of the PDZbd (Fig. 1A). We first asked if it is possible to effectively bypass the PDZbd using only a conserved ERM protein-binding domain (ERMbd). To do so we fused the carboxyl-terminal 39-residues derived from EBP50 (human NHERF1), which fully includes the previously mapped ERMbd (24), to the δOR tail (δOR-ERMbd receptor fusion protein, Fig. 1B).

We compared the trafficking behavior of the δOR-ERMbd to that of FLAG-tagged versions of the wild-type δOR and δOR-PDZbd constructs described previously (18). In the absence of agonist (control), tagged receptors labeled with anti-FLAG monoclonal antibody were observed in a peripheral pattern indicative of plasma membrane localization, demonstrating that all three tagged receptors were effectively delivered to the plasma membrane (Fig. 1C, left column). Within 25 minutes after adding the opioid agonist DADLE to the culture medium, labeled receptors redistributed to a punctate intracellular pattern, indicating that each of the constructs was able to undergo rapid agonist-induced endocytosis (Fig. 1C, middle column). 45 minutes after agonist

removal from the culture medium (Fig.1C, right column), wild type δOR remained predominantly in intracellular vesicles (top row), consistent with its failure to recycle efficiently. The δOR-PDZbd fusion receptor, as expected, returned to a predominantly plasma membrane localization pattern (middle row). Significantly, the δOR-ERMbd fusion receptor also returned to a predominantly plasma membrane localization pattern after agonist washout (bottom row), which was indistinguishable from efficient recycling of the δOR-PDZbd fusion receptor and visibly different from the endosomal retention observed for the wild type δOR. This selective return of both the δOR-PDZbd and δOR-ERMbd fusion receptors to the plasma membrane was emphasized in confocal optical sections imaged through the middle of the cell (Fig. 1D). Together, these observations suggest that the ERMbd is indeed sufficient to promote recycling of receptors in the absence of the PDZbd itself.

To quantify the trafficking properties of engineered receptors, a previously established flow cytometric assay was applied that allows evaluation of receptor trafficking in a large population of cells and in the absence of bound antibody (33). Substantial agonist-induced internalization of all receptor constructs tested was confirmed by the pronounced reduction in surface receptor immunoreactivity observed following incubation of cells in the presence of 10 μM DADLE for 25 min (Fig. 1E) (36). Assay of surface receptor recovery after agonist washout established clearly that fusion of the ERMbd promoted recycling of receptors, as indicated by a nearly complete recovery of surface receptors that was indistinguishable in magnitude from that produced by fusion of the PDZbd itself (Fig. 1F). The statistical significance of ERMbd-promoted recycling,

and its quantitative similarity to that promoted by the PDZbd, was confirmed by statistical analysis of flow cytometric data across multiple experiments (legend to Fig. 1).

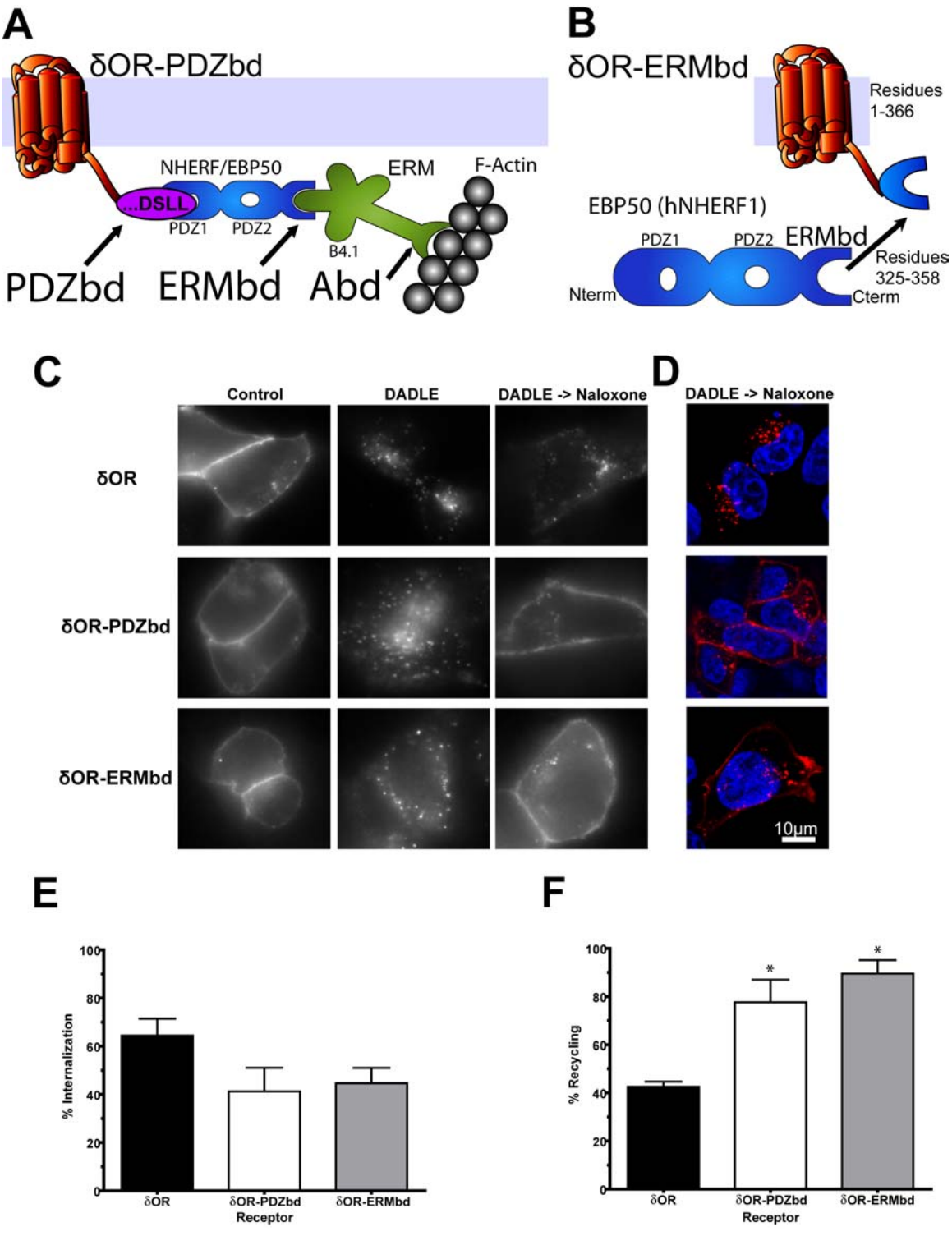

Figure 1: The conserved ERM-binding domain (ERMbd) derived from NHERF/EBP50 proteins is sufficient to promote endocytic recycling of the δOR when fused to the C-terminus.

(A) Schematic of the δOR-PDZbd fusion receptor containing the PDZ domain-interacting sequence and its proposed actin connectivity via the ERM protein-binding domain (ERMbd) present in PDZ-linked NHERF/EBP50 proteins and the F-actin-binding domain (Abd) present in ERM proteins. **(B)** Schematic of the δOR-ERMbd fusion receptor. **(C)** FLAG-tagged δOR, δOR-PDZbd, and δOR-ERMbd constructs were transiently expressed in HEK293 cells and surface-labeled with anti-FLAG monoclonal antibody. Cells were incubated in the absence of agonist (control), in the presence of 10 μM DADLE for 25 minutes (DADLE), or with 10 μM DADLE followed by washout and subsequent incubation for 45 minutes in the presence of 10 μM naloxone (DADLE->Naloxone). Cells were fixed and stained under permeabilized conditions to track the endocytic fate of surface-labeled receptors. **(D)** Representative confocal sections of receptor localization in cellular cross-sections following agonist washout. All images shown are representative of at least 4 independent experiments. **(E)** Agonist-induced internalization of FLAG-tagged receptors was quantified by flow cytometric assay of stably-transfected HEK293 cells by calculating the decrease in surface receptor immunoreactivity produced by incubation of cells in the presence of 10 μM DADLE for 25 minutes, as described in *Experimental Procedures*. **(F)** Recycling of FLAG-tagged receptors was quantified using the flow cytometric assay to determine the subsequent recovery of surface receptor immunoreactivity 45 minutes after agonist washout. Error bars reflect the standard error of the mean of at least 3 independent experiments. *denotes $p<0.05$ in Bonferroni post-hoc analysis relative to the δOR, additional analysis of the δOR-PDZbd relative to the δOR-ERMbd yielded $p>0.05$.

Direct association of receptors with the actin cytoskeleton is sufficient to promote efficient recycling. Having established that the isolated ERMbd promotes recycling of engineered receptors, we next tested the sufficiency of direct receptor connectivity to actin. A conserved F-actin-binding domain (Abd) has been mapped to the carboxyl-terminal 34 residues of human ezrin, and shown to confer specific actin binding in vitro when fused to the carboxyl-terminus of glutathione-S-transferase (26). Accordingly, we fused this sequence to the carboxyl-terminus of the δOR (δOR-Abd fusion receptor, Fig.

2A) and assessed effects on endocytic trafficking. Visualization of antibody-labeled δOR-Abd fusion receptors by fluorescence microscopy (Fig. 2B, middle row of images) revealed surface targeting and agonist-induced internalization similar to that of wild type receptors (left and middle panels). Remarkably, the δOR-Abd returned almost completely to the plasma membrane after agonist washout, suggesting that the isolated Abd indeed promotes receptor recycling similar to that mediated by both the PDZbd and ERMbd fusions. This was evident both in wide field micrographs (Fig 2B, middle, right image) and in confocal optical sections (Fig. 2C, middle row), and stood in marked contrast to the limited recycling observed for the wild type δOR (Fig. 1C). Two additional observations confirmed the specificity of recycling directed by the Abd. First, fusing a larger protein domain (full length EGFP) to the δOR tail (δOR-GFP fusion receptor) did not promote detectable recycling (Fig. 2B and C, top row of images). Second, deleting 6 residues from the extreme carboxyl-terminus of the Abd (δOR-Abdt[6] mutant receptor), which was shown previously to disrupt binding to actin filaments (26), abrogated the recycling activity of the Abd (Fig. 2B and C, bottom row).

These observations were quantified in stably transfected cells using the fluorescence flow cytometric assays of agonist-induced internalization (Fig. 2D) and recycling after agonist removal (Fig. 2E). Fusion of the Abd produced a pronounced increase in receptor recycling, which was similar in magnitude to that produced by the PDZbd or Ebd, and statistically significant when compared to the wild type δOR ($p<0.001$, Fig. 2E). Together, these results indicate that the Abd is itself sufficient to promote efficient recycling when fused to the δOR.

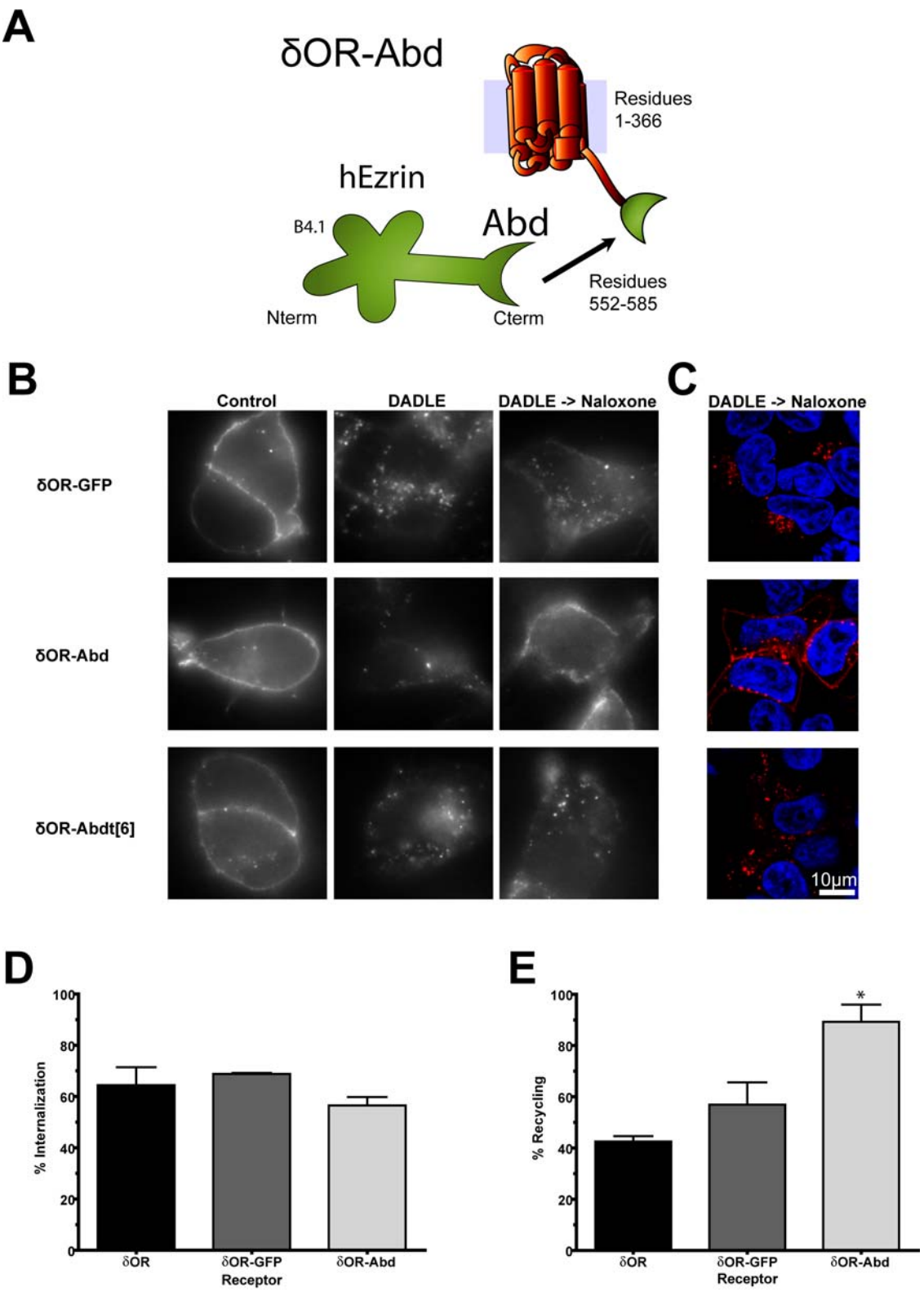

Figure 2: The conserved actin-binding domain (Abd) derived from ERM proteins is sufficient to promote endocytic recycling.

(A) Schematic of the δOR-Abd fusion receptor. **(B)** FLAG-tagged δOR-GFP, δOR-Abd, and δOR-Abdt[6] constructs were transiently expressed in HEK293 cells and surface- labeled with anti-FLAG monoclonal antibody. Cells were incubated and processed under the same conditions as described in Figure 1. **(C)** Confocal optical sections showing receptor localization following agonist washout. Images represent mid-focal planes and are representative of at least 4 independent experiments. Flow cytometric measurement of agonist-induced internalization **(D)** and recycling after agonist removal **(E)** were determined using the same procedure as described in Figure 1 (data for the δOR are re-displayed for comparison). Error bars reflect the standard error of the mean of at least 3 independent experiments. *denotes $p<0.05$ in Bonferroni post-hoc analysis relative to the δOR.

Recycling promoted by engineered protein connectivity is F-actin-dependent. To further test the actin connectivity hypothesis, we sought to determine if the δOR-Abd fusion receptor can truly bind actin filaments directly. FLAG-tagged receptor constructs were expressed in HEK293 cells, solubilized using nonionic detergent, and binding of receptors to purified F-actin was determined using an in vitro co-sedimentation assay (see Experimental Procedures). Wild type δOR and δOR-Abd constructs were expressed at similar levels in solubilized extracts, as detected specifically by anti-FLAG immunoblotting (Fig. 3A). The heterogeneous electrophoretic mobility observed for both receptors is consistent with previous studies indicating that the wild type δOR resolves as a mixture of complex-glycosylated forms (37). After a 10-minute incubation with purified F-actin on ice, actin polymers pelleted by ultracentrifugation (detected by Ponceau S staining, lower panel in Fig. 3B) co-sedimented the δOR-Abd (as detected by anti-FLAG immunoblot, upper panel in Fig. 3B). In parallel samples loaded with identical amounts of receptor (Fig. 3A) and actin (lower panels in Fig. 3B), FLAG-δOR

was detected at much lower levels in the actin pellet. The ratio of δOR-Abd compared to δOR co-sedimentation, as estimated across 3 independent experiments by scanning densitometry, was 3.0 $\pm$ 0.71. Titration of receptor input within individual experiments verified concentration-dependent association of δOR-Abd with F-actin (Fig. 3C).

We next asked if recycling promoted by the defined interaction domains requires an intact actin cytoskeleton in intact cells. Flow cytometric analysis indicated that the small fraction of wild type δOR recycling observed after removal of DADLE from the culture medium was not detectably affected by depolymerization of actin filaments by cytochalasin D (Fig. 3D, left set of bars), consistent with actin-independent recycling of a small fraction of the internalized δOR by default (14). The considerably enhanced recycling of receptors mediated by fusion of the PDZ ligand (δOR-PDZbd), in contrast, was markedly inhibited by cytochalasin D. This was indicated by the statistically significant reduction of surface receptor recovery after agonist washout in cytochalasin D-treated cells compared to the vehicle (DMSO) -treated control cells (Fig. 3D, second set of bars). Recycling promoted by both the ERMbd and Abd fusions was similarly sensitive to cytochalasin D (third and fourth set of bars). These results further confirm the importance of F-actin for PDZ-dependent recycling of GPCRs, as established previously in studies of the wild type β2AR (17), and indicate that bypassing the PDZbd using either of the proposed downstream protein-interaction domains preserves this actin-dependence in intact cells.

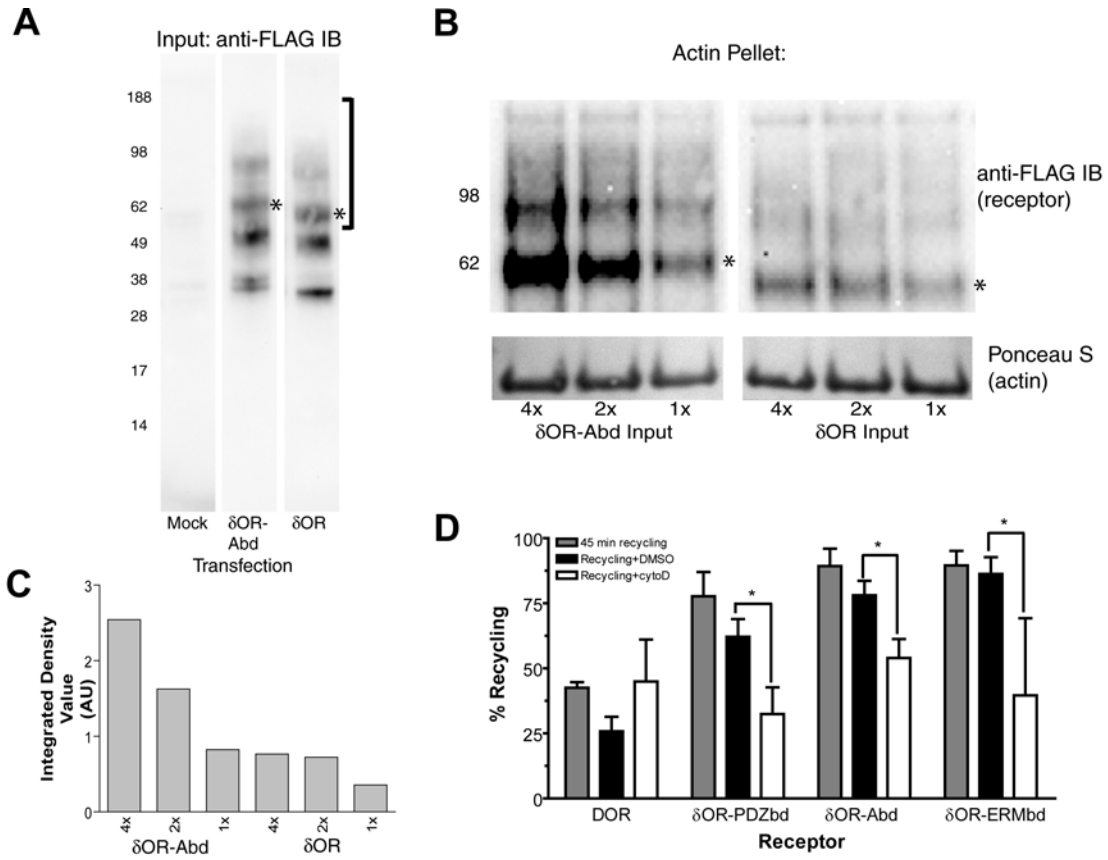

Figure 3: Verification of direct actin connectivity and actin-dependent recycling of engineered receptors.

(A) Anti-FLAG immunoblots of equal amounts (25 µg before clarification) of cell extracts prepared from mock-transfected HEK293 cells or cells transfected with either FLAG-tagged δOR or δOR-Abd fusion receptor. Bracket to the right of figure indicates the region of the blot shown in (B), and the band indicated by '*' indicates the species corresponding in electrophoretic mobility to the glycosylated receptor monomer. **(B)** Anti-FLAG immunoblot showing receptor (top panel) and Ponceau S stain showing actin (bottom panel) from actin co-sedimentation assay comparing δOR-Abd (left) and δOR (right) loaded in equal amount (as shown in A) and processed in parallel. **(C)** Background-corrected densitometry of the species corresponding to monomeric receptor. Data shown are representative of 3 independent experiments. **(D)** Flow cytometric analysis assessing the sensitivity of engineered receptor recycling to cytochalasin D. Recycling measured by flow cytometry in normal culture medium (gray bars) is displayed in comparison to that measured in the presence of vehicle (0.2 % DMSO, black bars) or 2 µg/mL cytochalasin D (cytoD,

white bars). *denotes $p<0.05$ by Student's t-test comparing vehicle-treated and cytochalasin D-treated cells, n=4-5 experiments.

Recycling promoted by either direct or indirect actin connectivity utilizes a similar membrane pathway. To further investigate the degree to which downstream actin connectivity mimics the recycling effect of the PDZbd, we compared the subcellular localization of engineered receptor constructs after agonist-induced endocytosis to that of internalized transferrin, which marks the shared recycling pathway traversed by the wild type β2AR (38). HEK293 cells were transfected with expression constructs encoding the FLAG-tagged δOR, δOR-PDZbd, δOR-ERMbd, or δOR-Abd, and surface-accessible receptors were labeled with anti-FLAG monoclonal antibody. Cells were then incubated at 37˚C in the presence of 10μM DAMGO to drive endocytosis of the GPCR, and with 10 μg/ml Texas Red-labeled transferrin (Tf-TR) to label endocytosed transferrin receptors in the same cells. After incubation at 37˚C for 40 minutes, a time period sufficient to achieve steady state labeling of transferrin receptors in the conserved recycling pathway (39), cells were fixed and the localization of the engineered receptors was compared by dual channel confocal microscopy (Fig. 4). Internalized δOR (top row of images, left panel) appeared in a vesicular pattern largely distinct from that of labeled transferrin (middle panel). This was confirmed in the merged image displaying δOR and transferrin in green and red, respectively (right panel). Examination of dual receptor localization at higher magnification (inset) emphasized that internalized δOR was present in endocytic compartments largely distinct from those mediating the conserved recycling pathway marked by internalized Tf-TR, while structures containing detectable amounts of both

receptors were relatively rare (an example is indicated by arrowhead in inset). Internalized δOR-PDZbd, in contrast, was visualized in a vesicular pattern more similar to that of Tf-TR (second row), with considerable overlap indicated by yellow structures observed in the merged color image (right, several examples are indicated by arrowheads in inset). Internalized δOR-ERMbd (third row) and δOR-Abd (fourth row) also exhibited substantial overlap with the shared recycling pathway. These observations indicate that fusion of either the ERMbd or Abd indeed promote trafficking of internalized receptors via a pathway similar to that promoted by the PDZbd, which overlaps significantly with the shared recycling pathway marked by transferrin receptors.

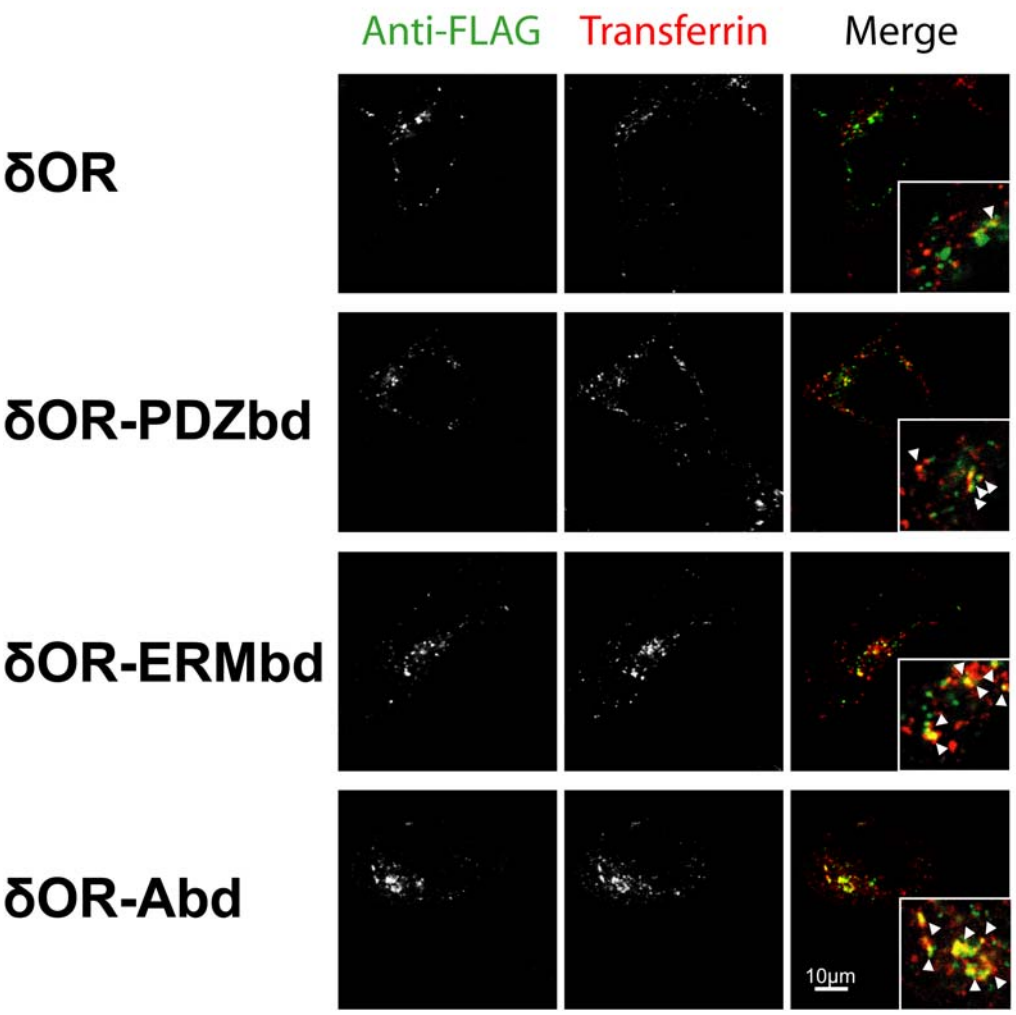

Figure 4: Engineered recycling sequences promote receptor trafficking via transferrin-containing endosomes.

The indicated δOR –derived constructs expressed in HEK293 cells were surface-labeled with M1 anti-FLAG monoclonal antibody, then cells were then incubated in the combined presence of 10 μM DADLE (to promote endocytosis of the labeled receptor constructs) and Texas Red-conjugated transferrin (to label endocytosed transferrin receptors marking the conserved recycling pathway). Cells were fixed and stained under permeabilized conditions with Alexa488-conjugated anti-mouse IgG and imaged by confocal fluorescence microscopy to selectively visualize internalized δOR –derived receptor constructs (green) and internalized transferrin (red) in the same cells. The corresponding merged images are shown to right, and insets show a region of the cytoplasm at 2.5x higher magnification in order to help distinguish endosomes labeled selectively with one receptor (appearing red or green) or co-labeled for both receptors (appearing

yellow, examples indicated by arrows). Images shown are representative of at 3 independent experiments for each receptor construct.

Downstream protein connectivity to actin fails to recapitulate Hrs-dependent regulation of recycling. Given that both the ERMbd and Abd promoted actin-dependent recycling with similarly high efficiency as the PDZbd, and did so via a similar vesicular pathway, we continued to investigate the degree to which downstream protein-interaction modules mimic PDZ-dependent recycling. Another characteristic feature of PDZ-dependent recycling of the β2AR is its regulation by Hrs (hepatocyte growth factor receptor substrate) (11), a conserved endosome-associating sorting protein (7,40). Over-expression of an epitope-tagged Hrs construct has been shown to inhibit PDZ-dependent recycling of the wild type β2AR, without detectably affecting 'default' recycling of either the transferrin receptor (40) or a mutant GPCR apparently devoid of all endocytic sorting determinants (11). Thus we examined the sensitivity of the engineered receptors to overexpression of Hrs. We first approached this question using dual channel, confocal microscopy to visualize trafficking of the δOR-PDZbd fusion receptor in stably transfected cells in which an EGFP-tagged Hrs construct (GFP-Hrs) was subsequently expressed by transient transfection. This allowed direct visualization of receptor trafficking in cells over-expressing tagged Hrs at various levels, as estimated by EGFP fluorescence intensity. We also noted that GFP-Hrs over-expression produced a visible increase in endosome diameter, characteristic of the dominant-negative inhibition of endosome function described previously (11,40). The δOR-PDZbd fusion receptor was targeted to the plasma membrane and exhibited rapid endocytosis upon addition of

DADLE, irrespective of GFP-Hrs expression level (Fig. 5A, left and middle panels). Following agonist washout, however, redistribution of the δOR-PDZbd fusion protein from endosomes to the plasma membrane was visibly reduced in cells over-expressing GFP-Hrs (Fig. 5A, right column, compare top and bottom panels). These observations suggested that Hrs over-expression inhibits recycling of the δOR-PDZbd fusion receptor, as it does the wild-type β2AR. A markedly different result was obtained in parallel experiments conducted on the δOR-ERMbd and δOR-Abd fusion receptors. While overexpression of GFP-Hrs again had no detectable effect on surface targeting or agonist-induced endocytosis (representative images of δOR-Abd – expressing cells are shown in Fig 5B, bottom row, left and middle columns), over-expression of GFP-Hrs, even at apparently high levels (as indicated by GFP fluorescence intensity and endosome enlargement), did not visibly interfere with recycling of either the δOR-ERMbd or δOR-Abd fusion receptors (δOR-Abd image Fig 5B, bottom right panel).

A pronounced difference in Hrs-sensitivity of the receptor constructs was confirmed quantitatively using a modification of the flow cytometric assay. Stably transfected cell clones expressing the indicated receptor were transiently transfected with the GFP-Hrs construct, producing a range of Hrs over-expression in cells expressing receptors uniformly (see Experimental Procedures). Receptor recycling was then analyzed simultaneously in two cell populations differing substantially in average fluorescence intensity of GFP-Hrs (region R2 vs. R3, respectively, Fig. 6A), allowing the inhibitory effect of increased Hrs expression to be assessed in an internally controlled manner. Inhibition of δOR-PDZbd recycling was substantially greater in cells expressing GFP-Hrs at higher levels (region R3) compared to lower levels (region R2) in the same

transfected samples (Fig. 6B, compare left and right bars), and recycling in both cell populations displayed a trend of inhibition relative to that observed in the parental cell clone not expressing GFP-Hrs (compare with Fig. 2B). By calculating the difference in receptor recycling observed in these internally controlled populations, dose-dependent inhibition of δOR-PDZbd recycling by Hrs overexpression was clearly confirmed. In contrast, increased expression of GFP-Hrs did not detectably inhibit recycling of the other engineered receptor constructs (Fig. 6C). Together these results indicate that, while both the ERMbd and Abd were sufficient to mimic the actin-dependent recycling activity of the PDZbd, neither of these proposed downstream interaction modules recapitulated Hrs-dependent regulation that is characteristic of PDZ-dependent recycling of both wild type β2AR (11) and engineered (δOR-PDZbd) receptors.

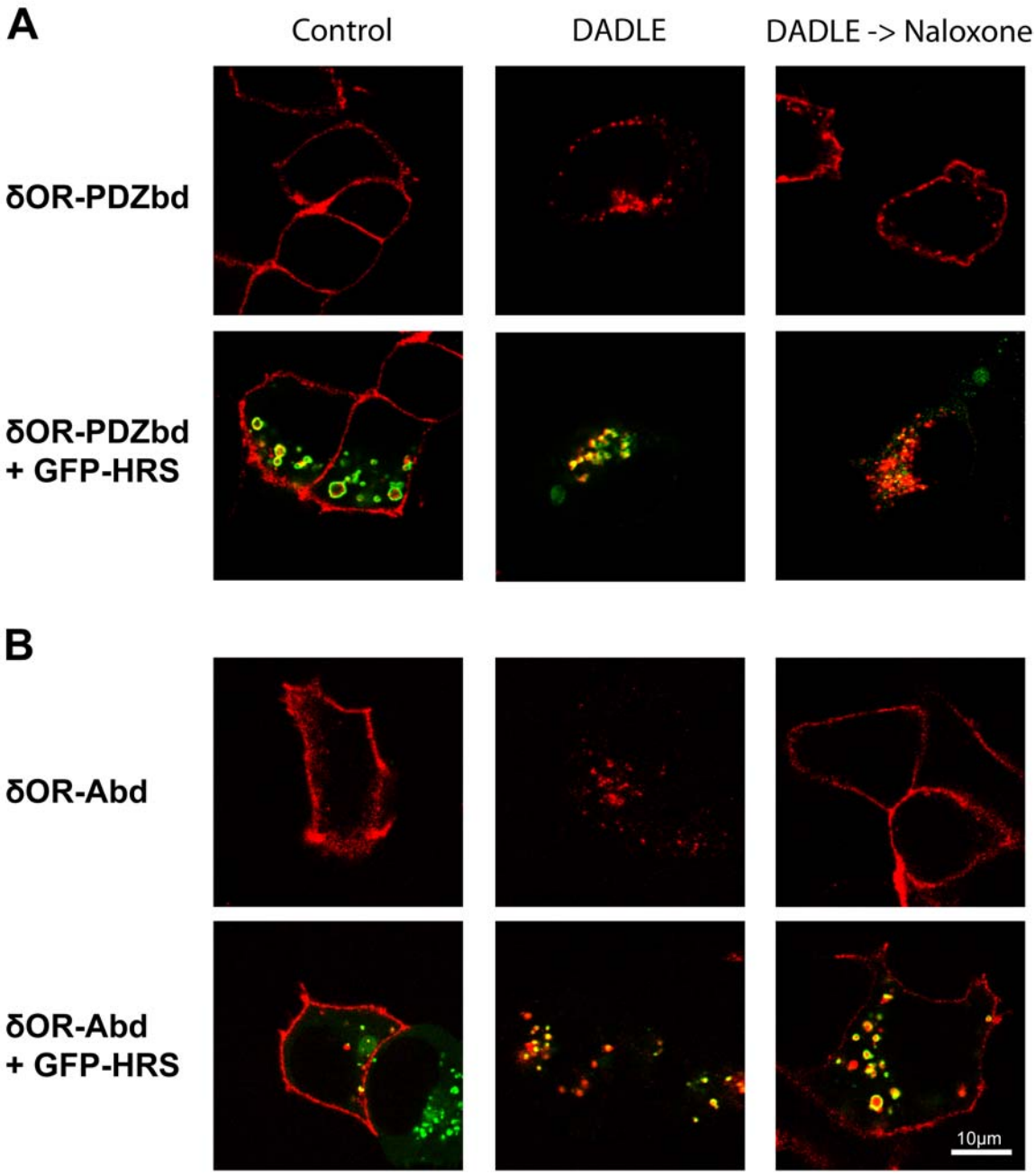

Figure 5: Receptors fused to distinct recycling sequences localize to Hrs-associated endosomes but differ in Hrs-dependent regulation of recycling.

(A) Confocal optical sections of cells showing the localization of labeled δOR-PDZbd (red) and co-expressed GFP-Hrs (green) in HEK293 cells incubated in the absence of ligand (control), in the presence of 10 μM DADLE for 25 minutes (DADLE), or with 10 μM DADLE followed by washout and subsequent incubation for 45 minutes in the presence of 10 μM naloxone (DADLE->Naloxone). Top panels show a

typical example of cells not expressing GFP-Hrs, and lower panels show a typical example of cells expressing GFP-Hrs at levels that produce visible endosome enlargement. (B) Similar experiments conducted in cells expressing the δOR-Abd. Images shown are representative of at least 4 independent experiments.

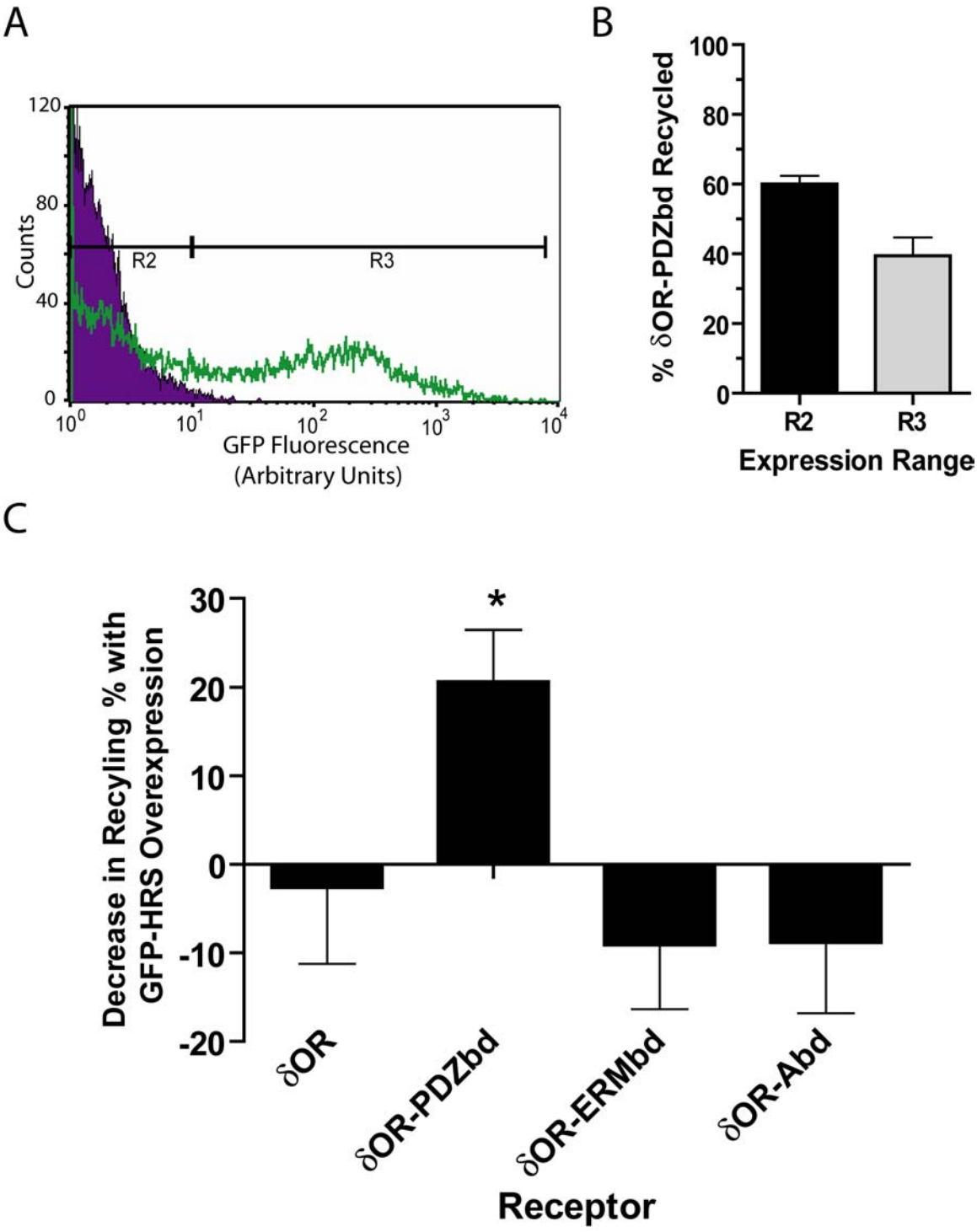

Figure 6: Hrs overexpression quantitatively inhibits recycling mediated by the PDZbd but not by the ERMbd or Abd.

(A) Dual label fluorescence flow cytometry was used to gate HEK293 cells stably transfected with the indicated FLAG-tagged receptor constructs and then transiently transfected with GFP-Hrs into two

populations (regions R2 and R3) differing in GFP fluorescence (indicating relative expression level of GFP-Hrs). Region R2 was defined according to the distribution of mock-transfected cells (purple distribution in the histogram shown), while region R3 was selected to exclude most of these cells and to include cells expressing GFP-Hrs over an approximately 100-fold range (green distribution). Data shown for calibration were derived from analysis of 15,000 mock-transfected and 15,000 GFP-Hrs –transfected cells. **(B)** Recycling of δOR-PDZbd measured in parallel in the indicated cell populations, confirming diminished surface recovery in the population over-expressing GFP-Hrs. Data represent results from 4 independent experiments, in which each recycling determination was made in triplicate samples containing 10-20,000 cells each. **(C)** Hrs dose-dependent regulation of recycling was determined by calculating the difference between surface recovery of the indicated receptor constructs measured in parallel in cells expressing relatively low (region R2) and high (region R3) levels of GFP-Hrs. Results are compiled from at least 4 independent experiments per receptor construct. Error bars represent the standard error of the mean difference in surface receptor recovery across experiments. *denotes $p<0.05$ in a paired t-test of recycling means between regions R2 and R3 for the indicated receptor.

3.5 Discussion

The present results indicate that a modular ERM protein-binding domain (ERMbd) conserved in NHERF/EBP50-family proteins, as well as an actin-binding domain (Abd) conserved in ERM proteins, is sufficient to promote recycling of an engineered GPCR with similarly high efficiency as the PDZbd derived from the β2AR. Because the recycling activity of the PDZbd can be effectively bypassed by either of these protein-interaction modules mediating the proposed downstream connectivity, and in the absence of any other known functional domains present in NHERF/EBP50 or ERM proteins, the present results suggest that a relatively simple architecture of actin connectivity is truly sufficient to promote sequence-directed recycling. Of particular interest, fusion of the Abd by itself was sufficient to promote efficient recycling of receptors, and we confirmed biochemically that the engineered receptor was indeed capable of direct binding to actin filaments. We further verified that recycling promoted by all of the interaction modules exhibited an actin-dependence that is characteristic of PDZ-dependent recycling of the wild type β2AR (17). Actin connectivity is known to affect the mobility of various signaling receptors in the plasma membrane (41) and can influence the clathrin-dependent endocytic mechanism (42) but, to our knowledge, the present results are the first to show that direct actin connectivity is sufficient to promote recycling of GPCRs after endocytosis.

An important question for future study, therefore, is precisely how protein connectivity to actin filaments mediates this endocytic sorting function. In principle, receptor linkage to actin structures could control the movement of individual GPCR-

containing endocytic vesicles directed back to the plasma membrane. Alternatively, actin linkage could influence the lateral partitioning of receptors in the endosome membrane, or bring endocytosed receptors in proximity to other proteins that subsequently dictate sorting fate (16).

While fusion of either the ERMbd or Abd promoted receptor recycling with similarly high efficiency as the PDZbd, 'bypassing' the proximal PDZ interaction using the engineered receptor approach did not fully recapitulate the features of PDZ-dependent recycling. In particular, manipulating the cellular concentration of the endosomal sorting protein Hrs, which is known to regulate recycling of the wild type β2AR (11), affected recycling of engineered receptors promoted by the PDZbd but not that promoted by the ERMbd or Abd. Thus, while the present results argue strongly that ERM-actin linkage is a sufficient basis for a core mechanism of sequence-dependent recycling, it is likely that additional regulation of recycling occurs physiologically at the level of the PDZ protein itself. It will be interesting in future studies to investigate the potential role of various PDZ proteins and non-ERM protein interactions with NHERF/EBP50 family members (22,23,25) in regulating, and perhaps conferring additional specificity on, PDZ-dependent recycling that occurs in a native cellular context (20, 43).

Considering that many PDZ proteins link directly or indirectly (via additional protein interactions) to actin (44), it is tempting to speculate that actin connectivity might play a rather general role in promoting GPCR recycling by various PDZ-linked protein complexes. We note, for example, that hScrib/ßPIX/GIT1-dependent recycling of thyrotropin receptors is regulated by the GTPase Arf6 and AKAP79/SAP97-dependent recycling of beta-1 adrenergic receptors involves PKA-mediated phosphorylation of the

receptor (30,31). Thus, if actin connectivity contributes to recycling via these alternative PDZ-linked protein complexes, it is likely that there exists additional cellular regulation dependent on the proximal PDZ domain-mediated interaction with the receptor that occurs naturally. Also, given that PDZ-dependent recycling of GPCRs occurs in diverse cell types, including neurons (43) and cardiac muscle cells (20), the possibility that distinct PDZ-linked complexes also confer cell type-specific regulation merits future study. It is also interesting to note that efficient recycling of the alpha-1b adrenergic receptor, although not directed by a PDZ-mediated protein interaction, is actin-dependent and requires an ERM protein-binding domain present in the receptor (33). Thus, it seems likely that actin connectivity represents a biochemical principle of receptor recycling that is deeply conserved.

In summary, the present results define a relatively simple network of protein connectivity that is sufficient to promote the core function of sequence-dependent recycling in the absence of a natural GPCR-derived recycling sequence. While indirect protein connectivity to actin likely represents a fundamental and conserved biochemical principle of sequence-dependent recycling, the present results also establish that such connectivity is not sufficient to fully recapitulate the cellular regulation observed in the endocytic trafficking of naturally occurring GPCRs.

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Chapter 4:

An Unexpected Requirement for Sorting Nexin 27 in PDZ-Mediated Recycling of the β2 Adrenoceptor in HEK293 Cells

B.E.L. Lauffer contributed work towards all figures and the writing of this text under the supervision of M. von Zastrow. Cristina Melero-Heras additionally contributed extensive work towards development and analysis of the knockdowns, and performed the majority of protein purification and biochemical analysis of PDZ domains. Tanja Kortemme provided additional advising on the progression of this project, and the SNX27 engineering work found in appendix III.

4.1 Summary

The majority of cellular response to extracellular stimuli is mediated through G protein-coupled receptors, receptors that are actively regulated by endocytic trafficking. Endocytic recycling of a model subtype, the β2 adrenergic receptor (β2AR), is dependent on its intracellular C-terminus in a process that is largely unclear. This distal C-terminus corresponds to a Post-Synaptic-Density-95/Discs-Large/Zonus-Occludens-1 (PDZ) interacting motif, or ligand. This PDZ ligand interacts with PDZ domains found in Na+/H+-Exchange-Regulatory-Factors (NHERF proteins). Two of these NHERF proteins indirectly link to Actin filaments, and we have previously shown the sufficiency of this connectivity network to promote recycling of a distinct receptor. Yet the relative necessities of these or any other proteins for β2 adrenergic receptor recycling have not been established. We have addressed this issue by examining the recycling of the β2 adrenergic receptor in HEK293 cells depleted of candidate PDZ proteins through the use of RNA interference. Our results surprisingly reveal a relative insensitivity of recycling to depletion of NHERF 1 and/or 2, whereas recycling is severely impaired in cells depleted of a NHERF PDZ relative, Sorting Nexin 27. We additionally show the capacity of this protein to engage in a previously-unreported PDZ interaction with the β2AR C-terminus, and localize to β2AR-containing endosomes. Recycling mediated by Sorting Nexin 27 is impaired by mutation of its PDZ domain, and recycling promoted by a distinct sequence not recognizable as a PDZ ligand is insensitive to depletion of Sorting Nexin 27. These results indicate that PDZ interactions between this protein and the β2AR mediate recycling that was attributed to other NHERF proteins.

4.2 Introduction

The sorting of endocytic components mediates a variety of cellular functions including nutrient uptake and the regulation of signal transduction. The largest class of pharmacologically-relevant, signaling receptors are the G protein-coupled receptors (GPCRs), which often undergo endocytosis in response to activation by a variety of agonists (Lefkowitz 2007). Upon traversing the same endosomes, different GPCR subtypes can preferentially sort into a recycling pathway destined for the cell surface or to a degradative fate in a lysosome (Tsao 2000). These fates influence opposite effects on subsequent cellular responsiveness my modulating functional, cell surface receptor numbers. In addition, these pathways allow for other spatiotemporal control of signaling events that are only beginning to be realized (von Zastrow and Sorkin, 2007). A major obstacle to our comprehensive understanding of these relationships is a lack of knowledge in the mechanisms underlying sorting of specialized membrane cargo like signaling receptors.

The mechanisms initiating endocytosis of GPCRs are relatively well known. Stabilization of receptors into active conformations by agonist binding stimulates G protein signal transduction while also making receptors good substrates for GPCR kinases. Arrestin proteins with affinity for phospho-receptors are recruited and provide a platform for switching signal transduction pathways and targeting receptors to clathrin-coated pits. From here, ubiquitination and various non-covalent interactions can additionally regulate events leading to the endosome and beyond (see (Lefkowitz 2007) and (Breann L. Wolfe 2007) for review). Indeed, ubiquitination and protein-protein

scaffolding at endosome membranes are integral to the lysosomal-targeting process (Hanyaloglu and von Zastrow 2008; Marchese, Paing et al. 2008). Much less is known about the molecular players and events directing GPCRs into the recycling pathway.

Classical studies of nutrient uptake into cells have identified recycling routes for nutrient receptors that follow the bulk majority of endocytosed lipid back to the plasma membrane. Receptors in this 'default' pathway recycle with similar kinetics to labeled lipids and seemingly lack a requirement for protein adaptors (Gruenberg and Maxfield 1995; Mukherjee, Ghosh et al. 1997). Recycling of the G protein-coupled, β2 adrenergic receptor (β2AR) on the other hand, revealed a new recycling paradigm when it was identified to require sequence at its intracellular, C-terminus for efficient recycling (Cao, Deacon et al. 1999). Since then, numerous recycling sequences have been identified in GPCRs that suggest the existence of almost as many adaptor proteins linking the receptors into a regulated, recycling pathway (or pathways) (Hanyaloglu and von Zastrow 2008). By and large, these adaptors remain unknown.

Recycling of the β2AR requires a PDZ (for the PSD-95, Discs Large, and ZO-1 founding domain members) ligand found at its intracellular C-terminus (Cao, Deacon et al. 1999). This distal sequence of DSLL constitutes a minimal ligand that can reroute δ opioid receptors from a lysosomal to a recycling pathway, though upstream sequence can additionally contribute to PDZ specificity and recycling function (Gage, Kim et al. 2001; Tonikian, Zhang et al. 2008). While a growing subset of GPCR recycling sequences are recognizable PDZ ligands, there appear to be separate PDZ-containing protein complexes responsible for the recycling of individual receptors (Lahuna, Quellari et al. 2005; Gardner, Tavalin et al. 2006). The PDZ adaptors proposed to mediate recycling of the

β2AR were the Ezrin-binding phosphoprotein of 50 kDa (EBP50) and, by homology, the Na+/H+ Exchanger 3, Kinase A regulatory protein (E3KARP), also known as NHERF1 and 2 for their redundant roles as factors in the aforementioned exchange regulation. These proteins link receptors and other membrane proteins to the actin cytoskeleton through their interaction with the ezrin-radixin-moesin (ERM) family (Bretscher, Chambers et al. 2000). We have recently shown that this network of protein connectivity is sufficient to drive recycling of the lysosomally-targeted, δ opioid receptor when downstream interaction domains are fused to the C-terminus (Lauffer, Chen et al. 2008). And recycling of these chimeric receptors, along with that of the wild type β2AR, is inhibited by toxins disrupting the actin cytoskeleton (Cao, Deacon et al. 1999; Tanowitz and von Zastrow 2003; Lauffer, Chen et al. 2008). While connectivity to actin might represent a fundamental downstream function of many recycling complexes, elucidation of the relevant complexes for particular receptors is paramount in validating this and revealing physiologic modes of regulation.

Despite the evidence for NHERF proteins in mediating β2AR recycling is additional work implicating a non-PDZ-domain-containing protein, the N-ethylmaleimide-Sensitive Factor (NSF), to be the relevant adaptor. This protein binds competitively with the NHERFs to the same β2AR PDZ ligand, though with different β2AR residue sensitivities (Cong, Perry et al. 2001; Gage, Matveeva et al. 2004; Wang, Lauffer et al. 2007). Since the β2AR PDZ ligand is not completely conserved between rodents and humans (most rodents possess a DSPL sequence), the murine β2AR recycling sequence does not pull down NSF from tissue extracts like it does EBP50

(Wang, Lauffer et al. 2007). While NSF does appear to be regulating β2AR trafficking, the *required* adaptors for β2AR recycling in any tissue have remained speculative.

In this study, we have overcome the caveats of potentially pleiotropic mutational approaches by examining β2AR recycling during RNA silencing of candidate, PDZ domain-encoding transcripts in the established Human Embryonic Kidney (HEK) 293 cell culture model. Surprisingly, we found that drastic reductions in either NHERF1 and/or 2 protein levels produced negligible effects on receptor recycling, which led us to consider contributions of other PDZ domain-containing proteins. A sorting nexin with an implicated role in cocaine sensitization, SNX27, stood out due to its possession of a PDZ domain with overlapping specificity to that of NHERF1 and its localization to endosomes (Kajii, Muraoka et al. 2003; Joubert, Hanson et al. 2004). We found sorting nexin 27 to colocalize with internalized β2ARs and exhibit PDZ compatibility with the β2AR C-terminus. Strikingly, β2AR recycling was almost completely abolished when SNX27 protein levels were reduced with siRNA transfection. This recycling defect could be rescued by cotransfection of the rat ortholog, and PDZ mutation of this ortholog would impair that ability to rescue recyling. In addition, a 'swap' of the β2AR PDZ ligand for a distinct recycling sequence not recognizable as a PDZ ligand rendered β2AR recycling insensitive to SNX27 protein levels. These results indicate that a previously unreported interaction between SNX27 and the β2AR serves as a specialized and required step in mediating endocytic recycling.

4.3 Materials and Methods

Construcs and special reagents. Information on the receptors used can be found in the *cell line and transfection* section below. Human EBP50/NHERF1 cDNA (accession # O14745) was generously provided by Anthony Bretscher (Cornell University) and subcloned from pcDNA3.0 (Invitrogen) into pEGFP-C3 (Clontech) using XmnI and HindIII digestion and subsequent ligation of the new fragments. A C-terminal HA-tag was encoded with EBP50 in the source vector that was subsequently removed by introduction of a stop codon into the appropriate site of the pEGFP-C3 construct, through Quikchange Site-Directed Mutagenesis (Stratagene). Human NHERF2 cDNA was obtained from Open Biosystems (#5296143) and subcloned into a modified pIREShyg3 vector (Clontech) encoding EGFP 5' of the cDNA insertion site. PCR amplification of NHERF 2 using NheI and BsiWI appendages on the forward and reverse primers, respectively, allowed digestion and ligation into the GFP vector. The *Rattus norvegicus mrt1a* cDNA was obtained from the ATCC (accession#NM_001110151) and subcloned into pENTR.D.TOPO (Invitrogen) according to the manufacturer's protocol. Subsequent recombination into pcDNA-DEST47 using the Gateway system (Invitrogen) resulted in the encoding of a C-terminal, cycle 3 GFP. The SNX27/*mrt* PDZ domain was transferred to the pBH4 vector for bacterial purification by PCR amplification and digestion using NcoI and XhoI sites. Sequence encoding the H112A SNX27 alteration was introduced into the *mrt1a* vectors by Quikchange Site-Directed Mutagenesis (Stratagene) according to the manufacturer's protocol. All coding sequence was verified by dideoxynucleotide sequencing (Elim Biopharmaceuticals, Inc., Hayward, CA). Control (non-silencing) and

silencing siRNAs were purchased from Qiagen's HP GenomeWide siRNA collection. The sense-strand, silencing siRNA sequences are as follows: r(GAA GGA GAA CAG UCG UGA A)dTdT (NHERF1), r(GAG ACA GAU GAA CAC UUC A)dTdT (NHERF2), and r(CCA GGU AAU UGC AUU UGA A)dTdT (*mrt1*/SNX27). Peptides corresponding to the C-terminal 6 residues of the β2AR (TNDSLL) and an alanine-extended version (TNDSLLA) were obtained from Genemed Synthesis containing a FITC conjugated to the N-terminus. Anti-FLAG, M1 primary antibody (Sigma) was conjugated with Alexa Fluor 594 using the kit and protocol from Molecular Probes/Invitrogen, and Alexa Fluor 647 conjugated to anti-mouse IgG secondary antibody was purchased from the same vendor as a conjugate. Anti-EBP50 rabbit polyclonal IgG (ab3452) was purchased from Abcam, Inc. (Cambridge, MA), Anti-NHERF2 (C-14) goat polyclonal IgG (sc-21117) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), whereas anti-SNX27 mouse monoclonal IgG was a generous gift from Wanjin Hong (Institue of Molecular and Cell Biology, Singapore). HRP-coupled, secondary antibodies were purchased from Amersham/GE Healthcare (anti-mouse and anti-rabbit IgG) and Pierce (anti-goat IgG).

Cell lines and Transfection. Human embryonic kidney 293 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (University of California, San Francisco Cell Culture Facility). For siRNA transfection, cells at ~30% confluency in 6 cm dishes were transfected with Lipofectamine RNAiMax and 40 pmol of siRNA according to the manufacturer's protocol, split into 12-well plates 48 hours following transfection, and assayed at 72

hours post-transfection. For co-transfection of DNA and siRNA, Lipofectamine 2000 (Invitrogen) was used with 40 pmol siRNA and ~2 μg DNA in the same plating format and recommended protocol. DNA transfection was performed in 6-well or 12-well plates at ~50% cell confluency using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Stably-transfected cells were selected in 500 μg/mL Geneticin (Life Technologies, Inc.), and cell clones expressing FLAG-tagged receptor constructs were chosen at similar levels based on average surface immunofluorescence/cell measured through flow cytometry, and found to have at least 90% of cells expressing surface immunoreactivity. Saturation binding at 10 nM [3H]dihydroalprenolol to membrane preparations of the cell clones were used to estimate receptor densities between 2-4 pmol/mg protein. All 3 receptor constructs used for transfection have been described previously (von Zastrow and Kobilka 1992; Cao, Deacon et al. 1999; Tanowitz and von Zastrow 2003).

Flow Cytometric Assays. The recycling protocol has been described previously (Hanyaloglu and von Zastrow 2007). These experiments utilize 25 minutes of agonist administration and 50 minutes of agonist washout with 10 μM alprenelol following labeling of mature, surface receptors with M1 anti-FLAG IgG2B (Sigma). Secondary, surface M1 labeling following these treatments was done using Alexa Fluor 647-conjugated, anti-mouse IgG (Invitrogen). Cells were mechanically lifted in this stain and shaken at 4˚C for at least half of an hour before cytometry. The analysis of separate cell populations based on GFP fluorescence levels has also been described previously

(Lauffer, Chen et al. 2008), and only amended here by the use of an expression construct of *mrt1a*-GFP as opposed to GFP-Hrs.

Fluorescence Polarization. The SNX27 PDZ domain (and mutated variant) were purified as 6His-tagged proteins from BL21 *E. coli* using chromatography and the indicated concentration of it was mixed with 10 nM FITC-peptide as described before (Harris, Hillier et al. 2001). Fluorescence polarization was measured using and Analys HT Fluorometer (LJL Biosystems/Molecular devices).

Fluorescence Microscopy. HEK293 cells stably-expressing signal sequence-FLAG-tagged receptors were transfected with the indicated, GFP-tagged expression vectors and plated onto coverslips 24 hours later. At 48 hours post-transfection, surface receptors were labeled with M1 conjugated to Alexa Fluor 594 in culture medium, and endocytosed during a 20-minute, agonist administration with a saturating, 10 μM dose of the β adrenergic agonist isoproterenol. Cells were then washed with phosphate-buffered saline and fixed in 4% formaldehyde/PBS for 10 minutes, followed by a Tris-buffered saline quench for 5 minutes. Specimens were then prepared for imaging with an inverted, Nikon Diaphot epifluorescence microscope with mercury arc lamp illumination and a 60X/NA1.4 oil objective. Images were captured using a cooled, CCD camera (Princeton Instruments) interfaced to a PC running MetaMorph acquisition and analysis software (Molecular Devices).

Western Blotting. HEK293 cells (2 wells of a 12-well plate) were lysed in 200 μL of lysis buffer (0.1% (v/v) Triton X-100, 150 mM NaCl, 25 mM KCl, and 10 mM Tris, pH 7.4, Complete protease inhibitor cocktail (Roche)) before clarification at full force for 10 minutes in a tabletop centrifuge. Clarified extracts were measured for protein concentrations and mixed 3:1 with 4x LDS sample buffer (Invitrogen) supplemented with 5% β-mercaptoethanol before heating to 95˚C for 5 minutes. Protein samples were separated by SDS-PAGE, transferred to nitrocellulose, and Ponceau S stained for loading evaluation before Western blotting. Antibodies were diluted as recommended by the manufacturer in TBST with 5% milk (SNX27 antibody was used at 1:1000). Exposures were caught on film or using a FluorChem 8000 imaging system (Alpha Innotech Corporation).

Statistical analysis. Internalization and recycling percentages and fold changes in surface receptor fluorescence were calculated for each individual experiment using mean fluorescence values from triplicate samples and collected as replicates in a Prism spreadsheet for statistical and graphing analysis (Graphpad, Inc.). Mean percentages were compared between siRNA transfections in the receptor cell clones by Student's t-test or ANOVA and analyzed post-hoc for pair-wise differences using the Bonferroni Multiple Comparisons test with a significance level of 0.01. Comparison of recycling means between cells expressing low and high amounts of *mrt1a*-GFP variants in the same sample were compared for each receptor using a paired t-test with a significance level of 0.05.

4.4 Results

The β2 adrenoceptor undergoes efficient, PDZ-dependent recycling in cells depleted of NHERF1/EBP50 and NHERF2/E3KARP. The PDZ domain-interacting sequence, DSLL, present in the distal C-terminal tail of the β2AR is essential for efficient recycling of this receptor after agonist-induced internalization and can promote efficient recycling when fused to a distinct GPCR (Cao, Deacon et al. 1999; Cong, Perry et al. 2001; Gage, Kim et al. 2001; Gage, Matveeva et al. 2004; Wang, Lauffer et al. 2007; Lauffer, Chen et al. 2008). This 'recycling sequence', in the context of the β2AR or δ opioid receptor 'tail' fused to GST proteins, or in the full length receptor, is known to mediate affinity for the PDZ proteins NHERF1/EBP50 and NHERF2/E3KARP (Hall, Premont et al. 1998; Cao, Deacon et al. 1999; He, Bellini et al. 2005), both of which also contain an ERM (ezrin-radixin-moesin) protein-binding domain. These PDZ and ERM-binding domains in NHERF1/EBP50 –family proteins are known to function as linkers between integral membrane proteins that contain the appropriate PDZ domain-interacting sequence and the cortical actin cytoskeleton (Bretscher, Chambers et al. 2000), and such indirect connectivity to actin has been proposed to be the biochemical basis for PDZ-dependent recycling of the β2AR (Cao, Deacon et al. 1999). It remains unknown, however, whether either NHERF1/EBP50 or NHERF2/E3KARP is actually required for PDZ-dependent recycling of the wild type β2AR.

Given the extensive sequence identity between NHERF1/EBP50 and NHERF2/E3KARP (~55%) (Bretscher, Chambers et al. 2000), and the ability of both to bind to receptors with high affinity, it is possible that both proteins function in the

recycling mechanism or that either protein can function redundantly. Therefore, to begin to address the question of functional necessity, we knocked down expression of both proteins simultaneously in HEK293 cells using dual siRNA transfection. Immunoblot analysis verified depletion of both NHERF1/EBP50 and NHERF2/E3KARP from the same cell extracts (a representative example is shown in Fig 1A). We then used an established flow cytometric assay to evaluate regulated trafficking of receptors by measuring ligand-dependent changes in surface receptor immunoreactivity.

Exposure of cells transfected with control (non-silencing) RNA duplexes to the adrenergic agonist isoproterenol (10 μM) decreased surface immunoreactivity of wild type β2ARs substantially within 25 minutes, consistent with rapid agonist-induced endocytosis of receptors shown previously. Surface receptors returned nearly to control levels after agonist washout, confirming efficient recycling of the wild type β2AR (Fig 1B, solid line). The same experiment conducted on cells expressing a mutant receptor construct (β2AR-Ala), which abrogates the detection of NHERF-β2AR interactions in GST pulldown assays (Cao, Deacon et al. 1999; Wang, Lauffer et al. 2007), revealed enhanced net internalization in the presence of agonist and greatly reduced recovery of surface receptor immunoreactivity after agonist washout (Fig 1B, dashed line). Both of these observations verify the substantially reduced recycling produced by disruption of PDZ-dependent protein interaction(s) with the β2AR.

Surprisingly, the flow cytometric profile of the wild type β2AR measured in cells depleted of both NHERF1/EBP50 and NHERF2/E3KARP resembled that observed in control-transfected cells (solid and dotted lines, respectively), and dual depletion of both candidate PDZ proteins did not mimic the flow cytometric profile of the PDZ binding-

defective β2AR-Ala mutant receptor (dotted and dashed lines, respectively). These observations were verified across multiple experiments when initial surface receptor immunoreactivity (Fig. 1B, -25 time point), agonist-induced internalization (Fig. 1C) and recycling after agonist removal (Fig. 1D) were calculated from the flow cytometric data. While dual knockdown of NHERF1/EBP50 and NHERF2/E3KARP produced a small increase in net internalization and small decrease in average recycling of the wild type β2AR, neither of these effects were statistical significant and the 13% decrease in recycling efficiency defect was not nearly as severe as the 67% decrease produced by mutational disruption of the PDZ-interaction sequence in the receptor itself.

Because it was not possible to fully deplete both NHERF1/EBP50 and NHERF2/E3KARP using RNA interference, we cannot completely exclude a function of one or both of these proteins in promoting β2AR recycling. Nevertheless, these results challenged our hypothesis that NHERF1/EBP50 and/or NHERF2/E3KARP represent the critical recycling proteins, and raised the question of whether the β2AR recycling mechanism might require a distinct PDZ domain-containing protein.

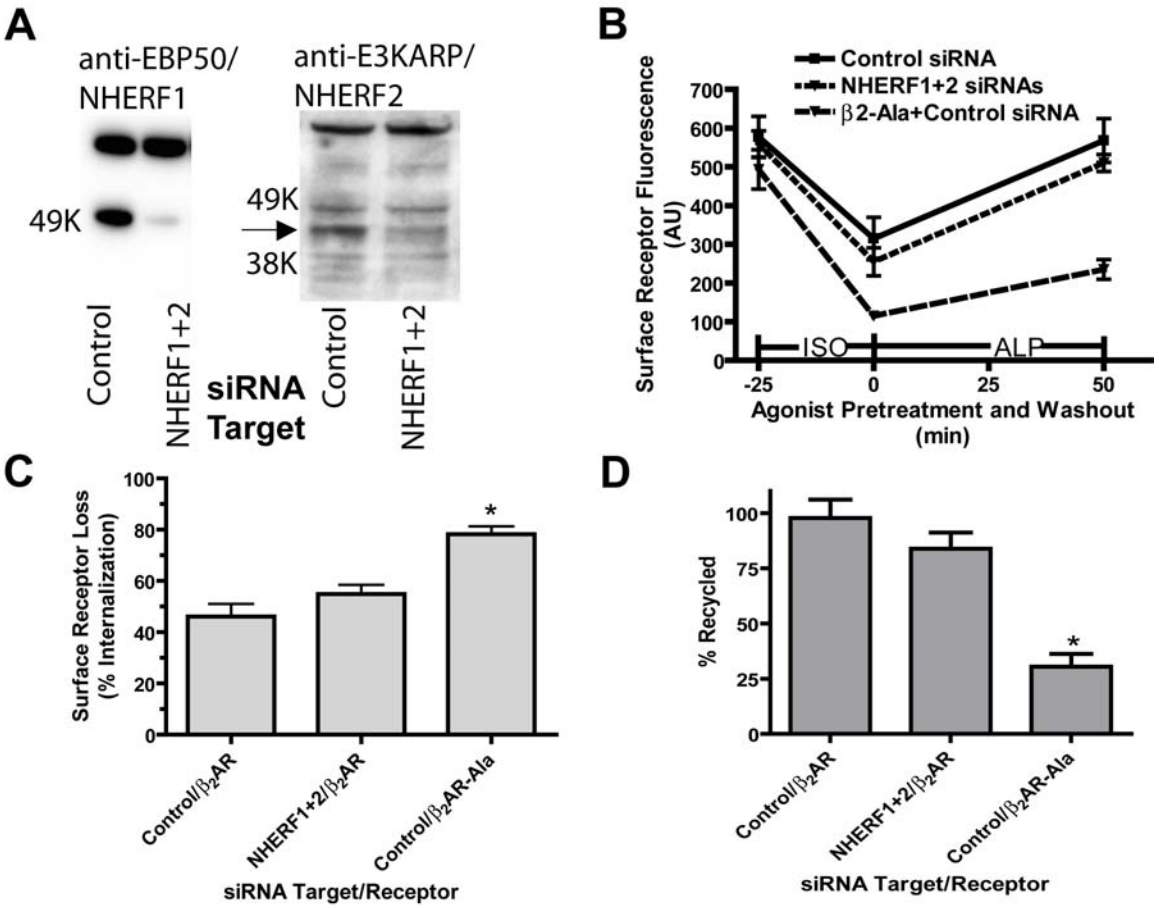

Figure 1. Efficient Internalization and Recycling of the β2AR in HEK293 cells depleted of both NHERF1 and 2.

(A) Extracts were prepared from β2AR-expressing HEK293 cells transfected with non-silencing (Control) or both NHERF1 and 2 (NHERF1+2) siRNAs. Immunoblotting for the indicated NHERF protein in these same extracts was performed in parallel, and single major species between 40 and 50K are shown along with non-specific bands used as loading controls. Images are representative of 3 experiments. **(B)** HEK293 cells expressing either a FLAG-β2AR or FLAG-β2AR-Ala PDZ mutant were transfected with the indicated siRNAs and assayed for surface receptor immunoreactivity before and after an agonist pretreatment and washout using fluorescence flow cytometry. Error bars reflect SEM fluorescence levels from 3-4 independent experiments. Agonist-induced internalization **(C)** and recycling **(D)** were calculated from this fluorescence data as described in the Materials and Methods. * indicates $p<0.01$ in post-Hoc analysis.

Sorting Nexin 27 interacts with the β2AR. If a distinct PDZ protein is required for efficient recycling of the β2AR, we reasoned that such a protein would likely have two properties. First, the putative recycling protein would likely associate with early endosome membranes, where internalized GPCRs are thought to undergo PDZ-dependent sorting into the recycling pathway. While many PDZ proteins associate with the cortical cytoskeleton underlying the plasma membrane, few are known to localize to endosomes. Second, this protein would be expected to have affinity for the PDZ-interacting sequence present in the β2AR tail. One candidate protein that potentially meets both criteria is sorting nexin-27 (SNX27). Like other sorting nexins, this protein contains a Phox homology (PX) domain mediating its association with endosomes (Lunn 2007). Unlike other sorting nexins however, SNX27 contains a PDZ domain that associates with a number of different C-terminal ligands (Joubert, Hanson et al. 2004; Lunn, Nassirpour et al. 2007). It has also been noted that the PDZ domain present in SNX27 is similar to those in NHERF/EBP50 –family proteins (Donowitz 2005), and BLAST search for protein homology to PDZ1 of NHERF1/EBP50 identified SNX27 as the closest relative outside of the immediate NHERF/EBP50 family.

We investigated subcellular localization of candidate PDZ proteins by expressing GFP-tagged fusions in HEK293 cells, and using dual channel fluorescence microscopy to visualize their subcellular distribution relative to β2AR-containing endosomes labeled with fluorescent M1 antibody. NHERF1/EBP50 did not visibly colocalize with β2AR-containing endosomes (Fig. 2A, left panels, compare top and bottom images) and, instead, was visualized throughout the cytoplasm and concentrated near filopodial projections of the plasma membrane (supplemental figure). In contrast both

NHERF2/E3KARP and SNX27 exhibited significant colocalization with β2AR-containing endosomes, and a particularly high degree of colocalization was apparent for SNX27 (Fig. 2A, middle and right panels, arrows indicate examples of colocalized structures).

We next tested if SNX27, like NHERF/EBP50 –family proteins, is capable of mediating PDZ-dependent binding to the β2AR tail. A fluorescence polarization assay revealed saturable binding of the β2AR-derived tail sequence with the PDZ domain derived from SNX27 (Fig. 2B). The specificity of this interaction was verified by parallel analysis of the alanine-extended sequence, which disrupts β2AR recycling in intact cells and was found to abrogate interaction with the PDZ domain (compare left graph to right graph).

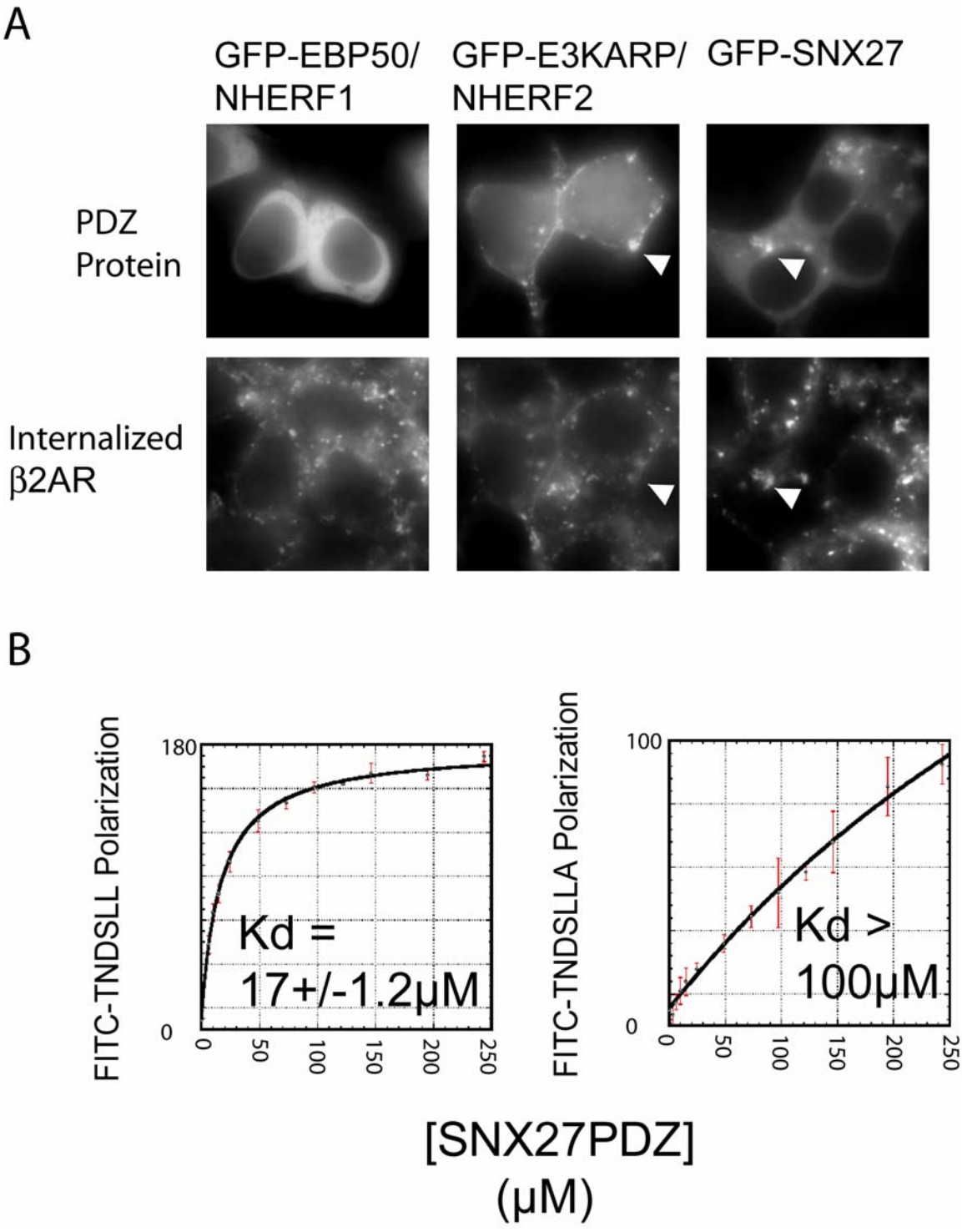

Figure 2. Association of SNX27 with the β2AR.

(A) HEK293 cells stably-expressing exogenous FLAG-β2AR were additionally transfected with a construct encoding the indicated GFP-tagged, PDZ protein. Following internalization of antibody-labeled receptors with 10 uM isoproterenol, cells were fixed and imaged using dual-channel microscropy to reveal

subcellular localization of the indicated protein. White arrows indicate points of colocalization. Representative of at least 3 experiments. **(B)** The SNX27 PDZ domain was purified from *E. coli* and mixed in increasing concentration with FITC-labeled peptides corresponding to the 6, C-terminal residues of the β2AR, or an alanine-extended version. A Kd was estimated where saturation of fluorescence polarization allowed. Representative of at least 3 experiments.

SNX27 knockdown severely impairs recycling of the β2AR. We then applied RNA interference and the previously described flow cytometric assay to assess the potential functional significance of SNX27 in endocytic trafficking of the β2AR. We compared effects of depleting cellular SNX27 and, given that NHERF1/EBP50 and NHERF2/E3KARP differed significantly in subcellular localization, compared the effects of depleting each of these proteins individually. The ability of the siRNAs utilized to drastically reduce protein products of the targeted transcripts was verified, and indicated that no secondary effects of one PDZ protein knockdown were exerted on the other PDZ relatives (Fig. 3A).

The first hint of a contributing role for any of these proteins in endocytic trafficking of the β2AR came from examination of the steady-state surface receptor levels in this stably-expressed receptor cell clone. Before the administration of any drugs, it was evident that SNX27 knockdown was substantially reducing the surface β2AR immunoreactivity compared to the same cell clone transfected with the negative control siRNA, consistent with an altered balance of exocytosis and endocytosis (Fig. 3B, last bar). On the other hand, depletion of NHERF1 levels alone had an opposite effect (Fig. 3B, 2nd bar). This analysis suggested distinct functional roles of individual PDZ proteins on β2AR homeostasis.

Subsequent evaluation of agonist-induced internalization and recycling again revealed some striking differences in the sensitivity of β2AR endocytic traffic to depletion of individual, PDZ domain-containing proteins. NHERF2 knockdown increased the relative reduction in surface immunoreactivity following agonist administration, consistent with enhanced endocytosis and/or reduced recycling (Fig. 3C, 3rd bar). Specific analysis of recycling after agonist washout again hinted at a relatively minor contribution of NHERF2 or 1 alone in the recycling mechanism, as the reduction was small in amount and not a statistically significant difference from control recycling (Fig. 3D, middle bars). Surprisingly, β2AR recycling was extremely sensitive to SNX27 knockdown despite a more subtle increase in internalization (Fig. 3C, right bar), bringing the recycling efficiency down to a background level seen by other, normally-inefficiently recycled receptors (Fig. 3D, right bar, and Fig. 1D right bar for reference). This suggested that β2AR recycling requires SNX27 in a largely non-redundant manner with other cellular factors.

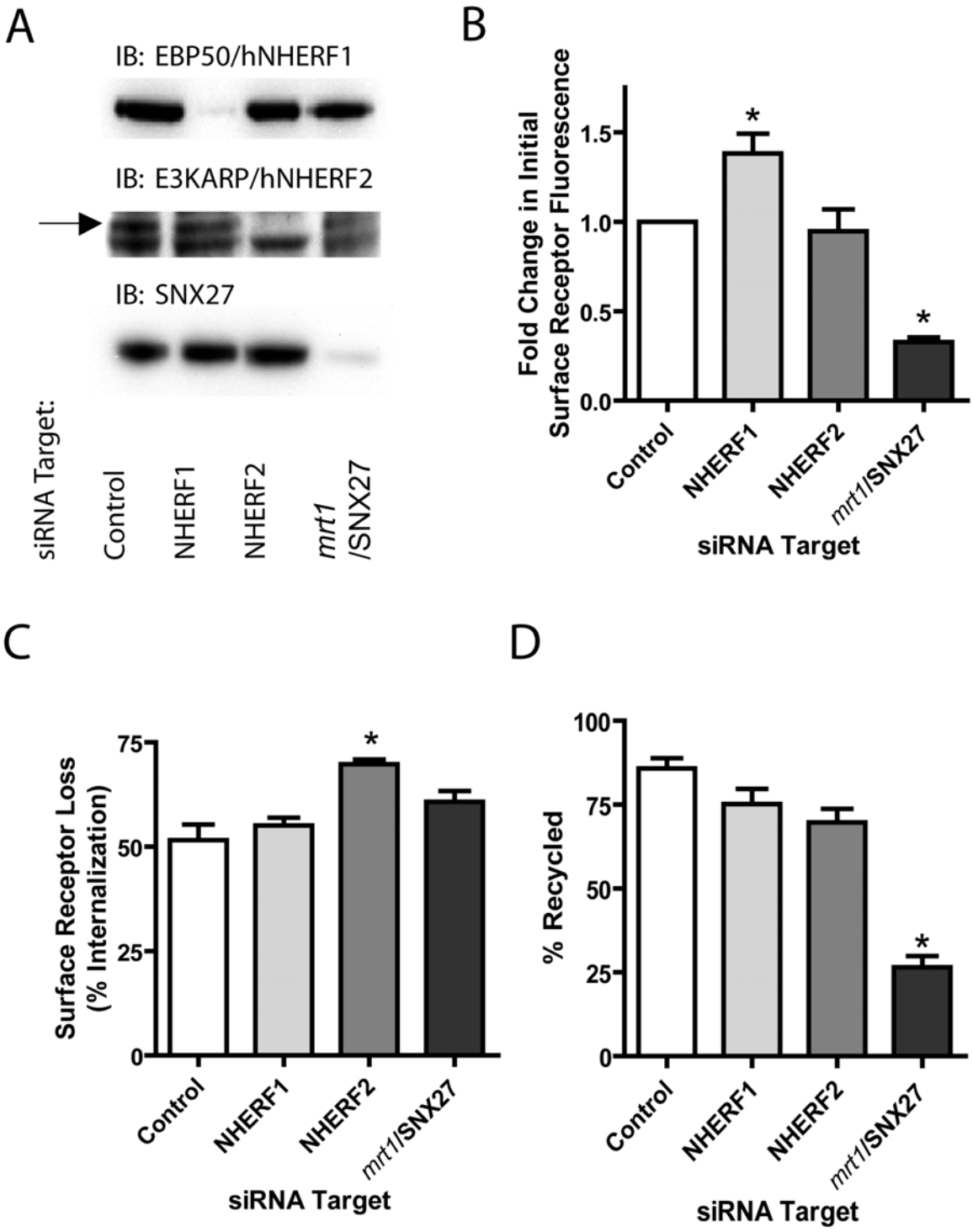

Figure 3. Depletion of β2AR-interacting, PDZ proteins reveal distinct roles in regulating surface numbers.

(A) Western analysis of β2AR-expressing HEK293 cells transfected with siRNA targeting the specified gene products. Arrow indicates specific NHERF2 band. Representative of 4 independent experiments.

Analysis of altered surface β2AR expression **(B)**, agonist-induced internalization **(C)**, and recycling following agonist withdrawal in the same transfected cell population depleted of the indicated transcripts and protein. Error bars represent SEM of at least 4 independent transfections and analyses.

Diminished β2AR recycling with SNX27/*mrt1* siRNA can be rescued by coexpression of rat *mrt1a*-GFP. The pronounced inhibitory effect of SNX27 depletion on β2AR recycling was verified with three independent RNA duplexes (not shown). As a further test of functional specificity we asked if the inhibitory effect of SNX27 depletion can be rescued by expression of the recombinant protein. To accomplish this we took advantage of the fact that our GFP-SNX27 construct was derived from rat, and used an RNA duplex specific to the human transcripts. Knockdown and replacement were verified by immunoblot analysis, in which the siRNA duplex efficiently knocked down the human SNX27 expressed endogenously in HEK293 cells and the recombinant rat protein (migrating at higher apparent molecular mass due to fusion of GFP) was expressed at levels similar to that of endogenous protein in control cells (Fig. 4A). We then tested rescue by using dual-channel fluorescence flow cytometry to measure β2AR recycling in knockdown cells gated according to expression of recombinant (non-silenced) SNX27, or of GFP alone used as a negative control (Fig. 4B). Whereas expression of GFP by itself minimally affected the β2AR steady-state and recycling after agonist washout (Fig. 4C and D, left two bars), we observed enhanced β2AR steady-state and recycling in cells expressing the recombinant SNX27 construct (right pair of bars). Analysis across multiple experiments confirmed this dose-dependent rescue (Fig. 4E).

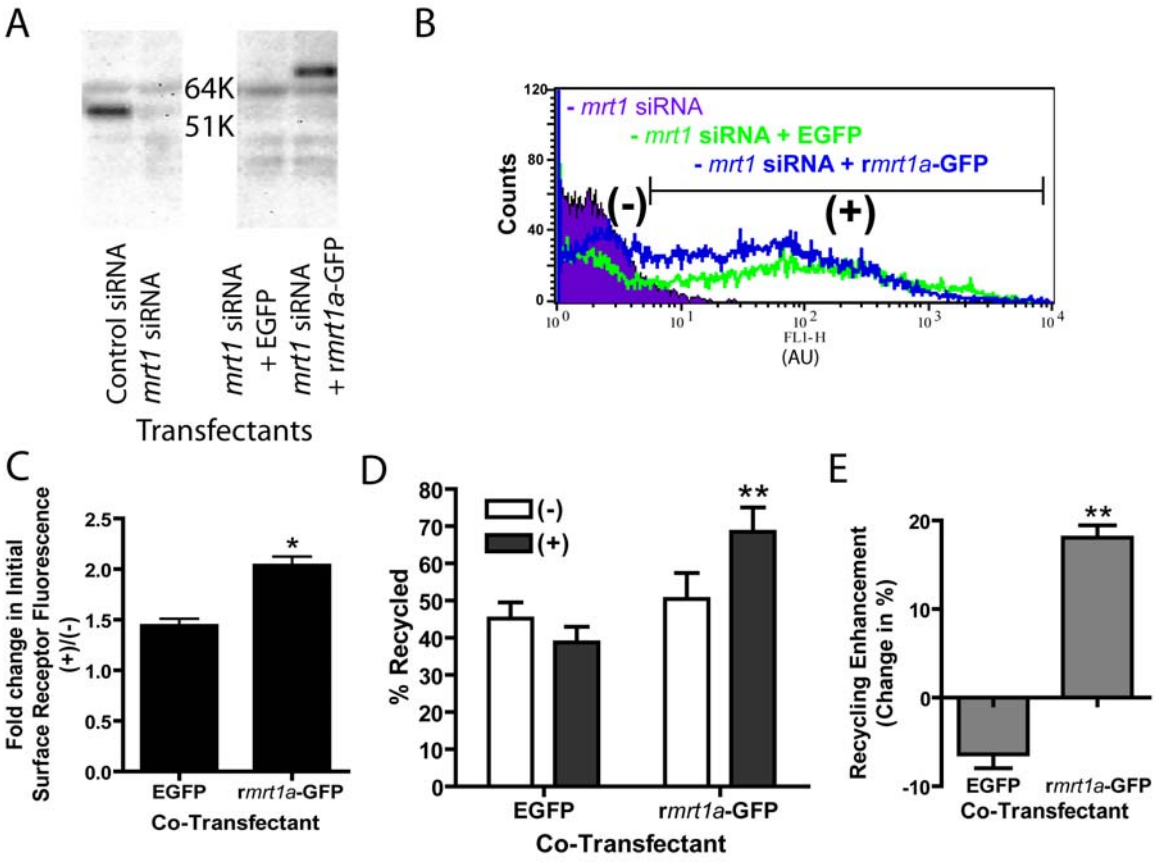

Figure 4. Recycling lost with *mrt1*/SNX27 depletion can be rescued by coexpression of a rat *mrt1a*-GFP construct.

(A) Extracts from HEK293 cells transfected with the indicated siRNA and cDNAs were immunoblotted for SNX27 protein. Image is representative of 4 independent experiments. **(B)** FLAG-β2AR-expressing HEK293 cells were additionally transfected with the indicated siRNA and cDNA, and green fluorescence was measured in the cell populations through flow cytometry. Gating for the cells represented in the (+) section allowed for receptor analysis in populations differing in GFP expression. Image is representative of the samples measured in at least 4 independent experiments. **(C)** Surface receptor fluorescence between the two GFP-expression-level populations from (B) was compared for each of the cDNAs transfected. Error bars reflect SEM and *indicates $p<0.05$ in Student's t-test. **(D)** Recycling in the same transfected cells was calculated based on GFP fluorescence levels shown in (B) and the average difference between expression groups for the indicated cDNA per experiment is shown in **(E)**. Error Bars represent SEM of at least 4 independent experiments and **denotes $p<0.05$ in a paired t-test of recycling means between (-) and (+) GFP expression levels.

SNX27-sensitive recycling of the β2AR is conferred by the receptor PDZ ligand. With elucidation of a central role of SNX27 in β2AR recycling, we wanted to distinguish between its potential role as a critical but general regulator of recycling pathways vs. a role as a more specialized PDZ adaptor directing specific cargo to their proper destinations. We thus examined whether recycling of a chimeric receptor was sensitive to SNX27 depletion. We chose a previously-characterized chimeric receptor composed mainly of the β2AR but with its distal C-terminus replaced by the C-terminal 17 residues of the μ-opioid receptor, which contains a unique, non-PDZ recycling sequence (mrs), (Tanowitz and von Zastrow 2003). This allowed treatment of the cells with the same drugs used for the wild type receptor, and allowed functional analysis of the requirement for a PDZ interaction in the context of other β2AR protein interactions. While we did not investigate any influence of NHERFs on β2-mrs trafficking, we did examine trafficking phenotypes of the inefficiently recycled β2-Ala mutant adrenoceptor under conditions of NHERF knockdown, and noticed that enhanced steady-state receptor levels again resulted from NHERF1 knockdown, despite the inability for this mutant to interact with NHERFs *in vitro* (Sfig2). Therefore, we attributed these results to an effect of NHERF1 on global membrane organization as opposed to a PDZ-mediated effect on the β2AR.

For better analysis of the recycling specificity of SNX27, we isolated a cell clone stably-expressing FLAG-β2-mrs receptors at similar levels to the FLAG-β2AR clone (compare initial fluorescence value in Figure 5B to the same point in Figure 1C) and verified the ability to effectively knock down SNX27 in these cells (Fig. 5A). Changes in surface immunoreactivity after agonist administration and washout were unaffected by control siRNA transfection (Fig. 5B, black line, untransfected comparison not shown).

Transfection with *mrt1* siRNA in this case had little effect on any of these changes despite the severe reduction in SNX27 protein level (Fig. 5B, dashed line). A small reduction in the steady-state receptor level was noted (Fig. 5C), however any inhibition in recycling efficiency was not apparent, especially when compared to the effect on the wild-type β2AR (Fig. 5D). This highlighted the need for the β2AR PDZ ligand to be involved in the SNX27-dependent recycling process.

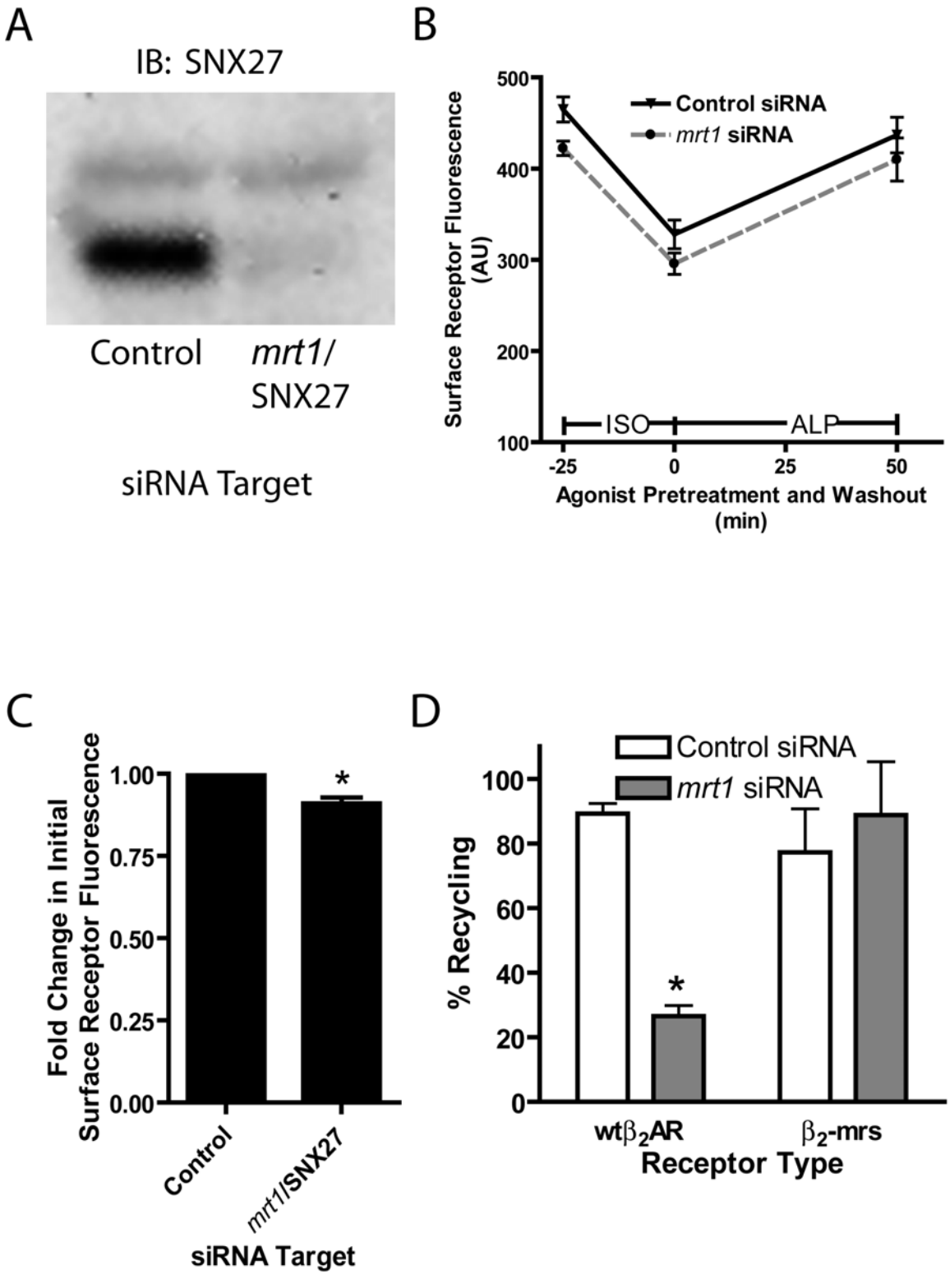

Figure 5. SNX27-dependent recycling requires the β2AR PDZ ligand.

HEK293 cells expressing the chimeric FLAG-β2-mrs were transfected with the indicated siRNA and split into samples for lysis and trafficking assays. **(A)** Immunoblot analysis of cell extracts showing SNX27 protein (lower band) and a non-specific upper band shown for loading control. Representative of 4 independent experiments. **(B)** Surface receptor flourescence in the cells coexpressing the FLAG-β2-mrs and the indicated siRNA is plotted vs. the trafficking time course applied. Error bars represent SEM from 4 independent experiements. The siRNA-specific change in steady-state receptor levels **(C)** and recycling **(D)** were additionally calculated. The effect of SNX27 knockdown in FLAG-β2AR-expressing cells is shown again for comparison. Error bars represent SEM of at least 4 independent experiments. *denotes $p<0.05$ in post-Hoc analysis.

SNX27-mediated recycling is impaired by PDZ mutation. While the recycling inhibition resulting from SNX27 knockdown required the receptor's PDZ ligand, we sought independent confirmation of the need for PDZ networking in promoting recycling. Thus we attempted to disrupt the PDZ interaction capability of SNX27 by point mutation, and analyzed the mutant's ability to rescue the β2AR recycling deficit under conditions of SNX27 knockdown. While recent studies of the PDZ domain family have highlighted a robustness in their ability to access ligands when mutated, there is still a highly-conserved histidine in the 2nd alpha helix of many PDZ domains that greatly impacts the specificity of PDZ domains for the –2 S/T position characteristic of the classical, type I ligands (Doyle, Lee et al. 1996; Tonikian, Zhang et al. 2008). We thus mutated the histidine at position 112 in the rSNX27 protein to alanine and purified this mutant PDZ domain for calculation of an affinity for the β2AR PDZ ligand. Analysis of FITC-TNDSLL polarization with increasing SNX27H112A protein revealed a detectable but substantially-reduced affinity reflected by a Kd of 65+/-20.5 μM when compared to the wild type PDZ domain (Fig. 6A compared to Figure 2B). When altering this PDZ

residue in the full-length, GFP-tagged SNX27, no evidence of gross structural defects were suspected, as the protein still localized in the endocytic pathway (Fig. 6B).

When using this mutant SNX27 for recycling 'rescue' over the knockdown phenotype, a small but significant increase in the β2AR recycling efficiency was observed in cell populations (+) for SNX27H112A-GFP compared to the (-) group (Fig. 6C). This enhancement in recyling was significantly reduced when compared to the ability of the wild-type SNX27-GFP to rescue β2AR recycling, however (Fig. 6D). This again supported the role of PDZ interactions between the β2AR and SNX27 proteins in promoting endocytic recycling of the receptor.

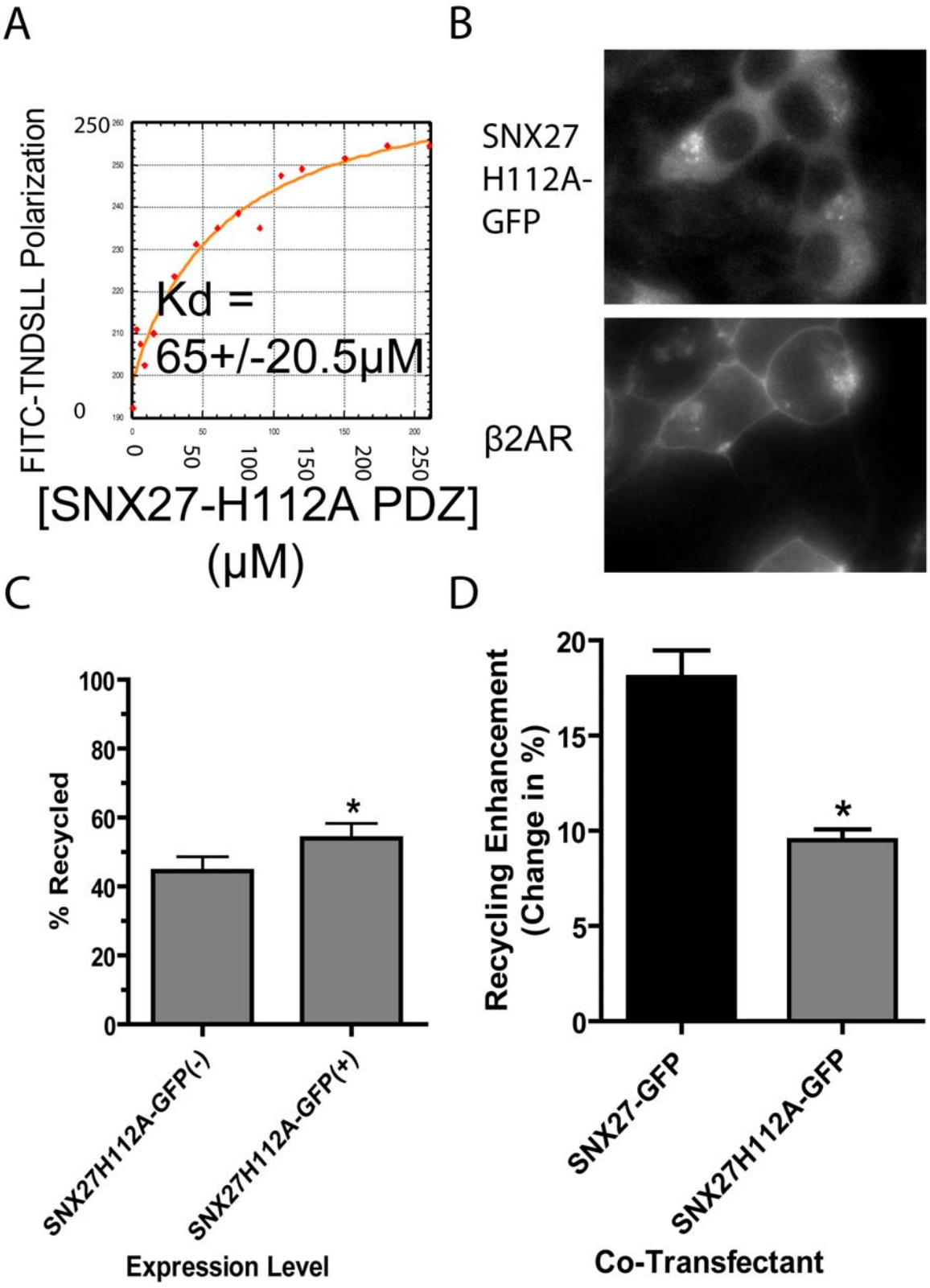

Figure 6. Mutation of the SNX27 PDZ domain impairs its recycling function.

(A) Determination of the affinity as in figure 2 of the β2AR PDZ ligand for a SNX27 PDZ mutant corresponding to a H112A change in the full length protein. **(B)** Evaluation of the subcellular localization of SNX27H112A-GFP in relation to internalized FLAG-β2AR as done in figure 2. **(C)** Recycling of FLAG-β2AR in HEK293 cells additionally transfected with SNX27/*mrt1* siRNA and SNX27H112A-GFP. Analysis was separated based on SNX27H112A-GFP fluorescence levels and the average recycling difference compared to the wild type rescue of recycling from figure 4 is shown in **(D)**. Error Bars represent the SEM of at least 4 independent experiments.

4.5 Discussion

The present study set out to determine the relative necessities of the NHERF/EBP50 pair of gene products in mediating endocytic recycling of the β2AR, given the high specificity of the receptor recycling sequence for these proteins and their link to the actin cytoskeleton, which has been linked to this trafficking process in several ways (Cao, Deacon et al. 1999; Bretscher, Chambers et al. 2000; He, Bellini et al. 2004; Lauffer, Chen et al. 2008). The remarkable results of β2AR trafficking analyses in cells depleted of both NHERFs simultaneously were the relative *lack* of any endocytic trafficking changes despite efficient knockdowns. While the analysis revealed a slight trend towards decreased recycling efficiency, the lack of statistical significance and the small magnitude of this decrease in comparison to the drastically reduced recycling of the β2AR-Ala PDZ-disrupting mutant challenged our model of solely NHERF-mediated recycling of the β2AR. While it is certainly conceivable that the small amount of NHERF/EBP50 protein remaining after knockdown is still sufficient to provide a critical or contributive sorting of receptors in the endocytic pathway, we consider this less likely due to the relatively efficient knockdowns we observed combined with the large load of receptors in our heterologous system that endocytose rapidly in response to agonist. In addition, NHERF association with endosomes was not as prominent as the association with cortical, juxtamembrane structures.

Endosomal localization turned out to be a critical clue in leading to the identification of a PDZ-domain-containing protein required for endocytic recycling of the β2AR. While NHERF2 showed a propensity to associate with β2AR-containing

endosomes, and while cytoplasmic concentration of NHERF1 could be masking the residence of this protein on endosomes, the more strict localization of SNX27 to endosomes traversed by the β2AR led us to wonder whether a functional interaction would be possible between these proteins. The C-terminal ligands previously reported as binding partners have so far shown a propensity for an E-S/T-x-V/F motif within the greater type I classification of PDZ ligands (also a D in the –1 'x' position appears detrimental) (Joubert, Hanson et al. 2004; Lunn, Nassirpour et al. 2007; MacNeil, Mansour et al. 2007; Rincon, Santos et al. 2007). The finding that the DSLL sequence from the β2AR shows affinity for the SNX27 PDZ domain, as well, expands the possible binding partners for this protein while maintaining a similar amino acid residue preference at each position. Recent comparison of the SNX27 PDZ domain to a homologous *C. elegans* domain with >70% identity in the ligand binding pocket predicted a SNX27 ligand specificity profile compatible with the motifs observed thus far and potentially expanding the possible binding partners (Tonikian, Zhang et al. 2008)While the *in vitro* Kd of the β2AR ligand with the SNX27 PDZ domain is slightly higher than that estimated in other studies of PDZ interactions, we suspect the endosomal concentration of the binding partners allow for a functional but transient interaction that can leave the β2AR partially available to interaction with overexpressed NHERF2 on endosomes, for example. Despite this possible endosomal promiscuity of the receptor PDZ ligand, we found little requirement for NHERFs in addition to SNX27 in regulating endosomal trafficking of the β2AR.

Even with an efficient knockdown of SNX27, we were surprised at what appeared to be as complete a block of recycling as is possible in our system. Not only was this

effect statistically significant, the recycling efficiency mimicked the 'background' level seen with the β2AR-Ala PDZ mutant. In addition, this poor recycling appeared to not only affect agonist-induced trafficking, but reduced the steady-state level of receptor as well. While these two observations could reflect separate trafficking functions of SNX27, reduced constitutive recycling would be predicted to increase receptor turnover and lower the basal surface equilibrium, explaining why we observed both of these effects. However, the role of SNX27 seems to regulate the trafficking of different binding partners in different ways. For example, it promotes endosome localization when coexpressed with the 5-HT4A receptor splice variant, and in the case of some inwardly-rectifying K+ channels this also results in enhanced degradation of the binding partners (Joubert, Hanson et al. 2004; Lunn, Nassirpour et al. 2007). To our knowledge, this is the first example of a SNX27-interacting protein that requires SNX27 for endocytic recycling. It appears that, like with other sorting machinery, the functional consequences of an interaction with SNX27 vary depending on the context of all the molecules and processes involved.

Sorting nexins comprise a large family of protein machinery governing various steps in membrane trafficking. An important distinction in relating the role of SNX27 to β2AR trafficking was thus to determine if the PDZ interaction specified the functional relationship between them. Luckily, a recycling sequence found in the μ opioid receptor, which does not conform to a known PDZ ligand, is able to promote recycling of the β2AR when replacing its PDZ ligand (Tanowitz and von Zastrow 2003). Recycling of this β2-mrs receptor was virtually unaffected by SNX27 knockdown, suggesting that SNX27 regulates the sorting of specific cargo that it engages through a PDZ interaction.

Indeed, mutation of the PDZ domain itself was able to weaken the functional rescue of recycling seen with coexpression of the rat mrt1a-GFP cDNA and the human-targeting siRNA. While this does not rule out the role of a larger PDZ complex in mediating the functional connection, it does highlight the specificity that the PDZ connectivity supplies. If there are other PDZ interactions required for β2AR recycling, it would appear they are not additive to or redundant with SNX27, as β2AR recycling with SNX27 knockdown appears as low as possible.

In conclusion, we have identified a novel PDZ interaction between the β2AR and SNX27 that appears to account for the recycling activity previously attributed to other protein interactions. The functional relationship is specific to the PDZ-mediated recycling of the β2AR, and expands the sorting roles for this interesting member of the sorting nexin family. This interaction also expands the ligand sequences compatible with the SNX27 PDZ domain, adding to a growing and versatile group of interacting proteins. Future studies will be needed to elucidate the mechanism by which SNX27 mediates sorting. And given that the SNX27 gene was originally characterized for its methamphetamine-responsive transcripts, it will be interesting to see how membrane trafficking contributes to the physiological sensitivity changes observed with prolonged use of many drugs.

4.6 Acknowledgements

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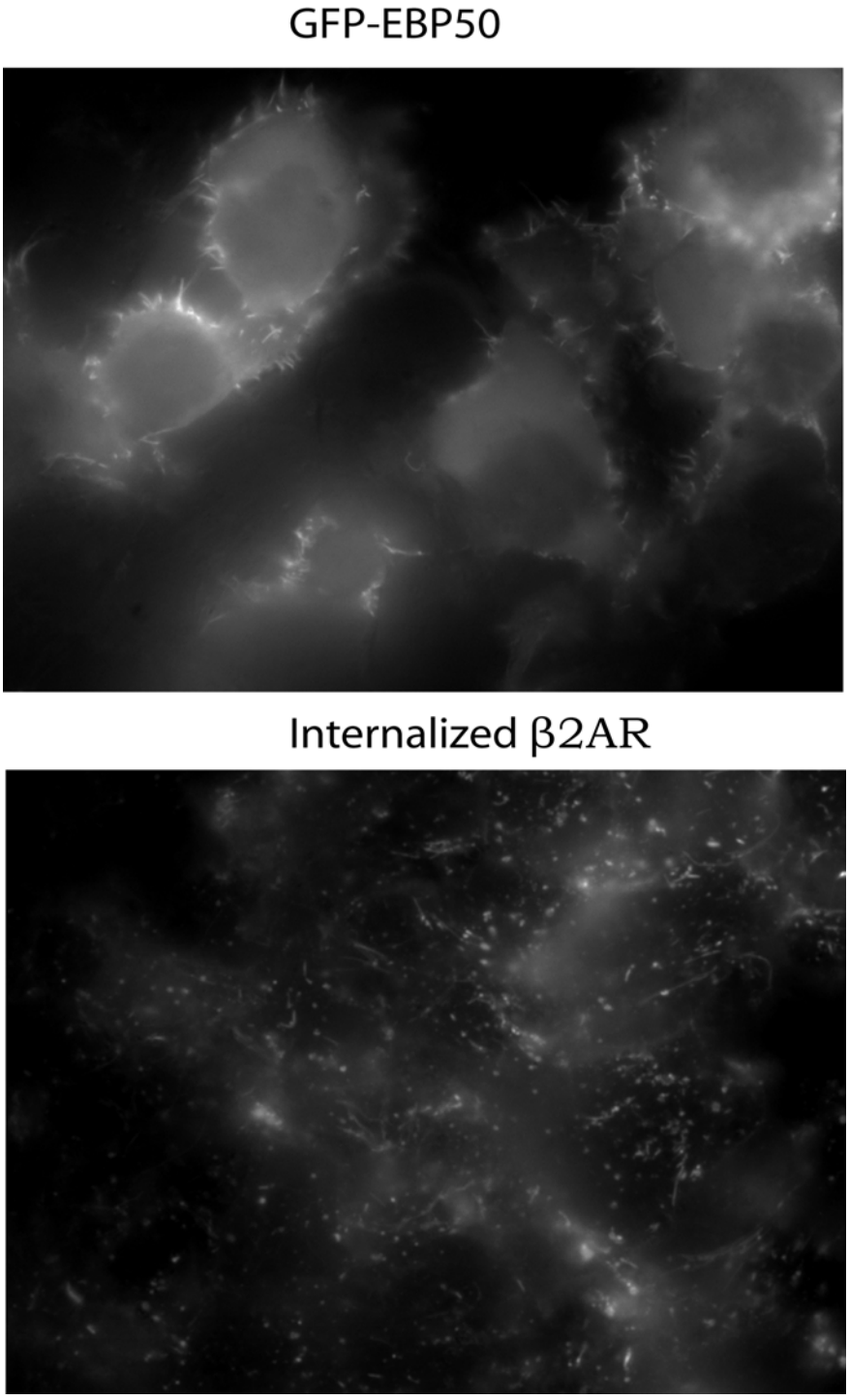
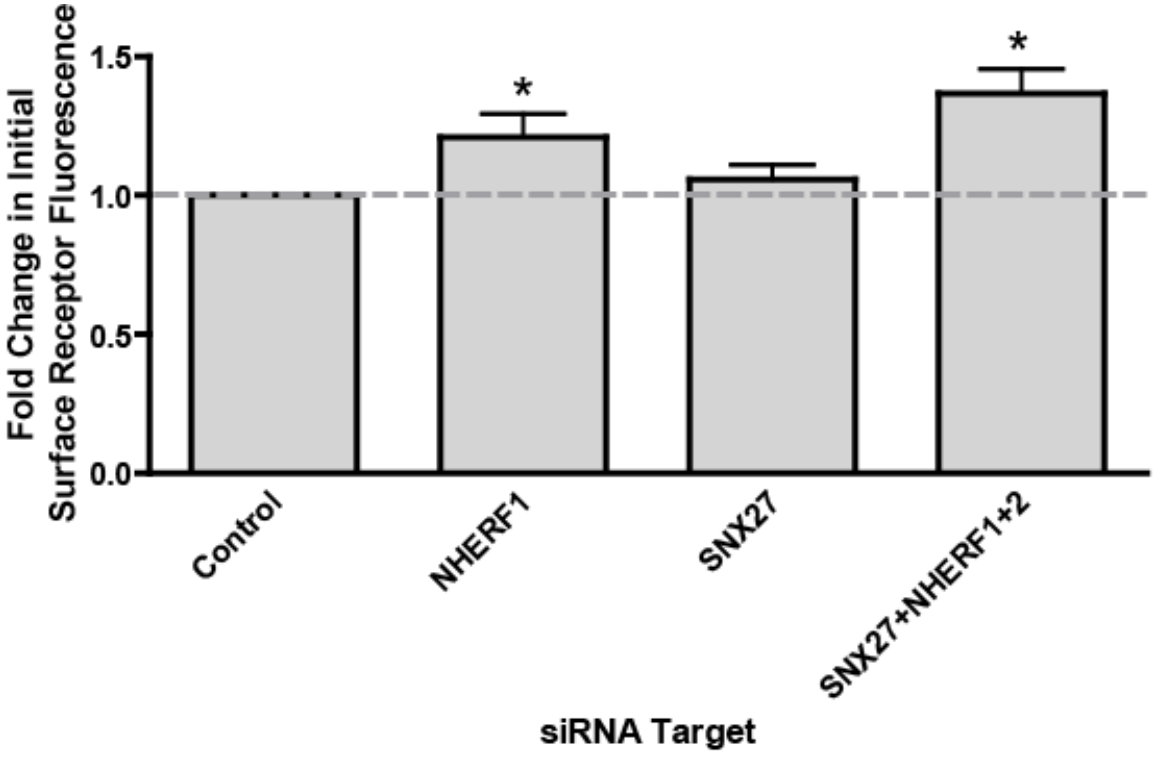
4.8 Supplemental Figures



Supplemental Figure 1. Concentration of EBP50 in Actin-rich structures in HEK293 cells.

HEK293 cells transfected with constructs encoding both GFP-EBP50 and FLAG-β2AR were labeled with M1 antibody conjugated to the Alexa Fluor-594. Receptors were then internalized for 20 minutes in the continued presence of 10 μM isoproterenol agonist. Cells were fixed and imaged for receptors and EBP50 using dual-channel fluorescence microscopy focused to the coverslip/cell boundary. Representative of 4 experiments.



Supplemental Figure 2. Effects of siRNA transfection on steady-state surface levels of the β2AR-Ala PDZ mutant.

HEK293 cells stably-expressing the FLAG-β2AR-Ala PDZ mutant were transfected with the indicated siRNA and labeled for surface receptors as described in the Materials and Methods. Fluorescence levels of cells naïve to drug treatments were normalized to that seen with control siRNA transfection. Error bars reflect SEM of 3-4 experiments. * denotes $p<0.05$ in post-Hoc comparison to control.

Chapter 5: Overall Conclusions

5.1 Summary of Conclusions

As discussed in Chapter 1, a growing number of GPCRs possess cytoplasm-accessible motifs that are required, and often sufficient on other receptors, to mediate endocytic recycling. The cytoplasmic factors recognizing these recycling sequences are largely unknown, though many recycling sequences conform to type I PDZ ligands. The PDZ ligand from the β2AR has shown relative specificity for the NHERF family of PDZ proteins, though an interaction with the non-PDZ-containing NSF confounded original interpretations of β2AR PDZ ligand function. Nevertheless, recycling inhibition observed during the administration of cytoskeletal toxins supported an integral and possibly more general contribution of actin filaments specifically in the sequence-directed recycling process. β2AR recycling mediated by Actin connectivity through NHERF1/EBP50 and/or NHERF2/E3KARP linking to ERM proteins thus presented a working model that incorporated this additional feature. As discussed in Chapter 2 and Appendix I, NSF did not fit into this model as more than a species-specific regulator, because the interaction with the mβ2AR was not conserved and the receptor exhibited efficient recycling in multiple cell types. The NHERF-Actin-mediated recycling model was supported by sufficient recycling achieved by linking a distinct receptor to ERM proteins or directly to Actin in Chapter 3. However, it now appears as if sufficiency does not translate into necessity for the β2AR. While actin connectivity remains an intriguing mechanism for achieving GPCR recycling, Chapter 4 revealed the striking necessity of Sorting Nexin 27 over NHERF1 and 2 for PDZ-mediated recycling of the β2AR, an adaptor protein with no obvious link to actin filaments. Thus it appears that the variety of

recycling sequences observed can direct a number of different recycling mechanisms that may or may not ultimately converge; two of these mechanisms of potential physiologic relevance are presented in the last 2 Chapters.

Before it was known that the β2AR PDZ ligand exhibited such species divergence in protein interaction biochemistry, particularly with NSF, it was determined that type I PDZ ligands and not NSF-specific peptides were sufficiently robust recycling sequences when affixed to the δOR (Gage, Matveeva et al. 2004). As such it seemed unlikely that an NSF interaction with the β2AR would be required for endocytic recycling, in contrast to previous inferences (Cong, Perry et al. 2001). The noted divergence of the human and rodent β2AR PDZ ligands offered an opportunity to examine this directly. Interestingly, an interaction between NSF and the mβ2AR tail was undetectable in several assays. This suggested that either the NSF interaction was more of a biochemical artifact than a physiologically-relevant connection, different species had evolved different reliance on NSF function, or that NSF function was conserved and insensitive to large, β2AR affinity differences. The different rates of internalization, recycling, and contraction rate regulation in cardiomyocytes observed when 'humanizing' the mβ2AR PDZ ligand suggested that the NSF affinity differences did matter by modulating β2AR trafficking and signaling regulation to different degrees. Though quite possibly the interaction is only relevant to human-like PDZ ligands. However, pleiotropic effects of PDZ ligand alteration may additionally explain the phenotypic changes observed, which leaves open the possibility of the NSF interaction being a biochemical artifact. As such, it is more important to note that the β2AR does *not* seem to rely on an evolutionarily-conserved

affinity for NSF to mediate recycling, and species differences within and outside the β2AR PDZ ligand result in different regulation in the same cellular background.

Although we cannot close the door completely on the involvement of NSF in β2AR trafficking, the weight of the evidence pointed toward a PDZ interaction with the EBP50-like NHERF proteins as the core interaction responsible for β2AR recycling, and presumably the recycling of other GPCRs and membrane proteins as well. In addition to validating this type of PDZ interaction as responsible for the observed recycling behavior of the β2AR, we were curious to know if the Actin connectivity mediated by this complex was the key, and perhaps more general, biochemical feature responsible for this type of recycling activity, for a non-default recycling mechanism was not encountered previously. Fortunately, protein interaction domains in the NHERF-ERM complex were shown to be relatively transferable structural modules, and protein engineering using the δOR was additionally amenable for trafficking studies (Turunen, Wahlstrom et al. 1994; Reczek, Berryman et al. 1997; Gage, Kim et al. 2001). Engineered δOR connectivity to actin, either through fusion of the ERMbd or direct F-Abd, was sufficient to induce recycling. And importantly, the recycling mediated through both of these domains (in addition to the β2AR PDZ ligand) was sensitive to the cytoskeletal toxin cytochalasin D, suggesting that both recycling mechanisms relied on F-actin linkage. In this way, the sequence-directed recycling process was recreated. However, in another aspect of this process just recently realized, ERM/Actin-linked receptors behave more like default recycling cargo. The multifunctional, endosomal protein Hrs has been shown to regulate the sequence-directed recycling processes of the β2AR and μOR (Hanyaloglu, McCullagh et al. 2005). When overexpressed, this protein inhibits recycling of a

δOR/β2AR-PDZ-ligand fusion, but not δORs linked more directly to actin. And seeing as how the discovery of the SNX27 requirement for β2AR recycling additionally demoted any NHERF requirement in this process, the role of Actin binding in β2AR-specific recycling remains more of an open question. However, the capacity for this Actin-linking biochemistry to mediate recycling may explain the recycling of the direct, ezrin-binding α1b adrenergic receptor, in addition to other PDZ-binding receptors, as PDZ proteins are common to actin-rich structures (Kim and Sheng 2004; Stanasila, Abuin et al. 2006). In addition, this has warranted the search for Actin-rich structures on endosomes, which is beginning to reveal a previously-unappreciated role for this part of the cytoskeleton in endosome structure and function (Manoj Puthenveedu, unpublished results).

The search for recycling-relevant PDZ interactions started as a process of elimination. NSF affinity did not correlate well, and knockdown of NHERF1 and 2 simultaneously or separately exhibited no significant effect on β2AR recycling in HEK293 cells. Because of the overwhelming number of PDZ protein combinations that could be involved, cracking the code of this possibly redundant process was daunting. Most PDZ domains, like those of NHERF proteins, are tuned towards particular ligands, however (Tonikian, Zhang et al. 2008). And knowing that NHERF PDZ domains show preference for the β2AR PDZ ligand allowed the identification of additional PDZ-domain-containing candidate recycling adaptors. While we had focused on the EBP50-like NHERF1 and 2, PDZ classification included the PDZ domains found in 2 other proteins as closely related, with the PDZ protein of SNX27 following close behind (Donowitz, Cha et al. 2005; Thelin, Hodson et al. 2005). Out of these 3 additional

candidates, SNX27 was the most intriguing, as it was considered an endosomal sorting protein already, though a role in recycling had not yet been demonstrated (Joubert, Hanson et al. 2004; Lunn, Nassirpour et al. 2007; Rincon, Santos et al. 2007). Even so, the seemingly complete necessity for SNX27 in β2AR recycling was a bit surprising. While this necessity does not exclude the role of other PDZ proteins in the process (and a role for NHERF1 and 2 could have been missed), the virtual block of recycling with SNX27 knockdown suggests that any contribution of other PDZ proteins would be non-redundant, working in series as opposed to parallel mechanisms.

Given that most membrane returns to the cell surface following endocytosis while most GPCRs seem to maintain a capacity for lysosomal sorting, the simplest role for sequence-directed recycling adaptors would be to protect receptors from lysosomal sorting and allow them to passively flow back to the cell surface. However, unpublished evidence in the lab suggests that the β2AR utilizes endosomal membrane tubules that contain a molecular identity distinct from that of other membrane tubules leaving the endosome. Thus it would seem that receptors are actively kept away from lysosomal retention by moving into specialized membrane tubules with the aid of SNX27 and Actin filaments. While there is no known physical link between SNX27 and Actin based on SNX27 domain structure, SNX27 contains primary structure predicted to hold overlapping Band4.1 and Ras-associating (RA) domains (NCBI). Since its PDZ domain presumably functions to recognize specific cargo, and its PX domain affords it endosomal residence, the Band4.1/RA region may coordinate with a macromolecular assembly utilizing actin for the maturation of specified tubules destined eventually for the cell surface (figure 1).

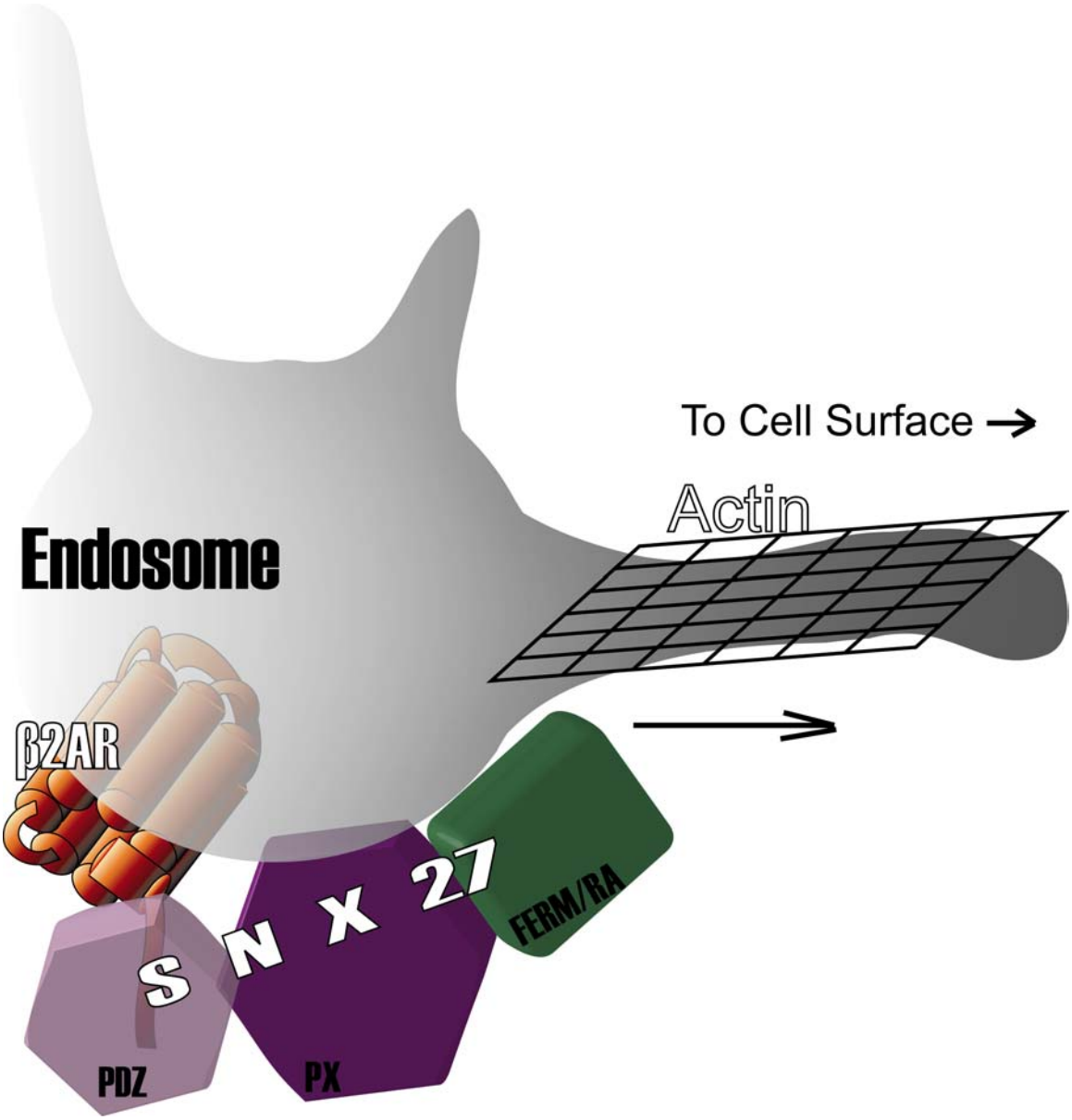


Figure 1. Model of SNX27 sorting.

Targeted to endosomes by the lipid affinity of its PX domain, SNX27 finds specific, endocytosed cargo through the use of its PDZ domain and facilitates their passage into a tubule marked by enhanced actin production. Components not to scale.

5.2 Future Directions

The fortuitous opportunity to find a specialized sorting adaptor like SNX27 has now offered the opportunity to ask questions central to GPCR sorting from the submolecular to the organismal level. The simplest question of "How does it work?" requires investigation of SNX27 biochemistry, an expanded characterization of the nebulous endosome, and improved understanding of the multi-faceted regulation of GPCRs. The other simple but sweeping question to ask is, "What is this orchestrated recycling for?" While the proximal cause is simply to move receptors to the cell surface, the ultimate gain in evolutionary fitness is likely achieved through regulation of cells in most physiologic systems. While mice with the *mrt1*/SNX27 gene disrupted are able to survive past birth, the producers of this line suspect that some severe neurological problems result from the functional absence of this gene (Wanjin Hong, personal communication). Since perturbations of other body systems often need directed examination, the physiological characterization of this line could greatly benefit from a comprehensive understanding of SNX27 functions in intracellular trafficking, and an expanded list of SNX27 interacting partners, with preference given to the range of receptors and other proteins it targets through its PDZ domain. This would likely create a list of neurotransmitter and electrochemical messenger systems to evaluate for disrupted function. It is likely that the answers to both of the 'simple' questions above will require much more extensive answers.

A superficial survey of the SNX27 domains required for β2AR recycling function is presented in Appendix III. Out of the three protein regions predicted to encompass one

or 2 domains, recycling function seems to require them all, supporting the model presented in the last section. Each domain analysis comes with caveats to interpretation, however. The problem common to all is the act of deleting large stretches of primary sequence, and interpreting the loss of function as a result of the lost sequence as opposed to a structural alteration of the remaining sequence. We do have one indication that SNX27 retains a functional PX domain when deleting the Band4.1/RA region or altering the PDZ domain, as these mutants retain endosomal localization when transfected into cells. The PX domain deletion did not localize to endosomes or promote recycling. And though the caveat remains as to whether this is due to the lost PX function, analogy to the function of other PX domains supports a critical role for this domain in procuring endosomal localization and related endosomal function. Future experiments should additionally mutate more targeted structural features to aid in evaluating the contribution of each of the domain regions to recycling function.

Some point mutations were made to SNX27 in an attempt to do corroborate the deletion mutant results. A point mutation in the PX domain was reported to accomplish the same loss of endosomal residence as seen with our PX deletion mutant, however it did not work as well in our setup to disrupt endosomal localization, and it did not impair recycling function. Mutations to the PDZ domain had additional ambiguities toward interpreting the PDZ/affinity relationship as discussed in Appendix III, though the ability of several different mutations to impair recycling ability independently argued for the domain's importance. It is interesting to note that a protein reported to engage SNX27 with a PDZ ligand also has a PDZ domain of its own that could conceivably be the interfacing domain with the β2AR (MacNeil, Mansour et al. 2007). This protein is

reported to interact with cytohesin proteins that activate Arf proteins, which in turn are involved in vesicle formation. This PDZ network may additionally provide for an interesting model of PDZ-directed recycling. On the other hand, no mutational dissection of the overlapping RA and Band4.1 domains of SNX27 were attempted. This would surely aid in exploring the full biochemical function of SNX27 itself.

Several PDZ-mediated interactions with SNX27 have already been reported, and the common functional implications have been related to endocytic trafficking. While SNX27 is thought to promote endocytic targeting (presumably from enhanced endocytosis and/or reduced recycling) of a 5-HT receptor, it is also thought to promote degradation of an ion channel, and perhaps even polarization of immune complexes (Joubert, Hanson et al. 2004; Lunn, Nassirpour et al. 2007; MacNeil and Pohajdak 2007). The only tie to endocytic recycling thus far has been through a PDZ interaction with a lipid-modifying enzyme that is itself linked to the default recycling process (Rincon, Santos et al. 2007). As such, it seems as if the greater protein complex that SNX27 finds itself in may dictate the ultimate trafficking consequence. However, when placing the highest affinity ligand for SNX27 so far tested on the β2AR, internalization is effectively blocked. This raises the possibility that different SNX27 affinity ranges dictate different trafficking consequences. The confounding factor in this instance, however, is the additionally high affinity of the aforementioned ligand for NHERF as well. As such, perhaps the relative competition for PDZ domains is what dictates the trafficking direction taken.

A trafficking comparison of the already-established SNX27 binding partners combined with a knockdown of SNX27 in the same cellular background will help

validate the full range of trafficking effects mediated by SNX27. In addition, the swapping of PDZ ligands between these proteins will help to distinguish the contribution of greater protein complexes from ligand specificity in dictating specific trafficking outcomes. Knockdowns of interacting PDZ proteins will further tease out the relative contributions of different PDZ protein affinities in these processes, as well. Future work in mapping the ligand specificity spectrum for SNX27 will additionally aid in this endeavor. And considering that many of the PDZ ligand recycling sequences identified thus far appear to show some affinity for SNX27, it will be important to verify if other PDZ interactions mediate recycling as well. This can be inferred from testing ligands with no apparent SNX27 affinity for recycling activity when fused to the β2AR or δOR.

In addition to a molecular, SNX27-centric view of sorting, it was discussed earlier that an expanded characterization of endosomes, GPCR regulation, and the analysis of SNX27-regulated physiology would also naturally extend from the studies presented previously. With indication that a specialized tubule might serve the sequence-dependent sorting process, further focus to its molecular identity would surely allow the evaluation of mechanism and reveal regulatory checkpoints. With Actin as a major player in this process, a look to Actin-associated or Actin-regulated proteins would be a logical next step. In terms of GPCR regulation, identification of the post-translational modification dynamics would aid in predicting the compatibility of adaptors at different points in the endocytic pathway. And with the blurred concept of the 'default' post-endocytic fate for a GPCR, increased understanding of the lysosomal sorting process may aid in understanding the 'override' provided by recycling sequences. As the list of SNX27 interaction partners and cellular roles grow, so should the potential impact on physiology.

Since the first identification of the SNX27 transcripts was revealed based on upregulation in response to chronic methamphetamine treatment, the potential role of this gene in drug adaptation will likely be the first exciting area to watch unfold. If the most basic recycling consequence of resensitization (or diminished desensitization) is promoted by upregulation of *mrt* transcripts, then this upregulation could not only underlie the *enhanced* sensitization of model mammals and applicable neurons to methamphetamine and cocaine following repeated use, but it may allow for an enhanced signaling exposure that triggers multiple 're-wiring' events resulting in other altered behaviors associated with addiction to these substances. Whether monoamine vs. catecholamine vs. other neurotransmission events would underlie these changes in neuronal and behavioral adaption would be a natural follow-up focus of study. It is interesting to speculate that the enhanced production of SNX27 could not only enhance regulation of certain signaling proteins, but expand its range of PDZ-compatible binding partners and begin to regulate additional pathways not influenced at basal levels of expression. In appendix III, we noted this effect in an artificial system. And while upregulation of *mrt in vivo* would likely not mimic regulation of a protein with an altered PDZ ligand like we studied *in vitro*, it may allow SNX27 to expand the number of type I PDZ ligands it can shuttle into their relative pathway, expanding the types of signaling systems regulated after chronic drug treatment, or regulating new effectors of the systems already impacted. In addition to neural studies, regulation of β2AR recycling and signaling in the many other tissues in which the receptors resides may reveal interesting modes of regulation, especially when considering the link between recycling and Gs/Gi switching in cardiomyocytes. And following this physiology and biochemistry into the

dynamics of live cell signaling and trafficking may lead back to a fundamental appreciation of the increasingly suspected ‘signaling’ endosome.

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Appendix I: PDZ, and not NSF, interaction is required for recycling of the β2AR

As discussed in Chapter 2, the divergent PDZ ligands found in human vs. several rodent β2AR species offered not just a look at divergent receptor function between important model species and our own, but a clue as to the contribution of PDZ v. the non-PDZ β2AR tail interactions with NSF, as the mβ2AR tail sequence was unable to pull down detectable NSF protein while an EBP50 pull down was robust. While the functional differences between the murine and human β2AR PDZ ligand sequence in murine cardiomyocytes were highlighted in Chapter 1, this appendix will highlight the relative *lack* of trafficking differences due to the divergent ligands as observed in the HEK 293 cell culture model. The role of other divergent receptor sequence will also become apparent in this culture system, however. Additional assays of NSF-β2AR interaction are provided to support the extreme differences in hβ2AR and mβ2AR, NSF-interaction capability. While it is virtually impossible and thus formally incorrect to determine that the mβ2AR does *not* directly coordinate with NSF *in vivo*, the negative results presented here predict just that. And certainly since both the hβ2AR and mβ2AR exhibit recycling phenotypes, this argues against a required interaction with NSF for recycling. However, the use of mβ2AR sequence in the HEK293 cell model in this appendix supports the generality of that assertion.

The mβ2AR does not induce NSF endosome recruitment or fluorescence polarization. While fusion protein of glutathione S-transferase and the intracellular C-terminus region of the mβ2AR is unable to pull NSF out of tissue extract, this may result from unanticipated consequences of separating the mβ2AR tail from the receptor, fusing it to another protein, and purifying it from bacteria. This type of assay may also have detection limits that miss physiologically-relevant interactions. As such, we investigated the interaction using two other complementary assays that do not completely overcome these concerns, but strengthen the relevance of such negative results.

The hβ2AR was shown to colocalize with a GFP-tagged NSF in a PDZ-ligand-dependent manner in HEK293 cells (Cong 2001). We thus wanted to see if the mβ2AR would be able to interact with NSF in the context of an intact cell. When cotransfected in HEK293 cells, NSF-GFP was recruited to hβ2AR-containing endosomes upon activation of the receptor with 10 μM isoproterenol (Figure 1, top row). It should be noted, however, that this effect was not as obvious when expressing NSF-GFP in a cell clone already stably-expressing the hβ2AR (data not shown). This suggests that the interaction is enhanced by higher expression levels than would be found physiologically (and even in some over-expressed culture systems). Nevertheless, we wanted to investigate any possibility of an evolutionarily-conserved interaction, and cotransfected NSF-GFP with the mβ2AR in HEK293 cells. Following activation and internalization of the receptors, NSF-GFP was not observed to recruit to endosomes Figure 1, 2nd row). In addition, the difference between these receptor species could be attributed to their divergent PDZ ligands, as a 'swap' of their PDZ ligands, a single amino acid change on either receptor, was sufficient to confer the colocalization phenotype of the other receptor species (figure

1, bottom 2 rows). These observations confirmed, using full-length receptors in a cellular context, the relative incompatibility of NSF with the mβ2AR due to the receptor's divergent PDZ ligand.

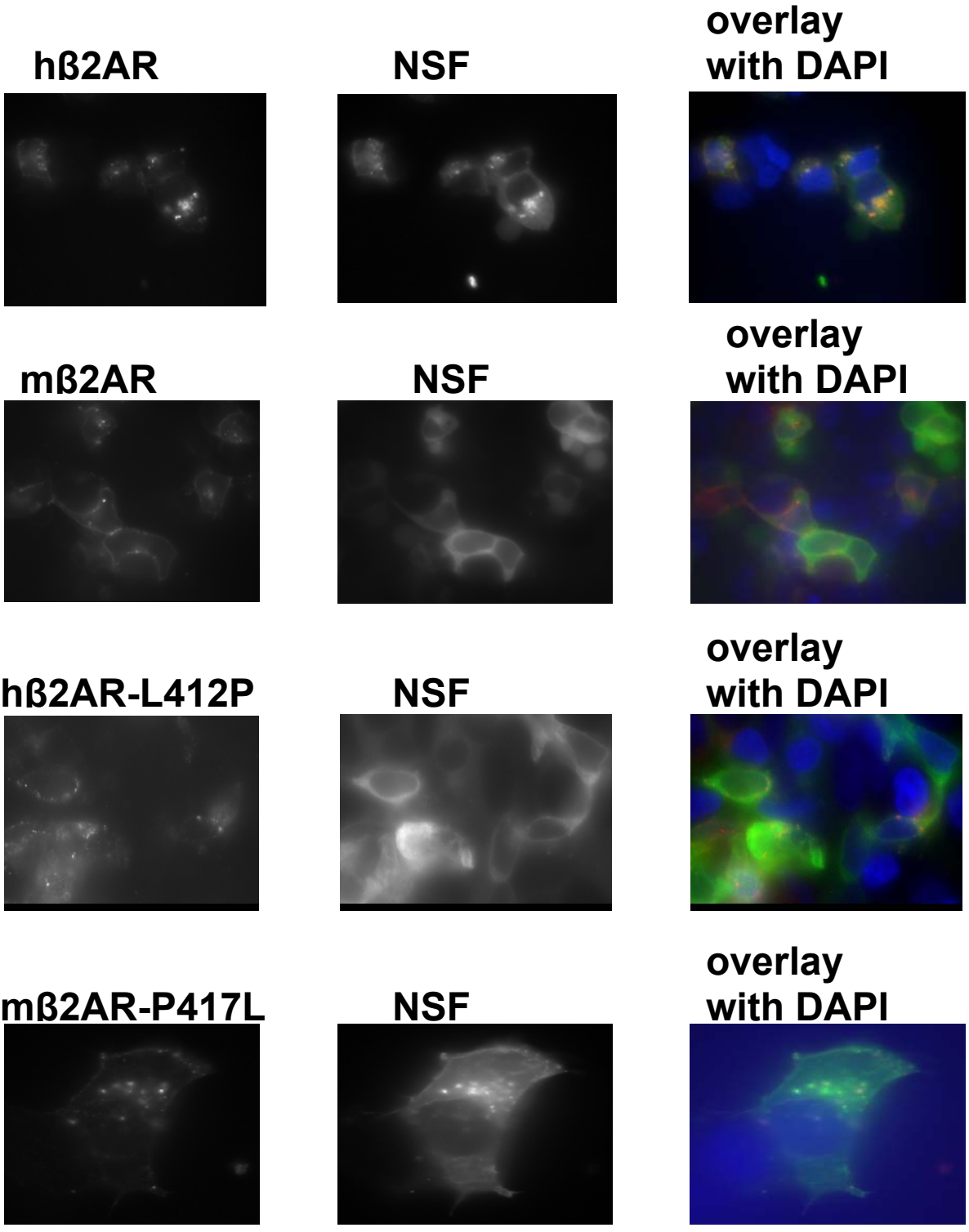


Figure 1. Colocalization of NSF with the β2AR is dependent on the receptor PDZ ligand sequence.

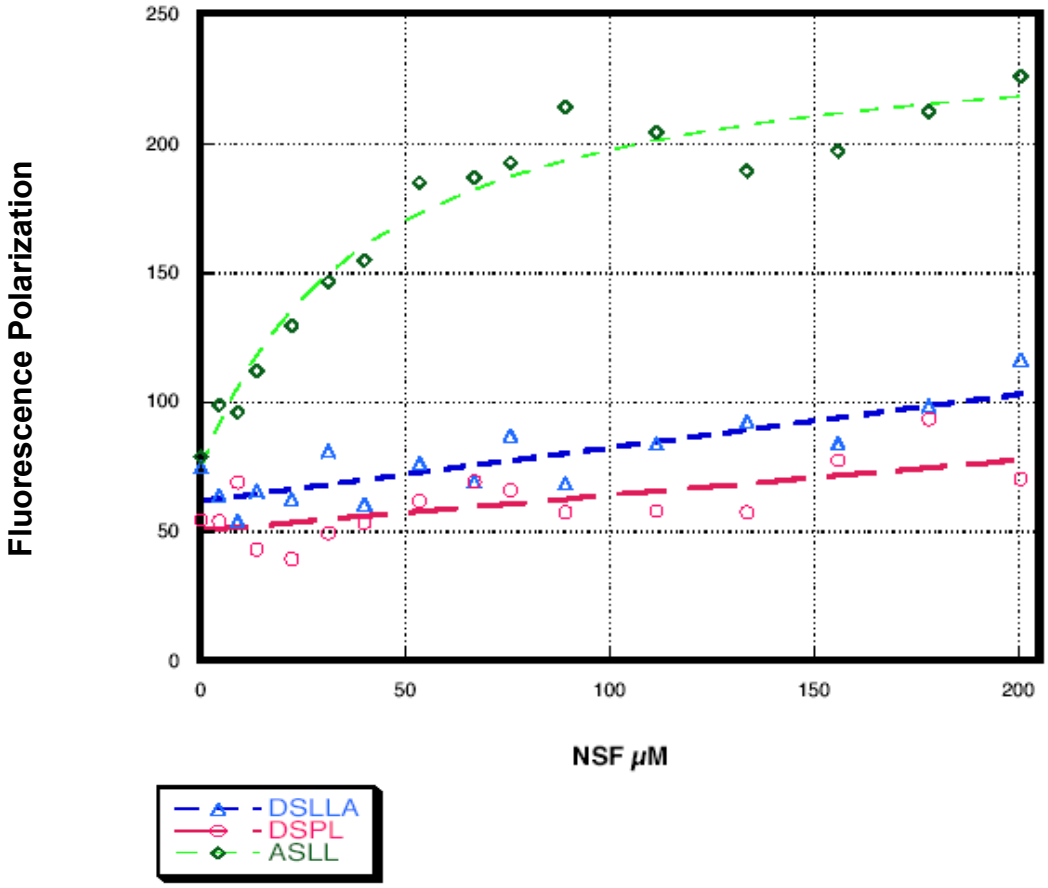
HEK 293 cells were transfected with GFP-NSF and the indicated receptor before labeling receptors with fluorescent M1 antibody and stimulating receptor internalization with the β adrenergic agonist isoproterenol. Cells were then fixed and prepared for wide-field fluorescent microscopy through the center of the cell.

The incompatibility of NSF and the mβ2AR in cell culture supported an idea proposed in Chapter 2 asserting that functional consequences of the interaction are species-specific. The data supporting that was presented more as a difference in the degree of the interaction between receptor species, suggesting that a weak interaction had functional consequences on mβ2AR-mediated signaling in cardiomyocytes. We thus wanted to utilize a protein interaction assay that would be able to detect weak affinities better than a GST pulldown. One attempt that was made to detect a weak interaction was to trap NSF in the ATP-bound state for colocalization. This has been reported to increase affinity for the receptor, and would halt any possible, enzyme-induced turnover of a receptor interaction (Gage 2004). While the use of EDTA in GST-pulldowns effectively did this (Mg++ is required for ATPase activity of NSF), we transfected an ATPase-abolishing, E329Q mutant NSF to replicate this in cells. This mutant was deleterious to trafficking (and cell survival) and did not allow internalization of receptors for a useful assay (not shown). As such, we turned to a fluorescence polarization assay that would be able to detect weaker affinities than pulldown assays with or without the use of enzyme disruption.

Cristina Melero in the laboratory of Tanja Kortemme had previously been able to detect an interaction between a purified, N-terminal region of NSF (or the 1st PDZ domain of NHERF1/EBP50) and a FITC-labeled hexapeptide of the hβ2AR PDZ ligand in a fluorescence polarization assay (the nucleotide-binding domains were not a factor in

this assay). We thus decided to investigate any affinity of the mβ2AR PDZ ligand for NSF, and compare it to other FITC-labeled ligands with preference for NSF over NHERFs (TNASLL) and with disruption of all NSF and PDZ binding activity (TNDSLLA). As expected, the NSF-specific ligand displayed saturable association with the NSF N-terminal region (predicting a Kd of around 40 μM, up from the hβ2AR Kd of around 18 μM (not shown)) whereas the negative control ligand did not produce a comparable or even saturable polarization in the 200-μM limit (Figure 2, top graph, green and blue lines). Rather surprisingly, the mβ2AR ligand also did not show any sign of specific binding in this range (figure 2, top graph, red line).

To confirm that this starkly contrasted the ability of this ligand to associate with PDZ domains when separated from the receptor, we repeated this series of ligand binding with a purified protein corresponding to the 1st PDZ domain of NHERF1/EBP50. Whereas the classical negative control ligand failed to show specific binding, the reported NSF-specific TNASLL ligand was actually able to show saturable PDZ binding with a calculated Kd of around 19 μM (figure 2, bottom graph, blue and green lines). As expected, the mβ2AR ligand showed saturable PDZ binding with a Kd of ~9 μM, more similar to but up from the 3 μM Kd seen with the hβ2AR ligand (figure 2, bottom graph, red line, hβ2AR data not shown). Taken together, this data shows that the mβ2AR ligand has high specificity for PDZ interactions over an NSF interaction (which was not even detected), whereas the specificity of the NSF-specific peptide is much more relative, with implications to be discussed at the end of this appendix.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200
150
100
50
0
0
50
100
150
200
NSF μM
DSLLA
DSPL
ASLL

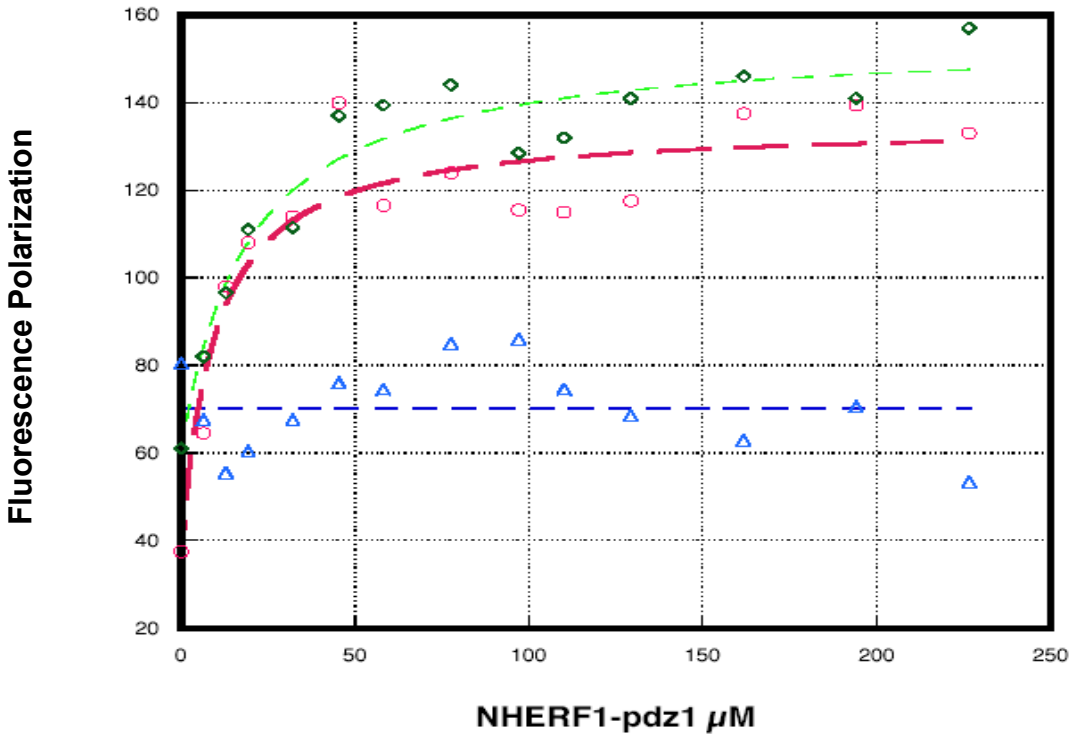

Fluorescence Polarization
160
140
120
100
80
60
40
20
0
50
100
150
200
250
NHERF1-pdz1 μM

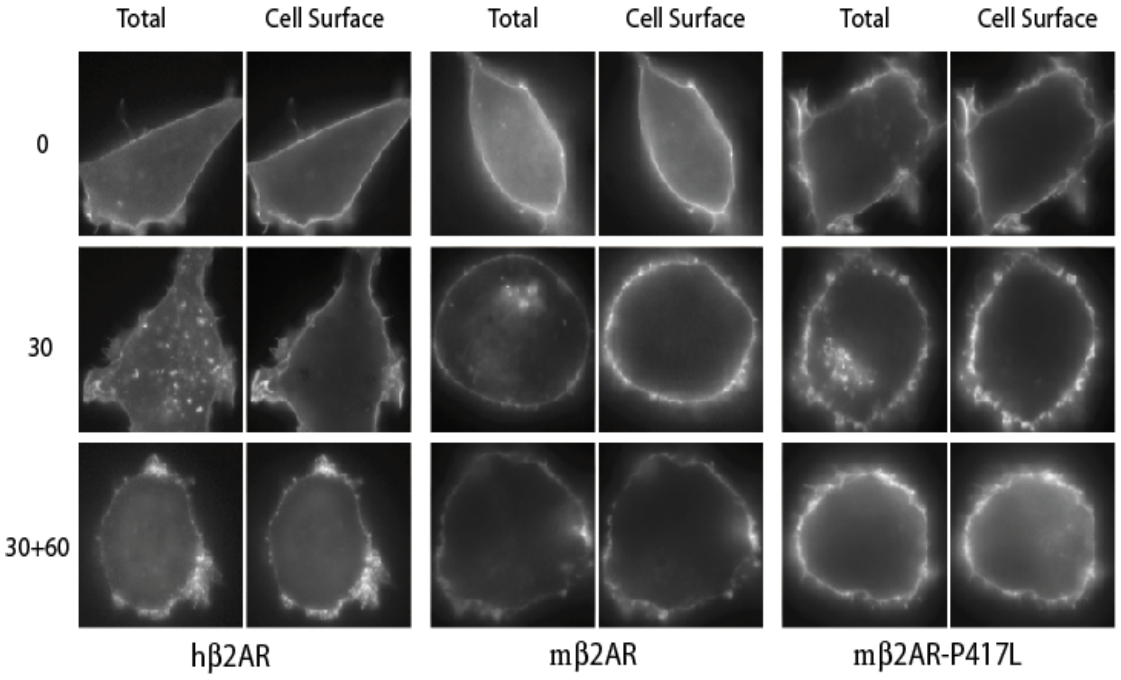
Figure 2. Interaction of β2AR-derived peptide ligands with NSF and NHERF PDZ protein. The indicated peptides were FITC-labeled at their N terminus and mixed with increasing concentrations of NSF N-terminal protein or the 1st PDZ domain of NHERF1/EBP50. Polarization of the FITC fluorescence indicated increased association of the peptide with the purified protein. Data is a representative experiment that was only repeated 1-3 times depending on the ligand used.

β2AR recycling efficiency correlates with PDZ affinity and not NSF affinity. Based on the work presented in previous chapters, a role for PDZ interactions in recycling of the β2AR should come as no surprise. However, the correlative work between protein-interaction affinity and recycling ability displayed in Gage et al., 2004, Chapter 2, and this appendix were so consistent, that a focus on individual PDZ interactions became warranted. As seen in the previous section, an NSF interaction with the mβ2AR was not detected in our most sensitive assays. Yet we knew the receptor qualitatively recycled in mouse cardiomyocytes, albeit with slower kinetics. We also wanted to investigate mβ2AR trafficking in HEK293 cells in order to make a direct comparison with the hβ2AR data in that cell type, and characterize any species-specific effects of the divergent PDZ ligands.

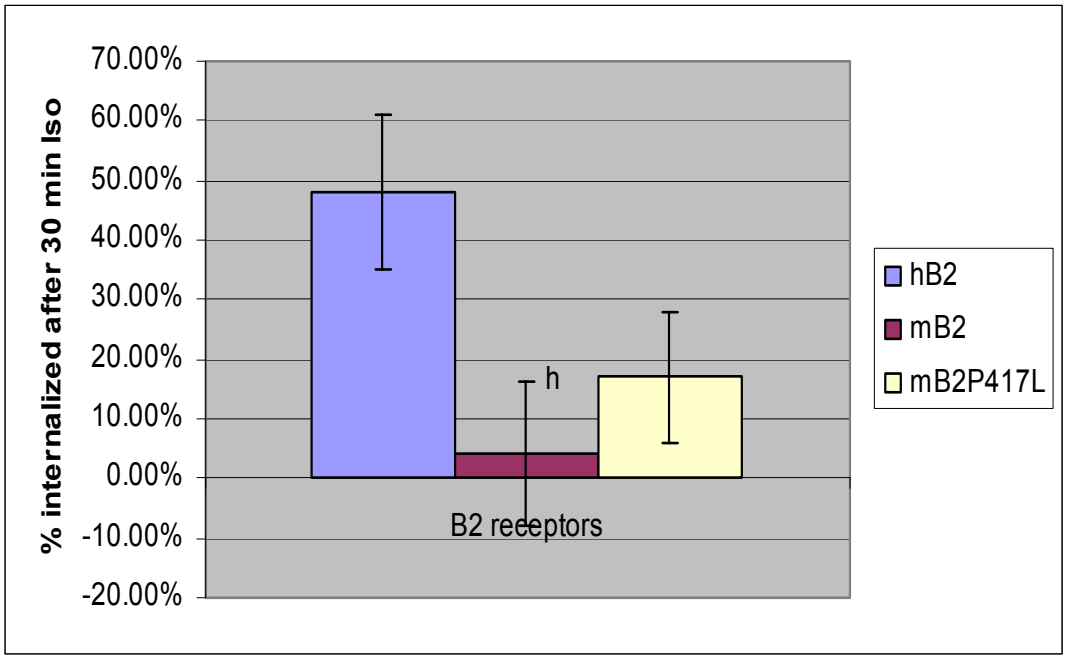
The first analysis of mβ2AR trafficking in HEK293 cells utilized stable cell clones expressing a FLAG-tagged mβ2AR, hβ2AR, or mβ2AR-P417L (humanized PDZ). Fluorescent M1 antibody was 'fed' to these cell clones before internalization of receptors with isoproterenol for 30' and subsequent agonist washout for 60' (controls of cells naïve to drugs, and only receiving agonist were prepared in parallel). Following drug incubations, surface M1 antibodies were labeled with a secondary antibody labeled with an alternate fluorophore for use in separating surface receptors from surface and internal receptors. The 'ratiometric' analysis of micrographs taken from these samples allowed

determination of a relative internalization and recycling as established previously (Tanowitz 2003). Figure 3 shows representative pictures from these experiments, which qualitatively did not evident much difference in the ability of any receptors to be delivered to the surface, internalize or recycle (though differences in the degree of internalization can be surmised). The following analysis of the internalization and recycling abilities of the receptor variants in figure 3 revealed some surprising differences, however. The first surprising result was the markedly reduced internalization *extent* of the mβ2AR when compared to the hβ2AR (figure 3B, first 2 bars). The second surprise was that this difference did not arise solely from the divergent PDZ ligand sequence, as the P417L mutant internalization was also much less than that of the hβ2AR (figure 3B, last bar). Despite these differences in internalization, though, was the efficient and indistinguishable recycling of all 3 receptors 60' after agonist washout, this time in agreement with the behavior observed in cardiac myocyte culture (figure 3C and Chapter 2, figure 2).

A


Total
Cell Surface
Total
Cell Surface
Total
Cell Surface
0
30
30+60
hβ2AR
mβ2AR
mβ2AR-P417L

B


% internalized after 30 min Iso
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
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h
B2 receptors
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mB2
mB2P417L

C

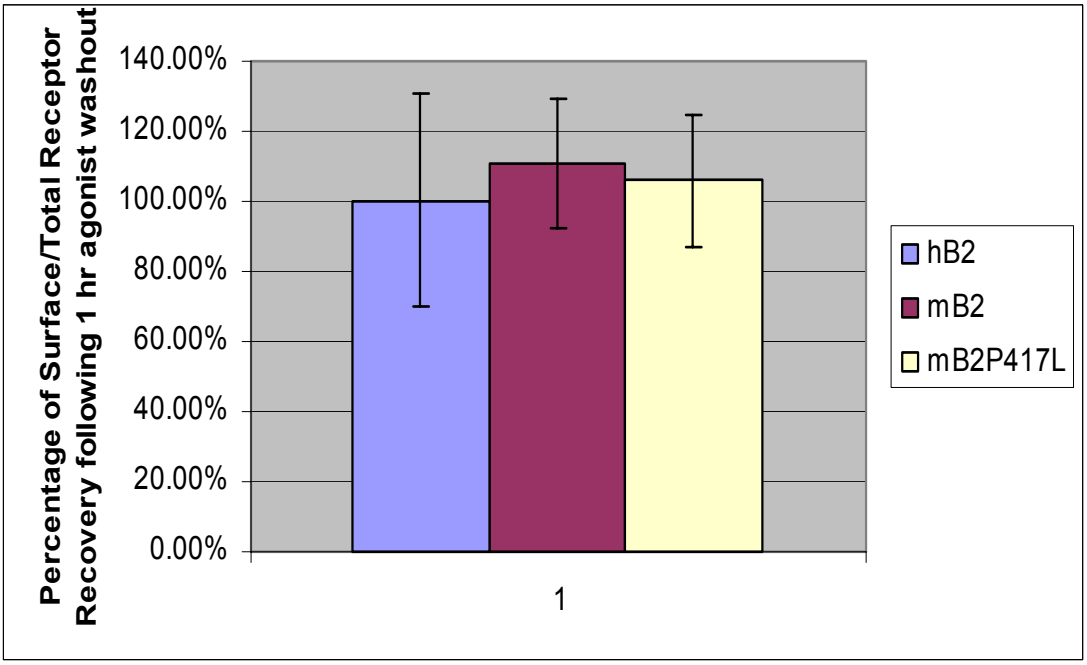


Figure 3. Murine β2ARs undergo reduced internalization but comparable recycling in HEK 293 cells.

(A) HEK 293 cells stably-transfected with the indicated receptor were given the recycling (30+60) or control (0 or 30) conditions described in the Materials and Methods section below and micrographs were taken at a central z plane to evaluate internal from surface receptor. **(B)** Internalization of receptors were calculated from the ratio of the integrated fluorescence of surface receptors to the integrated fluorescence of total labeled receptor in micrographs of single cells as shown in (A). **(C)** Recycling was calculated using similar ratiometric analysis described in the Materials and Methods section below. Images and analysis are representative of at least 4 experiments examining at least 15 cells per receptor expressed per condition. Error Bars represent the standard deviation from the mean.

To confirm the effects seen in these experiments in a larger population of cells, we employed a variation of the flow cytometric trafficking assays seen thus far. For these experiments, fluorescent M1 antibody was administered to stably-expressed

HEK293 cell clones in the culture medium before the familiar drug treatments. Following these incubations, cells were lifted in Ca++-chelating buffer which stripped remaining surface antibody. Internalization was thus evaluated by an increase in fluorescence, whereas recycling resulted in the loss of it. In addition, we suspected a confounding effect of multiple divergent sequences between the human and murine β2AR on internalization in HEK 293 cells, and thus we added not only a PDZ-mutated mβ2AR, but a hβ2AR-L412P mutant (murinized PDZ) as well.

Figure 4 shows an internalization time course for the 2 β2AR species and their PDZ-ligand-swapped counterparts in expression-matched cells. The 4 receptor types shown segregate into 2 groups based on their species of origin and not their PDZ ligand sequence. While no differences in kinetics or the extent of internalization were observed based on PDZ ligand sequence in this assay, separate assays with other cell clones did hint at such an effect (not shown). However, based on the inconsistency of this effect, and the relatively minor magnitude of it compared to the general species difference, it seems prudent to relegate the role of divergent PDZ ligands in β2AR internalization as a very cell-type-specific factor. And while the main factors accounting for the different internalization extents between receptor species in HEK293 cells remain unknown, a hotspot of sequence divergence occurs in the proximal receptor tail. This suggests an incompatibility of human GPCR kinases and/or arrestins with the mβ2AR, despite the converse not being applicable to the hβ2AR in murine cardiomyocytes (Chapter 2, figure 3).

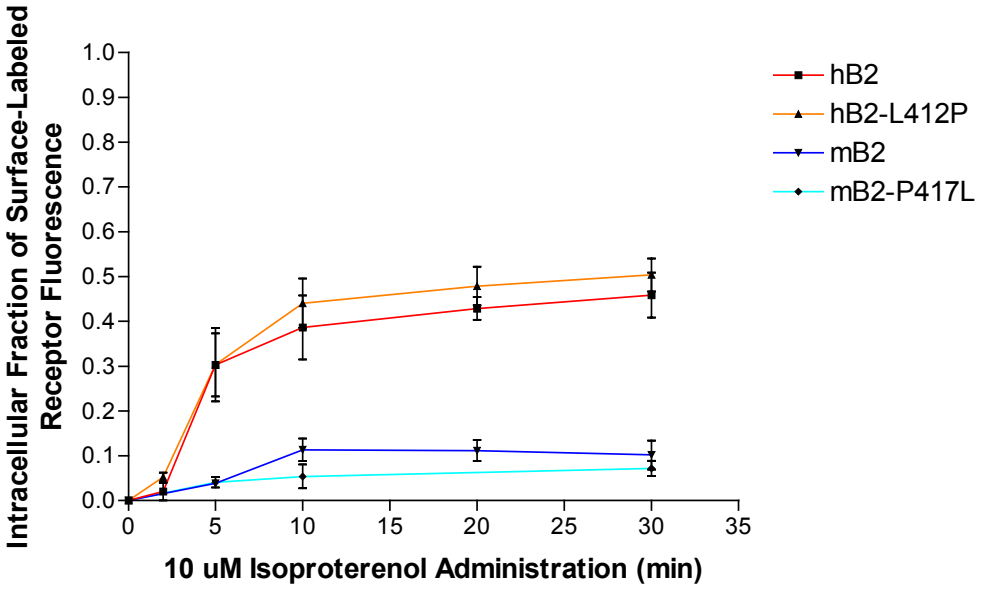


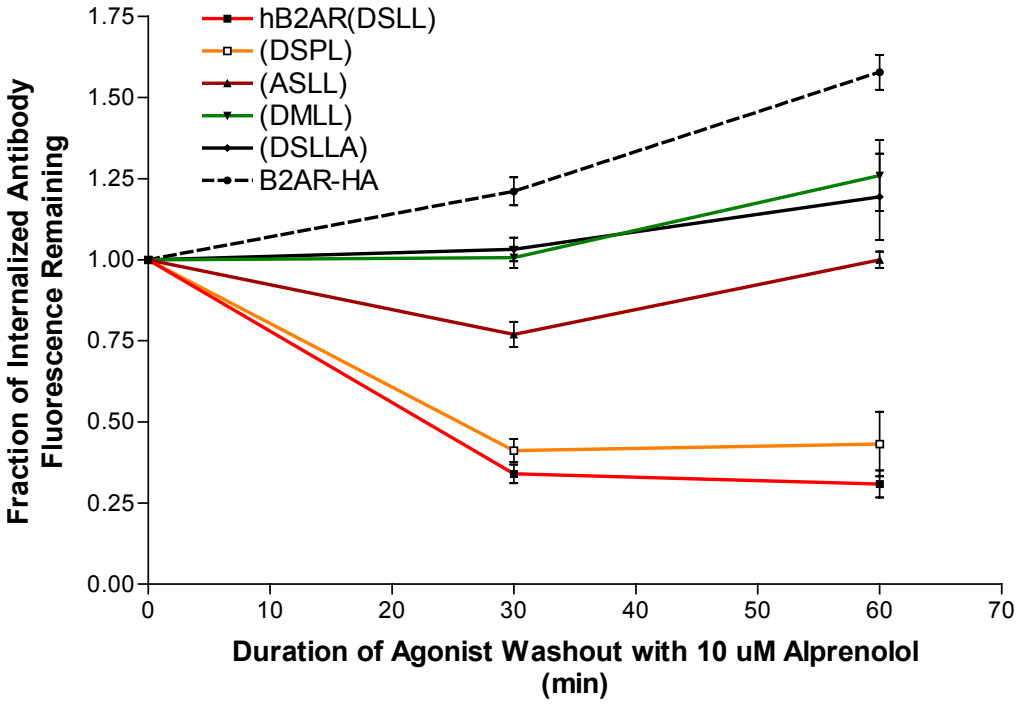
Figure 4. m β2ARs undergo less internalization in HEK 293 cells than hβ2ARs independent of their PDZ ligand divergence.

HEK 293 cells stably expressing the indicated receptor were administered M1 antibody conjugated to Alexa Fluor-647 in the culture medium followed by agonist for the indicated time period. Cells were then stripped of surface antibody and detected for fluorescence intensity by flow cytometry.

While we wanted to thoroughly characterize the effects of PDZ ligand variation on endocytic trafficking of the β2AR, particularly in regards to NSF v. PDZ contributions, the recycling route has been the main trafficking route linked to the β2AR PDZ ligand. And since we wanted to avoid confounding effects of the divergent β2AR receptors seen with internalization, we focused our secondary recycling assays on PDZ ligand mutation of the hβ2AR. In addition to the non-NSF-associating DSPL variation, we reexamined recycling mediated by the reportedly NSF-specific ASLL variant, and 3 non-specific, C-terminal disruptions: DMLL, DSLLA, and DSLL-HA (an HA tag fused to the C-terminus of the receptor). In this assay, internal fluorescence of wt hβ2ARs was reduced to around half of its agonist-pretreated state (figure 5A, red line). While 50%

recycling efficiency would seem low in comparison to other measurements, this can likely be explained by the propensity for remaining internal fluorescent antibodies to 'brighten' as they remain intracellular over time, as seen with the poorly-recycled DSLLA, DMLL, and DSLL-HA modified receptors (figure 5A, green, black, and dotted lines, respectively). Why the β2AR-HA receptor exerts the greatest brightening is unknown, but perhaps reflects a subtle decrease in recycling efficiency compared to the β2AR-Ala mutant that is not appreciable in other assays. Alternatively it could reflect the intracellular redistribution of the β2AR-HA receptors in an unanticipated way. In any event, and despite the sensitivity of this recycling assay, the murine and human β2AR PDZ ligand sequences displayed recycling that was virtually indistinguishable, despite their differential preference for NSF (figure 5A, red and orange lines). The NSF-favoring ASLL ligand, on the other hand promoted poor levels of recycling, but slightly better than the non-specific C-terminal variants (figure 5A, brown line). This suggested either a weak ability for decent NSF-binding, or a strong ability of weak PDZ interactions to induce recycling. Interestingly, correlating the Kd for selected ligand interactions with NHERF1PDZ1 to recycling ability (or fluorescence loss) created the sense of a 10 μM Kd threshold, after which recycling ability would be substantially reduced (figure 5B). Taken together, these results point to the ability of PDZ interactions to govern recycling more strongly than NSF, if NSF even plays a physiological role in this context at all.

A


hB2AR(DSLL)
(DSPL)
(ASLL)
(DMLL)
(DSLLA)
B2AR-HA
Fraction of Internalized Antibody Fluorescence Remaining
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
50
60
70
Duration of Agonist Washout with 10 uM Alprenolol (min)

B

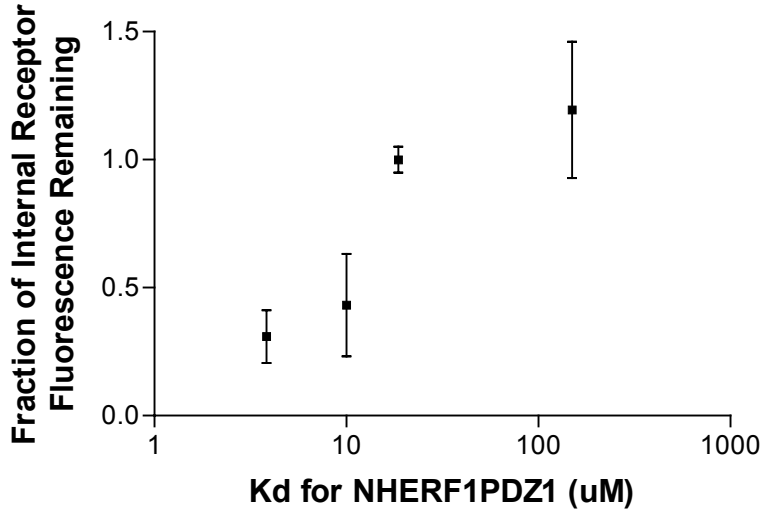

Fraction of Internal Receptor Fluorescence Remaining
1.5
1.0
0.5
0.0
1
10
100
1000
Kd for NHERF1PDZ1 (uM)

Figure 5. Recycling of β2AR PDZ ligand variants correlate to PDZ-binding affinity.
(A) Human β2ARs mutated to express the indicated PDZ ligand variations were stably expressed in HEK 293 cells. Following labeling of surface receptors with Alexa Fluor-647-conjugated M1 antibody, internalization was induced with agonist for 30 minutes, followed by 30 and 60 minute incubations with antagonist. Remaining surface antibody was stripped, and internalized receptor fluorescence was measured using flow cytometry. Error Bars represent SEM with n=4 **(B)** Affinities between 4 ligands chosen from (A) and the NHERF1PDZ1 domain were calculated in a fluorescence polarization assay and the corresponding Kd was plotted against the fluorescence remaining at time 60 in (A). Affinites used were only determined in one assay, though the wild type affinity of ~3 uM was independently confirmed. Error bars reflect the SEM for the normalized fluorescence.

In summary, we have failed to detect an interaction between NSF and the mβ2AR using endosome recruitment and fluorescence polarization assays. While the mβ2AR exhibits reduced internalization but qualitatively preserved recycling in HEK293 cells, the defect in internalization cannot be accounted for by its divergent PDZ ligand, as recycling of the hβ2AR with a 'murinized' PDZ ligand internalizes with similar kinetics to the wild type hβ2AR. In addition, recycling of this variant receptor is virtually identical to that of the wild type in the time points shown, despite any evidence for an NSF interaction capability. Recycling ability of the β2AR with PDZ ligand variations correlates with PDZ affinity for the NHERF1PDZ1, but not with NSF. This suggests that differences between mβ2AR and hβ2AR PDZ ligand function may actually be explained by variations in PDZ binding activity and not an NSF interaction. This also reaffirms the growing evidence for a required PDZ interaction involved in mediating endocytic recycling of the β2AR.

Appendix II: Ambiguities from the δOR-Fusion and Synthetic PDZ Interaction Extensions of Actin-Mediated GPCR Recycling

Chapter 3 highlighted the cytoskeletal-dependent recycling of chimeric δORs fused to the C-terminal domains of NHERF1/EBP50 and ezrin, recapitulating a complex between the β2AR and the actin cytoskeleton that was proposed to mediate endocytic recycling. While the ability of these domains to retain their interaction function on a receptor as they do in their native protein was supported by mutational disruption and/or cytoskeletal dependence of function, additional validation of the specificity v. generality in which actin connectivity promotes GPCR recycling was sought, with ambiguous results and a word of warning to further uses of the receptor fusion approach. In addition, a parallel strategy of utilizing an engineered PDZ interaction was functionally employed during the development of this project in an attempt to better recapitulate the proposed recycling complex, avoiding problems arising from covalent fusion of protein to receptor (which in the end did not present an issue with the first domains used) and gaining insight not only into protein networks sufficient for recycling, but also insight into the PDZ affinity ranges that could mediate them. The utilization of this system required a mutational loss of recycling function, a rescue with at least one engineered protein-binding partner, and then the establishment of specificity for the protein interactions involved. While the loss of recycling function was achieved, the rescue of it was not at this stage. This hurdle will be overcome in appendix III when applied to SNX27-

mediated recycling, and the additional challenge of determining specificity will be highlighted there.

Connection of GPCRs to the NHERF/ERM complex through a synthetic PDZ interaction does not induce receptor recycling. Before the activity of the ERMbd, Abd, or SNX27 were implicated in recycling function, the sufficiency of any protein interaction outside of the vague involvement of one or many PDZ interactions to promote GPCR recycling was unknown. The ability of multiple PDZ ligands with little overlap in their known binding partners to promote recycling suggested that potentially many proteins were sufficient to induce GPCR recycling (Gage 2004). However, since most ligands coordinate with multiple binding partners, the relative sufficiency of any of these complexes to promote recycling was a mystery. Knowledge of even one sufficient complex could provide insight into a recycling pathway that was largely uncharacterized. And further investigation of the sufficiency of proteins with related PDZ or downstream functional domains could provide insight into the redundancy, robustness, and regulation of the recycling process. What was needed was to investigate the possible sufficiency of individual PDZ proteins (or their downstream functions) in mediating recycling without the involvement of other confounding PDZ interactions. This was achieved in Chapter 3, but the alternative approach will be presented here.

Based on the crystal structure of the alpha1 sytnrophin-nNOS (nitric oxide synthase) PDZ interaction (Bredt and Lim, 1999), Tanja Kortemme had directed the computational selection of a new ligand-domain residue pair through 1) predicting destabilizing ligand mutations at a conserved ligand position providing one of the most

favorable energetic contributions towards interaction; 2) predicting compensatory mutations at the main interface position in the PDZ domain that would restore favorable energetics of interaction with the given ligand alteration; and 3) choosing a residue pair that not only offered an opportunity to destabilize and restore affinity when mixed with wild-type counterparts and matched together, respectively, but included a ligand alteration that did not conform to one of the evolutionarily-conserved consensus sequences. The only residue changes satisfying all these criteria will be dubbed ligand* and syn* (syn for the alpha1 syntrophin PDZ domain), as the method or application of this system has not yet been reported publicly. When applied to a C-terminally-derived ligand from a separate syntrophin PDZ interaction, fluorescence polarization assays confirmed a Kd increase from ~8 to >100 μM when introducing the * mutation into the ligand, and restoration of a Kd around ~12 μM when then compensating the ligand* with the syn* domain (personal communication).

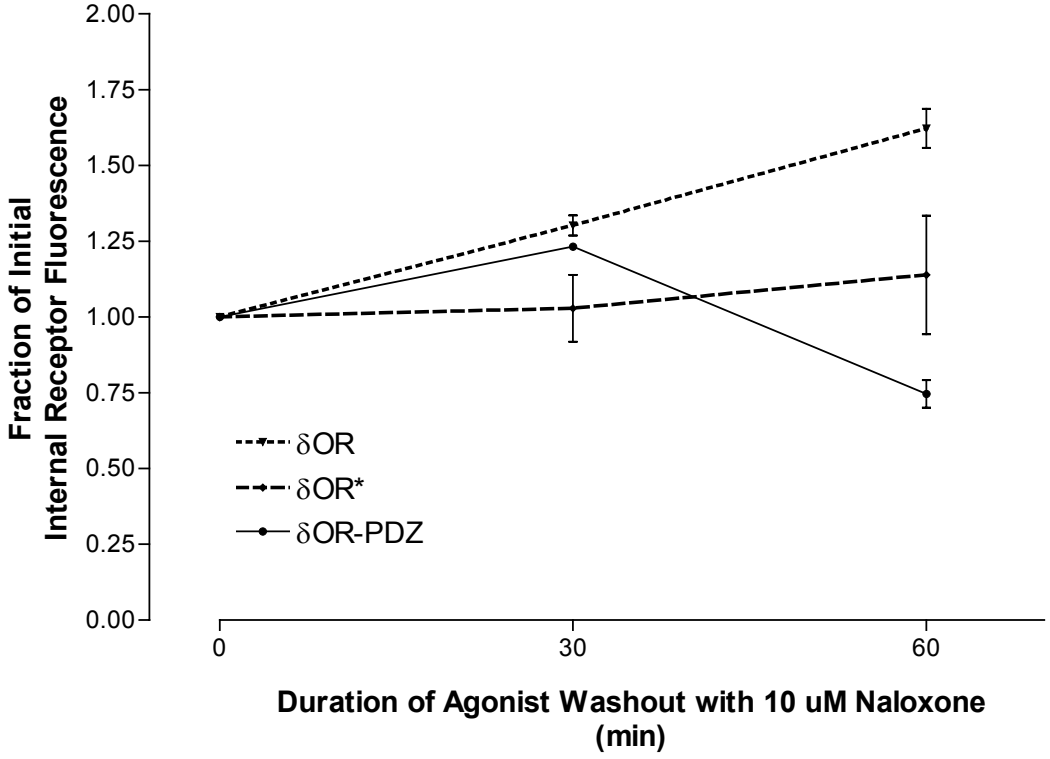
The first requirement for this system to be applied to GPCR recycling was for the ligand* to not induce recycling when attached to a receptor. As such, we added this ligand* to both the δOR C-terminus (δOR*) and replaced the last 6 residues of the β2AR with this sequence (β2AR*). Initially, δOR* was transiently expressed in HEK293 cells and recycling was evaluated by immunofluorescence microscopy. This yielded initially confusing results, as intracellular and surface receptor staining patterns were observed in the same cells administered the recycling experiment paradigm (data not shown). However, once a positive and negative control was employed (a δOR fused to the β2AR PDZ ligand and the wild type δOR), it became clear that the ability to recover surface staining distinguished δOR-β2[10] from δOR* and the wild type receptor. To measure

this effect, the internal fluorescence version of the flow cytometric, recycling assay (introduced in appendix I) was employed in HEK293 cells stably expressing wild type δOR, δOR*, or δOR-PDZ (containing a 6 residue PDZ ligand found in the NR2B glutamate receptor subunit). The internal fluorescence related to these internalized receptors in general underwent a muted decrease or enhanced increase following agonist washout when compared to results using the β2AR variants (figure 1A compared to appendix I, figure 5A). The positive control δOR-PDZ only barely dropped its fluorescence level while the wild type δOR dramatically increased its fluorescence level with agonist washout time (figure 1A, solid and dotted lines). The δOR* had quite variable change in its fluorescence, which averaged out somewhere between the 2 controls in an unsatisfying manner (figure 1A, dashed line). As such, we not only switched assays for better dynamic range, but began investigating the ability of the ligand* to mediate recycling of the β2AR.

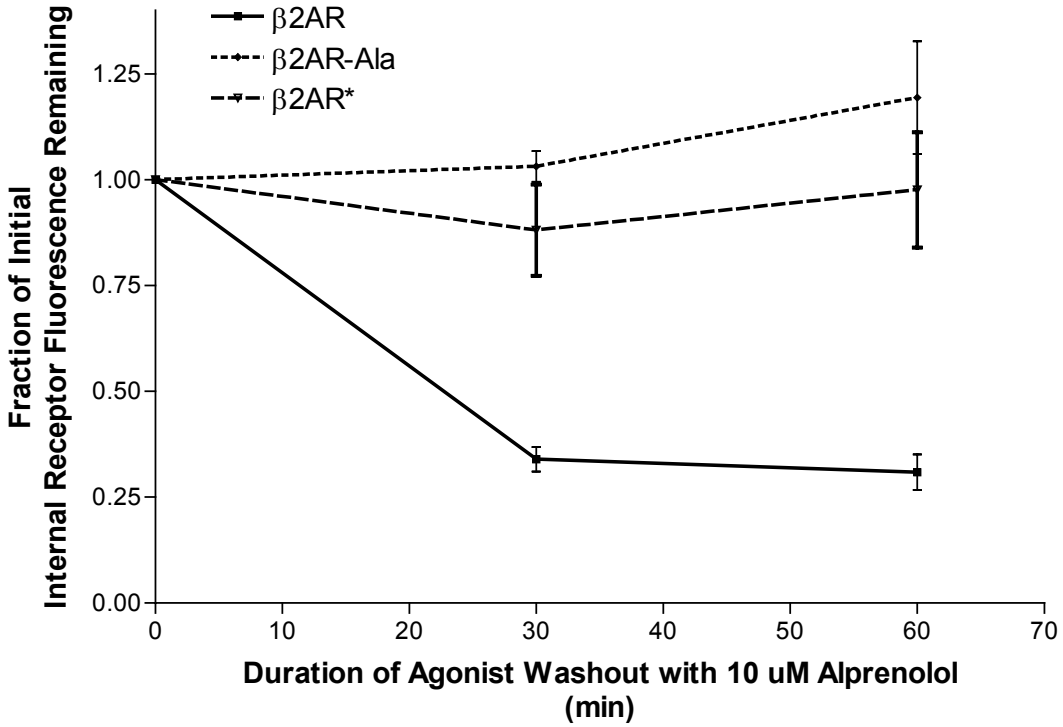
We first started employing the same internal fluorescence, flow cytometric assay in HEK293 cells stably expressing β2AR* or the wild type and β2AR-Ala controls. These results clearly distinguished the good recycling of the β2AR from that of the β2AR-Ala mutant and the β2AR* (figure 1B), suggesting that the ligand* is a poor recycling sequence in this receptor background. To confirm these results by tracking surface receptor changes over the same time course of drug treatments, we employed the surface-receptor, flow cytometric assay used for the β2AR in chapter 4. Again these results clearly established the β2AR* as a poorly recycling receptor, virtually indistinguishable from the Ala mutant in this regard (figure 1C). And microscopy experiments would show a strikingly more internal receptor remaining after agonist

washout for the β2AR* and Ala mutant than for the wild type (not shown). However, we were still curious to see if the ligand* was having a different effect on the δOR, so we employed the more sensitive surface-tracking, recycling assay utilized for δORs in Chapter 3. These experiments, using both the δOR-β2[10] and δOR-PDZ as positive controls, yielded varying but significantly higher recycling than the wild type negative control, whereas δOR* recycling again appeared enhanced relative to that of the δOR, but not as robust as normal PDZ ligand-mediated recycling (figure 1D). This established the ligand* as retaining residual recycling activity in the δOR but not in the β2AR context. As such, the β2AR background would provide our best system for the investigation of interaction-induced recycling.

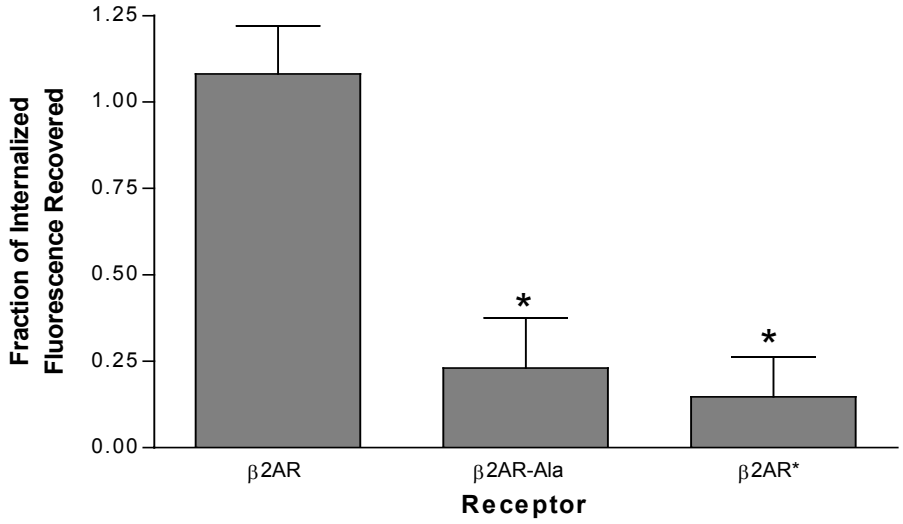
A



B


β2AR
β2AR-Ala
β2AR*
Fraction of Initial Internal Receptor Fluorescence Remaining
1.25
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
50
60
70
Duration of Agonist Washout with 10 uM Alprenolol (min)

C


Fraction of Internalized Fluorescence Recovered
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1.00
0.75
0.50
0.25
0.00
*
*
β2AR
β2AR-Ala
β2AR*
Receptor

D

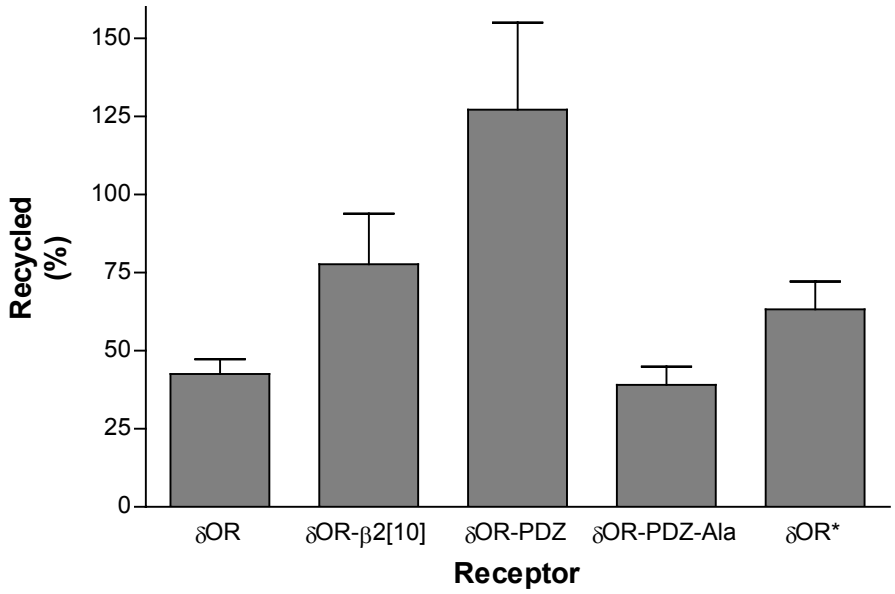


Figure 1. The ligand* confers weak recycling when fused to the δOR but not the β2AR.
(A) The δOR, PDZ-binding δOR-PDZ, and δOR* were stably expressed in HEK 293 cells. Following labeling of surface receptors with M1-647 fluorescent antibody, receptors were internalized with agonist and allowed recovery time periods without agonist. Remaining and returning receptors on the surface were stripped of antibody, and the internal fluorescence remaining was measured throw flow cytometry. Error bars represent the SEM of 3-4 experiments per receptor. **(B)** The β2AR, β2AR-Ala, and β2AR* were analyzed for recycling as in (A) (n=4). **(C)** The β2AR, β2AR-Ala, and β2AR* were stably expressed in HEK 293 cells and labeled at the cell surface with M1antibody. Receptors and antibodies were internalized and recycled for 30 and 60 minutes, respectively, and the proportion of internalized receptors recycled was calculated as described in Chapter 4. Error bars represent the SEM of 4-5 experiments. * above bars indicates a $p<0.05$ when compared to wild type. **(D)** The indicated receptors were examined for recycling by a surface receptor loss and recovery assay used in Chapter 3. Error bars represent SEM of 4-5 experiments.

With a ligand* working as an inert, recycling sequence on the β2AR, there was room to gain recycling function. The next step was to make a functional protein containing syn* that would provide this gain of function. Because of the prevailing NHERF hypothesis of the day, the 1st PDZ domain of NHERF1/EBP50 would be replaced with syn*, and tagged N-terminally with GFP for detection of cells containing it. Because the receptor fusion approach was giving supporting roles to the downstream ERM/actin complex, as well, we suspected PDZ fusions to domains from this complex would be good positive controls. In this series we ended up with 4 engineered proteins expected to yield recycling in addition to 6 negative control proteins (which became 10 negative controls after inspection of the 1st 4). The predicted 'recyclers' are summarized in the table below, with their domain architecture and subcellular localization (which suggested functionality of some domains in some cases).

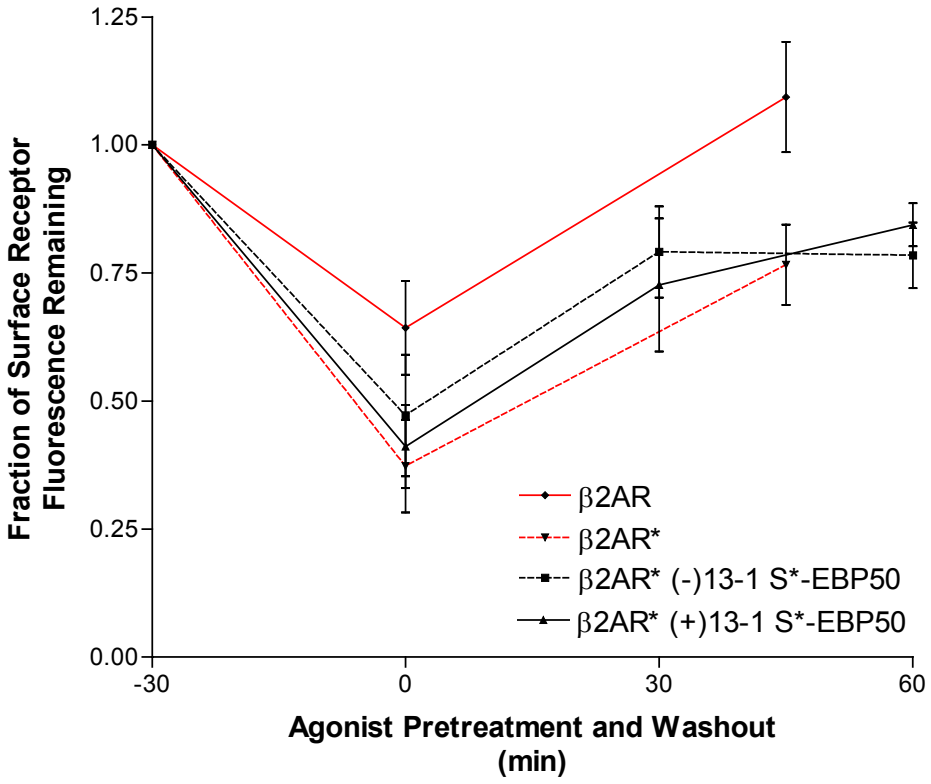
Table 1. Fusion Proteins with Predicted Recycling Activity

Code Name	Domain Architecture	Subcellular Localization
BL13-1 Syn*-EBP50	GFP-Syn*- NtruncEBP50(PDZ2+ERMbd)- HA tag	Cortical/filapodial and cytoplasmic
BL16-1 Syn*-ABD	Syn*-ABD(hEzrin)	Cortical/filapodial, nuclear, and cytoplasmic
BL19 2xSyn*-ERMbd (LL)	GFP-Syn*-Syn*-ERMbd(EBP50) (Glycine-rich 'Lim' linkers)	Internal puncta, cytoplasm
CM22 2xSyn*-ERMbd(NL)	GFP-Syn*-Syn*-ERMbd (EBP50) (NHERF linkers)	Internal puncta, cytoplasm

As alluded to before, these proteins did not induce recycling. Each one was assayed at least once using each of an internal fluorescence and surface fluorescence, flow-cytometric recycling assay. The analysis employed the same separation of cells into GFP (+) and (-) groups to look for the specific effect of the co-expressed protein, as seen

in Chapters 3 and 4. The normalized fluorescence data for these 2 groups, per engineered syn* protein, are shown in figure 2. The assay variation shown is the one with the greater replicates for the particular syn* protein. While we learned contemporaneously that the Abd and ERMbd could induce recycling when fused to the δOR, they were not compatible with a synthetic PDZ fusion. And since the ABD and ERMBD seemed to be inducing functionally-related subcellular localizations in the syn* proteins, it seems as if the problem related to the PDZ domain functioning in this setting. We will see more evidence for this as an issue in Appendix III, though the possible complications are great in number for any given protein engineered by blind domain juxtaposition.

A



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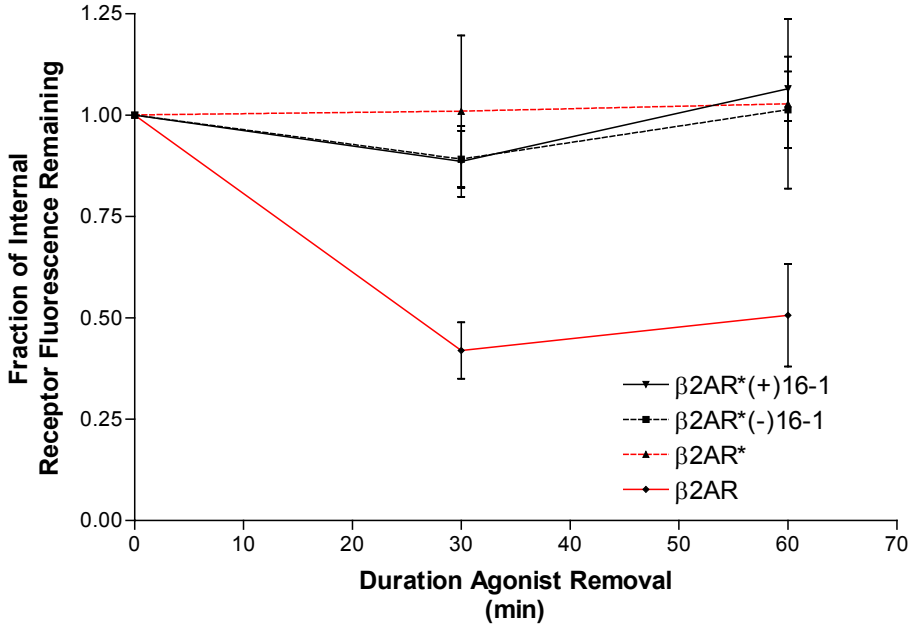
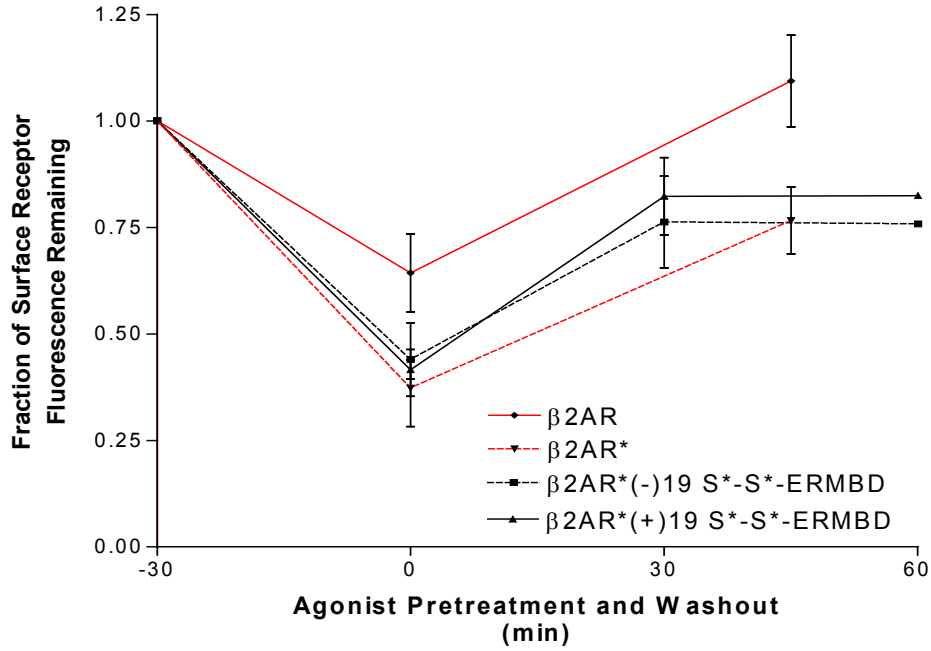

1.25
1.00
0.75
0.50
0.25
0.00
Fraction of Internal Receptor Fluorescence Remaining
0
10
20
30
40
50
60
70
Duration Agonist Removal (min)
β2AR*(+)16-1
β2AR*(-)16-1
β2AR*
β2AR

C


1.25
1.00
0.75
0.50
0.25
0.00
Fraction of Surface Receptor Fluorescence Remaining
-30
0
30
60
Agonist Pretreatment and Washout (min)
β2AR
β2AR*
β2AR*(-)19 S*-S*-ERMBD
β2AR*(+)19 S*-S*-ERMBD

D

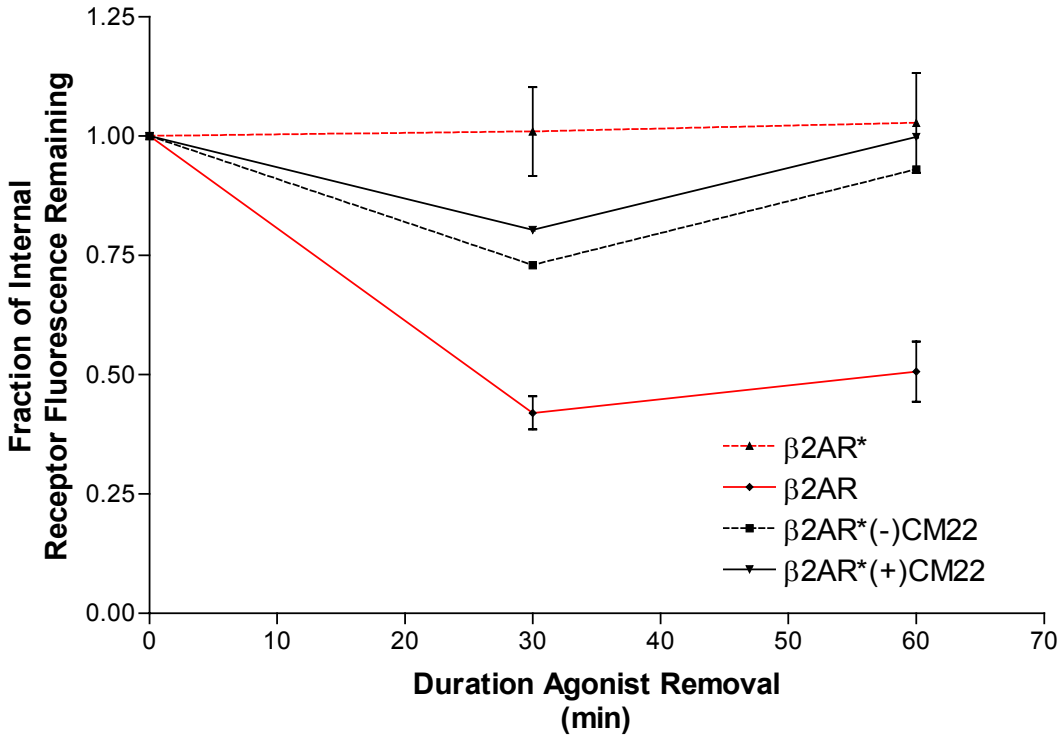


Figure 2. Attempts to Induce Recycling of β2AR*.

The proteins indicated in each legend were coexpressed with the β2AR* and receptors were tracked with fluorescent M1 antibody for recycling. N=2-4 for a particular staining method, of which 2 types were applied to each protein. The method shown is the one with the greater replicates. Error bars = SEM.

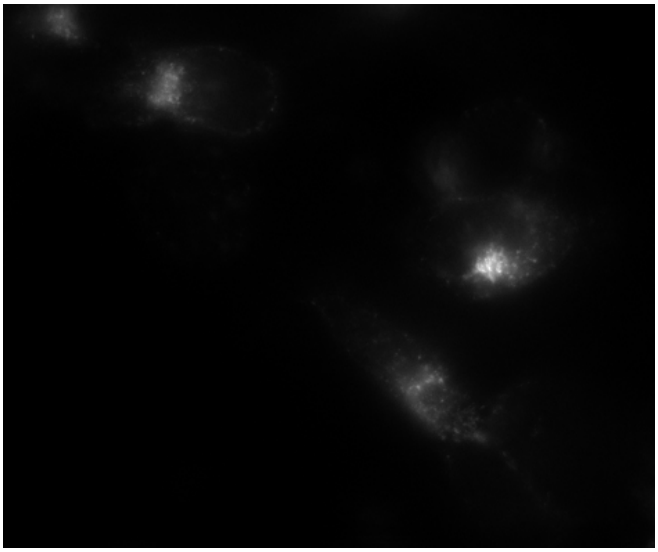
Recycling of the δOR can be induced by large protein additions to the C-terminus, unless that addition is a (-)end-directed motor. The success of promoting recycling of the δOR by linking it to actin with the Ezrin Abd was supported by the mutational loss of actin-binding activity and recycling activity, as well as the disruption of the Abd-mediated recycling by cytochalasin D (Chapter 3). This interaction dependence and sensitivity to actin cytoskeleton disruption distinguishes Abd and β2AR-mediated

recycling from that of the default-recycling transferrin receptor (Cao, 1999). However, Abd-mediated and transferrin recycling both are insensitive to endosomal Hrs manipulations, in contrast to the β2AR and other sequence-dependent recycling receptors (Hanyaloglu, Stenmark). This raised the question of whether Abd-mediated recycling utilized most of the same machinery as β2AR-mediated recycling, or whether the association with abundant cellular protein was simply a way to avoid degradative sorting and shunt receptors into the default recycling pathway? The low-level recycling seen by the wild type δOR, for example, is believed to be an inefficiency of the lysosomal sorting process to handle the receptor endosome load, possibly contributed to by overexpression of receptors (Hanyaloglu 2008).

To play with the idea of producing recycling by simple steric hindrance of degradative sorting, several large domains were fused to the δOR C-terminus. In addition to comprising 200+ residue domains, an alpha-actinin1 Abd (ACTN1Abd) and a KIFC1 microtubule-binding motor domain (400+ residue Kmd) would have the possibility of coordinating even larger protein complexes in the vicinity of the receptor, and thus be expected to shield receptors in the direction of following most membrane back to the cell surface. Initial microscopy studies provided a surprising result, which led to the realization of a large caveat of using a (-)-directed motor: the δOR-Kmd appeared to be clustering near the center of the cell after internalization and recycling treatments (figure 3A shows recycled condition). This intense clustering was so striking, that it led to the suspicion that the motor activity was working too well, transporting the receptors to a place where recycling was not possible. As such, additional fusion receptors were made with either of two halves of the motor domain, one predicted to retain microtubule

binding (an Mbd) and one predicted to retain nucleotide binding (dubbed Nbd). Surface-labeled flow cytometry of HEK 293 cells stably expressing these receptors were employed to evaluate recycling as consistent with the Chapter 3 experiments. These experiments confirmed a low level of δOR-Kmd recycling similar to the wild type receptor (though not brought to 0 as suspected might be possible), and validated observations obtained from microscopy examination of δOR-ACTN1Abd, that it exhibited robust recycling (figure 3B). Examination of δOR-Mbd and –Nbd recycling, however, also exhibited robust recycling and cast doubt on the actin-binding ability of the ACTN1Abd to promote recycling. Examination of the recycling of these receptors with actin cytoskeletal disruption was never revisited to validate the dependence of ACTN1Abd-mediated recycling on F-actin, but the important warning was made when considering the effect of the large Mbd and Nbd addition to the δOR C-terminus on recycling. Encouragingly for the use of the smaller Ezrin Abd and ERMbd, however, was the almost negligible effect of a GFP addition on recycling (figure 3).

A



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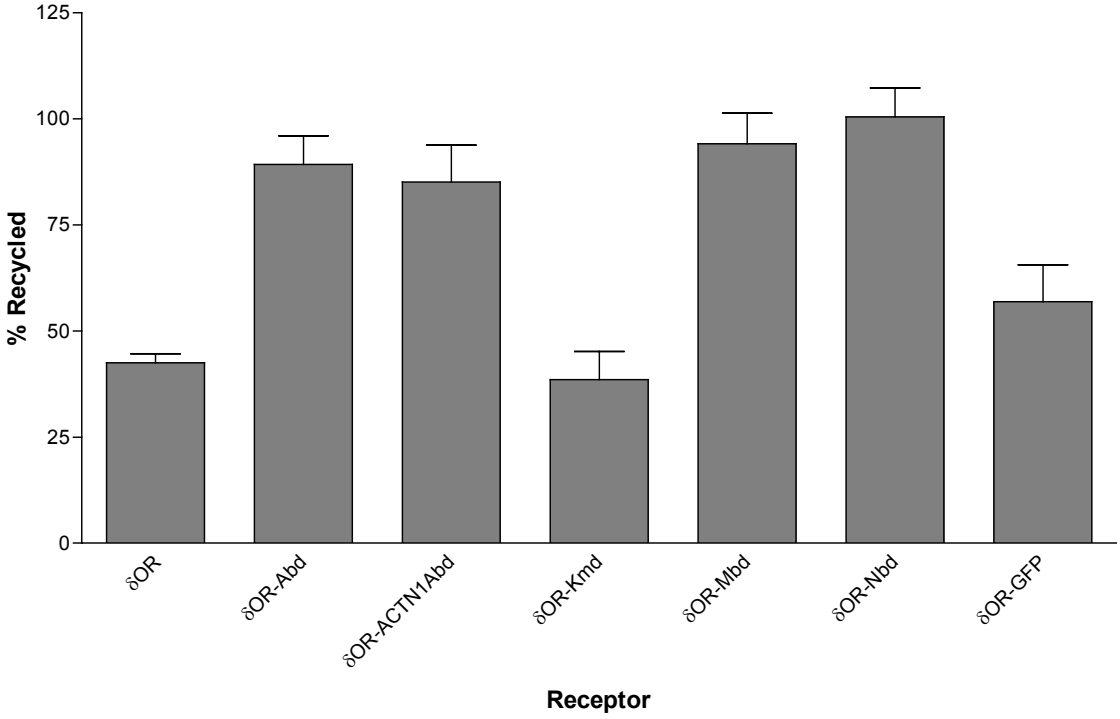

125
100
75
50
25
0
% Recycled
δOR
δOR-Abd
δOR-ACTN1Abd
δOR-Kmd
δOR-Mbd
δOR-Nbd
δOR-GFP
Receptor

Figure 3. Large additions of protein sequence to the δOR induce recycling, unless the sequence forms a (-)-end-directed motor.

(A) Transiently-transfected HEK 293 cells expressing the δOR-Kmd were administered recycling conditions as described in Chapter 3. A representative micrograph of 3 experiments, showing several cells is shown. **(B)** HEK 293 cells stably expressing the indicated receptor were assayed for recycling by the flow cytometric method employed in Chapter 3. Error bars represent the SEM of 3-5 independent experiments. Statistical significance is not highlighted, however the recycling levels fall into 2 groups distinct from eachother but not significantly different within the group: δOR, δOR-Kmd, and δOR-GFP vs. δOR-Abd, δOR-ACTN1Abd, δOR-Mbd, and δOR-Nbd. Results for several receptors are re-shown from Chapter 3.

Fusion of small villin headpiece domains to the δOR C-terminus promotes recycling, but cannot be disrupted by domain mutation. While the ability of actin-binding to promote recycling was supported by the experiments presented in Chapter 3, the use of alternate Abds to support the generality of this effect was sought. As we saw in the previous section, the recycling promoted by the use of an alpha actinin Abd could just as likely be explained by the size of the primary structure as it could be by any actin association. And these series of experiments were performed before the feasibility of testing recycling under conditions of actin disruption was optimized. Luckily, the well-characterized Villin headpiece (VHP) offered a good alternative as a relatively small F-Abd that could be impaired by mutation (Vardar 1999). As such, we fused 35, 40, and 68 residues from the greater VHP domain to the δOR C-terminus. It was predicted that the smaller the size, the weaker the actin affinity; though the relativity of this effect with respect to recycling was speculative. When examined by microscopy or flow cytometry, these δOR additions all promoted robust recycling (figure 4, microscopy not shown). As such, a reported amino acid substitution (W64A of the ABLIM3 VHP) was further

employed to attempt to disrupt actin affinity further. The use of the 35- and 68-residue domains containing this mutation product still promoted striking recycling when fused to the δOR, however (figure 4). The 'super recycling' (>100%) observed suggested the involvement of receptor surface delivery outside of the endocytosed pool, however pre-surface-labeled receptors also exhibited robust recycling in microscopy assays (not shown). As such, we could not be confident of an actin-promoted recycling process, and moved on to the other experiments highlighted in Chapter 3.

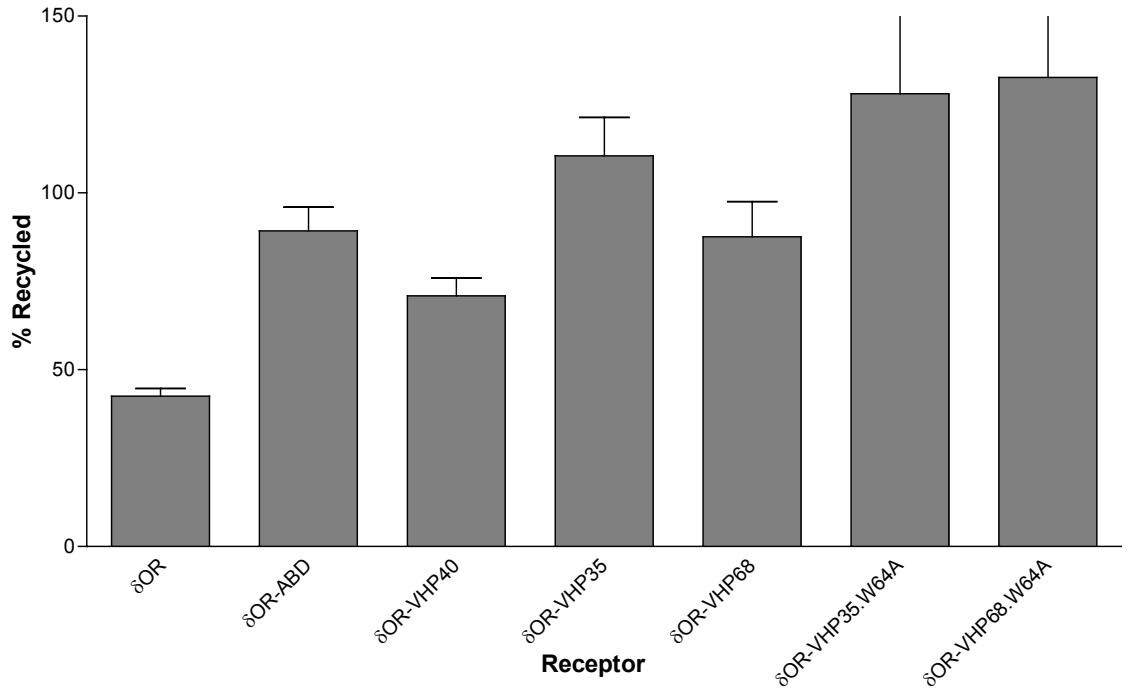


Figure 4. Villin headpiece domains and subdomains promote recycling of the δOR that cannot be abolished by actin-binding mutations.

HEK 293 cells stably expressing the indicated receptor were assayed for recycling by the flow cytometric method employed in Chapter 3. Error bars represent the SEM of 3-5 independent experiments. Results for the positive and negative control are taken from Chapter 3.

The experiments surveyed in this section all tried to extend the concept of the NHERF/ERM/F-Actin complex as a sufficient biochemical mediator of recycling. Seeing as these approaches were riddled with the potential for experimental artifact, no strong conclusions can be inferred from the lack of success in utilizing or controlling for these approaches. Seeing as the Abd from hEzrin was able to promote recycling when fused to the δOR, the lack of effect when trying to link through a synthetic PDZ interaction suggests an incompatibility of the PDZ domain to function in this format. However, seeing as knockdowns of NHERF protein were unable to dampen recycling in Chapter 4, perhaps the direct receptor fusion approach is the only way to make an actin-linking mechanism efficient. While additional actin-binding domains appeared to mediate recycling when fused to the receptor, the specificity of this effect for cytoskeletal coordination was not verified. Since other protein addition can seemingly promote default recycling in a presumably protein interaction-independent manner (Nbd) when fused to the δOR, it remains prudent to suspect that this is the case with the ACTN1Abd and even the smaller VHP, as mutational disruptions exert no disruption to recycling. However, addition of GFP to the δOR demonstrated that fusion of relatively large protein can be inert with respect to promoting recycling. And since the Ezrin Abd-mediated recycling is sensitive to mutation and cytoskeletal disruption, it would seem as if this protein module itself is unable to hinder degradative sorting. The question for future study is whether actin-binding itself serves as a steric inhibition of degradative sorting vs. an active promoter of recycling, and whether this mimics or contrasts to mechanisms explaining the sequence-dependent recycling of any GPCRs.

Appendix III: The Promiscuity and Expression-Level-Dependent Gain of Function of SNX27

Chapter 4 presented an unanticipated role for SNX27 in PDZ-mediated endocytic recycling of the β2AR. While this recycling role was dependent on the β2AR having its PDZ ligand recycling sequence, and could be hindered by PDZ mutation of a key, interaction-specificity residue in SNX27, this does not establish whether a direct interaction or PDZ protein network are responsible for this function. Additionally, because of the relatively weak *in vitro* affinity between SNX27 and the β2AR PDZ ligand, this raises questions as to the specificity of SNX27 for β2AR recycling versus other membrane proteins *in vivo*. SNX27 has been implicated to have degradative and endosomal targeting functions with some of its other binding partners (Lunn 2007, Joubert 2004). Another question of great importance, then, is how much does the PDZ affinity versus the environment in which the interaction takes place contribute to the functional sorting consequences? We have begun to explore these questions by investigating the specificity of the SNX27 PDZ domain *in vitro*, asking how different-affinity ligands affect β2AR trafficking as an appendage, and investigating the effects of overexpressing SNX27 variants with β2ARs containing low-affinity ligands. Additionally, we have examined the ability of SNX27-GFP to mediate recycling when one of the several predicted domains has been deleted. These data are largely preliminary, but together point toward an interesting relationship between PDZ affinity,

SNX27 domain structure, and recycling function that should be investigated more in order to expand our mechanistic understanding of SNX27-mediated sorting.

SNX27 is a promiscuous recycling protein. Because of the preference of various PDZ ligand recycling sequences for different PDZ proteins has come from the literature thus far, and the fact that several PDZ proteins have been shown to be required for recycling of different GPCRs, it has appeared as if there are almost as many 'recycling' PDZ proteins as there are ligands. Based on the SNX27-interacting ligands that have been published thus far, and the poor ability of the β2AR ligand to pull down *in-vitro*-translated SNX27 (not shown), SNX27 was not a great β2AR-interacting-protein candidate. However, as shown in Chapter 4, its subcellular residence and relation to the NHERFs made it worth the investigation of a possible affinity for the β2AR. And indeed, while not presenting as strong an affinity for the SNX27 PDZ domain in fluorescence polarization assays as it did for the NHERF1 version (Kd of ~18 μM vs. 3 μM, respectively), the data in Chapter 4 highly suggested the existence of a functionally-relevant PDZ interaction between the β2AR and SNX27 in the cellular environment. This raised the question of what affinity threshold was required for this functional interaction, and would various other SNX27 ligand partners serve as SNX27-dependent recycling sequences?

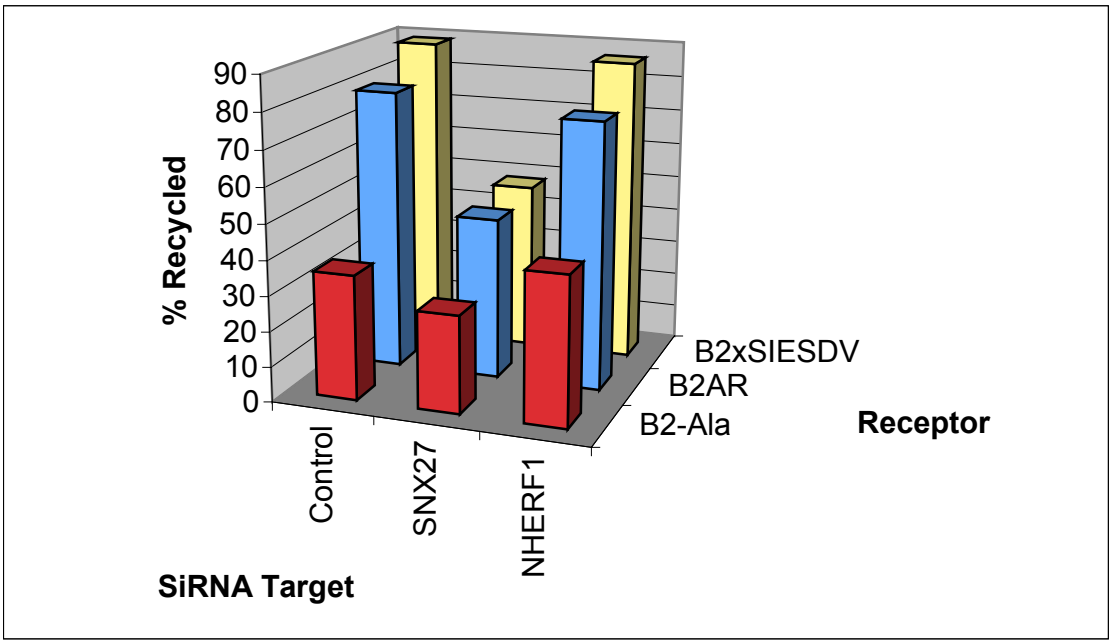
We decided to get an impression of the affinity of the SNX27 PDZ domain in fluorescence polarization assays for various reported PDZ ligand partners, and for recycling mutants analyzed in Appendix I. We also attached these various ligands to the β2AR, replacing its own ligand, and evaluated recycling ability of these ligands. Table 1

summarizes the results of these experiments, comparing the recycling ability of these ligands to the affinity for NHERF1 PDZ1 and SNX27 PDZ. As can be seen with this expanded ligand set compared to that of Appendix I, recycling ability of diverse ligands can be correlated to just SNX27 PDZ Kd < ~50 μM; on the other hand NHERF affinity does not work as a single correlative factor to recycling. This suggested that the relative SNX27 promiscuity for these PDZ ligands could explain the recycling activity of many PDZ-ligand-containing membrane proteins, with the caveat of not knowing the contribution of the greater protein complexes to the ultimate functional relationship between SNX27 and its naturally-occuring, interacting partners. Indeed, other sorting functions have been suggested for membrane proteins containing these or similar ligands (MacNeil 2007, Lunn 2007, Joubert 2004). While this may arise from different interaction contexts, it is interesting to note that the EEESRF ligand prevents internalization when attached to the β2AR, and its affinity for NHERF and SNX27 is the highest surveyed (Table 1, internalization data not shown). Thus, different combinations of varying PDZ affinity may result in different trafficking effects. However, to further establish that other ligands could mediate SNX27-dependent recycling when fused to the β2AR, we performed recycling analysis under conditions of SNX27 knockdown and rescue for the β2AR possessing the SIESDV ligand (Figure 1A and B). While not a comprehensive survey, these results predict that a low μM Kd between a ligand and SNX27's PDZ domain would allow a ligand to be a GPCR recycling signal. It will be interesting to see how particular receptor or membrane protein complexes and ligand promiscuity for several PDZ proteins complicate this.

Table 1. Correlation of PDZ affinity to recycling ability. The indicated peptide ligands were either FITC-labeled for fluorescence polarization analysis of PDZ affinity as described in Chapter 4, or attached to a SSF-β2AR for evaluation as a recycling sequence. A check indicates recycling efficiency >50% as calculated in Chapter 4, whereas an x marks recycling below that level. Not every analysis achieved an n=3.

β2AR tail sequence	Recycling Receptor	~Kd with NHERF1	~Kd with SNX27
EEESRF	N/A, no	1.3	0.5
ESESKV	✓	>100	5
SIESDV	✓	>100	5
TTESDL	✓	>100	15
TNDSLL	✓	4	18
TNDSPL	✓	9	30
SIEMDV	X	>100	50
TNASLL	X	19	>100

A


% Recycled
90
80
70
60
50
40
30
20
10
0
Control
SNX27
NHERF1
SiRNA Target
B2xSIESDV
B2AR
B2-Ala
Receptor

B

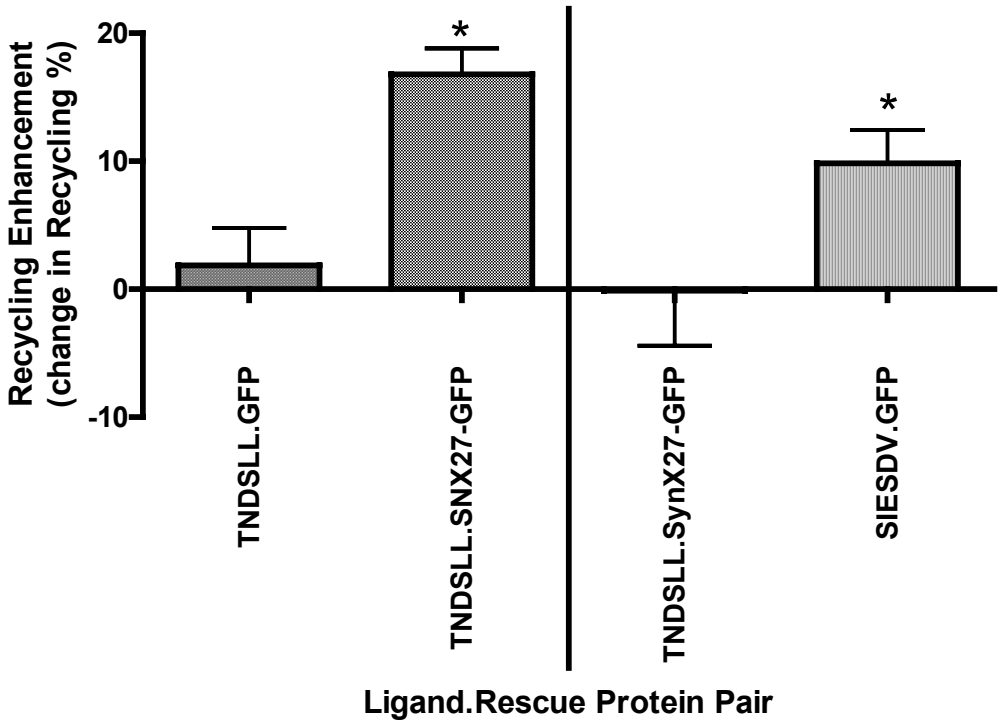

Recycling Enhancement
(change in Recycling %)
20
10
0
-10
*
*
TNDSLL.GFP
TNDSLL.SNX27-GFP
TNDSLL.SynX27-GFP
SIESDV.GFP
Ligand.Rescue Protein Pair

Figure 1. SNX27-dependent recycling mediated through diverse PDZ ligands.
(A) Recycling of hβ2ARs with the indicated ligands replacing the C-terminus was analyzed as in Chapter 4. **(B)** SNX27a-GFP-specific rescue of recycling was determined also as in Chapter 4. *denotes $p<0.05$ in paired t-test of recycling means between GFP (+) and (-) expression groups.

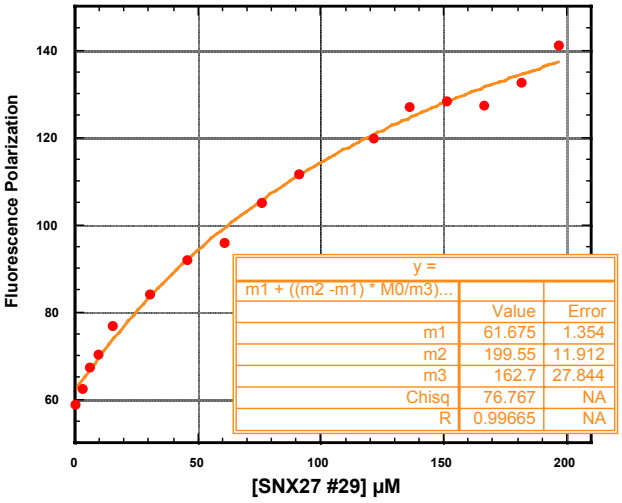
SNX27-dependent recycling requires predicted functional domains, and is sensitive to some, but not all, mutations to the PDZ domain. While the recycling function of SNX27 exhibits dependence on a highly conserved PDZ residue coordinating with the –2 ligand position, other PDZ mutations were utilized in an attempt to further establish this relationship. As shown in Figure 2A, 2 additional PDZ mutations decreased the SNX27-β2AR ligand affinity similarly to or greater than the H112A mutation shown in Chapter 4. Despite this similar hindrance of *in vitro* affinity, recycling function promoted by these mutations in the SNX27-GFP construct was preserved (Figure 2B). While this raises serious questions pertaining to the requirement of a PDZ interaction in SNX27-mediated recycling, it should be noted that these calculations were not repeated, and the affinity range that they fall in surround the detection limit of the assay, reducing confidence in relative comparisons between them. However, it appears safe to say that they all have reduced affinity for the β2AR ligand relative to the wild type PDZ protein. As such, it is surprising that any should function at all given the already low affinity of the β2AR ligand compared to other ligands we tested. Yet, the translation fidelity of all these affinity differences to the full length proteins in the cellular environment is not known, and thus this may not be as good a predictor of function as the variation of ligands. Though it does seem that SNX27 mutant overexpression seems to be able to produce functionally-relevant interactions with affinities lower than the functionally-relevant range observed with endogenous SNX27 expression in HEK293 cells (Fig. 2 and

Table 1). We will see a similar effect with low-affinity ligands in the next section, and because of this it is worth noting that the same shift in the affinity-function relationship with SNX27 overexpression may be at play here.

Before we move on, however, it should be noted that the SNX27 recycling function can be hurt by a PDZ replacement with the syntrophin PDZ domain, supporting the requirement for the PDZ domain, if not a classical PDZ interaction, in recycling function (Figure 3C). As a throwback to studies of NHERF sufficiency for recycling function, we additionally attempted to rescue recycling with overexpressed GFP-NHERF1 or 2, which did not prove successful in this situation (Figure 3 C, right bars). In addition, deletion of the other predicted functional domains in SNX27 (the lipid-binding PX domain and a multidomain, Band4.1/FERM and Ras-associating protein region) similarly produced functionally inept proteins, whereas a reported PX-debilitating point mutation, K220A did not (Figure 3C). While these deletions may produce adverse effects on the global protein function as opposed to hurting just a subcomponent of biochemical function, we can say that analysis of the mutants' subcellular localization revealed the retained ability to associate with endosomes for all but the PX deletion mutant (not shown), suggesting that the protein retains some functional structure when stripped of several domains. The retained endosomal localization of the PX point mutant indicated that the reported effect of this mutation was not as effective in our system, which could explain the preserved recycling function seen with this mutant. The results from the deletion mutants, however, suggest that SNX27 recycling function requires all predicted domain regions despite, in several cases, the preservation of endosomal localization.

Since several of these mutants were inert with respect to recycling function in a knockdown rescue experiment, we were curious to see whether they would interfere with the normal recycling of the β2AR. We thus coexpressed several of these mutants with the β2AR without the addition of a SNX27 knockdown, and analyzed protein-specific recycling as done with the rescue experiments. The mutants chosen were the Band4.1/RA region deletion and the H112A PDZ mutant. While coexpression of the H112A mutant hinted at a slight inhibition in β2AR recycling, the degree of this change or that of the other mutant and wild type SNX27-GFP recycling changes were so small that a substantial effect is not statistically significant (Figure 3D).

A

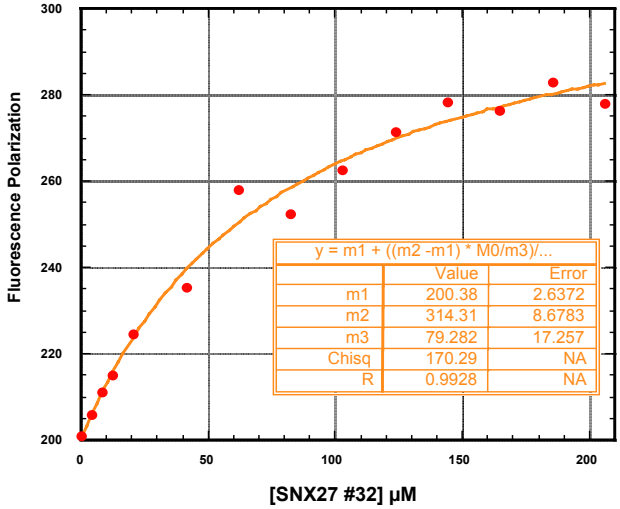


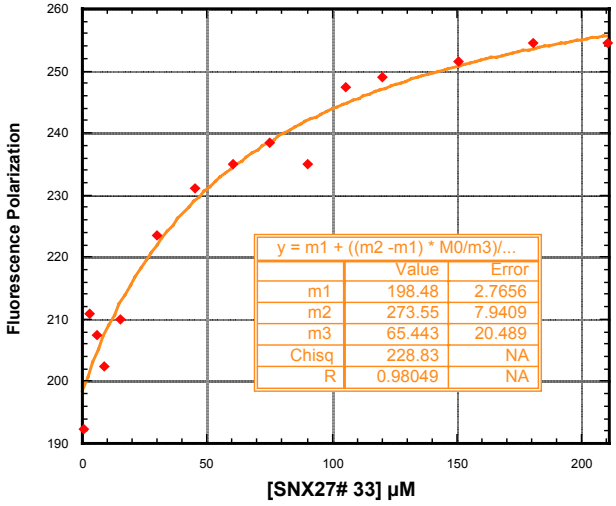
SNX27-mutants with FITC-*TNDSLL*

SNX27 #29 K46E targeting P_0

SNX27 #32 R56A targeting P_{-3}

SNX27 #33 H112A targeting P_{-2}





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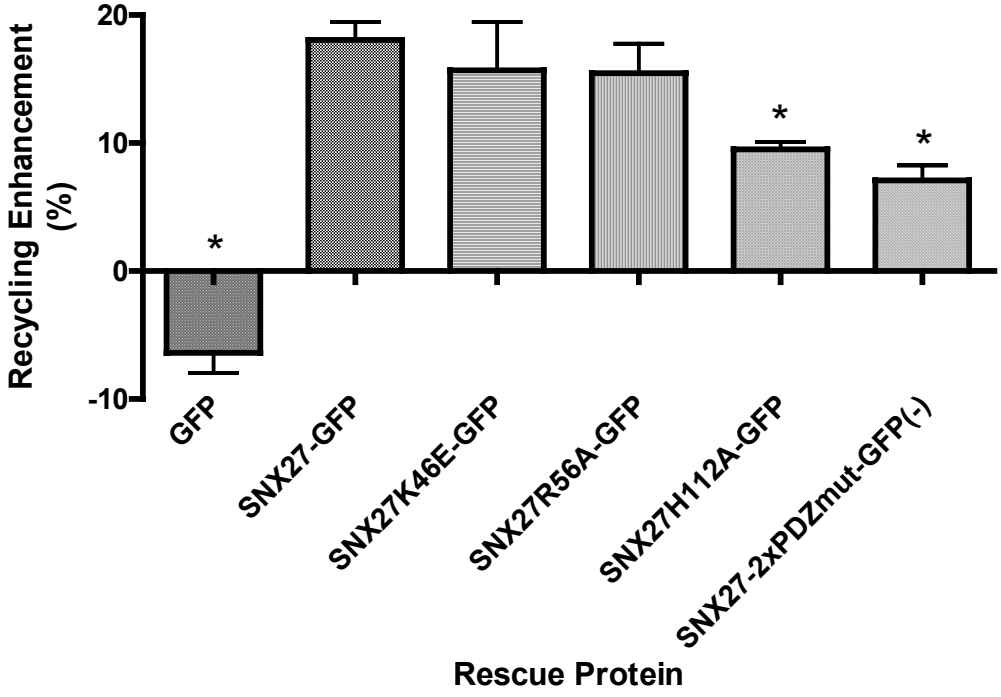
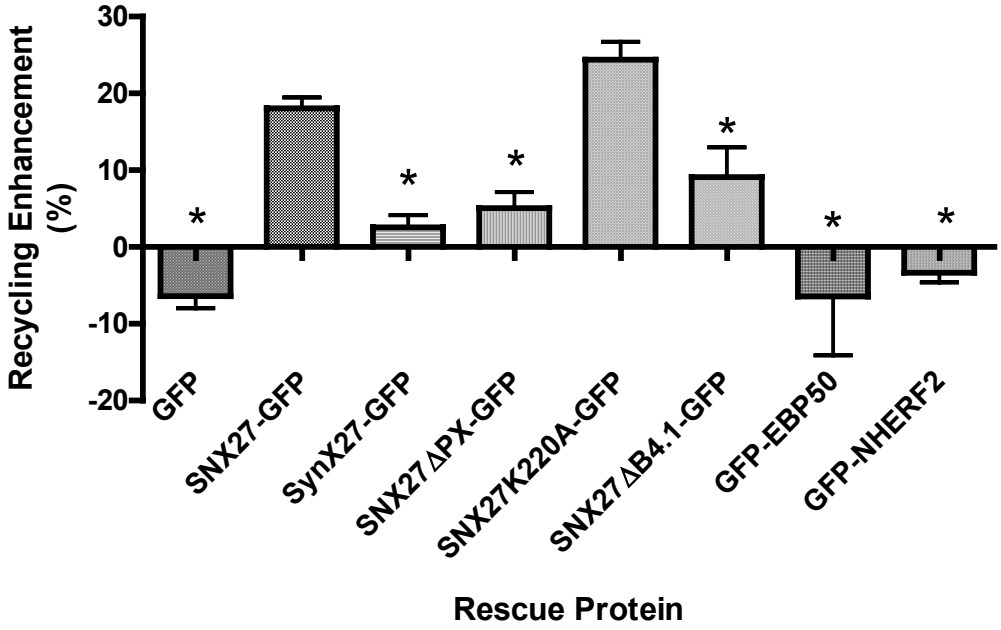

Recycling Enhancement (%)
20
10
0
-10
*
*
*
GFP
SNX27-GFP
SNX27K46E-GFP
SNX27R56A-GFP
SNX27H112A-GFP
SNX27-2xPDZmut-GFP(-)
Rescue Protein

C


Recycling Enhancement (%)
30
20
10
0
-10
-20
*
*
*
*
*
*
GFP
SNX27-GFP
SynX27-GFP
SNX27ΔPX-GFP
SNX27K220A-GFP
SNX27ΔB4.1-GFP
GFP-EBP50
GFP-NHERF2
Rescue Protein

D

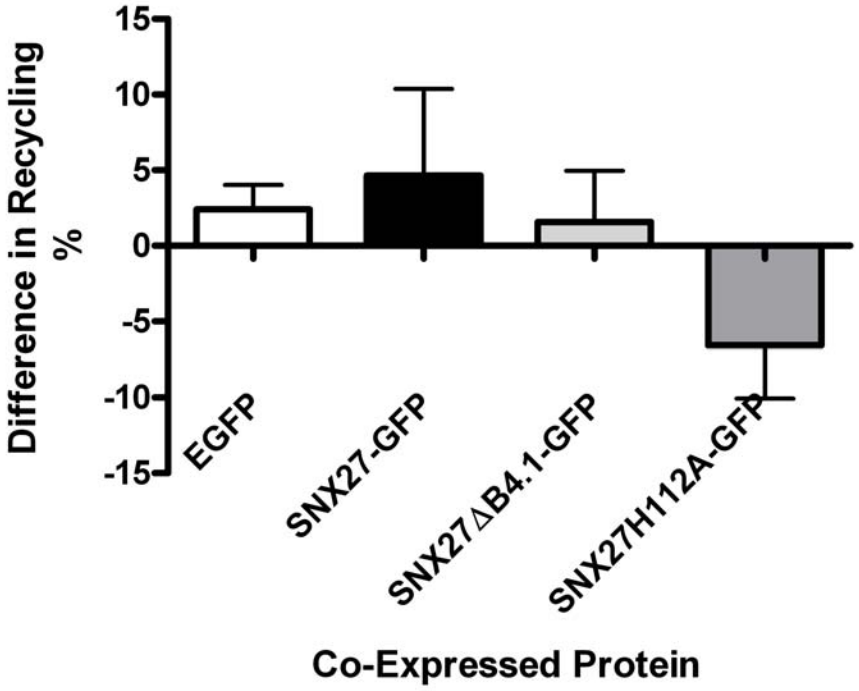


Figure 2. PDZ and other functional domain disruption of SNX27 have varying effects on recycling function.

(A) Fluorescence Polarization of the β2AR PDZ ligand with several SNX27 PDZ mutants. H112A is shown again for comparison. **(B)** Recycling rescue using coexpression of SNX27-GFP proteins containing the PDZ mutations from (A) were performed as in Chapter 4. The 2x PDZ mutant contains the #32 and 33 mutations from (A). GFP, wt SNX27, and the H112A mutant are shown again for comparison, as the first 2 are in **(C)** as well with SNX27 domain deletions, PDZ replacement (SynX27-GFP), the K220A point mutant and NHERF protein overexpression. **(D)** Wild type and SNX27 mutants were coexpressed with the wild type β2AR and analyzed for effects on wild type-level recycling without SNX27 knockdown. Error bars in all sections represent SEM and * denotes $p<0.05$ in post-Hoc comparison to the SNX27-GFP effect.

Overexpression of SNX27 can enhance the recycling of a β2AR with a weak-affinity ligand that normally does not promote recycling. Since the correlation of SNX27 PDZ mutation affinity and recycling function was not as clean as the previous survey utilizing ligand modification, we sought another validation for the role of PDZ

interaction(s) in conferring recycling of the β2AR. We first attempted to exploit the affinity of the SIESDV ligand for both SNX27 and the alpha1 syntrophin PDZ domain, by creating a hybrid SNX27 protein containing the syntrophin PDZ domain in place of the SNX27 PDZ (introduced as SynX27-GFP in figure 2). We then tried to rescue the recycling lost when knocking down SNX27 in cells expressing the β2ARxSIESDV with this SynX27-GFP. As seen in figure 2 and again in figure 3A, this SNX27 variant was unable to rescue recycling of the wild type receptor, in agreement with an incompatibility of the β2AR PDZ ligand with the syntrophin PDZ domain (affinity data not shown). Surprisingly, this SNX27 variant was also unable to rescue lost recycling of the β2ARxSIESDV (figure 3C). Unfortunately, it is not known if the PDZ domain is functional in this engineered protein, and thus we can not distinguish between the inability of this protein to produce a working PDZ interaction in the cellular environment vs. the functional need for an unconventional role of the SNX27 PDZ domain in recycling that is not dependent on a conventional PDZ interaction.

As another attempt to explore the relationship between SNX27 PDZ affinity and recycling function, we returned to the engineered PDZ interaction strategy introduced in Appendix II. Despite the lack of success in getting the SIESDV-containing β2AR to recycle with the SynX27-GFP protein expressed, we investigated whether this would hold true for the β2AR* coexpressed with Syn*X27-GFP. As presumed negative controls, we also expressed GFP, SynX27-GFP, and wt SNX27-GFP in parallel. Surprisingly, this series revealed the ability of the wt SNX27-GFP to induce mild but significant recycling (which wasn't replicated by the hybrid versions or just GFP), despite a Kd for the ligand* of ~50 μM (figure 3B, affinity data not shown). While this

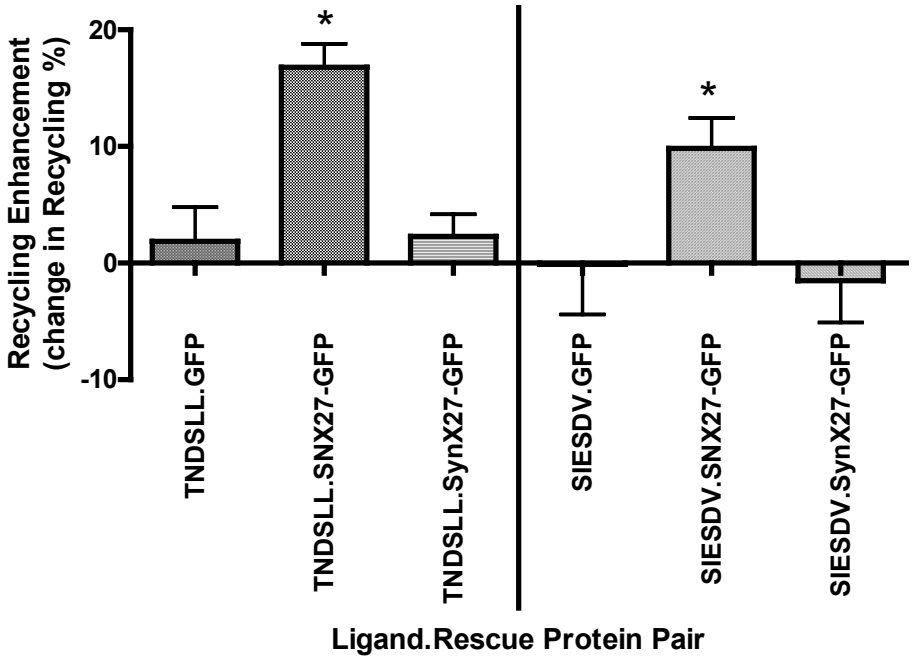
series again showed the inability of the syn/SNX27 hybrid to promote recycling, the contrast with the gain of recycling function produced by the wild type SNX27-GFP again highlighted the possible difficulty in creating functional transfers of PDZ domains in this system. While the SNX27 PDZ domain could conceivably be promoting a function distinct from a canonical PDZ interaction, the ability of the wild type SNX27 to work with a suboptimal ligand in promoting recycling suggests an affinity relationship seen with the SNX27 PDZ mutants, that of weak affinities becoming functionally relevant upon overexpression of SNX27.

In order to further probe this affinity relationship, we attempted to move the * mutation to the SNX27 PDZ domain, thus preserving the proper context of the domain. We also employed the H112A mutation as another variable. However, examination of the SNX27 PDZ* affinity for the ligand* revealed that the specificity of the interaction partners did not translate over from the syn interaction it was designed for; if anything the affinity between the * variants was weaker than the ligand* affinity for wild type SNX27, though all were in the range of detection. The ligand* affinity for the H112A mutant SNX27 PDZ was not measured, though surprisingly when assayed for recycling enhancement of the β2AR* in the SNX27-GFP protein, it showed recycling activity similar to, and perhaps greater that of wild type SNX27; this was also true of the SNX27*-GFP (figure 3B).

Since the recycling activity of these mutants suggests either a weak affinity limit for promoting recycling or an interaction-independent function of the SNX27 PDZ domain, we decided to find a weaker affinity range to use for testing the specificity of this gain of function. We made a mutation to the β2AR, an S416M substitution at the

critical –2 ligand position. This mutant ligand had no detectable affinity for SNX27 or SNX27* *in vitro* (not shown). When this mutant receptor was coexpressed with these SNX27 variants, an inconsistent and muted gain of recycling function was detected, slightly higher than the H112A mutant (figure 3C). While this suggests that some recycling activity is possible between these ligand and protein pairs (except for the H112A mutant), the enhancement of recycling was not statistically significant in a paired t-test of recycling between the GFP (+) and (-) expression levels. Thus it seems as though a detectable affinity *in vitro* is required for function with overexpressed protein partners in cells, even though the correlation between affinity and gain of recycling function is not as tight a relationship as originally suspected, likely contributed to by the limits of the assays employed and the superficial survey of data taken thus far.

A



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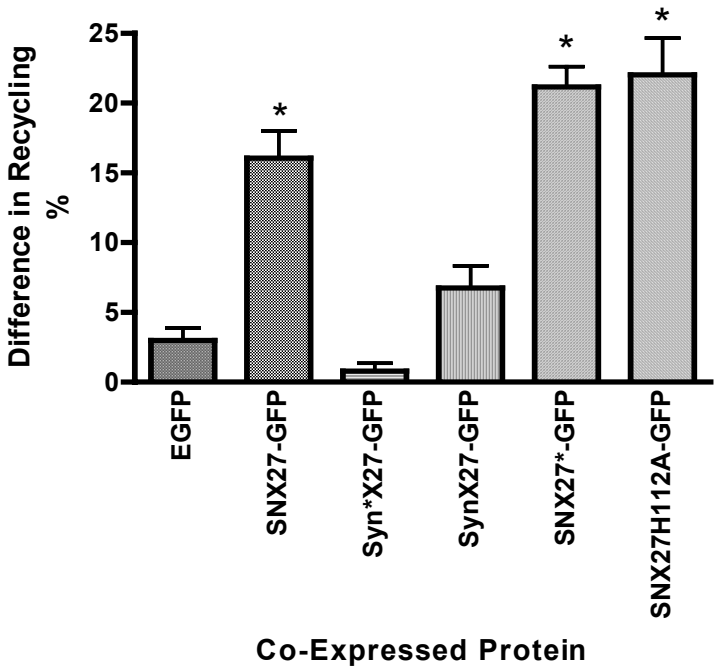
Difference in Recycling
%
25
20
15
10
5
0
*
*
*
EGFP
SNX27-GFP
Syn*X27-GFP
SynX27-GFP
SNX27*-GFP
SNX27H112A-GFP
Co-Expressed Protein

C

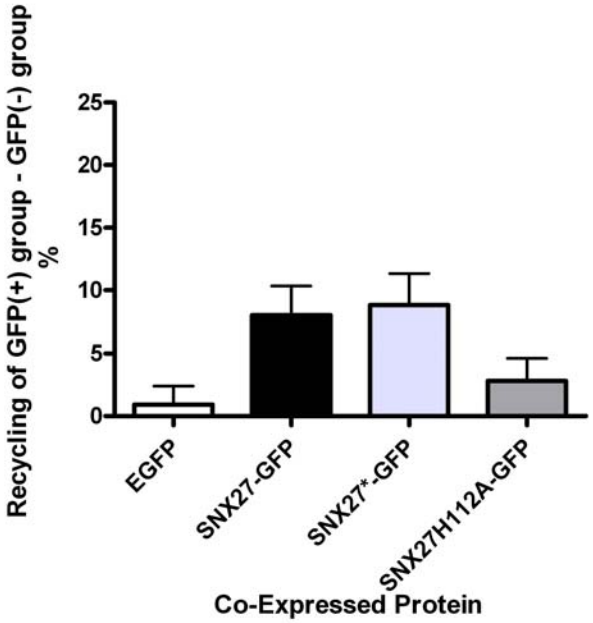
Recycling of GFP(+) group - GFP(-) group
%
25
20
15
10
5
0
EGFP
SNX27-GFP
SNX27*-GFP
SNX27H112A-GFP
Co-Expressed Protein

Figure 3. Weak SNX27 affinities can mediate recycling when overexpressing SNX27-GFP variants. **(A)** The wild type and SIESDV-ligand-modified β2AR were expressed in HEK293 cells along with the indicated GFP-tagged proteins and SNX27 siRNA. Recycling enhancement specific to the expressed GFP-tagged protein was determined as described in Chapter 4. **(B)** The β2AR* was expressed in HEK293 cells along with the indicated GFP-tagged protein and analyzed as with all GFP coexpression thus far. **(C)** An S416M mutant β2AR was expressed in HEK293 cells along with the indicated GFP-tagged protein and analyzed for recycling enhancement as in the other sections. Error bars represent SEM, * $p<0.05$ in paired t-test of (+) group recycling to (-) group recycling of the indicated coexpressed protein.

In summary, a short survey of peptide ligands with *in vitro* SNX27 affinity suggested that a Kd threshold of around 50 μM would constitute the point where recycling function would start to be lost, unless concentrations of SNX27 were enhanced. While measurements of the β2AR PDZ ligand affinity for SNX27 mutant PDZ domains did not provide a clean correlation with recycling function in the cellular environment, the requirement of the PDZ domain was further supported by the inability of a PDZ-replaced, hybrid SynX27 to promote recycling function of the wild type β2AR. In addition, deletion of other functional domains suggested that little, if any, of the SNX27 protein is dispensable for performing a role in recycling. Swapping of PDZ domains in the SNX27 protein was unable to promote recycling of receptors containing more 'optimal' ligands for the syn and syn* domains used. However, the wild type SNX27 was able to promote some recycling of these ligands, probably due to the weak but detectable affinities measured *in vitro*. While mutation of the PDZ domain in SNX27 was then unable to mute this recycling enhancement of the β2AR*, in particular, this likely is reflected by the retained affinity of this aberrant ligand to the mutant PDZ

domains. Altering the β2AR ligand in a way that prevents detectable affinities for these SNX27 variants also prevents detectable recycling enhancement when coexpressed. Collectively, these experiments point toward the need for a PDZ interaction to promote β2AR recycling, however whether this is through a direct interaction or a PDZ network remains a question. A pair of ligands and SNX27 PDZ mutants that do not share detectable affinities across combinations would greatly strengthen the argument for a required direct interaction.

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