Chapter 4
ESCRT and Membrane Protein Ubiquitination

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Contents

Abstract The ubiquitin-dependent degradation of membrane proteins via the multivesicular body (MVB) pathway requires the Endosomal Sorting Complexes Required for Transport (ESCRT). This molecular machinery is composed of five distinct multisubunit complexes. On the surface of endosomes, ESCRT-0, -I and -II bind to ubiquitinated membrane proteins, while ESCRT-III and Vps4 bud intraluminal vesicles (ILVs) into the lumen of the endosomes. By working together, ESCRTs package membrane proteins into ILVs and thereby generate MVBs. The fusion of mature MVBs with lysosomes delivers ILVs into the lysosomal lumen where the membrane proteins are degraded. Besides generating ILVs, the ESCRT machinery mediates for topologically related membrane budding processes at the plasma membrane and the nuclear envelop. In this chapter, we briefly discuss membrane protein ubiquitination, endocytosis, and summarize current knowledge on the ESCRT machinery in the MVB pathway.

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4.1 Ubiquitination

Ubiquitination is a modular, reversible and transferable protein modification (Piper and Lehner 2011). Ubiquitin itself is a highly conserved, cytoplasmic, 76-amino acid polypeptide. It can be covalently attached (conjugated) to lysine residues in target proteins by ubiquitin ligases and removed by deubiquitinating enzymes.

Ubiquitin conjugation is a three-step process. A ubiquitin-activating enzyme (E1) takes up the ubiquitin and transfers it to a ubiquitin-conjugating enzyme (E2). Ubiquitin-ligating (E3) enzymes conduct the final step of ubiquitination and conjugate the carboxy-terminal glycine of ubiquitin to the amino group of a lysine in the target protein (Fig. 4.1a).

E2 and E3 enzymes are directly involved in transferring ubiquitin to the target protein and therefore contribute to the specificity of the ligation process (Hicke et al. 2005; Hofmann and Pickart 2001; Komander 2009). Eukaryotic genomes encode only few E1 enzymes (1 in yeast, approx. 10 in human), while E2 enzymes are more abundant (11 in yeast, approx. 40 in human). E3 enzymes are even more diverse (54 in yeast, approx. 600 in human (Hershko and Ciechanover 1998; Hicke et al. 2005; Metzger et al. 2012). About 100 deubiquitinating, enzymes (DUBs) can cleave the isopeptide ubiquitin and the target protein and thereby make ubiquitination reversible and help to recycle ubiquitin (Komander 2009) (Figs. 4.1a, 4.2a, b).

E3 enzymes can be grouped in two families: HECT ligases and RING ligases. The HECT E3 ligase (homologous to the E6AP carboxyl terminus) family members pick up the ubiquitin from the E2 enzyme before they conjugate it to the target protein. Therefore, a prototypical HECT domain interacts with the E2 enzyme, as well as an active-site cysteine, that forms the thioester with ubiquitin (Metzger et al. 2012; Huang et al. 1999) and then transfers the ubiquitin to the target protein (Fig. 4.1a).

In contrast, the really interesting new gene (RING) E3 ligases mediate a direct transfer from the E2 carrier to the target protein. RING ligases function as a scaffold, that brings the E2 enzyme and the target protein in close proximity for the ubiquitin transfer process (Lorick et al. 1999; Metzger et al. 2012) (Fig. 4.1a).

From yeast to human ubiquitin has remained evolutionary almost invariable except for three conservative changes. It has an overall molecular mass of 8.5 kDA and contains a compact β-grasp fold with a flexible C-terminal tail. Ubiquitin contains seven lysine residues and they are ubiquitinated as well (Ravid and Hochstrasser 2007; Wang and Pickart 2005). This implies that target proteins can be mono- (one ubiquitin on one lysine), multi- (single ubiquitin molecules on several lysines within one protein), or poly-ubiquitinated (>10 ubiquitin moieties on one lysine) (Fig. 4.1b). Monoubiquitin can be extended into eight different homotypic polyubiquitin chains that always consist of the same linkage: K6, K11, K27, K29, K33, K48, K63 and Met1 (Dikic and Robertson 2012; Hofmann and Pickart 2001; Kerscher et al. 2006; Piper and Lehner 2011). Heterotypic polyubiquitin chains have mixed linkages. Moreover, conjugated ubiquitin molecules can be subject to posttranslational modifications, such as acetylation on 6 of 7 Lys and phosphorylation on Ser, Thr and Tyr residues (Swatek and Komander 2016; Mevissen and Komander 2016).

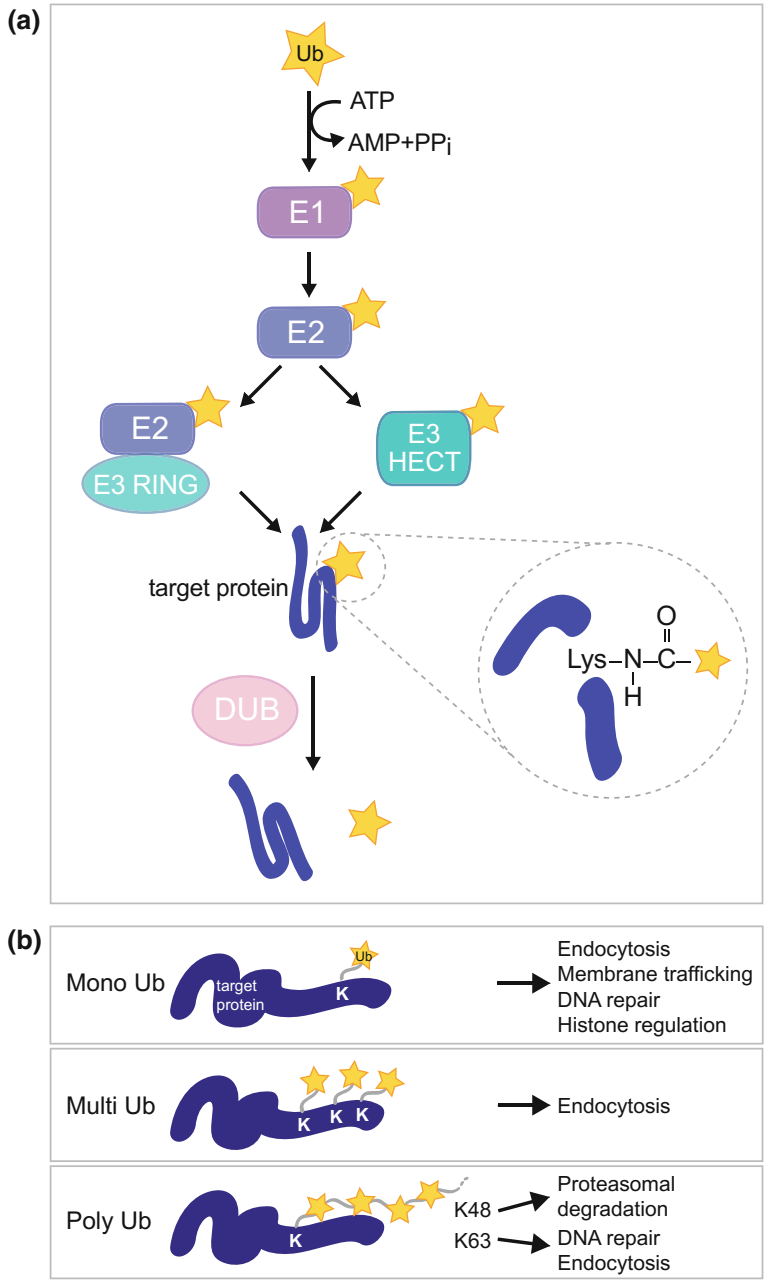

Fig. 4.1 **Ubiquitination of proteins a** Ubiquitination involves ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3). Ubiquitination can be reversed by deubiquitinating enzymes (DUBs). **b** Mono-, multi- and poly-ubiquitination of target proteins. Ubiquitin is covalently attached to lysine residues of the target protein via an isopeptide bond. The form of ubiquitination determines the destination of the protein

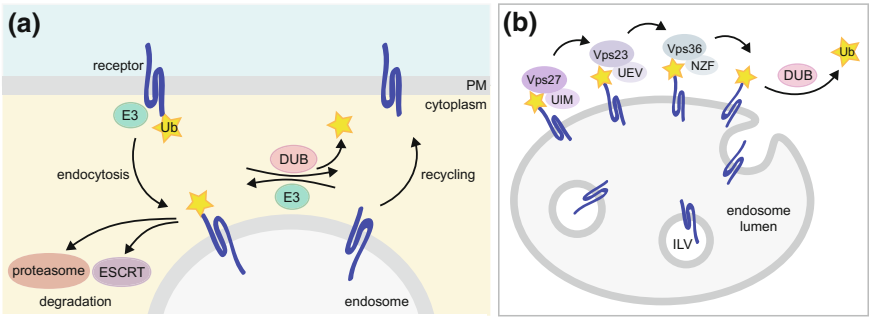

Fig. 4.2 Ubiquitination and deubiquitination of proteins a Ubiquitination and deubiquitination reactions at the plasma membrane and on endosomes. **b** Ubiquitination of membrane proteins induces ESCRT-dependent sorting into intraluminal vesicles (ILV) on endosomes. ESCRT subunits interact directly via their ubiquitin-binding domains. Deubiquitination takes place prior to ILV formation. Ubiquitin-interacting motiv (UIM), ubiquitin E2 variant (UEV), novel zink finger (NZF), plasma membrane (PM)

The flexible tails and the different ubiquitination modes provide a code that can be read by different ubiquitin-binding domains (UBDs) and thus determine the fate of the protein. UBDs recognize different forms of ubiquitination (mono- versus polyubiquitin chains) and/or different surface regions within the ubiquitin moiety and thus bind to ubiquitinated proteins with different affinities (Di Fiore et al. 2003; Schnell and Hicke 2003). Some examples of UBDs are: ubiquitin-interacting motif (UIM), ubiquitin-associated domain (UBA), ubiquitin-conjugating enzyme-like (UBC), ubiquitin E2 variant (UEV), Cue1-homologous (CUE), polyubiquitin-associated zinc finger (PAZ) and novel zinc finger (NZF).

Ubiquitination is essential for many cellular processes. In this chapter, we focus on the ubiquitination of plasma membrane proteins and the subsequent endocytosis and lysosomal degradation via the MVB pathway. We also briefly discuss some of the involved ubiquitin ligases and DUBs.

4.1.1 Ubiquitination of Membrane Proteins

The ubiquitination of membrane proteins also involves E1 and E2 enzymes and E3 ubiquitin ligases. Two major types of E3 ligation enzymes are involved: The HECT-type Nedd4 (neural precursor cell expressed developmentally down-regulated protein 4) family and the RING-type MARCH ligases (membrane associated RING-CH ligases).

The yeast Nedd4 homologue Rsp5 is one of the best-studied E3 ligases (Rotin and Kumar 2009; Staub et al. 2000; Belgareh-Touzé et al. 2008; Dupré et al. 2004).

The human genome encodes nine Nedd4 family members. Nedd4/Rsp5 have a characteristic domain organization with an N-terminal C2 domain, a variable number of WW domains (protein–protein interaction domains) and the HECT domain.

The C2 domain spans approximately 130 amino acids and functions as a protein–lipid interaction module (Nalefski and Falke 1996; Hurley and Misra 2000).

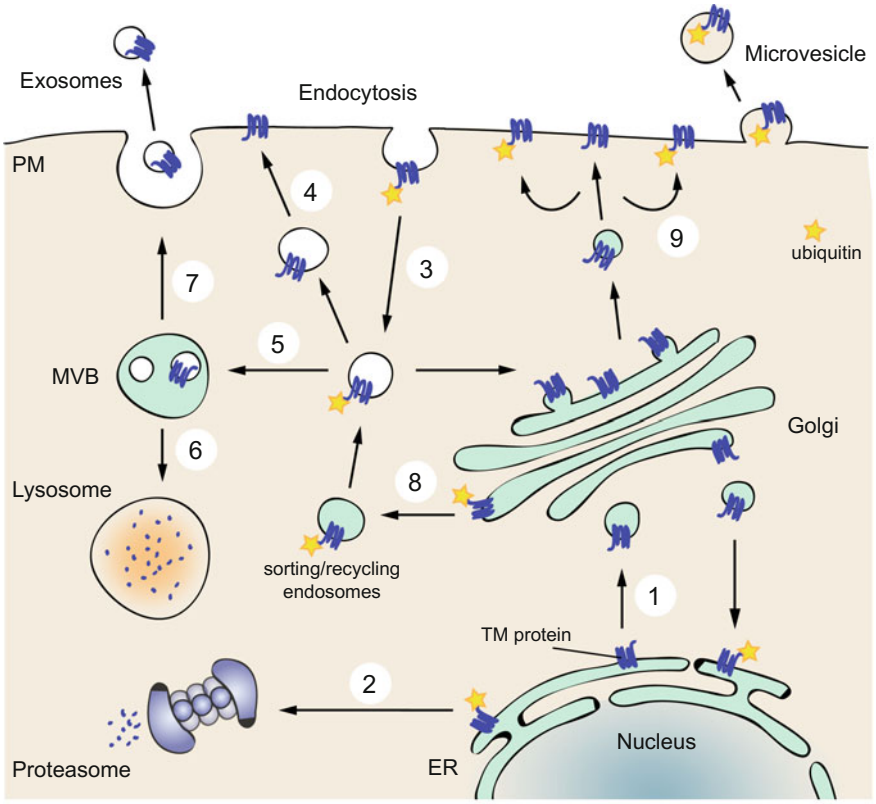

Fig. 4.3 Regulation of membrane proteins Ubiquitination influences the lifecycle and destination of transmembrane proteins. Membrane proteins are synthesized at the endoplasmatic reticulum (ER) and undergo quality control (ERQC). (1) Membrane proteins are transported to the Golgi, for modification and quality control. (2) Misfolded proteins are recognized by the ER-associated degradation (ERAD), ubiquitinated and a target for proteasomal degradation. (3) Ubiquitinated plasma membrane proteins undergo endocytosis. (4) Endocytosed proteins are either recycled back to the plasma membrane or to the Golgi. (5) Endosomes can mature via the ESCRT mediated multivesicular body (MVB) pathway, which (6) targets ubiquitinated membrane proteins for lysosomal degradation. (7) MVBs can fuse with the plasma membrane and release their vesicles as exosomes. (8) Ubiquitination at the Golgi contributes to the endosomal sorting or recycling. Proof read proteins are transported from the Golgi to the plasma membrane (PM). (9) Ubiquitination can be a signal for microvesicle formation

The C2 of Rsp5 interacts with phosphoinositides in membranes a function that is important for the ubiquitination of membrane proteins (Dunn et al. 2004).

The WW domains are about 40 amino acids long and bind to proline-rich sequences, so-called PY motifs (Kaliszewski and Zoładek 2008; Bedford et al. 2000). Group I binds to PXY, LPXY and PPXY motifs. group II binds to PPLP, group III binds PPR motifs, and group IV binds short sequences containing phospho-serine or phospho-threonine followed by proline. Some membrane proteins such as the epithelial sodium channel, ENaC, contain PPxY motifs and thus permit direct interaction with the WW domain of Nedd4-2, allowing its ubiquitination and endocytosis (Lu et al. 2007; Staub et al. 2000). The down-regulation of ENaC controls blood volume and pressure, and the defects in this process result in hereditary hypertension in humans (Liddle syndrome) (Hamilton 2014; Rotin et al. 2000; Kaliszewski and Zoładek 2008). Yet, most (yeast) PM proteins do not contain PY motifs. Therefore, adaptor proteins regulate the recruitment of Nedd4/Rsp5 to most membrane proteins.

These adaptor proteins are called α-arrestins. They contain arrestin-like domains that probably mediate cargo interaction and multiple PPxY motifs that interact with the WW domains of Rsp5/Nedd4 and thereby recruit Nedd4/Rsp5 to membrane proteins (Kang et al. 2014; Nikko et al. 2008; Becuwe et al. 2012b; Lin et al. 2008).

In humans, there are six α-arrestins and they are called arrestin domain containing proteins ARRDC1-5 and TXNIP. Little is known about their regulation and function in human cells. TXNIP (also known as VDUP1 for vitamin D3 up-regulated protein-1) functions together with Nedd4 to mediate the clathrin-dependent endocytosis of the glucose transporter GLUT1 (SLC2A1) (Wu et al. 2013). Other α-arrestins are involved in the endocytosis of transporters, GPCRs and integrins (Nabhan et al. 2010; Draheim et al. 2010; Wu et al. 2013; Rauch and Martin-Serrano 2011) and links to cancer have been reported (Draheim et al. 2010; Morrison et al. 2014). Their yeast orthologues are called arrestin-related trafficking adaptors (ARTs). They are better characterized. The core of this ubiquitin ligase adaptor network comprises a

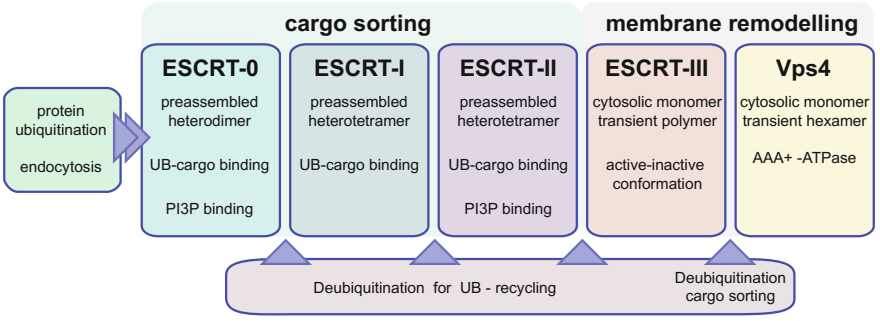

Fig. 4.4 Ubiquitination—deubiquitination and ESCRT complexes Overview on the most important features of the five ESCRT complexes. The ESCRT-0, -I and -II complexes bind to ubiquitinated cargo and PI3P. The ESCRT-III and Vps4 mediate membrane budding and scission. Deubiquitination can take place at different steps

family of 10 ART proteins (Art1-10), and Bul1 and Bul2. Art2/Art8 (Ecm21/Csr2), Art3/Art6 (Aly2/Aly1), Art4/Art7 (Rod1/Rog3) and Bul1/Bul2 are considered gene duplications.

The adaptor function for most yeast ART proteins was demonstrated in recent years. Under different growth conditions or under stress (mainly nutrient excess) ARTs regulate, in a partially redundant manner, several different transporters and thereby enable ubiquitin-dependent nutrient transporter endocytosis (Suzuki et al. 2013; O'Donnell et al. 2010, 2013; Alvaro et al. 2014; Marqués and Zamarbide-Forés 2015; O'Donnell et al. 2015; Becuwe et al. 2012a; Hatakeyama et al. 2010; Smardon and Kane 2014; Crapeau et al. 2014; Merhi and Andre 2012; Ghaddar et al. 2014; Nikko and Pelham 2009; Nikko et al. 2008; Lin et al. 2008; MacGurn et al. 2011; Zhao et al. 2013). This is exemplified by the regulation of Lyp1 (lysine transporter) endocytosis. Art1/Ldb19 is required for Lyp1 endocytosis in response to excess lysine, whereas Art2/Ecm21 is required for Lyp1 internalization in response to cycloheximide (CHX causes TORC1 hyper-activation) treatment. These findings suggested that ART function and transporter endocytosis are controlled by different stimuli (Lin et al. 2008). The regulation of ARTs is controlled by post-translation modifications (PTMs) (Becuwe et al. 2012b). One common regulatory concept is the ubiquitination of the ARTs by Rsp5. In addition, it is becoming clear that phosphorylation inhibits ART function whereas de-phosphorylation activates ART function. Some examples are described below: Under energy stress, mammalian cells activate the 5'AMP-activated protein kinase, AMPK. AMPK has many targets; among others, it phosphorylates the α-arrestin TXNIP, leading to its degradation. Low levels of TXNIP can no longer efficiently down-regulate the glucose transporter GLUT1 (SLC2A1), which in turn allows the influx of glucose to restore ADP/ATP homeostasis (Wu et al. 2013).

When yeast cells experience glucose starvation and grow in the presence of lactate, they also activate AMPK, Snf1. In turn, Snf1 phosphorylates Art4 (Rod1). 14-3-3 proteins bind to phosphorylated Art4 and inactivate it (Becuwe et al. 2012a). Thereby, Snf1-dependent inactivation of Art4 prevents endocytosis of the lactate transporter Jen1 and thus stimulates lactate influx. Upon addition of glucose, a preferred carbon source, Art4 is dephosphorylated by the PP1 phosphatase, Glc7, which releases the 14-3-3 proteins. Now, Art4-Rsp5 complexes become active and ubiquitinate Jen1, which results in endocytosis and degradation of Jen1 (Becuwe et al. 2012b). Conversely, glucose deprivation regulates the ART protein Csr2/Art8 at multiple levels to trigger high-affinity glucose transporter endocytosis (Hovsepian et al. 2017). Also, amino acid starvation triggers endocytosis (Muller et al. 2015; Jones et al. 2012), but how the ART proteins are regulated in response to nitrogen deprivation is not clear.

The nutrient-sensitive kinase complex—target of rapamycin complex 1 (TORC1)—also controls the ART-Rsp5 network via phospho-inhibition of the kinase Npr1. Inactivation of TORC1 by rapamycin treatment or by amino acid/nitrogen starvation activates Npr1. Activated Npr1 phosphorylates Art1 and Bul1/2 and thereby inhibits their function (Becuwe et al. 2012b; MacGurn et al. 2011; Merhi and Andre 2012; Crapeau et al. 2014). Phosphorylated Bul1/2 is bound by 14-3-3 proteins and

inactivated (Merhi and Andre 2012). Thus, inactivation of TORC1 should inhibit endocytosis of Art1- and Bul1/2-dependent PM proteins. Conversely, activation of TORC1 by nutrient excess or CHX treatment inactivates Npr1, which in turn stimulates endocytosis of the Art1 cargo, Can1 (arginine transporter) and the Bul1/2 cargo, general amino acid permease (Gap1). Activation of Bul1/2 and subsequent endocytosis of Gap1 additionally depends on Sit4 phosphates activity (Merhi and Andre 2012).

Interestingly, Gap1 is also down-regulated in rapamycin-treated cells, which shuts off TORC1 signalling. The rapamycin-induced endocytosis of Gap1 still requires Bul1/2 but additionally depends on Art3/6 (Aly2/1) (Crapeau et al. 2014). An additional layer of complexity may be added by stimulus-induced transporter phosphorylation, akin to the recognition of GPCRs by β-arrestins in mammalian cells (Shukla et al. 2014; Nikko et al. 2008). It seems that ART-Rsp5-mediated ubiquitin-dependent nutrient transporter endocytosis is controlled by central signalling networks (TORC1 and AMPK) to adjust nutrient influx with cellular metabolism and growth.

The second major family of membrane protein E3 ligases is represented by RING ligases mostly of the MARCH family. There are 11 MARCH proteins and they were originally discovered as structural homologues to the viral E3 ligases, K3 and K5 from Kaposi's sarcoma-associated herpesvirus (KSHV) that play an important role in immune evasion (Samji et al. 2014).

Most of the MARCH ligases have two transmembrane domains with some having up to 14 putative transmembrane domains (Nathan and Lehner 2009; Piper and Lehner 2011). The best-characterized member is MARCH1, which ubiquitinates MHC-II on the plasma membrane of antigen presenting cells, initiates its endocytosis, and thereby influences immune and inflammatory responses (Oh et al. 2013; Cho et al. 2015). Other surface receptors known to be down-regulated by MARCH are CD44, CD81, CD4, CD86, transferrin receptor and others mostly important for cell differentiation (Metzger et al. 2012; Nakamura 2011; Samji et al. 2014). Overall, it is becoming clear that MARCH proteins have a wide variety of substrates and binding partners, including other membrane-bound proteins, SNAREs and even cytoskeletal elements, like microtubules. Despite increasing evidence that MARCH proteins are important regulators of cellular physiology, they are currently understudied.

Another prominent family of the RING ligases involved in plasma membrane protein ubiquitination are the three CBL proteins (casitas B-lineage Lymphoma). c-CBL, CBL-b, CBL-3 contain a PTB domain (phosphotyrosine-binding domain or tyrosine kinase domain) and a RING finger domain. c-CBL and CBL-b additionally contain a proline-rich region and a C-terminal ubiquitin-associated domain (UBA) (Schmidt and Dikic 2005; Kozlov et al. 2007; Gay et al. 2008). CBL ubiquitinates numerous target proteins, including the activated epidermal growth factor receptor (EGFR) (Haglund 2003; Levkowitz et al. 1998, 1999; Sigismund et al. 2005, 2008; Yokouchi et al. 1999; Deshaies and Joazeiro 2009; Thien and Langdon 2001; Wee and Wang 2017; Mohapatra et al. 2013), which results in EGFR endocytosis and subsequent lysosomal degradation via the MVB pathway. This is most likely the best-studied ubiquitination of a membrane protein and has been subject to recent reviews (Haglund and Dikic 2012; Cruickshanks 2014).

So far, we have mainly discussed how membrane proteins are ubiquitinated once they have been properly folded and exported from the ER (Fig. 4.3). Early in their lifetime—still in the ER—membrane proteins undergo a ubiquitin-dependent quality control process that ensures, that misfolded proteins are not efficiently exported from the ER. Misfolded membrane proteins are retro-translocated into the cytoplasm, ubiquitinated and degraded by the proteasome. This ER-associated degradation pathway (ERAD) has been extensively studied in yeast. Two RING E3 ligases, Doa10p and Hrd1, have been discovered to target almost all substrates: Doa10 targeting cytosolic proteins and Hrd1 is ubiquitinating misfolded membrane proteins (Christianson and Ye 2014; Bays et al. 2001; Carvalho et al. 2006; Vashist and Ng 2004). Sometimes, misfolded proteins escape the ERAD system and arrive at the Golgi. The ubiquitin quality control at the Golgi and the E3 ligases involved is not yet characterized well. In fission yeast and in budding yeast, a transmembrane ubiquitin ligase complex called Dsc operates at the Golgi and can ubiquitinate misfolded membrane proteins which ensure that they are degraded in lysosomes (Reggiori and Pelham 2002). In addition, the Dsc complex is also required for the hypoxia-induced activation of yeast SREBP cleavage (Stewart et al. 2011). In budding yeast the E3 ligase of the Dsc complex, Tul1 also functions together with Rsp5 on the surface of the vacuole and there they ubiquitinate different membrane proteins and thereby target them for lysosomal degradation (Li et al. 2015a, b). Overall it is becoming clear that ubiquitin ligases target membrane proteins at the ER, the Golgi, at the PM and on endosomes, and even on lysosomes. In almost all cases, the ubiquitination of membrane proteins targets them for degradation (Foot et al. 2017) (Fig. 4.3).

Ubiquitination is reversed by approximately 100 DUBs in human cells and 20 DUBs in yeast. There are six structurally distinct DUB families that can deal with the vast complexity of the ubiquitination code and additionally contribute to the complexity of the system. Their structural features and functional properties have been summarized recently (Mevissen and Komander 2016). Later, we will briefly discuss DUBs that are involved in the degradation of membrane proteins (please see Sect. 4.3.1).

4.1.2 Endocytosis—As Consequence of Ubiquitination

Adjusting the cell surface abundance of ion channels, growth factor receptors, GPCRs and nutrient transporters is essential to control cell proliferation, migration, electrolyte balance, metabolism and differentiation (Lemmon and Schlessinger 2010). Frequently ubiquitination serves as signal for endocytosis, but there is also evidence that ubiquitin-independent endocytic pathways exist (Galan et al. 1996; Hicke and Riezman 1996; MacGurn et al. 2012). During endocytosis, the target plasma membrane proteins are sequestered in vesicles, which bud into the cytosol to form primary endosomes (Fig. 4.3).

The role of ubiquitin in endocytosis is complex and involves numerous endocytic adaptors. Such adaptors are the Epsin proteins. They bind to ubiquitinated membrane

proteins and to the clathrin machinery at the same time. The proteins are called Epsin1/2/3 (Ent1, Ent2 in yeast) and the Eps15 and Eps15R (Ede1, Ede3 in yeast). Their binding to ubiquitinated membrane proteins at the plasma membrane leads to the recruitment of the clathrin effector proteins and the actin cytoskeleton, and together with many other endocytic proteins, this ultimately results in endocytosis (Polo et al. 2002; Shih et al. 2002; Godlee and Kaksonen 2013).

4.2 Ubiquitin-Mediated Protein Sorting on Endosomes

Once ubiquitinated plasma membrane proteins have been internalized, they enter the endosomal network. From there, membrane proteins can either recycle back to the plasma membrane or are transported to the lumen of lysosomes for degradation via the MVB pathway. MVB formation requires the budding of the limiting endosomal membrane into the lumen of the organelle to generate intraluminal vesicles (ILVs). Membrane proteins—cargo—that are destined for degradation in lysosomes must be sorted into the growing ILVs. It is assumed that cargo molecules are typically ubiquitinated. In some case, cargo sorting along the MVB may also occur independent from cargo ubiquitination (e.g. for P2Y1 purinergic receptor) (Dores et al. 2012). Tetraspanins can support the sorting of non-ubiqutinated cargo molecules by providing a ubiquitination signal in trans (MacDonald et al. 2015). The formation of ILVs can be driven by lipids only or by the Endosomal Sorting Complexes Required for Transport (ESCRT) (for a review see Babst (2011).

The ESCRT machinery (Fig. 4.4) couples the sorting of ubiquitinated membrane proteins to the biogenesis of ILVs. ESCRT complexes bind directly to ubiquitinated membrane proteins, induce cargo crowing, ILV budding, cargo deubiquitination and finally scission of the cargo laden ILV from the limiting MVB membrane. The interplay between cargo, endosomal phospho-lipids and five different ESCRT complexes (ESCRT-0, -I, -II, -III and Vps4) ultimately leads to the biogenesis of MVBs.

4.3 The ESCRT Complexes—Domains, Conformation and Interaction

ESCRT-0, -I and -II contain specific ubiquitin-binding domains to recognize the ubiquitinated cargo of endosomes (Schmidt and Teis 2012) (for details see below) (Table 4.1).

ESCRT-0 consists of a 1:1 heterodimer consisting of Vps27/Hse1 (Hrs1/STAM in mammalian) (Ren et al. 2009). It is recruited to phosphatidylinositol 3-phosphate on endosomes via the FYVE zinc finger domain of HRS/Vps27, (Myromslien et al. 2006; Raiborg et al. 2001a, b; Wollert and Hurley 2010). The ESCRT-0 complex contains multiple ubiquitin-binding domains (UBDs). STAM/Hse1 contains one VHS (Vps27,

Table 4.1 ESCRT complexes on endosomes, co-factors, ubiquitin ligases and deubiquitinating enzymes

ESCRT protein	Yeast/human homologue		Domains and motives	Modification	Function
ESCRT-0	Vps27	HRS	UIM, FYVE, VHS, clathrin binding	Ubiquitinated	Binds to ubiquitinated membrane proteins and PI3P
	Hse1	STAM1, STAM2	VHS, UIM, GAT, coiled coil, CB	Ubiquitinated	Interaction with Hua1 and Rsp5
ESCRT-I	Vps23	TSG101	UEV, PRD, coiled coil	Ubiquitinated	Cargo and Vps27 interaction
	Vps28	VPS28	CTD		Binding to ESCRT-II (Vps36)
	Vps37	VPS37 A, B, C, D	Coiled coil, MOD(r)		
	Mvb12		MABP, UMA, UBD	Ubiquitin	Ubiquitin binding
ESCRT-II	Vps22	EAP30, SNF8	Coiled coil		
	Vps25	EAP20			Binding to ESCRT-III (Vps20)
	Vps36	EAP45	GLUE		Cargo and PI3P interaction; interaction with Vps28
ESCRT-III	Vps20	CHMP6	MIM2	Myristoylated	Binding to ESCRT-II (Vps25)
	Vps32/Snf7	CHMP4A, B, C	MIM1, MIM2		Membrane deformation; membrane invagination
	Vps2/Did4	CHMP2A, B	MIM1	Ubiquitinated	Vps4 recruitment
	Vps24	CHMP3	MIM1		

(continued)

Table 4.1 (continued)

ESCRT protein	Yeast/human homologue		Domains and motives	Modification	Function
	Ist1	IST1	MIM1, MIM2		Interacts with Did2 and Vps4
	Vps60/Mos10	CHMP5	MIM1, coiled coil		ESCRT-III like
	Vps46/Did2	CHMP1A, B	MIM1, coiled coil		ESCRT-III like
AAA+-ATPase	Vps4	VPS4A, B/SKD1	MIT, AAA+		ESCRT disassembly and recycling
	Vta1	LIP5	MIT, VSL, Coiled coil		Positive regulator of Vps4
Modulators and adaptors	Vps31/Bro1	ALIX/AIP1	Coiled coil, PRD, PTAP-like	Ubiquitin	Doa4 recruitment; ESCRT-III interaction
PI3 Kinase complex	Vps34	VPS34	C2, PIK		PI(3)P synthesis
	Vps15	PIK3R4(p150)	PKinase, HEAT, WD40		
Ubiquitin ligase	Rsp5	Nedd4/Cbl and many others	C2, WW, HECT		Cargo ubiquitination
De-ubiquitinase	Doa4	UBPY/USP8	Rhod, UBP		Cargo deubiquitination
	Ubp7	AMSH	MIT, JAMM		Cargo deubiquitination
	Hua1	Plant homologues			Links Rsp5 to Hse1
	Rup1	Plant homologues			Complex with Rsp5 and Ubp2

Abbreviations AAA+(ATPase associated with diverse cellular activities; CB (clathrin-box motive); CHMP (charged multivesicular body protein); CTD (C-terminal domain); FYVE (Fab1p/YOTB/Vac1p/EEA1 domain); GAT (GGA/TOM1 domain); GLUE (GRAM-like ubiquitin in EAP45 domain); HRS (hepatocyte growth factor-regulated tyrosine kinase substrate); JAMM (JAB1/MPN/MOV34 metalloenzyme); MABP (MVB12-associated β-prism domain); MIT-interacting motif (MIM); microtubule-interacting and transport (MIT) domain; MOD(r) (modifier of rudimentary domain); PI3P (phosphatidylinositol 3-phosphate); PIK (Phosphoinositide 3-kinas); PRD (proline-rich domain); Rhod (Rhodanese Homology Domain); STAM (signal-transducing adaptor molecule); ubiquitin-binding domain (UBD); ubiquitin-conjugated enzyme E2 variant (UEV); ubiquitin-interacting motif (UIM); UMA (UBAP1/MVB12-associated domain); VHS (Vps27/HRS/STAM domain); VSL (Vta1/SBP1/LIP1 domain)

HRS and STAM) and one ubiquitin interaction motif (UIM) domain. HRS/Vps27 contains a VHS domain plus a double UIM (Zakalskiy et al. 2002) or two UIMs (Vps27) (Babst 2005; Bilodeau et al. 2003; Hicke et al. 2005). Hence, a single ESCRT-0 complex could bind up to five different ubiquitinated membrane proteins or multiple ubiquitin moieties of poly-ubiquitinated cargo to sort these away from other membrane proteins (Prag et al. 2007; Raiborg and Stenmark 2009; Wollert and Hurley 2010). HRS of ESCRT-0 can also interact with flat clathrin coats on endosomes, which may further promote the ubiquitinated cargo crowding (Bilodeau and Urbanowski 2002; Clague 2002; Raiborg et al. 2002; Shih et al. 2002). The C-terminal region of HRS/Vps27 domains of both proteins contains PTAP motifs (proline, threonine, alanine, proline) required for the binding to ESCRT-I (Katzmann et al. 2003; Chu et al. 2006). STAM2 also interacts with ESCRT accessory protein HD-PTP (cytosolic protein tyrosine phosphatase). The N-terminal Bro1 domain of HD-PTP in turn interacts with ESCRT-III protein CHMP4B. In yeast, Bro1 binds ESCRT-I and -III and recruits and catalytically activates Doa4. HD-PTP might fulfil a similar role in establishing a connection between ubiquitinated cargo, ESCRT-III subunits and the coordination of deubiquitinating enzymes (Ali et al. 2013; Lee et al. 2016).

ESCRT-I is an elongated heterotetramer that spans approx. 18 nm. It consists of four subunits Vps23 (Tsg101), Vps28 (VPS28), Vps37 (VPS37A, B) and Mvb12 (MVB12A, B) or UBAP1 (Katzmann et al. 2001; Chu et al. 2006; Oestreich et al. 2007; Audhya et al. 2007; Curtiss et al. 2007; Wunderley et al. 2014). Together they form a soluble complex that is transiently recruited from the cytoplasm to the surface of endosomes. Different structural conformations of the ESCRT-I complex in solution have been resolved (Kostelansky et al. 2007; Boura et al. 2011). Together Vps23, Vps37 and Mvb12 form a long intertwined coiled–coiled stalk. The headpiece consists of Vps23, Vps28 and Vps37 (which binds to ESCRT-II). On its one end, the ubiquitin-conjugating enzyme E2 variant (UEV) domain of TSG101/Vps23 interacts with ESCRT-0 and with ubiquitinated membrane proteins. UBAP1 and yeast Mvb12 also bind ubiquitin. Thus, ESCRT-I could bind two ubiquitin moieties on endosomes. At the opposite end of the ESCRT-I rod, Vps28 binds to the GRAM-like ubiquitin binding in EAP45 (GLUE) domain of ESCRT-II (Gill et al. 2007).

Vps37 carries N-terminal helices, which interacts with the endosomal membrane and contributes to membrane association, which could be additionally enhanced by the β-prism fold—a hydrophobic membrane-anchoring loop and an electropositive phosphoinositide-binding patch of human MVB12b (Kostelansky et al. 2007; Boura and Hurley 2012). While Vps23 can interact with ESCRT-0, Vps28 interacts with the downstream ESCRT-II complex, thus potentially linking ESCRT-0 and ESCRT-II together. ESCRT-I can also interact with HD-PTP and it recruits the DUB UBPY/USP. This may facilitate the transfer of EGFR to the further ESCRT complexes (Ali et al. 2013; Doyotte et al. 2008; Stefani et al. 2011; Alwan and van Leeuwen 2007; Bowers et al. 2006; Pareja et al. 2012; Row et al. 2006; Wunderley et al. 2014). Overall, ESCRT-I recognizes ubiquitinated cargo and it may play a central role in the formation of a sorting domain by binding to ESCRT-0 and ESCRT-II.

ESCRT-II is a stable heterotetramer formed of Vps22 (EAP30), Vps36 (EAP45) and two copies of Vps25 (EAP20) (Hierro et al. 2004; Langelier et al. 2006; Teo et al. 2004). Vps22 and Vps36 constitute the main body of the Y-shaped structure, while the two copies of Vps25 form the flexible arms. The interaction with ESCRT-I (Vps28) and recruitment of ESCRT-II is mediated via the N-terminal GLUE domain of Vps36. The GLUE domain functions as a hub that also binds to PI3P in the endosomal membrane and it interacts with ubiquitin via its NZF (Np14-type zinc finger domains) (Teo et al. 2006). Membrane binding of ESCRT-II is additionally stabilized by the first helix of Vps22 (Im and Hurley 2008). Most likely ESCRT-0, -I and -II could interact simultaneously with up to eight different ubiquitinated membrane proteins and thereby generate a sorting domain on the surface of endosomes that matures into a site of MVB formation. While they collect cargo, ESCRT-I and ESCRT-II can form a super-complex that can adopt different shapes which may help to drive an initial phase of membrane budding (Wollert and Hurley 2010; Boura and Hurley 2012). At the same time, both Vps25 arms could already interact with one copy of the first ESCRT-III subunit, charged multivesicular body protein 6 (CHMP6)/Vps20, and convert them into an active nucleator for ESCRT-III assembly on endosomes (Im et al. 2009; Teis et al. 2010). In parallel, ESCRT-III assembly could be stimulated by ESCRT-0/Bro1 (Tang et al. 2016).

ESCRT-III consists of four core subunits: Vps20 (CHMP6), Snf7 (CHMP4A, B, C), Vps24 (CHMP3) and Vps2 (CHMP2A, B) and the associated subunits Did2 (Chmp1A, B), Vps60 (Chmp5) and the ESCRT-III-related protein Ist1 (hIst1) (Adell et al. 2016).

Unlike ESCRT-0, -I, -II, ESCRT-III subunits are monomers in the cytoplasm and only transiently assembly into poorly defined polymers, once recruited by upstream activators. So far, structural data on ESCRT-III polymers is available only from in vitro data and upon strong overexpression or Vps4 inhibition in situ. ESCRT-III polymers can adopt a variety of shapes, reaching from filaments, rings, spirals, helical filaments sheets and dome-shaped structures in vitro (Bajorek et al. 2009a; Ghazi-Tabatabai et al. 2008; Lata et al. 2008; Chiaruttini et al. 2015; Henne et al. 2012; Schöneberg et al. 2017). The architecture of the membrane scission competent ESCRT-III complex has not been visualized.

ESCRT-III subunits have a similar size, structure and domain organization, including a positively charged N-terminus and an acidic domain at their C-terminus (Muziol et al. 2006). The crystal structure of Vps24 for example revealed an antiparallel four-helix bundle (α1 to α4). In addition, the C-terminal helices α5 and α6 can fold back (and mediate autoinhibition in the cytoplasm or open up to allow polymerization on endosomes (Bajorek et al. 2009b; Shim et al. 2007; Zamborlini et al. 2006). In yeast, Snf7 is the most abundant subunit (Teis et al. 2008) and it undergoes even more dramatic conformational rearrangements that are required to promote the assembly of Snf7 arrays with ~30 Å periodicity into a membrane-sculpting filament (Tang et al. 2015). This involves the release of autoinhibition and the repositioning of α3 and α4 to mediate membrane binding and polymerization. Snf7 can also insert the terminal helix into the membrane (Buchkovich et al. 2013; Tang et al. 2015) a property that appears to be essential for membrane budding. In vitro Snf7 has been shown

to polymerize in flexible rings spirals (Henne et al. 2012; Shen et al. 2014), which may accumulate elastic energy to spring and deform membranes as a consequence of tension release (Chiaruttini et al. 2015).

The binding of Vps24 and Vps2 to Snf7 polymers changes the architecture of ESCRT-III from flat spirals into 3D helices in vitro and stimulates the recruitment of the AAA-ATPase Vps4 (Babst et al. 2002; Im et al. 2009; Teis et al. 2008, 2010; Adell et al. 2016; Henne et al. 2012). Binding of Vps4 is mediated by the microtubule-interacting and transport (MIT)-interacting motif (MIM) at the C-terminus of Vps2. The recruitment of Vps4 may additionally be regulated by Did2 (CHMP1A, B), Vps60 (CHMP5) and Ist1 (IST1). Did2 has been shown to form a complex with Ist1, promoting Vps4 binding and disassembly of ESCRT-III. Additionally, Vps60 interacts with Vta1 a co-factor that stabilizes Vps4 and stimulates the ATPase activity of Vps4 (Amerik et al. 2000b; Nickerson et al. 2006, 2010; Rue et al. 2008; Zhongzheng et al. 2012). On yeast endosomes, the ESCRT-III assemblies accumulated 75–200 Snf7 and 15–50 Vps24 molecules and more than 4 Vps4 hexamers during their 3–45s lifetimes (Adell et al. 2017). Instead of growing into stable filaments, the dynamic and largely stochastic exchange of ESCRT-III subunits and of Vps4 is one of the prerequisites for successful membrane budding (Adell et al. 2017; Mierzwa et al. 2017). Vps4 is recruited by ESCRT-III in the first second—and not at the end of the pathway—and is present for the entire lifetime until an all-or-none step led to final release of ESCRT-III and Vps4 from endosomes (Adell et al. 2017). How these dynamic properties of ESCRT-III and Vps4 assemblies drive membrane bulging and scission, is a major open question.

The AAA-ATPase **Vps4** is the only thermodynamic driving force of the ESCRT machinery (Babst et al. 1998). Vps4 belongs to the class of type I AAA+ (ATPase associated with a variety of cellular activities). Vps4 is largely a monomer in the cytoplasm.

Each Vps4 monomer contains an N-terminal MIT domain (microtubule-interacting and trafficking), which is crucial for the interaction with the c-terminal MIMs of ESCRT-III subunits in the membrane-bound polymer, the MIM of Vps2 being most critical. This MIT domain is connected by a ~40 residue flexible linker with the ATPase cassette (Gonciarz et al. 2008; Scott et al. 2005; Xiao et al. 2007). Vfa1, a recently identified regulator of Vps4 binds to the N-terminal MIT domain of Vps4 and has been shown to stimulate the ATPase activity of Vps4 (Arlt et al. 2011; Vild and Xu, 2014; Kojima et al., 2016). Once recruited to ESCRT-III, it most likely assembles into an active hexamer (Inoue et al. 2008; Xiao et al. 2007; Yang et al. 2015; Monroe et al. 2017; Su et al. 2017), that can bind to and hydrolyse ATP. The ATPase cassette typically consists of a large ATPase domain, a small ATPase domain and an β-domain insertion which is specific for Vps4 (Gonciarz et al. 2008; Inoue et al. 2008; Xiao et al. 2007).

The β-domain mediates the interaction of Vps4 with its co-factor Vta1. Vta1 promotes the oligomerization and hence the ATPase activity of Vps4 and it facilitates ESCRT-III binding, via its MIT domains (Azmi et al. 2006; Lottridge et al. 2006; Shiflett et al. 2004), (Yeo et al. 2003), ATP hydrolysis of Vps4 protomers leads to conformational changes within the hexamer, most likely leading to the translocation

of single ESCRT-III subunits trough the central pore of Vps4 (Monroe et al. 2017; Su et al. 2017; Adell et al. 2014; Landsberg et al. 2009; Scott et al. 2005; Yang et al. 2015; Yamada-Inagawa et al. 2003).

It is becoming clear that Vps4 is not simply an ESCRT-III disassembly factor that works at the end of each functional ESCRT cycle. The coordinated binding of Vps4 hexamers to Vps2 and Snf7 drives neck constriction (Adell et al. 2014) and Vps4 is immediately recruited to ESCRT and just at the end (Adell et al. 2017). How Vps4 and ESCRT-III function together during membrane budding is unclear but it may be that Vps4 constricts ESCRT-III filaments by pulling individual ESCRT-III subunits through the central pore of the Vps4 hexamer (Monroe et al. 2017; Su et al. 2017; Ghazi-Tabatabai et al. 2008; Yang et al. 2015; Shen et al. 2015; Babst et al. 1998; Shestakova et al. 2010; Adell et al. 2014). Repeating this process may help to concentrate cargo, buckle membrane and ultimately pull membranes together to drive membrane scission.

4.3.1 Deubiquitinating Enzymes and Co-factors on Endosomes

Degradation of alpha 4 (Doa4), or its mammalian orthologue ubiquitin-binding protein Y (UBPY), also known as ubiquitin-specific protease 8 (USP8) is required for the deubiquitination of membrane proteins during the ESCRT-dependent sorting process (Amerik et al. 2000a, b). UBPY and AMSH (associated molecule with the SH3 domain of STAM) are recruited to early endosome. They interact via the SH domain with STAM and via their MIT domains with ESCRT-III subunits (Kato et al. 2000; Agromayor and Martin-Serrano 2006; Row et al. 2007; McCullough et al. 2004; Nakamura et al. 2006). AMSH, also known as STAM-binding protein (STAMBP), is an endosome-associated isopeptidase (McCullough et al. 2004; Clague and Urbe 2006), which together with USP8 negatively regulates the degradation of proteins. They may stimulate early deubiquitination of cargos such as EGFR and therefore promote cargo recycling instead of degradation (Niendorf et al. 2007; Pareja et al. 2012; Clague and Urbe 2006).

But cargo deubiquitination can also occur later. In yeast, Doa4 is specifically recruited to ESCRT-III polymers by Bro1 (BCK1-like Resistance to Osmotic shock). Bro1 itself binds to Snf7 in ESCRT-III filaments and it is also directly involved in membrane budding, possible by delaying Vps4 mediated disassembly of ESCRT-III (Tanaka et al. 1999; Luhtala et al. 2004; McCullough et al. 2008; Richter et al. 2007; Wemmer et al. 2011). These mechanisms might help to ensure, that membrane proteins are de-ubiquitinated only when ESCRT-III filaments are already established. This may prevent their escape into recycling pathways.

Alix (formerly apoptosis-linked gene 2-interacting protein X; official name programmed cell death 6-interacting protein (PDCD6IP) also contains a Bro domain and interacts with Chmp4. Both, Bro1 and Alix can bind to ESCRT-0 or ESCRT-I and

stimulate parallel ESCRT-III assembly pathways that play a key role in ESCRT-III-mediated membrane budding events (Clague and Urbe 2006; Bissig and Gruenberg 2014; Tang et al. 2016). Cargo deubiquitination may contribute to enhance the plasticity and flexibility of the sorting system. It has also been proposed, that this early deubiquitination of cargo proteins may provide an additional proofreading and assure the specificity of the lysosomal degradation pathway (Piper and Lehner 2011; Wright et al. 2011). How cargo deubiquitation process is regulated (early or later) and how deubiquitination events are timed to allow efficient recycling or sorting into ILVs is not entirely clear.

Most importantly, cargo deubiquitination prevents ubiquitin wasting and thereby contributes to the recycling of ubiquitin, in analogy to deubiquitination prior to proteasomal degradation (Papa et al. 1999). This also highlights the important interplay between ubiquitinating and deubiquitinating enzymes, to maintain essential cellular processes.

4.4 Reverse Membrane Remodelling—Driven by ESCRT-III and Vps4

Additional cellular processes at the PM and the Nuclear envelop/ER require ESCRT-III and Vps4-mediated reverse membrane budding (Adell et al. 2016; Alfred and Vaccari 2016; Campsteijn et al. 2016; Christ et al. 2017; Schöneberg et al. 2017).

At the plasma membrane, the ESCRT system is required for repair, neuronal pruning, exosome and microvesicle shedding. The ESCRT machinery is also hijacked by retrovirus (e.g. HIV/Ebola) to cut the membrane stalk connecting the budding virus with the host cell during the release of retroviral particles (Zhang et al. 2014; Loncle et al. 2015; Jimenez et al. 2014; Scheffer et al. 2014; Nabhan et al. 2012; Colombo et al. 2013; Géminard et al. 2004; Baietti et al. 2012; Roucourt et al. 2015; Wehman et al. 2011; Martin-Serrano et al. 2005; Martin-Serrano and Neil 2011; Garrus et al. 2001; von Schwedler et al. 2003; Pornillos et al. 2003; Strack et al. 2003; Morita et al. 2011; Effantin et al. 2013; Cashikar et al. 2014; Prescher et al. 2015; Jouvenet et al. 2011; Baumgärtel et al. 2011).

Another ESCRT-dependent process at the PM is cytokinesis (Carlton and Martin-Serrano 2007; Morita et al. 2007; Lee et al. 2008; Morita et al. 2010; Carlton et al. 2008; Guizetti et al. 2011; Bajorek et al. 2009a; Yang 2008; Reid et al. 2005; Elia et al. 2011, 2012; Caballe et al. 2015; Capalbo et al. 2012). At the nuclear envelop, ESCRTs are involved in the removal of defective nuclear pore complexes and in the resealing of the nuclear envelop after mitosis or after rupture in interphase cells (Vietri et al. 2015; Olmos et al. 2015; Webster et al. 2014; Bauer et al. 2015; Denais et al. 2016; Raab et al. 2016). Not only damage of the nuclear envelop or the PM, but also lysosomal damage triggers the transient recruitment of the ESCRT machinery for repair (Skowyra et al. 2018). ESCRTs are also required for the biogenesis of double membrane compartments, so-called compartment of unconventional protein

secretion (CUPS), that mediate a conventional secretion pathway in yeast (Curwin et al. 2016). Remarkably, this process was largely independent of Vps4.

On endosomes, the ubiquitination signal on cargo proteins are the main recruiters of the ESCRT machinery, while all other ESCRTs depended processes require specific adaptor proteins. Some have been discovered such as Cep55 (Centrosomal protein of 55 kDa) (Carlton and Martin-Serrano 2007), that recruits ESCRT-III during cytokinesis, or Helix-Extension-Helix domain 2 (Heh2), Charged multivesicular body protein 7 (Chmp7), Lap2-emerin-man1 (Lem2) (Bauer et al. 2015; Olmos et al. 2015; Vietri et al. 2015; Webster et al. 2014; Gu et al. 2017; Webster et al. 2016; Olmos et al. 2016), which recruit ESCRT-III and Vps4 to the nuclear envelop. HIV-1 structural proteins such as GAG (Garrus et al. 2001; VerPlank et al. 2001; Martin-Serrano et al. 2001) hijack the hosts ESCRT proteins for budding. Many of the recruiting factors still need to be investigated, as well as the dynamic assembly and disassembly of ESCRT components at the distinct sides. Along with the open question, how ESCRT-III and Vps4 are capable of executing membrane budding in such a broad variety of cellular processes.

Summary

The basic concepts for membrane protein ubiquitination and subsequent degradation are emerging but many questions are not answered yet. Membrane proteins can constitute up to 20% of eukaryotic genomes but for the vast majority, it is completely unclear how they are ubiquitinated. What are the signals that mediate their ubiquitination and which ubiquitin ligase complexes are unsolved. How the ESCRT machinery sorts cargo proteins and coordinates this process with reverse membrane budding is mechanistically largely unresolved.

Answering these questions will provide a better understanding of how cells can adapt their repertoire of membrane proteins and thereby control cell growth and cellular homeostasis.

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