

## Monoubiquitylation: A Recurrent Theme in Membrane Protein Transport

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### Abstract

Polyubiquitylation of cellular proteins has long been recognized as a prelude to a degradative fate in proteasomes. In recent years, however, ubiquitin conjugation has emerged as a regulatory strategy of considerable versatility. Most notably, monoubiquitylation is attributed an intimate role in trafficking of membrane proteins between various cellular compartments. Diverse classes of transmembrane proteins from across the eukaryotic spectrum (e.g., epidermal growth factor-receptor and other receptor tyrosine kinases) become modified with monoubiquitin molecules. Monoubiquitylation of substrates, in turn, regulates both their endocytosis at the plasma membrane and sorting in endosomes for delivery to lysosomes or vacuoles. A mechanistic rationale lies in the identification of a growing list of ubiquitin-binding domains carried by a variety of endocytic adaptor proteins. Thus, ubiquitin-conjugated membrane proteins may form extensive contacts with the endocytic machinery. Further, ubiquitin-binding adaptors and other endocytic components are, likewise, often monoubiquitylated. In this case, ubiquitin conjugation may serve to enhance intermolecular avidity in cargo-bound endocytic complexes, or alternatively, to mediate timely inactivation of ubiquitin-binding adaptors. Interestingly, the ubiquitin/endocytosis interface is appropriated by pathogenic organisms, for instance, during budding of viruses from host-infected cells. Moreover, compromised ubiquitin-mediated transport of certain signaling receptors is associated with disease states, including oncogenic transformation.

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Ubiquitylation describes the covalent modification of proteins at lysine residues with ubiquitin, a globular polypeptide of 76 amino acids. The core reaction entails an ATP-dependent cascade drawing from a ubiquitin-activating enzyme (E1), a small repertoire of ubiquitin-conjugating enzymes (E2) and a plethora of ubiquitin ligases (E3), the last of which is largely responsible for substrate specificity [1]. Ubiquitin itself may form polymeric units, potentially utilizing as acceptor sites any of its seven lysines. Hence, proteins may be conjugated by polyubiquitin chains of different length and diverse topologies, or indeed, by monoubiquitin moieties alone. That ubiquitin is exquisitely conserved from yeast to humans attests to its evolutionary value. Most importantly, appendage of a globular entity confers to substrates an altered molecular landscape that manifests new possibilities for complex interactions. With the identification of a growing list of ubiquitin-binding domains in proteins operating in various functional realms, a mechanistic rationale is instated [2]. Moreover, substrate ubiquitylation is often inducible, and may be reversed by the action of de-ubiquitylating enzymes. Hence, ubiquitin in its

various guises, together with customized ubiquitin-associated players, constitutes a highly versatile mode of regulation.

In its most commonly ascribed role, ubiquitylation signals rapid degradation of substrates by the 26S proteasome, and here, the basis for recognition comprises polyubiquitin chains linked through Lys48 in ubiquitin. In contrast, non-proteolytic functions of alternative configurations are becoming ever apparent [3]. For example, Lys63-linked chains serve as interaction modules for protein kinase activation during NF $\kappa$ B signaling, among other roles. Monoubiquitylation is utilized in the regulation of histone function and in DNA repair pathways. Most prominently, though, monoubiquitylation has emerged as a pivotal feature in intracellular transport of membrane proteins.

Cell surface-resident transmembrane proteins are often subjected to an endocytic itinerary, e.g., upon binding cognate extracellular ligands. In one classical pathway, cargoes such as signal-transducing receptors are selectively recruited into clathrin-coated pits forming at the cell surface and delivered by vesicular transport to early endosomal compartments. Thereafter, receptor cargoes accumulate in large perinuclear endosomes, termed multi-vesicular bodies, where a critical sorting event determines their fate: active translocation into budding luminal vesicles of the MVB often preempts a degradative outcome in the lysosome (or vacuole, in yeast), while receptors remaining on the limiting membrane are thought to recycle back to the cell surface [4]. Thus, endocytic routing often leads to irreversible signal termination, but may also enable localization-dependent signal propagation. Here, we focus on ubiquitin in the realm of membrane protein transport. We review the role of monoubiquitylation of membrane proteins in endocytic trafficking between different membrane compartments, and highlight its conserved application in yeast and in metazoan organisms. Furthermore, key ubiquitin-associated players are described, particularly, various UBDs in endocytic adaptor proteins.

### Membrane protein monoubiquitylation in endocytic pathways: origins in yeast

A connection between ubiquitin and endocytic transport first emerged from studies in *Saccharomyces cerevisiae*. Ubiquitylation of various membrane proteins, including G-protein-coupled receptors and nutrient transporters, was correlated with their rapid internal-

MVB = multi-vesicular body

UBD = ubiquitin-binding domain

ization at the plasma membrane and subsequent degradation in the vacuole. Crucially, in certain cases, cytoplasmic lysines carried *in cis* were shown to be indispensable, which indicated that ubiquitin conjugation might drive receptor internalization at the plasma membrane [reviewed in 5]. The culpable ubiquitin ligase was commonly found to be Rsp5p, a member of the HECT family.

Subsequent works utilizing linear fusions of a non-extendable ubiquitin with different receptors demonstrated endocytic capacity to be intrinsically encoded in the ubiquitin moiety itself, and further, that monoubiquitin conjugation may be sufficient [5]. In this regard, critical surface residues in ubiquitin were mapped to two hydrophobic patches surrounding Phe4 and Ile44 [6,7]. The functional significance of the latter region would become clear with the discovery of ubiquitin-binding capabilities in the endocytic machinery (see below). In addition to operating at the plasma membrane, ubiquitylation-dependent sorting extends to the MVB [4]. Here, conclusive evidence was provided through the study of vacuole-resident proteins that undergo monoubiquitylation while traversing the biosynthetic pathway. Where abrogation of ubiquitylation by lysine acceptor site mutation inhibited cargo entry into MVB vesicles, proper vacuolar delivery could be restored by fusion of ubiquitin to the cytoplasmic tail [8]. Thus, ubiquitylation is critical for sorting cargoes into invaginating MVB vesicles and their subsequent transfer to vacuoles.

### Trafficking of receptor tyrosine kinases, and other cargoes

Ubiquitin-mediated transport is preserved in metazoan contexts, and best characterized is its role in the trafficking of receptor tyrosine kinases. RTKs, such as the epidermal growth factor receptor family, are primary mediators of fundamental cellular processes, including growth, differentiation and migration [9]. A central player in regulating RTK stability is the Cbl family of proto-oncoproteins. Cbl proteins were implicated in ligand-induced multi-ubiquitylation of EGF receptors, and their concomitant down-regulation in lysosomes [10]. Importantly, a truncated oncogenic variant of Cbl manifested increased recycling of receptors to the cell surface, thus leading to the notion of ubiquitylation-dependent sorting in endosomal compartments. Cbl was consequently shown to encode E3 ubiquitin ligase activity, mediated by its RING finger [11,12], and its substrate base extended to a range of tyrosine kinases and other signaling receptors. Interestingly, receptor multi-ubiquitylation by Cbl was resolved as multiple moieties of monoubiquitin, rather than polyubiquitin chains [13,14]. Hence, consistent with monoubiquitylation in yeast, RTK multi-ubiquitylation could explain evasion of proteasomes in favor of serving in MVB sorting towards lysosomal destruction.

Lysosomal degradation of RTKs constitutes an important homeostatic mechanism to restrain RTK signaling and, in particular, an inherent oncogenic potential. Accordingly, dysfunction in receptor ubiquitylation and ubiquitin recognition machineries

is associated with hyperactive signaling and cellular transformation [15]. Whether ubiquitin functions in metazoans during early endocytic steps at the plasma membrane, however, remains unclear. For example, while Cbl may already ubiquitylate EGF receptors at the plasma membrane [16], dominant-negative Cbl and Cbl<sup>-/-</sup> fibroblasts manifest no significant differences in EGF-receptor internalization rates [10,17]. In contrast, nerve growth factor-induced ubiquitylation of TrkA mediated by the ubiquitin ligase TRAF6 appears critical for receptor traffic from the cell surface to endosomal compartments, in turn, prompting endosome-specific signal generation [18].

Diverse classes of transmembrane proteins have emerged as clients for ubiquitin-dependent trafficking. For instance, ubiquitylation of some G-protein-coupled receptors, such as the  $\beta_2$ -adrenergic receptor, regulates MVB sorting and lysosomal targeting, though apparently not receptor internalization [19]. In another example, the metazoan orthologue of Rsp5p, Nedd4, controls the cell-surface stability of an epithelial sodium channel, and uncoupling mutations are associated with Liddle's syndrome, an inherited form of hypertension [20]. An interesting case is provided by the Notch receptor and its transmembrane ligands. Both Notch and Notch ligands are associated with multiple E3 ligases, which dictate recycling or lysosomal degradation, as well as endocytosis-dependent Notch activation [21]. Further types of cargo subject to ubiquitin-dependent sorting include junctional proteins (E-cadherin, connexin 43), ligand-gated ion channels and immune receptors [5].

### Ubiquitin-binding domains in endocytic adaptor proteins

The utility of substrate ubiquitylation predicted the likely existence of a ubiquitin-binding capability within the cellular repertoire of interactions, and indeed, recent years have seen a proliferation of UBDs annotated. Characterization of the first ubiquitin-binding sites, in the S5a subunit of the proteasome, was to herald a breakthrough in this field. Ubiquitin-binding regions in S5a were later matched via a bioinformatics screen to similar sequences arising in other proteins [22]. Remarkably, the conserved pattern, denoted the ubiquitin-interacting motif, was found in a number of adaptor proteins occupied in endocytic trafficking. These included Eps15 and Epsin, which interact with the clathrin adaptor, AP2, at the plasma membrane [23], and Hrs and STAM, which operate at sorting endosomes [4]. The UIM in several of these proteins was subsequently proven to bind ubiquitin, and interestingly, to mediate self-ubiquitylation in an autonomous manner [24,25]. Self-conjugation, in most cases with monoubiquitins, is thought to involve the Nedd4 family of E3s [24,25], however the precise mechanism and, indeed, its functional significance remains to be resolved. Importantly, the UIM is conserved from yeast and appears in orthologous endocytic adaptor proteins. Hence, a critical function in binding and trafficking of ubiquitylated membrane proteins might be envisaged. Several studies corroborate this notion; mutation

RTK = receptor tyrosine kinase  
EGF = epidermal growth factor

UIM = ubiquitin-interacting motif

**Table 1.** Ubiquitin-binding domains in endocytic adaptor proteins

UBD	Size (amino acids)	$K_d$ ( $\mu$ M)	Endocytic adaptor proteins	Subcellular compartment
UIM	~20	100–400 (mono- or polyubiquitin)	Eps15, Ent1p/Epsin, Vps27p/Hrs, Hse1p/STAM	PM, early endosome
UBA	45–55	~10–500 (monoubiquitin) ~0.03–10 (polyubiquitin)	Ede1p, Cbl-b	PM, early endosome
CUE	42–43	~2–160 (monoubiquitin)	Vps9p/Rabex-5, Vps9p, Tollip	Early endosome
UEV	~145	~100–500 (monoubiquitin)	Vps23p/Tsg101	MVB
GAT	135	~180 (monoubiquitin)	Gga/GGA, Tom1	Early endosome, TGN
GLUE	~135	~460 (monoubiquitin)	Eap45	MVB
NZF	~35	~100–400 (monoubiquitin)	Vps36p	MVB
VHS	150	Not determined	Vps27p/Hrs, Hse1p/STAM	Early endosome

Some features of ubiquitin-binding domains are presented, including affinity for ubiquitin and a listing of endocytic effectors that contain them.

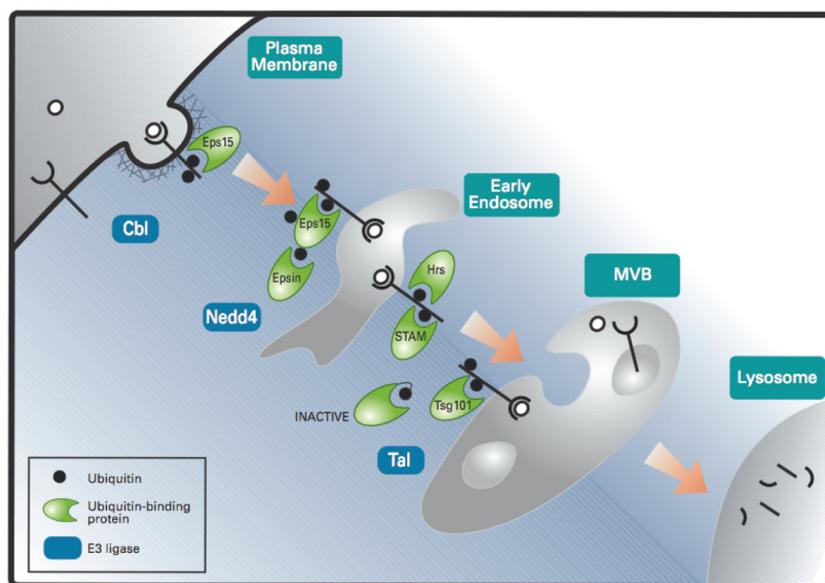
CUE = coupling of ubiquitin conjugation to endoplasmic reticulum degradation, GAT = Gga and Tom1, GLUE = GRAM-like ubiquitin binding in Eap45, MVB = multivesicular body, NZF = Np14 zinc finger, PM = plasma membrane, STAM = signal-transducing adaptor molecule, TGN = trans-Golgi network, UBA = ubiquitin-associated, UEV = ubiquitin-conjugating enzyme variant, UIM = ubiquitin-interacting motif; VHS, Vps27, Hrs, STAM.

of the UIM in yeast proteins is shown to perturb either endocytosis or MVB sorting, according to the proposed site of action of the host adaptor [26,27]. Likewise, the UIM of Hrs in mammalian cells is required to sort ubiquitylated cargo for lysosomal degradation [28].

Further advances came with the discovery of ubiquitin-recognition modules in molecular assemblages at the MVB. A subset of gene products attributed a role in vacuolar protein sorting were found to cluster into three major complexes, termed ESCRT-I, -II and -III, and to act sequentially in the transfer of incoming cargo to internalizing vesicles of the MVB [4]. In these studies, the ESCRT-I component, Vps23, was shown to bind ubiquitylated cargo [29]. Binding was contingent on an intact UEV domain, a ubiquitin-interacting module that resembles the catalytic domain of ubiquitin-conjugating enzymes but lacks the active site cysteine residue. The mammalian orthologue of Vps23, Tsg101, was also shown to bind ubiquitin. Interestingly, a novel UBD denoted the NZF domain is present in Vps36, a component of ESCRT-II [30].

Somewhat surprisingly, its metazoan counterpart, Eap45, appears instead to be endowed with yet another newly identified UBD, called the GLUE domain [31]. At the MVB, therefore, NZF or GLUE domains most likely functionally cooperate with the UEV domain in ESCRT-I.

Other UBDS have emerged in yeast two-hybrid screens using monoubiquitin as bait. They include CUE and GAT domains which, like the UIM, show a capacity for intramolecular monoubiquitylation [reviewed in 2]. CUE domain-containing proteins include Vps9, an activator of the Rab5 GTPase that, in turn, coordinates vesicular traffic from the cell surface to early endosomes. Accordingly, CUE::ubiquitin interactions also appear to regulate endocytic transition of ubiquitylated cargoes [32]. GAT domains, identified in the GGA family of coat proteins, highlight an emergent active role for ubiquitin in trans-Golgi network-to-endosome transport [33]. Further examples of UBDS include the VHS domain, present in Hrs and STAM, and the well-characterized UBA domain, found in Cbl proteins [2]. In brief, endocytic pathways are handsomely equipped with an assortment of ubiquitin docking sites, reinforcing the centrality of ubiquitin-dependent regulation within this realm [Table 1].



**Figure 1.** Roles for monoubiquitin during cargo endocytosis. Scheme showing endocytic routing of ligand-activated receptor tyrosine kinases. Cbl-mediated monoubiquitylation of receptor molecules at multiple sites promotes their progression along the endocytic pathway towards a degradative fate in lysosomes. Monoubiquitylated cargoes are recognized by several endocytic adaptors endowed with various ubiquitin-binding domains. Prominent examples are depicted, including Eps15 and Epsin, which operate at the plasma membrane-to-early endosome interface, and Hrs, STAM and Tsg101, functioning in endosome-to-MVB transport. Adaptor proteins, likewise, undergo monoubiquitylation, often in a manner dependent on their ubiquitin-binding capability. E3 ligases implicated here encompass the Nedd4 family whose targets include Eps15 and Hrs, and Tal, which monoubiquitylates Tsg101. Functionally, several scenarios for adaptor ubiquitylation are described: intermolecular ubiquitin::UBD interactions may cluster cargo-coupled endocytic complexes, in turn fostering efficient internalization of cargo. Alternatively, intramolecular ubiquitin::UBD binding may impose an inactive conformation, thereby restricting endocytic adaptors to appropriate schedules of activity.

## Ubiquitin: UBD interactions: structural considerations

Crystal and solution structures of ubiquitin complexed with currently known UBDs have been resolved in some cases [reviewed in 2]. Interestingly, UBDs exhibit highly divergent tertiary structure (with only CUE and UBA being closely related). In spite of this, all UBDs contact the hydrophobic patch surrounding Ile44 in ubiquitin, though certainly in differing manners. Further, these structures reveal unusual 'plasticity' of the mode of interaction; for instance, UEV domains appear to possess several ubiquitin-binding interfaces, while CUE::ubiquitin structures in different configurations have been observed [2]. Such apparent promiscuity is in line with empiric observations that ubiquitin::UBD interactions are generally of low affinity (dissociation constants in the micromolar range).

## Manipulation of ubiquitin-mediated transport by microorganisms

Pathogens evolve resourceful means of manipulating cellular machineries to sustain their life cycles, and ubiquitin-dependent trafficking offers some interesting examples. Budding of retroviruses at the plasma membrane shares similar topologic requirements as MVB formation. Accordingly, retroviral budding often entails recruitment of factors associated with MVB sorting, such as Tsg101 and Nedd4, and shows a dependence on the integrity of ubiquitin conjugation [34]. In another case, several plasma membrane molecules of the immune system are down-regulated by virus-encoded E3 ligases via the lysosomal pathway, thus serving as a mechanism of immune evasion [35]. Lastly, a recent report describes monoubiquitylation-dependent invasion by the bacterial pathogen *Listeria* of its target cells. Remarkably, the bacterial cell coopts the host's endocytic program by activating Cbl-mediated ubiquitylation of an RTK and binding to the internalizing complex [36]. Certainly, further such examples are likely to arise.

## Monoubiquitin-regulated traffic: towards an integrated view

A cohesive picture of ubiquitin-dependent traffic is emerging: monoubiquitylation of membrane proteins together with diverse UBD-containing endocytic adaptors constitutes a conserved eukaryotic strategy to regulate transport between various membrane compartments. Its impact is particularly prominent at the MVB, a major focal point of sorting decisions for cargoes arriving from both early endosomes and the trans-Golgi network. In contrast, cargo ubiquitylation during plasma membrane-to-early endosome transport may not be universally required in mammals. It may be that different sorting determinants such as di-leucine or tyrosine-based motifs are dominant in certain instances [37]. Alternatively, the monoubiquitin signal might be contracted out to cargo-coupled proxies. Candidates include self-ubiquitylated UBD-carrying proteins, for instance Eps15, or monoubiquitylated adaptors such as  $\beta$ -arrestin [19].

Considering trafficking of activated RTKs as a paradigm, one can envisage a sequential process in which suitably stationed UBD-containing proteins promote the progress of monoubiqui-

tylated cargo along a lysosomal trajectory [Figure 1]. Moreover, because the various UBDs all access overlapping regions in ubiquitin, this may facilitate efficient relaying of cargo. Such a scenario is corroborated in a recent study describing different kinetic profiles for interactions between various UBD-containing proteins and a receptor-ubiquitin chimera [38]. It is worth reflecting on the low affinity nature of monoubiquitin::UBD interactions. Ubiquitin recognition in endocytic effector complexes is complemented by a network of alternative connections that are, similarly, typically weak. Examples include EH or SH3 domain-mediated interactions, as well as membrane coupling via phospholipid-interacting domains [37]. One can conceive that this design allows flexibility in remodelling of effector complexes, as per the changing functional requirements of trafficking events. Thus, when operational, interaction avidity may be augmented locally, for instance via the presence of tandem copies of UBDs that is often observed, or by modification of cargoes with multiple moieties of monoubiquitin.

## Matters outstanding

Several questions remain unanswered. At the forefront, perhaps, is the role of adaptor protein auto-ubiquitylation in endocytic pathways. As mentioned, such activity may allow local aggregation of monoubiquitin::UBD combinations, and therefore aid in engendering endocytic momentum. In another plausible model, self-ubiquitylation may cause adaptors to adopt an auto-inhibited conformation through intramolecular ubiquitin::UBD coupling, and self-inhibition could be relieved by timely de-ubiquitylation, or competitively by the proximity of ubiquitylated cargo. Although yet to be proven, genetic evidence in flies that Epsin de-ubiquitylation promotes receptor endocytosis is consistent with this notion [39]. In a similar vein, multiple monoubiquitylation of Tsg101 by a novel E3 ligase, Tal, may mediate transient inactivation of Tsg101 during MVB sorting and viral budding [40].

An interesting recent proposal challenges fundamental conceptions regarding the degradative route fated for ubiquitylated cargo. Rather than originating in clathrin-coated pits, ubiquitylated RTKs destined to lysosomal destruction are reported to traffic via clathrin-independent lipid rafts [38]. It will be critical to establish direct or indirect links between cell-surface-peripheral endocytic adaptors, for instance Eps15 and Epsin, and raft-resident proteins such as caveolin. Further matters of interest relate to the possibility that ubiquitin conjugation per se may directly regulate substrate activity, irrespective of UBD function. Notably, the EGF receptor is modified by multiple monoubiquitins, yet the limited number of acceptor sites in its cytoplasmic tail would suggest targeting of the tyrosine kinase domain, which is far more lysine-rich [14]. In turn, kinase domain ubiquitylation might be expected to impact on enzymatic activity.

Another key unresolved issue is that of how substrate conjugation may be restricted to monoubiquitins on the one hand, and to polyubiquitins of varying connectivities on the other. One may speculate that ubiquitin-capping by UBDs may occlude further elongation from particular lysines in ubiquitin, or that ubiquitin chain topology might be intrinsically determined by the

particular E2/E3 combination involved. On a related final note, perfectly conserved surface residues of ubiquitin are shown to be important during endocytosis but are yet to be accounted for [7]. Thus, novel monoubiquitin docking sites associated with endocytic pathways might be anticipated. These exciting prospects await further investigation.

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