



# Resolution of Proteotoxic Stress in the Endoplasmic Reticulum by Ubiquitin Ligase Complexes

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# Declaration

I declare that this thesis has never been submitted, either in the same or a different form, to this or any other university, for a degree or any other qualification. The figures presented in Chapter III are the product of equally shared work between myself (FL) and Emma Fenech (EF, DPhil student in Christianson Lab), with the exception of the following: Figure 3.7 (generated by EF); Figure 3.10 (generated by FL). In terms of the division of labour for this project, I contributed more in the original design and project set-up (cloning and cell lines generation). EF contributed more to the optimisation of the LC-MS/MS sample preparation protocol and to the data analysis and annotation. Members of the Kessler Lab (TDI, Oxford) supported the LC-MS/MS sample preparation and data analysis.

The data presented in Chapter IV is the product of my own work, in collaboration with the van Anken Lab (San Raffaele Scientific Institute, Milan), who contributed by generating the cell lines used in the described experiments and Figure 4.3.

All the work was carried out while I was a graduate student at the Ludwig Institute for Cancer Research, University of Oxford, under the supervision of Dr. John Christianson.

# Abstract

The eukaryotic endoplasmic reticulum (ER) is a multifunctional organelle, primarily responsible for the folding and maturation of secretory proteins, as well as lipid metabolism, calcium homeostasis, ubiquitin-dependent signalling and cell fate decisions. ER-associated degradation (ERAD) oversees protein folding and delivers misfolded proteins for degradation by the proteasome via ubiquitin conjugation mediated by RING-type E3 ubiquitin ligases. An intact ERAD is crucial to cellular homeostasis, as unresolved protein imbalances cause ER stress that ultimately lead to apoptosis. The human ER accommodates at least 25 E3s, however our understanding is mostly limited to Hrd1 and AMFR/gp78, both of which have a defined function in ERAD. To understand the contribution of ER E3s to cellular and organelle homeostasis, this study used mass spectrometry of purified E3 complexes to identify cofactors and build interaction networks of ER-resident E3s. These findings will form the foundation for investigating the biological roles of these ubiquitin ligases. Transcriptional analysis highlighted the centrality of Hrd1 among all ER-resident E3s in response to protein misfolding in the ER. Additionally, the contribution of individual Hrd1 complex components to resolving proteotoxic stress was assessed using a misfolded antibody subunit (IgM heavy chain), rather than conventional pharmacological treatments. The ERAD components essential for substrate degradation and survival under proteotoxic stress were identified, highlighting the pivotal role of Hrd1, its cofactor SEL1L and the Derlin family members. Finally, it was demonstrated that autophagy induction in response to proteasome inhibition is key to relieve the burden of protein misfolding in the ER, as it sustained the survival of cells defective for ERAD. Importantly, this study proposes a potential involvement of Hrd1 in signalling from the ER to autophagy, suggesting potential crosstalk between the ERAD and autophagic pathways.

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# Abbreviations

|           |                                                             |
|-----------|-------------------------------------------------------------|
| 4F2       | 4F2 cell-surface antigen heavy chain                        |
| A1AT      | A-1-antitrypsin                                             |
| aa        | Amino acid                                                  |
| AAA       | ATPase associated with diverse cellular activities          |
| ACOD      | Cis-aconitate decarboxylase                                 |
| ACSL4     | Long-chain-fatty-acid-coA ligase 4                          |
| AD        | Alzheimer's disease                                         |
| ADAM      | Disintegrin and metalloproteinase domain-containing protein |
| AL1B1     | Aldehyde dehydrogenase X                                    |
| ALS       | Amyotrophic lateral sclerosis                               |
| AMBRA1    | Activating molecule in BECN1-regulated autophagy protein 1  |
| AMFR      | Autocrine motility factor receptor                          |
| AP-3      | Adaptor protein complex 3                                   |
| AP3B1     | AP-3 complex subunit beta-1                                 |
| APMAP     | Adipocyte plasma membrane-associated protein                |
| APP       | Amyloid precursor protein                                   |
| ASPH      | Aspartyl/asparaginyl beta-hydroxylase                       |
| ATAD1     | ATPase family AAA domain-containing protein 1               |
| ATF       | Activating transcription factor                             |
| ATG       | Autophagy-related gene                                      |
| ATP       | Adenosine triphosphate                                      |
| AUP1      | Ancient ubiquitous protein 1                                |
| A $\beta$ | Amyloid- $\beta$ peptides                                   |
| BACE      | Beta-secretase 1                                            |
| BAG6      | BCL2-associated athanogene 6                                |
| BAX       | Apoptosis regulator BAX                                     |
| Bcl-2     | B-cell lymphoma 2                                           |
| BCR       | B-cell receptor                                             |
| BFAR      | Bifunctional apoptosis regulator                            |
| BH3       | Bcl-2 homology 3                                            |
| BI1       | Bax inhibitor 1                                             |
| BIF1      | Bax-interacting factor 1                                    |
| Bim       | Bcl2-interacting mediator of cell death                     |
| BiP       | Binding immunoglobulin protein                              |
| BRI3B     | BRI3-binding protein                                        |
| BSCG      | Bait-specific control group                                 |

|               |                                                             |
|---------------|-------------------------------------------------------------|
| CBR1          | Carbonyl reductase [NADPH] 1                                |
| CD147         | Cluster of differentiation 147                              |
| CFTR          | Cystic fibrosis transmembrane conductance regulator         |
| CGRRF1        | Cell growth regulator with RING finger domain protein 1     |
| CHIP          | Carboxy terminus of Hsp70-interacting protein               |
| CHOP          | Transcription factor C/EBP homologous protein               |
| CLN6          | Ceroid-lipofuscinosis neuronal protein 6                    |
| CLPT1         | Cleft lip and palate transmembrane protein 1                |
| CNS           | Central nervous system                                      |
| CNX           | Calnexin                                                    |
| COPE          | Coatomer subunit epsilon                                    |
| CRT           | Calreticulin                                                |
| CSK           | Tyrosine-protein kinase CSK                                 |
| CYB5          | Cytochrome b5                                               |
| DC            | Dendritic cell                                              |
| DERLIN        | Degradation in ER protein 1                                 |
| DHCR7         | 7-dehydrocholesterol reductase                              |
| DIO2          | Type II iodothyronine deiodinase                            |
| DJC16         | Dnaj homolog subfamily C member 16                          |
| DPYL2         | Dihydropyrimidinase-related protein 2                       |
| DUB           | Deubiquitinating enzyme                                     |
| EDEM          | ER degradation-enhancing $\alpha$ -mannosidase-like protein |
| eIF2 $\alpha$ | Eukaryotic translation initiation factor-2                  |
| ER            | Endoplasmic reticulum                                       |
| ERAD          | ER-associated degradation                                   |
| ERdj          | ER-resident DNAJ                                            |
| ERFAD         | ER flavoprotein associated with degradation                 |
| ERGIC         | ER-Golgi intermediate compartment                           |
| ERLIN         | ER lipid raft-associated protein 2                          |
| ERO           | ER oxidoreductin                                            |
| ERp44         | ER resident protein 44                                      |
| ERQC          | ER quality control compartment                              |
| ERSE          | ER-stress element                                           |
| EXOS3         | Exosome complex component RRP40                             |
| FAF2          | FAS-associated factor 2                                     |
| FAM8A1        | Family with sequence similarity 8 member A1                 |
| GABARAP       | Gamma-aminobutyric acid receptor-associated protein         |
| GADD34        | Growth arrest and DNA damage-inducible 34                   |
| GEMI4         | Gem-associated protein 4                                    |



|         |                                                                   |
|---------|-------------------------------------------------------------------|
| GLG1    | Golgi apparatus protein 1                                         |
| gp78    | Glycoprotein 78                                                   |
| GPI     | Glycophosphatidylinositol                                         |
| GRP78   | 78 kDa glucose-regulated protein                                  |
| GRP94   | 94 kDa glucose-regulated protein                                  |
| HC      | Heavy chain                                                       |
| HCDH    | Hydroxyacyl-coenzyme A dehydrogenase                              |
| HCIP    | High confidence interactor protein                                |
| HD      | Huntington's disease                                              |
| HDAC6   | Histone deacetylase 6                                             |
| HERP    | Homocysteine-responsive ER-resident ubiquitin-like domain protein |
| HIV-1   | Human immunodeficiency virus-1                                    |
| HMG-CoA | 3-hydroxy-3-methylglutaryl-coenzyme A                             |
| HMGR    | HMG-coA reductase                                                 |
| HMOX2   | Heme oxygenase 2                                                  |
| HO-1    | Heme oxygenase-1                                                  |
| HPS2    | Hermansky Pudlak syndrome type 2                                  |
| Hrd1    | HMG-coA reductase degradation protein 1                           |
| HSP     | Heat shock protein                                                |
| Htt     | Huntingtin                                                        |
| IDHP    | Isocitrate dehydrogenase [NADP]                                   |
| Ig      | Immunoglobulin                                                    |
| IP3R    | Inositol 1,4,5-trisphosphate receptor                             |
| IRE1    | Inositol requiring enzyme 1                                       |
| ITCH    | E3 ubiquitin-protein ligase Itchy homolog                         |
| JAMP    | JNK-associated membrane protein                                   |
| JNK     | C-Jun N-terminal kinase                                           |
| KAI1    | Metastasis suppressor Kangai-1                                    |
| LC      | Light chain                                                       |
| LC-MS   | Liquid chromatography-mass spectrometry                           |
| LIR     | LC3-interacting region                                            |
| MARCH   | Membrane-associated RING-CH-type finger                           |
| Mcl-1   | Induced myeloid leukemia cell differentiation protein Mcl-1       |
| MHC-I   | Major histocompatibility complex class I                          |
| MMGT1   | Membrane magnesium transporter 1                                  |
| MRH     | Mannose-6-phosphate receptor homology                             |
| NB5R3   | NADH-cytochrome b5 reductase 3                                    |
| NBR1    | Next to BRCA1 gene 1 protein                                      |
| NCL     | Neuronal ceroid lipofuscinose                                     |

|               |                                                             |
|---------------|-------------------------------------------------------------|
| NDUS2/ NDUFS2 | NADH dehydrogenase [ubiquinone] iron-sulfur protein 2       |
| NED4L         | E3 ubiquitin-protein ligase NEDD4-like                      |
| N-Gly1        | <i>N</i> -glycanase 1                                       |
| NHK           | Null Hong Kong                                              |
| NICD          | Notch intracellular domain                                  |
| Notch2        | Neurogenic locus notch homolog protein 2                    |
| Npl4/NPLOC4   | Nuclear protein localization protein 4 homolog              |
| Nrf1          | Nuclear respiratory factor 1                                |
| Nrf2          | Nuclear factor (erythroid -derived 2)-like 2                |
| OS-9          | Osteosarcoma 9                                              |
| OST           | Oligosaccharyltransferase                                   |
| P4HA1         | Prolyl 4-hydroxylase subunit alpha-1                        |
| PaelR         | Parkin-associated endothelin receptor-like receptor         |
| PC            | Plasma cell                                                 |
| PD            | Parkinson's disease                                         |
| PDI           | Protein disulphide isomerase                                |
| PE            | Phosphatidylethanolamine                                    |
| PERK          | Double-stranded RNA-activated protein kinase-like ER kinase |
| PERK          | Eukaryotic translation initiation factor 2-alpha kinase 3   |
| PP1C          | Protein phosphatase 1C                                      |
| PPlase        | Peptidylprolyl isomerase                                    |
| PrP           | Prion protein                                               |
| PSME3         | Proteasome activator complex subunit 3                      |
| RA            | Rheumatoid arthritis                                        |
| RHBDL4        | Rhomboid-like protein 4                                     |
| RIDD          | Regulated Ire1-dependent decay                              |
| RING          | Really interesting new gene                                 |
| RMND1         | Required for meiotic nuclear division protein 1 homolog     |
| RNF           | RING finger protein                                         |
| RNFT1         | RING finger and transmembrane domain-containing protein 1   |
| ROS           | Reactive oxygen species                                     |
| S1P           | Site-1 protease                                             |
| S23IP         | SEC23-interacting protein                                   |
| S27A3         | Long-chain fatty acid transport protein 3                   |
| S2P           | Site-2 protease                                             |
| S39AA         | Zinc transporter ZIP10                                      |
| S39AE         | Zinc transporter ZIP14                                      |
| SAC1          | Phosphatidylinositide phosphatase SAC1                      |
| SCAP          | SREBP cleavage-activated protein                            |

|          |                                                              |
|----------|--------------------------------------------------------------|
| SEC23IP  | SEC23-interacting protein                                    |
| SEL1L    | Suppressor of lin-12-like protein 1                          |
| Sgk1     | Glucocorticoid-induced kinase 1                              |
| SGMR1    | Sigma non-opioid intracellular receptor 1                    |
| SLC1A5   | Sodium-dependent neutral amino acid transporter type 2       |
| SLC27A3  | Long-chain fatty acid transport protein 3                    |
| SLC30A6  | Zinc transporter 6                                           |
| SLC39A14 | Zinc transporter ZIP14                                       |
| SLMAP    | Sarcolemmal membrane-associated protein                      |
| SOD1     | Superoxide dismutase-1                                       |
| SopA     | Salmonella outer protein A                                   |
| SPCS2    | Signal peptidase complex subunit 2                           |
| SQLE     | Squalene monooxygenase                                       |
| SREBP-2  | Sterol response element binding protein 2                    |
| SRP      | Signal recognition particle                                  |
| STING    | Stimulator of interferon genes protein                       |
| SYSC     | Serine-trna ligase                                           |
| TCR      | T-cell receptor                                              |
| TFR1     | Transferrin receptor protein 1                               |
| TM9S2    | Transmembrane 9 superfamily member 2                         |
| TMD      | Transmembrane domain                                         |
| TMEM9    | Transmembrane protein 9                                      |
| TMUB1    | Transmembrane and ubiquitin-like domain-containing protein 1 |
| TMX2     | Thioredoxin-related transmembrane protein 2                  |
| TRAF2    | TNFR-associated factor 2                                     |
| TRC8     | Translocation in renal carcinoma on chromosome 8 protein     |
| TRIM     | Tripartite motif-containing protein                          |
| TTR      | Transthyretin                                                |
| Ub       | Ubiquitin                                                    |
| UBAC2    | Ubiquitin-associated domain-containing protein 2             |
| UBC12    | NEDD8-conjugating enzyme Ubc12                               |
| UBD      | Ub-binding domain                                            |
| Ube2g2   | E2 ubiquitin-conjugating enzyme G2                           |
| Ube2j1   | E2 ubiquitin-conjugating enzyme J1                           |
| UBXD8    | Ub-like (UBX)-domain-containing 8                            |
| UDP      | Uridine diphosphate                                          |
| Ufd1     | Ubiquitin fusion degradation protein 1                       |
| UFD1L    | Ubiquitin fusion degradation protein 1 homolog               |
| UGGT     | UDP-glucose:glycoprotein glucosyltransferase                 |

|        |                                                    |
|--------|----------------------------------------------------|
| ULK    | Unc-51-like kinase                                 |
| UPR    | Unfolded protein response                          |
| UPRE   | UPR element                                        |
| UPS    | Ubiquitin–proteasome system                        |
| Usp21  | Ubiquitin-specific-processing protease 25          |
| UVRAG  | Ultraviolet irradiation resistance-associated gene |
| VAMP7  | Vesicle-associated membrane protein 7              |
| VCP    | Valosin-containing protein                         |
| VIMP   | VCP-interacting membrane protein                   |
| VPP2   | V-type proton atpase catalytic subunit A           |
| VSP34  | Vacuolar sorting protein 34                        |
| WLS    | Protein wntless homolog                            |
| WWP2   | NEDD4-like E3 ubiquitin-protein ligase WWP2        |
| XPB1   | X-box binding protein 1                            |
| XTP3-B | Transactivated protein B                           |
| YOD1   | Ubiquitin thioesterase OTU1                        |
| ZIP14  | Zinc transporter ZIP14                             |
| ZNRF4  | Zinc/RING finger protein 4                         |
| ZNT6   | Zinc transporter 6                                 |
| β2m    | β2-microglobulin                                   |
| μS     | Secretory IgM heavy chain                          |

# Chapter I:

## Introduction

## 1.1 The secretory pathway

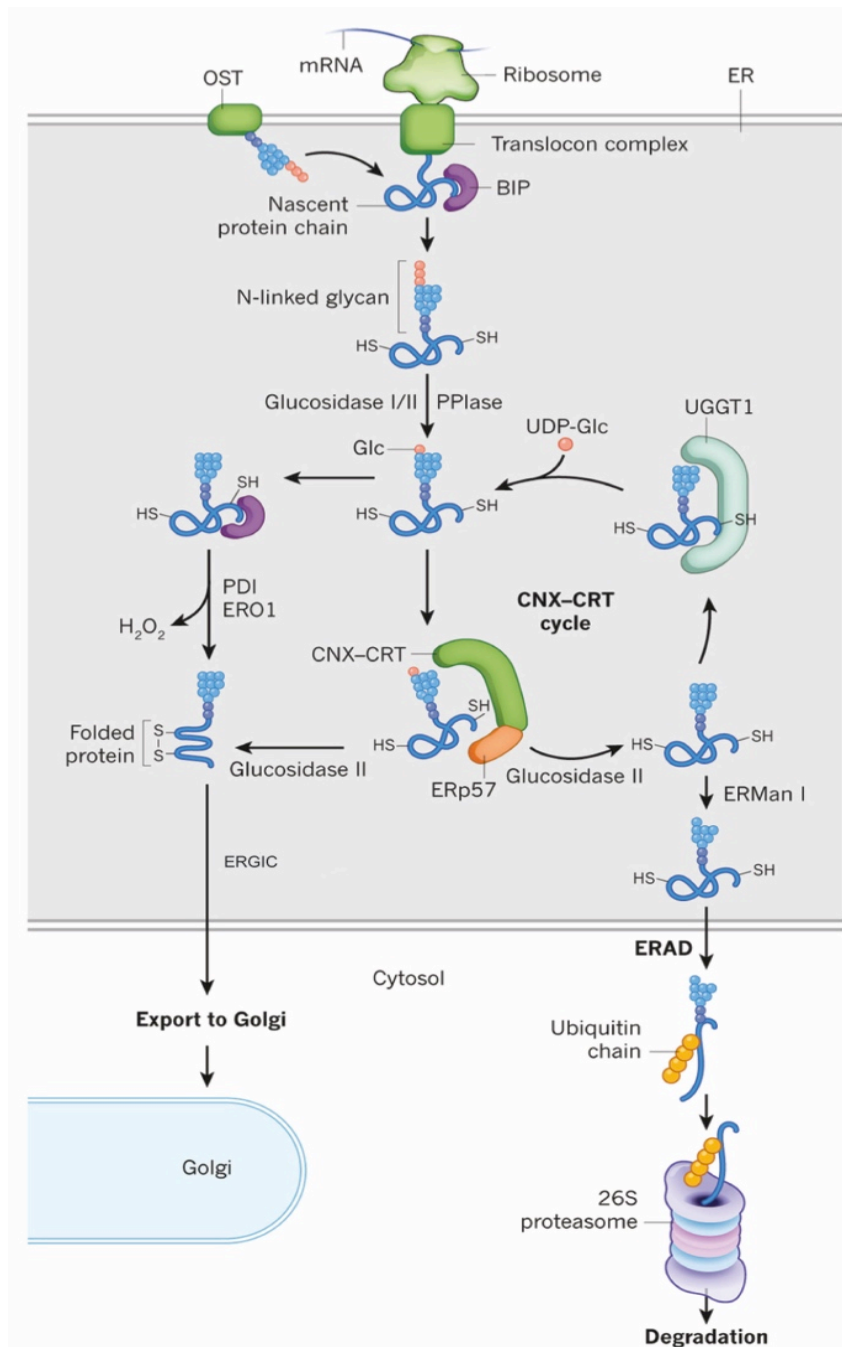
The secretory pathway of eukaryotic cells consists of an endomembrane system organised into distinct compartments, each with specialised functions. These include the endoplasmic reticulum (ER), the Golgi apparatus and the vesicles that travel in between them, as well as the plasma membrane and lysosomes. One third of the eukaryotic genome encodes for proteins destined for this pathway (Ghaemmaghami et al., 2003). These include secretory proteins that are delivered to the extracellular space, and resident proteins that can be either soluble or integrated into membranes via hydrophobic domains or glycosylphosphatidylinositol (GPI)-anchors. The ER is the port of entry to the secretory pathway, and is organised in an extensive network of flat layered cisternae, from which transport vesicles bud off for anterograde trafficking of their content to downstream compartments, namely the ER-Golgi intermediate compartment (ERGIC) and the Golgi apparatus. The Golgi apparatus is a polarised organelle consisting of three functionally distinct compartments: *cis*, medial and *trans* cisternae. From the *trans*-Golgi, vesicles shuttle proteins and fuse with the plasma membrane for insertion or secretion of their cargo, while from the *cis*-Golgi, immature or ER-resident proteins are returned to the ER via retrograde transport vesicles. This ensures that only mature proteins can proceed along the secretory pathway to reach their final destination [reviewed in (Pelham and Munro, 1993)].

Many proteins that enter the secretory pathway contain a characteristic ER signal sequence at their N-terminus. This hydrophobic sequence of 16–30 amino acids (aa) (Kapp *et al.*, 2009) is recognised by a signal recognition particle (SRP) that directs ribosomes that are synthesising the polypeptide to the translocon apparatus located in the ER membrane. The nascent polypeptide is co-translationally or post-translationally (Johnson et al., 2013) inserted into the ER lumen or membrane via the Sec61 complex (Görlich and Rapoport, 1993). During insertion, nascent chains

are modified by disulphide bonds and *N*-linked glycans that help promote folding and oligomerisation. Maturation of nascent chains is overseen by tight quality control mechanisms, which ensure that only properly folded and assembled proteins are exported from the organelle (Sitia and Braakman, 2003). In addition to protein folding, the ER plays key roles in other metabolic pathways by acting as the main intracellular site for calcium storage and lipid biosynthesis. All together, these functions are key to the preservation of cellular homeostasis.

## **1.2 Protein folding in the ER**

As the nascent chain enters the ER lumen, it is recognised directly by resident molecular chaperones that assist its folding, maturation and oligomeric assembly. Chaperones bind to hydrophobic regions of unfolded polypeptides, avoiding rapid collapse into inactive, aggregation-prone conformations. The ER harbours several classes of molecular chaperones and folding enzymes that include: (i) members of the heat shock protein (HSP)70 family, such as binding immunoglobulin protein (BiP) and its HSP40 family co-chaperones like ER-resident DNAJ (ERdj)1–5 (Hendershot, 2004), and the HSP90 family member GRP94 (94 kDa glucose-regulated protein) (Argon and Simen, 1999); (ii) the lectins calnexin (CNX) and calreticulin (CRT) (Helenius and Aebi, 2004); and (iii) the thiol-oxidoreductases of the PDI (protein disulphide isomerase) family (Ellgaard and Ruddock, 2005). The process of protein folding and associated post-translational modifications is illustrated in Figure 1.1 [adapted from (M. Wang and Kaufman, 2016)].



**Figure 1.1 Folding cycles in the ER**

Nascent polypeptides are inserted in the ER across the Sec61 translocon complex. ER-resident oligosaccharyltransferase (OST) transfers glycan moieties to the emerging polypeptides. Protein folding is assisted by chaperones (like BiP), oxidoreductases [like PDI, ERp57 and ER oxidoreductin 1 (ERO1)] and peptidylprolyl isomerases (PPIases). In the CNX/CRT cycle, addition and trimming of *N*-linked oligosaccharides, together with re-glucosylation by UGGT1 (UDP-glucose:glycoprotein glucosyltransferase), facilitate protein folding. Correctly folded proteins are trafficked to the Golgi apparatus by binding to shuttling factors such as ERGIC-53. Terminally misfolded proteins are retrotranslocated to the cytosol for ubiquitination and degradation by the 26S proteasome through ER-associated protein degradation (ERAD). Adapted from (M. Wang and Kaufman, 2016).



The major ER luminal chaperone is represented by BiP, also known as 78 kDa glucose-regulated protein (GRP78). It was originally discovered in non-covalent association with immunoglobulin heavy chains in myeloma cells defective for the synthesis of light chains (Haas and Wabl, 1983). BiP bears an N-terminal ATPase domain and a C-terminal domain with affinity for hydrophobic patches (Flynn et al., 1991). ATP (adenosine triphosphate) hydrolysis triggers a conformational change that favours the interaction of BiP with unfolded proteins. Upon several cycles of release and rebinding mediated by ATP hydrolysis, the protein likely attains its native conformation, in which hydrophobic regions are no longer exposed, allowing definitive release from BiP. In addition to its foldase activity, BiP plays a pivotal role in regulating ER homeostasis through the unfolded protein response (UPR) (Bertolotti et al., 2000; Pincus et al., 2010). Hydrophobic patches on unfolded polypeptides can also be recognised by the more selective chaperone Grp94 (Marzec et al., 2012).

Unlike cytosolic proteins, most secretory proteins are modified by *N*-linked oligosaccharides. *N*-linked glycosylation begins with the transfer of a core oligosaccharide ( $\text{Glc}_3\text{Man}_9\text{GlcNac}_2$ ) from a membrane-bound dolichol phosphate anchor to an Asn-X-Ser/Thr (where X is any amino acid except Pro) consensus sequence within the polypeptide chain (Hubbard and Ivatt, 1981). Then,  $\alpha$ -glucosidase I and  $\alpha$ -glucosidase II sequentially remove the glucose moieties until only one glucose remains, enabling the glycoprotein to be recognised by the lectin-like chaperones CNX/CRT, which assist folding and maturation. The CNX/CRT system associates with the thiol-disulphide oxidoreductase ERp57, which catalyses disulphide bond formation on the bound glycoprotein (Frickel et al., 2004). The monoglycosylated substrate shuttles through several cycles of de- and reglucosylation by  $\alpha$ -glucosidase II and uridine diphosphate (UDP)-glucose:glycoprotein glucosyl transferase (UGGT) (Ellgaard et al., 1999). If, after

removal of the terminal glucose by  $\alpha$ -glucosidase II, the polypeptide attains its native conformation, it can exit the CNX/CRT cycle to be exported to the Golgi compartment via ERGIC-53 (Appenzeller et al., 1999) or other systems. If improperly folded, reglucosylation by UGGT favours interaction with CNX for other folding attempts.  $\alpha$ -(1,2)-mannosidase I and EDEM (ER degradation-enhancing  $\alpha$ -mannosidase-like protein) family proteins participate in the multi-step removal of the mannose moieties from the terminally misfolded polypeptide (Ninagawa et al., 2014), which is then recognised by the lectin OS-9 and delivered to cytosolic proteasomes for degradation (Hosokawa et al., 2001). The position of the *N*-glycans determines which chaperone machinery will be preferentially employed: the closer they are to the N-terminus of the polypeptide, the more likely the CNX/CRT machinery is engaged over the BiP folding system (Molinari and Helenius, 2000). However, if the unfolded protein fails to engage one chaperone system, another can take over, highlighting the strong redundancy in the ER folding machinery (van Anken and Braakman, 2005).

The majority of the ER client proteins require disulphide bond formation to acquire a native conformation (Sitia and Braakman, 2003). PDI and family members catalyse this reaction. Structurally, PDI has four thioredoxin domains: two with oxidoreductase activity and two with hydrophobic features for binding of the substrates (Ellgaard and Ruddock, 2005). The oxidation occurs between thiol groups of two cysteine residues, and they can be either intra- or inter-molecular, in the latter case to promote oligomerisation. Upon disulphide bond formation, PDI is re-oxidised by Ero1 family members (Pagani et al., 2000; Pollard et al., 1998) or by other redox systems like peroxiredoxin-4 (Bulleid and Ellgaard, 2011). In addition to PDI, several other PDI-like oxidoreductases may be involved in thiol-oxidation, isomerisation and sometimes reduction (Jansen et al., 2012). Proteins carrying incomplete disulphide bonds are recognised for retention or degradation (Fra et al.,

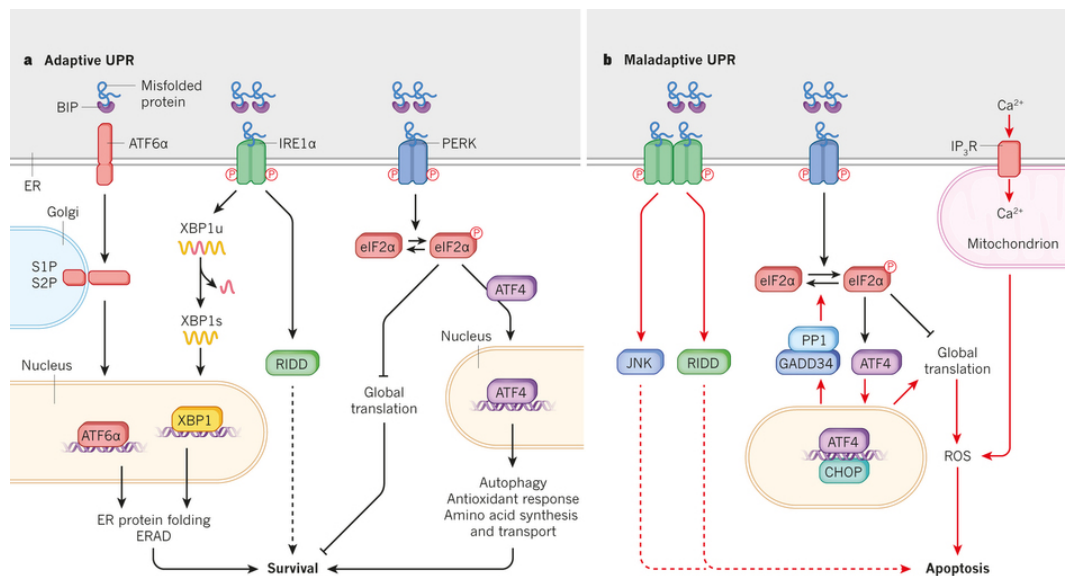
1993). Thiol-mediated retention is key to preventing exposed thiols from exiting the ER, and was originally described in the quality control of IgM (immunoglobulin M) (Sitia et al., 1990).

### **1.3 The unfolded protein response**

During the cell's lifespan, continuous protein misfolding occurs as the result of inefficient folding, genetic mutations, occasional misincorporation of amino acids, unassembled subunits of oligomeric complexes or failures in the quality control machinery (Balch et al., 2008; Voisine et al., 2010). In addition to these physiological sources of misfolded proteins, a number of pathological conditions, metabolic and environmental stresses, as well as aging, contribute to the accumulation of misfolded products (Haigis and Yankner, 2010). Some environmental stresses include hypoxia, exposure to chemicals, radiation, heavy metals and pathogen infections, while metabolic stresses are associated with nutrient imbalance, presence of reactive oxygen species (ROS) as well as mitochondrial dysfunction. Among different pathological conditions characterised by protein misfolding, cancer cells exhibit high levels of misfolded proteins due to their increased rates of cell division and mutation (Whitesell and Lindquist, 2005). In addition, aging is naturally associated with a decline in protein homeostatic capacity (Morley and Morimoto, 2004), which in turn catalyses further protein damage and the potential generation of toxic aggregates (Douglas and Dillin, 2010). Both protein misfolding and an excessive influx of unfolded proteins can challenge the folding and degradation capacity of the ER. Under these conditions, the ER undergoes stress and initiates a counteractive response mechanism, the UPR, which serves to restore homeostasis and sustain survival.

The UPR is a network of cellular pathways that transduce information about the protein folding status in the ER lumen to the nucleus to counteract the loss of

proteostatic capacity (Schröder and Kaufman, 2005). When the stress becomes unresolvable, the UPR ultimately triggers apoptosis, indicating the existence of mechanisms that integrate duration and intensity information of the stress stimuli (Woehlbier and Hetz, 2011). In mammals, three transmembrane proteins transduce the stress signal from the ER lumen: IRE1 (inositol requiring enzyme 1), ATF6 (activating transcription factor 6) and PERK (double-stranded RNA-activated protein kinase-like ER kinase). Together, these sensors concomitantly act to sense and respond to stress in the ER. The UPR branches and their associated pro-survival and pro-apoptotic outcomes are represented in Figure 1.2 (M. Wang and Kaufman, 2016).



**Figure 1.2 The UPR signalling pathway**

a) The UPR comprises three parallel signalling branches: ATF6α, IRE1α and PERK. BiP binds constitutively to the ER-luminal domains of ATF6α, IRE1α and PERK and sequesters them in an inactive form. Misfolded proteins that accumulate in the ER bind to BiP, causing its release from the UPR sensors and activation of their signalling pathways. ATF6α is trafficked to the Golgi for processing by the enzymes S1P and S2P to release a soluble cytosolic fragment that enters the nucleus to induce the expression of target genes. IRE1α and PERK oligomerise and *trans*-autophosphorylate. Direct interaction between unfolded/misfolded proteins and the UPR sensors might be required. b) Maladaptive UPR is induced by sustained activation of the PERK pathway, which is the result of prolonged severe ER stress and leads to apoptosis (solid red lines). The role of IRE1α-induced JNK (c-Jun N-terminal kinase) and RIDD (regulated IRE1-dependent decay) in ER-stress-induced apoptosis remains unclear (dashed lines) (M. Wang and Kaufman, 2016).

### 1.3.1 IRE1

IRE1 is the only branch conserved in all eukaryotes down to yeast, where the UPR was first discovered (Cox et al., 1993; Mori et al., 1993). IRE1 is a transmembrane protein that spans the ER membrane, with the N-terminal domain oriented towards the lumen and the C-terminal domain in the cytosol. The luminal domain contains the sensing region for interaction with unfolded proteins (Gardner and Walter, 2011) and a BiP binding site (Kimata et al., 2004). At the cytosolic side, IRE1 harbours kinase and endoribonuclease domains that are necessary for the propagation of its signalling pathway. Under non-stressed conditions, IRE1 is diffuse in the ER membrane in a monomeric state and associated with BiP, which maintains IRE1 in an inactive state. Upon binding of misfolded proteins to its luminal domain, an event that coincides with the dissociation of BiP (Kimata et al., 2004), IRE1 *trans*-autophosphorylates in its cytosolic domain and oligomerises into discrete clusters that confer full enzymatic activity (Aragón et al., 2009; Korennykh et al., 2009). IRE1 clusters recruit XBP1 (X-box binding protein) messenger RNA (mRNA), which is spliced by IRE1 endoribonuclease activity (H. Li et al., 2010; Walter and Ron, 2011). Spliced XBP1 is translated into a transcription factor that travels to the nucleus where it drives the expression of UPR target genes (Travers et al., 2000). These include ER chaperones, enzymes involved in lipid biosynthesis and components of ER-associated degradation (ERAD), such as the ubiquitin ligase Hrd1 (Friedlander et al., 2000; Hetz et al., 2011; A.-H. Lee et al., 2003). Altogether, the genetic programme initiated by IRE1 is responsible for the reinforcement of folding and degradation, as well as expansion of the ER membrane to accommodate the increased load of misfolded proteins. In addition to splicing XBP1 mRNA, IRE1 selectively degrades mRNAs encoding certain ER-located proteins, a process known as IRE1-dependent decay (RIDD), to reduce the influx of nascent proteins in the ER (Hollien et al., 2009). In situations of irreversible ER stress, IRE1 forms a

heterotrimeric complex with TRAF2 (TNFR-associated factor 2) and ASK1 (apoptosis signal-regulating kinase 1), which activates JNK (c-Jun N-terminal kinase) to promote apoptosis (Urano et al., 2000) IRE1-JNK signalling can also activate macroautophagy by activating beclin1 (Ogata et al., 2006).

### **1.3.2 ATF6**

The second branch of the UPR is governed by ATF6, a transmembrane stress sensor that may bind unfolded proteins via its luminal domain or, alternatively, be activated through dissociation of BiP (Xi Chen et al., 2002). Under ER stress, ATF6 translocates to the Golgi where it is processed by S1P and S2P (site-1 and site-2 protease), which cleave its luminal domain and the transmembrane anchor, respectively (J. Ye et al., 2000). Hence, the N-terminal fragment is released and travels to the nucleus where it drives the transcription of target genes encoding ERAD components and ER chaperones such as BiP, GRP94, PDI as well as XBP1 (Adachi et al., 2008; K. Lee et al., 2002; Yamamoto et al., 2007).

### **1.3.3 PERK**

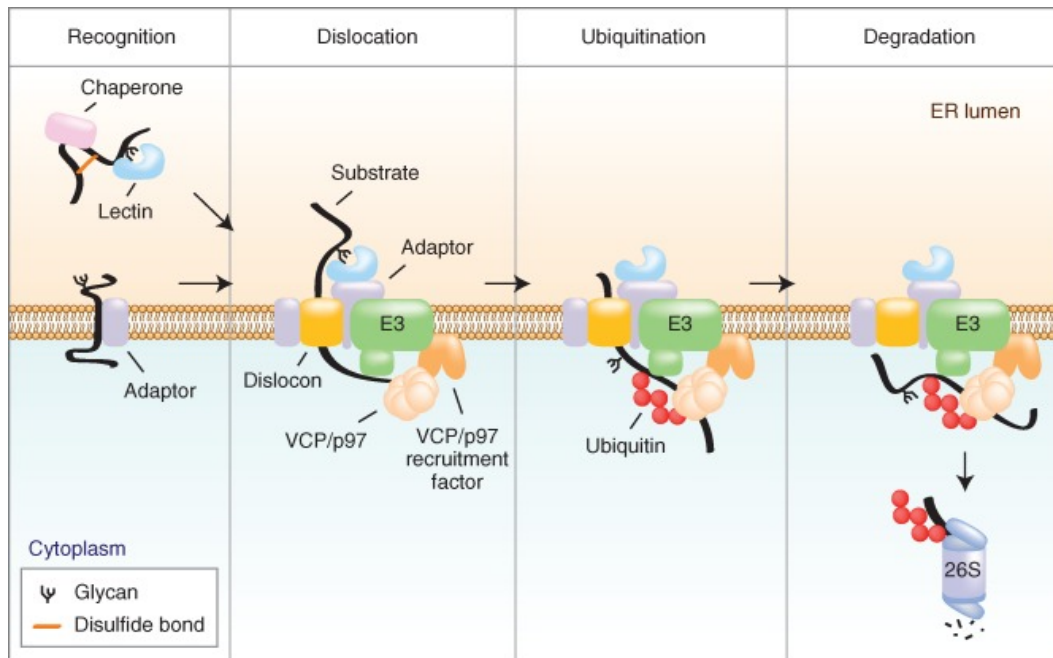
The third arm of the UPR is governed by PERK. PERK has a core sensing domain in the ER lumen and a kinase domain at the cytosolic side. Stress sensing triggers PERK oligomerisation and *trans*-autophosphorylation of its cytosolic domain. Activated PERK in turn phosphorylates the  $\alpha$ -subunit of eukaryotic translation initiation factor-2 (eIF2 $\alpha$ ), which results in two main events. First, phosphorylation of eIF2 $\alpha$  stalls mRNA translation (Harding et al., 1999), which reduces the amount of newly synthesised proteins, including those targeted to the ER. Second, phosphorylated eIF2 $\alpha$  allows selective translation of the transcription factor ATF4 (Activating transcription factor 4), which controls the expression of pro-survival genes involved in redox balance, amino acid metabolism, protein folding as well as autophagy (Ameri and Harris, 2008; Schröder and Kaufman, 2005). Along with the pro-survival programme, ATF4 also drives the expression of CHOP (transcription

factor C/EBP homologous protein) and GADD34 (growth arrest and DNA damage-inducible 34) (Harding et al., 2000). CHOP regulates pro-apoptotic genes, ultimately leading to cell death when the stress becomes chronic (Ron and Habener, 1992), while GADD34 encodes a regulatory subunit of protein phosphatase 1C (PP1C) that dephosphorylates eIF2 $\alpha$ , hence providing negative feedback on the PERK pathway (Yan-Jun Ma and Hendershot, 2003). Besides eIF2 $\alpha$ , PERK also phosphorylates Nrf2 (nuclear factor (erythroid -derived 2)-like 2), a pro-survival transcription factor involved in the antioxidant response (Cullinan et al., 2003).

In conclusion, the UPR represents a complex, dynamic signalling network, with both pro-survival and pro-apoptotic outcomes. On one side, it allows cellular adaptation to stress by reinforcing the folding and degradation capacity of the ER (adaptive UPR). On the other side, in conditions of irreversible stress and chronic activation, it promotes the removal of damaged cells by inducing apoptosis (maladaptive UPR).

## **1.4 ERAD**

ERAD is a vectorial, coordinated process that dispatches terminally misfolded proteins from the ER to the cytosol to be degraded by the ubiquitin–proteasome system (UPS). It consists of four sequential steps: (i) substrate recognition, (ii) substrate retrotranslocation, (iii) ubiquitin conjugation and (iv) degradation by 26S proteasomes. A schematic of the ERAD process is depicted in Figure 1.3 (Olzmann et al., 2013).



**Figure 1.3 Key steps in ERAD**

Recognition: ER luminal chaperones and lectins interact with unfolded clients and link them to the dislocation machinery by binding to membrane-embedded adaptors. Dislocation: substrates are dislocated across the bilayer, presumably through proteinaceous pores in an ATP-dependent process mediated by VCP/p97. Ubiquitination: on gaining access to the cytosol, substrates are K48-polyubiquitinated by E3 ligases. Degradation: ubiquitinated substrates are degraded by cytosolic 26S proteasomes (Olzmann et al., 2013).

#### 1.4.1 Substrate recognition

Glycoproteins that are unable to fold eventually escape the CNX/CRT cycle to be diverted to ERAD. Persistent retention in the folding cycle times the intervention of EDEMs to extract the misfolded protein from the CNX/CRT system. Processing of the terminal mannose residues attached to the glycoprotein results in a demannosylated form that is incompatible with UGGT-mediated reglucosylation. The mannosidases involved in the trimming reaction comprise EDEM1, EDEM2, EDEM3 (Hosokawa et al., 2010b; 2007) and ERManI (Gonzalez et al., 1999). The luminal lectins osteosarcoma 9 (OS-9) and transactivated protein B (XTP3-B)/Erlectin selectively recognise the trimmed oligosaccharides via mannose-6-phosphate receptor homology (MRH) domains (Hosokawa et al., 2009; 2010a). The two lectins appear to be functionally redundant, since only combined knockdown



has been shown to significantly affect luminal substrate degradation (Bernasconi et al., 2010).

Although glycoproteins represent the majority of the secretory proteome, folding-defective non-glycosylated proteins also need to be targeted for degradation. Association of BiP in complexes with XTP3-B/OS-9 may confer these lectins the capability to recognise unfolded proteins independent of *N*-glycan signatures (Christianson et al., 2008). Once the substrate is bound by OS-9/XTP3-B, it is shuttled to retrotranslocation sites at the ER membrane. Different recognition pathways coexist in the ER to target misfolded proteins for degradation. Some non-glycosylated, soluble BiP substrates are targeted to a Herp-Hrd1-Derlin-1 complex prior to retrotranslocation (Okuda-Shimizu and Hendershot, 2007). In one study, a mutant variant of the non-glycosylated protein transthyretin (TTR<sub>D18G</sub>) was post-translationally glycosylated to be recognised by EDEM3 for degradation (T. Sato et al., 2012). EDEM1 also binds unfolded, non-glycosylated proteins and delivers them to the adaptor molecule SEL1L (Suppressor of lin-12-like protein 1) for subsequent ubiquitination by Hrd1 (Cormier et al., 2009). In addition, interaction of ERdj5 with EDEM1-bound substrates mediates disulphide bond reduction to facilitate retrotranslocation (Hagiwara et al., 2011). EDEM2 has been found to interact with OS-9 for the recognition of non-glycosylated sonic hedgehog protein (H.-Y. Tang et al., 2014). It has recently been shown that exposure of hydrophobic patches is an important determinant for recognition of non-glycosylated substrates by EDEM1 and EDEM2 (Sokołowska et al., 2015). In conclusion, different mechanisms relying on *N*-glycan signatures as well as exposed hydrophobic regions ensure that all classes of misfolded substrates can be efficiently recognised and targeted for degradation.

### 1.4.2 Dislocation to the cytosol

Both luminal and membrane-integrated substrates need to reach the cytosol for proteasomal degradation. Although several mechanisms of dislocation (integral membrane proteins) or retrotranslocation (soluble proteins) have been proposed, conclusive mechanistic evidence *in vivo* is still missing. A unifying feature of retrotranslocation is the requirement for the AAA (ATPase associated with diverse cellular activities) ATPase valosin-containing protein (VCP)/p97, which provides energy for the extraction (discussed below). In order to initiate ubiquitination and retrotranslocation, substrates must approach the ubiquitin ligase complexes at the ER membrane. SEL1L has been shown to bridge luminal recognition lectins to Hrd1, the main E3 ubiquitin ligase in the ER and a candidate component of the dislocon (Iida et al., 2011). It is not clear whether complete unfolding of the substrate is required for dislocation (Tirosh et al., 2003). Several lines of evidence support the need for disulphide bond reduction prior to dislocation. The PDI family member ERp90, as well as ERFAD (ER flavoprotein associated with degradation), has been found in association with Hrd1, OS-9 and SEL1L (Riemer et al., 2009; 2011). Other oxidoreductases like ERdj5 (Ushioda et al., 2008), PDI family members (Spooner et al., 2004; B. Tsai et al., 2001) and ERO1 (Moore et al., 2010) participate in disulphide bond reduction for ERAD. Specifically, reduced PDI has been shown to bind unfolded substrates, while oxidised PDI, via the action of the oxidase ERO1, allows substrate release for subsequent retrotranslocation and degradation in the proteasomes (B. Tsai and Rapoport, 2002). For example, reduction of interchain disulphide bonds of IgM heavy chains is an essential prerequisite for retrotranslocation (Fagioli et al., 2001). Additionally, the ER transmembrane protein VIMP (VCP-interacting membrane protein) is implicated in the reduction of stable disulphide bonds that were not reduced prior to retrotranslocation (Christensen et al., 2012).

Several candidates have been proposed to function as dislocation/retrotranslocation systems. Sec61 $\alpha$  has been found in association with ERAD substrates (Wiertz et al., 1996), the Hrd1 ligase complex (Plemper et al., 1999; Schäfer and Wolf, 2009) and proteasomes (Ng et al., 2007), suggesting that it may act as a translocation channel. However, only small peptides can exit the ER by this route (Gillece et al., 2000), and several studies did not confirm its role in retrotranslocation (Garza et al., 2009; Wahlman et al., 2007).

Derlins have also been implicated in the retrotranslocation of misfolded proteins. In mammals, Derlin-1 has been shown to mediate degradation of MHC-I (major histocompatibility complex class I) heavy chains upon binding to the viral protein US11 via interaction with VIMP, a membrane protein that recruits VCP/p97 to the ER for substrate extraction (Lilley and Ploegh, 2004; Y. Ye et al., 2004). In a later study, Derlin-2, but not Derlin-1 or -3, was reported to interact with Hrd1 for retrotranslocation of glycosylated and non-glycosylated substrates, namely sonic hedgehog protein and NHK (null Hong Kong), a variant of  $\alpha$ -1-antitrypsin (Huang et al., 2013), in contrast to a previous study that reported the involvement of both Derlin-2 and -3 in the degradation of NHK (Oda et al., 2006).

Some studies have demonstrated the involvement of the Hrd1 complex in the retrotranslocation of misfolded substrates in yeast (Carvalho et al., 2010; B. K. Sato et al., 2009). Composed of six multi-spanning domains containing a high proportion of hydrophilic residues, Hrd1's structure may serve as a channel for misfolded peptides (B. K. Sato et al., 2009). *In vitro* reconstitution of the yeast Hrd1 complex components has revealed a requirement of Hrd1 for the retrotranslocation of luminal substrates (Stein et al., 2014), a process dependent on Hrd1 autoubiquitination (Baldrige and Rapoport, 2016). In mammals, retrotranslocation of the catalytic CTA1 subunit of cholera toxin is mediated by Hrd1 and the ER resident ubiquitin

ligase autocrine motility factor receptor (AMFR)/gp78 (glycoprotein 78) (Bernardi et al., 2010).

As an alternative mechanism, membrane-anchored substrates could instead be cleaved by rhomboid and rhomboid-like proteases that reside within the lipid bilayer (Urban and Dickey, 2011). In support of this hypothesis, the rhomboid protease RHBDL4, in addition to specifically binding the ATPase VCP/p97, has been reported to cleave both single- and multi-spanning membrane proteins, allowing their degradation through ERAD (Fleig et al., 2012). A striking feature that distinguishes RHBDL4 from other intramembrane proteases resides in its ubiquitin-interacting motif, reported to be required for its function in ERAD. Intramembrane proteolysis can be executed by a number of enzymatic complexes that reside in the ER membrane, including rhomboid and rhomboid-like proteases and  $\gamma$ -secretase [reviewed in (Lemberg, 2011)]. Despite the limited evidence in support of a role or intramembrane proteolysis in proteasome-dependent degradation, it represents one possibility by which membrane-embedded ERAD substrates are extracted.

In conclusion, a definitive retrotranslocation/dislocation mechanism remains elusive in mammals. To date, evidence points towards the engagement of diverse machineries depending on the nature of the substrate.

### **1.4.3 Ubiquitin conjugation**

Ubiquitin (Ub), a highly conserved protein of 76 aa, is conjugated to the substrate during its extraction from the ER and serves as targeting signal for proteasomal degradation. Ubiquitination occurs through the sequential action of three classes of proteins: E1 ubiquitin activating enzymes, E2 conjugating enzymes and E3 ubiquitin ligases [reviewed in (Komander, 2009)]. First, the C-terminal carboxyl group of Ub is activated via ATP hydrolysis and forms a high-energy thioester linkage with the active site cysteine in the E1. Second, Ub is *trans*-thiolated from the E1 to the active

site cysteine of the E2. Finally, Ub is transferred from the E2 to the  $\epsilon$ -amino group of a substrate lysine in an E3-dependent manner. Less commonly, Ub can be conjugated to Ser or Thr residues on the target protein (X. Wang et al., 2007). The E3s involved in ERAD belong to the group of RING (really interesting new gene) ligases, which are encoded by over 600 genes in mammals (W. Li et al., 2008). By simultaneously accommodating the substrate and the E2 enzyme to allow the direct transfer of Ub, RING E3s dictate the specificity of the ubiquitination reaction. Additional Ub moieties can be attached to the first Ub to form poly-Ub chains via lysine (K) residues. There are seven lysines on Ub, allowing the formation of diverse structures. Typically, poly-Ub chains of at least four molecules attached via K48, and less commonly K11 (M. Xu et al., 2009), target the substrate for proteasomal degradation (Chau et al., 1989; Thrower et al., 2000). Further processing by deubiquitinating enzymes (DUBs) has been suggested to regulate retrotranslocation (Bernardi et al., 2013; Ernst et al., 2009) and targeting to the 26S proteasome (J. Liu et al., 2014).

#### **1.4.4 Extraction and degradation via the 26S proteasome**

Polyubiquitinated substrates are extracted from the ER membrane by the action of the cytosolic AAA-ATPase VCP/p97 (Y. Ye et al., 2001). VCP/p97 contains two ATPase domains, which assemble into a barrel-like hexamer with a central pore (DeLaBarre et al., 2006; Q. Wang et al., 2004). Despite its cylindrical structure, passage of the substrate through the entire pore has not been demonstrated. The correct recognition of poly-Ub chains is facilitated by interaction with the cofactors Ufd1 and Npl4 (Meyer et al., 2000), which is crucial for VCP/p97 function (Y. Ye et al., 2003). VCP/p97 is recruited to the cytosolic side of the ER via interaction with ER proteins such as VIMP (Y. Ye et al., 2004), Ub-like (UBX)-domain-containing (UbxD8) (Suzuki et al., 2012) and Derlin-1 and -2 (Greenblatt et al., 2011). ATP hydrolysis promotes a conformational change in VCP/p97 (X. Zhang et al., 2000)

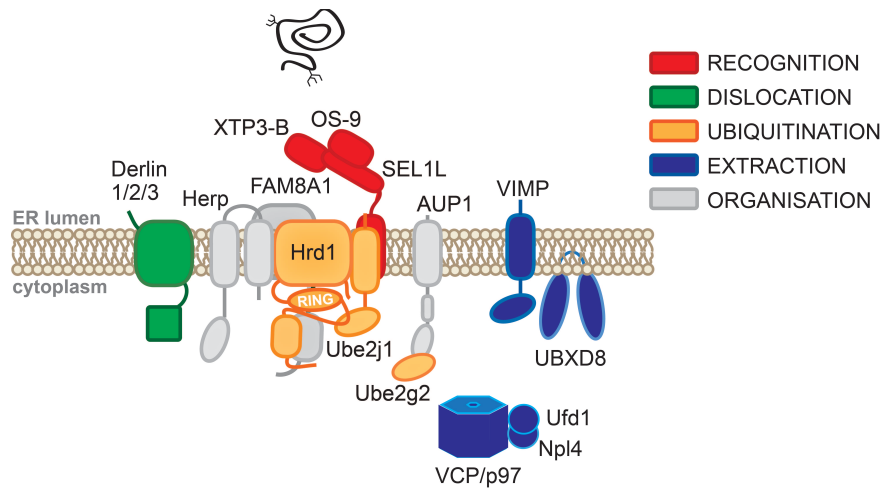
that provides the mechanical force to extract substrates from the ER (Y. Ye et al., 2001). In addition, VCP/p97 binds the cytoplasmic deglycosylating enzyme NGly1 (*N*-glycanase 1) that removes *N*-glycans from dislocating substrates to facilitate proteasomal degradation (Kim et al., 2006; G. Li et al., 2006). VCP/p97 also associates with various cytosolic DUBs, such as YOD1 and Ataxin-3, which regulate its activity (Y. Liu and Y. Ye, 2012). YOD1 is able to cleave multiple types of ubiquitin linkages, and dominant negative mutant YOD1 has been shown to prevent the dislocation of substrates through the VCP/p97 pore (Ernst et al., 2009). Ataxin-3, in complex with Derlin-1, VIMP and VCP/p97, promotes proteasomal degradation by deubiquitinating luminal and membrane substrates bound to the ATPase (Q. Wang et al., 2006).

The final step of ERAD is the delivery of the ubiquitinated substrate to the 26S proteasome. The proteasome is composed of a 20S catalytic core and two 19S regulatory caps. The 19S regulatory cap binds the poly-Ub chain via the subunits RNP10 and RNP13 (Deveraux et al., 1994; Husnjak et al., 2008), denatures the protein by ATP hydrolysis and feeds the protein into the proteasome's proteolytic core. The proteolytic core is a barrel-shaped structure composed of two inner  $\beta$  rings and two outer  $\alpha$  rings. The two  $\beta$  rings each contain three proteolytic sites with trypsin-like, caspase-like or chymotrypsin-like activity. Upon proteasomal degradation, peptides of 8–10 aa are released into the cytosol to be recycled.

## 1.5 The Hrd1 complex

The E3 ubiquitin ligases play a central role in ERAD, by providing organisation and functional activity to the ubiquitination machinery (Kostova et al., 2007). The best characterised complex involved in ERAD is the Hrd1 ubiquitin ligase complex (Figure 1.4). Hrd1 and MARCH6 (membrane-associated RING-CH-type finger 6, Doa10 in yeast) are the only two evolutionarily conserved ER-localised E3s present

in yeast, where Hrd1 was originally discovered as the E3 responsible for the degradation of the cholesterol biosynthetic enzyme HMG-CoA reductase (HMGR) (Hampton et al., 1996).



**Figure 1.4 The Hrd1 complex**

Misfolded proteins are recognised by the lectins OS-9 and XTP3-B, which deliver the substrate to Hrd1 via the adaptor protein SEL1L. Retrotranslocation may occur via Hrd1, Derlins or other candidate systems. Hrd1 ubiquitinates the substrate by interacting with the E2 Ube2j1. Less commonly, Hrd1 interacts with the E2 Ube2g2, which is recruited to the complex via AUP1 (ancient ubiquitous protein 1). Herp associates with the complex to facilitate ubiquitination. The substrate is extracted from the membrane via the ATPase VCP/p97 and its cofactors Nlp4 and Ufd1. VCP/p97 is recruited to the membrane by VIMP, UBXD8 and Derlin-1.

Structurally, Hrd1 is a polytopic protein containing six transmembrane domains with a C-terminal RING domain orientated towards the cytosol. It is not glycosylated and is capable of auto-ubiquitination *in vitro* (Nadav et al., 2003). Within the core complex, Hrd1 binds directly to SEL1L, which acts as an adaptor protein to mediate the interaction with the lectins OS-9 and XTP3-B (Bernasconi et al., 2008; Christianson et al., 2008; Hosokawa et al., 2008). This allows delivery of misfolded substrates from the OS-9/XTP3-B recognition machinery to the Hrd1 ubiquitination complex. FAM8A1 (family with sequence similarity 8 member A1) is a recently discovered Hrd1 exclusive accessory factor (Christianson et al., 2012) that may

facilitate substrate degradation by increasing the affinity of Hrd1 for the cofactor Herp (unpublished data).

ERAD substrates have been shown to accumulate in the ER quality control compartment (ERQC) (Leitman et al., 2013a) before delivery to the proteasome. While OS-9 and Herp are constitutively localised in this compartment [Leitman *et al.*, 2014], Hrd1 is recruited to this compartment by Herp only upon accumulation of misfolded proteins (Leitman et al., 2014). In this region, the ERAD components Derlin-1 and VCP/p97 are also concentrated under stress (Kondratyev et al., 2007), suggesting that it may function as a specialised site for retrotranslocation. While Herp interacts with Hrd1 directly (Schulze et al., 2005), Derlin-1 and Derlin-2 associate with the Hrd1 complex indirectly, possibly to facilitate substrate dislocation (Lilley and Ploegh, 2005; Y. Ye et al., 2005). The ATPase VCP/p97 is bound to Hrd1 via Derlin-1, Derlin-2 and VIMP. It has been shown that, during retrotranslocation, the substrate first interacts with Derlin-1 before VCP/p97 and other factors join the complex (Y. Ye et al., 2004). In addition, Hrd1 interacts with the E2 enzyme Ube2g2 through AUP1 to facilitate the degradation of some soluble misfolded proteins (Klemm et al., 2011), whereas interaction with Ube2j1 is required for the degradation of MHC-I heavy chains and a number of other substrates (Burr et al., 2011; Christianson et al., 2012).

SEL1L nucleates a protein complex in which AUP1, UbxD8, Ube2j1 and OS-9 are functionally important to degrade MHC-I heavy chains (Mueller et al., 2008). SEL1L plays a major role in ERAD, both by acting as an adaptor between ER lectins and Hrd1 and by preserving Hrd1 stability and activity (Christianson et al., 2008; Sun et al., 2014). A comprehensive proteomics study on the ERAD network has revealed a consistent degree of heterogeneity with the ERAD complexes required for the degradation of specific substrates (Christianson et al., 2012). It appears that



different substrates rely on a unique set of Hrd1-associated cofactors, where the only common interactors are VCP/p97 and the proteasome.

Hrd1 recognises a broad range of luminal, membrane and cytoplasmic substrates. In cells depleted for  $\beta$ 2-microglobulin ( $\beta$ 2m), MHC-I heavy chains are targeted for degradation via Hrd1 (Burr et al., 2011). Hrd1 also mediates the retrotranslocation and degradation of the catalytic CTA1 subunit of cholera toxin (Bernardi et al., 2010). CD147 (cluster of differentiation 147) and the sonic hedgehog protein are constitutive Hrd1-dependent substrates (Xin Chen et al., 2011; Tyler et al., 2012). Other substrates include  $\alpha$ 1-antitrypsin (A1AT), T-cell receptor  $\alpha$  (TCR- $\alpha$ ) and CD3- $\delta$  (Kikkert et al., 2004; Nadav et al., 2003; H. Wang et al., 2011). Hrd1 is also involved in immunomodulation by regulating the amounts of CD44 and the recently identified cyclin-dependent kinase inhibitor p27Kip1 in T-cells (K. A. Lee et al., 2011; Y. Xu et al., 2016). Secretory IgM heavy chains ( $\mu$ S) have been reported as Hrd1 and SEL1L substrates, although their stabilisation upon Hrd1 depletion was incomplete, suggesting the involvement of other ubiquitin ligases (Cattaneo et al., 2008). A recent study showed the engagement of Hrd1 and SEL1L in the turnover of the UPR sensor IRE1 $\alpha$ , highlighting the important regulation of UPR signalling by ERAD (Sun et al., 2015). In the brain, Hrd1 has been shown to degrade amyloid precursor protein (APP), preventing the formation of amyloid- $\beta$  peptides (A $\beta$ ) that are found in plaque deposits in Alzheimer's disease patients (Kaneko et al., 2010). Hrd1 can also ubiquitinate cytosolic clients. Among these are the serum- and glucocorticoid-induced kinase 1 (Sgk1), a regulatory protein of ion channels and transporters in epithelial cells (Arteaga et al., 2006), and the tumour suppressor p53 (Yamasaki et al., 2007). Another Hrd1 cytosolic client is the transcription factor Nrf1 (nuclear respiratory factor 1). Nrf1 translocates to the nucleus in response to stimuli to maintain cellular homeostasis, but is normally degraded by ERAD under basal conditions (Tsuchiya et al., 2011).

## 1.6 The Hrd1 complex in disease

The broad nature of the Hrd1 substrates indicates a pivotal role of the Hrd1 complex in cellular homeostasis. Hrd1 is ubiquitously expressed but its levels are particularly high in the pancreas (Kaneko et al., 2002), where expression of SEL1L (Biunno et al., 1997) and IRE1 $\alpha$  (Tirasophon et al., 1998) is also elevated. In the initial characterisation of human Hrd1, it was found that IRE1 $\alpha$ -dependent upregulation of Hrd1 exerted a protective role against ER stress-induced apoptosis (Kaneko et al., 2002). However, it must be noted that in this study, ER stress was induced with high doses of pharmacological agents (1  $\mu$ M Thapsigargin and 10  $\mu$ g/ml Tunicamycin) for a long duration (48 hours), a condition far from any physiological situation. In a later study, Hrd1 (renamed synoviolin after this work) was found to be highly expressed in synovial cells from patients with rheumatoid arthritis (RA) (Amano et al., 2003). RA is a common articular disease affecting about 1% of the world's population (Feldmann et al., 1996). Clinically, RA features chronic inflammation of systemic joints associated with an overgrowth of synovial cells, which eventually cause cartilage and bone destruction leading to severe locomotive disability (Aarvak and Natvig, 2001; Schett et al., 2001). In RA, Hrd1 acts as a causative factor by preventing the apoptosis of synovial cells, which leads to hyperplasia and consequent arthritis (Amano et al., 2003). Homozygous deletion of Hrd1 results in mouse embryonic lethality, whereas heterozygous ablation increases the apoptosis of synovial cells and protects from collagen-induced arthritis in mice (Amano et al., 2003; Yagishita et al., 2005). More recently, the pivotal role of Hrd1 in cellular homeostasis has been confirmed by conditional deletion of Hrd1 in dendritic cells (DC). DC-specific Hrd1 knockout mice exhibit splenomegaly with accumulation of B-cells and defects in the autoimmune inflammatory response (Heeyoung Yang et al., 2014). Like Hrd1, homozygous mutation of its essential cofactor SEL1L is also embryonically lethal (Francisco et al., 2010). Acute loss of SEL1L in adult mice

causes striking abnormalities of the ER leading to severe pathologies of secretory tissues and premature lethality, highlighting the importance of SEL1L-dependent degradation, especially in highly secretory organs (Sun et al., 2014). In addition to RA (Amano et al., 2003), Hrd1-dependent degradation has been directly linked to neurodegeneration, autoimmune diseases (Y. Xu et al., 2016), diabetes (Hartley et al., 2010) and cancer (Y.-M. Xu et al., 2015).

Diabetes mellitus arises either from the insufficient production of insulin or from a defective response to insulin, resulting in hyperglycaemia. The hormone insulin is produced by pancreatic  $\beta$ -cells and stimulates the clearance of circulating glucose by muscle, fat and liver cells (O'Rahilly, 2009). Insulin matures across the secretory pathway, and its production is associated to high levels of ER stress in pancreatic  $\beta$ -cells (Volchuk and Ron, 2010). Unresolved ER stress leads to apoptosis, contributing to  $\beta$ -cell death in diabetes type I and II (Butler et al., 2003; Fonseca et al., 2010). Moreover, mutant insulin is found in familial forms of diabetes. Hrd1 is implicated in the degradation of mutant insulin (C96Y), suggesting that the ERAD pathway is involved in the pathogenesis of genetically-linked diabetes (Allen et al., 2004). In agreement, depletion of Hrd1 complex components has been shown to enhance the apoptosis of  $\beta$ -cells in a mouse model with inducible expression of mutant insulin (Hartley et al., 2010).

A number of Hrd1 substrates are associated with neurodegenerative diseases. Among these are the mutant polyQ-Htt protein (Hui Yang et al., 2007), the prion protein (PrP) (Apodaca et al., 2006) and the Parkin-associated endothelin receptor-like receptor (PaelR), which is a pathogenic factor in Parkinson's disease (PD) (Omura et al., 2006). For example, Huntington's disease (HD) is associated with an increased number of polyglutamine (polyQ) repeats in the mutant protein Huntingtin (Htt), which propends to aggregate in the cytosol and nucleus where it causes cytotoxicity and neuronal cell death (Duennwald and Lindquist, 2008). Despite the

cytosolic and nuclear localisation of the polyQ-Htt aggregates, forced expression of the ER-resident ubiquitin ligase Hrd1 has been shown to enhance the ubiquitination and clearance of the pathogenic protein, correlating with reduced neuronal cell death (Hui Yang et al., 2007). This finding expands the role of ERAD from quality control restricted to ER clients to a broader cellular quality control mechanism.

Alzheimer's disease (AD) is characterised by extracellular deposition of A $\beta$  peptides and hyperphosphorylated, cleaved microtubule-associated protein tau in the brain (O'Brien and Wong, 2011). Generation of neurotoxic A $\beta$  derives from inefficient degradation of the ER-localised APP, which is cleaved by  $\beta$ - and  $\gamma$ -secretases to generate A $\beta$  peptides that ultimately deposit in A $\beta$  plaques, typically found in the brain of AD patients (Gandy, 2005). The pivotal role of ERAD in the development of AD is demonstrated by the observation of decreased levels of Hrd1 in the cerebral cortex of AD patients. In this study, forced expression of Hrd1 was able to increase the degradation of APP resulting in decreased A $\beta$  deposition and lower neurotoxicity (Kaneko et al., 2010). In addition, gene polymorphisms in the SEL1L intron have been identified as susceptibility factors in AD (Saltini et al., 2006).

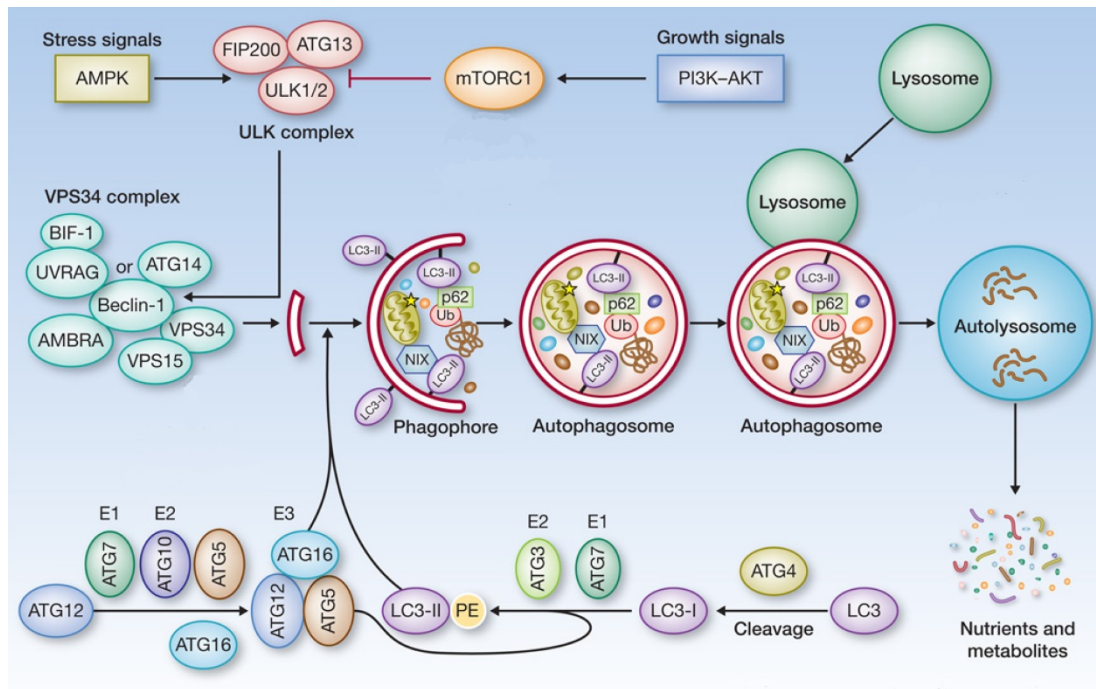
A definitive role for Hrd1 in cancer is not clear. In a recent study, it emerged that Hrd1 was significantly downregulated in breast cancer tissues from 170 patients (Y.-M. Xu et al., 2015). Hrd1 overexpression had an inhibitory effect on the growth, migration and invasion of breast cancer cells *in vitro* and *in vivo*, demonstrating a direct role of Hrd1 in breast cancer progression. On the other hand, evidence that the tumour suppressor p53 is degraded by Hrd1 supports a pro-proliferative role of Hrd1 (Yamasaki et al., 2007), although studies on Hrd1-p53 regulation in cancer are missing. A polymorphism in the SEL1L gene has been involved in the pathogenesis of pancreatic ductal adenocarcinoma (Q. Liu et al., 2012), and aberrant expression

levels of SEL1L have been found in breast and oesophageal cancer, correlating with poor prognosis (Granelli et al., 2004; Orlandi et al., 2002).

Overall, the implication of Hrd1-dependent ERAD in a growing number of pathologies, ranging from loss-of-function to gain-of-function diseases, emphasizes the essential requirement for an intact degradation machinery in physiology.

## **1.7 Protein quality control via autophagy**

Macroautophagy (hereafter called autophagy) is a quality control mechanism that delivers intracellular material to the lysosome for degradation. It constitutes the other facet of cellular catabolism, with a much wider spectrum of substrates than the UPS, being able to degrade soluble proteins, protein complexes, aggregates and even whole cellular organelles (Feng et al., 2014). By exerting this function, autophagy is key to the preservation of cellular homeostasis, allowing the recycling of intracellular material and energy to sustain survival, particularly in conditions of metabolic stress and starvation. During periods of starvation, autophagy is thought to be a non-specific process that engulfs cytoplasmic proteins and organelles in bulk to provide quick energy turnover (Ciechanover, 2005). However, increasing evidence has revealed the existence of selective mechanisms by which intracellular material and organelles are specifically delivered to the lysosome (Klionsky, 2007). Selectivity can be achieved either by Ub signals on the target cargoes recognised by autophagy receptors, or by Ub-independent mechanisms employing protein–protein interactions or sugar- or lipid-based signals (Khaminets et al., 2016). In both cases, the autophagy receptors interact simultaneously with the cargo and LC3/GABARAP-like proteins on autophagosome membranes (Rogov et al., 2014; Stolz et al., 2014). The canonical autophagic pathway is illustrated in Figure 1.5 (Cicchini et al., 2015).



**Figure 1.5 Overview of the autophagic pathway**

Several signals sensing cellular nutrient and energy status lead to activation of the ULK complex. Phosphorylated components of the ULK complex in turn phosphorylate Beclin-1, triggering VPS34 activation and phagophore formation. Phagophore elongation is catalyzed by the ATG proteins, which possess E1-, E2- and E3-like activity. ATG5 and ATG7 catalyse conjugation of LC3-I to phosphatidylethanolamine (PE) to generate PE-LC3-II, which is bound to the phagophore membrane to serve as a docking site for adaptor proteins and cargos. Final maturation of the elongated phagophore results in autophagosome formation. In the final step, lysosomes fuse with autophagosomes, releasing their hydrolases for cargo degradation and the recycling of nutrients and metabolites. Modified from (Cicchini et al., 2015).

Canonical autophagy is initiated by the formation of a double-layered membrane, called the phagophore, which is proposed to originate from various organelles including the ER and the plasma membrane (Mizushima et al., 2011). The phagophore enwraps cytoplasmic portions containing the autophagic substrates to form the autophagosome, whose formation is regulated by a number of autophagy-related genes (ATG). In most cases, the induction of autophagy requires the repression of the mTOR (mechanistic target of rapamycin) kinase, which induces a change in the phosphorylation of the ULK (unc-51-like kinase) complex components, including ULK-1 and Atg13 (Russell et al., 2013). Activated ULK-1 phosphorylates Beclin-1, stimulating the recruitment of VSP34 (vacuolar sorting

protein 34) to form an initiation complex. Additional factors associate to the complex: VPS15, activating molecule in BECN1-regulated autophagy protein 1 (AMBRA1), ATG14 or ultraviolet irradiation resistance-associated gene (UVRAG) and Bax-interacting factor 1 (BIF1) (Funderburk et al., 2010). The activity of the complex is regulated by stimulatory and inhibitory signals. For instance, Bcl-2 (B-cell lymphoma 2) prevents the induction of autophagy by inhibitory binding of Beclin-1 and AMBRA1. Displacement of Bcl-2 by Bcl-2 homology 3 (BH3)-only proteins activates the Beclin-1-VPS34 complex (Pattingre et al., 2005). Two ubiquitin-like conjugation systems participate in vesicle elongation. One system involves the conjugation of Atg12 to Atg5, catalysed by the E1 enzyme Atg7 and the E2 enzyme Atg10. The resulting Atg12–Atg5 conjugate then forms a complex with Atg16. Cytosolic LC3 is cleaved at the C-terminus by the protease Atg4 to generate LC3-I. The second ubiquitin-like system involves the conjugation of phosphatidylethanolamine (PE) to cytosolic LC3-I by the E1-like protein Atg7 and the E2-like enzyme Atg3. Lipid conjugation converts soluble LC3-I to LC3-II, which is inserted in the phagophore membrane where it mediates phagophore expansion and cargo recognition (Nakatogawa, 2013). Several autophagy receptors interact directly with LC3-II on the phagophore to bring the cargo into the vesicle. Once the autophagosome is completed, the Atg proteins that were associated with the growing phagophore are released into the cytoplasm and are reused for the biogenesis of new vesicles, while LC3-II remains attached. For this reason, LC3 is used as a reliable marker for autophagosome formation and degradation (Tanida et al., 2008). Mature autophagosomes finally fuse with lysosomes to create autolysosomes, where cargoes are degraded by lysosomal hydrolases (Füllgrabe et al., 2014).

### 1.7.1 ER-selective autophagy: ER-phagy

Selective autophagy is mediated by autophagy receptors that simultaneously interact with cargo and LC3-II on autophagosomes via LC3-interacting regions (LIR). In Ub-dependent selective autophagy, autophagy receptors recognise Ub chains conjugated to the cargo via Ub-binding domains (UBDs). For instance, ubiquitinated protein aggregates are recognised by p62, NBR1 (next to BRCA1 gene 1 protein) and OPTN (optineurin) (Kirkin et al., 2009b; Korac et al., 2013; Pankiv et al., 2007). Other specific receptors mediate the Ub-dependent autophagy of intracellular organelles, such as mitochondria and peroxisomes [reviewed by (Khaminets et al., 2016)]. In addition to Ub-dependent autophagy, cellular cargo can be delivered to autophagosomes independently of Ub. An example is represented by the recently discovered FAM134 family, which mediates autophagy of the ER, a process known as ER-phagy (Khaminets et al., 2015). In this mechanism, the ER-associated protein FAM134B executes remodelling of the ER membrane and simultaneously binds to LC3 protein on the autophagosome membrane, promoting ER fragmentation and delivery into the autophagic pathway. By controlling the size of the ER, FAM134B is crucial for maintaining cellular homeostasis. *In vivo*, FAM134B knockout mice display ER abnormalities in sensory neurons resulting in neuronal loss and defective nociception (Khaminets et al., 2015). A parallel study identified the yeast orthologue of FAM134B, named Atg40, responsible for the degradation of cytoplasmic and cortical ER in yeast (Mochida et al., 2015).



## **1.8 Autophagy–proteasome crosstalk**

The degradation of soluble misfolded proteins represents a functional overlap between the proteasomal and autophagic pathways (Ding and Yin, 2008). It has been observed that impairment of the UPS leads to increased autophagic flux as a compensatory mechanism (Ding et al., 2007; Iwata et al., 2005). Indeed, proteasome inhibition causes the accumulation of ubiquitinated proteins and aggregates that are cleared by autophagy (Kirkin et al., 2009a) to alleviate the burden of misfolded substrates. In addition, experimental induction of autophagy by rapamycin has been shown to protect against cell death caused by proteasome inhibition (Pan et al., 2008). Despite the general consensus on the compensatory role of autophagy following proteasome inhibition, little is known about the exact mechanism. Autophagy activation in response to the UPR (Høyer-Hansen and Jäättelä, 2007) has been linked to ATF4 stabilisation (Milani et al., 2009; Rzymiski et al., 2009), but with controversial results, and to the downstream effector of IRE1, JNK-1, which may induce autophagy by phosphorylating Bcl-2 (Wei et al., 2008). Compensatory regulation in proteasome-inhibited cells has also been suggested to depend on histone deacetylase 6 (HDAC6), which mediates the delivery of inclusion bodies containing misfolded proteins (called aggresomes) into autophagosomes (Iwata et al., 2005). Autophagy also contributes to the clearance of toxic polymers of neuroserpin and A1AT when ERAD is inhibited (Kroeger et al., 2009). In the case of muscular dystrophy, native dysferlin is degraded by ERAD while the mutant form aggregates in the ER from where it is targeted to autophagy (Fujita et al., 2007). In addition, concerted activation of the ERAD and autophagic pathways allows the elimination of misfolded procollagen (Ishida and Nagata, 2009) and mutant fibrinogen (Kruse et al., 2006), highlighting the importance of the coordination between these two catabolic processes for the maintenance of protein homeostasis.

## **1.9 Proteostatic failure in aging and disease**

Proteostasis surveillance is ensured by the coordinated action of chaperones and proteolytic systems. Cells have developed the ability to respond to cellular and environmental challenges, such as hypoxia or starvation, by mounting adaptive signalling pathways that increase cellular fitness and support viability in new conditions. However, if the stressors become chronic, homeostasis can be compromised and proteotoxicity develops [reviewd by (Morimoto and Cuervo, 2014)]. Loss of proteostasis is a feature of many pathological conditions, including neurodegenerative diseases like AD and PD (Morimoto and Cuervo, 2014), metabolic diseases such as diabetes mellitus type 2 (U. Ozcan et al., 2004), atherosclerosis (Tabas, 2010) and cancer (Moenner et al., 2007). It is not a coincidence that most of these diseases are age-related; indeed, increasing evidence demonstrates the physiological deterioration of the proteostasis machinery with age (Labbadia and Morimoto, 2015; Vilchez et al., 2014). On one side, poor cellular energetics associated with aging result in reduced levels of available ATP, which can change the chaperones landscape and reduce their function (Brehme et al., 2014; Yina Ma and J. Li, 2015). On the other side, both autophagy and proteasome activity decrease with aging (Rubinsztein et al., 2011). Another important feature associated with aging is the increase in protein abundance, leading to proteome imbalance, perturbed stoichiometry and aggregation (Walther et al., 2015). In addition, despite the ability of the autophagic pathway to compensate for impaired proteasomal activity (Korolchuk et al., 2010), this crosstalk is lost in aged organisms (Gavilán et al., 2015; Schneider et al., 2015). In general, disturbance of proteostasis can lead to protein aggregation, which in turn triggers a cascade of pathological processes that could mark the progression of chronic degenerative diseases (J.-H. Lee et al., 2014). To date, more than 60 pathologies have been associated with failures of ERAD (Guerriero and Brodsky, 2012), many

of which are associated with aging, particularly those affecting the nervous system. Indeed, neuronal cells have a relatively low regenerative capacity and an age-dependent decay in their ability to sustain homeostasis, rendering them particularly sensitive to toxic aggregates that result in apoptosis (Bernhardi et al., 2010).

In line with a loss of proteostasis, activation of the UPR is found in a number of neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), PD, HD and AD, as well as in demyelinating neurodegenerative autoimmune diseases such as multiple sclerosis (Doyle et al., 2011; Matus et al., 2011). Interestingly, in neurodegeneration, the pathogenic contribution of the UPR appears to be disease specific. For instance, the deletion of XBP1 delayed ALS disease onset and increased survival rate following autophagy induction (Hetz et al., 2009), whereas it did not affect neuronal loss or survival in a prion-related disease model (Hetz and Soto, 2006).

One example of a disease associated with toxic aggregate formation is AD, where the A $\beta$  peptide and tau protein undergo conformational changes and form toxic amyloid fibrils. These, in turn, trigger ER stress, which elicits both the IRE1 and PERK pathways, culminating in the activation of CHOP and consequent neuronal death (L. Ozcan and Tabas, 2012). In familial ALS, several mutated genes lead to the formation of aberrantly folded proteins, which aggregate and form inclusion bodies, actively impairing proteostasis (Soo et al., 2015). Additionally, dominant mutations affecting components of the protein quality control machinery, including protein trafficking, autophagy and the UPS, directly perturb components of the proteolytic systems: ubiquilin-2, a protein that recruits proteasome complexes to ubiquitinated proteins; sequestosome-1 (p62), a ubiquitin-binding protein required for autophagy of aggregates; and VCP/p97, the ATPase that functions in ERAD (Robberecht and Philips, 2013).

Proteasomal degradation has been involved in the progression of HD, an autosomal dominant disease that affects muscle coordination and leads to progressive cognitive decline. HD results from abnormally high numbers of polyQ repeats in the Htt protein, which ultimately promote the formation of Htt aggregates. It has emerged that the generation of Htt polyQ aggregates decreases UPS activity, leading to further accumulation of unprocessed UPS substrates and amplification of aggregate toxicity (Bence et al., 2001). In addition to aggregate formation, mutant Htt can also oligomerise into toxic oligomers that sequester components of the ERAD machinery, such as VCP/p97, therefore generating ER stress and ultimately leading to apoptosis (Leitman et al., 2013b).

PD results from the loss of dopaminergic neurons. The endothelin receptor-like receptor PaelR (Parkin-associated endothelin receptor-like receptor) is a putative G protein-coupled receptor that is highly expressed in the central nervous system (CNS) (Leng et al., 1999). The connection between PaelR and PD is supported by the observation that PaelR accumulates in Lewy bodies, electrondense inclusions that serve as a histological indicator of PD. Both the cytosolic ubiquitin ligase Parkin and Hrd1 contribute to PaelR degradation (Imai et al., 2001; Omura et al., 2006), conferring protection against PaelR-induced cell death. Additionally, loss-of-function mutations in the ubiquitin ligase PARKIN and the PARKIN-related kinase PINK1 lead to early-onset PD (Kitada et al., 1998; Leroy et al., 1998). Since PARKIN cooperates with PINK1 in the selective autophagy of mitochondria (mitophagy), deficiencies in this pathway result in the accumulation of damaged mitochondria, aberrant calcium release and oxidative stress [reviewed in (Winklhofer, 2014)].

In solid cancers, the hypoxic microenvironment is responsible for chronic activation of the UPR (Rouschop et al., 2010), which promotes tumour progression and survival. Both PERK and IRE1 pathways are implicated in pro-survival mechanisms,

by protecting from hypoxia and sustaining angiogenesis (Ghosh et al., 2010). Selective inhibitors of IRE1's RNase activity (Mimura et al., 2012; Papandreou et al., 2011), as well as proteasome inhibitors (Dimopoulos et al., 2011), have been shown to strongly suppress multiple myeloma cell growth, supporting the idea that targeting of proteostasis may be therapeutic in a number of cancers associated with excessive expression of secretory proteins. However, in some cases, activation of autophagy as a consequence of sustained ER stress has been reported to confer tumour resistance to therapy (Luo and A. S. Lee, 2013). One example of proliferative disease in which the UPS plays an important role is multiple myeloma. Current treatment includes the use of immunomodulatory agents as well as proteasome inhibitors (Moreau et al., 2012) that target the degradative capacity of these highly secretory cells. However, proteasome inhibitor treatment is associated with a number of primary and secondary resistance mechanisms (Orlowski, 2013). For example, lower levels of ER stress, decreased plasma cell markers, lower Ig production and consequent lower proteasome load correlate with resistance to treatment (Leung-Hagesteijn et al., 2013). In another study, malignant plasma cells featuring decreased proteasome expression or workload negatively correlated with response to therapy (Bianchi et al., 2009), highlighting the need for the degradative capacity to be targeted in alternative ways.

# Chapter II:

## Materials and Methods

## **2.1 Cell biology techniques**

### **Mammalian cell culture**

HeLa cells were cultured in Dulbecco's modified eagle medium (DMEM, Lonza) supplemented with 5% foetal bovine serum (FBS) (Sigma Aldrich) and 2 mM L-Glutamine (Gibco® by Life Technologies) and maintained into a ventilated incubator at 37°C, 5% v/v CO<sub>2</sub>. FlpIn™ T-Rex™ HEK293 cells were cultured in the same medium but supplemented with 10% FBS. Cells were regularly subcultured every 3 days (on average) by washing with sterile phosphate-buffered saline (PBS) followed by incubation with trypsin-EDTA (ethylenediaminetetraacetic acid) (Gibco® by Life Technologies) until detached. Cells were resuspended in fresh medium, counted and seeded at the desired density. For long-term storage, cell pellets were resuspended in FBS containing 10% dimethyl sulfoxide (DMSO) and kept at -80°C for 1–2 days before being transferred to liquid nitrogen for long-term maintenance. For cell revival, the cryovials were thawed at 37°C, cells were resuspended in medium and spun down at 1,200 rpm for 3 min to wash off the DMSO. The supernatant was discarded and cells were seeded in fresh medium.

### **Mammalian cell transfection**

Cells were transiently transfected with plasmid DNA using Lipofectamine® 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions:  $1 \times 10^6$  cells were seeded into a 6 cm dish the day prior to transfection. Confluency was about 80% on the day of transfection. 4 µg of DNA was diluted in 250 µl of OptiMEM (Gibco® by Life Technologies) and mixed with 250 µl OptiMEM containing 10 µl of Lipofectamine® 2000. The mix was incubated for 5 min at room temperature (RT) and then added to the cells for a final volume of 5 ml. Cells were incubated with the transfection mix for 24 hours before subculture or expansion.

## **Flow cytometry**

Cell supernatant was collected in FACS (fluorescence-activated cell sorting) tubes (BD Falcon) to retain floating dead cells. Adherent cells were detached with 10 mM EDTA in PBS by scraping and pooled in the corresponding FACS tubes. Cells were centrifuged at 1,500 rpm at 4°C for 3 min and the supernatant was discarded. Cell pellets were resuspended by vortexing in 300 µl of PBS/EDTA. 3 µl of Propidium Iodide (PI) (Sigma-Aldrich) solution was added to the cell suspension, vortexed and measured by FACS (BD FACSCanto™). 10,000 cells per sample were recorded and the percentage of PI-positive cells was calculated using FlowJo software.

## **siRNA transfection**

150,000 cells were seeded into a 12-well plate the day prior to transfection. Transfection was performed according to the manufacturer's instructions: 4 µl of Dharmafect1 was diluted in 96 µl of HBSS (Hanks' balanced salt solution, Gibco® by Life Technologies) and incubated at RT for 10 min. 10 µl of 10 µM siRNA (small interfering RNA) was diluted in 90 µl of HBSS. The transfection mix was added to the siRNA mix and incubated at RT for 20 min. 200 µl of transfection mixture were added to 800 µl of freshly changed medium on the cells. 24 hours after transfection, cells were expanded according to experimental conditions. Knockdown efficiency was analysed 48–72 hours after transfection.

## **Cell treatments**

Table 1 summarises the drugs and doses (when a range is indicated, it depends on the experimental setting) that were used. Drug dilutions were prepared in a master mix using fresh medium and the master mix was applied to the cells to allow homogenous treatment.



| Drug               | Abbreviation       | Dose            | Source             |
|--------------------|--------------------|-----------------|--------------------|
| Cycloheximide      | CHX                | 100 µg/ml       | Abcam              |
| Doxycycline        | DOX                | 1 ng/ml-1 µg/ml | Sigma-Aldrich      |
| Mifepristone       | Mif                | 0.5 nM          | Invitrogen         |
| Kifunensine        | Kif                | 20 µM           | Sigma-Aldrich      |
| MG132              | -                  | 1–10 µg/ml      | Merck Millipore    |
| Dithiothreitol     | DTT                | 5 mM            | Sigma-Aldrich      |
| Tunicamycin        | TM                 | 100–500 ng/ml   | Sigma-Aldrich      |
| Dimethyl sulfoxide | DMSO               | <0.1%           | Sigma-Aldrich      |
| SubtilaseAB5       | SubAB <sub>5</sub> | 10 ng/ml        | J. Paton           |
| Bortezomib         | BTZ                | 50 nM           | Cell Signalling    |
| Torin1             | -                  | 1 µM            | Tocris Bioscience  |
| NMS-873            | -                  | 5 µM            | Sigma-Adrich       |
| CB-5083            | -                  | 5 µM            | Cleave Biosciences |

**Table 1 List of drugs and doses used for cell treatments**

### **Clonogenic assay and crystal violet staining**

HeLa cells were seeded in a 24-well plate at the following dilutions: 5,000, 1,000, 200 or 40 cells in 500 µl of medium. Cells were allowed to adhere for at least 6 hours before medium was replaced with drug-free or drug-containing medium. Medium was changed after 3 days, and cells were incubated for a further 4 days (7 days in total) prior to fixation and staining. For crystal violet staining, cells were fixed with methanol:acetone (1:1) for 10 min and then stained with crystal violet solution (0.1% crystal violet in 10% ethanol) for 20 min at RT. Cells were carefully washed with ultrapure water and allowed to dry. Images were acquired using a Typhoon™ FLA 9000 laser scanner (GE Healthcare Life Sciences) using 647 nm wavelength excitation and 50 µm resolution. Images were processed with ImageJ software.

### **Colony counting**

Colonies from the third dilution of the clonogenic assay (from approximately 200 cells) were quantified using an ImageJ macro to allow automatic analysis. In summary, the colonies-containing well was cropped from the original image and the plate background was subtracted. Automated thresholding was used to identify colonies. The acquired measurements were: number of colonies, area, perimeter, solidity, roundness, integrated density and raw integrated density (the sum of the values of the pixels in the image or selection). Colours were defined to distinguish

colonies from the background and an overlay image was produced to confirm that the identified colonies correlated with actual colonies.

## Immunofluorescence

Cells were grown on coverslips during exposure to treatment for the time required and then fixed with 4% paraformaldehyde (PFA) in PBS for 20 min at RT. Cells were then permeabilised in 0.2% TritonX-100 in PBS for 5 min, followed by blocking in 0.2% fish skin gelatin (Sigma-Aldrich) in PBS for 30 min. Primary and secondary antibodies were diluted in blocking solution and incubated on coverslips for 1 hour. DAPI staining was performed for 10 min and coverslips were mounted using Fluoromount (Sigma) or ProLong® Gold Antifade reagent (Thermo Fisher Scientific). Coverslips were analysed via confocal microscopy using a LSM 710 Zeiss confocal microscope. Table 2 summarises the antibodies and dilutions used in immunofluorescence.

| Antibodies for Immunofluorescence      | Host   | Source        | Dilution |
|----------------------------------------|--------|---------------|----------|
| Primary                                |        |               |          |
| Alexa Fluor® 546 anti-mouse IgM HC     | Goat   | Thermo Fisher | 1:200    |
| Calnexin                               | Rabbit | EnzoLifeTech  | 1:200    |
| LC3B                                   | Rabbit | Abcam         | 1:500    |
| PDI                                    | Rabbit | Abcam         | 1:200    |
| KDEL                                   | Rabbit | Abcam         | 1:200    |
| Secondary                              |        |               |          |
| Alexa Fluor® 488 anti-rabbit IgG (H+L) | Goat   | Thermo Fisher | 1:400    |
| Alexa Fluor® 647 anti-mouse IgG (H+L)  | Goat   | Thermo Fisher | 1:400    |

**Table 2 Antibodies used for immunofluorescence**

## Generation of FlpIn T-REx HEK293\_FLAG-HA-E3 cell lines

Flp-In™T-REx™ HEK293 cells (Thermo Fisher Scientific) were transfected via the Calcium Phosphate method (Sambrook and Russell, 2006) or using Lipofectamine® 2000 transfection reagent (Thermo Fisher Scientific). 48 hours after transfection, the positive cells were selected with Hygromycin B (100 µg/ml) and maintained in antibiotic-containing medium. Expression of FLAG-HA-tagged E3s was tested upon treatment with Doxycycline (DOX) at increasing concentrations (0, 1, 10, 100 and 1,000 ng/ml) for 18 hours. MG132 (10 µg/ml) was added 4 hours prior to harvesting,

in combination with 1  $\mu\text{g/ml}$  of DOX to rescue the E3s potentially targeted to the proteasome upon overexpression.

### **Generation of HeLa\_ $\mu\text{S}$ cell lines**

HeLa\_ $\mu\text{S}$  cell lines were previously generated in Dr van Anken Lab (San Raffaele Scientific Institute, Milan) by lentiviral infection. Briefly, wild-type HeLa cells were first made TetON by insertion of a rTTA element under a PGK (phosphoglycerate kinase) promoter, and a single insertion was verified by TaqMan PCR (polymerase chain reaction). Cells were subsequently made MifON by infection with pSwitch (GeneSwitch™ System, Invitrogen) lentiviral vector (Sirin and Park, 2003). Finally, the murine IgM heavy chain ( $\mu\text{S}$ ) coding sequence was cloned into the pGene3 viral vector and integrated into the genome of HeLa\_TetON\_MifON under the Mif promoter using the same method. Single cell clones were selected and tested for  $\mu\text{S}$  expression.

### **Generation of Hrd1-reconstituted HeLa\_ $\mu\text{S}$ \_Hrd1 KO cell lines**

Using the protocol described above, the HeLa\_ $\mu\text{S}$ \_Hrd1 knockout (KO) cell line was reconstituted with either wild-type HRD1-S or the mutant C294A HRD1-S, under the TetON promoter. These cell lines also express  $\mu\text{S}$  under the Mif promoter. The reconstituted cell lines were generated by our collaborator Dr. Eelco van Anken.

### **Generation of CRISPR-Cas9 stable cell lines**

HeLa cells were transfected in 6 cm dishes with 3.5  $\mu\text{g}$  of clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 constructs and incubated with the transfection mixture for 24 hours. Cells were expanded to 10 cm dishes with fresh medium and incubated for an additional 24 hours. Puromycin (2  $\mu\text{g/ml}$ ) selection was applied for 3 days. Significant cell death was observed upon selection. Cells were allowed to recover in drug-free medium to confluency. The selected population was tested for expression of the target protein and either used as a pool or single clones were isolated to generate a clonal population. In the case

where a pool population was used, cells were assayed at early passage (up to 5) to limit propagation of negative clones. When clonal selection was performed, cells were seeded in a 96-well plate at a concentration of 0.5 cells per well and allowed to grow until colonies became visible and ready to expand.

### **Validation of Hrd1 CRISPR-Cas9 knockout cell lines**

An average of 30 clones was screened in immunoblots for the expression of the target protein, and at least 3 clones were tested in phenotypic assays to exclude off-target effects.

## 2.2 Molecular biology techniques

### Design and generation of CRISPR-Cas9 constructs

Single guide RNA (sgRNA) target sequences for Hrd1, FAM8A1 and HERP (homocysteine-induced ER protein) were designed and cloned into the PX459 vector (Addgene) by Dr Andrew Bassett (Dunn School of Pathology, Oxford). PX459 contained the Cas9 cassette and the puromycin resistance gene to allow selection of the positive cells. For deletion of the other genes, CRISPR-Cas9 constructs were generated in this study and all target sequences are summarised in Table 3.

| Gene     | sgRNA ID | sgRNA Target Sequence    | Source                              |
|----------|----------|--------------------------|-------------------------------------|
| Derlin-1 | #1       | TCCCGGCGATCACGCGCTAT     | van den Boomen <i>et al.</i> , 2014 |
|          | #2       | GGTCTGAAGATGTCGGACAT     |                                     |
| Derlin-2 | #1       | GGCTGACCGGTGGGATCTGC     | Ma <i>et al.</i> , 2015             |
|          | #2       | GAGGACGCAGGCAGTGGTGT     |                                     |
| Hrd1     | #407     | GGCCAGGGCAATGTTCCGCACGG  | Christianson Lab                    |
|          | #408     | CTTGGTCTAGGTACACCACAGTGG |                                     |
| FAM8A1   | #396     | TTCGGCCTGGGGGTCTGTCGCGGG | Christianson Lab                    |
|          | #397     | CACTTGCCGGGAGTACTCGCGGG  |                                     |
| HERP     | #398     | CCGCTCTACCCGACGCTCGGGG   | Christianson Lab                    |
|          | #399     | AAGTCGCGGTGGCGCTGGTTGGG  |                                     |
| OS-9     | #2       | TTACCCGCAAGTCTGACCGG     | Ron Kopito                          |
|          | #3       | CGGTCAGACTTGCGGGTAAC     |                                     |
| Ube2j1   | #1       | GGTTTCATGGGATACTCTGG     | Ma <i>et al.</i> , 2015             |
|          | #2       | CACCATGGAGGAGTTTATCACGGG |                                     |
| XTP3-B   | #1       | CCGCAGAGGACCAGTAACAC     | Ron Kopito                          |
|          | #2       | CCCGGGCGGGCCGGTGTTAC     |                                     |

**Table 3 sgRNA sequences used for CRISPR-Cas9 cloning**

Cloning of sgRNAs into the PX459 vector was executed according to the protocol described in Ran *et al.*, 2013. Final CRISPR plasmids were sequenced using the U6-Fwd primer to validate the correct insertion of the sgRNA.

### Protein extraction and quantification

Adherent cells were detached in 10 mM EDTA/PBS using a cell scraper and then centrifuged at 6,000 rpm at 4°C for 1 min. For Western blot (WB) applications on whole cell lysate (WCL), cells were extracted in RIPA (radioimmunoprecipitation assay) buffer [50 mM Tris-HCl pH 7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS (sodium dodecyl sulfate), 150 mM NaCl and 2 mM EDTA] supplemented with

protease inhibitors (cOmplete tablets, Roche), vortexed and incubated on ice for 30 min. For immunoprecipitation (IP) applications, cells were lysed in IP buffer (50 mM Tris pH 7.4, 150 mM NaCl and 5 mM EDTA) typically containing 1% lauryl maltose neopentyl glycol (LMNG, Anatrace) detergent and protease inhibitors and incubated on ice for 1 hour. Cell lysates were centrifuged at 14,000 rpm for 30 min and the supernatant was collected in a new tube for protein quantification. Cell lysates were stored at -80°C. Protein amounts from WCLs were determined with Bradford reagent (Biorad) according to the manufacturer's instructions in a 96-well plate. A standard curve was generated for every experiment using known dilutions of bovine serum albumin (BSA) (Thermo Fisher Scientific). The plate was read in a spectrophotometer (Thermo Fisher Scientific) at 595 nM and protein concentration was extrapolated from the standard curve. The appropriate amount of lysate was prepared by addition of Laemmli buffer (1 M Tris HCl pH 6.8, 2% SDS, 10% glycerol and 0.004% bromophenol blue) containing 100 mM, heated at 56°C for 10 min and stored at -80°C.

### **Immunoprecipitation**

WCL was pre-cleared using Sepharose<sup>®</sup> CL-4B beads (Pharmacia) on rotation at 4°C for 1 hour. 400 µg of total proteins were used to immunoprecipitate Hrd1 (mouse hybridoma raised against the peptide PAQSPPPPEP) and SEL1L (mouse hybridoma raised against the peptide EGPPEQQPPQ) from HeLa cells, 1 mg of total proteins were used to pull-down Hrd1 from FlpIn HEK293 cells. The appropriate amount of pre-cleared lysate was adjusted to 500 µl and incubated with 1 µl of antibody on rotation at 4°C for 1 hour, followed by incubation with protein G beads (Roche) for 1 additional hour. Beads were washed three times with lysis buffer without detergent, resuspended in 2× Laemmli buffer containing DTT (dithiothreitol) and heated at 56°C for 10 min prior to freezing at -80°C.

## **SDS-PAGE**

Protein samples heated in Laemmli buffer were separated by SDS-PAGE (SDS-polyacrylamide gel electrophoresis). Homemade gels (acrylamide:bis-acrylamide 29:1) consisted of 4% stacking and 7–15% resolving. Gels were run in 1× running buffer (National Diagnostics) in a BioRad gel apparatus at 100 V in the stacking phase and 130 V in the resolving phase. Alternatively, 4–12% NuPAGE® Novex® Bis-Tris Midi precast gels (Thermo Fisher Scientific) were used with NuPAGE® MOPS [3-(*N*-morpholino)propanesulfonic acid] or MES [2-(*N*-morpholino)ethanesulfonic acid] buffer (Thermo Fisher Scientific) and run in an Invitrogen apparatus at 160 V. All blue pre-stained protein ladder (BioRad) was used as molecular weight standard.

## **Silver staining**

SDS-PAGE gels were stained with Pierce™ Silver Stain for Mass Spectrometry (Thermo Fisher Scientific) according to the manufacturer's instructions.

## **Western blotting**

Gels were transferred onto a Hybond P PVDF (polyvinylidene fluoride) membrane (GE Healthcare Life Sciences) previously activated in methanol for 2 min, and then assembled in a BioRad blotting apparatus with Tris-glycine transfer buffer. Transfer was carried out at 100 V for 90 min at 4°C. Membranes were blocked in 5% milk PBS-0.1%Tween (PBST) at RT for 1 hour. Primary antibodies were diluted in either 5% milk or 5% BSA in PBST and incubated for 1 hour at RT or overnight at 4°C. Membranes were washed in PBST for 30 min. Secondary antibodies were diluted in 5% milk PBST, incubated with the membranes for 1 hour at RT, and finally washed off with PBST for 40 min. Protein signal from horseradish peroxidase (HRP)-conjugated secondary antibodies was revealed using Clarity™ ECL (enhanced chemiluminescence) substrate (BioRad) and detected either on X-ray films (Fujifilm) or using the ChemiDoc™ MP imaging system (BioRad). Protein signal from Alexa

Fluor<sup>®</sup>-conjugated secondary antibodies was imaged directly in the ChemiDoc<sup>™</sup> system using the appropriate excitation wavelength. Antibodies used for WB are listed in Table 4.

| Antibodies for WB                                  | Host             | Source                          | Dilution |
|----------------------------------------------------|------------------|---------------------------------|----------|
| Primary (antigen)                                  |                  |                                 |          |
| $\alpha$ -tubulin                                  | Mouse mAb        | Sigma-Aldrich                   | 1:10,000 |
| Alexa Fluor <sup>®</sup> 546 anti-mouse IgM HC     | Goat pAb         | Thermo Fisher Scientific        | 1:1,000  |
| ATF4                                               | Rabbit pAb       | Santa Cruz Biotech              | 1:1,000  |
| Bcl-2 HRP-conjugated                               |                  | Santa Cruz Biotech              | 1:1,000  |
| CHOP                                               | Mouse mAb        | Cell Signaling                  | 1:1,000  |
| Derlin-1                                           | Rabbit pAb       | Gift from Dr. Ye (NIH Bethesda) | 1:5,000  |
| Derlin-2                                           | Rabbit pAb       | Gift from Dr. Ye (NIH Bethesda) | 1:1,000  |
| Derlin-3                                           | Rabbit pAb       | GeneTex                         | 1:1,000  |
| FAM8A1                                             | Rabbit pAb       | Purified serum                  | 1:10,000 |
| FLAG                                               | Mouse mAb, M2    | Sigma-Aldrich                   | 1:4,000  |
| HA                                                 | Rabbit pAb, HA-7 | Sigma-Aldrich                   | 1:2,000  |
| HERP                                               | Rabbit pAb       | Abcam                           | 1:10,000 |
| Hrd1                                               | Rabbit pAb       | Bethyl Laboratories             | 1:2,000  |
| KDEL                                               | Rabbit pAb       | Enzo Life Sciences              | 1:1,000  |
| LC3B                                               | Rabbit pAb       | Abcam                           | 1:1,000  |
| Mcl-1                                              | Rabbit pAb       | Santa Cruz Biotech              | 1:1,000  |
| Mouse IgM HC                                       | Rabbit pAb       | Thermo Fisher Scientific        | 1:1,000  |
| OS-9                                               | Rabbit pAb       | Purified serum                  | 1:3,000  |
| PERK                                               | Rabbit pAb       | Cell Signaling                  | 1:1,000  |
| Ube2j1                                             | Rabbit pAb       | Abcam                           | 1:5,000  |
| Ubiquitin                                          | Mouse mAb        | Cell Signaling                  | 1:1,000  |
| S-tag                                              | Mouse mAb        | Thermo Fisher Scientific        | 1:10,000 |
| SEL1L                                              | Goat pAb         | Santa Cruz Biotech              | 1:2,000  |
| XTP3-B                                             | Rabbit pAb       | Purified serum                  | 1:1,000  |
| Secondary                                          |                  |                                 |          |
| Alexa Fluor <sup>®</sup> 488 anti-rabbit IgG (H+L) | Goat             | Thermo Fisher Scientific        | 1:1,000  |
| Alexa Fluor <sup>®</sup> 647 anti-mouse IgG (H+L)  | Goat             | Thermo Fisher Scientific        | 1:1,000  |
| Alexa Fluor <sup>®</sup> 488 anti-mouse IgG (H+L)  | Goat             | Thermo Fisher Scientific        | 1:1,000  |
| Anti-rabbit IgG HRP                                | Goat             | BioRad                          | 1:10,000 |
| Anti-mouse IgG HRP                                 | Goat             | Santa Cruz Biotech              | 1:10,000 |
| Anti-goat IgG HRP                                  | Donkey           | Santa Cruz Biotech              | 1:10,000 |

**Table 4 List of antibodies used in western blot (WB) analysis**

### Membrane stripping

Membranes were incubated in stripping buffer (Thermo Fisher Scientific) for 5 min at RT and then washed with PBST for 10 min. Membranes were blocked for 30 min before incubation with primary antibody.



## Purification of ubiquitinated proteins

Cells from one 10 cm dish were lysed in 600 µl of TUBE (Tandem ubiquitin binding entity) buffer (20 mM sodium phosphate pH 7.4, 1% NP-40 and 2 mM EDTA) supplemented with 1 mM DTT, 20 mM NEM (*N*-ethylmaleimide, Fluka Analytical) and protease inhibitors. Lysates were incubated at 4°C on rotation for 1 hour, centrifuged at 16,000× g for 20 min and transferred into a new tube. 5% of lysate was collected to run as WCL. 40 µg of (glutathione S transferase) GST-1× UBA-His6 (with the UBA being derived from the human protein Ubiquilin-1, isoform 3) were used for IP at 4°C on rotation overnight. The resin was then washed three times with TUBE buffer and each sample was split into two tubes for deubiquitination treatment. Laemmli buffer with DTT was added to the resin, samples were heated at 56°C for 10 min and stored at -80°C.

## Deubiquitination assay

Immunoprecipitates from the ubiquitin pull-down were resuspended in 20 µl of DUB buffer (50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.5, 100 mM NaCl, 2 mM DTT, 1 mM MnCl<sub>2</sub>, and 0.01% (w/v) Brij-35) and incubated with 0.5 µM of Usp21-GST (Ubiquigent) at 30°C for 1 hour. The reaction was inhibited by addition of Laemmli buffer with DTT and heating at 56°C.

## Deglycosylation assay

Protein samples in 1× Laemmli buffer were treated with EndoH (NEB) according to the manufacturer's instructions for 1 hour at 37°C. Samples were subsequently heated at 56°C for 10 min and analysed via SDS-PAGE.

## Sucrose gradient sedimentation

Cells were lysed in 1% LMNG in IP lysis buffer as previously described. Sucrose solutions were prepared as follows:

| Stock | 5M NaCl | 1M Tris pH 7.4 | 0.5M EDTA | Sucrose | ddH <sub>2</sub> O |
|-------|---------|----------------|-----------|---------|--------------------|
| 10%   | 1.5 ml  | 2.5 ml         | 0.5 ml    | 5 g     | 35 ml              |
| 40%   | 1.5 ml  | 2.5 ml         | 0.5 ml    | 20 g    | 5 ml               |

Solutions were dissolved by rotation at 4°C and adjusted to 50 ml with ddH<sub>2</sub>O. 5 ml of 10% LMNG detergent and PMSF (phenylmethyl sulfonyl fluoride) protease inhibitor (Boehringer Mannheim) were added to 50 ml of solution. 10–40% gradients were obtained using Gradient Master (Biocomp) following the manufacturer's instructions. 800 µg of total proteins in 300 µl were loaded onto the gradient, while 10% of WCL was prepared in Laemmli buffer to run as input. Gradients were positioned in a SW.41 swing bucket rotor (Beckman) and centrifuged at 39,000 rpm at 4°C for 16 hours. Thirteen fractions of 940 µl each were collected in separate tubes from the top of the gradient. Proteins were precipitated with 190 µl of 50% TCA (trichloroacetic acid) and incubated on ice for 1 hour. Samples were centrifuged at 14,000× g at 4°C for 5 min. The supernatant was discarded and the protein pellet was washed twice with ice-cold acetone and allowed to dry. Pellets were resuspended in 100 µl of 2× Laemmli buffer with DTT and the pH was neutralized with Tris pH 9 if necessary. Samples were vortexed briefly, heated at 56°C for 10 min and then frozen at -80°C. 20 µl from each fraction were run in SDS-PAGE along with 25 µg of proteins from WCL as input.

### **RNA extraction and reverse transcription**

RNA was extracted using the QIAgen RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. 500 ng of RNA was used in most applications for transcription into cDNA (complementary DNA) using the QuantiTect<sup>®</sup> Reverse transcription kit (QIAGEN) according to the manufacturer's instructions.

### **Nanostring assay**

RNA was extracted using a QIAgen RNeasy mini kit according to the manufacturer's instructions, including digestion of genomic DNA with DNase I (Qiagen). The Nanostring system is based on a hybridisation reaction between the input mRNA and colour-coded probes bound to the surface of a chip, followed by imaging for

quantitation of the hybridized transcripts. The hybridization reaction was composed of: 10 µl Reporter CodeSet, 10 µl hybridisation buffer, 5 µl of sample RNA (150 ng) and 5 µl Capture ProbeSet. The Reporter Probe carried the colour-coded barcode, while the Capture Probe allowed the complex to be immobilised for data collection. The reaction was incubated in a thermocycler at 65°C for 18 hours, after which samples were loaded in the Prep Station following the manufacturer's instructions (Nanostring Technologies). Here, the excess probes were removed and the probe/target complexes aligned and immobilised in the nCounter Cartridge. Sample Cartridges were finally imaged in the Digital Analyser for data collection. Colour codes on the surface of the cartridge were counted and tabulated for each target molecule. Data were analysed following the user guide (nCounter<sup>®</sup> Nanostring Technology). Target transcripts chosen for the analysis are listed in Table 5.

| Gene Name | Target ID      | Gene Name   | Target ID      |
|-----------|----------------|-------------|----------------|
| AMFR      | NM_001144.4    | SEL1L       | NM_005065.5    |
| MARCH6    | NM_005885.2    | FAM8A1      | NM_016255.2    |
| RNF103    | NM_005667.3    | HERP        | NM_014685.3    |
| RNF139    | NM_007218.3    | ERdj5       | NM_018981.2    |
| RNF170    | NM_001160223.1 | GRP94       | NM_003299.2    |
| RNF185    | NM_152267.3    | AUP1        | NM_181575.3    |
| RNF5      | NM_006913.3    | VIMP        | NM_203472.1    |
| SYVN1     | NM_032431.2    | VCP/p97     | NM_007126.3    |
| TRIM13    | NM_001007278   | CPVL        | NM_031311.3    |
| BFAR      | NM_016561.2    | DERLIN1     | NM_024295.5    |
| CGRRF1    | NM_006568.2    | DERLIN2     | NM_016041.3    |
| MARCH2    | NM_016496.4    | DERLIN3     | NM_001135751.1 |
| MARCH3    | NM_178450.3    | ERLIN1      | NM_006459.3    |
| MARCH4    | NM_020814.2    | ERLIN2      | NM_007175.6    |
| MARCH8    | NM_001002265.1 | UBAC2       | NM_001144072.1 |
| RNF128    | NM_194463.1    | EDEM1       | NM_014674.2    |
| RNF13     | NM_007282.4    | EDEM2       | NM_018217.2    |
| RNF145    | NM_001199380.1 | EDEM3       | NM_025191.3    |
| RNF148    | NM_198085.1    | UBXD8       | NM_014613.2    |
| RNF150    | NM_020724.1    | ERFAD       | NM_024955.5    |
| RNF26     | NM_032015.4    | TXD16/ERp90 | NM_020784.2    |
| RNFT1     | NM_016125.3    | PDIA6       | NM_005742.2    |
| TRIM59    | NM_173084.2    | UBE2J1      | NM_016021.2    |
| ZNRF4     | NM_181710.3    | UBE2G2      | NM_003343.5    |
| KIAA0090  | NM_015047.2    | ATF6        | NM_007348.3    |
| C15ORF24  | NM_020154.2    | PERK        | NM_004836.5    |
| MMGT1     | NM_173470.1    | IRE1        | NM_001433.3    |
| TMEM85    | NM_016454.2    | ATF4        | NM_001675.2    |
| TMEM93    | NM_001014764.1 | CHOP        | NM_001195053.1 |
| C14ORF122 | NM_016049.3    | XBP1        | NM_005080.3    |
| COX4NB    | NM_006067.4    | OS9 exon11  | NM_006812.3    |
| TTC35     | NM_014673.3    | OS9 exon13  | NM_006812.3    |
| C19ORF63  | NM_206538.2    | MT1X        | NM_005952.3    |
| TMEM111   | NM_018447.2    | MT1H        | NM_005951.2    |
| CANX      | NM_001746.3    | ACTB        | NM_001101.2    |
| BiP/HSP5A | NM_005347.4    | GAPDH       | NM_002046.3    |
| OS9       | NM_006812.3    | TUBB        | NM_178014.2    |
| XTP3B     | NM_015701.4    |             |                |

**Table 5 nCounter™ CodeSet**

### **XBP1 splicing assay**

PCR for XBP1 mRNA was performed as described previously (J. H. Lin et al., 2007). Primer sequences for human XBP1 were derived from the same paper. The PCR product was run on a 2.5% agarose gel and imaged via ChemiDoc™ (BioRad) using UV excitation.

## Real-time quantitative PCR (qRT-PCR)

cDNA obtained from reverse transcription was diluted 1:10 and used as a template for qRT-PCR. Reactions were performed using GoTaq® qPCR Master Mix (Promega) according to the manufacturer's instructions. Annealing and elongation temperature was set at 60°C for all reactions. Oligos used for qRT-PCR were validated for amplification efficiency and sequences are listed in Table 6.

| Target  | Forward Oligo             | Reverse Oligo            |
|---------|---------------------------|--------------------------|
| ACTIN   | GAGGCACTCTTCCAGCCTT       | AAGGTAGTTTCGTGGATGCC     |
| ATF6    | GGATTTGATGCCTTGGGAGTCAGAC | ATTTTTTCTTTGGAGTCAGTCCAT |
| AMFR    | AGCTGACACGCTCAGTTGAA      | GCTCGGTCTGAGCAGAAGTT     |
| BFAR    | CTCCTCAGGGATGCCATTGAAA    | TCCCTGCTGCTCCAGTGATA     |
| CGRRF1  | GGCCTGGTATTGGGATGTTTT     | TTTTTGAACACTGCACCCCC     |
| DERLIN3 | GTGGACCTGCTGGGGATT        | CTCTGCTGTGTCCAGATGGT     |
| HRD1    | CGTGTGGACTTTATGGAACGC     | AGGGGTCTCTACTCTGGAGG     |
| MARCH2  | ACACCGAGTGATGGTCCTTTCTG   | GCACAGTGTCGGCTTCTCCG     |
| MARCH3  | TGCTGTCAACAGTAGTGCGG      | TCAGCCACTCCACTAACGG      |
| MARCH4  | CCGAGGATCGCTACTCACTG      | CCTGCCACTGCAGAGGATTTT    |
| MARCH6  | TCAATGGCTGAAACACAGTCG     | AGATGCGGCATGCTGTAAGA     |
| MARCH8  | TAGCTTGAAATCCATGGCCCCAA   | CGGAGGACTCCCAGCCTTAG     |
| RNF5    | GTTTGGAGACTGCTCGGGAA      | TGGAATCCCCCTCTGCTCTC     |
| RNF13   | GGCCGGACTTCAAGAGAGAA      | GGAGAGCAGCATCTCGTTGT     |
| RNF26   | GAATCCCCTCCTACCCCTGT      | GAGGAGAGCCACAGCAAAT      |
| RNF103  | CGAGAAGAAGGATGTCCGGG      | GCTATGACCTGAACCAGCCA     |
| RNF128  | CTGCTCGAAGGCTACGGAAT      | GGGCCAATTTCTTGTCTCCT     |
| RNF139  | AGCAGCAGGTGCGGATG         | AATACACCAAAGAGCCGGAGG    |
| RNF145  | CCATGAATCGGGGCATGACA      | AGCACGGAAGTGTTCACACA     |
| RNF148  | AGCAACCTGAAAGGCATGGA      | CAATTGTGGCAGCCAGGAAG     |
| RNF150  | AGGGTGACAAGGAAACAGAGT     | AGAGAGCCCTCGAAGTCAGT     |
| RNF170  | GGCTCTAGACCTGGAATGGC      | GCTCCTGGTTTTCTGGGTGA     |
| RNF185  | GTAACTGTGCGGAGGC          | CGGCCAACAGAAGAGGTG       |
| RNFT1   | TCTAAGAGAAAGGTCCTCAAAGATT | TCCCAGAAGCTCAAATGGTCC    |
| TMEM129 | CTATCTGGTGTTCCCGTGT       | ATGCCACATAGTAGCCGAG      |
| TRIM13  | CTGTCTGACTTTGAGACCAT      | TTTGAAAGCCTCAGCAATGT     |
| TRIM59  | AGACCACTTCCTGAGGTCCA      | ATCAGACCAGGAACATGGCG     |
| ZNRF4   | GAGGGCGACCAACTCAAGAT      | CAAAGCTGTCTTCTGTGGCG     |

**Table 6 Oligo sequences for RT-qPCR**

## Molecular cloning of FLAG-HA-E3s

pcDNA™5/FRT/TO (Invitrogen) was modified to carry a FLAG-HA sequence in the multiple cloning site to allow subsequent fusion of the tag either at the N- or C-terminal of the gene of interest. To this aim, a DNA duplex containing the FLAG-HA sequence flanked by specific restriction sites (RSs) was generated as follow. Forward and Reverse oligos (100 µM) were diluted 1:10 in annealing buffer (10 mM

TrisCl pH 7.4, 1 mM EDTA and 50 mM NaCl) in a 1:1 ratio. The PCR programme for the annealing was: 94°C for 5 min, reduction of 1°C/min x 70 cycles starting at 94°C to a final temperature of 24°C. The duplex was then phosphorylated using T4 PNK (polynucleotide kinase) enzyme (Invitrogen) following the manufacturer's instructions. The reaction was inhibited at 65°C for 10 min. The duplex was cloned into pcDNA™5/FRT/TO using NotI and HindIII RSs to generate the constructs pFL001 (N-terminal tag) and pFL002 (C-terminal tag). The sequence of the duplex and position of the tag relative to the ORF (open reading frame) are summarised in Table 7.

| FLAG-HA | Duplex Sequence                                                                                                    | E3 ORFs                                                                                                                                                 |
|---------|--------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------|
| N-term  | AGCTTGCCACCATGGACTACAAGGAT<br>GACGATGACAAGCTCGATGGAGGATA<br>CCCATACGATGTTCCAGATTACGCTGG<br>TGAACCGGTGGATCCGCTAGCGC | MARCH6, RNF5, RNF185,<br>BFAR, RNFT1                                                                                                                    |
| C-term  | AGCTTGCTAGCACCGGTGGATCCGGT<br>ACCGACTACAAGGATGACGATGACAA<br>GCTCGATGGAGGATACCCATACGATG<br>TTCCAGATTACGCTTGAGC      | HRD1, TRIM59, RNF128,<br>RNF13, RNF103, AMFR,<br>CGRRF1, MARCH2, MARCH3,<br>MARCH4, MARCH8, ZNRF4,<br>RNF139, RNF148, RNF170,<br>RNF26, TRIM13, TMEM129 |

**Table 7 Cloning strategy for FLAG-HA-tagged E3s**

ORF sequences for each E3 were obtained from the IMAGE collection (I.M.A.G.E Consortium) and the sources of the cDNAs are listed in Table 8.

| E3 Name | Accession #  | Uniprot ID | Clone Source                                 |
|---------|--------------|------------|----------------------------------------------|
| AMFR    | NM_001144    | Q9UKV5     | Thermo Fisher Scientific                     |
| BFAR    | NM_016561    | Q9NZS9     | SGC (Structural Genomics Consortium)         |
| CGRRF1  | NM_006568    | Q99675     | SGC                                          |
| HRD1    | NM_032431    | Q86TM6     | Lab collection                               |
| MARCH2  | NM_016496    | Q9P0N8     | Paul Lenher                                  |
| MARCH3  | NM_178450    | Q86UD3     | Paul Lenher                                  |
| MARCH4  | NM_020814    | Q9P2E8     | Paul Lenher                                  |
| MARCH6  | NM_005885    | O60337     | Wiertz Emmanuel                              |
| MARCH8  | NM_145021    | Q5T0T0     | Lenher Paul                                  |
| RNF5    | NM_006913    | Q99942     | Lenher Paul                                  |
| RNF13   | NM_007282    | O43567     | SGC                                          |
| RNF26   | NM_032015    | Q9BY78     | SGC                                          |
| RNF103  | NM_005667    | O00237     | SGC                                          |
| RNF128  | NM_194463    | Q8TEB7     | SGC                                          |
| RNF139  | NM_007218    | Q8WU17     | Paul Lenher                                  |
| RNF145  | NM_001199380 | Q96MT1     | SGC                                          |
| RNF148  | NM_198085    | Q8N7C7     | SGC                                          |
| RNF150  | NM_020724    | Q9ULK6     | Neuroblastoma CHP-212                        |
| RNF170  | NM_001160223 | Q96K19     | PlasmID Repository at Harvard Medical School |
| RNF185  | NM_152267    | Q96GF1     | SGC                                          |
| RNFT1   | NM_016125    | Q5M7Z0     | SGC                                          |
| TMEM129 | NM_001127266 | A0AVI4     | Lenher Paul                                  |
| TRIM13  | NM_005798    | O60858     | SGC                                          |
| TRIM59  | NM_025863    | Q8IWR1     | SGC                                          |
| ZNRF4   | NM_181710    | Q8WWF5     | SGC                                          |

**Table 8 List of the ORFs and plasmid sources used for cloning FLAG-HA-E3s**

The ORFs were amplified with primers containing RSs at their 5'-end to enable subsequent cloning into pFL001/2. For the PCR reactions, the following mix was prepared: 10 µl 5× Phusion HF Buffer, 0.5 µl Phusion® High-Fidelity DNA Polymerase (Invitrogen), 1 µl 100 mM dNTPs (deoxynucleotides, Invitrogen), 2.5 µl 10µM forward oligo, 2.5 µl 10uM reverse oligo, 1 µl DNA (diluted 1:10), 1.5 µl DMSO and 31 µl H<sub>2</sub>O. In some cases, 12.5 µl of 4 M Betaine was used instead of DMSO. The PCR programme was as follow: 98°C for 2 min, (98°C for 10 seconds, calculated annealing T° for 30 seconds and 72°C for 75 seconds) × 30 cycles, 72°C for 5 min. After ligation of the digested PCR products with the vector, DH5α competent bacteria were transformed and plated on ampicillin Luria Broth (LB)-agar plates. Final constructs were analysed by restriction digest and sequencing.

Sequences were analysed using Sequence Scanner Software 2.0 (Applied Biosystems) and Serial Cloner 2.6.1 Software. Where the E3 was not expressed or did not localise correctly in the final cell line, the ORF was re-cloned with the tag at the opposite end. Table 9 summarises the oligos used for sequencing and cloning.

| Name   | Oligo Sequence                       | Target            |
|--------|--------------------------------------|-------------------|
| oFL001 | GAAACAGCTATGACCATGTTC                | pDNR-LIB 362-383R |
| oFL002 | TATAGGGAGACCGGCAGATCT                | pOTB7 278-299R    |
| oFL003 | TCTACCTGTACCTGTTTGAC                 | BFAR 850-870F     |
| oFL004 | ATCTGCCTTTAATTGCCTCT                 | RNF128 730-750R   |
| oFL005 | AAGTAGGGAAAAACATGGC                  | RNF128 580-600F   |
| oFL006 | TTCATGATCACAAAATTTGT                 | RNF13 600-620F    |
| oFL007 | ATCCCCCAGTCGGCGCTGGT                 | RNF150 730-750R   |
| oFL008 | CAGAAATATGTGAGCCGCAC                 | RNF150 600-620F   |
| oFL009 | TGGGGTGTCCCAGGGACAGC                 | RNF185 480-500R   |
| oFL010 | GACCCCAGAGAGAAGACCCC                 | RNF185 300-320F   |
| oFL011 | CTGGTCAACATCTGCCTCAT                 | RNF26 450-470F    |
| oFL012 | TTTGAAAGCCTCAGCAATGT                 | TRIM13 700-720R   |
| oFL013 | CTGTCTGACTTTGAGACCAT                 | TRIM13 600-620F   |
| oFL014 | CACAGGAAGCCTCCACTTCG                 | MARCH8 1140-1159F |
| oFL015 | TTTGAACATAGATCACTCTA                 | MARCH8 1542-1561R |
| oFL016 | CCCTTGAACCTCCTCGTTCGACC              | pLXSN5' F         |
| oFL021 | AATATACCGGTGACACCGCGGAGGAAGACAT      | MARCH6_AgeI F     |
| oFL022 | CTTGCGGCCGCTTATTCTTGGGATGACTGTG      | MARCH6 NotI R     |
| oFL023 | AATATACCGGTGCAAAATTTGAGGAAGAGTT      | TRIM59 AgeI F     |
| oFL024 | CTTGCGGCCGCTCAATGGGAACTATTTTCC       | TRIM59 NotI R     |
| oFL025 | AATATACCGGTGGGCCCGCCGCTGGGGCCGG      | RNF128 AgeI F     |
| oFL026 | CTTGCGGCCGCTTAAGATTTAATTTCTCGAA      | RNF128 NotI R     |
| oFL027 | AATATACCGGTCTGCTCTCCATAGGGATGCT      | RNF13 AgeI F      |
| oFL028 | CTTGCGGCCGCTCAAACAGTATTTGCTATGT      | RNF13 NotI R      |
| oFL029 | AATATACCGGTGCAATGTCTCTCATCCAAGC      | RNF150 AgeI F     |
| oFL030 | CTTGCGGCCGCTCAAGATTTCACTTCTTCAC      | RNF150 NotI R     |
| oFL031 | AATATACCGGTGCAGCAGCGGAGGAGGAGGA      | RNF5 AgeI F       |
| oFL032 | CTTGCGGCCGCTCAAATACTGAGCAGCCAAA      | RNF5 NotI R       |
| oFL033 | AATATACCGGTGCTGCAAAGGAGAACTGGA       | RNF145 AgeI F     |
| oFL034 | CTTGCGGCCGCTAGGCTGATTCAACAGGAT       | RNF145 NotI R     |
| oFL035 | AATAAGCTTGCCACCATGCCGCTGCTTCTCCTCGA  | AMFR HindIII F    |
| oFL036 | TTGAATATTACCGGTGAGGCTGCTGCTTCTGAA    | AMFR AgeI R       |
| oFL037 | AATAAGCTTGCCACCATGGCTGCGGTGTTTCTGGT  | CGRRF1 HindIII F  |
| oFL038 | TTGAATATTACCGGTAAGAGTCTTCGGTTTGTCTT  | CGRRF1 AgeI R     |
| oFL039 | AATAAGCTTGCCACCATGAGCATGCCACTGCATCA  | MARCH8 HindIII F  |
| oFL040 | TTGAATATTACCGGTGACGTGAATGATTTCTGCTC  | MARCH8 AgeI R     |
| oFL041 | AATAAGCTTGCCACCATGCCGCTCTGCCGTCCGGA  | ZNRF4 HindIII F   |
| oFL042 | TTGAATATTACCGGTCTGACCAGGGGCTCAGGAG   | ZNRF4 AgeI R      |
| oFL043 | AATGCTAGCGCCACCATGGCGGCCGTGGGGCCCCC  | RNF139 Nhe1 F     |
| oFL044 | TTGAATATTGGATCCGTCAGTATCATCATTAAATT  | RNF139 BamHI R    |
| oFL045 | AATAAGCTTGCCACCATGAGCTTCTTAGAATTAC   | RNF148 HindIII F  |
| oFL046 | TTGAATATTACCGGTAGTTTTTCAGGATGTCACACT | RNF148 AgeI R     |
| oFL047 | AATAAGCTTGCCACCATGGCCAAATATCAAGGTGA  | RNF170 HindIII F  |
| oFL048 | TTGAATATTACCGGTACCTGAGAACCAAAGGATGG  | RNF170 AgeI R     |
| oFL049 | AATAAGCTTGCCACCATGACGACGGGTGACTGCTG  | MARCH2 HindIII F  |
| oFL050 | TTGAATATTACCGGTACTGGTGTCTCCTCTGCCA   | MARCH2 AgeI R     |
| oFL051 | AATAAGCTTGCCACCATGACAACCGGCTGCAG     | MARCH3 HindIII F  |
| oFL052 | TTGAATATTACCGGTAACAACCTGTCTCCTTTGAGT | MARCH3 AgeI R     |
| oFL053 | AATAAGCTTGCCACCATGCTGATGCCCTGTGTGG   | MARCH4 HindIII F  |
| oFL054 | TTGAATATTACCGGTCACTGTCGTGACTCTCATGA  | MARCH4 AgeI R     |
| oFL055 | AATAAGCTTGCCACCATGGCAAGCAAGGGGCCCTC  | RNF185 HindIII F  |
| oFL056 | TTGAATATTACCGGTGCAATCAGGAGCCAGAACA   | RNF185 AgeI R     |



|        |                                      |                       |
|--------|--------------------------------------|-----------------------|
| oFL057 | AATAAGCTTGCCACCATGGAGGCAGTGTACCTGGT  | RNF26 HindIII F       |
| oFL058 | TTGAATATTACCGGTGAGGTAGACATTGAGGGTCT  | RNF26 AgeI R          |
| oFL059 | AATGCTAGCGCCACCATGGAGGAACCTCAGAAAAG  | BFAR NheI F           |
| oFL060 | TTGAATATTGGATCCCAACACCTGGGTTTCCAGCC  | BFAR BamHI R          |
| oFL061 | AATAAGCTTGCCACCATGGAGCTGCTTGAAGAAGA  | TRIM13 HindIII F      |
| oFL062 | TTGAATATTACCGGTAAATAGTTTATATTTGCACA  | TRIM13 AgeI R         |
| oFL063 | AATAAGCTTGCCACCATGTTCCGCACGGCAGTGAT  | SYVN1 HindIII F       |
| oFL064 | TTGAATATTACCGGTGTGGCAACAGGAGACTCCA   | SYVN1 AgeI R          |
| oFL065 | AATAAGCTTGCCACCATGCCGCTGTTCTTGCTGTC  | RNFT1 HindIII F       |
| oFL066 | TTGAATATTACCGGTATATATTTGAAGGTGTGATG  | RNFT1 AgeI R          |
| oFL067 | AATATACCGGTTGGCTGAAGCTTTTTTCTT       | RNF103 AgeI F         |
| oFL068 | CTTGCGGCCGCTTAAGATGGGACATCATTG       | RNF103 NotI R         |
| oFL069 | GTTGCTGCTGATCAGCCTGC                 | MARCH6 607-627F       |
| oFL070 | ACATAGAGACCACAAGCAGC                 | MARCH6 2032-2051R     |
| oFL071 | GAATTTATGGATTACAGTTA                 | RNF139 650-669F       |
| oFL072 | GCCTCTCAACATTTTCTGCC                 | TRIM13 300-319F       |
| oFL073 | TAAATGGATAACTGCTCATG                 | RNF103 1600-1620F     |
| oFL074 | CCACGGAATGCACACCTTGG                 | AMFR 630-650F         |
| oFL075 | AAGTTCTCTATTTTCTTGGG                 | RNF145 544-564F       |
| oFL076 | TGGCTTATATGATTTGCCAG                 | RNF145 1186-1206F     |
| oFL077 | TCTCGTTGGTGTGGAGCCC                  | RNF150 444-464R       |
| oFL078 | GTGAATGAAAGTTCAGTCAC                 | RNF150 1084-1103F     |
| oFL079 | GCCTCCTTCCCGGTGGAGAC                 | RNF170 280-299F       |
| oFL080 | AATAAGCTTGCCACCATGGCCAAATATCAAGGTGA  | RNF170 Harv HindIII F |
| oFL081 | TTGAATATTACCGGTTCTAGTTAGCCTTTGGGTTA  | RNF170 Harv AgeI R    |
| oFL082 | AATGCTAGCGCCACCATGGGGCCCGCCTGGGGC    | RNF128 NheI F         |
| oFL083 | TTGAATATTGGATCCAGATTTAATTTCTCGAACAG  | RNF128 BamHI R        |
| oFL084 | AATATACCGGTGCAAGCAAGGGGCCCTCGGC      | RNF185 AgeI F         |
| oFL085 | CTTGCGGCCGCTTAGGCAATCAGGAGCCAGA      | RNF185 NotI R         |
| oFL086 | AATAAGCTTGCCACCATGCACAATTTTGAGGAAGA  | TRIM59 HindIII F      |
| oFL087 | TTGAATATTACCGGTATGGGAACTATTTTCCACA   | TRIM59 AgeI R         |
| oFL088 | AATATACCGGTCCGCTGTTCTTGCTGTCGCT      | RNFT1 AgeI F          |
| oFL089 | CTTGCGGCCGCTTAATATATTTGAAGGTGTG      | RNFT1 NotI R          |
| oFL090 | AATAAGCTTGCCACCATGGACACCGCGGAGGAAGA  | MARCH6 HindIII F      |
| oFL091 | TTGAATATTACCGGTTTCTTGGGATGACTGTGGAG  | MARCH6 AgeI R         |
| oFL092 | AATAAGCTTGCCACCATGGACAGCCCCGAGGTGAC  | TMEM129 HindIII F     |
| oFL093 | TTGAATATTGGATCCGCGCACGGTGCACACATCCA  | TMEM129 BamHI R       |
| oFL094 | AATAAGCTTGCCACCATGCTGCTCTCCATAGGGAT  | RNF13 HindIII F       |
| oFL095 | TTGAATATTACCGGTAACAGTATTTTGCTATGTTGT | RNF13 AgeI R          |
| oFL096 | AATGCTAGCGCCACCATGTGGCTGAAGCTTTTTTT  | RNF103 NheI F         |
| oFL097 | TTGAATATTGGTACCAGATGGGACATCATTTGACA  | RNF103 KpnI R         |
| oFL098 | GCGATTTGTGGTTCTCATAA                 | RNF103 1053-1073F     |
| oFL099 | CTCTGCGAGAAGCTCCGGGC                 | TMEM129 706-726F      |
| oFL100 | CCAACGACATTGAGGTAC                   | RNF13 1390-1407F      |
| oFL101 | AATATACCGGTGAGGAACCTCAGAAAAGCTA      | BFAR AgeI F           |
| oFL102 | CTTGCGGCCGCTCACAAACCTGGGTTTCCA       | BFAR NotI R           |
| oFL103 | AATAAGCTTGCCACCATGGCTGCAAAGGAGAACT   | RNF145 HindIII F      |
| oFL104 | TTGAATATTACCGGTGGCTGATTCAACAGGATGTA  | RNF145 AgeI R         |
| oFL105 | AATATACCGGTGAGCTGCTGAAGAAGATCT       | TRIM13 AgeI F         |
| oFL106 | CTTGCGGCCGCTTATAATAGTTTATATTTGC      | TRIM13 NotI R         |
| oFL107 | GGTTCAGGTAGTGGAGGT                   | R145 615-632          |
| oFL108 | CTGGGAGCATCTAGAGAC                   | R145 1102-1119        |
| oFL109 | AATATACCGGTGAGGCAGTGTACCTGGTAGT      | RNF26 AgeI F          |
| oFL110 | CTTGCGGCCGCTCAGAGGTAGACATTGAGGG      | RNF26 NotI R          |
| oFL111 | CTTGCGGCCGCTCAGAGTGCGACATTGAGGG      | R26_FH_V-A_mut        |

**Table 9 Primers used for cloning and sequencing**

### **Polymerase chain reaction (PCR)**

DNA target sequences were amplified using Phusion<sup>®</sup> High-Fidelity DNA Polymerase (Invitrogen) according to the manufacturer's instructions. Either DMSO or Betaine was used to increase the performance of the reaction. The annealing temperature for the primers to the DNA template was calculated using Serial Cloner 2.6 Software and the reaction was carried out in a thermocycler (BioRad).

### **Restriction endonuclease digest**

30 µl of PCR product were digested with 1 µl (20,000 U/ml) of restriction enzyme (NEB) in a total volume of 50 µl. The reaction was set up following the manufacturer's instructions and incubated at 37°C for 1 hour. Digested products were purified using a QIAquick PCR purification Kit (QIAGEN) following the manufacturer's instructions. For preparative applications, approximately 1 µg of plasmid DNA was digested with 1 µl (20,000 U/ml) of restriction enzyme in a final volume of 20 µl and incubated at 37°C for 3 hours. For analytical applications, 1 µl of plasmid DNA was digested with 0.5 µl (20,000 U/ml) of restriction enzyme in a final volume of 10 µl and incubated at 37°C for 1 hour. The digested products were analysed by electrophoresis on an agarose gel.

### **Agarose gel electrophoresis**

Agarose (at the appropriate concentration) was heated in TAE (Tris-acetate-EDTA) buffer until dissolved and DNA/RNA dye peqGREEN (VWR) was subsequently added 1:20,000 to the gel. DNA samples containing loading buffer (Invitrogen) were loaded onto the gel and run in TAE buffer at 135 V. A 1 kb DNA ladder (Invitrogen) was run next to the samples as a size marker. Bands were visualized in a UV imaging system (UVP Bio Imaging Systems).

### **DNA purification from agarose gel**

DNA fragments separated by electrophoresis were visualized with UV light. Bands of interest were cut out of the gel to be purified using the QIAquick PCR purification Kit (QIAGEN) according to the manufacturer's instructions.

### **Ligation of DNA fragments**

Digested vector and insert DNA were mixed in a 3:1 molar ratio and ligated using T4 DNA ligase (Invitrogen) at RT for 1 hour.

### **Transformation of competent bacteria**

DH5 $\alpha$  competent bacteria were thawed on ice for 10 min. 10  $\mu$ l of ligation reaction or 1  $\mu$ l of plasmid DNA were added to 100  $\mu$ l of bacteria and incubated on ice for 15 min, followed by heat shock at 42°C for 45 seconds. The transformation mix was incubated on ice for 2 min and then plated on LB agar containing the appropriate antibiotic. Plates were incubated at 37°C overnight to allow colony formation.

### **Purification of plasmid DNA from bacteria**

Single bacterial colonies were inoculated in 5 ml of antibiotic-containing LB medium and grown at 37°C under agitation overnight. Bacteria were collected by centrifugation at 14,000 rpm for 1 min. DNA was extracted using a QIAprep<sup>®</sup> Spin Miniprep Kit (QIAGEN) following the manufacturer's instructions.

### **Determination of nucleic acid concentration**

Concentration and purity of DNA or RNA was determined by measuring their absorbance at 260 nm and 280 nm with a Nanodrop spectrophotometer (Thermo Fisher Scientific).

### **Validation of DNA constructs**

DNA constructs were validated by restriction digest and by sequencing. DNA sequences were analysed using Sequence Scanner Software 2.0 and aligned against the predicted sequence using Serial Cloner 2.6 Software.

## **2.3 Sample preparation for mass spectrometry**

### **FLAG immunoprecipitation**

FLAGHA\_ Flp-In™293 cell lines were seeded in 3 × 15 cm dishes and treated with the appropriate dose of DOX (Table 3) for 18 hours. For harvesting, cells were washed with PBS and detached by scraping with PBS containing 10 mM EDTA. Cell pellets were snap frozen in dry ice/EtOH and stored at -80°C. For protein extraction, pellets were resuspended in 2 ml of LMNG lysis buffer [50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA and 1% (w/v) LMNG] supplemented with protease inhibitors (Roche) and incubated at 4°C for 1 hour. Cell lysates were centrifuged at 17,000× g at 4°C for 30 min and supernatants were re-centrifuged for 15 min. Clear supernatants were pre-cleared using 100 µl sepharose CL-4B beads (Pharmacia) and incubated at 4°C overnight. Beads were sedimented by centrifugation at 3,000× g for 2 min and proteins were quantified using Bradford reagent (BioRad). 10 mg of total proteins were prepared in 1.8 ml of LMNG lysis buffer and immunoprecipitated using 75 µl of anti-FLAG M2 affinity gel (Sigma-Aldrich) while rotating at 4°C for 2 hours. The remaining proteins were prepared in Laemmli buffer/DTT, heated at 56°C for 10 min and stored at -80°C to be subsequently run as inputs in SDS-PAGE. FLAG resin was collected by centrifugation at 5,000× g at 4°C for 30 seconds and washed twice in lysis buffer without detergent, and then twice in TBS (Tris-buffered saline) for buffer exchange.

### **SDS protein elution**

Immunoprecipitated protein complexes were eluted with 100 µl 2× SDS with 10% BME (β-mercaptoethanol) at 95°C for 10 min. The eluate was collected upon centrifugation at 5,000× g for 30 seconds.

### **Protein reduction and alkylation**

Eluates were adjusted with ddH<sub>2</sub>O to 175 µl. 5 µl of 200 mM DTT were added to the samples and incubated at RT for 30 min. Alkylation was performed by addition of 20 µl 200 mM IAA (iodoacetamide) and incubation at RT for 30 min.

### **Double methanol/chloroform precipitation**

Proteins were precipitated by addition of 600 µl methanol, 150 µl chloroform and 450 µl ddH<sub>2</sub>O, then vortexed and centrifuged at 17,000× g at RT for 2 min. The aqueous phase was removed, 450 µl methanol were added to the samples, then they were vortexed and centrifuged for 5 min. Supernatant was removed and the protein pellet was resuspended in 200 µl ddH<sub>2</sub>O for a second precipitation as before.

### **Tryptic digestion**

Protein pellets were resuspended by vortexing in 50 µl 6 M Urea and 0.1 M Tris HCl pH7.8 and sonicated for 2 min. 250 µl ddH<sub>2</sub>O were added to reduce urea concentration and ice-cold trypsin was added at a 1:50 ratio for digestion at 37°C overnight while shaking at 300 rpm. Trypsin was inactivated by addition of 3 µl of 100% TFA (trifluoroacetic acid) to a 1% final concentration.

### **C18 column purification**

Trypsinised peptides were purified through C18 Sep-Pak cartridges (Waters). Solution B (65% acetonitrile and 0.1% TFA in ultrapure water) was used for cartridge conditioning and 10 ml of solution A (2% acetonitrile and 0.1% TFA in ultrapure water) were used for the equilibration step. The acidified tryptic peptides were bound to the cartridge prior to a wash with 10 ml solution A. The peptide elution was performed by adding 2 × 600 µl of Solution B to the cartridge. The samples were speed-vac dried and resuspended in 20 µl of solution A, vortexed, sonicated and centrifuged at 17,000× g for 20 min. The clear suspension was transferred in a glass tube and stored at -20°C.

## **LC-MS/MS settings**

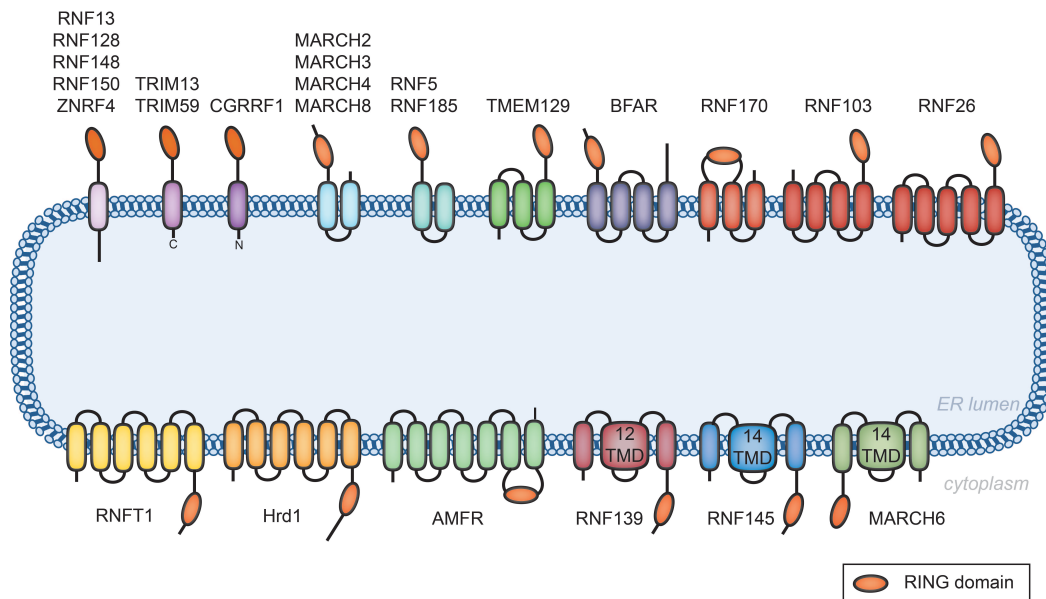
2 µl of peptides were analysed by tandem mass spectrometry (Q Exactive-HF, Thermo Fisher Scientific, Bremen, Germany) coupled with ultra-high performance liquid chromatography (UHPLC, Dionex Ultimate 3000, Thermo Fisher Scientific) using a PepMap RSLC column (C18, 2µm, 100 Å and 75µm × 50 cm). The samples were loaded in 0.1% TFA (Trifluoroacetic acid) buffer and analysed using a 1 hour linear gradient at a flow rate of 250 nl/min. The gradient used to elute the peptides started at 3 min with 2% buffer B (0.1% FA and 5% DMSO in acetonitrile), increasing to 5% buffer B by 6 min followed by an increase of up to 35% by 63 min. The column was washed twice for 5 and 6 min respectively, with 99% buffer B. The total length of the analysis was 100 min to allow column re-equilibration. The data were acquired with a resolution of 70,000 full-width half maximum at mass/charge 400 with lockmass enabled (445.120025 m/z), top 15 precursor ion selection, dynamic exclusion of 27 seconds and fragmentation performed in higher-energy C-trap dissociation (HCD) mode with normalized collision energy of 28.

Chapter III:

Interaction Networks of ER-Resident  
Ubiquitin Ligases and their  
Participation in ER Homeostasis

### 3.1 Background

The human ER contains at least 25 E3 ubiquitin ligases, including Hrd1 (Maruyama et al., 2008; Neutzner et al., 2011; van den Boomen and Lehner, 2015). They are single pass or multispanning membrane proteins with varying topology, bearing a RING finger domain oriented towards the cytosol (Figure 3.1).



**Figure 3.1 Topology of the ER-resident E3 ubiquitin ligases**

About half of the ER-embedded E3s have been reported to play role in ERAD by regulating turnover of different substrates, including: AMFR (Fang et al., 2001), BFAR (Rong et al., 2011), MARCH6 (Kreft et al., 2006), RNF5 (Younger et al., 2006), TRIM13 (Lerner et al., 2007), TMEM129 (van den Boomen et al., 2014), MARCH8 (Rui Chen et al., 2012), ZNRF4 (Neutzner et al., 2011), RNF103 (Maruyama et al., 2008), RNF128 (Ichikawa et al., 2011), RNF139 (Stagg et al., 2009), RNF170 (Lu et al., 2011) and RNF185 (Khouri et al., 2013). The MARCH family proteins MARCH2 (Nakamura et al., 2005) and MARCH4 (Bartee et al., 2004), as well as RNF13 (Arshad et al., 2013) and RNF26 (Qin et al., 2014), have reported roles in Ub-dependent regulation of different cellular processes



independent of ERAD. In particular, MARCH2 and MARCH4 control lysosomal trafficking of transmembrane receptors such as CFTR (cystic fibrosis transmembrane conductance regulator) and MHC-I, respectively (Bartee et al., 2004; Cheng and Guggino, 2013). RNF13 regulates JNK activation and downstream apoptotic signalling in response to ER stress (Arshad et al., 2013). Finally, RNF26 participates in the antiviral response by regulating type I interferon induction, and most recently has been reported to be involved in orchestration of the endosomal system for cargo trafficking (Jongsma et al., 2016). The remaining ER-localised E3s (CGRRF1, RNFT1, TRIM59, RNF148, RNF150, MARCH3 and RNF145) are still uncharacterised in terms of their catalytic activity and potential function. Table 11 (Appendix) summarises the currently known interactors and substrates of the ER-resident E3s.

MARCH6/TEB4 is the human orthologue of yeast Doa10 and, together with Hrd1, they represent the most evolutionarily conserved E3s in the ER of metazoans (Kreft et al., 2006). MARCH6 contains an estimated 14 transmembrane domains (TMDs) and it is capable of autoubiquitination, promoting its own proteasomal degradation (Hassink et al., 2005). Only two substrates have been identified for MARCH6: the thyroid hormone-activating type 2 deiodinase (DIO2) (Zavacki et al., 2009) and the more recently discovered squalene monooxygenase (SQLE) (Foresti et al., 2013). By mediating SQLE degradation, MARCH6 participates in the regulation of sterol homeostasis, alongside Hrd1 and AMFR (Bays et al., 2001; Song et al., 2005).

AMFR/gp78 is the first ubiquitin ligase integral to the ER membrane to be shown to participate in ERAD in mammalian cells (Fang et al., 2001). Topologically, it contains seven TMDs and shares a high degree of homology with Hrd1's N-terminal region (28% similarity and 18% identity) and RING finger domain (69% similarity

and 54% identity) (Kikkert et al., 2004). It is involved in the degradation of a number of substrates, among which are the TCR subunits CD3- $\delta$  and TCR- $\alpha$ , and the Z variant of A1AT (Fang et al., 2001; Shen et al., 2006). It is implicated in the degradation of Apolipoprotein B-100 (APOB), Insig-1 and HMG-CoA reductase (J. N. Lee et al., 2006; Song et al., 2005), highlighting its role in cholesterol metabolism. AMFR mediates substrate ubiquitination via binding to the E2 enzyme Ube2g2 (Das et al., 2009), the ATPase VCP/p97 and Derlin-1 (Y. Ye et al., 2005). Disease-associated substrates of AMFR include mutant CFTR $\Delta F508$ , whose degradation occurs in cooperation with the E3 ligase RNF5 (Morito et al., 2008), as well as SOD1 $G94A$  (superoxide dismutase-1) and Ataxin-3, which are associated with ALS and Machado–Joseph disease, respectively (Ying et al., 2009). AMFR has been reported to promote sarcoma metastasis by targeting the metastasis suppressor KAI1/CD82 for degradation (Y. C. Tsai et al., 2007). Consistent with this finding, depletion of AMFR in cancer cells promotes apoptosis. The pathological relevance of AMFR in cancer is supported by the observation that AMFR expression inversely correlates with the levels of KAI1 in human sarcoma samples (Y. C. Tsai et al., 2007).

RNF139 (RING finger protein 139)/TRC8 (translocation in renal carcinoma on chromosome 8 protein) was originally identified as a tumour suppressor gene associated with hereditary renal cell carcinoma (Gemmell et al., 1998). RNF139 is a polytopic protein consisting of 12 TMDs and a sterol-sensing domain (SSD). In the presence of sterols, RNF139 promotes its own ubiquitination and degradation, whereas it is stabilised under conditions of sterol depletion. It binds SREBP-2 (sterol response element binding protein 2) and SCAP (SREBP cleavage-activated protein) to inhibit the translocation of the SREBP-1/SCAP complex from the ER to the Golgi for proteolytic processing, thereby reducing SREBP-2 target gene expression

(Irisawa et al., 2009). RNF139 can also destabilise SREBP-1 and SREBP-2 by promoting their ubiquitination and degradation (J. P. Lee et al., 2010). Due to these activities, this ligase regulates lipid biosynthesis during periods of cholesterol limitation. Other substrates of RNF139 include MHC-I through US2 binding (Stagg et al., 2009) and heme oxygenase-1 (HO-1) (P.-H. Lin et al., 2013). Degradation of HO-1 in renal carcinoma induces mitotic arrest, highlighting the role of RNF139 in the control of cancer growth (P.-H. Lin et al., 2013). Additionally, RNF139 has been reported to inhibit kidney tumour growth in a RING-dependent manner via cell cycle arrest and the induction of apoptosis (Brauweiler et al., 2007).

RNF5/RMA1 is an ER ubiquitin ligase containing two TMDs, originally found in association with paxillin (Didier et al., 2003), a focal adhesion protein involved in several signalling pathways (Schaller, 2001). RNF5 catalyses K63-linked ubiquitination of paxillin, reducing its concentration at focal adhesions. By regulating paxillin's localisation, RNF5 regulates cancer cell motility. In addition, RNF5 mediates the degradation of mutant CFTR in cooperation with AMFR or CHIP (Morito et al., 2008; Younger et al., 2006). Interestingly, it has been shown to have an inhibitory effect on ERAD prior to and after ER stress. At the ER membrane, RNF5 binds to and ubiquitinates JAMP (JNK-associated membrane protein). Non-canonical ubiquitination of JAMP prevents its interaction with VCP/p97 and the proteasome, resulting in impaired recruitment of the degradation machinery at the ER (Tcherpakov et al., 2009). It has been reported that RNF5 also has roles in immunity and during pathogen infection. In particular, it is required by *Salmonella* to escape to the cytosol via ubiquitination of the *Salmonella* effector SopA (*Salmonella* outer protein A), therefore contributing to bacterial proliferation and enteropathogenicity (Y. Zhang et al., 2005). It has been shown that, during viral infection, RNF5 relocates from the ER to the mitochondria, where it targets the

immune modulator MITA/STING (Stimulator of interferon genes protein) to degradation via ubiquitination at K150, resulting in a reduced antiviral response (B. Zhong et al., 2009). High levels of this ligase are associated with poor prognosis and metastasis in breast cancer, melanoma and other cancers, while RNF5 depletion inhibits cell proliferation (Bromberg et al., 2007). Finally, by mediating ubiquitination and the degradation of the glutamine transporter SLC1A5 (Sodium-dependent neutral amino acid transporter type 2), RNF5 predicts the response to ER stress-inducing chemotherapies in breast cancer (Jeon et al., 2015).

In summary, E3-mediated ubiquitination at the ER is responsible for governing both protein turnover and degradation of misfolded substrates, as well as proteasome-independent pathways such as lysosomal trafficking and immune signalling. The majority of substrates and biological processes regulated by the ER-resident E3s remain unknown. The characterisation of E3 substrates and cofactors may help to elucidate the contribution of this class of enzymes to ERAD and cellular homeostasis.

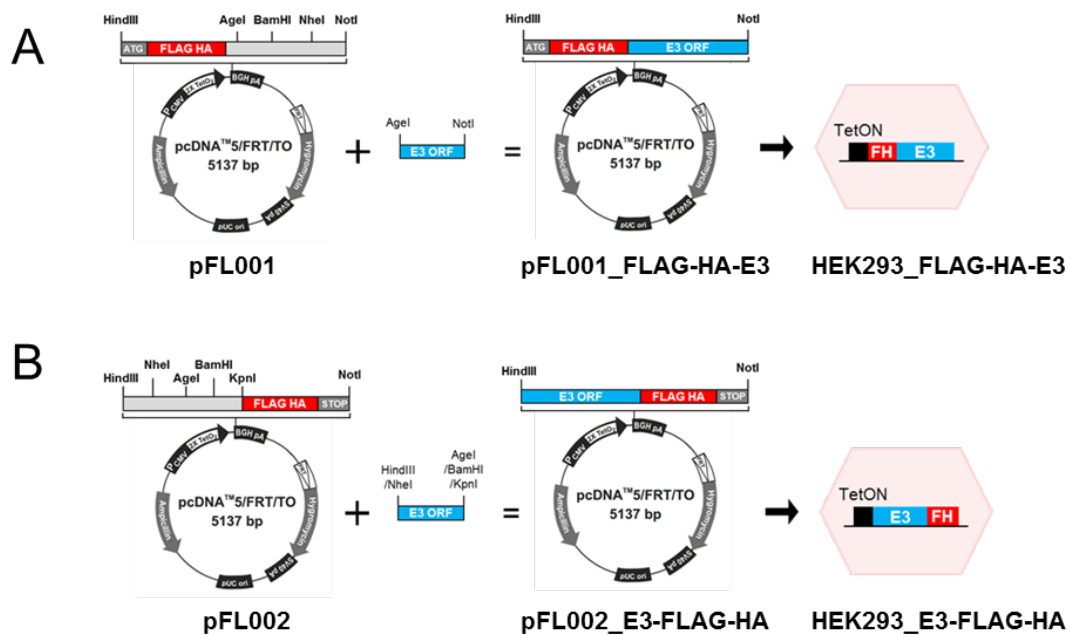
## 3.2 Aim

Almost one third of the human proteome (approximately 7,000 proteins) matures in the ER, where the ERAD machinery is responsible for overseeing protein folding and targeting misfolded variants for degradation. In this process, ER-resident E3 ubiquitin ligases play a central role in mediating ubiquitination and the subsequent disposal of misfolded substrates by cytoplasmic proteasomes. While yeast only expresses two E3s in the ER (Hrd1 and Doa10), the human ER contains at least 25 E3 ubiquitin ligases. This evolutionary diversification may have been required to respond to the more sophisticated nature of secretory proteins in metazoans. In addition to a role in degradation, Ub signalling from the ER has the potential to control a number of cellular functions, including the immune response signalling, lipid and calcium homeostasis, as well as cell fate decisions. Our understanding of the biological functions of the ER-resident E3s is still limited, with Hrd1 and AMFR having received most attention over the last years. Identifying the interactors and substrates of these ER E3s would be a first step in determining their physiological roles within the cell. This project aims to elucidate the participation of the ER-localised E3s in ERAD and other physiological processes by analysing the organisation of the E3 complexes with components of the degradation machinery, as well as by identifying novel cofactors and potential substrates. An affinity purification LC-MS/MS strategy is used to identify high confidence interactors and candidate substrates of each E3. Participation of these ligases in maintaining ER homeostasis is assessed by measuring UPR induction upon siRNA-mediated depletion of individual E3s. Finally, transcriptional regulation in response to ER stress-inducing drugs is also addressed to highlight the engagement of ER-resident E3s in the resolution of ER stress.

### **3.3 Results**

#### **3.3.1 Generation of FlpIn T-REx HEK293\_FLAG-HA-E3 cell lines**

In order to determine the composition of the ER-resident E3 complexes, each ubiquitin ligase was affinity purified and analysed by mass spectrometry. To do so, cell lines were generated encoding FLAG-HA tagged versions of the E3s under a doxycycline (DOX) inducible promoter. This strategy allowed controllable expression levels while circumventing the lack of available antibodies for robust IP of most of these E3s. To limit the risk of aberrant integration of the constructs of interest, FlpIn T-REx HEK293 cell lines were chosen as the expression system. These cells already contain a single Flp Recombination Target (FRT) site integrated in the genome to allow recombination with the expression vector carrying the cDNA of the individual E3s. Two versions of the expression vector pcDNA5/FRT/TO were created to encode a FLAG-HA sequence in-frame with the multiple cloning site, so that each E3 ORF could be subsequently cloned upstream or downstream of the FLAG-HA, to generate either N- or C-terminally-tagged versions. The position of the tag was chosen for each E3 based on previous studies (Maruyama et al., 2008; Neutzner et al., 2011; van den Boomen et al., 2014), predicted topology and/or the presence of a signal sequence. The final constructs containing FLAG-HA-tagged E3s were transfected into FlpIn T-REx HEK293 cells, with the clones containing the cassette properly integrated into the FRT site selected using Hygromycin B. The final selected population was an isogenic pool, which has the advantage of avoiding potential clone-specific off-target effects. A schematic of the cloning strategy and process used to generate the stable cell lines is depicted in Figure 3.2.

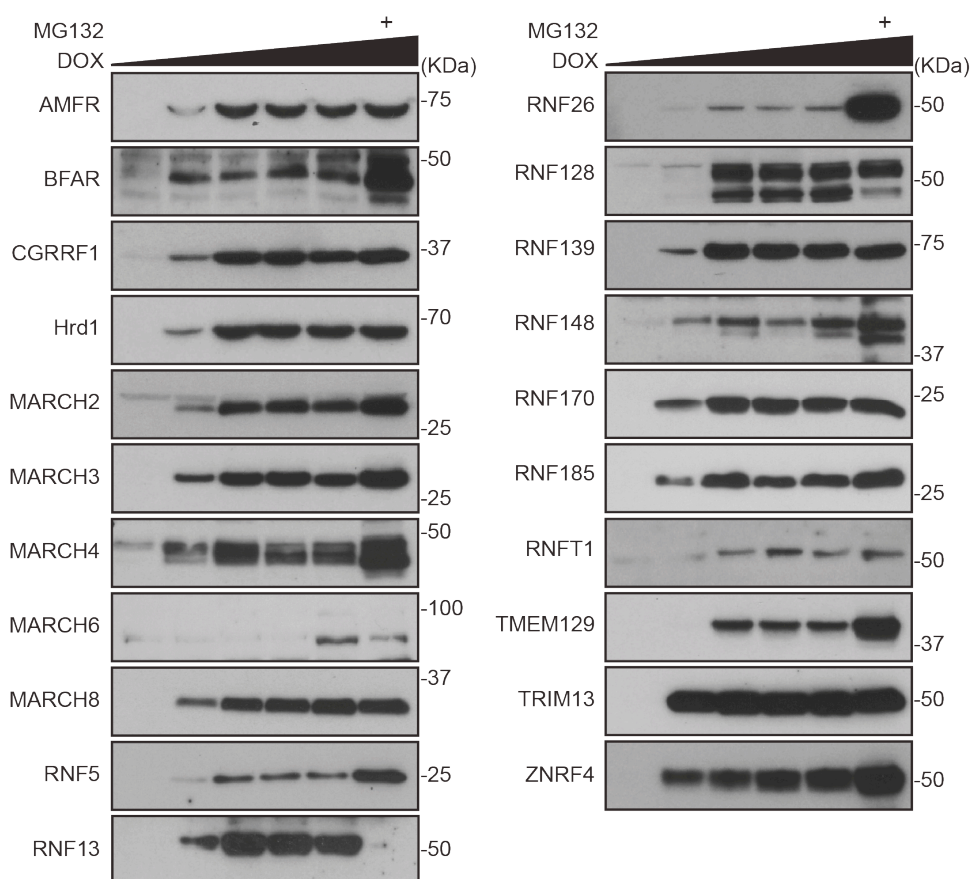


**Figure 3.2 Generation of FlpIn T-Rex HEK293\_FLAG-HA-tagged E3 cell lines**

Schematic of the cloning strategy and generation of FlpIn T-REx HEK293 cell lines with N-terminal (A) or C-terminal (B) FLAG-HA-tagged E3s stably integrated.

### 3.3.2 Determination of FLAG-HA-E3 expression and localisation

A DOX titration was performed on HEK293\_FLAG-HA\_E3 cell lines to determine the inducibility of the different E3s and choose the concentration that allowed sufficient detectable expression. Cells were stimulated with four doses of DOX (1, 10, 100 or 1,000 ng/ml) for 18 hours. In addition, 4 hours before harvesting, the proteasome inhibitor MG132 (10 µg/ml) was added to the highest dose of DOX to rescue the E3s that may have been intrinsically unstable and targeted to degradation, possibly as a consequence of overexpression. Total proteins were extracted and expression of the individual tagged E3s was analysed by Western blot (WB) using antibodies against the FLAG or HA tag (Figure 3.3).



**Figure 3.3 DOX titration of FLAG-HA-E3s**

WB for FLAG (or HA in the case of MARCH3) from WCL of HEK293\_FLAG-HA\_E3 cell lines induced with DOX. DOX was used at 1, 10, 100 or 1,000 ng/ml for 18 hours. MG132 (10 µg/ml) was added to the highest dose of DOX for 4 hours.

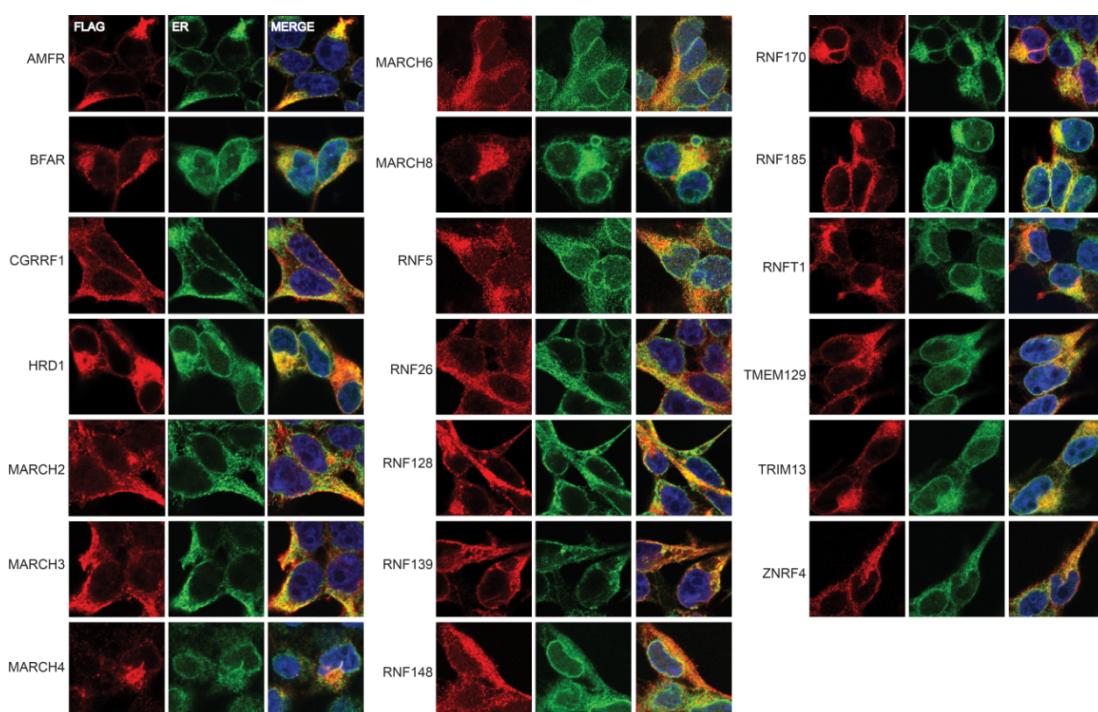
The expected molecular weight of each expressed E3 was confirmed by comparison to molecular size markers as a reference. In cases where no expression was detected, the tag was repositioned at the opposite terminus of the ORF and the new cell line was tested for E3 expression and ER localisation. All FLAG-HA-tagged E3s were detectable from WCL, with the exception of RNF103 and TRIM59, which could only be resolved by mass spectrometry. Despite several attempts, RNF145 and RNF150 could not be expressed nor detected, and we therefore decided to exclude these two ligases from the analysis. Table 10 summarises the DOX concentrations that were chosen to induce each E3 prior to immunoprecipitation for mass spectrometry.



| E3 name   | DOX (ng/ml) | E3 name   | DOX (ng/ml) | E3 name   | DOX (ng/ml) | E3 name    | DOX (ng/ml) |
|-----------|-------------|-----------|-------------|-----------|-------------|------------|-------------|
| AMFR-FH   | 1           | MARCH4-FH | 10          | RNF103-FH | 100         | FH-RNFT1   | 10          |
| FH-BFAR   | 1           | FH-MARCH6 | 1000        | RNF128-FH | 10          | TMEM129-FH | 1           |
| CGRRF1-FH | 1           | MARCH8-FH | 1           | RNF139-FH | 10          | TRIM13-FH  | 1           |
| HRD1-FH   | 1           | FH-RNF5   | 10          | RNF148-FH | 1           | TRIM59-FH  | 10          |
| MARCH2-FH | 10          | RNF13-FH  | 1           | RNF170-FH | 1           | ZNRF4-FH   | 1           |
| MARCH3-FH | 1           | RNF26-FH  | 1000        | RNF185-FH | 1           |            |             |

**Table 10 Dose of DOX used to induce FLAG-HA-E3s for affinity purification**

To confirm localisation of the overexpressed E3s to the ER, immunofluorescence analysis was performed after 18 hours of DOX induction. Co-localisation with the ER marker CNX or KDEL was monitored by confocal microscopy (Figure 3.4).



**Figure 3.4 Immunofluorescence for FLAG-HA-E3s**

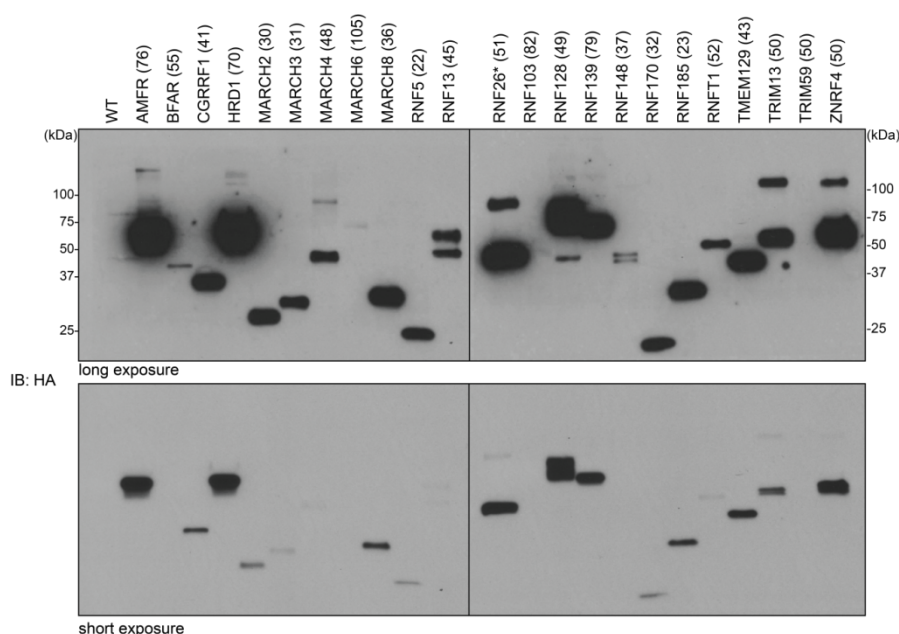
FLAG-HA-E3 expression was obtained by DOX treatment for 18 hours. Nuclei were stained with DAPI,  $\alpha$ -calnexin or  $\alpha$ -KDEL were used as ER markers and  $\alpha$ -FLAG was used against the E3s. Secondary Alexa-conjugated antibodies were used to detect localisation. Images were acquired by confocal microscopy, objective 63 $\times$ .

It was established that all FLAG-HA-E3s were in the ER based on significant colocalisation with ER markers. Only RNF103 and TRIM59 could not be detected due to low expression levels. Importantly, all ligases displayed a diffuse, soluble

pattern and no punctae that could reflect aggregation, an important consideration when the aim is to analyse native complexes.

### 3.3.3 Affinity purification of E3 protein complexes

Each step in the sample preparation process for mass spectrometry was optimised for efficient pull-down and the preservation of native complexes. Upon inducing each individual E3 with the selected DOX concentration for 18 hours, all samples were prepared simultaneously for mass spectrometry to reduce potential variations arising from sample processing. Selected ionic and non-ionic detergents were used for solubilisation followed by immunoprecipitation of Hrd1, and were thereafter tested for their capability to preserve weak interactors of the Hrd1 complex (data not shown). LMNG was chosen as the mildest detergent in which the weakest Hrd1 interactors OS-9 and Herp were retained. Therefore, all E3 complexes were extracted from the generated cell lines in 1% LMNG detergent. WB analysis of the E3s from WCL revealed a significant heterogeneity in the expression levels (Figure 3.5).



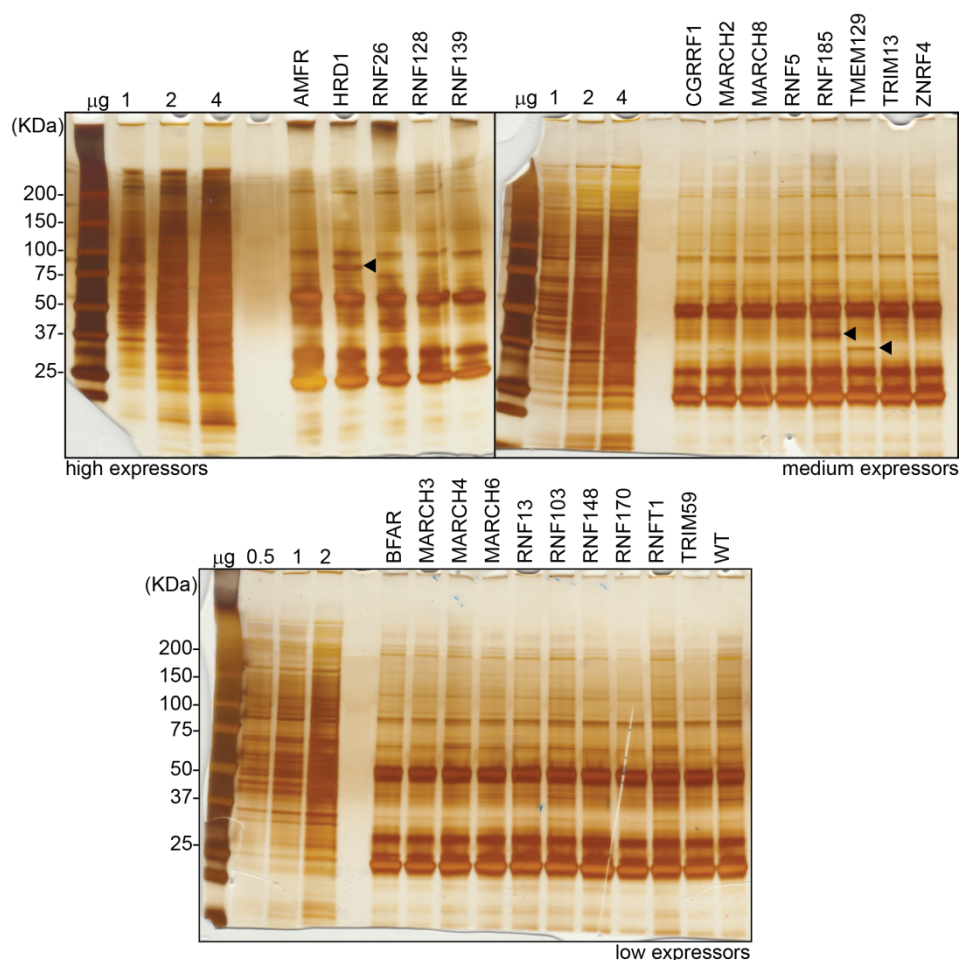
**Figure 3.5 Expression levels of FLAG-HA-E3s**

WB for HA on WCL from HEK293\_FLAG-HA-E3 cell lines induced with the chosen DOX concentration (see Table 10) for 18 hours.

A range of steady-state levels of the E3s was observed: high (AMFR, Hrd1, RNF26, RNF128, RNF139 and ZNRF4), medium (CGRRF4, MARCH2, MARCH8, RNF5, RNF185, TMEM129 and TRIM13) and low (BFAR, MARCH3, MARCH4, MARCH6, RNF13, RNF103, RNF148, RNF170, RNFT1 and TRIM59), which could reflect differences in intrinsic stability between E3s. Most E3s expressed as a single band and exhibited a molecular weight close to what was expected, while additional bands were detected in several instances (e.g. RNF128, MARCH4, RNF13, TRIM13 and ZNRF4). Double bands appearing for RNF13 and RNF148 most likely represent different glycosylated forms, as previously reported for RNF13 (Q. Zhang et al., 2009) and predicted for RNF148 (UniProt source). RNF26, TRIM13 and ZNRF4 displayed a major band at the correct molecular weight and a minor band of approximately double size, possibly reflecting the formation of DTT- or SDS-resistant dimers. The same was observed for RNF128, although in this case the dimeric form was more prominent than the monomeric protein. RNF170 was resolved at a significantly lower molecular weight than expected (approximately 22 kDa instead of 32 kDa); however, this observation is in agreement with previously published data (Wright et al., 2015). As mentioned above, the expression of RNF103 and TRIM59 could not be detected in WCL. Overall, 21 out of 23 FLAG-HA-tagged E3s were successfully detected by WB.

Following lysis of each cell line in 1% LMNG, FLAG-HA-tagged E3s were affinity purified by FLAG agarose beads. FLAG beads were chosen over the HA beads as they exhibited higher protein yield in pull-downs. Mild wash conditions using IP buffer without detergent were used to preserve weak interactions, and therefore the integrity of the complexes. Bead-bound complexes were eluted using 2× SDS-10% BME, since it showed a greater efficiency of elution than the FLAG peptide (data not shown). Although elution by SDS typically produces more complex samples with

higher non-specific backgrounds in mass spectrometry analysis, these non-specific binders were used in our data analysis to normalise samples (see below). To compare the background intensities of the different immunoprecipitated E3 complexes, a fraction corresponding to approximately 8% of each eluate was analysed by SDS-PAGE followed by silver staining (Figure 3.6).



**Figure 3.6 Total protein intensities from induced HEK293\_FLAG-HA-E3 cell lines**

Silver staining of 8% eluate from immunoprecipitated FLAG-HA-E3 samples. Eluates were run in three batches according to the E3 expression levels (high, medium or low). Black arrows indicate the band corresponding to the E3 in those cases where expression was detectable. 0.5, 1, 2 and 4 µg of denatured HEK293 WCL was loaded as a reference.

Despite heterogeneous E3 expression levels, similar background intensities were observed across all samples, an important prerequisite for chromatogram alignment and downstream comparative data analysis. The eluted protein complexes were

reduced and alkylated to irreversibly break disulphide bonds between cysteines, allowing optimal unfolding for subsequent enzymatic cleavage. Double methanol-chloroform precipitation was carried out to remove excess SDS prior to tryptic digestion and final purification on C18 Sep pak columns. The 23 samples were run in two batches that were subsequently merged, and the batch effects were removed using an algorithm developed for microarray analysis (Limma package, R). For each sample, the purified peptides were analysed in duplicate in a tandem mass spectrometer coupled with UHPLC.

### **3.3.4 MS/MS raw data processing for generation of *p*-values**

LC-MS/MS of all samples identified  $>1.5 \times 10^6$  spectra. Raw spectral data were uploaded onto Progenesis QI software for chromatogram alignment, a prerequisite of comparative expression profiling. Progenesis QI uses raw peptide abundance values to calculate peptide intensities. Importantly, protein quantification is based on unique peptides only. Peptide identity was assessed by the SwissProt Human database, which generated a matrix of 2,274 hits across the 23 baits. Mean intensities from the two technical replicates were calculated for each protein hit in every sample and subsequently  $\log_2$  transformed. The standard deviation (SD) of each protein across all samples was calculated and plotted against the SD rank. A 90% cut-off was applied to this plot to define the background proteome, which comprised the proteins exhibiting the least variation in intensity across all samples. The background proteome was used for median normalisation of the  $\log_2$ -transformed mean intensities of each sample and the Cyclic Loess method was employed to standardise sample distributions without forcing equal distribution. The intensity of each protein in each sample was divided by the mean intensity of that protein in all other samples to generate fold change values for every identified hit. Fold changes were then converted to *p*-values using MaxQuant Significance A (Cox and Mann, 2008), a method used to derive *p*-values from two replicates instead of

the classical Student's *t*-test, which requires a minimum of three replicate values. The higher the fold change of a particular protein across the samples, the lower its corresponding *p*-value. Final *p*-values were FDR (false discovery rate)-corrected (Benjamini–Hochberg correction).

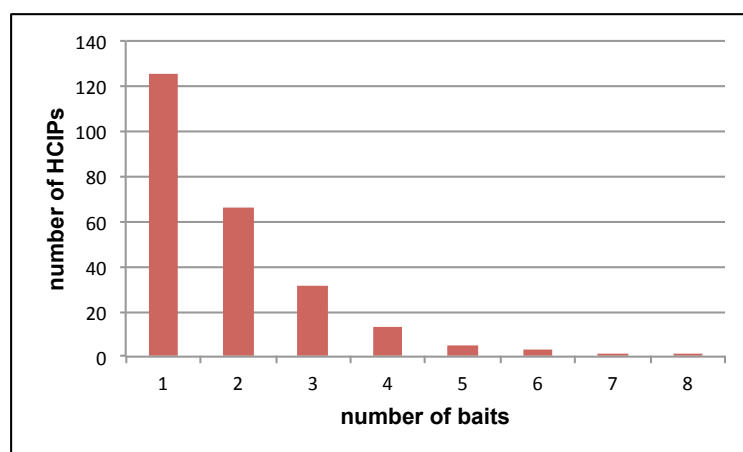
### **3.3.5 Identification of high confidence interactors**

A series of filtering steps was performed to define the High Confidence Interactor Proteins (HCIPs) for each bait. Firstly, a threshold of <0.05 was applied to the *p*-value scores, resulting in an average of 100 protein hits per E3. Subsequently, contaminants (defined as nuclear or cytoskeletal proteins, see contaminant list in the Appendix) and proteins with a negative fold change and only identified by one peptide were removed. On average, this resulted in the identification of about 20 HCIPs for each E3-bait. The UniProt Retrieve/ID mapping function was used to annotate the HCIPs. Given the specialised localisation of these E3 protein complexes, the following features were used to classify the HCIPs: signal peptide, *N*-linked glycosylation, disulphide bonds and TMDs. Therefore, the HCIPs were classified as either cytosolic or transmembrane and/or ER-resident proteins. Due to the comparative nature of the Progenesis Q1 analysis (based on peptide intensity), protein hits could only be assigned a *p*-value in one sample if they were present in at least one other sample. For this reason, interactors unique to only one E3 may risk being disregarded. To this aim, the SING tool (Trudgian et al., 2011) was employed, which uses spectral count index quantitation to score protein hits. Unique interactors to each E3 were ranked according to their SING score and a cut-off of  $>1 \times 10^{-8}$  was used to define the most significant HCIPs. These were annotated as cytosolic, membrane and/or ER proteins as described above.

### **3.3.6 A comparative method for MS/MS data analysis**

One of the main problems in analysing proteomic datasets is the difficulty in distinguishing between background proteins and true interactors. In the case of

large datasets, it becomes particularly important to employ a rigorous pipeline for data normalisation, so that specific interactors are enriched over the non-specific binders. Building on a previous study (Keilhauer et al., 2015), this comparative methodology was modified to take advantage of the high number of background binders to enrich for bait-specific interactors. Instead of employing standard normalisation techniques based on a control group represented by an untagged control strain, the large number of samples allowed the definition of a background proteome to use for median normalisation as a bait-specific control group (BCSG) (Keilhauer et al., 2015). For this reason, stringent immunoprecipitation conditions were not required, as the non-specific interactors common to all or many samples are then identified as background and used for normalisation. Despite the majority of the identified HCIPs being specific for single baits, this analytical method retained the capability of distinguishing background interactors from HCIPs shared by up to eight different E3s (Figure 3.7).



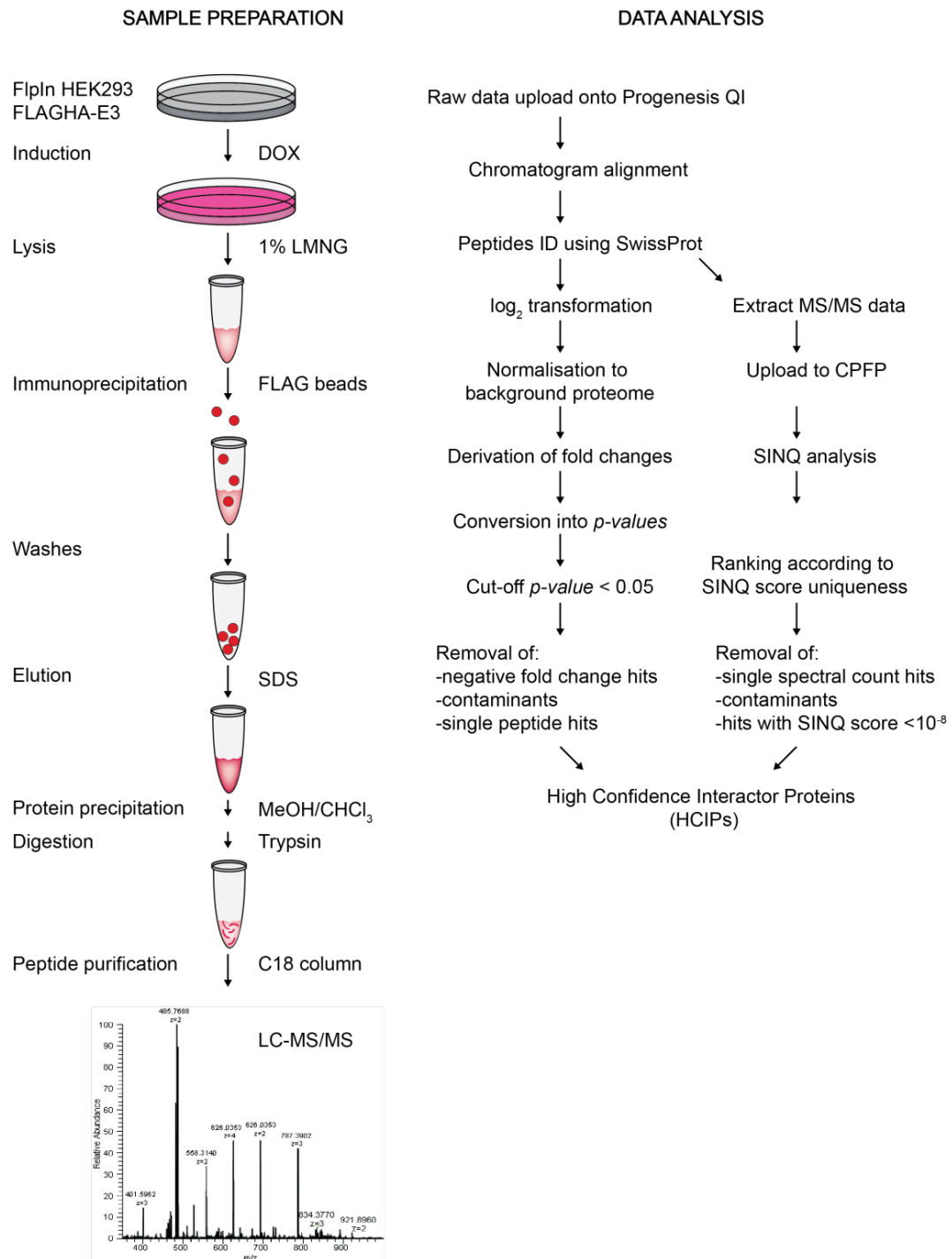
**Figure 3.7 Distribution of the frequency of unique and shared HCIPs**

The majority of HCIPs (125/460) were found to be specific interactors of a single E3-bait. However, HCIPs shared by up to eight baits were still assigned as specific interactors.

Due to the comparative nature of this type of analysis, the SINQ software was additionally employed to find unique interactors that could have been disregarded in the Progenesis data processing. The combination of peptide intensity-based

analysis (by Progenesis) and spectral count-based analysis (by SING) allowed the generation of a complete list of unique and shared HCIPs. Starting from a matrix of more than 2,000 proteins identified per bait, the list was narrowed down to an average of 20 HCIPs per E3. Of the 460 HCIPs across all samples, 95% of these proteins were interactors discovered in this study. Figure 3.8 summarizes the pipeline used to generate the E3 interaction network, from the construction of the cell lines to data analysis.



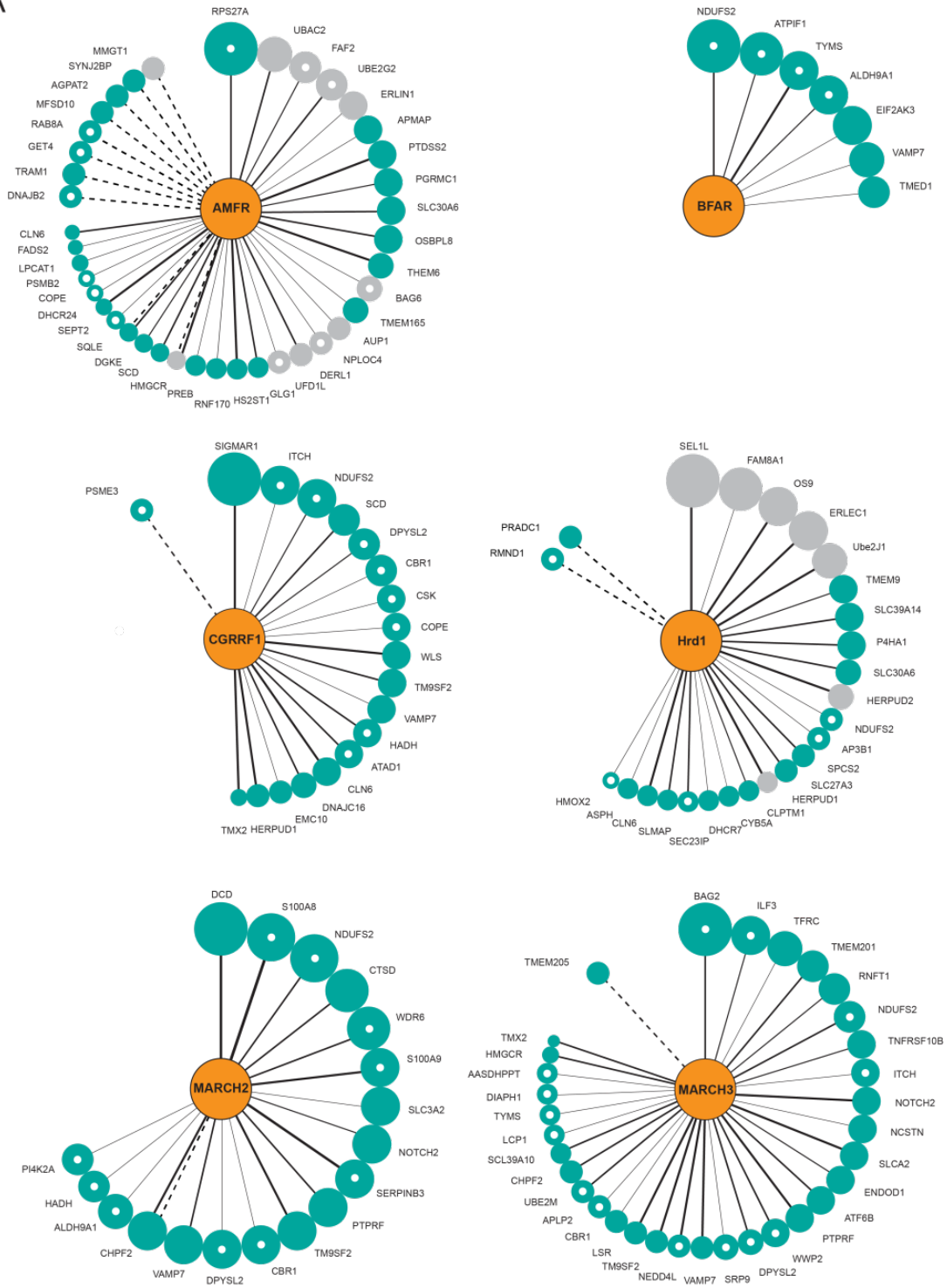


**Figure 3.8 Workflow for sample preparation and MS/MS data analysis**

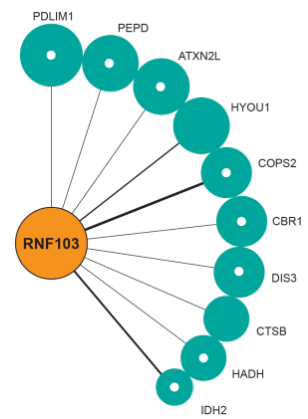
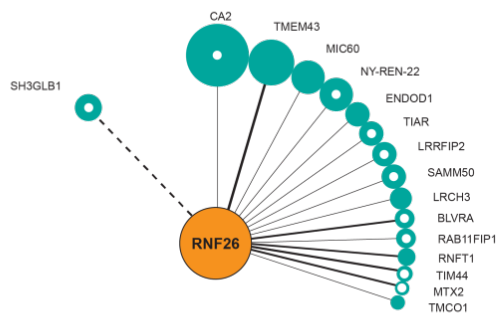
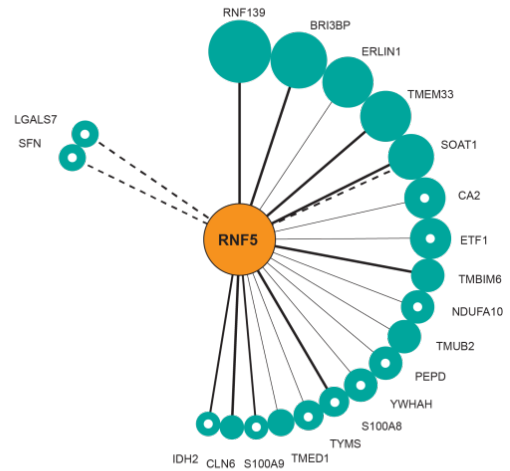
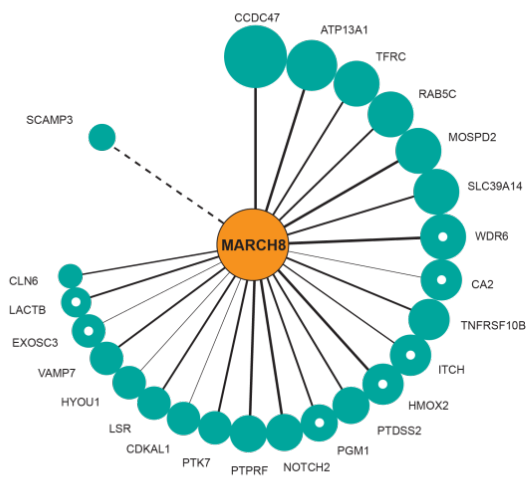
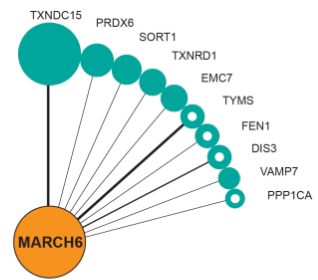
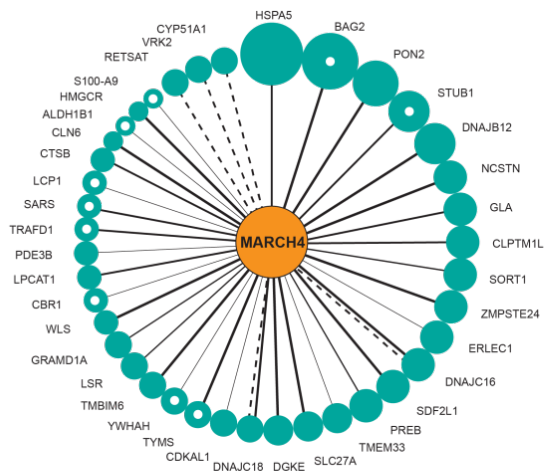
### 3.3.7 Interaction networks of E3 ubiquitin ligases

High confidence interactors of 23 ER-resident E3 ubiquitin ligases were mapped (Figure 3.9 A–D).

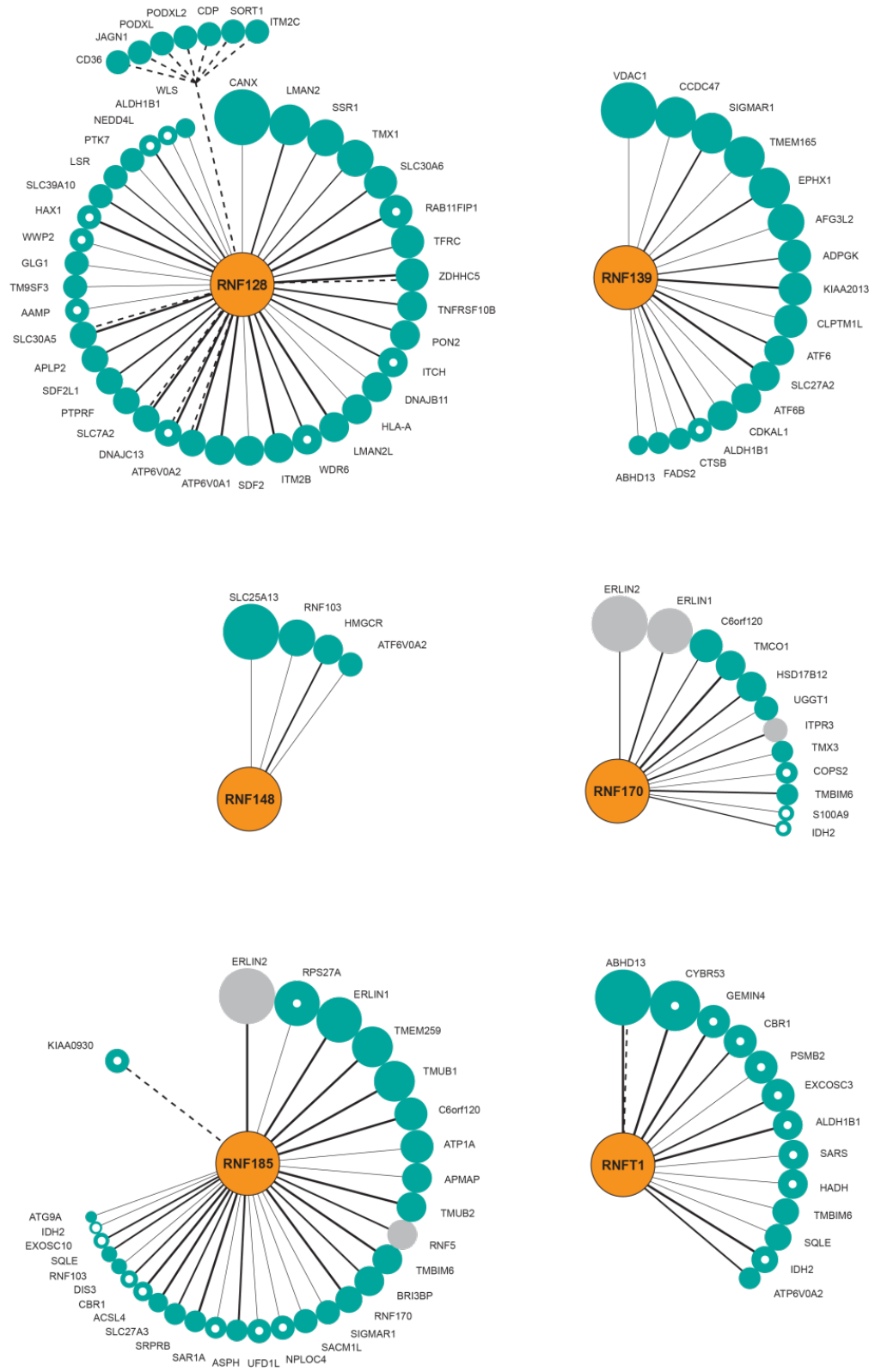
A



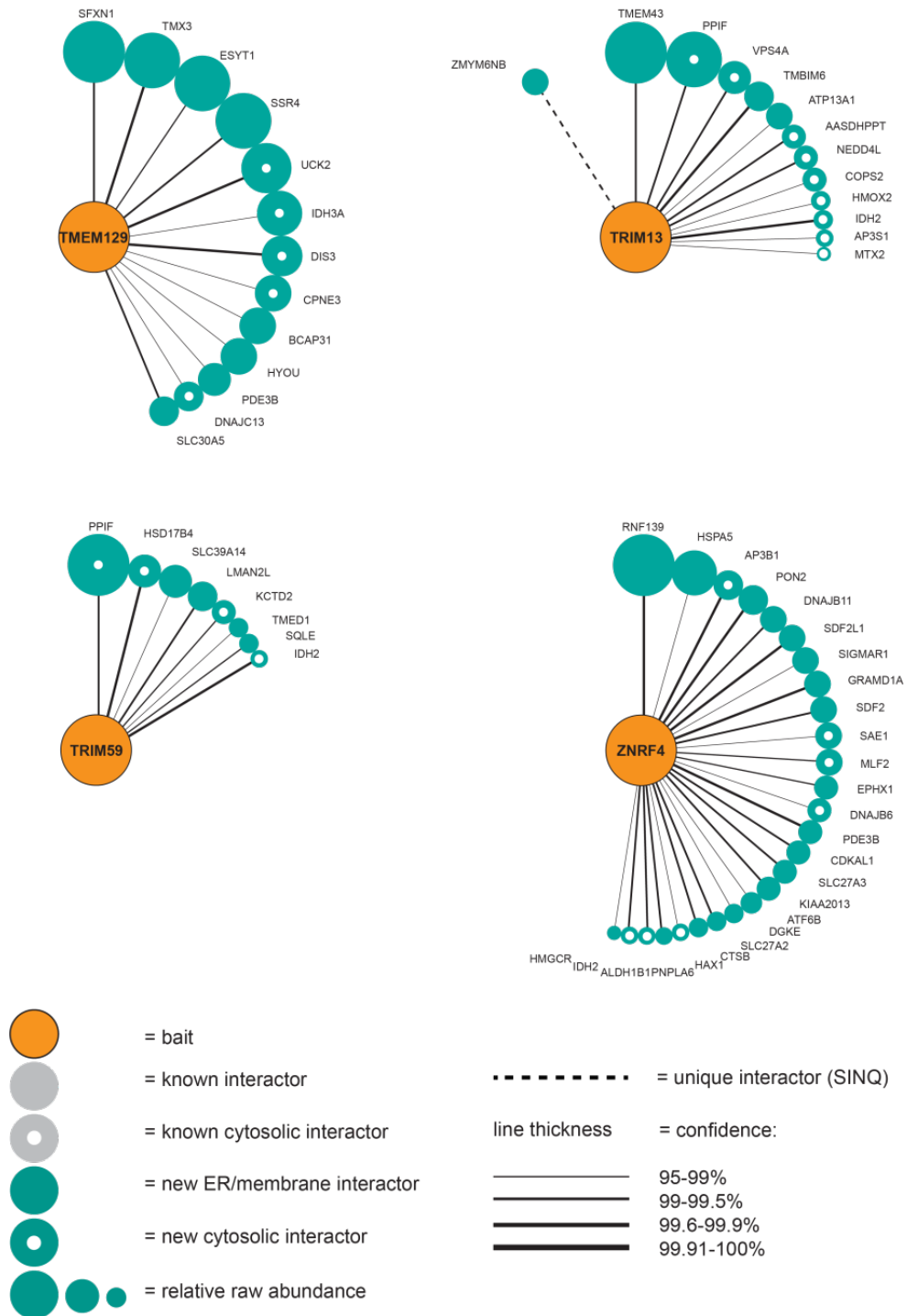
B



C



D



**Figure 3.9 Interaction network of ER-resident E3s**

Each bait (E3) is represented by an orange circle, connected to its HCIPs. Solid lines represent HCIPs found by Progenesis, whereas dashed lines represent those found by SINQ. The line thickness reflects the confidence of the interaction, and the size of the HCIPs reflects raw abundance normalized to the most abundant interactor in each bait. Coloured circles are new interactors, grey circles are known interactors. The white dot indicates cytosolic proteins, whereas the absence of the white dot indicates ER-localised or transmembrane proteins.

Each E3-bait is represented as a central node (in orange), connected to the HCIPs by lines, the thickness of which reflects the confidence of the interaction (the thicker the line, the lower the *p*-value). Interactors identified by Progenesis (solid line) and SINQ (dashed line) are indicated. In several instances, unique interactors found by SINQ were found to also possess a significant *p*-value (i.e. RNF5-SOAT1 interaction). This situation can arise due to a discrepancy between the peptide cut-off number of the two analytical techniques. Specifically, a peptide cut-off of one was applied to the Progenesis data processing, while two peptides were required for analysis by SINQ. Therefore, if a HCIP identified by Progenesis only possesses one peptide in all other samples, it will be identified as a unique interactor by SINQ. The final list of HCIPs for each bait was ranked according to the raw abundance. The size of each HCIP circle reflects the relative abundance of that protein normalised to the most abundant HCIP in that bait, which is depicted as the biggest circle. Based on the range of normalised abundances, 18 subgroups were defined, each represented by different circle sizes. For instance, in the case of Hrd1, the abundances of its HCIPs are normalised to the most abundant interactor, SEL1L. Newly found and previously reported interactors are coloured in blue and in grey, respectively. Finally, cytosolic proteins are distinguished from integral membrane/ER proteins by the presence of a white dot inside the circle.

In general, fewer HCIPs were identified from the lowest expressed E3s (BFAR, MARCH6, RNF103, RNF148 and TRIM59). Importantly, previously identified interactors of the well characterised E3s Hrd1 and AMFR were found, supporting the validity of the experimental and analytical methods used. Despite the extensive attention that these E3s have received in the past, 18 and 29 additional HCIPs were identified for Hrd1 and AMFR, respectively.

In the case of Hrd1, in addition to the extensively described SEL1L, FAM8A1, OS-9, ERLEC1, Ube2j1 and Herp, 10 out of the 16 new HCIPs found by Progenesis possessed a high confidence score with a  $p$ -value  $<0.01$  (TMEM9, SLC39A14, P4HA1, SLC30A6, SPCS2, SLC27A3, CLPTM1, SEC23IP, SLMAP and CLN6), and 7 of these HCIPs were also found to be highly abundant. Table 12 (Appendix) summarises the newly found HCIPs for Hrd1, with associated biological processes (modified from UniProt and GO annotations database). Of interest, enzymes and transporters responsible for ion homeostasis (ZIP14, ZNT6, ASPH and HMOX2) and protein trafficking across the secretory pathway (SPCS2 and S23IP) were found, in addition to mitochondrial proteins (NDUS2, S27A3, CYB5 and RMND1) as well as proteins regulating the lysosomal proteolytic pathway (AP3B1 and CLN6). Importantly, two interactors of Hrd1 are implicated in cell differentiation and development (CLPT1 and DHCR7), supporting the requirement for Hrd1 in embryogenesis and development, as homozygous deletion of Hrd1 results in embryonic lethality (Yagishita et al., 2005). Although physiological validation of these interactions is required to confirm *bona fide* interactors, they may reveal new roles for Hrd1 in the regulation of fundamental cellular processes independent from degradation, possibly involving different Ub linkages.

Among the uncharacterised E3s (CGRRF1, MARCH3, RNF145, RNF148, RNF150, RNFT1 and TRIM59), many significant interactors were obtained for CGRRF1, MARCH3 and RNFT1, and only a few interactors for RNF148 and TRIM59, possibly due to their low expression levels.

In the case of CGRRF1, the 20 newly discovered HCIPs (Table 13, Appendix) were found in association with five main biological processes: (i) lipid transport and metabolism (SGMR1, ACOD, HCDH and CLN6), (ii) protein trafficking (COPE, WLS, TM9S2 and VAMP7), (iii) redox regulation (NDUS2, CBR1, DJC16 and TMX2), (iv) immune response signalling (ITCH and CSK) and (v) neuronal development

(SGMR1, DPYL2, CSK and ATAD1). Interestingly, the association of CGRRF1 with the ERAD cofactor Herp and the proteasome subunit PSME3 (Proteasome activator complex subunit 3) may suggest the participation of this ligase in the ERAD pathway.

MARCH3 co-precipitated 32 HCIPs associated with multiple biological processes (Table 14, Appendix), including protein trafficking (VAMP7, TM9S2 and SRP09) and ion transport (TFR1, 4F2, NED4L and S39AA), among others. Interestingly, MARCH3 was found in association with a number of ubiquitin ligases and ubiquitin-conjugating enzymes (ITCH, WWP2, NED4L and UBC12), highlighting the potential involvement of MARCH3 in ubiquitin signalling from the ER. Several noteworthy interactors include Notch2, whose signalling is involved in proliferation and development (Bray, 2006), and Nicastrin, a subunit of the  $\gamma$ -secretase complex that mediates intramembrane proteolysis of Notch receptors and  $\beta$ APPs (Yu et al., 2000). The fact that two functionally related proteins (Notch2 and Nicastrin) were found to interact with the same E3, increases the confidence that they may represent *bona fide* interactors. Upon binding to Delta and Jagged ligands, Notch receptor is sequentially cleaved by ADAM family metalloproteases and by the  $\gamma$ -secretase complex, leading to the release of the Notch intracellular domain (NICD), which translocates to the nucleus to promote transcription. This signalling cascade is key to sustain cellular homeostasis, proliferation and development. Importantly, deregulated Notch signalling is found in several cancer types, including breast, colorectal, lung and prostate cancer, T-cell leukaemia, as well as malignancies of the CNS (reviewed in (Yuan et al., 2015). Since inhibitors of the Notch pathway are proving effective as anticancer therapies, the potential involvement of MARCH3 in the regulation of this signalling could be important.

We identified 14 HCIPs for RNFT1 (Table 15, Appendix), of which some are proteins involved in RNA processing (GEMI4, EXOS3 and SYSC), mitochondrial



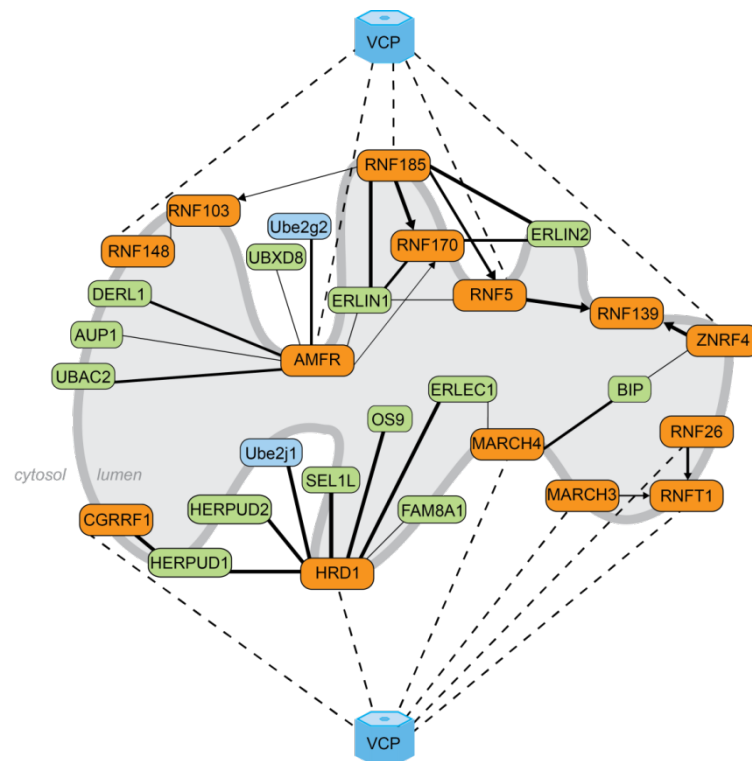
metabolic processes such as fatty acid oxidation (NB5R3 and HCDH) and carbohydrate metabolism (AL1B1, IDHP). The interaction with ERG1/SQLE suggests a potential involvement of RNFT1 in cholesterol metabolism. Among the most interesting RNFT1 interactors, we found BI1 (Bax inhibitor 1), a negative regulator of autophagosome formation (Castillo et al., 2011), and VPP2, a V-type proton ATPase subunit that mediates lysosomal acidification, which may indicate an additional role for RNFT1 in protein catabolism by autophagy.

### **3.3.8 The E3-ERAD interaction network**

The central aim of this study was to identify new interactors of ER-resident E3s that may highlight a potential role of these ligases in ERAD. The well-studied Hrd1 and AMFR/gp78 assemble into functional complexes wherein interactions with specific cofactors (e.g. SEL1L and ERLINs) promote substrate binding and catalytic activity. Given the enzymatic similarity of the ER-resident E3s to Hrd1 and AMFR, it was hypothesised that they may likewise share cofactors or ERAD components. Therefore an interaction network was generated of the E3s found in association with each other, with ERAD components (including the p97/ATPase for substrate extraction) or with E2 conjugating enzymes (Figure 3.10).

Hrd1 and AMFR appear to be central hubs for coordination of the known ERAD machinery, particularly by virtue of their interaction with the E2 conjugating enzymes Ube2j1 and Ube2g2, respectively. Strikingly, the majority of the ERAD factors do not interact with other E3s, indicating that they may assemble into distinct functional complexes. Although no association of E2 enzymes involved in ERAD with other ER-resident E3s could be found, several E3s were found in association with VCP/p97 interactors and adaptor proteins, suggesting that they may require substrate extraction from the ER as part of their activities.

Among the few E3s interacting with known ERAD components, we found CGRRF1 (Herp), MARCH4 (XTP3-B/ERLEC1; BiP), RNF185 and RNF170 (ERLIN1 and ERLIN2), and RNF5 (ERLIN1). Additionally, ZNRF4 was found to interact with the ER chaperone BiP, in agreement with its previously reported role in ER homeostasis by regulating ubiquitination and degradation of CNX (Neutzner et al., 2011). However, we cannot exclude the possibility that BiP interaction simply reflects chaperone-assisted folding of ZNRF4.



**Figure 3.10 Interaction network of ER-resident E3 with ERAD components**

E3s found to interact with each other or with ERAD components are shown in orange. HCIPs known to participate in ERAD are shown in green. The E2 ubiquitin-conjugating enzymes are presented in light blue. The position of each protein reflects its localisation to either the cytosol, ER membrane or lumen. Thickness of the line reflects confidence of the interaction. Arrowhead lines indicate the relationship with the bait. Dashed lines indicate that the interaction with VCP was not found directly but by means of association with VCP adaptors or VCP-binding proteins.

Interestingly, a subset of E3s cluster with one another by means of direct interactions or shared interaction partners (e.g. via ERLINS). A major cluster of E3s represented by RNF185, RNF103, RNF148, RNF170, RNF5, RNF139, ZNRF4 and

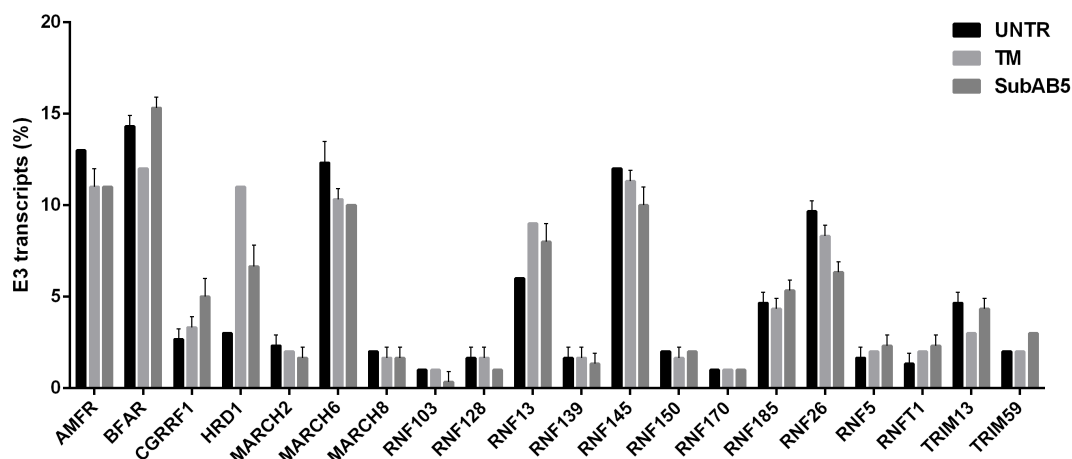
AMFR was identified. However, further experiments on the endogenous E3s are necessary to validate the existence of such a cluster. Co-sedimentation patterns of these E3s in a sucrose gradient may prove useful to support their residency in the same complexes. If validated on the endogenous proteins, the existence of an E3 cluster within the ER membrane could reflect cooperation in the same signalling pathway. Despite the fact that RNF185 was not the most highly expressed E3 among the others in the cluster, it co-purified with four other E3s, suggesting that it may constitute the core complex component. Within this network, ERLIN1, and to a less extent ERLIN2, are shared partners. The ERLIN1/2 complex was first identified as the complex responsible for degradation of inositol 1,4,5-trisphosphate receptors (IP3Rs), whose primary role is to control calcium release from the ER (Pearce et al., 2009). A later study implicated ERLIN1/2 in the regulation of cholesterol homeostasis by promoting the stability of SREBPs in complex with Scap and Insig-1 (Huber et al., 2013). In addition to ERLIN1 and ERLIN2, which were the most abundant and significant interactors of RNF185 (together with Ub), a significant association was also found with ERG1/SQLE, pointing towards a possible role for this ligase in cholesterol regulation. RNF185 is implicated in the degradation of CFTR (Khouri et al., 2013) and in the regulation of mitophagy (F. Tang et al., 2011). The role of RNF185 in ERAD is further supported by the data presented here, which found it associated with the VCP/p97 adaptors Npl4 and Ufd1. In addition to SQLE, other components of cholesterol metabolism and fatty acid regulation were also enriched among RNF185 HCIPs (TMUB1, APMAP, SAC1, S27A3 and ACSL4), suggesting a potential new role of this ligase in lipid metabolism. In agreement with a regulatory role of RNF185 in mitophagy, we found the new interactor ATG9A, a known factor in autophagic degradation. Intriguingly, RNF185 has been reported to localise both to the ER and the mitochondria. Interestingly, we were able to identify several interaction partners localised at the mitochondria (BI1, BRI3B, S27A3,

ACSL4 and IDHP), which may broaden the existing role of RNF185 in this organelle. Table 16 (Appendix) summarises the newly found HCIPs for RNF185 with associated biological processes (modified from UniProt and GO annotations).

### **3.3.9 E3 transcriptional regulation under ER stress**

ER chaperones and ERAD components, including Hrd1, are induced by ER stress in an attempt to restore homeostasis (Friedlander et al., 2000). Therefore, it was hypothesised that other ER-resident E3s may also be induced by stress, reflecting a potential role in protein degradation and regulation of ER homeostasis. To investigate the transcriptional response of these E3s to ER stress, we stimulated wild-type FlpIn T-REx HEK293 cells with Tunicamycin (TM) and Subtilase AB<sub>5</sub> (SubAB<sub>5</sub>). While the former blocks *N*-linked glycosylation of nascent proteins undergoing maturation, the latter is an active toxin that inactivates the principal ER chaperone BiP (Paton et al., 2006), resulting in substantial protein misfolding in the ER lumen. Dose and length of treatment were titrated to achieve maximal transcriptional induction of known stress markers (BiP, HERP and CHOP) without causing cell death (data not shown). TM was used at 500 ng/ml for 8 hours, while SubAB<sub>5</sub> was administered at 10 ng/ml for 8 hours. Following treatment, cells were harvested and the RNA was extracted for expression analysis. mRNA transcripts of all ER-resident E3s (except TMEM129, which was included later in the study) and several other ERAD genes were measured using the Nanostring transcript quantification platform (Geiss et al., 2008). This multiplexed system provides a highly sensitive method for direct detection of mRNAs via hybridisation between transcripts and molecular barcodes (nCounter Reporter Probes), which are then digitally imaged and quantified. The final output is absolute number of transcript molecules per sample. At basal levels, a marked heterogeneity in E3 expression could be observed, where Hrd1 transcript represented only 3% of the total ER-resident E3s transcriptome (Figure 3.11). The most highly expressed E3s were

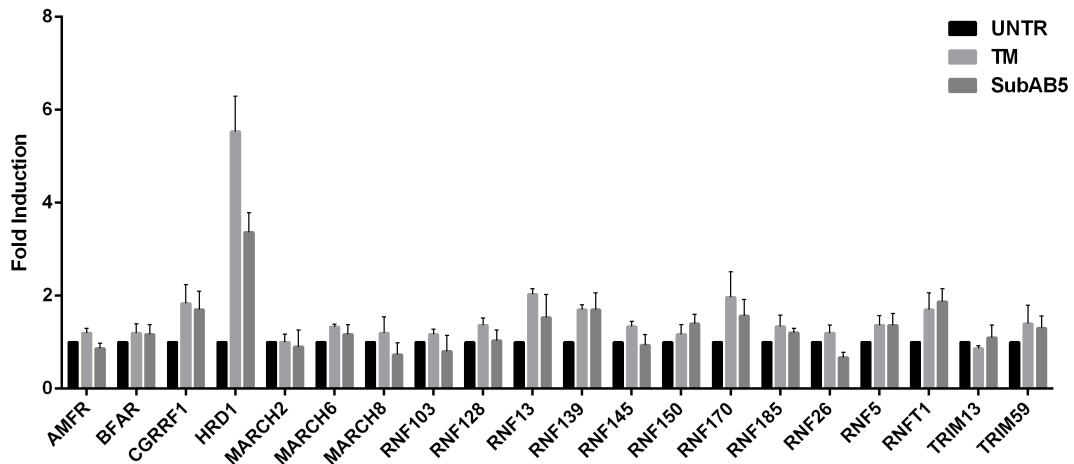
AMFR, BFAR, MARCH6, RNF145 and RNF26, together constituting over 50% of the total E3 transcriptome analysed.



**Figure 3.11 Transcriptome of ER-resident E3s at basal and stressed conditions**

Nanostring analysis on total mRNA extracted from wild-type FlpIn T-REx HEK293 cells either untreated, treated with TM (500 ng/ml) or with SubAB<sub>5</sub> (10 ng/ml) for 8 hours. Data are expressed as % of each transcript relative to the total number of E3 transcripts. Error bars indicate SD from three biological replicates.

Following treatment with either TM or SubAB<sub>5</sub>, Hrd1 and RNF13 contributed significantly to the most prominent E3s constituting, together with AMFR, BFAR, MARCH6, RNF145 and RNF26, over 70% of the total. The transcript levels of MARCH3, MARCH4, RNF148 and ZNRF4 were at the threshold of detection in every condition tested. Therefore quantitative measurements for these ligases could not be obtained. Importantly, Hrd1 was the most strongly upregulated E3 in response to stress, undergoing an increase of almost six-fold upon TM treatment and three-fold under SubAB<sub>5</sub> addition (Figure 3.12).

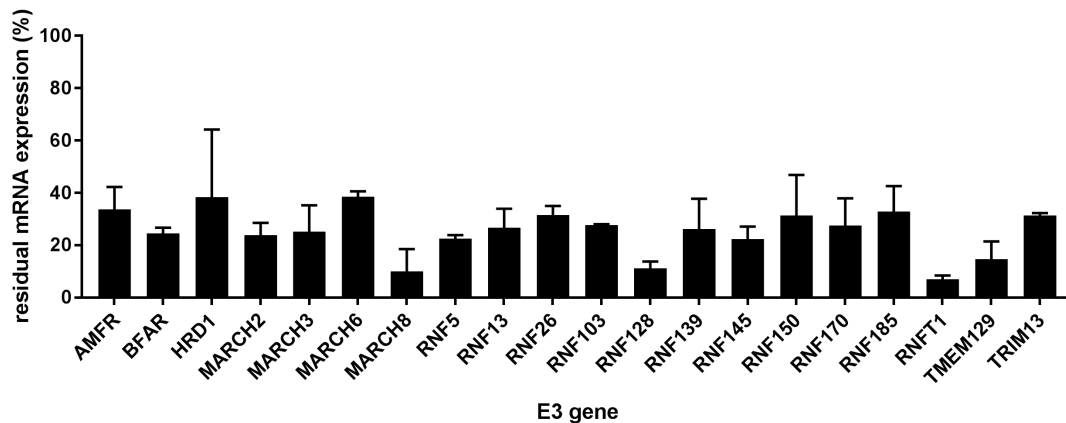


**Figure 3.12 Fold induction of ER-resident E3s upon TM and SubAB<sub>5</sub> treatment**  
Calculation of fold change induction relative to untreated from the same transcripts shown in Figure 3.11. Error bars indicate SD from three biological replicates.

Since an increased demand for degradation requires reinforcement of ERAD components, also reflected by transcriptional induction, we can conclude that Hrd1 underlies the principal degradation mechanism for responding to and resolving ER stress. Consequently, it would appear that the remaining ER-resident E3s are responsible for functions beyond the resolution of proteotoxic stress.

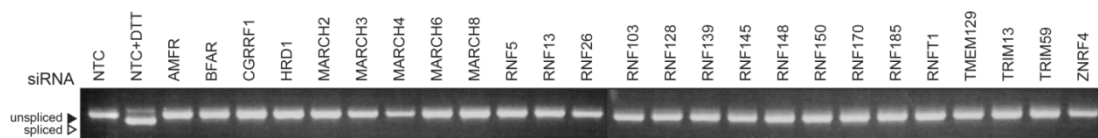
### 3.3.10 ER homeostasis in response to E3 depletion

ER-resident E3s may play essential roles in the maintenance of ER homeostasis. To investigate their potential contribution, we depleted each individual E3 in FlpIn HEK293 cells and measured ER stress induction at basal levels by monitoring IRE1-dependent splicing of XBP1 mRNA, an event that occurs early in UPR signalling. siRNA-mediated knockdown resulted in a 60–90% depletion of the E3s at mRNA level, measured 48 hours after transfection (Figure 3.13). Surprisingly, individual depletion of any ER-resident E3s did not appear to disrupt ER homeostasis and activate the UPR (Figure 3.14).



**Figure 3.13 Knockdown efficiency of the E3s in HEK293 cells**

Residual mRNA expression is measured 48 hours after silencing. Error bars indicate SD from n=2.



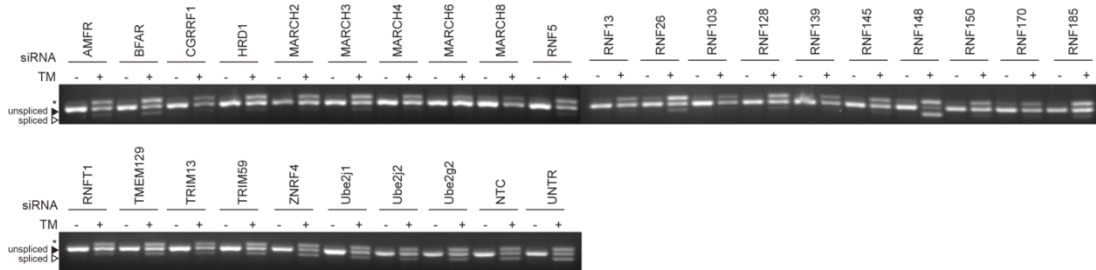
**Figure 3.14 XBP1 splicing in cells depleted for individual E3s**

PCR for unspliced (black arrow) and spliced (white arrow) XBP1 from cDNA obtained by retrotranscription of mRNA extracted from wild-type cells 48 hours after silencing of individual E3s. Non-targeting control (NTC) siRNA is used as negative control. Cells treated with DTT (5 mM) for 4 hours are used as positive control.

The lack of effect could be the result of functional redundancy or possibly insufficient depletion. Due to their similar enzymatic nature, and assuming some E3s may not have stringent substrate specificity, it is possible that when the activity of one E3 is impaired, others would compensate. Alternatively, residual enzymatic activity (10–40%) would be sufficient for the E3 function, at least under basal conditions. Notably, depletion of individual E3s from Hrd1 KO FlpIn HEK293 cells was not sufficient to induce ER stress either (data not shown).

It was reasoned that a stress phenotype might become apparent by depleting individual E3s in cells sensitised to mild ER stress, a condition that may reveal the necessity of select E3s. After silencing E3s for 48 hours, a sub-maximal dose of TM (100 ng/ml), sufficient to trigger partial splicing of XBP1, was applied for 4 hours.

Under this mild stress paradigm, any perturbation of ER homeostasis due to depletion of individual E3s would exacerbate XBP1 splicing (Figure 3.15).



**Figure 3.15 XBP1 splicing in cells sensitised to stress and depleted for the E3s**  
PCR for unspliced (black arrow) and spliced (white arrow) XBP1 from cDNA obtained by retrotranscription of mRNA extracted from wild-type cells 48 hours after silencing of individual E3s. 4 hours before harvesting, cells were treated with 100 ng/ml of TM. Non-targeting control (NTC) siRNA and untransfected (UNTR) cells were used as negative control. “\*” indicates a hybrid band consisting of half spliced and half unspliced XBP1.

Unexpectedly, there was no change to XBP1 splicing, except in the case of RNF148 knockdown. However, further deconvolution of the siRNA pool for RNF148 could not confirm this finding (data not shown), leading to the conclusion that this phenotype was likely the result of an off-target effect. Remarkably, not even depletion of Hrd1 itself was sufficient to sensitise cells to ER stress, probably due to activity of the residual ligase molecules that escaped silencing. Overall, it was concluded that attenuating expression of any individual E3 ligase in the ER does not perturb ER homeostasis, nor does it sensitise cells to ER stress. However, due to the limited knockdown efficiency achieved in our screening, further investigation may require employment of knockout technology (i.e. CRISPR-Cas9) to definitively rule out the potential impact of residual E3 activity.

## 3.4 Discussion

### 3.4.1 Exploring ER-resident E3 ubiquitin ligases

The ER accommodates over 7,000 proteins, broadly divided between ER-resident and client proteins, the latter only travelling across the ER for folding and maturation before reaching their final destination elsewhere in the secretory pathway or in the



extracellular space [reviewed in (Braakman and Hebert, 2013)]. A significant challenge faced by ER quality control mechanisms is the vast heterogeneity of ER clients that need to be folded and degraded. To initiate their journey to the proteasome, misfolded proteins or proteins that are simply undergoing turnover are first recognised by resident lectins such as OS-9 and XTP3-B. Upon recognition, substrates are brought into the proximity of transmembrane E3 ubiquitin ligases for ubiquitin chain conjugation. Substrate delivery is facilitated by E3 cofactors, like SEL1L, that scaffold misfolded proteins to the membrane-embedded ubiquitin ligase Hrd1. During VCP/p97-mediated dislocation (for transmembrane proteins) or retrotranslocation (for luminal proteins), the substrate is polyubiquitinated by a RING finger E3 via K48-linked Ub, the targeting signal for proteasomal degradation. The E3 ligases act as organisational nexuses by scaffolding luminal, cytosolic and membrane cofactors required for the delivery, dislocation and ubiquitination of selected substrates [reviewed in (Christianson and Y. Ye, 2014)]. During the ubiquitination reaction, the E3 ligase simultaneously accommodates substrate and E2 conjugating enzyme while the RING domain catalyses ubiquitin transfer from the active site cysteine of the E2 directly to the substrate, typically on a lysine residue (Deshaies and Joazeiro, 2009). By directly interacting with the substrate, the E3 retains the intrinsic capacity to deal with a striking variety of proteins, whose number greatly exceeds the number of ER-resident E3 enzymes.

To meet the high demand for protein degradation, the mammalian ER has evolved at least 25 E3 ubiquitin ligases spanning the ER membrane with single or polytopic domains. Despite the largely different topology among these enzymes, they all share a catalytic RING finger domain towards the cytosol, suggesting that they may all play a role in Ub-dependent pathways, including ERAD. Whether there is functional redundancy or stringent substrate specificity among these 25 E3s is largely unknown. In a few reported cases, multiple E3s can regulate turnover of the

same substrate, such as for CFTR (AMFR, RNF5 and RNF185) (Khoury et al., 2013; Morito et al., 2008) and MHC-I (Hrd1, MARCH4, TMEM129 and RNF139) (Bartee et al., 2004; Burr et al., 2011; Stagg et al., 2009; van den Boomen et al., 2014). On the other hand, the discovery that different versions of the same protein are selectively targeted to different E3s raises the idea of a high degree of specificity within each enzyme. Such is the case in neuroserpin mutants, retained as polymers in the ER of neurons where they cause a form of familial dementia. Interestingly, the G392E mutant neuroserpin is degraded by the complex Hrd1-Ube2j1, whereas truncated neuroserpin, lacking 132 aa, is degraded by AMFR-Ube2g2 (Roussel et al., 2013). Similarly, same misfolded polypeptides are engaged in different degradation pathways depending on the presence of a membrane-tethering domain (Bernasconi et al., 2010). In particular, while disposal of membrane-anchored BACE and CD3- $\delta$  requires Hrd1, removal of their TMD targets them to ubiquitination by AMFR/gp78. Likewise, anchoring soluble NHK A1AT to the ER membrane relieves its dependency to Hrd1-mediated degradation (Bernasconi et al., 2010). In addition to substrate specificity, cofactor specificity is also crucial for the E3 catalytic activity. For instance, substrate degradation via Hrd1 requires a retrotranslocation machinery involving VCP/97-Ufd1-Npl4, while AMFR-mediated ERAD only requires VCP/p97-Npl4 (Ballar et al., 2011). The organisation of these 25 E3s into functional hubs is also largely unknown. It is attractive to speculate that some of these enzymes may cluster together to enhance degradation capacity, but evidence is still missing. An example of cooperation between E3s is represented by AMFR and RNF5, which synergise to ubiquitinate mutant CFTR  $\Delta$ F508 (Morito et al., 2008).

Among the 25 ER-resident E3s, only Hrd1 and AMFR/gp78 have been extensively studied, and a number of substrates and cofactors important for their function in ERAD have been characterised. A few substrates and interaction partners have been assigned to the E3s TMEM129 and RNF185, highlighting their participation in

protein degradation. Of the remaining E3s, about half (BFAR, MARCH2, MARCH4, MARCH6, MARCH8, RNF128, RNF139, RNF26, RNF5, TRIM13, TRIM59 and ZNRF4) have a validated role in protein degradation for at least one known substrate, but the cofactors necessary for their function are largely unknown. Finally, CGRRF1, MARCH3, RNF13, RNF103, RNF145, RNF148, RNF150, RNF170 and RNFT1 remain largely unexplored. Our understanding of ERAD is predominantly centred on the study of Hrd1. The identification of Hrd1 interaction partners greatly contributed to defining its function in protein degradation. For example, the association of Hrd1 with the cofactors SEL1L and the E2 enzyme Ube2j1 is essential for the stability and functional activity of the whole complex (Burr et al., 2011; Iida et al., 2011; Mueller et al., 2008). Along this line, elucidating the interaction partners of ER-resident E3s may help define their involvement in ERAD or participation in other cellular processes.

### **3.4.2 A comparative strategy to build the E3 interaction network**

This study set out to identify novel interaction partners of all ER-resident E3s via mass spectrometry of the purified E3 complexes. Similar approaches have previously identified the components of the Hrd1 and AMFR networks (Christianson et al., 2012), as well as the deubiquitinating enzymes and autophagy networks (Behrends et al., 2010; Sowa et al., 2009). Due to the lack of available antibodies against the majority of the ER-resident E3s, FLAG-HA-tagged versions of each protein were generated, cloned under Tet-ON inducible promoter and stably integrated into a single genomic locus of FlpIn T-REx HEK293 cells. An advantage offered by the use of a tag for immunoprecipitation is represented by the greater homogeneity between samples, which is key for a comparative analytical method. Endogenous tagging via CRISPR-Cas9-mediated technology would have offered a suitable alternative to the ectopic expression approach used here, by recapitulating native endogenous complexes without disturbing the stoichiometry of the

components. In addition, it would have avoided potential competition in complex formation between endogenous and overexpressed E3. However, despite the technical challenge of tagging 25 genomic loci given the existing technology, it was reasoned that this approach might have come with at least two caveats. Firstly, generation of endogenously tagged E3s via CRISPR-Cas9 transfection would have required clonal selection, with potential off-target associated risks. Secondly, some E3s may be expressed at very low levels endogenously, as suggested by this study's transcriptional analysis, in which about a quarter of the E3s came under the detection limit. Insufficient protein expression could have hindered the affinity purification of the E3 complexes. A possible future optimisation step using overexpressed E3s, could include knockdown of the endogenous counterparts to maximise complex formation. In essence, the ectopic, inducible expression system used here allowed successful immunoprecipitation and detection in mass spectrometry of 22 out of 25 E3 complexes. None of the overexpressed E3s led to detectable puncta that would reflect aggregation in immunofluorescence, and they all proved to localise in the ER.

The pipeline for sample preparation prior to mass spectrometry was carefully optimised in every step using Hrd1 as a positive control. A selection of 10 different detergents with varying properties was tested for protein extraction (data not shown, EF), with LMNG chosen as the best at preserving native, weak interactions of the Hrd1 complex. The interaction profile of exogenously expressed, tagged Hrd1 was comparable to that of endogenous Hrd1 from IP and WB analysis using an antibody raised against Hrd1 (data not shown, EF). Additionally, endogenous and exogenous Hrd1 complexes exhibited comparable sedimentation patterns in sucrose gradient centrifugation (data not shown, EF). These observations led to the conclusion that

C-terminally tagged Hrd1 was likely to form (at least partly) complexes resembling the endogenous forms. Affinity purification of complexes using FLAG beads demonstrated higher efficiency than the HA resin, as less residual protein was observed in the post-IP fraction analysed by WB. Elution of the E3 complexes from the beads was carried out using SDS, showing a higher protein recovery than upon elution with FLAG peptides. While elution from beads by SDS did produce more non-specific interactors, we exploited this side-effect by using the background proteome to normalise between datasets.

Over 2,000 proteins were identified by mass spectrometry from all samples. We developed a pipeline for data analysis using the Progenesis QI platform and the SING software to assess unique and shared high confidence interactors of each E3. Thanks to the relatively high number of samples, we were able to perform a comparative analysis based on the principle that non-specific binders would display the least variability across the baits, while specific interactors would be preferentially enriched in individual baits versus all other pull-downs. This strategy enables a more robust identification of specific interactors than standard normalisation techniques based on a separate control sample (Keilhauer et al., 2015).

Overall, 460 HCIPs were identified among the 22 E3 complexes, corresponding to 245 protein identities. About half of the identified proteins (125) were unique interactors of single E3s, while the remaining HCIPs were shared by two to eight E3s, demonstrating that the comparative analysis was able to preserve HCIPs common to multiple E3s. Additionally, spectral count index calculation was employed using SING software to identify protein hits only found in one sample by at least two peptides. Due to the intrinsic nature of this type of analysis, *p*-values could not be assigned to the SING interactors; however, spectral count index was used to score the protein hits in order to assess the most confident interactors. The

combination of peptide-based intensity analysis and spectral count calculation allowed the generation of a comprehensive list of HCIPs for each E3 complex.

### **3.4.3 Functional diversification of the E3 complexes**

High confidence interactors of all known ER-resident E3s were identified except for RNF13, RNF145 and RNF150. All E3 complexes were significantly different from one another, suggesting a functional diversification among the E3s. This contributed significantly to the discovery of a large number of potential substrates or cofactors, which, upon validation, may open new avenues of research into this large class of enzymes. Preliminary GO (gene ontology) annotation analysis (by use of GO Slim) revealed an enrichment of interactors involved in metabolic pathways, among which were several enzymes implicated in lipid and cholesterol metabolism (not shown). Of the identified HCIPs, known interactors and substrates of the well-studied ubiquitin ligase AMFR/gp78 (UBAC2, FAF2, UBE2G2, ERLIN1, BAG6, AUP1, NPLOC4, DERLIN-1, UFD1L, GLG1, HMGCOA and MGMT1) were confirmed (Cao et al., 2007; Christianson et al., 2012; Jo et al., 2011; Pearce et al., 2007; Song et al., 2005; T. Zhang et al., 2015). Several cofactors of the Hrd1 complex (SEL1L, FAM8A1, OS-9, ERLEC1, UBE2J1, HERP1 and HERP2) and a few reported interactors of the ubiquitin ligases RNF170 (ERLIN1, ERLIN2, ITPR3) and RNF185 (ERLIN2, RNF5) were also identified (Khouri et al., 2013; Lu et al., 2011), supporting the validity of the methodology used. Besides Hrd1 and AMFR, a very limited interaction network of the remaining E3s with known components of the ERAD machinery was observed, suggesting that they may participate in other regulatory functions. Alternatively, they may participate in ERAD by means of uncharacterised cofactors. Among the few E3s within the ERAD system, CGRRF1 was found to interact with Herp; RNF185 and RNF170 with both ERLIN1 and ERLIN2; and RNF5 in association with ERLIN1, and MARCH4, with ERLEC1/XTP3-B. The data suggest that RNF185, RNF103, RNF148, RNF170, RNF5, RNF139 and

ZNRF4 may be part of the same interaction nexus with AMFR by virtue of the shared partner ERLIN1. Whether or not this is a *bona fide* interaction hub will require validation, however, it is possible that multiple E3s cooperate for ubiquitin signalling. Alternatively, ERLIN1 may be a shared interactor of several E3s, but present in distinct complexes. On the other hand, CGRRF1 and MARCH4 were found to be part of the Hrd1 network via interaction with Herp and XTP3-B, respectively. These findings suggest that diverse E3s are able to share the same cofactors for their activity. For instance, MARCH4, as well as Hrd1, may require the lectin XTP3-B for substrate recognition, while Herp may promote substrate degradation by assisting both Hrd1 and CGRRF1, although the ubiquitination capacity of the latter remains to be established. Despite direct interaction with the ATPase VCP/p97 was not found among the HCIPs, half of the E3s analysed co-purified with VCP adaptor proteins, suggesting they may participate in proteasomal degradation. This study represents a powerful discovery platform that lays down the foundations for functional studies on selected E3s. Biochemical validation on the endogenous proteins will confirm *bona fide* interactors and guide the exploration of new functional activities for these E3s.

#### **3.4.4 Hrd1 role in ERAD and beyond**

The data processing pipeline proved successful in the identification of the core Hrd1 complex components SEL1L, FAM8A1, OS-9, ERLEC1/XTP3-B, Ube2j1, Herp and Herp2. With the exception of Herp, they were among the most abundant interactors of Hrd1, possibly the reason underlying their identification in previous studies (Christianson et al., 2012). The validated *bona fide* interactors of Hrd1 represent a good proof-of-principle for this analysis, increasing the confidence that the identified HCIPs of all the other E3s are most likely native interactors. Despite the great amount of attention that Hrd1 has attracted over the past few years, 18 new Hrd1 interactors were identified. Importantly, their nature suggests that they may

represent substrates rather than cofactors. Among the top hits, zinc ion transporters (S39AE and ZNT6) and two proteins localised in the lysosomal membrane (TMEM9 and AP3B1) were found, as well as mitochondrial components (NDUFS2, SLC27A3 and CYB5A), highlighting potential new regulatory functions for Hrd1. Although Hrd1 localisation has been so far restricted to the ER, the possibility that it interacts with proteins localised in other cellular compartments is supported by the evidence of ER-mitochondria and -endosome contact sites [reviewed in (Rowland and Voeltz, 2012)], which have been implicated in the regulation of several biological processes, including autophagy [(Hamasaki et al., 2013) and reviewed in (Phillips and Voeltz, 2016)].

AP3B1 is part of the adaptor protein complex 3 (AP-3), which is involved in sorting proteins from the Golgi apparatus to the lysosome for degradation (Huttenhower et al., 2009). Notably, loss of function mutations of the AP3B1 gene cause a rare autosomal recessive disorder known as Hermansky Pudlak syndrome type 2 (HPS2), characterised by partial albinism, prolonged bleeding occurrence and immunodeficiency (Jones et al., 2013; Wenham et al., 2010). In addition, the AP-3 complex has been reported to be a key promoter of HIV-1 (human immunodeficiency virus-1) assembly and release as cells expressing mutant AP-3 fail to propagate mature HIV-1 particles (L. Liu et al., 2012). Given the crucial involvement of the AP-3 complex in viral infections, the potential regulation of AP3B1 by Hrd1 may have important implications.

Another interesting HCIP of Hrd1 is Ceroid-lipofuscinosis neuronal protein 6 (CLN6). Autosomal recessive mutations in one of the nine CLN genes, including CLN6, are the cause of neuronal ceroid lipofuscinoses (NCLs), a group of progressive, neurodegenerative lysosomal storage diseases biochemically characterised by the accumulation of autofluorescent material in the lysosomes that leads to neuronal



cell death. Clinically, it is characterised by seizures, dementia, visual loss and/or cerebral atrophy (Weimer et al., 2002). CLN6 is an ER transmembrane protein whose function is not well understood. Some of the mutations in the CLN6 protein that are found in patient samples (G123D and M241T) are predicted to be in the CLN6 transmembrane domain, where they cause protein misfolding and subsequent proteasomal degradation via SEL1L, Derlin-1 and VCP/p97 (Oresic et al., 2009). However, the E3 ligase for CLN6 has not yet been reported, and this study strongly points towards Hrd1.

A new noteworthy Hrd1 interactor is Prolyl 4-hydroxylase subunit alpha-1 (P4HA1), a component of prolyl 4-hydroxylase, a key enzyme for collagen synthesis. Prolyl 4-hydroxylase catalyses the formation of 4-hydroxyproline, required for correct three-dimensional folding of newly synthesised procollagen chains. A recent study showed that hypoxia-induced expression of collagen prolyl hydroxylases promotes collagen deposition by breast cancer cells, resulting in enhanced invasiveness and metastasis to lymphonodes and lungs (Gilkes et al., 2013). In the same study, the use of a prolyl hydroxylase inhibitor reduced breast tumour fibrosis and metastasis in a mouse model. P4HA1 expression increases in response to the hypoxic tumour microenvironment and its levels are elevated in patient samples, correlating with poor prognosis. An independent study demonstrated downregulation of Hrd1 in several breast cancer tissues, and highlighted the role of Hrd1 as a tumour suppressor (Y.-M. Xu et al., 2015). Low expression of Hrd1 and high expression of P4HA1 in the two independent studies is in line with an E3 substrate relationship. One of the contributing factors to tumour progression could be the stabilisation of P4HA1 as a consequence of Hrd1 downregulation. If proven, this would represent an important mechanistic insight into breast cancer malignancy and could potentially underlie the progression of other similar solid tumours.

In conclusion, the nature of several newly found Hrd1 interactors suggests that they may represent new substrates of this ligase. The involvement of these interactors in a wide range of cellular processes, ranging from ion transporters, lysosomal and mitochondrial components, as well as disease-associated factors, largely expands the existing understanding of Hrd1 functions. Further investigations are required to validate the dependency of these factors on Hrd1 and its cofactors. If verified, they may reveal new roles of the Hrd1 complex in the pathogenicity of certain diseases.

### **3.4.5 Hrd1 is the central player in resolution of ER stress**

The UPR regulates the transcription of components important to reinforce folding and degradation capacities in the ER, with the aim of restoring homeostasis (Ron and Walter, 2007). To investigate the participation of the ER-resident E3s in the resolution of ER stress, their transcriptional regulation was analysed under stress. To gain quantitative transcriptional data on a large number of targets (in total, 72 ERAD/UPR-related genes were included) this study employed the Nanostring platform for expression analysis. Given the similar nature of this class of enzymes, a certain extent of uniformity was predicted in the expression levels at basal conditions and/or under stress. In contrast, quantification of the absolute number of transcripts for each E3 revealed a wide heterogeneity in the expression patterns. In particular, those E3s that were found within the same interaction network by MS/MS displayed expression levels highly divergent. Surprisingly, the Hrd1 transcript was among the least abundant, while AMFR, BFAR, MARCH6, RNF145 and RNF26 dominated the expression landscape.

Next, the engagement of these E3s in the resolution of acute ER stress was addressed. TM and the toxin SubAB<sub>5</sub> were used to induce general protein misfolding in the ER, and transcriptional activation of the E3s was measured. It was found that only the ubiquitin ligase Hrd1 was transcriptionally induced at significant levels, undergoing an average five-fold change from basal conditions. This finding

confirms previous reports that argue for the pivotal role of Hrd1 in the resolution of ER stress. Transcriptional upregulation of Hrd1 upon UPR activation following ER stress is in agreement with the presence of ER-stress elements (ERSE) and UPR elements (UPRE) in the promoter of the Hrd1 gene (Kaneko et al., 2007; Yamamoto et al., 2008). It also suggests that the remaining ER-resident E3s are not targets of the canonical UPR, meaning that they may be engaged in response to different challenges. What is rather surprising is the lack of induction of E3s with an established role in ERAD, primarily AMFR/gp78, despite the presence of a canonical ERSE-II element in its promoter (Kokame et al., 2001). It is possible that, despite their capacity to ubiquitinate proteins for degradation, they function predominantly under basal conditions, when the flux of misfolded proteins is maintained under a certain threshold. It can be speculated that, under physiological conditions, all E3s contribute to maintaining ER homeostasis by regulating abundance and turnover of different substrates. However, when global misfolding occurs, the added ERAD capacity is entirely funnelled towards Hrd1, possibly due to its broad substrate recognition capability. It is also possible that the ancient Hrd1-SEL1L nexus evolved as the most efficient degradation platform of the eukaryotic ER, and therefore represents the preferential route under misfolding emergency. This finding reinforces the centrality of Hrd1 in resolution of ER stress, and minimises the contribution of the other ER-resident E3s to the reinforcement of the ERAD capacity under stress.

Additionally, this study found that the depletion of individual E3s by siRNA-mediated knockdown is insufficient to perturb ER homeostasis, measured by splicing of XBP1 mRNA, which occurs under acute UPR induction via IRE1. Depletion of single E3s also does not exacerbate ER stress when cells are sensitised to stress by mild doses of TM. The same result was observed upon E3 depletion in cells stably depleted for Hrd1 by shRNA (short hairpin RNA). The surprising observation that

depletion of Hrd1 itself did not affect ER homeostasis under any of the tested conditions may be due to the activity of residual Hrd1 left from incomplete knockdown. In support of this hypothesis, in other experimental settings (not shown) it was found that depletion of Hrd1 levels was not sufficient to reveal the phenotype that was otherwise detected upon complete knockout of the ligase. This could be true for the other ligases as well, therefore it can only be concluded that depletion of individual E3 ligases does not affect ER homeostasis. However, the possibility that deletion of an E3 would impact protein homeostasis in the ER remains open. Additionally, it should be acknowledged that perturbation of ER homeostasis was monitored only by canonical activation of IRE1-dependent UPR, which is generally the first event in the stress response. It could be that XBP1 splicing may have recovered by the time its induction was measured (48 hours after E3 silencing), and that the correct window of detection was missed. Another possibility is that depletion of specific E3s may have perturbed other cellular pathways, while preserving ER homeostasis. To avoid all these potential caveats in future research, complete knockout, for instance via CRISPR-Cas9 technology, and employment of multiple stress readouts would certainly increase the chances of detecting phenotypic consequences.

In conclusion, of all 25 ER-resident E3 ubiquitin ligases, Hrd1 is the most significantly induced by canonical ER stress, suggesting its centrality in the maintenance of ER homeostasis and the resolution of stress. Therefore, this study suggests that the regulation of the remaining E3s may occur in response to different stimuli. Additionally, it was found that impairment of individual E3 ligase activity is insufficient to perturb ER homeostasis or exacerbate ER stress.

### 3.5 Appendix

| E3 Name | Alias               | TMD | ERAD/E3 Interactors                                                                         | Substrates                                                                                                                                                                                  |
|---------|---------------------|-----|---------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| AMFR    | gp78, RNF45         | 7   | DERL1, p97/VCP, Ube2g2, RNF5, UBXD8, ERLIN1,                                                | <b>Membrane:</b> INSIG-1, HMGCR, CFTR, CD3- $\delta$ , TCR- $\alpha$ , KAI1<br><b>Luminal:</b> AAT, APOB<br><b>Cytosolic:</b> SOD1, ATXN3                                                   |
| BFAR    | BAR, RNF47          | 4   |                                                                                             | <b>Membrane:</b> BCAP31, BCL2, BCL2L1<br><b>Cytosolic:</b> CASP8, HIP1, IFT57                                                                                                               |
| CGRRF1  | CGR19, RNF197       | 1   |                                                                                             |                                                                                                                                                                                             |
| Hrd1    | SYNV1               | 6   | DERL1, DERL2, p97/VCP, SEL1L, HERP, OS-9, XTP3-B, VIMP, AUP1, Ube2j1, Ube2g2, FAM8A1, UBXN6 | <b>Membrane:</b> MHC-I, CD147, TC3- $\alpha$ , CD3- $\delta$ , CD44, IRE1 $\alpha$ , APP, PaelR<br><b>Luminal:</b> AAT, $\mu$ S, insulin<br><b>Cytosolic:</b> p53, HTT, p27Kip1, SGK1, NRF1 |
| MARCH2  | RNF172, HSPC240     | 2   | MARCH3                                                                                      | <b>Membrane:</b> TFRC, CD86, CFTR (via CAL, STX6)                                                                                                                                           |
| MARCH3  | RNF173              | 2   | MARCH2                                                                                      | <b>Membrane:</b> STX6                                                                                                                                                                       |
| MARCH4  | RNF174              | 2   |                                                                                             | <b>Membrane:</b> MHC-I, CD4                                                                                                                                                                 |
| MARCH6  | TEB4, RNF176        | 14  |                                                                                             | <b>Membrane:</b> DIO2, SQLE                                                                                                                                                                 |
| MARCH8  | MIR, MIR, RNF178    | 2   |                                                                                             | <b>Membrane:</b> CD44, CD86, MHC-II (HLA-DR), VSV-G, TRAILR1, TFRC, IL1RAP                                                                                                                  |
| RNF103  | KF-1                | 4   | DERL1, p97/VCP                                                                              |                                                                                                                                                                                             |
| RNF128  | GRAIL               | 1   |                                                                                             | <b>Membrane:</b> CD3, CD151, CD81<br><b>Cytosolic:</b> ARPC5, COR1A                                                                                                                         |
| RNF13   | RZF                 | 1   |                                                                                             | <b>Membrane:</b> IRE1 $\alpha$ (interaction)<br><b>Cytosolic:</b> snapin                                                                                                                    |
| RNF139  | TRC8                | 12  | DERL1                                                                                       | <b>Membrane:</b> MHC-I, HM13, INSIG-1, SREBP-2, SCAP<br><b>Cytosolic:</b> uXBP1, VHL                                                                                                        |
| RNF145  |                     | 14  |                                                                                             |                                                                                                                                                                                             |
| RNF148  |                     | 1   |                                                                                             |                                                                                                                                                                                             |
| RNF150  |                     | 1   |                                                                                             |                                                                                                                                                                                             |
| RNF170  |                     | 3   |                                                                                             | <b>Membrane:</b> ITPR1                                                                                                                                                                      |
| RNF185  |                     | 2   | Ube2j1, Ube2j2, ERLIN2                                                                      | <b>Membrane:</b> BNIP1, CFTR<br><b>Cytosolic:</b> DVL2                                                                                                                                      |
| RNF26   |                     | 5   |                                                                                             | <b>Membrane:</b> MITA<br><b>Cytosolic:</b> p62                                                                                                                                              |
| RNF5    | RMA1, G16           | 2   | ERLIN2                                                                                      | <b>Membrane:</b> SLC1A5, JAMP, CFTR<br><b>Luminal:</b> SOPA,<br><b>Cytosolic:</b> PXN, ATG4B                                                                                                |
| RNFT1   |                     | 6   |                                                                                             |                                                                                                                                                                                             |
| TMEM129 |                     | 3   | DERL1, DERL2, Ube2j2, VIMP, p97/VCP, SEL1L, HRD1                                            | <b>Membrane:</b> HLA-I, US11, MHC-I                                                                                                                                                         |
| TRIM13  | LEU5, RFP2, RNF77   | 1   | p97/VCP                                                                                     | <b>Cytosolic:</b> MDM2, AKT1, NEMO                                                                                                                                                          |
| TRIM59  | Mrf1, TSBF1, RNF104 | 1   |                                                                                             | <b>Cytosolic:</b> P53                                                                                                                                                                       |
| ZNRF4   |                     | 1   |                                                                                             | <b>Membrane:</b> CNX                                                                                                                                                                        |

Table 11 E3 interactors and substrates from published literature

| Entry  | Name  | Protein name                                                               | Gene ontology<br>(cellular component)                                                              | Gene ontology<br>(biological process)                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
|--------|-------|----------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Q9P0T7 | TMEM9 | Transmembrane protein 9                                                    | lysosomal membrane                                                                                 | transport                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
| Q15043 | S39AE | Zinc transporter ZIP14                                                     | ER; Golgi apparatus;<br>plasma membrane                                                            | zinc II ion transmembrane import                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| P13674 | P4HA1 | Prolyl 4-hydroxylase subunit<br>alpha-1                                    | ER lumen;<br>mitochondrion;<br>procollagen-proline 4-<br>dioxygenase complex                       | collagen fibril organization; peptidyl-proline<br>hydroxylation to 4-hydroxy-L-proline                                                                                                                                                                                                                                                                                                                                                                                                                          |
| Q6NXT4 | ZNT6  | Zinc transporter 6                                                         | Golgi membrane                                                                                     | regulation of sequestering of zinc ion                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
| O75306 | NDUS2 | NADH dehydrogenase<br>[ubiquinone] iron-sulfur protein<br>2, mitochondrial | mitochondrial respiratory<br>chain complex I;<br>nucleoplasm                                       | mitochondrial electron transport, NADH to<br>ubiquinone ; mitochondrial respiratory chain<br>complex I assembly; response to oxidative stress                                                                                                                                                                                                                                                                                                                                                                   |
| O00203 | AP3B1 | AP-3 complex subunit beta-1                                                | axon cytoplasm; clathrin-<br>coated vesicle<br>membrane; Golgi<br>apparatus; lysosomal<br>membrane | anterograde synaptic vesicle transport; antigen<br>processing and presentation; blood coagulation;<br>intracellular protein transport; melanosome<br>organization; NK T cell differentiation; protein<br>targeting to lysosome                                                                                                                                                                                                                                                                                  |
| Q15005 | SPCS2 | Signal peptidase complex<br>subunit 2                                      | ER membrane; signal<br>peptidase complex                                                           | protein targeting to ER; signal peptide processing                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
| Q5K4L6 | S27A3 | Long-chain fatty acid transport<br>protein 3                               | mitochondrial membrane                                                                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |
| O96005 | CLPT1 | Cleft lip and palate<br>transmembrane protein 1                            | plasma membrane                                                                                    | cell differentiation; multicellular organism<br>development; regulation of T cell differentiation in<br>thymus                                                                                                                                                                                                                                                                                                                                                                                                  |
| P00167 | CYB5  | Cytochrome b5                                                              | cytoplasm; ER<br>membrane; extracellular<br>exosome;mitochondrial<br>outer membrane                | L-ascorbic acid metabolic process; response to<br>cadmium ion                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| Q9UBM7 | DHCR7 | 7-dehydrocholesterol reductase                                             | ER membrane; nuclear<br>outer membrane                                                             | blood vessel development; cell differentiation;<br>cholesterol biosynthetic process; lung<br>development; multicellular organism growth; post-<br>embryonic development; regulation of cell<br>proliferation                                                                                                                                                                                                                                                                                                    |
| Q9Y6Y8 | S23IP | SEC23-interacting protein                                                  | cytoplasm; ER; ER to<br>Golgi transport vesicle<br>membrane; Golgi<br>membrane                     | COPII vesicle coating; Golgi organization;<br>intracellular protein transport                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| Q14BN4 | SLMAP | Sarcolemmal membrane-<br>associated protein                                | plasma membrane;<br>microtubule organizing<br>center; sarcolemma;<br>smooth ER                     | muscle contraction                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
| Q9NWW5 | CLN6  | Ceroid-lipofuscinosis neuronal<br>protein 6                                | ER lumen;ER membrane                                                                               | cellular macromolecule catabolic process;<br>cholesterol metabolic process; ganglioside<br>metabolic process; glycosaminoglycan metabolic<br>process; locomotion; lysosomal lumen<br>acidification; positive regulation of proteolysis;<br>visual perception                                                                                                                                                                                                                                                    |
| Q12797 | ASPH  | Aspartyl/asparaginyl beta-<br>hydroxylase                                  | calcium channel complex;<br>ER membrane; plasma<br>membrane; sarcoplasmic<br>reticulum             | activation of endopeptidase activity; calcium ion<br>transmembrane transport; face and limb<br>morphogenesis; muscle contraction ; negative<br>regulation of cell proliferation; palate<br>development; pattern specification process;<br>peptidyl-aspartic acid hydroxylation; protein<br>transport; regulation of: proteolysis; transcription;<br>cardiac conduction; cell communication; protein<br>depolymerization; protein stability; release of<br>calcium ion into cytosol by sarcoplasmic<br>reticulum |
| P30519 | HMOX2 | Heme oxygenase 2                                                           | ER membrane; plasma<br>membrane                                                                    | cellular iron ion homeostasis; heme oxidation;<br>response to hypoxia                                                                                                                                                                                                                                                                                                                                                                                                                                           |
| Q9NWS8 | RMND1 | Required for meiotic nuclear<br>division protein 1 homolog                 | mitochondrion                                                                                      | positive regulation of mitochondrial translation                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| Q9BSG0 | PADC1 | Protease-associated domain-<br>containing protein 1                        | extracellular exosome                                                                              |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |

**Table 12 New HCIPs for Hrd1 and associated biological processes (GO)**

| Entry  | Name  | Protein name                                                                                  | Gene ontology<br>(cellular component)                                                                                                                                                             | Gene ontology(biological process)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
|--------|-------|-----------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Q99720 | SGMR1 | Sigma non-opioid intracellular receptor 1                                                     | cell junction; er membrane; plasma membrane; lipid particle; nuclear envelope                                                                                                                     | lipid transport; nervous system development; regulation of neuron apoptotic process                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
| Q96J02 | ITCH  | E3 ubiquitin-protein ligase Itchy homolog                                                     | cytoplasmic vesicle; exosome; membrane; nucleus; plasma membrane                                                                                                                                  | inflammatory response; innate immune response; negative regulation of: alpha-beta T cell proliferation, cell growth, apoptotic process, defense response to virus; JNK cascade; NF-kappaB transcription factor activity; type I interferon production; Notch signaling pathway; K29-, K48-, K63-linked ubiquitination; protein deubiquitination                                                                                                                                                                                                                  |
| O75306 | NDUS2 | NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial                          | mitochondrial respiratory chain complex I; nucleoplasm                                                                                                                                            | mitochondrial electron transport, NADH to ubiquinone; mitochondrial respiratory chain complex I assembly; response to oxidative stress                                                                                                                                                                                                                                                                                                                                                                                                                           |
| O00767 | ACOD  | Acyl-CoA desaturase                                                                           | er membrane                                                                                                                                                                                       | long-chain fatty-acyl-CoA biosynthetic process; unsaturated fatty acid biosynthetic process                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
| Q16555 | DPYL2 | Dihydropyrimidinase-related protein 2                                                         | cytosol; dendrite; exosome; membrane; mitochondrion; myelin sheath; neuronal cell body                                                                                                            | cytoskeleton organization; endocytosis; nervous system development; positive regulation of glutamate secretion; regulation of axon extension; response to drug; spinal cord development; synaptic vesicle transport                                                                                                                                                                                                                                                                                                                                              |
| P16152 | CBR1  | Carbonyl reductase [NADPH] 1                                                                  | cytosol; exosome                                                                                                                                                                                  | drug metabolic process; epithelial cell differentiation; oxidation-reduction process; vitamin K metabolic process                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| P41240 | CSK   | Tyrosine-protein kinase CSK                                                                   | cell-cell junction; cytoplasm; exosome; membrane raft; plasma membrane                                                                                                                            | adaptive immune response; adherens junction organization; cell differentiation; cell migration; central nervous system development; EGFR signaling pathway; innate immune response; negative regulation of: bone resorption, cell proliferation, ERK1 and ERK2 cascade, Golgi to plasma membrane protein transport, IL-6 production, phagocytosis; oligodendrocyte differentiation; positive regulation of MAP kinase activity; TCR signaling pathway; transmembrane receptor protein tyrosine kinase signaling pathway                                          |
| O14579 | COPE  | Coatamer subunit epsilon                                                                      | COP1 vesicle coat; er membrane; Golgi membrane; nucleoplasm; transport vesicle                                                                                                                    | ER to Golgi vesicle-mediated transport                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
| Q5T9L3 | WLS   | Protein wntless homolog                                                                       | dendrite; early endosome membrane; endocytic vesicle membrane; ER membrane; exosome; Golgi membrane; plasma membrane; TGN                                                                         | exocrine pancreas development; protein transport; mesoderm formation; midbrain development; positive regulation of Wnt signaling pathway, I-KB kinase/NF-KB signaling                                                                                                                                                                                                                                                                                                                                                                                            |
| Q99805 | TM9S2 | Transmembrane 9 superfamily member 2                                                          | endosome membrane; exosome; plasma membrane                                                                                                                                                       | transport                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
| P51809 | VAMP7 | Vesicle-associated membrane protein 7                                                         | cell junction; cytoplasm; ER membrane; exosome; late endosome membrane; lysosomal membrane; phagocytic vesicle membrane; plasma membrane; secretory granule membrane; SNARE complex; synapse; TGN | calcium ion regulated exocytosis; endocytosis; endosome to lysosome transport; eosinophil degranulation; ER to Golgi vesicle-mediated transport; Golgi to plasma membrane protein transport; NK cell degranulation; neutrophil degranulation; phagocytosis; positive regulation of: dendrite morphogenesis, histamine secretion by mast cell; SNARE complex assembly; triglyceride transport                                                                                                                                                                     |
| Q16836 | HCDH  | Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial                                           | cytoplasm; mitochondrial inner membrane; mitochondrial matrix; nucleoplasm                                                                                                                        | fatty acid beta-oxidation; negative regulation of insulin secretion                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
| Q8NBU5 | ATAD1 | ATPase family AAA domain-containing protein 1                                                 | cell junction; mitochondrion; nucleus; peroxisomal membrane; postsynaptic membrane                                                                                                                | cytoplasmic microtubule organization; learning; memory; negative regulation of synaptic transmission                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
| Q9NWW5 | CLN6  | Ceroid-lipofuscinosis neuronal protein 6                                                      | ER lumen; ER membrane                                                                                                                                                                             | cholesterol metabolic process; glycosaminoglycan metabolic process; locomotion; lysosomal lumen acidification; positive regulation of proteolysis                                                                                                                                                                                                                                                                                                                                                                                                                |
| Q9Y2G8 | DJC16 | DnaJ homolog subfamily C member 16                                                            | membrane                                                                                                                                                                                          | cell redox homeostasis                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
| Q5UCC4 | EMC10 | ER membrane protein complex subunit 10                                                        | ER membrane protein complex; extracellular region                                                                                                                                                 |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
| Q15011 | HERP1 | Homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein | calcium channel complex; ER membrane                                                                                                                                                              | ER calcium ion homeostasis; negative regulation of ER stress-induced intrinsic apoptotic signaling pathway; PERK-mediated UPR; positive regulation of ER-associated ubiquitin-dependent protein catabolic process; regulation of protein ubiquitination; response to UPR; retrograde protein transport, ER to cytosol                                                                                                                                                                                                                                            |
| Q9Y320 | TMX2  | Thioredoxin-related transmembrane protein 2                                                   | membrane                                                                                                                                                                                          | cell redox homeostasis                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
| P61289 | PSME3 | Proteasome activator complex subunit 3                                                        | cytoplasm; membrane; nucleoplasm; proteasome activator complex                                                                                                                                    | anaphase-promoting complex-dependent catabolic process; antigen processing and presentation of exogenous peptide antigen via MHC class I; MAPK cascade; Wnt signaling pathway; negative regulation of: extrinsic apoptotic signaling pathway, ubiquitin-protein ligase activity involved in mitotic cell cycle; NIK/NF-KB signaling; proteasome-mediated ubiquitin-dependent protein catabolic process; amino acid metabolic process; G1/S transition of mitotic cell cycle; regulation of mRNA stability; TCR signaling pathway; TNF-mediated signaling pathway |

**Table 13 HCIPs for CGRRF1 and associated biological processes (GO)**

| Entry  | Name  | Protein names                                                             | Gene ontology (cellular component)                                                                                                                                                     | Gene ontology (biological process)                                                                                                                                                                                                                                                                                                                                                                                                                       |
|--------|-------|---------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Q95816 | BAG2  | BAG family molecular chaperone regulator 2                                | cytosol                                                                                                                                                                                | protein folding; regulation of cellular response to heat                                                                                                                                                                                                                                                                                                                                                                                                 |
| Q12906 | ILF3  | Interleukin enhancer-binding factor 3                                     | cytoplasm; ribonucleoprotein complex; membrane; mitochondrion; nucleoplasm                                                                                                             | regulation of transcription; negative regulation of: translation, viral genome replication; protein phosphorylation                                                                                                                                                                                                                                                                                                                                      |
| P02786 | TFR1  | Transferrin receptor protein 1                                            | plasma membrane; endosome membrane; exosome; HFE-transferrin receptor complex; mitochondrion                                                                                           | aging; cellular iron ion homeostasis; osteoclast differentiation; regulation of bone resorption; response to: hypoxia, manganese ion, nutrient, retinoic acid; transferrin transport                                                                                                                                                                                                                                                                     |
| Q5SNT2 | TM201 | Transmembrane protein 201                                                 | nuclear membrane                                                                                                                                                                       | fibroblast migration; nuclear migration along microtubule                                                                                                                                                                                                                                                                                                                                                                                                |
| Q5M7Z0 | RNFT1 | RING finger and transmembrane domain-containing protein 1                 | membrane                                                                                                                                                                               |                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
| O75306 | NDUS2 | NADH dehydrogenase [ubiquinone] iron-sulfur protein 2                     | mitochondrial matrix; mitochondrial respiratory chain complex I; nucleoplasm                                                                                                           | mitochondrial electron transport, NADH to ubiquinone; mitochondrial respiratory chain complex I assembly; response to oxidative stress                                                                                                                                                                                                                                                                                                                   |
| O14763 | TR10B | Tumor necrosis factor receptor superfamily member 10B                     | plasma membrane                                                                                                                                                                        | cellular response to mechanical stimulus; immune response; inflammatory response; intrinsic apoptotic signaling pathway in response to ER stress; multicellular organism development; positive regulation of I-KB kinase/NF-KB signaling; regulation of cell proliferation; response to ER stress; response to lipopolysaccharide                                                                                                                        |
| Q96J02 | ITCH  | E3 ubiquitin-protein ligase Itchy homolog                                 | cytoplasmic vesicle; exosome; nucleus; plasma membrane                                                                                                                                 | defense response to virus; inflammatory response; innate immune response; negative regulation of alpha-beta T cell proliferation; negative regulation of: apoptotic process; JNK cascade, NF-KB transcription factor activity, type I interferon production; Notch signaling pathway; K29-, K48-, K63-linked ubiquitination; regulation of cell growth; protein deubiquitination                                                                         |
| Q04721 | NOTC2 | Neurogenic locus notch homolog protein 2                                  | ER membrane; extracellular region; Golgi membrane; plasma membrane; nucleoplasm                                                                                                        | bone remodeling; cell growth; hemopoiesis; marginal zone B cell differentiation; multicellular organism development; negative regulation of: apoptotic process, cell proliferation; nervous system development; Notch signaling involved in heart development; positive regulation of Ras signal transduction; regulation of transcription; stem cell population maintenance; tissue regeneration                                                        |
| Q92542 | NICA  | Nicastrin                                                                 | ER; exosome; Golgi apparatus; plasma membrane; lysosomal membrane                                                                                                                      | amyloid precursor protein catabolic process; beta-amyloid metabolic process; epithelial cell proliferation; membrane protein intracellular domain proteolysis; myeloid cell homeostasis; Notch signaling pathway; positive regulation of apoptotic process; T cell proliferation                                                                                                                                                                         |
| P08195 | 4F2   | 4F2 cell-surface antigen heavy chain                                      | plasma membrane; cytoplasm; exosome; nucleus                                                                                                                                           | amino acid transport; calcium ion transport; carbohydrate metabolic process; cell growth; leucine import; leukocyte migration; response to exogenous dsRNA; tryptophan transport                                                                                                                                                                                                                                                                         |
| O94919 | ENDD1 | Endonuclease domain-containing 1 protein                                  | exosome; membrane                                                                                                                                                                      |                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
| Q99941 | ATF6B | Cyclic AMP-dependent transcription factor ATF-6 beta                      | ER membrane; Golgi apparatus; nucleus; protein-DNA complex                                                                                                                             | ATF6-mediated UPR; positive regulation of transcription from RNA polymerase II promoter in response to ER stress                                                                                                                                                                                                                                                                                                                                         |
| P10586 | PTPRF | Receptor-type tyrosine-protein phosphatase F                              | exosome; plasma membrane; neuronal cell body                                                                                                                                           | cell adhesion; cell migration; axon regeneration; synaptic transmission; transmembrane receptor protein tyrosine phosphatase signaling pathway                                                                                                                                                                                                                                                                                                           |
| O00308 | WWP2  | NEDD4-like E3 ubiquitin-protein ligase WWP2                               | cytoplasm; exosome; membrane; nucleus; ubiquitin ligase complex                                                                                                                        | negative regulation of: gene expression, protein transport, transcription; proteasome-mediated ubiquitin-dependent protein catabolic process; protein autoubiquitination; protein K63-linked ubiquitination; regulation of: membrane potential, potassium ion transmembrane transporter activity; viral entry into host cell                                                                                                                             |
| Q16555 | DPYL2 | Dihydropyrimidinase-related protein 2                                     | cytosol; dendrite; exosome; membrane; mitochondrion; myelin sheath; neuronal cell body                                                                                                 | cytoskeleton organization; endocytosis; nervous system development; positive regulation of glutamate secretion; regulation of axon extension; spinal cord development; synaptic vesicle transport                                                                                                                                                                                                                                                        |
| P49458 | SRP09 | Signal recognition particle 9 kDa protein                                 | cytosol; exosome; signal recognition particle receptor complex                                                                                                                         | negative regulation of translational elongation; SRP-dependent cotranslational protein targeting to membrane                                                                                                                                                                                                                                                                                                                                             |
| P51809 | VAMP7 | Vesicle-associated membrane protein 7                                     | cell junction; ER membrane; exosome; late endosome membrane; lysosomal membrane; phagocytic vesicle membrane; plasma membrane; secretory granule membrane; SNARE complex; synapse; TGN | calcium ion regulated exocytosis; endocytosis; endosome to lysosome transport; eosinophil degranulation; ER to Golgi vesicle-mediated transport; Golgi to plasma membrane protein transport; NK cell degranulation; neutrophil degranulation; phagocytosis; positive regulation of histamine secretion by mast cell; SNARE complex assembly                                                                                                              |
| Q96PU5 | NED4L | E3 ubiquitin-protein ligase NEDD4-like                                    | cytoplasm; exosome; nucleoplasm                                                                                                                                                        | sodium ion homeostasis; negative regulation of: potassium ion transmembrane transport, protein localization to cell surface, sodium ion transmembrane transport, transcription from RNA polymerase II promoter; positive regulation of caveolin-endocytosis; protein K48-linked ubiquitination; regulation of membrane potential                                                                                                                         |
| Q99805 | TM9S2 | Transmembrane 9 superfamily member 2                                      | endosome membrane; exosome; plasma membrane                                                                                                                                            | transport                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| Q86X29 | LSR   | Lipolysis-stimulated lipoprotein receptor                                 | exosome; plasma membrane; tight junction                                                                                                                                               | establishment of blood-brain barrier; liver development; regulation of lipid metabolic process; tight junction assembly                                                                                                                                                                                                                                                                                                                                  |
| P16152 | CBR1  | Carbonyl reductase [NADPH] 1                                              | cytosol; exosome                                                                                                                                                                       | cyclooxygenase pathway; epithelial cell differentiation; oxidation-reduction process; vitamin K metabolic process                                                                                                                                                                                                                                                                                                                                        |
| Q06481 | APLP2 | Amyloid-like protein 2                                                    | exosome; nucleus; plasma membrane                                                                                                                                                      | G-protein coupled receptor signaling pathway                                                                                                                                                                                                                                                                                                                                                                                                             |
| P61081 | UBC12 | NEDD8-conjugating enzyme Ubc12                                            | cytoplasm; exosome                                                                                                                                                                     | NIK/NF-KB signaling; regulation of neuron apoptotic process; protein neddylation                                                                                                                                                                                                                                                                                                                                                                         |
| Q9P2E5 | CHPF2 | Chondroitin sulfate glucuronyltransferase                                 | Golgi membrane                                                                                                                                                                         | chondroitin sulfate biosynthetic process                                                                                                                                                                                                                                                                                                                                                                                                                 |
| Q9ULF5 | S39AA | Zinc transporter ZIP10                                                    | plasma membrane                                                                                                                                                                        | negative regulation of B cell apoptotic process; positive regulation of B cell proliferation; positive regulation of BCR signaling pathway; zinc II ion transmembrane import                                                                                                                                                                                                                                                                             |
| P04818 | TYSY  | Thymidylate synthase                                                      | cytoplasm; mitochondrial inner membrane; mitochondrial matrix; nucleoplasm                                                                                                             | aging; cell proliferation; circadian rhythm; developmental growth; DNA biosynthetic process; immortalization of host cell by virus; intestinal epithelial cell maturation; organ regeneration; pyrimidine nucleoside biosynthetic process; regulation of transcription involved in G1/S transition of mitotic cell cycle; response to: cytokine, ethanol, folic acid, glucocorticoid, progesterone, toxic substance, vitamin A; uracil metabolic process |
| Q9NRN7 | ADPPT | L-aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase | cytosol; exosome                                                                                                                                                                       | lysine biosynthetic process; pantothenate metabolic process                                                                                                                                                                                                                                                                                                                                                                                              |
| P04035 | HMDH  | 3-hydroxy-3-methylglutaryl-coenzyme A reductase                           | ER membrane; peroxisomal membrane                                                                                                                                                      | aging; cholesterol biosynthetic process; coenzyme A metabolic process; negative regulation of: insulin secretion, MAP kinase activity, striated muscle cell apoptotic process, vasodilation, wound healing; positive regulation of: cardiac muscle cell apoptotic process, ERK1 and ERK2 cascade, skeletal muscle tissue development, stress-activated MAPK cascade; response to ethanol, nutrient; ubiquinone metabolic process                         |
| Q9Y320 | TMX2  | Thioredoxin-related transmembrane protein 2                               | membrane                                                                                                                                                                               | cell redox homeostasis                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| Q6UW68 | TM205 | Transmembrane protein 205                                                 | exosome; membrane                                                                                                                                                                      |                                                                                                                                                                                                                                                                                                                                                                                                                                                          |

**Table 14 HCIPs for MARCH3 and associated biological processes (GO)**



| Entry  | Name  | Protein name                                        | Gene ontology<br>(cellular component)                                                                                 | Gene ontology (biological process)                                                                                                                                                                                                                                                                                                                                                                                                                                                               |
|--------|-------|-----------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Q7L211 | ABHDD | Protein ABHD13                                      | membrane                                                                                                              |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
| P00387 | NB5R3 | NADH-cytochrome b5 reductase 3                      | cytoplasm; ER membrane; exosome; hemoglobin complex; lipid particle; mitochondrial membrane                           | blood circulation; cholesterol biosynthetic process; L-ascorbic acid metabolic process                                                                                                                                                                                                                                                                                                                                                                                                           |
| P57678 | GEM14 | Gem-associated protein 4                            | cytoplasm; exosome; membrane; nucleoplasm; small nuclear ribonucleoprotein complex                                    | nuclear import; rRNA processing; spliceosomal snRNP assembly                                                                                                                                                                                                                                                                                                                                                                                                                                     |
| P16152 | CBR1  | Carbonyl reductase [NADPH] 1                        | cytosol; exosome                                                                                                      | cyclooxygenase pathway; drug metabolic process; epithelial cell differentiation; oxidation-reduction process ; vitamin K metabolic process                                                                                                                                                                                                                                                                                                                                                       |
| P49721 | PSB2  | Proteasome subunit beta type-2                      | cytosol; exosome; membrane; nucleoplasm; proteasome core complex                                                      | anaphase-promoting complex-dependent catabolic process; antigen processing and presentation of exogenous peptide antigen via MHC class I; MAPK cascade; regulation of Wnt signaling pathway; NIK/NF-kappaB signaling; regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle transition; proteasome-mediated ubiquitin-dependent protein catabolic process; cellular amino acid metabolic process; mRNA stability; TCR signaling pathway; TNF-mediated signaling pathway |
| Q9NQ75 | EXOS3 | Exosome complex component RRP40                     | cytoplasmic exosome (RNase complex); nuclear exosome (RNase complex); transcriptionally active chromatin              | DNA deamination; isotype switching; nuclear tRNA catabolic process; nuclear retention of pre-mRNA with aberrant 3'-ends; nuclear-transcribed mRNA catabolic process; regulation of mRNA stability; rRNA processing                                                                                                                                                                                                                                                                               |
| P30837 | AL1B1 | Aldehyde dehydrogenase X, mitochondrial             | membrane-bounded organelle; mitochondrial matrix; nucleoplasm                                                         | carbohydrate metabolic process; ethanol oxidation                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| P49591 | SYSC  | Serine-tRNA ligase, cytoplasmic                     | cytoplasm; exosome                                                                                                    | selenocysteine metabolic process; translation; tRNA aminoacylation for protein translation                                                                                                                                                                                                                                                                                                                                                                                                       |
| Q16836 | HCDH  | Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial | cytoplasm; mitochondrial inner membrane; mitochondrial matrix; nucleoplasm                                            | fatty acid beta-oxidation; negative regulation of insulin secretion                                                                                                                                                                                                                                                                                                                                                                                                                              |
| P55061 | BI1   | Bax inhibitor 1                                     | cytoplasm; ER membrane; plasma membrane; mitochondrial membrane; nucleus                                              | autophagy; ER calcium ion homeostasis; lung development; negative regulation of: apoptotic process, ER stress-induced intrinsic apoptotic signaling pathway, hypoxia-induced intrinsic apoptotic signaling pathway, immunoglobulin secretion, mRNA endonucleolytic cleavage involved in UPR,transcription from RNA polymerase II promoter in response to ER stress; response to L-glutamate spermatogenesis                                                                                      |
| Q14534 | ERG1  | Squalene monooxygenase                              | ER membrane                                                                                                           | cholesterol biosynthetic process                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |
| P48735 | IDHP  | Isocitrate dehydrogenase [NADP], mitochondrial      | cytosol; exosome; mitochondrial inner membrane; mitochondrial matrix; peroxisome                                      | 2-oxoglutarate metabolic process; carbohydrate metabolic process; isocitrate metabolic process; TCA cycle                                                                                                                                                                                                                                                                                                                                                                                        |
| Q9Y487 | VPP2  | V-type proton ATPase 116 kDa subunit a isoform 2    | cytoplasm; endosome membrane; lysosomal membrane; plasma membrane; vacuolar proton-transporting V-type ATPase complex | ATP hydrolysis coupled proton transport; ATP synthesis coupled proton transport; immune response; insulin receptor signaling pathway; ion transmembrane transport; phagosome acidification; regulation of macroautophagy; transferrin transport; vacuolar proton-transporting V-type ATPase complex assembly                                                                                                                                                                                     |

**Table 15 HCIPs for RNFT1 and associated biological processes (GO)**

| Entry  | Name  | Protein names                                                | Gene ontology (cellular component)                                                                            | Gene ontology (biological process)                                                                                                                                                                                                                                                                                                                                                                                                                                               |
|--------|-------|--------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| P62979 | RS27A | Ubiquitin-40S ribosomal protein S27a                         | cytosolic small ribosomal subunit; endocytic vesicle membrane; exosome; nucleoplasm; plasma membrane          | Ubiquitin-related processes                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
| O75477 | ERLN1 | Erlin-1                                                      | ER membrane                                                                                                   | ER-associated ubiquitin-dependent protein catabolic process; negative regulation of cholesterol biosynthetic process and fatty acid biosynthetic process; SREBP signaling pathway                                                                                                                                                                                                                                                                                                |
| Q4ZIN3 | MBRL  | Membralin                                                    | ER membrane                                                                                                   | negative regulation of neuron death; positive regulation of ERAD pathway; response to ER stress                                                                                                                                                                                                                                                                                                                                                                                  |
| Q9BVT8 | TMUB1 | Transmembrane and ubiquitin-like domain-containing protein 1 | cell junction; cytoplasm; membrane; microtubule organizing center; nucleoplasm; endosome                      | ER-associated ubiquitin-dependent protein catabolic process                                                                                                                                                                                                                                                                                                                                                                                                                      |
| Q7Z4R8 | CF120 | UPF0669 protein C6orf120                                     | extracellular exosome                                                                                         | apoptotic process                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| P05023 | AT1A1 | Sodium/potassium-transporting ATPase subunit alpha-1         | plasma membrane; ER; endosome; exosome; Golgi apparatus; plasma membrane; sarcolemma;                         | ATP hydrolysis coupled proton transport; potassium ion homeostasis; response to mechanical stimulus; response to steroid hormone; sodium ion homeostasis; membrane hyperpolarization; cell action potential; negative regulation of glucocorticoid biosynthetic process; regulation of heart contraction; potassium ion import; regulation of blood pressure, cardiac conduction; sodium ion transport                                                                           |
| Q9HDC9 | APMAP | Adipocyte plasma membrane-associated protein                 | cell surface; ER; exosome; membrane                                                                           | biosynthetic process                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
| Q71RG4 | TMUB2 | Transmembrane and ubiquitin-like domain-containing protein 2 | membrane                                                                                                      |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
| P55061 | BI1   | Bax inhibitor 1                                              | cytoplasm; ER membrane; plasma membrane; mitochondrial membrane; nucleus                                      | autophagy; ER calcium ion homeostasis; lung development; negative regulation of: apoptotic process, ER stress-induced intrinsic apoptotic signaling pathway, hypoxia-induced intrinsic apoptotic signaling pathway, immunoglobulin secretion, mRNA endonucleolytic cleavage involved in UPR, transcription from RNA polymerase II promoter in response to ER stress; response to L-glutamate spermatogenesis                                                                     |
| Q8WY22 | BRI3B | BRI3-binding protein                                         | mitochondrial outer membrane                                                                                  |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
| Q96K19 | RN170 | E3 ubiquitin-protein ligase RNF170                           | ER membrane                                                                                                   | protein ubiquitination                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
| Q99720 | SGMR1 | Sigma non-opioid intracellular receptor 1                    | cell junction; ER membrane; plasma membrane; lipid particle; nuclear membrane                                 | lipid transport; nervous system development; regulation of neuron apoptotic process                                                                                                                                                                                                                                                                                                                                                                                              |
| Q9NTJ5 | SAC1  | Phosphatidylinositol phosphatase SAC1                        | ER membrane; Golgi membrane                                                                                   | phosphatidylinositol biosynthetic process                                                                                                                                                                                                                                                                                                                                                                                                                                        |
| Q8TAT6 | NPL4  | Nuclear protein localization protein 4 homolog               | cytosol; ER; nuclear outer membrane-ER membrane network; VCP-NPL4-UFD1 AAA ATPase complex                     | ER-associated ubiquitin-dependent protein catabolic process; Golgi organization; retrograde protein transport, ER to cytosol                                                                                                                                                                                                                                                                                                                                                     |
| Q92890 | UFD1  | Ubiquitin fusion degradation protein 1 homolog               | cytosol; nucleoplasm; VCP-NPL4-UFD1 AAA ATPase complex                                                        | ER-associated misfolded protein catabolic process; retrograde protein transport, ER to cytosol; skeletal system development                                                                                                                                                                                                                                                                                                                                                      |
| Q12797 | ASPH  | Aspartyl/asparaginyl beta-hydroxylase                        | calcium channel complex; ER membrane; sarcoplasmic reticulum membrane; plasma membrane                        | activation of endopeptidase activity; calcium ion transmembrane transport; face and limb morphogenesis; muscle contraction; negative regulation of cell proliferation; palate development; pattern specification process; peptidyl-aspartic acid hydroxylation; protein transport; regulation of: proteolysis; transcription; cardiac conduction; cell communication; protein depolymerization; protein stability; release of calcium ion into cytosol by sarcoplasmic reticulum |
| Q9NR31 | SAR1A | GTP-binding protein SAR1a (COPII-associated small GTPase)    | ER; exosome; Golgi                                                                                            | vesicle-mediated transport                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
| Q9Y5M8 | SRPRB | Signal recognition particle receptor subunit beta            | cytoplasm; ER membrane; signal recognition particle receptor complex                                          | IRE1-mediated UPR; small GTPase mediated signal transduction; SRP-dependent cotranslational protein targeting to membrane                                                                                                                                                                                                                                                                                                                                                        |
| Q5K4L6 | S27A3 | Long-chain fatty acid transport protein 3                    | mitochondrial membrane                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
| O60488 | ACSL4 | Long-chain-fatty-acid--CoA ligase 4                          | cytoplasm; ER membrane; ER-mitochondrion membrane contact site; exosome; lipid particle; peroxisomal membrane | dendritic spine development; fatty acid transport; lipid biosynthetic process; negative regulation of prostaglandin secretion; positive regulation of cell growth; response to interleukin-15; response to nutrient; triglyceride metabolic process                                                                                                                                                                                                                              |
| P16152 | CBR1  | Carbonyl reductase [NADPH] 1                                 | cytosol; extracellular exosome                                                                                | cyclooxygenase pathway; epithelial cell differentiation; oxidation-reduction process; vitamin K metabolic process                                                                                                                                                                                                                                                                                                                                                                |
| Q9Y2L1 | RRP44 | Exosome complex exonuclease RRP44                            | cytoplasmic exosome (RNase complex); nuclear exosome (RNase complex)                                          | mRNA catabolic process; rRNA catabolic process                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| O00237 | RN103 | E3 ubiquitin-protein ligase RNF103                           | ER membrane                                                                                                   | central nervous system development; ER-associated ubiquitin-dependent protein catabolic process                                                                                                                                                                                                                                                                                                                                                                                  |
| Q14534 | ERG1  | Squalene monooxygenase                                       | ER membrane                                                                                                   | cholesterol biosynthetic process; sterol biosynthetic process                                                                                                                                                                                                                                                                                                                                                                                                                    |
| Q01780 | EXOSX | Exosome component 10                                         | cytoplasm; exosome (RNase complex); membrane; nuclear exosome (RNase complex)                                 | nuclear retention of unspliced pre-mRNA at the site of transcription; nuclear-transcribed mRNA catabolic process; nonsense-mediated decay; rRNA processing                                                                                                                                                                                                                                                                                                                       |
| P48735 | IDHP  | Isocitrate dehydrogenase [NADP], mitochondrial               | cytosol; exosome; mitochondrion; peroxisome                                                                   | 2-oxoglutarate metabolic process; carbohydrate metabolic process; isocitrate metabolic process; tricarboxylic acid cycle                                                                                                                                                                                                                                                                                                                                                         |
| Q7Z3C6 | ATG9A | Autophagy-related protein 9A                                 | ER membrane; endosome; pre-autophagosomal structure; trans-Golgi network                                      | autophagosome assembly; late nucleophagy; mitophagy; protein localization to pre-autophagosomal structure; protein transport                                                                                                                                                                                                                                                                                                                                                     |

**Table 16 New HCIPs for RNF185 and associated biological processes (GO)**

| Entry  | Entry name | Protein names                                         | Entry  | Entry name | Protein names                                               |
|--------|------------|-------------------------------------------------------|--------|------------|-------------------------------------------------------------|
| P06753 | TPM3       | Tropomyosin alpha-3 chain                             | Q14166 | TTL12      | Tubulin-tyrosine ligase-like protein 12                     |
| Q9BXP3 | CND3       | Condensin complex subunit 3                           | Q43795 | MYO1B      | Unconventional myosin-Ib                                    |
| P67936 | TPM4       | Tropomyosin alpha-4 chain                             | P49643 | PR12       | DNA primase large subunit                                   |
| P61927 | RL37       | 60S ribosomal protein L37                             | Q76013 | KRT36      | Keratin, type I cuticular Ha6                               |
| Q9H254 | SPTN4      | Spectrin beta chain, non-erythrocytic 4               | P62304 | RUXE       | Small nuclear ribonucleoprotein E                           |
| Q9NRH3 | TBG2       | Tubulin gamma-2 chain                                 | Q9B7X1 | NDC1       | Nucleoporin NDC1                                            |
| Q16181 | SEPT7      | Septin-7                                              | Q9BSJ2 | GCP2       | Gamma-tubulin complex component 2                           |
| Q9Y2U8 | MAN1       | Inner nuclear membrane protein Man1                   | P18615 | NELFE      | Negative elongation factor E                                |
| Q01105 | SET        | Protein SET                                           | P82673 | RT35       | 28S ribosomal protein S35, mitochondrial                    |
| P35579 | MYH9       | Myosin-9                                              | Q7L014 | DDX46      | Probable ATP-dependent RNA helicase DDX46                   |
| P52701 | MSH6       | DNA mismatch repair protein Msh6                      | P19387 | RPB3       | DNA-directed RNA polymerase II subunit RPB3                 |
| Q9NYL9 | TMOD3      | Tropomodulin-3                                        | Q2NL82 | TSR1       | Pre-rRNA-processing protein TSR1 homolog                    |
| Q8WXH0 | SYNE2      | Nesprin-2                                             | Q53GS9 | SNUT2      | U4/U6.U5 tri-snRNP-associated protein 2                     |
| Q14929 | HAT1       | Histone acetyltransferase type B catalytic subunit    | P10155 | RO60       | 60 kDa SS-A/Ro ribonucleoprotein                            |
| Q08554 | DSC1       | Desmocollin-1                                         | P08754 | GNAI3      | Guanine nucleotide-binding protein G                        |
| Q02413 | DSG1       | Desmoglein-1                                          | P50570 | DYN2       | Dynamin-2                                                   |
| Q5D862 | FILAI      | Filaggrin-2                                           | Q07065 | CKAP4      | Cytoskeleton-associated protein 4                           |
| Q92734 | TFG        | Protein TFG                                           | P36954 | RPB9       | DNA-directed RNA polymerase II subunit RPB9                 |
| Q95232 | LC7L3      | Luc7-like protein 3                                   | Q7Z333 | SETX       | Probable helicase senataxin                                 |
| P02533 | K1C14      | Keratin, type I cytoskeletal 14                       | Q86VZ3 | HORN       | Hornerin                                                    |
| Q15813 | TBCE       | Tubulin-specific chaperone E                          | P13646 | K1C13      | Keratin, type I cytoskeletal 13                             |
| Q92541 | RTF1       | RNA polymerase-associated protein RTF1 homolog        | Q9NVJ2 | ARL8B      | ADP-ribosylation factor-like protein 8B                     |
| P60228 | EIF3E      | Eukaryotic translation initiation factor 3 subunit E  | Q2TAY7 | SMU1       | WD40 repeat-containing protein SMU1                         |
| Q9BQ67 | GRWD1      | Glutamate-rich WD repeat-containing protein 1         | P18754 | RCC1       | Regulator of chromosome condensation                        |
| P49321 | NASP       | Nuclear autoantigenic sperm protein                   | P0C0S8 | H2A1       | Histone H2A type 1                                          |
| P20700 | LMNB1      | Lamin-B1                                              | Q71D13 | H32        | Histone H3.2                                                |
| Q95347 | SMC2       | Structural maintenance of chromosomes protein 2       | P11532 | DMD        | Dystrophin                                                  |
| Q29RF7 | PDS5A      | Sister chromatid cohesion protein PDS5 homolog A      | P62805 | H4         | Histone H4                                                  |
| Q96019 | ACL6A      | Actin-like protein 6A                                 | P16401 | H15        | Histone H1.5                                                |
| P42766 | RL35       | 60S ribosomal protein L35                             | P16403 | H12        | Histone H1.2                                                |
| Q8TEM1 | PO210      | Nuclear pore membrane glycoprotein 210                | Q7L576 | CYFP1      | Cytoplasmic FMR1-interacting protein 1                      |
| Q9P0K7 | RAH4       | Ankyrin                                               | Q43790 | KRT86      | Keratin, type II cuticular Hb6                              |
| P14649 | MYL6B      | Myosin light chain 6B                                 | Q9BWJ5 | SF3B5      | Splicing factor 3B subunit 5                                |
| Q7L2J0 | MEPCE      | 7SK snRNA methylphosphate capping enzyme              | Q969T9 | WBP2       | WW domain-binding protein 2                                 |
| Q6UN15 | FIP1       | Pre-mRNA 3'-end-processing factor FIP1                | Q14529 | CUX2       | Homeobox protein cut-like 2                                 |
| P15927 | RFA2       | Replication protein A 32 kDa subunit                  | Q9NTK5 | OLA1       | Olg-like ATPase 1                                           |
| Q9NZR1 | TMOD2      | Tropomodulin-2                                        | Q6P48  | SYDM       | Aspartate-tRNA ligase, mitochondrial                        |
| P28289 | TMOD1      | Tropomodulin-1                                        | Q60237 | MYPT2      | Protein phosphatase 1 regulatory subunit 12B                |
| P29692 | EF1D       | Elongation factor 1-delta                             | Q95684 | FR1OP      | FGFR1 oncogene partner                                      |
| Q15393 | SF3B3      | Splicing factor 3B subunit 3                          | Q9BUQ8 | DDX23      | Probable ATP-dependent RNA helicase DDX23                   |
| A7E2Y1 | MYH7B      | Myosin-7B                                             | Q9Y3A5 | SBDS       | Ribosome maturation protein SBDS                            |
| Q9UM54 | MYO6       | Unconventional myosin-VI                              | Q96EA4 | SPDLY      | Protein Spindly                                             |
| Q66K74 | MAP1S      | Microtubule-associated protein 1S                     | Q9NRG9 | AAAS       | Aladin                                                      |
| Q92804 | RBP56      | TATA-binding protein-associated factor 2N             | Q9NWZ8 | GEM8       | Gem-associated protein 8                                    |
| P17480 | UBF1       | Nucleolar transcription factor 1                      | Q9UIA9 | XPO7       | Exportin-7                                                  |
| Q9UH99 | SUN2       | SUN domain-containing protein 2                       | Q95071 | UBR5       | E3 ubiquitin-protein ligase UBR5                            |
| Q14186 | TFDP1      | Transcription factor Dp-1                             | P17096 | HMGAI      | High mobility group protein HMG-I/HMG-Y                     |
| Q96P70 | IPO9       | Importin-9                                            | Q9NZ72 | STMN3      | Stathmin-3                                                  |
| Q94901 | SUN1       | SUN domain-containing protein 1                       | Q8N5G2 | MACOI      | Macoilin                                                    |
| Q8TEX9 | IPO4       | Importin-4                                            | Q60548 | FOX2       | Forkhead box protein D2                                     |
| Q92621 | NU205      | Nuclear pore complex protein Nup205                   | Q9NUJ7 | DD19A      | ATP-dependent RNA helicase DDX19A                           |
| Q9NV11 | FANCI      | Fanconi anemia group I protein                        | Q14530 | TXND9      | Thioredoxin domain-containing protein 9                     |
| P42695 | CNDD3      | Condensin-2 complex subunit D3                        | Q9P1Z2 | CACO1      | Calcium-binding and coiled-coil domain-containing protein 1 |
| Q95793 | STAU1      | Double-stranded RNA-binding protein Staufen homolog 1 | Q16342 | PDCD2      | Programmed cell death protein 2                             |
| Q75122 | CLAP2      | CLIP-associating protein 2                            | Q9UJC5 | SH3L2      | SH3 domain-binding glutamic acid-rich-like protein 2        |
| Q9GZS3 | WDR61      | WD repeat-containing protein 61                       | Q9Y5B9 | SP16H      | FACT complex subunit SPT16                                  |
| Q96K17 | BT3L4      | Transcription factor BTF3 homolog 4                   | Q9BRK4 | LZTS2      | Leucine zipper putative tumor suppressor 2                  |
| P35609 | ACTN2      | Alpha-actinin-2                                       | Q08945 | SSRP1      | FACT complex subunit SSRP1                                  |
| P00491 | PNPH       | Purine nucleoside phosphorylase                       | Q60610 | DIAP1      | Protein diaphanous homolog 1                                |
| P13796 | PLSL       | Plastin-2                                             |        |            |                                                             |

**Table 17 List of contaminants**

## Chapter IV:

### Contribution of the Hrd1 complex to resolving $\mu$ S-driven proteotoxic stress

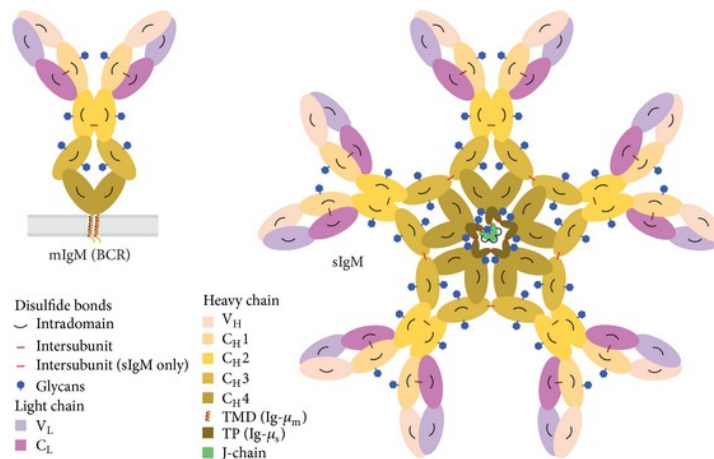
## **4.1 Background**

### **4.1.1 IgM physiology in B-lymphocytes**

Among all secretory and membrane proteins that fold and mature within the secretory pathway, IgM constitutes one of the most challenging examples, representing the most complex antibody that B-lymphocytes produce. In resting B-cells, IgM is expressed as a membrane-bound form at the cell surface as part of the B-cell receptor complex (BCR). B-cells differentiate into plasma cells (PCs) upon antigen recognition and undergo transformation into a cellular folding factory, facilitating the maturation and secretion of thousands of antibodies per second to mediate humoral immunity. During differentiation, B-cells undergo major functional and morphological changes to adapt the existing secretory machinery to meet the needs of increased demand. In particular, consistent ER expansion and upregulation of ER chaperones provide the optimal environment to accommodate the imminent bulk of IgM synthesis (van Anken et al., 2003). The UPR plays a critical role during B-cell differentiation, allowing reinforcement of the folding and degradation capacity in the ER to ensure correct assembly and abundance of the secreted antibodies. Indeed, XBP1 has been shown to be strictly required for B-cell differentiation (Reimold et al., 2001). Upon differentiation, B-cells secrete pentameric IgM that differs from the membrane-bound form only in the C-terminus of the heavy chain (HC). IgM represents the first line of defence in humoral immunity, containing up to 12 antigen-binding sites and therefore being very efficient in the activation of the complement and downstream responses. After a few days of intense secretion, most PCs go into apoptosis as a result of excessive activity.

#### 4.1.2 Quality control on IgM maturation and trafficking

While membrane-bound IgM (mIgM) is monomeric ( $\mu_2L_2$ ), secretory IgM (sIgM) is composed of five monomers and one J chain ( $5 \mu_2L_2 + J$ ), linked together via disulphide bonds (Figure 4.1) (Anelli and van Anken, 2013).

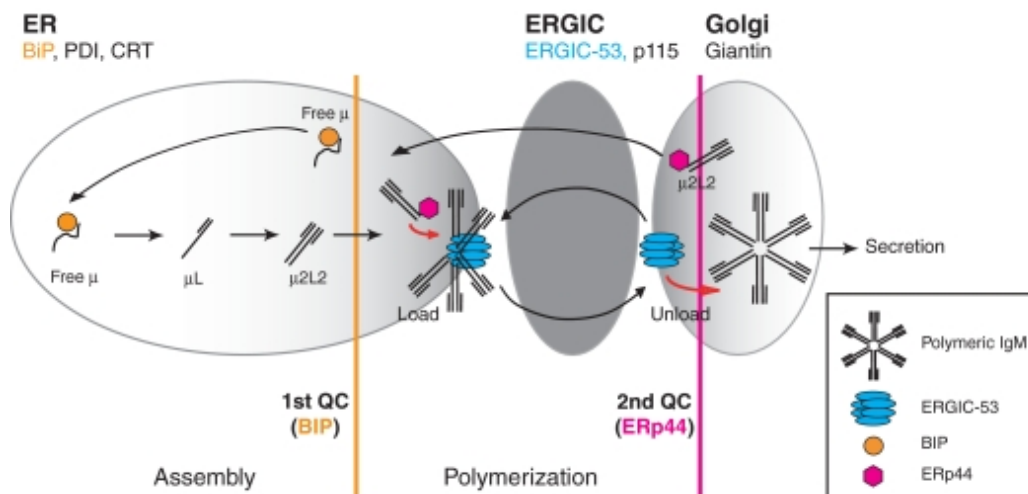


**Figure 4.1 Schematic of membrane and secretory IgM**

Membrane-bound IgM (mIgM) is part of the BCR exposed on B-lymphocytes. Upon antigen activation, PCs secrete secretory IgM (sIgM) in a pentameric (or hexameric, not shown) form. HCs and LCs consist of various domains: V<sub>H</sub>, C<sub>H</sub>1–4; V<sub>L</sub> and C<sub>L</sub>. Glycans, intra- and intersubunit disulfide bonds, and the J-chain are illustrated. The HC in mIgM differs from the HC in sIgM at the C-terminus, having a TMD or a cysteine-containing tailpiece (TP), respectively (Anelli and van Anken, 2013).

Each pentameric structure contains more than 50 *N*-linked oligosaccharides and 100 disulfide bonds (Hendershot and Sitia, 2005). IgM assembly is a multi-step process that engages almost all ER chaperones (Figure 4.2) (Anelli et al., 2007). As soon as the HC is synthesised and enters the ER lumen, the CH1 domain is bound by BiP to maintain the solubility of the IgM HC ( $\mu$ ). Interaction with the light chain (LC) subsequently displaces BiP and  $\mu_2L_2$  monomers can be assembled in the ER lumen (Haas and Wabl, 1983). These monomers are prevented from trafficking by virtue of thiol-mediated retention; PDI and its family members, in particular ERp44 (Anelli et al., 2003), associate with cysteine 575 (C575) in the  $\mu$  tailpiece. The same cysteine is used to form the disulphide bonds between monomers that lead to

assembly of the mature pentameric structure (Sitia et al., 1990). ERGIC-53 is a hexameric transmembrane lectin that recognizes the *N*-linked glycan at asparagine 563 (N563) in the C-terminal tailpiece of  $\mu$ , and serves as a platform on which polymerisation of IgM takes place (Anelli et al., 2007). Therefore, stringent quality control machinery from the ER to the Golgi ensures the secretion of correctly assembled antibodies, while misfolded intermediates are retained and disposed of by ERAD.



**Figure 4.2 Schematic of IgM maturation**

In the ER, BiP ensures that  $\mu$  chains do not proceed to the downstream compartments unless assembled with  $L$  chains (1st QC). ERGIC-53, a hexameric lectin, captures  $\mu_2L_2$  subunits, aligning them in a planar conformation suitable for IgM polymerisation. ERp44 (the distribution of which is reflected by the gradient of grey) ensures that unassembled subunits are retrieved into the assembly line (2nd QC). By virtue of its interaction with IgM subunits and ERGIC-53, ERp44 may recruit and locally concentrate  $\mu_2L_2$  to promote polymerization (Anelli et al., 2007).

IgM polymerisation in B-lymphocytes is an inefficient process (Carelli et al., 1997). While LCs can be secreted when unpaired to the HCs (Reddy et al., 1996), unassembled HCs are retained in the ER bound to BiP and eventually degraded by cytosolic proteasomes (Mancini et al., 2000). In resting B-cells, ERAD has been shown to constitutively degrade free  $\mu$  chains, therefore reducing their secretion or presentation at the cell surface (Elkabetz et al., 2003). Among the constituents of

the ERAD machinery, SEL1L and Hrd1 have been implicated in degradation of secretory IgM HC ( $\mu$ S) (Cattaneo et al., 2008). However, the mechanism by which  $\mu$ S is delivered to the cytosol for proteasomal degradation and the contribution of this clearance to cellular homeostasis remain unknown. A recent study highlighted the importance of autophagy in regulating ER homeostasis in differentiated PCs during IgM secretion (Pengo et al., 2013). Here, intense autophagic activity is key to restrict IgM synthesis to sustainable amounts, hence optimising energy metabolism and supporting survival.

#### **4.1.3 IgM HCs in disease**

HC diseases (HCDs) are rare B-cell proliferative disorders associated with the synthesis and secretion of monoclonal, incomplete Ig HCs.  $\mu$ -HCDs represent the rarest form, classified as chronic lymphocytic leukaemia (Bianchi et al., 2014). A variety of genetic alterations, mostly generated in the course of somatic hypermutation, are responsible for the production of secreted HCs (Corcos et al., 2011). In heavy chain deposition diseases (HCDDs), aberrant Ig HC are secreted by PCs due to defective retention and assembly with LCs. Tissue deposition of these chain fragments ultimately leads to organ failure and disease onset (Rane et al., 2012). In addition to HCDs, misfolded Ig chains accumulate in so-called Russell Bodies, particularly prevalent in some severe pathologies like multiple myeloma (MM), autoimmune and autoinflammatory diseases (Kopito and Sitia, 2000). The most frequent aberration in the HCs associated with Russell Bodies is a deletion in the CH1 domain, leading to the formation of detergent-insoluble aggregates in the malignant PCs. However, the most common disease associated with Ig production is MM, a plasma cell neoplasia characterised by uncontrolled production of monoclonal Igs. Currently, proteasome inhibitors represent the frontline treatment for this incurable malignancy (Moreau et al., 2012). Importantly, the balance between proteasome workload and degradation capacity is a critical determinant of



MM sensitivity to proteasome inhibition. In particular, higher proteasome activity inversely correlates with apoptotic sensitivity to Bortezomib (BTZ), highlighting the importance of proteasomal degradation in the regulation of cellular homeostasis and response to treatments (Bianchi et al., 2009).

Besides the pivotal role of Igs in immunity and their involvement in disease, IgMs represent an invaluable model to dissect the folding and degradation machineries in the ER. Firstly, because of IgM's challenging nature, and secondly because unpaired  $\mu$ S subunits are retained in the ER, offering a unique opportunity to investigate the ERAD mechanism in response to accumulation of an individual luminal misfolded protein.

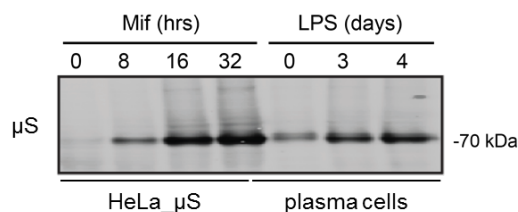
## 4.2 Aim

The contribution of the ERAD components, in particular of Hrd1, to cell viability in response to ER stress has conventionally been assessed via the use of potent ER-stress inducing drugs like Thapsigargin or Tunicamycin (Kaneko et al., 2002). High doses of these chemicals, such as those used in the cited study, can lead to broad pleiotropic effects and often do not represent any physiological situation ever encountered in a biological system. In other experimental settings, knockout mouse models for individual ERAD components do not always elucidate the role of these proteins in the maintenance of cellular homeostasis as a consequence of impaired ERAD activity, due to the high complexity of the organism as a system. For example, homozygous deletion of Hrd1 and Derlin-1 results in embryonic lethality (Amano et al., 2003; Eura et al., 2012), but how their loss affects ER homeostasis or cellular adaptation to stress has not been addressed. In this study, the aim is to mimic physiological ER stress arising from the inducible expression of a secretory misfolded protein. The secretory IgM heavy chain ( $\mu$ S) is overexpressed in HeLa cells to recapitulate the physiological burden of Igs experienced by activated PCs. In this system,  $\mu$ S represents a proteotoxic stress in the ER, rather than a pharmacologically-induced ER stress. Here, attempts are made to elucidate the contribution of individual ERAD components to the restoration of ER homeostasis following proteotoxic stress, and how loss of these components impacts cell viability as a direct consequence of impaired protein degradation.

## 4.3 Results

### 4.3.1 HeLa\_μS cells as a model for proteotoxic stress

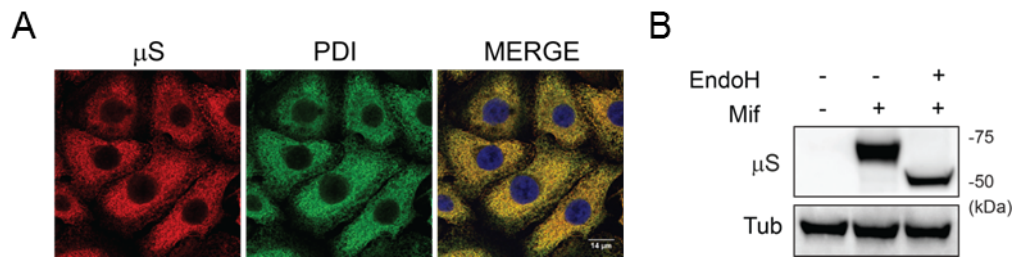
HeLa cells were previously demonstrated to be a suitable model to investigate the folding and maturation of secretory IgM, displaying all the intracellular requirements for its multi-step assembly into polymeric structures (Anelli et al., 2007; Cortini and Sitia, 2010). For this reason, and given their amenability to be manipulated compared to immortalized B-cells, HeLa cells were employed as a model system, where μS expression was controlled by a Mifepristone (Mif)-inducible promoter. To do so, the μS gene was integrated downstream of the Mif promoter in the genome of HeLa cells by lentiviral infection. A clonal population was selected and tested for μS expression by treatment with 0.5 nM Mif for the time indicated (Figure 4.3). Ectopic μS expression in HeLa cells was comparable to endogenous production of μS by differentiating murine i29+ B-lymphocytes upon lipopolysaccharide (LPS) treatment.



**Figure 4.3 Immunoblot for μS on HeLa\_μS and murine i29+ cells**

HeLa cells were stimulated with Mifepristone (Mif) (0.5 nM) and murine i29+ cells were induced to differentiate with LPS (lipopolysaccharide) for the time indicated. Figure generated by van Anken Lab.

This observation supports the validity of using μS-expressing HeLa cells to mimic the high burden of antibodies generated by activated PCs. To confirm the presence of μS in the ER upon overexpression, HeLa\_μS cells were treated with Mif (12 hours) and co-localisation of μS with the ER-resident chaperone PDI was observed by immunofluorescence (Figure 4.4 A).



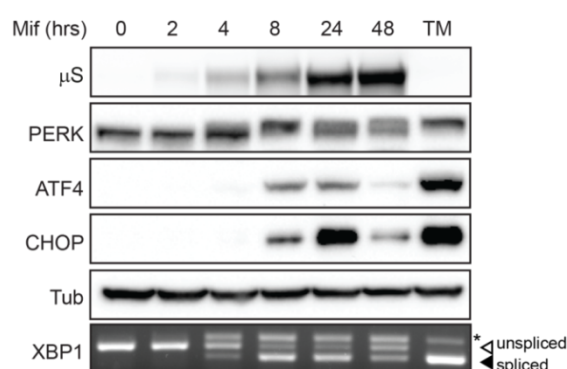
**Figure 4.4  $\mu$ S localises to the ER**

A) Immunofluorescence of HeLa\_ $\mu$ S cells treated with Mif (0.5 nM) for 12 hours.  $\mu$ S was detected using an  $\alpha$ -IgM HC conjugated to Alexa546. The ER was stained with an anti-PDI antibody and revealed using an  $\alpha$ -mouse Alexa488 secondary antibody. Images were acquired by confocal microscopy, objective 63 $\times$ . B) Immunoblot for  $\mu$ S on WCL. HeLa\_ $\mu$ S were stimulated with Mif (0.5 nM) for 24 hours. Lysates were incubated with EndoH to digest *N*-glycans. Tubulin was used as a loading control.

ER localisation was also assessed by treating WCL from  $\mu$ S-expressing HeLa cells with EndoH, which resulted in  $\mu$ S being fully converted to a slower migrating band that reflects specific trimming of the high mannose *N*-linked glycan (Figure 4.4 B). Together, these data confirm that intracellular  $\mu$ S glycoprotein is localised and retained in the ER.

#### 4.3.2 $\mu$ S expression induces the UPR and ERAD components

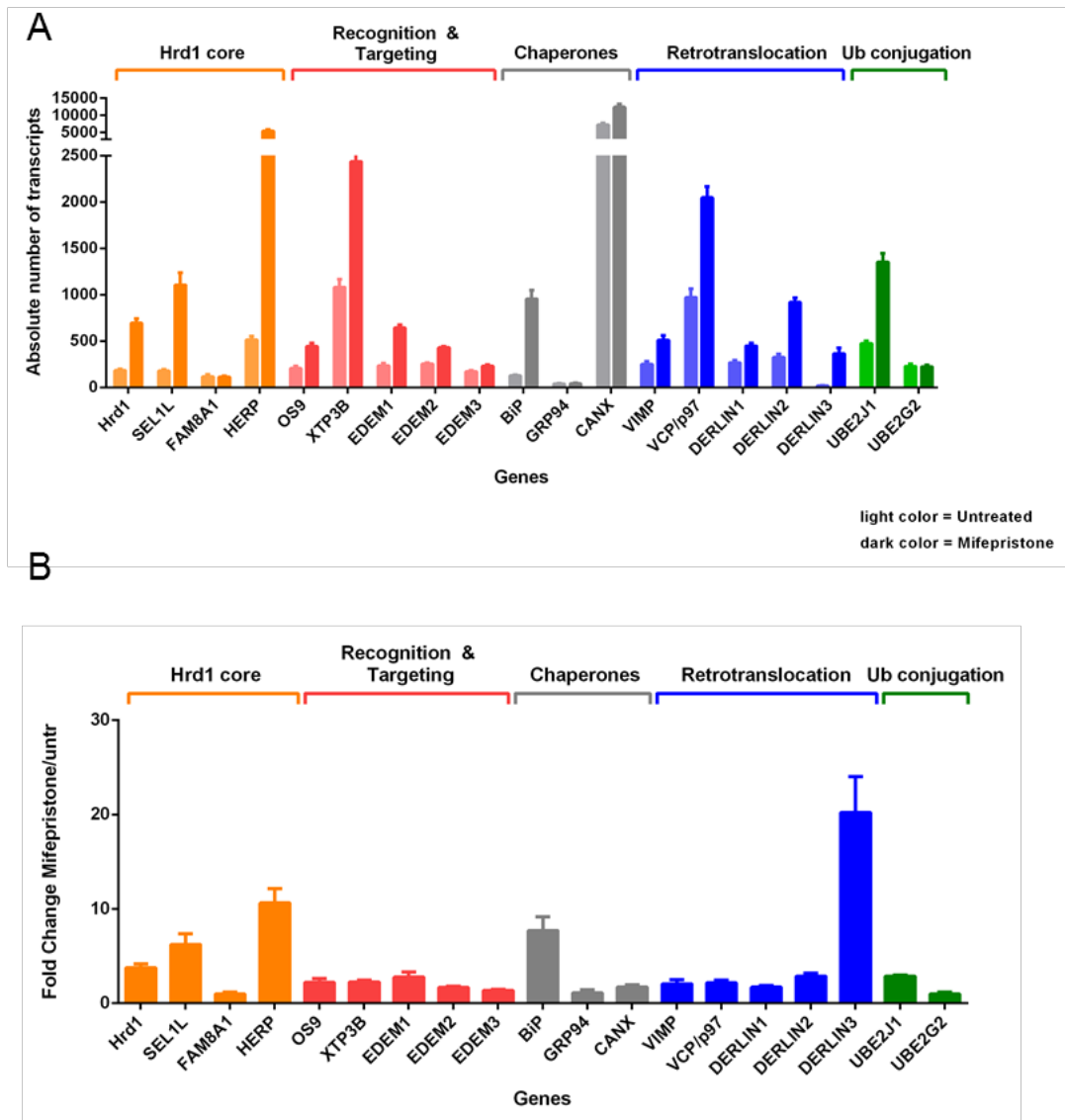
The inability of  $\mu$ S to assemble and oligomerise, and its subsequent accumulation in the ER, make it a suitable model with which to recapitulate protein misfolding in the ER; therefore, whether  $\mu$ S expression was sufficient to induce ER stress was investigated. Activation of the UPR was tested by monitoring the induction of the PERK and IRE1 pathways while inducing  $\mu$ S expression over a 48-hour time-course. While XBP1 mRNA (downstream of IRE1) was already spliced by 4 hours, PERK activation and its downstream targets ATF4 and CHOP appeared after 8 hours of induction, corresponding to greater detectable  $\mu$ S expression (Figure 4.5).



#### Figure 4.5 $\mu$ S induces the UPR

Immunoblot for  $\mu$ S and the ER stress markers PERK, ATF4 and CHOP (Top). PCR for XBP1 mRNA (Bottom). '\*' indicates a hybrid band consisting of half spliced and half unspliced XBP1. HeLa\_ $\mu$ S cells were treated with Mif (0.5 nM) for the time indicated. Cells were treated with TM (1  $\mu$ g/ml) for 8 hours as a positive control.

Importantly, UPR signalling downstream of PERK and IRE1 started to recover at 48 hours, reflecting the capability of the UPR to terminate after acute signalling. It was reasoned that attenuation of  $\mu$ S-driven ER stress would likely be mediated by upregulation of ER chaperones and ERAD genes following UPR activation. Hence, transcriptional analysis was performed using the Nanostring platform (Geiss et al., 2008) to gain quantitative insight into the expression of selected ERAD components and ER chaperone genes after 16 hours of  $\mu$ S induction. Figure 4.6 A shows absolute abundance of ER chaperones and ERAD transcripts before (steady-state) and after  $\mu$ S expression in HeLa cells, highlighting significant diversity within the expression patterns of factors participating in the same machinery.



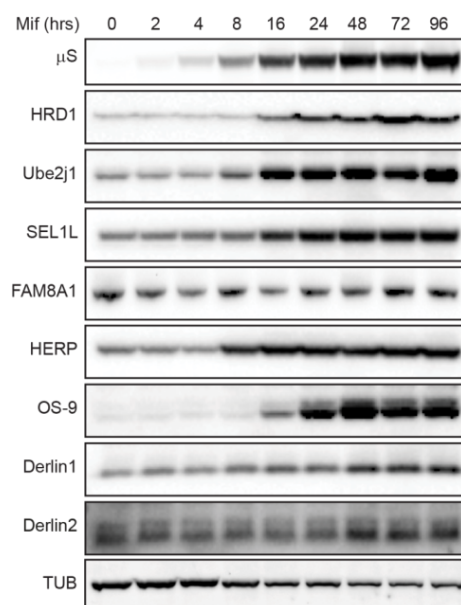
**Figure 4.6 Transcriptional analysis of ERAD genes by Nanostring technology**

A) Absolute number of transcripts are represented on the y-axis. Error bars indicate SD from three biological replicates. HeLa<sub>μ</sub>S cells were treated with Mif (0.5 nM) for 16 hours prior to analysis. B) Fold change induction from transcriptional analysis in A. Transcripts measured from Mif treatment were normalised to the untreated condition. Error bars indicate SD from three biological replicates.

Notably, it was found that the most highly transcribed genes induced by  $\mu$ S expression corresponded to the ubiquitin ligase Hrd1, its cofactors SEL1L, Herp, and Ube2j1, the chaperone BiP and Derlin-3 (Figure 4.6 B).

Next, it was asked whether transcriptional upregulation corresponded with increased protein expression, crucial for executing the reinforcement of folding and degradation capacities in the ER.

To do so, selected ERAD components were monitored by immunoblot over a time-course of  $\mu$ S induction. Concomitant upregulation of all Hrd1 complex components was observed during 16 hours of  $\mu$ S expression, with the exception of the Hrd1 interaction partner FAM8A1 (Christianson et al., 2012), which remained unchanged. Interestingly, Derlin-1 and -2 levels also rose but with a slight delay in onset (Figure 4.7).



**Figure 4.7 ERAD components are upregulated by  $\mu$ S at the protein level**  
Immunoblot for  $\mu$ S and selected Hrd1 complex components from WCL of HeLa\_ $\mu$ S treated with Mif (0.5 nM) for the times indicated.

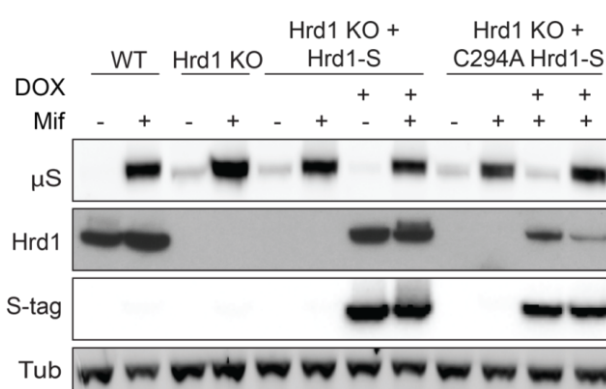
These results demonstrate that  $\mu$ S can serve as a model misfolded protein, suitable to induce the canonical UPR that leads to increased folding and degradation capacities within the ER.

### 4.3.3 $\mu$ S degradation requires Hrd1 catalytic activity

To understand the functional implications of increased ERAD capacity in response to  $\mu$ S accumulation, CRISPR-Cas9 genomic editing was used to generate HeLa\_ $\mu$ S cells that lacked Hrd1. Two sgRNAs targeting independent sites of the Hrd1 locus were designed to reduce potential off-target effects of genomic manipulation.

Individual clones from the puromycin-resistant population were evaluated by WB for Hrd1 expression and at least three positive clones were tested in biochemical and phenotypic assays to exclude clone-dependent effects. In addition, C-terminally S-tagged wild-type (WT) or RING mutant (C294A) Hrd1 were reintroduced into a clonal Hrd1 KO HeLa\_μS cell line to gain deeper insight into the functional activity of the ligase. Stable integration of Hrd1-S (WT or C294A) was accomplished by lentiviral infection, while a DOX inducible promoter enabled controlled Hrd1 expression. In all experiments, expression of Hrd1 was induced 24 hours prior to μS expression by addition of DOX (500 ng/ml). Reconstitution of Hrd1 KO HeLa\_μS cell lines with Hrd1-S (WT or C294A) and expressing μS (+Mif, 8 hours) is shown in

Figure 4.8. Comparable Hrd1-S expression between WT and C294A was observed when WBs were probed with an antibody toward the S-tag but not with anti-Hrd1, where mutation of the RING domain may interfere with the availability of the epitope recognised by this antibody.

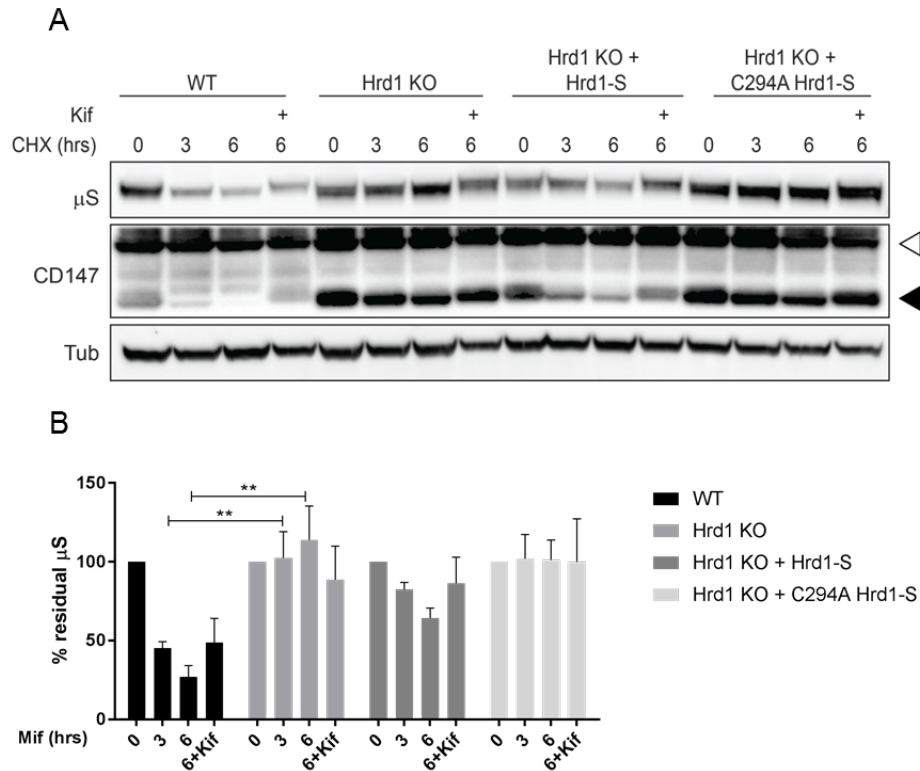


**Figure 4.8 Reconstitution of Hrd1 KO HeLa\_μS with WT and Hrd1-S C294A**

Immunoblot for μS, Hrd1 and S-tag from WCL. Clonal Hrd1 KO HeLa\_μS cells are restored with lentivirus carrying either WT Hrd1-S or mutant Hrd1-S C294A. Ectopic expression of Hrd1-S is induced with DOX (500 ng/ml) for 24 hours before treatment with Mif (0.5 nM) for an additional 8 hours.



To determine whether Hrd1 was responsible for degrading  $\mu$ S, we compared the stability of  $\mu$ S in WT, Hrd1 KO, Hrd1 KO + Hrd1-S WT and Hrd1 KO + Hrd1-S C294A HeLa cells using translational shutoff assays. Each cell line that was induced to express  $\mu$ S (+Mif, 4 hours) was subsequently treated with CHX (0, 3 and 6 hours) to arrest protein synthesis, and the remaining  $\mu$ S was quantified at each time point. Cells treated with the mannosidase inhibitor kifunensine (KIF, 20  $\mu$ M, 6 hours) were also included as a positive control, as KIF prevents initial recognition of  $\mu$ S and subsequent targeting for degradation. WT HeLa\_ $\mu$ S displayed 27% residual  $\mu$ S after 6 hours of CHX treatment, showing relatively fast degradation kinetics. In contrast,  $\mu$ S degradation was completely abrogated in cells lacking Hrd1. Reintroduction of WT Hrd1-S but not the RING mutant was able to partially restore degradation, leading to a 64% residual  $\mu$ S after 6 hours of chase (Figure 4.9 A and B). Therefore, it can be concluded that  $\mu$ S degradation relies on catalytically active Hrd1. Importantly, the endogenous Hrd1 substrate CD147 (Tyler et al., 2012) exhibited a similar degradation pattern compared to  $\mu$ S, confirming the integrity and reliability of the cell lines generated.



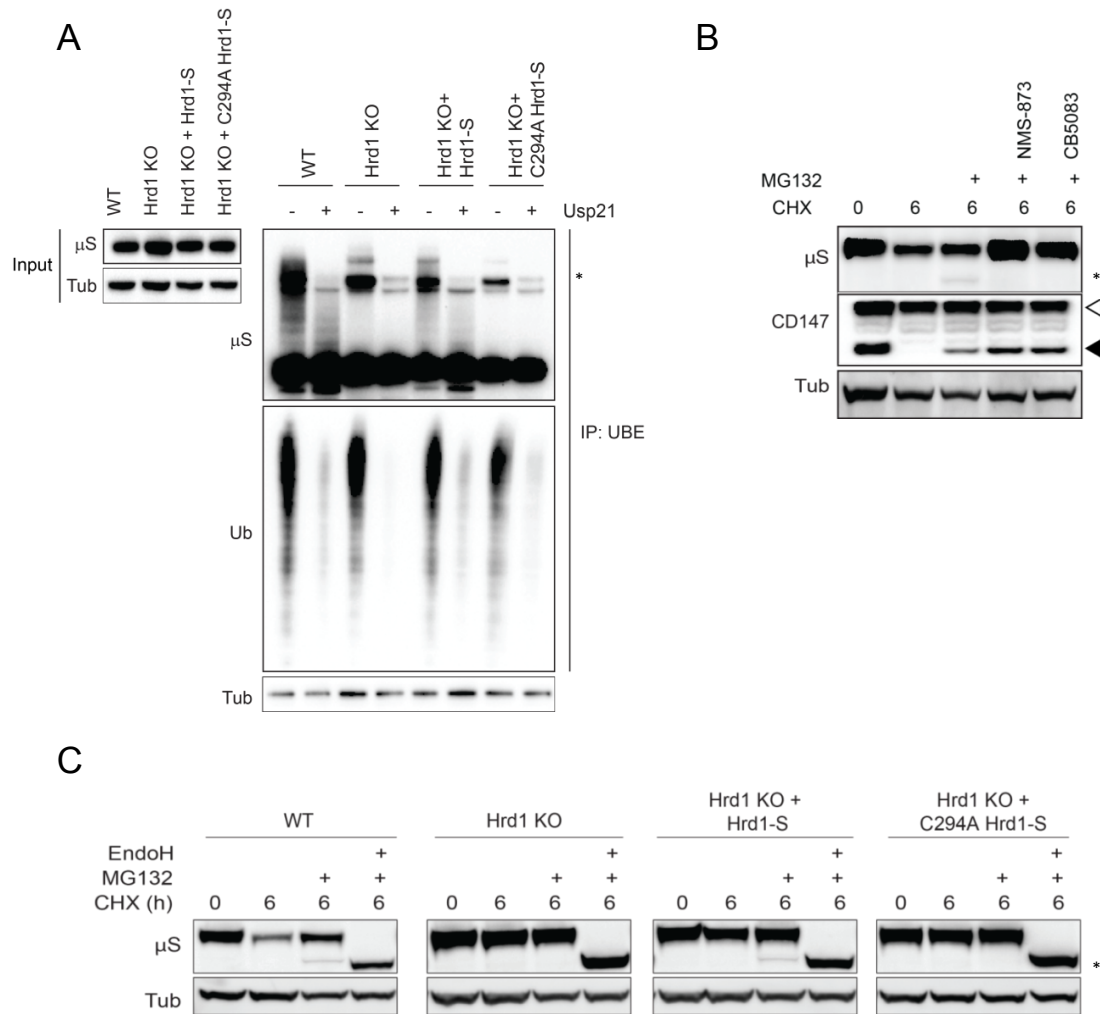
**Figure 4.9  $\mu$ S degradation is impaired in the absence of functional Hrd1**

A) Immunoblot for  $\mu$ S and endogenous CD147 from WCL of HeLa\_ $\mu$ S cells treated with Mif (0.5 nM) for 4 hours and then incubated with CHX (100  $\mu$ g/ml) for the time indicated. Kif (20  $\mu$ M) was used in combination with CHX for 6 hours.  $\mu$ S was revealed with Alexa546  $\alpha$ -IgM heavy chain, tubulin was detected by mouse  $\alpha$ -tubulin antibody followed by Alexa488  $\alpha$ -mouse antibody. White arrow: mature, fully glycosylated CD147; black arrow: immature, ER-retained CD147. B) Quantitation of residual  $\mu$ S from A. Error bars indicate SD from three biological replicates. Statistical significance is calculated using Student's *t*-test. \* *p*-value < 0.05, \*\* *p*-value < 0.01.

Given that  $\mu$ S degradation was dependent upon the activity of Hrd1, it was next asked whether direct ubiquitination of  $\mu$ S by Hrd1 could be detected. Therefore,  $\mu$ S ubiquitination was analysed in the cell lines described above. To this aim,  $\mu$ S was induced for 24 hours with a lower dose of Mif (50 pM) to prevent saturation of the ubiquitination machinery. Enrichment of total ubiquitinated proteins was achieved by blocking proteasome activity with MG132 for 1 hour prior to harvesting. Ubiquitinated proteins from WCL were captured by a GST-ubiquitin-His6 entity (Ub-binding entity, UBE), with the UBA (Ub associated) domain being derived from the human protein Ubiquilin-1.

Enrichment of ubiquitinated species was assessed by anti-ubiquitin WB. Comparable amounts of ubiquitinated proteins were affinity purified from all samples, as demonstrated by the equal presence of a canonical high molecular weight smear of polyUb immunoreactive material that was sensitive to treatment with the deubiquitinating enzyme Usp21 (Ubiquitin-specific-processing protease 25) (Figure 4.10 A). WBs probed for  $\mu$ S revealed the presence of Usp21-sensitive high molecular weight smears in WT and Hrd1 KO cells reconstituted with WT Hrd1-S. These smears were absent in cells lacking Hrd1 or reconstituted with the catalytically inactive form. This result indicates that polyubiquitination of  $\mu$ S occurs in a Hrd1-dependent manner via its catalytic domain.

Luminal substrates targeted to ERAD require transport across the ER membrane prior to their degradation in the cytoplasm, a process suspected to occur via Hrd1 (Baldrige and Rapoport, 2016; Stein et al., 2014). To determine whether Hrd1 was necessary for retrotranslocation of  $\mu$ S from the ER, the accumulation of deglycosylated forms was monitored during CHX chase assays. It was found that concomitant treatment of HeLa\_ $\mu$ S cells with CHX and MG132 permitted detection of deglycosylated  $\mu$ S, reflecting the exposure of *N*-linked oligosaccharides to the NGly1 enzyme localised in the cytoplasm (Figure 4.10 B). The faster migrating  $\mu$ S band in MG132 treatment displayed a molecular weight comparable to that of  $\mu$ S treated with EndoH. Treatment with either of the VCP/p97 inhibitors, NMS-873 or CB-5083, prevented the appearance of these faster migrating forms, in accordance with the ERAD model where activity of VCP/p97 drives substrate retrotranslocation (Y. Ye et al., 2004).



**Figure 4.10 Hrd1 ubiquitinates and mediates  $\mu$ S retrotranslocation**

A) Immunoblot for  $\mu$ S and Ub. HeLa\_ $\mu$ S cells were treated with Mif (50 pM) for 24 hours and incubated with MG132 (5  $\mu$ g/ml) for 1 hour before harvesting. Total ubiquitinated proteins were immunoprecipitated using UBE conjugated to GSH-beads and then treated with the deubiquitinating enzyme Usp21 to confirm that the high molecular weight smear corresponds to Ub. (\*) aspecific band. 4% of WCL is loaded as input. 8% of UBE IP is loaded for Ub blot.

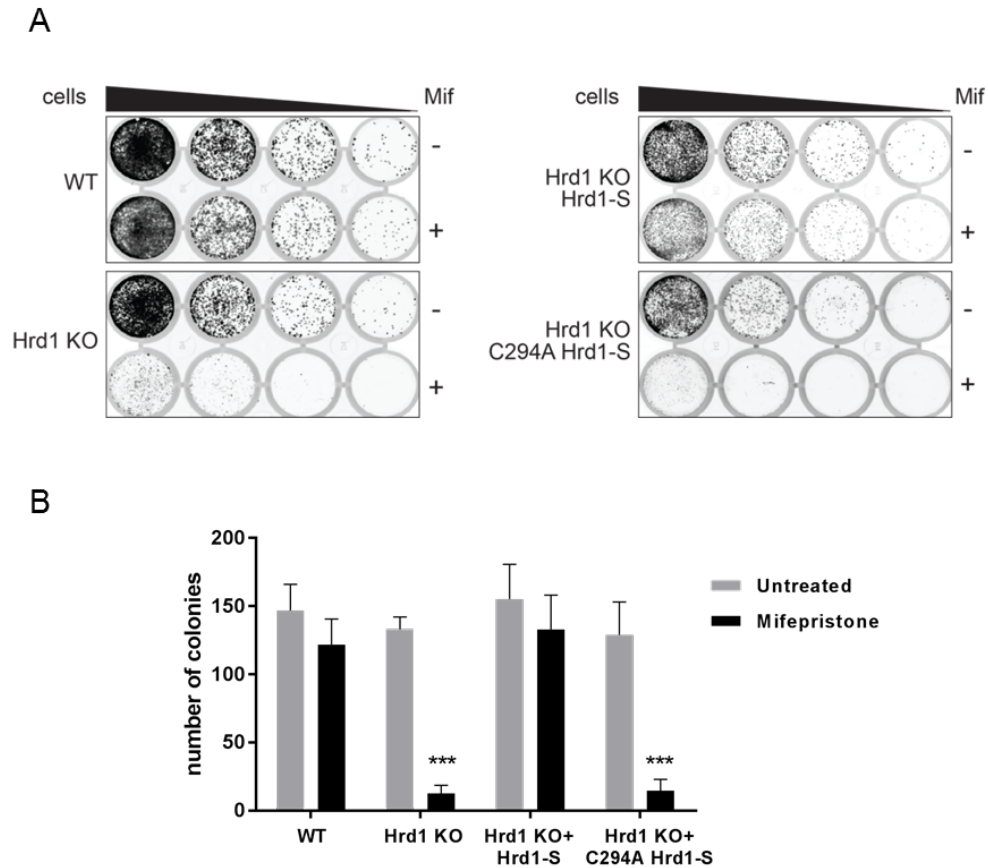
B) Immunoblot for  $\mu$ S and endogenous CD147 from WCL of WT HeLa\_ $\mu$ S treated with Mif (0.5 nM) for 4 hours and then incubated with CHX (100  $\mu$ g/ml) for 6 hours. MG132 (10  $\mu$ g/ml) and the VCP inhibitors NMS-873 (5  $\mu$ M) and CB5083 (5  $\mu$ M) were used in combination with CHX for 6 hours.  $\mu$ S was revealed with Alexa546  $\alpha$ -IgM heavy chain, tubulin was detected by mouse  $\alpha$ -tubulin antibody followed by Alexa488  $\alpha$ -mouse antibody. White arrow: mature, fully glycosylated CD147; black arrow: immature, ER-retained CD147, (\*) deglycosylated  $\mu$ S.

C) Immunoblot for  $\mu$ S and tubulin from WCL of HeLa\_ $\mu$ S cells treated as in B (without VCP inhibitors). EndoH treatment was performed on lysates and used as a control for deglycosylated  $\mu$ S (\*).

To address the question of direct involvement of Hrd1 in  $\mu$ S retrotranslocation, cells lacking Hrd1 and reconstituted with WT or mutant Hrd1-S were tested under the same conditions. It was found that only reconstitution with WT Hrd1-S was sufficient to produce  $\mu$ S in a deglycosylated form that was comparable to EndoH treated  $\mu$ S from the same lysates (Figure 4.10 C). This indicates that ubiquitination by Hrd1 of either itself (Baldrige et al., 2016) or of  $\mu$ S (or both) is necessary for the transport of luminal misfolded proteins from the ER to the cytosol in a p97/VCP-dependent manner.

#### **4.3.4 Hrd1 is essential for survival under $\mu$ S proteotoxic stress**

Since Hrd1 seemed to be the only ubiquitin ligase involved in  $\mu$ S ubiquitination at basal levels, the phenotypic consequence of loss of Hrd1 in cells expressing  $\mu$ S was assessed. A clonogenic assay was employed to measure the capability of Hrd1-deficient cells to survive and proliferate upon  $\mu$ S expression. HeLa\_ $\mu$ S WT, Hrd1 KO and reconstituted lines were seeded at decreasing densities and grown for 7 days while induced to express  $\mu$ S. Colonies were detected by crystal violet staining (Figure 4.11 A) and subsequently counted to measure cell proliferation (Figure 4.11 B).



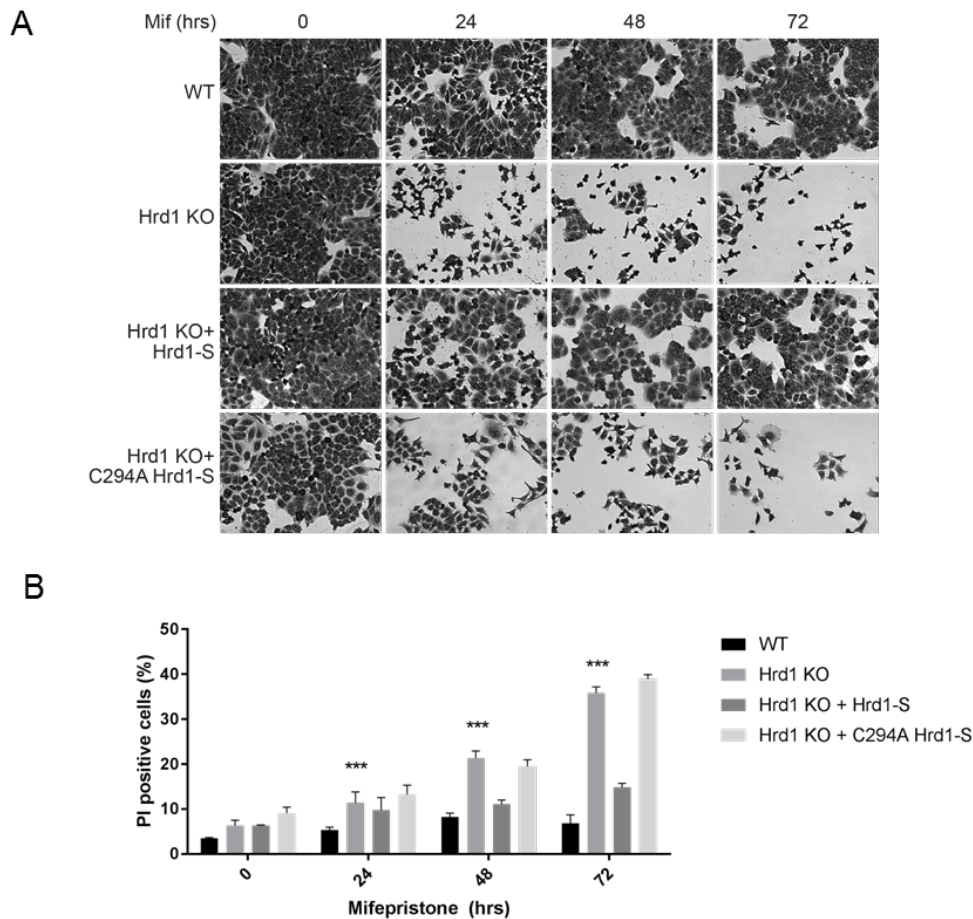
**Figure 4.11 Clonogenic assay of WT and Hrd1 KO HeLa\_μS cells**

A) Cells were serially diluted 1:5, starting with 5,000 cells/well, and incubated with Mif (0.5 nM) for 7 days before fixation and crystal violet staining. Images were acquired at Typhoon FLA9000. B) Quantitation of colonies from A. Colonies were quantified from the third dilution (approx. 200 cells). Error bars indicate SD from six biological replicates. Statistical significance was calculated using two-way ANOVA test comparing WT to Hrd1 KO and reconstituted cells in the Mif condition. \*\*\*  $p$ -value < 0.001.

HeLa\_μS cells displayed only a mild impairment in colony formation upon μS expression (88% colonies compared to untreated), but predominantly remained viable and able to proliferate. Notably, the absence of Hrd1 was tolerated and cells remained viable under non-induced conditions. This result suggests that cells can withstand 'physiological' fluxes of misfolded proteins that spontaneously arise, even when a key ERAD component is missing. In other words, lack of Hrd1-mediated ERAD does not overload the ER with toxic species at basal levels. However, when challenged with μS expression, cells lacking functional Hrd1 were significantly impaired in survival and proliferation, resulting in reduced colony formation (11%

colonies compared to untreated). Restoring ligase activity by inducing the expression of Hrd1-S was able to rescue growth (86% colonies compared to untreated), in contrast to the reintroduction of the RING mutant (11%). These results confirm that the ubiquitin ligase activity of Hrd1 is essential for cells to survive when challenged with proteotoxic ER stress.

Colony formation is a complex and integrated phenotype: the cumulative result of multiple cellular events over an extended time scale (7 days). To gain insight into the toxicity induced by  $\mu$ S, cell growth was monitored over shorter time periods. HeLa\_ $\mu$ S cells were induced with Mif for 24, 48 and 72 hours and then stained with crystal violet (Figure 4.12 A). Notable impairment to cell growth could be seen by 24 hours of  $\mu$ S expression when Hrd1 activity was absent from HeLa\_ $\mu$ S cells (Hrd1 KO).



**Figure 4.12  $\mu$ S is toxic in cells lacking functional Hrd1**

A) Inverted light microscopy of HeLa\_ $\mu$ S cells incubated with Mifepristone (0.5 nM) for the time indicated followed by fixation and crystal violet staining. B) Quantitation of HeLa\_ $\mu$ S cells positive for Propidium Iodide (PI) staining treated with Mif as in A. Cells were harvested after the time indicated, stained with PI and analysed by flow cytometry (FACS). Error bars indicate SD from three biological replicates. Statistical significance is calculated using a two-way ANOVA test comparing WT to Hrd1 KO cells. \*\*\*  $p$ -value < 0.001.

Under the same growth conditions, cell death was evaluated by measuring incorporation of PI into cells by flow cytometry (Figure 4.12 B). Similarly, WT cells were only mildly affected by  $\mu$ S expression, displaying a percentage of cell death ranging between 3 and 8% over the whole period of treatment. Notably, cell death peaked at 48 hours (8%) and started decreasing at 72 hours (7%), possibly reflecting adaptation to stress achieved by a reinforcement of folding and degradation capacities. In contrast, early increase in the frequency of cell death was



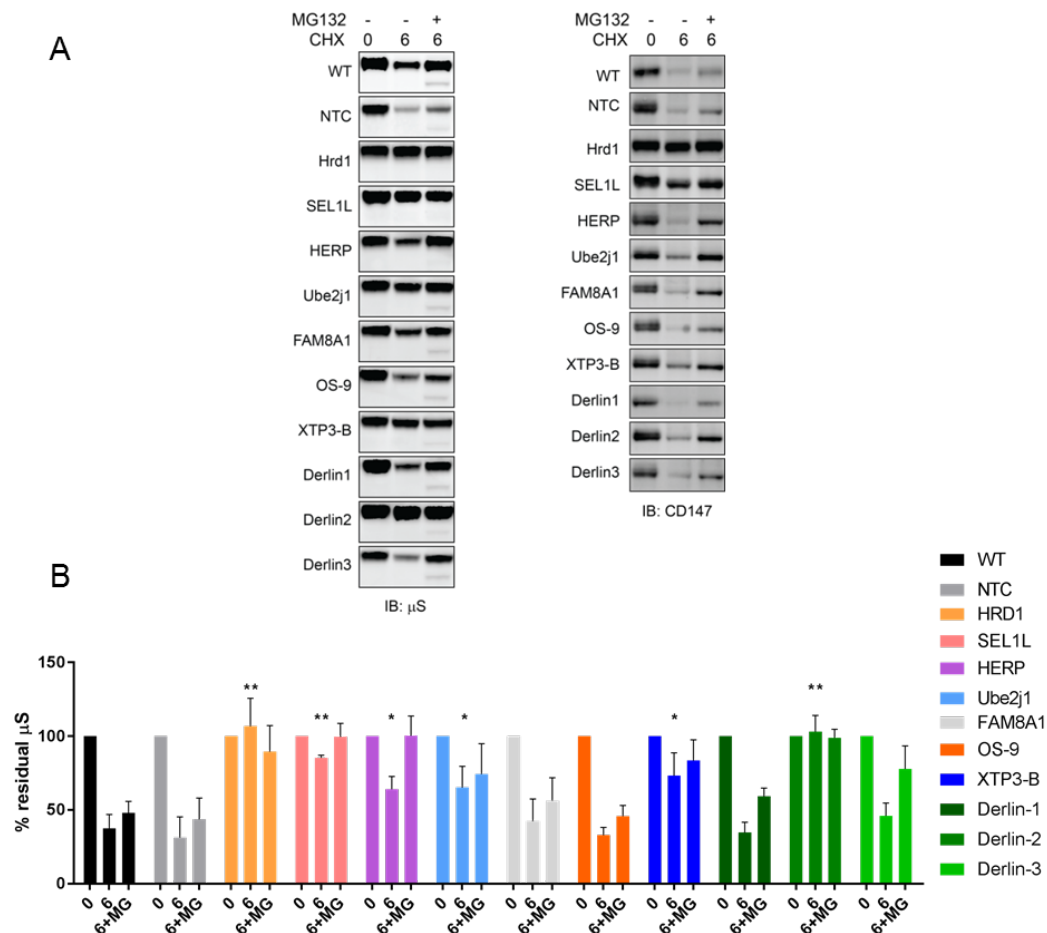
observed by 24 hours of  $\mu$ S expression in cells lacking Hrd1 (11%), where the death rate continued to rise to 36% after 72 hours of  $\mu$ S induction. Cell viability could be rescued by reintroducing WT Hrd1-S (15% cell death at 72 hours) but not the C294A RING mutant (39% cell death at 72 hours). These data confirm the necessity of Hrd1 activity for cell survival under conditions of proteotoxic ER stress.

#### **4.3.5 Identification of key ERAD components under proteotoxic stress**

Having established the pivotal role of Hrd1 under conditions of proteotoxic challenge, whether or not other components of the Hrd1 complex also play a critical role in the maintenance of cellular homeostasis under proteotoxic stress was investigated. To this aim, CRISPR-Cas9 genomic editing was used to generate deletions of the Hrd1 interactors Herp, Ube2j1, FAM8A1, OS-9 and XTP3-B. Derlin-1, -2 and -3 were also included, with the aim of elucidating their controversial role in ERAD. Two unique sgRNAs per target gene were designed to introduce deletions while minimising potential off-target effects. sgRNAs were cloned into a vector containing the puromycin resistance gene to allow selection of positive clones. In some cases, the selected pools, rather than single cell clones, were used for further investigation. Therefore, these pools are referred to as 'depleted' for the selected ERAD component, rather than as knockouts. Depletion of the individual proteins was confirmed by WB from WCL and by immunoprecipitation of Hrd1 or SEL1L in the case of XTP3-B (Figure 4.18). Moreover, because experiments performed on the two cell lines from individual sgRNAs elicited similar phenotypes, the data presented hereafter may have been carried out using either cell line. CRISPR-Cas9-mediated deletions of either SEL1L or Derlin-3 could not be obtained, hence a siRNA knockdown strategy was instead employed for these targets. Knockdown efficiencies of SEL1L and Derlin-3 were validated by WB and qPCR, respectively, confirming a significant depletion (Figure 4.18).

To assess  $\mu$ S stability in cells lacking individual components of the ERAD machinery,  $\mu$ S expression was induced (4 hours) and a CHX chase (6 hours) was conducted in the absence or presence of MG132 (Figure 4.13 A).  $\mu$ S levels were determined by quantification of WBs from three biological replicates for each cell line (Figure 4.13 B). After CHX treatment, residual  $\mu$ S was significantly higher in cells lacking SEL1L (85%), Herp (64%), Ube2j1 (65%), XTP3-B (73%) or Derlin-2 (103%), compared to controls (37% in WT and 31% in NTC), highlighting the important contribution of Hrd1 cofactors to the degradation of this luminal misfolded glycoprotein. To assess their contribution to disposal of a membrane-bound substrate, WBs of the same samples were probed for endogenous CD147 (Figure 4.13 A, right). It was found that turnover of immature CD147 was markedly impaired by the absence of Hrd1 and SEL1L, while degradation was only mildly attenuated in cells depleted for Ube2j1, XTP3-B and Derlin-2. Interestingly, Herp appeared to be dispensable for the degradation of membrane CD147. These results emphasise the specificity that some Hrd1 cofactors have towards different substrate classes, whereas Hrd1 and SEL1L are necessary for the degradation of both luminal and membrane substrates.

Additionally, it was asked whether the same components required for  $\mu$ S degradation may also participate in retrotranslocation of  $\mu$ S from the ER lumen. Using the experimental paradigm discussed previously (Figure 4.13), deglycosylated  $\mu$ S was not observed in cells depleted for SEL1L, Herp or Derlin-2 (partially), reflecting  $\mu$ S accumulation in the ER lumen and impairment of substrate retrotranslocation. Notably, the lack of Ube2j1 did not affect  $\mu$ S retrotranslocation, in line with the role of the E2 conjugating enzyme in substrate ubiquitination upon retrotranslocation.

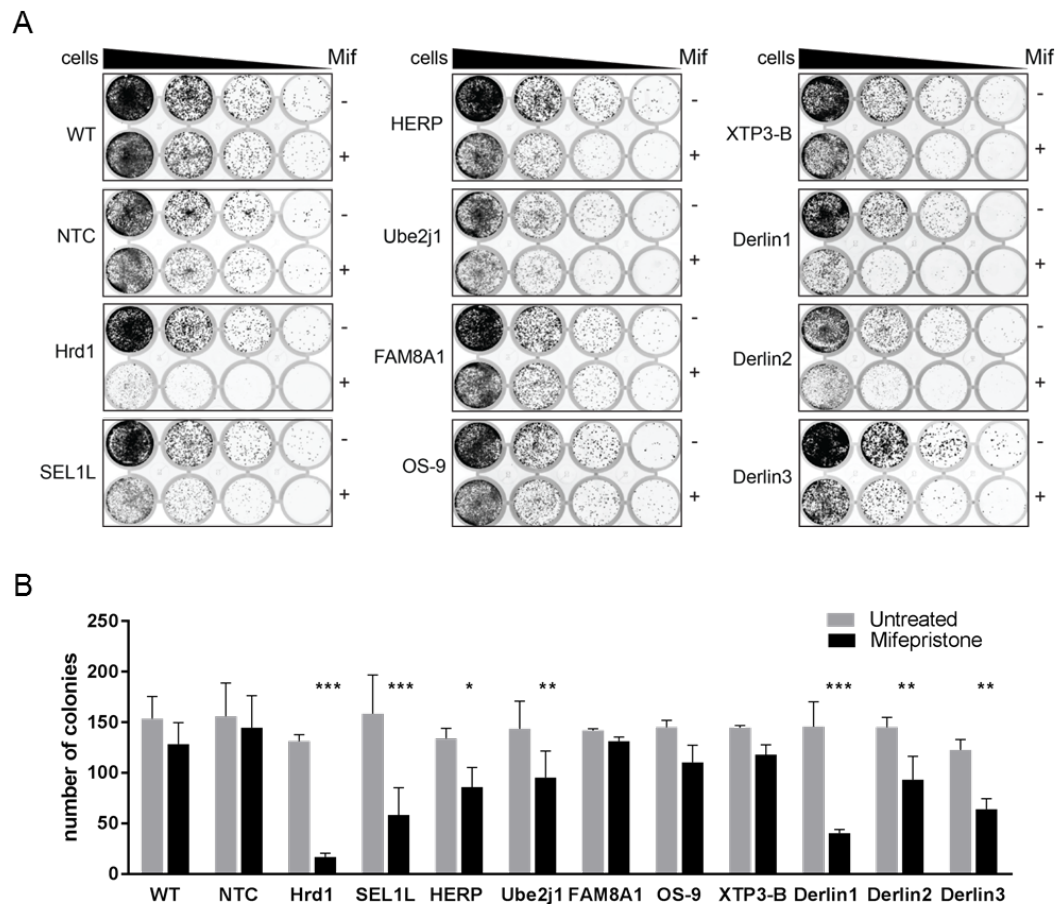


**Figure 4.13  $\mu$ S degradation is impaired in cells depleted for SEL1L, Herp, Ube2j1, XTP3-B and Derlin-2**

A) Immunoblot for  $\mu$ S (left) and endogenous CD147 (right) from WCL of HeLa\_ $\mu$ S cells depleted for the indicated components. Both  $\mu$ S and CD147 are visualized using fluorescent antibodies. Cells were treated with Mif (0.5 nM) for 4 hours, followed by CHX addition alone or in combination with MG132 (10  $\mu$ g/ml) for 6 hours. B) Quantitation of residual  $\mu$ S (%) from A. Error bars indicate SD from three biological replicates. Statistical significance is calculated using the Student's *t*-test comparing each cell line to WT or NTC at 6 hours of CHX treatment. \* *p*-value < 0.05, \*\* *p*-value < 0.01, \*\*\* *p*-value < 0.001.

Having assessed which Hrd1 complex components are required for  $\mu$ S degradation, it was next asked whether their absence would impact survival during  $\mu$ S expression. To this aim, clonogenic assays were performed on all cell lines depleted for Hrd1 cofactors (Figure 4.14 A), followed by crystal violet staining and colony quantification (Figure 4.14 B). Colony formation was severely impaired in cells depleted for SEL1L (35%), Herp (64%), Ube2j1 (66%), Derlin-1 (28%), Derlin-2 (64%) or Derlin-3 (53%), compared to the untreated condition. Notably, the

depletion of any of the above components did not significantly affect cell viability when unchallenged, indicating an acute requirement for activity of the Hrd1 complex and its associated ERAD machinery during stress conditions.

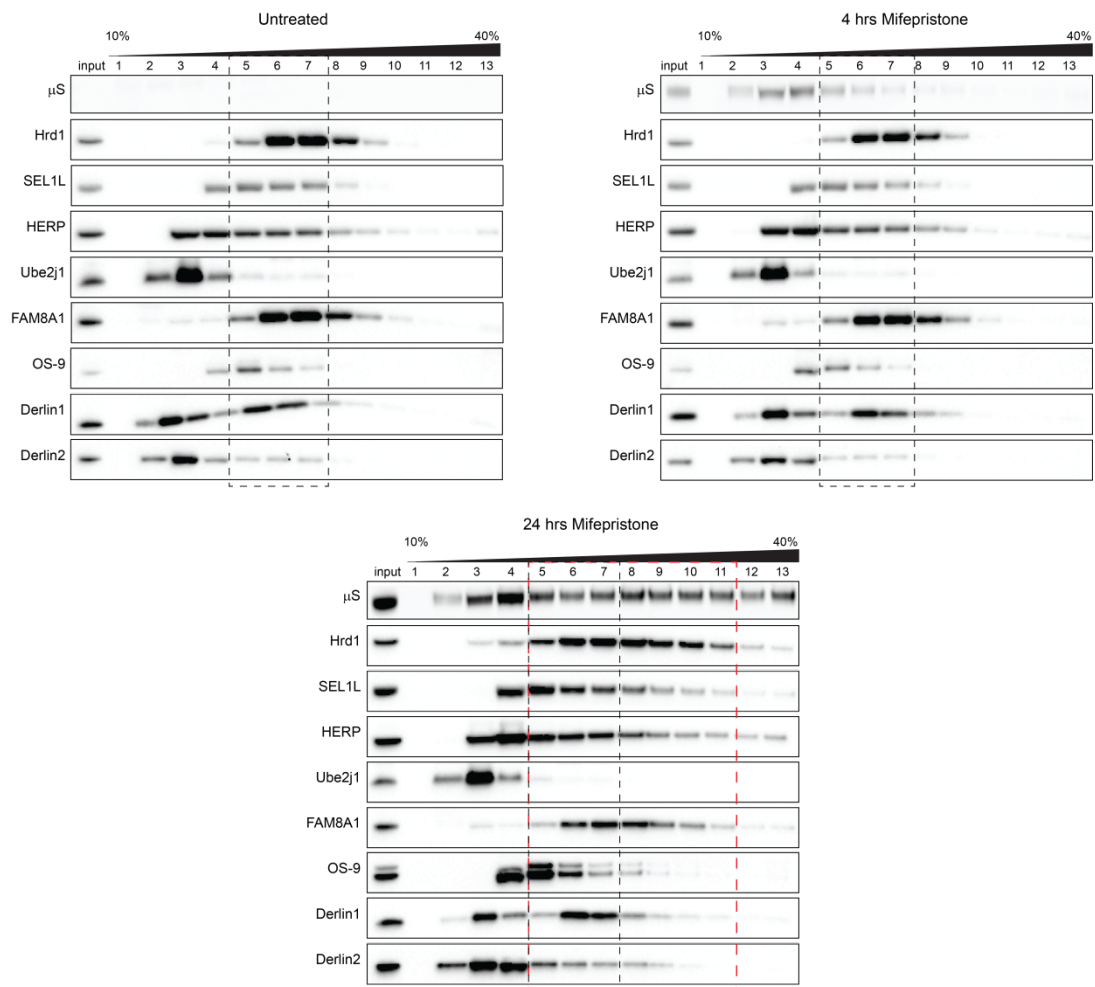


**Figure 4.14  $\mu$ S is toxic in cells depleted for SEL1L, Herp, Ube2j1 and Derlins**

A) Clonogenic assay of HeLa\_ $\mu$ S cells depleted for the indicated components. Cells were serially diluted 1:5 starting with 5000 cells/well and incubated with Mif (0.5 nM) for 7 days before fixation and crystal violet staining. Images were acquired with a Typhoon FLA9000. B) Quantitation of colonies from clonogenic assay in A. Colonies were quantified from the third dilution (approx. 200 cells). Error bars indicate SD from three biological replicates. Statistical significance is calculated using a two-way ANOVA test comparing each cell line to WT in the Mif condition. \*  $p$ -value < 0.05, \*\*  $p$ -value < 0.01, \*\*\*  $p$ -value < 0.001.

#### **4.3.6 Differential Hrd1 complex assembly under proteotoxic stress**

It was hypothesised that the composition of Hrd1 complexes may vary between basal and stress conditions, with the rationale that an increased demand for degradation might necessitate a different complex assembly. To gain insight into Hrd1 complex dynamics during proteotoxic stress, lysates from HeLa\_μS cells untreated or expressing μS (Mif for 4 and 24 hours) were sedimented on 10–40% sucrose gradients. Thirteen individual fractions were collected, precipitated, separated by SDS-PAGE and the resulting WBs probed for Hrd1 complex components (Figure 4.15). Under basal conditions (untreated), the core complex components (Hrd1, SEL1L, Herp, FAM8A1 and OS-9) and Derlin-1 co-sedimented between fractions 5 and 7. When low levels of proteotoxic stress were induced (Mif, 4 hours), no change was observed in the sedimentation patterns of the Hrd1 complex. Under these conditions, the pre-existing Hrd1 complexes were sufficient to degrade the low amounts of μS (and other misfolded proteins) without undergoing significant rearrangement (as shown in Figure 4.9). In contrast, 24 hours of μS expression caused the whole Hrd1 core complex (except OS-9) to shift to heavier fractions (up to fraction 11), suggesting that higher molecular weight complexes may form in response to proteotoxic stress. This effect was unlikely to be due to an upregulation of these proteins, since neither OS-9 nor Derlin-2 underwent the same shift, despite being upregulated.

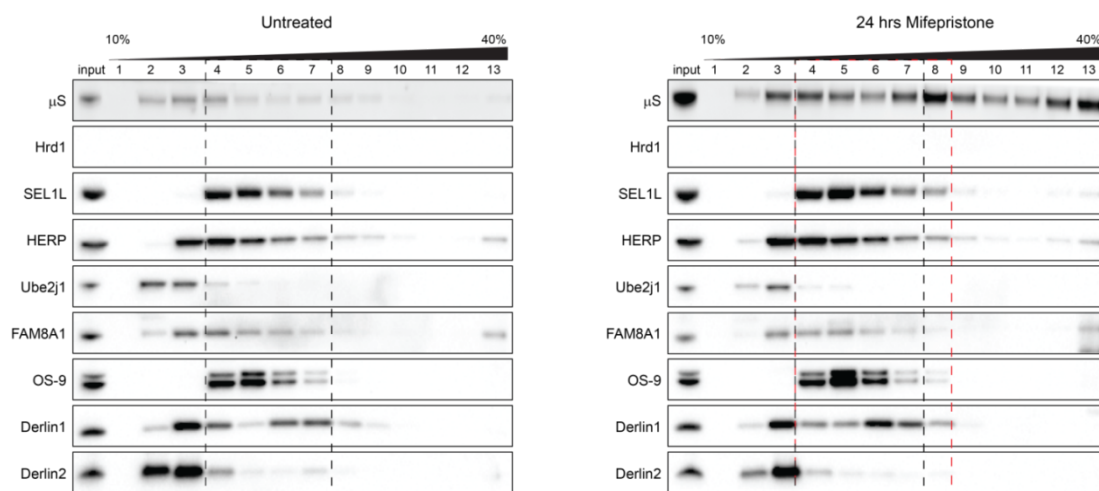


**Figure 4.15 Sucrose gradient of the Hrd1 complex from WT cells**

Immunoblot for selected ERAD components from 13 fractions of a 10-40% sucrose gradient. HeLa\_μS cells were either untreated or induced with Mif (0.5 nM) for 4 and 24 hours. 25 μg of total proteins from WCL were loaded as input.

To determine the importance of Hrd1 in the maintenance of functional integrity within the ERAD machinery, complex assembly was investigated upon loss of Hrd1. Sucrose gradient centrifugation was performed on lysates from Hrd1 KO HeLa\_μS cells following the same procedure. Since no detectable changes in component sedimentation patterns were observed after 4 hours of μS expression, μS was induced for 24 hours using a lower dose of Mif (50 pM) to prevent toxicity. At basal conditions, lack of Hrd1 did not cause any remarkable perturbation in the sedimentation profile of its core components (SEL1L, Herp and FAM8A1) (Figure 4.16), even though both FAM8A1 and Ube2j1 were destabilised, as has been

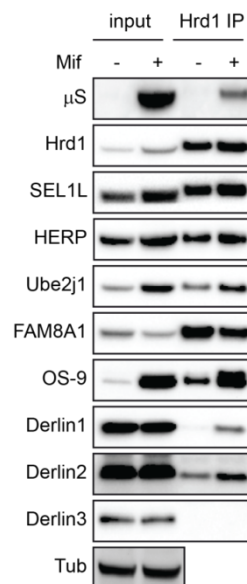
observed previously (unpublished results). However, upon  $\mu$ S expression, sedimentation of the core components in higher fractions was completely abrogated, as opposed to WT cells. In particular, the Hrd1 interactors SEL1L and Herp remained concentrated between fractions 4 and 8, possibly reflecting their assembly into complex intermediates. As in WT HeLa\_ $\mu$ S cells,  $\mu$ S was found to sediment across all fractions, which could reflect polymerisation with itself or ER-resident chaperone complexes owing to unresolved  $\mu$ S accumulation. More importantly, the absence of Hrd1 precluded a shift to heavier fractions of complex components during overexpression of  $\mu$ S. Although Ube2j1 and FAM8A1 were destabilised in the absence of Hrd1, possibly contributing to the destabilisation of the whole complex, it is most likely that Hrd1 is the key driver for the assembly of functional degradation complexes under proteotoxic stress.



**Figure 4.16 Sucrose gradient of the Hrd1 complex from Hrd1 KO cells**

Immunoblot for selected ERAD components from 13 fractions of a 10-40% sucrose gradient. HeLa\_ $\mu$ S cells were either untreated or induced with Mif (50 pM) for 24 hours. 25  $\mu$ g of total proteins from WCL were loaded as input.

To determine the composition of Hrd1 complexes under proteotoxic stress, Hrd1 was immunoprecipitated from WT cells, either untreated or expressing  $\mu$ S (24 hours), and the co-IP components were analysed by WB (Figure 4.17). While interactions with the core components SEL1L, Herp, FAM8A1 and Ube2j1 were already present under basal conditions, Derlin-1 appeared to be selectively recruited to the Hrd1 complex only under  $\mu$ S-induced ER stress. Derlin-2 interaction with Hrd1 also seemed to be reinforced in the presence of  $\mu$ S, suggesting an increased engagement of Derlin-1 and -2 within the Hrd1 complex under proteotoxic stress, possibly resulting in increased ERAD capacity.



**Figure 4.17 Composition of the Hrd1 complex in WT HeLa  $\mu$ S cells**

Immunoblot for ERAD components upon immunoprecipitation of endogenous Hrd1. Cells were treated with Mif (0.5 nM) for 24 hours. 400  $\mu$ g of WCL were used to IP Hrd1 and 25% of the sample was loaded onto an SDS-PAGE gel. 30  $\mu$ g of total proteins from WCL were loaded as input.

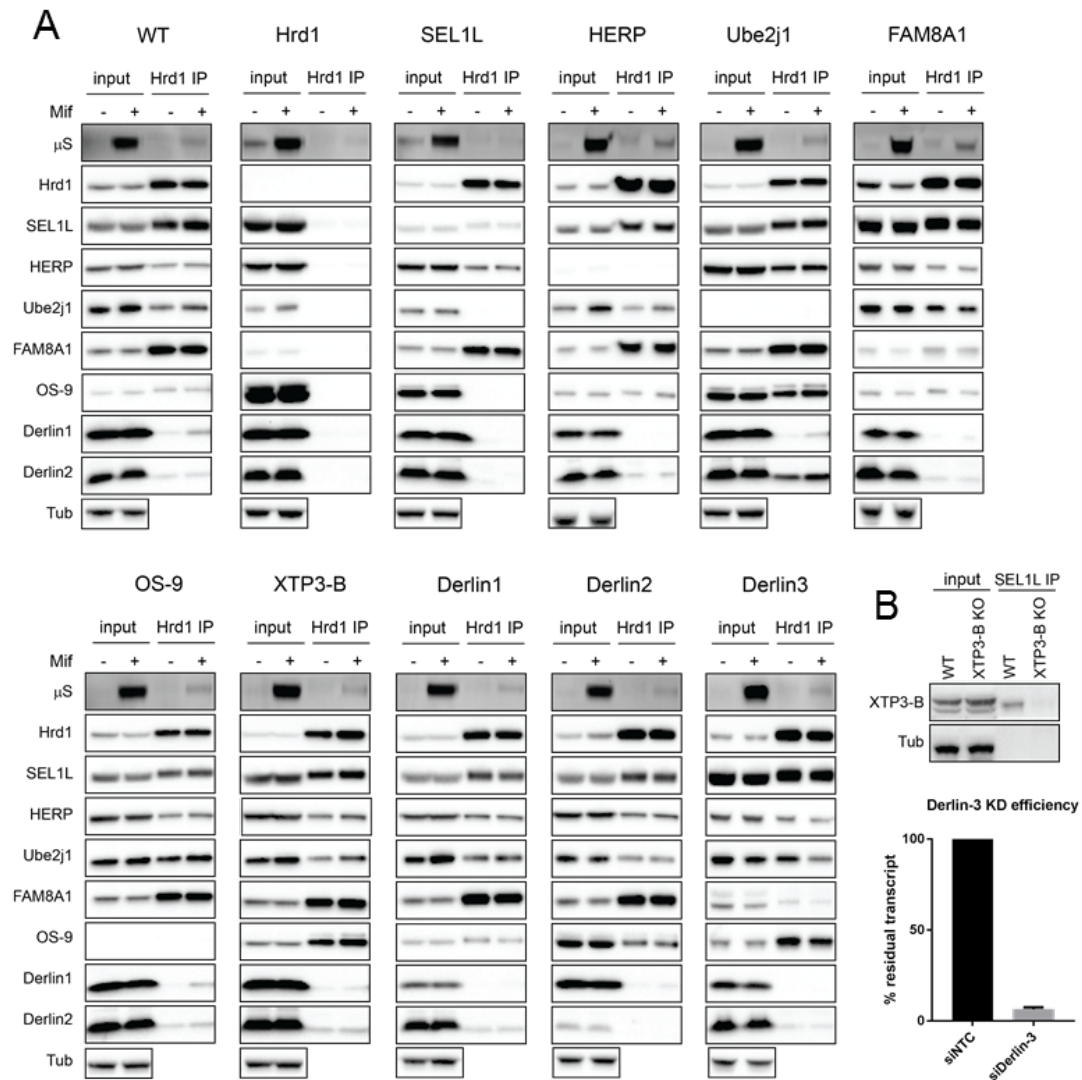
To correlate the phenotypic toxicity of  $\mu$ S with potential defects in Hrd1 complex integrity, the interactions within the Hrd1 complex upon ablation of individual core components were investigated. Cell lines depleted for individual ERAD components were each induced to express  $\mu$ S (4 hours). A shorter induction time was chosen to avoid UPR activation and consequently the transcriptional upregulation of the ERAD



machinery. In this way, any enhanced interaction with Hrd1 observed in response to the increasing  $\mu$ S burden could be attributable to the recruitment of existing components rather than to their upregulation. WBs of Hrd1 complex components co-immunoprecipitated with Hrd1 revealed co-dependent stabilisation of selected factors (Figure 4.18).

Specifically, SEL1L depletion decreased steady-state levels of Hrd1, Ube2j1 and FAM8A1, while depletion of Ube2j1, XTP3-B and Derlin-1 each reduced steady-state levels of Hrd1. Steady-state OS-9 levels were higher in cells lacking SEL1L, Hrd1, Ube2j1 or Derlin-2, suggesting that OS-9 could be a substrate of the Hrd1 complex as well as a cofactor. As expected, loss of SEL1L prevented the interaction of Ube2j1, OS-9, Derlin-1 and Derlin-2 with Hrd1, compromising the integrity of the complex and correlating with reduced viability during  $\mu$ S expression. In addition, attenuating expression of Derlin-2, Herp and XTP3-B (partially) compromised the interaction of Derlin-1 with Hrd1 under proteotoxic stress. Steady-state SEL1L levels were higher in all conditions with reduced FAM8A1 levels (e.g. loss of Hrd1, FAM8A1 or Derlin-3), while Derlin-1 levels decreased in the absence of Herp, Derlin-3 and possibly FAM8A1.

These results highlight the complicated interconnectivity within the Hrd1 complex, and emphasise the pivotal role of select components for maintaining complex integrity, which is required to resolve proteotoxic stress. In summary, cells lacking Hrd1, SEL1L, Herp, Ube2j1, Derlins and partially XTP3-B corresponded to those exhibiting the most severe phenotypes in response to accumulating  $\mu$ S (Figure 4.14).

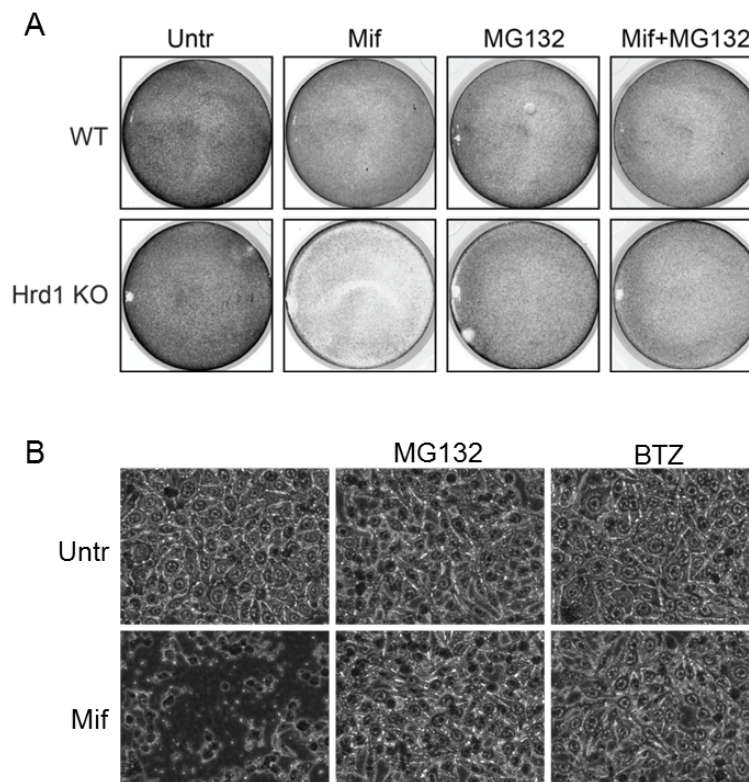


**Figure 4.18 The Hrd1 complex in cells depleted for ERAD components**

A) Immunoblot for ERAD components upon immunoprecipitation of endogenous Hrd1. HeLa $_{\mu}$ S cells depleted for the indicated ERAD components were treated with Mifepristone (0.5 nM) for 4 hours. 400  $\mu$ g of WCL were used to immunoprecipitate Hrd1 and 25% of the sample was loaded on SDS-PAGE. 30  $\mu$ g of total proteins from WCL were loaded as input. B) Validation of XTP3-B and Derlin-3 depletion: Immunoblot for XTP3-B upon immunoprecipitation of SEL1L from XTP3-B-depleted cells (top); RT qPCR for Derlin-3 in HeLa $_{\mu}$ S knocked down for Derlin-3, 48 hours after transfection (bottom). Values are normalised to actin and error bars indicate SD from three biological replicates.

#### 4.3.7 Autophagy activation protects from proteotoxic stress

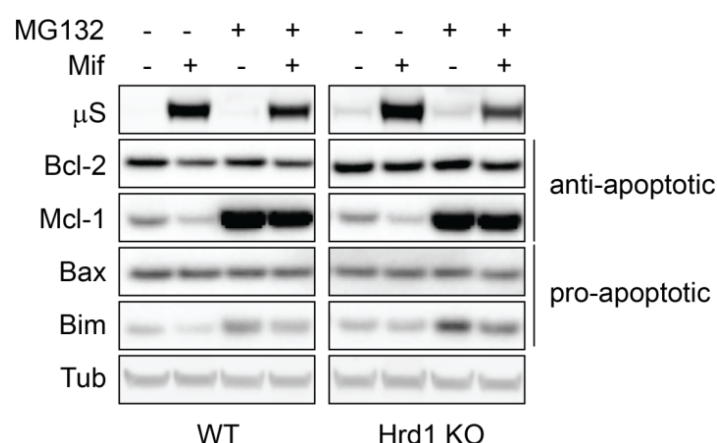
To confirm the role of the proteasome in  $\mu$ S degradation,  $\mu$ S expression was induced for 16 hours in WT and Hrd1 KO HeLa\_ $\mu$ S cells under concomitant treatment with MG132 (1  $\mu$ g/ml), and growth was monitored using crystal violet staining (Figure 4.19 A). Surprisingly, while  $\mu$ S expression was lethal in Hrd1-deficient HeLa\_ $\mu$ S cells, blockage of proteasomal degradation was able to rescue viability. To confirm the phenotype and exclude the possibility that the rescue was due to pleiotropic effects of MG132, Hrd1 KO cells were treated with the proteasome specific inhibitor BTZ (10 nM, 16 hours). Both proteasome inhibitors had an equally protective impact on the viability of Hrd1-deleted cells under proteotoxic stress (Figure 4.19 B).



**Figure 4.19 Proteasome inhibitor rescues growth of Hrd1 KO HeLa\_ $\mu$ S cells**

A) Cells were seeded at the same density and incubated with the indicated drugs for 16 hours (Mif 0.5 nM, MG132 1  $\mu$ g/ml). Cells were then fixed and stained with crystal violet. Images were acquired with a Typhoon FLA9000. B) Inverted microscopy of Hrd1 KO HeLa\_ $\mu$ S cells treated for 16 hours with the indicated drugs (Mif 0.5 nM, MG132 1  $\mu$ g/ml, BTZ 10 nM). Cells were then fixed and stained with crystal violet. Images were acquired by light inverted microscopy, objective 40 $\times$ .

To further investigate the underlying cytoprotective mechanism, it was reasoned that proteasome inhibition either: i) stabilised anti-apoptotic factors or ii) induced autophagy as a compensatory degradation pathway. Changes in the abundance of the most common regulators of apoptosis were monitored in response to proteasome inhibition and  $\mu$ S expression for 12 hours. It was found that both anti-apoptotic Mcl-1 and pro-apoptotic Bim were stabilised, while levels of Bcl-2 and Bax were not significantly altered (Figure 4.20). Importantly, a marked reduction in total  $\mu$ S levels was observed upon MG132 treatment, suggesting that  $\mu$ S was either being degraded, secreted or having its detergent solubility altered. This prompted the conclusion that MG132 interference with the apoptotic machinery was not likely to be the mechanism underlying the growth rescue.

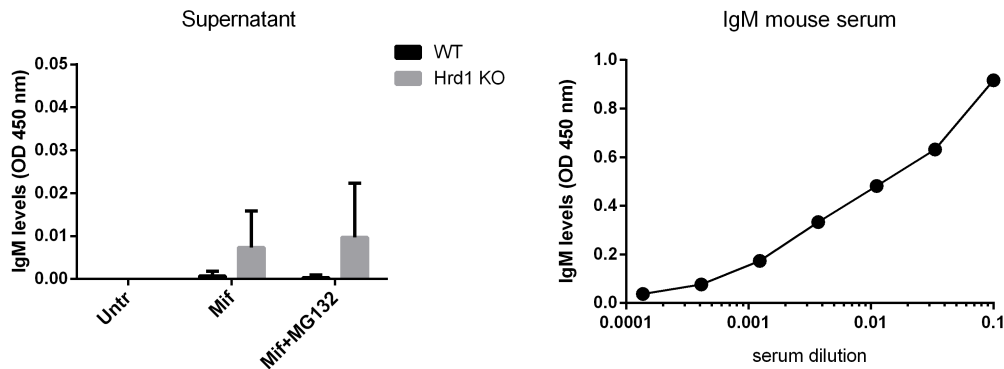


**Figure 4.20 Apoptotic signatures upon proteasome inhibition**

Immunoblot for anti-apoptotic and pro-apoptotic markers. HeLa\_ $\mu$ S cells were treated with Mif (0.5 nM) and MG132 (1  $\mu$ g/ml) for 12 hours. Protein expression was analysed from WCL.

To exclude the possibility that the decrease in intracellular  $\mu$ S observed with proteasome inhibition resulted from promoting the clearance of  $\mu$ S through secretion,  $\mu$ S levels present in the media were measured by ELISA (Figure 4.21). The levels of secreted  $\mu$ S from Hrd1-deleted cells showed negligible differences

with MG132 treatment and were markedly less than intracellular levels. These data indicate that  $\mu$ S is not secreted when proteasome activity is impaired.

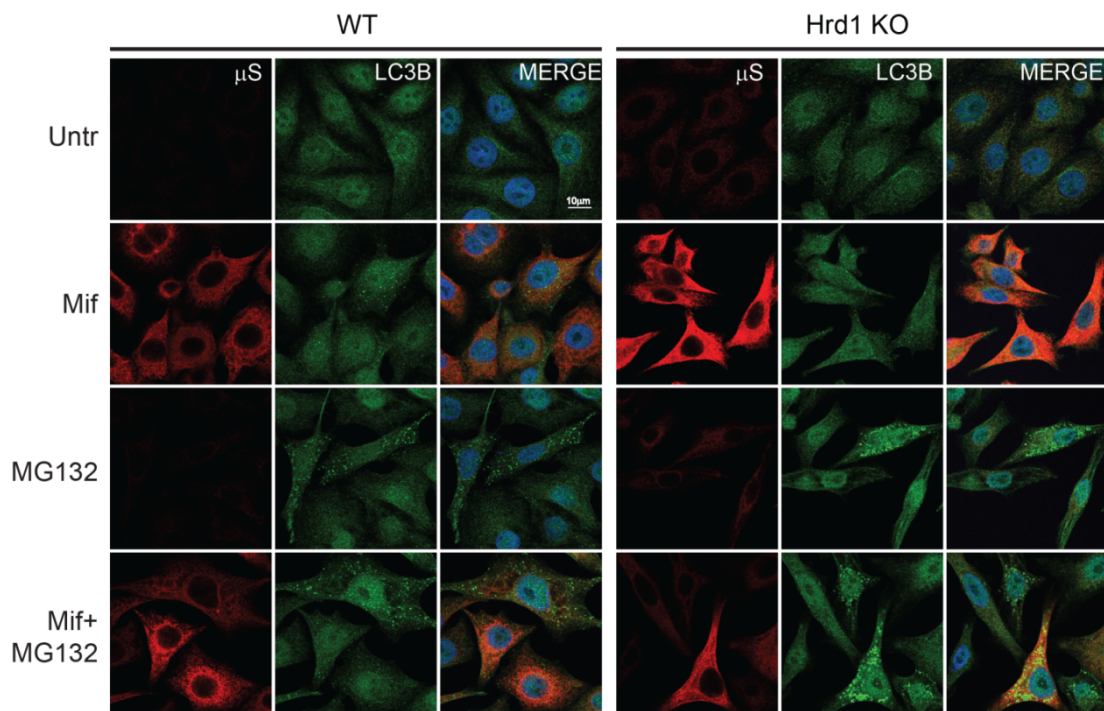


**Figure 4.21 ELISA assay for mouse IgM**

Left: Quantitation of secreted IgM from HeLa\_ $\mu$ S cells treated with the indicated drugs for 12 hours (same doses as in Figure 4.20). Right: standard curve of mouse IgM from mouse serum serially diluted 1:10.

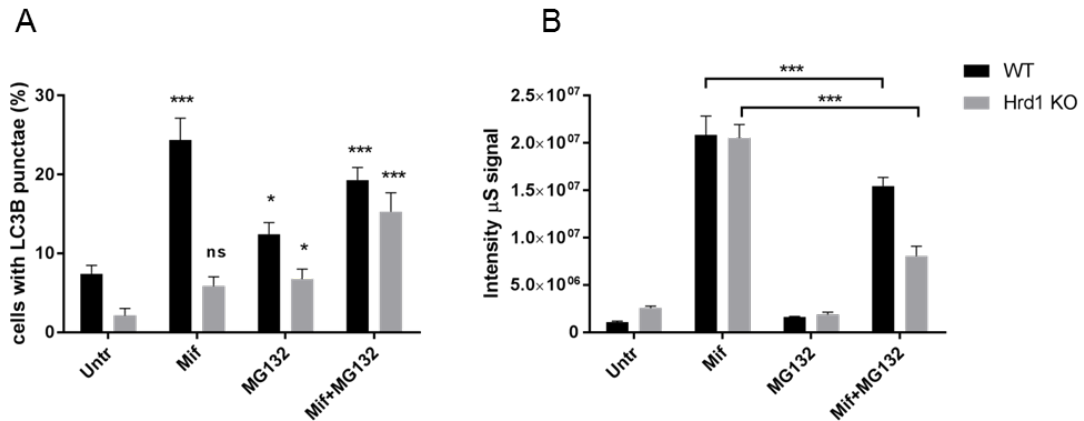
To ask whether the activation of autophagy could serve as a compensatory degradation pathway for  $\mu$ S triggered by proteasome impairment, autophagy activation upon MG132 treatment in  $\mu$ S-expressing cells was quantified by LC3B immunofluorescence in WT and Hrd1-deleted HeLa\_ $\mu$ S cells (Figure 4.22).  $\mu$ S signal intensity was concominantly quantified to monitor differences in its intracellular accumulation. Activation of the autophagic pathway was reflected by the increased frequency of cells positive for LC3B punctae (Figure 4.23 A). A high percentage (24%) of cells positive for LC3B punctae was observed in  $\mu$ S-expressing WT cells, reflecting early autophagic induction and suggesting that both proteasomal and autophagic degradation could contribute to the clearance of ER luminal misfolded proteins. In contrast,  $\mu$ S expression alone was not sufficient to activate autophagy in Hrd1-deleted cells, with only 6% of the cells positive for LC3 punctae. This suggests that defects in ERAD processing prior to accessing the

proteasome may not be sensed by the cells and, as a result, do not activate compensatory pathways. However, proteasome inhibition was able to significantly increase autophagy via LC3B, and this correlated with a consistent reduction in  $\mu$ S signal in both WT and Hrd1-deleted cells (Figure 4.23 B). In particular, WT and Hrd1-deleted cells displayed a reduction of  $\mu$ S protein levels of about 30% and 60%, respectively, under proteasome inhibition.



**Figure 4.22 Immunofluorescence for  $\mu$ S and LC3B**

WT and Hrd1 KO HeLa\_ $\mu$ S cells were treated for 12 hours with Mif (0.5 nM) and MG132 (1  $\mu$ g/ml), alone or in combination.  $\mu$ S was revealed with  $\alpha$ -IgM heavy chain conjugated to Alexa546, LC3B was detected using  $\alpha$ -LC3B rabbit antibody followed by  $\alpha$ -rabbit Alexa488 secondary antibody. Images were acquired by confocal microscopy, objective 63 $\times$ .

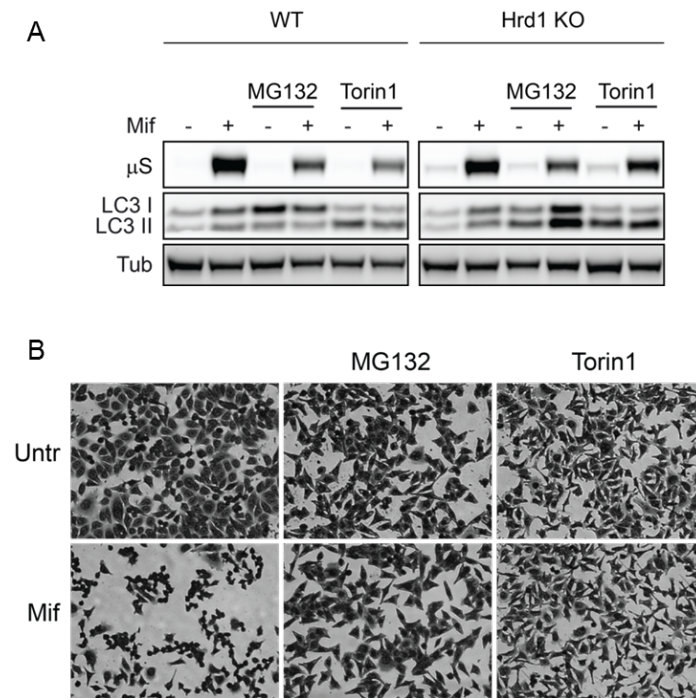


#### Figure 4.23 Quantitation of autophagy and $\mu\text{S}$ expression

A) LC3B punctae were quantified from the experiment in Figure 4.22: images were analysed with ImageJ and cells positive for LC3B punctae were automatically counted using a customised macro. Error bars indicate SD from three biological replicates, 300 cells from each replicate. Statistical significance was calculated using two-way ANOVA compared to the untreated condition. B) Quantitation of  $\mu\text{S}$  signal from same images as in A. Total  $\mu\text{S}$  intensity/cell was automatically calculated with Image J. Error bars indicate SD from three biological replicates, 300 cells from each replicate. Statistical significance was calculated using two-way ANOVA.

These data suggest that misfolded proteins accumulating in the ER may be degraded by autophagy. Using the specific autophagy activator Torin1, it was found that directly inducing autophagy was sufficient to rescue the growth and viability of Hrd1-deficient cells expressing proteotoxic levels of  $\mu\text{S}$  (Figure 4.24 B), mimicking the effect of proteasome inhibitors. Importantly, cells treated with Torin1 for 12 hours displayed reduced amounts of  $\mu\text{S}$  (Figure 4.24 A), indicating that autophagy was capable of degrading misfolded proteins from the ER lumen. LC3-I to LC3-II conversion was monitored alongside to confirm autophagy induction upon Torin1 treatment.





**Figure 4.24 Autophagy reduces  $\mu$ S levels and rescues growth of Hrd1 KO cells**

A) Immunoblot for  $\mu$ S and LC3B from WCL.  $\mu$ S was revealed with Alexa546  $\alpha$ -IgM heavy chain, LC3B was detected by rabbit  $\alpha$ -LC3B antibody followed by Alexa488  $\alpha$ -rabbit. HeLa\_ $\mu$ S cells were treated with the indicated drugs for 12 hours (Mif 0.5 nM, MG132 1  $\mu$ g/ml, Torin1 1  $\mu$ M). B) Inverted microscopy of Hrd1 KO HeLa\_ $\mu$ S cells. Cells were treated as in A for 16 hours, then fixed and stained with crystal violet. Images were acquired by light inverted microscopy, objective 40 $\times$ .

Given these observations, it can be concluded that induction of the autophagic pathway can compensate for deficient proteasomal degradation of misfolded ER proteins, therefore alleviating proteotoxicity and promoting survival.



## **4.4 Discussion**

### **4.4.1 A model of proteotoxic stress to dissect ERAD**

The contribution of mammalian ERAD to cellular homeostasis and the resolution of ER stress has been mostly assessed by the use of chemical agents or deletions of ERAD encoding genes in whole organisms, often in a way that is rather unrepresentative of physiological situations (Doroudgar et al., 2015; Eura et al., 2012; Kaneko et al., 2002). In many cases, the relationship between ERAD and the UPR and the importance of ERAD factors in the degradation of model substrates have been addressed separately, and the phenotypic consequences have been analysed in only a few cases (Belal et al., 2012; Sun et al., 2014). The transcriptional regulation of the ERAD genes has largely been analysed in response to TM, DTT or other canonical ER stress-inducing drugs (Murray et al., 2004; Shen et al., 2007), demonstrating the dependency of ERAD on the UPR (Travers et al., 2000). In most cases, microarray analysis did not provide enough sensitivity to gain information on the lowest transcripts, therefore limiting the power of the analysis (Dombroski et al., 2010; Murray et al., 2004). A quantitative proteomic study on TM-treated neuroblastoma cells revealed upregulation of many ER chaperones and foldases, but disregarded ERAD components, probably due to the relatively low abundance of these factors in complex samples (Bull and Thiede, 2012). More recently, quantitative transcriptional induction of the main ERAD genes has been shown by real-time qPCR in response to TM treatment (Christianson et al., 2012). The relationship between ER stress and ERAD is unquestionable, as is the regulation of ERAD genes by the UPR transcription factors. However, the UPR is commonly defined as the response to misfolded proteins in the ER, where in most experimental settings misfolded proteins are induced by chemical agents that simultaneously affect almost the entire secretome. Such massive perturbation of ER homeostasis is unlikely to ever be encountered in a physiological system. Possibly

one of the greatest perturbations that a cell can encounter is during its high secretory activity, for instance during antibody or insulin secretion by B-lymphocytes and pancreatic beta cells, respectively. In a more physiological system, it has been shown that, upon antigen presentation, B-cells prepare for the high burden of antibody secretion by upregulating ER chaperones and ERAD components (van Anken et al., 2003). A subsequent study revealed the participation of SEL1L and Hrd1 in the degradation of IgM heavy chains in HeLa cells, highlighting the role of ERAD in antibody quality control (Cattaneo et al., 2008). More recently, the involvement of SEL1L and Hrd1 in pre-BCR turnover has been confirmed in developing B-cells (Ji et al., 2016). However, how ERAD is functionally implemented to respond to the increased demand for degradation, as well as the requirement of ERAD for survival under basal and high secretory activity, remain largely unknown. On the other hand, studies focusing on the functional requirement of ERAD for degradation of misfolded substrates mostly addressed the dependency of the substrates on specific components of the degradation machinery from an exclusively biochemical perspective. For example, several ERAD components, including SEL1L and Hrd1, have been extensively reported as necessary for the degradation of model substrates such as mutant A1AT NHK-QQQ (Christianson et al., 2008; Hosokawa et al., 2003; Y. Zhong et al., 2015), mutant TTR (Susuki et al., 2009) and endogenous CD147 (Tyler et al., 2012). These and similar studies are key in the understanding of the molecular mechanisms by which substrates are recognised and delivered for degradation. However, in most cases, the phenotypic impact of such regulation is not addressed.

*In vivo* experiments in animal models have highlighted the requirement of specific ERAD components for development or tissue homeostasis as a whole. For instance, homozygous deletions of Hrd1, SEL1L or Derlin-1 lead to embryonic lethality (Eura et al., 2012; Francisco et al., 2010; Yagishita et al., 2005), Derlin-2 deletion results

in premature death after birth (Dogan et al., 2011), whereas Derlin-3 and Hrd1 deficiencies are dispensable for development (Eura et al., 2012). SEL1L conditional deletion in adult mice provokes early lethality and pancreatic atrophy. Mechanistically, SEL1L deficiency in the exocrine pancreas perturbs cellular homeostasis by destabilising Hrd1 levels, impairing translation and inducing fragmentation of the ER. However, why only the pancreas is affected, and how acute loss of SEL1L affects other cell types remains an open question.

This study aimed to elucidate both the functional and physiological requirements of ERAD in a model of proteotoxic stress. By combining functional readouts to monitor substrate degradation, and phenotypic analysis to monitor cell viability, the physiological contribution of individual ERAD components to the clearance of misfolded proteins and survival was defined. To this end, ER stress was elicited via the overexpression of unpaired secretory IgM heavy chain ( $\mu$ S) in HeLa cells, followed by the interrogation of each ERAD component in terms of  $\mu$ S degradation and the requirement for viability under basal conditions and under  $\mu$ S-driven proteotoxic stress.

#### **4.4.2 Proteotoxic stress reinforces degradation capacity**

In the absence of its light chain, IgM HC is not able to assemble to form a functional antibody with the classical pentameric structure. Unpaired  $\mu$ S has previously been shown to be retained in the ER lumen bound to BiP, which prevents further trafficking along the secretory pathway. Whether or not orphan  $\mu$ S elicits the UPR as a consequence of its ER accumulation has recently been addressed by our collaborators (van Anken Lab, San Raffaele Scientific Institute, Milan), who showed that overexpressed  $\mu$ S triggers the UPR in HeLa cells in a time- and dose-dependent manner (unpublished). Physiological UPR activation in response to an individual misfolded protein in a cellular system represents a unique opportunity to interrogate the downstream pathways engaged in the stress response. In this

system, a single copy of  $\mu$ S was stably integrated into the genome of HeLa cells, under a Mif-inducible promoter. In the study described here,  $\mu$ S retention within the ER lumen could be confirmed, as well as UPR activation by use of classical markers. Transcriptional analysis by Nanostring technology after 16 hours of  $\mu$ S expression revealed a robust induction of ER chaperones and ERAD genes.  $\mu$ S-driven ERAD expression is in agreement with previously reported expression data on cells treated with ER stress-inducing agents like DTT or TM (Christianson et al., 2012). This observation confirms the validity of the model used in this study, which relies on a single misfolded protein to mimic proteotoxic stress. Of all ERAD genes analysed, Hrd1, SEL1L, Herp, BiP and Derlin-3 underwent the greatest fold change. This study was able to confirm upregulation of Hrd1 complex components at the protein level in a fashion that followed  $\mu$ S accumulation. Notably, while the UPR (via PERK and IRE1 pathways) was triggered as early as 8 hours after  $\mu$ S induction and then recovered to nearly basal levels over time, levels of the ERAD components were raised after 16 hours and their levels persisted over the whole period of  $\mu$ S expression. This result suggests that acute UPR activation is sufficient to elicit an ERAD response that is maintained over time to sustain the continuous accumulation of misfolded protein. It is known that shutdown of UPR signalling is key to promote survival, while prolonged activation trigger apoptosis, mainly via the PERK-ATF4-CHOP axis [reviewed in (Tabas and Ron, 2011)]. Sustained expression of ERAD components in the absence of a continuous UPR is most likely due to the existence of positive feedback loops (Majumder et al., 2012; Takayanagi et al., 2013).

#### 4.4.3 Hrd1 sustains viability under proteotoxic stress

Soluble  $\mu$ S was significantly stabilised in the absence of Hrd1, and reintroduction of WT but not the RING mutant (C294A) Hrd1 was able to restore  $\mu$ S degradation, suggesting that Hrd1 is indeed the ubiquitin ligase of  $\mu$ S. Notably, in WT cells,  $\mu$ S was never completely rescued by either proteasome inhibition (MG132) or KIF, suggesting that parallel degradation pathways may be engaged to clear the misfolded protein. It is likely that autophagy participates in the clearance of  $\mu$ S in WT cells, since significant autophagy activation was observed by counting cells positive for LC3B punctae. If that is the case, simultaneous inhibition of both pathways should result in the complete rescue of  $\mu$ S. In a previous study (Cattaneo et al., 2008), depletion of Hrd1 by siRNA-mediated knockdown did not prevent  $\mu$ S degradation, but overexpression of dominant negative mutant Hrd1 stabilised  $\mu$ S. Therefore, the authors concluded that  $\mu$ S is a substrate of Hrd1, and that either the residual enzymatic activity of silenced Hrd1 is sufficient for  $\mu$ S degradation or other ubiquitin ligases are involved in  $\mu$ S turnover. In the Hrd1 knockout model used in this study, it was confirmed that Hrd1 is the only ubiquitin ligase of  $\mu$ S under basal conditions, as indicated by  $\mu$ S stabilisation as well as complete lack of ubiquitination in the absence of Hrd1, excluding the possibility that other E3s ubiquitinate  $\mu$ S as a compensatory mechanism.

Additionally, it was found that Hrd1 is required for  $\mu$ S retrotranslocation from the ER to the cytosol prior to degradation. Reintroduction of WT but not RING mutant Hrd1 was able to restore  $\mu$ S retrotranslocation, meaning that Hrd1 is essential in this process. A recent *in vitro* study on reconstituted Hrd1 complex components from yeast has shown that Hrd1 auto-ubiquitination is necessary to allow retrotranslocation of a model substrate (Baldrige and Rapoport, 2016). Whether this applies *in vivo* to mammalian cells is unknown, but if it is, it is possible that the RING mutant Hrd1 fails to retrotranslocate  $\mu$ S because it lacks its auto-ubiquitination

activity. Whether  $\mu$ S retrotranslocation occurs directly through Hrd1, which may organise as a translocon channel itself (Carvalho et al., 2010), or through a complex that is assembled or stabilised by Hrd1, remains to be assessed. In any case, this study represents the first evidence of the requirement of the catalytic activity of mammalian Hrd1 for retrotranslocation of a misfolded luminal glycoprotein in live cells.

Phenotypically, loss of Hrd1 in cells undergoing proteotoxic stress is lethal and can be rescued by reintroduction of WT Hrd1, but not the RING mutant. The protective role of Hrd1 is exerted by virtue of its ubiquitination activity, as mutation in the catalytic domain disables the ligase from alleviating  $\mu$ S proteotoxicity. This observation demonstrates the essential requirement of Hrd1 in the resolution of proteotoxic stress arising from an individual misfolded protein in the ER, rather than from global misfolding induced by pharmacological agents. This finding elucidates the functional implications of the reinforced ERAD capacity under stress. Interestingly, Hrd1 deficiency is tolerated in the absence of proteotoxic stress, suggesting that the ER is able to manage basal flux of nascent proteins when degradation is impaired. This study did not identify any markers of UPR activation in Hrd1-deleted cells in the absence of  $\mu$ S (data not shown), further supporting the idea that the role of ERAD under unchallenged conditions is dispensable in cell line models. It is possible that our constitutive Hrd1 KO model induced some adaptive mechanisms, therefore preventing UPR activation. The possibility that acute removal of Hrd1 may lead to proteostatic imbalances leading to characteristic UPR signatures cannot be excluded. However, Hrd1 requirement may be essential in activated secretory cells, when ERAD activity is needed to sustain the high secretory demand. It is exciting to speculate that acute Hrd1 inhibition may have beneficial effects in the treatment of cancers associated with high secretory activity, such as multiple myeloma (M. Wang and Kaufman, 2014).

In principle, impairment of Hrd1 activity may preferentially affect the defective, malignant cells, as they are already exposed to high levels of stress, while normal cells would remain able to survive in the absence of functional Hrd1.

#### **4.4.4 SEL1L and Derlins are essential under proteotoxic stress**

In addition to the findings discussed above, this study interrogated the main components of the Hrd1 complex and the Derlin family members, to elucidate the contribution of individual ERAD factors to the resolution of proteotoxic stress. It was found that depletion of SEL1L and Derlin-2 had the most dramatic effect on  $\mu$ S degradation, correlating with impaired  $\mu$ S retrotranslocation and significant lethality. This finding agrees with the pivotal role of SEL1L in ERAD, but additionally highlights the requirement of SEL1L (and partially Derlin-2) for substrate retrotranslocation (Huang et al., 2013; Williams et al., 2013). SEL1L depletion leads to destabilisation of Hrd1, as previously reported (Sun et al., 2014), and Ube2j1. It is possible that SEL1L is directly involved in substrate retrotranslocation or, more likely, that impaired  $\mu$ S retrotranslocation and degradation are a consequence of decreased Hrd1 stability in the absence of SEL1L. Additionally, this study found decreased levels of the E2 enzyme Ube2j1 in SEL1L-depleted cells, which (to our knowledge) has not previously been reported. Loss of Ube2j1 alone only mildly affected  $\mu$ S degradation but not retrotranslocation. Impaired  $\mu$ S degradation in cells depleted for Ube2j1 is lethal, confirming that Ube2j1 is the preferential, if not the exclusive, E2 partner of Hrd1 for ubiquitination of soluble glycoproteins. As in SEL1L-depleted cells, cells lacking Ube2j1 display lower levels of Hrd1 and increased OS-9, suggesting that impaired survival may be a consequence of Hrd1 instability. Notably, OS-9 was significantly stabilised in the absence of Hrd1, SEL1L, Ube2j1, Derlin-2 and, partially, XTP3-B and Derlin-3, suggesting that OS-9 may be a substrate of the Hrd1 complex as well as an integral cofactor. Additionally, OS-9 failed to bind Hrd1 in cells depleted for SEL1L, confirming the role of SEL1L in

scaffolding this luminal lectin to the E3 ligase complex for substrate ubiquitination (Iida et al., 2011).

The remarkable accumulation of  $\mu$ S upon Derlin-2 deletion is most likely a direct consequence of Derlin-2 loss rather than a consequence of Hrd1 destabilisation. Indeed, cells lacking Derlin-2 displayed only mildly decreased levels of Hrd1, correlating with higher OS-9, but overall showed functional Hrd1 activity as degradation of the Hrd1 substrate CD147 was not significantly affected. Binding of Derlin-1 to Hrd1 was attenuated in the absence of Derlin-2, which may indicate a role for Derlin-2 in the stability of the Hrd1 complex under proteotoxic stress. In a recent study, Derlin-2 was shown to facilitate retrotranslocation of other luminal Hrd1 substrates: the sonic hedgehog protein and NHK A1AT (Huang et al., 2013) (Huang et al., 2013). These results suggest that Derlin-2 is a key component of the Hrd1 complex for the degradation of luminal substrates, while it is dispensable for the degradation of membrane protein CD147. The requirement for Derlin-2 in  $\mu$ S degradation may implicate an important role of this ERAD component in B-cell development. However, a study on Derlin-2 conditional KO mice revealed no impairment in B-cell maturation and antibody secretion (Dougan et al., 2011). These variations may be due to species- or cell-specific differences.

Absence of Herp caused mild impairment of  $\mu$ S degradation, retrotranslocation and survival, while degradation of membrane CD147 was unaffected. This is in agreement with the previously reported role of Herp in facilitating the degradation of Hrd1 luminal substrates (Huang et al., 2014; Kny et al., 2011). In the same study, Herp was shown to be required for the recruitment of Derlin-2 to the Hrd1-SEL1L complex to allow substrate retrotranslocation. Impairment of Derlin-2 binding to Hrd1 in the absence of Herp was not seen in this study, but reduced levels of Derlin-1 were observed. Whether Herp effectively participates in the stability of Derlin-1 will require further validation. From the results described here, it can be concluded that



Herp plays an important but not essential role in the resolution of proteotoxic stress, perhaps due to compensatory mechanisms by the constitutively expressed homologue Herp2 (Huang et al., 2014).

Depletion of either Derlin-1 or Derlin-3 led to significant lethality under proteotoxic stress, however the degradation and retrotranslocation of  $\mu$ S were unaffected. Likewise, degradation of CD147 was not perturbed in the absence of either protein. Depletion of Derlin-1 did not cause significant aberrations in the expression and composition of the Hrd1 complex, while depletion of Derlin-3 led to increased levels of SEL1L and OS-9 and decreased levels of FAM8A1 and Derlin-1. The common feature between the two depleted cell lines was represented by a reduction in Derlin-1, which may underlie the phenotypic similarity. A possibility that was not investigated in this study is the formation of insoluble aggregates in the absence of Derlin-1 and Derlin-3, which could perturb homeostasis leading to lethality. Alternatively, it is possible that depletion of either Derlin-1 or -3, combined with sequestration of critical ERAD components by  $\mu$ S, may affect the clearance of other proteins, hence impairing viability.

An intermediate phenotype was observed in the case of XTP3-B, its depletion causing partial stabilisation of  $\mu$ S but not significant toxicity. In addition, degradation of endogenous CD147 was mildly impaired. This suggests that XTP3-B activity may be compensated for by other lectins like OS-9 (Bernasconi et al., 2010; Christianson et al., 2008), therefore it is concluded that XTP3-B is not an essential component in the resolution of proteotoxic stress. Depletion of either OS-9 or FAM8A1 did not have any detectable consequence on  $\mu$ S degradation or cell viability, indicating that both components are dispensable for cellular homeostasis and survival.

Of all of the tested components of the ERAD machinery, none is essential to sustain cell viability under basal conditions. However, the possibility that removal of

individual components leads to cell adaptation to enable survival cannot be excluded. Hrd1, SEL1L and Derlin-1, -2 and -3 are essential in the resolution of  $\mu$ S-driven proteotoxic stress, allowing cells to survive. Ube2j1 and Herp also play important roles by sustaining degradation and survival, although their loss leads to a less severe phenotype. Finally, FAM8A1 and the lectins XTP3-B and OS-9 are dispensable components in our model of proteotoxic stress, possibly due to partial redundancy between OS-9 and XTP3-B and engagement of FAM8A1 in other cellular responses.

#### **4.4.5 Proteotoxic stress rearranges the Hrd1 complex**

Sucrose sedimentation revealed which ERAD components rearrange following proteotoxic stress. This study found that overexpression of misfolded  $\mu$ S triggers a profound rearrangement of the Hrd1 complex, reflected in the shift of Hrd1, SEL1L, Herp and FAM8A1 towards heavier fractions. Importantly, complex rearrangement appears to be nucleated by Hrd1, since cells lacking Hrd1 do not display significant redistribution of the above components following  $\mu$ S expression. Sedimentation in heavier fractions could reflect the formation of larger complexes, as a consequence of the upregulation of the ERAD components by  $\mu$ S expression. It was recently reported that Hrd1 complex components are recruited to specific ER compartments during stress to facilitate substrate degradation (Leitman et al., 2014). It can be speculated that ERAD compartmentalisation under stress would result in the formation of higher molecular weight complexes, possibly reflecting a greater efficiency of disposal of misfolded proteins. Quantification of signal intensity in each fraction of the gradient from multiple biological replicates will help solidify the hypothesis of complex rearrangement. Additionally, proper stoichiometric analysis will be required to assess the exact composition of these larger complexes.

FAM8A1 was not upregulated by  $\mu$ S, but redistributed together with Hrd1, SEL1L and Herp towards heavier fractions. What the functional contribution of FAM8A1 is

to the integrity or functionality of the complex is not clear at the moment. This study was able to confirm that  $\mu$ S is engaged by the Hrd1 complex during its degradation. Importantly, all tested components except Derlin-1 and -2 already interact with Hrd1 under basal conditions, meaning that they form the steady-state degradation machinery. However, proteotoxic stress resulted in Derlin-1 recruitment to Hrd1 complexes and the reinforcement of interactions with Derlin-2. These results suggest that protein misfolding in the ER leads to rearrangement of the degradation machinery by: (i) increasing the abundance of the ERAD components through the UPR, (ii) increasing the size of Hrd1 complexes, and (iii) recruiting additional components or reinforcing existing interactions. Taken together, these changes may help increase the degradation efficiency of the ERAD machinery, thus avoiding toxic accumulation of misfolded proteins. It would be interesting to analyse the composition and stoichiometry of the Hrd1 complex under proteotoxic stress, and identify the regions of Hrd1 that are necessary for nucleating these extended complexes. Additionally, it will be important to determine whether this phenomenon extends generally to other cellular models under different proteotoxic challenges, or in disease models. If the formation of specialised complexes is a requirement to survive under consistent ER stress, but not under basal conditions, it would be important to define the key regions in the Hrd1 protein that are necessary for nucleation of these structures. As a future intervention, specific inhibition of such regions may facilitate the selective lethality of stressed, aberrant cells, while maintaining the viability of the population of normal cells.

#### **4.4.6 Autophagy engagement under stress requires Hrd1**

The failure of proteasome inhibitors to completely impair  $\mu$ S degradation, alongside significant autophagy activation in cells expressing  $\mu$ S, strongly suggests that both catabolic pathways take part in the degradation of the misfolded protein. Strikingly, cells lacking Hrd1 did not activate autophagy upon  $\mu$ S expression. When treated with a proteasome inhibitor, the activation of autophagy indicates the existence of direct crosstalk between the UPS and autophagic pathways, in agreement with previous reports [reviewed in (Cohen-Kaplan et al., 2016)]. Importantly, autophagy activation correlated with decreased intracellular levels of soluble  $\mu$ S in both WT and Hrd1-deficient cells. Autophagy activation and decreased  $\mu$ S levels sustained the survival of cells lacking Hrd1, demonstrating the protective role of this alternative degradation route under stress. Although direct participation of autophagy in the clearance of  $\mu$ S is missing, this evidence is supported by the fact that  $\mu$ S is neither secreted nor aggregated in the ER, as observed by ELISA and immunofluorescence, respectively. Degradation of ER material by autophagy is supported by a few studies reporting the engagement of macro-ER-phagy in the degradation of ER aggregates (Sha et al., 2014) or ER transmembrane proteins (Houck et al., 2014). Additionally, activation of autophagy in antibody-secreting PCs has been shown to regulate antibody secretion, therefore sustaining viability (Pengo et al., 2013).

What is particularly exciting about this finding is the fact that cells lacking Hrd1 do not mount an autophagic response in the presence of proteotoxic stress, unlike WT cells. This observation proposes the existence of a signalling mechanism that operates via Hrd1 towards the autophagic compartment. A recent study revealed the presence of an ER transmembrane protein, FAM134B, that functions as an autophagic receptor by interacting with LC3B, which is bound to autophagosomes (Khaminets et al., 2015). By virtue of this interaction, FAM134 recruits

autophagosomal membranes to initiate ER-phagy, a process essential to regulate ER turnover and cellular homeostasis. At the moment, this reticulon protein represents the only known receptor that mediates ER-phagy in mammalian cells. It is possible that, under conditions of proteotoxic stress, other receptors are involved in ER-phagy signalling, and one of these could be Hrd1. Future experiments will be directed towards elucidating this mechanism, by addressing the interaction of the Hrd1 complex with LC3B and potentially FAM134. In order to confirm the involvement of ER-phagy in the clearance of luminal  $\mu$ S, autophagy activation and  $\mu$ S accumulation would need to be measured upon FAM134B knockdown, as well as depletion of canonical Atg proteins such as Atg5 or Beclin-1 (Pengo et al., 2013; Quiroga et al., 2013).

Lack of autophagy in Hrd1-deleted cells can represent an important advantage for future therapeutic interventions towards Hrd1, where selective inhibition of the ligase is aimed at inducing lethality. For instance, treatment of multiple myeloma is based on the use of proteasome inhibitors, well known to encounter problems due to the development of resistance mechanisms in the long term, including the induction of autophagy as a way to escape cell death (Niewerth et al., 2015). It is tempting to speculate that selective inhibition of Hrd1 may be a potential strategy to treat these types of cancers, and could offer a better alternative in terms of lower toxicity and lack of a compensatory mechanism by autophagy.

In conclusion, this study was able to identify the ERAD components necessary for survival under IgM HC-driven proteotoxic stress. Among these were the Hrd1 ubiquitin ligase, its cofactors SEL1L and HERP, the E2 enzyme Ube2j1, and Derlin-1, -2 and -3. Significantly impaired degradation kinetics of  $\mu$ S was shown in the absence of Hrd1, SEL1L, Derlin-2 and (partially) Herp, Ube2j1 and the lectin XTP3-B. Additionally, the pivotal role of Hrd1 in  $\mu$ S ubiquitination and retrotranslocation

prior to degradation was demonstrated. This study demonstrated the dynamics of the Hrd1 complex under proteotoxic stress, reflected by a rearrangement of the sedimentation pattern of core components in a sucrose gradient. Importantly, both ERAD and autophagy were engaged in the disposal of the misfolded luminal glycoprotein, sustaining cell survival under proteotoxic stress. Deletion of Hrd1 abrogated autophagy, proposing a new signalling mechanism for the induction of ER-phagy arising from the ER.

# Chapter V:

## Conclusions and Perspectives

This study aimed to elucidate the participation of ER-resident E3 ubiquitin ligases in ERAD and in the maintenance of ER homeostasis. Firstly, the composition of the E3 complexes in the ER were characterised to evaluate their association with known ERAD components, as well as with substrates and new cofactors (Chapter III). This study was combined with transcriptional analysis and knockdown experiments on all 25 ER-resident E3s to evaluate their regulation upon stress and their participation in ER homeostasis. Secondly, the contribution of individual Hrd1 complex components to the resolution of proteotoxic stress generated by a single misfolded antibody subunit was assessed (Chapter IV). This system facilitated the identification of individual ERAD factors that are essential for cell survival under proteotoxic insults and provided evidence for the contribution of autophagy in the clearance of misfolded proteins from the ER.

The composition of the E3 complexes was determined by use of mass spectrometry on purified FLAG-HA-E3s, which were generated from constructs stably integrated in Flp-IN HEK293T-REx cell lines under a doxycycline-inducible promoter. A comparative analysis on the successful E3 datasets was developed to distinguish HCIPs from the background proteome, and enabled the identification of 460 HCIPs in total. The interaction networks of 22 ER-resident ubiquitin ligases were generated, with an average of 20 HCIPs per E3. Known cofactors of the well-characterised E3s Hrd1 and AMFR/gp78 were positively identified, as well as several candidate substrates across all networks, which may help to elucidate the functions of the E3s. An enrichment in the HCIPs for proteins involved in metabolic pathways, such as cholesterol biosynthesis, was observed, supporting the participation of these E3s in the regulation of lipid homeostasis and, more broadly, cell metabolism. The majority of the known ERAD components were found in association with Hrd1 and AMFR/gp78, indicating that the other E3s may have alternative functions or use different cofactors to participate in ERAD. A potential



role in degradation is supported by the interaction of several ubiquitin ligases with VCP/p97 adaptor molecules, suggesting their engagement in substrate degradation. Besides a potential role in the UPS, some E3s could be involved in ubiquitin signalling from the ER by virtue of different ubiquitin chain linkages, as previously suggested for RNF26 (Qin et al., 2014). The identification of over 430 novel, high confidence interactors sets the foundations to investigate the biological role of the ER-resident E3s in terms of: (i) which substrates they regulate, (ii) which cofactors they utilise for their function and (iii) which cellular processes they participate in.

Biochemical validation of candidate interactors will be required to assess *bona fide* binders and will involve the analysis of endogenous complexes primarily by means of co-IP of the endogenous E3s followed by immunoblotting for the candidate interactors. Validation of substrate turnover will be assessed by protein translation shutdown experiments in the context of cells deleted for the specific E3 (for instance by siRNA or CRISPR/Cas9 knock-out) or by expressing dominant negative versions of the ligase. In cases where the substrate may be regulated via Ub modifications not targeting for proteasomal degradation, analysis of the Ub linkages by use of specific Ub-binding elements will help to elucidate the signalling involved in substrate regulation. *In vitro* ubiquitination assays may also be required to confirm the catalytic activity of the E3s that are still uncharacterised. Once the *bona fide* interactors can be ascertained, phenotypic assays will be designed to assess the biological implications of the interactions. Specific readouts will be chosen depending on the nature of the candidates being investigated. For instance, RNF26 has been reported to modulate virus-triggered interferon release by promoting K11 polyubiquitination of MITA (mediator of IRF3 activation) (Qin et al., 2014). Identification of the RNF26 cofactors that impact its activity will enable us to better understand how this pathway is regulated. The characterisation of new substrates regulated by the E3s and the elucidation of the cofactors implicated in their function

will advance the understanding of this class of enzymes, as well as the orchestration of the activities that take place in the ER.

Analysis of disease association may be used to guide the selection of the HCIPs that will be further validated and investigated. The discovery of new E3s regulating pathogenic factors (via Ub modifications) may advance the understanding of pathological conditions where these factors are involved and, in the long term, lay the foundations for alternative therapeutic strategies targeting the E3s.

Among the 25 ER-resident E3s, Hrd1 is the only ligase that is significantly upregulated by stress, highlighting its central position in responding to protein misfolding, deriving from either pharmacological treatment (TM/SubAB<sub>5</sub>) or from an accumulation of unpaired antibody subunits. This finding emphasises the pivotal role of Hrd1, not only in ERAD but especially in the resolution of proteotoxic stress, for which the other resident E3s do not seem to be engaged nor able to compensate in the absence of Hrd1. The lack of compensatory activity, along with the identification of distinct E3 complexes by mass spectrometry, strongly suggests a diversification in the E3 functions and a low degree of redundancy, at least with respect to the degradation of luminal proteins. In the future, it will be important to assess the regulation of the other E3s upon different cellular stimuli, ranging from lipid stress, oxidative stress, pathogen infection and nutrient starvation. We can envision that different E3s may respond to different challenges, likely correlating with their biological functions. We can think of this 'labour division' as a result of evolution responding to the higher complexity of the human proteome.

This study was able to demonstrate the requirement of Hrd1 and its cofactors for survival under proteotoxic stress generated by the accumulation of a single misfolded protein. Although ablation of Hrd1 correlated with the most severe phenotype under stress, depletion of SEL1L, Derlin-1 and Derlin-3 was comparably

detrimental. Ube2j1, Derlin-2 and HERP are important for survival under proteotoxic stress, but their depletion did not lead to complete lethality, suggesting that their function is partially dispensable. Loss either of OS-9, XTP3-B or FAM8A1 did not affect cell viability under the same conditions, suggesting the existence of redundant and/or dispensable functions within the ERAD machinery. Strikingly, a significant stabilisation of soluble  $\mu$ S was only observed in the absence of Hrd1, SEL1L or Derlin-2, suggesting that the mechanism leading to toxicity (for instance in cells lacking Derlin-1 or -3) may be different upon loss of different ERAD components. As a follow up to my investigation, it would be important to monitor substrate solubility and evaluate the formation of insoluble toxic aggregates. Accumulation of K48-linked ubiquitinated proteins would also need to be addressed to assess global impairment of proteasomal degradation. Highlighting these mechanisms will be important to clarify the role of the Derlins in substrate degradation, hence advancing our general understanding of specific ERAD machineries in the context of proteotoxic stress. In the long term, these findings may prove useful for the development of new interventions for conditions associated with proteostatic imbalance, such as age-related diseases (Cuanalo-Contreras et al., 2013; Hipp et al., 2014).

This study showed that the catalytic activity of Hrd1 is essential to mediate  $\mu$ S ubiquitination, retrotranslocation and subsequent degradation by the proteasome. Additionally, Hrd1 is required to nucleate reassembly of the core complex components SEL1L, HERP and FAM8A1 under proteotoxic stress. Sucrose gradient co-sedimentation of the Hrd1 core complex components showed a pattern shifted towards heavier fractions, possibly reflecting assembly into larger complexes, which may be required in conditions of high degradation demand. Stoichiometric analysis of the Hrd1 complex under stress compared to basal conditions may demonstrate the potential existence of different Hrd1 complexes depending on the load of

misfolded proteins. Reorganisation of the ERAD factors is likely sustained by transcriptional and translational upregulation of the ERAD genes under stress. Such rearrangement could be part of an adaptive program that cells mount to increase their degradation capacity in order to sustain survival under proteotoxic stress. If this is the case, prevention of upregulation, for instance by mutating the ERSE/UPRE elements in the promoters of ERAD genes, would result in failed assembly into larger complexes and inefficient disposal of misfolded proteins. If proven, it will represent a new mechanistic insight into the dynamics of the ERAD machinery during ER stress.

Finally, the growth of Hrd1-deficient cells under proteotoxic stress could be rescued by proteasome inhibition or autophagy activation, both conditions correlating with decreased  $\mu$ S levels. The data reported here indicate that, in addition to the UPS, WT cells engage autophagy for the degradation of luminal misfolded proteins in the ER, as reflected by significant autophagy induction upon  $\mu$ S expression. Measurement of substrate accumulation upon knockdown of the autophagy genes Atg5 and the specific ER-phagy receptor FAM134B could be used to demonstrate the engagement of autophagy in the clearance of misfolded proteins from the ER lumen, and will elucidate the involvement of canonical autophagy (via Atg5) or selective ER-phagy (via FAM134B). By simultaneously assessing cell viability, the functional implications of protein clearance by autophagy may be determined. More importantly, cells lacking Hrd1 are unable to mount an autophagic response, suggesting the involvement of Hrd1 in the signalling of a 'misfolding status' from the ER to the autophagosomal compartment. In support of this hypothesis, the mass spectrometry analysis in this study revealed the association of Hrd1 with two lysosomal proteins (AP3B1 and CLN6). Hrd1 signalling to the autophagy system will be interrogated by reintroducing WT and RING mutant Hrd1 in Hrd1-deficient cells and by monitoring the subsequent activation of autophagy. The dependency of such

signalling upon an ubiquitination reaction will be revealed by the restoration of autophagy only in the presence of catalytically active Hrd1. The proximity of Hrd1 with autophagosomal membranes could also be evaluated by co-localisation with the autophagy receptor LC3B via immunofluorescence. To investigate a role for Hrd1 cofactors in autophagy signalling, autophagy activation should be monitored in cells lacking other complex components.

Although the present data only provide an indication of the requirement of Hrd1 for autophagic signalling, validation of this mechanism will represent a fundamental discovery in the field. As a long term application, inhibition of Hrd1 or its cofactors as a strategy to treat diseases associated with high secretory activity, such as multiple myeloma, may provide an alternative to proteasome inhibition. This idea is supported by the evidence that an impairment of Hrd1 activity is detrimental only in cells experiencing proteotoxic stress. Additionally, lack of autophagy induction in Hrd1-defective cells would circumvent the resistance mechanisms deriving from autophagy activation upon proteasome inhibitor treatments. Further experiments testing the impairment of Hrd1 activity in multiple myeloma model cell lines will be required to assess the validity of such a strategy.

# Bibliography

- Aarvak, T., Natvig, J.B., 2001. Cell-cell interactions in synovitis: antigen presenting cells and T cell interaction in rheumatoid arthritis. *Arthritis Res.* 3, 13–17. doi:10.1186/ar135
- Adachi, Y., Yamamoto, K., Okada, T., Yoshida, H., Harada, A., Mori, K., 2008. ATF6 is a transcription factor specializing in the regulation of quality control proteins in the endoplasmic reticulum. *Cell Struct. Funct.* 33, 75–89.
- Allen, J.R., Nguyen, L.X., Sargent, K.E.G., Lipson, K.L., Hackett, A., Urano, F., 2004. High ER stress in beta-cells stimulates intracellular degradation of misfolded insulin. *Biochem. Biophys. Res. Commun.* 324, 166–170. doi:10.1016/j.bbrc.2004.09.035
- Amano, T., Yamasaki, S., Yagishita, N., Tsuchimochi, K., Shin, H., Kawahara, K.-I., Aratani, S., Fujita, H., Zhang, L., Ikeda, R., Fujii, R., Miura, N., Komiya, S., Nishioka, K., Maruyama, I., Fukamizu, A., Nakajima, T., 2003. Synoviolin/Hrd1, an E3 ubiquitin ligase, as a novel pathogenic factor for arthropathy. *Genes Dev.* 17, 2436–2449. doi:10.1101/gad.1096603
- Ameri, K., Harris, A.L., 2008. Activating transcription factor 4. *Int. J. Biochem. Cell Biol.* 40, 14–21. doi:10.1016/j.biocel.2007.01.020
- Anelli, T., Alessio, M., Bachi, A., Bergamelli, L., Bertoli, G., Camerini, S., Mezghrani, A., Ruffato, E., Simmen, T., Sitia, R., 2003. Thiol-mediated protein retention in the endoplasmic reticulum: the role of ERp44. *EMBO J.* 22, 5015–5022. doi:10.1093/emboj/cdg491
- Anelli, T., Ceppi, S., Bergamelli, L., Cortini, M., Masciarelli, S., Valetti, C., Sitia, R., 2007. Sequential steps and checkpoints in the early exocytic compartment during secretory IgM biogenesis. *EMBO J.* 26, 4177–4188. doi:10.1038/sj.emboj.7601844
- Anelli, T., van Anken, E., 2013. Missing links in antibody assembly control. *Int J Cell Biol* 2013, 606703–9. doi:10.1155/2013/606703
- Apodaca, J., Kim, I., Rao, H., 2006. Cellular tolerance of prion protein PrP in yeast involves proteolysis and the unfolded protein response. *Biochem. Biophys. Res. Commun.* 347, 319–326. doi:10.1016/j.bbrc.2006.06.078
- Appenzeller, C., Andersson, H., Kappeler, F., Hauri, H.P., 1999. The lectin ERGIC-53 is a cargo transport receptor for glycoproteins. *Nat. Cell Biol.* 1, 330–334. doi:10.1038/14020
- Aragón, T., van Anken, E., Pincus, D., Serafimova, I.M., Korennykh, A.V., Rubio, C.A., Walter, P., 2009. Messenger RNA targeting to endoplasmic reticulum stress signalling sites. *Nature* 457, 736–740. doi:10.1038/nature07641
- Argon, Y., Simen, B.B., 1999. GRP94, an ER chaperone with protein and peptide binding properties. *Semin. Cell Dev. Biol.* 10, 495–505. doi:10.1006/scdb.1999.0320
- Arshad, M., Ye, Z., Gu, X., Wong, C.K., Liu, Y., Li, D., Zhou, L., Zhang, Y., Bay, W.P., Yu, V.C., Li, P., 2013. RNF13, a RING finger protein, mediates endoplasmic reticulum stress-induced apoptosis through the inositol-requiring enzyme (IRE1α)/c-Jun NH2-terminal kinase pathway. *J. Biol. Chem.* 288, 8726–8736. doi:10.1074/jbc.M112.368829
- Arteaga, M.F., Wang, L., Ravid, T., Hochstrasser, M., Canessa, C.M., 2006. An amphipathic helix targets serum and glucocorticoid-induced kinase 1 to the endoplasmic reticulum-

- associated ubiquitin-conjugation machinery. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11178–11183. doi:10.1073/pnas.0604816103
- Balch, W.E., Morimoto, R.I., Dillin, A., Kelly, J.W., 2008. Adapting proteostasis for disease intervention. *Science* 319, 916–919. doi:10.1126/science.1141448
- Baldridge, R.D., Rapoport, T.A., 2016. Autoubiquitination of the Hrd1 Ligase Triggers Protein Retrotranslocation in ERAD. *Cell* 166, 394–407. doi:10.1016/j.cell.2016.05.048
- Ballar, P., Pabuccuoglu, A., Kose, F.A., 2011. Different p97/VCP complexes function in retrotranslocation step of mammalian ER-associated degradation (ERAD). *Int. J. Biochem. Cell Biol.* 43, 613–621. doi:10.1016/j.biocel.2010.12.021
- Bartee, E., Mansouri, M., Hovey Nerenberg, B.T., Gouveia, K., Früh, K., 2004. Downregulation of major histocompatibility complex class I by human ubiquitin ligases related to viral immune evasion proteins. *J. Virol.* 78, 1109–1120. doi:10.1128/JVI.78.3.1109-1120.2004
- Bays, N.W., Gardner, R.G., Seelig, L.P., Joazeiro, C.A., Hampton, R.Y., 2001. Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. *Nat. Cell Biol.* 3, 24–29. doi:10.1038/35050524
- Behrends, C., Sowa, M.E., Gygi, S.P., Harper, J.W., 2010. Network organization of the human autophagy system. *Nature* 466, 68–76. doi:10.1038/nature09204
- Belal, C., Ameli, N.J., Kommos, El, A., Bezalel, S., Al'Khafaji, A.M., Mughal, M.R., Mattson, M.P., Kyriazis, G.A., Tyrberg, B., Chan, S.L., 2012. The homocysteine-inducible endoplasmic reticulum (ER) stress protein Herp counteracts mutant  $\alpha$ -synuclein-induced ER stress via the homeostatic regulation of ER-resident calcium release channel proteins. *Hum. Mol. Genet.* 21, 963–977. doi:10.1093/hmg/ddr502
- Bence, N.F., Sampat, R.M., Kopito, R.R., 2001. Impairment of the ubiquitin-proteasome system by protein aggregation. *Science* 292, 1552–1555. doi:10.1126/science.292.5521.1552
- Bernardi, K.M., Williams, J.M., Inoue, T., Schultz, A., Tsai, B., 2013. A deubiquitinase negatively regulates retro-translocation of nonubiquitinated substrates. *Mol. Biol. Cell* 24, 3545–3556. doi:10.1091/mbc.E13-06-0332
- Bernardi, K.M., Williams, J.M., Kikkert, M., van Voorden, S., Wiertz, E.J., Ye, Y., Tsai, B., 2010. The E3 ubiquitin ligases Hrd1 and gp78 bind to and promote cholera toxin retro-translocation. *Mol. Biol. Cell* 21, 140–151. doi:10.1091/mbc.E09-07-0586
- Bernasconi, R., Galli, C., Calanca, V., Nakajima, T., Molinari, M., 2010. Stringent requirement for HRD1, SEL1L, and OS-9/XTP3-B for disposal of ERAD-LS substrates. *J. Cell Biol.* 188, 223–235. doi:10.1083/jcb.200910042
- Bernasconi, R., Pertel, T., Luban, J., Molinari, M., 2008. A dual task for the Xbp1-responsive OS-9 variants in the mammalian endoplasmic reticulum: inhibiting secretion of misfolded protein conformers and enhancing their disposal. *J. Biol. Chem.* 283, 16446–16454. doi:10.1074/jbc.M802272200
- Bernhardi, von, R., Tichauer, J.E., Eugénin, J., 2010. Aging-dependent changes of microglial cells and their relevance for neurodegenerative disorders. *J. Neurochem.* 112, 1099–1114. doi:10.1111/j.1471-4159.2009.06537.x
- Bertolotti, A., Zhang, Y., Hendershot, L.M., Harding, H.P., Ron, D., 2000. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat. Cell Biol.* 2, 326–332. doi:10.1038/35014014

- Bianchi, G., Anderson, K.C., Harris, N.L., Sohani, A.R., 2014. The heavy chain diseases: clinical and pathologic features. *Oncology* (Williston Park, N.Y.) 28, 45–53.
- Bianchi, G., Oliva, L., Cascio, P., Pengo, N., Fontana, F., Cerruti, F., Orsi, A., Pasqualetto, E., Mezghrani, A., Calbi, V., Palladini, G., Giuliani, N., Anderson, K.C., Sitia, R., Cenci, S., 2009. The proteasome load versus capacity balance determines apoptotic sensitivity of multiple myeloma cells to proteasome inhibition. *Blood* 113, 3040–3049. doi:10.1182/blood-2008-08-172734
- Biunno, I., Appierto, V., Cattaneo, M., Leone, B.E., Balzano, G., Socci, C., Saccone, S., Letizia, A., Valle, Della, G., Sgaramella, V., 1997. Isolation of a pancreas-specific gene located on human chromosome 14q31: expression analysis in human pancreatic ductal carcinomas. *Genomics* 46, 284–286.
- Braakman, I., Hebert, D.N., 2013. Protein folding in the endoplasmic reticulum. *Cold Spring Harb Perspect Biol* 5, a013201–a013201. doi:10.1101/cshperspect.a013201
- Brauweiler, A., Lorick, K.L., Lee, J.P., Tsai, Y.C., Chan, D., Weissman, A.M., Drabkin, H.A., Gemmill, R.M., 2007. RING-dependent tumor suppression and G2/M arrest induced by the TRC8 hereditary kidney cancer gene. *Oncogene* 26, 2263–2271. doi:10.1038/sj.onc.1210017
- Bray, S.J., 2006. Notch signalling: a simple pathway becomes complex. *Nat. Rev. Mol. Cell Biol.* 7, 678–689. doi:10.1038/nrm2009
- Brehme, M., Voisine, C., Rolland, T., Wachi, S., Soper, J.H., Zhu, Y., Orton, K., Vilella, A., Garza, D., Vidal, M., Ge, H., Morimoto, R.I., 2014. A chaperome subnetwork safeguards proteostasis in aging and neurodegenerative disease. *Cell Rep* 9, 1135–1150. doi:10.1016/j.celrep.2014.09.042
- Bromberg, K.D., Kluger, H.M., Delaunay, A., Abbas, S., DiVito, K.A., Krajewski, S., Ronai, Z., 2007. Increased expression of the E3 ubiquitin ligase RNF5 is associated with decreased survival in breast cancer. *Cancer Res.* 67, 8172–8179. doi:10.1158/0008-5472.CAN-07-0045
- Bull, V.H., Thiede, B., 2012. Proteome analysis of tunicamycin-induced ER stress. *Electrophoresis* 33, 1814–1823. doi:10.1002/elps.201100565
- Bulleid, N.J., Ellgaard, L., 2011. Multiple ways to make disulfides. *Trends Biochem. Sci.* 36, 485–492. doi:10.1016/j.tibs.2011.05.004
- Burr, M.L., Cano, F., Svobodova, S., Boyle, L.H., Boname, J.M., Lehner, P.J., 2011. HRD1 and UBE2J1 target misfolded MHC class I heavy chains for endoplasmic reticulum-associated degradation. *Proc. Natl. Acad. Sci. U.S.A.* 108, 2034–2039. doi:10.1073/pnas.1016229108
- Butler, A.E., Janson, J., Soeller, W.C., Butler, P.C., 2003. Increased beta-cell apoptosis prevents adaptive increase in beta-cell mass in mouse model of type 2 diabetes: evidence for role of islet amyloid formation rather than direct action of amyloid. *Diabetes* 52, 2304–2314.
- Cao, J., Wang, J., Qi, W., Miao, H.-H., Wang, J., Ge, L., DeBose-Boyd, R.A., Tang, J.-J., Li, B.-L., Song, B.-L., 2007. Ufd1 is a cofactor of gp78 and plays a key role in cholesterol metabolism by regulating the stability of HMG-CoA reductase. *Cell Metab.* 6, 115–128. doi:10.1016/j.cmet.2007.07.002
- Carelli, S., Ceriotti, A., Sitia, R., 1997. Stringent thiol-mediated retention in B lymphocytes



- and *Xenopus* oocytes correlates with inefficient IgM polymerization. *Eur. J. Immunol.* 27, 1283–1291. doi:10.1002/eji.1830270533
- Carvalho, P., Stanley, A.M., Rapoport, T.A., 2010. Retrotranslocation of a misfolded luminal ER protein by the ubiquitin-ligase Hrd1p. *Cell* 143, 579–591. doi:10.1016/j.cell.2010.10.028
- Castillo, K., Rojas-Rivera, D., Lisbona, F., Caballero, B., Nassif, M., Court, F.A., Schuck, S., Ibar, C., Walter, P., Sierralta, J., Glavic, A., Hetz, C., 2011. BAX inhibitor-1 regulates autophagy by controlling the IRE1 $\alpha$  branch of the unfolded protein response. *EMBO J.* 30, 4465–4478. doi:10.1038/emboj.2011.318
- Cattaneo, M., Otsu, M., Fagioli, C., Martino, S., Lotti, L.V., Sitia, R., Biunno, I., 2008. SEL1L and HRD1 are involved in the degradation of unassembled secretory Ig-mu chains. *J. Cell. Physiol.* 215, 794–802. doi:10.1002/jcp.21364
- Chau, V., Tobias, J.W., Bachmair, A., Marriott, D., Ecker, D.J., Gonda, D.K., Varshavsky, A., 1989. A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* 243, 1576–1583.
- Chen, Rui, Li, M., Zhang, Y., Zhou, Q., Shu, H.-B., 2012. The E3 ubiquitin ligase MARCH8 negatively regulates IL-1 $\beta$ -induced NF- $\kappa$ B activation by targeting the IL1RAP coreceptor for ubiquitination and degradation. *Proc. Natl. Acad. Sci. U.S.A.* 109, 14128–14133. doi:10.1073/pnas.1205246109
- Chen, Xi, Shen, J., Prywes, R., 2002. The luminal domain of ATF6 senses endoplasmic reticulum (ER) stress and causes translocation of ATF6 from the ER to the Golgi. *J. Biol. Chem.* 277, 13045–13052. doi:10.1074/jbc.M110636200
- Chen, Xin, Tukachinsky, H., Huang, C.-H., Jao, C., Chu, Y.-R., Tang, H.-Y., Mueller, B., Schulman, S., Rapoport, T.A., Salic, A., 2011. Processing and turnover of the Hedgehog protein in the endoplasmic reticulum. *J. Cell Biol.* 192, 825–838. doi:10.1083/jcb.201008090
- Cheng, J., Guggino, W., 2013. Ubiquitination and degradation of CFTR by the E3 ubiquitin ligase MARCH2 through its association with adaptor proteins CAL and STX6. *PLoS ONE* 8, e68001. doi:10.1371/journal.pone.0068001
- Christensen, L.C., Jensen, N.W., Vala, A., Kamarauskaite, J., Johansson, L., Winther, J.R., Hofmann, K., Teilum, K., Ellgaard, L., 2012. The human selenoprotein VCP-interacting membrane protein (VIMP) is non-globular and harbors a reductase function in an intrinsically disordered region. *J. Biol. Chem.* 287, 26388–26399. doi:10.1074/jbc.M112.346775
- Christianson, J.C., Olzmann, J.A., Shaler, T.A., Sowa, M.E., Bennett, E.J., Richter, C.M., Tyler, R.E., Greenblatt, E.J., Harper, J.W., Kopito, R.R., 2012. Defining human ERAD networks through an integrative mapping strategy. *Nat. Cell Biol.* 14, 93–105. doi:10.1038/ncb2383
- Christianson, J.C., Shaler, T.A., Tyler, R.E., Kopito, R.R., 2008. OS-9 and GRP94 deliver mutant  $\alpha$ 1-antitrypsin to the Hrd1-SEL1L ubiquitin ligase complex for ERAD. *Nat. Cell Biol.* 10, 272–282. doi:10.1038/ncb1689
- Christianson, J.C., Ye, Y., 2014. Cleaning up in the endoplasmic reticulum: ubiquitin in charge. *Nat. Struct. Mol. Biol.* 21, 325–335. doi:10.1038/nsmb.2793
- Cicchini, M., Karantza, V., Xia, B., 2015. Molecular pathways: autophagy in cancer--a matter

- of timing and context. *Clin. Cancer Res.* 21, 498–504. doi:10.1158/1078-0432.CCR-13-2438
- Ciechanover, A., 2005. Proteolysis: from the lysosome to ubiquitin and the proteasome. *Nat. Rev. Mol. Cell Biol.* 6, 79–87. doi:10.1038/nrm1552
- Cohen-Kaplan, V., Livneh, I., Avni, N., Cohen-Rosenzweig, C., Ciechanover, A., 2016. The ubiquitin-proteasome system and autophagy: Coordinated and independent activities. *Int. J. Biochem. Cell Biol.* doi:10.1016/j.biocel.2016.07.019
- Corcos, D., Osborn, M.J., Matheson, L.S., 2011. B-cell receptors and heavy chain diseases: guilty by association? *Blood* 117, 6991–6998. doi:10.1182/blood-2011-02-336164
- Cormier, J.H., Tamura, T., Sunryd, J.C., Hebert, D.N., 2009. EDEM1 recognition and delivery of misfolded proteins to the SEL1L-containing ERAD complex. *Mol. Cell* 34, 627–633. doi:10.1016/j.molcel.2009.05.018
- Cortini, M., Sitia, R., 2010. ERp44 and ERGIC-53 synergize in coupling efficiency and fidelity of IgM polymerization and secretion. *Traffic* 11, 651–659. doi:10.1111/j.1600-0854.2010.01043.x
- Cox, J., Mann, M., 2008. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* 26, 1367–1372. doi:10.1038/nbt.1511
- Cox, J.S., Shamu, C.E., Walter, P., 1993. Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. *Cell* 73, 1197–1206.
- Cuanalo-Contreras, K., Mukherjee, A., Soto, C., 2013. Role of Protein Misfolding and Proteostasis Deficiency in Protein Misfolding Diseases and Aging. *Int J Cell Biol* 2013, 1–10. doi:10.1155/2013/638083
- Cullinan, S.B., Zhang, D., Hannink, M., Arvisais, E., Kaufman, R.J., Diehl, J.A., 2003. Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Mol. Cell. Biol.* 23, 7198–7209. doi:10.1128/MCB.23.20.7198-7209.2003
- Das, R., Mariano, J., Tsai, Y.C., Kalathur, R.C., Kostova, Z., Li, J., Tarasov, S.G., McFeeters, R.L., Altieri, A.S., Ji, X., Byrd, R.A., Weissman, A.M., 2009. Allosteric activation of E2-RING finger-mediated ubiquitylation by a structurally defined specific E2-binding region of gp78. *Mol. Cell* 34, 674–685. doi:10.1016/j.molcel.2009.05.010
- DeLaBarre, B., Christianson, J.C., Kopito, R.R., Brunger, A.T., 2006. Central pore residues mediate the p97/VCP activity required for ERAD. *Mol. Cell* 22, 451–462. doi:10.1016/j.molcel.2006.03.036
- Deshaies, R.J., Joazeiro, C.A.P., 2009. RING Domain E3 Ubiquitin Ligases. *Annu. Rev. Biochem.* 78, 399–434. doi:10.1146/annurev.biochem.78.101807.093809
- Deveraux, Q., Ustrell, V., Pickart, C., Rechsteiner, M., 1994. A 26 S protease subunit that binds ubiquitin conjugates. *J. Biol. Chem.* 269, 7059–7061.
- Didier, C., Broday, L., Bhoumik, A., Israeli, S., Takahashi, S., Nakayama, K., Thomas, S.M., Turner, C.E., Henderson, S., Sabe, H., Ronai, Z., 2003. RNF5, a RING finger protein that regulates cell motility by targeting paxillin ubiquitination and altered localization. *Mol. Cell. Biol.* 23, 5331–5345. doi:10.1128/MCB.23.15.5331-5345.2003
- Dimopoulos, M.A., San Miguel, J.F., Anderson, K.C., 2011. Emerging therapies for the treatment of relapsed or refractory multiple myeloma. *Eur. J. Haematol.* 86, 1–15.

- Ding, W.-X., Ni, H.-M., Gao, W., Yoshimori, T., Stolz, D.B., Ron, D., Yin, X.-M., 2007. Linking of autophagy to ubiquitin-proteasome system is important for the regulation of endoplasmic reticulum stress and cell viability. *Am. J. Pathol.* 171, 513–524. doi:10.2353/ajpath.2007.070188
- Ding, W.-X., Yin, X.-M., 2008. Sorting, recognition and activation of the misfolded protein degradation pathways through macroautophagy and the proteasome. *Autophagy* 4, 141–150.
- Dombroski, B.A., Nayak, R.R., Ewens, K.G., Ankener, W., Cheung, V.G., Spielman, R.S., 2010. Gene expression and genetic variation in response to endoplasmic reticulum stress in human cells. *Am. J. Hum. Genet.* 86, 719–729. doi:10.1016/j.ajhg.2010.03.017
- Doroudgar, S., Völkers, M., Thuerauf, D.J., Khan, M., Mohsin, S., Respress, J.L., Wang, W., Gude, N., Müller, O.J., Wehrens, X.H.T., Sussman, M.A., Glembotski, C.C., 2015. Hrd1 and ER-Associated Protein Degradation, ERAD, are Critical Elements of the Adaptive ER Stress Response in Cardiac Myocytes. *Circ. Res.* 117, 536–546. doi:10.1161/CIRCRESAHA.115.306993
- Dougan, S.K., Hu, C.-C.A., Paquet, M.-E., Greenblatt, M.B., Kim, J., Lilley, B.N., Watson, N., Ploegh, H.L., 2011. Derlin-2-deficient mice reveal an essential role for protein dislocation in chondrocytes. *Mol. Cell. Biol.* 31, 1145–1159. doi:10.1128/MCB.00967-10
- Douglas, P.M., Dillin, A., 2010. Protein homeostasis and aging in neurodegeneration. *J. Cell Biol.* 190, 719–729. doi:10.1083/jcb.201005144
- Doyle, K.M., Kennedy, D., Gorman, A.M., Gupta, S., Healy, S.J.M., Samali, A., 2011. Unfolded proteins and endoplasmic reticulum stress in neurodegenerative disorders. *J. Cell. Mol. Med.* 15, 2025–2039. doi:10.1111/j.1582-4934.2011.01374.x
- Duennwald, M.L., Lindquist, S., 2008. Impaired ERAD and ER stress are early and specific events in polyglutamine toxicity. *Genes Dev.* 22, 3308–3319. doi:10.1101/gad.1673408
- Elkabetz, Y., Kerem, A., Tencer, L., Winitz, D., Kopito, R.R., Bar-Nun, S., 2003. Immunoglobulin light chains dictate vesicular transport-dependent and -independent routes for IgM degradation by the ubiquitin-proteasome pathway. *J. Biol. Chem.* 278, 18922–18929. doi:10.1074/jbc.M208730200
- Ellgaard, L., Molinari, M., Helenius, A., 1999. Setting the standards: quality control in the secretory pathway. *Science* 286, 1882–1888.
- Ellgaard, L., Ruddock, L.W., 2005. The human protein disulphide isomerase family: substrate interactions and functional properties. *EMBO Rep.* 6, 28–32. doi:10.1038/sj.embor.7400311
- Ernst, R., Mueller, B., Ploegh, H.L., Schlieker, C., 2009. The otubain YOD1 is a deubiquitinating enzyme that associates with p97 to facilitate protein dislocation from the ER. *Mol. Cell* 36, 28–38. doi:10.1016/j.molcel.2009.09.016
- Eura, Y., Yanamoto, H., Arai, Y., Okuda, T., Miyata, T., Kokame, K., 2012. Derlin-1 deficiency is embryonic lethal, Derlin-3 deficiency appears normal, and Herp deficiency is intolerant to glucose load and ischemia in mice. *PLoS ONE* 7, e34298. doi:10.1371/journal.pone.0034298
- Fagioli, C., Mezghrani, A., Sitia, R., 2001. Reduction of interchain disulfide bonds precedes the dislocation of Ig-μ chains from the endoplasmic reticulum to the cytosol for proteasomal degradation. *J. Biol. Chem.* 276, 40962–40967.

- Fang, S., Ferrone, M., Yang, C., Jensen, J.P., Tiwari, S., Weissman, A.M., 2001. The tumor autocrine motility factor receptor, gp78, is a ubiquitin protein ligase implicated in degradation from the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U.S.A.* 98, 14422–14427. doi:10.1073/pnas.251401598
- Feldmann, M., Brennan, F.M., Maini, R.N., 1996. Rheumatoid arthritis. *Cell* 85, 307–310.
- Feng, Y., He, D., Yao, Z., Klionsky, D.J., 2014. The machinery of macroautophagy. *Cell Res.* 24, 24–41. doi:10.1038/cr.2013.168
- Fleig, L., Bergbold, N., Sahasrabudhe, P., Geiger, B., Kaltak, L., Lemberg, M.K., 2012. Ubiquitin-dependent intramembrane rhomboid protease promotes ERAD of membrane proteins. *Mol. Cell* 47, 558–569. doi:10.1016/j.molcel.2012.06.008
- Flynn, G.C., Pohl, J., Flocco, M.T., Rothman, J.E., 1991. Peptide-binding specificity of the molecular chaperone BiP. *Nature* 353, 726–730. doi:10.1038/353726a0
- Fonseca, S.G., Ishigaki, S., Osowski, C.M., Lu, S., Lipson, K.L., Ghosh, R., Hayashi, E., Ishihara, H., Oka, Y., Permutt, M.A., Urano, F., 2010. Wolfram syndrome 1 gene negatively regulates ER stress signaling in rodent and human cells. *J. Clin. Invest.* 120, 744–755. doi:10.1172/JCI39678
- Foresti, O., Ruggiano, A., Hannibal-Bach, H.K., Ejsing, C.S., Carvalho, P., 2013. Sterol homeostasis requires regulated degradation of squalene monooxygenase by the ubiquitin ligase Doa10/Teb4. *Elife* 2, e00953. doi:10.7554/eLife.00953
- Fra, A.M., Fagioli, C., Finazzi, D., Sitia, R., Alberini, C.M., 1993. Quality control of ER synthesized proteins: an exposed thiol group as a three-way switch mediating assembly, retention and degradation. *EMBO J.* 12, 4755–4761.
- Francisco, A.B., Singh, R., Li, S., Vani, A.K., Yang, L., Munroe, R.J., Diaferia, G., Cardano, M., Biunno, I., Qi, L., Schimenti, J.C., Long, Q., 2010. Deficiency of suppressor enhancer Lin12 1 like (SEL1L) in mice leads to systemic endoplasmic reticulum stress and embryonic lethality. *J. Biol. Chem.* 285, 13694–13703. doi:10.1074/jbc.M109.085340
- Frickel, E.-M., Frei, P., Bouvier, M., Stafford, W.F., Helenius, A., Glockshuber, R., Ellgaard, L., 2004. ERp57 is a multifunctional thiol-disulfide oxidoreductase. *J. Biol. Chem.* 279, 18277–18287. doi:10.1074/jbc.M314089200
- Friedlander, R., Jarosch, E., Urban, J., Volkwein, C., Sommer, T., 2000. A regulatory link between ER-associated protein degradation and the unfolded-protein response. *Nat. Cell Biol.* 2, 379–384. doi:10.1038/35017001
- Fujita, E., Kouroku, Y., Isoai, A., Kumagai, H., Misutani, A., Matsuda, C., Hayashi, Y.K., Momoi, T., 2007. Two endoplasmic reticulum-associated degradation (ERAD) systems for the novel variant of the mutant dysferlin: ubiquitin/proteasome ERAD(I) and autophagy/lysosome ERAD(II). *Hum. Mol. Genet.* 16, 618–629. doi:10.1093/hmg/ddm002
- Funderburk, S.F., Wang, Q.J., Yue, Z., 2010. The Beclin 1-VPS34 complex--at the crossroads of autophagy and beyond. *Trends Cell Biol.* 20, 355–362. doi:10.1016/j.tcb.2010.03.002
- Füllgrabe, J., Klionsky, D.J., Joseph, B., 2014. The return of the nucleus: transcriptional and epigenetic control of autophagy. *Nat. Rev. Mol. Cell Biol.* 15, 65–74. doi:10.1038/nrm3716

- Gandy, S., 2005. The role of cerebral amyloid beta accumulation in common forms of Alzheimer disease. *J. Clin. Invest.* 115, 1121–1129. doi:10.1172/JCI25100
- Gardner, B.M., Walter, P., 2011. Unfolded proteins are Ire1-activating ligands that directly induce the unfolded protein response. *Science* 333, 1891–1894. doi:10.1126/science.1209126
- Garza, R.M., Sato, B.K., Hampton, R.Y., 2009. In vitro analysis of Hrd1p-mediated retrotranslocation of its multispinning membrane substrate 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase. *J. Biol. Chem.* 284, 14710–14722. doi:10.1074/jbc.M809607200
- Gavilán, E., Pintado, C., Gavilan, M.P., Daza, P., Sánchez-Aguayo, I., Castaño, A., Ruano, D., 2015. Age-related dysfunctions of the autophagy lysosomal pathway in hippocampal pyramidal neurons under proteasome stress. *Neurobiol. Aging* 36, 1953–1963. doi:10.1016/j.neurobiolaging.2015.02.025
- Geiss, G.K., Bumgarner, R.E., Birditt, B., Dahl, T., Dowidar, N., Dunaway, D.L., Fell, H.P., Ferree, S., George, R.D., Grogan, T., James, J.J., Maysuria, M., Mitton, J.D., Oliveri, P., Osborn, J.L., Peng, T., Ratcliffe, A.L., Webster, P.J., Davidson, E.H., Hood, L., Dimitrov, K., 2008. Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat. Biotechnol.* 26, 317–325. doi:10.1038/nbt1385
- Gemmill, R.M., West, J.D., Boldog, F., Tanaka, N., Robinson, L.J., Smith, D.I., Li, F., Drabkin, H.A., 1998. The hereditary renal cell carcinoma 3;8 translocation fuses FHIT to a patched-related gene, TRC8. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9572–9577.
- Ghaemmaghami, S., Huh, W.-K., Bower, K., Howson, R.W., Belle, A., Dephoure, N., O'Shea, E.K., Weissman, J.S., 2003. Global analysis of protein expression in yeast. *Nature* 425, 737–741. doi:10.1038/nature02046
- Ghosh, R., Lipson, K.L., Sargent, K.E., Mercurio, A.M., Hunt, J.S., Ron, D., Urano, F., 2010. Transcriptional regulation of VEGF-A by the unfolded protein response pathway. *PLoS ONE* 5, e9575. doi:10.1371/journal.pone.0009575
- Gilkes, D.M., Chaturvedi, P., Bajpai, S., Wong, C.C., Wei, H., Pitcairn, S., Hubbi, M.E., Wirtz, D., Semenza, G.L., 2013. Collagen prolyl hydroxylases are essential for breast cancer metastasis. *Cancer Res.* 73, 3285–3296. doi:10.1158/0008-5472.CAN-12-3963
- Gillece, P., Pilon, M., Römisch, K., 2000. The protein translocation channel mediates glycopeptide export across the endoplasmic reticulum membrane. *Proc. Natl. Acad. Sci. U.S.A.* 97, 4609–4614. doi:10.1073/pnas.090083497
- Gonzalez, D.S., Karaveg, K., Vandersall-Nairn, A.S., Lal, A., Moremen, K.W., 1999. Identification, expression, and characterization of a cDNA encoding human endoplasmic reticulum mannosidase I, the enzyme that catalyzes the first mannose trimming step in mammalian Asn-linked oligosaccharide biosynthesis. *J. Biol. Chem.* 274, 21375–21386.
- Görlich, D., Rapoport, T.A., 1993. Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane. *Cell* 75, 615–630.
- Granelli, P., Cattaneo, M., Ferrero, S., Bottiglieri, L., Bosari, S., Fichera, G., Biunno, I., 2004. SEL1L and squamous cell carcinoma of the esophagus. *Clin. Cancer Res.* 10, 5857–5861. doi:10.1158/1078-0432.CCR-04-0075
- Greenblatt, E.J., Olzmann, J.A., Kopito, R.R., 2011. Derlin-1 is a rhomboid pseudoprotease required for the dislocation of mutant  $\alpha$ -1 antitrypsin from the endoplasmic reticulum. *Nat. Struct. Mol. Biol.* 18, 1147–1152. doi:10.1038/nsmb.2111

- Guerriero, C.J., Brodsky, J.L., 2012. The delicate balance between secreted protein folding and endoplasmic reticulum-associated degradation in human physiology. *Physiol. Rev.* 92, 537–576. doi:10.1152/physrev.00027.2011
- Haas, I.G., Wabl, M., 1983. Immunoglobulin heavy chain binding protein. *Nature* 306, 387–389.
- Hagiwara, M., Maegawa, K.-I., Suzuki, M., Ushioda, R., Araki, K., Matsumoto, Y., Hoseki, J., Nagata, K., Inaba, K., 2011. Structural basis of an ERAD pathway mediated by the ER-resident protein disulfide reductase ERdj5. *Mol. Cell* 41, 432–444. doi:10.1016/j.molcel.2011.01.021
- Haigis, M.C., Yankner, B.A., 2010. The aging stress response. *Mol. Cell* 40, 333–344. doi:10.1016/j.molcel.2010.10.002
- Hamasaki, M., Furuta, N., Matsuda, A., Nezu, A., Yamamoto, A., Fujita, N., Oomori, H., Noda, T., Haraguchi, T., Hiraoka, Y., Amano, A., Yoshimori, T., 2013. Autophagosomes form at ER-mitochondria contact sites. *Nature* 495, 389–393. doi:10.1038/nature11910
- Hampton, R.Y., Gardner, R.G., Rine, J., 1996. Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. *Mol. Biol. Cell* 7, 2029–2044.
- Harding, H.P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., Ron, D., 2000. Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol. Cell* 6, 1099–1108.
- Harding, H.P., Zhang, Y., Ron, D., 1999. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature* 397, 271–274. doi:10.1038/16729
- Hartley, T., Siva, M., Lai, E., Teodoro, T., Zhang, L., Volchuk, A., 2010. Endoplasmic reticulum stress response in an INS-1 pancreatic beta-cell line with inducible expression of a folding-deficient proinsulin. *BMC Cell Biol.* 11, 59. doi:10.1186/1471-2121-11-59
- Hassink, G., Kikkert, M., van Voorden, S., Lee, S.-J., Spaapen, R., van Laar, T., Coleman, C.S., Bartee, E., Früh, K., Chau, V., Wiertz, E., 2005. TEB4 is a C4HC3 RING finger-containing ubiquitin ligase of the endoplasmic reticulum. *Biochem. J.* 388, 647–655. doi:10.1042/BJ20041241
- Helenius, A., Aebi, M., 2004. Roles of N-linked glycans in the endoplasmic reticulum. *Annu. Rev. Biochem.* 73, 1019–1049. doi:10.1146/annurev.biochem.73.011303.073752
- Hendershot, L.M., 2004. The ER function BiP is a master regulator of ER function. *Mt. Sinai J. Med.* 71, 289–297.
- Hendershot, L.M. and Sitia R., 2005. Immunoglobulin assembly and secretion in *Molecular Biology of B Cells*, T. Honjo, F. W. Alt, and M. S. Neuberger, Eds., pp. 261–273, Elsevier Academic Press, Amsterdam, The Netherlands.
- Hetz, C., Martinon, F., Rodriguez, D., Glimcher, L.H., 2011. The unfolded protein response: integrating stress signals through the stress sensor IRE1 $\alpha$ . *Physiol. Rev.* 91, 1219–1243. doi:10.1152/physrev.00001.2011
- Hetz, C., Thielen, P., Matus, S., Nassif, M., Court, F., Kiffin, R., Martinez, G., Cuervo, A.M., Brown, R.H., Glimcher, L.H., 2009. XBP-1 deficiency in the nervous system protects against amyotrophic lateral sclerosis by increasing autophagy. *Genes Dev.* 23, 2294–2306. doi:10.1101/gad.1830709
- Hetz, C.A., Soto, C., 2006. Stressing out the ER: a role of the unfolded protein response in

- prion-related disorders. *Curr. Mol. Med.* 6, 37–43.
- Hipp, M.S., Park, S.-H., Hartl, F.U., 2014. Proteostasis impairment in protein-misfolding and -aggregation diseases. *Trends Cell Biol.* 24, 506–514. doi:10.1016/j.tcb.2014.05.003
- Hollien, J., Lin, J.H., Li, H., Stevens, N., Walter, P., Weissman, J.S., 2009. Regulated Ire1-dependent decay of messenger RNAs in mammalian cells. *J. Cell Biol.* 186, 323–331. doi:10.1083/jcb.200903014
- Hosokawa, N., Kamiya, Y., Kamiya, D., Kato, K., Nagata, K., 2009. Human OS-9, a lectin required for glycoprotein endoplasmic reticulum-associated degradation, recognizes mannose-trimmed N-glycans. *J. Biol. Chem.* 284, 17061–17068. doi:10.1074/jbc.M809725200
- Hosokawa, N., Kato, K., Kamiya, Y., 2010a. Mannose 6-phosphate receptor homology domain-containing lectins in mammalian endoplasmic reticulum-associated degradation. *Meth. Enzymol.* 480, 181–197. doi:10.1016/S0076-6879(10)80010-2
- Hosokawa, N., Tremblay, L.O., Sleno, B., Kamiya, Y., Wada, I., Nagata, K., Kato, K., Herscovics, A., 2010b. EDEM1 accelerates the trimming of alpha1,2-linked mannose on the C branch of N-glycans. *Glycobiology* 20, 567–575. doi:10.1093/glycob/cwq001
- Hosokawa, N., Tremblay, L.O., You, Z., Herscovics, A., Wada, I., Nagata, K., 2003. Enhancement of endoplasmic reticulum (ER) degradation of misfolded Null Hong Kong alpha1-antitrypsin by human ER mannosidase I. *J. Biol. Chem.* 278, 26287–26294. doi:10.1074/jbc.M303395200
- Hosokawa, N., Wada, I., Hasegawa, K., Yorihuzi, T., Tremblay, L.O., Herscovics, A., Nagata, K., 2001. A novel ER alpha-mannosidase-like protein accelerates ER-associated degradation. *EMBO Rep.* 2, 415–422. doi:10.1093/embo-reports/kve084
- Hosokawa, N., Wada, I., Nagasawa, K., Moriyama, T., Okawa, K., Nagata, K., 2008. Human XTP3-B forms an endoplasmic reticulum quality control scaffold with the HRD1-SEL1L ubiquitin ligase complex and BiP. *J. Biol. Chem.* 283, 20914–20924. doi:10.1074/jbc.M709336200
- Hosokawa, N., You, Z., Tremblay, L.O., Nagata, K., Herscovics, A., 2007. Stimulation of ERAD of misfolded null Hong Kong alpha1-antitrypsin by Golgi alpha1,2-mannosidases. *Biochem. Biophys. Res. Commun.* 362, 626–632. doi:10.1016/j.bbrc.2007.08.057
- Houck, S.A., Ren, H.-Y., Madden, V.J., Bonner, J.N., Conlin, M.P., Janovick, J.A., Conn, P.M., Cyr, D.M., 2014. Quality control autophagy degrades soluble ERAD-resistant conformers of the misfolded membrane protein GnRHR. *Mol. Cell* 54, 166–179. doi:10.1016/j.molcel.2014.02.025
- Huang, C.-H., Chu, Y.-R., Ye, Y., Chen, X., 2014. Role of HERP and a HERP-related protein in HRD1-dependent protein degradation at the endoplasmic reticulum. *J. Biol. Chem.* 289, 4444–4454. doi:10.1074/jbc.M113.519561
- Huang, C.-H., Hsiao, H.-T., Chu, Y.-R., Ye, Y., Chen, X., 2013. Derlin2 protein facilitates HRD1-mediated retro-translocation of sonic hedgehog at the endoplasmic reticulum. *J. Biol. Chem.* 288, 25330–25339. doi:10.1074/jbc.M113.455212
- Hubbard, S.C., Ivatt, R.J., 1981. Synthesis and processing of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 50, 555–583. doi:10.1146/annurev.bi.50.070181.003011
- Huber, M.D., Vesely, P.W., Datta, K., Gerace, L., 2013. Erlins restrict SREBP activation in the ER and regulate cellular cholesterol homeostasis. *J. Cell Biol.* 203, 427–436. doi:10.1083/jcb.201305076

- Husnjak, K., Elsasser, S., Zhang, N., Chen, X., Randles, L., Shi, Y., Hofmann, K., Walters, K.J., Finley, D., Dikic, I., 2008. Proteasome subunit Rpn13 is a novel ubiquitin receptor. *Nature* 453, 481–488. doi:10.1038/nature06926
- Huttenhower, C., Haley, E.M., Hibbs, M.A., Dumeaux, V., Barrett, D.R., Collier, H.A., Troyanskaya, O.G., 2009. Exploring the human genome with functional maps. *Genome Res.* 19, 1093–1106. doi:10.1101/gr.082214.108
- Høyer-Hansen, M., Jäättelä, M., 2007. Connecting endoplasmic reticulum stress to autophagy by unfolded protein response and calcium. *Cell Death Differ.* 14, 1576–1582. doi:10.1038/sj.cdd.4402200
- Ichikawa, D., Mizuno, M., Yamamura, T., Miyake, S., 2011. GRAIL (gene related to anergy in lymphocytes) regulates cytoskeletal reorganization through ubiquitination and degradation of Arp2/3 subunit 5 and coronin 1A. *J. Biol. Chem.* 286, 43465–43474. doi:10.1074/jbc.M111.222711
- Iida, Y., Fujimori, T., Okawa, K., Nagata, K., Wada, I., Hosokawa, N., 2011. SEL1L protein critically determines the stability of the HRD1-SEL1L endoplasmic reticulum-associated degradation (ERAD) complex to optimize the degradation kinetics of ERAD substrates. *J. Biol. Chem.* 286, 16929–16939. doi:10.1074/jbc.M110.215871
- Imai, Y., Soda, M., Inoue, H., Hattori, N., Mizuno, Y., Takahashi, R., 2001. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. *Cell* 105, 891–902.
- Irisawa, M., Inoue, J., Ozawa, N., Mori, K., Sato, R., 2009. The sterol-sensing endoplasmic reticulum (ER) membrane protein TRC8 hampers ER to Golgi transport of sterol regulatory element-binding protein-2 (SREBP-2)/SREBP cleavage-activated protein and reduces SREBP-2 cleavage. *J. Biol. Chem.* 284, 28995–29004. doi:10.1074/jbc.M109.041376
- Ishida, Y., Nagata, K., 2009. Autophagy eliminates a specific species of misfolded procollagen and plays a protective role in cell survival against ER stress. *Autophagy* 5, 1217–1219.
- Iwata, A., Riley, B.E., Johnston, J.A., Kopito, R.R., 2005. HDAC6 and microtubules are required for autophagic degradation of aggregated huntingtin. *J. Biol. Chem.* 280, 40282–40292. doi:10.1074/jbc.M508786200
- Jansen, G., Määttänen, P., Denisov, A.Y., Scarffe, L., Schade, B., Balghi, H., Dejgaard, K., Chen, L.Y., Muller, W.J., Gehring, K., Thomas, D.Y., 2012. An interaction map of endoplasmic reticulum chaperones and foldases. *Mol. Cell Proteomics* 11, 710–723. doi:10.1074/mcp.M111.016550
- Jeon, Y.J., Khelifa, S., Ratnikov, B., Scott, D.A., Feng, Y., Parisi, F., Ruller, C., Lau, E., Kim, H., Brill, L.M., Jiang, T., Rimm, D.L., Cardiff, R.D., Mills, G.B., Smith, J.W., Osterman, A.L., Kluger, Y., Ronai, Z.A., 2015. Regulation of glutamine carrier proteins by RNF5 determines breast cancer response to ER stress-inducing chemotherapies. *Cancer Cell* 27, 354–369. doi:10.1016/j.ccell.2015.02.006
- Ji, Y., Kim, H., Yang, L., Sha, H., Roman, C.A., Long, Q., Qi, L., 2016. The Sel1L-Hrd1 Endoplasmic Reticulum-Associated Degradation Complex Manages a Key Checkpoint in B Cell Development. *Cell Rep* 16, 2630–2640. doi:10.1016/j.celrep.2016.08.003
- Jo, Y., Sguigna, P.V., DeBose-Boyd, R.A., 2011. Membrane-associated ubiquitin ligase complex containing gp78 mediates sterol-accelerated degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *J. Biol. Chem.* 286, 15022–15031.



- Johnson, N., Powis, K., High, S., 2013. Post-translational translocation into the endoplasmic reticulum. *Biochim. Biophys. Acta* 1833, 2403–2409. doi:10.1016/j.bbamcr.2012.12.008
- Jones, M.L., Murden, S.L., Brooks, C., Maloney, V., Manning, R.A., Gilmour, K.C., Bharadwaj, V., la Fuente, de, J., Chakravorty, S., Mumford, A.D., 2013. Disruption of AP3B1 by a chromosome 5 inversion: a new disease mechanism in Hermansky-Pudlak syndrome type 2. *BMC Med. Genet.* 14, 42. doi:10.1186/1471-2350-14-42
- Jongsma, M.L.M., Berlin, I., Wijdeven, R.H.M., Janssen, L., Janssen, G.M.C., Garstka, M.A., Janssen, H., Mensink, M., van Veelen, P.A., Spaapen, R.M., Neefjes, J., 2016. An ER-Associated Pathway Defines Endosomal Architecture for Controlled Cargo Transport. *Cell* 166, 152–166. doi:10.1016/j.cell.2016.05.078
- Kaneko, M., Ishiguro, M., Niinuma, Y., Uesugi, M., Nomura, Y., 2002. Human HRD1 protects against ER stress-induced apoptosis through ER-associated degradation. *FEBS Lett.* 532, 147–152.
- Kaneko, M., Koike, H., Saito, R., Kitamura, Y., Okuma, Y., Nomura, Y., 2010. Loss of HRD1-mediated protein degradation causes amyloid precursor protein accumulation and amyloid-beta generation. *J. Neurosci.* 30, 3924–3932. doi:10.1523/JNEUROSCI.2422-09.2010
- Kaneko, M., Yasui, S., Niinuma, Y., Arai, K., Omura, T., Okuma, Y., Nomura, Y., 2007. A different pathway in the endoplasmic reticulum stress-induced expression of human HRD1 and SEL1 genes. *FEBS Lett.* 581, 5355–5360. doi:10.1016/j.febslet.2007.10.033
- Keilhauer, E.C., Hein, M.Y., Mann, M., 2015. Accurate protein complex retrieval by affinity enrichment mass spectrometry (AE-MS) rather than affinity purification mass spectrometry (AP-MS). *Mol. Cell Proteomics* 14, 120–135. doi:10.1074/mcp.M114.041012
- Khaminets, A., Behl, C., Dikic, I., 2016. Ubiquitin-Dependent And Independent Signals In Selective Autophagy. *Trends Cell Biol.* 26, 6–16. doi:10.1016/j.tcb.2015.08.010
- Khaminets, A., Heinrich, T., Mari, M., Grumati, P., Huebner, A.K., Akutsu, M., Liebmman, L., Stolz, A., Nietzsche, S., Koch, N., Mauthe, M., Katona, I., Qualmann, B., Weis, J., Reggiori, F., Kurth, I., Hübner, C.A., Dikic, I., 2015. Regulation of endoplasmic reticulum turnover by selective autophagy. *Nature* 522, 354–358. doi:10.1038/nature14498
- Khoury, El, E., Le Pavec, G., Toledano, M.B., Delaunay-Moisan, A., 2013. RNF185 is a novel E3 ligase of endoplasmic reticulum-associated degradation (ERAD) that targets cystic fibrosis transmembrane conductance regulator (CFTR). *J. Biol. Chem.* 288, 31177–31191. doi:10.1074/jbc.M113.470500
- Kikkert, M., Doolman, R., Dai, M., Avner, R., Hassink, G., van Voorden, S., Thanedar, S., Roitelman, J., Chau, V., Wiertz, E., 2004. Human HRD1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum. *J. Biol. Chem.* 279, 3525–3534. doi:10.1074/jbc.M307453200
- Kim, I., Ahn, J., Liu, C., Tanabe, K., Apodaca, J., Suzuki, T., Rao, H., 2006. The Png1-Rad23 complex regulates glycoprotein turnover. *J. Cell Biol.* 172, 211–219. doi:10.1083/jcb.200507149
- Kimata, Y., Oikawa, D., Shimizu, Y., Ishiwata-Kimata, Y., Kohno, K., 2004. A role for BiP as an adjustor for the endoplasmic reticulum stress-sensing protein Ire1. *J. Cell Biol.* 167, 445–456. doi:10.1083/jcb.200405153
- Kirkin, V., Lamark, T., Johansen, T., Dikic, I., 2009a. NBR1 cooperates with p62 in selective

autophagy of ubiquitinated targets. *Autophagy* 5, 732–733.

- Kirkin, V., Lamark, T., Sou, Y.-S., Bjørkøy, G., Nunn, J.L., Bruun, J.-A., Shvets, E., McEwan, D.G., Clausen, T.H., Wild, P., Bilusic, I., Theurillat, J.-P., Øvervatn, A., Ishii, T., Elazar, Z., Komatsu, M., Dikic, I., Johansen, T., 2009b. A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. *Mol. Cell* 33, 505–516. doi:10.1016/j.molcel.2009.01.020
- Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., Shimizu, N., 1998. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 392, 605–608. doi:10.1038/33416
- Klemm, E.J., Spooner, E., Ploegh, H.L., 2011. Dual role of ancient ubiquitous protein 1 (AUP1) in lipid droplet accumulation and endoplasmic reticulum (ER) protein quality control. *J. Biol. Chem.* 286, 37602–37614. doi:10.1074/jbc.M111.284794
- Klionsky, D.J., 2007. Autophagy: from phenomenology to molecular understanding in less than a decade. *Nat. Rev. Mol. Cell Biol.* 8, 931–937. doi:10.1038/nrm2245
- Kny, M., Standera, S., Hartmann-Petersen, R., Kloetzel, P.-M., Seeger, M., 2011. Herp regulates Hrd1-mediated ubiquitylation in a ubiquitin-like domain-dependent manner. *J. Biol. Chem.* 286, 5151–5156. doi:10.1074/jbc.M110.134551
- Kokame, K., Kato, H., Miyata, T., 2001. Identification of ERSE-II, a new cis-acting element responsible for the ATF6-dependent mammalian unfolded protein response. *J. Biol. Chem.* 276, 9199–9205. doi:10.1074/jbc.M010486200
- Komander, D., 2009. The emerging complexity of protein ubiquitination. *Biochem. Soc. Trans.* 37, 937–953. doi:10.1042/BST0370937
- Kondratyev, M., Avezov, E., Shenkman, M., Groisman, B., Lederkremer, G.Z., 2007. PERK-dependent compartmentalization of ERAD and unfolded protein response machineries during ER stress. *Exp. Cell Res.* 313, 3395–3407. doi:10.1016/j.yexcr.2007.07.006
- Kopito, R.R., Sitia, R., 2000. Aggresomes and Russell bodies. Symptoms of cellular indigestion? *EMBO Rep.* 1, 225–231. doi:10.1093/embo-reports/kvd052
- Korac, J., Schaeffer, V., Kovacevic, I., Clement, A.M., Jungblut, B., Behl, C., Terzic, J., Dikic, I., 2013. Ubiquitin-independent function of optineurin in autophagic clearance of protein aggregates. *J. Cell. Sci.* 126, 580–592. doi:10.1242/jcs.114926
- Korennykh, A.V., Egea, P.F., Korostelev, A.A., Finer-Moore, J., Zhang, C., Shokat, K.M., Stroud, R.M., Walter, P., 2009. The unfolded protein response signals through high-order assembly of Ire1. *Nature* 457, 687–693. doi:10.1038/nature07661
- Korolchuk, V.I., Menzies, F.M., Rubinsztein, D.C., 2010. Mechanisms of cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems. *FEBS Lett.* 584, 1393–1398. doi:10.1016/j.febslet.2009.12.047
- Kostova, Z., Tsai, Y.C., Weissman, A.M., 2007. Ubiquitin ligases, critical mediators of endoplasmic reticulum-associated degradation. *Semin. Cell Dev. Biol.* 18, 770–779. doi:10.1016/j.semcdb.2007.09.002
- Kreft, S.G., Wang, L., Hochstrasser, M., 2006. Membrane topology of the yeast endoplasmic reticulum-localized ubiquitin ligase Doa10 and comparison with its human ortholog TEB4 (MARCH-VI). *J. Biol. Chem.* 281, 4646–4653. doi:10.1074/jbc.M512215200
- Kroeger, H., Miranda, E., MacLeod, I., Pérez, J., Crowther, D.C., Marciniak, S.J., Lomas, D.A., 2009. Endoplasmic reticulum-associated degradation (ERAD) and autophagy

- cooperate to degrade polymerogenic mutant serpins. *J. Biol. Chem.* 284, 22793–22802. doi:10.1074/jbc.M109.027102
- Kruse, K.B., Dear, A., Kaltenbrun, E.R., Crum, B.E., George, P.M., Brennan, S.O., McCracken, A.A., 2006. Mutant fibrinogen cleared from the endoplasmic reticulum via endoplasmic reticulum-associated protein degradation and autophagy: an explanation for liver disease. *Am. J. Pathol.* 168, 1299–308– quiz 1404–5. doi:10.2353/ajpath.2006.051097
- Labbadia, J., Morimoto, R.I., 2015. The biology of proteostasis in aging and disease. *Annu. Rev. Biochem.* 84, 435–464. doi:10.1146/annurev-biochem-060614-033955
- Lee, A.-H., Iwakoshi, N.N., Glimcher, L.H., 2003. XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Mol. Cell. Biol.* 23, 7448–7459. doi:10.1128/MCB.23.21.7448-7459.2003
- Lee, J.-H., Jeong, J., Jeong, E.M., Cho, S.-Y., Kang, J.W., Lim, J., Heo, J., Kang, H., Kim, I.-G., Shin, D.-M., 2014. Endoplasmic reticulum stress activates transglutaminase 2 leading to protein aggregation. *Int. J. Mol. Med.* 33, 849–855. doi:10.3892/ijmm.2014.1640
- Lee, J.N., Song, B., DeBose-Boyd, R.A., Ye, J., 2006. Sterol-regulated degradation of Insig-1 mediated by the membrane-bound ubiquitin ligase gp78. *J. Biol. Chem.* 281, 39308–39315. doi:10.1074/jbc.M608999200
- Lee, J.P., Brauweiler, A., Rudolph, M., Hooper, J.E., Drabkin, H.A., Gemmill, R.M., 2010. The TRC8 ubiquitin ligase is sterol regulated and interacts with lipid and protein biosynthetic pathways. *Mol. Cancer Res.* 8, 93–106. doi:10.1158/1541-7786.MCR-08-0491
- Lee, K., Tirasophon, W., Shen, X., Michalak, M., Prywes, R., Okada, T., Yoshida, H., Mori, K., Kaufman, R.J., 2002. IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. *Genes Dev.* 16, 452–466. doi:10.1101/gad.964702
- Lee, K.A., Hammerle, L.P., Andrews, P.S., Stokes, M.P., Mustelin, T., Silva, J.C., Black, R.A., Doedens, J.R., 2011. Ubiquitin ligase substrate identification through quantitative proteomics at both the protein and peptide levels. *J. Biol. Chem.* 286, 41530–41538. doi:10.1074/jbc.M111.248856
- Leitman, J., Ron, E., Ogen-Shtern, N., Lederkremer, G.Z., 2013a. Compartmentalization of endoplasmic reticulum quality control and ER-associated degradation factors. *DNA Cell Biol.* 32, 2–7. doi:10.1089/dna.2012.1889
- Leitman, J., Shenkman, M., Gofman, Y., Shtern, N.O., Ben-Tal, N., Hendershot, L.M., Lederkremer, G.Z., 2014. Herp coordinates compartmentalization and recruitment of HRD1 and misfolded proteins for ERAD. *Mol. Biol. Cell* 25, 1050–1060. doi:10.1091/mbc.E13-06-0350
- Leitman, J., Ulrich Hartl, F., Lederkremer, G.Z., 2013b. Soluble forms of polyQ-expanded huntingtin rather than large aggregates cause endoplasmic reticulum stress. *Nat Commun* 4, 2753. doi:10.1038/ncomms3753
- Lemberg, M.K., 2011. Intramembrane proteolysis in regulated protein trafficking. *Traffic* 12, 1109–1118. doi:10.1111/j.1600-0854.2011.01219.x
- Leng, N., Gu, G., Simerly, R.B., Spindel, E.R., 1999. Molecular cloning and characterization of two putative G protein-coupled receptors which are highly expressed in the central

- nervous system. *Brain Res. Mol. Brain Res.* 69, 73–83.
- Lerner, M., Corcoran, M., Cepeda, D., Nielsen, M.L., Zubarev, R., Pontén, F., Uhlén, M., Hober, S., Grandér, D., Sangfelt, O., 2007. The RBCC gene RFP2 (Leu5) encodes a novel transmembrane E3 ubiquitin ligase involved in ERAD. *Mol. Biol. Cell* 18, 1670–1682. doi:10.1091/mbc.E06-03-0248
- Leroy, E., Anastasopoulos, D., Konitsiotis, S., Lavedan, C., Polymeropoulos, M.H., 1998. Deletions in the Parkin gene and genetic heterogeneity in a Greek family with early onset Parkinson's disease. *Hum. Genet.* 103, 424–427.
- Leung-Hagesteijn, C., Erdmann, N., Cheung, G., Keats, J.J., Stewart, A.K., Reece, D.E., Chung, K.C., Tiedemann, R.E., 2013. Xbp1s-negative tumor B cells and pre-plasmablasts mediate therapeutic proteasome inhibitor resistance in multiple myeloma. *Cancer Cell* 24, 289–304. doi:10.1016/j.ccr.2013.08.009
- Li, G., Zhao, G., Zhou, X., Schindelin, H., Lennarz, W.J., 2006. The AAA ATPase p97 links peptide N-glycanase to the endoplasmic reticulum-associated E3 ligase autocrine motility factor receptor. *Proc. Natl. Acad. Sci. U.S.A.* 103, 8348–8353. doi:10.1073/pnas.0602747103
- Li, H., Korennykh, A.V., Behrman, S.L., Walter, P., 2010. Mammalian endoplasmic reticulum stress sensor IRE1 signals by dynamic clustering. *Proc. Natl. Acad. Sci. U.S.A.* 107, 16113–16118. doi:10.1073/pnas.1010580107
- Li, W., Bengtson, M.H., Ulbrich, A., Matsuda, A., Reddy, V.A., Orth, A., Chanda, S.K., Batalov, S., Joazeiro, C.A.P., 2008. Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling. *PLoS ONE* 3, e1487. doi:10.1371/journal.pone.0001487
- Lilley, B.N., Ploegh, H.L., 2005. Multiprotein complexes that link dislocation, ubiquitination, and extraction of misfolded proteins from the endoplasmic reticulum membrane. *Proc. Natl. Acad. Sci. U.S.A.* 102, 14296–14301. doi:10.1073/pnas.0505014102
- Lilley, B.N., Ploegh, H.L., 2004. A membrane protein required for dislocation of misfolded proteins from the ER. *Nature* 429, 834–840. doi:10.1038/nature02592
- Lin, J.H., Li, H., Yasumura, D., Cohen, H.R., Zhang, C., Panning, B., Shokat, K.M., Lavail, M.M., Walter, P., 2007. IRE1 signaling affects cell fate during the unfolded protein response. *Science* 318, 944–949. doi:10.1126/science.1146361
- Lin, P.-H., Lan, W.-M., Chau, L.-Y., 2013. TRC8 suppresses tumorigenesis through targeting heme oxygenase-1 for ubiquitination and degradation. *Oncogene* 32, 2325–2334. doi:10.1038/onc.2012.244
- Liu, J., Han, L., Li, B., Yang, J., Huen, M.S.Y., Pan, X., Tsao, S.W., Cheung, A.L.M., 2014. F-box only protein 31 (FBXO31) negatively regulates p38 mitogen-activated protein kinase (MAPK) signaling by mediating lysine 48-linked ubiquitination and degradation of mitogen-activated protein kinase kinase 6 (MKK6). *J. Biol. Chem.* 289, 21508–21518. doi:10.1074/jbc.M114.560342
- Liu, L., Sutton, J., Woodruff, E., Villalta, F., Spearman, P., Dong, X., 2012. Defective HIV-1 particle assembly in AP-3-deficient cells derived from patients with Hermansky-Pudlak syndrome type 2. *J. Virol.* 86, 11242–11253. doi:10.1128/JVI.00544-12
- Liu, Q., Chen, J., Mai, B., Amos, C., Killary, A.M., Sen, S., Wei, C., Frazier, M.L., 2012. A single-nucleotide polymorphism in tumor suppressor gene SEL1L as a predictive and prognostic marker for pancreatic ductal adenocarcinoma in Caucasians. *Mol. Carcinog.* 51, 433–438. doi:10.1002/mc.20808

- Liu, Y., Ye, Y., 2012. Roles of p97-associated deubiquitinases in protein quality control at the endoplasmic reticulum. *Curr. Protein Pept. Sci.* 13, 436–446.
- Lu, J.P., Wang, Y., Sliter, D.A., Pearce, M.M.P., Wojcikiewicz, R.J.H., 2011. RNF170 protein, an endoplasmic reticulum membrane ubiquitin ligase, mediates inositol 1,4,5-trisphosphate receptor ubiquitination and degradation. *J. Biol. Chem.* 286, 24426–24433. doi:10.1074/jbc.M111.251983
- Luo, B., Lee, A.S., 2013. The critical roles of endoplasmic reticulum chaperones and unfolded protein response in tumorigenesis and anticancer therapies. *Oncogene* 32, 805–818. doi:10.1038/onc.2012.130
- Ma, Yanjun, Hendershot, L.M., 2003. Delineation of a negative feedback regulatory loop that controls protein translation during endoplasmic reticulum stress. *J. Biol. Chem.* 278, 34864–34873. doi:10.1074/jbc.M301107200
- Ma, Yina, Li, J., 2015. Metabolic shifts during aging and pathology. *Compr Physiol* 5, 667–686. doi:10.1002/cphy.c140041
- Majumder, M., Huang, C., Snider, M.D., Komar, A.A., Tanaka, J., Kaufman, R.J., Krokowski, D., Hatzoglou, M., 2012. A novel feedback loop regulates the response to endoplasmic reticulum stress via the cooperation of cytoplasmic splicing and mRNA translation. *Mol. Cell. Biol.* 32, 992–1003. doi:10.1128/MCB.06665-11
- Mancini, R., Fagioli, C., Fra, A.M., Maggioni, C., Sitia, R., 2000. Degradation of unassembled soluble Ig subunits by cytosolic proteasomes: evidence that retrotranslocation and degradation are coupled events. *FASEB J.* 14, 769–778.
- Maruyama, Y., Yamada, M., Takahashi, K., Yamada, M., 2008. Ubiquitin ligase Kf-1 is involved in the endoplasmic reticulum-associated degradation pathway. *Biochem. Biophys. Res. Commun.* 374, 737–741. doi:10.1016/j.bbrc.2008.07.126
- Marzec, M., Eletto, D., Argon, Y., 2012. GRP94: An HSP90-like protein specialized for protein folding and quality control in the endoplasmic reticulum. *Biochim. Biophys. Acta* 1823, 774–787. doi:10.1016/j.bbamcr.2011.10.013
- Matus, S., Glimcher, L.H., Hetz, C., 2011. Protein folding stress in neurodegenerative diseases: a glimpse into the ER. *Curr. Opin. Cell Biol.* 23, 239–252. doi:10.1016/j.ceb.2011.01.003
- Meyer, H.H., Shorter, J.G., Seemann, J., Pappin, D., Warren, G., 2000. A complex of mammalian ufd1 and npl4 links the AAA-ATPase, p97, to ubiquitin and nuclear transport pathways. *EMBO J.* 19, 2181–2192. doi:10.1093/emboj/19.10.2181
- Milani, M., Rzymiski, T., Mellor, H.R., Pike, L., Bottini, A., Generali, D., Harris, A.L., 2009. The role of ATF4 stabilization and autophagy in resistance of breast cancer cells treated with Bortezomib. *Cancer Res.* 69, 4415–4423. doi:10.1158/0008-5472.CAN-08-2839
- Mimura, N., Fulciniti, M., Gorgun, G., Tai, Y.-T., Cirstea, D., Santo, L., Hu, Y., Fabre, C., Minami, J., Ohguchi, H., Kiziltepe, T., Ikeda, H., Kawano, Y., French, M., Blumenthal, M., Tam, V., Kertesz, N.L., Malyankar, U.M., Hokenson, M., Pham, T., Zeng, Q., Patterson, J.B., Richardson, P.G., Munshi, N.C., Anderson, K.C., 2012. Blockade of XBP1 splicing by inhibition of IRE1 $\alpha$  is a promising therapeutic option in multiple myeloma. *Blood* 119, 5772–5781. doi:10.1182/blood-2011-07-366633
- Mizushima, N., Yoshimori, T., Ohsumi, Y., 2011. The role of Atg proteins in autophagosome

- formation. *Annu. Rev. Cell Dev. Biol.* 27, 107–132. doi:10.1146/annurev-cellbio-092910-154005
- Mochida, K., Oikawa, Y., Kimura, Y., Kirisako, H., Hirano, H., Ohsumi, Y., Nakatogawa, H., 2015. Receptor-mediated selective autophagy degrades the endoplasmic reticulum and the nucleus. *Nature* 522, 359–362. doi:10.1038/nature14506
- Moenner, M., Pluquet, O., Bouchecareilh, M., Chevet, E., 2007. Integrated endoplasmic reticulum stress responses in cancer. *Cancer Res.* 67, 10631–10634. doi:10.1158/0008-5472.CAN-07-1705
- Molinari, M., Helenius, A., 2000. Chaperone selection during glycoprotein translocation into the endoplasmic reticulum. *Science* 288, 331–333.
- Moore, P., Bernardi, K.M., Tsai, B., 2010. The Ero1 $\alpha$ -PDI redox cycle regulates retro-translocation of cholera toxin. *Mol. Biol. Cell* 21, 1305–1313. doi:10.1091/mbc.E09-09-0826
- Moreau, P., Richardson, P.G., Cavo, M., Orlowski, R.Z., San Miguel, J.F., Palumbo, A., Harousseau, J.-L., 2012. Proteasome inhibitors in multiple myeloma: 10 years later. *Blood* 120, 947–959. doi:10.1182/blood-2012-04-403733
- Mori, K., Ma, W., Gething, M.J., Sambrook, J., 1993. A transmembrane protein with a cdc2+/CDC28-related kinase activity is required for signaling from the ER to the nucleus. *Cell* 74, 743–756.
- Morimoto, R.I., Cuervo, A.M., 2014. Proteostasis and the aging proteome in health and disease. *J. Gerontol. A Biol. Sci. Med. Sci.* 69 Suppl 1, S33–8. doi:10.1093/gerona/glu049
- Morito, D., Hirao, K., Oda, Y., Hosokawa, N., Tokunaga, F., Cyr, D.M., Tanaka, K., Iwai, K., Nagata, K., 2008. Gp78 cooperates with RMA1 in endoplasmic reticulum-associated degradation of CFTR $\Delta$ F508. *Mol. Biol. Cell* 19, 1328–1336. doi:10.1091/mbc.E07-06-0601
- Morley, J.F., Morimoto, R.I., 2004. Regulation of longevity in *Caenorhabditis elegans* by heat shock factor and molecular chaperones. *Mol. Biol. Cell* 15, 657–664. doi:10.1091/mbc.E03-07-0532
- Mueller, B., Klemm, E.J., Spooner, E., Claessen, J.H., Ploegh, H.L., 2008. SEL1L nucleates a protein complex required for dislocation of misfolded glycoproteins. *Proc. Natl. Acad. Sci. U.S.A.* 105, 12325–12330. doi:10.1073/pnas.0805371105
- Murray, J.I., Whitfield, M.L., Trinklein, N.D., Myers, R.M., Brown, P.O., Botstein, D., 2004. Diverse and specific gene expression responses to stresses in cultured human cells. *Mol. Biol. Cell* 15, 2361–2374. doi:10.1091/mbc.E03-11-0799
- Nadav, E., Shmueli, A., Barr, H., Gonen, H., Ciechanover, A., Reiss, Y., 2003. A novel mammalian endoplasmic reticulum ubiquitin ligase homologous to the yeast Hrd1. *Biochem. Biophys. Res. Commun.* 303, 91–97.
- Nakamura, N., Fukuda, H., Kato, A., Hirose, S., 2005. MARCH-II is a syntaxin-6-binding protein involved in endosomal trafficking. *Mol. Biol. Cell* 16, 1696–1710. doi:10.1091/mbc.E04-03-0216
- Nakatogawa, H., 2013. Two ubiquitin-like conjugation systems that mediate membrane formation during autophagy. *Essays Biochem.* 55, 39–50. doi:10.1042/bse0550039
- Neutzner, A., Neutzner, M., Benischke, A.-S., Ryu, S.-W., Frank, S., Youle, R.J., Karbowski, M., 2011. A systematic search for endoplasmic reticulum (ER) membrane-associated

- RING finger proteins identifies Nixin/ZNRF4 as a regulator of calnexin stability and ER homeostasis. *J. Biol. Chem.* 286, 8633–8643. doi:10.1074/jbc.M110.197459
- Ng, W., Sergeyenko, T., Zeng, N., Brown, J.D., Römisch, K., 2007. Characterization of the proteasome interaction with the Sec61 channel in the endoplasmic reticulum. *J. Cell. Sci.* 120, 682–691. doi:10.1242/jcs.03351
- Niewerth, D., Jansen, G., Assaraf, Y.G., Zweegman, S., Kaspers, G.J.L., Cloos, J., 2015. Molecular basis of resistance to proteasome inhibitors in hematological malignancies. *Drug Resist. Updat.* 18, 18–35. doi:10.1016/j.drug.2014.12.001
- Ninagawa, S., Okada, T., Sumitomo, Y., Kamiya, Y., Kato, K., Horimoto, S., Ishikawa, T., Takeda, S., Sakuma, T., Yamamoto, T., Mori, K., 2014. EDEM2 initiates mammalian glycoprotein ERAD by catalyzing the first mannose trimming step. *J. Cell Biol.* 206, 347–356. doi:10.1083/jcb.201404075
- O'Brien, R.J., Wong, P.C., 2011. Amyloid precursor protein processing and Alzheimer's disease. *Annu. Rev. Neurosci.* 34, 185–204. doi:10.1146/annurev-neuro-061010-113613
- O'Rahilly, S., 2009. Human genetics illuminates the paths to metabolic disease. *Nature* 462, 307–314. doi:10.1038/nature08532
- Oda, Y., Okada, T., Yoshida, H., Kaufman, R.J., Nagata, K., Mori, K., 2006. Derlin-2 and Derlin-3 are regulated by the mammalian unfolded protein response and are required for ER-associated degradation. *J. Cell Biol.* 172, 383–393. doi:10.1083/jcb.200507057
- Ogata, M., Hino, S.-I., Saito, A., Morikawa, K., Kondo, S., Kanemoto, S., Murakami, T., Taniguchi, M., Tani, I., Yoshinaga, K., Shiosaka, S., Hammarback, J.A., Urano, F., Imaizumi, K., 2006. Autophagy is activated for cell survival after endoplasmic reticulum stress. *Mol. Cell. Biol.* 26, 9220–9231. doi:10.1128/MCB.01453-06
- Okuda-Shimizu, Y., Hendershot, L.M., 2007. Characterization of an ERAD pathway for nonglycosylated BiP substrates, which require Herp. *Mol. Cell* 28, 544–554. doi:10.1016/j.molcel.2007.09.012
- Olzmann, J.A., Kopito, R.R., Christianson, J.C., 2013. The mammalian endoplasmic reticulum-associated degradation system. *Cold Spring Harb Perspect Biol* 5, a013185–a013185. doi:10.1101/cshperspect.a013185
- Omura, T., Kaneko, M., Okuma, Y., Orba, Y., Nagashima, K., Takahashi, R., Fujitani, N., Matsumura, S., Hata, A., Kubota, K., Murahashi, K., Uehara, T., Nomura, Y., 2006. A ubiquitin ligase HRD1 promotes the degradation of Pael receptor, a substrate of Parkin. *J. Neurochem.* 99, 1456–1469. doi:10.1111/j.1471-4159.2006.04155.x
- Oresic, K., Mueller, B., Tortorella, D., 2009. Cln6 mutants associated with neuronal ceroid lipofuscinosis are degraded in a proteasome-dependent manner. *Biosci. Rep.* 29, 173–181. doi:10.1042/BSR20080143
- Orlandi, R., Cattaneo, M., Troglio, F., Casalini, P., Ronchini, C., Ménard, S., Biunno, I., 2002. SEL1L expression decreases breast tumor cell aggressiveness in vivo and in vitro. *Cancer Res.* 62, 567–574.
- Orlowski, R.Z., 2013. Why proteasome inhibitors cannot ERADicate multiple myeloma. *Cancer Cell* 24, 275–277. doi:10.1016/j.ccr.2013.08.014
- Ozcan, L., Tabas, I., 2012. Role of endoplasmic reticulum stress in metabolic disease and other disorders. *Annu. Rev. Med.* 63, 317–328. doi:10.1146/annurev-med-043010-144749

- Ozcan, U., Cao, Q., Yilmaz, E., Lee, A.-H., Iwakoshi, N.N., Ozdelen, E., Tuncman, G., Görgün, C., Glimcher, L.H., Hotamisligil, G.S., 2004. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* 306, 457–461. doi:10.1126/science.1103160
- Pagani, M., Fabbri, M., Benedetti, C., Fassio, A., Pilati, S., Bulleid, N.J., Cabibbo, A., Sitia, R., 2000. Endoplasmic reticulum oxidoreductin 1- $\beta$  (ERO1- $\beta$ ), a human gene induced in the course of the unfolded protein response. *J. Biol. Chem.* 275, 23685–23692. doi:10.1074/jbc.M003061200
- Pan, T., Kondo, S., Zhu, W., Xie, W., Jankovic, J., Le, W., 2008. Neuroprotection of rapamycin in lactacystin-induced neurodegeneration via autophagy enhancement. *Neurobiol. Dis.* 32, 16–25. doi:10.1016/j.nbd.2008.06.003
- Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.-A., Outzen, H., Øvervatn, A., Bjørkøy, G., Johansen, T., 2007. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J. Biol. Chem.* 282, 24131–24145. doi:10.1074/jbc.M702824200
- Papandreou, I., Denko, N.C., Olson, M., Van Melckebeke, H., Lust, S., Tam, A., Solow-Cordero, D.E., Bouley, D.M., Offner, F., Niwa, M., Koong, A.C., 2011. Identification of an Ire1 $\alpha$  endonuclease specific inhibitor with cytotoxic activity against human multiple myeloma. *Blood* 117, 1311–1314. doi:10.1182/blood-2010-08-303099
- Paton, A.W., Beddoe, T., Thorpe, C.M., Whisstock, J.C., Wilce, M.C.J., Rossjohn, J., Talbot, U.M., Paton, J.C., 2006. AB5 subtilase cytotoxin inactivates the endoplasmic reticulum chaperone BiP. *Nature* 443, 548–552. doi:10.1038/nature05124
- Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X.H., Mizushima, N., Packer, M., Schneider, M.D., Levine, B., 2005. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 122, 927–939. doi:10.1016/j.cell.2005.07.002
- Pearce, M.M.P., Wang, Y., Kelley, G.G., Wojcikiewicz, R.J.H., 2007. SPFH2 mediates the endoplasmic reticulum-associated degradation of inositol 1,4,5-trisphosphate receptors and other substrates in mammalian cells. *J. Biol. Chem.* 282, 20104–20115. doi:10.1074/jbc.M701862200
- Pearce, M.M.P., Wormer, D.B., Wilkens, S., Wojcikiewicz, R.J.H., 2009. An endoplasmic reticulum (ER) membrane complex composed of SPFH1 and SPFH2 mediates the ER-associated degradation of inositol 1,4,5-trisphosphate receptors. *J. Biol. Chem.* 284, 10433–10445. doi:10.1074/jbc.M809801200
- Pelham, H.R., Munro, S., 1993. Sorting of membrane proteins in the secretory pathway. *Cell* 75, 603–605.
- Pengo, N., Scolari, M., Oliva, L., Milan, E., Mainoldi, F., Raimondi, A., Fagioli, C., Merlini, A., Mariani, E., Pasqualetto, E., Orfanelli, U., Ponzoni, M., Sitia, R., Casola, S., Cenci, S., 2013. Plasma cells require autophagy for sustainable immunoglobulin production. *Nat. Immunol.* 14, 298–305. doi:10.1038/ni.2524
- Phillips, M.J., Voeltz, G.K., 2016. Structure and function of ER membrane contact sites with other organelles. *Nat. Rev. Mol. Cell Biol.* 17, 69–82. doi:10.1038/nrm.2015.8
- Pincus, D., Chevalier, M.W., Aragón, T., van Anken, E., Vidal, S.E., El-Samad, H., Walter, P., 2010. BiP binding to the ER-stress sensor Ire1 tunes the homeostatic behavior of the unfolded protein response. *PLoS Biol.* 8, e1000415. doi:10.1371/journal.pbio.1000415



- Plempner, R.K., Bordallo, J., Deak, P.M., Taxis, C., Hitt, R., Wolf, D.H., 1999. Genetic interactions of Hrd3p and Der3p/Hrd1p with Sec61p suggest a retro-translocation complex mediating protein transport for ER degradation. *J. Cell. Sci.* 112 ( Pt 22), 4123–4134.
- Pollard, M.G., Travers, K.J., Weissman, J.S., 1998. Ero1p: a novel and ubiquitous protein with an essential role in oxidative protein folding in the endoplasmic reticulum. *Mol. Cell* 1, 171–182.
- Qin, Y., Zhou, M.-T., Hu, M.-M., Hu, Y.-H., Zhang, J., Guo, L., Zhong, B., Shu, H.-B., 2014. RNF26 temporally regulates virus-triggered type I interferon induction by two distinct mechanisms. *PLoS Pathog.* 10, e1004358. doi:10.1371/journal.ppat.1004358
- Quiroga, C., Gatica, D., Paredes, F., Bravo, R., Troncoso, R., Pedrozo, Z., Rodriguez, A.E., Toro, B., Chiong, M., Vicencio, J.M., Hetz, C., Lavandero, S., 2013. Herp depletion protects from protein aggregation by up-regulating autophagy. *Biochim. Biophys. Acta* 1833, 3295–3305. doi:10.1016/j.bbamcr.2013.09.006
- Rane, S., Rana, S., Mudrabettu, C., Jha, V., Joshi, K., 2012. Heavy-chain deposition disease: a morphological, immunofluorescence and ultrastructural assessment. *Clin Kidney J* 5, 383–389. doi:10.1093/ckj/sfs062
- Reddy, P., Sparvoli, A., Fagioli, C., Fassina, G., Sitia, R., 1996. Formation of reversible disulfide bonds with the protein matrix of the endoplasmic reticulum correlates with the retention of unassembled Ig light chains. *EMBO J.* 15, 2077–2085.
- Reimold, A.M., Iwakoshi, N.N., Manis, J., Vallabhajosyula, P., Szomolanyi-Tsuda, E., Gravalles, E.M., Friend, D., Grusby, M.J., Alt, F., Glimcher, L.H., 2001. Plasma cell differentiation requires the transcription factor XBP-1. *Nature* 412, 300–307. doi:10.1038/35085509
- Riemer, J., Appenzeller-Herzog, C., Johansson, L., Bodenmiller, B., Hartmann-Petersen, R., Ellgaard, L., 2009. A luminal flavoprotein in endoplasmic reticulum-associated degradation. *Proc. Natl. Acad. Sci. U.S.A.* 106, 14831–14836. doi:10.1073/pnas.0900742106
- Riemer, J., Hansen, H.G., Appenzeller-Herzog, C., Johansson, L., Ellgaard, L., 2011. Identification of the PDI-family member ERp90 as an interaction partner of ERFAD. *PLoS ONE* 6, e17037. doi:10.1371/journal.pone.0017037
- Robberecht, W., Philips, T., 2013. The changing scene of amyotrophic lateral sclerosis. *Nat. Rev. Neurosci.* 14, 248–264. doi:10.1038/nrn3430
- Rogov, V., Dötsch, V., Johansen, T., Kirkin, V., 2014. Interactions between autophagy receptors and ubiquitin-like proteins form the molecular basis for selective autophagy. *Mol. Cell* 53, 167–178. doi:10.1016/j.molcel.2013.12.014
- Ron, D., Habener, J.F., 1992. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes Dev.* 6, 439–453.
- Ron, D., Walter, P., 2007. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat. Rev. Mol. Cell Biol.* 8, 519–529. doi:10.1038/nrm2199
- Rong, J., Chen, L., Toth, J.I., Tcherpakov, M., Petroski, M.D., Reed, J.C., 2011. Bifunctional apoptosis regulator (BAR), an endoplasmic reticulum (ER)-associated E3 ubiquitin ligase, modulates BI-1 protein stability and function in ER Stress. *J. Biol. Chem.* 286, 1453–1463. doi:10.1074/jbc.M110.175232

- Rouschop, K.M.A., van den Beucken, T., Dubois, L., Niessen, H., Bussink, J., Savelkoul, K., Keulers, T., Mujcic, H., Landuyt, W., Voncken, J.W., Lambin, P., van der Kogel, A.J., Koritzinsky, M., Wouters, B.G., 2010. The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5. *J. Clin. Invest.* 120, 127–141. doi:10.1172/JCI40027
- Roussel, B.D., Newton, T.M., Malzer, E., Simecek, N., Haq, I., Thomas, S.E., Burr, M.L., Lehner, P.J., Crowther, D.C., Marciniak, S.J., Lomas, D.A., 2013. Sterol metabolism regulates neuroserpin polymer degradation in the absence of the unfolded protein response in the dementia FENIB. *Hum. Mol. Genet.* 22, 4616–4626. doi:10.1093/hmg/ddt310
- Rowland, A.A., Voeltz, G.K., 2012. Endoplasmic reticulum-mitochondria contacts: function of the junction. *Nat. Rev. Mol. Cell Biol.* 13, 607–625. doi:10.1038/nrm3440
- Rubinsztein, D.C., Mariño, G., Kroemer, G., 2011. Autophagy and aging. *Cell* 146, 682–695. doi:10.1016/j.cell.2011.07.030
- Russell, R.C., Tian, Y., Yuan, H., Park, H.W., Chang, Y.-Y., Kim, J., Kim, H., Neufeld, T.P., Dillin, A., Guan, K.-L., 2013. ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. *Nat. Cell Biol.* 15, 741–750. doi:10.1038/ncb2757
- Rzymiski, T., Milani, M., Singleton, D.C., Harris, A.L., 2009. Role of ATF4 in regulation of autophagy and resistance to drugs and hypoxia. *Cell Cycle* 8, 3838–3847. doi:10.4161/cc.8.23.10086
- Saltini, G., Dominici, R., Lovati, C., Cattaneo, M., Michelini, S., Malferrari, G., Caprera, A., Milanesi, L., Finazzi, D., Bertora, P., Scarpini, E., Galimberti, D., Venturelli, E., Musicco, M., Adorni, F., Mariani, C., Biunno, I., 2006. A novel polymorphism in SEL1L confers susceptibility to Alzheimer's disease. *Neurosci. Lett.* 398, 53–58. doi:10.1016/j.neulet.2005.12.038
- Sato, B.K., Schulz, D., Do, P.H., Hampton, R.Y., 2009. Misfolded membrane proteins are specifically recognized by the transmembrane domain of the Hrd1p ubiquitin ligase. *Mol. Cell* 34, 212–222. doi:10.1016/j.molcel.2009.03.010
- Sato, T., Sako, Y., Sho, M., Momohara, M., Suico, M.A., Shuto, T., Nishitoh, H., Okiyoneda, T., Kokame, K., Kaneko, M., Taura, M., Miyata, M., Chosa, K., Koga, T., Morino-Koga, S., Wada, I., Kai, H., 2012. STT3B-dependent posttranslational N-glycosylation as a surveillance system for secretory protein. *Mol. Cell* 47, 99–110. doi:10.1016/j.molcel.2012.04.015
- Schaller, M.D., 2001. Paxillin: a focal adhesion-associated adaptor protein. *Oncogene* 20, 6459–6472. doi:10.1038/sj.onc.1204786
- Schäfer, A., Wolf, D.H., 2009. Sec61p is part of the endoplasmic reticulum-associated degradation machinery. *EMBO J.* 28, 2874–2884. doi:10.1038/emboj.2009.231
- Schett, G., Tohidast-Akrad, M., Steiner, G., Smolen, J., 2001. The stressed synovium. *Arthritis Res.* 3, 80–86. doi:10.1186/ar144
- Schneider, J.L., Villarroja, J., Diaz-Carretero, A., Patel, B., Urbanska, A.M., Thi, M.M., Villarroja, F., Santambrogio, L., Cuervo, A.M., 2015. Loss of hepatic chaperone-mediated autophagy accelerates proteostasis failure in aging. *Aging Cell* 14, 249–264. doi:10.1111/acer.12310
- Schröder, M., Kaufman, R.J., 2005. The mammalian unfolded protein response. *Annu. Rev.*

- Schulze, A., Standera, S., Buerger, E., Kikkert, M., van Voorden, S., Wiertz, E., Koning, F., Kloetzel, P.-M., Seeger, M., 2005. The ubiquitin-domain protein HERP forms a complex with components of the endoplasmic reticulum associated degradation pathway. *J. Mol. Biol.* 354, 1021–1027. doi:10.1016/j.jmb.2005.10.020
- Sha, H., Sun, S., Francisco, A.B., Ehrhardt, N., Xue, Z., Liu, L., Lawrence, P., Mattijssen, F., Guber, R.D., Panhwar, M.S., Brenna, J.T., Shi, H., Xue, B., Kersten, S., Bensadoun, A., Péterfy, M., Long, Q., Qi, L., 2014. The ER-associated degradation adaptor protein Sel1L regulates LPL secretion and lipid metabolism. *Cell Metab.* 20, 458–470. doi:10.1016/j.cmet.2014.06.015
- Shen, Y., Ballar, P., Apostolou, A., Doong, H., Fang, S., 2007. ER stress differentially regulates the stabilities of ERAD ubiquitin ligases and their substrates. *Biochem. Biophys. Res. Commun.* 352, 919–924. doi:10.1016/j.bbrc.2006.11.121
- Shen, Y., Ballar, P., Fang, S., 2006. Ubiquitin ligase gp78 increases solubility and facilitates degradation of the Z variant of alpha-1-antitrypsin. *Biochem. Biophys. Res. Commun.* 349, 1285–1293. doi:10.1016/j.bbrc.2006.08.173
- Sitia, R., Braakman, I., 2003. Quality control in the endoplasmic reticulum protein factory. *Nature* 426, 891–894. doi:10.1038/nature02262
- Sitia, R., Neuberger, M., Alberini, C., Bet, P., Fra, A., Valetti, C., Williams, G., Milstein, C., 1990. Developmental regulation of IgM secretion: the role of the carboxy-terminal cysteine. *Cell* 60, 781–790.
- Sokołowska, I., Piłka, E.S., Sandvig, K., Węgrzyn, G., Słomińska-Wojewódzka, M., 2015. Hydrophobicity of protein determinants influences the recognition of substrates by EDEM1 and EDEM2 in human cells. *BMC Cell Biol.* 16, 1. doi:10.1186/s12860-015-0047-7
- Song, B.-L., Sever, N., DeBose-Boyd, R.A., 2005. Gp78, a membrane-anchored ubiquitin ligase, associates with Insig-1 and couples sterol-regulated ubiquitination to degradation of HMG CoA reductase. *Mol. Cell* 19, 829–840. doi:10.1016/j.molcel.2005.08.009
- Soo, K.Y., Halloran, M., Sundaramoorthy, V., Parakh, S., Toth, R.P., Southam, K.A., McLean, C.A., Lock, P., King, A., Farg, M.A., Atkin, J.D., 2015. Rab1-dependent ER-Golgi transport dysfunction is a common pathogenic mechanism in SOD1, TDP-43 and FUS-associated ALS. *Acta Neuropathol.* 130, 679–697. doi:10.1007/s00401-015-1468-2
- Sowa, M.E., Bennett, E.J., Gygi, S.P., Harper, J.W., 2009. Defining the human deubiquitinating enzyme interaction landscape. *Cell* 138, 389–403. doi:10.1016/j.cell.2009.04.042
- Spooner, R.A., Watson, P.D., Marsden, C.J., Smith, D.C., Moore, K.A.H., Cook, J.P., Lord, J.M., Roberts, L.M., 2004. Protein disulphide-isomerase reduces ricin to its A and B chains in the endoplasmic reticulum. *Biochem. J.* 383, 285–293. doi:10.1042/BJ20040742
- Stagg, H.R., Thomas, M., van den Boomen, D., Wiertz, E.J.H.J., Drabkin, H.A., Gemmill, R.M., Lehner, P.J., 2009. The TRC8 E3 ligase ubiquitinates MHC class I molecules before dislocation from the ER. *J. Cell Biol.* 186, 685–692. doi:10.1083/jcb.200906110
- Stein, A., Ruggiano, A., Carvalho, P., Rapoport, T.A., 2014. Key steps in ERAD of luminal ER proteins reconstituted with purified components. *Cell* 158, 1375–1388.

- Stolz, A., Ernst, A., Dikic, I., 2014. Cargo recognition and trafficking in selective autophagy. *Nat. Cell Biol.* 16, 495–501. doi:10.1038/ncb2979
- Sun, S., Shi, G., Han, X., Francisco, A.B., Ji, Y., Mendonça, N., Liu, X., Locasale, J.W., Simpson, K.W., Duhamel, G.E., Kersten, S., Yates, J.R., Long, Q., Qi, L., 2014. Sel1L is indispensable for mammalian endoplasmic reticulum-associated degradation, endoplasmic reticulum homeostasis, and survival. *Proc. Natl. Acad. Sci. U.S.A.* 111, E582–91. doi:10.1073/pnas.1318114111
- Sun, S., Shi, G., Sha, H., Ji, Y., Han, X., Shu, X., Ma, H., Inoue, T., Gao, B., Kim, H., Bu, P., Guber, R.D., Shen, X., Lee, A.-H., Iwawaki, T., Paton, A.W., Paton, J.C., Fang, D., Tsai, B., Yates, J.R., Wu, H., Kersten, S., Long, Q., Duhamel, G.E., Simpson, K.W., Qi, L., 2015. IRE1 $\alpha$  is an endogenous substrate of endoplasmic-reticulum-associated degradation. *Nat. Cell Biol.* 17, 1546–1555. doi:10.1038/ncb3266
- Susuki, S., Sato, T., Miyata, M., Momohara, M., Suico, M.A., Shuto, T., Ando, Y., Kai, H., 2009. The Endoplasmic Reticulum-associated Degradation of Transthyretin Variants Is Negatively Regulated by BiP in Mammalian Cells. *J. Biol. Chem.* 284, 8312–8321. doi:10.1074/jbc.M809354200
- Suzuki, M., Otsuka, T., Ohsaki, Y., Cheng, J., Taniguchi, T., Hashimoto, H., Taniguchi, H., Fujimoto, T., 2012. Derlin-1 and UBXD8 are engaged in dislocation and degradation of lipidated ApoB-100 at lipid droplets. *Mol. Biol. Cell* 23, 800–810. doi:10.1091/mbc.E11-11-0950
- Tabas, I., 2010. The role of endoplasmic reticulum stress in the progression of atherosclerosis. *Circ. Res.* 107, 839–850. doi:10.1161/CIRCRESAHA.110.224766
- Tabas, I., Ron, D., 2011. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat. Cell Biol.* 13, 184–190. doi:10.1038/ncb0311-184
- Takayanagi, S., Fukuda, R., Takeuchi, Y., Tsukada, S., Yoshida, K., 2013. Gene regulatory network of unfolded protein response genes in endoplasmic reticulum stress. *Cell Stress Chaperones* 18, 11–23. doi:10.1007/s12192-012-0351-5
- Tang, F., Wang, B., Li, N., Wu, Y., Jia, J., Suo, T., Chen, Q., Liu, Y.-J., Tang, J., 2011. RNF185, a novel mitochondrial ubiquitin E3 ligase, regulates autophagy through interaction with BNIP1. *PLoS ONE* 6, e24367. doi:10.1371/journal.pone.0024367
- Tang, H.-Y., Huang, C.-H., Zhuang, Y.-H., Christianson, J.C., Chen, X., 2014. EDEM2 and OS-9 are required for ER-associated degradation of non-glycosylated sonic hedgehog. *PLoS ONE* 9, e92164. doi:10.1371/journal.pone.0092164
- Tanida, I., Ueno, T., Kominami, E., 2008. LC3 and Autophagy. *Methods Mol. Biol.* 445, 77–88. doi:10.1007/978-1-59745-157-4\_4
- Tcherpakov, M., Delaunay, A., Toth, J., Kadoya, T., Petroski, M.D., Ronai, Z.A., 2009. Regulation of endoplasmic reticulum-associated degradation by RNF5-dependent ubiquitination of JNK-associated membrane protein (JAMP). *J. Biol. Chem.* 284, 12099–12109. doi:10.1074/jbc.M808222200
- Thrower, J.S., Hoffman, L., Rechsteiner, M., Pickart, C.M., 2000. Recognition of the polyubiquitin proteolytic signal. *EMBO J.* 19, 94–102. doi:10.1093/emboj/19.1.94
- Tirasophon, W., Welihinda, A.A., Kaufman, R.J., 1998. A stress response pathway from the

endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase/endoribonuclease (Ire1p) in mammalian cells. *Genes Dev.* 12, 1812–1824.

- Tirosh, B., Furman, M.H., Tortorella, D., Ploegh, H.L., 2003. Protein unfolding is not a prerequisite for endoplasmic reticulum-to-cytosol dislocation. *J. Biol. Chem.* 278, 6664–6672. doi:10.1074/jbc.M210158200
- Travers, K.J., Patil, C.K., Wodicka, L., Lockhart, D.J., Weissman, J.S., Walter, P., 2000. Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* 101, 249–258.
- Trudgian, D.C., Ridlova, G., Fischer, R., Mackeen, M.M., Ternette, N., Acuto, O., Kessler, B.M., Thomas, B., 2011. Comparative evaluation of label-free SING normalized spectral index quantitation in the central proteomics facilities pipeline. *Proteomics* 11, 2790–2797. doi:10.1002/pmic.201000800
- Tsai, B., Rapoport, T.A., 2002. Unfolded cholera toxin is transferred to the ER membrane and released from protein disulfide isomerase upon oxidation by Ero1. *J. Cell Biol.* 159, 207–216. doi:10.1083/jcb.200207120
- Tsai, B., Rodighiero, C., Lencer, W.I., Rapoport, T.A., 2001. Protein disulfide isomerase acts as a redox-dependent chaperone to unfold cholera toxin. *Cell* 104, 937–948.
- Tsai, Y.C., Mendoza, A., Mariano, J.M., Zhou, M., Kostova, Z., Chen, B., Veenstra, T., Hewitt, S.M., Helman, L.J., Khanna, C., Weissman, A.M., 2007. The ubiquitin ligase gp78 promotes sarcoma metastasis by targeting KAI1 for degradation. *Nat. Med.* 13, 1504–1509. doi:10.1038/nm1686
- Tsuchiya, Y., Morita, T., Kim, M., Iemura, S.-I., Natsume, T., Yamamoto, M., Kobayashi, A., 2011. Dual regulation of the transcriptional activity of Nrf1 by  $\beta$ -TrCP- and Hrd1-dependent degradation mechanisms. *Mol. Cell. Biol.* 31, 4500–4512. doi:10.1128/MCB.05663-11
- Tyler, R.E., Pearce, M.M.P., Shaler, T.A., Olzmann, J.A., Greenblatt, E.J., Kopito, R.R., 2012. Unassembled CD147 is an endogenous endoplasmic reticulum-associated degradation substrate. *Mol. Biol. Cell* 23, 4668–4678. doi:10.1091/mbc.E12-06-0428
- Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H.P., Ron, D., 2000. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 287, 664–666.
- Urban, S., Dickey, S.W., 2011. The rhomboid protease family: a decade of progress on function and mechanism. *Genome Biol.* 12, 231. doi:10.1186/gb-2011-12-10-231
- Ushioda, R., Hoseki, J., Araki, K., Jansen, G., Thomas, D.Y., Nagata, K., 2008. ERdj5 is required as a disulfide reductase for degradation of misfolded proteins in the ER. *Science* 321, 569–572. doi:10.1126/science.1159293
- van Anken, E., Braakman, I., 2005. Versatility of the endoplasmic reticulum protein folding factory. *Crit. Rev. Biochem. Mol. Biol.* 40, 191–228. doi:10.1080/10409230591008161
- van Anken, E., Romijn, E.P., Maggioni, C., Mezghrani, A., Sitia, R., Braakman, I., Heck, A.J.R., 2003. Sequential waves of functionally related proteins are expressed when B cells prepare for antibody secretion. *Immunity* 18, 243–253.
- van den Boomen, D.J.H., Lehner, P.J., 2015. Identifying the ERAD ubiquitin E3 ligases for viral and cellular targeting of MHC class I. *Mol. Immunol.* 68, 106–111. doi:10.1016/j.molimm.2015.07.005

- van den Boomen, D.J.H., Timms, R.T., Grice, G.L., Stagg, H.R., Skødt, K., Dougan, G., Nathan, J.A., Lehner, P.J., 2014. TMEM129 is a Derlin-1 associated ERAD E3 ligase essential for virus-induced degradation of MHC-I. *Proc. Natl. Acad. Sci. U.S.A.* 111, 11425–11430. doi:10.1073/pnas.1409099111
- Vilchez, D., Saez, I., Dillin, A., 2014. The role of protein clearance mechanisms in organismal ageing and age-related diseases. *Nat Commun* 5, 5659. doi:10.1038/ncomms6659
- Voisine, C., Pedersen, J.S., Morimoto, R.I., 2010. Chaperone networks: tipping the balance in protein folding diseases. *Neurobiol. Dis.* 40, 12–20. doi:10.1016/j.nbd.2010.05.007
- Volchuk, A., Ron, D., 2010. The endoplasmic reticulum stress response in the pancreatic  $\beta$ -cell. *Diabetes Obes Metab* 12 Suppl 2, 48–57. doi:10.1111/j.1463-1326.2010.01271.x
- Wahlman, J., DeMartino, G.N., Skach, W.R., Bulleid, N.J., Brodsky, J.L., Johnson, A.E., 2007. Real-time fluorescence detection of ERAD substrate retrotranslocation in a mammalian in vitro system. *Cell* 129, 943–955. doi:10.1016/j.cell.2007.03.046
- Walter, P., Ron, D., 2011. The unfolded protein response: from stress pathway to homeostatic regulation. *Science* 334, 1081–1086. doi:10.1126/science.1209038
- Walther, D.M., Kasturi, P., Zheng, M., Pinkert, S., Vecchi, G., Ciryam, P., Morimoto, R.I., Dobson, C.M., Vendruscolo, M., Mann, M., Hartl, F.U., 2015. Widespread Proteome Remodeling and Aggregation in Aging *C. elegans*. *Cell* 161, 919–932. doi:10.1016/j.cell.2015.03.032
- Wang, H., Li, Q., Shen, Y., Sun, A., Zhu, X., Fang, S., Shen, Y., 2011. The ubiquitin ligase Hrd1 promotes degradation of the Z variant alpha 1-antitrypsin and increases its solubility. *Mol. Cell. Biochem.* 346, 137–145. doi:10.1007/s11010-010-0600-9
- Wang, M., Kaufman, R.J., 2016. Protein misfolding in the endoplasmic reticulum as a conduit to human disease. *Nature* 529, 326–335. doi:10.1038/nature17041
- Wang, M., Kaufman, R.J., 2014. The impact of the endoplasmic reticulum protein-folding environment on cancer development. *Nat. Rev. Cancer* 14, 581–597. doi:10.1038/nrc3800
- Wang, Q., Li, L., Ye, Y., 2006. Regulation of retrotranslocation by p97-associated deubiquitinating enzyme ataxin-3. *J. Cell Biol.* 174, 963–971. doi:10.1083/jcb.200605100
- Wang, Q., Song, C., Li, C.-C.H., 2004. Molecular perspectives on p97-VCP: progress in understanding its structure and diverse biological functions. *J. Struct. Biol.* 146, 44–57. doi:10.1016/j.jsb.2003.11.014
- Wang, X., Herr, R.A., Chua, W.-J., Lybarger, L., Wiertz, E.J.H.J., Hansen, T.H., 2007. Ubiquitination of serine, threonine, or lysine residues on the cytoplasmic tail can induce ERAD of MHC-I by viral E3 ligase mK3. *J. Cell Biol.* 177, 613–624. doi:10.1083/jcb.200611063
- Wei, Y., Sinha, S., Levine, B., 2008. Dual role of JNK1-mediated phosphorylation of Bcl-2 in autophagy and apoptosis regulation. *Autophagy* 4, 949–951.
- Weimer, J.M., Kriscenski-Perry, E., Elshatory, Y., Pearce, D.A., 2002. The neuronal ceroid lipofuscinoses: mutations in different proteins result in similar disease. *Neuromolecular Med.* 1, 111–124. doi:10.1385/NMM:1:2:111
- Wenham, M., Grieve, S., Cummins, M., Jones, M.L., Booth, S., Kilner, R., Ancliff, P.J.,

- Griffiths, G.M., Mumford, A.D., 2010. Two patients with Hermansky Pudlak syndrome type 2 and novel mutations in AP3B1. *Haematologica* 95, 333–337. doi:10.3324/haematol.2009.012286
- Whitesell, L., Lindquist, S.L., 2005. HSP90 and the chaperoning of cancer. *Nat. Rev. Cancer* 5, 761–772. doi:10.1038/nrc1716
- Wiertz, E.J., Tortorella, D., Bogoy, M., Yu, J., Mothes, W., Jones, T.R., Rapoport, T.A., Ploegh, H.L., 1996. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature* 384, 432–438. doi:10.1038/384432a0
- Williams, J.M., Inoue, T., Banks, L., Tsai, B., 2013. The ERdj5-Sel1L complex facilitates cholera toxin retrotranslocation. *Mol. Biol. Cell* 24, 785–795. doi:10.1091/mbc.E12-07-0522
- Winklhofer, K.F., 2014. Parkin and mitochondrial quality control: toward assembling the puzzle. *Trends Cell Biol.* 24, 332–341. doi:10.1016/j.tcb.2014.01.001
- Woehlbier, U., Hetz, C., 2011. Modulating stress responses by the UPProsome: a matter of life and death. *Trends Biochem. Sci.* 36, 329–337. doi:10.1016/j.tibs.2011.03.001
- Wright, F.A., Lu, J.P., Sliter, D.A., Dupré, N., Rouleau, G.A., Wojcikiewicz, R.J.H., 2015. A Point Mutation in the Ubiquitin Ligase RNF170 That Causes Autosomal Dominant Sensory Ataxia Destabilizes the Protein and Impairs Inositol 1,4,5-Trisphosphate Receptor-mediated Ca<sup>2+</sup> Signaling. *J. Biol. Chem.* 290, 13948–13957. doi:10.1074/jbc.M115.655043
- Xu, M., Skaug, B., Zeng, W., Chen, Z.J., 2009. A ubiquitin replacement strategy in human cells reveals distinct mechanisms of IKK activation by TNFalpha and IL-1beta. *Mol. Cell* 36, 302–314. doi:10.1016/j.molcel.2009.10.002
- Xu, Y., Zhao, F., Qiu, Q., Chen, K., Wei, J., Kong, Q., Gao, B., Melo-Cardenas, J., Zhang, B., Zhang, J., Song, J., Zhang, D.D., Zhang, J., Fan, Y., Li, H., Fang, D., 2016. The ER membrane-anchored ubiquitin ligase Hrd1 is a positive regulator of T-cell immunity. *Nat Commun* 7, 12073. doi:10.1038/ncomms12073
- Xu, Y.-M., Wang, H.-J., Chen, F., Guo, W.-H., Wang, Y.-Y., Li, H.-Y., Tang, J.-H., Ding, Y., Shen, Y.-C., Li, M., Xuan, W.-Y., Liu, L.-H., Wang, J., Wang, X.-R., Gao, Z.-J., Liang, X.-B., Su, D.-M., 2015. HRD1 suppresses the growth and metastasis of breast cancer cells by promoting IGF-1R degradation. *Oncotarget* 6, 42854–42867. doi:10.18632/oncotarget.5733
- Yagishita, N., Ohneda, K., Amano, T., Yamasaki, S., Sugiura, A., Tsuchimochi, K., Shin, H., Kawahara, K.-I., Ohneda, O., Ohta, T., Tanaka, S., Yamamoto, M., Maruyama, I., Nishioka, K., Fukamizu, A., Nakajima, T., 2005. Essential role of synoviolin in embryogenesis. *J. Biol. Chem.* 280, 7909–7916. doi:10.1074/jbc.M410863200
- Yamamoto, K., Sato, T., Matsui, T., Sato, M., Okada, T., Yoshida, H., Harada, A., Mori, K., 2007. Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6alpha and XBP1. *Dev. Cell* 13, 365–376. doi:10.1016/j.devcel.2007.07.018
- Yamamoto, K., Suzuki, N., Wada, T., Okada, T., Yoshida, H., Kaufman, R.J., Mori, K., 2008. Human HRD1 promoter carries a functional unfolded protein response element to which XBP1 but not ATF6 directly binds. *J. Biochem.* 144, 477–486. doi:10.1093/jb/mvn091
- Yamasaki, S., Yagishita, N., Sasaki, T., Nakazawa, M., Kato, Y., Yamadera, T., Bae, E., Toriyama, S., Ikeda, R., Zhang, L., Fujitani, K., Yoo, E., Tsuchimochi, K., Ohta, T.,

- Araya, N., Fujita, H., Aratani, S., Eguchi, K., Komiya, S., Maruyama, I., Higashi, N., Sato, M., Senoo, H., Ochi, T., Yokoyama, S., Amano, T., Kim, J., Gay, S., Fukamizu, A., Nishioka, K., Tanaka, K., Nakajima, T., 2007. Cytoplasmic destruction of p53 by the endoplasmic reticulum-resident ubiquitin ligase 'Synoviolin'. *EMBO J.* 26, 113–122. doi:10.1038/sj.emboj.7601490
- Yang, Heeyoung, Qiu, Q., Gao, B., Kong, S., Lin, Z., Fang, D., 2014. Hrd1-mediated BLIMP-1 ubiquitination promotes dendritic cell MHCII expression for CD4 T cell priming during inflammation. *J. Exp. Med.* 211, 2467–2479. doi:10.1084/jem.20140283
- Yang, Hui, Zhong, X., Ballar, P., Luo, S., Shen, Y., Rubinsztein, D.C., Monteiro, M.J., Fang, S., 2007. Ubiquitin ligase Hrd1 enhances the degradation and suppresses the toxicity of polyglutamine-expanded huntingtin. *Exp. Cell Res.* 313, 538–550. doi:10.1016/j.yexcr.2006.10.031
- Ye, J., Rawson, R.B., Komuro, R., Chen, X., Davé, U.P., Prywes, R., Brown, M.S., Goldstein, J.L., 2000. ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. *Mol. Cell* 6, 1355–1364.
- Ye, Y., Meyer, H.H., Rapoport, T.A., 2003. Function of the p97-Ufd1-Npl4 complex in retrotranslocation from the ER to the cytosol: dual recognition of nonubiquitinated polypeptide segments and polyubiquitin chains. *J. Cell Biol.* 162, 71–84. doi:10.1083/jcb.200302169
- Ye, Y., Meyer, H.H., Rapoport, T.A., 2001. The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature* 414, 652–656. doi:10.1038/414652a
- Ye, Y., Shibata, Y., Kikkert, M., van Voorden, S., Wiertz, E., Rapoport, T.A., 2005. Recruitment of the p97 ATPase and ubiquitin ligases to the site of retrotranslocation at the endoplasmic reticulum membrane. *Proc. Natl. Acad. Sci. U.S.A.* 102, 14132–14138. doi:10.1073/pnas.0505006102
- Ye, Y., Shibata, Y., Yun, C., Ron, D., Rapoport, T.A., 2004. A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature* 429, 841–847. doi:10.1038/nature02656
- Ying, Z., Wang, H., Fan, H., Zhu, X., Zhou, J., Fei, E., Wang, G., 2009. Gp78, an ER associated E3, promotes SOD1 and ataxin-3 degradation. *Hum. Mol. Genet.* 18, 4268–4281. doi:10.1093/hmg/ddp380
- Younger, J.M., Chen, L., Ren, H.-Y., Rosser, M.F.N., Turnbull, E.L., Fan, C.-Y., Patterson, C., Cyr, D.M., 2006. Sequential quality-control checkpoints triage misfolded cystic fibrosis transmembrane conductance regulator. *Cell* 126, 571–582. doi:10.1016/j.cell.2006.06.041
- Yu, G., Nishimura, M., Arawaka, S., Levitan, D., Zhang, L., Tandon, A., Song, Y.Q., Rogaeva, E., Chen, F., Kawarai, T., Supala, A., Levesque, L., Yu, H., Yang, D.S., Holmes, E., Milman, P., Liang, Y., Zhang, D.M., Xu, D.H., Sato, C., Rogaev, E., Smith, M., Janus, C., Zhang, Y., Aebbersold, R., Farrer, L.S., Sorbi, S., Bruni, A., Fraser, P., St George-Hyslop, P., 2000. Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing. *Nature* 407, 48–54. doi:10.1038/35024009
- Yuan, X., Wu, H., Xu, H., Xiong, H., Chu, Q., Yu, S., Wu, G.S., Wu, K., 2015. Notch signaling: an emerging therapeutic target for cancer treatment. *Cancer Lett.* 369, 20–27. doi:10.1016/j.canlet.2015.07.048
- Zavacki, A.M., Arrojo E Drigo, R., Freitas, B.C.G., Chung, M., Harney, J.W., Egri, P., Wittmann, G., Fekete, C., Gereben, B., Bianco, A.C., 2009. The E3 ubiquitin ligase



- TEB4 mediates degradation of type 2 iodothyronine deiodinase. *Mol. Cell. Biol.* 29, 5339–5347. doi:10.1128/MCB.01498-08
- Zhang, Q., Meng, Y., Zhang, L., Chen, J., Zhu, D., 2009. RNF13: a novel RING-type ubiquitin ligase over-expressed in pancreatic cancer. *Cell Res.* 19, 348–357. doi:10.1038/cr.2008.285
- Zhang, T., Xu, Y., Liu, Y., Ye, Y., 2015. gp78 functions downstream of Hrd1 to promote degradation of misfolded proteins of the endoplasmic reticulum. *Mol. Biol. Cell* 26, 4438–4450. doi:10.1091/mbc.E15-06-0354
- Zhang, X., Shaw, A., Bates, P.A., Newman, R.H., Gowen, B., Orlova, E., Gorman, M.A., Kondo, H., Dokurno, P., Lally, J., Leonard, G., Meyer, H., van Heel, M., Freemont, P.S., 2000. Structure of the AAA ATPase p97. *Mol. Cell* 6, 1473–1484.
- Zhang, Y., Higashide, W., Dai, S., Sherman, D.M., Zhou, D., 2005. Recognition and ubiquitination of Salmonella type III effector SopA by a ubiquitin E3 ligase, HsRMA1. *J. Biol. Chem.* 280, 38682–38688. doi:10.1074/jbc.M506309200
- Zhong, B., Zhang, L., Lei, C., Li, Y., Mao, A.-P., Yang, Y., Wang, Y.-Y., Zhang, X.-L., Shu, H.-B., 2009. The ubiquitin ligase RNF5 regulates antiviral responses by mediating degradation of the adaptor protein MITA. *Immunity* 30, 397–407. doi:10.1016/j.immuni.2009.01.008
- Zhong, Y., Shen, H., Wang, Y., Yang, Y., Yang, P., Fang, S., 2015. Identification of ERAD components essential for dislocation of the null Hong Kong variant of  $\alpha$ -1-antitrypsin (NHK). *Biochem. Biophys. Res. Commun.* 458, 424–428. doi:10.1016/j.bbrc.2015.01.133

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