Developmental Cell
Review

Governance of Endocytic Trafficking and Signaling by Reversible Ubiquitylation

Michael J. Clague,[1,*] Han Liu,[1] and Sylvie Urbé[1,*]
[1]Physiological Laboratory, Institute for Translational Research, University of Liverpool, Crown Street, L69 3BX Liverpool, UK
*Correspondence: clague@liv.ac.uk (M.J.C.), urbe@liv.ac.uk (S.U.)
http://dx.doi.org/10.1016/j.devcel.2012.08.011

The endosomal pathway provides a major platform for ubiquitin-modifying enzymes, which act upon membrane-associated proteins in transit. Ubiquitylated cargo proteins are recognized by ubiquitin-binding domains inherent to key adaptor proteins at the plasma membrane and sorting endosome. A balance between ubiquitylation and deubiquitylation activities may govern the efficiency of recycling from endosomes to the plasma membrane versus lysosomal sorting through the multivesicular body pathway. We discuss the current knowledge of the properties of adaptors and ubiquitin-modifying proteins and their effects upon the trafficking and signaling of receptors and ligands associated with pathways fundamental to development.

Introduction

Ubiquitylation is a reversible protein modification with critical roles associated with each of the cell's major degradative pathways, which include proteasomes, lysosomes, and autophagolysosomes (Clague and Urbé, 2010). Plasma membrane proteins traverse the endocytic pathway en route to degradation in lysosomal compartments. This normally first entails encapsulation in clathrin-coated vesicles (CCVs), which are delivered to a tubulovesicular compartment referred to as the early or sorting endosome. From here cargo may be either recycled to the plasma membrane via tubular recycling endosomes or captured by small vesicles that bud from the limiting membrane into the endosomal lumen to create multivesicular bodies (MVBs). This step, which is generally considered the point of no return, is mediated by the endosomal complex required for transport (ESCRT) machinery (Henne et al., 2011; Hurley and Stenmark, 2011). Mature MVBs then deliver their content to lysosomes by undergoing direct fusion (Futter et al., 1996). Ubiquitylation can be used as an initial internalization signal from the plasma membrane, but it is more stringently required for inclusion into lumenal vesicles of the MVB. ESCRT proteins interpret ubiquitin as a sorting signal and promote the inward vesicular budding into the vacuolar core of the sorting endosome.

Ubiquitylated proteins are recognized by specific ubiquitin-binding domains, of which more than 20 types have been identified in the human genome, found within hundreds of proteins (Dikic et al., 2009). In the absence of a ubiquitin signal directing them toward MVBs, endocytosed proteins may recycle to the plasma membrane by simple bulk flow of membrane, though in some cases active sorting mechanisms can also operate. This recycling pathway is important for determining not just the abundance of channels and receptors at the plasma membrane, but also their spatial distribution, by the deposition of cargo at discrete sites on the plasma membrane (Palamidessi et al., 2008). In this review we shall consider the dynamics and multiple forms of ubiquitin associated with endosomes, the proteins that recognize them, and the enzymes that make and break them. We shall then discuss their influence upon key receptor-mediated signaling pathways, which play major roles in developmental biology.

Ubiquitin: Form and Function

Ubiquitin is a 76 amino acid polypeptide that is added onto primary amino groups of the acceptor protein through an enzymatic cascade involving sequential actions of E1, E2, and E3 conjugating proteins. It is the E3s that are primarily involved in substrate recognition, and, accordingly, these are more numerous than their E1 and E2 counterparts. They can be subdivided into the really interesting new gene (RING) family (~300 in the human genome) and the homologous to E6-associated protein carboxy terminus (HECT) family (28 in the human genome) (Li et al., 2008). Both types of E3 link E2 enzymes with substrates, but they differ in that RINGs do not themselves directly transfer ubiquitin, whereas the HECT family forms an intermediate thioester linkage with the ubiquitin C terminus. Although the RING-E2 interaction occurs at some distance from the E2 catalytic site, it is proposed to facilitate ubiquitin transfer from the E2 by an allosteric mechanism (Budhidarmo et al., 2012; Ozkan et al., 2005).

There are seven lysine residues intrinsic to ubiquitin itself, offering the opportunity to synthesize polyubiquitin chains of various topologies through specific isopeptide linkages, in addition to linear chains. In principal there are two advantages to polyubiquitin chains over monoubiquitylation: increased avidity for interaction partners due to multiple binding sites and increased potential for selectivity offered by the palette of chain linkage topologies. It is the E2 enzyme, in combination with an E3, that determines the species of ubiquitin chain that are made (David et al., 2011), and each linkage type, as well as monoubiquitylation, is substantively represented within the proteome of yeast and mammalian cells (Kim et al., 2011; Ziv et al., 2011). Appendage of a single noncleavable monoubiquitin to the C terminus of a protein can be sufficient to direct both internalization and MVB sorting (Reggiori and Pelham, 2001; Sigismund et al., 2005). However, an extensive body of literature now suggests that the efficacy of the internalization and MVB sorting steps can be increased by polyubiquitylation, commonly of the Lys63-linkage type.

Rigorous studies have compared endosomal trafficking in yeast strains expressing similar levels of wild-type or Lys63Arg

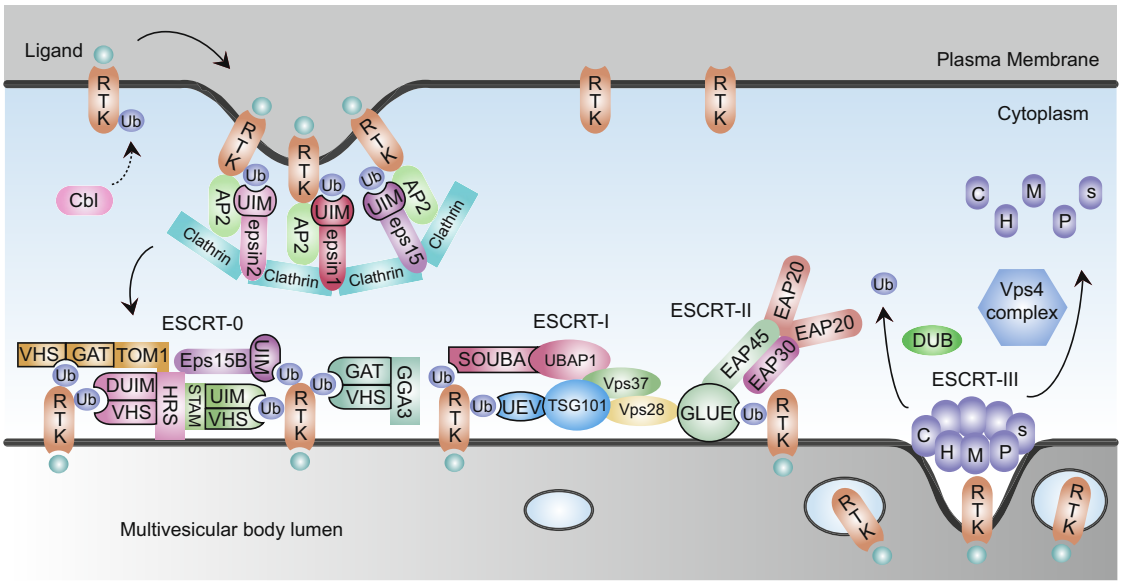

Figure 1. Receptor Tyrosine Kinase Sorting on the Endocytic Pathway Involves Interactions with Multiple Ubiquitin-Binding Domain Proteins
Upon ligand-dependent activation, RTKs are ubiquitylated by the E3 ligase c-CBL. UIM-containing proteins act as accessory linkers between receptors, the endocytic hub protein AP-2, and clathrin. At the sorting endosome, the ESCRT machinery captures ubiquitylated cargo through multiple interactions with proteins containing ubiquitin-binding domains. This leads to their inclusion into small vesicles that bud from the limiting membrane into the lumen, thereby creating MVBs destined to fuse with lysosomes. The first point of engagement is generally considered to be through the core components of the ESCRT-0 complex, HRS, and STAM, but other options indicated are available and discussed in the main text.

mutated ubiquitin. They show that, while Lys63-linked polyubiquitylation of cargo is not required for MVB formation per se, it is necessary for MVB sorting of both the plasma membrane-derived Gap1 permease and Carboxypeptidase S, which is routed from the biosynthetic pathway (Lauwers et al., 2009). Furthermore, Rsp5, the yeast E3 ubiquitin ligase that confers ubiquitylation upon a variety of plasma membrane proteins, exclusively generates Lys63-linked chains (Kee et al., 2006). Mass spectrometry and other studies using linkage-specific antibodies or Lys63Arg mutant forms of ubiquitin have also indicated that Lys63 linkages are predominant on several mammalian membrane proteins known to traffic to lysosomes, such as activated epidermal growth factor receptor (EGFR), TrkA, and dopamine transporters (Geetha et al., 2005; Huang et al., 2006; Vina-Vilaseca and Sorkin, 2010). MHC class I molecules are downregulated following the conjugation of Lys63 only or mixed Lys11/Lys63-linked chains, respectively, induced by the K3 and K5 ubiquitin ligases derived from Kaposi's sarcoma-associated herpesvirus (Boname et al., 2010; Duncan et al., 2006; Goto et al., 2010). In the case of the K3-ligase catalyzed reaction, UBC13, which specializes in making Lys63-linked chains, has been identified as a requisite E2 enzyme.

Despite some countervailing evidence (Saeki et al., 2009), it is believed that Lys63-linked ubiquitin chains are not usually involved in proteasomal degradation. They are the only linkage type that does not accumulate in the first few hours following proteasome inhibition, yet they preferentially accrue following perturbation of the endo/lysosomal pathway with an inhibitor of the v-ATPase proton pump (Dammer et al., 2011). An outstanding question is how such Lys63-linked chains are protected from proteasomal degradation, given that they are able to target proteins to the proteasome in vitro (Kim et al., 2007; Saeki et al., 2009). Our preferred possibility is that the high concentration of ubiquitin-binding domain-containing proteins associated with endosomal membranes ensures effective local competition with proteasomes for binding.

Ubiquitin-Binding Domains and Endocytic Trafficking

The best-characterized pathway for internalization from the plasma membrane involves CCVs, which are able to concentrate cargo proteins through a range of adaptor proteins linking to clathrin directly and to the hub protein, AP2 (Figure 1). Tandem ubiquitin-interaction motifs (UIM) are found in the Epsin 1 and 2, Eps15, and Eps15R CCV adaptors and in yeast epsin homologs Ent1p and Ent2p, while the yeast Eps15 homolog Ede1p bears a single ubiquitin-associated (UBA) domain (Traub and Lukacs, 2007). Their functional relevance has been assessed by genetic and siRNA approaches. For example, an Ent1p mutant lacking UIM domains, in contrast to the full-length protein, fails to complement a Ste2p internalization defect in *ent1Δent2Δede1Δ* yeast cells (Shih et al., 2002). Epsin contributes to the internalization of activated receptors such as the protease-activated receptor 1 (Par1) and the EGFR, while in *Drosophila melanogaster* the epsin homolog Liquid facets is required for internalization of the Notch-receptor ligand Delta (Chen et al., 2011; Kazazic et al., 2009; Overstreet et al., 2004). Structural and in vitro binding studies have suggested a preference of Epsin for Lys63-linked ubiquitin chains, due to the spacing and orientation between tandem ubiquitin-binding domains that is similar to a Lys63-specific functional ubiquitin-binding unit in the DNA repair protein RAP80 (Sato et al., 2009). Reports also suggest that in some instances ubiquitylated EGFR can be internalized via a non-clathrin-dependent route

that nevertheless requires interaction with the UIM domains of Eps15s and Epsin (Sigismund et al., 2005).

The ESCRT-0 complex is proposed to represent the first point of engagement of the MVB sorting machinery with ubiquitylated cargo (Henne et al., 2011; Hurley and Stenmark, 2011). The core complex is comprised of the constitutively associated components HRS and STAM (Vps27 and Hse1 in yeast), which together provide five ubiquitin-binding sites. HRS contributes two such sites by a double-sided ubiquitin-interaction motif (DUIM) (Hirano et al., 2006), while STAM contains a conventional UIM. Both proteins also contain N-terminal VHS domains that provide additional ubiquitin-binding sites (Hong et al., 2009; Ren and Hurley, 2010) (Figure 1). In-vitro-binding studies of ubiquitin to an intact ESCRT-0 complex revealed a modest selectivity for Lys63- over Lys48-linked chains, 3.7-fold for diubiquitin forms and 2.4-fold for tetraubiquitin chains. Mutational analysis in yeast has suggested that all of the ESCRT-0 ubiquitin-binding domains contribute to MVB sorting of a model cargo protein (Ren and Hurley, 2010).

Many of the proteins containing ubiquitin-binding domains that are described above are themselves monoubiquitylated, a process that has been termed coupled monoubiquitylation (Woelk et al., 2006). This affords them the opportunity to generate a network of interactions that may play a role in organizing the machinery and allows for regulatory mechanisms through which the attached ubiquitin can occupy the ubiquitin-binding site (Hoeller et al., 2006). Data obtained on yeast mutants of Ent UIMs and Ede1 UBA domain support this view, as they play roles in the internalization of both ubiquitylated and non-ubiquitylated receptors (Dores et al., 2010). However, it has been suggested that this is not essential for the yeast ESCRT machinery function in endocytic sorting, based on measurements of sorting following the elimination of ESCRT-0 ubiquitylation (Stringer and Piper, 2011).

Other components of the ESCRT machinery contain ubiquitin-binding domains. The ESCRT-I proteins TSG101 (Vps23 in yeast) and UBAP1 contain an N-terminal ubiquitin E2 variant (UEV) domain and a solenoid of overlapping UBAs (SOUBA) domain, respectively, while the ESCRT-II protein EAP45 (Vps36) contains a GRAM-like ubiquitin-binding in EAP45 (GLUE) domain (Agromayor et al., 2012; Hurley and Stenmark, 2011). The C terminus of the yeast ESCRT-I protein Mvb12 also binds to ubiquitin, but this protein has diverged significantly in humans and may be functionally replaced by UBAP1 (Shields et al., 2009; Stefani et al., 2011). At present, the precise choreography of these interactions is unclear. The nomenclature anchors the notion of sequential action of ESCRT complexes. However, mutational studies in yeast support a more cooperative mechanism, perhaps involving a supercomplex with numerous components contributing to ubiquitin recognition in a partially redundant manner (Shields et al., 2009). The later events of MVB budding are controlled by the ESCRT-III complex, which contains no known ubiquitin-binding domains, although it does recruit the endosomal deubiquitylating enzymes (DUBs) USP8 and AMSH (Clague and Urbé, 2006). Conceivably, at this point, cargo is committed to the pathway and a sorting signal is no longer required.

Another group of ubiquitin-binding proteins either act in concert with the HRS/STAM ESCRT-0 complex or provide a parallel link between ubiquitylated cargo and ESCRT-I in mammalian cells. TOM1, TOM1 like-1 (TOM1L1), and TOM1 like-2 (TOM1L2) constitute a small family of proteins with an N-terminal VHS domain followed by a GAT domain, which binds to ubiquitin. The complex between TOM1, Tollip, and Endofin could act as an alternative ESCRT-0. It possesses all of the salient features of the HRS/STAM complex, which include PtdIns*P*, clathrin, and TSG101 binding, as well as ubiquitin recognition (Urbé, 2005). TOM1L1 can be recruited to endosomes by direct binding to HRS through its VHS domain, and it similarly binds to TSG101 (Puertollano, 2005). However, TOM1L1 has also been proposed to transiently associate with activated EGFR at the plasma membrane before redistributing to endosomes along with an internalized receptor. siRNA-mediated depletion of TOM1L1 reduces the activated EGFR internalization rate, consistent with a proposed role as an accessory factor for its packaging into clathrin-coated vesicles (Liu et al., 2009). Another HRS binding protein, the Eps15 isoform Eps15B, contains tandem UIMs, localizes to endosomes, and seems to contribute to the efficiency of EGFR degradation (Roxrud et al., 2008). Golgi-localized, γ-ear-containing ARF-binding protein 3 (GGA3) possesses a GAT domain that, like the HRS DUIM, presents two binding sites for ubiquitin, as well as a VHS ubiquitin-binding domain (Kawasaki et al., 2005; Puertollano and Bonifacino, 2004; Ren and Hurley, 2010). Like HRS, it can bind to ubiquitylated EGFR and also to the ESCRT-I component TSG101. Depletion of GGA3 inhibits EGFR downregulation via the MVB pathway (Puertollano and Bonifacino, 2004) but enhances that of another receptor tyrosine kinase, c-Met (Parachoniak et al., 2011). Parachoniak et al. proposed an active role for GGA3 in promoting recycling of the Met receptor, in concert with the adaptor protein CrkA and the small GTPase Arf6, that is likely to be independent of ubiquitylation.

Rabex-5 is an exchange factor for the GTPase Rab5, widely regarded as a master regulator of early endosomal properties, including motility and membrane fusion capacity. Its catalytic domain is homologous to the yeast nucleotide exchange factor Vps9, which is responsible for activating the endosomal rab protein, Vps21. Rabex-5 also contains an A20 Zn-finger domain and an adjacent inverted UIM (MIU), both of which bind directly to distinct surfaces of ubiquitin (Lee et al., 2006; Penengo et al., 2006) and are critical for endosomal recruitment. In contrast, a monoubiquitylated form of Rabex-5 is enriched in the cytosol, leading to the suggestion that a cycle of ubiquitin-dependent recruitment, followed by monoubiquitylation-driven release, controls the dynamic association of Rabex-5 with endosomes (Mattera and Bonifacino, 2008). A physical association between Rabex-5 and ubiquitylated EGFR has been established by coimmunoprecipitation experiments, providing a potential mechanism to couple EGFR activation to Rab5 activation (Penengo et al., 2006). An analogous system may operate in yeast, whereby Vps9 is also recruited to endosomes and activated by virtue of ubiquitin interaction with its CUE domain (Carney et al., 2006; Davies et al., 2003; Donaldson et al., 2003). The ZnF-finger domain also confers E3 ubiquitin ligase activity upon Rabex-5, perhaps by recruiting ubiquitin-conjugated E2 enzymes (Mattera et al., 2006). One key substrate of Rabex-5-mediated ubiquitylation is Ras. In *Drosophila*, Rabex-5 serves to restrict Ras signaling in order to establish organism size,

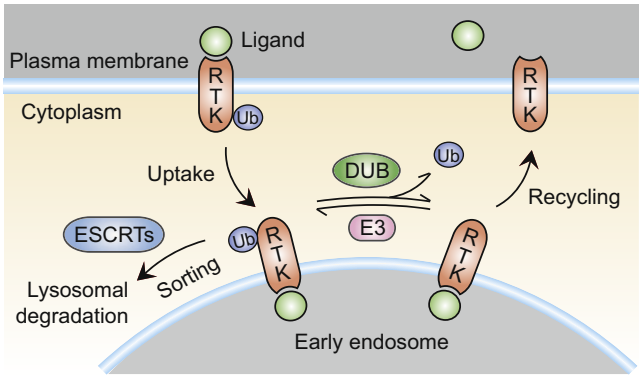

Figure 2. A Balance between Ubiquitylation and Deubiquitylation Determines the Fate of Endocytic Cargo
Proteins in transit at the sorting endosome are subject to competing ubiquitylation and deubiquitylation activities, the balance of which may determine the efficiency of selection by the ESCRT machinery for lysosomal degradation versus recycling to the plasma membrane.

wing vein pattern, and eye versus antennal fate. Overexpression of Rabex-5 can suppress the eye phenotypes of oncogenic Ras expression (Yan et al., 2010). This mechanism of Ras regulation is conserved in human cells as Rabex-5 ubiquitin ligase activity acts to restrict the activation of ERK by H-Ras (Xu et al., 2010).

Lysosomal Sorting Efficiency Is Determined by a Balance of Ubiquitin Ligase and Deubiquitylating Activities

In mammalian cells, the requirement for ubiquitin as a sorting signal is often more stringent at the MVB stage rather than at the plasma membrane. Because ubiquitin is a reversible post-translational modification, the efficiency of this sorting step reflects a balance between the relevant E3-ligase activities and that of DUBs (Figure 2). One attractive notion is that this provides for a kind of ''proofreading'' system that can selectively regulate the fate of ubiquitylated proteins at the endosome according to substrate identity or ubiquitin chain type, analogous to a similar system associated with the proteasome (Finley, 2009).

For many receptor tyrosine kinases (RTKs), including EGFR, c-Met, and PDGFR, the relevant E3-ligase activity is the proto-oncogene c-CBL, which is recruited via interactions with a specific pTyr residue on the receptor and with the receptor-associated molecule GRB2 (Joazeiro et al., 1999; Levkowitz et al., 1998; Waterman et al., 2002). In the case of EGFR, UBC4/5 has been suggested to provide the complementary E2-conjugating activity (Umebayashi et al., 2008). v-CBL acts in a dominant-negative fashion to inhibit RTK downregulation, and various oncogenic mutations in receptors are associated with loss of CBL binding, exhibiting similar defects in downregulation (Abella et al., 2005; Peschard and Park, 2003; Thien and Langdon, 2005). Thus, the RTK lysosomal pathway can be considered as a tumor-suppressor pathway (Mosesson et al., 2008). In the case of the large family of seven *trans*-membrane G protein-coupled receptors, the E3 palette appears to be more diverse (Hislop and von Zastrow, 2011). c-CBL has been shown to ubiquitylate and promote downregulation of human protease-activated receptor 2 (Jacob et al., 2005), but stimulus-dependent ubiquitylation of some other GPCRs is mediated by HECT family E3 ligases (Rotin and Kumar, 2009), such as Nedd4 for β2-adrenergic receptor (Nabhan et al., 2010; Shenoy et al., 2008) and AIP4 for CXCR4 receptors (Marchese et al., 2003).

In yeast, a family of Arrestin-like trafficking adaptors (ARTs) that harbor essential PY motifs were proposed to recruit the HECT E3 ligase Rsp5 to transporters in yeast cells following changes to the environmental conditions that require their removal from the plasma membrane (Lin et al., 2008; Nikko and Pelham, 2009). This results from ubiquitylation of both cargo proteins and the ARTs themselves. Human homologs of this family may also act as adaptors for Nedd4-like enzymes (Draheim et al., 2010; Nabhan et al., 2010). In general, this adaptor function is thought to be regulated by specific signaling cascades. For example, Art1 is negatively regulated by Npr1 kinase, which in turn is negatively regulated by TORC1 kinase, a critical sensor of a yeast cell's nutritional status (MacGurn et al., 2011). ARTs have also been proposed to bind directly to ESCRT components TSG101 (Vps23) and ALIX (Herrador et al., 2010; Rauch and Martin-Serrano, 2011), suggesting that this may provide a means to couple HECT E3 ligases to the endosomal sorting machinery.

Three DUBs are known to influence EGFR trafficking and degradation. AMSH is a metalloprotease DUB with high selectivity for Lys63-linked chains, while USP8 is highly active but shows minimal chain-type discrimination in vitro (Faesen et al., 2011; Komander et al., 2009; McCullough et al., 2006; Sato et al., 2008). Both compete for binding to the Src homology 3 (SH3) domain of the ESCRT-0 protein STAM and also bind to distinct complements of charged multivesicular body (CHMP) proteins that make up ESCRT-III, through their respective microtubule interacting and trafficking (MIT) domains (Clague and Urbé, 2006; McCullough et al., 2006; Row et al., 2007; Solomons et al., 2011). Based on siRNA-depletion experiments, AMSH is proposed to negatively regulate EGFR downregulation and consequently to enhance recycling of the receptor (Bowers et al., 2006; McCullough et al., 2004; Pareja et al., 2011). However, the exquisite selectivity of AMSH for Lys63-linked chains may preclude removal of the proximal ubiquitin moiety attached to a cargo molecule, leaving a monoubiquitin residue to either act as a weaker sorting signal or be subsequently removed by a less discriminatory DUB, such as USP8. The effects of USP8 depletion are more pleiotropic and may depend upon the extent of knockdown. They include inhibition of RTK degradation, clustering of tethered MVBs, and destabilization of ESCRT-0 components (Mizuno et al., 2006; Row et al., 2006). Conditional USP8 knockout mice show reduced levels of EGFR and ESCRT-0 components (Niendorf et al., 2007). AMSH and USP8, along with AMSH-like protein (AMSH-LP), represent the most clear-cut examples of endosomal DUBs based on a localization screen of more than 60 GFP-DUBS in HeLa cells (Urbé et al., 2012). However, others may influence ubiquitin dynamics at endosomes through transient interactions. A recent siRNA screen across the DUB family further identified Cezanne-1 as a negative regulator of EGFR degradation and showed that it interacts directly with the ubiquitylated receptor through its ubiquitin-binding domains (Pareja et al., 2011).

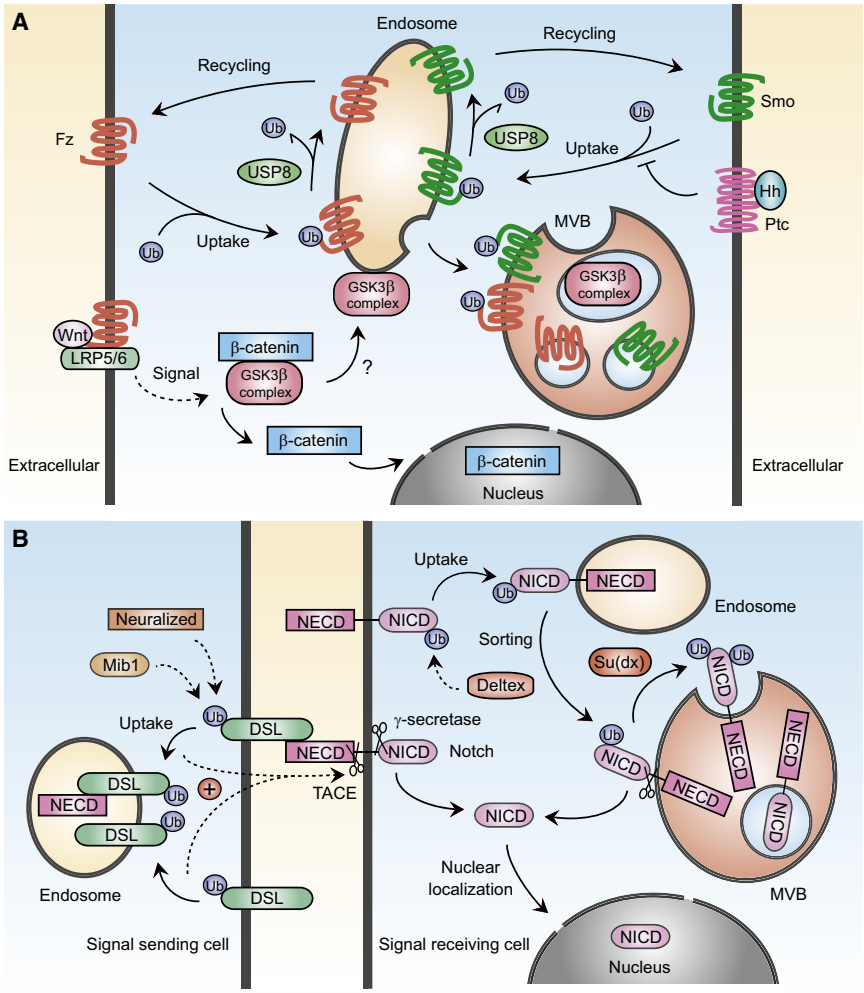

Figure 3. Models for the Governance of Wnt, Hedgehog, and Notch Signaling Pathways by Ubiquitin-Dependent Endocytosis

(A) The Frizzled (Fz) receptor for Wnt ligands undergoes constitutive endocytosis. Levels of surface receptor are regulated by the DUB enzyme USP8, which suppresses receptor ubiquitylation at the endosome, thereby ensuring the recycling of the receptor. The kinase GSK3β is a critical component of a protein complex (not shown), which suppresses β-catenin levels through a proteasomal degradative pathway. Wnt signaling inhibits the activity of this destruction complex, thereby enabling the nuclear accumulation of β-catenin. In the face of sustained Wnt signaling, GSK3β may be sequestered into MVBs through the ESCRT machinery, although a role for ubiquitylation has not been formally established. The seven-transmembrane receptor Smoothened (Smo) is a critical factor in the Hedgehog (Hh) signaling pathway that is also subject to a ubiquitylation-dependent endosomal trafficking itinerary. Hh signaling (through binding to its receptor Patched [Ptc]) acts in conjunction with USP8 activity at endosomes to promote the cell-surface accumulation of Smo by deflecting it from lysosomal degradation.

(B) The E3 ligases Mib1 and Neuralized ubiquitylate DSL ligands for Notch in signal-sending cells and promote their endocytosis. This is required for activity in inducing Notch cleavage by TACE/ADAM on the surface of signal-receiving cells to generate the Notch extracellular domain (NECD). This is then followed by γ-secretase-mediated cleavage to liberate the Notch intracellular domain (NICD) from the membrane that allows for its nuclear accumulation. NICD can also be generated in a ligand-independent manner. The E3 ligase Deltex is proposed to promote endocytosis of Notch through ubiquitylation, with the effect that endosomal localization renders Notch sensitive to cleavage. This modus can be opposed at the endosome by further ubiquitylating activity provided by Su(dx), which ensures efficient sequestration into MVBs before cleavage can occur. DUB activities also regulating this pathway are discussed in the main text.

The principal of negative regulation of lysosomal sorting by deubiquitylation described above has been extended to other DUBs and ubiquitylated cargos. For example, USP-46 negatively regulates the degradation of glutamate receptors in *Caenorhabditis elegans* ventral nerve chord (Kowalski et al., 2011). USP8 is proposed to negatively regulate degradation of Smoothened (Smo) and Frizzled receptor (Fz) associated with the Hedgehog (Hh) and Wingless (Wnt) signaling pathways, respectively (see below). The second principal of regulation through governance of endosomal protein stability, exemplified by USP8, has been extended to the vasopressin-induced DUB, USP10. It stabilizes Sorting Nexin 3 (SNX3), a positive regulator of endosomal recycling, and increases the amount of amiloride-sensitive epithelial sodium channels (ENaC) at the plasma membrane (Boulkroun et al., 2008). Finally, another important proposed role is to maintain ubiquitin homeostasis by recycling ubiquitin from receptors that have been committed to degradation at some point before full sequestration from the cytosol occurs. In yeast, this function is performed by Doa4, which may closely overlap in function with mammalian USP8 (Clague et al., 2012; Kimura et al., 2009).

Signaling Pathways Influenced by Ubiquitin-Dependent Endocytosis

The trafficking of receptors and associated signaling effectors through the endocytic pathway exposes them to a changing spectrum of substrates, while their inclusion into lumenal vesicles of MVBs terminates signaling through sequestration away from the cytosol. In addition, ubiquitylation may itself act as a signaling device through the dynamic assembly of protein interaction networks, much akin to phosphorylation (Grabbe et al., 2011). In this section, we will focus on key signaling pathways fundamental to developmental cell biology, which are regulated by ubiquitin-dependent sorting processes at the endosome.

Wnt promotes various morphogenic outputs depending on the local strength of the signal, which is determined by the levels of Fz receptor. In both mammalian cells and *Drosophila*, Fz undergoes continuous rounds of constitutive endocytosis and recycling (Figure 3A). This itinerary is associated with monoubiquitylation, which is reversed by the endosomal DUB, USP8/UBPY (dUBPY in *Drosophila*). In the absence of this activity, the receptor is diverted to the MVB/lysosomal pathway, and

plasma membrane levels decline. Consequently, dUBPY is required for sensory bristle formation in the *Drosophila* wing (Mukai et al., 2010). Many other factors associated with this pathway are also regulated by reversible ubiquitylation but are most likely independent of endosomes (Tauriello and Maurice, 2010). However, recent data has suggested that inhibition of GSK3β, which is required for Wnt signaling, is achieved through sequestration into MVBs. Although this process requires the ESCRT-0 component HRS, the means by which GSK3β is targeted to endosomes is presently unclear (Metcalfe and Bienz, 2011; Taelman et al., 2010). The seven-transmembrane receptor Smo is also subject to ubiquitin-mediated lysosomal degradation. Hedgehog binding to Patched (Ptc) increases the levels of Smo at the plasma membrane by inhibiting its ubiquitylation. Similar to the situation described above for Fz, gains and loss of USP8 lead to reciprocal changes in Smo levels at the cell surface (Li et al., 2012; Xia et al., 2012) (Figure 3A).

TGF-β and other related cytokines regulate a diverse set of developmental and homeostatic functions through heterotetrameric complexes of type I and type II serine/threonine kinase receptors. The principal substrates of activated receptors are the SMAD transcription factors. The turnover of TGFβ type I receptor (TβRI) via lysosomal degradation is promoted by ubiquitylation effected by the Smad7-dependent recruitment of the related HECT family E3 ligases SMURF1, SMURF2, or NEDD4-2 (Ebisawa et al., 2001; Kavsak et al., 2000; Kuratomi et al., 2005). Two closely related DUBs, USP4 and USP15, have recently been proposed to positively regulate signaling output through extension of TβRI half-life, augmenting an earlier report invoking similar activity for UCH37 (Eichhorn et al., 2012; Wicks et al., 2005; Zhang et al., 2012). Both USP4 and USP15 are proposed to deubiquitylate the receptor, through direct interaction in the case of USP4, but requiring Smad7 for recruitment of USP15. Smad7 can engage in simultaneous interaction with countervailing E3-ligase (SMURF2) and DUB (USP15) activities. This association of USP15 declines with increasing concentration of TGF-β stimulation, promoting receptor degradation. This provides a rheostat-like feature that limits hyperactivation of the pathway (Eichhorn et al., 2012). One further link between this pathway and endosomes concerns the parallel findings that the endosomal DUBs AMSH and AMSH-LP positively regulate the related bone morphogenic protein (BMP) and TGFβ signaling pathways, respectively (Ibarrola et al., 2004; Itoh et al., 2001). These both interact with various SMAD family members, but their molecular mechanisms of action have not been further characterized since these initial studies, which predated the realization of their DUB activity.

Notch is a single-pass transmembrane receptor that is activated through association with membrane-bound ligands on neighboring cells to provide contextual information influencing cell differentiation pathways. These ligands belong to the DSL family and are subdivided into the Delta (Dl) or Serrate (Ser) classes in higher metazoans. Two RING E3 ligases, Neuralized and Mind-Bomb 1 (Mib1), have been shown to ubiquitylate and activate DSL proteins, and there is a notable correlation between ligase expression, DSL internalization, and signaling capacity (Daskalaki et al., 2011) (Figure 3B). Furthermore, the ubiquitin-binding endocytic adaptor protein Epsin is also required in Notch-signal-sending cells in a variety of organisms. The reason for the requirement for endocytosis in signal-sending cells is still not completely resolved beyond dispute. However, one compelling idea is that the mechanical force placed on Notch by endocytosis of its ligand into a neighboring cell promotes a conformation that is susceptible to an activating proteolytic cleavage adjacent to the extracellular face of the plasma membrane by TACE/ADAM metalloproteases (Weinmaster and Fischer, 2011). This then enables a second cleavage of Notch by γ-secretase to release its intracellular domain (NICD).

A number of studies have suggested that endocytosis of Notch itself contributes to activation, but there are many uncertainties. A key player in some contexts is the RING E3 ligase Deltex, expressed in Notch-signal-receiving cells, which promotes Notch ubiquitylation (Baron, 2012; Vaccari et al., 2008). In *Drosophila*, overexpression of Deltex leads to Notch activation and accumulation in late endosomes, but, critically, this mode of signaling is independent of DSL ligand activation and probably generates the NICD by a different proteolytic cleavage mechanism (Hori et al., 2004). Coexpression of the HECT E3-ligase family protein Suppressor of Deltex, Su(dx) (AIP4/ITCH in mammalian cells), opposes this activation mechanism by shifting the distribution of Notch from the limiting membrane of MVBs to lumenal vesicles such that NICD cannot be released into the cytosol (Chastagner et al., 2008; Hori et al., 2011) (Figure 3B). Intriguingly, one further role of Deltex established in mammalian cells is to recruit a DUB enzyme EIF3F, more commonly recognized as a translation initiation factor and hitherto presumed to be an inactive member of the JAMM family of metalloprotease DUBs. EIF3F and another DUB, USP12 in complex with its activator UAF1, have been proposed to act as positive and negative regulators of Notch signaling by suppressing the ubiquitylation of ligand activated and constitutively endocytosed Notch, respectively (Moretti et al., 2010, 2012). Thus, a complex interplay between various ubiquitin-modifying enzymes is likely to govern the positioning of both ligand-activated and constitutively trafficking Notch within the endocytic pathway, providing the capacity for fine control of the signaling strength.

Control of Receptor Trafficking through Regulated Transcription of E3s and DUBs

The plasma membrane levels of channels and receptors are often rapidly regulated by alterations to their trafficking itinerary, in which ubiquitin dynamics play a central role. However, cells sometimes respond to prevailing conditions over a slower timescale through transcriptional mechanisms. Interesting examples of transcriptional regulation of a RING E3 and of DUBs have been shown to influence endocytic trafficking and impact on key processes essential to wellbeing.

The cellular response to elevated cholesterol is transcriptionally regulated through ER retention of sterol response element-binding protein (SREBP) transcription factor precursor, leading to reduced transcription of genes involved in cholesterol synthesis and uptake (low-density lipoprotein receptors, LDLRs). In addition, the sterol responsive nuclear liver X receptor (LXR) promotes cholesterol efflux and inhibits LDLR-mediated uptake. LXR upregulates the expression of the RING E3 ligase Inducible degrader of the LDLR (IDOL) (Figure 4A). The FERM domain (F-4.1 protein, Ezrin, Radixin, Moesin) of IDOL binds

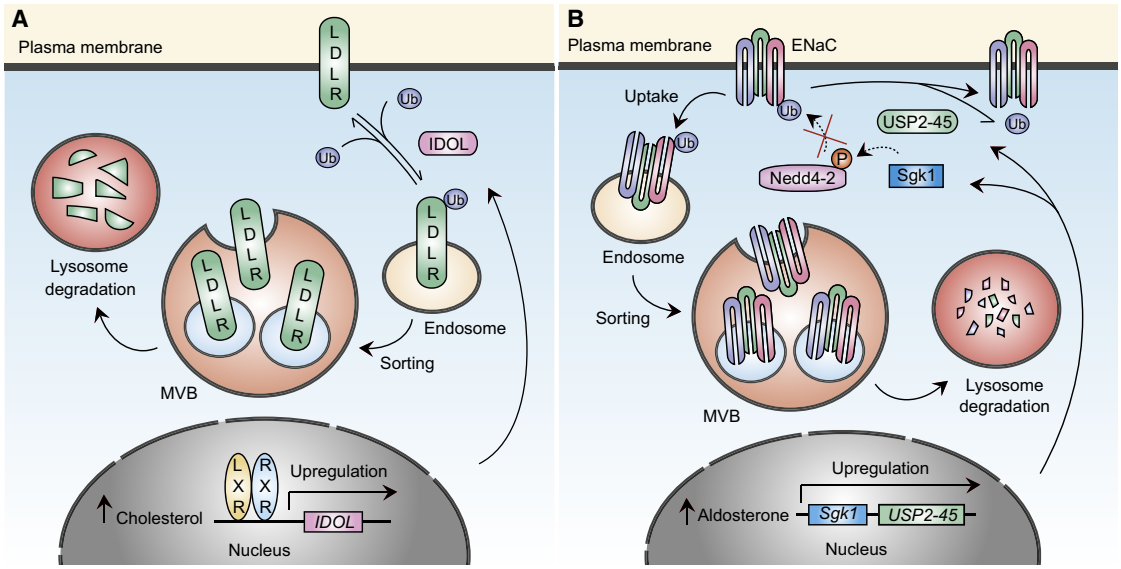

Figure 4. Models for Regulation of Endocytic Trafficking through Transcriptional Control of Ubiquitin-Modifying Enzymes

(A) The LDL receptor (LDLR) takes up cholesterol in the form of LDL particles, which dissociate from the receptor at a low pH in the sorting endosome. LDL particles are transported to lysosomes for processing, while the receptor constitutively recycles. However, under conditions of excess cholesterol, the sterol-responsive liver X receptor (LXR) combines with the retinoid X receptor (RXR) to drive transcription of the E3 ligase IDOL, which specifically ubiquitylates LDLR, leading to its downregulation through lysosomal degradation.

(B) The ubiquitylation-dependent trafficking itinerary of the ENaC sodium channel determines surface activity. In response to the blood-pressure-regulated hormone aldosterone, ENaC surface levels are increased by suppression of ubiquitylation. This is accomplished through the combined effects of transcriptional upregulation of both an isoform of the DUB USP2 (USP2-45) and the kinase Sgk1, which inhibits the E3 ligase Nedd4-2 by phosphorylation.

directly to LDLR, promoting its ubiquitylation and lysosomal degradation through the conjugation of Lys63-linked ubiquitin chains, leading to reduced receptor levels (Sorrentino et al., 2011; Zelcer et al., 2009). IDOL preferentially associates with the UBE2D family of E2 ubiquitin-conjugating enzymes, for which expression of a dominant-negative form inhibits LDLR degradation (Zhang et al., 2011).

The ENaC channel plays a fundamental role in salt and fluid homeostasis. It is usually a short-lived protein that is turned over by ubiquitin- and ESCRT-dependent lysosomal degradation (Staub et al., 1997; Zhou et al., 2010). The relevant E3 ligase is Nedd4-2, and defects in this interaction give rise to an inherited hypertensive condition known as Liddle syndrome. This process can be reversed by deubiquitylation. Two DUBs have been implicated in governing ENaC levels, which are under transcriptional control by established hormonal regulators of blood pressure (Figure 4B). Aldosterone and vasopressin induce expression of USP2-45 and USP10, respectively. Both enhance plasma membrane ENaC levels but have different modes of action. As discussed earlier, USP10 stabilizes the regulator of trafficking SNX3 (Boulkroun et al., 2008), while USP2-45 interacts with and deubiquitylates the channel directly (Fakitsas et al., 2007). A second layer of control is proposed to be provided by the aldosterone-induced Sgk1 kinase that interacts with and phosphorylates Nedd4-2, leading to reduced interaction with ENaC (Debonneville et al., 2001).

Conclusions and Outlook

Endocytosis, and its intimate connection with reversible ubiquitylation, pervades nearly all aspects of cellular regulation. It provides the major mode of protein turnover for plasma membrane proteins but also coordinates spatial distribution and signaling outputs. One emerging aspect is the crosstalk with other protein degradation pathways provided by the proteasome and autophagy routes, which goes beyond a shared reliance upon ubiquitylation (Clague and Urbé, 2010). Proteasomes are found to be associated with endosomes, and their function is required for the lysosomal trafficking of certain receptors such as growth hormone receptor, TrkA, and Met receptor (Carter et al., 2004; Geetha and Wooten, 2008; Gorbea et al., 2010; van Kerkhof et al., 2000). The abundant p97/valosin-containing protein that controls many aspects of cellular proteostasis, including the ERAD pathway, has recently been associated with endosomal functions (Meyer et al., 2012). For example, binding of p97 to Lys48-modified mannose receptor in dendritic cells is required for translocation of antigens from the endosomal lumen, a critical step for cross-presentation by MHC class I molecules (Zehner et al., 2011). p97 has a variety of cofactor partners, many of which contain UBX or UBX-like domains. When complexed with UBXD1, p97 binds to the plasma membrane and endosome-associated protein, caveolin, and is implicated in its trafficking to MVBs, as well as having more general effects on the MVB pathway. Interestingly, mutations in p97 associated with the late-onset degenerative disorder inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia (IBMPFD) compromise the interaction with caveolin. Individuals with IBMPFD with corresponding mutations show deviations in the distribution of caveolin isoforms from control subjects that may underpin clinically observed symptoms (Ritz et al., 2011).

Regulation of signal strength plays a critical role in the development of multicellular organisms. The balance between

endosomal ubiquitylation and deubiquitylation governing receptor number and activity is emerging as a common device for fine-tuning of key signaling pathways. Certain regulators, such as c-Cbl, Nedd4, and USP8, are used in this context by multiple signaling systems, but many more specific partnerships remain to be discovered.

REFERENCES

Abella, J.V., Peschard, P., Naujokas, M.A., Lin, T., Saucier, C., Urbé, S., and Park, M. (2005). Met/Hepatocyte growth factor receptor ubiquitination suppresses transformation and is required for Hrs phosphorylation. Mol. Cell. Biol. *25*, 9632–9645.

Agromayor, M., Soler, N., Caballe, A., Kueck, T., Freund, S.M., Allen, M.D., Bycroft, M., Perisic, O., Ye, Y., McDonald, B., et al. (2012). The UBAP1 subunit of ESCRT-I interacts with ubiquitin via a SOUBA domain. Structure *20*, 414–428.

Baron, M. (2012). Endocytic routes to Notch activation. Semin. Cell Dev. Biol. *23*, 437–442.

Boname, J.M., Thomas, M., Stagg, H.R., Xu, P., Peng, J., and Lehner, P.J. (2010). Efficient internalization of MHC I requires lysine-11 and lysine-63 mixed linkage polyubiquitin chains. Traffic *11*, 210–220.

Boulkroun, S., Ruffieux-Daidié, D., Vitagliano, J.J., Poirot, O., Charles, R.P., Lagnaz, D., Firsov, D., Kellenberger, S., and Staub, O. (2008). Vasopressin-inducible ubiquitin-specific protease 10 increases ENaC cell surface expression by deubiquitylating and stabilizing sorting nexin 3. Am. J. Physiol. Renal Physiol. *295*, F889–F900.

Bowers, K., Piper, S.C., Edeling, M.A., Gray, S.R., Owen, D.J., Lehner, P.J., and Luzio, J.P. (2006). Degradation of endocytosed epidermal growth factor and virally ubiquitinated major histocompatibility complex class I is independent of mammalian ESCRTII. J. Biol. Chem. *281*, 5094–5105.

Budhidarmo, R., Nakatani, Y., and Day, C.L. (2012). RINGs hold the key to ubiquitin transfer. Trends Biochem. Sci. *37*, 58–65.

Carney, D.S., Davies, B.A., and Horazdovsky, B.F. (2006). Vps9 domain-containing proteins: activators of Rab5 GTPases from yeast to neurons. Trends Cell Biol. *16*, 27–35.

Carter, S., Urbé, S., and Clague, M.J. (2004). The met receptor degradation pathway: requirement for Lys48-linked polyubiquitin independent of proteasome activity. J. Biol. Chem. *279*, 52835–52839.

Chastagner, P., Israël, A., and Brou, C. (2008). AIP4/Itch regulates Notch receptor degradation in the absence of ligand. PLoS ONE *3*, e2735.

Chen, B., Dores, M.R., Grimsey, N., Canto, I., Barker, B.L., and Trejo, J. (2011). 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. *286*, 40760–40770.

Clague, M.J., and Urbé, S. (2006). Endocytosis: the DUB version. Trends Cell Biol. *16*, 551–559.

Clague, M.J., and Urbé, S. (2010). Ubiquitin: same molecule, different degradation pathways. Cell *143*, 682–685.

Clague, M.J., Coulson, J.M., and Urbé, S. (2012). Cellular functions of the DUBs. J. Cell Sci. *125*, 277–286.

Dammer, E.B., Na, C.H., Xu, P., Seyfried, N.T., Duong, D.M., Cheng, D., Gearing, M., Rees, H., Lah, J.J., Levey, A.I., et al. (2011). Polyubiquitin linkage profiles in three models of proteolytic stress suggest the etiology of Alzheimer disease. J. Biol. Chem. *286*, 10457–10465.

Daskalaki, A., Shalaby, N.A., Kux, K., Tsoumpekos, G., Tsibidis, G.D., Muskavitch, M.A., and Delidakis, C. (2011). Distinct intracellular motifs of Delta mediate its ubiquitylation and activation by Mindbomb1 and Neuralized. J. Cell Biol. *195*, 1017–1031.

David, Y., Ternette, N., Edelmann, M.J., Ziv, T., Gayer, B., Sertchook, R., Dadon, Y., Kessler, B.M., and Navon, A. (2011). E3 ligases determine ubiquitination site and conjugate type by enforcing specificity on E2 enzymes. J. Biol. Chem. *286*, 44104–44115.

Davies, B.A., Topp, J.D., Sfeir, A.J., Katzmann, D.J., Carney, D.S., Tall, G.G., Friedberg, A.S., Deng, L., Chen, Z., and Horazdovsky, B.F. (2003). Vps9p CUE domain ubiquitin binding is required for efficient endocytic protein traffic. J. Biol. Chem. *278*, 19826–19833.

Debonneville, C., Flores, S.Y., Kamynina, E., Plant, P.J., Tauxe, C., Thomas, M.A., Münster, C., Chraïbi, A., Pratt, J.H., Horisberger, J.D., et al. (2001). Phosphorylation of Nedd4-2 by Sgk1 regulates epithelial Na(+) channel cell surface expression. EMBO J. *20*, 7052–7059.

Dikic, I., Wakatsuki, S., and Walters, K.J. (2009). Ubiquitin-binding domains - from structures to functions. Nat. Rev. Mol. Cell Biol. *10*, 659–671.

Donaldson, K.M., Yin, H., Gekakis, N., Supek, F., and Joazeiro, C.A. (2003). Ubiquitin signals protein trafficking via interaction with a novel ubiquitin binding domain in the membrane fusion regulator, Vps9p. Curr. Biol. *13*, 258–262.

Dores, M.R., Schnell, J.D., Maldonado-Baez, L., Wendland, B., and Hicke, L. (2010). The function of yeast epsin and Ede1 ubiquitin-binding domains during receptor internalization. Traffic *11*, 151–160.

Draheim, K.M., Chen, H.B., Tao, Q., Moore, N., Roche, M., and Lyle, S. (2010). ARRDC3 suppresses breast cancer progression by negatively regulating integrin beta4. Oncogene *29*, 5032–5047.

Duncan, L.M., Piper, S., Dodd, R.B., Saville, M.K., Sanderson, C.M., Luzio, J.P., and Lehner, P.J. (2006). Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. EMBO J. *25*, 1635–1645.

Ebisawa, T., Fukuchi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T., and Miyazono, K. (2001). Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. J. Biol. Chem. *276*, 12477–12480.

Eichhorn, P.J., Rodón, L., Gonzàlez-Juncà, A., Dirac, A., Gili, M., Martínez-Sáez, E., Aura, C., Barba, I., Peg, V., Prat, A., et al. (2012). USP15 stabilizes TGF-β receptor I and promotes oncogenesis through the activation of TGF-β signaling in glioblastoma. Nat. Med. *18*, 429–435.

Faesen, A.C., Luna-Vargas, M.P.A., Geurink, P.P., Clerici, M., Merkx, R., van Dijk, W.J., Hameed, D.S., El Oualid, F., Ovaa, H., and Sixma, T.K. (2011). The differential modulation of USP activity by internal regulatory domains, interactors and eight ubiquitin chain types. Chem. Biol. *18*, 1550–1561.

Fakitsas, P., Adam, G., Daidié, D., van Bemmelen, M.X., Fouladkou, F., Patrignani, A., Wagner, U., Warth, R., Camargo, S.M., Staub, O., and Verrey, F. (2007). Early aldosterone-induced gene product regulates the epithelial sodium channel by deubiquitylation. J. Am. Soc. Nephrol. *18*, 1084–1092.

Finley, D. (2009). Recognition and processing of ubiquitin-protein conjugates by the proteasome. Annu. Rev. Biochem. *78*, 477–513.

Futter, C.E., Pearse, A., Hewlett, L.J., and Hopkins, C.R. (1996). Multivesicular endosomes containing internalized EGF-EGF receptor complexes mature and then fuse directly with lysosomes. J. Cell Biol. *132*, 1011–1023.

Geetha, T., and Wooten, M.W. (2008). TrkA receptor endolysosomal degradation is both ubiquitin and proteasome dependent. Traffic *9*, 1146–1156.

Geetha, T., Jiang, J., and Wooten, M.W. (2005). Lysine 63 polyubiquitination of the nerve growth factor receptor TrkA directs internalization and signaling. Mol. Cell *20*, 301–312.

Gorbea, C., Pratt, G., Ustrell, V., Bell, R., Sahasrabudhe, S., Hughes, R.E., and Rechsteiner, M. (2010). A protein interaction network for Ecm29 links the 26 S proteasome to molecular motors and endosomal components. J. Biol. Chem. *285*, 31616–31633.

Goto, E., Yamanaka, Y., Ishikawa, A., Aoki-Kawasumi, M., Mito-Yoshida, M., Ohmura-Hoshino, M., Matsuki, Y., Kajikawa, M., Hirano, H., and Ishido, S. (2010). Contribution of lysine 11-linked ubiquitination to MIR2-mediated major histocompatibility complex class I internalization. J. Biol. Chem. *285*, 35311–35319.

Grabbe, C., Husnjak, K., and Dikic, I. (2011). The spatial and temporal organization of ubiquitin networks. Nat. Rev. Mol. Cell Biol. *12*, 295–307.

Henne, W.M., Buchkovich, N.J., and Emr, S.D. (2011). The ESCRT pathway. Dev. Cell *21*, 77–91.

Herrador, A., Herranz, S., Lara, D., and Vincent, O. (2010). Recruitment of the ESCRT machinery to a putative seven-transmembrane-domain receptor is mediated by an arrestin-related protein. Mol. Cell. Biol. *30*, 897–907.

Hirano, S., Kawasaki, M., Ura, H., Kato, R., Raiborg, C., Stenmark, H., and Wakatsuki, S. (2006). Double-sided ubiquitin binding of Hrs-UIM in endosomal protein sorting. Nat. Struct. Mol. Biol. *13*, 272–277.

Hislop, J.N., and von Zastrow, M. (2011). Role of ubiquitination in endocytic trafficking of G-protein-coupled receptors. Traffic *12*, 137–148.

Hoeller, D., Crosetto, N., Blagoev, B., Raiborg, C., Tikkanen, R., Wagner, S., Kowanetz, K., Breitling, R., Mann, M., Stenmark, H., and Dikic, I. (2006). Regulation of ubiquitin-binding proteins by monoubiquitination. Nat. Cell Biol. *8*, 163–169.

Hong, Y.H., Ahn, H.C., Lim, J., Kim, H.M., Ji, H.Y., Lee, S., Kim, J.H., Park, E.Y., Song, H.K., and Lee, B.J. (2009). Identification of a novel ubiquitin binding site of STAM1 VHS domain by NMR spectroscopy. FEBS Lett. *583*, 287–292.

Hori, K., Fostier, M., Ito, M., Fuwa, T.J., Go, M.J., Okano, H., Baron, M., and Matsuno, K. (2004). Drosophila deltex mediates suppressor of Hairless-independent and late-endosomal activation of Notch signaling. Development *131*, 5527–5537.

Hori, K., Sen, A., Kirchhausen, T., and Artavanis-Tsakonas, S. (2011). Synergy between the ESCRT-III complex and Deltex defines a ligand-independent Notch signal. J. Cell Biol. *195*, 1005–1015.

Huang, F., Kirkpatrick, D., Jiang, X., Gygi, S., and Sorkin, A. (2006). Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain. Mol. Cell *21*, 737–748.

Hurley, J.H., and Stenmark, H. (2011). Molecular mechanisms of ubiquitin-dependent membrane traffic. Annu Rev Biophys *40*, 119–142.

Ibarrola, N., Kratchmarova, I., Nakajima, D., Schiemann, W.P., Moustakas, A., Pandey, A., and Mann, M. (2004). Cloning of a novel signaling molecule, AMSH-2, that potentiates transforming growth factor beta signaling. BMC Cell Biol. *5*, 2.

Itoh, F., Asao, H., Sugamura, K., Heldin, C.H., ten Dijke, P., and Itoh, S. (2001). Promoting bone morphogenetic protein signaling through negative regulation of inhibitory Smads. EMBO J. *20*, 4132–4142.

Jacob, C., Cottrell, G.S., Gehringer, D., Schmidlin, F., Grady, E.F., and Bunnett, N.W. (2005). c-Cbl mediates ubiquitination, degradation, and down-regulation of human protease-activated receptor 2. J. Biol. Chem. *280*, 16076–16087.

Joazeiro, C.A., Wing, S.S., Huang, H., Leverson, J.D., Hunter, T., and Liu, Y.C. (1999). The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. Science *286*, 309–312.

Kavsak, P., Rasmussen, R.K., Causing, C.G., Bonni, S., Zhu, H., Thomsen, G.H., and Wrana, J.L. (2000). Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. Mol. Cell *6*, 1365–1375.

Kawasaki, M., Shiba, T., Shiba, Y., Yamaguchi, Y., Matsugaki, N., Igarashi, N., Suzuki, M., Kato, R., Kato, K., Nakayama, K., and Wakatsuki, S. (2005). Molecular mechanism of ubiquitin recognition by GGA3 GAT domain. Genes Cells *10*, 639–654.

Kazazic, M., Bertelsen, V., Pedersen, K.W., Vuong, T.T., Grandal, M.V., Rødland, M.S., Traub, L.M., Stang, E., and Madshus, I.H. (2009). Epsin 1 is involved in recruitment of ubiquitinated EGF receptors into clathrin-coated pits. Traffic *10*, 235–245.

Kee, Y., Muñoz, W., Lyon, N., and Huibregtse, J.M. (2006). The deubiquitinating enzyme Ubp2 modulates Rsp5-dependent Lys63-linked polyubiquitin conjugates in Saccharomyces cerevisiae. J. Biol. Chem. *281*, 36724–36731.

Kim, H.T., Kim, K.P., Lledias, F., Kisselev, A.F., Scaglione, K.M., Skowyra, D., Gygi, S.P., and Goldberg, A.L. (2007). Certain pairs of ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s) synthesize nondegradable forked ubiquitin chains containing all possible isopeptide linkages. J. Biol. Chem. *282*, 17375–17386.

Kim, W., Bennett, E.J., Huttlin, E.L., Guo, A., Li, J., Possemato, A., Sowa, M.E., Rad, R., Rush, J., Comb, M.J., et al. (2011). Systematic and quantitative assessment of the ubiquitin-modified proteome. Mol. Cell *44*, 325–340.

Kimura, Y., Yashiroda, H., Kudo, T., Koitabashi, S., Murata, S., Kakizuka, A., and Tanaka, K. (2009). An inhibitor of a deubiquitinating enzyme regulates ubiquitin homeostasis. Cell *137*, 549–559.

Komander, D., Reyes-Turcu, F., Licchesi, J.D.F., Odenwaelder, P., Wilkinson, K.D., and Barford, D. (2009). Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains. EMBO Rep. *10*, 466–473.

Kowalski, J.R., Dahlberg, C.L., and Juo, P. (2011). The deubiquitinating enzyme USP-46 negatively regulates the degradation of glutamate receptors to control their abundance in the ventral nerve cord of Caenorhabditis elegans. J. Neurosci. *31*, 1341–1354.

Kuratomi, G., Komuro, A., Goto, K., Shinozaki, M., Miyazawa, K., Miyazono, K., and Imamura, T. (2005). NEDD4-2 (neural precursor cell expressed, developmentally down-regulated 4-2) negatively regulates TGF-beta (transforming growth factor-beta) signalling by inducing ubiquitin-mediated degradation of Smad2 and TGF-beta type I receptor. Biochem. J. *386*, 461–470.

Lauwers, E., Jacob, C., and André, B. (2009). K63-linked ubiquitin chains as a specific signal for protein sorting into the multivesicular body pathway. J. Cell Biol. *185*, 493–502.

Lee, S., Tsai, Y.C., Mattera, R., Smith, W.J., Kostelansky, M.S., Weissman, A.M., Bonifacino, J.S., and Hurley, J.H. (2006). Structural basis for ubiquitin recognition and autoubiquitination by Rabex-5. Nat. Struct. Mol. Biol. *13*, 264–271.

Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W.Y., Beguinot, L., Geiger, B., and Yarden, Y. (1998). c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. Genes Dev. *12*, 3663–3674.

Li, W., Bengtson, M.H., Ulbrich, A., Matsuda, A., Reddy, V.A., Orth, A., Chanda, S.K., Batalov, S., and Joazeiro, C.A. (2008). 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 *3*, e1487.

Li, S., Chen, Y., Shi, Q., Yue, T., Wang, B., and Jiang, J. (2012). Hedgehog-regulated ubiquitination controls smoothened trafficking and cell surface expression in Drosophila. PLoS Biol. *10*, e1001239.

Lin, C.H., MacGurn, J.A., Chu, T., Stefan, C.J., and Emr, S.D. (2008). Arrestin-related ubiquitin-ligase adaptors regulate endocytosis and protein turnover at the cell surface. Cell *135*, 714–725.

Liu, N.S., Loo, L.S., Loh, E., Seet, L.F., and Hong, W. (2009). Participation of Tom1L1 in EGF-stimulated endocytosis of EGF receptor. EMBO J. *28*, 3485–3499.

MacGurn, J.A., Hsu, P.C., Smolka, M.B., and Emr, S.D. (2011). TORC1 regulates endocytosis via Npr1-mediated phosphoinhibition of a ubiquitin ligase adaptor. Cell *147*, 1104–1117.

Marchese, A., Raiborg, C., Santini, F., Keen, J.H., Stenmark, H., and Benovic, J.L. (2003). The E3 ubiquitin ligase AIP4 mediates ubiquitination and sorting of the G protein-coupled receptor CXCR4. Dev. Cell *5*, 709–722.

Mattera, R., and Bonifacino, J.S. (2008). Ubiquitin binding and conjugation regulate the recruitment of Rabex-5 to early endosomes. EMBO J. *27*, 2484–2494.

Mattera, R., Tsai, Y.C., Weissman, A.M., and Bonifacino, J.S. (2006). The Rab5 guanine nucleotide exchange factor Rabex-5 binds ubiquitin (Ub) and functions as a Ub ligase through an atypical Ub-interacting motif and a zinc finger domain. J. Biol. Chem. *281*, 6874–6883.

McCullough, J., Clague, M.J., and Urbé, S. (2004). AMSH is an endosome-associated ubiquitin isopeptidase. J. Cell Biol. *166*, 487–492.

McCullough, J., Row, P.E., Lorenzo, O., Doherty, M., Beynon, R., Clague, M.J., and Urbé, S. (2006). Activation of the endosome-associated ubiquitin isopeptidase AMSH by STAM, a component of the multivesicular body-sorting machinery. Curr. Biol. *16*, 160–165.

Metcalfe, C., and Bienz, M. (2011). Inhibition of GSK3 by Wnt signalling—two contrasting models. J. Cell Sci. *124*, 3537–3544.

Meyer, H., Bug, M., and Bremer, S. (2012). Emerging functions of the VCP/p97 AAA-ATPase in the ubiquitin system. Nat. Cell Biol. *14*, 117–123.

Mizuno, E., Kobayashi, K., Yamamoto, A., Kitamura, N., and Komada, M. (2006). A deubiquitinating enzyme UBPY regulates the level of protein ubiquitination on endosomes. Traffic *7*, 1017–1031.

Moretti, J., Chastagner, P., Gastaldello, S., Heuss, S.F., Dirac, A.M., Bernards, R., Masucci, M.G., Israël, A., and Brou, C. (2010). The translation initiation factor 3f (eIF3f) exhibits a deubiquitinase activity regulating Notch activation. PLoS Biol. *8*, e1000545.

Moretti, J., Chastagner, P., Liang, C.C., Cohn, M.A., Israël, A., and Brou, C. (2012). The Ubiquitin specific protease 12 (USP12) is a negative regulator of Notch signaling acting on receptor trafficking towards degradation. J. Biol. Chem., in press.

Mosesson, Y., Mills, G.B., and Yarden, Y. (2008). Derailed endocytosis: an emerging feature of cancer. Nat. Rev. Cancer *8*, 835–850.

Mukai, A., Yamamoto-Hino, M., Awano, W., Watanabe, W., Komada, M., and Goto, S. (2010). Balanced ubiquitylation and deubiquitylation of Frizzled regulate cellular responsiveness to Wg/Wnt. EMBO J. *29*, 2114–2125.

Nabhan, J.F., Pan, H., and Lu, Q. (2010). Arrestin domain-containing protein 3 recruits the NEDD4 E3 ligase to mediate ubiquitination of the beta2-adrenergic receptor. EMBO Rep. *11*, 605–611.

Niendorf, S., Oksche, A., Kisser, A., Löhler, J., Prinz, M., Schorle, H., Feller, S., Lewitzky, M., Horak, I., and Knobeloch, K.P. (2007). Essential role of ubiquitin-specific protease 8 for receptor tyrosine kinase stability and endocytic trafficking in vivo. Mol. Cell. Biol. *27*, 5029–5039.

Nikko, E., and Pelham, H.R. (2009). Arrestin-mediated endocytosis of yeast plasma membrane transporters. Traffic *10*, 1856–1867.

Overstreet, E., Fitch, E., and Fischer, J.A. (2004). Fat facets and Liquid facets promote Delta endocytosis and Delta signaling in the signaling cells. Development *131*, 5355–5366.

Ozkan, E., Yu, H., and Deisenhofer, J. (2005). Mechanistic insight into the allosteric activation of a ubiquitin-conjugating enzyme by RING-type ubiquitin ligases. Proc. Natl. Acad. Sci. USA *102*, 18890–18895.

Palamidessi, A., Frittoli, E., Garré, M., Faretta, M., Mione, M., Testa, I., Diaspro, A., Lanzetti, L., Scita, G., and Di Fiore, P.P. (2008). Endocytic trafficking of Rac is required for the spatial restriction of signaling in cell migration. Cell *134*, 135–147.

Parachoniak, C.A., Luo, Y., Abella, J.V., Keen, J.H., and Park, M. (2011). GGA3 functions as a switch to promote Met receptor recycling, essential for sustained ERK and cell migration. Dev. Cell *20*, 751–763.

Pareja, F., Ferraro, D.A., Rubin, C., Cohen-Dvashi, H., Zhang, F., Aulmann, S., Ben-Chetrit, N., Pines, G., Navon, R., Crosetto, N., et al. (2011). Deubiquitination of EGFR by Cezanne-1 contributes to cancer progression. Oncogene.

Penengo, L., Mapelli, M., Murachelli, A.G., Confalonieri, S., Magri, L., Musacchio, A., Di Fiore, P.P., Polo, S., and Schneider, T.R. (2006). Crystal structure of the ubiquitin binding domains of rabex-5 reveals two modes of interaction with ubiquitin. Cell *124*, 1183–1195.

Peschard, P., and Park, M. (2003). Escape from Cbl-mediated downregulation: a recurrent theme for oncogenic deregulation of receptor tyrosine kinases. Cancer Cell *3*, 519–523.

Puertollano, R. (2005). Interactions of TOM1L1 with the multivesicular body sorting machinery. J. Biol. Chem. *280*, 9258–9264.

Puertollano, R., and Bonifacino, J.S. (2004). Interactions of GGA3 with the ubiquitin sorting machinery. Nat. Cell Biol. *6*, 244–251.

Rauch, S., and Martin-Serrano, J. (2011). Multiple interactions between the ESCRT machinery and arrestin-related proteins: implications for PPXY-dependent budding. J. Virol. *85*, 3546–3556.

Reggiori, F., and Pelham, H.R. (2001). Sorting of proteins into multivesicular bodies: ubiquitin-dependent and -independent targeting. EMBO J. *20*, 5176–5186.

Ren, X., and Hurley, J.H. (2010). VHS domains of ESCRT-0 cooperate in high-avidity binding to polyubiquitinated cargo. EMBO J. *29*, 1045–1054.

Ritz, D., Vuk, M., Kirchner, P., Bug, M., Schütz, S., Hayer, A., Bremer, S., Lusk, C., Baloh, R.H., Lee, H., et al. (2011). Endolysosomal sorting of ubiquitylated caveolin-1 is regulated by VCP and UBXD1 and impaired by VCP disease mutations. Nat. Cell Biol. *13*, 1116–1123.

Rotin, D., and Kumar, S. (2009). Physiological functions of the HECT family of ubiquitin ligases. Nat. Rev. Mol. Cell Biol. *10*, 398–409.

Row, P.E., Prior, I.A., McCullough, J., Clague, M.J., and Urbé, S. (2006). The ubiquitin isopeptidase UBPY regulates endosomal ubiquitin dynamics and is essential for receptor down-regulation. J. Biol. Chem. *281*, 12618–12624.

Row, P.E., Liu, H., Hayes, S., Welchman, R., Charalabous, P., Hofmann, K., Clague, M.J., Sanderson, C.M., and Urbé, S. (2007). The MIT domain of UBPY constitutes a CHMP binding and endosomal localization signal required for efficient epidermal growth factor receptor degradation. J. Biol. Chem. *282*, 30929–30937.

Roxrud, I., Raiborg, C., Pedersen, N.M., Stang, E., and Stenmark, H. (2008). An endosomally localized isoform of Eps15 interacts with Hrs to mediate degradation of epidermal growth factor receptor. J. Cell Biol. *180*, 1205–1218.

Saeki, Y., Kudo, T., Sone, T., Kikuchi, Y., Yokosawa, H., Toh-e, A., and Tanaka, K. (2009). Lysine 63-linked polyubiquitin chain may serve as a targeting signal for the 26S proteasome. EMBO J. *28*, 359–371.

Sato, Y., Yoshikawa, A., Yamagata, A., Mimura, H., Yamashita, M., Ookata, K., Nureki, O., Iwai, K., Komada, M., and Fukai, S. (2008). Structural basis for specific cleavage of Lys 63-linked polyubiquitin chains. Nature *455*, 358–362.

Sato, Y., Yoshikawa, A., Mimura, H., Yamashita, M., Yamagata, A., and Fukai, S. (2009). Structural basis for specific recognition of Lys 63-linked polyubiquitin chains by tandem UIMs of RAP80. EMBO J. *28*, 2461–2468.

Shenoy, S.K., Xiao, K., Venkataramanan, V., Snyder, P.M., Freedman, N.J., and Weissman, A.M. (2008). Nedd4 mediates agonist-dependent ubiquitination, lysosomal targeting, and degradation of the beta2-adrenergic receptor. J. Biol. Chem. *283*, 22166–22176.

Shields, S.B., Oestreich, A.J., Winistorfer, S., Nguyen, D., Payne, J.A., Katzmann, D.J., and Piper, R. (2009). ESCRT ubiquitin-binding domains function cooperatively during MVB cargo sorting. J. Cell Biol. *185*, 213–224.

Shih, S.C., Katzmann, D.J., Schnell, J.D., Sutanto, M., Emr, S.D., and Hicke, L. (2002). Epsins and Vps27p/Hrs contain ubiquitin-binding domains that function in receptor endocytosis. Nat. Cell Biol. *4*, 389–393.

Sigismund, S., Woelk, T., Puri, C., Maspero, E., Tacchetti, C., Transidico, P., Di Fiore, P.P., and Polo, S. (2005). Clathrin-independent endocytosis of ubiquitinated cargos. Proc. Natl. Acad. Sci. USA *102*, 2760–2765.

Solomons, J., Sabin, C., Poudevigne, E., Usami, Y., Hulsik, D.L., Macheboeuf, P., Hartlieb, B., Göttlinger, H., and Weissenhorn, W. (2011). Structural basis for ESCRT-III CHMP3 recruitment of AMSH. Structure *19*, 1149–1159.

Sorrentino, V., Scheer, L., Santos, A., Reits, E., Bleijlevens, B., and Zelcer, N. (2011). Distinct functional domains contribute to degradation of the low density lipoprotein receptor (LDLR) by the E3 ubiquitin ligase inducible Degrader of the LDLR (IDOL). J. Biol. Chem. *286*, 30190–30199.

Staub, O., Gautschi, I., Ishikawa, T., Breitschopf, K., Ciechanover, A., Schild, L., and Rotin, D. (1997). Regulation of stability and function of the epithelial Na+ channel (ENaC) by ubiquitination. EMBO J. *16*, 6325–6336.

Stefani, F., Zhang, L., Taylor, S., Donovan, J., Rollinson, S., Doyotte, A., Brownhill, K., Bennion, J., Pickering-Brown, S., and Woodman, P. (2011). UBAP1 is a component of an endosome-specific ESCRT-I complex that is essential for MVB sorting. Curr. Biol. *21*, 1245–1250.

Stringer, D.K., and Piper, R.C. (2011). A single ubiquitin is sufficient for cargo protein entry into MVBs in the absence of ESCRT ubiquitination. J. Cell Biol. *192*, 229–242.

Taelman, V.F., Dobrowolski, R., Plouhinec, J.L., Fuentealba, L.C., Vorwald, P.P., Gumper, I., Sabatini, D.D., and De Robertis, E.M. (2010). Wnt signaling requires sequestration of glycogen synthase kinase 3 inside multivesicular endosomes. Cell *143*, 1136–1148.

Tauriello, D.V., and Maurice, M.M. (2010). The various roles of ubiquitin in Wnt pathway regulation. Cell Cycle *9*, 3700–3709.

Thien, C.B., and Langdon, W.Y. (2005). c-Cbl and Cbl-b ubiquitin ligases: substrate diversity and the negative regulation of signalling responses. Biochem. J. *391*, 153–166.

Traub, L.M., and Lukacs, G.L. (2007). Decoding ubiquitin sorting signals for clathrin-dependent endocytosis by CLASPs. J. Cell Sci. *120*, 543–553.

Umebayashi, K., Stenmark, H., and Yoshimori, T. (2008). Ubc4/5 and c-Cbl continue to ubiquitinate EGF receptor after internalization to facilitate polyubiquitination and degradation. Mol. Biol. Cell *19*, 3454–3462.

Urbé, S. (2005). Ubiquitin and endocytic protein sorting. Essays Biochem. *41*, 81–98.

Urbé, S., Liu, H., Hayes, S.D., Heride, C., Rigden, D.J., and Clague, M.J. (2012). Systematic survey of deubiquitinase localization identifies USP21 as a regulator of centrosome- and microtubule-associated functions. Mol. Biol. Cell *23*, 1095–1103.

Vaccari, T., Lu, H., Kanwar, R., Fortini, M.E., and Bilder, D. (2008). Endosomal entry regulates Notch receptor activation in Drosophila melanogaster. J. Cell Biol. *180*, 755–762.

van Kerkhof, P., Govers, R., Alves dos Santos, C.M., and Strous, G.J. (2000). Endocytosis and degradation of the growth hormone receptor are proteasome-dependent. J. Biol. Chem. *275*, 1575–1580.

Vina-Vilaseca, A., and Sorkin, A. (2010). Lysine 63-linked polyubiquitination of the dopamine transporter requires WW3 and WW4 domains of Nedd4-2 and UBE2D ubiquitin-conjugating enzymes. J. Biol. Chem. *285*, 7645–7656.

Waterman, H., Katz, M., Rubin, C., Shtiegman, K., Lavi, S., Elson, A., Jovin, T., and Yarden, Y. (2002). A mutant EGF-receptor defective in ubiquitylation and endocytosis unveils a role for Grb2 in negative signaling. EMBO J. *21*, 303–313.

Weinmaster, G., and Fischer, J.A. (2011). Notch ligand ubiquitylation: what is it good for? Dev. Cell *21*, 134–144.

Wicks, S.J., Haros, K., Maillard, M., Song, L., Cohen, R.E., Dijke, P.T., and Chantry, A. (2005). The deubiquitinating enzyme UCH37 interacts with Smads and regulates TGF-beta signalling. Oncogene *24*, 8080–8084.

Woelk, T., Oldrini, B., Maspero, E., Confalonieri, S., Cavallaro, E., Di Fiore, P.P., and Polo, S. (2006). Molecular mechanisms of coupled monoubiquitination. Nat. Cell Biol. *8*, 1246–1254.

Xia, R., Jia, H., Fan, J., Liu, Y., and Jia, J. (2012). USP8 promotes smoothened signaling by preventing its ubiquitination and changing its subcellular localization. PLoS Biol. *10*, e1001238.

Xu, L., Lubkov, V., Taylor, L.J., and Bar-Sagi, D. (2010). Feedback regulation of Ras signaling by Rabex-5-mediated ubiquitination. Curr. Biol. *20*, 1372–1377.

Yan, H., Jahanshahi, M., Horvath, E.A., Liu, H.Y., and Pfleger, C.M. (2010). Rabex-5 ubiquitin ligase activity restricts Ras signaling to establish pathway homeostasis in Drosophila. Curr. Biol. *20*, 1378–1382.

Zehner, M., Chasan, A.I., Schuette, V., Embgenbroich, M., Quast, T., Kolanus, W., and Burgdorf, S. (2011). Mannose receptor polyubiquitination regulates endosomal recruitment of p97 and cytosolic antigen translocation for cross-presentation. Proc. Natl. Acad. Sci. USA *108*, 9933–9938.

Zelcer, N., Hong, C., Boyadjian, R., and Tontonoz, P. (2009). LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. Science *325*, 100–104.

Zhang, L., Fairall, L., Goult, B.T., Calkin, A.C., Hong, C., Millard, C.J., Tontonoz, P., and Schwabe, J.W. (2011). The IDOL-UBE2D complex mediates sterol-dependent degradation of the LDL receptor. Genes Dev. *25*, 1262–1274.

Zhang, L., Zhou, F., Drabsch, Y., Gao, R., Snaar-Jagalska, B.E., Mickanin, C., Huang, H., Sheppard, K.A., Porter, J.A., Lu, C.X., and Ten Dijke, P. (2012). USP4 is regulated by AKT phosphorylation and directly deubiquitylates TGF-β type I receptor. Nat. Cell Biol. *14*, 717–726.

Zhou, R., Kabra, R., Olson, D.R., Piper, R.C., and Snyder, P.M. (2010). Hrs controls sorting of the epithelial Na+ channel between endosomal degradation and recycling pathways. J. Biol. Chem. *285*, 30523–30530.

Ziv, I., Matiuhin, Y., Kirkpatrick, D.S., Erpapazoglou, Z., Leon, S., Pantazopoulou, M., Kim, W., Gygi, S.P., Haguenauer-Tsapis, R., Reis, N., et al. (2011). A perturbed ubiquitin landscape distinguishes between ubiquitin in trafficking and in proteolysis. Mol Cell Proteomics *10*, M111 009753.