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UBIQUITINATION AND THE REGULATION OF MEMBRANE PROTEINS

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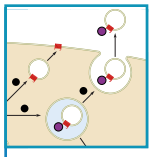

Foot N, Henshall T, Kumar S. Ubiquitination and the Regulation of Membrane Proteins. *Physiol Rev* 97: 253–281, 2017. Published December 7, 2016; doi:10.1152/physrev.00012.2016.—Newly synthesized transmembrane proteins undergo a series of steps to ensure that only the required amount of correctly folded protein is localized to the membrane. The regulation of protein quality and its abundance at the membrane are often controlled by ubiquitination, a multistep enzymatic process that results in the attachment of ubiquitin, or chains of ubiquitin to the target protein. Protein ubiquitination acts as a signal for sorting, trafficking, and the removal of membrane proteins via endocytosis, a process through which multiple ubiquitin ligases are known to specifically regulate the functions of a number of ion channels, transporters, and signaling receptors. Endocytic removal of these proteins through ubiquitin-dependent endocytosis provides a way to rapidly downregulate the physiological outcomes, and defects in such controls are directly linked to human pathologies. Recent evidence suggests that ubiquitination is also involved in the shedding of membranes and associated proteins as extracellular vesicles, thereby not only controlling the cell surface levels of some membrane proteins, but also their potential transport to neighboring cells. In this review, we summarize the mechanisms and functions of ubiquitination of membrane proteins and provide specific examples of ubiquitin-dependent regulation of membrane proteins.

I. INTRODUCTION

All cells contain a large number of membrane proteins, including signaling and recognition receptors, ion transporters and channels, structural proteins, and enzymes. Some of these proteins are integral to the membrane, such as the α-helical transmembrane (TM) proteins, whereas others interact with the membrane peripherally through various types of anchors (reviewed by Cournia et al., Ref. 56). Here we will largely focus on ubiquitin-mediated regulation of TM proteins, which are estimated to be ~20% of a typical cellular proteome (56).

Ubiquitination (or ubiquitylation) is one of the most common forms of posttranslational protein modification, with thousands of proteins targeted for ubiquitination at some time during their life (53, 128). Ubiquitin, as the name suggests, is a highly conserved ubiquitously expressed protein of ~8 kDa (76 amino acids) which, during the process of ubiquitination, is attached in single or multiple chains to the target protein in a multistep ATP-dependent manner (53, 128). Powerful mass spectrometric analyses have identified ~20,000 ubiquitination events, with many cellular proteins ubiquitinated at multiple sites (171, 360). Although initially identified as a signal for degradation by the 26S proteasome of proteins which are damaged, misfolded, or no longer required, ubiquitination is now known to be important for protein localization, trafficking, and recognition by signaling or regulatory complexes, thus affecting all aspects of cellular signaling and homeostasis (103, 265). Ubiquitination is critical in determining the trafficking, abundance, and localization of many TM proteins, thereby directly regulating their physiological functions. In this review, we will describe the roles of ubiquitination in the life cycle of membrane proteins; how ubiquitination maintains quality control at the ER and Golgi, how it regulates the transport of newly synthesized mature proteins to the membrane, and finally, how ubiquitination regulates membrane proteins via endocytic and exocytic pathways **(FIGURE 1)**. We will also discuss how dysfunction in the ubiquitin pathway can lead to disease and how it may be used for therapeutic intervention.

II. THE UBIQUITINATION MACHINERY

Ubiquitin attachment involves the formation of an isopeptide bond between the COOH-terminal glycine in ubiquitin and the ε-amino group of lysine residues in substrates. The

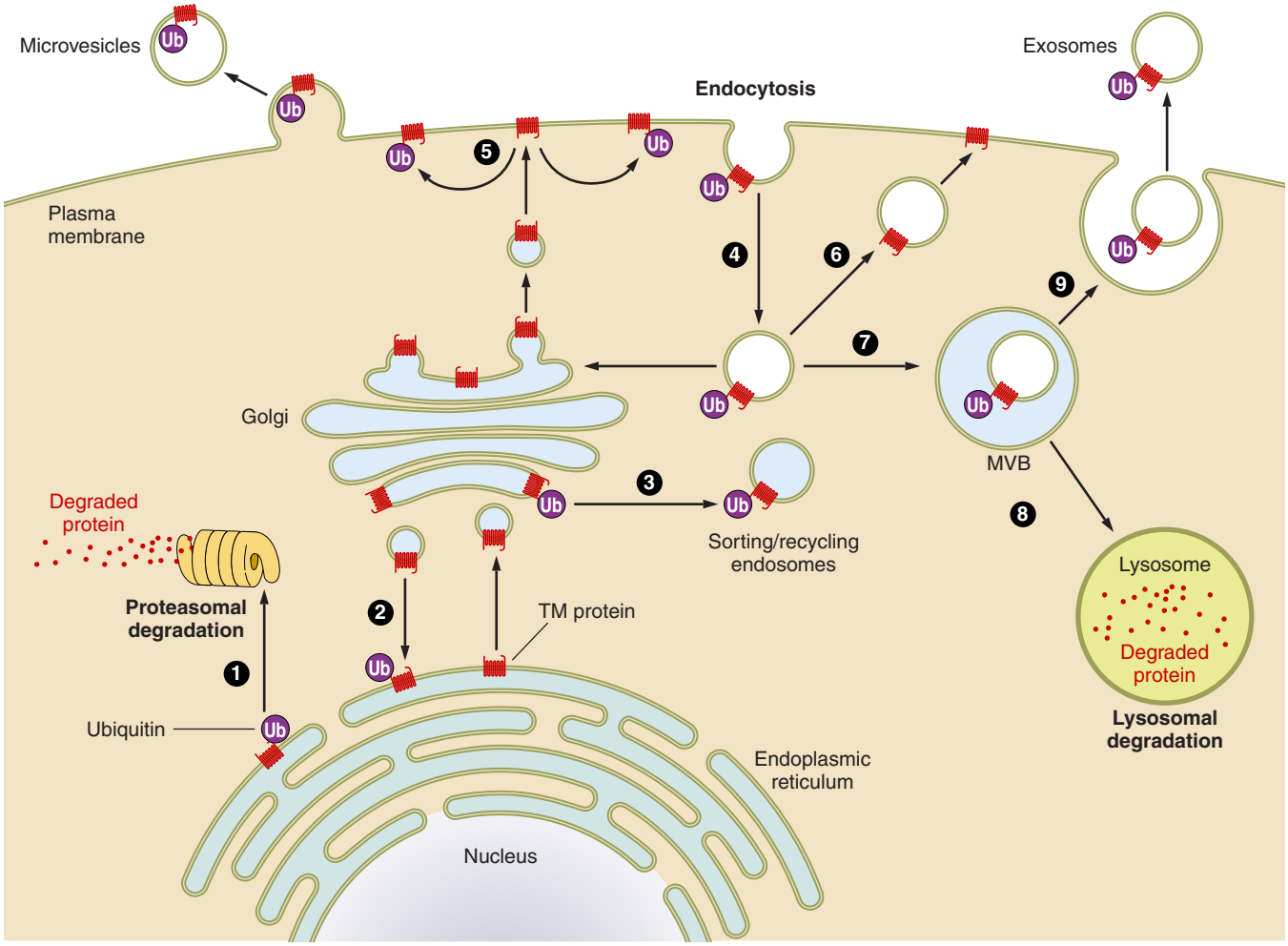

FIGURE 1. The role of ubiquitin in the life cycle of a membrane protein. Membrane proteins are first synthesized at the endoplasmic reticulum, where they are subjected to endoplasmic reticulum quality control (ERQC). Misfolded or unrequired proteins are recognized by ERAD and ubiquitinated to target them for proteasomal degradation (*1*). Once they pass ERQC, they are trafficked to the Golgi, where the second quality control system is present. Misfolded proteins that reach the Golgi after escaping ER quality control may be recognized and sent back to the ER via retrograde transport of coat protein I (COPI) vesicles (*2*), or can be ubiquitinated at the Golgi and targeted to endosomes for lysosomal degradation (*3*). If they pass the Golgi quality control system, proteins are trafficked to their required destination (in this case the plasma membrane) where they can perform their function. Once no longer required, membrane proteins are ubiquitinated, and this targets them for internalization (*4*). Alternatively, the ubiquitin signal can target the substrate to be released directly from the plasma membrane via microvesicles (*5*). Internalized proteins can either be deubiquitinated and recycled back to either the plasma membrane or the Golgi (*6*), or sent to the MVB for lysosomal degradation (*7*). Once in the MVB, ubiquitinated proteins are either degraded by the lysosome (*8*) or released into the extracellular space as exosomes (*9*).

entire process leading to ubiquitination is carried out in a number of steps **(FIGURE 2)**; the initial step involves activation of ubiquitin mediated by the ubiquitin-activating enzyme (E1), which links glycine 76 of ubiquitin to a cysteine residue in the E1 via a thioester bond (53, 128). Then, ubiquitin is transferred to the cysteine residue of a ubiquitin conjugating enzyme (E2; **FIGURE 2*A***). In the final step, a ubiquitin ligase (E3) facilitates the transfer of ubiquitin from the charged E2 to an internal lysine residue in the substrate **(FIGURE 2*B*)**. Substrates can be monoubiquitinated or polyubiquitinated at one or more lysine residues (**FIGURE 2*C***). Ubiquitin can also be removed from ubiquitinated proteins through the action of deubiquitinatinating enzymes (DUBs) (177). There are over 1,000 proteins involved in the ubiquitin pathway, including over 600 E3s and ~100 DUBs.

There are seven lysine residues in ubiquitin (K6, K11, K27, K29, K33, K48, K63), and each of these lysine residues can form isopeptide bonds with the COOH terminus of other ubiquitin molecules (335). Linear chains of varying combinations of ubiquitin linkages can therefore form and lead to different functional outcomes. For example, K48-linked chains are most common and lead to proteasomal degrada-

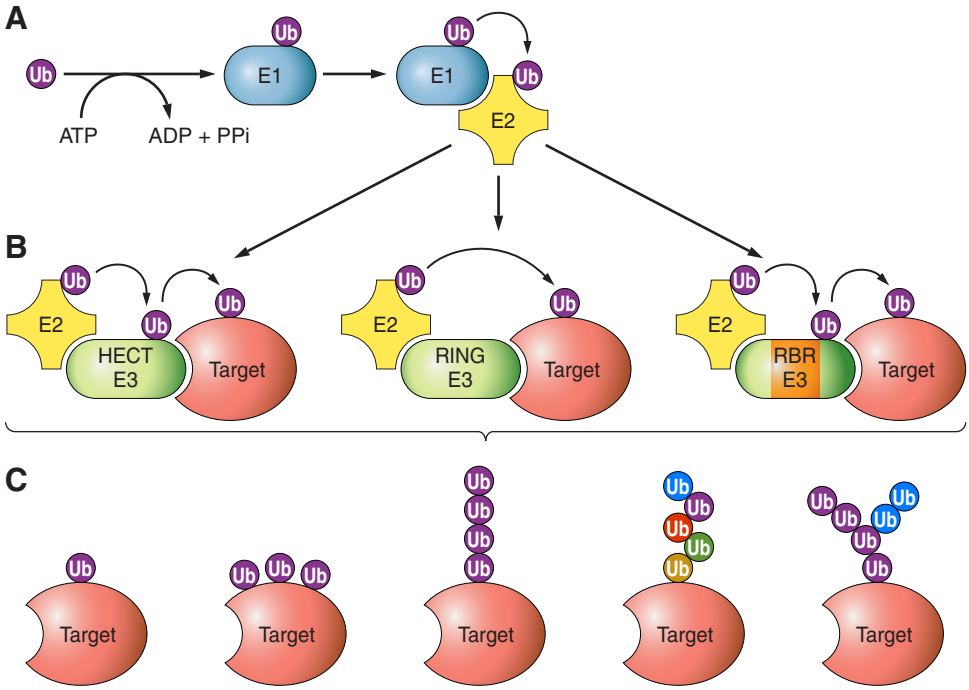

FIGURE 2. The ubiquitination cascade. *A:* ubiquitination begins with the activation of ubiquitin in an energy-dependent manner by the ubiquitin-activating enzyme (E1). Activated ubiquitin is then transferred to the ubiquitin conjugating enzyme (E2). *B:* in a final step, a ubiquitin ligase (E3) catalyzes the transfer of ubiquitin to the target substrate. There are three known families of E3s: HECT E3s, which first accept ubiquitin onto a catalytic cysteine residue, then transfer the ubiquitin to the substrate; RING E3s, which facilitate the transfer of ubiquitin directly from the E2 to the substrate; and RBR E3s, which function like RING/HECT hybrids in that they bind E2s via a RING domain, but transfer ubiquitin to a catalytic cysteine in their RING2 domain before subsequent transfer to the substrate. *C:* substrates can be modified in a variety of ways including monoubiquitination, multi-monoubiquitination, homotypic polyubiquitination, heterotypic polyubiquitination, and branched [see Swatek and Komander (335) for a comprehensive review of ubiquitin modifications].

tion of proteins, whereas substrates with K63 chains often undergo nonproteasomal fates. Recent discoveries that ubiquitin can also undergo phosphorylation, acetylation, and modification by the attachment of ubiquitin-like proteins, such as SUMO, suggest that this process and its regulation are far more complex than initially envisaged (168, 182, 333, 335, 367). The myriad of modifications, often termed the "ubiquitin code" (283), along with the large number of E3s and DUBs with different specificity and affinity for ubiquitin attachment and removal, give rise to vast complexity in the way ubiquitination can control cellular signaling and protein fate.

The E3s provide the substrate specificity to the ubiquitination process, and as such, they are the largest group of proteins involved in the ubiquitin system (61, 286, 318). These enzymes can be grouped into three families based on the presence of specific domains, as well as the mechanisms they utilize to ubiquitinate the substrate proteins (**FIGURE 2*B***). The HECT E3s (29 encoded by the human genome) contain the HECT (homologous to E6-AP COOH terminus) domain and use a two-step process to attach ubiquitin: first, by accepting the ubiquitin onto a catalytic cysteine, and second, transferring it to the substrate. The RING E3s constitute the largest family (over 600 in the human genome) and are characterized by the presence of a RING (Really Interesting New Gene) domain. These ligases act as a scaffold, binding to a charged E2 and facilitating the transfer of ubiquitin from the E2 directly to the substrate. The third group, the RBR (RING-between-RING) E3s (13 in humans), mediate ubiquitin transfer in a two-step process similar to the HECT E3s (318).

The roles of various E3s and the TM proteins they target for ubiquitin-mediated regulation are discussed within this article and summarized in **TABLE 1**. We will follow the ubiquitin-mediated regulation of newly synthesized membrane proteins in the secretary pathway, at the ER and Golgi, at the plasma membrane, and through endocytosis and extracellular vesicles. We will also discuss the pathological consequences of misregulated ubiquitination and the promising new therapies that are emerging to target the ubiquitin pathway.

III. REGULATION IN THE SECRETORY PATHWAY: ENDOPLASMIC RETICULUM AND THE GOLGI

Under normal conditions, transmembrane proteins are synthesized by the endoplasmic reticulum (ER), packaged into

TABLE 1. *List of E3 ligases and their transmembrane substrates*

Ligase	Substrate	Function	Location	Associated Pathology	Reference Nos.
		RING E3s			
AMFR (gp78, RNF45, Doa10p-yeast)	KAI1, STING, CFTR, CD3δ	An integral ER membrane protein within the VCP/p97-AMFR/gp78 complex and functions in ER-associated degradation (ERAD). Promotes tumor metastasis through ubiquitination of the metastasis suppressor, KAI1, and regulates innate immunity through ubiquitination of STING.	Endoplasmic reticulum	Cancer, tumor metastasis	349, 364
Cbl (RNF55)	EGFR, IGF1R, PDGFRA/B, CSF1R, c-KIT, FLT1, VEGFR-1, FGFR1/2, TrkA, c-MET, EPHA8, KDR, EPHB1, RET, Notch, Ron	Ubiquitinates numerous activated RTKs, such as EGFR, T-cell and B-cell receptors, as well as integrin receptors. Cbl proteins play an important role in T-cell receptor signaling pathways.	Cytoplasm, cell membrane	Cancer, acute myeloid leukemia, cystic fibrosis, Noonan syndrome-like disorder with or without juvenile myelomonocytic leukemia	71, 81, 141, 150, 194, 220, 229, 263, 345
CBLL1 (Hakai, RNF188)	E-cadherin	Ubiquitinates the phosphorylated form of E-cadherin, causing its endocytosis and degradation and loss of cell-cell adhesions.	Nucleus, nucleoplasm	Modulation of cell adhesion and regulation of epithelial-mesenchymal transitions in development or metastasis	93
MARCH-I (RNF171)	TFRC, FAS, CD86, CD98, MHC-II, proteins including HLA-DRα/β	Ubiquitinates MHC class II proteins on antigen presenting cells and downregulates their cell surface expression.	Golgi, lysosome, endosome, cell membrane, cytoplasmic vesicle		16, 79, 148, 313
MARCH-II (HSPC240, RNF172)	TFRC, CD86	Regulates vesicle trafficking by association with syntaxin6. Promotes endocytosis and degradation of TFRC and CD86.	Endoplasmic reticulum, endosome, lysosome		16, 243
MARCH-III (MGC48332, RNF173)		Associates with Syntaxin6 in the endosomes and helps to regulate vesicle trafficking.	Cytoplasmic vesicle, early endosomes		94
MARCH-IV (KIAA1399, RNF174)	MHC class I, CD4	Ubiquitinates MHC class I proteins and downregulates their cell surface expression.	Golgi		17
MARCH-VI (TEB4, RFN176)	MARCH-VI	Localizes to the endoplasmic reticulum and participates in ER-associated protein degradation.	Endoplasmic reticulum		121, 183
MARCH-VII (Axotrophin, RNF177)	gp190	Plays a role in LIF signaling in T lymphocytes through degradation of the LIF-receptor subunit gp190.			84, 248, 336
MARCH-VIII (c-MIR, RNF178)	MHC class II, CD86, CD98, TRAIL-R1	Causes the ubiquitination/degradation of CD86 and MHC class II proteins, affecting antigen presentation. Also plays a role in trafficking and recycling of proteins after clathrin-independent endocytosis.	Cytoplasmic vesicle, endosomes, lysosomes		16, 79, 255, 354
MARCH-IX (RNF179, FLJ36578)	MHC class I, CD4, ICAM-1	Ubiquitinates MHC class I proteins and downregulates their cell surface expression.	Lysosome, Golgi		17, 136, 235

Continued

Table 1.—*Continued*

Ligase	Substrate	Function	Location	Associated Pathology	Reference Nos.
MDM2	IGF-IR, β2-AR	Ubiquitinates p53. Its interaction with p53 sequesters MDM2 from another substrate, IGF-IR, which regulates cell growth. Ubiquitinates β-arrestin2, which then mediates the internalization of GPCRs, such as β2-AR.	Cell membrane, nucleoplasm, nucleolus, and cytosol		102, 123
MIB1	Dll, Jagged	Facilitates the ubiquitination and subsequent endocytosis of the Notch ligands, Dll and Jagged.	Cell membrane, cytoplasm		47, 50, 147, 156
MIB2	Dll, Jagged	Positively regulates ligand-mediated Notch signaling by ubiquitinating the intracellular domain of Dll, leading to endocytosis of Dll receptors. MIB2 ubiquitinates NMDAR subunits to help regulate synaptic plasticity in neurons.	Cell membrane, cytoplasm, endosome	Multiple myeloma	37, 47, 50, 147, 156
Neurl1	Jagged1	Monoubiquitinates Jagged1 (in vitro), thereby regulating the Notch pathway. Acts as a tumor suppressor by inhibiting malignant cell transformation of medulloblastoma cells by inhibiting the Notch signaling pathway.	Cytoplasm, cell membrane, postsynaptic membrane		181, 344
Neurl2	Dll1	Stimulates Dll1 internalization, localization into Hrs-positive vesicles, and recycling to the apical plasma membrane of polarized cells.	Cytoplasm		20, 322
NRDP1 (RNF41)	IL-3, EPO, ErbB3	Involved in the control of hematopoietic progenitor cell differentiation into myeloerythroid lineages by maintaining basal levels of cytokine receptors. Ubiquitinates an EGFR homolog, ErbB3, in a ligand (neuregulin)-independent manner, but this ubiquitination is augmented by neuregulin stimulation.	Cytoplasm		34
Parkin (Park2)	VDAC1/2/3, MFN1/2 and RHOT1/2, Miro (*Dros.*)	Plays a central role in mitochondrial homeostasis and mitophagy. It is recruited to the mitochondrial outer membrane via its regulatory kinase PINK1, where it is credited with resculpting the MOM proteome.	Cytoplasm, nucleus, ER mitochondrial membrane	Autism spectrum disorder and Parkinson's disease	49, 174, 294, 379
RNF5 (RMA5, NG2)	JAMP, paxillin	Plays a role in cell motility and localization of paxillin. Ubiquitination of JAMP affects its role in ER-associated degradation of misfolded proteins and function at the proteasome.	Cell membrane, mitochondrial membrane, ER membrane		63, 343

Continued

Table 1.—*Continued*

Ligase	Substrate	Function	Location	Associated Pathology	Reference Nos.
SCF/β-TrCP	β-Catenin	Mediates the ubiquitination of β-catenin and participates in Wnt signaling.	Cytoplasm, nucleus		117, 201
SIAH1	β-Catenin, DCC, FLT3	Plays a role in inhibition of Wnt signaling through ubiquitination of β-catenin. Triggers the ubiquitin-mediated degradation of cell surface receptor, DCC and receptor tyrosine kinase, FLT3 to affect cell signaling and proliferation.	Cytoplasm, nucleus	Possible roles in Parkinson's disease and hepatocellular carcinomas	64, 96, 139
UBR1 (N-recognin)	CFTR (yeast), Ste6 (yeast)	An additional ubiquitin ligase that can participate in ERAD of Ste6 and CFTR (yeast).	Cytoplasm	Johanson-Blizzard syndrome and urethral obstruction	9, 328
		HECT E3s			
AIP4 (Itch–Ms, Su(dx)-*Dros.*)	Notch, CXCR4, HGS, AIP4, Adipophilin, Notch (*Dros.*), ptc (*Dros.*)	Plays a role in regulation of plasma membrane signaling through interaction with CXCR4 and Notch, as well as intracellular signal transduction mediated by cytokines and growth factors through regulation of HGS in the ESCRT-0 complex.	Cell membrane, cytoplasm, nucleus	Syndromic multisystem autoimmune disease	89, 137, 219, 274
HUWE1 (UREB1, HECTH9, ARF-BP1, MULE, E3 Histone, LASU1)	ABCG1, ABCG4	Downregulates cell surface levels of ABCG1 and ABCG4 to attenuate cholesterol export from cells.	Cell membrane, cytoplasm	Turner type X-linked syndromic mental retardation	8, 292
NEDD4 (Rsp5-yeast)	IGF1R, VEGFR-2, Notch, ENaC, NCC, B2-AR, Cav1.2, Cnx43, EGFR, FGFR1, ErbB3, ErbB4, LAPTM5, LMP2A, Nav1.2, Nav1.7, NHE1, ABCG1, ABCG4, Ptc (*Dros.*), α-synuclein	Downregulates a variety of ion channels, protein tyrosine kinases, and growth factor receptors.	Cell membrane, cytoplasm, endosome	Liddle syndrome, Alzheimer's disease, α-synuclein proteopathies	8, 65, 89, 103, 347; see Ref. 24 for a thorough review
NEDD4L (Nedd4-2-Ms)	TrkA, TGFbR1, Occludin, CFTR, ATA2, AQP2, CLC-5, CLC-Ka, DAT, OAT3, EAAT1/2, ENaC, Dlg3, KCNQ, Navs, SGLT1, Tweety, SNAT2, LAT1, hERG	Downregulates a variety of ion channels to regulate sodium, chloride, and potassium homeostasis.	Cell membrane, cytoplasm	Liddle syndrome, hereditary hypertension, peripheral neuropathic pain	12, 48, 160, 285, 289, 377; see Ref. 106 for a thorough review
Smurf1 (dSmurf-*Dros.*)	WFS1	Ubiquitinates WFS1 at the endoplasmic reticulum to negatively regulate the ER stress response.	Endoplasmic reticulum, cytoplasm, cell membrane	Wolfram syndrome	85, 110
WWP1 (AIP5)	ErbB4	Ubiquitinates and degrades the RTK, ErbB4 (both full-length and m80, the product of the first proteolytic cleavage).	Cell membrane, cytoplasm, nucleus	Commonly found to be overexpressed in breast cancer	36, 82, 198, 386
WWP2 (AIP2)	ENaC, DMT1, KCNH6, PAR1	Interacts with adaptor proteins to promote ubiquitination and degradation of target proteins.	Plasma membrane		45, 67, 86, 227
UBE3A (E6AP)	Progesterone receptor	Functions as a ubiquitin ligase as well as a transcriptional coactivator of steroid hormone receptors.	Cytoplasm, nucleus	Angelman syndrome, autism spectrum disorder, and Prader-Willi syndrome	104

Associated pathologies include those caused by mutations in either the ligase itself or in the substrate whereby ubiquitination is affected. Alternate names are indicated in parentheses. Ms, mouse; Dros, *Drosophila*; yeast, *Saccharomyces cerevisiae*.

coat protein complex II (COPII) vesicles, and transported to the *cis* face of the Golgi complex, where they are processed and then further transported to their final destination from the *trans*-Golgi network. While ubiquitin modification does not play a direct role in regulating cargo sorting for ER-to-Golgi transport, abnormal/misfolded or excess proteins are subject to quality control ubiquitination at both the ER and Golgi.

A. Quality Control of Membrane Proteins in the ER

Much of what we know about ER-associated degradation (ERAD) has come from studies in yeast. ERAD is the cellular pathway that targets misfolded or unwanted proteins in the ER for ubiquitination and subsequent degradation by the proteasome (51). The process of ERAD can be divided into multiple steps: *1*) recognition of aberrant substrate, *2*) ubiquitination, *3*) the movement of ubiquitinated proteins from the ER back to the cytosol in a process called retrotranslocation, *4*) delivery to the proteasome, and *5*) degradation (51) **(FIGURE 3*A*)**. Ubiquitin conjugation is essential for processing of ERAD substrates, as inhibiting this process prevents retrotranslocation and degradation (51).

In yeast, two RING E3s, Hrd1p and Doa10p, are responsible for almost all substrate ubiquitination in ERAD (18, 334), with Hrd1p ubiquitinating misfolded luminal or membrane proteins, and Doa10p targeting cytosolic proteins (39, 358). These ligases are highly conserved throughout evolution; however, mammalian cells use a larger cohort of E3s in ER quality control, possibly due to the larger proteome, cellular and tissue complexity, and longer lifespans of mammals, which together result in a more pronounced risk of protein misfolding (51).

The ERAD process is important in the normal regulation of many membrane proteins, and the nature of the ubiquitin linkages is critical for correct recognition of substrates. TM proteins must be polyubiquitinated, as demonstrated by Shamu et al. (303), who showed that monoubiquitination of the major histocompatibility complex (MHC) class I heavy chain is insufficient for retrotranslocation. More recently, it has been discovered that K48-linked, but not K63-linked, ubiquitin chains are required for degradation of the inositol 1,4,5-trisphosphate receptor (316) and the γ-aminobutyric acid type B receptors (387). p97, an ATPase that provides the energy required for the retrotranslocation of proteins prior to destruction by the proteasome, interacts with K11- and K48-linked, but not K63 ubiquitin chains (208).

Several diseases are caused by mutated proteins being directed for degradation by ERAD. A classic example of this is in cystic fibrosis, where deletion of phenylalanine 508 (ΔF508) of cystic fibrosis transmembrane conductance regulator (CFTR) causes misfolding of the protein, leading to its degradation by ERAD and a reduction of CFTR channel at the plasma membrane (15, 76, 366) **(FIGURE 3*B*)**. Under normal conditions, up to 70% of wild-type CFTR is degraded by ERAD (365). The ΔF508 mutation reduces the folding efficiency, resulting in nearly 99% of mutant CFTR being degraded before it can reach the plasma membrane (306). There are five mammalian E3s reported to play a role in CFTR degradation at the ER: Hrd1, gp78, CHIP, RmaI/RNF5, and RNF185 (15, 76). RNF5 cooperates with RNF185, along with the E2 Ubc6 and the transmembrane protein Derlin1, to control CFTR stability by "priming" CFTR by ubiquitination during translation, with gp78 subsequently elongating the ubiquitin chain and targeting CFTR to ERAD (76, 234, 382) **(FIGURE 3*B*)**. CHIP, a RING E3 ligase with a cytosolic U-box domain, is recruited to CFTR by the heat shock protein Hsc70 after translation (186, 322) (**FIGURE 3*C***). Hrd1 regulates CFTR indirectly by targeting gp78 for degradation (15) **(FIGURE 3*B*)**.

Further examples of the role of ERAD in disease include Niemann-Pick disease, where mutations in the Niemann-Pick disease, type C1 intracellular cholesterol transporter (NPC1), destabilize the protein and lead to its degradation by ERAD in a manner dependent on CHIP (244). Prion protein (PrP) is normally removed by ERAD following ubiquitination by gp78 (304). The misfolded version PrP^{Sc} is also targeted to the proteasome by ERAD, but then inhibits proteasome function by blocking substrate entry, resulting in the accumulation of other targeted proteins in the cytosol leading to cell death (60). Viruses can also hijack the ERAD system to evade detection. The human cytomegalovirus proteins US2 and US11 recruit the E3 ligases TRC8 and TMEM129, respectively, to ubiquitinate MHC class I molecules and target them for proteasomal degradation (325, 355). This stops viral peptides from being presented at the cell surface for recognition by cytotoxic T cells.

To further enhance the stringency of this system, misfolded proteins that reach the Golgi after escaping ER quality control may be recognized and sent back to the ER via retrograde transport of coat protein I (COPI) vesicles through a process that seems to depend on ubiquitination (149) (**FIGURE 1,** step 2). In Charcot-Marie-Tooth disease, the peripheral myelin protein 22 (PMP22), a membrane protein crucial in the development and maintenance of myelin sheaths in Schwann cells, is normally regulated at the Golgi and targeted for lysosome degradation if not required. Mutations in PMP22 cause its retrograde transport from the Golgi back to the ER where it is ubiquitinated by the E3s, Hrd1 and gp78, and degraded by ERAD (115).

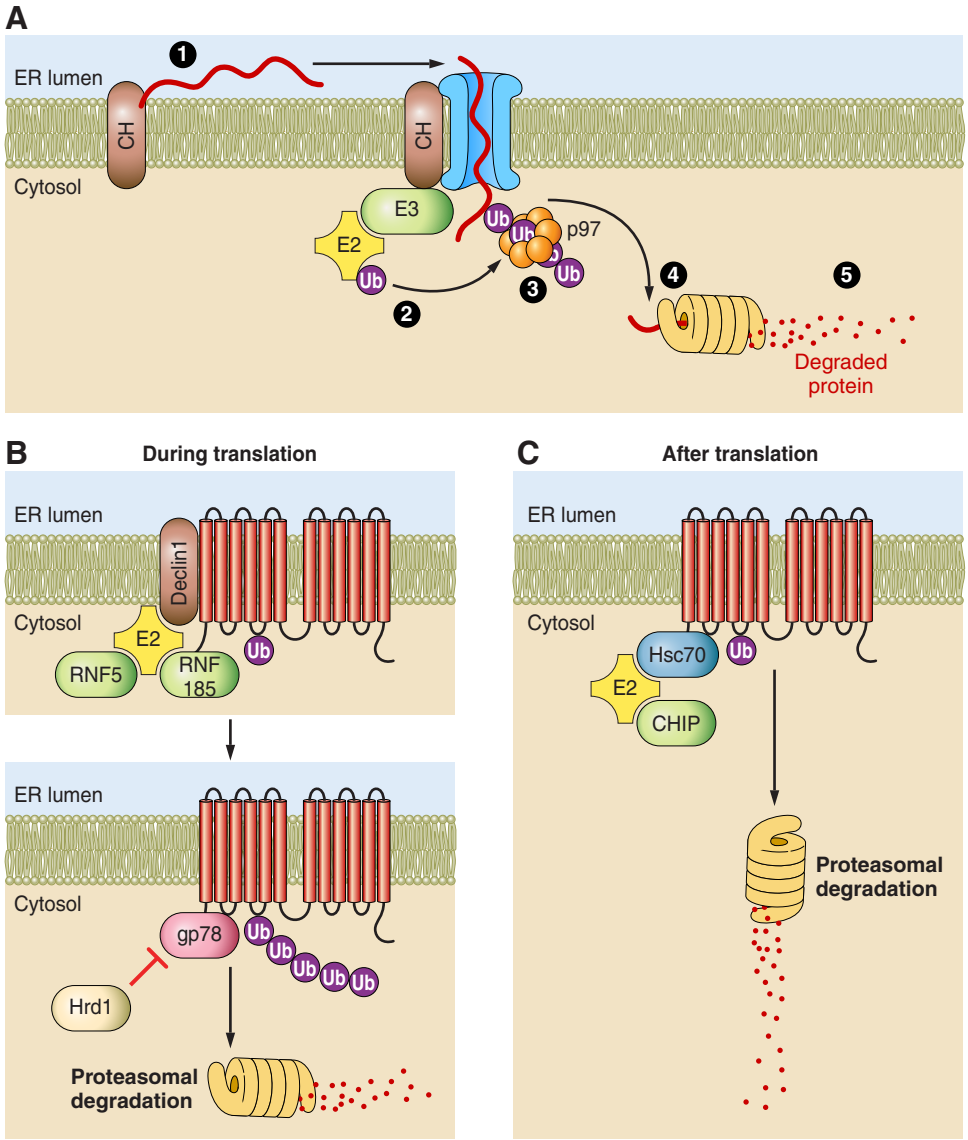

FIGURE 3. ER-associated degradation (ERAD). *A:* the process of ERAD is divided into multiple steps: *1*) recognition of aberrant substrate by chaperone proteins (Ch), *2*) ubiquitination, *3*) the retrotranslocation of ubiquitinated proteins in an energy-dependent process requiring the ATPase p97, *4*) delivery to the proteasome, and *5*) degradation (51). *B:* the regulation of CFTR by ERAD. During translation, the ER-associated E3s RNF5 and RNF185 are recruited to misfolded CFTR by Derlin1 and the E2 Ubc6 to initiate its ubiquitination. Gp78 is then recruited to elongate the ubiquitin chain, enabling recognition by the ERAD pathway. Hrd1 indirectly regulates CFTR by ubiquitinating gp78, thereby blocking its function. *C:* any CFTR misfolded after translation is recognized by Hsc70, which recruits the CHIP E3 ligase complex to ubiquitinate CFTR and target it to the proteasome for degradation.

B. Regulation of Membrane Proteins at the Golgi

A second level of protein quality control exists at the Golgi to control misfolded proteins that escape the ER. In contrast to proteins in the ER or cytosol, which are degraded by the proteasome, the majority of membrane proteins, including those at the Golgi, are degraded by the lysosome. This process is ubiquitin-dependent and involves selective recognition of cargo for ubiquitination, capture of ubiquitinated cargo for trafficking to the endosome, endosomal sorting by the ESCRT (endosomal sorting complexes required for transport) machinery into multivesicular bodies (MVBs), and fusion of MVBs with lysosomal/vacuolar compartments, resulting in cargo degradation (125, 214, 390) (**FIGURE 1,** step 3).

Golgi-localized, gamma adaptin ear domain homology, ARF-binding (GGA) coat proteins that regulate the trafficking of substrates between the *trans*-Golgi network and the lysosome, are involved in the trafficking of cargo from the *trans*-Golgi network to the endosomal compartment (26,

134). GGAs contain a GAT domain which binds ubiquitin moieties on substrate proteins (27, 101, 260). Ubiquitination and GGA-mediated intracellular traffic are associated with the degradation of a number of receptors. GGA3 binds to ubiquitinated cation-independent mannose-6-phosphate receptors (CI-MPR), the epidermal growth factor receptor (EGFR), and the transmembrane aspartic acid protease BACE1 to promote their trafficking to the lysosome (271, 337). This process is highly conserved, as evidenced in yeast where a number of ubiquitinated membrane proteins are targeted to the vacuole by GGAs (23, 78, 124, 172, 205, 267, 281, 288, 320). Interestingly, the ubiquitination of GGAs themselves has also been suggested to play a role in the trafficking of cargo to the endosomal compartment. RNF11, a RING E3 found at the plasma membrane, binds to GGA3 and recruits the HECT E3, Itch, to ubiquitinate GGA3 and facilitate correct intracellular sorting (291).

While ubiquitination is critical for protein quality control at the Golgi, the E3 ligases involved in this process are not very well characterized. Tul1, a Golgi-localized E3 in *Saccharomyces cerevisiae* (Dsc1 in *Schizosaccharomyces pombe*), has been shown to function in the Golgi protein quality control of Pep12D, a mutant form of the endosomal SNARE protein, and as a result, Pep12D is sorted into the MVB pathway and degraded rather than normally localizing to the limiting membrane of the vacuole (280). In zebrafish, *alligator* (encoding the mammalian ortholog of the RING E3, RNF121) is involved in the quality control of voltage-gated sodium channels (Na_vs) at the *cis*-Golgi (254). In mammals, RNF126 is important for the retrograde transport of CI-MPR, but not cholera toxin, furin, or TGN38 (319), suggesting that there are other E3 ligases involved in this process to confer specificity to the system.

IV. REGULATION AT THE PLASMA MEMBRANE

Ubiquitination and endocytic removal of plasma membrane proteins have a long evolutionary history as a means to alter the abundance of proteins at the cell surface, and extinguish receptor signaling (249). The earliest advances in understanding the important role of ubiquitin in regulating plasma membrane proteins came from studies in yeast, with the discovery of two E3 ligases, Ubr1p and Rsp5p (83, 144, 152), and the first evidence to show that ubiquitin is required for endocytosis of membrane proteins (74, 97, 131, 176). Since that time, studies in mammalian cells have established a major role for ubiquitination in endocytosis, recycling, and/or degradation of transmembrane proteins (111, 245, 276, 329). By adjusting the residence time and relative abundance of membrane proteins including ion channels, signaling receptors, and nutrient transporters at the plasma membrane, the cell can alter activation of downstream signaling pathways with varying effects on cellular physiology, such as proliferation, migration, electrolyte balance, and differentiation (193).

The Casitas B-lineage lymphoma (Cbl) family members are the best known of all membrane-associated E3 ligases and function predominantly at the plasma membrane (189, 190). Cbl, comprised of three members (c-Cbl, Cbl-b, and Cbl-c), is a highly evolutionarily conserved family of RING E3 ligases, recognized for their ability to bind to and ubiquitinate several receptor tyrosine kinases (RTKs) including EGFR, platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), and ephrin receptors (EphR) (71, 81, 194, 220, 229). RTKs are high-affinity cell surface receptors which, following ligand-dependent activation, can drive critical signaling pathways affecting cell growth, survival, differentiation, and proliferation; therefore, perturbation in Cbl activity is highly correlated with the onset of many types of cancers (246).

EGF-dependent EGFR activation involves receptor dimerization and autophosphorylation at specific tyrosine residues in the cytoplasmic domain. Cbl is then recruited to this phosphorylated tyrosine residue either directly through the tyrosine-kinase-binding domain, or indirectly through interaction with an adaptor protein, such as growth factor receptor-bound protein 2 (GRB2). For FGFR, an additional adaptor protein, fibroblast growth factor receptor substrate 2 (Frs2), is also required for Cbl binding (153, 374). Once located at the site of the activated receptor, the RING domain of Cbl can then recruit an E2 enzyme and catalyze transfer of ubiquitin to the receptor (reviewed by Swaminathan et al., Ref. 332).

Ion channels and transporters are present on the membrane of all cells and have essential roles in regulation of membrane potential, cell-cell communication, body electrolyte/water homeostasis, and also in uptake of essential nutrients and electrolytes (including metal ions). Many ion channels and transporters are regulated by ubiquitination-dependent endocytosis, and members of the highly conserved Nedd4 family of HECT E3s are commonly found to be involved in their ubiquitination. The Nedd4 family of E3 ligases contains nine members in humans (NEDD4, NEDD4-2/NEDD4L, AIP4, SMURF1, SMURF2, WWP1, WWP2, NEDL1, and NEDL2; Ref. 378), all characterized by a unique domain architecture consisting of a NH_2-terminal lipid binding domain (C2 domain), followed by two to four WW domains, and a COOH-terminal HECT domain. Interaction of Nedd4 family members with their substrates and regulators is often mediated through the WW-domains, which interact with PPxY or similar proline-containing motifs in the target proteins. The Nedd4 E3s are closely related to yeast Rsp5p (144), where it is the predominant E3 ligase for membrane proteins (70, 130).

A well-known example of an ion channel regulated by a Nedd4 E3 is the epithelial sodium channel (ENaC). Essential for sodium homeostasis, ENaC is expressed in the epi-

thelia of kidney, colon, lung, and sweat glands where its activity is required for maintenance of blood volume and blood pressure in adults, and also for lung fluid clearance in newborns (24, 25, 106). ENaC exists as a complex of three subunits, each with a PY motif in their cytoplasmic tail. The WW domains of Nedd4-2 bind to all three subunits of this complex to enable ubiquitination and subsequent clathrin-mediated endocytosis and degradation (90, 119, 157, 158) **(FIGURE 5*A*)**. Dysregulation of ubiquitin-mediated internalization of ENaC results in Liddle syndrome, a form of early-onset hypertension caused by deletion or disruption of the PY motifs in ENaC subunits. The resulting abrogation in the recruitment of Nedd4-2 leads to accumulation of ENaC at the surface of the renal tubular epithelia, and excess sodium reabsorption leading to hypertension (119, 158, 310). Nedd4-2 knockout mice show increased cell surface retention of ENaC in the lung and kidney, leading to altered sodium absorption and lung and kidney pathologies (25, 284, 310).

Various other ion channels and transporters are among the ever-growing list of Nedd4 and Nedd4-2 plasma membrane substrates, including the renal Na^+/Cl^- cotransporter (NCC), voltage-gated sodium channels (Na_v), chloride channels (CFTR, CLC-5, CLC-Ka), potassium channels (KCNQ, hERG1), the divalent metal ion transporter (DMT1), and amino acid transporters (SNAT2 and LAT1) (24, 48, 106, 285). Perturbations in the ubiquitination and/or degradation pathways leading to aberrant expression of these channels cause a variety of pathological conditions including hypertension (13), cystic fibrosis (365), and cardiac arrhythmias (reviewed by Abriel and Staub, Ref. 3).

The Nedd4 family of E3s has also been implicated in ubiquitination of the G protein-coupled receptors (GPCRs), the largest group of membrane receptors in eukaryotes (68). GPCRs play important roles in transmission of messages/stimuli from the external environment, and from other cells. Ubiquitination-dependent regulation of GPCRs at the plasma membrane typically occurs subsequent to ligand binding and receptor phosphorylation (22, 218, 307). Subsequent interaction of the E3 with the phosphorylated receptor occurs either directly through binding of the GPCR to the WW-domain of the E3 ligase (22) or indirectly through interactions involving adaptor proteins, called β-arrestins (126, 232, 257, 309). Two known GPCRs that are regulated by Nedd4 family of E3s include β2-adrenergic receptor (β2-AR), which interacts with Nedd4 via the adaptor protein β-arrestin-2 (309), and the C-X-C chemokine receptor 4 (CXCR4), which interacts directly with AIP4 (22, 219).

Nedd4 is also known to regulate plasma membrane levels of various cell signaling receptors including insulin-like growth factor I receptor (IGF-IR), EGFR, and vascular endothelial growth factor receptor-2 (VEGFR2), as well as FGFR1 and Notch (two important regulators of cell differentiation and proliferation) (33, 163, 231, 240, 261, 290, 359). In addition to Nedd4, the Notch receptor is also regulated by another member of the Nedd4 family, AIP4 [the human homolog of *Drosophila* Supressor of Deltex, Su(dx) and mouse Itch]. Nedd4 and AIP4 both ubiquitinate the intracellular domain of the nonactivated Notch receptor, part of the constitutive cycling of the receptor from the membrane. For the activated receptor, it is believed that ubiquitination and endocytosis are required for successful downstream signaling of the receptor; however, as yet the responsible E3 ligase is unknown (233).

On the ligand side, additional E3s are involved in regulation of Notch signaling. Mind bomb (Mib) and Neuralized (Neurl) are two E3 ligases involved in endocytosis of the Notch ligands, Delta-Like and Jagged, in mammals (Delta and Serrate in *Drosophila*). The necessity of these ligases for Notch signaling was demonstrated in *Drosophila* where binding of Delta to Notch is insufficient to activate signaling from the receptor alone, and signaling only ensues when coexpressed with either Mib or Neurl E3 ligases (59, 187, 188, 191, 259). These studies demonstrate that Notch signaling is regulated by a complex interplay between various ligases and adaptor proteins whereby the common mechanism of ubiquitin-mediated endocytosis can yield opposing and context-specific outcomes (233).

In addition to the regulation of protein abundance, ubiquitination at the plasma membrane can also be used for protein quality control. It has been demonstrated recently that when yeast cells undergo heat-mediated stress, partially denatured and misfolded integral membrane proteins accumulate at the plasma membrane, where these toxic and damaged proteins are cleared by Rsp5 and a network of adaptor proteins, called ARTs (arrestin-related trafficking adaptors) (390). Failure of the ART-Rsp5 complexes to clear damaged proteins from the membrane results in a build-up of proteins, loss of cellular integrity, and ultimately cell death. This observation was supported by another study showing that the same quality control mechanism exists for cytosolic proteins damaged during heat-mediated stress in yeast and mammalian cells (80). Fang et al. (80) demonstrate that both yeast Rsp5 and its mammalian counterpart Nedd4 are important E3s responsible for the increased ubiquitination upon heat stress, and that removal of proteins in this manner is particularly important in the brain, where cell death due to buildup of damaged proteins can lead to neurodegeneration. In fact, Nedd4 and Rsp5 were shown to play a major role in α-synuclein proteopathies, as mammalian Nedd4 targets α-synuclein for degradation (347). Significantly, a potential druggable target, *N*-aryl benzimidazole (NAB2), has been identified which re-

duces α-synuclein toxicity in mammalian and yeast cells by targeting Nedd4/Rsp5 (342).

The Nedd4 family members also play important roles in survival and function of neurons (62, 75, 212). Ubiquitination of the TrkA neurotrophic receptor by Nedd4-2 plays a critical role in nerve growth factor-mediated functions, including neuronal survival and sensitivity to pain (12, 384, 385). In addition, peripheral neuropathic pain, a condition associated with hyperexcitability of neurons following nerve injury, is associated with altered ubiquitination of voltage-gated sodium channels (Na_vs) by Nedd4-2 (91, 185, 353).

For communication, neurons rely on the dynamic presentation of receptors on postsynaptic protrusions, which are linked to the cytoskeleton and downstream pathways via a network of proteins called the postsynaptic density (PSD). Glutamate receptors (GluRs) are part of this network, and when a nerve is stimulated, glutamate is released at the excitatory synapse by vesicles at the presynaptic terminal, resulting in either of the following outcomes: *1*) activation of downstream cascades by binding to metabotrophic glutamate receptors (mGluRs), or *2*) opening of ion channels by binding to ionotrophic glutamine receptors (iGluRs). There are two subtypes of iGluR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs; GluA1-4) and *N*-methyl-D-aspartate receptors (NMDARs; GluN1; GluN2A-D; GluN3A-B). E3s play a critical role in regulating the density and composition of available GluRs, ultimately altering the strength of the synapse. These include Uch-L1, Fbx2, Mib-2, and Nedd4 which ubiquitinate the NMDAR subunits GluN1, GluN2B, and GluN2D, respectively (38, 99, 156, 161), and APCCdh1, Nedd4-1, Uch-L1, and RNF167 which have been demonstrated to ubiquitinate the subunits of AMPAR (31, 38, 92, 200, 209, 210, 300, 301, 372). Multiple lines of evidence suggest a link between the ligase activity of Uch-L1, Nedd4, and Fbx2, and the development of Alzheimer's disease and Parkinson's disease, either through direct regulation of synaptic neurotransmitter release and receptor turnover, β-amyloid plaque formation, or α-synuclein dysfunction in the brain (283; and reviewed by Gong et al., Ref. 107). In fact, dysfunction of the ubiquitin system is associated with many neurological diseases, including Alzheimer's disease, Parkinson's disease, transmissible spongiform encephalopathies and Huntington's disease (see sect. VIII) (40, 146, 174, 351).

V. SORTING OF MEMBRANE PROTEINS

A. Endocytosis

The canonical method by which a cell removes membrane proteins, including cell surface receptors, is via endocytosis, a process where the plasma membrane and its integral membrane proteins bud inward and are transported to the endosome, where they are either sorted for degradation in the lysosome or recycled back to the membrane via recycling endosomes **(FIGURE 1)**. Endocytosis via clathrin-coated pits (clathrin-mediated endocytosis) is the major mechanism of removal of cell surface proteins in most cells and is the canonical method for all activated RTKs; however, endocytosis can also proceed by clathrin-independent mechanisms (114, 223).

Selection of ubiquitinated proteins for endocytosis is carried out with the help of conserved families of endocytic adaptors, which can bind to both the clathrin machinery and also to ubiquitinated proteins. The Epsin family (Epsin 1/2/3; Ent1 and Ent2 in yeast) and the Eps15 and Eps15R proteins (Ede1/End3 in yeast) are two such groups of endocytic adaptors which serve redundant functions in endocytosis of ubiquitinated cargo by binding to ubiquitin through their ubiquitin interacting domains. Epsins also bind to clathrin through their NH_2-terminal homology domains (105, 268, 311, 314).

The direct role of ubiquitin in the process of endocytosis is complex. Early evidence in invertebrate systems suggested that ubiquitin is necessary for endocytosis [Ste6, 2 and Fur4 in *S. cerevisiae* (97, 131, 151, 176, 312) and EGFR in *Drosophila* and *C. elegans* (305, 319)]. However, subsequent studies have revealed ubiquitin-independent pathways (46, 338). In the invertebrate systems therefore, it appears that ubiquitination can drive endocytosis of some membrane proteins, and not others. In mammalian cells, the situation appears to be more complex. Ubiquitination of activated receptors is a characteristic feature of all types of RTKs, and studies have shown that alteration of Cbl activity can inhibit internalization of EGFR and c-Kit, among others (142, 192, 194, 388), demonstrating that Cbl is a key mediator of RTK endocytosis. Interestingly, mutation of major ubiquitination sites of EGFR and FGFR2 does not prevent their endocytosis (122, 142), and activated EGFR can be endocytosed independently of their ubiquitination status (69, 140). In these studies however, Cbl is still required to be present at the activated receptor and can ubiquitinate the receptor at any point along the way to, or even at the endosome prior to sorting (140). To explain these results, it may be possible that the E3 ligase, when recruited to the activated receptor, has additional functions other than ubiquitination to drive endocytosis, or that it may ubiquitinate an undiscovered accessory protein. For example, most GPCRs are internalized via a β-arrestin-dependent mechanism and likely do not require direct ubiquitination for internalization (159). In fact, a ubiquitin-deficient β2-AR mutant internalizes just as efficiently as the wild-type receptor (307). To date, studies have revealed multiple pathways through which endocytosis may proceed, some of which do not require ubiquitin. In mammalian cells therefore, the process of endocytosis is

complex and ubiquitination is often sufficient, but not always required, for internalization (214).

B. Endosomal Sorting of Ubiquitinated Proteins

Following budding off from the membrane, clathrin is removed from the internalized vesicles and the cargo is transported to the early endosome, where the proteins undergo sorting to decide their fate. Some proteins will bud off from the endosomal membrane to be recycled back to the plasma membrane, either directly or via the *trans*-Golgi network (73, 230, 297) (**FIGURE 1,** step 6). Other proteins will be recognized by the ESCRT machinery and will bud inward from the endosome into intraluminal vesicles, forming MVBs and committing the proteins to lysosomal degradation (164, 276) (**FIGURE 1,** step 7). A ubiquitin tag is necessary for the sorting of proteins (165, 275, 276) and as long as ubiquitin is added before sorting in the early endosome, the protein is able to be degraded.

The sorting function for proteins at the surface of the endosome is carried out by a core group of five evolutionarily conserved protein complexes: ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III, and the Vps4 AAA-ATPase complex. The ESCRT-0 complex, comprised of constitutively associated components, HRS and STAM (Vps27 and Hse1 in yeast), is the crucial element in recruitment of the subsequent sorting machinery to the endosome and is the first point of contact for the sorting machinery with ubiquitinated cargo (125, 145). At this point, the ubiquitin tag is critical for sorting at the endosome as a protein without a bound ubiquitin tag cannot be recognized by the ESCRT-0 complex (214). ESCRT I and II also require ubiquitin to associate with the endosomes and function to initiate the budding of the intraluminal vesicle into the MVB. ESCRT III, however, does not bind ubiquitin; it functions to recruit additional proteins to dissociate ESCRT complexes from the endosome and for final scission of the vesicle after budding into the lumen.

In addition to the E3 ligases acting at the plasma membrane, other E3s can be recruited to the early endosome and ubiquitinate receptors prior to sorting. The E3, UBE4B, is recruited to endosomal membranes by Hrs within the ESCRT-0 complex, where it can bind to and ubiquitinate proteins, including EGFR, at the surface of the endosome. The interaction of UBE4B with ESCRT-0 is necessary for EGFR degradation, expression, and downstream signaling following EGFR stimulation by ligand (315).

After recognition by ESCRT-0, and just prior to internalization in the MVB, the ubiquitin tag is removed by the action of DUBs to enable recycling of the ubiquitin molecule to the cytoplasmic pool. The ~100 DUBs in mammals (20 in yeast) belong to 5 families, 4 of which are cysteine proteases, including ubiquitin specific proteases (USPs), ubiquitin COOH-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), and Josephins, while the JAB1/MPN/MOV34 family (JAMMs) are Zn^{2+} metalloproteases (177). Together, DUBs function to reverse the ubiquitination of proteins enabling spatiotemporal control of protein ubiquitination.

Two DUBs, AMSH and USP8, are present at the site of the early endosome and can associate with ESCRT-0 component, STAM, through their interaction with its SH3 domain, and with ESCRT-III through their MIT domains (4, 162, 225, 287, 321, 341, 350). By their interaction with ESCRT machinery, both AMSH and USP8 negatively regulate the degradation of proteins, such as EGFR, by deubiquitinating the receptor prior to sorting, therefore favoring its recycling (28, 54, 225, 251, 258). The same has also been seen for USP8 function with Frizzled receptor (Fz) and Smoothened (Smo), as well as key components of Wnt and Hedgehog signaling pathways (196, 238, 376).

In addition to direct protein deubiquitination, USP8 also regulates protein stability through its interaction with components of the sorting machinery. By binding to STAM and Hrs, USP8 can stabilize the ESCRT-0 complex, providing more effective capture of substrates, such as chemokine receptor 4 (CXCR4), and direct it towards lysosomal degradation (21, 226, 287).

The site of the early endosome is a hotbed of interactions of E3 ligases; ESCRT machinery and DUBs are recruited simultaneously to regulate the final moments in the life of a protein, or save it from destruction (315). However, in a further demonstration of the complexity of control of the ubiquitin system, DUBs and E3 ligases have been shown to act in reciprocal regulation. USP8 has been shown to stabilize the levels of the E3 ligase, RNF41, by deubiquitination (34), while RNF41 also ubiquitinates, destabilizes, and relocalizes USP8, ultimately destabilizing the ESCRT-0 complex (58). Thus the balance between functions of ubiquitin ligases and DUBs determines protein stability, signaling, and the efficiency of lysosomal sorting.

VI. UBIQUITINATION AND THE BUDDING OF EXTRACELLULAR VESICLES

As discussed above, the role of ubiquitin in the internalization and degradation of membrane proteins is well established; however, new evidence now suggests that proteins can also be secreted from cells via membrane-bound vesicles known collectively as extracellular vesicles (EVs). EVs are released by eukaryotic and prokaryotic cells, both constitutively and in response to various signals (356). EVs mediate the release of a wide variety of proteins, microRNAs, mRNAs, and lipids (346). They can interact with neighboring cells to mediate cell-cell communication and are in-

volved in normal physiological processes (e.g., immune signaling and development), as well as pathological processes, such as cancer, atherosclerosis, and inflammation (11). EVs can be divided into three main groups: exosomes, generated by the release of intraluminal vesicles from MVBs when they fuse with the plasma membrane; microvesicles (MVs), that bud directly from the plasma membrane; and apoptotic bodies, released from cells undergoing apoptosis (278). A role for ubiquitin in directing proteins towards extracellular secretion by both exosomes and MVs is now being established.

A. Ubiquitination in Exosomal Targeting

It is well recognized that ubiquitin is an important signaling molecule for targeting proteins to the MVB, recruiting the ESCRT machinery and forming intraluminal vesicles (213). Exosomes are formed when the MVB fuses with the plasma membrane and releases its intraluminal vesicles into the extracellular space (55). Ubiquitination has been implicated in sorting proteins into exosomes; however, the precise mechanism remains unclear. Fas ligand, a potent mediator of apoptosis in cytotoxic T cells and natural killer cells, is sorted into intraluminal vesicles for secretion in exosomes via a mechanism that requires its monoubiquitination (392). The Nedd4 E3 adaptor protein Ndfip1 is released in exosomes and is necessary for targeting both Nedd4 E3s and the tumor suppressor protein PTEN to exosomes (272, 273). Another line of evidence suggesting the importance of ubiquitination in exosomal sorting comes from studies on the COP9-associated DUB CSN5. Mutations that attenuate the DUB activity of CSN5 enhance the sorting of proteins, such as HSP70 and HIV Gag into exosomes (204).

For other proteins, ubiquitination appears to be unnecessary or even inhibitory for trafficking to exosomes. Exosomal trafficking of the small integral membrane protein of the lysosome/late endosome (SIMPLE) is enhanced by mutations in its PY motif, which prevent its ubiquitination (391). In antigen-presenting cells, MHC-II accumulates in MVBs and is then secreted in exosomes. While ubiquitination by the RING E3 MARCH8 is required for its internalization from the plasma membrane, it is not required for the packaging of MHC-II into exosomes (100).

B. Ubiquitination in Microvesicle Targeting

While still a matter of debate within the field, MVs (also termed ectosomes, shedding vesicles, or microparticles) are generally defined as membrane-enclosed vesicles released directly by budding of the plasma membrane. First described 20 years ago as a way for removing unwanted transferrin receptor during maturation of reticulocytes (116, 155), the mechanisms behind how cells shed MVs and how contents are selectively targeted to MVs for release are poorly understood. Much of the knowledge gained into this process comes from work on virus egress. Many viruses take advantage of the cells ability to release proteins in MVs to promote viral particle release and evade degradation. Viruses, such as Ebola and Rabies, recruit Nedd4 family E3s and ESCRT components, such as Tsg101 and VPS4 via binding motifs in their structural proteins to facilitate the release of infectious virus (118, 199). Small molecule drugs designed to block the Nedd4-late budding domain interaction can inhibit the release of active virus particles that require this interaction for budding (88). In a similar manner, ESCRT components are recruited to and required for the release of Arrdc1-positive MVs, and this requires the E3 ligase WWP2 (241).

Our own recent work has found that membrane proteins can be specifically targeted to MVs (215). Members of the α-arrestin family of proteins, Arrdc1 and Arrdc4, facilitate the release of DMT1 in MVs by acting as adaptors between DMT1 and the E3 ligase Nedd4-2 **(FIGURE 4)**. Interestingly, while the biogenesis of both Arrdc1- and Arrdc4-positive vesicles relies on the recruitment of Nedd4 family E3s, alternative accessory proteins are utilized. Arrdc1 requires the ESCRT components Tsg101 and VPS4 (241), whereas Arrdc4 requires Rab11a (215), a small GTPase previously shown to be involved in VPS4-independent viral budding and EV formation in the reticulocyte-like cell line K562 (30, 295, 296). Thus ubiquitination appears to be important for both the biogenesis of MVs and for cargo selection; however, much more work is required to further delineate this process.

VII. ADAPTOR PROTEINS THAT REGULATE UBIQUITINATION OF MEMBRANE PROTEINS

While many ubiquitin ligases can bind directly to their target substrates, others utilize adaptor or accessory proteins to facilitate binding to targets. RING E3s are scaffolding proteins themselves to bring the E2 and substrate together for transfer of ubiquitin from the E2 to the target protein. RING ligases also function as large regulatory complexes with other adaptor proteins (264). Many HECT E3s directly interact with their substrates to transfer ubiquitin; however, it is becoming increasingly apparent that some HECT E3s require adaptor proteins to modulate the interaction.

A. Adaptor Proteins of RING E3 Ligases

A well-characterised example of a RING E3 adaptor complex is the SCF (Skp1-Cullin-F-box) complex. SCF complexes are known to ubiquitinate a wide variety of membrane proteins to regulate processes, such as cell cycle, signal transduction, and protein quality control (264). In these

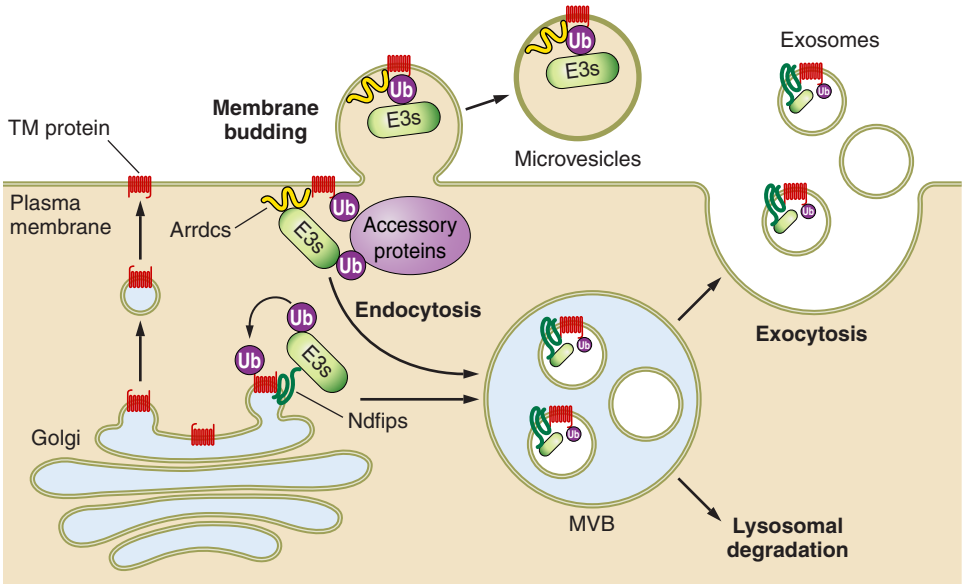

FIGURE 4. The conserved role of the Nedd4 adaptor protein families, Ndfips and Arrdcs, in regulating ubiquitin-mediated protein trafficking. Ndfips, located at the Golgi and MVB, recruit the Nedd4 family E3s and mediate the ubiquitination of TM proteins. These can then be targeted for lysosomal degradation (e.g., DMT1), or for release into the extracellular space in membrane bound vesicles called exosomes (e.g., PTEN). Alternatively, the Arrdc family members are primarily localized to the plasma membrane where they function in a similar manner by recruiting Nedd4 family E3s and other accessory proteins (such as ESCRT components or small GTPases) to mediate the ubiquitination of TM proteins. From here, proteins can either be internalized and targeted for lysosomal degradation (e.g., PAR1), or released directly by budding of the plasma membrane (e.g., DMT1).

complexes, the Cullin-RING ligase mediates the transfer of ubiquitin from the E2 to the substrate, while substrate recognition is achieved by the adaptor subunits of the complex (61, 264). For example, the F-box adaptor protein Fbxw7 mediates the interaction between Notch family members and the Skp1-Cul1-F-box ubiquitin ligase complex. In neural stem cells, loss of Fbxw7 leads to an increase in Notch1 and Notch3 levels, resulting in a loss of differentiation (135, 222).

Cbl E3s are another major group of RING ligases that form adaptor complexes. APS (adapter protein with a pleckstrin homology and an src homology 2 domain) has been shown in vitro to regulate insulin signaling by recruiting c-Cbl to the insulin receptor and GLUT4 and mediating their ubiquitination and subsequent downregulation (6, 203). This observation, however, was not supported in mice, where deletion of APS does not alter adiposity, energy balance, or glucose metabolism (195). CIN85 (Cbl-interacting protein of 85 kDa) has been shown to regulate EGFR through the recruitment of Cbl (298, 224), and overexpression of CIN85 promotes EGFR-mediated tumor development and progression in head and neck squamous cell carcinoma (361). A third adaptor protein SLAP (src-like adaptor protein) aids in the ubiquitination by Cbl of Flt3 (167), the RTK responsible for myeloid leukemia development following loss of Cbl function (279, 293).

B. Adaptor Proteins of HECT E3 Ligases

Among the best characterized HECT ligase adaptors are the Nedd4 family interacting proteins (Ndfips). Ndfips are highly conserved throughout evolution, with a single ortholog in yeast (Bsd2) and *Drosophila* (dNdfip), and two family members in mammals (Ndfip1 and Ndfip2). These proteins can bind to several members of the Nedd4 E3 family through PY-WW domain interactions (120, 306). Initial work in *S. cerevisiae* found that Bsd2 recruits Rsp5 to ubiquitinate transmembrane proteins, such as Cps1, Phm5, and Smf1 (129, 330). This was also found to be the case in mammalian cells, with Ndfip1 and Ndfip2 regulating the degradation of the Smf1 homolog DMT1 through recruitment of WWP2 and Nedd4-2 (86-88, 98, 138). Loss of Ndfip1 causes dysregulation of DMT1, leading to an iron overload phenotype in mice (71), and metal toxicity in human neurons (138). In *Drosophila*, dNdfip was also shown to enhance Notch ubiquitination by dNedd4 and Su(dx) (57). By facilitating the interaction between the E3 ligase Itch and JunB, Ndfip1 has also been shown to regulate Th2 cell differentiation and pancreatic β-cell apoptosis (19, 256). Aside from their adaptor role in E3 recognition of substrates, Ndfips can facilitate ubiquitination by recruiting the E3s to specific subcellular compartments. Recently, Ndfips have been shown to regulate the potassium channel human ether-à-go-go-related gene (hERG) by directing Nedd4-2 to either the Golgi or

MVBs to ubiquitinate the mature form of hERG (160). Ndfips have also been shown to regulate E3s by sequestering the E3 away from its intended target, as in the case of ENaC regulation in a *Xenopus* oocyte system (128), or by releasing the E3 from an autoinhibitory state to an activated state, which was shown to facilitate the ubiquitination of endophilin (239).

Another group of proteins that have recently been identified as adaptor proteins for E3s are the arrestins. Comprising the α, β, and visual arrestins, these proteins are structurally similar in that they all contain arrestin N and C domains; however, only the α-arrestins (Arrdc1-4 and TXNIP, but not Arrdc5) contain PY motifs in their COOH-terminal tail (10, 270). β-Arrestins have been extensively studied for their role in the regulation of G protein-coupled receptors (GPCR), where they act as adaptors for both RING and HECT E3s. The best known is β2-AR. Upon stimulation by ligand, β2-AR is rapidly phosphorylated, leading to recruitment of β-arrestin-2 and the E3 ligase Mdm2. Mdm2 ubiquitinates β-arrestin-2 (not the receptor) which then leads to the internalization of the receptor (308) **(FIGURE 5*B*)**. Once in the endosomal compartment, Nedd4 displaces Mdm2, with β-arrestin-2 bridging the interaction between β2-AR and Nedd4, thereby enabling ubiquitination of the receptor (169, 309). This process is essential for β2-AR degradation by the lysosome. The α-arrestin proteins have also been implicated in the regulation of β2-AR; however, there are still some discrepancies around whether Arrdcs recruit Nedd4 to β2-AR after binding to β-arrestin-2, or if β-arrestin-2 recruits Nedd4 first, followed by secondary recruitment of Arrdcs (112, 242, 305). In spite of this, all studies show that α-arrestins play an important role in the regulation of β2-AR.

Recently, α-arrestins have been further implicated in the regulation of GPCRs through their role in the lysosomal degradation of the protease-activated receptor-1 (PAR1) (67). Following the activation of PAR1, Arrdc3 is recruited to the plasma membrane to mediate the internalization of the receptor **(FIGURE 4)**. Once inside the endosomal compartment, Arrdc3 facilitates the ubiquitination of the ESCRT protein ALIX by WWP2. Ubiquitinated ALIX can then bind to PAR1 and regulate its sorting to the lysosome (67).

Ubiquitination of the TGF-β receptor (TGFβR1) is also mediated by adaptor proteins. Following prolonged TGF-β stimulation, the E3 ligases Smurf1 or Smurf2 interacts with the inhibitory Smad, Smad7. This complex exits the nucleus and binds to activated TGFβR1. Smurf1/2 is then able to ubiquitinate the receptor, causing its rapid proteosomal degradation (72, 166). This ubiquitination can be reversed by Smad7 recruitment of the DUB, UCH37, which rescues TGFβR1 from proteasomal degradation (370, 371).

VIII. PATHOLOGICAL CONSEQUENCES OF DEREGULATED UBIQUITINATION

Considering how many pathways and membrane proteins are targeted by the ubiquitin system **(TABLE 1)**, it is not surprising that aberrations in the system underlie many human diseases. Pathological states associated with the ubiquitin system can be loosely divided into two groups: *1*) those with mutations (resulting in either gain or loss of function) in the ubiquitin machinery, and *2*) those with mutations in the target substrates that prevent ubiquitination from occurring. While a number of mutations have been deciphered in human disease, animal models can also provide insight into the function of the ubiquitin system in the pathology of disease.

A. Cancer

Ubiquitination plays an important role in the regulation of signaling receptors, and cancer cells often acquire mutations that allow proteins to evade this downregulation and promote cell transformation. An example of this is in RTK signaling, where the loss of negative regulation by ubiquitin results in enhanced receptor signaling (206, 236). Under normal conditions, RTKs are multiubiquitinated and K63-linked polyubiquitinated by Cbl E3 ligases, resulting in recognition by Tsg101 and other components of the ESCRT pathway, which leads to targeting to the MVB and degradation by the lysosome (111, 165, 237). Mutations that lead to the loss of Cbl binding or loss of Cbl ubiquitin ligase activity causing defective downregulation of the receptor are often associated with cancer (32, 206, 236, 339). For example, the hepatocyte growth factor receptor HGFR/Met is an RTK that, following phosphorylation triggered by ligand binding, is ubiquitinated by c-Cbl and targeted for lysosomal degradation. An oncogenic mutant Met receptor that cannot be phosphorylated fails to bind c-Cbl, and therefore is not ubiquitinated (154, 262) **(FIGURE 5*C*)**. Mutant Met can then be recycled back to the plasma membrane, where it is free to continue signaling to downstream Ras/MAPK pathways. In acute myeloid leukemia, mutations in c-Cbl itself inhibit the ubiquitination and subsequent downregulation of the RTK FLT3, leading to leukemic transformation (1, 32, 279, 293).

FBW7 is a component of the SCF E3 ligase complex and is commonly mutated in cancers. Substrates of FBW7 include some of the most well-known oncogenes, such as cyclin E, Myc, Jun, and Notch (368); thus FBW7 is classed as a tumor suppressor gene. Notch proteins are membrane-sequestered transcription factors, which translocate to the nucleus after cleavage of the intracellular domain by the γ-secretase proteolytic complex. Mutations in FBW7 itself lead to dysregulation of the Notch activation pathway in leukaemic cells (216, 253), and mutations within either the PEST domain of Notch or in the phospho-degron motif,

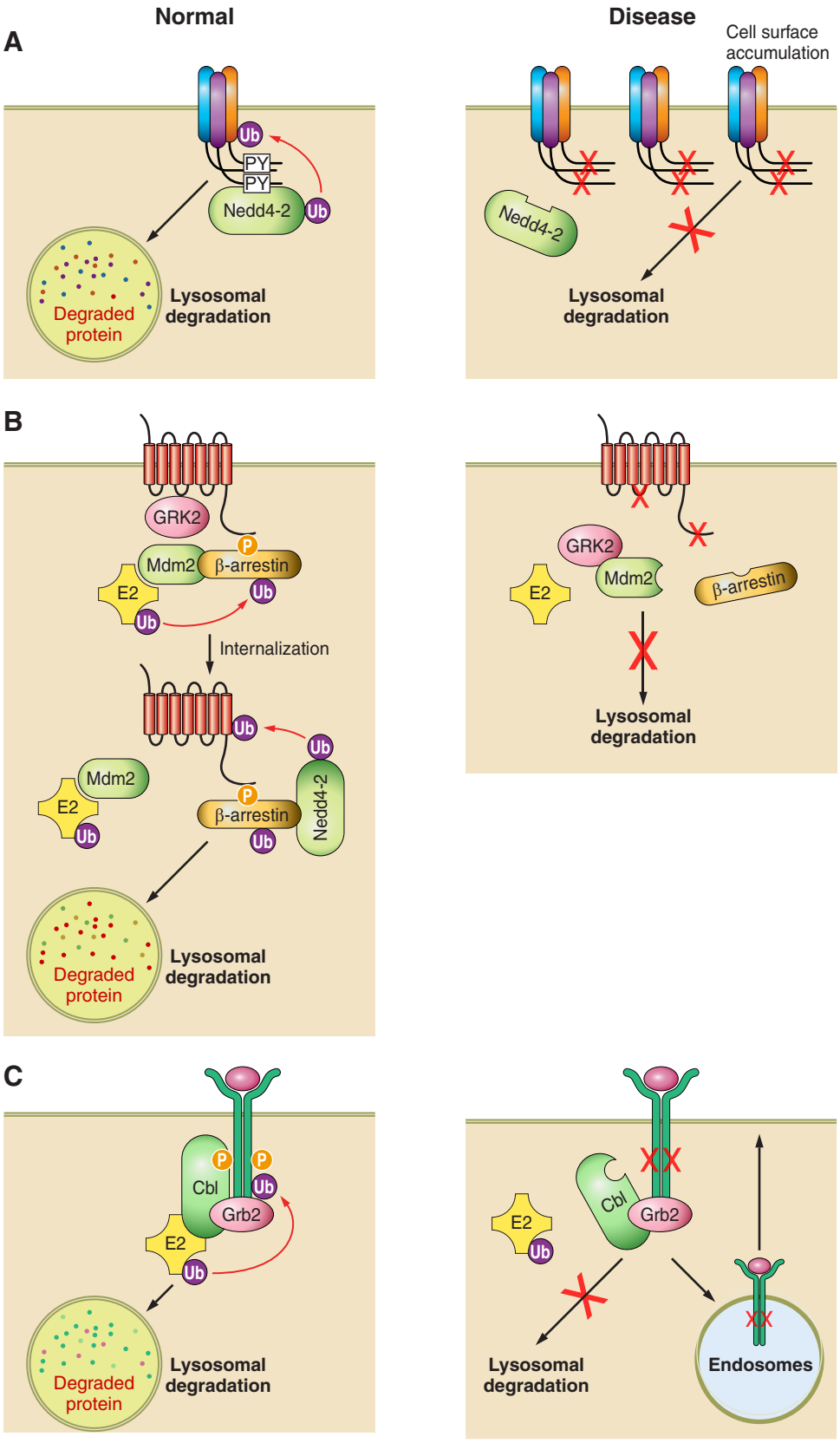

FIGURE 5. Examples of ubiquitination of membrane proteins in normal physiology and disease. *A:* some ion channels, such as ENaC, contain PY motifs that can directly bind to the WW domains in Nedd4 family of HECT E3s. Upon Nedd4-2 binding to the COOH termini of ENaC subunits, the channel is ubiquitinated, leading to its internalization and lysosomal degradation. In Liddle syndrome, the PY motifs of the ENaC β or γ subunits is mutated or deleted, preventing Nedd4-2 binding. This leads to the accumulation of ENaC at the cell surface, causing an excess of sodium reabsorption and hypertension. *B:* GPCRs, such as the adrenergic receptors, are regulated in a multistep manner. Upon activation, the receptor is phosphorylated by G protein receptor kinases, such as GRK2. β-Arrestin2 is then able to bind to the phosphoresidue and recruit the RING E3 Mdm2. This leads to the ubiquitination of β-arrestin2, which is the signal for the internalization of the complex. Once inside the cell, Mdm2 is displaced by Nedd4, which ubiquitinates the receptor to target it for degradation in the lysosome. Mutations that inhibit the binding and phosphorylation of receptor by GRKs prevent the recruitment of β-arrestins and subsequent ubiquitination, leading to accumulation of receptor at the surface. This hyperactivation of receptor can lead to diseases, such as cardiac dysfunction and hypertension. *C:* RTKs, such as Met, are phosphorylated following activation. This phosphorylation causes a conformational change that allows Cbl and its partners to bind and ubiquitinate the receptor. This complex is then internalized and trafficked to the lysosome for degradation. When the receptor is mutated at the phosphorylation site, Cbl is no longer able to bind the receptor. This prevents ubiquitination, thereby causing the receptor to be maintained on the endocytic membrane or recycled back to the plasma membrane where it is free to continue signaling. Mutant Met is therefore tumorigenic due to sustained signaling of downstream Ras/MAPK pathways.

which abrogate FBW7 binding (221, 368), cause a dysregulation of Notch signaling, which possibly contributes to the pathogenesis of T-cell acute lymphoblastic leukemia.

B. Hypertensive and Cardiac Diseases

Liddle syndrome is an inherited hypertensive disease caused by an increase in the activity of ENaC (269, 327). This hyperactivity is caused by gain-of-function mutations at the COOH termini of either β- or γ-ENaC subunits, at or near the PY motifs. This results in an abrogation of ENaC binding to the HECT E3 Nedd4-2, preventing ENaC ubiquitination and subsequent degradation of the channel (2, 326) **(FIGURE 5*A*)**. Salt-dependent hypertension has recently been demonstrated in a Nedd4-2 knockout mouse (25, 310). The Nedd4-2-deficient mice developed increased blood pressure partly due to increased expression and activity of ENaC, which could be reduced using the ENaC inhibitor amiloride. This idea was supported by a later study where Nedd4-2 was specifically knocked out in the nephrons of the kidney (284). In this model, loss of Nedd4-2 results in the upregulation of both ENaC and NCC at the plasma membrane. NCC has been shown to bind to and be ubiquitinated by Nedd4-2 (13, 175); however, this interaction does not occur through the canonical PY motif-WW domain interaction, suggesting a possible role for adaptor proteins. Nedd4-2 has also been shown in vitro to ubiquitinate other sodium transporters, such as the cotransporter NKCC2 and the sodium/hydrogen exchanger NHE1, which are also involved in regulating blood pressure (252, 375). Consistent with its central role in maintenance of blood pressure, NEDD4-2 (NEDD4L) variants in human families are linked to essential hypertension and salt-sensitive hypertension (282).

Pseudohypoaldosteronism type IIE is a severe form of familial hyperkalemia and hypertension. This is caused by mutations in Cullin-3 (CUL3) or Kelch-like 3 (KLHL3), components of the Cullin-RING-ubiquitin-ligase complex (29). Normally, CUL3 binds KLHL3 and the RING protein RBX1 to ubiquitinate WNK kinases, promoting their proteasomal degradation. WNK kinases activate NCC to promote salt retention. Loss of functional CUL3 or KLHL3 results in disruption of WNK ubiquitination, leading to increased WNK activity and, by extension, increased NCC activity (29, 224, 299).

Cardiac and hypertensive disorders can also be caused by mutations in GPCR signaling pathways (as reviewed by Lymperopoulos and Bathgate, Ref. 211). An example of this is in the α_2 adrenergic receptors, where a deleterious mutation of residues 301–303 prevents the binding of the G protein-coupled receptor kinase GRK2 and subsequent phosphorylation of the receptor (317). This then prevents β-arrestin2/Mdm2 binding and ubiquitination, and the attenuation of α_2 adrenergic receptor signaling (169, 211) **(FIGURE 5*B*)**. The resultant increase in signaling can then lead to an increased risk of hypertension and acute coronary events

C. Cystic Fibrosis

As previously discussed, cystic fibrosis is caused by mutations in the CFTR chloride channel which cause it to be recognized by the ERAD pathway and degraded rather than trafficked to the plasma membrane. Because of this, much work has been carried out to find therapeutic targets of the ubiquitin pathway that allow CFTR to traffic to the plasma membrane and thereby restore chloride balance. CFTR(ΔF508) can be rescued by low temperature or small molecular chaperones (357) and has some activity at the plasma membrane; however, its stability is decreased due to its misfolded nature and recognition by c-Cbl (52). Simultaneous knockdown of the RING E3 ligases RNF5 and RNF185 block CFTR(ΔF508) degradation, but have no effect on other classical ERAD substrates, making them ideal targets for therapeutic intervention (76, 348). In addition to this, another study found that activating the PDK1/SGK phosphorylation pathway with the small molecule C4-ceramide also rescued CFTR(ΔF508) through the phosphorylation and subsequent inactivation of Nedd4-2 through binding of 14-3-3 proteins (35).

D. Neurodegenerative Disorders

Ubiquitination of membrane proteins has also been implicated in a number of neurodegenerative disorders. Hereditary forms of Parkinson's disease are caused by mutations in the RBR-type E3 Parkin (174) or its activating kinase, PTEN-induced putative kinase 1 (PINK1) (352). In healthy neurons, cleaved PINK1 is recognized by E3 ubiquitin ligases and targeted to the proteasome. Damage to mitochondria circumvents the cleavage of PINK1 resulting in the accumulation of PINK1 on the mitochondrial outer membrane. This then recruits Parkin to the mitochondria, where it ubiquitinates a number of outer membrane proteins (294) and targets them for ERAD-like degradation by the proteasome (41, 170, 247, 381). This removal of mitochondrial proteins then leads to the degradation of the mitochondria by mitophagy (41, 340). In autosomal recessive forms of Parkinson's disease, mutations in Parkin or PINK1 reduce the cell's ability to remove damaged mitochondria, leading to the early onset of Parkinson's (170, 173, 266).

Alzheimer's disease is a neurodegenerative disorder characterized by the accumulation of the neurotoxic peptide β-amyloid, a cleavage product of the synaptic membrane protein amyloid precursor protein (APP). Ubiquitination has been shown to play a role in regulating both APP and β-amyloid. F-box only protein 2 (Fbxo2), a component of the SCF E3 ligase complex, is important for regulating APP

levels, and lack of Fbxo2 in knockout mice results in increased levels of APP as well as β-amyloid (14). Another underlying mechanism of Alzheimer's disease is the dysfunction of AMPA receptor trafficking, a critical component of learning and memory. Nedd4 has been shown to ubiquitinate the AMPA receptor GluA1 to mediate its internalization from the plasma membrane (20, 300). This results in reduced GluA1 surface expression and suppressed excitatory synaptic transmission (200). Furthermore, Nedd4 has also been implicated in the regulation of the ATP binding cassette transporter ABCB1 which is involved in the export of β-amyloid from endothelial cells of the blood brain barrier (7). This suggests a multifunctional role for Nedd4 in Alzheimer's disease.

As previously mentioned, misfolded prion protein (PrP^{Sc}) blocks proteasomal function by preventing entry by other substrates (60). This leads to cell death after accumulation of proteins in the cytosol and is associated with a variety of cognitive deficiencies and neurodegenerative diseases, such as Creutzfeldt-Jakob disease, bovine spongiform encephalopathy, fatal familial insomnia, and Kuru (5, 207).

Angelman syndrome is a severe disorder of postnatal brain development caused by neuron-specific loss of the HECT domain E3 ubiquitin ligase Ube3a/E6AP; however, the mechanisms by which Ube3a leads to neuronal dysfunction are still unclear. Ube3a has been shown to regulate several substrates involved in neuronal development and synaptic function. Ube3a directly ubiquitinates the small-conductance potassium channel SK2, leading to its endocytosis (331). Ube3a-deficient mice show increased SK2 levels that lead to a decrease in NMDA receptor activation, thereby impairing synaptic plasticity and memory (331). In *Drosophila*, Ube3a ubiquitinates the type I BMP receptor Thickveins (Tkv), promoting its proteasomal degradation (197). In Ube3a mutant flies, increased BMP signaling leads to an increased number of boutons in the neuromuscular junction, indicating a role for Ube3a in regulating synapse development by repressing BMP signaling (197). Ube3a also regulates the activity-regulated cytoskeleton-associated protein Arc, although how this occurs is a matter of some debate. Loss of Ube3a in neurons leads to an increase in Arc expression, leading to a concomitant decrease in AMPA receptors at the excitatory synapse (109), which has been proposed to contribute to the cognitive dysfunction that occurs in Angelman syndrome and possibly other autism spectrum disorders. In Ube3a-deficient mice, reducing the expression of Arc prevents seizurelike activity (217). It has been suggested, however, that Arc is not a direct substrate of Ube3a, but rather is regulated transcriptionally (184).

E. Metabolic Diseases

The muscle-specific ubiquitin ligase MG53/TRIM72 is markedly upregulated in models of insulin resistance and causes metabolic syndrome also characterized by obesity, hypertension, and dyslipidemia. MG53 mediates the ubiquitin-dependent degradation of the insulin receptor and insulin receptor substrate 1, and ablation of MG53 prevents this metabolic syndrome by preserving insulin receptor and subsequent signaling integrity (323).

Wolfram syndrome, a disease characterized by diabetes mellitis and optic atophy, is caused by mutations in the ER-localized Wolfram syndrome protein 1 (WFS1) that render the protein resistant to ubiquitination by the Nedd4 E3 Smurf1, triggering the ER stress response and apoptosis (85, 110).

F. Other

The HECT E3 ligase E6-AP controls mammary gland development by regulating progesterone and estrogen receptor ubiquitination and subsequent proteasomal degradation (277). With the use of mouse models, it has been shown that overexpression of wild-type E6-AP results in impaired mammary gland development, and loss of E6-AP increases lateral branching and alveolus-like protuberances in the mammary gland (277).

Mice deficient in Fbx2, a component of the SCF complex, develop an age-related hearing loss with cellular degeneration of the epithelial support cells of the organ of Corti, due to the dysregulation of the gap junction protein connexin26 (127, 250).

G. The Ubiquitin Pathway as a Promising Target of Therapeutic Intervention

Unlike the rapid progress achieved with kinase inhibitors, only a small number of drugs have been produced to target the UPS system (143). Progress has so far been hampered by technical difficulties of targeting small molecules to components of the UPS, due to the lack of a well-defined catalytic pocket to which small molecules may be targeted and the difficulty in disrupting the protein-protein interactions intrinsic to the UPS system. Despite these challenges, pharmaceutical companies are searching for ways to perturb the system to treat diseases. Many of the best-known UPS modulating drugs to date used for the treatment of diseases include bortezomib (Velcade), carfilzomib (Kyprolis), MLN9708 (ixazomib citrate), and CEP-18770 (delanzomib), which have shown dramatic results in treatment of multiple myeloma (44, 133, 180, 302). These drugs target the proteasome and regulate proteins involved in apoptosis and the cell cycle, such as $p27^{KIP1}$, p53, p21, PUMA, BAX, and NOXA (43, 44, 132, 202, 302).

Only a handful of drugs to date are available to target ubiquitin-dependent regulation of membrane proteins, and

most are in early stages of development. One promising target is the estrogen receptor, which is overexpressed in ~70% of breast cancer patients. Eventual resistance to current endocrine therapies, such as Tamoxifen, is a common occurrence, and therefore therapies targeting this receptor will be a welcome addition to the treatment arsenal. Fulvestrant, a selective ER downregulator (SERD), has been approved for use in patients (362); however, it has inferior pharmaceutical properties compared with other available drugs such as aromatase inhibitors and therefore has not progressed clinically. Fulvestrant binds to estrogen receptor monomers to inhibit receptor dimerization and translocation of receptor to the nucleus, and promote the receptor. Recent work at Genentech/Seragon has focused on identification and optimization of a number of SERDs with one (GDC-0810) showing promising results in phase I/IIa clinical trials (186).

New technological advances are also showing promise to unlock the UPS system to new therapeutics. For example, ubiquitin variants have been developed that can block or enhance the activity of E3 ligases and DUBs (77, 389), while computer-aided drug design can also help to develop new, targeted therapies. Currently, the UPS system remains a relatively unexplored world for therapeutics, but one that has the promise of significant advancements in the near future.

IX. SUMMARY AND FUTURE PERSPECTIVES

As listed in **TABLE 1**, numerous membrane proteins are likely to be regulated by the ubiquitin system. Through specific examples in this review, we have summarized how ubiquitination controls trafficking and abundance of a membrane protein from the time of its birth, through its functional life, and finally to degradation. Given that many thousands of proteins in the cellular proteome are likely to be ubiquitinated at some point in their lives, it is reasonable to expect that many more membrane proteins are modified by ubiquitination. For example, recent studies have uncovered that many GPCRs are regulated by ubiquitin-dependent mechanisms in various subcellular compartments, and thus ubiquitination may be a critical control system in determining the functional outcomes of this largest family of receptors, involved in a vast array of physiological signaling pathways (159). Also, although not thoroughly discussed in this review, many membrane proteins are regulated indirectly by the ubiquitination of accessory proteins in the membrane proximal complexes. Examples of this include regulation of IGF1R in IR signaling through Nedd4 and PTEN-mediated ubiquitination of Grb10 and IRS (33, 95), and regulation of TNFR1 and TLR signaling in inflammation through various types of ubiquitination including linear chains, and multiple specific substrates and E3s (66, 369). Given that our knowledge of ubiquitination-dependent control of membrane proteins is still rather limited, a lot more work is required in this field to fully appreciate the intricate mechanisms involved.

Initially thought to be simply a signal for protein degradation, the complexity of ubiquitination and how it may regulate signaling outcomes through various types of ubiquitin chains and posttranslation modifications of ubiquitin itself have only become apparent in recent years. In the context of regulation of membrane proteins through trafficking, recycling, and degradation, the mechanistic insight gained from further studies will be critical in targeting the ubiquitin system for therapeutic purposes. We have provided a few examples of pathological consequences of aberrant ubiquitination of membrane proteins, but given the ubiquitous role of this protein-modification system in cell signaling, numerous human pathologies can be linked directly or indirectly to the ubiquitin system. As briefly discussed above and reviewed recently elsewhere (143), various novel strategies are currently underway for drug development to target the ubiquitin system.

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