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Ubiquitination of GPCRs, Functional Implications and Drug Discovery

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ABBREVIATIONS: AT₁R, angiotensin-II type 1 receptor; D₅R, dopamine D₅ receptor; DOR, δ -opioid receptor; ER, endoplasmic reticulum; ESCRT, endosomal sorting complex required for transport; GPCR, G protein-coupled receptor; HRS, hepatocyte growth factor-regulated tyrosine kinase substrate; ILVs, intraluminal vesicles; MOR, μ -opioid receptor; MVBs, multivesicular bodies; PAR, protease-activated receptor; PTH, parathyroid hormone; S1P, sphingosine-1-phosphate; UBDs, ubiquitin-binding domains; USP, ubiquitin-specific protease

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ABSTRACT

G protein-coupled receptors (GPCRs) comprise the largest and most diverse family of signaling receptors and control a vast array of physiological responses. Modulating the signaling responses of GPCRs therapeutically is important for the treatment of various diseases and discovering new aspects of GPCR signal regulation is critical for future drug development. Posttranslational modifications are integral to the regulation of GPCR function. In addition to phosphorylation, many GPCRs are reversibly modified with ubiquitin. Ubiquitin is covalently attached to lysine residues within the cytoplasmic domains of GPCRs by ubiquitin ligases and removed by ubiquitin-specific proteases. In many cases, ubiquitin functions as a sorting signal that facilitates trafficking of mammalian GPCRs from endosomes to lysosomes for degradation, but not all GPCRs utilize this pathway. Moreover, there are distinct types of ubiquitin conjugations that are known to serve diverse functions in controlling a wide range of cellular processes, suggesting broad roles for GPCR ubiquitination. In this review, we highlight recent studies that illustrate various roles for ubiquitin in regulation of GPCR function. Ubiquitination is known to target many GPCRs for lysosomal degradation, and current studies now indicate that basal ubiquitination, deubiquitination and transubiquitination of certain GPCRs are important for controlling cell surface expression and cellular responsiveness. In addition, novel functions for ubiquitin in regulation of GPCR dimers and in mediating differential GPCR regulation induced by biased agonists have been reported. We will discuss the implications of these new discoveries for ubiquitin regulation of GPCR function in the context drug development.

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Introduction

Mammalian G protein-coupled receptors (GPCRs) are a large and diverse family of seven transmembrane signaling proteins that control numerous physiological processes. The precise signaling response induced by activation of GPCRs is critical for appropriate cellular behavior and function of many organ systems. GPCRs represent a major pharmacological target for the treatment of many pathological conditions including immunological, endocrine, renal and pulmonary diseases, pain, cancer, cardiovascular and neurological disorders (Mason et al., 2012). Signaling by GPCRs is a highly dynamic and regulated process in which agonists induce receptor coupling to heterotrimeric G-proteins and interaction with adaptor proteins that direct the trafficking of receptors to subcellular compartments. In addition, GPCRs respond to multiple agonists, each potentially initiating unique signaling cascades by directing receptor interaction with heterotrimeric G-proteins or β -arrestins. The ubiquitously expressed β -arrestin-1 and -2 isoforms mediate desensitization and internalization of most classic GPCRs and also serve as scaffolds that promote non-G-protein-dependent signaling (Reiter et al., 2012). The ability of specific agonists to activate distinct G-protein or β -arrestin-mediated signaling pathways through the same receptor is known as biased agonism (Urban et al., 2007). GPCRs also associate with each other forming homodimers or heterodimeric complexes that elicit distinct signaling responses and are likely differentially regulated. Understanding the molecular mechanisms that govern GPCR signaling is essential for the discovery of new regulatory processes that can be targeted for drug development.

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Posttranslational modifications are essential to the regulation of all GPCRs. In addition to phosphorylation, glycosylation and palmitoylation, many GPCRs are posttranslationally modified with ubiquitin (Table 1). Ubiquitin is a 76 amino acid protein that is covalently linked to lysine residues of substrate proteins by the sequential actions of three distinct enzymes (Macgurn et al., 2012). Ubiquitin is processed by an E1 activating enzyme, forming a thioester bond with ubiquitin that is required for transfer to E2 conjugating enzymes and E3 ubiquitin ligases facilitate the covalent attachment of ubiquitin to substrate proteins. Ubiquitination is reversible and the removal of ubiquitin is mediated by a family of deubiquitinating enzymes, including the ubiquitin-specific proteases (USPs) that are known to target ubiquitinated GPCRs (Alonso et al., 2011; Milojevic et al., 2006). There are distinct types of ubiquitin conjugation that are generated through the covalent attachment of a single ubiquitin moiety to an individual lysine or several lysine residues, i.e. monoubiquitination and multi-monoubiquitination (Fig. 1). Ubiquitin also contains 7 lysine residues that are subject to ubiquitin conjugation and form different configurations of ubiquitin chains including K48- and K63-linked polyubiquitin (Fig. 1). Ubiquitinated substrates are recognized by ubiquitin-binding domains (UBDs), which are a large structurally diverse class of protein modules that bind non-covalently to ubiquitin and have been identified in hundreds of proteins. Some UBDs display selectivity towards different types of ubiquitin conjugations and function in multiple cellular processes including protein degradation by the proteasome, protein trafficking to lysosomes, DNA repair, cell-cycle progression, autophagy and gene transcription (Dikic et al., 2009). Despite extensive knowledge of ubiquitin function in growth factor and cytokine receptor signaling, the role of ubiquitin in regulation of

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GPCRs is best characterized to function as a sorting signal for lysosomal degradation.

In this review, we discuss additional roles of ubiquitin in the regulation of GPCR function, including new findings on GPCR constitutive ubiquitination, deubiquitination, transubiquitination, and the role of ubiquitin in mediating differential GPCR regulation induced by biased agonists.

Ubiquitin-dependent and -independent lysosomal sorting of GPCRs

Studies of the yeast *S. cerevisiae* Ste2 GPCR demonstrated that ubiquitin can act as both an internalization signal (Hicke and Riezman, 1996) and a lysosomal sorting signal (Katzmann et al., 2001). In mammalian cells, ubiquitin is not essential for GPCR internalization, rather ubiquitin is best known to target agonist-activated GPCRs to lysosomes for degradation via the highly conserved endosomal-sorting complex required for transport (ESCRT) pathway (Fig. 2) (Marchese et al., 2008). This process is important for ridding cells of receptors following chronic agonist stimulation and for disposing of irreversibly activated receptors (Marchese et al., 2008; Trejo et al., 1998). Several mammalian GPCRs have been shown to undergo modification with ubiquitin within minutes of ligand binding (Table 1) (Hislop and von Zastrow, 2010), although the types of ubiquitin conjugation are largely undefined. Ubiquitination of receptors is required for engagement of the ESCRT machinery at early endosomes (Hurley and Hanson, 2010), the site at which the trafficking fate of receptors is determined. The ESCRTs are comprised of four distinct complexes, three of which contain components with UBDs and facilitate the trafficking of ubiquitinated receptors from early endosomes

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to late endosomes/multivesicular bodies (MVBs) where receptors are sorted into intraluminal vesicles (ILVs) prior to degradation within the lumen of the lysosome (Fig. 3). ESCRT- and ubiquitin-dependent sorting of GPCRs to lysosomes has been best characterized for the chemokine CXCR4 receptor and protease-activated receptor-2 (PAR2). In these studies, ubiquitination-deficient receptor mutants failed to degrade following prolonged agonist stimulation and disruption of canonical ESCRT components impaired agonist-promoted receptor degradation (Hasdemir et al., 2007; Malerod et al., 2007; Marchese et al., 2003). Many other mammalian GPCRs also require ubiquitination for ligand-induced lysosomal degradation (Table 1).

An exclusive function for ubiquitin in ESCRT-mediated GPCR MVB/lysosomal sorting has been recently challenged. An ubiquitination-deficient δ -opioid receptor (DOR) mutant and the calcitonin receptor-like receptor, a non-ubiquitinated GPCR, have been shown to sort to lysosomes independent of ubiquitination (Cottrell et al., 2007; Tanowitz and Von Zastrow, 2002). Surprisingly, lysosomal degradation of DOR and calcitonin receptor-like receptor does require hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), an ubiquitin-binding component of the ESCRT machinery (Fig. 3) (Hasdemir et al., 2007; Hislop et al., 2004), suggesting that not all GPCRs require direct ubiquitination for interaction with the canonical ESCRT machinery. In contrast to these receptors, protease-activated receptor-1 (PAR1), a GPCR for thrombin, is sorted to lysosomes independent of ubiquitination and the ubiquitin-binding ESCRT-0 and -I components, HRS and Tsg101 (Gullapalli et al., 2006; Wolfe et al., 2007). In recent work, we discovered that PAR1 sorts to ILVs of MVBs through a pathway mediated by ESCRT-III components, which do not contain any known UBDs.

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We further demonstrated that ALIX, an ESCRT-III interacting protein, bound to a YPX₃L motif within the second intracellular loop of PAR1 via its central V-domain to facilitate MVB/lysosomal degradation (Fig. 3) (Dores et al., 2012). In addition, seven other GPCRs contained conserved YPX₃L motifs within their second intracellular loop raising the possibility that ALIX mediates ubiquitin-independent lysosomal sorting of a subset of GPCRs in mammalian cells. Intriguingly, both PAR1 and DOR are ubiquitinated following agonist stimulation (Chen et al., 2011; Dores et al., 2012; Hislop et al., 2009), although ubiquitin is not required for lysosomal sorting indicating that ubiquitin has other functions in GPCR regulation that have yet to be discovered. A conserved role for ubiquitin as a lysosomal sorting signal for certain GPCRs is essential for proper downregulation, however recent evidence now suggests that alternative degradation pathways exist and ubiquitination of receptors may play a wider role in signal regulation than previously considered.

Ubiquitination and de-ubiquitination of GPCRs and cell surface expression

Non-ligand mediated or basal ubiquitination of GPCRs appears to serve distinct functions compared to ligand-induced receptor ubiquitination. Basally ubiquitinated GPCRs have been reported to exist at the endoplasmic reticulum (ER) and plasma membrane (Fig. 2). Ubiquitination of GPCRs during *de novo* synthesis in the ER is mainly related to the clearance of misfolded receptors, however some properly folded GPCRs are targeted to the proteasome at steady-state and are released to the cell surface by deubiquitination. In addition, certain GPCRs expressed on the cell surface

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exist at steady-state as ubiquitinated proteins. In both cases, the ubiquitination and deubiquitination of the receptors is a dynamic process that can drastically alter the surface expression and thus sensitivity of cells to ligand stimulation.

During biogenesis of GPCRs and other transmembrane proteins at the ER, proteins are folded and adopt distinct conformations, which is mediated by interactions with chaperones before export to the Golgi for further processing. Calnexin, calreticulin and receptor activity modifying proteins are chaperones that function broadly to facilitate the transport of numerous GPCRs through the ER. Other chaperones appear to act selectively on specific GPCRs to regulate trafficking through the biosynthetic pathway (Achour et al., 2008). A quality control system ensures proper protein folding and clearance of misfolded proteins via polyubiquitin-mediated proteasomal degradation (Fig. 2), an ER-associated degradation pathway that involves retrotranslocation and extraction of transmembrane proteins. Several studies indicate that during biogenesis, GPCRs are polyubiquitinated and recognized by the proteasomal system and degraded, a process attributed to misfolding of a fraction of the newly synthesized receptors. This has been illustrated for the δ - and μ -opioid receptors and thyrotropin-releasing hormone receptor as well as many other GPCRs in model systems that involved overexpression (Chaturvedi et al., 2001; Cook et al., 2003; Petaja-Repo et al., 2001). The importance of this pathway is further highlighted by the discovery of naturally occurring point mutations in the rhodopsin, vasopressin type 2 receptor and gonadotropin hormone-releasing hormone receptor that cause misfolding, loss of cell surface expression and contribute to disease progression (Conn et al., 2007). Pharmacological agents that bind specifically to misfolded gonadotropin-releasing hormone receptor have been

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discovered and shown to rescue defects in sorting at the ER by inducing proper receptor folding resulting in increased cell surface expression (Conn and Ulloa-Aguirre, 2011). The discovery of pharmacological agents that function as chaperones represents a major area of therapeutic development. In addition, wildtype non-mutated GPCRs that are basally ubiquitinated can be targeted for deubiquitination during biosynthesis, which enhances receptor transport through the ER (Fig. 2). USP4 has been shown to bind to and deubiquitinate the adenosine receptor A_{2A} during biogenesis and results in increased A_{2A} receptor cell surface expression (Milojevic et al., 2006). Therefore, modulating the ubiquitination status of GPCRs at the ER can directly affect the density of functional cell surface receptors and cellular responsiveness and demonstrate that ER-related GPCR ubiquitination is important for regulating both misfolded and properly folded GPCRs.

GPCRs expressed at the cell surface also appear to be constitutively ubiquitinated (Fig. 2). Ubiquitination of the platelet-activating factor receptor occurs at steady-state and it is not modulated by agonist stimulation, but appears to regulate receptor degradation following agonist stimulation (Dupre et al., 2003). In contrast, CXCR7 is basally ubiquitinated, but upon agonist stimulation becomes deubiquitinated in a process that requires receptor phosphorylation and β -arrestin recruitment (Canals et al., 2012). Interestingly, ubiquitination of CXCR7 is restored upon removal of ligand, resulting in recycling of internalized receptor back to the cell surface. Hence, ubiquitination controls the expression of CXCR7 at the cell surface, rather than lysosomal sorting and degradation. Basal ubiquitination of PAR1 is also necessary for receptor retention at the cell surface. An ubiquitin-deficient PAR1 mutant exhibited a

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higher rate of constitutive internalization as compared to wildtype receptor (Wolfe et al., 2007), whereas fusion of a single ubiquitin moiety to the cytoplasmic tail of the receptor mutant restored normal cell surface expression. Interestingly, PAR1 constitutive internalization is mediated by the clathrin adaptor protein complex-2 (AP-2), which binds to a cytoplasmic tail localized tyrosine “YKKL” motif interlaced with lysine (K) residues that are sites of ubiquitination (Paing et al., 2006; Wolfe et al., 2007). These findings suggest that ubiquitination precludes AP-2 binding to PAR1 and thereby maintains receptors at the cell surface. It is possible that ubiquitin similarly regulates cell surface expression of CXCR7 by preventing its association with endocytic adaptor proteins. Thus, ubiquitination *per se* is not sufficient to induce internalization of mammalian GPCRs and in fact, is capable of negatively regulating this process.

Ubiquitination of GPCRs is dynamic. When internalization of PAR1 is inhibited by expression of a dominant-negative mutant form of dynamin, a GTPase essential for clathrin-mediated endocytosis, agonist stimulation induces PAR1 deubiquitination (Wolfe et al., 2007). However, after activation *and* internalization, PAR1 ubiquitination is markedly increased (Chen et al., 2011). These findings suggest that ubiquitination of PAR1 is tightly regulated in a spatio-temporal manner through the actions of ubiquitin ligases and deubiquitinating enzymes. Ubiquitination and deubiquitination of Frizzled-4, a seven transmembrane receptor for Wnt ligands, is also important for regulating surface expression and cellular responsiveness (Mukai et al., 2010). Constitutive ubiquitination of Frizzled-4 promotes internalization and lysosomal degradation, whereas deubiquitination mediated by USP8 leads to recycling and increased surface expression, events that occur independent of stimulation with Wnt ligands (Mukai et al.,

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2010). Deubiquitination of agonist-activated β 2-adrenergic receptor by USP33 and USP20 also switches the receptor fate from lysosomal degradation to recycling and enhances cellular resensitization (Berthouze et al., 2009). These studies illustrate diverse roles for ubiquitin in regulating GPCR retention at the cell surface, recycling and endocytic sorting (Fig. 2) and suggest that influencing the receptor ubiquitination status at the plasma membrane or within internal endosomal compartments can considerably alter GPCR surface expression.

GPCRs and transubiquitination

GPCRs are known to transactivate other receptors such as growth factor tyrosine kinase receptors via release of membrane-anchored ligands or through modulation of receptor cytoplasmic domains (Wetzker and Bohmer, 2003). New studies now indicate that signaling by GPCRs can instigate the ubiquitination of other GPCRs, which appears to negatively regulate their expression. Thus, GPCRs at the cell surface can be ubiquitinated in *trans* following activation of another receptor, and this transubiquitination can drastically change cell and tissue responsiveness. Dimers of GPCRs are also capable of directing the co-ubiquitination and co-degradation of their unactivated binding partners. The studies discussed below reveal new mechanisms by which GPCR function is regulated through transubiquitination and co-ubiquitination.

The phenomenon of transubiquitination of an unstimulated GPCR that is induced by signaling from another receptor was first described for the angiotensin-II type 1 receptor (AT₁R) in response to dopamine D₅ receptor (D₅R) activation (Li et al., 2008).

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Angiotensin-II and dopamine hormones counterregulate hypertensive responsiveness, where AT₁R signaling is prohypertensive (Kobori et al., 2007) and D₅R signaling is antihypertensive (Hollon et al., 2002). Disruption of the D₅R gene in mice resulted in increased blood pressure and AT₁R expression, suggesting that D₅R negatively regulates AT₁R expression *in vivo*. Moreover, activation of D₅R induced ubiquitination of AT₁R at the cell surface and receptor degradation via the proteasome and not the lysosome (Li et al., 2008). In another study, the orexin receptor OX₂, a component of the hypocretin system responsible for sleep/wake cycle regulation, was shown to be ubiquitinated and degraded in response to signaling by the proinflammatory cytokine tumor necrosis factor- α (Zhan et al., 2011), suggesting that different signaling receptors can stimulate GPCR transubiquitination and degradation. A clinically relevant mechanism of ubiquitination and downregulation of an unstimulated GPCR is elegantly demonstrated by the sphingosine-1-phosphate (S1P) receptor antagonist FTY720. Also known as Fingolimod, FTY720 is an immunomodulatory drug used for the treatment of multiple sclerosis (Pelletier and Hafler, 2012). FTY720 binds to the S1P receptor and promotes phosphorylation, polyubiquitination and degradation by the proteasome, resulting in functional antagonism of S1P signaling (Oo et al., 2011; Oo et al., 2007). Intriguingly, direct agonist stimulation of either the AT₁R or S1P receptors does not result in ubiquitination or degradation of these receptors (Li et al., 2008; Oo et al., 2011), indicating that posttranslational modification by ubiquitin can be selectively regulated. These studies suggest that novel therapeutics capable of directly or indirectly inducing ubiquitination and downregulation of certain GPCRs could be beneficial in certain disease scenarios.

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The existence of many GPCRs as functional homodimers or heterodimers is well established. Of particular interest is the regulation and trafficking of dimerized GPCRs following activation of one of the receptor protomers. A recent study showed that depending on the activating ligand, internalized DOR and μ -opioid receptor (MOR) heterodimers are either recycled or sorted to lysosomes for degradation (He et al., 2011). DOR is known to internalize and traffic to lysosomes following ligand stimulation (Tsao et al., 2001), whereas agonist-activated MOR is internalized and efficiently recycled, which is important for cellular resensitization (Qiu et al., 2003). DOR and MOR form heterodimers, and stimulation with a DOR specific agonist directs the ubiquitination but not the phosphorylation of MOR, causing degradation of both DOR and the non-stimulated MOR (He et al., 2011). Consequently, MOR is depleted from the cell surface and cells are no longer responsive to MOR agonists. Disruption of the heterodimer restores MOR to the cell surface and increases sensitivity to opiate agonists, suggesting that therapies that isolate MOR from DOR can improve patient responses to analgesics (He et al., 2011). This study illustrates the importance of heterodimer-mediated GPCR transubiquitination, and future studies will undoubtedly reveal additional roles for ubiquitin in various aspects of GPCR dimer regulation.

GPCR ubiquitination and biased agonism

Activation of the same GPCR by two or more distinct ligands can elicit differential responses and is a phenomena referred to as “biased agonism” (Urban et al., 2007). Biased agonism is an emerging area in pharmacology that has important implications in

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drug development. Thus, elucidating the molecular basis of biased agonism is important for the development of newer selective drugs with less adverse side effects.

Two recent studies have shown that ubiquitination is important in determining differential GPCR regulation induced by biased agonists. The type 1 parathyroid hormone (PTH) receptor regulates bone growth and mineral ion balance and is responsive to distinct ligands. Stimulation with the activating ligand PTH [1-34] results in PTH receptor ubiquitination (Alonso et al., 2011). The PTH receptor is internalized and then recycled following deubiquitination. In contrast, PTH receptor binding to the non-activating ligand PTH [7-34] causes sustained ubiquitination and receptor degradation (Alonso et al., 2011). The different trafficking behaviors exhibited by the PTH receptor appear to be regulated by the deubiquitinating enzyme USP2, which is upregulated in response to stimulation by the PTH [1-34] agonist (Alonso et al., 2011). These findings indicate that specific PTH receptor ligands distinctly modulate the activity of the ubiquitination machinery, resulting in differential receptor ubiquitination and trafficking.

The μ -opioid receptor, a major target of opiate drugs that are used clinically as analgesics, displays biased agonism (Raehal et al., 2011). Previous studies showed that morphine and D-enkephalin analog [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]enkephalin (DAMGO) differentially regulate MOR internalization and recycling. A recent study has now reported that morphine and DAMGO cause differential β -arrestin recruitment and ubiquitination of MOR. Stimulation of MOR with DAMGO induces β -arrestin-1 mediated receptor ubiquitination and dephosphorylation (Groer et al., 2011). In contrast, activation of MOR with morphine results in β -arrestin-2-dependent internalization, sustained MOR

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phosphorylation and slower resensitization (Groer et al., 2011). Compared to DAMGO, morphine fails to promote β -arrestin-1 recruitment and MOR ubiquitination. These observations suggest that agonist-promoted ubiquitination of MOR is mediated specifically by β -arrestin-1 and not β -arrestin-2, and is important for receptor dephosphorylation and cellular resensitization. Thus, delineating the molecular mechanisms by which MOR is differentially ubiquitinated by DAMGO *versus* morphine is important for understanding cellular responsiveness to different opiate drugs and will further enhance our knowledge of the mechanisms that underlie biased agonism.

Summary

The posttranslational modification of GPCRs is essential for proper receptor signaling and appropriate cellular responses. Unlike phosphorylation, our knowledge of ubiquitin function in regulation of GPCR signaling and trafficking is vastly limited. Recent studies have provided new insight into the various mechanisms by which ubiquitin functions to regulate GPCR expression at the cell surface. Other work suggests an important role for ubiquitin in differential GPCR regulation induced by biased agonists. We will now need to explore how ubiquitin distinctly regulates these processes by defining the types of ubiquitin conjugation, proteins that recognize ubiquitinated GPCRs as well as the ubiquitin machinery. By regulating the activity of specific proteins involved in the ubiquitination of GPCRs, we will be able to modulate the manner in which GPCR signaling occurs by controlling GPCR surface expression and degradation. In addition, several fundamental questions remain regarding the mechanisms by which

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polyubiquitinated GPCRs at the cell surface are targeted and degraded by the proteasome, since integral membrane proteins are generally not accessible to the proteasome. Ubiquitin can also influence signaling through multiple mechanisms beyond regulating receptor subcellular localization and degradation by influencing the assembly of receptor signaling complexes as has been described for cytokine receptor activation of mitogen-activated protein kinases (Laine and Ronai, 2005). Thus, future studies are needed to explore new functions of ubiquitin in GPCR signaling.

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Footnotes

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Figure legends

Fig. 1. Distinct types of ubiquitin conjugation. GPCRs are potentially modified with distinct types of ubiquitin conjugations including monoubiquitin, multiple monoubiquitin and either K48- or K63-linked polyubiquitin. The different types of ubiquitin modifications are likely to serve distinct functions such as GPCR lysosomal sorting, cell surface retention, proteasomal degradation and signaling.

Fig. 2. Model of ubiquitin function in GPCR trafficking. Ubiquitination of GPCRs regulates transport through the endoplasmic reticulum to the *trans*-Golgi network and trafficking to the plasma membrane. GPCR ubiquitination has been recently implicated in mediating receptor retention at the plasma membrane and receptor recycling. The function of ubiquitin as a signal for sorting GPCRs from endosomes to multivesicular bodies (MVBs)/lysosomes after ligand stimulation is well established. Some GPCRs appear to be ubiquitinated at the plasma membrane and targeted to the proteasome for degradation through a poorly understood process, as indicated by the question mark.

Fig. 3. Ubiquitin-dependent and –independent lysosomal sorting of GPCRs. In the classic paradigm, agonist stimulation induces GPCR ubiquitination and internalization to early endosomes. The ubiquitinated GPCR is then recognized by ESCRT-0/HRS harboring an ubiquitin-interacting motif and ESCRT-I and –II containing UBDs. GPCRs are deubiquitinated prior to sorting into the intraluminal vesicles (ILV) of the late endosome/multivesicular body, the later process is regulated by ESCRT-III/charged

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MVB protein-4 (CHMP4) and the AAA-ATPase Vps4A,B. In contrast to the canonical ESCRT pathway, PAR1 is sorted to lysosomes independent of ubiquitin and the canonical ESCRT machinery. New studies indicate that activated and internalized PAR1 is recognized by the adaptor protein ALIX, which binds to a YPX₃L motif within the second intracellular loop of the receptor, and links PAR1 to ESCRT-III/CHMP4 to facilitate sorting to ILVs of MVBs/lysosomes. Interestingly, PAR1 is modified with ubiquitin, although ubiquitin is not essential for PAR1 lysosomal sorting, suggesting additional functions for ubiquitin in GPCR regulation that remain to be discovered.

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TABLE 1

Ubiquitinated GPCRs

Receptor Family	IUPHAR Name	Ubiquitination	E3 Ligase	DUB	Function	References
Class A						
Adenosine Receptors	A _{2A}	Constitutive	N.D.	Usp4	Deubiquitination required for surface expression	(Milojevic et al., 2006)
Adrenoceptors	β ₂ AR	Agonist-induced	Nedd4-1	USP20, USP33	Lysosomal degradation, Regulation of arrestin-mediated signaling	(Berthouze et al., 2009; Shenoy et al., 2009; Shenoy et al., 2008)
Angiotensin Receptors	AT ₁	Via D ₅ R Activation	N.D.	N.D.	Proteosomal degradation	(Li et al., 2008)
Chemokine Receptors	CXCR4	Agonist-induced	AIP4	USP14	Lysosomal degradation	(Marchese and Benovic, 2001; Marchese et al., 2003; Mines et al., 2009)
Chemokine Receptors	CXCR7	Constitutive	N.D.	N.D.	Ubiquitination required for surface expression	(Canals et al., 2012)
Dopamine Receptors	D ₁ , D ₂	Constitutive	N.D.	N.D.		(Rondou et al., 2008)
Dopamine Receptors	D ₄	Constitutive	Roc1-Cul3-KLHL12 Complex	N.D.	Not required for degradation, K48-linked ubiquitination	(Rondou et al., 2008; Rondou et al., 2010)
Dopamine Receptors	D ₅	Constitutive, Agonist-Induced	N.D.	N.D.	Directs AT ₁ R degradation	(Li et al., 2008; Rondou et al., 2008)
Glycoprotein Hormone Receptors	FSH Receptor	Constitutive	N.D.	N.D.	ICL-3 ubiquitination regulates surface	(Cohen et al., 2003)

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					expression	
Lysophospholipid receptors	LPA ₂	Agonist-induced	N.D.	N.D.	Promotes cell survival via co-degradation of Siva-1	(Lin et al., 2007)
Lysophospholipid receptors	S ₁ P	Induced by inhibitor FTY720	WWP2	N.D.	Promotes pulmonary vascular leakage	(Oo et al., 2011; Oo et al., 2007)
Melanocortin receptors	MCR ₂	Agonist-induced	Mahogunin	N.D.	Multiple-mono ubiquitination	(Cooray et al., 2011)
Opioid Receptors	μ	Agonist-induced	N.D.	N.D.	Agonist-specific ubiquitination, co-degradation with δ	(Groer et al., 2011; He et al., 2011; Henry et al., 2011)
Opioid Receptors	δ	Agonist-induced	AIP4	N.D.	Non-ubiquitin mediated degradation, Co-degradation with μ	(He et al., 2011; Henry et al., 2011; Hislop et al., 2009; Tanowitz and Von Zastrow, 2002)
Opioid Receptors	κ	Constitutive, enhanced by agonist	N.D.	N.D.	K63-linked ubiquitination	(Li et al., 2008)
Orexin Receptors	OX ₂	Via TNF-α Stimulation	N.D.	N.D.	TNF-α stimulation causes degradation	(Zhan et al., 2011)
Platelet-activating Receptor	PAF Receptor	Constitutive	N.D.	N.D.	Proteosomal and lysosomal degradation	(Dupre et al., 2003)
Prostinoid Receptors	IP ₁	Agonist-induced	N.D.	N.D.	Lysosomal degradation	(Donnellan and Kinsella, 2009)
Protease-Activated Receptors	PAR1	Constitutive, enhanced by agonist	N.D.	N.D.	Not required for degradation	(Chen et al., 2011; Dores et al., 2012; Wolfe et al., 2007)

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Protease-Activated Receptors	PAR2	Agonist-induced	c-Cbl	AMSH, UBPY	Lysosomal degradation	(Hasdemir et al., 2009; Jacob et al., 2005)
Tachykinin Receptors	NK ₁	Agonist-induced	N.D.	N.D.	Desensitization to sustained Substance-P stimulation	(Cottrell et al., 2006)
Thyrotropin-Releasing Hormone Receptor	THR ₁	Constitutive	N.D.	N.D.	Biosynthetic turnover, not required for internalization.	(Cook et al., 2003)
Vasopressin and Oxytocin Receptors	V ₂	Agonist-induced	N.D.	N.D.	Arrestin-mediated ubiquitination	(Martin et al., 2003)
Class B						
Glucagon Receptors	GIP Receptor	Agonist-induced	N.D.	N.D.	Proteasome dependent	(Zhou et al., 2007)
Parathyroid Hormone Receptors	PTH1	Agonist-induced	N.D.	N.D.	Differential ubiquitination and deubiquitination by two ligands	(Alonso et al., 2011)
Class C						
Calcium-sensing Receptors	CaS	Constitutive	Dorphin	N.D.	Biosynthetic turnover	(Huang et al., 2006)
Metabotropic glutamate Receptors	mGlu _{1a} , mGlu ₅	Constitutive	Siah1A	N.D.	Via a binding peptide motif (SID) in c-tail	(Moriyoshi et al., 2004)

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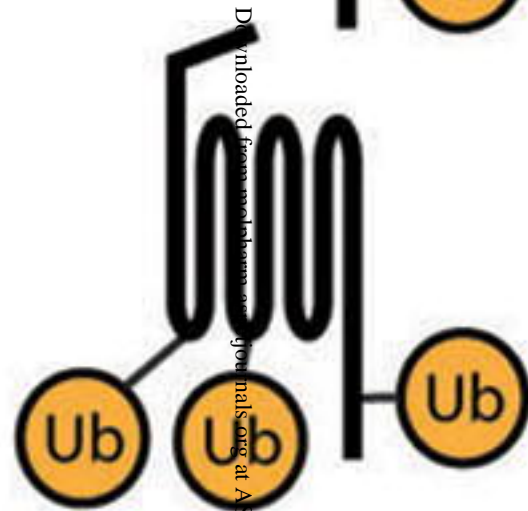
Table 1 legend.

Ubiquitinated GPCRs. A search of the literature for all known ubiquitinated mammalian Class A, B and C GPCRs was performed. This list includes the GPCR family name, the International Union of Basic and Clinical Pharmacology (IUPHAR) designated nomenclature, the nature of GPCR ubiquitination, including constitutive or agonist-activated, the ubiquitin E3 ligases and ubiquitin-specific proteases (USPs), the reported function and references. N.D. indicates not determined

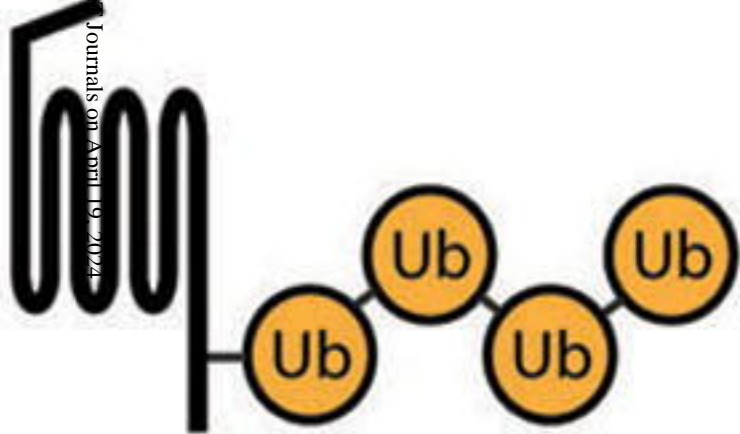
Monoubiquitin



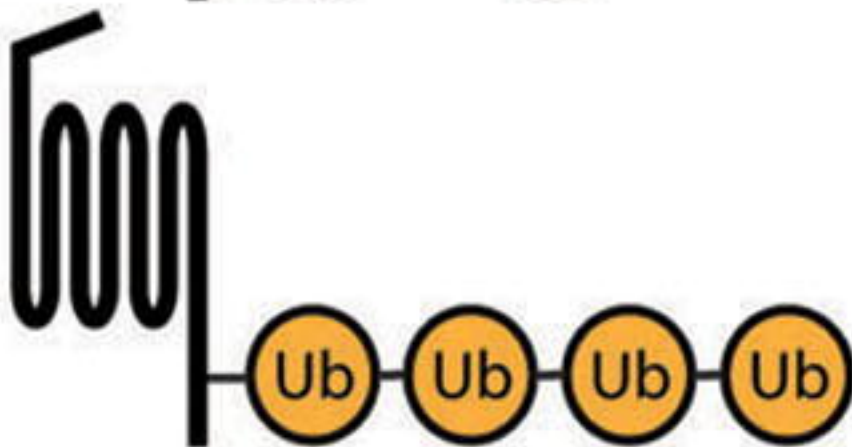
Multiple monoubiquitin



K48-linked polyubiquitin



K63-linked polyubiquitin

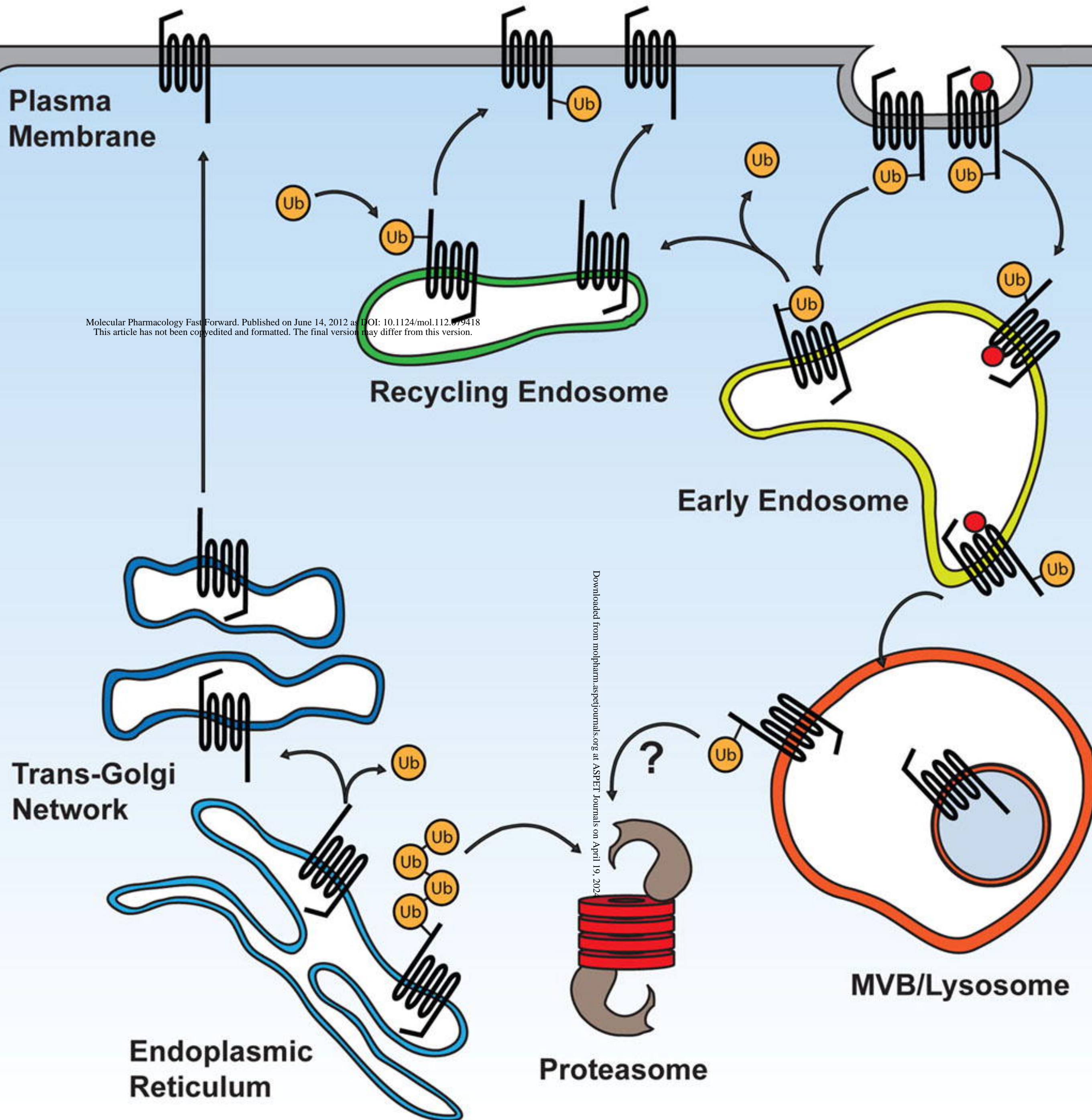


**Lysosomal sorting
Cell surface retention
Signaling?**

Proteasomal degradation

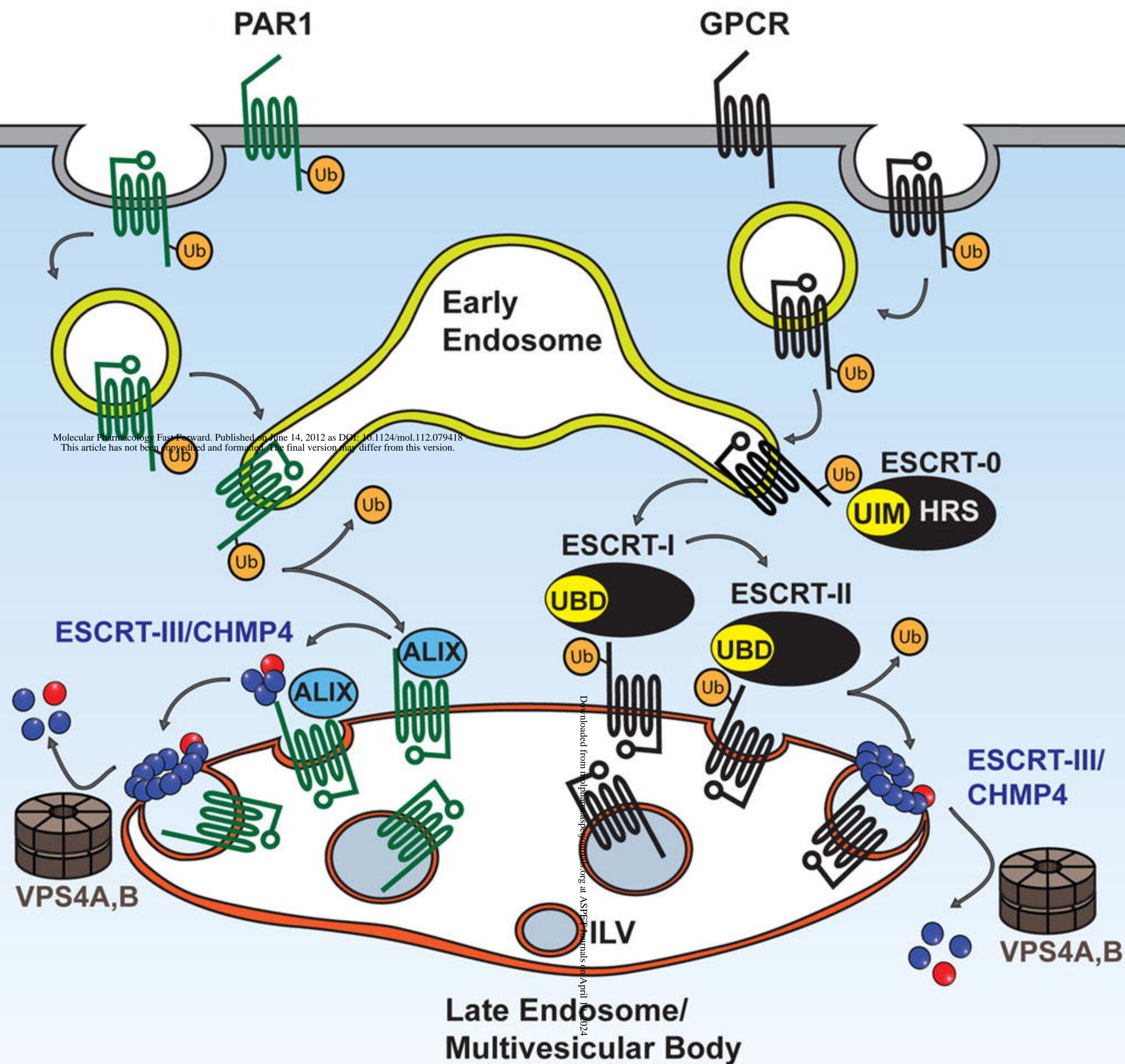
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