Proteasome-Independent Functions of Ubiquitin in Endocytosis and Signaling

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Ubiquitination is a reversible posttranslational modification of cellular proteins, in which a 76–amino acid polypeptide, ubiquitin, is primarily attached to the ε-amino group of lysines in target proteins. Ubiquitination is a major player in regulating a broad host of cellular processes, including cell division, differentiation, signal transduction, protein trafficking, and quality control. Aberrations in the ubiquitination system are implicated in pathogenesis of some diseases, certain malignancies, neurodegenerative disorders, and pathologies of the inflammatory immune response. Here, we discuss the proteasome-independent roles of ubiquitination in signaling and endocytosis.

Ubiquitin (Ub) is a highly conserved protein of 8 kilodaltons that becomes covalently attached to lysine (Lys) residues of target proteins. Protein-attached Ub is a substrate for the attachment of further Ub residues, which leads to the formation of a polyubiquitin chain. Classically, polyubiquitination is a signal that directs proteins to the proteasome, where the Ub is recycled and the protein is degraded (*1*). Ubiquitination also remodels the surface of substrate proteins and thereby potentially affects properties such as stability and activity (*2*), drives interactions with other proteins, and plays roles in subcellular localization. Diverse forms of Ub modifications exist: Monoubiquitination is the attachment of a single Ub to a protein; multiubiquitination occurs when several Lys residues of the target protein are tagged with single Ub molecules; and polyubiquitination denotes the addition of a Ub chain made of several ubiquitins that are linked through the C-terminal glycine residue of each Ub unit and a specific internal Lys of the previously attached Ub. Monoubiquitination or multiubiquitination has been shown to be required for the entry of certain cargo proteins into vesicles at different stages of the secretory/endocytic pathway (Fig. 1) (*3*), whereas polyubiquitination has been mainly associated with proteasomal degradation, although it certainly has a broader function (*4*). In the case of polyubiquitination, there can be at least seven different linkages between ubiquitins, because there are seven internal lysines in Ub. The role of different linkages in polyubiquitins has begun to be elucidated in recent years. Linkage through Lys^{48} (Ub^{K48}) is mainly used for targeting to the proteasome, and Lys^{63} (Ub^{K63}) linkages seem to play important roles in DNA damage tolerance, inflammatory response, the endocytic pathway, and ribosomal protein synthesis (*4*). Ub can also be removed from proteins, and different Ub hydrolases that regenerate free Ub have been identified and implicated in regulation of various cellular events (*5*–*7*). Protein modules containing Ub-binding domains (UBDs) have also been identified and are being characterized. The identification of many UBDs with diverse structural folds, by which specific Ub modifications can be recognized, has helped in understanding the role of ubiquitination (*8*). The presence of several structurally distinct Ub modifications, the exquisite specificity of Ub conjugation, and the existence of UBDs, in which the different Ub modifications are capable of being distinguished, make Ub a multifunctional signal.

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Ubiquitination as an Endocytic Signal

One of the first nonproteasomal functions of ubiquitination was its implication in the process of endocytosis in yeast (*9*, *10*), where monoubiquitination is sufficient as an endocytic internalization signal (*11*) and Ub^{K63} branches facilitate endocytosis (*12*). Many plasma membrane proteins depend on their ubiquitination for internalization in yeast (*13*). On the other hand, the situation is much less clear in animal cells. Early evidence clearly demonstrated a role for the process of ubiquitination for internalization of growth hormone receptor, but ubiquitination of the receptor itself was not required (*14*). Studies on the transforming growth factor–β (TGF-β) receptor suggest that receptor ubiquitination may determine the endocytic internalization pathway that is used. When the TGF-β receptor associated with the Smad7-Smurf2 E3 Ub ligase (*15*), it localized to caveolae and was rapidly degraded after internalization. On the other hand, when the TGF-β receptor associated with Smad anchor for receptor activation, it was internalized via clathrin-coated pits where signaling proceeds from an endosomal compartment (*16*). Therefore, modulation of the endocytic internalization route by Ub could play a critical role in deciding between signal transduction or receptor down-regulation.

Receptor ubiquitination in endocytic internalization is most studied for its potential role in the down-regulation of activated mammalian receptor tyrosine kinases (*17*, *18*), although the situation is still not entirely clear. When epidermal growth factor receptor (EGFR) was stimulated at low epidermal growth factor (EGF) concentrations in HeLa cells, EGFR ubiquitination was not detected, and the receptor localized with clathrin; however, at high EGF concentrations, EGFR was ubiquitinated, and the receptor localized with both caveolae and clathrin. It is not clear that caveolae were sites of internalization in this study. Moreover, with the use of a chimeric protein, EGFR-Ub-mut, that could not be extended by polyubiquitination, a single Ub was sufficient to drive internalization (*19*) in a clathrin-independent manner. Three proteins with UBDs—Eps15, Eps15R, and epsin—that normally localize to clathrin-coated pits were required. In another study, epsin, which is considered to be an endocytic adaptor between ubiquitinated cargo and clathrin, was not able to interact with ubiquitinated cargo in the presence of clathrin (*20*). These results invoked a working hypothesis in which ubiquitination directs clathrin-independent endocytosis and where epsin and Eps15 might be important for coupling ubiquitinated cargo to clathrin-independent internalization. However, in porcine aortic endothelial cells, EGFR is normally polyubiquitinated (Ub^{K63}) before endocytosis on multiple lysines in the kinase domain under low EGF conditions, which should lead to internalization through clathrin-coated pits (Fig. 1). Mutation of the target lysines preventing ubiquitination abrogated down-regulation but not receptor internalization (*21*). Thus, a single Ub can act as an internalization signal in animal cells, but receptor ubiquitination is probably not required for clathrin-mediated internalization of EGFR.

The idea that a single Ub can act as an autonomous endocytic signal is difficult to reconcile with the relative low affinity of UBDs (*8*). However, the low-affinity interactions between monoubiquitin and UBDs could be physiologically relevant in the protein networks formed that drive vesicle budding at the plasma membrane. There are many possibilities for the formation of multiple interactions. Most UBD-containing proteins have additional modules that recognize other proteins or specific phospholipids. For example, epsins have a domain that recognizes phosphoinositides in addition to their three UBDs (*22*). Dual binding to phosphoinositides, generated at specific sites on the membrane, and Ub should allow epsins to stably recognize a monoubiquitin at the plasma membrane in the presence of a pool of cytosolic

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Ub. Another possibility is that many Ub-binding proteins have multiple UBDs, and the addition of rather low binding affinities can lead to a cooperative binding reaction and higher avidity. In this case, multiubiquitinated or multimeric cargo molecules would probably be a better substrate. It is possible that the successful demonstration of single ubiquitins working as endocytic signals is related to the fact that both EGFR and the alpha factor receptor are internalized as dimers. Thus, although monoubiquitination may sometimes be sufficient for endocytic internalization, generally productive recognition and uptake of ubiquitinated cargoes requires stabilization of cargo-adaptor complexes through multivalent Ub-UBD interactions. One can speculate how Ub^{K63} polyubiquitination might be particularly adapted to act as an efficient endocytic signal. The extended conformation of Ub^{K63} chains has a linear topology, lining up adjacent Ub chains and making their hydrophobic surfaces available for binding, which should increase the avidity of binding to UBDs (*23*). The higher avidity can thus promote productive internalization of Ub^{K63}-conjugated proteins. Especially for those receptors that have a limited number of cytoplasmic Lys residues and thus cannot be multiubiquitinated, Ub^{K63} polyubiquitination offers an alternate mechanism.

Endocytosis of certain membrane proteins seems to require polyubiquitination. For example, in the case of the β_2-adrenergic receptor (β_2AR), a mammalian heterotrimeric guanine nucleotide–binding protein–coupled receptor, agonist stimulation leads to rapid polyubiquitination of both the receptor and the receptor regulatory protein β-arrestin. It is probably the polyubiquitination of β-arrestin that is necessary for β_2AR internalization (*24*). The yeast protein general amino acid permease (Gap1p) requires the formation of short Ub^{K63} chains for efficient internalization in response to changes in nitrogen status in the medium (*25*). Similarly, Ub^{K63} polyubiquitination of the nerve growth factor (NGF) receptor tyrosine receptor kinase A (TrkA) is required for TrkA internalization and signaling (*26*). It could be that the role of Ub chain topology in vivo is currently underestimated, with Ub^{K63} chains being more important than previously thought. The presence of Ub^{K63} chains may also allow selective recognition by particular UBDs.

Ub and Sorting into Multivesicular Bodies

Ubiquitination also functions as a sorting signal at endosomes targeting its substrates to the interior of the multivesicular body (MVB) (*27*), which is the first step leading to their transport to lysosomes. This pathway is used not only in plasma membrane–receptor down-regulation but also in lysosome biogenesis (Fig. 1). Most proteins that are not ubiquitinated follow other pathways, and those proteins are recycled from endosomes to other compartments. Monoubiquitination or multiubiquitination is critical for sorting of membrane proteins to the interior of the multivesicular endosome in a process that depends on endosomal sorting complex required for transport (ESCRT) complexes, which is detailed in an excellent review (*28*). Ub on receptors or cargo is recognized by the vacuolar protein sorting–associated protein

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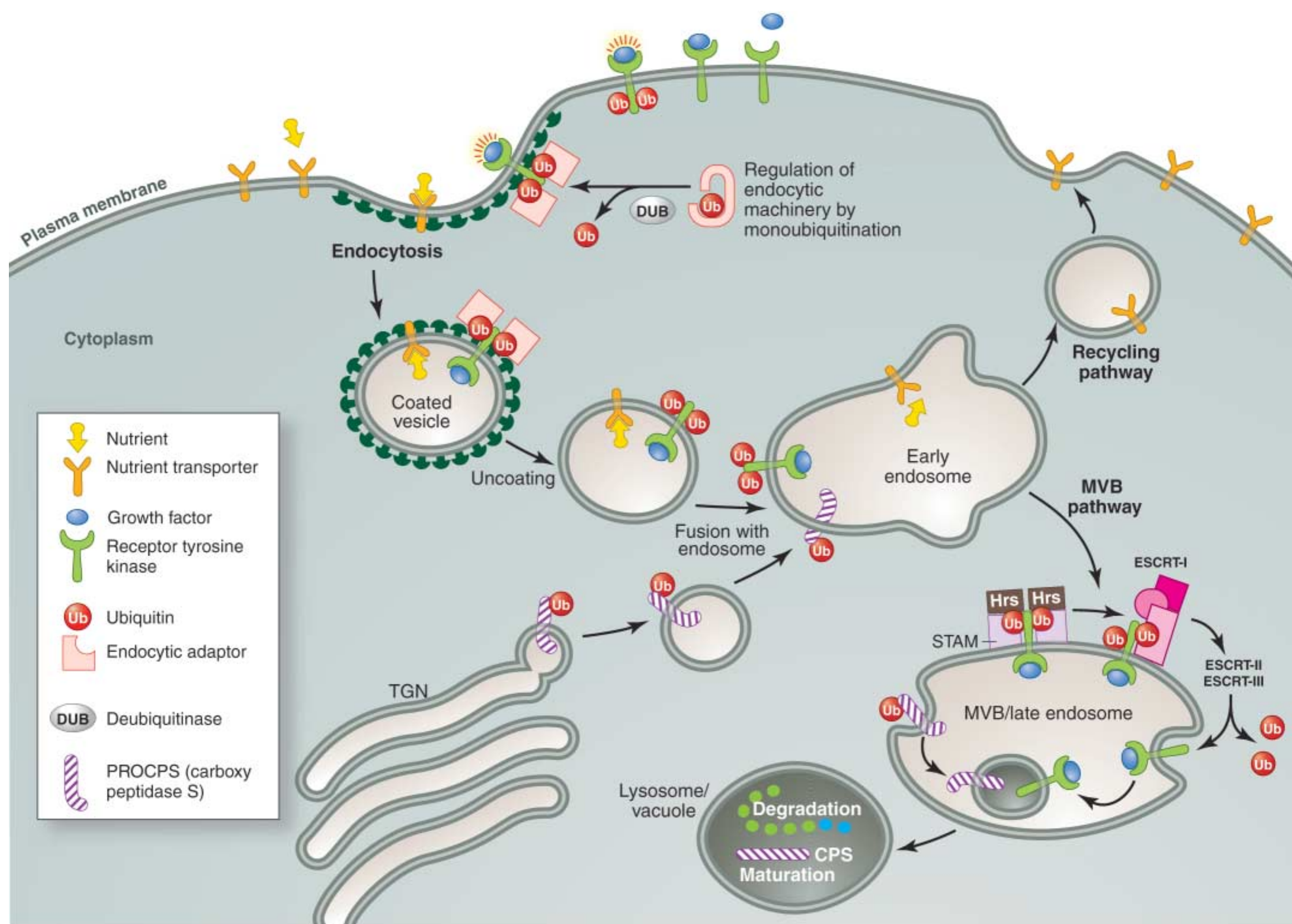

Fig. 1. Ub plays a major role at two steps of the secretory/endocytic pathways. Many plasma membrane proteins and receptors are ubiquitinated on their cytoplasmic domains. In animal cells, this may affect the choice of endocytic pathway (not shown). Ub also acts as a signal into the MVB pathway, where Ub is recognized by Vps27/Hrs and passed onto ESCRT complexes. Ub is removed before entry into internal vesicles of the MVB. Non-ubiquitinated proteins, such as nutrient transporters, recycle from endosomes back to the cell surface. TGN, trans-Golgi network; STAM, signal-transducing adaptor molecule.

(Vps27) complex through its Ub-interaction motif domains, and the complex is anchored on endosomes via the Vps27 FYVE domain, which binds phosphatidylinositol (PI) 3-phosphate (*29*). The Vps27 complex and ubiquitinated substrates are then thought to interact with ESCRT-I complex, from where the ubiquitinated cargoes are passed on to ESCRT-II and then to ESCRT-III, which is required for the concentration of cargoes into the MVB vesicles. ESCRT-III also regulates the accessory factors Bro1p and Doa4p, a Ub hydrolase that removes Ub from the cargo before it is sequestered into the MVB vesicles. Removal of Ub before receptor incorporation into MVBs is not mandatory because Ub that is fused in frame with cargo molecules gets sorted into MVBs, but such removal seems to be required for the maintenance of the free Ub pool, upon which receptor trafficking depends. Recent findings point to a regulatory role of deubiquitination in receptor down-regulation, because depletion of Ub hydrolases by RNA interference caused accelerated degradation of ligand-activated receptors (*30*, *31*). The topology of MVB vesicle budding is similar to biosynthetic viral budding from the cell surface, and several components are conserved (*32*).

Viruses also exploit the Ub-dependent entry into MVBs to escape immune recognition. Down-regulation of the major histocompatibility complex I (MHC I) by Kaposi's sarcoma–associated herpesvirus (KSHV) involves a virally encoded E3 Ub ligase that associates with MHC I molecules in the late secretory pathway, recruiting host Ub-conjugating enzymes to modify MHC class I molecules with Ub^{K63} chains. This ubiquitination targets the MHC I molecules for degradation through the MVB pathway (*33*).

Protein Regulation by Monoubiquitination

Many of the UBD-containing proteins that interact with ubiquitinated targets are themselves monoubiquitinated, especially the endocytic adaptor proteins (*8*). Insight into the role of monoubiquitination has recently been obtained (*34*). Sts1 and Sts2 bind through their N-terminal UBDs to ubiquitinated EGFR complexes and inhibit their endocytosis. Monoubiquitination of Sts2 leads to an intramolecular interaction between the UBD and its Ub, preventing Sts2 from interacting with exogenous Ub and EGFR. This intramolecular inhibitory reaction is functionally relevant because an Sts2-Ub chimera caused a substantial increase in EGFR down-regulation. Other endocytic adaptors with UBDs, such as Eps15 and hepatocyte growth factor–regulated tyrosine kinase substrate (Hrs), showed similar intramolecular interactions with their monoubiquitin. Only a small fraction of these proteins are monoubiquitinated at any one time (*34*, *35*), but this would be consistent with a regulatory role, where monoubiquitination of the UBD-containing proteins could act as a conformational switch, promoting dissociation from their targets. Once dissociated, the proteins could be rapidly deubiquitinated and reused. Therefore, changes in ubiquitination status could act as a rapid switch between active and inactive forms, permitting the highly dynamic exchange of ubiquitinated cargoes between the UBD-harboring proteins that is required by the endocytic sorting machinery.

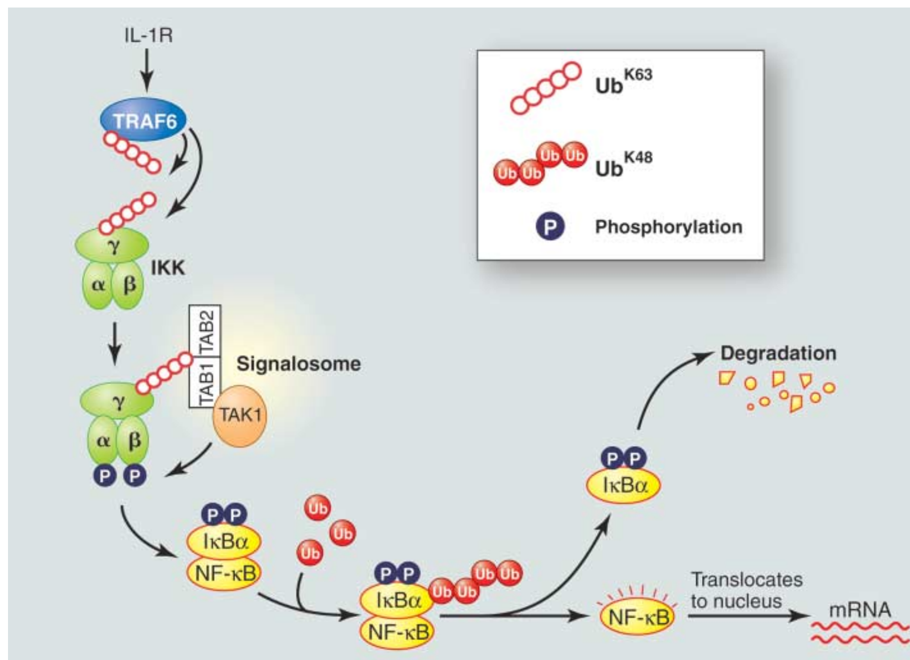

Fig. 2. Activated IL-1 receptor (IL-1R) stimulates TRAF6-dependent Ub^{K63} polyubiquitination of IKKγ (NEMO). The TAB/TAK1 complex is recruited, forming a "signalosome" that phosphorylates IKK, which in turn phosphorylates IκB, making it a substrate for Ub^{K48} polyubiquitination and subsequent degradation by the proteasome (Fig. 3). NF-κB is free to migrate to the nucleus and induce transcription.

Role of Ub in Signaling

Ubiquitination has been found to serve as a signal for directing internalization of signaling receptors and their incorporation into MVBs for targeting to lysosomal degradation. However, several receptors can transmit signals from endocytic compartments, and these signals might be qualitatively different from those initiated at the plasma membrane. For example, TrkA promotes NGF-mediated cell survival at the cell surface, whereas it induces differentiation when internalized (*36*). TrkA is modified by Ub^{K63} polyubiquitination upon stimulation with NGF in a p75 neurotrophin receptor–dependent manner. This modification is necessary for TrkA internalization and TrkA signaling from endosomes because abrogation of ubiquitination prevents NGF-mediated differentiation of neuronal cells (*26*). TrkA is multiubiquitinated by Nedd4-2, an E3 ligase, in an NGF-dependent manner, which is important for down-regulating the levels of activated receptor (*37*). Unexpectedly, this multiubiquitination did not show any effect on TrkA internalization. One can speculate a hierarchy of differential ubiquitination, whereby Ub^{K63} chains promote internalization while multiubiquitination regulates receptor degradation.

Monoubiquitination and endocytosis are prerequisites for activation and signaling of Notch receptor, which is a regulator of cell development and cell fate in metazoans (*38*). Recently, it has emerged that the ubiquitination of Notch ligands Delta and Serrate (type I membrane proteins and ligands of Notch) is necessary for their endocytosis in signal-sending cells and thus is a key event in activating the Notch cascade in the signal-receiving cells (*39*). Presently, the mechanism by which endocytosis of Notch ligands activates Notch signaling in neighboring cells remains elusive. An attractive model for the role of ubiquitination in regulating activity of the Notch ligands has been proposed. Ubiquitination is required for ligand activation but also makes the ligand prone to degradation. This would prevent the endless recycling of activated ligand and thereby limit the temporal extent of Notch-pathway activation (*39*). Thus, ubiquitination could couple activation with down-regulation, which allows for precise temporal control of signaling by limiting the lifetime of the activated signaling components.

Ub, Replication, and Transcription

Proliferating cell nuclear antigen (PCNA) is a replicative processivity factor that undergoes two types of Ub modifications. PCNA forms a polymerase sliding clamp around DNA, and the type of ubiquitination influences DNA-polymerase recruitment. Monoubiquitination of PCNA seems to shift recruitment from replica-

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tive to translesion polymerases (*40*, *41*), which are error prone, whereas Ub^{K63} modification signals error-free DNA repair. The same residue in PCNA is also sumoylated, which is involved in normal DNA synthesis during S phase (*42*). Modification of PCNA with monoubiquitin or Ub^{K63} chains may thus regulate the engagement of low-fidelity lesion-bypass polymerases or high-fidelity replicases to the site of replication (*43*).

Ubiquitination has come to light as a major regulator of the nuclear factor (NF) κB signaling pathway. NF-κB transcription factors are activated by the proinflammatory cytokines [tumor necrosis factor–α (TNF-α) and interleukin-1β (IL-1β)], resulting in degradation of an inhibitor of NF-κB (IκB), which binds to NF-κB and retains it in the cytoplasm (Fig. 2). IκB is phosphorylated by a kinase [IκB kinase (IKK)], which has a regulatory subunit called NF-κB essential modulator (NEMO). NEMO is modified by Ub^{K63} polyubiquitination by the TNF-α receptor–associated factor 6 (TRAF6) (*44*). Ub^{K63} on NEMO activates IKK, which phosphorylates IκB, targeting it for polyubiquitination and proteasomal degradation (*45*). This action liberates NF-κB. Another kinase that activates IKK is TGF-β–activated kinase (TAK1). TAK1-binding proteins (TAB1, TAB2, and TAB3) bind to TRAF6 and TAK1, stimulating its kinase activity, and preferentially bind Ub^{K63} (*46*). Furthermore, the TRAF6-TAB2-TAK1 complex interacts with IKK. So, Ub^{K63} may function as part of a scaffold to assemble a TAK1-IKK–containing signaling complex (*47*). When NF-κB activation is induced by TNF-α, receptor-interacting protein (RIP), which is important for receptor complex assembly, undergoes Ub^{K63} polyubiquitination. NEMO binds to Ub^{K63}-polyubiquitinated RIP and thereby recruits IKK to the TNF receptor. This recruitment could explain the propagation of signal from the activated TNF receptor to the activation of IKK and NF-κB. NEMO also stabilizes RIP through interaction with its Ub^{K63} chains, and thus the binding of NEMO could impair RIP interaction with another protein, A20 (*48*). A20 inhibits NF-κB signaling by sequentially removing Ub^{K63} chains from RIP and targeting it for degradation through ligation of Ub^{K48} chains (*6*).

In general, it is assumed that Ub^{K48} polyubiquitin chains act to target modified substrates for proteasomal degradation and that Ub^{K63} linkages are used for other functions. However, for a budding yeast transcription factor Met4p, Ub^{K48} polyubiquitination represses its activity but does not affect Met4p stability (Fig. 3) (*49*). Further work on Met4p has shown that it contains a UBD, which interacts in cis with the Ub^{K48} chains, preventing their elongation. Because multiple Ub^{K48} linkages are required for recognition by the proteasome (*50*), this leads to protection from proteolysis. In this case, Ub^{K48} chains act to switch Met4p to an inactive state but do not lead to Met4p degradation. Rapid deubiquitination of Met4p, in response to stimulus, recreates a large pool of active Met4p and thus allows a rapid transcriptional response to changing environmental conditions (*51*).

Ubiquitination and Disease

Ubiquitination is a widely used posttranslational modification to regulate cellular processes. Thus, aberrations in the ubiquitination system lead to disease and malignancies. Enhanced degradation of tumor suppressor proteins by the proteasomal pathway or abrogation of degradation of oncogenic proteins is the basis of oncogenesis in several cancers. Several pathogens and viruses have evolved mechanisms to subvert the Ub system for their own ends. For instance, anthrax toxin triggers ubiquitination of its receptor to facilitate efficient and rapid endocytosis of the toxin-receptor complex. Endocytosis of the toxin-receptor complex, in turn, is important for toxin action, because passage through low-pH endosomal compartments makes the toxin competent to induce toxicity in cells (*52*). *Yersinia* produces a virulence factor, YopJ, that acts as a deubiquitination enzyme, preventing activation of NF-κB (*53*). On the other hand, many viruses encode for E3 Ub ligases in order to down-regulate proteins of the immune pathway. The E3 Ub ligase, named modulator of immune recognition 1 of KSHV, can mediate ubiquitination of cysteine residues in the MHC class I molecule. This form of ubiquitination is found to be sufficient for endocytosis and degradation of MHC I molecules (*54*). The finding that nonlysine residues are also targets of ubiquitination extends the number of potential substrates to molecules that do not contain an accessible Lys or an accessible N terminus. Also, transient ubiquitination of substrates may occur and may be harder to detect than other forms of ubiquitination because thiol ester bonds of Ub-cysteine are more labile than isopeptide bonds of Ub-Lys. Thus, the regulatory processes rendered by Ub might be more complex than previously thought.

Liddle's syndrome, which affects sodium transport, is a disease in which ubiquitination clearly plays a role in regulating sodium channel activity by controlling its down-regulation via endocytosis (*55*). Ub is also thought to play a role in neurodegenerative diseases. Accumulation of insoluble proteinaceous deposits enriched with Ub and components

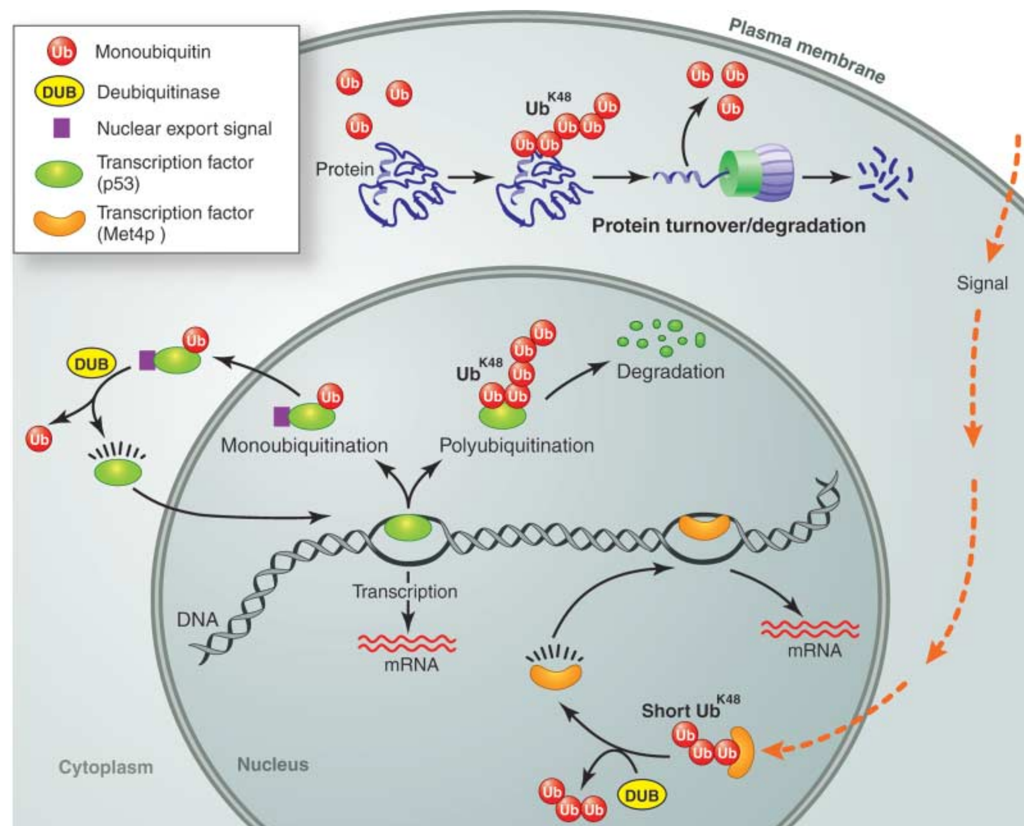

Fig. 3. Protein modified by long (more than four ubiquitins) Ub^{K48} chains is targeted to the proteasome for degradation. Short Ub^{K48} chains can inhibit activity of a transcription factor (Met4p) without leading to its degradation. Deubiquitination activates the transcription factor. A transcription factor (p53) is imported into the nucleus. Monoubiquitination leads to its nuclear export, whereas polyubiquitination leads to its degradation (*59*).

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of the Ub-proteasome system has been reported in a broad array of human neurodegenerative disorders. Notably in Parkinson's disease, defects in ubiquitination are thought to play a crucial role in generation of the disease state. Parkin, a protein implicated in familial Parkinson's disease, is an E3 Ub ligase that contains a Ub-like domain that interacts in a regulated manner with the UBD of Eps15. This interaction seems to negatively affect EGFR endocytosis and down-regulation and to promote signaling through PI 3-kinase–Akt, because parkin mutant cells show increased EGFR down-regulation (*56*), and this could have an effect on progression of the disease. Parkin has also been found to synthesize Ub^{K63} polyubiquitin chains on proteins, including synphilin-1, and on the modified proteins that are found in Lewy-like inclusion bodies (*57*). Considering the suggested neuroprotective role of inclusion bodies, it is tempting to speculate that Ub^{K63} modification represents a protective route by which unfolded or aggregated proteins could be diverted away from an overloaded proteasomal machinery.

Ubiquitination may also provide an entry point for therapy against previously difficult targets. Myc is a critical transcription factor that is misregulated in tumors but is a difficult chemotherapeutic target because it has no enzymatic activity. Ub^{K63} is added to Myc by the E3 ligase HectH9, and the modification is essential for tumor proliferation (*58*). As an enzyme, HectH9 should be a more suitable target than Myc for chemotherapy.

Conclusions

Ub is a chemically complex molecule that offers a large surface area to interact with other proteins and can form chains with distinct conformations, which makes it a very versatile modification. These properties of Ub make it an ideal regulator of very diverse biological processes. The multiple steps required for protein ubiquitination, the specificity involved, and the process of deubiquitination make it subject to control at many levels. We are only at the beginning of understanding the wide array of Ub functions in cellular processes. Protein ubiquitination is best compared to protein phosphorylation. In both cases, proteins are specifically, diversely, and reversibly modified. The modifications can directly affect activity or conformation but can also act as interaction surfaces to recruit other proteins containing domains that recognize the specific modification. The assemblies then confer function to the modification. Understanding the functions of proteins that recognize ubiquitinated substrates will be the key to understanding the function of Ub in a particular cellular process. Also, because of the similarities between ubiquitination and phosphorylation, it is likely that ubiquitination can positively or negatively affect cellular processes, depending on the particular substrate and the context. Care should thus be taken when comparing experiments performed in different contexts. Finally, the fact that many proteins that are targets for ubiquitination are also phosphorylated increases the complexity of regulation that can be achieved.

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