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A CULLINary ride across the secretory pathway: more than just secretion

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Abstract

Multimeric cullin-RING ubiquitin ligases (CRLs) represent the largest class of ubiquitin ligases in eukaryotes. However, most CRL ubiquitylation pathways remain uncharacterized. CRLs control a myriad of functions by catalyzing mono- or poly-ubiquitylation of target proteins. Recently, novel CRLs have been identified along the secretory pathway where they modify substrates involved in diverse cellular processes such as vesicle coat assembly and cell cycle progression. This review discusses our current understanding of CRL ubiquitylation within the secretory pathway, with special emphasis on the emerging role of the Golgi as a ubiquitylation platform. CRLs are also implicated in endosome function, where their specific roles are less well understood.

Keywords

Cullin proteins; ubiquitylation; Golgi complex; cullin-RING ligases; post-translational modification

Ubiquitylation in the secretory pathway

All proteins are likely subject to post-translational modification (PTM) to regulate their functions. An important class of PTM is ubiquitylation, which involves the covalent attachment of the small protein ubiquitin ($M_r=8.5$ kDa) to a lysine or methionine residue in target proteins. The addition of one or more ubiquitin moieties has different consequences for the substrate. In general, addition of a single ubiquitin or addition of a single ubiquitin to different residues (multi-monoubiquitylation) regulates protein function and trafficking [1]. In contrast, polyubiquitylation signals proteasomal degradation; however, different types of ubiquitin chains, as defined by the Lys or Met residue to which they are attached, serve as alternative signals for different processes [1, 2].

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Protein ubiquitylation is achieved via a series of sequential reactions carried out by three types of enzymes: E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases. The ubiquitylation cascade begins with ATP-dependent activation of ubiquitin mediated by an E1 enzyme, which then transfers it to an E2 enzyme via a transesterification reaction. Subsequently, the charged E2 enzyme is recruited by an E3 ligase, which specifically recognizes and binds to a target protein that ultimately becomes ubiquitylated by the coordinated activities of both enzymes. Thus, ubiquitylation substrate specificity is dictated by E3 ubiquitin ligases. E3 ubiquitin ligases can be divided into two main families: those containing a “homologous with E6-associated C-terminus” (HECT) domain or those containing a so-called RING (Really Interesting New Gene) domain within their structures [3]. HECT domains form a catalytic intermediate with ubiquitin to mediate its direct transfer to the substrate. In contrast, RING domains function as scaffolds that bring a charged E2 enzyme into close proximity with the substrate to transfer activated ubiquitin from it.

While the mammalian genome encodes ~30 HECT E3s, the RING E3 family predominates and includes more than 600 different ligases. Of these, the so-called cullin RING ubiquitin ligases (CRLs) represent the largest class (Box 1; [4]). Recently, novel CRL complexes have been found in association with the secretory pathway; however, some of these ubiquitylated substrates do not appear to have apparent roles in classical secretion. Here we review recent advances in our understanding of CRL ubiquitylation pathways localized at the endoplasmic reticulum (ER), Golgi complex and plasma membrane (PM).

Box 1

Molecular architecture of cullin RING ubiquitin ligases

CRLs are multimeric protein complexes comprised of a cullin scaffold that bridges a C-terminal domain (CTD)-bound, RING E3 enzyme, and an N-terminal domain (NTD)-interacting substrate adaptor module (Figure 1A and B). The seven cullin scaffold proteins (CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7 and CUL9 (formerly PARC [97]) share a common, 150 amino acid-long fold in their CTD. CRL catalytic activity relies on either RBX1 or RBX2 proteins, two RING E3 ligases known to interact with cullins [98]. Substrate specificity is determined by a substrate receptor and, with the exception of CUL3-based CRLs, at least one adaptor protein (Figure I A–C).

Early structural studies predicted a gap of ~50Å between the E2 enzyme, recruited by RBX1/2, and the substrate receptor [99], which made efficient ubiquitylation difficult to explain (Figure I B). Later studies revealed the ubiquitin-like molecule Nedd8 modifies CRLs via neddylation, which induces a major conformational change in the cullin CTD, causing the RBX RING domain to spring out of its interaction with it (Figure I B; [100]). As a consequence, the E2 enzyme is brought closer to the substrate, increasing the probability of ubiquitylation (Figure I B) [101].

Each cullin associates with a different family of substrate receptors (Figure I C). For example, CUL3 specifically binds a Bric-a-brac, Tramtrack-Broad complex (BTB) domain-containing protein [102–104]. In humans the BTB protein family alone provides

over 300 potential substrate receptors [4, 105]. Thus, the combinatorial assembly of CRL complexes accounts for the long and diverse list of substrates for these enzymes [4, 94].

Figure I. Structure of cullin-RING ubiquitin ligases (CRLs). **(A)** Side and front views of the Cul1/Rbx1/Skp1/F-box Skp2 ubiquitin ligase complex depicted as surface and ribbon diagrams. The N- and C-termini are shown; subunit coloring is maintained in both views. Diagrams generated using Protein Workshop visualization tool for the Protein Data Bank entry, RCSB [99]. **(B)** Regulation of CRLs by neddylation. In the unneddylated form of CRLs, the RBX1 RING domain contacts broadly the CTD and is less outwardly exposed (inactive or closed conformation). Consequently, the distance between the ubiquitin (Ubi)-charged E2 enzyme (bound to RBX1) and the substrate is ~50Å, which makes ubiquitylation rather difficult. Upon cullin Neddylation, the CTD undergoes a dramatic reorientation that makes the RBX1 RING domain to spring out (active or open

conformation), diminishing the gap between the ubiquitin E2 and the substrate, thus increasing CRL-mediated ubiquitylation efficiency [99–101]. (C) Table listing cullin scaffolds (green) and respective adaptors (blue) and substrate receptors (grey).

CRLs in transport from the ER to the Golgi

Regulation of cargo secretion by ubiquitylation

Proteins destined for secretion start their journey at the ER. After translation by ER-bound ribosomes and membrane translocation, properly folded cargoes are packaged into nascent COP-II vesicles and transported to the Golgi (Figure 1 A). Cargo export from the ER involves the collection of cargo, cargo receptors, and vesicle coat components into COP-II vesicles at so-called ER exit sites [5]. A longstanding question in protein secretion is how large cargoes such as collagen, which is ~300–400 nm in length, are accommodated in COP-II transport vesicles (typically ~60–80 nm diameter). Recent work suggests that ubiquitylation by CRL complexes may play a role in this process: CUL3 was found to aid collagen secretion from embryonic stem (ES) cells [6]. Specifically, a CUL3-based CRL complex utilizes the BTB protein, KLHL12 to bind and mediate mono-ubiquitylation of the COP-II coat component, Sec31p (Figure 1 A), resulting in a ~5-fold increase in the size of export vesicles and increased collagen export. However, apparent vesicle enlargement was only observed upon KLHL12-overexpression, which also increased endogenous collagen levels. It will be important to demonstrate the presence of collagen in these vesicles to confirm that they are bona fide cargo carriers. It will also be important to determine the mechanism by which ubiquitylation regulates the size of COP-II carriers, to facilitate transport of large cargoes from the ER to the Golgi.

A less well-characterized CRL ubiquitylation pathway has been proposed to play a role in the secretion of another large protein, fibronectin. Fibronectin co-immunoprecipitated with a CRL complex comprised of the substrate receptor, von Hippel-Lindau (VHL), two adaptor proteins, Elongin B and C, and CUL2 [7]. Mice lacking both VHL alleles showed gross defects in fibronectin extracellular matrix deposition. Since fibronectin remains in the lumen of the secretory pathway and VHL is primarily found in the cytosol, it is unlikely that fibronectin interacts directly with VHL. However, a fraction of VHL associates with ER membranes [7], suggesting that VHL could mediate the ubiquitylation of a currently unidentified transmembrane protein in the ER that regulates fibronectin exit. This notion would support the hypothesis that CRL ubiquitylation pathways exert general control over large cargo export from the ER. Another possibility is that CUL2^{VHL} targets misfolded FN as it is retro-translocated to the cytosol by protein quality control mechanisms (see below).

In *Saccharomyces cerevisiae*, the COP-II coat component, Sec23p and the COP-I protein, β ¹-COP (involved in retrograde transport from the Golgi to the ER), undergo polyubiquitylation followed by proteasomal degradation [8, 9]. The yeast ubiquitin protease, Ubp3, forms a complex with Bre5 that removes the initial monoubiquitin moiety added to these coat proteins, thereby rescuing them from degradation [8, 9]. This type of ubiquitylation may enable the cell to regulate tightly, the rates of secretion and retrograde transport in relation to the cell's overall physiological state. Alternatively, the Ubp3/Bre5

complex may distinguish properly assembled COP vesicle components from aggregates of these proteins, and spare their premature destruction.

Defects in COP-II function have been associated with various diseases related to improper extracellular matrix deposition including cranio-lenticulo-sutural dysplasia, a collagen deposition disease [10, 11]. Also, dominant mutations in a single *VHL* allele cause VHL syndrome, in which deletion of the remaining allele results in the development of multiple renal cysts [12]. Furthermore, an abnormal extracellular matrix may be the cause of cyst formation associated with polycystic kidney disease [13]. Nonetheless, it remains to be shown if CRL ubiquitylation pathways play a role in these extracellular matrix-related conditions.

CRL-mediated protein quality control at the ER

Proteins entering the secretory pathway are folded and assembled into oligomeric complexes. Misfolded proteins are recognized by the ER protein quality control machinery and targeted for ERAD. Mis-folded proteins are retro-translocated from the ER lumen (and membrane) back to the cytosol through pore-like structures (“Dislocons”) in a process coupled to ATP hydrolysis-assisted, substrate pulling by the AAA+ ATPase, p97/valosin-containing protein (VCP), which interacts with ubiquitin moieties added to retro-translocated substrates (Figure 1 B; [14, 15]). Once in the cytoplasm, polyubiquitylated mis-folded proteins are degraded by the proteasome [15].

p97/VCP interacts with the neddylated form of most cullins via its adaptor, UBXD7 [16, 17], and this interaction seems required for efficient CRL-mediated, substrate degradation [16]. Thus, p97/VCP appears to couple ER-substrate retro-translocation to CRL-mediated ubiquitylation in the cytoplasm. Although most ERAD substrates are subject to ubiquitylation by non-cullin RING E3 ligases [15], a CUL1-dependent pathway targeting misfolded glycoproteins has been described [18].

The majority of newly synthesized proteins in the ER are N-glycosylated, a PTM that is recognized by lectin-like chaperones in the ER and aids the folding process [19]. After retro-translocation, misfolded glycoproteins are de-glycosylated by the cooperative action of p97/VCP and the N-glycanase NGly1/PNG1 prior to proteasome degradation [20, 21]. In some cases, denatured glycoproteins may fail to be deglycosylated by NGly1. In such cases, the chitobiose core of the oligosaccharides is recognized by the F-box domain-containing proteins, FBXO2 and FBXO6, which target them for polyubiquitylation via a p97/VCP-associated, SCF (SKP1-CUL1-F-box) E3 ligase complex (Figure 1 B) [18, 22, 23]. The role of these CRL complexes in ERAD was demonstrated by the expression of dominant-negative mutants of either F-box protein, which led to the accumulation of typical ERAD substrates [18, 22]. Interestingly, degradation of BACE1, the major β -secretase involved in the generation of amyloid β -peptides in Alzheimer’s disease [24], is mediated by an ER-localized SCF^{FBXO2} complex [25].

Additional, uncharacterized CRL-dependent ERAD pathways may also exist. Evidence for this comes from studies on pathogens that hijack this protein clearance system. The human immunodeficiency virus type 1 transmembrane protein, VPU recruits the host F-box protein,

β -transducin repeat-containing protein 1 (β -TrCP1) to the ER, which in turn, binds and targets its host cell surface receptor CD4 to an SCF-mediated degradation pathway [26], thereby preventing super-infection. Elucidation of the full repertoire of substrates targeted by SCF $^{\beta$ -TrCP1 and other CRL complexes (Table 1) will provide further insight into the role of these ubiquitin ligases in the ERAD pathway.

The Golgi as a platform for cullin-mediated ubiquitylation

Regulation of Golgi structure

Secreted proteins are transported from the ER to the Golgi, en route to the cell surface. The Golgi is comprised of three subcompartments: early (cis), middle (medial) and late (trans), through which secreted cargos traffic sequentially before exiting in transport carriers (Figure 2 A; [27]). Each of these compartments contains distinct sets of proteins and unique lipid compositions; they are stacked together in close apposition by mechanisms only beginning to be understood [27].

Trans-oligomerization of the peripheral Golgi proteins, GRASP55 and GRASP65 appears to be required for Golgi stacking (Figure 2 B) [28]. siRNA-depletion of either GRASP homolog leads to cisternal unstacking. This phenotype is also observed under physiological conditions: during G2 phase of the mammalian cell cycle, GRASPs are phosphorylated, which interferes with their oligomerization, resulting in Golgi stack disassembly [28], a step required before cells can enter mitosis. Ubiquitylation of GRASP proteins was recently reported, but the exact role of this modification is not clear [29]. A Golgi-localized CUL7-dependent pathway mediates ubiquitylation of GRASP65 in neurons [30]. CUL7 is recruited to the Golgi by the cytoskeleton adaptor protein, Obscurin-like 1 (OBSL1). Once on the Golgi, CUL7 forms a CRL complex with the substrate receptor, FBXW8, which engages GRASP65 (but not GRASP55) for polyubiquitylation and proteasomal degradation (Figure 2B). siRNA-depletion of OBSL1, CUL7 or FBXW8 resulted in increased GRASP65 protein levels, Golgi fragmentation, reduced secretion and defects in dendritic arborization, while simultaneous depletion of GRASP65 rescued these phenotypes [30]. In light of these findings, regulation of GRASP65 turnover by ubiquitylation may have important implications for Golgi morphogenesis and protein secretion during dendrite outgrowth [30–32]. Since GRASP65 is a stacking factor, its upregulation would not be expected to trigger Golgi fragmentation [33, 34]. The Golgi may be more sensitive to perturbations in neurons compared with other cell types [35]. Alternatively, efficient dendritic outgrowth may require unconventional secretion that is mediated by GRASP proteins [36–39] and modulated by ubiquitylation.

Other studies further support a role for CRL pathways in Golgi membrane dynamics (Figure 2 A). A genome-wide siRNA screen for genes required for Ricin and Pseudomonas exotoxin cytotoxicity found that loss of Nedd8 or CUL3 leads to Golgi fragmentation; Ricin susceptibility, which requires reverse transport through the Golgi (towards the ER), is also compromised [40]. In addition, RhoBTB3, a member of the RhoBTB family of atypical Rho GTPases, acts together with CUL3 and RBX1 to form a peripherally-associated Golgi complex involved in Golgi structure regulation (Figure 2 A, C) [41]. Either depletion or overexpression of RhoBTB3 disrupts Golgi architecture, suggesting that an uncharacterized

RhoBTB3 substrate is critical for this process. Similar morphological alterations have also been reported upon depletion of the kelch repeat-containing protein KBTBD8 [42], which localizes to the Golgi and is a putative CUL3 substrate receptor (Table 1).

As mentioned earlier, the Golgi undergoes a series of structural changes during the cell cycle. One of the key molecular machineries regulating these changes is p97/VCP, which together with the adaptor proteins p37 and p47, regulates Golgi membrane dynamics during interphase and late mitosis, respectively [43]. Since p97 interacts with and modulates the activity of CRL complexes [16], it is likely that p97 assists in controlling Golgi morphology. Finally, other E3 ubiquitin ligases exist on the Golgi, and in particular, the HECT domain-containing ubiquitin ligase HACE1, is required for Golgi disassembly before mitosis [44]. In summary, a complex network of ubiquitylation pathways is essential for Golgi structure maintenance.

Regulation of protein secretion at the Golgi by ubiquitylation

CRL complexes play an important role in regulating protein secretion at the Golgi. Using a thermosensitive mutant of vesicular stomatitis virus glycoprotein (VSV-G-ts045 [49]), RhoBTB2, another RhoBTB family member that interacts with CUL3 as a putative substrate receptor [45, 46], was found to be required for proper secretory pathway transport [47]. Depletion of RhoBTB2, but not RhoBTB3, leads to accumulation of VSV-G-ts045 in the ER [47, 48]. It is not clear whether RhoBTB2 facilitates the exit of VSV-G-ts045 from the ER or in post-ER events, partly because there are not yet, known substrates for this BTB protein. While RhoBTB2 was reported to localize to perinuclear vesicles [47, 49], another study suggested that it co-localizes and hetero-dimerizes with RhoBTB3 [46], which resides primarily on the Golgi [48]. Further support for a role for RhoBTB2 in vesicle trafficking comes from a microarray analysis that revealed differences in the expression profiles of genes involved in membrane traffic, cell cycle regulation, apoptosis and cytoskeleton remodeling in cells lacking this protein [50].

Vesicle budding and export from the Golgi is aided by local cytoskeleton rearrangements [51]. CUL3 pathways have also been implicated in regulation of cytoskeletal dynamics [52, 53]. It is noteworthy that several Golgi-localized BTB proteins contain kelch motifs that mediate interactions with the cytoskeleton, including gigaxonin, LZTR-1 and KBTBD8 (Table 1). Gigaxonin regulates the turnover of several cytoskeletal components via a CUL3-mediated ubiquitylation pathway [54, 55]. Loss-of-function mutations in gigaxonin lead to aberrant accumulation of different microtubule-associated and intermediate filament proteins, a hallmark of giant axonal neuropathy [54, 55]. However, it is unclear whether modification of any of these cytoskeletal substrates takes place on Golgi, since several studies have failed to confirm Golgi localization of gigaxonin as originally described [56]

Unconventional cell cycle control

In addition to their roles in regulating the structure of the Golgi complex and transport from the ER to this compartment, RhoBTB proteins regulate the cell cycle and have been proposed to function as tumor suppressors [57]. Mutations in and down-regulation of *RhoBTB* genes have been described in a variety of tumors [46, 57]. Yet the precise

mechanisms by which these proteins regulate cell proliferation remain unknown. Depletion of RhoBTB2 results in elevated Cyclin D levels [58], a phenotype frequently observed in cancer [59]. Whether RhoBTB2 plays a direct role in Cyclin D turnover remains to be determined. It was recently shown that during S-phase of the mammalian cell cycle, RhoBTB3 targets unphosphorylated Cyclin E for ubiquitylation via a Golgi-associated CUL3-dependent pathway (Figure 2 C, [41]), which seems to co-exist with a major SCF pathway that targets phosphorylated Cyclin E within the nucleolus [60, 61]. Deregulation of either of these pathways increases Cyclin E levels and triggers abnormal cell cycle progression, an oncogenic hazard for the cell [62]. Golgi localization is required for RhoBTB3-mediated Cyclin E turnover [41]. Since the Golgi is closely associated with the centrosome, CUL3^{RhoBTB3} may serve to locally regulate centromeric Cyclin E [63]. It is particularly intriguing that RhoBTB3, which is also required for the transport of mannose 6-phosphate receptors from late endosomes to the TGN [48], controls cell cycle progression as well as Golgi structure homeostasis. Identification of the full range of substrates of the RhoBTB family should provide important clues underlying protein coordination during membrane trafficking and cell cycle regulation.

Dividing cells need to increase their net cell surface area to reach a cell size threshold before entering S phase [64]. During G1, Golgi-mediated secretion provides de novo membrane supply, which is rate limiting for cell growth [65]. Therefore, it is likely that secretion is somehow coupled to cell cycle progression. Golgi resident proteins like RhoBTB3 may function as a cell size threshold sensor before targeting Cyclin E and other Golgi substrates.

Doubling of membrane in proliferating cells requires phospholipid synthesis, which is regulated by enzymes such as CTD:phosphocholine cytidyltransferase (CCT α) in close relationship with the Golgi [66, 67]. CCT α plays a key role in phosphatidylcholine production, a precursor needed for production of membranes and pulmonary surfactant. It has been reported that *Pseudomonas aeruginosa* infection of lung epithelial cells triggers calcium influx that activates a Golgi-localized SCF-pathway that consequently inhibits surfactant production by targeting CCT α . On the Golgi, the CUL1 substrate receptor FBXL2 mediates CCT α monoubiquitylation (Figure 2 D) [68]. Overexpression of FBXL2 decreased CCT α protein levels, which were restored upon treatment with lysosomal but not proteasomal inhibitors, suggesting that monoubiquitylation targets CCT α for lysosomal-mediated degradation [1, 69]. FBXL2 also interacts with Calmodulin, and this interaction antagonizes FBXL2-mediated downregulation of CCT α . Since both Calmodulin binding to FBXL2 and CCT α ubiquitylation are Ca²⁺-dependent, intracellular Ca²⁺ elevation may regulate ubiquitylation of CCT α by this pathway. Important molecular clues to these events may be obtained by vitro reconstitution of CCT α ubiquitylation by recombinant SCF^{FBXL2} in the presence or absence of Calmodulin and Ca²⁺.

FBXL2 contains a unique, lipidated CAAX motif required for membrane anchorage [70]. On membranes, it engages ubiquitylation substrates, such as CCT α or the PI(3)K regulatory subunit p85 β [68, 71]. At least some FBXL2 is also targeted to other compartments, such as the centrosome or the midbody, to mediate ubiquitylation of specific substrates during mitosis [72, 73]. Phosphorylation of a number of peripherally-associated Golgi proteins triggers their release from membranes during mitosis, when the Golgi undergoes

fragmentation. It will be interesting to test whether this CRL substrate receptor shows similar cell cycle dependent regulation and how this impacts phosphatidylcholine production that is ultimately needed for cell growth [66].

In summary, the Golgi does much more than just drive secretion; indeed, it plays an important role controlling the mammalian cell cycle [74]. Since multiple ubiquitylation pathways, including CRL-mediated pathways, are essential for various aspects of cell cycle control, the characterization of Golgi-localized CRL complexes represents an important area for future research.

A Golgi-associated CRL pathway in *Xenopus* development

In *Xenopus*, a Golgi-localized CUL5 complex controls Wnt signaling, which is essential for embryogenesis [75]. The *Xenopus* homolog of the small GTPase, Rab40 (XRab40) localizes to the Golgi, where it binds CUL5 and adaptor proteins, Elongin B and C (Figure 2 E). Rab40 differs from other Rab family members in that it contains a C-terminal SOCS-box that interacts with a cullin scaffold and adaptors and is required for Golgi recruitment. Silencing of XRab40 led to gastrulation defects, and rescue experiments revealed that Golgi targeting is required for proper XRab40 function. The Rap2 small GTPase that is important for Wnt signaling [76] was identified as an XRab40 substrate. Rather than targeting Rap2 for proteasomal degradation, polyubiquitylation was shown to regulate the recruitment of Rap2 to the plasma membrane where it exerts its function [75, 76]. Accordingly, a ubiquitylation-deficient mutant of Rap2 was unable to recruit the effector protein Misshapen/Nck-interacting kinase (XMINK), which is required for normal development [75, 77], to the plasma membrane. In cells depleted of XRab40 or CUL5, XMINK and its binding partner Dishevelled, a key downstream molecule in the Wnt signaling pathway [78], were unable to localize to the plasma membrane, further implying a role for this Golgi-associated CRL in regulating key signaling events in *Xenopus* development.

Rab GTPases are master regulators of membrane trafficking [79], and it is surprising that XRab40 has evolved to localize Cul5. It is not yet known whether the mammalian homolog of XRab40 plays a similar role during development. If so, it would be unlikely to occur on the Golgi, since none of the mammalian Rab40 isoforms studied so far localize to this compartment [80–82].

CRL-pathways at the plasma membrane: a role in receptor-mediated endocytosis

Secretion terminates at the plasma membrane, where exocytic vesicles deliver their content to the cell surface (transmembrane receptors) or outside the cell (secreted cargoes). In contrast, endocytosis involves the internalization of membrane and solutes from the plasma membrane and extracellular space. To date, the majority of plasma membrane-associated ubiquitylation events are involved in endocytosis, and in some cases are catalyzed by CRLs.

Upon interferon- α (IFN α) stimulation, the type I IFN receptor subunit, IFN α R1 undergoes phosphorylation, which facilitates its interaction with the SCF substrate receptor, β -TrCP2, also named Homolog of Slimb (HOS) [83]. SCF β -TrCP2-mediated ubiquitylation of IFN α R1

resulted in its reduced total and cell surface expression. Abrogation of β -TrCP2-assisted ubiquitylation or inhibition of lysosomal function increased the stability of IFN α R1 and IFN α -associated downstream signaling. These results suggest that SCF $^{\beta}$ -TrCP2 regulates type I IFN receptor endocytosis and downregulation via lysosomal degradation (Figure 3 A). A genetic screen based on accumulation of glutamate receptors (GluRs) at postsynaptic membranes in *Caenorhabditis elegans* revealed a potential CUL3 pathway involved in receptor-mediated endocytosis [84]. Mutations of the BTB-protein, KEL-8 impaired ubiquitin-dependent turnover of the GLR-1 subunit of AMPA-type GluRs, which consequently accumulated at the cell surface. Furthermore, *kel-8* mutants had a higher frequency of a locomotive behavior associated with signaling through this receptor. KEL-8 binds CUL3 and localizes to adjacent GLR-1-positive regions of the plasma membrane. Although no interaction between KEL-8 and GLR-1 was reported, it was suggested that a CUL3^{KEL8} pathway targets GLR-1 for ubiquitylation to promote its endocytosis and lysosomal degradation. CUL3 is activated at the plasma membrane by the Nedd8-E3 ligase, defective in Cul1 neddylation 1 (DCN1)-like 3 (DCNL3) [85]. Therefore, after glutamate-induced activation of AMPA receptor, DCNL3 may be locally recruited to activate CUL3^{KEL8}, which could then mediate GLR-1 ubiquitylation (Figure 3 B).

In *Arabidopsis*, a plasma membrane-localized CRL pathway controls endocytosis to modulate an adaptive response to changes in the quantity of light. Low-intensity blue light stimulates a CUL3 pathway in which the substrate receptor, NPH3, targets the photoreceptor, phot1 for mono-/multi-monoubiquitylation and subsequent clathrin-mediated endocytosis. In contrast, exposure to high-intensity blue light induces CUL3^{NPH3}-mediated polyubiquitylation of phot1 followed by proteasome degradation [86]. In this case, mono-/multi-monoubiquitylation may permit endocytosed phot1 to recycle back to the plasma membrane, while polyubiquitylation may represent desensitization by destroying phot1. Other examples of CUL3's influence on the endocytic pathway are summarized in Box 2.

Box 2

Regulation of endosomal traffic and maturation by CUL3

Recent work suggests that CUL3-mediated ubiquitylation may regulate trafficking along the endosomal pathway [90]. Cells devoid of CUL3 show defects in the endocytic transport of influenza A virus (IAV) and epidermal growth factor receptor (EGFR) [106]. Upon CUL3-depletion, infection of IAV but not VSV, decreases dramatically. CUL3-mediated ubiquitylation appears to be needed for IAV penetration from late endosomes and/or subsequent viral uncoating. Moreover, EGFR degradation was significantly delayed in CUL3-depleted cells that also showed altered LE and lysosome morphology. The BTB domain-substrate receptors involved in CUL3-endosomal transport remain unidentified. Rabankyrin is a Rab5 effector with an N-terminal BTB domain that promotes endosomal fusion [107]. The precise role of Rabankyrin's BTB domain is not yet known. Finally, RhoBTB3 is an established CUL3 substrate receptor required for transport of mannose 6-phosphate receptors from LEs back to the TGN [48]. CUL3 endocytic substrates have yet to be identified. Ubiquitylation regulates endosomal sorting of internalized transmembrane cargos such as EGFR [108]. The ESCRT machinery

recognizes specific ubiquitin signatures in ubiquitylated cargoes to target these into the lumen of LEs [108]. Thus, CUL3 likely contributes to ubiquitylation of cargoes to be sorted into late endosomal intraluminal vesicles. Alternatively, CUL3-mediated monoubiquitylation could also modulate the activities of certain ESCRT components, as previously described [109, 110]. Further studies will be necessary to define the exact role of CUL3-mediated ubiquitylation in the endocytic pathway.

Regulation of Arf6 activation by ubiquitylation

Arf small GTPases regulate membrane traffic, cytoskeleton remodeling and cell migration [87]. While most Arfs are located on the Golgi, the most divergent family member, Arf6, is located on the inner leaflet of the plasma membrane. There, GTP-bound Arf6 functions in actin remodeling, secretion and tumor invasion [87]. The SCF substrate receptor, FBXO8 binds Arf6 via its sec7 domain [88] and localizes to the plasma membrane via its sec7 and F-box domains. SCF^{FBXO8} mediates polyubiquitylation of Arf6 (but not other Arf GTPases), and this modification interferes with the interaction between Arf6 activator and effector proteins. Down-regulation of this CRL pathway may correlate with increased tumor invasion and metastasis [88–90]. Other Arf isoforms are also ubiquitylated [29], but whether those are CRL-mediated is not known. It will be interesting to explore the potential role of ubiquitylation in regulating Golgi-associated Arf isoforms.

Concluding Remarks

Discoveries in the secretory pathway have led to multiple Nobel prizes [91, 92]. Nevertheless, much remains to be discovered regarding the function of compartments that comprise this pathway. Beyond secretion, multiple secretory pathway-associated CRL ubiquitylation machines control an array of cellular processes, of which many remain uncharacterized (Table 1). Furthermore, additional CRL complexes and pathways may exist [93, 94]. Indeed, global surveys have demonstrated the ubiquitylation of a large number of proteins known to regulate the structure and function of secretory organelles [29, 94]; many of these are likely CRL substrates. What upstream signals and molecular mechanisms regulate CRL function and how are they localized? Does secretory pathway activity influence CRL ubiquitylation pathways? Recently, the first pharmacological intervention to inhibit a specific CRL complex was reported [95]. Since CRL enzymes are implicated in multiple pathological processes, development of additional CRL-specific drugs will surely impact both the laboratory and the clinic.

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Highlights

Certain Cullin-RING ubiquitin ligases (CRLs) are localized to the secretory pathway

At the ER, CRLs modify vesicle coat proteins and may aid large cargo export

CRLs aid ER-associated destruction of misfolded proteins

Unidentified CRL substrates regulate maintenance of normal Golgi structure

CRLs may regulate secretory flux, cell cycle progression and Wnt signaling

Figure 1.

Regulation of endoplasmic reticulum functions by CRLs. **(A)** CRL-mediated control of large cargo secretion. CUL3^{KLHL12} complex binds and ubiquitylates the COP-II coat component Sec31p [6]. This PTM is proposed to increase the size of COP-II vesicles, enabling large cargo such as collagen (~300 nm) to fit inside. **(B)** A CRL-dependent ERAD pathway. Misfolded glycoproteins are translocated from the ER lumen to the cytoplasm via the cooperative roles of the dislocon complex and p97/VCP [14, 15], which interacts with ubiquitin moieties on the substrate to pull it out of the ER lumen. In the cytoplasm, some denatured glycoproteins fail to undergo deglycosylation by NGly1. In such cases a p97/VCP-associated SCF^{FBXO2/6} pathway engages these substrates and targets them for further ubiquitylation followed by proteasomal degradation [18, 22, 23].

Figure 2.

The Golgi as a CRL ubiquitylation platform. **(A)** Regulation of Golgi structure by CRLs. At steady state, the Golgi exists as a ribbon-like structure comprised of laterally linked stacks of trans, medial and cis cisternae (left). Secreted cargo exits the Golgi in post-Golgi transport vesicular carriers. The Golgi ribbon fragments into mini-stacks upon siRNA-depletion of different CRL components (shown at right) [30, 40–42]. Confocal micrographs ©Lu and Pfeffer, 2013. Originally published in *J. Cell Biol.* doi: 10.1083/jcb.201305158 [41]. **(B)** In neurons, Golgi structure and dendritic outgrowth are regulated by a CUL7^{FBXW8} complex that targets the Golgi stacking factor, GRASP65, for polyubiquitylation and proteasomal degradation [30]. The cytoskeleton adaptor protein OBSL1 links this CRL to the Golgi. **(C)**

In mammalian cells, a CUL3^{RhoBTB3} ubiquitylation pathway regulates turnover of free Cyclin E. How RhoBTB3 associates with Golgi membranes is not yet known [41]. **(D)** In mammals, an SCF^{FBXL2} pathway regulates surfactant production by controlling turnover of CCT α , a key enzyme in surfactant biosynthesis [68]. Intracellular Ca²⁺ elevation promotes SCF^{FBXL2}-mediated CCT α ubiquitylation. On the other hand, Ca²⁺ also mediates an interaction between calmodulin (CaM) and FBXL2, which interferes with CCT α ubiquitylation. Finally, Golgi association of SCF^{FBXL2} is likely mediated by FBXL2, which contains a lipid modification that enables membrane association [70]. **(E)** In *Xenopus*, Rab40 is a substrate receptor for a Golgi-localized CUL5-based CRL complex that modulates Rap2 localization and function by ubiquitylation [75]. This pathway has important implications for *Xenopus* embryogenesis. Note that both XRab40 and XRap2 are small GTPases that interact with membranes via their lipidated C-terminal CAAX motifs [75, 96]. Golgi localization of XRab40 also relies on its association with the CUL5 complex [96].

Figure 3.

Regulation of receptor-mediated endocytosis by CRL pathways. **(A)** Upon IFN α stimulation, the type I IFN receptor subunit IFN α R1 is phosphorylated and downstream signaling occurs [83]. Phosphorylated-IFN α R1 interacts with β -TrCP2, which targets it to an SCF-mediated ubiquitylation pathway [83]. Ubiquitylated type I IFN receptor undergoes endocytosis and, ultimately, lysosomal degradation. **(B)** In *C. elegans*, the GLR-1 subunit of AMPA-type glutamate receptors (AMPA) undergoes clathrin-mediated endocytosis upon ubiquitylation by a CUL3^{KEL8} pathway [84]. It is unclear whether upon glutamate stimulation, AMPAR signaling leads to plasma membrane translocation of the CUL3-neddylating enzyme DCNL3 (red question mark). Once at the plasma membrane, DCNL3 mediates neddylation of CUL3 complexes (i.e. CUL3^{KEL8}) [85], thus enhancing ubiquitylation of corresponding substrates (i.e. GLR-1).

Table 1

Cullin RING-ubiquitin ligases in the secretory pathway

Compartment	Substrate receptor	Cullin	Substrate	Organism	References
ER	FBXO2	CUL1	Misfolded Glycoproteins, BACE1	mammals	[18, 22–24]
	FBXO6	CUL1	Misfolded Glycoproteins	mammals	[18, 22, 23]
	FBXW7 ^β	CUL1	?	mammals	[111]
	KLHL12	CUL3	Sec31	mammals	[6]
	β-TTCP1 ^a	CUL1	CD4	mammals	[26]
	pVHL	CUL2	?	mammals	[7]
Golgi	FBXL2	CUL1	CCT α	mammals	[68]
	FBXW8	CUL7	GRASP65	mammals	[30]
	Gigaxonin ^a	CUL3	MAPIB, vimentin, peripherin	mammals	[55, 56]
	KBTBD8	CUL3 ^c	?	mammals	[42]
	KCTD5 ^a	CUL3	GRASP55 ^b	mammals	[112, 113]
	LZTR-1	CUL3	?	mammals	[114]
	PRAME	CUL2	?	mammals	[115]
	XRab40	CUL5	XRap2	<i>Xenopus l.</i>	[75]
	RhoBTB2 ^a	CUL3	Cyclin D1 ^b	mammals	[45, 46, 58]
	RhoBTB3	CUL3	Cyclin E1	mammals	[41, 46]
Plasma membrane	FBXO8	CUL1	Arf6	mammals	[88]
	KEL-8	CUL3	GLR-1	<i>C. elegans</i>	[84]
	NPH3	CUL3	Phototropin 1	<i>Arabidopsis t.</i>	[86]
	β-TTCP2 ^a	CUL1	IFN α R1	mammals	[83]

^aLocalization unclear

^bUndetermined putative substrate

^cUndetermined putative cullin partner