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Regulation of GPCR Trafficking by Ubiquitin

Justine E. Kennedy and Adriano Marchese¹

Department of Molecular Pharmacology and Therapeutics, Loyola University Chicago, Health Sciences Division, Maywood, Illinois, USA

Abstract

G protein-coupled receptor (GPCR)-promoted signaling mediates cellular responses to a variety of stimuli involved in diverse physiological processes. In addition, GPCRs are also the largest class of target for many drugs used to treat a variety of diseases. Despite the role of GPCR signaling in health and disease, the molecular mechanisms governing GPCR signaling remain poorly understanding. Classically, GPCR signaling is tightly regulated by GPCR kinases and β -arrestins, which act in a concerted fashion to govern GPCR desensitization and also GPCR trafficking. Ubiquitination has now emerged as an important posttranslational modification that has multiple roles, either directly or indirectly, in governing GPCR trafficking. Recent studies have revealed a mechanistic link between GPCR phosphorylation, β -arrestins, and ubiquitination. Here, we review recent developments in our understanding of how ubiquitin regulates GPCR trafficking within the endocytic pathway.

1. INTRODUCTION

G protein-coupled receptor (GPCR) signaling is classically known to be mediated through the associated guanine nucleotide (G)-binding proteins (G proteins) that are heterotrimers comprised of an α -subunit and a $\beta\gamma$ heterodimer.¹ Agonist binding to GPCRs induces a conformational change in the associated G protein, thereby facilitating the exchange of GDP for GTP on the α -subunit and reversible disassociation of the $\beta\gamma$ heterodimer.^{1,2} Both the GTP-bound α -subunit and the released $G\beta\gamma$ heterodimer can signal to a diverse array of effector molecules involved in many signaling pathways leading to cellular responses.¹ Importantly, to ensure that the cellular responses are of the appropriate magnitude and duration, signaling is highly regulated to maintain normal cellular homeostasis.³ The mechanisms that regulate GPCR signaling are complex and occur at every level of the signaling pathway, including at the level of the receptor itself. Two main families of proteins that regulate GPCRs directly include G protein-coupled receptor kinases (GRKs) and the multifaceted adaptor proteins referred to as β -arrestins.³ β -Arrestins also regulate signaling by controlling receptor proximal degradation of classical second messengers cAMP and diacylglycerol (DAG) through interactions with phosphodiesterases or DAG enzymes, respectively.^{4,5} In addition to their role as negative regulators of GPCR signaling, β -arrestins are now also commonly recognized as positive regulators or transducers of signaling.⁶

¹Corresponding author: amarchese@luc.edu.

Direct phosphorylation of GPCRs is a common posttranslational modification that governs their signaling. Agonist activation usually results in rapid phosphorylation by GRKs on serine or threonine amino acid residues located within the intracellular domains of GPCRs.³ GRK-mediated phosphorylation provides a binding surface for the adaptor proteins, β -arrestins,^{7,8} which are recruited from the cytoplasm to the phosphorylated receptor at the plasma membrane.⁹ This serves to uncouple the receptor from the associated G protein through a process that involves steric hindrance, thereby terminating or preventing further G protein signaling from the receptor.⁸ This culminates in a process referred to as desensitization, a process in which even in the continued presence of stimulus the receptor is unable to signal.

In addition, activated GPCRs are typically removed from the cell surface via a complex process leading to their endocytosis or internalization into intracellular compartments known as endosomes.¹⁰ Once in an endocytic compartment, GPCRs can be dephosphorylated by an endosomal-associated phosphatase and recycled to the cell surface whereby the GPCR again has access to the extracellular ligand leading to functional resensitization of receptor signaling.¹¹ Alternatively, GPCRs can be targeted to a terminal degradative compartment known as the lysosome, leading to degradation and a loss in the total cellular complement of a GPCR giving rise to a phenomenon known as downregulation culminating in long-term attenuation of signaling.^{10,12} The mechanisms dictating whether a GPCR recycles or is targeted to lysosomes for degradation remain poorly understood, but recent advances have revealed a role for ubiquitin in this sorting decision.¹³ Direct ubiquitination of GPCRs themselves in which ubiquitin acts in a *cis* manner or ubiquitination of adaptor proteins in which ubiquitin acts in a *trans* manner has been shown to regulate various steps of the itinerary that GPCRs follow along the endocytic pathway. Here, we focus on recent advances that have led to our current understanding of the mechanisms by which ubiquitin regulates GPCR trafficking.

2. UBIQUITINATION MACHINERY

Ubiquitin is a 76-amino acid protein that is generally covalently attached to protein substrates through the formation of an isopeptide bond between the C-terminal glycine (Gly76) residue of ubiquitin and the epsilon amino group of internal lysine residues on target substrates.^{14,15} In certain circumstances, ubiquitin can also be attached to the free amino group at the N-terminus of a substrate^{16,17} or other internal amino acid residues,¹⁸ but whether this applies to GPCRs remains to be determined, to our knowledge.

Ubiquitin conjugation of proteins is carried out by an enzymatic cascade involving the sequential activity of three enzymes that are dedicated to ubiquitination reactions: E1, E2, and E3.^{14,15} There is a single conserved E1 enzyme and approximately 40 identified E2 enzymes in the human genome.¹⁹ In contrast, E3 ubiquitin ligases represent a diverse family of over 600 identified proteins in the mammalian genome.²⁰ A typical ubiquitination reaction can be divided into discrete steps. Ubiquitin is first activated at its C-terminus in an ATP-dependent manner by the E1-activating enzyme. This first step can be divided into two distinct events in which ubiquitin is initially activated via a ubiquitin-adenylate intermediate, which then reacts with a cysteine residue on the E1 to form an E1-ubiquitin intermediate. In

the second step, ubiquitin is transferred from the E1 cysteine residue to a cysteine residue on the E2-conjugating enzyme. The E2 interacts with E3, which also binds to the substrate, and the E3 either directly or indirectly transfers ubiquitin to a nearby lysine residue on the substrate. Ubiquitination of protein substrates is typically transient and is reversed by deubiquitinating enzymes (DUBs).²¹ DUBs have selective protease activity and mediate cleavage of the isopeptide bond between ubiquitin and its substrates. DUBs have been implicated in regulating the trafficking of GPCRs.^{22–30}

Because E3s mediate the interaction with their substrates, they typically provide the specificity to an ubiquitination reaction. E3s that indirectly attach ubiquitin to substrate proteins essentially serve as a scaffold or bridging molecule for E2 and the substrate.¹⁹ E3s that serve as scaffolds for ubiquitination reactions fall into two general families: RING domain or F box E3s.¹⁹ RING domain ligases do not possess intrinsic catalytic activity; however, their ligase activity stems from the fact that the RING domain binds the E2 enzyme, while the substrate binds to another region in a manner that facilitates transfer of ubiquitin moieties from the ubiquitin-loaded E2 to the substrate.¹⁹ The RING domain E3s form the largest family of E3s, and several RING domain E3s have been implicated in GPCR trafficking, via either ubiquitination of GPCRs or adaptor molecules.^{31–34}

In contrast to RING domain E3s, HECT (homologous to E6-AP C-terminus) domain E3s are directly involved in ubiquitination reactions because they form a direct thioester intermediate with ubiquitin in which the ubiquitin-loaded E2 transfers ubiquitin to an active site cysteine residue on the E3 before the ubiquitin is transferred to a lysine residue on the substrate protein.³⁵ HECT domain E3s represent a smaller family (~30 members) within the large family of E3 ligases.³⁶ HECT domain E3 can be divided into three discrete groups.³⁶ The group known as the Nedd4-like HECT domain family of E3s has been implicated in GPCR trafficking.³⁷ The Nedd4 family comprises nine mammalian members: Nedd4, Nedd4-2, AIP4 (a.k.a. Itch), WWP1, WWP2, SMURF1, SMURF2, NEDL1, and NEDL2.³⁸ Nedd4-like E3s contain a N-terminal calcium-dependent phospholipid-binding domain, two to four tandemly linked WW domains, and a conserved C-terminal HECT domain.³⁶ The Nedd4-like E3 AIP4 uniquely contains a proline-rich region that can bind to SH3 domains.³⁹ AIP4 is the human ortholog of the mouse E3 ubiquitin ligase referred to as Itch.⁴⁰ WW domains are protein–protein interaction modules that contain two conserved tryptophan residues and they typically interact with PPXY or PPPY motifs, where X represents any amino acid.^{41,42} Nedd4-like E3s typically interact directly with their substrates by interacting with PPXY motifs; however, not all substrates have these motifs and in such cases the interaction is indirect via an adaptor protein that has such a motif.^{41–43} Nedd4-like E3 ubiquitin ligases have been shown to interact with GPCRs either directly through noncanonical WW-domain-mediated interactions⁴⁴ or indirectly through interactions involving adaptor proteins.^{45–47} The HECT domain is located at the C-terminal end of Nedd4-like E3s and contains a highly conserved cysteine residue that directly accepts ubiquitin and therefore facilitates substrate ubiquitination directly.³⁵

3. MECHANISMS OF GPCR UBIQUITINATION

GPCR ubiquitination can be regulated by agonist activation or it can occur in an agonist-independent manner. To the best of our knowledge, the first mammalian GPCRs shown to be ubiquitinated in an agonist-dependent manner were the β_2 -adrenergic receptor (β_2 AR) and the C-X-C receptor 4 (CXCR4) chemokine receptor.^{32,48} Several GPCRs have since been shown to be ubiquitinated in an agonist-dependent manner.⁴⁹ GPCRs can also be ubiquitinated in an agonist-independent manner. For example, GPCRs such as GPR37⁵⁰ or the δ -opioid receptor (DOR)⁵¹ can be ubiquitinated during biosynthesis, as a quality control measure to target misfolded receptors for ubiquitination and degradation by the proteasome. The trigger for ubiquitination during biosynthesis is likely detected as a conformational change in the misfolded GPCR, leading to its removal via endoplasmic reticulum protein degradation.⁵² Interestingly, limiting the amount of ubiquitination during biosynthesis can enhance the cell surface levels of certain GPCRs. The DUB ubiquitin-specific protease 4 associates with the C-terminus of the A_{2A} adenosine receptor and possibly regulates its ubiquitination status, thereby facilitating its passage via the biosynthetic pathway to the plasma membrane.³⁰ GPCRs can also be constitutively ubiquitinated postsynthesis in a ligand-independent manner, but the trigger for this type of ubiquitination remains unknown, although it may be dependent upon the compartment to which the receptor localizes.⁵³ In this case, it appears that agonist activation can induce GPCR deubiquitination.^{37,53} Surprisingly, given that there are many GPCRs, a relatively small number have been shown to be ubiquitinated.³⁷ The reason for this is not clear, but it is possible that not all GPCRs are regulated by ubiquitination.^{53,54} Another possibility is that it may be due to technical difficulties in detecting GPCR ubiquitination.⁵⁵

Lysine residues within any of the intracellular domains of GPCRs can be subject to ubiquitination. For example, the μ -opioid receptor (MOR) is mostly ubiquitinated on two lysine residues located within the first intracellular loop of the receptor, as determined by mutational analysis.⁵⁶ Although lysine residues are present on the other intracellular domains of MOR, and in particular the C-terminal tail, these lysine residues are not sufficient to support receptor ubiquitination.⁵⁶ The β_2 AR appears to be ubiquitinated on lysine residues within the third intracellular loop, but in contrast to MOR, β_2 AR is also ubiquitinated on lysine residues in the C-terminal tail.⁵⁷ Other GPCRs, such as CXCR4 and protease-activated receptor 1 (PAR1), seem to be mostly ubiquitinated on lysine residues located within the C-terminal tail, despite the fact that there are lysine residues located on other intracellular domains.^{48,58} It remains unclear why certain lysine residues are subject to ubiquitination while others are not, but it is likely related to the structural constraints adopted by distinct ligand-induced receptor conformations that restrict E3 ligase access to certain intracellular domains and hence lysine residues. Further work will be required to understand this process in greater detail.

4. GPCR REGULATION BY E3 UBIQUITIN LIGASES

Agonist-dependent ubiquitination of GPCRs typically occurs at the plasma membrane and it typically requires receptor phosphorylation.^{32,44,48} This is particularly well characterized for CXCR4. The E3 ubiquitin ligase AIP4 mediates agonist-dependent ubiquitination of CXCR4 at the plasma membrane.⁵⁹ The mechanism by which AIP4 recognizes and

ubiquitinates CXCR4 was only recently elucidated.⁴⁴ A receptor mutant in which two consecutive serine residues (S324 and S325) are mutated to alanine residues is not ubiquitinated as efficiently as wild-type CXCR4.^{44,48} These residues are rapidly phosphorylated by agonist activation at the plasma membrane, as assessed by confocal microscopy using a phospho-specific antibody directed against dually phosphorylated S324 and S325.⁴⁴ Phosphorylation of these residues is likely mediated by GRK6 and/or PKC δ .⁶⁰ Phosphorylation of these residues promotes the recruitment of the E3 ubiquitin ligase AIP4 to the plasma membrane following agonist stimulation, as assessed by TIRF microscopy.⁴⁴ Therefore, AIP4 binding to the phosphorylated receptor at the plasma membrane is required for ubiquitinating nearby lysine residues (Fig. 1A).

The interaction between AIP4 and CXCR4 occurs directly via a noncanonical interaction.⁴⁴ As described above, AIP4 is a member of the Nedd4-like family of E3 ubiquitin ligases.³⁸ Similar to other members of the Nedd4-like family, AIP4 typically interacts with its substrates via the WW domains.⁴² WW domains are protein–protein interaction modules approximately 40 amino acids residues, and as the name implies, they are defined by the presence of two conserved tryptophan residues.⁶¹ The WW domains found in AIP4 interact with proline-rich sequences, such as PPXY or PPPY motifs.^{41,42} However, the intracellular domains of CXCR4, or GPCRs in general, do not contain such motifs, suggesting that AIP4 interacts with CXCR4 via an indirect mechanism. However, biochemical interaction studies using purified proteins revealed that the C-terminal tail of CXCR4 interacts directly with full-length AIP4.⁴⁴ This is mediated by AIP4 WW domains I and II, but not III and IV, which appear to interact directly with the tandemly phosphorylated amino acid residues S324 and S325,⁴⁴ thereby establishing a noncanonical mode of interaction of WW domains with phosphorylated serine and possibly threonine residues. This may not be restricted to CXCR4 as AIP4 may also mediate ubiquitination of DOR; however, the mechanism by which AIP4 recognizes and ubiquitinates DOR remains to be defined.²⁶ AIP4 is likely not going to be required for ubiquitination of all GPCRs, at least not for β_2 AR,⁴⁶ MOR⁴⁷ nor the S1P receptor,⁴⁵ which have been shown to not require AIP4 for their ubiquitination. Interestingly, the S1P receptor may be regulated by phosphorylation and ubiquitination by WWP2 (a.k.a. AIP2), a member of the Nedd4-like family of E3 ligases, although the interaction between S1P and WWP2 may not require prior phosphorylation.⁴⁵ Therefore, GPCR ubiquitination is likely going to be regulated by diverse mechanisms via distinct E3 ubiquitin ligases.

Although CXCR4 can interact directly with AIP4, other GPCRs appear to interact indirectly with E3 ubiquitin ligases via an adaptor protein, although phosphorylation of the receptor is also required. For example, β_2 AR interacts with the E3 ubiquitin ligase Nedd4 via the adaptor protein β -arrestin-2⁴⁶ (Fig. 1B). Nedd4 is a member of the Nedd4-like family of E3 ubiquitin ligases.³⁸ Knockdown of Nedd4 by siRNA, but not other related E3s such as AIP4 or Nedd4-2, attenuates ubiquitination of β_2 AR.⁴⁶ However, β_2 AR ubiquitination is also attenuated in mouse embryonic fibroblasts (MEFs) isolated from β -arrestin-2 knockout mice,³² suggesting a role of β -arrestin-2 in β_2 AR ubiquitination.⁴⁶ Consistent with this idea, recruitment of Nedd4 to the β_2 AR complex is attenuated in cells in which β -arrestin-2 is reduced by targeted siRNA.⁴⁶ These data suggest that it is likely that β -arrestin-2 serves as an adaptor for recruitment of Nedd4 to the activated β_2 AR. This may be generalizable to

other GPCRs such as MOR because ubiquitination of MOR is impaired in MEFs isolated from β -arrestin-1 knockout mice.⁶² This is in contrast to CXCR4 in which ubiquitination of CXCR4 is not impaired in cells in which β -arrestin-1 and β -arrestin-2 have been silenced by siRNA.⁶³ This is likely because AIP4 can interact directly with CXCR4,⁴⁴ while other E3 ligases cannot and require an adaptor protein.⁴⁶ Why certain GPCRs such as CXCR4 can interact directly with Nedd4-like E3 ubiquitin ligases, while others require an adaptor protein remains to be explored.

In addition to β -arrestins, α -arrestins may also mediate recruitment of E3 ligases to GPCRs. α -Arrestins were recently discovered by bioinformatics analysis to be distantly related to β -arrestins.^{64,65} There is very little amino acid sequence identity between β -arrestins and α -arrestins, but they share structural similarities.^{64,66} Computational methods predict that α -arrestins share a so-called arrestin-fold with β -arrestins.^{64,66} In contrast to β -arrestins, α -arrestins have a long C-terminal region, which has two or three PPXY motifs,⁶⁴ which as described above commonly interact with WW domains.⁴¹ Indeed, α -arrestins have been shown to interact with WW domains of several Nedd4-like E3s via their PPXY motifs.^{67,68} In this regard, the α -arrestin protein ARRDC3 has been shown to serve as an adaptor for β_2 AR ubiquitination at the plasma membrane by Nedd4.⁶⁹ Knockdown of ARRDC3 by siRNA attenuates agonist-stimulated β_2 AR ubiquitination and Nedd4 binds directly to the PPXY motifs of ARRDC3 through its WW domains.⁶⁹ α -Arrestins can also regulate the ubiquitination and trafficking of other GPCRs.⁶⁷ Therefore, in common with β -arrestins, α -arrestins seem to link the same E3 ligase to the same GPCR, suggesting a complex and possibly context-dependent regulation of GPCRs by ubiquitination. However, a recent study has suggested that ARRDC3 is not involved in β_2 AR ubiquitination, but instead may be required at a later step of β_2 AR trafficking.⁷⁰ Interestingly, β -arrestin-1 has been shown to regulate CXCR4 trafficking on endosomes,⁷¹ suggesting that β -arrestins and α -arrestins may share a common function in GPCR trafficking at the level of the endosome.

Unlike α -arrestins, β -arrestins do not have PPXY motifs, although they can interact with Nedd4 and other members of the WW-domain containing Nedd4-like family of E3s, including AIP4 and Smurf2.^{46,47,63} Despite a lack of PPXY motifs, the interaction between β -arrestin-1 and AIP4 is mediated by the WW domains of AIP4, suggesting a noncanonical WW-domain-mediated interaction.⁶³ In contrast, the interaction between β -arrestin-2 and Nedd4 is not mediated by the WW domains of Nedd4, suggesting that it occurs via another domain on Nedd4.⁴⁶ The C2 domain is a Ca^{2+} -dependent phospholipid-binding domain that can also mediate protein–protein interactions with Nedd4.⁷² Whether the C2 domain of Nedd4 mediates the interaction with β -arrestin-2, to our knowledge, remains to be determined.

5. ROLE OF UBIQUITIN IN GPCR INTERNALIZATION

One function of GPCR ubiquitination is to promote GPCR internalization. In general, most GPCRs are internalized via a β -arrestin-dependent mechanism and likely do not require direct ubiquitination for internalization.⁷³ For example, a ubiquitin-deficient β_2 AR mutant internalizes just as efficiently as the wild-type receptor³² likely because internalization is efficiently promoted by β -arrestin-2.⁷⁴ However, not all GPCRs require β -arrestins for

internalization,⁷⁵ indicating that there are additional mechanisms and adaptors to promote GPCR internalization. For example, internalization of the thrombin receptor, PAR1, is not impaired in MEFs isolated from embryos of β -arrestin knockout mice, indicating that PAR1 internalization occurs via a β -arrestin-independent mechanism.⁷⁵ Recently, AP2 was shown to act as an adaptor that in part mediates agonist-induced internalization of PAR1.⁷⁶ AP2 is a clathrin adaptor and can link certain types of receptors to clathrin-coated pits for clathrin-mediated endocytosis.⁷⁷ AP2 is a heterotetrameric protein complex comprised of α -, β_2 -, μ_2 -, and σ_2 -adapting subunits.⁷⁷ The β_2 -adapting subunit interacts with β -arrestins and this interaction is generally required for β -arrestin-mediated internalization of GPCRs.^{73,78} However, the μ_2 -adapting subunit is essential for PAR1 internalization upon agonist activation.⁷⁶ The μ_2 -adapting subunit is believed to interact directly with phosphorylated serine and threonine residues within the C-terminal tail, thereby linking PAR1 to clathrin for internalization via clathrin-coated pits. In addition, PAR1 may also require the ubiquitin-binding adaptor protein epsin-1 for internalization.⁷⁶ PAR1 internalization via epsin requires ubiquitination of C-terminal lysine residues and an intact ubiquitin-binding domain (UBD) in epsin-1, suggesting that the ubiquitin moieties attached to PAR1 serve to link the receptor to epsin-1 via an ubiquitin-UBD-mediated interaction. This may not be unique to PAR1 because internalization of MOR also requires receptor ubiquitination and epsin-1, likely via a similar ubiquitin-UBD-mediated interaction.^{47,56} Further work will be required to establish whether ubiquitin-mediated internalization can be generalizable to other GPCRs.

Ubiquitin also plays an indirect role in GPCR internalization via ubiquitination of β -arrestins. Agonist activation of β_2 AR promotes ubiquitination of β -arrestin-2 by the RING domain E3 ubiquitin ligase Mdm2.³² Ubiquitination of β -arrestins does not occur in Mdm2-null MEFs and agonist-promoted internalization of β_2 AR is attenuated, although ubiquitination of β_2 AR is not impaired.³² While the precise role for ubiquitin remains to be elucidated, it appears that discrete ubiquitin moieties on β -arrestin may stabilize its interaction with GPCRs and clathrin, thereby facilitating its ability to promote GPCR internalization.⁷⁹ Interestingly, β -arrestins are modified by other types of posttranslational modifications that may have an impact on its role in GPCR internalization.⁷³ For example, agonist-dependent SUMOylation of β -arrestin-2 may facilitate its binding to AP2, thereby facilitating β_2 AR internalization.⁸⁰ Similarly, S-nitrosylation of β -arrestin-2 may also enhance its interactions with clathrin and AP2 to facilitate β_2 AR internalization.⁸¹ SUMOylation and S-nitrosylation occur on distinct amino acid residues located on the C-terminal tail of β -arrestins, which becomes exposed once β -arrestins bind to activated and phosphorylated receptors.^{80,81} This region also contains the binding sites for clathrin and the β_2 -adapting subunit of AP2.⁷³ Therefore, it is possible that SUMOylation and/or S-nitrosylation may somehow facilitate GPCR internalization by enabling access to the exposed tail to proteins of the internalization machinery. Further work will be required to delineate exactly if and how this occurs.

6. ROLE OF UBIQUITIN IN GPCR ENDOSOME TO LYSOSOME SORTING

In addition to a role in GPCR internalization, ubiquitin moieties attached to GPCRs promote GPCR sorting into the degradative pathway.¹³ Sorting into this pathway typically occurs on early-to-late endosomes or maturing multivesicular bodies (MVBs).^{13,82,83} MVBs are

endocytic vesicular intermediates between early and late endosomes defined by the presence of many intraluminal vesicles (ILVs).^{84,85} GPCRs destined for lysosomal degradation can be found on the limiting membrane of these structures and accumulate on the membranes of the ILVs^{86–88} (Fig. 2). Because MVBs fuse with lysosomes where their contents are degraded,⁸⁴ targeting into the ILVs is likely required for complete degradation of GPCRs. The formation of ILVs and targeting of ubiquitinated GPCRs into ILVs occurs via the endosomal sorting complex required for transport (ESCRT) pathway, which comprises four protein complexes (ESCRT-0, -I, -II, and -III) and the AAA-ATPase-Vps4 complex.⁸⁹ In the canonical model of ESCRT sorting, the ESCRT complexes act in a coordinated fashion to recognize and concentrate ubiquitinated transmembrane proteins on membranes of the ILVs.⁸⁹ ESCRTs are multimeric protein complexes, and three ESCRTs complexes (-0, -I, and -II) exist as stable complexes with subunits that have UBDs. These ESCRTs interact directly with the ubiquitin moieties on cargo via their UBDs, thereby directing the cargo into the forming ILVs. This process is initiated by ESCRT-0, comprised of HRS and STAM1, which concentrates ubiquitinated cargo in microdomains of early endosomes, and with the help of ESCRT-I and -II sorts the ubiquitinated cargo into ILVs. This is followed by the recruitment of the individual monomeric subunits that comprise ESCRT-III which assemble on the limiting membrane of endosomes and act at the scission step to form ILVs-containing receptors destined for degradation on their membranes. The AAA-ATPase Vps4 complex acts to disassemble ESCRT-III subunits, thereby allowing for recycling of the ESCRT components to initiate additional rounds of ESCRT-mediated sorting.

The role of ESCRTs in targeting GPCRs into the degradative pathway is particularly well characterized for CXCR4⁹⁰ (Fig. 2). Although CXCR4 is ubiquitinated at the plasma membrane, it is internalized in a ubiquitin-independent manner, but the ubiquitin moiety is required for sorting CXCR4 into the degradative pathway.⁴⁸ CXCR4 ubiquitination-deficient mutants when transiently expressed in HEK293 cells show attenuated agonist-induced degradation.^{44,48} Internalized CXCR4 is found within specialized microdomains of endosomes together with ESCRT-0 where sorting into ILVs likely begins.⁵⁹ Treating cells with siRNA directed against ESCRT-0 (i.e., against HRS subunit) or a dominant-negative Vps4 attenuates CXCR4 degradation.⁵⁹ This established for the first time, to our knowledge, a role of ESCRTs in lysosomal trafficking of mammalian GPCRs.⁵⁹

The ubiquitin moieties attached to CXCR4 likely initially interact with UBDs present on subunits of ESCRT-0 for entry into the ESCRT pathway (Fig. 2A). The other UBD-containing ESCRTs, ESCRT-I and -II, have also been implicated in CXCR4 trafficking to lysosomes.^{91,92} CXCR4 sorting to lysosomes is very efficient because only a small fraction of CXCR4 escapes and recycles to the plasma membrane.⁷¹ In other cell types, such as T cells, CXCR4 is also efficiently degraded following exposure to agonist⁴⁸ and only a small portion recycles back to the plasma membrane via Rab11-positive recycling endosomes.⁹³ Interestingly, CXCR4 lysosomal sorting may be compromised in breast cancer cells leading to greater recycling and may explain in part why CXCR4 levels are upregulated in a subset of breast tumors.^{87,94,95} Other GPCRs such as DOR, PAR2, and β_2 AR also sort to lysosomes via the ESCRT pathway, suggesting that this pathway may be generalizable to most GPCRs.⁸⁶

Although most GPCRs likely require direct ubiquitination for entry into ILVs, for some GPCRs direct ubiquitination may not be required. For example, ubiquitination of PAR1 is not required for its sorting into ILVs of MVBs as a ubiquitination-deficient mutant can be easily detected on the membrane of ILVs⁸⁶ (Fig. 2B). Accordingly, UBD-containing ESCRTs, such as ESCRT-0 and ESCRT-I, and likely ESCRT-II, are not required for PAR1 lysosomal degradation.⁹⁶ However, ESCRT-III is required, as siRNA targeting ESCRT-III subunits blocks agonist-induced degradation of PAR1.⁸⁶ The connectivity of PAR1 to ESCRT-III is bridged by the adaptor protein ALIX.⁸⁶ ALIX binds to ESCRT-III and to a tyrosine-based (YPX₍₃₎L) motif found on the second intracellular loop of PAR1. Mutation of the tyrosine residue within this motif disrupts binding to ALIX and attenuates PAR1 degradation. ALIX is not involved in lysosomal degradation of PAR2, a GPCR that is dependent upon ubiquitin-binding ESCRTs for delivery to lysosomes.^{86,97} The YPX₍₃₎L motif is not unique to PAR1 and is found on the intracellular loops of several GPCRs, suggesting that these GPCRs may be sorted for lysosomal degradation via an ALIX/ESCRT-III-dependent mechanism.⁸⁶ Therefore, as shown schematically in Fig. 2, direct ubiquitination of GPCRs may be required for some (e.g., CXCR4) but not all GPCRs (e.g., PAR1) for sorting in ILVs.

Ubiquitin also regulates GPCR sorting into the degradative pathway in an indirect manner. The E3 ubiquitin ligase AIP4 that mediates CXCR4 ubiquitination at the plasma membrane is also found on microdomains of early endosomes where internalized CXCR4 and ESCRT-0 are present.⁵⁹ CXCR4 activation induces ubiquitination of ESCRT-0 subunits HRS and STAM, which may be mediated by endosomally localized AIP4. The role of ESCRT-0 ubiquitination on CXCR4 sorting remains to be clearly defined, but the attached ubiquitin moieties on HRS and STAM1 may be linked to terminating ESCRT-0 sorting activity.⁷¹ This is based on the unexpected finding in CXCR4 degradation experiments that suggest that ESCRT-0 subunits HRS and STAM have opposing roles in targeting CXCR4 for lysosomal degradation.^{59,71} While siRNA-mediated knockdown of HRS inhibits CXCR4 degradation,⁵⁹ STAM1 knockdown has the opposite effect and accelerates CXCR4 degradation.⁷¹ This may be explained by the fact that STAM1 interacts with β -arrestin-1 to regulate ubiquitination of HRS.⁷¹ Expression of a dominant-negative β -arrestin-1 that disrupts the β -arrestin-1/STAM1 interaction attenuates HRS ubiquitination while accelerating or enhancing CXCR4 degradation.⁷¹ Because β -arrestin-1 also interacts with AIP4, it is likely that β -arrestin-1 serves as an adaptor that binds to STAM1 to promote AIP4-mediated ubiquitination of HRS and likely STAM1.⁶³ To counter this, AIP4-mediated ubiquitination of HRS and STAM1 is negatively regulated by the RING domain E3 ubiquitin ligase DTX3L.³³ Ultimately, it appears that the ubiquitination status of ESCRT-0 is tightly linked to the amount of CXCR4 that is sorted into ILVs eventually degraded in lysosomes (see model in Fig. 2A). Ubiquitination of ESCRT-0 may induce intra- or intermolecular interactions between the ubiquitin moieties and its own UBDs, possibly inhibiting its sorting activity by preventing it from interacting with ubiquitin moieties on cargo.³³ Although this remains to be tested directly, it does represent a unique process by which GPCRs can regulate their own sorting efficiency by regulating the activity of the sorting machinery.

Proteins can also interact with the ESCRT machinery to modulate GPCR lysosomal targeting. A multidomain protein termed G protein-coupled receptor interacting scaffold protein (GISP) was identified in a yeast two-hybrid screen of a rat brain cDNA library to interact with the C-terminal domain of the GABA_{B1} subunit, but not the GABA_{B2} subunit, of the GABA_B receptor.⁹⁸ Overexpression of GISP increased the surface expression of the GABA_{B1} subunit, possibly by promoting or enhancing its surface expression via the biosynthetic pathway.⁹⁸ In another yeast two-hybrid screen, Tsg101, a subunit of ESCRT-I, was identified to be an interacting partner of GISP.⁹⁹ The interaction between GISP and Tsg101 was confirmed in the adult rat brain and HEK293 cells, as assessed by coimmunoprecipitation studies and GISP and Tsg101 were shown to colocalize in neurons by fluorescence microscopy. Overexpression of GISP delays lysosomal degradation of the GABA_{B2} subunit in HEK293 cells, while a mutant of GISP that is unable to bind to Tsg101 does not prevent degradation of this subunit.⁹⁹ Therefore, GISP binding to Tsg101 can inhibit the sorting activity of ESCRT-I, possibly by preventing ESCRT-I from interacting with ubiquitinated receptors or other elements of the ESCRT pathway, thereby leading to increased GABA_{B2} levels in cells and possibly increased GABAergic responsiveness.⁹⁸ This highlights the fact that proteins like GISP can regulate ESCRT function and underscores the notion that together with ubiquitination the ESCRT machinery is susceptible to multiple modes of regulation. Ultimately, this is important because changes in the sorting efficiency or the sorting itinerary of GPCRs can impact signaling and therefore impact physiological processes.

7. ROLE OF DEUBIQUITINATION IN GPCR LYOSOMAL SORTING

Although ubiquitin attachment to GPCRs is required for their degradation, the removal of ubiquitin seems to be equally important. Removal of ubiquitin can occur before or after ubiquitinated GPCRs enter the ESCRT pathway.⁵⁹ Two DUBs, known as USP8 (ubiquitin-specific protease 8; a.k.a. UBPY) and AMSH (associated molecule with the SH3 domain of STAM) are found on endosomes and are linked to the ESCRT machinery.¹⁰⁰ USP8 and AMSH have also been linked to GPCR deubiquitination. For example, both USP8 and AMSH have been shown to regulate the ubiquitination status of PAR2.²⁴ USP8 or AMSH knockdown by siRNA results in somewhat greater ubiquitination of PAR2, suggesting that these DUBs regulate PAR2 deubiquitination.²⁴ Deubiquitination may be necessary to efficiently target PAR2 to lysosomes because USP8 or AMSH knockdown moderately attenuated PAR2 degradation.²⁴ In the case of CXCR4, knockdown of USP8 by siRNA attenuates agonist-induced degradation of CXCR4,²³ however, knockdown of AMSH does not.⁷¹ In contrast to PAR2, USP8 (or AMSH) does not impact CXCR4 ubiquitination but instead modulates the ubiquitin status of ESCRT-0 that is ubiquitinated by the E3 ligase AIP4,²³ reinforcing the idea that ubiquitination of the transport machinery represents an important regulatory event in GPCR trafficking. Although AMSH or USP8 does not seem to regulate the ubiquitination status of CXCR4, CXCR4 may be deubiquitinated by another DUB.²⁵ Interestingly, USP8 may regulate the deubiquitination and trafficking of Frizzled²⁷ and Smoothened,²⁸ other members of the GPCR/7-transmembrane domain superfamily of receptors. Therefore, ubiquitin removal from either the GPCR or sorting machinery is essential to sort GPCRs into ILVs for eventual degradation in lysosomes.

Deubiquitination of GPCRs may also represent an important regulatory event dictating their recycling and resensitization of receptor signaling. Two highly related DUBs, USP20 and USP30, have been shown to regulate the ubiquitination status of β_2 AR possibly by interacting directly with the third intracellular loop.²⁹ The interactions may occur on endosomal compartments where deubiquitination of β_2 AR may promote its recycling and resensitization of receptor signaling.²⁹ Deubiquitination of Smoothed by USP8 may regulate trafficking of Smoothed from an intracellular compartment to the cell surface, thereby enhancing cellular responsiveness to the ligand hedgehog.²⁸ Interestingly, β_2 AR recycling may also be governed by ubiquitination of Rab11, a GTPase involved in recycling.¹⁰¹ In any event, deubiquitination of GPCRs may serve as a generalizable mechanism to regulate cell surface expression of GPCRs and hence hormonal responsiveness. Dysregulation of deubiquitination could potentially be a contributing factor to certain diseases. For example, decreased ubiquitination of CXCR4 contributes to CXCR4 recycling and increased responsiveness of tumor cells to the CXCR4 agonist CXCL12, which is a contributing factor to metastasis.⁹⁵ While to our knowledge defective deubiquitination of CXCR4 has not been shown, it does highlight the fact that the ubiquitination status of GPCRs is under tight control and any perturbations could be deleterious.

8. EFFECT OF BIASED AGONISM ON GPCR TRAFFICKING: ROLE OF UBIQUITIN

Given that biased agonism dictates β -arrestin versus G protein-dependent signaling,¹⁰² and since as discussed above, β -arrestins can serve as adaptors for E3 ubiquitin ligases, it is therefore likely that GPCR ubiquitination may also be influenced by ligand bias. This is likely best demonstrated by MOR, with which two distinct MOR agonists, morphine and DAMGO, have differential effects on promoting β -arrestin-mediated MOR ubiquitination.⁶² Remarkably, DAMGO is able to promote MOR ubiquitination, while morphine is not. DAMGO-promoted MOR ubiquitination is attenuated in β -arrestin-1-null MEFs, indicating a role of β -arrestin-1 in MOR ubiquitination. The difference in the MOR ubiquitination profile induced by DAMGO and morphine may be linked to the fact that DAMGO is better able to induce β -arrestin recruitment to the receptor than morphine. It is likely that DAMGO and morphine induce distinct receptor conformations that are differentially recognized by GRKs and β -arrestin-1. Interestingly, although DAMGO also promotes robust β -arrestin-2 recruitment, β -arrestin-2 is not required for DAMGO-induced MOR ubiquitination.⁶² In contrast, DADLE, another opioid agonist, induces rapid ubiquitination of MOR that may require β -arrestin-2.⁴⁷ In this case, β -arrestin-2 may serve as an adaptor for Smurf2, a member of the Nedd4-family of E3 ubiquitin ligases.⁴⁷ The ligase that promotes DAMGO-dependent ubiquitination of MOR remains to be determined, as far as we know. But this highlights the fact that distinct ligands can regulate the recruitment of E3 ligases likely by regulating differential recruitment of β -arrestins to activated GPCRs.

Ligand-biased ubiquitination of GPCRs may be linked to ligand-biased interactions between β -arrestins and E3 ubiquitin ligases. The β -blocker carvedilol is a biased agonist for β -arrestin-dependent signaling but is an antagonist for G protein-dependent signaling.¹⁰³ Recently, carvedilol was shown to regulate ubiquitination of β_2 AR by the E3 ubiquitin ligase MARCH2 (membrane-associated RING-CH2).⁷⁰ Carvedilol, but not the β -blocker

propranolol, induces rapid ubiquitination of β_2 AR, similar to the β -agonist isoproterenol.⁷⁰ Accordingly, carvedilol induces lysosomal trafficking of endogenous β_2 AR in vascular smooth muscle cells or overexpressed in HEK293 cells, very similar to what is observed with the β -agonist isoproterenol.⁷⁰ However, in contrast to isoproterenol, β -arrestins and Nedd4 are not required for carvedilol-mediated ubiquitination of β_2 AR, suggesting that carvedilol-promoted β_2 AR ubiquitination is mediated by another E3 ubiquitin ligase. A mass spectrometry-based approach to identify binding partners of β_2 AR after carvedilol treatment compared with isoproterenol treatment revealed that the E3 ubiquitin ligase MARCH2 prefers to bind to β_2 AR in cells treated with carvedilol compared with isoproterenol.⁷⁰ Carvedilol promotes recruitment of a GFP-tagged MARCH2 to the plasma membrane leading to a stable interaction with β_2 AR. Interestingly, this stable interaction may hinder β_2 AR deubiquitination and thereby limits receptor recycling and facilitates lysosomal degradation of β_2 AR leading to attenuation of signaling. The fact that carvedilol mediates receptor ubiquitination via MARCH2 and limits receptor recycling, and receptor signaling may explain some of the beneficial effects that carvedilol has in treating heart failure.⁷⁰

9. CONCLUSION

Ubiquitin has emerged as an important posttranslational modification that regulates GPCR trafficking. Recent developments have led to a greater understanding of the mechanisms governing the role of ubiquitin in GPCR internalization and endosomal sorting. It is apparent that GPCRs are differentially regulated by ubiquitin, either directly or indirectly, and it is now clear that ligand bias can differentially dictate GPCR ubiquitination and also possibly regulate deubiquitination. However, a large gap exists in our mechanistic understanding of these processes. Future studies aimed at identifying the underlying mechanisms governing the ubiquitination status of GPCRs will be essential in understanding the role that ubiquitin has in GPCR trafficking. Given that GPCRs are the targets for many drugs used to treat a variety of diseases, it is likely that elucidating these mechanisms will translate into a better understanding of GPCR signaling in human health and disease.

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REFERENCES

1. Oldham WM, Hamm HE. Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat Rev Mol Cell Biol.* 2008; 9(1):60–71. [PubMed: 18043707]
2. Pierce KL, Premont RT, Lefkowitz RJ. Seven-transmembrane receptors. *Nat Rev Mol Cell Biol.* 2002; 3(9):639–650. [PubMed: 12209124]
3. Moore CA, Milano SK, Benovic JL. Regulation of receptor trafficking by GRKs and arrestins. *Annu Rev Physiol.* 2007; 69:451–482. [PubMed: 17037978]
4. Perry SJ, Baillie GS, Kohout TA, et al. Targeting of cyclic AMP degradation to beta 2-adrenergic receptors by beta-arrestins. *Science.* 2002; 298(5594):834–836. [PubMed: 12399592]
5. Nelson CD, Perry SJ, Regier DS, Prescott SM, Topham MK, Lefkowitz RJ. Targeting of diacylglycerol degradation to M1 muscarinic receptors by beta-arrestins. *Science.* 2007; 315(5812):663–666. [PubMed: 17272726]

6. Shenoy SK, Lefkowitz RJ. Beta-arrestin-mediated receptor trafficking and signal transduction. *Trends Pharmacol Sci.* 2011; 32(9):521–533. [PubMed: 21680031]
7. Gurevich VV, Dion SB, Onorato JJ, et al. Arrestin interactions with G protein-coupled receptors. Direct binding studies of wild type and mutant arrestins with rhodopsin, beta 2-adrenergic, and m2 muscarinic cholinergic receptors. *J Biol Chem.* 1995; 270(2):720–731. [PubMed: 7822302]
8. Lohse MJ, Benovic JL, Codina J, Caron MG, Lefkowitz RJ. Beta-arrestin: a protein that regulates beta-adrenergic receptor function. *Science.* 1990; 248(4962):1547–1550. [PubMed: 2163110]
9. Barak LS, Ferguson SS, Zhang J, Caron MG. A beta-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. *J Biol Chem.* 1997; 272(44):27497–27500. [PubMed: 9346876]
10. Marchese A, Chen C, Kim YM, Benovic JL. The ins and outs of G protein-coupled receptor trafficking. *Trends Biochem Sci.* 2003; 28(7):369–376. [PubMed: 12878004]
11. Pitcher JA, Payne ES, Csontos C, DePaoli-Roach AA, Lefkowitz RJ. The G-protein-coupled receptor phosphatase: a protein phosphatase type 2A with a distinct subcellular distribution and substrate specificity. *Proc Natl Acad Sci USA.* 1995; 92(18):8343–8347. [PubMed: 7667292]
12. Tsao P, von Zastrow M. Downregulation of G protein-coupled receptors. *Curr Opin Neurobiol.* 2000; 10(3):365–369. [PubMed: 10851176]
13. Marchese A, Trejo J. Ubiquitin-dependent regulation of G protein-coupled receptor trafficking and signaling. *Cell Signal.* 2013; 25(3):707–716. [PubMed: 23201781]
14. Hershko A, Ciechanover A. The ubiquitin system. *Annu Rev Biochem.* 1998; 67:425–479. [PubMed: 9759494]
15. Pickart CM. Mechanisms underlying ubiquitination. *Annu Rev Biochem.* 2001; 70:503–533. [PubMed: 11395416]
16. Varshavsky A. The ubiquitin system, an immense realm. *Annu Rev Biochem.* 2012; 81:167–176. [PubMed: 22663079]
17. Kirisako T, Kamei K, Murata S, et al. A ubiquitin ligase complex assembles linear polyubiquitin chains. *EMBO J.* 2006; 25(20):4877. [PubMed: 17006537]
18. Wang X, Herr RA, Hansen TH. Ubiquitination of substrates by esterification. *Traffic.* 2012; 13(1):19–24. [PubMed: 21883762]
19. Metzger MB, Hristova VA, Weissman AM. HECT and RING finger families of E3 ubiquitin ligases at a glance. *J Cell Sci.* 2012; 125(pt 3):531–537. [PubMed: 22389392]
20. Li W, Bengtson MH, Ulbrich A, et al. Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling. *PLoS One.* 2008; 3(1):e1487. [PubMed: 18213395]
21. Reyes-Turcu FE, Ventii KH, Wilkinson KD. Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. *Annu Rev Biochem.* 2009; 78:363–397. [PubMed: 19489724]
22. Sierra MI, Wright MH, Nash PD. AMSH interacts with ESCRT-0 to regulate the stability and trafficking of CXCR4. *J Biol Chem.* 2010; 285(18):13990–14004. [PubMed: 20159979]
23. Berlin I, Higginbotham KM, Dise RS, Sierra MI, Nash PD. The deubiquitinating enzyme USP8 promotes trafficking and degradation of the chemokine receptor 4 at the sorting endosome. *J Biol Chem.* 2010; 285(48):37895–37908. [PubMed: 20876529]
24. Hasdemir B, Murphy JE, Cottrell GS, Bunnett NW. Endosomal deubiquitinating enzymes control ubiquitination and down-regulation of protease-activated receptor 2. *J Biol Chem.* 2009; 284(41):28453–28466. [PubMed: 19684015]
25. Mines MA, Goodwin JS, Limbird LE, Cui FF, Fan GH. Deubiquitination of CXCR4 by USP14 is critical for both CXCL12-induced CXCR4 degradation and chemotaxis but not ERK activation. *J Biol Chem.* 2009; 284(9):5742–5752. [PubMed: 19106094]
26. Hislop JN, Henry AG, Marchese A, von Zastrow M. Ubiquitination regulates proteolytic processing of G protein-coupled receptors after their sorting to lysosomes. *J Biol Chem.* 2009; 284(29):19361–19370. [PubMed: 19433584]
27. Mukai A, Yamamoto-Hino M, Awano W, Watanabe W, Komada M, Goto S. Balanced ubiquitylation and deubiquitylation of Frizzled regulate cellular responsiveness to Wg/Wnt. *EMBO J.* 2010; 29(13):2114–2125. [PubMed: 20495530]

28. Xia R, Jia H, Fan J, Liu Y, Jia J. USP8 promotes smoothened signaling by preventing its ubiquitination and changing its subcellular localization. *PLoS Biol.* 2012; 10(1):e1001238. [PubMed: 22253573]
29. Berthouze M, Venkataramanan V, Li Y, Shenoy SK. The deubiquitinases USP33 and USP20 coordinate beta2 adrenergic receptor recycling and resensitization. *EMBO J.* 2009; 28(12):1684–1696. [PubMed: 19424180]
30. Milojevic T. The ubiquitin-specific protease USP4 regulates the cell surface level of the A_{2A} receptor. *Mol Pharmacol.* 2006; 69(4):1083–1094. [PubMed: 16339847]
31. Jacob C, Cottrell GS, Gehringer D, Schmidlin F, Grady EF, Bunnett NW. c-Cbl mediates ubiquitination, degradation, and down-regulation of human protease-activated receptor 2. *J Biol Chem.* 2005; 280(16):16076–16087. [PubMed: 15708858]
32. Shenoy SK, McDonald PH, Kohout TA, Lefkowitz RJ. Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. *Science.* 2001; 294(5545):1307–1313. [PubMed: 11588219]
33. Holleman J, Marchese A. The ubiquitin ligase Deltex-3l regulates endosomal sorting of the G protein-coupled receptor CXCR4. *Mol Biol Cell.* 2014; 25(12):1892–1904. [PubMed: 24790097]
34. Moriyoshi K, Iijima K, Fujii H, Ito H, Cho Y, Nakanishi S. Seven in absentia homolog 1A mediates ubiquitination and degradation of group 1 metabotropic glutamate receptors. *Proc Natl Acad Sci USA.* 2004; 101(23):8614–8619. [PubMed: 15163799]
35. Huijbrechtse JM, Scheffner M, Beaudenon S, Howley PM. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proc Natl Acad Sci USA.* 1995; 92(7):2563–2567. [PubMed: 7708685]
36. Rotin D, Kumar S. Physiological functions of the HECT family of ubiquitin ligases. *Nat Rev Mol Cell Biol.* 2009; 10(6):398–409. [PubMed: 19436320]
37. Does MR, Trejo J. Ubiquitination of G protein-coupled receptors: functional implications and drug discovery. *Mol Pharmacol.* 2012; 82(4):563–570. [PubMed: 22700696]
38. Ingham RJ, Gish G, Pawson T. The Nedd4 family of E3 ubiquitin ligases: functional diversity within a common modular architecture. *Oncogene.* 2004; 23(11):1972–1984. [PubMed: 15021885]
39. Malik R, Soh UJ, Trejo J, Marchese A. Novel roles for the E3 ubiquitin ligase atrophin-interacting protein 4 and signal transduction adaptor molecule 1 in G protein-coupled receptor signaling. *J Biol Chem.* 2012; 287(12):9013–9027. [PubMed: 22275353]
40. Perry WL, Hustad CM, Swing DA, O'Sullivan TN, Jenkins NA, Copeland NG. The itchy locus encodes a novel ubiquitin protein ligase that is disrupted in a18H mice. *Nat Genet.* 1998; 18(2):143–146. [PubMed: 9462742]
41. Chen HI, Sudol M. The WW domain of Yes-associated protein binds a proline-rich ligand that differs from the consensus established for Src homology 3-binding modules. *Proc Natl Acad Sci USA.* 1995; 92(17):7819–7823. [PubMed: 7644498]
42. Ingham RJ, Colwill K, Howard C, et al. WW domains provide a platform for the assembly of multiprotein networks. *Mol Cell Biol.* 2005; 25(16):7092–7106. [PubMed: 16055720]
43. Shearwin-Whyatt L, Dalton HE, Foot N, Kumar S. Regulation of functional diversity within the Nedd4 family by accessory and adaptor proteins. *BioEssays.* 2006; 28(6):617–628. [PubMed: 16700065]
44. Bhandari D, Robia SL, Marchese A. The E3 ubiquitin ligase atrophin interacting protein 4 binds directly to the chemokine receptor CXCR4 via a novel WW domain-mediated interaction. *Mol Biol Cell.* 2009; 20(5):1324–1339. [PubMed: 19116316]
45. Oo ML, Chang S-H, Thangada S, et al. Engagement of S1P1-degradative mechanisms leads to vascular leak in mice. *J Clin Invest.* 2011; 121(6):2290–2300. [PubMed: 21555855]
46. Shenoy SK, Xiao K, Venkataramanan V, Snyder PM, Freedman NJ, Weissman AM. Nedd4 mediates agonist-dependent ubiquitination, lysosomal targeting, and degradation of the beta2-adrenergic receptor. *J Biol Chem.* 2008; 283(32):22166–22176. [PubMed: 18544533]
47. Henry AG, Hislop JN, Grove J, Thorn K, Marsh M, von Zastrow M. Regulation of endocytic clathrin dynamics by cargo ubiquitination. *Dev Cell.* 2012; 23(3):519–532. [PubMed: 22940114]

48. Marchese A, Benovic JL. Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. *J Biol Chem*. 2001; 276(49):45509–45512. [PubMed: 11641392]
49. Does MR, Trejo J. Ubiquitination of GPCRs, functional implications and drug discovery. *Mol Pharmacol*. 2012; 82:563–570. [PubMed: 22700696]
50. Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y, Takahashi R. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. *Cell*. 2001; 105(7):891–902. [PubMed: 11439185]
51. Petaja-Repo UE, Hogue M, Laperriere A, Bhalla S, Walker P, Bouvier M. Newly synthesized human delta opioid receptors retained in the endoplasmic reticulum are retrotranslocated to the cytosol, deglycosylated, ubiquitinated, and degraded by the proteasome. *J Biol Chem*. 2001; 276(6):4416–4423. [PubMed: 11054417]
52. Petaja-Repo UE, Hogue M, Bhalla S, Laperriere A, Morello JP, Bouvier M. Ligands act as pharmacological chaperones and increase the efficiency of delta opioid receptor maturation. *EMBO J*. 2002; 21(7):1628–1637. [PubMed: 11927547]
53. Canals M, Scholten DJ, de Munnik S, Han MK, Smit MJ, Leurs R. Ubiquitination of CXCR7 controls receptor trafficking. *PLoS One*. 2012; 7(3):e34192. [PubMed: 22457824]
54. Baugher PJ, Richmond A. The carboxyl-terminal PDZ ligand motif of chemokine receptor CXCR2 modulates post-endocytic sorting and cellular chemotaxis. *J Biol Chem*. 2008; 283(45):30868–30878. [PubMed: 18755694]
55. Caballero A, Marchese A. Ubiquitination of GPCRs. *Methods Mol Biol*. 2011; 746:251–259. [PubMed: 21607861]
56. Hislop JN, Henry AG, von Zastrow M. Ubiquitination in the first cytoplasmic loop of mu-opioid receptors reveals a hierarchical mechanism of lysosomal down-regulation. *J Biol Chem*. 2011; 286(46):40193–40204. [PubMed: 21953467]
57. Xiao K, Shenoy SK. Beta2-adrenergic receptor lysosomal trafficking is regulated by ubiquitination of lysyl residues in two distinct receptor domains. *J Biol Chem*. 2011; 286(14):12785–12795. [PubMed: 21330366]
58. Wolfe BL, Marchese A, Trejo J. Ubiquitination differentially regulates clathrin-dependent internalization of protease-activated receptor-1. *J Cell Biol*. 2007; 177(5):905–916. [PubMed: 17535965]
59. Marchese A, Raiborg C, Santini F, Keen JH, Stenmark H, Benovic JL. The E3 ubiquitin ligase AIP4 mediates ubiquitination and sorting of the G protein-coupled receptor CXCR4. *Dev Cell*. 2003; 5(5):709–722. [PubMed: 14602072]
60. Busillo JM, Armando S, Sengupta R, Meucci O, Bouvier M, Benovic JL. Site-specific phosphorylation of CXCR4 is dynamically regulated by multiple kinases and results in differential modulation of CXCR4 signaling. *J Biol Chem*. 2010; 285(10):7805–7817. [PubMed: 20048153]
61. Sudol M, Chen HI, Bougeret C, Einbond A, Bork P. Characterization of a novel protein-binding module—the WW domain. *FEBS Lett*. 1995; 369(1):67–71. [PubMed: 7641887]
62. Groer CE, Schmid CL, Jaeger AM, Bohn LM. Agonist-directed interactions with specific beta-arrestins determine mu-opioid receptor trafficking, ubiquitination, and dephosphorylation. *J Biol Chem*. 2011; 286(36):31731–31741. [PubMed: 21757712]
63. Bhandari D, Trejo J, Benovic JL, Marchese A. Arrestin-2 interacts with the ubiquitin-protein isopeptide ligase atrophin-interacting protein 4 and mediates endosomal sorting of the chemokine receptor CXCR4. *J Biol Chem*. 2007; 282(51):36971–36979. [PubMed: 17947233]
64. Alvarez CE. On the origins of arrestin and rhodopsin. *BMC Evol Biol*. 2008; 8:222. [PubMed: 18664266]
65. Lin CH, MacGurn JA, Chu T, Stefan CJ, Emr SD. Arrestin-related ubiquitin-ligase adaptors regulate endocytosis and protein turnover at the cell surface. *Cell*. 2008; 135(4):714–725. [PubMed: 18976803]
66. Aubry L, Guetta D, Klein G. The arrestin fold: variations on a theme. *Curr Genomics*. 2009; 10(2): 133–142. [PubMed: 19794886]

67. Shea FF, Rowell JL, Li Y, Chang TH, Alvarez CE. Mammalian alpha arrestins link activated seven transmembrane receptors to Nedd4 family e3 ubiquitin ligases and interact with beta arrestins. *PLoS One*. 2012; 7(12):e50557. [PubMed: 23236378]
68. Rauch S, Martin-Serrano J. Multiple interactions between the ESCRT machinery and arrestin-related proteins: implications for PPXY-dependent budding. *J Virol*. 2011; 85(7):3546–3556. [PubMed: 21191027]
69. Nabhan JF, Pan H, Lu Q. Arrestin domain-containing protein 3 recruits the NEDD4 E3 ligase to mediate ubiquitination of the beta2-adrenergic receptor. *EMBO Rep*. 2010; 11(8):605–611. [PubMed: 20559325]
70. Han SO, Kommaddi RP, Shenoy SK. Distinct roles for beta-arrestin2 and arrestin-domain-containing proteins in beta2 adrenergic receptor trafficking. *EMBO Rep*. 2013; 14(2):164–171. [PubMed: 23208550]
71. Malik R, Marchese A. Arrestin-2 interacts with the endosomal sorting complex required for transport machinery to modulate endosomal sorting of CXCR4. *Mol Biol Cell*. 2010; 21(14):2529–2541. [PubMed: 20505072]
72. Vecchione A, Marchese A, Henry P, Rotin D, Morrione A. The Grb10/Nedd4 complex regulates ligand-induced ubiquitination and stability of the insulin-like growth factor I receptor. *Mol Cell Biol*. 2003; 23(9):3363–3372. [PubMed: 12697834]
73. Kang DS, Tian X, Benovic JL. Role of beta-arrestins and arrestin domain-containing proteins in G protein-coupled receptor trafficking. *Curr Opin Cell Biol*. 2014; 27:63–71. [PubMed: 24680432]
74. Ahn S, Nelson CD, Garrison TR, Miller WE, Lefkowitz RJ. Desensitization, internalization, and signaling functions of beta-arrestins demonstrated by RNA interference. *Proc Natl Acad Sci USA*. 2003; 100(4):1740–1744. [PubMed: 12582207]
75. Paing MM, Stutts AB, Kohout TA, Lefkowitz RJ, Trejo J. Beta-arrestins regulate protease-activated receptor-1 desensitization but not internalization or down-regulation. *J Biol Chem*. 2002; 277(2):1292–1300. [PubMed: 11694535]
76. Chen B, Dores MR, Grimsey N, Canto I, Barker BL, Trejo J. Adaptor protein complex-2 (AP-2) and epsin-1 mediate protease-activated receptor-1 internalization via phosphorylation- and ubiquitination-dependent sorting signals. *J Biol Chem*. 2011; 286(47):40760–40770. [PubMed: 21965661]
77. Traub LM. Tickets to ride: selecting cargo for clathrin-regulated internalization. *Nat Rev Mol Cell Biol*. 2009; 10(9):583–596. [PubMed: 19696796]
78. Kim YM, Benovic JL. Differential roles of arrestin-2 interaction with clathrin and adaptor protein 2 in G protein-coupled receptor trafficking. *J Biol Chem*. 2002; 277(34):30760–30768. [PubMed: 12070169]
79. Shenoy SK, Barak LS, Xiao K, et al. Ubiquitination of beta-arrestin links seven-transmembrane receptor endocytosis and ERK activation. *J Biol Chem*. 2007; 282(40):29549–29562. [PubMed: 17666399]
80. Wyatt D, Malik R, Vesecky AC, Marchese A. Small ubiquitin-like modifier modification of arrestin-3 regulates receptor trafficking. *J Biol Chem*. 2011; 286(5):3884–3893. [PubMed: 21118812]
81. Ozawa K, Whalen EJ, Nelson CD, et al. S-nitrosylation of β -arrestin regulates β -adrenergic receptor trafficking. *Mol Cell*. 2008; 31(3):395–405. [PubMed: 18691971]
82. Hislop JN, von Zastrow M. Role of ubiquitination in endocytic trafficking of G-protein-coupled receptors. *Traffic*. 2011; 12(2):137–148. [PubMed: 20854416]
83. Dores MR, Trejo J. Atypical regulation of G protein-coupled receptor intracellular trafficking by ubiquitination. *Curr Opin Cell Biol*. 2014; 27:44–50. [PubMed: 24680429]
84. Piper RC, Katzmann DJ. Biogenesis and function of multivesicular bodies. *Annu Rev Cell Dev Biol*. 2007; 23:519–547. [PubMed: 17506697]
85. Gruenberg J, Stenmark H. The biogenesis of multivesicular endosomes. *Nat Rev Mol Cell Biol*. 2004; 5(4):317–323. [PubMed: 15071556]
86. Dores MR, Chen B, Lin H, et al. ALIX binds a YPX(3)L motif of the GPCR PAR1 and mediates ubiquitin-independent ESCRT-III/MVB sorting. *J Cell Biol*. 2012; 197(3):407–419. [PubMed: 22547407]

87. Slagsvold T, Marchese A, Brech A, Stenmark H. CISK attenuates degradation of the chemokine receptor CXCR4 via the ubiquitin ligase AIP4. *EMBO J*. 2006; 25(16):3738–3749. [PubMed: 16888620]
88. Henry AG, White IJ, Marsh M, von Zastrow M, Hislop JN. The role of ubiquitination in lysosomal trafficking of delta-opioid receptors. *Traffic*. 2011; 12(2):170–184. [PubMed: 21106040]
89. Henne WM, Buchkovich NJ, Emr SD. The ESCRT pathway. *Dev Cell*. 2011; 21(1):77–91. [PubMed: 21763610]
90. Marchese A. Endocytic trafficking of chemokine receptors. *Curr Opin Cell Biol*. 2014; 27:72–77. [PubMed: 24680433]
91. Valiathan RR, Resh MD. Differential control of CXCR4 and CD4 downregulation by HIV-1 Gag. *Virology*. 2008; 5:23. [PubMed: 18267010]
92. Malerod L, Stuffers S, Brech A, Stenmark H. Vps22/EAP30 in ESCRT-II mediates endosomal sorting of growth factor and chemokine receptors destined for lysosomal degradation. *Traffic*. 2007; 8(11):1617–1629. [PubMed: 17714434]
93. Kumar A, Kremer KN, Dominguez D, Tadi M, Hedin KE. Galpha13 and Rho mediate endosomal trafficking of CXCR4 into Rab11+ vesicles upon stromal cell-derived factor-1 stimulation. *J Immunol*. 2011; 186(2):951–958. [PubMed: 21148034]
94. Benovic JL, Marchese A. A new key in breast cancer metastasis. *Cancer Cell*. 2004; 6(5):429–430. [PubMed: 15542424]
95. Li YM, Pan Y, Wei Y, et al. Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis. *Cancer Cell*. 2004; 6(5):459–469. [PubMed: 15542430]
96. Gullapalli A, Wolfe BL, Griffin CT, Magnuson T, Trejo J. An essential role for SNX1 in lysosomal sorting of protease-activated receptor-1: evidence for retromer-, Hrs-, and Tsg101-independent functions of sorting nexins. *Mol Biol Cell*. 2006; 17(3):1228–1238. [PubMed: 16407403]
97. Hasdemir B, Bunnett NW, Cottrell GS. Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) mediates post-endocytic trafficking of protease-activated receptor 2 and calcitonin receptor-like receptor. *J Biol Chem*. 2007; 282(40):29646–29657. [PubMed: 17675298]
98. Kantamneni S, Correa SA, Hodgkinson GK, et al. GISP: a novel brain-specific protein that promotes surface expression and function of GABA(B) receptors. *J Neurochem*. 2007; 100(4):1003–1017. [PubMed: 17241134]
99. Kantamneni S, Holman D, Wilkinson KA, et al. GISP binding to TSG101 increases GABA receptor stability by down-regulating ESCRT-mediated lysosomal degradation. *J Neurochem*. 2008; 107(1):86–95. [PubMed: 18643869]
100. Clague MJ, Coulson JM, Urbe S. Cellular functions of the DUBs. *J Cell Sci*. 2012; 125(pt 2):277–286. [PubMed: 22357969]
101. Lachance V, Degrandmaison J, Marois S, et al. Ubiquitylation and activation of a Rab GTPase is promoted by a beta(2)AR-HACE1 complex. *J Cell Sci*. 2014; 127(pt 1):111–123. [PubMed: 24190883]
102. Reiter E, Ahn S, Shukla AK, Lefkowitz RJ. Molecular mechanism of β -arrestin-biased agonism at seven-transmembrane receptors. *Annu Rev Pharmacol Toxicol*. 2012; 52(1):179–197. [PubMed: 21942629]
103. Wisler JW, DeWire SM, Whalen EJ, et al. A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. *Proc Natl Acad Sci USA*. 2007; 104(42):16657–16662. [PubMed: 17925438]

Figure 1.

Mechanisms of GPCR ubiquitination at the plasma membrane by E3 ubiquitin ligases. E3 ubiquitin ligases belonging to the HECT domain Nedd4-like family can interact with GPCRs directly (A) or indirectly (B). (A) Upon binding to its cognate ligand CXCL12 (yellow oval), CXCR4 is phosphorylated by GRK6 on serine residues 324 and 325 at the plasma membrane. This is followed by recruitment of AIP4, a Nedd4-like E3 ubiquitin ligase, to the receptor, resulting in ubiquitination of nearby lysine residues. The WW domains of AIP4 mediate the interaction with CXCR4 via a noncanonical mechanism with phosphorylated serine residues 324 and 325. (B) Upon binding to the selective β -agonist isoproterenol (red oval) β_2 AR is rapidly phosphorylated, likely by GRK2, leading to recruitment of β -arrestin-2. β -Arrestin-2 interacts with Nedd4, a Nedd4-like E3 ubiquitin ligase, and serves to bridge the interaction between β_2 AR and Nedd4, thereby enabling ubiquitination of the receptor. The α -arrestin protein ARRDC3 has also been implicated in this event, but it may in fact act at a later step in the endocytic pathway.

Figure 2.

Mechanisms of GPCR sorting into intraluminal vesicles (ILVs) of endosomes or multivesicular bodies (MVBs). GPCRs targeted for lysosomal degradation can be found on the limiting membrane of endosomes or MVBs and on the membrane of ILVs. A mature MVB fuses with lysosomes where degradation of its contents occurs. The ILVs are formed by a complex process that involves the ESCRT (endosomal sorting complex required for transport) pathway. ESCRTs also deliver ubiquitinated GPCRs or other ubiquitinated transmembrane proteins into the invaginating regions of the limiting membrane that eventually give rise to the ILVs. (A) For GPCRs such as CXCR4, the attached ubiquitin moieties interact with ubiquitin-binding ESCRTs such as ESCRT-0. ESCRT-I, -II, and -III are also required and act in a coordinated manner to deliver CXCR4 into ILVs, but for clarity are not shown in the figure. ESCRT-0 recognizes and recruits ubiquitinated CXCR4 into the pathway. This step is regulated by ubiquitination of ESCRT-0. β-Arrestin-1 likely serves as an adaptor for ESCRT-0 ubiquitination by the Nedd4-like E3 ubiquitin ligase AIP4. Ubiquitination of ESCRT-0 is believed to inhibit its sorting activity, possibly to allow ubiquitinated CXCR4 to interact with the other ubiquitin-binding ESCRTs. AIP4 ligase activity is inhibited by the RING-finger E3 ubiquitin ligase DTX3L through a mechanism that is not completely understood, but this is thought to reduce ESCRT-0 ubiquitination and facilitate CXCR4 sorting into ILVs. (B) For some GPCRs, for which PAR1 serves as an example, delivery into ILVs does not require receptor ubiquitination and nor are the ubiquitin-binding ESCRTs (ESCRT-0, -I, and -II) required. Instead, the adaptor protein ALIX is required to target PAR1 into ILVs. ALIX interacts with a YP(X)₃L motif present in the second intracellular loop of PAR1 and it also interacts with ESCRT-III, thereby delivering PAR1 into ILVs.