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Direktorin: Prof. Dr. Britta Eickholt

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Functional aspects of the ubiquitin-proteasome system

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Contents

1. Summary

Synthesis and maturation of endoplasmic reticulum (ER) proteins is tightly regulated by an extensive quality control system. Perturbation of ER homeostasis may lead to an accumulation of aberrant proteins that trigger a number of signaling pathways known as unfolded protein response (UPR). The UPR results in a transient inhibition of general translation followed by an enhanced expression of genes that encode molecular chaperones and factors involved in ER-associated protein degradation (ERAD). One of the genes that are induced by the UPR encodes a protein called Herp. Herp resides at the ER membrane and associates with the ubiquitin-protein ligase Hrd1, which is a central component of membrane complexes required for ERAD. In the absence of Herp, Hrd1-dependent ubiquitylation and degradation of specific ER proteins is compromised, while the positive effect of Herp on ERAD requires its N-terminal ubiquitin-like (UBL) domain. Hence, it is suggested that Herp acts as a positive regulator of Hrd1-mediated protein degradation, counteracting the accumulation of aberrant proteins in the ER.

Hrd1-mediated ubiquitylation enables extraction of proteins from the ER to the cytosol by p97, as well as their proteasome dependent degradation. Specificity of the p97 ATPase complex towards certain cellular processes is ensured by a number of cofactors. One of these cofactors is UBXD6/Rep8, a transmembrane protein that binds p97 as well as Hrd1. Inhibition of UBXD6 expression leads to a reduced amount of ER membrane-associated p97, accompanied by an impairment of ERAD. It is therefore proposed that UBXD6 recruits p97 to Hrd1 based ERAD complexes, enabling efficient extraction of ubiquitylated ER proteins to the cytosol and their degradation by the 26S proteasome.

The 26S proteasome comprises the barrel shaped 20S proteasome that is connected to one or two 19S regulator complexes. The 19S regulator complex is responsible for substrate protein binding, deubiquitylation, as well as ATP dependent unfolding and translocation to the catalytic sites in the lumen of the 20S proteasome. Recognition and binding of multi-ubiquitylated proteins by the 26S proteasome has been attributed to the ubiquitin interacting motif (UIM) of the 19S regulator subunit Rpn10/Pus1. Experiments in fission yeast revealed that ubiquitin-associated (UBA) domains also display a binding preference for multi-ubiquitin chains. The proteins Rhp23 and Dph1 contain such UBA

domains as well as a UBL domain, which, in contrast to the UBL domain of Herp, is able to bind the proteasome. While phenotypes of fission yeast cells carrying single deletions of Pus1, Rhp23 or Dph1 are similar to wild type cells, double deletions of Rhp23 and either Pus1 or Dph1 lead to a stabilization of proteasome substrates, accumulation of ubiquitylated proteins and severe growth defects. The data therefore suggest that Rhp23 and Dph1 represent a group of proteins that act as adapters to recruit multi-ubiquitylated substrate proteins to the proteasome.

2. Introduction

Synthesis of proteins, their maturation, transport, secretion as well as their degradation contribute to proteostasis. The coordinated regulation of these processes is therefore crucial to control the cellular protein load, and prevent the development of diseases, such as diabetes, cancer and neurodegenerative disorders (Wang & Kaufman 2012). Protein synthesis and secondary damage result in a fair amount of aberrant proteins. To eliminate these dysfunctional species, the cell exploits the ubiquitin proteasome system (UPS) and autophagy pathways (Ding & Yin, 2008).

2.1 The ubiquitin-proteasome system

Apart from its role in the disposal of aberrant proteins, the UPS is responsible for the timely degradation of regulatory proteins in order to control cellular processes (Hershko & Chiechanover, 1998). Substrate proteins are targeted for degradation by the modification with a multi-ubiquitin chain, which is a prerequisite for their recognition and degradation by the 26S proteasome (Chau et al., 1989).

2.1.1   The ubiquitin-like domain

Ubiquitin is a eukaryotic protein consisting of 78 amino acids. It displays a characteristic structural feature called ubiquitin- (fold) like (UBL) domain, also found in other proteins that

participate in a variety of cellular processes including proteasome-dependent proteolysis and autophagy (Vijay-Kumar et al., 1985, Orengo et al., 1994). Proteins that contain a UBL domain can be assigned to two different groups: ubiquitin-like modifiers and UBL-domain-containing proteins (UDPs) (Jentsch & Pyrowolakis, 2000). Ubiquitin-like modifiers, such as SUMO, FAT10, ISG15, NEDD8, ATG12 and others, contain just one or two UBL-domains and, in most cases, a C-terminal di-glycine motif. They are covalently attached to other proteins in a multistep process that confers specificity (van der Veen & Ploegh, 2012). In contrast, UDPs do not act as modifiers and mostly contain additional structural features apart from their UBL domain. The ubiquitin-superfold present in UBL domains has also been described in so-called ubiquitin related X (UBX) domains, although their similarity to ubiquitin with respect to the primary structure is rather limited (Hofmann & Bucher, 1996). In general, the ubiquitin-like fold appears to provide a basic scaffold for the formation of binding surfaces that enable specific interactions involved in the regulation of various cellular pathways (Dikic et al., 2009).
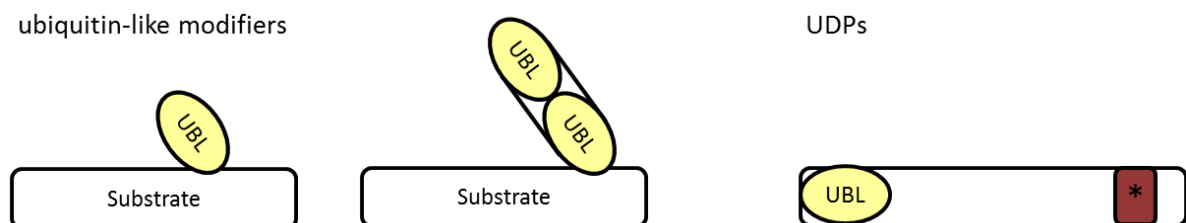
Figure 1. Ubiquitin-like modifiers and ubiquitin-like-domain-containing proteins (UDPs). Ubiquitin-like modifiers comprise one or two UBL-domains and can be linked covalently to substrate proteins via their C-terminus. UDPs are mostly unrelated proteins that contain a UBL-domain along with other structural features (*), such as UBA- or RING-domains (Jentsch and Pyrowolakis, 2000).

2.1.2   Ubiquitin and ubiquitylation

Ubiquitylation or ubiquitination is the covalent attachment of ubiquitin to substrate proteins. It is employed by many cellular pathways to target proteins for specific processes. Ubiquitylation is initiated by the ATP-dependent formation of a thioester-intermediate, involving the carboxy-terminus of ubiquitin and a cysteine residue of a ubiquitin activating
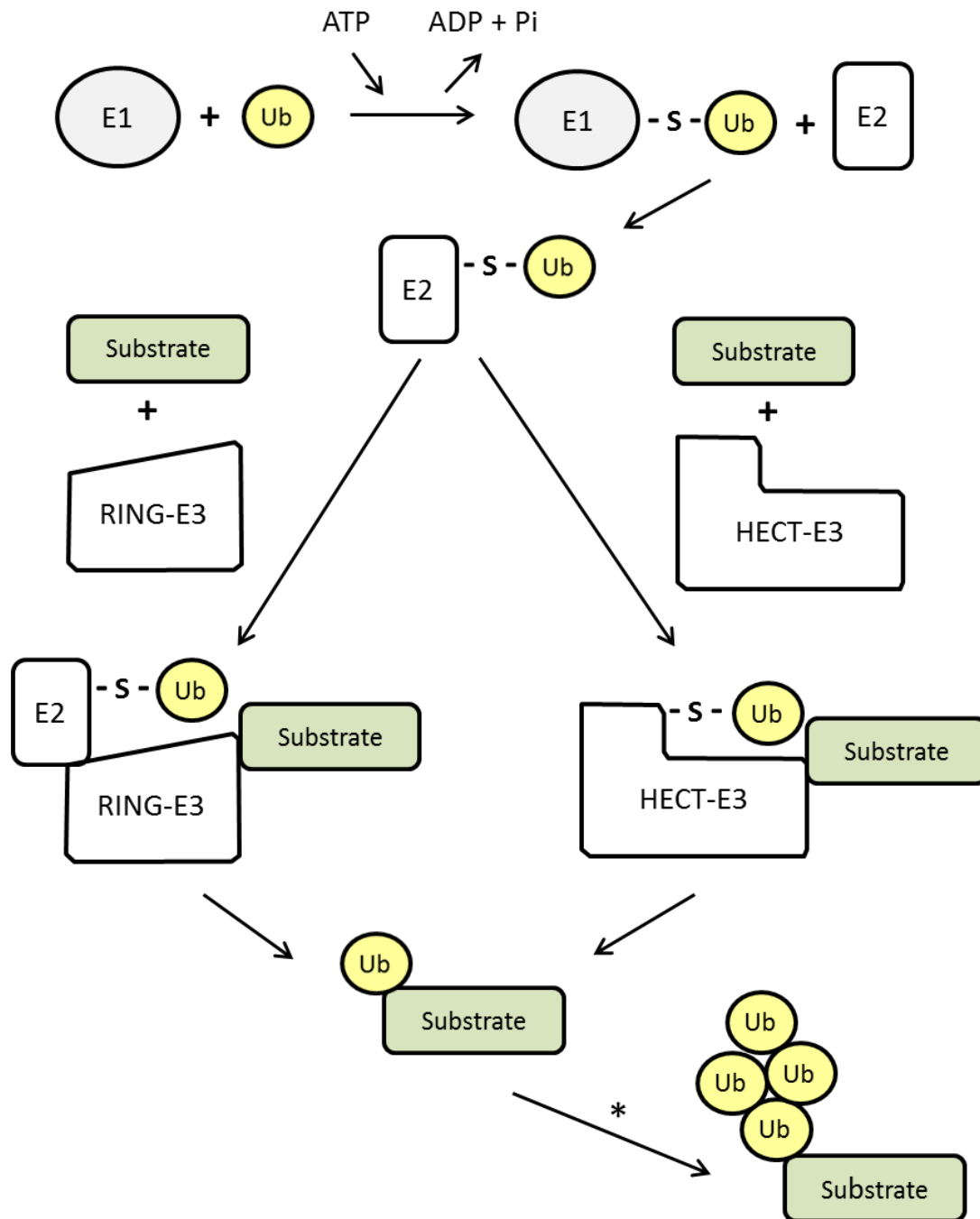
Figure 2. The ubiquitylation process. In an ATP-dependent step, the C-terminus of ubiquitin (Ub) is linked to an E1-enzyme via a thioester-bond, followed by transthiolation to an E2. Transfer of the ubiquitin molecule to the acceptor site at the substrate protein is then mediated by an E3. While HECT-E3s form a thioester-intermediate with ubiquitin, RING-E3 rather act as scaffolds enabling the direct transfer of ubiquitin from the E2 to the substrate. *Multi-ubiquitin chains are either built sequentially, by the attachment of a second ubiquitin to one of the lysine residues of the ubiquitin that has been already attached to the substrate, or by preassembly of the chain while still bound to the E2 or E3 (Metzger et al., 2012; Wang & Pickart, 2005; Li et al., 2009).

enzyme (E1). In a second step, ubiquitin is transferred to the active site cysteine of a ubiquitin conjugating enzyme (E2). The E2 then co-operates with a ubiquitin protein ligase (E3), enabling formation of an isopeptide bond between the C-terminus of ubiquitin and a lysine residue of the substrate protein. E3 enzymes that belong to the HECT (homologous to the E6AP carboxyl terminus) family also form a thioester intermediate with ubiquitin to catalyse this step. The majority of ubiquitin ligases are members of the RING (really interesting new gene), RING-related, PHD (plant homeodomain), LAP (leukemia associated protein) finger protein or U-box families, which mediate the direct transfer of ubiquitin from the E2 to the acceptor site of the substrate protein (Rotin & Kumar, 2009; Deshaies & Joazeiro, 2009, Metzger et al., 2009). Although in most cases the ε-amino group of a lysine residue serves as an acceptor site for ubiquitin, also non-canonical ubiquitylation sites, such as N-terminal amino-groups or non-lysine residues, have been described (Kommander & Rape, 2012).

Multi- or poly-ubiquitylation is the attachment of a ubiquitin chain to a single lysine of the substrate. Within these chains, ubiquitin molecules are either linked via isopeptide bonds involving one of the seven lysine residues present within the modifier or, in case of linear chains, by conventional peptide bonds. Specificity is conferred mainly by the three-dimensional structure adopted by the chain, which depends on the position of the lysine residues involved in intra-chain linkages as well as on the cellular context (Chau et al., 1989; Thrower et al., 2000; Dikic et al., 2009). The attachment of chains, consisting of at least four ubiquitin molecules that are mostly linked via Lys48 or Lys11, has been described to serve as a signal for proteasome dependent degradation. (Pickart, 1997; Ciechanover & Stanhill 2014).

2.1.3   Degradation of ubiquitylated proteins by the 26S proteasome

Proteasomes are modular proteolytic complexes responsible for the degradation of non-aggregated cellular proteins, which also provide peptides for MHC class I restricted antigen presentation. They are localized abundantly in the cytosolic and nuclear compartment of eukaryotic cells. While most substrates of the proteasome require modification with a

specific ubiquitin chain, there are also examples for ubiquitin independent degradation (Chau et al., 1989; Murakami et al., 1992; Hipp et al., 2005; Erales & Coffino, 2014).

The 20S proteasome is the core complex of the system, containing the catalytic sites. It associates with regulatory subcomplexes such as the 19S regulator and the PA28 complex. The 20S proteasome is a barrel shaped structure comprising 28 alpha and beta type subunits that are organized in 4 stacked rings arranged $\alpha_7\beta_7\beta_7\alpha_7$. The catalytic chamber is formed by the beta rings, each of them containing 3 subunits that possess an active site threonine. Access to the catalytic chamber is provided by the alpha rings via a gated pore at either end of the particle (Tomko & Hochstrasser, 2013).

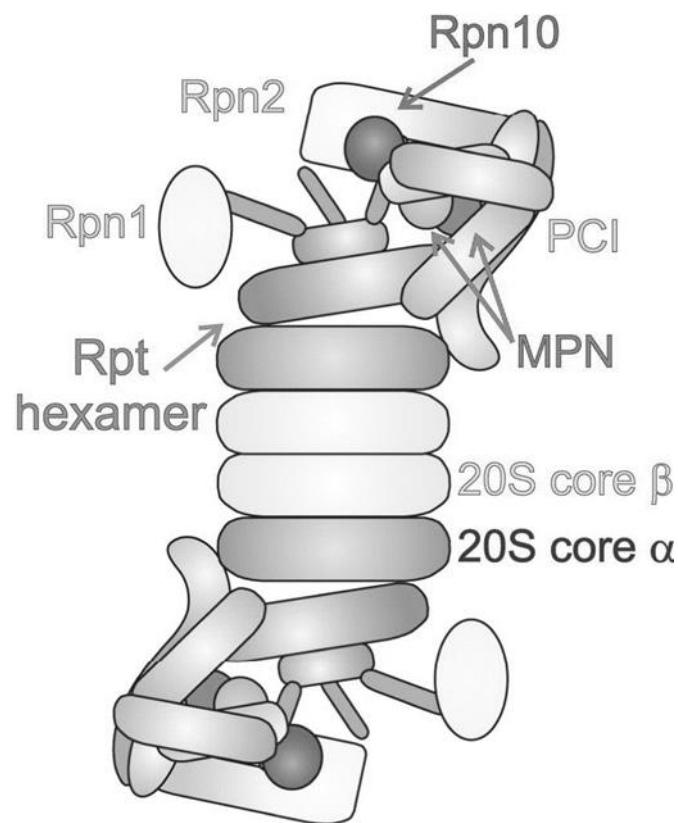
Figure 3. The 26S proteasome. The 20S proteasome core complex, which consists of four stacked seven-membered rings (20S core alpha and beta), associates with two 19S regulator complexes comprising the base with Rpt- (regularory particle ATPase) subunits, Rpn1, Rpn2 and Rpn10, as well as the lid structure with its MPN- and PCI-domain-containing subunits (taken from da Fonseca et al., 2012).

Docking of the 19S regulator complex at one or both ends of the 20S proteasome results in the 26S proteasome, which is able to degrade multi-ubiquitylated proteins in an ATP-dependent manner. The 19S regulator is required for the recruitment of ubiquitylated substrate proteins, their deubiquitylation, unfolding and their translocation to the catalytic chamber within the 20S proteasome core complex (Tomko & Hochstrasser, 2013). The regulator comprises two multimeric substructures dubbed lid and base. Subunits of the lid contain either a so-called PCI- (proteasome, COP9, Initiation factor 3) domain or an MPN- (Mpr1p and Pad1p N-terminus) domain, which in case of Rpn11 possesses deubiquitylating activity (Hofmann & Bucher, 1998; Verma et al., 2001). The base contains a ring of six ATPase subunits that interact with alpha subunits of the 20S core complex, thus linking both modules. Gating of the 20S proteasome involves ubiquitylated substrates as well as another deubiquitylating subunit called Usp14/Ubp6, while activity of the ATPases is required for substrate protein unfolding and translocation to the core complex (Peth et al., 2009; Bech-Otschir et al., 2011; Sledz et al., 2013)

Work done in the labs of Martin Rechsteiner and Richard Viestra revealed that the S5a/Rpn10 subunit of the 19S proteasome regulator complex binds ubiquitin chains by means of its ubiquitin interacting motifs (UIM)(Deveraux et al., 1994; Hofmann and Falquet, 2001; van Nocker et al., 1996). Remarkably, there is a considerable degree of variation in the Rpn10 C-terminus between different species. In comparison to higher eukaryotes, yeast Rpn10 is about 110 amino acids shorter, containing a single UIM, while two of these motifs are present in mammals (Haracska and Udvardy, 1995; van Nocker et al., 1996; Wilkinson et al., 2000). Apart from being a component of the regulator complex, Rpn10 has also been found in a monomeric state, which is likely to facilitate its function in the recruitment of ubiquitylated substrate proteins (Haracska and Udvardy, 1995; van Nocker et al., 1996; Wilkinson et al., 2000). As deletion of Rpn10 in budding yeast has been shown to destabilize the interaction between lid and base, it was concluded that the protein is also critical for the structural integrity of the 19S regulator (Glickman et al., 1998). However, despite its reported functional properties, Rpn10 is not essential for cell survival (van Nocker et al., 1996; Wilkinson et al., 2000).

2.2   Protein quality control in the endoplasmic reticulum

The endoplasmic reticulum (ER) is the compartment involved in synthesis and maturation of secretory proteins, which account for about 30% of translation products in an average cell and may reach up to 70% in cells with high secretion rates (Benyair et al., 2011). Therefore the ER harbors an elaborate quality control system, to ensure that only proteins that meet certain structural criteria leave the compartment by means of the secretory pathway.

The majority of proteins synthesized into the ER is modified co-translationally with a branched N-linked carbohydrate chain ($Glc_3Man_9GlcNAc_2$) by oligosaccharyltransferase. Specific modifications of the N-glycan serve as a targeting signal for chaperons and mark the folding state of the glycoprotein as well as its localization along the secretory pathway. Removal of the two distal glucose residues from the A-branch enables binding of the calcium-dependent chaperones calreticulin and calnexin. Upon completion of the folding process the third glucose residue is removed, resulting in the dissociation of the glycoprotein from the chaperone. The folding state is monitored by a glucosyltransferase, which re-glucosylates incompletely folded proteins for re-targeting to calnexin/calreticulin, while glycoproteins that have been folded correctly leave the ER towards the Golgi. Repeated folding attempts increase the probability that the acceptor mannose residue, required for re-glucosylation of the misfolded protein, is removed by mannosidases with the help of so-called EDEMs. Misfolded glycoproteins that have been subjected to excessive mannose trimming are recognized by the lectins OS9 and XTB3, which direct them to ER-associated protein degradation (ERAD). In addition, BiP, a member of the Hsp70 family, and the disulfide isomerase ERdj5 are involved in targeting of terminally misfolded glycosylated as well as non-glycosylated proteins to the ERAD machinery. (Benyair et al., 2011; Ushioda et al., 2013)

Carbohydrate-independent protein folding is supported by BiP, the first ER chaperone to bind nascent peptides translated into the ER lumen. Together with the co-chaperone ERdj3, BiP also promotes the assembly of oligomeric complexes, which is a precondition for certain ER proteins to acquire their native state. In addition, oxidoreductases facilitate the formation of intra- and inter-molecular disulfide bonds. (Benyair et al., 2011)

2.3 The unfolded protein response

Overload of the folding machinery caused by high translation rates, expression of mutant genes, or perturbation of calcium levels that affect protein folding, may lead to an increased concentration of aberrant proteins in the ER. This accumulation of misfolded ER-proteins triggers a number of signaling pathways know as unfolded protein response (UPR). The UPR results in a transient block of translation, accompanied by an induction of factors that enforce protein folding and disposal. (Wang & Kaufman 2012)

There are three signaling proteins of the ER membrane called Perk, IRE1 and ATF6 that act as ER stress sensors by detecting an accumulation of ER proteins. Binding of BiP seems to keep these proteins in an inactive state. It has been suggested that an increased concentration of misfolded proteins that bind BiP might have a competitive effect that leads to the dissociation of the chaperone from the sensor proteins, resulting in their activation (Pincus et al., 2010; Liu et al., 2000; Bertolotti et al., 2000; Ye et al., 2000). In addition, misfolded proteins appear to bind directly to IRE1 (Credle et al., 2005). Consequently, synthesis of ER proteins is inhibited by Perk-dependent phosphorylation of eIF2alpha as well as with the help of the IRE1 ribonuclease activity, which destroys signal-sequence-containing mRNAs. Genes that encode proteins required for ER protein folding and ERAD are induced via three major mechanisms: IRE1-mediated unconventional splicing to generate the transcription factor XTB1s, the eIF2-dependent increase in ATF4 translation that accompanies the inhibition of general translation, as well as transcriptional activation upon ATF6 cleavage (Wang & Kaufman 2012). Prolonged activity of the UPR results in apoptosis mediated via JNK, pro-apoptotic CHOP and GADD34 (Shore et al., 2011).

2.4 ER-associated protein degradation (ERAD)

ERAD is crucial for the disposal of aberrant proteins, which have been detected by the ER quality control system, as well as for the inactivation of proteins in the course of regulatory processes. In order to degrade ER-derived substrates, the cell has adopted the ubiquitin proteasome system. Thus, ER proteins are multi-ubiquitylated at the ER membrane, enabling

their extraction from the endosomal compartment and degradation by the 26S proteasome. (Hiller et al., 1996; Hampton & Sommer, 2012)

2.4.1 Ubiquitylation of ER proteins

As ubiquitin is only found in the cytosol and the nucleus, multi-ubiquitylation of ER proteins is facilitated by specific protein complexes that are localized at the ER membrane. These ERAD complexes contain a ubiquitin ligase as a central component, which is associated with a ubiquitin conjugating enzyme and variety of other factors that confer the recruitment of substrate proteins, their multi-ubiquitylation at the cytosolic surface of the ER membrane as well as their dislocation to the cytosol. In budding yeast, two E3s called Hrd1 and Doa10 have been shown to ubiquitylate ER derived substrate proteins. In mammals, there is a number of various ubiquitin ligases associated with ERAD function, including a Doa10 orthologue called Teb4/March6, the Hrd1 orthologues Hrd1/synoviolin and gp78/AMFR (Hampton et al., 1996; Bordallo & Wolf, 1999; Swanson et al., 2001; Fang et al., 2001; Kikkert et al., 2004; Hassink et al., 2005). Although both mammalian Hrd1 counterparts have been implicated in ER quality control, only Hrd1 seems to be regulated by the UPR (Allen et al., 2004). During the last decade, Hrd1 has been studied extensively with the help of a variety of model substrates. It has been described to ubiquitylate a wide variety of substrates, including soluble proteins derived from the ER lumen, transmembrane proteins and cytoplasmic proteins that are associated with the ER membrane (Christianson et al. 2008; Yamasaki et al., 2006; Artega et al., 2006; Kikkert et al., 2004). In order to direct misfolded luminal proteins to Hrd1-dependent ERAD ubiquitylation, BiP, ERdj5, XTB3 and OS9 associate with Sel1L, a component of Hrd1-based ERAD complexes, which seems to provide access of these substrates to the ligase (Christianson et al. 2008). Hrd1 encompasses five transmembrane helices located at its N-terminal region, while its C-terminal cytosolic portion contains the catalytically active RING domain. Considering that the homologous gp78 as well as yeast Hrd1 have been shown to require dimerization for their function, a similar structural arrangement is also conceivable for human Hrd1 (Li et al., 2009; Horn et al., 2009). Linkage of ubiquitin chains to the substrate protein is then enabled in concert with the

ubiquitin conjugating enzyme UBC6e/UBE2J1 at the cytosolic part of the ERAD complex, requiring at least partial dislocation of the substrate (Lenk et al., 2002; Mueller et al., 2008).

2.4.2   Extraction of ubiquitin conjugates from the ER

Complete retro-translocation of ER-derived substrate proteins to the cytosol requires additional ERAD complex components called Derlin and Vimp, as well as the activity of p97/VCP (Ye et al., 2001; Lilley & Ploegh, 2004; Ye et al., 2004).

P97, which is called Cdc48 in yeast, acts as a segregase, dissociating ubiquitylated proteins from interacting molecules in an ATP-dependent manner (Jentsch & Rumpf, 2007). Its activity is therefore involved in many pathways ranging from ERAD to replication. Targeting of p97 function to all these different processes is achieved with the help of a number of cofactors that bind the ATPase by means of specific structural features (Madsen et al. 2009). While heterodimeric Ufd1/Npl4 has been implicated in binding of ubiquitylated substrates for p97, a group of UBX proteins appears to direct the ATPase to specific ubiquitin ligases (Meyer et al., 2002; Schuberth & Buchberger, 2008). With the exception of UBXD1, all these proteins employ their UBX domain to bind the N-domain of p97 (Schuberth & Buchberger, 2005,; Alexandru et al., 2008; Madsen et al., 2008; Kern et al., 2009).

Recruitment of cdc48/p97 to the ERAD pathway requires Ubx2 in yeast, while in higher eukaryotes the Ubx2 homologues UBXD8 and UBXD2 have been implicated in this function (Schuberth et al., 2005, Neubert et al. 2005; Liang et al., 2006, Mueller et al., 2008). In addition, the seleno-protein VIMP has been identified as an ERAD component that binds p97 (Ye et al., 2004). Upon extraction to the cytosol, ubiquitylated glycoproteins are deglycosylated by a PNGase, which is able to bind the C-terminus of p97 (Li et al., 2005). Once their sugar chains have been removed, ER-derived ubiquitin conjugates are degraded by the 26S proteasome, along with other multi-ubiquitylated proteins.

3. Objectives

A detailed knowledge of the UPS is crucial to comprehend proteostasis as well as the regulation of specific cellular pathways. Therefore, the studies presented here were aimed to characterize functional aspects of the system.

Selectivity of the UPS has been shown to largely depend on the ubiquitylation machinery. Thus, the mammalian genome encodes about 40 E2 enzymes and more than 600 E3 protein ligases (Metzger et al., 2012). In addition, ubiquitin conjugates may get modified further by ubiquitin-specific proteases (Eletr & Wilkinson, 2014). A critical step towards proteasome-dependent degradation of ubiquitylated substrate proteins is their recruitment to the 26S proteasome, which has also been a matter of extensive research. The first breakthrough was achieved in M. Rechsteiners lab by the identification of S5a/Rpn10/Pus1 as a multi-ubiquitin-binding subunit of the 19S regulator (Deveraux et al., 1994). However, as deletion of Rpn10 in yeast cells did not severely compromise their viability, it was concluded that there are additional proteins that are able to recruit ubiquitylated substrates for the proteasome (van Nocker et al., 1996; Wilkinson et al., 2000). Thus the first project described here was initiated to better understand processes and factors involved in the recruitment of ubiquitylated substrate proteins for the proteasome.

The discovery that ER-proteins are degraded by the proteasome has been fundamental for our current concept of cellular proteolysis, which is in part due to the fact that this pathway requires dislocation of substrates from the ER (Hiller et al., 1996). The studies on ERAD presented here were prompted by the observation that the UDP Herp interacts with the ER-resident E3 Hrd1. Consequently, further work was aimed to characterize molecular mechanisms that are required for the ubiquitylation of ER proteins, their dislocation as well as their degradation (Schulze et al., 2005; Kny et al., 2011).

4.   Results and discussion

4.1   Proteins containing a UBA domain are able to bind to multi-ubiquitin chains (this chapter is based on Wilkinson et al., 2001)

The proteasome selectively degrades proteins that have been modified with specific ubiquitin chains. Here we describe a group of adapters that are able to recruit these ubiquitylated substrate proteins for the proteasome.

In a screen for multi-copy suppressors of a proteasome mutation in fission yeast, we identified the gene mud1+, encoding a protein that is able to interact with multi-ubiquitin chains by means of its C-terminal ubiquitin-associated (UBA) domain. The high affinity for potential proteasome substrates is indicated by a K_D of 30nM for the interaction between Mud1 and tetra-ubiquitin, as compared to 8µM for the interaction between the budding yeast Mud1 orthologue and mono-ubiquitin (Bertolaet et al., 2001). UBA-domain-dependent binding of multi-ubiquitin chains was also observed for Rhp23 and Dph1, the *S.pombe* homologues of Rad23 and Dsk2 respectively. In addition, both proteins contain an N-terminal ubiquitin-like (UBL) domain, which is able to interact with the 19S regulator complex of the 26S proteasome. In accordance with genetic data, the results suggest an involvement of Rhp23 and Dph1 in the recruitment of multi-ubiquitin modified substrate proteins for the proteasome, together with the S5a/Rpn10 fission yeast orthologue Pus1.

4.2   Interaction of ubiquitin-chain-binding proteins with components of the proteasome and the anaphase-promoting complex/cyclosome (this chapter is based on Seeger et al., 2003)

Although the ubiquitin-chain-binding proteins Rpn10 and Rad23 both appear to act as substrate receptors for the proteasome, they do not share similar structural features (Di Fiore et al., 2003). While Rad23 employs a UBL domain to bind the proteasome, Rpn10 is devoid of a similar ubiquitin fold. Their *S.pombe* orthologues Rhp23 and Pus1 respectively were found to interact with the mts-4/Rpn1 subunit of the proteasome. While Pus1 binds within a region of mts4 forming so-called PC repeat structures, the binding site for Rhp23 was mapped to a stretch of amino acids N-terminal of this site. Apart from the proteasome, PC repeats are found only in Cut4, which is part of a multi-subunit ubiquitin ligase called anaphase-promoting complex or cyclosome (APC/C). In accordance with genetic data we demonstrate that Pus1 binds to Cut4 PC repeats, indicating a possible involvement of Pus1 in the recruitment of APC/C dependent substrates for the proteasome.

4.3 **Herp interacts with a Hrd1-based protein complex and promotes ERAD** (this chapter is based on Schulze et al., 2005)

Herp is an UPR-inducible UDP that resides in the ER membrane, with its UBL domain facing the cytoplasm. As UBL domains in Rad23, Dsk2 and other UDPs have been shown to bind the proteasome, we hypothesized that Herp employs its UBL domain to facilitate the localisation of a proteasome population to the ER, to ensure efficient ERAD (Schauber et al.,1998; Hartmann-Petersen & Gordon, 2004).

Interaction studies revealed that despite its UBL domain, Herp is not able to bind the proteasome when compared to human Rad23. However, we found that Herp interacts with a multimeric complex comprising the proteins Derlin-1, VIMP, Hrd1, and p97, which have been demonstrated to participate in ERAD. Our studies with recombinant proteins revealed direct binding of the ATPase p97 to Derlin-1 and the ubiquitin ligase Hrd1, as well as UBL-domain-independent association of Herp with Hrd1. Inhibition of Herp synthesis by shRNA resulted in a stabilization of the ERAD substrate CD3-delta, indicating its involvement in ERAD. We propose that Herp and its interaction partners are organized in an ER-membrane-resident multimeric complex that facilitates the ubiquitylation of ER proteins and their extraction to the cytosol, enabling proteasome-dependent degradation.

4.4   The UBL domain of Herp enables Hrd1-dependent ubiquitylation and degradation

(this chapter is based on Kny et al., 2011)

To elucidate the function of Herp as an ER-stress-regulated component of Hrd1-based complexes, we monitored its fate upon induction of the unfolded protein response (UPR) pathway. Elevated Herp expression under ER stress conditions was accompanied by its binding to pre-existing Hrd1, where it was continuously displaced by *de novo* synthesized copies and degraded in a proteasome dependent manner. Co-precipitation studies, facilitating tagged versions of the E3, indicate the presence of multiple Hrd1 molecules within a single complex, resembling gp78, an ERAD E3 implicated in the degradation of mutant CFTR and the regulation of cholesterol synthesis (Li et al., 2009). Experiments facilitating the alpha-1-antitrypsin mutant Null-Hong-Kong (NHK) as a Hrd1-specific model substrate revealed that the Herp UBL domain is crucial for substrate ubiquitylation and degradation. Based on these data a model is envisioned, in which Herp activates Hrd1-based ERAD complexes upon its induction by the UPR, permitting efficient clearance of misfolded proteins from the ER.

4.5 UbxD6 contributes to ERAD by recruiting p97 to the ER membrane (this chapter is based on Madsen et al. 2011)

Extraction of ubiquitylated proteins from the ER by the p97 ATPase complex is required for their cytoplasmic degradation by the proteasome. Although several lines of evidence suggested that human p97 binds the ER-associated degradation machinery directly via Hrd1, derlin-1 and Vimp, data generated in the yeast system indicated, that cofactors containing a UBX-domain are required for the recruitment of p97 to the ER (Schulze et al., Schuberth et al., Neuber et al.). Here we characterize UbxD6/Rep8 as a p97 cofactor, which is anchored at the ER membrane. We show that UbxD6 facilitates its cytoplasmic UBX-domain to interact directly with the N-domain of the ATPase, while it seems to be associated with Hrd1 in an indirect fashion. Inhibition of UbxD6 expression in human cells results in a decrease of ER-associated p97 and impairs the disposal of the ERAD substrate CD3delta. In mice, expression of UbxD6 appears to be restricted to the gonads, specifically round spermatozoids and granulosa cells. The data suggest that UbxD6 complements UbxD8-dependent recruitment of p97 to ERAD complexes in a tissue-specific manner.

4.6   Comprehensive discussion and outlook

4.6.1   Recruitment of ubiquitylated substrate proteins for the proteasome

The knowledge, how the proteasome is able to selectively degrade proteins that carry a ubiquitin chain, is crucial in order to understand proteostasis and thus the etiology of diseases caused by their perturbation.

As deletion of the ubiquitin-chain-binding proteasome subunit Rpn10 in budding yeast only resulted in a modest impairment of cellular functions, the existence of additional mechanisms that facilitate substrate recruitment for the proteasome has been postulated (van Nocker et al., 1996). By demonstrating that UBA domains possess a strong binding preference for multi-ubiquitin chains over mono-ubiquitin, we provided evidence that proteins that contain such a domain are candidate substrate receptors of the proteasome (Chau et al., 1989; Thrower et al., 2000; Wilkinson et al.; 2001). Furthermore, we have shown that the UBA-domain-containing UDPs Rhp23 and Dph1, which are fission yeast orthologues of Rad23 and Dsk2 respectively, are able to associate with the proteasome by means of their UBL-domains. We observed severe growth defects displayed by fission yeast double mutants lacking Rhp23 and either Pus1 or Dph1, which was accompanied by the stabilization of the proteasome substrate Rum1 as well as accumulation of ubiquitin conjugates. This data indicated that these UBA-domain proteins share essential functions in proteasome dependent degradation. We proposed that UBA-UBL-proteins represent a distinct class of proteasome substrate receptors that act as adapters, linking multi-ubiquitylated substrate proteins with the proteasome. Among others, a study by Verma et al. corroborated our results by using a cell free system, demonstrating that Rpn10 and Rad23 are required for the degradation of the cell cycle inhibitor Sic1 (Verma et al., 2004). In this work, screening of additional substrate proteins revealed a degree of specificity that can be assigned to different substrate receptors. The data are also in agreement with the more severe phenotypes displayed by the Rhp23/Pus1 and Rhp23/Dph1 double mutants as compared to the strain lacking Dph1 and Pus1 (Wilkinson et al., 2001). Hence it was suggested that substrate recruitment to the proteasome by a variety of receptors represent a further level of selectivity within the ubiquitin proteasome system (Verma et al., 2004). Interestingly, it also appears that binding of ubiquitylated substrate proteins to either of the

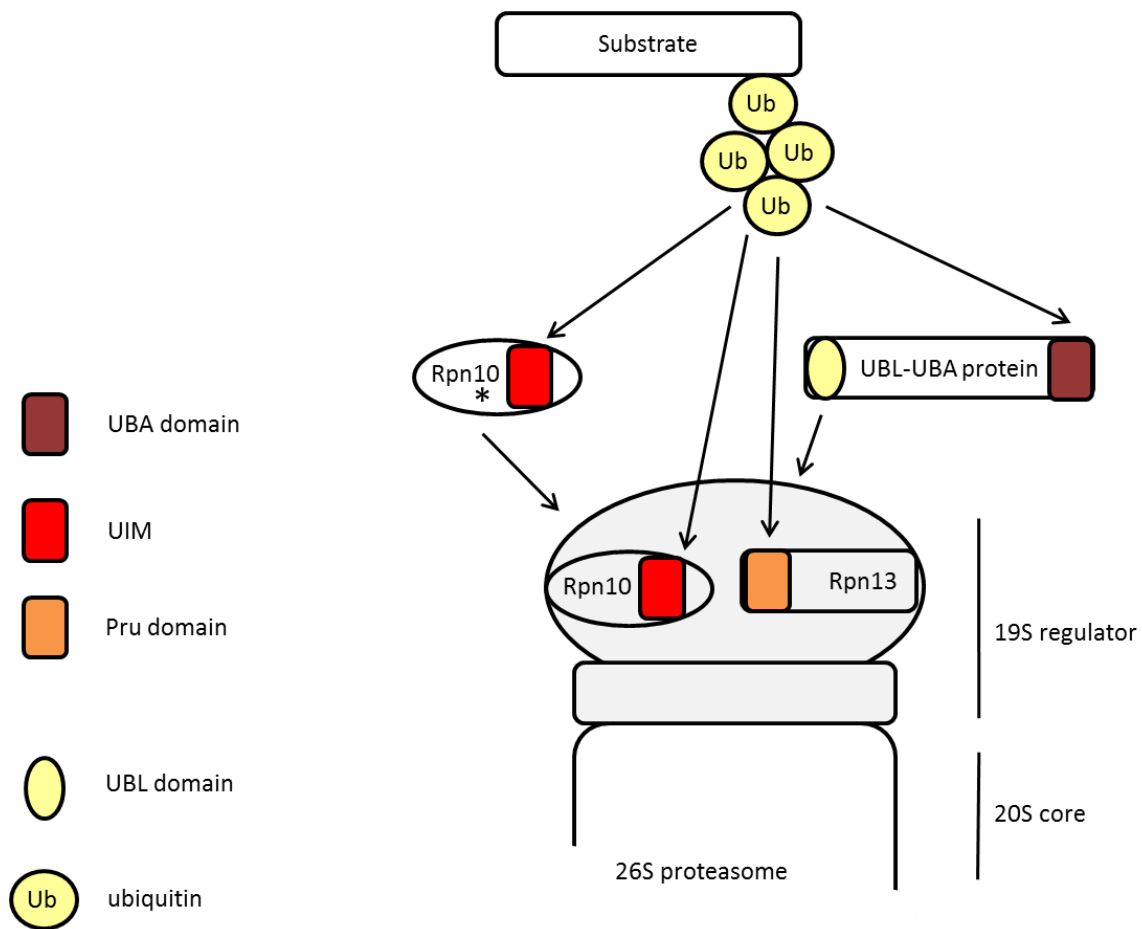recehttptor proteins, protects these substrates from deubiquitylation (Hartmann-Petersen et al., 2003).

Figure 4. Recruitment of ubiquitylated substrates for the proteasome. Substrate proteins that have been modified with a ubiquitin chain either access the proteasome directly by binding the subunits Rpn10 or Rpn13, or indirectly via a group of adapter proteins that contain a UBA as well as a UBL domain. In addition, it is conceivable that free Rpn10 (*) also shuttles ubiquitin conjugates to the proteasome. Rpn13 binds ubiquitin chains by its Pru domain while Rpn10 employs UIMs. Adapters, such as Rad23 and Dsk2, bind ubiquitin conjugates via their UBA domains while interacting with the proteasome in a UBL-domain-dependent fashion. (Deveraux et al., 1994; Wilkinson et al., 2001; Seeger et al., 2003; Husnjak et al., 2008)

In budding yeast, a mutant strain deleted for all UBL-UBA-domain-containing adapter proteins, which was also lacking the UIM-domain of Rpn10, was still viable. This data by Husnjak et al. indicated the presence of at least one additional protein that is able to bind ubiquitylated substrates for the proteasome. Accordingly, proteasome subunit Rpn13 has

been identified as a further substrate receptor. In contrast to Rpn10 and the UBL-UBA-domain proteins, Rpn13 interacts with ubiquitin via a structure related to pleckstrin-homology domains, which was therefore called pleckstrin-like receptor for ubiquitin (Pru) domain. (Husnjak et al., 2008)

Including the UIM, the UBA domain and the Pru domain, which contribute to substrate binding for the proteasome, there are about 20 different ubiquitin binding domains known to date that interact specifically with a variety of ubiquitin-like structures (Dikic et al., 2009). Although most of these ubiquitin binding domains target a hydrophobic binding surface encompassing Leu8, Ile44 and Val70 within the beta-sheet of the ubiquitin fold, the diversity of surrounding residues permits specific interactions with a wide variety of surfaces. Thus ubiquitin binding domains are rather diverse structures permitting specific interactions with different ubiquitin modifications (Lam et al. 1997; Beal et al. 1998; Raasi et al., 2005; Dikic et al., 2009, Kommander & Rape, 2012).

In summary, the proteasome recruits multi-ubiquitylated substrates by means of its subunits Rpn10 and Rpn13 as well as group of adapter proteins that bind ubiquitin conjugates via their UBA domain, while associating with the protease complex in an UBL-domain-dependent manner. In addition, substrates proteins do not only require a structure, such as a ubiquitin chain, that is recognized by substrate receptors of the proteolytic machinery, they must also contain an unstructured region that serves as an initiation site for degradation (Prakash et al., 2004).

Spatial organization of substrate recruitment processes at the 19S regulator complex of the 26S proteasome appears therefore to be crucial for the system, especially with regard to its substrate selectivity (Inobe et al., 2011). As Rpn10 has been reported to bind Rpn1/Mts4, we sought to characterize this interaction in more detail (Xie & Varshavsky, 2000). Apart from being able to verify the data for the *S.pombe* system, we found that the first of two PC repeat regions present in Mts4 is sufficient to bind the N-terminal region Pus1/Rpn10 in vitro. As PC repeat regions derived from proteasome subunit Rpn2 and the Cut9 component of the APC/C also interacted with Pus1 in a similar experimental setup, we suggested that these structures might function as a general binding site for Rpn10/Pus1. Recent electron-microscopy- and NMR-based studies on the topology of the 26S proteasome

however, demonstrate that, within the 26S proteasome, Rpn10 binds to the lid-component Rpn12, bridging Rpn11 and Rpn9 via its N-terminal region, without interacting directly with Rpn1 (Riedinger et al., 2010; Lander et al., 2012). Thus, there is a possibility that the alpha-solenoid-like structures, formed by PC repeat regions of Rpn1, Rpn2 and Cut9, enable contact of free Rpn10 to the proteasome and the APC/C respectively. Considering the reported stabilizing function of Rpn10 as a component of the 19S regulator, additional transient binding of free Rpn10 to PC repeat structures may contribute to substrate dynamics (Glickman et al., 1998; Kajava, 2002; Effantin et al., 2009).

In mammals the UBL domain of the Rad23 orthologues HHR23A and HHR23B have been shown to bind the second, more C-terminal UIM of Rpn10/S5a (Hiyama et al., 1999). In yeast Rpn10/Pus1 the homologous C-terminal region is missing, which is likely to explain that we did not see such an interaction in *S.pombe*. Instead, we were able to detect binding of Rhp23 to the Rpn1 orthologue Mts4 in a yeast-two-hybrid screen, which was verified by an *in vitro* binding assay. In addition, we identified Rhp23 as a multi-copy suppressor of the temperature sensitive mts4-1 mutant, representing genetic evidence for the specificity of the interaction. These data are in accordance with a study performed in budding yeast, demonstrating binding of the Rad23 UBL-domain to Rpn1 (Elsasser et al., 2002). A presumably species-specific difference was observed only with respect to the localization of the of the UBL-domain protein binding site, which was mapped to the more N-terminal of the two PC-repeat regions of *S. cerevisiae* Rpn1, while we found that the PC-repeat structures are not involved in Rhp23 binding to Mts4 (Elsasser et al., 2002, Seeger et al., 2003).

As UBL domains present in UBA-UBL-proteins, such as Rad23, Dsk2, Ddi1, Usp6, Bag1 and their orthologues, are able to interact with the 26S proteasome, it was hypothesized that this function can be assigned to all members of this group (Schauber et al.,1998; Hartmann-Petersen & Gordon, 2004). However, when we studied the binding properties of the UDP Herp in comparison to the human Rad23 orthologue HHR23B, no interaction of Herp with the proteasome was detected (Schulze et al., 2005). On the other hand, a hybrid protein, consisting of Herp, carrying the UBL domain of HHR23B instead of its own, was able to bind the proteasome as well as recombinant human Rpn10 (Schulze, 2007). Considering the close

resemblance of their UBL domains, binding properties of UDPs are likely to follow similar principles as discussed for the interactions between ubiquitin modifications and their receptors (Dikic et al., 2009). Thus affinities of certain ubiquitin folds to a variety of ubiquitin receptors may vary considerably due to specific differences in their primary structures, while others are in a similar range, resulting in competitive effects. The UBL domains present in HHR23 proteins for instance only bind the second UIM within human Rpn10/S5a, while not interacting with the first more N-terminal motif, although both are involved in binding ubiquitin (Hiyama et al., 1999). Remarkably, HHR23UBL domains are not only able to bind the second UIM in S5a, but also a U-box domain present in the ubiquitin ligase Ufd2 as well as one of their own UBA domains via an intra- or intermolecular interaction. As the HHR23 UBL domain binds these interaction partners with similar affinities as compared to ubiquitin, this may lead to competitive effects important to regulate the system (Ryu et al., 2003; Goh et al., 2008). In addition, Rpn10 has been demonstrated to get ubiquitylated itself, affecting UIM-dependent binding (Isasa et al., 2010; Lipinszki et al., 2012). Considering these multiple inter- and intramolecular interactions it seems therefore not surprising that HHR23/Rad23 has also been described to exhibit dominant negative effects on ubiquitin chain elongation and proteasome-dependent degradation of ubiquitin conjugates (Hiyama et al., 1999; Ortolan et al., 2000, Raasi et al., 2003).

A model envisioned for the function of substrate recruiting adapter proteins suggests that they acquire an inactive state by forming intra- or intermolecular UBA-UBL domain interactions. Changing conditions, perhaps an increased concentration of ubiquitin conjugates, may then lead to a conformational rearrangement of the UBA-UBL protein, resulting in ubiquitin chain binding by the UBA domain. Hence the UBL domain becomes available to bind one of its interaction partners, e.g. the proteasome, permitting substrate delivery and degradation. (Goh et al., 2008; Su & Lau, 2009)

Whether the recruitment of ER-derived ubiquitin conjugates by the proteasome requires a specific set of proteins is not fully understood. In yeast, Rad23 and Dsk2 were found to be essential for the degradation of the ERAD substrate CPY* (Medicherla et al., 2004). Moreover, Rad23 has been demonstrated to bind the degylcosylating enzyme PNG1 independent of its UBL domain, regulating the turnover of the glycosylated ricin A chain (Kim

et al., 2006). While these data clearly indicate an involvement of budding yeast Rad23 and Dsk2 in ERAD, evidence that supports such a cellular function in higher eukaryotes is limited to Dsk2 orthologues called ubiquilins. In *Caenorhabditis elegans* ubiquilin binds the p97-interacting protein erasin/UBXD2, while a pathogenic mutation in human ubiquilin2 linked to amyotrophic lateral sclerosis (ALS) impairs its interaction with UBXD8, resulting in disruption of ERAD (Lim et al., 2009, Xia et al., 2013). Interestingly, all human ubiquilin isoforms have been shown to bind an N-terminal tagged Herp fragment, which has been expressed ectopically (Kim et al., 2008). Although we have been able to reproduce these reported interactions, co-precipitation of the endogenous proteins would prove the physiological relevance of the observed interactions more unambiguously (unpublished). Furthermore, the localization of proteasome complexes at the sites of ER protein retro-translocation to the cytosol may also contribute to an efficient targeting of these ER-derived ubiquitin conjugates. Although co-precipitation of proteasome subunits with gp78 as well as Hrd1 has been described, an in depth characterization of proteasome recruitment to the ER membrane will be matter of further investigations (Christianson et al., 2012).

4.6.2 The role of Herp in ERAD

The detection of microsome-associated proteasomes suggested that a subpopulation of the protease complex is recruited to the ER membrane to ensure efficient ERAD as well as a sufficient supply of peptides for MHC class I restricted antigen presentation (Brooks et al., 2000). Considering that UBL domains present in UDPs such as Rad23 and Dsk2 have been identified as proteasome-binding structures, it was tempting to hypothesize that ER membrane proteins with a cytoplasmic UBL domain fulfil such a function (Schauber et al., 1998; Hartmann-Petersen & Gordon, 2004). To this end an *in silico* screen for proteins that possess a UBL domain as well as potential transmembrane helices was performed. In this screen we identified homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein (Herpud1/Herp). Herp is a transmembrane protein of the ER that possesses an N-terminal cytosolic UBL domain. The expression of Herp is induced strongly by the UPR (van Laar et al., 2000).

While our initial hypothesis was slain by data from interaction studies as described above, we established that Herp binds to Hrd1-based ERAD complexes by interacting directly with the ubiquitin ligase (Schulze et al., 2005). We demonstrated that the Herp UBL domain is necessary to promote Hrd1-dependent ubiquitylation and degradation of mutant alpha-1-antitrypsin, even though it is not required to bind Hrd1 (Schulze et al., 2005; Kny et al., 2011). Furthermore, we and others provided evidence that Herp is crucial for the degradation of various additional ERAD substrates, such as mutant CD3delta, connexin43, polycystin2, non-glycosylated substrates of the chaperone BiP and mutant transthyretin (Hori et al., 2004; Schulze et al., 2005; Liang et al., 2008; Okuda-Shimizu & Hendershot, 2007; Christianson et al., 2013). In addition, it has been suggested that upon exposure of cells to ER stress, Herp is crucial for the degradation of inositol triphosphate receptor and ryanodine receptor, which are supposed to account for calcium leakage from the ER (Belal et al 2012). Cells in which Herp synthesis is blocked, display destabilization of calcium homeostasis and early onset of apoptosis upon exposure to ER-stress conditions (Chan et al., 2004). Therefore a model has been proposed, suggesting that in an ER stress situation induction of Herp promotes the destruction of these channels, therefore sustaining calcium homeostasis and preventing apoptosis (Belal et al 2012).

Although both proteins are induced by the UPR, ER stress did not result in an obvious change in Hrd1 steady state levels, while the concentration of Herp increased dramatically under these conditions (Kny et al. 2011). This observation can be explained by the fact that Herp has a half-life of approximately 2.5h, while the half-life of Hrd1 is about 15h (Sai et al. 2003; Kikkert et al., 2004). In line with these data, we observed that Herp is constantly exchanged at Hrd1 complexes (Kny et al., 2011). Upon its UPR-mediated induction, Herp associates with Hrd1, permitting efficient substrate degradation and therefore rapid adaptation of the ERAD machinery to changing cellular requirements (Kny et al., 2011). Degradation of Herp appears to be controlled by two different sets of ubiquitylating enzymes. While the E2 Ube2J1/Ubc6e and the E3 Rnf5 mediate turnover of Herp in unstressed cells, ER stress not only results in the induction of Herp synthesis, but also in the engagement of Ube2g2- and gp78-dependent Herp degradation (Bernasconi et al., 2013; Yan et al., 2014). Possibly, usage of an alternative set of ubiquitylating enzymes is required to cope with higher Herp levels, present upon UPR induction.

Identification of direct interactions between ERAD components permitted further insight into the spatial arrangement of these proteins within the complex as well as at the interface linking ubiquitylation of substrate proteins and their p97-mediated extraction into the cytosol. Hrd1 appears to be arranged in a homo-oligomeric structure that is able to interact directly with Herp as well as Derlin1, while both, Hrd1 and Derlin1 are, along with VIMP, able to bind p97 (Schulze et al., 2005; Ye et al., 2004). However, recruitment of the p97 complex to the ER membrane seems to involve also UBXD2, UBXD6 and/or UBXD8, even though the degree of functional overlapping is unclear yet (Liang et al., 2006, Mueller et al., 2008; Madsen et al., 2011).
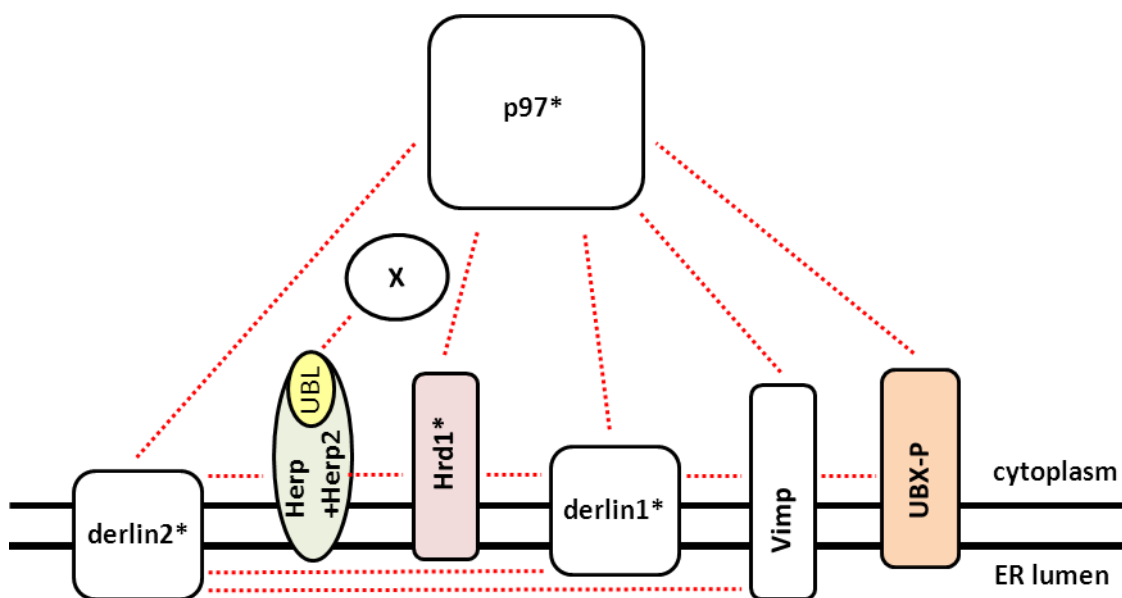

Figure 5. Interaction map of selected ERAD components. Simplified model showing reported interactions (red dotted lines) between Herp proteins, UBX-domain proteins (UBX-P) and other ERAD pathway components. Components marked with an asterisk have been described to form homooligomeric structures. UBX-domain proteins, such as UBXD8 and UBXD6 recruit the p97 complex to the ER membrane, where it interacts with Hrd1, derlins and Vimp. Herp proteins link derlin2 with Hrd1 in a UBL-domain-independent manner, while the UBL-domains may bind yet unidentified factors (X) to promote Hrd1-dependent ERAD. More detailed information is provided in the main text. (Ye et al., 2004; Lilley et al., 2004; Schulze et al., 2005, Mueller et al., 2009; Kny et al., 2011; Madsen et al., 2011, Christianson et al., 2012; Huang et al., 2013)

Despite the fact that there is no budding yeast protein possessing significant sequence homology to Herp, a UBL-domain containing protein called Usa1 has been suggested as a functional equivalent, due to the ability of Herp to partially rescue a phenotype caused by

Usa1 deletion (Carvalho et al., 2006). However, in contrast to the transient association of Herp with Hrd1-based ERAD complexes caused by its continuous degradation, Usa1 appears to be a stable scaffold protein of yeast ERAD complexes (Horn et al., 2009). It is crucial for oligomerization of yeast Hrd1 as well as for connecting the E3 to the Der1 component of the complex, thereby enabling ubiquitylation and degradation of ERAD substrates (Horn et al., 2009). Remarkably, the UBL domain of Usa1 is not required for the reported ERAD function of the protein (Rao et al., 2009; Carroll & Hampton, 2010). Interestingly, a recently published report demonstrates that a Herp paralogue called Herp2 is constitutively expressed in human cells (Huang et al., 2013). Herp2 appears to co-operate with Herp to recruit the derlin2 protein to Hrd1, while it does not affect the oligomerization of Hrd1 (Huang et al., 2013). In addition, this study further complements our data by demonstrating that the Herp UBL-domain is required for the Hrd1-dependent degradation of the C-terminal processing product of sonic hedgehog, while it is dispensable for derlin2-recruitment. According to the data presented, Herp- and Herp2-dependent recruitment of derlin2 appears to be necessary but not sufficient for substrate degradation (Huang et al., 2013). The requirement of the Herp-UBL domain for substrate ubiquitylation and degradation prompted us to propose that the domain may recruit an additional factor that is essential for Hrd1-dependent ubiquitylation (Kny et al., 2011). In a recent study it has been proposed that Herp mediates localization of Hrd1-dependent ERAD to an ER-derived quality control compartment (Leitman et al., 2014). Hence, it is conceivable, that the Herp UBL domain binds a factor that is critical for this compartmentalisation. It is also noteworthy that degradation of Herp itself involves binding of the gp78 Cue domain to the Herp UBL domain (Yan et al., 2014). Whether or not this interaction is also required for Herp-mediated Hrd1 activation requires further investigation. By employing a yeast two-hybrid approach as well as mass spectrometry analysis, we have been able to identify two other proteins that interact with the Herp UBL domain (unpublished). Due the limited overlap of functional data at the time being, further experiments are necessary to address the relevance of these interactions for the role of Herp in Hrd1-dependent ubiquitylation. Hence, it is possible that there are additional yet unidentified binding partners of the Herp UBL domain that are essential for the function of Herp in ERAD.

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6. Abbreviations

APC/C	anaphase-promoting complex / cyclosome
ATP	adenosine triphosphate
ER	endoplasmic reticulum
ERAD	endoplasmic-reticulum-associated protein degradation
HECT	homologous to the E6AP carboxyl terminus
LAP	leukemia associated protein
MHC	major histocompatibility complex
MPN	Mpr1p and Pad1p N-terminus
NHK	alpha-1-antitrypsin Null-Hong-Kong
PCI	proteasome, COP9, Initiation factor 3
Pru	pleckstrin-like receptor for ubiquitin
RING	really interesting new gene
S	Svedberg
UBA	ubiquitin-pathway-associated
UBL	ubiquitin-like
UBX	ubiquitin-related x
UDP	UBL-domain-containing protein
UIM	ubiquitin-interacting motif
UPR	unfolded protein response
UPS	ubiquitin proteasome system

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8. Erklärung

gemäß § 4 Abs. 3 k der HabOMed der Charité

Hiermit erkläre ich, dass

- ich weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder

  angemeldet habe,

- die vorgelegte Habilitationsschrift ohne fremde Hilfe verfasst, die beschriebenen

  Ergebnisse selbst gewonnen sowie die verwendeten Hilfsmittel, die Zusammenarbeit

  mit anderen Wissenschaftlern/Wissenschaftlerinnen und mit technischen Hilfskräften

  sowie die verwendete Literatur vollständig in der Habilitationsschrift angegeben

  wurde und

- mir die geltende Habilitationsordnung bekannt ist.

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