Protein Degradation from the Mammalian Endoplasmic Reticulum

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Hiermit erkläre ich, dass ich die Arbeit selbst verfasst habe, und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe.

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1 Abbreviations

β_2m,	β_2-microglobulin or class I MHC light chain
BMDC	bone marrow derived dendritic cell
CHIP	C-terminus of Hsc70-interacting protein
CFTR	cystic fibrosis transmembrane conductance regulator
CPY	carboxypeptidase yscY
CPY*	misfolded variant of CPY
CRT	calreticulin
CTL	cytotoxic T lymphocyte ($CD8^+$ T cell)
CXN	calnexin
DC	dendritic cell
Der	degradation from the endoplasmic reticulum
DHFR	dehydrofolate reductase
Doa	degradation of transcription factor Matα2
DRiPs	defective ribosomal products
DTT	dithithreitol
DUB	deubiquitinating enzyme
EDEM	ER degradation-enhancing α-mannosidase-like protein
eGFP	enhanced green fluorescent protein
ENU	N-ethyl-N-nitrosurea
ER	Endoplasmic Reticulum
ERAD	endoplasmic reticulum associated degradation
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme (UBC)
E3	ubiquitin ligase
HC	class I MHC heavy chain
HCMV	Human cytomegalovirus
HECT	homologous to E6-AP carboxyl terminus
HLA	Human leukocyte antigen
HMGR	3-hydroxy-3-methylglutaryl coenzyme A reductase
Hrd	HMGR degradation
HSP	heat shock protein
IFN	interferon
Ig	immunoglobulin
IKK	inhibitor of IκB kinase
IL	interleukin
ISG15	IFN-stimulated gene product of 15 kDa
KO	knock-out
LPS	lipopolysaccharide
LRR	leucine rich repeats
MALP2	macrophage activating lipopeptide
MHC	Major Histocompatibility Complex
MAPK	mitogen activated protein kinase
NEDD8	neuronal precursor cell-expressed developmentally down-regulated 8
NK	natural killer
Nemo	NFκB essential modulator

OS	osteosarcoma
PDI	protein disulphide isomerase
PAGE	polyacrylamide gel electrophoresis
PAM_3CSK_4	N- Palmitoyl- S-[2,3- bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]- seryl-[S]-lysyl-[S]- lysyl-[S]- lysyl-[S]-lysine
PAMPS	pathogen associated molecular patterns
PRR	pattern recognition receptor
RING	really interesting new gene
RNAi	RNA interference
ROS	reactive oxygen species
SCF	Skp1-Cullin1-F-box
SDS	sodium dodecyl sulfate
ShRNA	short hairpin RNA
SPP	signal peptide peptidase
SUMO	small ubiquitin-like modifier
TAP	transporter associated with antigen presentation
TCR	T cell receptor
TLR	toll like receptor
TMD	transmembrane domain
TNF	tumor necrosis factor
TPRs	tetratricopeptide repeats
Ub	ubiquitin
UBC	ubiquitin-conjugating enzyme (E2)
Uba	Ubiquitin associated domain
Ubl	ubiquitin-like domain
U-box	UFD2-homology box
UGGT	UDP-Glucose:glycoprotein glucosyltransferase
UPR	unfolded protein response
US	unique short region of the HCMV genome
VCP	valosin-containing protein (p97)
VIMP	VCP-interacting membrane protein
WT	wild type
ZL_3VS	carboxyl benzyl-leucyl-leucyl-leucyl vinylsulfone

2 Summary

2.1 Summary

In eukaryotes, one third of all proteins enter the secretory pathway. They do so in the endoplasmic reticulum (ER), an organelle that provides an oxidative environment filled with enzymes and chaperones that aid in protein folding. In the ER, secretory and transmembrane proteins undergo a productive folding cycle, acquire N-linked glycans, and assemble into multisubunit complexes prior to their exit to the Golgi. The surveillance mechanism in the ER that monitors the folding status of a protein is called ER quality control. Quality control ensures that only properly folded and assembled proteins can exit the ER. Accumulation of irreversibly misfolded proteins presents a threat to proper ER homeostasis and function and therefore to cell survival. Proteins that fail quality control are considered misfolded and are selectively retrotranslocated or dislocated into the cytosol for degradation by the cytosolic proteasome, in a process also referred to as ER associated degradation.

Two type I ER transmembrane proteins, US2 and US11, encoded by human cytomegalovirus (HCMV), co-opt the cellular dislocation pathway by catalyzing the dislocation of class I MHC (major histocompatibility complex) heavy chain (HC) molecules from the ER to avoid detection by the immune system. US2 and US11 both target the same molecules but do so by recruiting different sets of proteins. US2 uses signal peptide peptidase (SPP) to degrade class I MHC, whereas US11 seems to engage a more conserved pathway superficially similar to that used by yeast: The polytopic transmembrane protein Derlin-1, the homolog of yeast Der1p, is required for dislocation of HCs in US11, but not in US2 expressing cells. Derlin-1 can oligomerize with another member of the Derlin family, Derlin-2. Derlin-2 forms a complex with p97 and with orthologs of the Hrd1p/Hrd3p ligase complex.

In this thesis I investigated the contribution of the human ortholog of Hrd3p, SEL1L, to heavy chain dislocation as catalyzed by US11. SEL1L is a type I transmembrane protein with a large globular domain containing multiple repeated regions of the tetratricopeptide repeat (TPR) family, suggesting that it might be involved in substrate recognition. Here I provide evidence that SEL1L is essential for HC

dislocation in US11, but not in US2 cells. Furthermore I was able to show that SEL1L is required not only for the degradation of a truncated thereby misfolded version of ribophorin (RI_{332}), but also binds only to misfolded RI_{332}, and not to properly folded ribophorin. To further investigate SEL1L's role in dislocation, I performed a biochemical search for SEL1L-interacting proteins. I identified OS9, AUP1, and UBXD8 as part of the dislocation machinery. Furthermore, I identified an ER membrane bound ubiquitin conjugating E2 enzyme, UBC6e, as required for HC dislocation in US11 cells. We designed dominant negative versions of UBXD8 and AUP1 by constructing C-terminal fusions with GFP. Expression of these constructs leads to inhibition of HC dislocation in US11 cells. OS9, the ortholog of yeast Yos9p, a protein involved in dislocation of glycosylated proteins, does not appear to be involved in HC dislocation. Overexpression of OS9 mutants in which the putative lectin binding domain of OS9 was disrupted, as well as overexpression of wild-type OS9 frustrate dislocation of RI_{332}, but have only a very mild effect on HC dislocation. This result suggests that US11 assumes the role of OS9 in substrate recognition and selection.

Further analysis of UBC6e led to an intriguing connection between proteins involved in ER dislocation and immune function, possibly cross-presentation. UBC6e is highly expressed in dendritic cells and can be phosphorylated upon ER stress. I found that UBC6e can be quantitatively phosphorylated upon stimulation with various Toll-like receptor (TLR) ligands, and that this phosphorylation is dependent on an intact toll-like receptor signaling pathway. Dendritic cells from mice with compromised TLR signaling do not show phosphorylation of UBC6e. The observed cross-talk between chemicals that induce ER stress and between agonists of TLR signaling uncovers new aspects of TLR biology.

To study ubiquitination pathways in primary cells, we typed and characterized a knock-in mouse that expresses HA-tagged ubiquitin. This mouse was used in a study to investigate the contribution of the UbC promoter locus of ubiquitin to the overall pool of ubiquitin in the cell.

2.2 Zusammenfassung

In Eukaryoten werden ein Drittel aller Proteine ins endoplasmatische Retikulum (ER) eingeschleust. Bei diesen Proteinen handelt es sich um sekretorische oder membrangebundene Proteine. Im Inneren oder Lumen des ER herrscht ein oxidatives Milieu, das zusammen mit bestimmten Enzymen und Chaperonen die richtige Proteinfaltung begünstigt. Dabei durchlaufen Proteine einen Faltungszyklus, erhalten N-verknüpfte Glykane und verbinden sich zu Multiproteinkomplexen bevor sie das ER in Richtung Golgi verlassen dürfen. Der Faltungszustand jedes einzelnen Proteins wird genau überwacht und überprüft, und nur solche Proteine, die einen gewissen Qualitätsstandard erfüllen, dürfen das ER verlassen. Eine Anhäufung irreversibel misgefalteter Proteine bedroht das Gleichgewicht im ER und kann zum Zelltod führen. Deswegen werden solche Proteine, die irreversibel missgefaltet sind, aus dem ER heraus ins Zytosol zurücktransportiert (disloziert) und dort vom Proteasom abgebaut. Dieser Prozess heisst auch ER assozierte Degradation.

Zytomegaloviren, die den Menschen befallen (human cytomegalo virus, HCMV), kodieren für zwei Transmembranproteine (Typ I) im ER, US2 und US11. Diese beiden Proteine machen sich die zelluläre Dislokationsmaschinerie zu eigen, um den Abbau der schweren Kette des Histokompatibilitätskomplexes (major histocompatibility complex, MHC) Klasse I zu initiieren. Durch die Entfernung von MHC Produkten von der Zelloberfläche können virusbefallene Zellen nicht mehr von den zytotoxischen T Zellen des Immunsystems erkannt und eliminiert werden. US2 und US11 haben beide zum Ziel, die gleichen Proteine (die schweren Ketten des MHC Klasse I) zu entfernen. Dieses Ziel erreichen sie allerdings mit verschiedenen Methoden: US2 rekrutiert SPP (signal peptide peptidase) um Klasse I MHC zu entfernen, während US11 einen von Hefe zu Mensch konservierteren Weg wählt: das polytopische Transmembranprotein Derlin-1, ein Homolog von Der1p aus Hefe, bindet an US11 und wird für die Zerstörung des Klasse I Moleküls benötigt. Derlin-1 kann mit einem weiteren Protein aus der Derlin-Familie, Derlin-2, Oligomere bilden. Derlin-2 bildet einen Komplex mit der ATPase p97 und mit den Orthologen des Hrd1p/Hrd3p Ligasekomplexes aus Hefe. Die vorliegende Doktorarbeit untersucht das humane Ortholog von Hrd3p, SEL1L, und welche Rolle

dieses Protein in US11-induzierter und allgemeiner Dislokation spielt. SEL1L ist ein fünffach glykosyliertes Transmembranprotein vom Typ1. Der Grossteil des Proteins befindet sich im Lumen des ER, und besteht aus wiederholten Domänen der Tetratricopeptidfamilie, die an der Erkennung misgefalteter Peptide beteiligt sind. In dieser Doktorarbeit konnte ich zeigen, dass SEL1L essentiell für den Abbau von Klasse I MHC Molekülen in Zellen ist, die US11 exprimieren, aber nicht in Zellen, die US2 exprimieren. Desweiteren konnte ich zeigen, dass SEL1L unabhängig von Virusprodukten für die Degradation einer misgefalteten kürzeren Version von Ribophorin, RI_{332}, verantwortlich ist, und ausserdem spezifisch an das misgefaltete RI_{332} und nicht an das normal gefaltete Ribophorin bindet. Mit biochemischen Methoden bestimmte ich Interaktionspartner von SEL1L. Die neuen Proteine OS9, AUP1, und UBXD8 sind alle Teil der Dislokaktionsmaschinerie, die sich US11 zu eigen macht. Ausserdem identifizierte ich ein membrangebundenes Ubiquitinkonjugations-enzym UBC6e, das nötig ist für die Zerstörung von Klasse I MHC Molekülen in US11-exprimierenden, aber nicht in US2-exprimierenden Zellen. Wir entwarfen dominant-negative Versionen von AUP1 und UBXD8, indem wir den C-terminus mit einem GFP tag fusionierten. Diese Konstrukte führen zu einem Arrest der Klasse I MHC Moleküle in der ER Membran. OS9, das ortholog von Hefe Yos9p, ein Protein das an der Degradation von glykosilierten Proteinen beteiligt ist, scheint keine Rolle am Abbau von Klasse I Molekülen zu spielen. Die Überexpression von OS9 Mutanten, die die angebliche Lektinbindedomäne zerstören, sowie Überexpression von Wildtyp OS9 –wahrscheinlich aufgrund eines Ungleichgewichts des Dislokationskomplexes- inhibieren die Dislokation von RI_{332}, aber haben nur einen sehr geringen Effekt auf den Abbau von Klasse I MHC Molekülen. Dieses Resultat könnte bedeuten, dass US11 die Rolle von OS9 in Subtratselektion übernimmt.

Eine weiterführende Analyse von UBC6e erbrachte eine interessante Verbindung zwischen Proteinen, die eine Rolle beim Abau von ER Proteinen spielen, und Proteinen, die eine Rolle in der Immunfunktion spielen. UBC6e wird stark in dendritischen Zellen exprimiert und kann nach der Induktion von ER Stress phosphoryliert werden. Wir fanden heraus dass UBC6e quantitativ phosphoryliert werden kann, wenn man dendritische Zellen mit verschiedenen Liganden von Toll-like Rezeptoren (TLR) stimuliert. Dendritische Zellen aus Mäusen die eine Mutation

in einem der Proteine der TLR Signalkaskade haben, zeigen keine UBC6e Phosphorylierung mehr. Ein Zusammenhang zwischen Chemikalien, die ER Stress induzieren, und Liganden, die TLRs aktivieren, ist noch unbekannt.
Um Ubiquitin in Primärzellen untersuchen zu können, charakterisierten wir eine HA-Ubiquitin Knock-in Maus. Diese Maus wurde auch in einer Studie verwendet, die den Beitrag des UbC Promotors des Ubiquitinlokus zum allgemeinen Pool an Ubiquitin untersucht.

3 Introduction

3.1 Protein translation and translocation

Prokaryotes consist of a single compartment surrounded by the plasma membrane. Eukaryotes contain - in addition to the plasma membrane - several membrane-delimited compartments such as the lysosomes, endosomes, mitochondria and the organelles of the secretory pathway, the endoplasmic reticulum (ER) and the Golgi. These compartments and the cytosol contain the proteins that fulfill all major tasks of the cell (Rapoport, 1986).

Proteins are polymers of amino acids that need to fold into a distinct three-dimensional structure to be functional. Proteins that are translated on free ribosomes fold in the cytosol with the help of cytosolic chaperones, a specialized set of proteins that bind to hydrophobic patches and mainly prevent aggregation through a regulated ATP-driven release of bound polypeptides (Ellis, 1987). Proteins translated on membrane-bound ribosomes are translocated through the membrane via a protein-conducting channel with a hydrophilic interior (Crowley et al., 1993; Simon and Blobel, 1991). This channel is called Sec YEG in prokaryotes and Sec61 in eukaryotes (Osborne et al., 2005). The Sec YEG channel, located in the plasma membrane, releases proteins into the periplasmic space or anchors them in the lipid bilayer. The details of this process are beyond the scope of this thesis and have been reviewed elsewhere (Rapoport, 2007).

The Sec61 channel is a protein complex present in the ER membrane. Transmembrane proteins, secreted proteins and ER luminal or Golgi proteins enter the ER through Sec61 (Rapoport, 2007). The lumen of the ER provides an oxidizing environment by maintaining the requisite balance between oxidized and reduced glutathione (Hwang et al., 1992). This supports the formation of disulfide bonds, thereby stabilizing the proteins in an extracellular environment. Many ER resident proteins are calcium binding proteins and depend on calcium for their function, consistent with the elevated calcium levels in the ER (Michalak et al., 2002). This thesis focuses on the ER.

3.2 ER quality control: protein folding and protein degradation

3.2.1 Protein maturation in the ER

Over one-third of all proteins enter the secretory pathway. They are segregated either partially or completely from the cytoplasm in a membrane-delimited compartment (Ghaemmaghami et al., 2003). Therefore, the first step of the secretory pathway is the translocation or insertion of the nascent polypeptide into the ER membrane or the ER lumen, depending on the presence of hydrophobic stretches in the polypeptide. Most secretory proteins are directed to the ER by virtue of an N-terminal cleavable signal sequence (Rapoport, 2007). As soon as the signal sequence or a hydrophobic sequence on the nascent polypeptide emerges from the translating ribosome, signal recognition particle (SRP) binds to it, stalls translation and redirects the ribosome-nascent polypeptide complex to the SRP receptor at the ER membrane. The ribosome then associates with the Sec61 complex which forms a proteinaceous pore in the ER membrane, through which the polypeptide is co-translationally translocated into the ER (Clemons et al., 2004; Matlack et al., 1997; Van den Berg et al., 2004). Folding of the polypeptide starts during translation and translocation. A number of chaperone complexes immediately bind to the nascent polypeptide and facilitate import and proper folding, and mediate co-translational modifications such as addition of N-linked glycans (Brodsky, 2007; Fewell et al., 2001). Proteins can also be modified post-translationally with the formation of disulfide bonds and the attachment of glycosylphosphatidylinositol (GPI) anchors (Ellgaard and Helenius, 2003). The chaperones in the ER must be present at high concentrations and they must be versatile, as the various polypeptides are topologically and structurally very distinct. These chaperones can be categorized into three distinct groups: a) chaperones of the heat shock family of proteins (HSP), which includes BiP or GRP78 and GRP90, b) members of the lectin family of chaperones, such as calnexin (CNX), calreticulin (CRT), and EDEM, (and probably Yos9p) and c) substrate-specific chaperones such as HSP47 (Ni and Lee, 2007). In addition, there are a number of folding enzymes in the ER, such as the thiol oxidoreductase of the protein disulfide isomerase (PDI) family, and peptidyl prolyl isomerases that catalyze the

cis-trans isomeration of peptide bonds N-terminal to proline residues (Ni and Lee, 2007). One of the most abundant chaperones is BiP, an HSP70 family member, equipped with an ATPase at the N-terminus and a C-terminal substrate binding domain (McKay, 1993). BiP acts early during translocation in a complex with its cofactor HSP40. BiP binds to hydrophobic regions in the nascent polypeptide, and prevents the protein's aggregation by repeated ATP-driven cycles of binding and release (Gething, 1999). These cycles are catalyzed by co-chaperones of the DnaJ family (e.g. HSP40). One of the 5 BiP co-chaperone proteins or ERdj proteins, ERdj5, contains not only a DnaJ domain but also a PDI-like domain and thioredoxin domains. Thus, while BiP prevents aggregation and promotes folding, ERdj5 catalyzes the formation of disulfide bonds (Cunnea et al., 2003).

Although glutathione sustains the oxidizing milieu of the ER lumen, it is not sufficient for disulfide bond formation. For this purpose, the ER contains several thiol-oxidoreductases that bear the thioredoxin CXXC motif. PDI is another extremely abundant chaperone of the ER, with local concentrations approaching millimolar levels (Lyles and Gilbert, 1991a; Lyles and Gilbert, 1991b). In vitro, PDI can act as an oxidase, reductase, and isomerase (Freedman et al., 2002). PDI also has a peptide binding site. PDI is essential for oxidative protein folding in yeast (LaMantia and Lennarz, 1993). Reduced PDI is generated to its oxidized state by the activity of its own oxidase, Ero1 (**ER o**xidation 1) (Frand and Kaiser, 1998; Frand and Kaiser, 1999). Mammals possess two isoforms of yeast Ero1p, Ero1α and Ero1β (Pagani et al., 2000). Only Ero1α is constitutively expressed, Ero1β is induced when misfolded proteins accumulate (Pagani et al., 2000).

3.2.2 ER Quality Control:

Incorrectly folded or incompletely assembled proteins are believed to be harmful to the cell if allowed to proceed along the secretory pathway. Therefore, all ER proteins are subject to stringent quality control (QC). This QC assesses the protein's folding status and allows more time for proteins to reach their final and proper conformation (Ellgaard et al., 1999). Several distinct steps comprise QC:

3.2.2.1 Glycoprotein quality control

3.2.2.1.1 N-linked glycosylation

In the ER, many nascent polypeptide chains are modified with a branched 14-hexose core glycan, Glc3Man9GlcNac2, at asparagine (N) residues (N-linked glycosylation). The modification occurs within the consensus sequence Asn-Xxx-Ser/Thr, where Xxx can be any amino acid except proline (Gavel and von Heijne, 1990). Oligosaccharide assembly begins in the cytosol as a dolycholpyrophosphate-linked precursor, which is flipped to face the ER lumen and assembled onto the nascent polypeptide by the flippase RFT1 and the action of the oligosaccharyl transferase complex (Burda and Aebi, 1999; Helenius and Aebi, 2002; Kornfeld and Kornfeld, 1985). The oligosaccharide can modulate protein structure and function by increasing solubility and stability (Drickamer and Taylor, 1998; Dwek et al., 1996; O'Connor and Imperiali, 1996; Wormald and Dwek, 1999; Wormald et al., 2002). At the cell surface, the glycan can modulate protein-protein interaction and protect proteins from proteases (Moody et al., 2001). In the ER, apart from modulating folding of the nascent polypeptide, N-linked glycans can also act as sorting signals to direct trafficking in the secretory pathway (Hauri et al., 2000; Helenius and Aebi, 2001; Schrag et al., 2001).

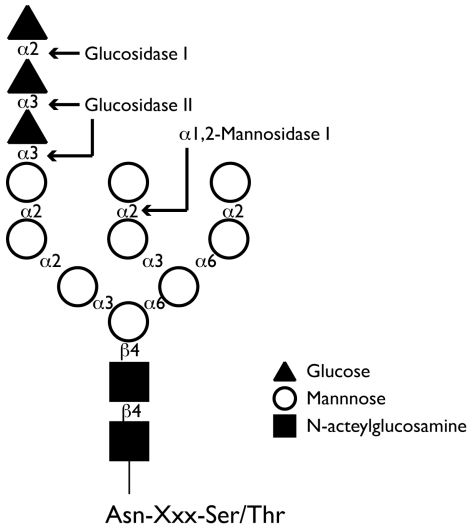

Figure 1: Structure of the N-linked oligosaccharide. Linkage type between glucosyl residues and cleavage sites for enzymes are indicated. (Adapted from Ellgaard and Frickel, 2003.)

3.2.2.1.2 The calnexin/calreticulin cycle

The calcium-binding lectins calnexin (CNX, a type I transmembrane protein) and calreticulin (CRT, a soluble ER luminal protein) bind to the monoglucosylated glycan, Glc1Man9GlcNac2, present on nascent polypeptide chains, and assist in the folding of most glycoproteins (Ellgaard and Helenius, 2003; Hebert and Molinari, 2007). Nascent polypeptides that have N-linked glycans within the first 50 residues of their amino-terminus interact with CNX/CRT prior binding to BiP (Molinari and Helenius, 2000). Soon after the core oligosaccharide, is added onto the nascent polypeptide, it is exposed to the successive action of the ER-resident enzymes glucosidase I and II (Helenius and Aebi, 2004). Glucosidase I trims the two outermost glucose residues. While CNX/CRT associate with the client protein, glucosidase II removes the innermost glucose residue and releases the glycoprotein from the CNX/CRT cycle (Helenius and Aebi, 2004). The release likely occurs irrespective of the folding state of the glycoprotein and prevents renewed association with CNX/CRT. The enzyme UDP-glucose:glycoprotein glucosyltransferase (UGGT) works as a folding sensor and reglucosylates glycoproteins that are incompletely or incorrectly folded. UGGT ensures that proteins in a misfolded conformation can re-associate with CNX/CRT (Parodi, 2000; Trombetta and Helenius, 2000). The thiol-oxidoreductase ERp57 associates with CNX/CRT and catalyzes the formation of disulfide bonds in the nascent polypeptide chain (Frickel et al., 2004). Through its association with CNX/CRT, ERp57 functions as a specialized thiol-disulfide oxidoreductase for glycoproteins. The glucosylation cycle retains incorrectly folded proteins in the ER. Once the protein has reached its final folding state, it exits the ER via COPII coated vesicles to reach its destination, the plasma membrane or other subcellular compartments (Hebert and Molinari, 2007). Oligomeric proteins must pass another level of quality control involving glycoprotein GP96 (a member of the HSP90 family of chaperones) to ensure that only properly assembled structures leave the ER. GP96 is essential for the proper multimeric assembly and export of some members of the integrin and toll-like receptor protein families, which in the absence of GP96, are retained unassembled in the ER (Randow and Seed, 2001).

3.2.2.2 Recognizing misfolded proteins

When is a protein misfolded? There are no experimental assays to determine whether a protein has reached its final conformation. A protein can be considered as misfolded due to a frameshift, mutations, oxidative stress, lack of partner subunits, or when the amount of proteins exceeds the folding capacity of the cell. Distinguishing between proteins that are in the process of reaching their final conformation and those that are permanently misfolded presents an intriguing conundrum for the cell.

It has been proposed that terminally misfolded proteins are recognized by the mannose content of their N-linked glycans (Helenius and Aebi, 2004). The ER enzyme α 1,2 mannosidase is a slow-acting membrane-bound exo-mannosidase that removes the terminal mannose from the oligosaccharide to yield the Man8GlcNAc2 structure. This structure is thought to be the signal that targets some glycoproteins for degradation (Ellgaard and Helenius, 2003; Helenius and Aebi, 2004). Proteins with this tag are transferred from CNX to lectins of the EDEM (**E**R **d**egradation **e**nhancing α−**m**annosidase-like protein) family (Ermonval et al., 2001; Hosokawa et al., 2001; Kitzmuller et al., 2003). EDEM1 plays a dual role: it escorts substrates out of the CNX cycle for degradation (Molinari et al., 2003; Oda et al., 2003) and it maintains substrate solubility by acting as a lectin chaperone (Kanehara et al., 2007). EDEM1, EDEM2, and EDEM3 seem to have overlapping functions (Hirao et al., 2006; Hosokawa et al., 2003; Hosokawa et al., 2001; Mast et al., 2005; Mast and Moremen, 2006; Olivari et al., 2005). Although it was initially thought that EDEMs lack any mannosidase activity due to a missing cysteine otherwise conserved in mannosidases, it has now been shown that EDEMs themselves can trim mannoses of misfolded proteins (Olivari et al., 2006).

Yos9p represents a second class of lectin-like factors that play a role in the recognition of misfolded proteins. Its lectin-like domain is homologous to the mannose-6-phosphate receptor domain. It can bind to glycosylated and non-glycosylated proteins, so it remains unclear whether it in fact represents a true lectin (Bhamidipati et al., 2005). Yeast Yos9p has two mammalian orthologues, XTP-3B and OS9. OS9 is described in the results section.

The chaperones PDI and BiP not only play a role in proper folding, they have also been proposed to aid in removal of misfolded proteins: BiP by preventing aggregation (Kabani et al., 2003; Nishikawa et al., 2001) and PDI by unfolding disulfide bonds (Tsai et al., 2001; Tsai et al., 2002). It remains unclear whether EDEMs or Yos9p, analogous to BiP or PDI, can also play a role in protein folding.

3.2.3 The unfolded protein response

A significant increase in secretory load may exceed the folding capacity of the ER. Therefore, a stress response called the unfolded protein response (UPR) is initiated when misfolded proteins accumulate (Rutkowski and Kaufman, 2004). The UPR serves two purposes: it initially decreases the rate of protein translation and then increases the quantities of chaperones that aid in folding, and of proteins that are involved in the degradation of misfolded proteins. The secretory load is decreased by the action of the transmembrane protein kinase PERK, which, under conditions of ER stress, phosphorylates the translation initiation factor eIF2α. This causes an arrest in protein synthesis (Harding et al., 2000). There are two phospho-eIF2α phosphatases, GADD34 and CreP, in a complex with protein phosphatase PP1c, that provide a feedback mechanism to alleviate translational arrest (Jousse et al., 2003; Novoa et al., 2001; Novoa et al., 2003).

The UPR also acts on transcriptional level through two transmembrane sensors, ATF6 and IRE1. Upon accumulation of misfolded proteins, ATF6 travels from the ER to the Golgi, where two proteases, S1P and S2P, cleave its transmembrane region (Ye et al., 2000). Intramembrane cleavage results in the release of the cytosolic bZIP transcription factor that is imported to the nucleus where it activates transcription of chaperone genes (Ye et al., 2000).

IRE1 (**i**nsotitol **re**quiring) is a kinase located in the ER membrane (Cox et al., 1993). It autophosphorylates upon ER stress, thereby activating its cytosolic endonuclease activity (Shamu and Walter, 1996). The endonuclease splices the mRNA of the transcriptional activator XBP-1 (X-box-binding protein 1), resulting in a 26 nucleotide shorter mRNA (Calfon et al., 2002; Shen et al., 2001; Yoshida et al., 2001). Unspliced XBP-1 encodes a highly unstable protein that lacks the

transcriptional activation domain. Spliced XBP-1 yields a more stable protein that has a DNA binding domain and a transcriptional activation domain (Calfon et al., 2002). XBP-1 upregulates the transcription of chaperones and of proteins involved in the disposal of misfolded ER proteins (Lee et al., 2003). BiP has been proposed to play a major role in the activation of all three UPR transducers (Bertolotti et al., 2000; Okamura et al., 2000; Shen et al., 2002) in that it binds to the luminal regions of e.g. Ire1a, but dissociates when misfolded proteins accumulate, thereby allowing activation of Ire1 and PERK (Bertolotti et al., 2000) and ATF6 to travel to the Golgi (Shen et al., 2002). This model, however, does not explain the fast initiation of the UPR when just a few misfolded proteins arise, since BiP attains millimolar concentrations in the ER. The crystal structure of the luminal domain of yeast Ire1p also suggests a different model: the luminal domain forms a peptide binding groove very similar to that of class I MHC (see Chapter 3.2.3.7.1) and might recognize misfolded proteins directly (Credle et al., 2005). In addition, when mutating the BiP binding site on IRE1, recognition of misfolded proteins continues (Oikawa et al., 2007).

When misfolded proteins persist, high levels of reactive oxygen species are generated by attempts at oxidative folding (Tu and Weissman, 2004) . The reactive oxygen species will eventually lead to cellular damage and apoptosis (Haynes et al., 2004). Cells with a high secretory load, such as hepatocytes, antibody-producing plasma cells, or insulin-producing β-cells of the pancreas are more sensitive to the effects of ER stress and to mutations that interfere with proper UPR induction (Harding et al., 2001; Ozcan et al., 2004; Reimold et al., 2000). Eventually, prolonged UPR signaling induces apoptosis, presumably through ER-associated caspases (Rao et al., 2001). The three UPR inducers attenuate with different time frames: IRE1 is switched off the earliest, already after 8hours, whereas PERK signaling persist the longest (30hrs) with ATF6 being intermediate (Lin et al., 2007). If IRE1 signaling is artificially enhanced, cells cannot undergo apoptosis, making it an important determinant of cell fate (Lin et al., 2007).

3.2.4 The ubiquitin-proteasome system

All cellular proteins are subject to turnover. Proteins are targeted for degradation through tagging with multiple molecules of ubiquitin, a polypeptide of 76 amino acids (Hershko and Ciechanover, 1998; Varshavsky, 1997). Ubiquitin-tagged proteins are degraded by the cytosolic proteasome with concomitant release of free ubiquitin (Glickman and Ciechanover, 2002; Heinemeyer et al., 1991; Wolf and Hilt, 2004).

The ubiquitin-proteasome system is pivotal to numerous cellular processes such as cell cycle control, transcriptional regulation, signal transduction, membrane trafficking, and many others (Pickart, 2001). Ubiquitin is highly conserved in all eukaryotes. It is encoded by four genes, either as poly-ubiquitin fusion genes (UbB and UbC) or as ubiquitin fusions to the small ribosomal subunit (UbA52, UbA80) (Finley et al., 1989; Redman and Rechsteiner, 1989). Ubiquitin is transcribed at relatively low levels (see results section, Ryu et al., 2007). Deubiquitinating enzymes process the ubiquitin fusion proteins immediately after their synthesis to generate mono-ubiquitin.

The attachment of ubiquitin to target proteins follows a complex enzymatic cascade: The ubiquitin-activating enzyme E1 activates ubiquitin in an ATP-dependent reaction by forming a thioester between a cysteine residue in the enzyme and the c-terminus of ubiquitin (G76). One of several E2 enzymes (ubiquitin-conjugating enzymes or UBCs) transfer the activated ubiquitin via another thioester, now between a cysteine residue in the E2 and ubiquitin, to the NH_2 group of a lysine residue (in special cases the NH_2 group at the amino-terminus of the substrate can be used) on the substrate. At this stage the substrate and the E2-ubiquitin complex interacts with one of hundreds of E3 enzymes or ubiquitin ligases, which facilitate the transfer of ubiquitin to the substrate by creating a covalent amide bond between ubiquitin and the substrate, and are therefore responsible for substrate selection (Glickman and Ciechanover, 2002; Hershko and Ciechanover, 1998). Most of the more than 500 known E3's in mammals belong to two classes, the HECT (homologous to E6-AP carboxyl terminus) domain ligases or the RING (really

interesting new gene) domain ligases. HECT domain E3's form (analogous to the E1 and E2's) a catalytic thioester intermediate with ubiquitin and a conserved cysteine residue in the HECT domain through transesterification (Pickart, 2001). There are only 6 known HECT E3's in Saccharomyces cerevisiae and less than 30 in mammals. The majority of E3 ligases belongs to the RING and RING finger like ligases. RING finger E3s recognize E2's through the coordination of two zinc ions (Kostova et al., 2007). RING E3s do not necessarily form a thioester intermediate with ubiquitin, but mainly bring the ubiquitin-loaded E2 in close proximity to the substrate (Pickart, 2001).

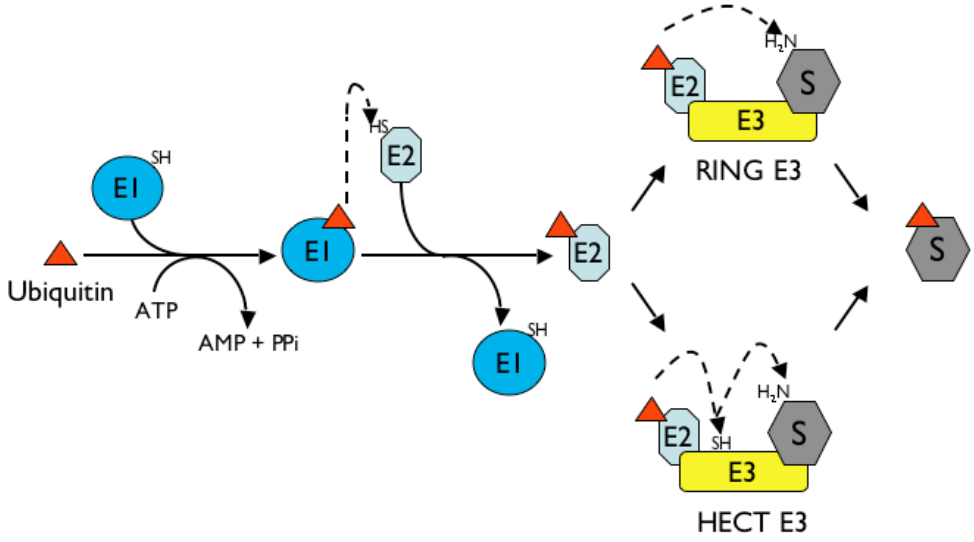

Figure 2: The ubiquitin cascade. E1= ubiquitin activating enzyme, E2=ubiquitin conjugating enzyme, E3= ubiquitin ligase, S= Substrate. For details see text.

By successively adding ubiquitin molecules through their C-terminal carboxy group onto lysine residues on previously added ubiquitin molecules, a poly-ubiquitin chain is synthesized. The presence of a fourth class of enzymes, the E4 proteins, that assist in the elongation of the ubiquitin chain, has been suggested (Hoppe, 2005). As there are multiple lysines on ubiquitin, chains with different linkage types can be created.

Targeting of proteins for proteasomal degradation generally requires poly-ubiquitin chains in a K48-linkage type, and a minimum of 4 ubiquitin to build the chain.
Other ubiquitin linkage types exits: K63 and K29 linked ubiquitin chains as well as mono-ubiquitin usually have non-proteolytic functions in DNA repair, endocytosis, transcriptional regulation and proteasomal function (Pickart and Eddins, 2004). Mono-ubiquitin, which can occur on a single lysine residue in the target protein, or on several lysine residues, represents an important sorting signal in the endocytic pathway (Hicke, 2001; Hicke and Dunn, 2003).
Proteins can not only be covalently tagged with ubiquitin itself, but also with molecules that share structural homology to ubiquitin, such as SUMO, NEDD8, Fat10, or ISG15. These ubiquitin-like modifiers (UBLs) usually do not tag proteins for degradation but have more specialized roles (Love et al., 2007; Schwartz and Hochstrasser, 2003).
Deubiquitinating enzymes (DUBs) cleave the isopeptide bond between ubiquitin and substrate or between ubiquitin (Ub) and another ubiquitin molecule. This function serves to rescue substrate proteins from proteasomal degradation (Rumpf and Jentsch, 2006), to generate or recycle ubiquitin (Swaminathan et al., 1999), or to control trafficking (Nikko and Andre, 2007). DUBs belong to a diverse group of cysteine and metallo proteases (Love et al., 2007; Nijman et al., 2005), and can be divided into 5 distinct classes in mammals: (i) Ub C-terminal hydrolases (UCHs), (ii) ub-specific proteases (USPs), (iii) Machado-Joseph disease protein domain proteases (MJDs), (iv) ovarian tumor proteases (OTUs), and (v) JAMM motive (zink metallo) proteases (Love et al., 2007).
Since the ubiquitin conjugation pathway involves a remarkable diversity of its substrates, the activities of DUBs, E2, and E3 ligases are regulated. For example E3 ligases can bind to more than one E2, and their activity, in addition, is controlled by post-translational modification, localization, oligomerization, and degradation (Pickart and Eddins, 2004).

3.2.4.1 The proteasome

The proteasome is a multisubunit protease in the cytosol. It consists of the 19S Cap or regulatory particle (RP) and the 20S core particle (CP), a barrel-shaped proteolytic chamber. Both the 19S Cap and the 20S core particle confer selectivity to the degradation process. The 19S regulatory particle recognizes substrates selectively, and controls entry into the barrel of the core particle. In addition, only previously unfolded substrates are able to enter the core particle, as access to the core particle occurs through a narrow pore with a diameter of about 2nm (Glickman et al., 1998; Wolf and Hilt, 2004). The 20S core consists of a barrel or cylinder composed of four stacked rings with seven distinct subunits each. The two outer rings are composed of α subunits and the two innermost rings of β subunits, forming in total three chambers (Groll et al., 1997). The β subunits line the inner cavity of the proteolytic core particle. Only three of the seven β subunits in each ring are catalytically active, with a chymotrypsin-like, trypsin-like, and peptidyl-glutamyl-like hydrolyzing activity (Wolf and Hilt, 2004). The α subunits are catalytically inactive. In addition, alternative interferon-γ inducible β genes exist that encode for β subunits that proteolyze peptides for improved display on MHC class I (see chapter 3.2.3.7). The 19 S Cap has to perform a multitude of functions: (i) it has to selectively bind to the ubiquitinated protein, (ii) unfold it, (iii) assist in the deubiquitination of the substrate, (iv) open the gates into the core particle, and (v) drive the unfolded protein into the proteolytic chamber (Wolf and Hilt, 2004).

3.2.5 Removal of misfolded proteins from the ER

ER-associated protein degradation (ERAD) involves at least three distinct steps: (i) recognition of the respective protein as terminally misfolded (see chapter 3.2.2.2), (ii) dislocation or retrotranslocation of the protein through the ER membrane into the cytoplasm, and (iii) its ubiquitin-dependent degradation in the cytosol by the proteasome (Ahner and Brodsky, 2004).

3.2.5.1 ER protein degradation in yeast: early findings through a genetic approach

Evidence that proteins from the ER might be degraded in the cytosol emerged when an ER-bound UBC enzyme, Ubc6p, with its catalytic center located in the cytosol, was discovered in yeast (Chen et al., 1993). Furthermore, disruption of the *ubc6* locus rescues the protein translocation defect caused by a mutation in the translocation channel Sec61 (Biederer et al., 1996; Sommer and Jentsch, 1993), suggesting that the mutant subunit is stabilized if the ubiquitin-proteasome pathway is compromised.

Further evidence that the ubiquitin-proteasome pathway is involved in the degradation of misfolded ER proteins emerged from genetic screens in yeast. The genetic screens were performed using two substrates: CPY*, a point-mutant (G255R) of the vacuolar protein carboxypeptidase yscY (CPY), that is soluble and glycosylated, and HMG-CoA-Reductase (3-hydroxy 3-methylglutary coenzyme A reductase), a polytopic transmembrane ER protein that is a crucial enzyme in cholesterol biosynthesis of the mevalonate pathway. The enzyme is degraded when cholesterol and its precursors reach a certain threshold (Hampton and Rine, 1994). Two genetic screens - one screening for stabilization of CPY* (Hiller et al., 1996; Knop et al., 1996), yielding the DER genes (for **d**egradation in the **ER**), the other screening for mutants that are defective in the regulated degradation of HMGCoA-Reductase (Hampton et al., 1996), yielding the HRD genes (for **H**MG CoA **r**eductase **d**egradation) – revealed the existence of a conserved group of proteins required for protein degradation from the ER.

One of the major findings of this screen for CPY* stabilization was a surprise: Much research had been dedicated to finding an ER resident protease that would degrade entirely luminal ER proteins, such as CPY*, which do not expose any domains into the cytosol. The screen for a defect in the degradation of CPY* revealed that the cytosolic proteasome and the ubiquitin-conjugating enzyme Ubc7p play a major role in this degradation (Hiller et al., 1996), and not an ER-resident protease (Wolf and Schafer, 2005).

3.2.5.2 ER protein degradation in mammalian cells

The involvement of the ubiquitin-proteasome pathway in the degradation of aberrantly folded proteins in the mammalian ER was first established in studies involving the mutant form of an ion channel, the cystic fibrosis transmembrane conductance regulator (CFTR). A mutation in this channel leads to the deletion of a phenylalanine residue at position 508, thereby preventing proper folding and assembly of the channel (Cheng et al., 1990). The mutant CFTR (CFTR delta F508) does not traffic to the plasma membrane and, in homozygous causes one of the most common lethal genetic diseases in Caucasians, called cystic fibrosis (Pilewski and Frizzell, 1999). Proteasome inhibitors and dominant-negative forms of ubiquitin (K48R) uncovered the role of the ubiquitin-proteasome pathway in the disposal of this protein (Jensen et al., 1995; Ward et al., 1995).
Around the same time, two viral proteins, US2 and US11, were uncovered that triggered the destruction of class I MHC heavy chains from the ER to avoid immune recognition (Wiertz et al., 1996a; Wiertz et al., 1996b). These heavy chains are stabilized when cells are exposed to proteasome inhibitor, giving further evidence that ER-resident proteins, with or without transmembrane domains, are degraded by the cytosolic proteasome.

3.2.5.3 Ubiquitin conjugation of dislocation substrates – different substrates, different E3 complexes

3.2.5.3.1 Yeast dislocation E3s

The main ubiquitin ligases studied in yeast are the RING-finger E3's Hrd1p/Der3p and Doa10p. Both are part of multiprotein "dislocation" complexes and can assemble with different adaptor factors, depending on the nature of the protein that is being dislocated. However, the Hrd1p and Doa10p pathways seem to converge at the cytosolic side of the ER: they require the ATPase p97/Cdc48p for substrate removal from the ER membrane (Kostova et al., 2007).

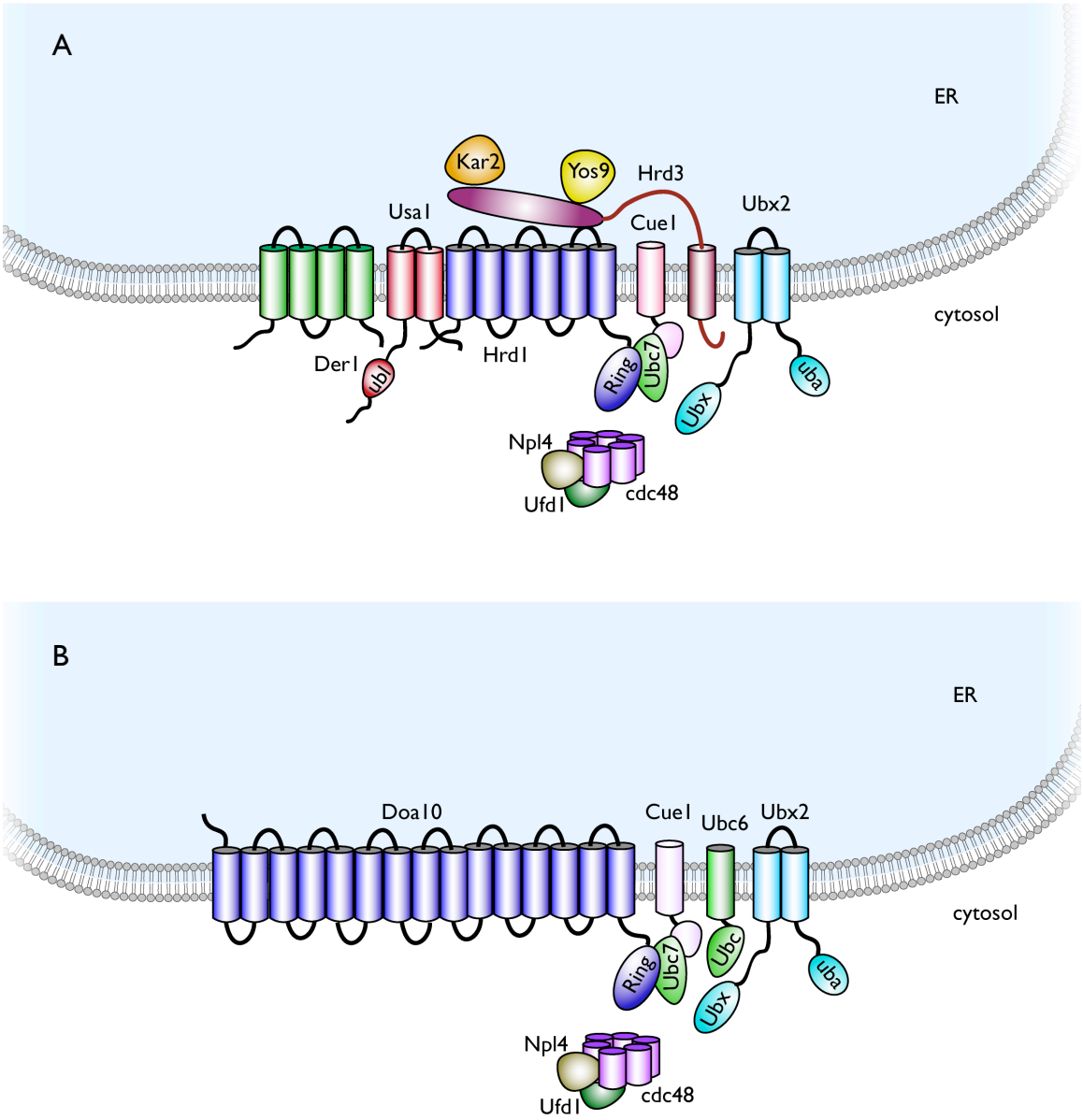

Figure 3: Schematic representation of the Hrd1p (upper panel, A) and Doa10p (lower panel, B) ligase complexes in yeast.

In yeast, a distinction between ER dislocation substrates is being made not only according to the overall topology and localization of the substrate, but also according to the site where misfolding occurs on the substrate (Ahner and Brodsky, 2004). The ERAD-C pathway (**ER a**ssociated **d**egradation, **c**ytosolic) requires the ligase Doa10p and acts on proteins with misfoldings in cytoplasmically exposed domains. The ERAD-L pathway (**ER** associated **d**egradation, **l**uminal) uses the Hrd1p ligase complex to clear proteins that have defects in luminal domains (Vashist and Ng,

2004). These substrates usually have to shuttle to the Golgi, presumably to acquire a modification that will target them for degradation once they rereach the ER, providing one explanation for why luminal dislocation substrates have a much longer half-life than transmembrane substrates. Proteins that have lesions in their transmembrane regions are supposed to be degraded through the ERAD-M (**ER a**ssociated **d**egradation, **m**embrane) pathway. ERAD-M also requires the ligase Hrd1p but uses different adaptor proteins to form the degradation complex (Carvalho et al., 2006). It remains unclear however, how a lesion in one domain might affect the overall structure of the protein, and whether in the case of multiple lesions, one is dominant over the other. For example, in polytopic transmembrane proteins, interactions between transmembrane helices are essential for proper folding and assembly (Curran and Engelman, 2003). The aforementioned dislocation substrate HMGR is presumably targeted for degradation due to structural alterations in its transmembrane region (Shearer and Hampton, 2005), leaving unexplained why such restructuring would make this protein a target for the dislocation machinery.

3.2.5.3.1.1 The Hrd1 complex

Hrd1p spans the ER six times, with both termini exposed to the cytosol (Bordallo and Wolf, 1999; Deak and Wolf, 2001; Gardner et al., 2000). It lacks a glycosylation side and an ER retention motif, but it seems to be localized exclusively to the ER (Kostova et al., 2007). Hrd1p can associate with the ubiquitin conjugating enzymes Ubc7p, Ubc1p, and with the transmembrane anchored Ubc6p. *In vivo*, the principal E2 for Hrd1p catalyzed polyubiquitination is Ubc7p, which is tethered to the ER membrane via a CUE domain containing protein, Cue1p (Biederer et al., 1997).

Ubc1p and Ubc7p both physically associate with Hrd1p through its RING finger, with the catalytically dead mutant C399S incapable of binding (Bays et al., 2001a; Deak and Wolf, 2001). There are some substrates that utilize Ubc6p, but generally it does not seem specifically required for Hrd1p function (Bays et al., 2001a; Kostova et al., 2007).

Hrd1p ligase activity depends critically on its co-factor Hrd3p, a type I glycosylated transmembrane protein that forms a 1:1 stochiometric complex with Hrd1p (Gardner et al., 2000). The bulk of Hrd3p is in the lumen of the ER and carries 5 N-linked

glycans. In the absence of Hrd3p, the normally stable Hrd1p itself turns into an ER dislocation substrate, using Ubc7p and Cue1p and its own ligase activity for its destruction (Gardner et al., 2000; Plemper et al., 1999). Hrd3p's N-terminal region is essential for ER degradation, and its central region is required for binding and stabilizing Hrd1p (Gardner et al., 2000). The luminal domain contains several repetitive structural and functional domains (sel1-like repeats of the tetratricopeptide (tpr) family) that might be involved in binding of misfolded proteins or in recruitment of chaperones. The human homologue of Hrd3p, SEL1L, binds to the misfolded protein RI_{332} and not to its properly folded parent protein ribophorin (Mueller et al., 2006). This is discussed in detail in the results chapter.

The finding that the ER degradation defect in a Δ*hrd3* deletion strain could be circumvented by overexpression of Hrd1p (Gardner et al., 2000; Plemper et al., 1999) is confusing. More insight into Hrd3p's function emerged with the discovery of an ER luminal lectin-like protein, Yos9p, involved in the degradation of glycosylated proteins (Bhamidipati et al., 2005; Buschhorn et al., 2004; Kim et al., 2005; Szathmary et al., 2005). Yos9p interacts with Kar2p/BiP and with the luminal domain of Hrd3p (Denic et al., 2006; Gauss et al., 2006b). In yeast, Hrd3p, Yos9p, and Kar2p can all independently of each other bind to misfolded proteins, yet, in the absence of Yos9p and Hrd3p, Hrd1p-mediated degradation proceeds uncompromised (Denic et al., 2006). Therefore, it has been proposed that Hrd3p acts as a gate-keeper of the Hrd1p ligase complex: Although Yos9p and Hrd3p can bind to substrates independently of their glycosylation state, only those bearing the GlcNAc2-Man8 tag are handed over from Hrd3p to Hrd1p and proceed to degradation (Ismail and Ng, 2006). Moreover, wild-type cells degrade Hrd1p if it is not complexed with Hrd3p, presumably to prevent indiscriminate degradation of ER proteins (Gardner et al., 2000; Gauss et al., 2006a; Plemper et al., 1999).

Hrd3p and Hrd1p interact biochemically with the small, non-glycosylated transmembrane protein Der1p, which spans the membrane four times (Gauss et al., 2006a; Knop et al., 1996). Although Der1p is essential for ERAD-L in yeast, its function remains obscure (Taxis et al., 2003; Vashist and Ng, 2004). Its human homologue, Derlin-1, is required for US11-mediated dislocation of the transmembrane protein class I MHC heavy chain (Lilley and Ploegh, 2004; Ye et al., 2004), but appears to be dispensable for the function of US2, which also assists in

the degradation of class I MHC products (Lilley and Ploegh, 2004). In yeast, the interaction between Der1p and the ligase complex is mediated through Usa1p, a protein of unknown function (Carvalho et al., 2006). The link to the cytosolic ATPase Cdc48p is provided by the UBX domain containing proteins Ubx2p (Neuber et al., 2005; Schuberth and Buchberger, 2005).

3.2.5.3.1.2 The Doa10 complex

Doa10p spans the ER membrane 14 times and associates with Ubc6p, Ubc7p, Cue1p, Ubx2p and Cdc48p (Carvalho et al., 2006). Doa10p (degradation of α2) was identified in a screen with a soluble fusion substrate, consisting of the deg1 domain, which targets the transcription factor Matα2 for degradation (Chen et al., 1993; Swanson et al., 2001). In vivo, Matα2 is degraded with two distinct pairs of UBCs, Ubc4/5p and Ubc6/7p (Chen et al., 1993). Doa10p participates only in the Ubc6/7p dependent degradation (Swanson et al., 2001). Doa10p is not only localized to the ER, but also to the inner and outer nuclear membrane, explaining how the nuclear protein Matα2 can be a substrate (Deng and Hochstrasser, 2006). Another substrate of Doa10p is the polytopic transmembrane protein Ste6*p. Here, the Doa10p complex associates with the cytosolic chaperones Hsp70/40p, presumably to unfold the protein (Nakatsukasa et al., 2008). Cdc48p, tethered via Ubx2p to the ligase complex, is only required for membrane anchored proteins, not soluble proteins, discovered in a screen with the deg1 domain fused to a membrane anchored protein (Ravid et al., 2006).

3.2.5.3.2 Mammalian E3's

Hrd1p has two mammalian orthologs, gp78 and HRD1 (Fang et al., 2001; Kikkert et al., 2004). Gp78 was the first mammalian ER bound E3 ligase that plays a role in the removal of ubiquitinated proteins from the ER. Subunits of the T cell receptor complex, TCRα and CD3δ are unstable when expressed in isolation, presumably due to the presence of unpaired charged residues in their transmembrane regions (Cosson et al., 1991). Gp78 interacts with the mammalian ortholog of Ubc7p (Ube2g2) via its CUE domain and ubiquitinates TCRα and CD3δ (Fang et al., 2001). Gp78 also

interacts with the AAA ATPase p97/Cdc48p (Zhong et al., 2004). The CUE domain is required for proper ubiquitination *in vivo*, but the p97 binding domain at the C-terminus is not (Chen et al., 2006; Zhong et al., 2004). This may be caused by gp78's ability to bind to a p97-interacting protein, Ubxd2, and to Derlin-1 (Liang et al., 2006). Apart from ubiquitinating T cell receptor subunits, gp78 has a wide range of substrates: it plays a role in cholesterol metabolism by degrading INSIG-1 and HMGR depending on sterol levels in the cell (Lee et al., 2006; Song et al., 2005). Other substrates include apolipoprotein B100 and the Z variant of α1-antitrypsin (Chen et al., 2006; Shen et al., 2006).

3.2.5.3.2.1 HRD1/Synoviolin

HRD1 and gp78 share more than 50% homology in their transmembrane regions. HRD1 derives its name from its overall similarity to yeast Hrd1p (Kostova et al., 2007). HRD1, like gp78, plays a role in ubiquitination of the unpaired T cell receptor subunits, TCRα and CD3δ (Kikkert et al., 2004; Nadav et al., 2003). Additionally, it ubiquitinates a misfolded G-protein coupled receptor, Pael-R, that plays a role in Parkinson's disease (Omura et al., 2006). Recent evidence suggests that it also aids in the distruction of cytosolic p53 (Yamasaki et al., 2007). In vitro, HRD1 can function together with Ube2G2/UBC7, but it remains unclear what E2 complexes it forms in vivo (Kostova et al., 2007).

In the results chapter of this thesis, protein complexes of HRD1 were analyzed. Interestingly, HRD1 was found as an integral member of a complex containing the Der1p orthologs Derlin-1 and Derlin-2, and SEL1L, the human homolog of Hrd3p. Derlin-1 and SEL1L/Hrd3p both play a crucial role in the dislocation of class I MHC heavy chains (Lilley and Ploegh, 2004; Lilley and Ploegh, 2005a; Mueller et al., 2006). The mammalian HRD1 complex therefore seems to be superficially similarly organized as the yeast Hrd1p complex. Many open questions remain: HRD1 itself was reported to not be required for ubiquitination of class I MHC heavy chains (Kikkert et al., 2004), the cognate E3 for this reaction has not been found, although analysis of the complexes involved in this process all hint towards HRD1 (Lilley and Ploegh, 2005a; Mueller et al., 2006). Another surprise came with the discovery of the E2 enzyme catalyzing the ubiquitination of heavy chains: E2-25K was uncovered

using a permeabilized cell system to analyze HC dislocation (Flierman et al., 2006; Shamu et al., 1999). However, analysis of the complexes containing HRD1 and SEL1L together with functional data uncovered the ortholog of yeast Ubc6p, Ube2J1 (see results section of this thesis). Due to the more numerous E2 and E3 enzymes present in mammalian cells, and the difficulties in a genetic approach, a simple analogy to the yeast system cannot be made.

3.2.5.3.2.2 March IV/Teb4

Teb4 is the human homolog of Doa10p with a similar topology (Hassink et al., 2005; Kreft et al., 2006). Similarly to yeast Hrd1p, Teb4 catalyzes its autoubiquitination together with Ube2g2/UBC7, but no ER dislocation substrates have been analyzed so far (Hassink et al., 2005).

3.2.5.3.2.3 Trc8

Trc8 is a polytopic transmembrane ER protein with a sterol sensing domain (Gemmill et al., 2002). It is involved in cholesterol and fatty acid biosynthesis, both of which are regulated by SREBPs (sterol response element binding proteins), but the mechanism is unknown (Brauweiler et al., 2007).

3.2.5.3.2.4 Cytosolic E3's

Entirely cytosolic E3's that regulate ER degradation are unknown in yeast. In mammals CFTR is ubiquitinated by the concerted action of multiple E3's. First the ring domain protein RMA1 together with Ube2j1 ubiquitinates CFTR ΔF508 and forms a complex with Derlin-1. Then, the cytosolic U-box protein CHIP binds to HSC70, Hdj2, and UbcH5a (an E2) to form a ubiquitin ligase complex that senses the folding state of the cytosolic portion of CFTR and induces ubiquitination of that portion (Meacham and Cyr, 2002; Meacham et al., 1999; Meacham et al., 2001; Younger et al., 2006). Another cytosolic ubiquitin ligase is Parkin, which together with HRD1, ubiquitinates Pael-R (Imai et al., 2002b; Imai et al., 2001).

F-box containing ligases have also been implicated in ER protein degradation in mammals. They bind to N-linked glycans of glycoproteins that have been dislocated

into the cytosol, prior to glycan removal by N-glycanase (Yoshida, 2005; Yoshida, 2007; Yoshida et al., 2005; Yoshida et al., 2002).

3.2.5.4 Crossing the lipid bilayer

The involvement of the ubiquitin-proteasome pathway in the degradation of ER proteins leads to one of the most pressing questions in the field: how do proteins cross the lipid bilayer?

3.2.5.4.1 Sec61

Yeast genetics, using CPY* as the dislocation substrate, suggested the involvement of Sec61 (Plemper et al., 1997). In addition to its role in translocation, it might be capable of performing the reverse reaction and act as a channel to release certain dislocation substrates into the cytosol (Plemper et al., 1997; Plemper et al., 1999; Zhou and Schekman, 1999). Additionally, purified microsomes from Sec61 mutant temperature-sensitive yeast cells are incapable of dislocation gpαF, which remains in the ER lumen bound to Sec61 (Pilon et al., 1998; Pilon et al., 1997). Early biochemical analysis of a variety of mammalian dislocation substrates also pointed to the involvement of Sec61 in dislocation (Bebok et al., 1998; de Virgilio et al., 1998; Wiertz et al., 1996b). Recently, proteasomes tethered to the Sec61 translocon were found (Kalies et al., 2005; Ng et al., 2007). However, the involvement of Sec61 remains controversial: The dislocation substrates examined appear to exit the ER fully glycosylated (de Virgilio et al., 1999; de Virgilio et al., 1998; Hiller et al., 1996; Wiertz et al., 1996a; Wiertz et al., 1996b), suggesting that the pore size of the channel must be larger than that of the import channel, which must accommodate only a single polypeptide chain. Moreover, mutants that affect an essential process such as translocation might lead to indirect effects on dislocation. Defects in dislocation might thus be the result not so much of a direct effect on the channel as the dislocation pore, but rather be due to an incomplete set of proteins present in the ER lumen. The biochemistry data also are difficult to interpret, as proteins in the Sec61 complex are rather abundant and might bind non-specifically to the overexpressed substrate proteins, depending on the ionic strength of the buffers used (Lilley thesis 2005). More recent, extensive biochemical analysis of yeast and

mammalian dislocation did not reveal Sec61 subunits (Carvalho et al., 2006; Huyer et al., 2004; Lilley and Ploegh, 2004; Ye et al., 2004, this thesis). Although the direct involvement of the Sec61 complex in the US2- mediated dislocation reaction was proposed earlier (Wiertz et al., 1996b), our proteomic analyses for US2 and US11 interacting partners have yet to confirm the presence of Sec61 subunits (Lilley and Ploegh, 2004; Lilley and Ploegh, 2005a; Loureiro et al., 2006).

3.2.5.4.2 Transmembrane proteins that could constitute the dislocon

The existence of a proteinacous pore, consisting of Hrd1p/HRD1 and/or Der1p/Derlin has been proposed (Gauss et al., 2006b; Ye et al., 2004). Der1p spans the ER membrane four times (Knop et al., 1996), its human homologs can oligomerize, and additional interacting partners also contain transmembrane segments (Lilley and Ploegh, 2005a, this thesis). This complex generically is referred to by the term dislocon, not only to distinguish it from the translocon involved in protein import into the ER, but also to convey the sense of direction (from ER to cytosol) inherent in the process and to suggest its involvement in removal of proteins no longer suitable for use. The organization of a putative dislocon could include numerous transmembrane segments that might contribute to the construction of a protein conducting channel. If all of the proteins that interact with Derlin-1, -2 and with SEL1L are part of a dislocon, then its complexity approximates or exceeds that of the Sec61 complex (See results chapter of this thesis).

The question of whether complete substrate unfolding necessarily precedes dislocation, or whether (partly) folded structures can be ferried across the ER as well, remains controversial. Based on the use of dihydrofolate reductase (DHFR) and GFP N-terminal fusions with Class I MHC heavy chains, dislocation assisted by US11 and US2 is not impeded by these bulky and stably folded attachments (Fiebiger et al., 2002; Tirosh et al., 2003), based on arrival in the cytoplasm of the tagged Class I MHC heavy chains in fluorescent or protease resistant form. Further, the radius of gyration of a typical high mannose oligosaccharide and its structure in solution are such that the mere presence of a single N-linked high mannose glycan would effectively preclude dislocation through protein conducting channels such as Sec61 (Van den Berg et al., 2004), although it remains to be investigated whether a

channel consisting of HRD1 and Derlin heterodimers could accommodate such a large pore. Finally, the involvement of Derlin-2 in infection by polyoma virus reinforces the concept that elements of (glyco)protein quality control may be used also to allow this virus to escape from the ER and access the cytoplasm (Lilley et al., 2006). The available evidence suggests that complete deconstruction of the polyoma capsid structure may not occur until its arrival in the cytoplasm (Lilley et al., 2006). The details of how proteins involved in quality control exert their effect in infection with polyoma virus is still not clear. It was recently reported that simian virus 40 (SV40) requires the oxidoreductases PDI and ERp57 prior to its exit from the ER, presumably for its partial uncoating. For release from the ER, the proteins SEL1L and Derlin-1 are essential, maybe for recognition and in forming some form of a channel (Schelhaas et al., 2007).

Establishing the identity of the channel through which proteins or (sub)viral particles leave the ER thus remains an important target. The identification of additional proteins that participate in these reactions, as reported in this thesis, is an obvious step towards that goal.

3.2.5.4.3 Lipid droplets

The conundrum of how folded entities or even large globular particles such as viruses can cross the lipid bilayer has been explained by an interesting alternative involving the formation of lipid droplets (Ploegh, 2007). Lipid droplets consist of a hydrophobic core of neutral lipids (triacylglycerides (TAG), cholesterol esters), surrounded by a monolayer of polar lipids (phospholipids). The surface of the droplet is populated with proteins, the majority of which await further characterization. There is some debate whether the core of the lipid droplet can hold proteins as well (Robenek et al., 2005; Wan et al., 2007; Welte, 2007). Lipid droplet purification is prone to artifacts, as exposure of the hydrophobic core might induce binding of hydrophobic stretches in proteins non-specifically (Fujimoto and Ohsaki, 2006a). Nevertheless, calnexin and BiP have been found in lipid droplets, and other, equally abundant ER proteins are not part of the lipid droplet repertoire (Brasaemle et al., 2004). Lipid droplets are present in all eukaryotes and are thought to bud off the ER, induced by certain lipid-droplet inducing proteins or an excess of lipids. The

bulk of the lipids necessary for formation of the droplet derives from the cytoplasmically exposed part of the ER lipid bilayer. Lipid droplets have been proposed to act as protein storage organelles, since lipid droplet localization of some proteins occurs in response to their elevated levels (Welte, 2007). The localization of these proteins is elsewhere in the cell, but when overexpressed, the droplet provides "shelter" to these proteins and removes them from their usual site of action, possibly to prevent aggregation (Fujimoto et al., 2006; Ohsaki et al., 2006b) or even for refolding by droplet associated chaperones (Cermelli et al., 2006; Jiang et al., 2007). Therefore, the term "refugee proteins" has been proposed (Welte, 2007). This idea can be taken a step further, in a way that excess proteins that will not be of use at a later time point might be degraded at the lipid droplet. The mechanism of this action has been hypothesized in detail (Ploegh, 2007, Figure 4). It can be envisioned that the droplet, although usually a round structure easily recognized in fluorescent microscopy, can have creases and curvatures that can accommodate the transmembrane domain of the protein to be degraded, or that viral particles "slip through" membrane curvatures at the site where lipid droplets form (Ploegh, 2007). This would help explain how for example the class I MHC heavy chain molecule, devoid of all cytoplasmically exposed lysines, can still be ubiquitinated (Furman et al., 2003). Additionally, why would the cell make the effort to relocalize ER dislocation substrates such as apolipoprotein B (ApoB) to lipid droplets prior to their destruction? Maybe because the lipid droplet itself is the site where dislocation can take place. Apolipoprotein B is normally secreted as part of the very low density lipoprotein (VLDL) particles that are part of the body's internal transport mechanism that carry cholesterol to tissues. When VLDL assembly is inefficient or when the proteasome is compromised, ApoB accumulates in lipid droplets. In proteasome inhibitor-treated cells, proteasomal subunits are found on lipid droplets and ApoB is present at the droplet in polyubiquitinated state (Fujimoto and Ohsaki, 2006b; Ohsaki et al., 2006a), suggesting a direct role for lipid droplets in dislocation (Ploegh, 2007). Multiple different modes of extraction likely exist. For example, as described earlier, misfolded CPY* in yeast clearly requires Hrd1p and Der1p for its degradation (Wolf and Schafer, 2005). When CPY* is expressed at high levels, it no longer requires Hrd1p. This pathway was called HRD/DER-independent or HIP pathway (Haynes et al., 2002). It critically depends on proteasome activity, an intact

UPR, and ER-to-Golgi transport. It is important to keep in mind that in mammalian cells most substrates, apart from class I in the US2 or US11 background, have to be overexpressed to be able to study their half life, raising the interesting question whether a form of HIP pathway is involved in the degradation of such substrates (Lilley thesis 2005). The HIP pathway itself might represent some form of dislocation pathway mediated by lipid droplets.

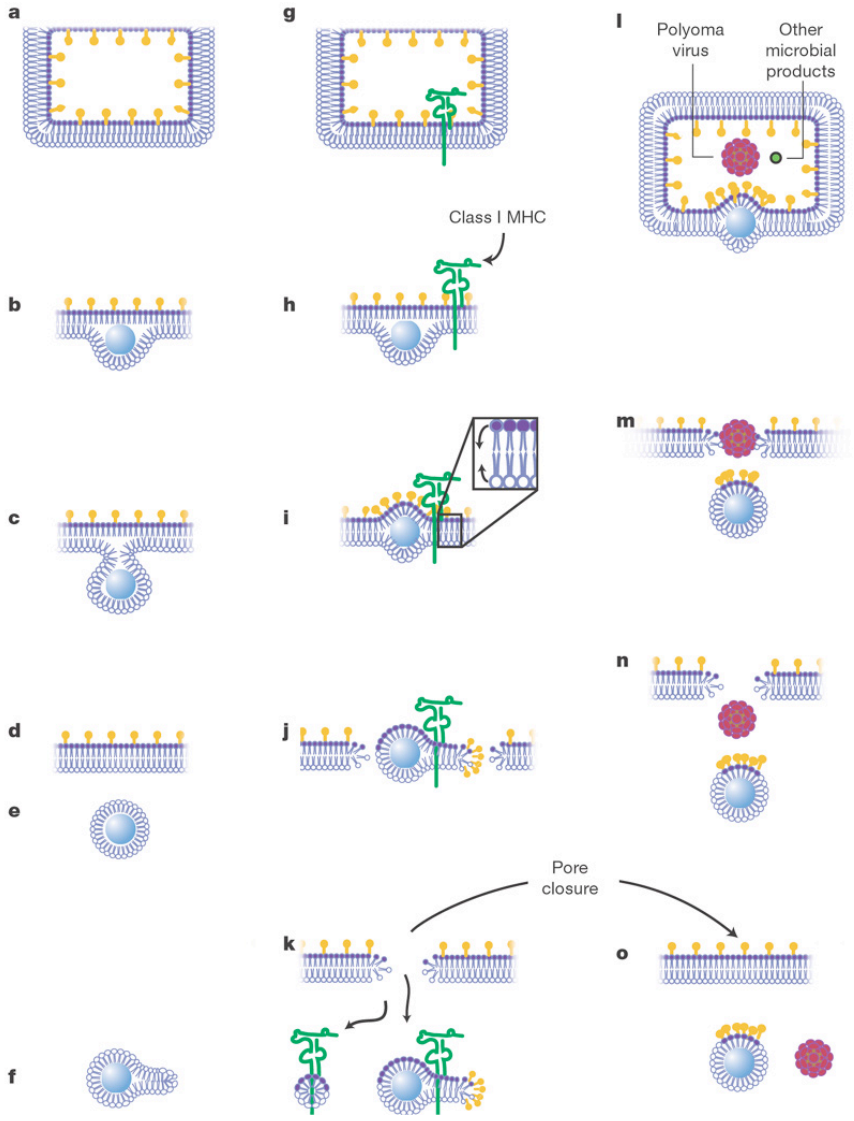

Figure 4: Biogenesis of lipid droplets and a model for their involvement in escape of macromolecules and viruses from the ER. a–f, Lipid droplet formation. Phospholipids on the luminal face of the ER lipid bilayer are depicted with purple headgroups; phospholipids on the cytoplasmic face are depicted with white headgroups. The yellow lollipops represent lysophospholipids and/or glucosylceramides. b, Lipid droplet formation starts with the formation of a neutral lipid 'lens' in between the two sheets of phospholipids. e, A lipid droplet consists of a neutral lipid core surrounded by a monolayer of phospholipids, derived by scission from the cytoplasmic face of the ER membrane. f, 'Wrinkles' on the surface of a lipid droplet impart local phospholipid

bilayer character. g–k, Resolution of lipid droplet intermediates by bicelle formation. The route taken by the class I MHC molecule (green) is representative of the fate proposed for a type I membrane protein about to be discharged from the ER, either as part of a lipid droplet (k, right) or as a structure composed solely of the class I MHC molecule and phospholipid (k, left). l–o, Polyoma virus leaves the ER in the slipstream of a lipid droplet, through a bicelle-stabilized hole created by the departure of the lipid droplet. Other microbial products (perhaps muramyldipeptide) might use the same route to access the cytosol. n, o, The hole created transiently (n) closes by coalescence of the luminal and cytoplasmic phospholipid leaflets, leaving polyoma virus in the cytoplasm (o). Taken from Ploegh, 2007.

3.2.5.5 The p97/Cdc48p complex

Removal of ubiquitinated substrates from the ER membrane requires the driving force of an AAA (ATPase associated with various cellular activities) ATPase. This ATPase, termed Cdc48p in yeast and p97 in mammals, is conserved from archea to mammals and is implicated in diverse cellular processes, such as spindle assembly after mitosis, membrane trafficking and fusion, nucleic acid repair and replication, and Ub-dependent degradation (Woodman, 2003). p97 forms a homohexameric barrel structure, with the 6 subunits forming a ring with a pore in the center (Zhang et al., 2000). Each subunit contains two AAA domains (D1 and D2) with the Walker A and B motifs essential for ATPase binding and hydrolysis, respectively (Zhang et al., 2000). The function of p97 is regulated through its adaptor proteins (Dreveny et al., 2004). The two p97 binding proteins Npl4 (nuclear binding protein 4, a zinc finger protein) and Ufd1 (ubiquitin fusion degradation 1), both of which contain poly-ubiquitin-binding domains, direct the activity of p97 towards ER degradation (Lord et al., 2002; Romisch, 2005; Stirling and Lord, 2006; Ye et al., 2001a; Ye et al., 2003). p97 on its own can recognize denatured proteins (Thoms, 2002) and bind to poly ubiquitin chains (Ye et al., 2003). The name “segregase” has been suggested for Cdc48p because its involvement in the disassembly and reassembly of protein complexes (Jentsch and Rumpf, 2007; Raasi and Wolf, 2007; Wang et al., 2004a).

p97/Cdc48p, together with Npl4 and Ufd1, associates strongly with the membrane fraction in yeast (Hitchcock et al., 2001; Jarosch et al., 2002b; Rabinovich et al., 2002; Ye et al., 2001a). Some ER degradation substrates arrest in a membrane-associated poly-ubiquitinated state when Npl4 and Ufd1 are mutated (Bays and Hampton, 2002; Bays et al., 2001b; Jarosch et al., 2002a; Jarosch et al., 2002b).

Most yeast substrates characterized require the Cdc48p/Npl4p/Ufd1p complex, suggesting that the different ER pathways converge at the cytosolic side of the ER (Huyer et al., 2004; Taxis et al., 2003). In case of the ER degradation protein pre-pro-α-factor, an entirely luminal and non-glycosylated protein, the ATPases of the 19S Cap of the proteasome take over the ATPase driving force of the Cdc48p complex (Lee et al., 2004). Cytoslic ATP-driven chaperones such as HSP70, HSP40, and HSP90 also play a role in the removal from the ER membrane (Taxis et al., 2003; Youker et al., 2004). The ATPase activity of p97 might provide the driving force for crossing the lipid bilayer, or they might be simply required for liberating dislocated substrates from the cytosolic face of the ER (Braun et al., 2002; Flierman et al., 2003; Kostova and Wolf, 2003; Meusser et al., 2005). In the case of the removal of CFTR from the ER membrane, p97 has been suggested to function as a non-essential factor that liberates thermodynamically stable domains from the membrane (Carlson et al., 2006). In mammals, p97 is always present when purifying Derlin or SEL1L complexes (Lilley and Ploegh, 2005a) Proteins that can recruit p97 to these complexes have been identified, one of which is VIMP (Ye et al., 2004). Another one, UBXD8, will be discussed in the results chapter of this thesis.

3.2.5.6 From the membrane to the proteasome: association with ubiquitin escort factors and deglycosylation

In US2 or US11 expressing cells, the class I MHC heavy chain is completely dislocated to the cytosol when the proteasome is inhibited (Wiertz et al., 1996a; Wiertz et al., 1996b). This is not usually the case, as there are only few other examples for complete removal from the ER when the proteasome is blocked. The truncated version of ribophorin, RI_{332}, can be arrested deglycosylated in the cytosol upon treatment with proteasome inhibitors (Kitzmuller et al., 2003), and misfolded class I MHC heavy chains (independently of US2 or US11) are detectable as deglycosylated intermediates in the cytosol as well (Hughes et al., 1997).
Other substrates, such as tyrosinase, TCRα, CD3δ arrest at the ER membrane in a partially dislocated state when the proteasome is inhibited (Elkabetz et al., 2004; Halaban et al., 1997; Menendez-Benito et al., 2005; Misaghi et al., 2004; Tiwari and

Weissman, 2001; Yang et al., 1998) or –in yeast- when the Cdc48p cofactors Npl4p or Ufd1p are mutated (Bays et al., 2001b; Jarosch et al., 2002b). These data suggest tight coupling between extraction (by the p97 ATPase) and degradation by the proteasome. It has been proposed that this coupling is achieved via ubiquitin-escort factors that can bind poly-ubiquitin (via their ubiquitin-associated or Uba domain) and the proteasome (via their ubiquitin-like or Ubl domain) (Elsasser et al., 2004; Elsasser et al., 2002; Hartmann-Petersen and Gordon, 2004; Hartmann-Petersen et al., 2003; Medicherla et al., 2004; Richly et al., 2005). The proteins Rad23 and Dsk2 have been shown to escort and shuttle ubiquitinated proteins to the proteasome (Elsasser et al., 2004; Medicherla et al., 2004), maybe preventing premature ubiquitin chain disassembly by cellular DUBs and/or aggregation (Meusser et al., 2005). The Rad23/Dsk2 proteins convey proteins from the site of ubiquitination to the site of destruction by increasing affinity the longer the ubiquitin chains are (Meusser et al., 2005). Cdc48p can associate with other co-factors that influence the processing of the substrate: Ufd2p, Ufd3p, and Otu1p. Ufd2p and Ufd3p are are ubiquitin receptors themselves, while Otu1p has deubiquitinating activity (Rumpf and Jentsch, 2006). Ufd2p consists of a U-box domain structurally similar to a RING finger and extends preassembled ubiquitin chains on the substrate (Aravind and Koonin, 2000). Because of this ubiquitin elongation activity, Ufd2p belongs to the class of E4 enzymes (Hoppe, 2005). Ufd3p competes for the same binding site with Ufd2p on Cdc48p (Rumpf and Jentsch, 2006). Ufd3p binds ubiquitin chains via an uncharacterized ubiquitin binding domain (Mullally et al., 2006). With these adaptor factors in hand, p97/Cdc48p exerts control over the size of the ubiquitin chain, thereby even capable of preventing degradation (Richly et al., 2005; Rumpf and Jentsch, 2006).

3.2.5.6.1 Deglycosylation

Another protein capable of binding to p97/Cdc48p is peptide N-glycanase (PNGase) 1. PNGase1 is expressed in all eukaryotes and removes N-linked glycans from misfolded proteins once they become accessible in the cytosol prior to proteasomal degradation (Hirsch et al., 2003; Suzuki et al., 2000). In a yeast two hybrid assay, the

sole binding partner of yeast PNGase is Rad23p (Suzuki et al., 2001). Png1p-Rad23p occurs as a distinct subcomplex from Ufd2p-Rad23p. In mammals, PNGase binds to multiple proteins, including the Rad23p orthologs HR23A/B, p97, the proteasome (Katiyar et al., 2004), gp78 (Li et al., 2005), and UBX domain containing proteins (Raasi and Wolf, 2007). PNGase has not been found to be physically associated with the ER degradation complexes (Suzuki, 2007). Surprisingly, removal of PNG1 or inhibition of the activity of PNGase (Suzuki et al., 2000) with the chemical inhibitor Z-Vad-fmk (Misaghi et al., 2004) causes no general degradation defect, although PNGase is clearly involved in the deglycosylation of heavy chains after their dislocation into the cytosol (Blom et al., 2004; Hirsch et al., 2003). The only substrate whose degradation clearly depends on PNGase activity is the A-chain of ricin toxin (Kim et al., 2006). It is possible that not all proteins require deglycosylation before they enter the proteasome. Instead, the glycans might be removed for entirely different reasons, for example for recycling of hexoses in lysosomes (Suzuki, 2007), or because of the preferential presentation of non-glycosylated peptides by class I MHC (Kario et al., 2008).

3.3 Viruses can co-opt the cellular dislocation machinery: class I MHC dislocation

Viruses depend entirely on their hosts for replication. The mammalian immune system has developed strategies to detect virally infected cells and eliminate them. Therefore, many viruses have evolved unique methods to manipulate the host response (Tortorella et al., 2000c).

An effective method for a virus to evade the immune system is to interfere with antigen presentation of the host cell. All nucleated cells present peptides (antigen) on Class I MHC products at the cell surface. These peptides are generated in the cytosol from cellular or virally-derived proteins through normal protein turnover, are loaded onto Class I MHC products and are then presented to specialized immune cells, the CD8+ cytotoxic T-lymphocytes (CTLs) on the plasma membrane (Tortorella et al., 2000c). If the peptide is virally derived, the CTLs may recognize the antigen as foreign and eliminate the infected cell (Shresta et al., 1998). CTLs are therefore

crucial for control of viral dissemination (Harty et al., 2000). CTLs recognize the class I MHC as a trimeric complex consisting of the class I MHC heavy chain (HC), the β2-microglobulin (β2m, light chain) and an 8-10 residue antigenic peptide. The α1 and α2 domains of the HC form a groove or cleft that displays the peptide, while α3 (the immunoglobulin (Ig)-like domains of HC) and β2m support the peptide-binding groove (Bouvier, 2003).

3.3.1 Assembly of class I MHC products and antigen presentation

The 43 kDa Class I MHC HCs enter the ER co-translationally via the Sec61 channel. HCs acquire a single N-linked glycan upon insertion into the ER membrane. Subsequent folding obeys the normal rules for glycoprotein biosynthesis and involves calnexin (CNX) and calreticulin (CRT) and the thiol oxidoreductase ERp57. The association with CNX prevents HCs from aggregation and also promotes their assembly with b2m (Bouvier, 2003; Degen et al., 1992). Surprisingly, the assembly and cell surface expression of class I MHCs is normal in a cell line lacking CNX (Sadasivan et al., 1995; Scott and Dawson, 1995). There has been some speculation that other ER chaperones such as BiP, can substitute for CNX (Degen et al., 1992; Nossner and Parham, 1995).

The HC β2m heterodimer is a rather unstable complex in vitro (Bouvier and Wiley, 1998). In vivo, the majority of "empty" class I HC β2m complexes are associated with CRT, ERp57, and two MHC encoded chaperones, TAP and tapasin to form the "class I loading complex", or "peptide loading complex (PLC)". The **t**ransporter **a**ssociated with antigen **p**resentation (TAP) is an ATP-dependent pump with the two subunits, TAP1 and TAP2, that translocates antigenic peptides into the ER (Kelly et al., 1992). Tapasin, a type I transmembrane glycoprotein that belongs to the immunoglobulin superfamily (Mayer and Klein, 2001) mediates binding between the Tap transporter and the "empty" HC/β2m heterodimers (Sadasivan et al., 1996). Tapasin also stabilizes and retains the HC/β2m complexes in the ER (Bouvier, 2003; Ortmann et al., 1997). Recently, PDI has been implicated in regulating the state of the disulfide bond in the peptide binding groove (Park et al., 2006).

Upon loading of the peptide into the MHC binding groove, the assembled class I HC/β2m/peptide complex enters the secretory pathway and travels to the plasma membrane. On the cell surface, the peptides are subject to inspection by the TCR or by invariant receptors on NK cells (Cresswell, 2005; Heemels and Ploegh, 1995; Lanier, 2005; Rammensee, 2004).

3.3.2 Recognition of virally infected cells by CTLS and NK cells

The vast majority of antigenic peptides are derived from **d**efective **ri**bosomal **p**roducts or DRiPs. These are prematurely terminated translation products (up to 30% of all proteins translated in the cytosol) on free ribosomes in the cytoplasm that never reach their final protein length. DRiPs half a very short half-life of about 20 min and are rapidly degraded by the cytosolic proteasome (Schubert et al., 2000; Turner and Varshavsky, 2000; Yewdell et al., 2001). Therefore, they deliver a continuous supply of antigenic peptides for display by class I MHC products (Princiotta et al., 2003). Degradation of misfolded ER proteins can also provide source material for class I MHC antigen presentation (Ostankovitch et al., 2005), but the extent to which these misfolded ER proteins contribute to the peptide pool remains unknown.

In a virally infected cell, the virus hijacks the cellular translation machinery. In the course of the infection, mainly “non-self” or “foreign” peptides are loaded onto class I MHC molecules and displayed to the TCR, which ultimately leads to the destruction of the infected cells by the CTL (Andersen et al., 2006). Cells that lose their ability to display class I MHC molecules on their cell surface, are instead examined by another set of immune cells, the natural killer (NK) cells, that will eliminate “suspicious” cells without class I MHC complexes in a manner similar to CTLs (Lanier, 2005).

3.3.3 Virally encoded immunoevasins

Intracellular pathogens have developed means to counteract MHC class I antigen presentation, thereby evading immune surveillance (Tortorella et al., 2000a; Tortorella et al., 2000c). Virally encoded immunoevasins target almost every aspect of antigen presentation. They can inhibit peptide loading by binding to TAP (Herpes Simplex virus [HSV] ICP47, human cytomegalovirus [HCMV] US6, bovine herpes virus 1 UL49.5), retain HC molecules in the ER (adenovirus E3/19K, HCMV US3), block ER to Golgi transport of HCs (mouse cytomegalovirus [MCMV] m152), misdirect MHC molecules to lysosomal compartments (MCMV m06, human herpes virus [HHV] U21), inititate the internalizing and degradation of MHC molecules from the cell surface (kaposi's sarcoma associated HV [KSHV k3 and k5], human immunodeficiency virus [HIV] Nef), or catalyze the dislocation of HC molecules form the ER to the cytosol (HCMV US2, US11, US10, mouse herpes virus 68 [MHV] mK3). Some immunoevasins hinder generation of antigenic peptides in the cytosol by inhibiting proteolysis of viral proteins (Epstein barr virus [EBV] nuclear antigen (EBVNA-1, HCMV E protein pp65, HIV Tat) or encode class I MHC decoy molecules to impede CTL or NK cell recognition (HCMV UL18, UL40, UL142, MCMV m04), see figure 5 (Arase and Lanier, 2004; Hengel and Koszinowski, 1997; Lybarger et al., 2003; Mocarski, 2004; Orange et al., 2002; Tortorella et al., 2000a; Yewdell and Hill, 2002).

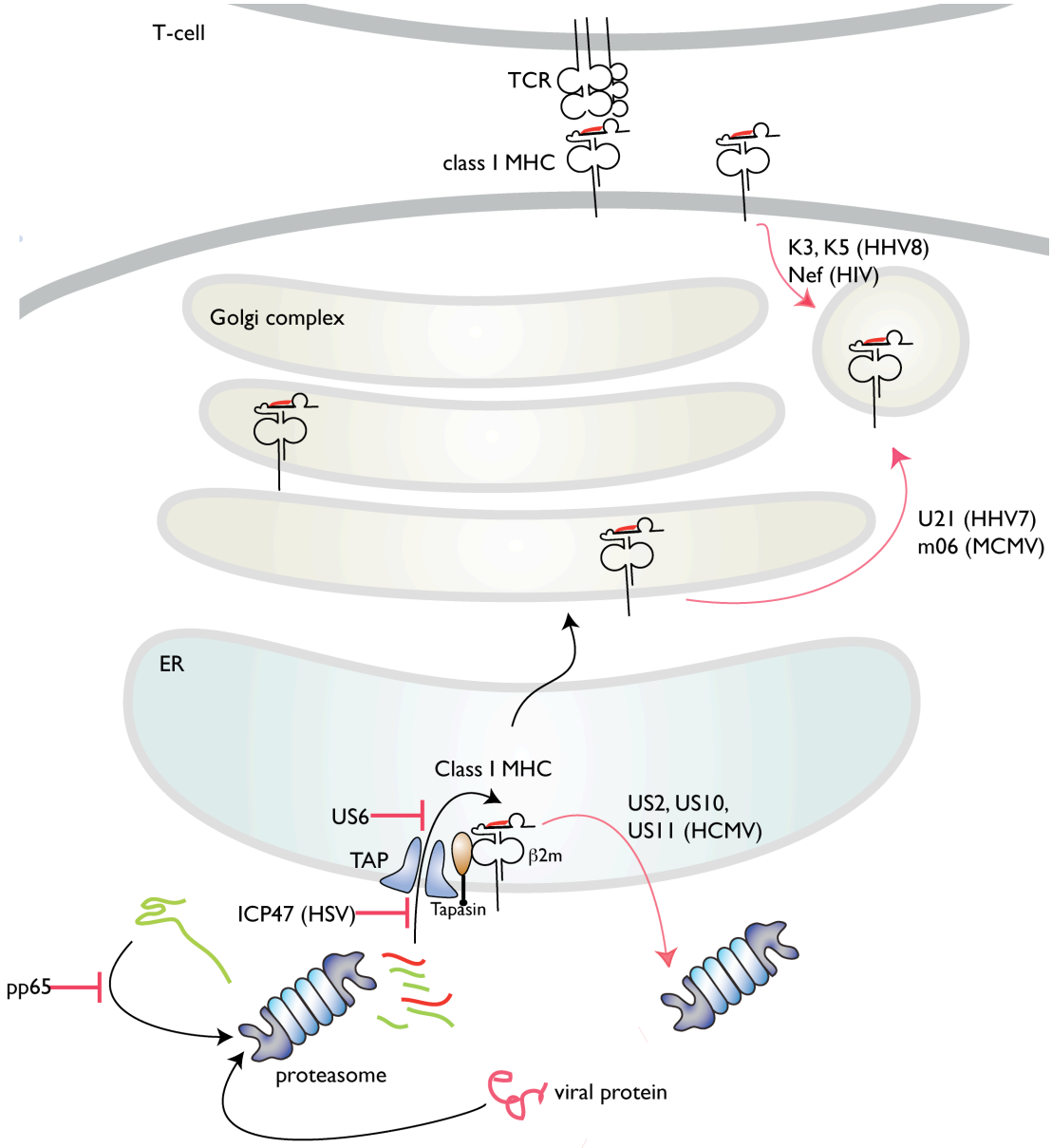

Figure 5: Schematic representation of class I MHC antigen presentation to the TCR on cytotoxic T-cells. Viral proteins that interfere with this process are indicated.

3.3.4 HCMV immune evasion

Whereas many viruses broadly target cellular factors in a number of subcellular compartments, human cytomegalovirus has confined its efforts to evade immune surveillance mainly to the ER. HCMV possesses a number of ER-resident type I transmembrane glycoproteins that disrupt MHC class I MHC antigen presentation

(Ahn et al., 1996). They are encoded within the unique short (US) region of its genome, and belong to the set of immediate early or early US genes, that are synthesized at early times after infection (Ahn et al., 1996; Jones et al., 1995).
US3 is the first immunoevasin that is synthesized. It binds and retains class I MHC HCs in the ER, thereby preventing their egress to the cell surface (Jones et al., 1996). US6, also an immediate early gene, binds to TAP from within the ER and blocks peptide transport (Ahn et al., 1996; Ahn et al., 1997; Hengel et al., 1997; Lehner et al., 1997). US10 retains HCs in the ER (Furman et al., 2002a) and catalyses their dislocation in a poorly understood mechanism (Boyoun Park, and Hidde Ploegh, unpublished observation). US2 and US11 are the best characterized immunoevasins of HCMV. They are both synthesized at early times after infection and, like US10, but incomparably faster, catalyze the dislocation of class I MHC HCs from the ER to the cytosol, where they are degraded by the cytosolic proteasome (Ahn et al., 1996; Jones and Sun, 1997; Wiertz et al., 1996a; Wiertz et al., 1996b).

3.3.4.1 US2- and US11- mediated dislocation

US2 and US11 are small type I transmembrane glycoproteins without any obvious cellular homologs or sequence motifs that would help predict their function. In HCMV-infected cells or in U373MG astrocytoma cells expressing US2 or US11, class I MHC HCs after completion of their translocation into the ER membrane and their glycosylation, are destroyed with rapid kinetics (Wiertz et al., 1996a; Wiertz et al., 1996b). In US11-expressing cells, the half-life of HCs after their synthesis is a mere 2-5min (Wiertz et al., 1996a). US11 seems to be slightly more efficient at targeting HCs than US2 (Wiertz et al., 1996b). Still, this rate of degradation is an order of magnitude faster than any other known mammalian model substrates.
US2 and US11 both associate with HCs in the ER shortly after their synthesis and subsequently initiate their transport across the ER membrane into the cytosol (Wiertz et al., 1996a; Wiertz et al., 1996b). In the cytosol, their N-linked glycan is removed by PNGase, and the deglycosylated HC molecule is ubiquitinated and subsequently degraded by the cytosolic proteasome (Figure 6, Hirsch et al., 2003; Wiertz et al., 1996a; Wiertz et al., 1996b). Since US2 and US11 accomplish this rapid dislocation

and destruction without the help of any other viral accessories, US2 and US11 hijack the cellular machinery that dislocates proteins that fail to fold into their proper conformation (Romisch, 2005; Tsai et al., 2002).

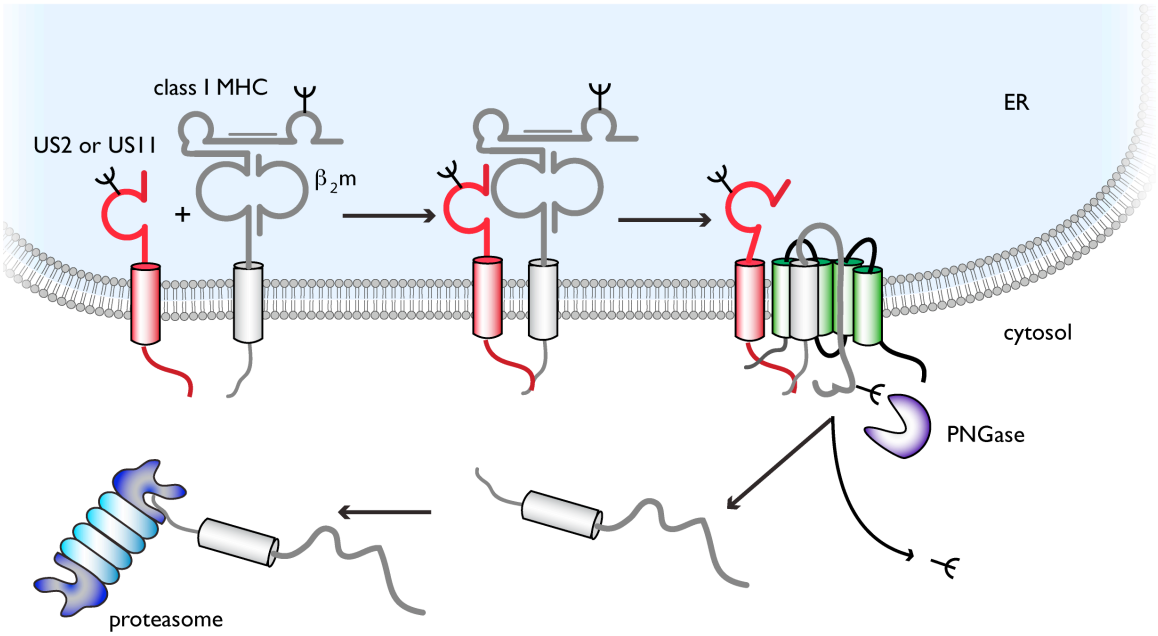

Figure 6: US2 and US11-mediated dislocation. Both proteins associate with newly synthesized HCs and target them to the dislocation machinery, here represented as a putative dislocon in green.

3.3.4.1.1 US2 structure and function

US2 is a type I transmembrane glycoprotein of 199 amino acids with a single N-linked glycan (Wiertz et al., 1996b). US2 is retained in the ER without any obvious ER-retention motifs. It is targeted to the ER membrane via a non-cleavable signal sequence. This unusual mode of insertion into the ER membrane is somewhat inefficient, and leads to a significant amount of US2 that is non-glycosylated in the cytosol where it is rapidly degraded (Gewurz et al., 2002). Nonetheless, this non-cleavable signal sequence, when appended on other type I transmembrane proteins is

sufficient to direct them to the ER where it is also refractory to cleavage (Gewurz et al., 2002). Replacing the signal sequence of US2 with that of another transmembrane protein does not affect its function (Gewurz et al., 2002). In the ER, US2 assumes an Ig-type fold consisting of 7 β strands. The luminal domain associates with the lumenal domain of the class I MHC heavy chain (Gewurz et al., 2001a). ER-resident US2 is a relatively short-lived protein: it is also a substrate of the cellular dislocation machinery and is dislocated and degraded in the cytosol with a half-life of 1 hr (Gewurz et al., 2002; Lilley and Ploegh, 2004). US2 binds to sites on the HC molecule not used by other class I MHC interacting proteins, the α3 domain and the peptide binding region (Gewurz et al., 2001a). The association of US2 with HC is allele-specific, but the mere interaction with HC is not sufficient for dislocation (Barel et al., 2003b; Gewurz et al., 2001b). Truncated forms of US2 lacking all or part of its14 amino acid long tail can bind to HCs but do not initiate their dislocation (Chevalier et al., 2002; Furman et al., 2002b; Oresic et al., 2006). When replacing the US3 tail sequence with that of US2 the chimeric molecule is capable of catalyzing dislocation (Chevalier and Johnson, 2003).

US2 mutants incapable of dislocating HCs stably interact with properly folded class I MHC complexes in the ER but then exit together with class I through the secretory pathway to the cell surface (Furman et al., 2003; Gewurz et al., 2001b). A biochemical search for proteins that interact specifically with full-length and therefore dislocation competent US2, and not with tailless US2 incapable of dislocating class I molecules, afforded the identification of signal peptide peptidase (SPP) as a cellular factor required for dislocation (Loureiro et al., 2006).

The crystal structure of US2 in contact with HLA-A2, peptide and b2m has been solved (Gewurz et al., 2001a). US2 binding does not alter the quaternary structure of the trimeric class I molecule. US2 can only bind to properly folded class I MHC complexes, as US2 is incapable of targeting HC for degradation in astrocytoma U373 cells in which the β2m levels have been reduced (Blom et al., 2004). The residues in HC that contact US2, which are between the peptide binding region and the α3 domain, are present only in certain groups of HLA locus alleles, notably HLA-A, most HLA-B products and HLA-G, although HLA-G with much reduced kinetics (Barel et al., 2006b; Barel et al., 2003a; Barel et al., 2003b; Gewurz et al.,

2001a; Pizzato et al., 2004). Only those class I molecules are dislocated that display the HLA-A2 consensus binding site, for example HLA-B7 or HLA-Cw3 do not conform to the US2 binding site and are therefore spared form degradation (Rehm et al., 2002; Schust et al., 1998). Initially, the HLA-A2 tail was thought to be required for US2 mediated dislocation. Truncated versions of HC expressed in astrocytoma U373 cells proved to be resistant to dislocation by US2 contrary to their full-length counterparts (Story et al., 1999). In contrast, HLA-A2 molecules und truncations thereof were expressed together with b2m in a murine J26 cell line. In those cells, the tail is not a requisite of dislocation (Barel et al., 2003b). Presumably the amount of HCs already endogenously present in the human U373 cells together with the overexpressed HLA-A2 constructs exceeded the capacity of the US2 dislocation machinery, whereas the murine cells, lacking any endogenous class I MHC HC molecules, where capable of dislocation (Lilley and Ploegh, 2005b).
Once HCs reach the cytosol, they have access to the ubiquitination machinery. Removal of HC's tail lysines or of the whole tail in some systems does not block dislocation (Barel et al., 2003b; Furman et al., 2003), but a functional ubiquitin system is required (Furman et al., 2003; Hassink et al., 2006). Mutation of all lysines in the HC molecule together with a "protection group" at its N-terminus to block all free amino groups eliminates dislocation by US2 (Hassink et al., 2006).

3.3.4.1.2 US11 structure and function

US11 is a type I transmembrane glycoprotein of 215 amino acids with a single N-linked glycan (Wiertz et al., 1996a). Just like US2, US11 lacks an ER-retention motif, despite being targeted to the ER by a signal sequence. US11's signal sequence is also unusual in that it is cleaved post-translationally (Rehm et al., 2001) and the rate of its cleavage depends on certain residues in the transmembrane domain (Lilley et al., 2003; Rehm et al., 2001). There are no structural data for US11, but the luminal domain of US11 is thought to assume an Ig-like fold similar to US2 and mediate interaction with the luminal domain of class I MHC heavy chain (Gewurz et al., 2001a; Lilley et al., 2003). US11 can interact with properly folded and –unlike US2- also with free uncomplexed HCs, independently of their state of synthesis

(Lilley and Ploegh, unpublished, and (Barel et al., 2006a). In general, US11 seems to have a broader range of HC substrates than US2: not only can it initiate dislocation of improperly folded mutant class I molecules (Furman et al., 2003), but in addition to the extensive array of HLA-A and HLA-B locus products, it can even induce dislocation of several murine class I MHC molecules (Barel et al., 2003a; Machold et al., 1997). US11 appears to bind to HC at its α1 and α2 regions, those regions that also mediate peptide binding (Barel et al., 2003a). The class I cytosolic tail is essential for US11-mediated dislocation from both human U373 and mouse J26 cells (Barel et al., 2006b; Barel et al., 2003a; Story et al., 1999). The determining attribute of the tail is not so much its sequence as its length: removal of 10 amino acids or even just 2 amino acids (Barel et al., 2006b; Barel et al., 2003a) render the HC molecule resistant to dislocation, as does the addition of the green fluorescent protein (GFP) at its C-terminus (Barel et al., 2003a). HLA-E gains sensitivity to dislocation by US11 when its 29 amino acid tail is extended by 2 residues (Barel et al., 2006b). Replacement of HC's C-terminal 10 amino acids with the sequence of the 10 amino acid hemagglutinin (HA) peptide transform this molecule again into a substrate for US11-mediated dislocation (Margot Furman and Hidde Ploegh, unpublished). Therefore, the existence of a cellular factor in the cytosol that binds to the HC tail in a length-dependent manner and is recruited by US11, has been proposed, but has not yet been identified (Patrick Stern Thesis 2003).

Analysis of mutations in the US11 protein itself showed that the US11 tail (US11 200) is dispensable for dislocation, but the transmembrane domain is essential. A truncated US11 protein lacking its tail and transmembrane region is incapable of initiating dislocation, as is a chimeric protein consisting of the luminal region and tail of US11 separated by the transmembrane domain of the unrelated protein CD4. A single point mutant in the transmembrane region of US11 renders it dislocation incompetent: Mutation of the polar glutamine (Q) residue at position 192 to the hydrophobic leucine (L) residue (US11 Q192L) frustrates the dislocation reaction (Lilley and Ploegh, 2004; Lilley et al., 2003), presumably because the polar residue Q is essential for forming inter-helical hydrogen bonds within the lipid bilayer that would allow interaction with cellular factors of the dislocation machinery (Lilley et al., 2003). In contrast to US2, all non-functional US11 mutants examined to date

bind to HCs and retain them in the ER (Lilley and Ploegh, 2005b). A polar residue in the transmembrane region of US2 (asparagine 172) is not essential for dislocation, as its replacement with leucine had no effect on dislocation (Lilley and Ploegh, 2005b). A search for proteins that bind to dislocation competent US11, but not to the incompetent mutant US11QL led to the discovery of proteins that are conserved to yeast, such as the Derlin proteins and the SEL1L/HRD1 complex (Lilley and Ploegh, 2004; Lilley and Ploegh, 2005a).

The cellular mechanism used to dispose of misfolded proteins in the secretory pathway is a sequential multi-step process, consisting of the dislocation reaction from the ER to the cytosol, and subsequent degradation of the dislocated protein in the cytosol by the cytosolic proteasome (Hirsch et al., 2004). The superficially similar dislocation reactions catalyzed by US2 and US11 provides an excellent model system to study retrotranslocation. These two viral proteins provide the unique opportunity to study the cellular ER dislocation reaction mediated by two distinct effectors that utilize two distinct disposal pathways.

3.4 Aim of this work

The aim of this work was to gain insight into the process of ER associated degradation, or dislocation, in mammalian cells.

The process of dislocation is likely to be fundamental to cell survival, as many diseases are linked to a defect in ER homeostasis. Although the characterization of dislocation in yeast has helped identify a lot of the proteins responsible for dislocation, the mammalian counterparts often remain elusive, due to multiple orthologs, the difficulty of a genetic approach, and few dislocation substrates. The protein Hrd3p in yeast is required for proper function of the yeast dislocation ubiquitin ligase Hrd1p, but its direct role in dislocation was less well understood. The aim of this thesis was to analyze the human ortholog of Hrd3p, SEL1L, and investigate its contribution to dislocation. I was able to show that SEL1L is indeed the human homolog of Hrd3p, and that it is essential for US11-mediated dislocation of class I MHC heavy chains, and for the dislocation of truncated ribophorin RI_{332}. This demonstrated that US11 indeed co-opts a conserved pathway, and that proteins involved in US11-mediated dislocation are also used in the dislocation of substrates independently of viral accessories. Furthermore, I report that SEL1L is involved in substrate recognition. To gain more inside into SEL1L's function, I identified interaction partners of SEL1L and analyzed their contribution to dislocation.

4 Results and Discussion

4.1 SEL1L, the homolog of yeast Hrd3p, is involved in protein dislocation from the mammalian ER

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SEL1L is part of a multiprotein complex consisting of Derlin-1, Derlin-2, HRD1, and p97 (Lilley and Ploegh, 2005a). To address its role in dislocation, we used a model dislocation system consisting of the viral proteins US2 and US11 (Wiertz et al., 1996a; Wiertz et al., 1996b). US2 and US11 both mediate the rapid dislocation of class I MHC products but do so by recruiting different sets of proteins (Lilley and Ploegh, 2004; Loureiro et al., 2006).

We established first that SEL1L is part of a conserved complex present in all tested cell lines (HeLa, astrocytoma, rat glioblastoma cells). To address whether SEL1L plays a role in dislocation, we designed knockdown constructs targeting SEL1L or an unrelated protein not present in the cell (GFP) as a control. US11 cells stably transduced with SEL1L knockdown constructs were compromised in their ability to dislocate class I MHC. US2 cells, which target the exact same proteins for dislocation (class I MHC products) remained dislocation competent, thereby serving as a control for proper ER function in the presence of reduced levels of SEL1L.

To confirm that SEL1L plays a general role in dislocation independently of viral accessories we tested several mammalian dislocation substrates (TCRα, RI_{332}) in HeLa cells stably transduced with shRNAs against SEL1L. We observed a delay in dislocation for RI_{332} in the knockdown background, but not for TCRα, suggesting that –as has been proposed in yeast (Vashist and Ng, 2004)- different substrates are retrotranslocated by different sets of proteins.

The bulk of the SEL1L protein is in the lumen of the ER and contains several repeat regions (Biunno et al., 1997) that might be involved in substrate recognititon. Therefore, we analyzed whether SEL1L could discriminate between properly folded

proteins and those that are terminally misfolded. To this effect we used the misfolded version of ribophorin, RI_{332} (de Virgilio et al., 1999; de Virgilio et al., 1998; Kitzmuller et al., 2003) after we demonstrated that RI_{332} requires SEL1L for its dislocation. In re-immunoprecipitation experiments, we showed that SEL1L binds to misfolded RI_{332}, but not to properly folded ribophorin. These results establish a role for SEL1L in substrate selection.

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SEL1L, the homologue of yeast Hrd3p, is involved in protein dislocation from the mammalian ER

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Protein quality control in the endoplasmic reticulum (ER) involves recognition of misfolded proteins and dislocation from the ER lumen into the cytosol, followed by proteasomal degradation. Viruses have co-opted this pathway to destroy proteins that are crucial for host defense. Examination of dislocation of class I major histocompatibility complex (MHC) heavy chains (HCs) catalyzed by the human cytomegalovirus (HCMV) immunoevasin US11 uncovered a conserved complex of the mammalian dislocation machinery. We analyze the contributions of a novel complex member, SEL1L, mammalian homologue of yHrd3p, to the dislocation process. Perturbation of SEL1L function discriminates between the dislocation pathways used by US11 and US2, which is a second HCMV protein that catalyzes dislocation of class I MHC HCs. Furthermore, reduction of the level of SEL1L by small hairpin RNA (shRNA) inhibits the degradation of a misfolded ribophorin fragment (RI_{332}) independently of the presence of viral accessories. These results allow us to place SEL1L in the broader context of glycoprotein degradation, and imply the existence of multiple independent modes of extraction of misfolded substrates from the mammalian ER.

Introduction

Quality control of newly synthesized glycoproteins involves recognition of misfolded proteins in the ER, where they are either returned to a productive folding pathway or are targeted for degradation (Ellgaard and Helenius, 2003). Terminally misfolded glycoproteins are transferred to the cytoplasm for proteasomal proteolysis, a process termed dislocation (Wiertz et al., 1996a,b).

How the cell distinguishes between newly synthesized proteins that have not yet acquired their correct folding state and proteins that are terminally misfolded remains a mystery. In yeast, genetic analysis has shown the involvement of a limited set of proteins that contribute to recognition of misfolded proteins and their subsequent degradation. The secretory protein carboxypeptidase Y (CPY), when engineered to yield a misfolded product, CPY*, has served as a substrate to identify the genetic factors that interfere with its disposal. Der1p was identified as a key player in clearing the yeast ER of misfolded CPY* (Knop et al., 1996; Hill and Cooper, 2000; Walter et al., 2001; Haynes et al., 2002). HMG-CoA reductase, which is a transmembrane protein, has similarly served as a reporter substrate, allowing Hampton et al. (1996) to define HRD1 and HRD3 as essential for its degradation (Gardner et al., 2000, 2001).

Hrd1p/Der3p has ubiquitin ligase activity (E3) and forms a complex predominantly with the ubiquitin-conjugating enzymes (E2s) Ubc7p and Ubc1p (Bays et al., 2001a), which are themselves recruited by the protein Cue1p (Biederer et al., 1997) to the site of degradation. Hrd3p is required for regulating the activity and stability of Hrd1p/Der3p (Plemper et al., 1999), but the function of Hrd3p in protein degradation remains obscure. Hrd3p has a large luminal domain that contains different sets of repeated regions that might be involved in substrate recognition or form complexes with chaperones. Apart from the Ring-H2 ligase Hrd1p/Der3p, there are additional ER membrane–resident E3s, such as Doa10p (Swanson et al., 2001).

Depending on the topology of the ER degradation substrates, different proteins are required for their clearance (Ahner and Brodsky, 2004). Substrates with defects in their cytosolic domain require Doa10p in yeast. Substrates with defects in their luminal portion require the ER lectin Htm1p/Mnl1p, the ubiquitin ligase Hrd1p/Der3p-Hrd3p, Der1p, and proteins involved in ER–Golgi trafficking (Vashist and Ng, 2004). The two pathways

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Abbreviations used in this paper: CPY, carboxypeptidase Y; EDEM, ER degradation-enhancing mannosidase-like; HC, heavy chain; MHC, major histocompatibility complex; NHK, null Hong Kong; PDI, protein disulfide isomerase; shRNA, small hairpin RNA; HCMV, human cytomegalovirus; RI, ribophorin; TPR, tetratricopeptide repeat.

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merge when leaving the ER; extraction of the ubiquitin-modified substrate occurs with the assistance of Cdc48p/p97 and its cofactors Ufd1p and Npl4p, culminating in delivery to the proteasome and proteolysis of the substrate (Meyer et al., 2000, 2002; Ye et al., 2001, 2003; Wang et al., 2004; Park et al., 2005). Recent studies analyzed the composition of the protein complexes involved. The Doa10p complex contains Ubc7p, Cue1p, Ubx2p, Cdc48p, and its cofactors Ufd1p and Npl4p. These proteins are mainly cytosolic, supporting Doa10p's role in clearing proteins with defects in their cytosolic domain. In addition to these proteins, the Hrd1p complex consists of Hrd3p, Der1p, the ER lectin Yos9p, and Usa1p (Carvalho et al., 2006; Denic et al., 2006). Yos9p has been shown to specifically bind misfolded glycoproteins (Bhamidipati et al., 2005; Kim et al., 2005; Szathmary et al., 2005). Ubx2p recruits Cdc48p to the membrane (Neuber et al., 2005). Usa1p is thought to link Der1p to the Hrd1p ligase and thereby assist in clearing luminally misfolded proteins from the ER (Ismail and Ng, 2006).

In mammalian cells, the dislocation pathway is more complex. Because of the lack of a genetic approach, the dissection of the degradation pathway in mammalian cells relies on the use of substrates, such as mutant versions of the cystic fibrosis chloride conductance channel (Ward et al., 1995; Bebok et al., 1998; Xiong et al., 1999; Kiser et al., 2001), of proteins considered terminally misfolded, such as the mutant null Hong Kong (NHK) version of the secretory glycoprotein α1 antitrypsin (Liu et al., 1999) or the truncated and misfolded version of the ER-resident glycoprotein ribophorin (RI), termed RI_{332} (Tsao et al., 1992; de Virgilio et al., 1998). Many membrane proteins fail to fold properly for lack of the correct partner subunits, such as the unpaired T cell receptor α chain (TCRα; Huppa and Ploegh, 1997a,b) or the free immunoglobulin μ chain (Fagioli et al., 2001).

Many parallels exist between the yeast and the mammalian glycoprotein quality control systems; however, the players in the mammalian system are more numerous. The mammalian version of HRD1 is also an ER membrane–resident ubiquitin ligase (Kaneko et al., 2002; Nadav et al., 2003; Kikkert et al., 2004), which forms a complex with SEL1L, a mammalian orthologue of yeast Hrd3p (Lilley and Ploegh, 2005). Additional ubiquitin ligases exist, such as gp78, which has similarity to HRD1 in its Ring finger and interacts with UBC7 via its CUE domain to ubiquitinate TCRα (Fang et al., 2001). There are at least three Der1p homologues in mammals, Derlin-1, -2, and -3, which play roles in the disposal of proteins from the ER (Lilley and Ploegh, 2004, 2005; Oda et al., 2006).

Among the better-studied routes of membrane glycoprotein degradation are the pathways used by human cytomegalovirus (HCMV) to destroy the class I major histocompatibility complex (MHC) heavy chains (HCs; Ahn et al., 1996). Class I MHC products serve as a warning system to alert cytotoxic T cells to the presence of virus-derived polypeptides inside the cell. The infected cell, thus, invites attack by the cytotoxic T cell as a means of eradicating the source of the virus (Tortorella et al., 2000). Large DNA viruses, such as HCMV, are under strong selective pressure to avoid recognition by the immune system. Although widespread amongst the [herpesviridae as a strategy to avoid detection, HCMV in particular has amassed a set of genes whose products evolved to interfere with assembly and intracellular transport of class I MHC products. Amid the HCMV-encoded immunoevasins, two, US2 and US11, which are small membrane glycoproteins that assist in the degradation of class I MHC HCs, stand out. US2 and US11 catalyze superficially similar reactions, characterized by complex formation of US2 or US11 with their target class I MHC HCs, and subsequent extraction of the class I MHC HCs from the ER, a process referred to as dislocation (Wiertz et al., 1996a,b). After dislocation, the class I HC is destroyed by the proteasome. In the presence of proteasome inhibitors, a diagnostic intermediate in this pathway is the result of attack by N-glycanase on the newly dislocated class I MHC HCs (Wiertz et al., 1996a,b; Hirsch et al., 2003; Blom et al., 2004). This intermediate consists of a fully cytoplasmic, yet intact, class I MHC HC, devoid of its N-linked glycan (Misaghi et al., 2004). It occurs not only in cells that express US2 or US11, but also in Daudi cells, which are unable to assemble class I MHC products for lack of the light chain β2-microglobulin (Hughes et al., 1997; Radcliffe et al., 2002). Similar deglycosylated intermediates have been reported for a misfolded fragment of RI (de Virgilio et al., 1998, 1999), but for most other glycoproteins examined, degradation occurs without the obvious production of deglycosylated intermediates.

These observations raise the question as to whether the pathways exemplified by US2- and US11-dependent degradation are indeed emblematic of glycoprotein turnover, as we have argued in the past. The furthest advanced is the characterization of the US11 pathway, for which we identified the Derlin-1 protein as an essential participant (Lilley and Ploegh, 2004; Ye et al., 2004). Derlin-1, in turn, forms a complex not only with itself and other members of the Derlin family, but also with the mammalian Hrd1p and Hrd3p (SEL1L) homologues, as well as with Cdc48p/p97 (Lilley and Ploegh, 2005; Ye et al., 2005). The US2 pathway is impervious to the action of a dominant-negative version of Derlin-1, whereas the US11 pathway is inhibited by it (Lilley and Ploegh, 2004). Direct involvement in mammalian glycoprotein degradation has now been suggested for components of this ER-resident complex, based on interference with degradation of α1 antitrypsin NHK by means of overexpression and knockdowns of Derlins (Oda et al., 2006). Still, in mammalian cells, much of the relevant data stem from the analysis of reactions that depend on the action of the viral accessories US11 and US2.

We analyze the contributions of a mammalian Hrd3p homologue, SEL1L. We show that reduction of the levels of SEL1L by RNA interference results in impairment of US11-mediated dislocation of class I MHC HC molecules. Expression of the same interfering small hairpin RNAs (shRNAs) against SEL1L, however, does not affect the US2 pathway. Expression of SEL1L shRNAs inhibits the degradation of RI_{332}, which is a process that occurs independently of the presence of viral accessories. These results allow us to place SEL1L in a more general context of glycoprotein degradation, and suggest that SEL1L might be involved in substrate recognition of misfolded proteins in the ER.

Results

SEL1L is part of a mammalian ER multiprotein complex involved in dislocation (Lilley and Ploegh, 2005; Ye et al., 2005). SEL1L associates with Derlin-1, -2, p97, and HRD1, as well as with additional proteins that remain to be identified, some or all of which may also play a role in dislocation. Some of these proteins are presumably recruited to the site of dislocation through SEL1L. SEL1L is predicted to be a type I transmembrane protein (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200605196/DC1) with 5 N-linked glycans (Biunno et al., 1997). Because the bulk of the SEL1L protein is predicted to be in the ER lumen, it is possible that SEL1L first plays a role in substrate recognition and identification of misfolded proteins, and then recruits them to the site of dislocation. We performed coimmunoprecipitations from steady-state radioactive [^{35}S]methionine/cysteine–labeled cells using mild lysis conditions (2% digitonin) to preserve ER membrane protein complexes, to analyze whether composition of protein complexes with SEL1L are conserved for the cell lines used in this study.

Our earlier observations concern the composition of protein complexes that include the Derlins in the astrocytoma cell line U373 (Lilley and Ploegh, 2005). We first verified that the types of complexes detected in U373 cells are not cell type–specific, but occur in other cell lines as well (Fig. 1). We were particularly interested in cell lines that would lend themselves to transient transfection experiments (Fig. 1, HeLa cells, lanes 1–4) and in multiple myeloma cells (Fig. 1, MM1.S cells, lanes 5–8), which represent cells with a high secretory capacity, and, presumably, a correspondingly pronounced requirement to clear misfolded proteins from the ER.

When we performed immunoprecipitations with antibodies against Derlin-1, -2, and SEL1L, the composition of the complexes obtained was very similar to that reported for U373 cells (Lilley and Ploegh, 2005). We conclude that the multiprotein complexes, which were initially identified for U373 cells, are representative of the complexes detected in other, unrelated cell types. The pattern of the protein complexes is equivalent in all tested cell lines, including U373 (astrocytoma), HeLa (epithelial), 293T, MM1.S (multiple myeloma), and C6 (Rat glioma) cells. SEL1L is likely to be a crucial component of a ubiquitous ER-dislocation complex.

When we analyzed the half-life of SEL1L, we found that it decays with a half-life of ~180 min (Fig. 2, lanes 1, 3, and 5). SEL1L degradation appears to be proteasome-dependent because it is inhibited by inclusion of the proteasome inhibitor ZL_3VS (Fig. 2, lanes 7, 9, and 11). SEL1L remains completely susceptible to endoglycosidase H (EndoH) treatment, suggesting that SEL1L is an ER protein and does not traffic through the secretory pathway (Fig. 2, lanes 2, 4, 6, 8, 10, and 12).

To address whether SEL1L plays a direct role in the process of dislocation from the ER, we generated small hairpin RNAs (shRNAs) that target SEL1L or a control unrelated protein (GFP) using the pRETRO-SUPER in vivo expression system (Brummelkamp et al., 2002). The shRNA plasmids reduced SEL1L protein levels to 30% compared with control cells (Fig. 3 a).

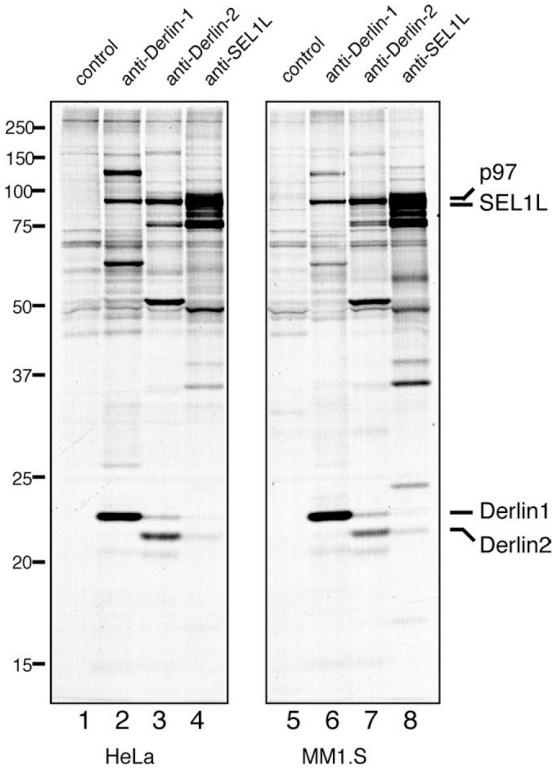

Figure 1. **SEL1L is part of a conserved complex in many cell types.** Immunoprecipitation from digitonin lysates of HeLa (lanes 1–4) or MM1.S (lanes 5–8) cells pulse-labeled for 16 h (steady state) were performed with control rabbit IgG, anti–Derlin-1, anti–Derlin-2, and anti-SEL1L antibodies, respectively. The positions of the relevant proteins are indicated.

We analyzed the stability of class I MHC HCs in cells expressing US11 and the SEL1L shRNAs. The fate of HC is best analyzed in a pulse-chase experiment in view of the rate of dislocation ($t_{1/2}$ = 2–5 min) in US11 cells (Wiertz et al., 1996a). In cells expressing US11 and shRNAs against GFP (control cells), HC disappears completely when proteasome inhibitors are not included (Fig. 3 b, 1, lanes 2 and 3). In cells expressing SEL1L shRNA, the rate of degradation of class I MHC HC is much reduced (Fig. 3 b, 1, lanes 8 and 9).

In the presence of the proteasome inhibitor ZL_3VS, the deglycosylated class I MHC HC accumulates as the diagnostic intermediate that characterizes the dislocation reaction. In control shRNA US11 cells, complete conversion to the deglycosylated form of HC is seen after 30 min of chase (lane 6). In SEL1L knockdown cells, >50% of HC remains in its fully glycosylated form. This persistence of HC is attributable to compromised dislocation, as only ~50% of HC accesses the cytosolically disposed N-glycanase (Fig. 3, b, 1 [lanes 11 and 12], and c).

This impairment in dislocation is not caused by reduced levels of US11 in SEL1L knockdown cells, as US11 is expressed at comparable levels, nor is it caused by aberrant ER insertion and processing of US11, as determined by normal cleavage of

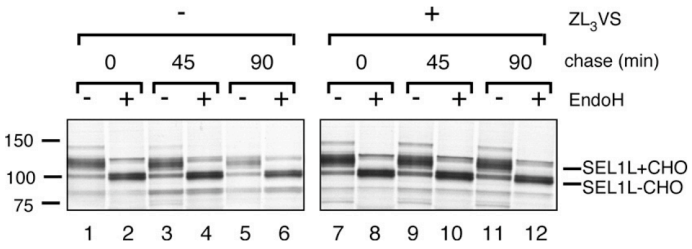

Figure 2. **SEL1L is an unstable ER membrane glycoprotein.** Immunoprecipitation from NP-40 lysates of U373 cells pulse-labeled for 20 min and chased for the indicated time points in the absence (lanes 1–6) or presence (lanes 7–12) of ZL_3VS were performed with anti-SEL1L antibodies. Half of the eluates were treated with EndoH (lanes 2, 4, 6, 8, 10, and 12).

US11's signal sequence (Rehm et al., 2001) in SEL1L knockdown cells (Fig. 3 b, 2).

Because SEL1L is a mammalian homologue of yeast Hrd3p, we looked for possible parallels between the two systems that could account for the observed phenotype. Hrd1p, which is a yeast E3 ligase known to be necessary for degradation of some substrates (Bays et al., 2001b; Bordallo and Wolf, 1999; Hampton et al., 1996; Kikkert et al., 2004; Nadav et al., 2003), is regulated by Hrd3p and destabilized in *Δhrd3* yeast (Gardner et al., 2000; Plemper et al., 1999). Therefore, we analyzed HRD1 levels in SEL1L knockdown cells to examine whether inhibition of dislocation is primarily caused by reduced levels of SEL1L or, alternatively, attributable to a reduction in HRD1 levels. We find that HRD1 is stable throughout the chase periods over which HC dislocation occurs (Fig. 3 b, 3).

A comparable reduction of SEL1L levels in US2 cells (Fig. 4 a) has no observable effect on HC dislocation. In US2 cells exposed to proteasome inhibitor, we see conversion of almost all class I MHC HCs to the deglycosylated species after 30 min (Fig. 4 b, 1, lane 6). In US2 cells, knockdown of SEL1L results in a rate of dislocation equal to that in control cells and yields a pattern noticeably distinct from that seen for the SEL1L knockdown in US11 cells (Fig. 4, b [1] and c). US2 levels are also comparable for control and SEL1L knockdown cells (Fig. 4 b, 2). Unimpaired degradation in US2 cells of class I MHC HCs is consistent with the idea that US11 co-opts a conserved complex to catalyze degradation of class I MHC HC, whereas US2 utilizes a distinct pathway (Lilley and Ploegh, 2004, 2005; Loureiro et al., 2006). This result is all the more striking because US2 and US11 target the same set of substrates, class I MHC products.

Given the homology of SEL1L to Hrd3p, it is likely that SEL1L plays a general role in glycoprotein turnover, similar to what was shown for the Der1p homologues of the Derlin proteins (Lilley and Ploegh, 2004; Oda et al., 2006). Thus far, the only substrates that for their dislocation depend on Derlin-1 are class I MHC HCs in US11-expressing cells and US2 itself (Lilley and Ploegh, 2004). Overexpression and knockdowns of Derlin-2 and -3 have been reported to affect the half-life of α1 antitrypsin NHK (Oda et al., 2006).

Therefore, we examined other model substrates of ER dislocation for their susceptibility to inhibition by a knockdown of SEL1L. We analyzed the effect of SEL1L shRNAs on the degradation of a mutant version of RI, RI_{332}. The RI_{332} mutant lacks

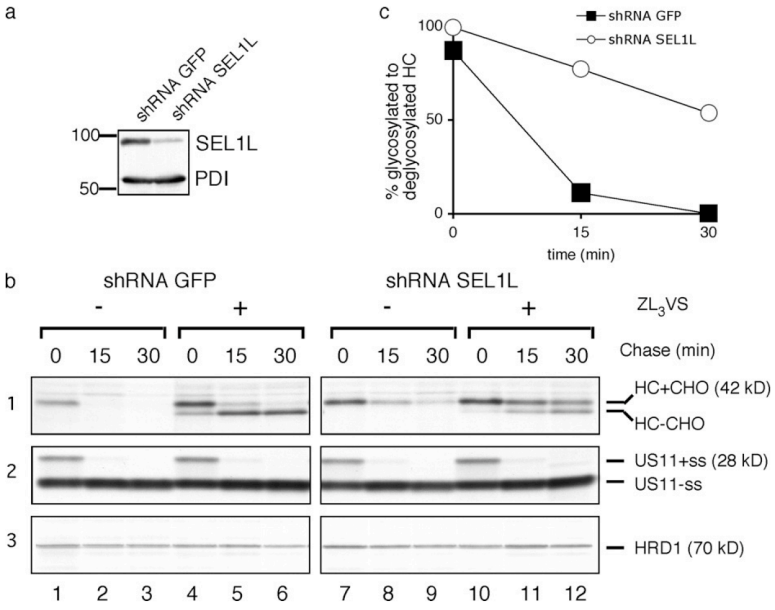

Figure 3. **shRNAs targeting SEL1L impair US11-mediated dislocation of class I MHC HC.** (a) US11 cells were transduced with virus encoding shRNAs against GFP (control cells) or against SEL1L (knockdown cells) and analyzed for levels of SEL1L by immunoblotting with anti-SEL1L antibodies and anti–protein disulfide isomerase (PDI) antibodies as a loading control. (b) Control and knockdown cells were pulse-labeled for 10 min and chased for the indicated time points. Immunoprecipitation from SDS lysates for class I MHC HC (1), US11 (2), and HRD1 (3) were performed using respective antibodies. (c) Quantitation of the amount of glycosylated to deglycosylated HC from shRNA GFP (control) and shRNA SEL1L (knockdown) cells treated with ZL_3VS.

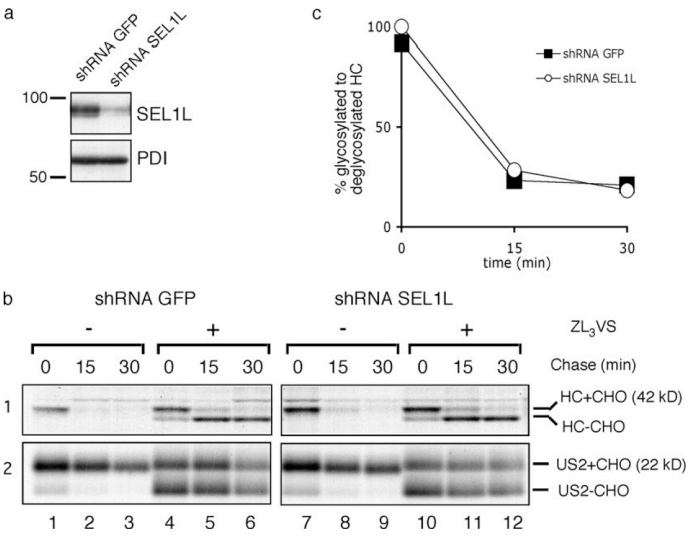

Figure 4. **shRNAs targeting SEL1L do not impair US2-mediated dislocation of class I MHC HC.** (a) US2 cells were transduced with virus encoding shRNAs against GFP (control cells) or against SEL1L (knockdown cells) and analyzed for levels of SEL1L by immunoblotting with anti-SEL1L antibodies and anti-PDI antibodies as a loading control. (b) Control and knockdown cells were pulse-labeled for 10 min and chased for the indicated time points. Immunoprecipitation from SDS lysates for class I MHC HC (1) and US2 (2) were performed using respective antibodies. (c) Quantitation of the amount of glycosylated to deglycosylated HC from shRNA GFP (control) and shRNA SEL1L (knockdown) cells treated with ZL_3VS.

the C terminus of RI; unlike the very stable RI protein, the RI_{332} fragment has a half-life of ~90 min (Tsao et al., 1992; de Virgilio et al., 1998, 1999). We chose RI_{332} as substrate because its mode of disposal includes an intermediate that is the product of N-glycanase activity (Kitzmuller et al., 2003). We transiently transfected RI_{332} into HeLa cells, which had been stably transduced with shRNAs against either GFP or SEL1L (Fig. 5 a). SEL1L knockdown cells showed a reduction of SEL1L levels to 40% of controls. We transfected a GFP expression plasmid into both GFP shRNA and SEL1L shRNA HeLa cells as a transfection control, as well as a control for shRNA-mediated knockdown in the HeLa shRNA GFP control cells. The percentage of cells expressing GFP was equivalent (~40%), but, as expected, the intensity of the GFP signal was much lower in GFP shRNA cells (unpublished data).

Two days after transfection, cells were harvested, pulsed for 20 min with [^{35}S]methionine, and chased for 0, 90, and 180 min. Immunoprecipitation with anti-RI antibody retrieves both the truncated and wild-type RI. In control shRNA GFP HeLa cells, ~90% of truncated RI_{332} is degraded after 180 min, whereas in SEL1L knockdown cells, we see a considerable stabilization of RI_{332}; ~40% of RI_{332} remains (Fig. 5, b [lanes 3 and 6] and c). The observed stabilization of RI_{332} is all the more noteworthy as the achieved stable knockdown of SEL1L in HeLa cells is ≥40% (Fig. 5 a). Wild-type RI (a stable protein in the ER) remains unaffected, and expression of SEL1L shRNA does not affect overall glycosylation as assessed by class I MHC HC and US11 maturation and glycosylation earlier.

Does SEL1L specifically interact with misfolded proteins in the ER?

Because SEL1L has a long luminal domain with tetratricopeptide repeats (TPRs; Sel1 repeats of the TPR family; Fig. S1), a domain structure suggested to mediate protein–peptide interactions, we determined whether SEL1L interacts with misfolded proteins, and whether SEL1L can discriminate between properly folded and misfolded proteins. We examined association of RI_{332} with SEL1L by immunoprecipitating for SEL1L from extracts of cells transiently transfected with RI_{332} and lysed under mild conditions. We found that RI_{332} interacts with SEL1L (Fig. 6 b, lane 1), and we confirmed this interaction in re-immunoprecipitation experiments (Fig. 6 b, lane 2). We were unable to detect the full length, and presumably properly folded, form of RI in the re-immunoprecipitation, although the presence of trace amounts of full-length RI remains a possibility, as misfolded RI_{332} is in excess over endogenous RI (Fig. 6 a). As a second specificity control, we confirmed that the deglycosylated dislocation intermediate of RI_{332}, which accumulates in the cytosol when cells are treated with proteasome inhibitor, does not interact with SEL1L (Fig. 6 and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200605196/DC1).

We transiently transfected the same HeLa cell lines that had been stably transduced with shRNAs targeting either SEL1L or GFP with TCRα. TCRα is likely degraded because of the unpaired charged residues in its transmembrane domain when its proper partner subunits (TCRβ and CD3 complex) are not coexpressed (Cosson et al., 1991). Cells were labeled for 20 min with [^{35}S]methionine and chased for 0, 2, and 4 h (Fig. 7 a), and the amount of TCRα remaining at each time point was quantitated (Fig. 7 b). We see a moderate effect of a SEL1L knockdown on TCRα degradation; after 4 h of chase, 25% of TCRα remains in the shRNA GFP control cells, whereas in SEL1L knockdown cells, 35% remains (Fig. 7 b). The effect is likely to be less pronounced for TCRα than for RI_{332} because TCRα would be classified as misfolded because of recognition of unpaired charges in its transmembrane domain, whereas RI_{332} is an entirely luminal protein. From yeast, we know that the site of misfolding is crucial for the recruitment of specific substrate recognition proteins (Ahner and Brodsky, 2004; Carvalho et al., 2006). Another explanation for the poor interference with TCRα degradation could be the level of SEL1L knockdown to no more than 40%. Interference with the degradation of TCRα might

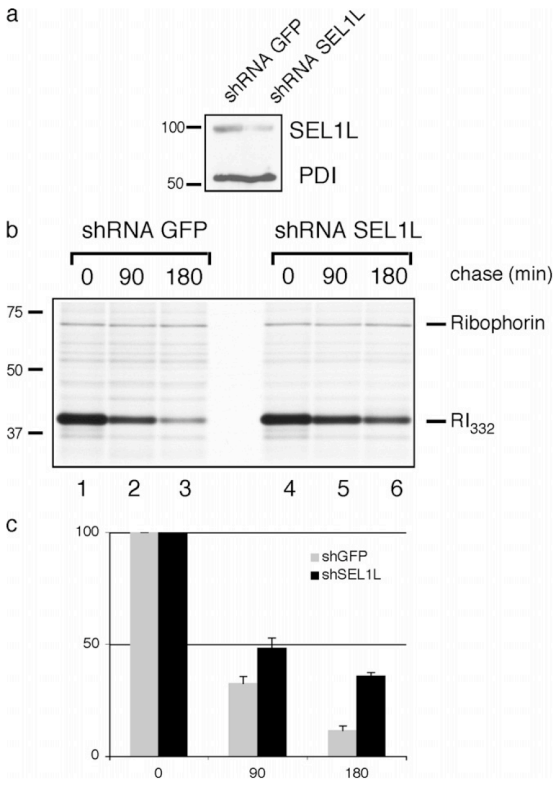

Figure 5. **Dislocation of truncated RI (RI_{332}) is impaired by shRNAs targeting SEL1L.** (a) HeLa cells were stably transduced with either shRNAS against GFP (control cells) or SEL1L (knockdown cells) and analyzed for levels of SEL1L by immunoblotting with anti-SEL1L antibodies and anti-PDI antibodies as a loading control. (b) Control and knockdown cells were transiently transfected with RI_{332}. 36 h after transfection, these cells were pulse-labeled for 20 min and chased for the indicated time points. Immunoprecipitation from SDS lysates were performed using anti-RI I antibodies. (c) The bar diagram shows quantitation of the experiment. Error bars represent the SD of three independent experiments.

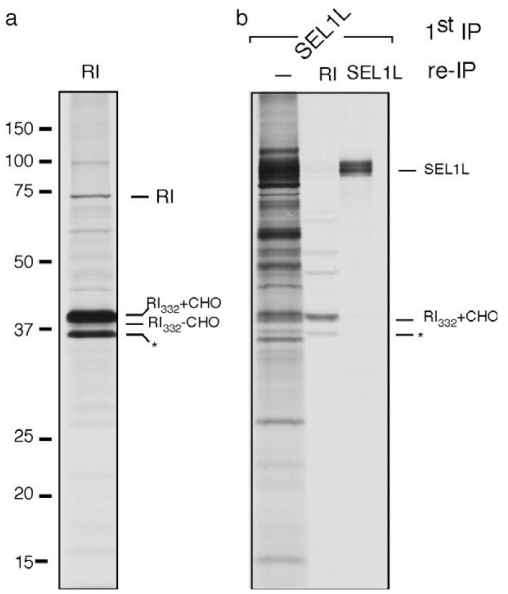

Figure 6. **SEL1L interacts with RI_{332} 293T cells transfected with RI_{332} that were pulse-labeled to steady-state for 5 h in the presence of 10 μM of the proteasome inhibitor ZL_3VS.** Immunoprecipitations from digitonin lysates were performed with anti-SEL1L antibodies. The anti-SEL1L immunoprecipitate was either analyzed directly (b, lane 1) or reimmunoprecipitated sequentially using anti-RI I antibodies (b, lane 2) or anti-SEL1L antibodies (b, lane 3). (a) Input levels of endogenous RI and overexpressed RI_{332}. Immunoprecipitates were resolved on a 12% SDS polyacrylamide gel. Asterisk represents a presumably processed version of RI_{332} in the ER.

require a greater reduction in SEL1L levels than attained with the present knockdown strategies.

Discussion

SEL1L is essential for dislocation of class I MHC molecules from the ER in cells that express US11. Upon reduction of the levels of the SEL1L protein, fully glycosylated, EndoH-sensitive class I MHC HCs accumulate in the ER. In contrast, the US2 pathway is not perturbed by reduction of SEL1L protein levels. These results underscore the relevance of other observations that place US11 and US2 in distinct pathways, e.g., based on their susceptibility to interference with Derlin-1 function; a Derlin-1^{GFP} fusion protein impairs degradation of class I MHC molecules via the US11-dependent, but not via the US2-dependent, pathway (Lilley and Ploegh, 2004).

There are several lines of evidence to suggest that US2 and US11 use different principles to accelerate degradation of class I MHC molecules, in addition to the aforementioned difference in Derlin-1 dependency. US2 must exploit features in its relatively short cytoplasmic tail, whereas tailless US11 remains dislocation-competent (Furman et al., 2002; Loureiro et al., 2006). The preference of the US2 and US11 pathways for folded and unfolded class I MHC molecules appears to be different (Blom et al., 2004), as is the requirement for elements in the tail of the class I MHC molecules themselves (Wiertz et al., 1996b). Our data now establish that the US11, but not the US2, pathway is sensitive to a SEL1L knockdown. These distinctions are all the more remarkable given the similarities of the substrates targeted for degradation, the identical class I MHC HCs in the same parent cell line.

We also report that down-regulation of SEL1L affects the degradation of a RI fragment whose route of degradation is similar to what we have described for class I MHC products targeted by the HCMV immunoevasins (Hughes et al., 1997; Kitzmuller et al., 2003). In the presence of proteasome inhibitors, a deglycosylated RI_{332} species, the product of N-glycanase digestion, is observed (Kitzmuller et al., 2003). Our data, thus, support the notion that human SEL1L is the orthologue of yeast Hrd3p because reduction of SEL1L levels perturbs the degradation of a misfolded substrate, RI_{332}.

We conclude that SEL1L and its associated partners operate in a pathway that is neither restricted to class I MHC products, nor strictly dependent on the involvement of virus-encoded

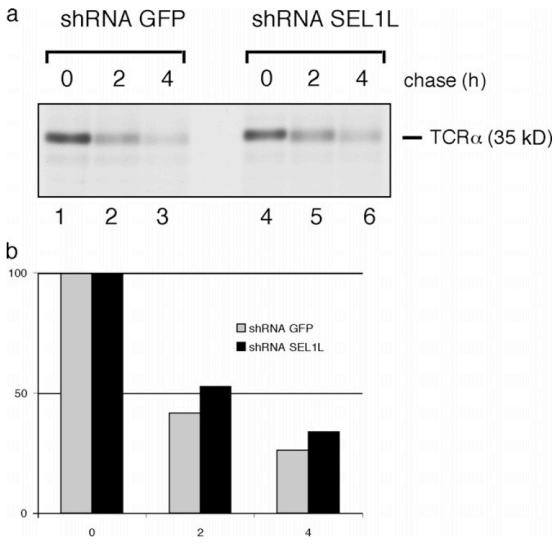

Figure 7. **TCRα degradation is moderately slowed by shRNAs targeting SEL1L.** The same control and knockdown cells used in Fig. 5 were transiently transfected with TCRα. 36 h after transfection these cells were pulse-labeled for 20 min and chased for the indicated time points. Immunoprecipitation from SDS lysates were performed using anti–TCRα-antibodies. (c) The histogram shows mean quantitation of two independent experiments.

proteins. The concept of physically extracting proteins from the ER and their delivery to the cytosolic proteasome achieves the compartmentalization required to spare nascent and properly folded ER proteins from premature degradation. The advantages of studying the pathways for the HCMV US2 and US11 proteins are the speed with which degradation occurs and the involvement of well-characterized substrates, the class I MHC products. The available antibodies allow an easy distinction between various folding intermediates, and the occurrence of the tell-tale deglycosylated degradation intermediate is an accurate reporter for its localization and for the dislocation reaction per se (Wiertz et al., 1996a,b). We have taken advantage of the fact that US2 and US11 attract very similar substrates in one and the same cell, yet apparently do so by mechanistically different pathways (Furman et al., 2002; Lilley and Ploegh, 2004; Loureiro et al., 2006).

This does raise the question, however, of whether the results obtained for these HCMV immunoevasins and class I MHC substrates can be extrapolated to other substrates, and to pathways that operate independently of viral accessories.

To our knowledge, the observation that SEL1L is involved in the degradation of RI_{332}, a known dislocation substrate, is the first example for mammalian cells that directly places SEL1L in a pathway of protein degradation. The dominant-negative version of Derlin-1, Derlin-1^{GFP}, had no effect on RI_{332} degradation (unpublished data), making the mammalian degradation pathways even more complex. The different degradation pathways that, in yeast, process distinct types of substrates (luminally vs. cytoplasmically exposed proteins) likely operate in mammals, too, but involve more factors that comprise the different complexes. In addition to the multiple Derlin proteins, ubiquitin ligases, and lectins involved in mammalian ER degradation, there is an additional SEL1L-like protein in mammals that bears homology to Hrd3p (Fig. S1). Whether this protein binds to HRD1 and participates in the degradation of misfolded proteins remains to be determined.

In yeast, substrates that require Der1p for degradation require Hrd1p/Hrd3p and usually belong to the set of completely luminal substrates. In mammals, depending on the substrate, Derlin-1 and SEL1L can act in concert with each other, as we show for class I MHC HC, but SEL1L can also assist in the degradation of substrates independently of Derlin-1, as is the case for RI_{332}. A recent study suggests that, in yeast, Hrd3p and Der1p can recruit substrates independently of each other (Gauss et al., 2006b).

We also examined a substrate that is recognized as misfolded by means of charged residues in its transmembrane domain, TCRα. We observed modest stabilization when SEL1L expression is compromised. The lesser effect on this substrate compared with RI_{332}, which is an entirely luminal substrate, further suggests that the nature of the substrate determines recruitment of an otherwise conserved complex. We cannot exclude that different degradation substrates require different levels of SEL1L. Perhaps TCRα degradation usually proceeds in the presence of very few SEL1L molecules.

The ER machinery involved in recognition of substrates is likely to be specific for certain substrates, but the details of recognition of misfolded substrates in yeast are no better resolved than in mammalian cells. In yeast, Yos9p, a putative lectin protein, is involved in the degradation of a membrane-bound version of CPY* (Buschhorn et al., 2004) and might be involved in identifying and targeting substrates for degradation (Bhamidipati et al., 2005; Kim et al., 2005; Szathmary et al., 2005). A recent study suggests that proteins that are misfolded in yeast bind to Hrd3p, which itself binds to Yos9p. Yos9p then ensures that only terminally misfolded proteins are being degraded (Gauss et al., 2006a). Another avenue to identification of misfolded glycoproteins might be through their high-mannose–containing, N-linked glycan modifications (Helenius and Aebi, 2004). Mannose residues are trimmed in the ER by the enzyme α-mannosidase I (Hosokawa et al., 2003; Wu et al., 2003), generating a Man8GlcNac2 tag implicated in targeting glycoproteins for degradation (Ellgaard and Helenius, 2003). Proteins with this tag bind to calnexin and other lectins, the ER degradation-enhancing mannosidase-like (EDEM) proteins, EDEM1 and EDEM2 (Hosokawa et al., 2001, 2003; Mast et al., 2005; Olivari et al., 2005), which then target some glycoproteins for degradation (Molinari et al., 2003; Ahner and Brodsky, 2004; Oda et al., 2006) There is a mammalian orthologue of Yos9p, OS-9, whose function is unknown but might be critical in degradation of certain substrates.

Similar to EDEM, SEL1L might be involved in substrate recognition and bind to a subset of misfolded proteins. SEL1L is remarkably conserved, and contains several repetitive structural and functional domains (sel1-like repeats of the TPR family) and a type II fibronectin domain in its large luminal part that

could bind to chaperones or misfolded proteins. Alternatively, SEL1L might recruit substrate recognition proteins such as EDEM or OS-9 through its type II fibronectin domain at its N terminus. In yeast, Hrd3p's N-terminal domain is essential for its function in ER degradation, and its central region required for interaction with Hrd1p (Gardner et al., 2001).

SEL1L forms a 1:1 stoichiometric complex with HRD1 in mammalian cells (Lilley and Ploegh, 2005), but until now, SEL1L's direct involvement in protein degradation has not been shown. Thus, we conclude that SEL1L, based on its associated partners, its direct involvement in glycoprotein degradation, and its structural relationship to yeast Hrd3p, helps select proteins for entry into a degradative pathway. Our data provide the first functional evidence to support the notion that SEL1L is indeed the orthologue of yeast Hrd3p.

Materials and methods

Antibodies, DNA constructs, and cell lines

Anti-HC, anti-US2, anti-US11, anti–Derlin-1, anti–Derlin-2, and anti-SEL1L antibodies have been described previously (Tortorella et al., 1998; Gewurz et al., 2002; Lilley et al., 2003; Lilley and Ploegh, 2004, 2005).

Anti-RI_{332} antibodies and the RI_{332} DNA construct were a gift from N.E. Ivessa (University of Vienna, Vienna, Austria) and G. Kreibich (New York University Medical Center, New York, NY), anti-TCRα antibodies were purchased from Sigma-Aldrich, and anti-HRD1 antibodies were a gift from E. Wiertz (Leiden University, Leiden, Netherlands; Kikkert et al., 2004).

The TCRα expression construct was described previously (Huppa and Ploegh, 1997a).

For stable shRNA expression, the pRETRO-SUPER vector was used (Brummelkamp et al., 2002).

U373-MG astrocytoma/glioblastoma cells, US2, US11, 293T, and HeLa cells were cultured as previously described (Rehm et al., 2001; Lilley et al., 2003).

MM1.S cells were cultured in RPMI containing 10% FCS.

Metabolic labeling, immunoprecipitation, endoglycosidase H digestion, gel electrophoresis, immunoblotting, and transient transfections

These procedures were similar to those described in Lilley and Ploegh (2005). Treatment of cells with the proteasome inhibitor ZL_3VS has been previously described (Shamu et al., 1999). Analysis of class I MHC HC stability in US11 and US2 cells, pulse-labeling, cell lysis (SDS and digitonin), and re-immunoprecipitations have been previously described (Lilley et al., 2003). NEM (N-ethylmaleimide) was included in digitonin and SDS lysis at a concentration of 2.5 mM. SDS PAGE has been previously described (Ploegh, 1995).

293T cells were transiently transfected with Trans-IT (Takara Mirus Bio) according to the manufacturer, using 2–5 μg of DNA for transient transfections and 10 μg of total DNA for virus production in 10-cm dishes. HeLa cells were transiently transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer, using 2–5 μg for transient transfections in 10-cm dishes.

Immunoprecipitates were treated with EndoH according to the manufacturer (New England Biolabs).

The program ImageJ (National Institutes of Health) was used for quantitating bands on film.

SEL1L knockdown

19-nt target sequences in SEL1L were chosen according to the online design software available from the Whitehead Institute webpage (http://jura.wi.mit.edu/siRNAext/). Sense and antisense strands were annealed to form the shRNA template insert and ligated into the retroviral vector pRETRO SUPER (Brummelkamp et al., 2002) to generate shRNA construct s2 for stable shRNA expression and for transient transfection (19mer sequences; GGCTATACTGTGGCTAGAA and TTCTAGCCACAGTATAGCC, respectively). As a control, the unrelated construct (GFP shRNA) targeting the enhanced GFP reporter was used (Lilley and Ploegh, 2005). All constructs were sequence-confirmed and used for virus production in HEK 293T cells (Soneoka et al., 1995). pRETRO-transduced U373 cells and HeLa cells were selected with 0.375 μg/ml and 1 μg/ml puromycin, respectively, as previously described (Lilley and Ploegh, 2005).

Online supplemental material

Fig. S1 shows a schematic of the Hrd3p homologues in mammals. Fig. S2 shows SEL1L interacting with RI_{332} 293T cells transfected with RI_{332}. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200605196/DC1.

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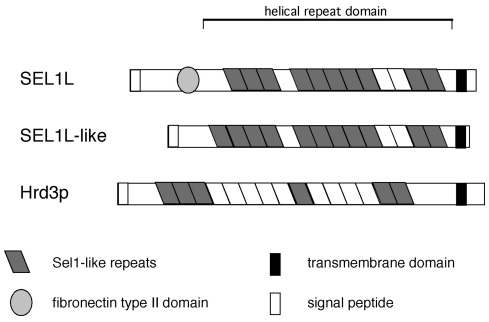
Supplementary figure S1

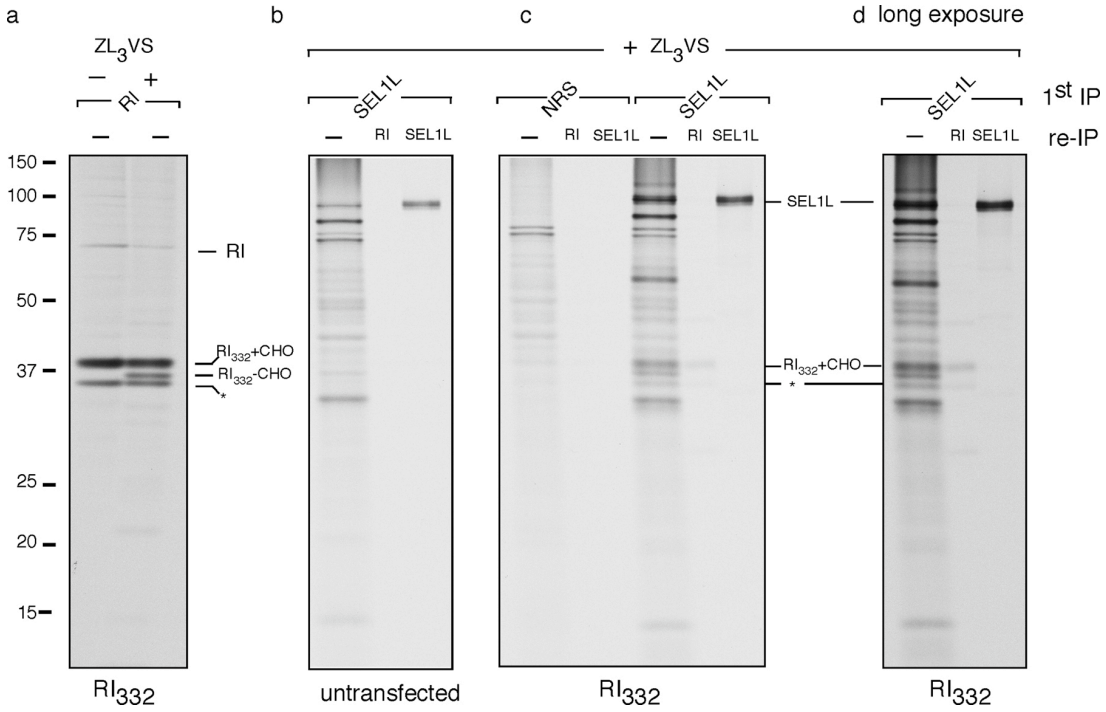
Schematic of the Hrd3p homologs in mammals.

Supplementary figure S2:

SEL1L interacts with RI_{332}

293T cells transfected with RI_{332} were pulse-labeled for 5hrs in the presence of proteasome inhibitor ZL_3VS (50 μM). The digitonin lysates were split in two and immunoprecipitated with SEL1L antibodies and normal rabbit serum (NRS), respectively. Both immunoprecipitates were analyzed for ribophorin and SEL1L in re-immunoprecipitation experiments (panel c). Panel b shows the same immunoprecipitates for untransfected cells as a control. A fraction of the transfected cells were lysed in SDS and analyzed for levels of RI_{332} and endogenous ribophorin, with cells that had not been exposed to proteasome inhibitor as a control for the deglycosylated intermediate of RI_{332} (panel a). Panel d is an overexposure of the SEL1L immunoprecipitates from panel c. Shown is a 10% SDS polyacrylamide gel.

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4.2 An extended mammalian membrane protein complex that mediates glycoprotein dislocation

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SEL1L plays a crucial role in dislocation of class I MHC in the US11 system and in the dislocation of truncated ribophorin (Mueller et al., 2006). To analyze mammalian dislocation complexes, we performed a large-scale immunoprecipitation from HeLa cells transduced with tagged SEL1L and from control cells. We found several polypeptides specifically bound to SEL1L. We cloned four proteins (OS9, UBXD8, UBC6e, and AUP1) made antibodies against them, established that they are ER-resident proteins, and analyzed their contribution to US11-mediated dislocation of Class I MHC products.

We showed that UBXD8 recruits p97 to the ER membrane. We designed a C-terminally GFP-tagged version of UBXD8 which acts as a dominant-negative inhibitor of class I MHC dislocation when overexpressed in US11 cells. UBXD8-GFP frustrates US11-mediated dislocation, but has no effect on US2 mediated dislocation, suggesting that the ER is not grossly perturbed upon overexpression of this construct. AUP1, a protein that contains a CUE domain which might be involved in recruitment of ubiquitin conjugating enzymes to the ER membrane, also disturbs US11-mediated dislocation when expressed with a GFP tag, but has no effect on US2-mediated dislocation. Furthermore we established a role for the ubiquitin conjugating enzyme UBC6e in HC dislocation. Overexpression of this enzyme impairs HC dislocation in US11 cells. Again, US2 cells served as a control. When dislocation is hindered, class I MHC HCs accumulate in the ER. We designed point mutations in the proposed lectin binding domain of OS9 in addition to installing a GFP tag onto the N-terminus of OS9. Overexpression of mutant versions of OS9 or of GFP-OS9 did not have an effect on HC dislocation. We therefore analyzed the fate of RI_{332} in HeLa cells transduced with the same constructs. Overexpression of WT or mutant OS9 delayed RI_{332} dislocation significantly, whereas a non-

glycolsylated version of RI_{332}, RI_{332}-thr (de Virgilio et al., 1999), remained unaffected, again serving as a control for proper dislocation. We concluded that OS9 might be -together with SEL1L- involved in substrate selection of glycosylated proteins, and therefore be dispensable for US11-mediated dislocation. In this model, US11 itself would be responsible for targeting HCs to the dislocation machinery.

An extended mammalian membrane protein complex that mediates glycoprotein dislocation

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Abstract:

Membrane and secretory proteins that fail to pass quality control in the endoplasmic reticulum (ER) are discharged into the cytosol and degraded by the proteasome. Many of the mammalian components involved in this process remain to be identified. We performed a biochemical search for proteins that interact with SEL1L, a protein that is part of the mammalian HRD1 ligase complex and involved in substrate recognition. SEL1L is crucial for dislocation of Class I Major Histocompatibility Complex (MHC) heavy chains (HCs) by the human cytomegalovirus (HCMV) US11 protein. We identified AUP1, UBXD8, UBC6e, and OS9 as new and functionally important components of the degradation complex in mammalian cells, confirmed by mutagenesis and dominant negative versions of these proteins. Overexpression of OS9 compromises degradation of a glycosylated ribophorin fragment, but does not significantly affect operation of the US11 pathway, consistent with the notion that US11 assumes the role of a substrate recognition module and may be functionally equivalent to OS9. Turnover of a nonglycosylated ribophorin fragment is largely unaffected by manipulation of OS9, consistent with a role for the carbohydrate moiety in substrate processing.

Key words: Class I MHC heavy chain/ Dislocation/ SEL1L/ RI_{332}/ US11

Introduction

Terminally misfolded membrane or secretory proteins that have entered the endoplasmic reticulum (ER) are typically transported back across the ER membrane into the cytosol, a process referred to as dislocation or retrotranslocation. Once in the cytosol, the proteasome degrades these misfolded proteins in a ubiquitin-dependent manner (Ellgaard and Helenius, 2003).

How the cell distinguishes between terminally misfolded polypeptides and those that may still acquire their final conformation remains a mystery. We know of several proteins that participate in substrate recognition, but how they interact with other parts of the dislocation machinery is not clear. A protein marked for degradation crosses the ER membrane and accesses ER-associated components of the ubiquitination machinery. The AAA ATPase Cdc48p (yeast)/p97 (mammals) provides the energy required for extraction in a complex with its co-factors Ufd1p and Npl4p (Bays and Hampton, 2002).

In broad outline, most dislocation substrates take this path, but the specific route used depends on the nature of the substrate itself and on the species examined. Model substrates and their corresponding pathways have been characterized most extensively in yeast, and have suggested the existence of three distinct groups of degradation substrates: proteins with misfolded cytosolic domains, proteins with misfolded luminal domains, and proteins where misfolding occurs in their transmembrane region(s) (Vashist and Ng, 2004).

In mammalian cells, most of the components of the dislocation machinery remain to be clarified in molecular detail. Although some components show some sequence similarities to yeast proteins, their contribution to dislocation is not always clear. There are often several mammalian orthologues for each yeast component of the dislocation machinery, thus precluding direct identification of mammalian components by study in yeast.

Analysis of two viral proteins encoded by human cytomegalovirus (HCMV), US2 and US11, has helped define the composition of the protein complexes involved in dislocation (Ahn et al., 1996) and emphasizes the complexity of mammalian dislocation compared to simpler eukaryotes. Both US2 and US11 facilitate dislocation of newly synthesized Class I MHC heavy chains (HCs), presumably to evade recognition by cytotoxic T cells at the appropriate stages of the virus' lifecycle (Tortorella et al., 2000a). US2 and US11 are ER-resident type I transmembrane proteins that interact with Class I MHC HC in the ER lumen and from there initiate their destruction (Wiertz et al., 1996a; Wiertz et al., 1996b). US2 and US11 achieve this by recruiting different sets of proteins: US2 uses signal peptide peptidase (SPP) (Loureiro et al., 2006) and other proteins that remain to be identified, whereas US11 engages a pathway that includes Derlin-1 (Lilley and Ploegh, 2004). Derlin-1 itself associates with the ubiquitin ligase HRD1 and gp78, both of which share sequence similarities with yeast Hrd1p (Lilley and Ploegh, 2005a; Ye et al., 2005). Whether HRD1 and gp78 are involved in the ubiquitination of Class I MHC HC is an open question (Kikkert et al., 2004), although the human homologue of yeast Hrd3p, SEL1L, is involved in Class I MHC HC dislocation (Mueller et al., 2006). Derlin-1, HRD1 and the transmembrane protein VIMP form a complex with p97 and its cofactors UFD1/NPL4, and might be involved in their recruitment to the ER membrane (Lilley and Ploegh, 2004; Lilley and Ploegh, 2005a; Ye et al., 2005; Ye et al., 2004).

How a luminal protein can cross the lipid bilayer is not known, and the existence of a proteinacous pore, consisting of Hrd1p and/or Der1p has been suggested (Gauss et al., 2006a; Gauss et al., 2006b; Lilley and Ploegh, 2004; Lilley and Ploegh, 2005a; Ye et al., 2005; Ye et al., 2004). Alternative modes of extraction of proteins from the ER that involve lipid rearrangements have also been proposed (Ploegh, 2007).

US11 hijacks a pathway that contributes to the degradation of aberrantly folded proteins independently of viral accessories, as shown by examination of the mammalian dislocation substrates α1-antitrypsin null Hong Kong (NHK), truncated ribophorin RI_{332} and misfolded cystic fibrosis transmembrane conductance regulator

(CFTR) ΔF508 (Mueller et al., 2006; Oda et al., 2006; Younger et al., 2006). Here we identified new components of the mammalian dislocation machinery that are essential for degradation, including an ER luminal protein important for substrate recognition, the E2 ligase that cooperates with HRD1/SEL1L, and two ER transmembrane proteins that act downstream of the substrate selection process.

Results:

Isolation and identification of proteins that interact with SEL1L.

We conducted a large-scale immunopurification of SEL1L using HA-TEV-tagged SEL1L transduced into HeLa cells (Figure 1). The HA-TEV tag was fused to the N-terminus of SEL1L, for which we replaced its signal sequence with that of the murine Class I MHC molecule H2-K^b. HA-TEV-SEL1L was isolated by immunoprecipitation with anti-HA antibody-coated beads from digitonin extracts. Materials eluted with TEV protease were subjected to SDS-PAGE, and SEL1L-interacting polypeptides were identified by tandem mass-spectrometry (LC/MS/MS, Figure 1). We recovered several proteins already known to be SEL1L interactors: HRD1, a ubiquitin E3-ligase involved in ER dislocation (Kikkert et al., 2004; Lilley and Ploegh, 2005a), Derlin-2, a multispanning transmembrane protein required for exit of polyomavirus from the ER (Lilley et al., 2006), the ATPase p97 and several other proteins involved in protein folding, such as PDI, BiP, and calnexin (Figure 1b). The latter bind to many different proteins in the ER, and their contribution, if any, to dislocation is not always clear.

We identified four additional proteins not previously known to be part of the mammalian dislocation machinery: OS9, ancient ubiquitous protein 1 (AUP1), and UBXD8 (Figure 1a and b). In addition, we identified UBC6e, an enzyme that serves as a ubiquitin conjugating enzyme (E2) (Lenk et al., 2002). Because of its physical association, we propose that UBC6e is the E2-type activity that acts in concert with the ubiquitin ligase HRD1.

OS9 was originally identified as a protein amplified in osteosarcoma. OS9 is ubiquitously expressed and has alternative splice versions (Kimura et al., 1998). The C-terminus of OS9 interacts with HIF1α, a subunit of the protein hypoxia inducible factor (HIF) 1. HIF1α is ubiquitinated and degraded, depending on oxygen levels in the cell. OS9 regulates HIF1α levels by increasing the rate of prolyl hydroxylation in HIF1α, thereby initiating ubiquitination (Baek et al., 2005).

The presence of an N-terminal signal sequence suggests that OS9 is targeted to the ER lumen. OS9 has a glucosidase type II (Mannose-6 phosphate receptor homology, MRH) domain involved in binding to misfolded proteins. In yeast, its homolog Yos9p is a luminal ER protein that binds to the luminal domain of Hrd3p, the homolog of SEL1L. Yeast Yos9p targets terminally misfolded ER proteins to the dislocation machinery, consisting of Hrd3p and Der1p (Carvalho et al., 2006; Denic et al., 2006; Gauss et al., 2006a). We now show that OS9 is also part of the mammalian dislocation machinery by binding to SEL1L, and that OS9 is an ER-resident glycosylated protein (Figure 1a and 2b). OS9 has been previously located to the cytosol (Litovchick et al., 2002, Baek et al., 2005). It remains unclear whether there is a pool of OS-9 that is active in the cytosol or whether OS9 could regulate HIF1α indirectly from within the ER.

AUP1 is proposed to interact with integrins (Kato et al., 2002), but its function is obscure. AUP1 has a CUE domain, involved in ubiquitin binding or in recruitment of ubiquitin conjugating enzymes to the site of dislocation (Hurley et al., 2006). AUP1 has a transmembrane anchor at its N-terminus, with the bulk of the protein predicted to be in the cytosol (Kato et al., 2002). AUP1 has not previously been implicated in any aspect of (glyco) protein quality control, and is without an obvious homolog in yeast.

UBXD8 (ETEA) was initially identified among proteins highly upregulated in T-cells obtained from patients with atopic dermatitis (Imai et al., 2002a). UBXD8 has a UBX domain, a UBA domain, a UAS domain, and a transmembrane domain according to prediction programs (Figure 1b). The UBA domain is found in many proteins of otherwise divergent structure and function, and mediates binding to ubiquitin. The UBX domain is structurally similar to ubiquitin despite the lack of a high degree of sequence homology. UBX domains may serve as adaptors for the multi-functional AAA ATPase p97 (Buchberger, 2002). The UAS domain is a domain of > 100 amino acids of unknown function, which assumes a thioredoxin-type fold (InterPro database). The closest relative of UBXD8 in yeast cannot immediately be inferred, because of the limited extent of overall sequence identity and lack of functional data.

UBC6e (UBE2J1) is an ortholog of yeast Ubc6p, a transmembrane ER-bound ubiquitin conjugating enzyme (E2). In yeast, Ubc6p can function together with the ubiquitin ligase Doa10p and the cytosolic E2 Ubc7p (Chen et al., 1993; Swanson et al., 2001). In a Ubc6p deletion strain the half-life of the yeast model substrate, CPY*, is extended two-fold. The *Δubc6p Δubc7p* double mutants are indistinguishable from the *Δubc7* single mutant and delay CPY* degradation, a substrate of the Hrd1p/Hrd3p dependent degradation pathway, more than three-fold (Hiller et al., 1996). There are two Ubc6p orthologs in mammalian cells, UBE2J1 and UBE2J2 (Lenk et al., 2002). UBE2J1 was termed UBC6e, and UBE2J2 is called UBC6 (Lenk et al., 2002). For simplicity we shall refer to UBE2J1 as UBC6e. UBC6e and UBC6 are both involved in the degradation of TCRα and CFTRΔF508 (Lenk et al., 2002, Younger et al., 2006). UBC6e forms a complex with Derlin-1 for CFTRΔF508 disposal (Younger et al., 2006). UBC6e displays less sequence identity (25%) to the yeast protein than does UBC6 (40%) (Lenk et al., 2002). Unlike yeast Ubc6p, human UBC6e is a stable protein (Oh et al., 2006).

ER localization of the newly identified proteins.

We performed immunofluorescence microscopy with affinity purified anti-AUP1 and anti-UBXD8 antibodies in HeLa cells. Immunofluorescence shows the diagnostic reticular ER staining pattern for AUP1 and UBXD8 and co-localization with the ER marker PDI (Figure 2a). We thus localize endogenous AUP1 and UBXD8 proteins to the ER, the site where dislocation occurs. Our antibodies directed against UBC6e and OS9 failed to show specific staining in immunofluorescence.

Immunoblotting of microsomal fractions showed that UBXD8, AUP1, and UBC6e all readily co-sediment with the microsomes in the absence of detergent, and are largely resistant to extraction with alkaline sodium carbonate and urea (Figure 2b). OS9, consistent with its predicted characterization as a soluble ER luminal protein, is readily extracted from the microsomes by alkaline sodium carbonate. For OS9, we observe the presence of two splice variants (Kimura et al., 1998), both of which are

sensitive to digestion with endoglycosidase H (EndoH, Figure 2c), consistent with ER residency and the presence of the single N-linked glycan predicted by the amino acid sequence of OS9.

OS9 overexpression perturbs dislocation of RI_{332} but not nonglycosylated RI_{332}-Thr or Class I MHC via US11

Based on the sequence coverage of OS9 recovered in the SEL1L pull-down, we are confident that OS9 is a member of the same complex. We then performed experiments aimed to demonstrate a possible role for OS9 in dislocation. We designed an N-terminal GFP-tagged version and two mutant versions (R188A; E212D) of OS9. These point mutations are predicted to disrupt the mannose receptor homology (glucosidase II) domain which has been implicated in OS9 substrate interaction (Bhamidipati et al., 2005; Hancock et al., 2002). There is a marginal effect on class I MHC HC dislocation when introducing the mutant versions of OS9 or the GFP-tagged OS9 into US11-expressing cells (Figure 3 and Figure 6a, lanes 10-12). The effect is very small compared to the effect seen with overexpression of UBC6e, AUP1-GFP or UBXD8-GFP (Figure 5 and 6). We do not consider such a low level of inhibition as evidence that implicates OS9 in US11-mediated dislocation of Class I MHC HC. As OS9 might be involved in substrate recognition, a plausible explanation for the comparative dispensability of OS9 in US11-expressing cells is that US11 might be directly responsible for substrate recognition, and target Class I MHC HC directly to the dislocation machinery.

To address whether OS9 plays a role in dislocation independently of viral proteins, we generated HeLa cells that stably overexpress wild-type OS9, GFP-OS9, or the mutant versions OS9 R188A, and OS9 E212D. We then transiently transfected these cell lines with a truncated version of ribophorin, RI_{332} (Kitzmuller et al., 2003), a protein that is dislocated in a SEL1L-dependent manner (Mueller et al., 2006). For all constructs examined, we observe a delay in RI_{332} degradation (Figure 4a and 4b). The stable endogenous ribophorin is electrophoretically distinct and serves as a control for recovery in the immunoprecipitation. We conclude that OS9 does play an important role in the dislocation of the soluble glycoprotein, RI_{332}.

In addition, we generated a truncated ribophorin mutant that lacks its glycosylation site, RI_{332}-Thr, (de Virgilio et al., 1999) RI_{332}-Thr is also a short-lived protein, but its degradation is not significantly impaired by the introduction of the OS9 constructs (Figure 4c and 4d). This experiment demonstrates that OS9 is involved specifically in the disposal of glycosylated proteins. Since the change in amino acid sequence required to eliminate the N-linked attachment site might also alter the three dimensional structure of RI_{332}, we cannot attribute the difference in sensitivity to OS9 level exclusively to the glycosylation status of the RI_{332} substrate.

Verification of involvement in dislocation for the SEL1L-interacting proteins UBXD8, AUP1 and UBC6e.

Since US11 and US2 both target Class I MHC molecules but apparently do so by initially recruiting different proteins, we used US2-mediated dislocation as a control for proper ER function (Lilley and Ploegh, 2004; Loureiro et al., 2006; Mueller et al., 2006). Manipulations that perturb ER function non-specifically should affect dislocation via both the US2 and the US11 pathways. Our criterion is thus to score as specific those manipulations that interfere with US11-mediated dislocation only.

Amongst the set of SEL1L-interacting proteins, UBC6e was the only protein known to act as an enzyme and whose catalytic center could be ascertained (Lenk et al., 2002). Thus for the E2 enzyme UBC6e, a catalytically inactive version was generated by replacement of cysteine 91 with serine (Oh et al., 2006).

We installed a GFP tag onto the C-terminus of AUP1 and UBXD8 and onto the N-terminus of OS9. We reasoned that the GFP domain may interfere with, but not completely abolish, the function or recruitment capabilities of flanking domains and thus yield inhibitory effects for the corresponding GFP fusion proteins (Lilley and Ploegh, 2004). Specifically, since UBXD8 has a UBX domain that might recruit p97 to the site of dislocation, the attachment of a globular GFP-sized domain in close proximity to the C-terminal UBX domain might interfere with this interaction. Similarly, the GFP-tagged version of Derlin-1 inhibits Class I MHC HC degradation in US11-dependent fashion (Lilley and Ploegh, 2004).

In pulse-chase experiments on US11 cells that overexpress UBC6e C91S, AUP1-GFP, and UBXD8-GFP, we observe strong inhibition of class I MHC HC degradation (Figures 5 and 6). In cells that express the empty vector (pLHCX, control cells), most Class I MHC HCs have lost their N-linked glycan at the 30 min chase point, due to the activity of the cytoplasmically disposed PNGase (Hirsch et al., 2003) (Figure 5a and b). In the presence of proteasome inhibitor (ZL_3VS), the diagnostic deglycosylated dislocation intermediate accumulates and is recognized by its distinct mobility on SDS PAGE (Wiertz et al., 1996a). When overexpressing catalytically inactive UBC6e (C91S) or wild-type UBC6e, we see a pronounced delay in the degradation of Class I MHC HC: more than 75% of HC remains in the ER (Figure 5a-1, lanes 4-9). US11 is expressed to comparable levels in all three cell types, and displays the typical delayed cleavage of its signal peptide (Rehm et al., 2001) (Figure 5a-3, lanes 1-9).

In US2 cells transduced with the very same constructs, degradation continues unperturbed: Class I MHC HCs are dislocated at rates very similar to those observed in control cells, compared to cells that overexpress UBC6e or UBC6e C91S (Figure 5c-1, lanes 1-9). US2 itself is expressed at similar levels in all three cell lines and shows its usual mobility on SDS-PAGE: in addition to ER-membrane inserted glycosylated US2, a faster migrating US2 occurs that represents US2 lacking its N-linked glycan, as US2 is inefficiently translocated into the ER (Gewurz et al., 2002). Both the US2 and the US11 cell lines were obtained by viral transduction of UBC6e C91S and WT UBC6e and show equivalent levels of expression of UBC6e (Figure 5a-2, lanes 4-9 and Figure 5c-2, lanes 4-9). We conclude that the ubiquitin-activating enzyme UBC6e is involved in Class I MHC HC dislocation in US11 cells, but not in US2 cells. Because US2 cells remain capable of proper dislocation, ER function as such is not compromised.

We then examined the fate of Class I MHC HC when expressing the GFP-tagged versions of the three newly identified proteins AUP1, UBXD8 and OS9 (Figure 6). Cells that express AUP1-GFP showed inhibition of dislocation: 50% of Class I MHC HC remains in the ER after 30min of chase (Figure 6a, lanes 4-6, and Figure 6b). The effect is even more pronounced when using cells that express UBXD8-GFP:

more than 75% of HCs fail to reach the cytosol (Figure 6a, lanes 7-9 and Figure 6b, compared with lanes 1-3). GFP-OS9 did not significantly inhibit dislocation of Class I MHC HC. Again, US2 cells served as a control (Figure 6c). After introduction of the same GFP-constructs into US2-expressing cells, we observed no effect on Class I MHC HC dislocation (Figure 6c and d).

Based on data describing the recruitment of Cdc48p to the site of dislocation by Ubx2p, we wondered if the GFP-tag hinders recruitment of p97 by the UBX domain of UBXD8. We compared the ability of UBXD8 and UBXD8-GFP to recruit p97 into the dislocation complex. To this end, we overexpressed UBXD8 and UBXD8-GFP to the same levels in 293T cells and performed an immunoprecipitation with anti-UBXD8 antibodies from digitonin lysates. The recovered material was then analyzed by immunoblotting with anti-p97 antibodies (Figure 6f). The amount of p97 recovered in immunoprecipitates from cells expressing UBXD8-GFP is much reduced compared to cells expressing wild type UBXD8. We conclude that the GFP tag hinders recruitment of p97 to the ER membrane and therefore blocks dislocation. The residual p97 recovered is attributable to the endogenous UBXD8 present in the cells.

AUP1 has not been previously implicated in ER dislocation. The Cue domain-containing protein Cue1p in yeast recruits the cytosolic E2 enzyme Ubc7p to the ER membrane (Biederer et al., 1997). AUP1 has a CUE domain, but it remains unclear whether this domain is used to recruit the E2 UBC6e to the ER as the site of dislocation. We consider this unlikely, since UBC6e itself has a membrane anchor (Figure 1c) and localizes to the ER (Lenk et al., 2002). We did not observe a complex between SEL1L and UBC7 in human cells by affinity purification under mild conditions using SEL1L antibodies and subsequent interrogation of the immunoprecipitate with UBC7 antibodies (data not shown), even when overexpressing UBC7. For this reason, we did not further pursue the possible involvement of UBC7 in US11-mediated Class I MHC HC dislocation.

The dominant negative constructs of UBC6e, AUP1 and UBXD8 retain Class I MHC HC in the ER

We used the monoclonal antibody W6/32, which recognizes only correctly assembled Class I MHC molecules in their fully native conformation (Barnstable et al., 1978), to explore whether inhibition of dislocation is accompanied by an increase in the amount of correctly folded Class I MHC molecules. We indeed found this to be the case (Figure 7, cell lines used were those from Figure 5 and Figure 6) and conclude that the intermediates that accumulate when dislocation is inhibited retain their typical orientation within the ER. In pulse chase experiments, the W6/32-reactive Class I MHC molecules do not undergo conversion of their high mannose to the complex type glycans, as inferred by a lack of a shift in mobility assessed by SDS-PAGE. This observation is consistent with the ability of US11 to retain Class I MHC molecules in the ER, also when dislocation is blocked, as observed for the single point mutant in the transmembrane segment of US11 (Lilley et al., 2003). This experiment also demonstrates that UBC6e C91S, UBC6e WT, AUP1-GFP, and UBXD8-GFP do not disrupt dislocation merely by preventing the association of US11 with Class I MHC HC: as with the empty vector control, US11 co-immunoprecipitates with W6/32-reactive Class I MHC HC in all of the cell lines constructed (Figure 7).

Discussion:

We have identified four new components of the mammalian dislocation machinery. We used as the point of departure the isolation of SEL1L-interacting partners. We reasoned that via SEL1L we should recover additional proteins involved in ER dislocation: both ER luminal components that may be involved in substrate recognition, and -through its binding partner HRD1- additional cytosolic components that act downstream.

We chose a cell line that did not express US11 to isolate the SEL1L complex to avoid possible bias that might derive from remodeling of the dislocation machinery by US11 itself. We then verified the significance of the isolated proteins by returning to our model dislocation cell lines, those expressing US11 or US2. US11 uses a pathway that is superficially similar to the Hrd1p/Hrd3p pathway in yeast (Lilley and Ploegh, 2004; Lilley and Ploegh, 2005a; Mueller et al., 2006). The complex that degrades CFTR Δ F508 has also been analyzed in some detail and consists of the E3 ubiquitin ligase RMA1, unrelated to Hrd1/SEL1L, and the ubiquitin-conjugating enzyme, UBC6e (Lenk et al., 2002; Younger et al., 2006). UBC6e is involved in the degradation of TCRα (Lenk et al., 2002). UBC6e shows only 25% sequence identity with yeast Ubc6p, and the human ortholog cannot complement yeast Ubc6p (Lenk et al., 2002).

The extent of sequence coverage obtained in our proteomic analyses suggests that OS9 may be a stoichiometric partner of SEL1L. From our analysis of the US11 pathway, the role of OS9 in dislocation is not immediately apparent. We thus turned to an examination of the fate of the ribophorin fragment RI_{332} to assess a possible contribution of OS9 to dislocation, because RI_{332} is removed from the ER and destroyed in a SEL1L-dependent manner. When interpreting whether or not there is an effect on dislocation, it is imperative to keep in mind the timescale of dislocation of each of the substrates. Since US11-mediated dislocation proceeds at a rapid pace (Class I MHC HC half-life is only 2-5 minutes), it may well be more sensitive to minor perturbations in metabolism than the dislocation of other, longer-lived,

substrates such as RI_{332} or RI_{332}-Thr. This sensitivity is valuable for identification of members of the dislocation complex, but requires a more stringent threshold when discussing significance. Thus the same magnitude of stabilization of RI_{332} may well be more significant than for Class I MHC HC. Since the effect of the OS9 mutants and GFP-OS9 on US11-mediated dislocation is much lower than AUP1-GFP and UBXD8-GFP, we consider the role of OS9 to be comparatively minor (Figure 6). In contradistinction, the moderate effect of manipulating OS9 level on RI_{332} degradation is sufficient to implicate OS9 in the quality control mechanism of RI_{332}, as is the case in SEL1L-dependent degradation (Mueller et al., 2006).

Since overexpression of wildtype OS9 inhibits dislocation of RI_{332}, excess OS9 likely disrupts the architecture of the complex by titrating away components and rendering the dislocon incapable of efficiently processing substrates. Interestingly, we do not see such a difference in US11 cells that overexpress OS9 to similar levels. Why does a disruptive level of OS9 not disrupt the geometry of the dislocon in US11-expressing cells? We attribute this discrepancy to the fact that US11 itself may stabilize the complex in a way that is insensitive to excess OS9 levels. Perhaps the rapidity of US11-mediated dislocation in itself also points to a stabilized dislocon and a streamlined substrate recognition method.

Since overexpression of wildtype OS9 perturbed dislocation, we were unable to study the importance of the postulated lectin capacity of OS9 with the MRH point mutants of OS9 (Bhamidipati et al., 2005). We therefore examined the fate of a non-glycosylated, but nonetheless short-lived version of RI_{332}, RI_{332}-Thr (de Virgilio et al., 1999). The degradation of RI_{332}-Thr is not greatly impaired by overexpression of wildtype or mutant OS9, suggesting that the glycan is important for OS9 associated dislocation (Figure 8b). Much like the US2 system, the fact that the fate of RI_{332}-Thr is unaffected argues against gross disturbance of the ER as the reason for stabilization of RI_{332}. Since we see little effect of OS9 on RI_{332}-Thr degradation, we conclude that this nonglycosylated substrate must use a pathway that does not involve the OS9-associated complex. There is evidence for multiple pathways in both yeast and mammalian cells and the pathways in yeast discriminate between substrates based on the location of the misfolded lesion (Vashist and Ng, 2004).

Likewise, there may be an as yet to be identified mammalian dislocon that does not contain OS9 and dislocates nonglycosylated terminally misfolded proteins.

Apart from this study, there is no data linking OS9 to protein degradation from the ER in mammalian cells. In this case, extrapolation from the yeast to the mammalian system is especially tricky, as yeast lack a functional calnexin/calreticulin (CNX/CRT) cycle. In mammalian cells, terminally misfolded glycosylated proteins are usually re-directed from the CNX/CRT cycle to other putative lectins in the ER, such as members of the EDEM (ER degradation enhancer, mannosidase a-like) family (Molinari et al., 2003). The mannose content of the misfolded proteins' N-linked glycans are thought to be what is recognized by EDEM (Brodsky, 2007; Hebert and Molinari, 2007). EDEM proteins fulfill a dual role: they first escort substrates out of the CNX/CRT cycle, and then maintain their solubility for subsequent degradation (Kanehara et al., 2007). EDEM proteins also interact with members of the Derlin family, providing a link to the dislocation pathway (Oda et al., 2006). Surprisingly, we did not find EDEM proteins in our biochemical analysis of SEL1L-interacting proteins. Instead, OS9, the ortholog of Yos9p, seems to play a role as "degradative" chaperone. Whether EDEMs are part of the SEL1L complex, but in a more transient way than OS9, remains to be investigated.

Combined, these results are consistent with a model in which US11 serves the specific function of delivering Class I MHC HC to the HRD1/SEL1L complex and accelerates their removal from the ER and degradation (Figure 8a). In HeLa cells, OS9 is an integral part of this complex, and contributes to substrate recognition. For neither mammalian OS9 nor for its yeast homolog, Yos9p, is it clear what (sets of) endogenous substrates each of them recognize. The example of US11 shows that other proteins can assume a substrate recognition function in the context of the larger HRD1/SEL1L complex and deliver substrates to the ligase complex.

Furthermore, we show that UBC6e is involved in the degradation of Class I MHC HCs in US11 cells. The identification of the ubiquitin-conjugating enzyme (E2) that mediates this process has been an important goal, and one possible E2, the E2-25K protein, was uncovered using a permeabilized cell system (Flierman et al., 2006) to

assay for its activity. However, this assay does not allow exchange or removal of membrane-bound molecules, and so it is not surprising that UBC6e, an E2-type enzyme equipped with a transmembrane anchor, escaped detection. From the *in vitro* data in intact cells presented here, we believe that UBC6e is the primary E2 enzyme that catalyzes the ubiquitination of Class I MHC HCs in US11 cells. Other E2s, especially if present in excess, might nonetheless be capable of performing the same reaction.

Ubc6p in yeast is a type IV ER-resident membrane protein, thought to be integrated into the ER membrane without the help of Sec61p (Sommer and Jentsch, 1993). Its levels are carefully controlled, as it is a short-lived substrate of ER degradation itself, and overexpression has an inhibitory effect on dislocation (Lenk et al., 2002; Walter et al., 2001). Analysis of complexes assembled around Hrd1p/Hrd3p in yeast did not yield Ubc6p (Carvalho et al., 2006, Denic et al., 2006), and previous studies report binding of yeast Hrd1p to Ubc7p or Ubc1p *in vivo,* not to Ubc6p (Bays et al., 2001a). Ubc7p has no transmembrane anchor and requires the CUE-domain containing protein Cue1p for recruitment to the ER membrane (Biederer et al., 1997; Bordallo and Wolf, 1999). While we did not detect the human homologue of Ubc7p through co-immunoprecipitation with SEL1L, we did find a CUE domain containing protein, AUP1 (Kato et al., 2002), as an interactor of SEL1L. The AUP1-GFP fusion caused a delay in Class I MHC HC degradation in US11, but not in US2 cells. Therefore, in this model AUP1 makes an essential contribution to US11-dependent dislocation. AUP1 might be necessary to target UBC6e specifically to the dislocation complex within the ER by mediating the association of UBC6e and SEL1L.

We also identified two UBX domain-containing proteins, UBXD2 and UBXD8, both of which associate with SEL1L. UBXD2 (Erasin), the first mammalian UBX-containing protein linked to dislocation, participates in the degradation of CD3δ (Liang et al., 2006), but does so through unknown mechanisms. Could UBXD8 be the possible homolog of Ubx2p, a protein that spans the ER membrane twice and is involved in recruiting p97 to the ER membrane (Neuber et al., 2005; Schuberth and Buchberger, 2005)? We do see strong inhibition of US11-mediated HC dislocation when overexpressing UBXD8-GFP. However, UBXD8 shares only 17% sequence

identity with Ubx2p. Curiously, UBXD8 shares the same level of homology with Ubx3p (another Cdc48p cofactor of unknown function). Ubx3p was not reported to be part of the dislocation complex in yeast (Carvalho et al., 2006; Denic et al., 2006). UBXD8 and Ubx3p share similar organization, reflected by the order of the distinct domains that are present: both are predicted to have a UAS and a UBX domain C-terminal to a single transmembrane domain. In contrast, Ubx2p has two transmembrane domains and lacks the UAS domain, but does have a UBA domain at its N-terminus. If UBXD8 were to be inserted as a type I or type II ER transmembrane protein, either the UBA or the UBX domain would reside within the ER lumen. Domains that specify involvement in the ubiquitination pathway are not usually found inside the ER. We see clear ER–localization of UBXD8 in immunofluorescence and by sedimentation analysis of microsomes (Figure 2), so we propose a similar mechanism of ER insertion as has been shown for Erasin or UBXD2. UBXD8 might be inserted in the ER membrane by dipping into the outer leaflet of the lipid bilayer (Figure 8a) with both tails exposed to the cytosol (Liang et al., 2006). UBXD8 and UBXD2 might both be involved in recruitment of p97 to the site of dislocation, together or separately, depending on the topology of the substrate. The GFP tag installed on UBXD8 hinders recruitment of p97 which might account for the slowed dislocation (Figure 6f).

It is now clear that UBC6e, AUP1, and UBXD8 are required for the exit of a type I ER-membrane protein from the ER (Figures 5, 6, 7, 8a). UBC6e and AUP1 each have one transmembrane segment and UBXD8 may dip into the cytosolic face of the ER membrane, all of which may contribute to the formation of a proteinacious channel. Each of these three proteins also contain conserved functional domains with cytoplasmic exposure (Figure 1c, 8a). A schematic representation of the putative organization and composition of this complex is shown in figure 8. The initial step of the dislocation pathway involves recognition of the substrate. In the case of Class I MHC HC, this is primarily done by US11, but for RI_{332}, a glycosylated misfolded protein, OS9 is involved in the process. The other three proteins described in this paper, AUP1, UBXD8 and UBC6e, also act prior to cytoplasmic disposition of the dislocation substrate (Figure 4). UBC6e acts as an E2 ubiquitin ligase and UBXD8 appears to play a role in the recruitment of the AAA ATPase p97. The role of AUP1

remains elusive, but its CUE domain may be involved in recruitment of another ubiquitin conjugating enzyme. The identification of additional proteins that participate in these reactions, as reported here, is an important step towards a better understanding of the essential cellular process of dislocation.

Experimental Procedures

Antibodies, cell lines, constructs

Antibodies:

The cytosolic parts of the three proteins AUP1 (aa 62-411), UBC6e (aa 1-232), and UBXD8 (aa 361-445) were expressed as N-terminal His-tagged fusions in *E. coli BL21 (DE3) Rosetta* cells and purified. The recombinant His-tagged fusion proteins were sent to Covance Research Products to generate rabbit polyclonal antibodies. Antibodies against AUP1, UBC6e, and UBXD8 were affinity purified as described (Lilley and Ploegh, 2004). Antibodies to Class I MHC HC, US2, US11 have been described (Gewurz et al., 2002; Lilley et al., 2003). The anti-GFP, anti-PDI, and anti-OS9 antibodies were purchased from Abcam. Alexa 488-conjugated goat anti-mouse and alexa 568-conjugated goat anti-rabbit were from Molecular Probes. Anti-Ribophorin antibody and the RI_{332} cDNA were a generous gift from N. Erwin Ivessa.

Cell lines:

U373, US2, US11 cell lines have been described (Mueller et al., 2006). HeLa and 293T cells were purchased from ATCC. Cells transduced with pLHCX based vectors were selected and maintained in 125ug/ml hygromycin B (Roche).

Protein Constructs:

The murine H2-K^b signal sequence was fused to the N-terminal HA-TEV tag of SEL1L to ensure proper ER localization. SEL1L was cloned from cDNA using standard methods. The SEL1L sequence is unstable in bacteria and several mutations occured that were removed by single point mutagenesis (Strategene). cDNA clones for UBXD8, OS9, UBC6e, and AUP1 were obtained from Open Biosystems and the open reading frame was cloned into pcDNA3.1(+), pLHCX (clontech), and pEGFP-N1 (clontech). GFP-OS9 was cloned with the OS9 signal sequence replaced by the murine H2-K^b signal sequence followed by GFP.

Anti-HA-affinity purification and MS/MS analysis

$5*10^8$ HeLa cells were lysed for 30 min in 24 ml of ice-cold lysis buffer (2% digitonin, 25mM Tris-HCl pH 7.4, 150mM NaCl, 5mM $MgCl_2$, complete protease

inhibitor tablets (Roche), 2.5mM N-ethylmaleimide). The nuclei and cell debris were pelleted at 16000g for 15min, and the cleared lysate was incubated with 250ul anti-HA-agarose beads (clone 3F10, Roche) for 3hours at 4C with gentle agitation. The beads were washed with 50ml wash buffer (0.1%digitonin, 25mM Tris-HCl pH 7.4, 150mM NaCl, 5mM $MgCl_2$) and eluted with 100 units of tobacco etch virus (TEV) protease (Invitrogen, AcTEV) in 250ul wash buffer at 4C overnight. The eluted material was collected, and the beads were washed with 500 ul wash buffer. The washes and eluted materials were pooled and exchanged into 20 mM NH_4CO_3 pH 8.0, 0.1% SDS by using MicroSpin G-25 Columns (Amersham Biosciences). The eluate was concentrated in a speed-vac and separated by SDS-PAGE (10% acrylamide). Polypeptides were revealed by Coomassie Blue staining, excised, and trypsinized as described (Lilley and Ploegh, 2004). Peptides were sequenced by LC/MS/MS.

Pulse-chase experiments, immunoblotting, SDS-PAGE

Methods for pulse-labeling, cell lysis, immunoprecipitation, pulse-chase regarding Class I MHC HCs in US11 and US2 cells, viral transduction of cells, transfection of cells with RI_{332}, SDS-PAGE, and fluorography have been described (Mueller et al., 2006). All quantitation was performed on a phosphoimager.

Immunofluorescence, microsomal preparation

Cells were seeded onto glass coverslips and allowed to attach overnight. Fixation was achieved with 4% paraformaldehyde for 20 minutes at room temperature. Cells were permeabilized with 0.1% Triton X-100 for 10 minutes at room temperature and incubated with the affinity purified antibody as described (Lilley and Ploegh, 2004). Imaging was performed on a spinning disk confocal microscope at 100x magnification. Microsomes were prepared from U373 cells as previously described (Furman et al., 2002c). Microsomes were incubated in the indicated buffer conditions for 30 minutes and centrifuged at 20,000xg for 20 minutes. The pellet was resuspended directly in reducing sample buffer and the supernatant was first TCA precipitated.

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Figure legends:

Figure 1. Isolation of SEL1L associated proteins.

a. Immunoprecipitation of HA-TEV-tagged SEL1L. HeLa cells (ctrl) and HA-TEV-SEL1L expressing HeLa cells were lysed in 2% digitonin, and the lysate was subjected to immunoprecipitation with anti-HA antibody beads. Bound material was eluted from the beads with TEV protease and separated on SDS PAGE (10% acrylamide). Polypeptides were visualized by Coomassie Blue staining.
b. Proteins that interact with SEL1L, the peptides identified by LC/MS/Ms are shown in grey together with the sequence coverage (in %, number on the right).
c. Proposed domain structure of the isolated SEL1L-interacting partners. CHO = N-linked glycan, ss = signal sequence, TM = transmembrane domain, UBA = ubiquitin associated, UAS = thioredoxin fold, UBX = ubiquitin fold, PlsC = phosphate acyltransferase domain, UBC = ubiquitin conjugating domain. CUE = domain involved in E2 or ubiquitin binding. Numbers on the right represent length in amino acids.

Figure 2. AUP1, UBXD8, OS9 and UBC6e are found in the ER

a. HeLa cells were fixed and incubated with anti-PDI antibody and affinity-purified antibodies against AUP1 (upper panels) or UBXD8 (lower panels) for immunofluorescence analysis. Right panels show the merged images. Scale bars= 10μm.
b. Microsomes from U373 cells were incubated with homogenization buffer, 3M Urea, sodium carbonate pH11.6, or 1% SDS, pelleted and immunoblotted as indicated. T=total, P=pellet, S=supernatant. Proteins were separated by 10% (UBC6e and OS9) or 15% (PDI, Calnexin, AUP1, and UBXD8) SDS-PAGE. Cross-reactive bands are indicated with a #.
c. US11 cells were pulse-labeled for 10min with ^{35}S and chased for indicated time points. The cells were lysed in 1% SDS and the lysate was subjected to immunoprecipitation with anti-OS9-antibodies. The bound material was eluted off the beads and half of the eluate was incubated with EndoH for

1hour at 37^0C. The eluates were separated on SDS-PAGE (8% acrylamide) and visualized on film.

Figure 3. OS9 is not involved in US11-mediated dislocation

a. US11-expressing cells were transduced with the following virus preparations: empty vector (pLHCX), wild type OS9, OS9 R188A, OS9 E212D, or GFP-OS9. The cell lines were treated with ZL3VS and pulse-labeled for 10min with 35S and chased for indicated time points. The cells were then lysed in 1% SDS and immunoprecipitated with anti-HC antibody. The eluates were separated on SDS-PAGE (12% acrylamide) and visualized on film.

b. Quantitation of the amount of glycosylated Class I MHC HC to total HC counts.

Figure 4. OS9 is crucial for dislocation of the terminally misfolded glycoprotein, RI_{332}, but not the nonglycosylated RI_{332}-Thr

a. HeLa cells were transduced with either empty vector (pLHCX), wild type OS9, OS9 R188A, OS9 E212D, or GFP-OS9, using the same virus preparation as in Figure 3. The five cell lines were then transfected with a construct that specifies truncated ribophorin, RI_{332}. The five cell lines were pulse-labeled 36 hrs post transfection for 15min with ^{35}S and chased for indicated time points. The cells were then lysed in 1% SDS and immunoprecipitated with antibody raised against the luminal portion of ribophorin. The eluates were separated on SDS-PAGE (10% acrylamide) and visualized by autoradiography. The # indicates a faster migrating version of RI_{332}.

b. Quantitation of the amount of RI_{332} remaining at the indicated time points.

c. Cells from (a) were transfected with a construct that encodes non-glycosylated truncated ribophorin, RI_{332}-Thr-HA, and a pulse-chase experiment was performed as for (b).

d. Quantitation of the amount of RI_{332}-Thr-HA remaining at the indicated time points.

Figure 5. UBC6e is crucial for US11 mediated dislocation.

a. US11-expressing cells were transduced with either empty vector (pLHCX), UBC6e WT, or UBC6e C91S mutant. The three cell lines were treated with ZL_3VS and pulse-labeled for 10min with ^{35}S and chased for indicated time points. The cells were then lysed in 1% SDS and the lysate was immunoprecipitated with anti-HC (1), anti-UBC6e (2), and anti-US11 (3) antibodies sequentially. The eluates were separated on SDS-PAGE (12% acrylamide) and visualized on film. The asterix represents a slower migrating band that occurs upon overexpression of UBC6e and is likely a phosphorylated version of UBC6e (Oh et al., 2006).
b. Quantitation of the amount of glycosylated HC to total HC counts. US2-expressing cells were transduced with the same virus preparation used in (a). The experiment was performed as in (a).
c. Quantitation of the amount of glycosylated Class I MHC HC to total HC counts.

4.3 Figure 6. AUP1 and UBXD8 are crucial for US11-mediated dislocation

a. US11-expressing cells were transduced with either empty vector (pLHCX), AUP1-GFP, UBXD8-GFP, or GFP-OS9. The four cell lines were treated with ZL_3VS and pulse-labeled for 10min with ^{35}S and chased for indicated time points. The cells were then lysed in 1% SDS and immunoprecipitated with anti-HC antibody. The eluates were separated on SDS-PAGE (12% acrylamide) and visualized on film.
b. Quantitation of the amount of glycosylated Class I MHC HC to total HC counts.
c. US2-expressing cells were transduced with the same virus preparation used in (a) and a pulse-chase experiment was performed as for (a).
d. Quantitation of the amount of glycosylated Class I MHC HC to total HC counts.
e. GFP-fusion constructs are expressed to similar levels and do not affect the level of US11 or US2. The cell lines used in (a) and (c) were lysed in 1%

SDS, separated by SDS-PAGE, and transferred to a PVDF membrane. The membrane was immunoblotted for GFP, calnexin (cnx, loading control), US11, and US2.

f. UBXD8 WT and UBXD8-GFP were expressed in 293T cells and immunoprecipitated with anti-UBXD8 antibodies from digitonin extracts. The eluates were separated on a 10% SDS-PAGE and immunoblotted with anti-p97 antibodies.

Figure 7. Dominant negative versions of UBC6e, AUP1, and UBXD8 retain Class I MHC HC in the ER

a. Cell lines from Figure 5 (a) were pulse labeled for 10 minutes with ^{35}S and chased for the indicated time points. The cells were then lysed in 0.5% NP-40 and properly folded Class I MHC HC was immunoprecipitated with the W6/32 antibody. Eluates were separated on SDS-PAGE (12%) and visualized on film.
b. Cell lines from Figure 6 (a) were treated as in (a).

Figure 8. Schematic of US11- and OS9-mediated dislocation

a. Depicted is the complex that US11 uses to dislocate class I MHC HCs. Fn II = fibronectin type II domain, Glc II = glucosidase II domain. Domains are italicized.

b. OS-9 only assists in the dislocation of misfolded RI_{332} that carries its N-linked glycan. An unglycosylated version of RI_{332}, RI_{332}-Thr remains unaffected by OS9 mutants.

FIGURE I

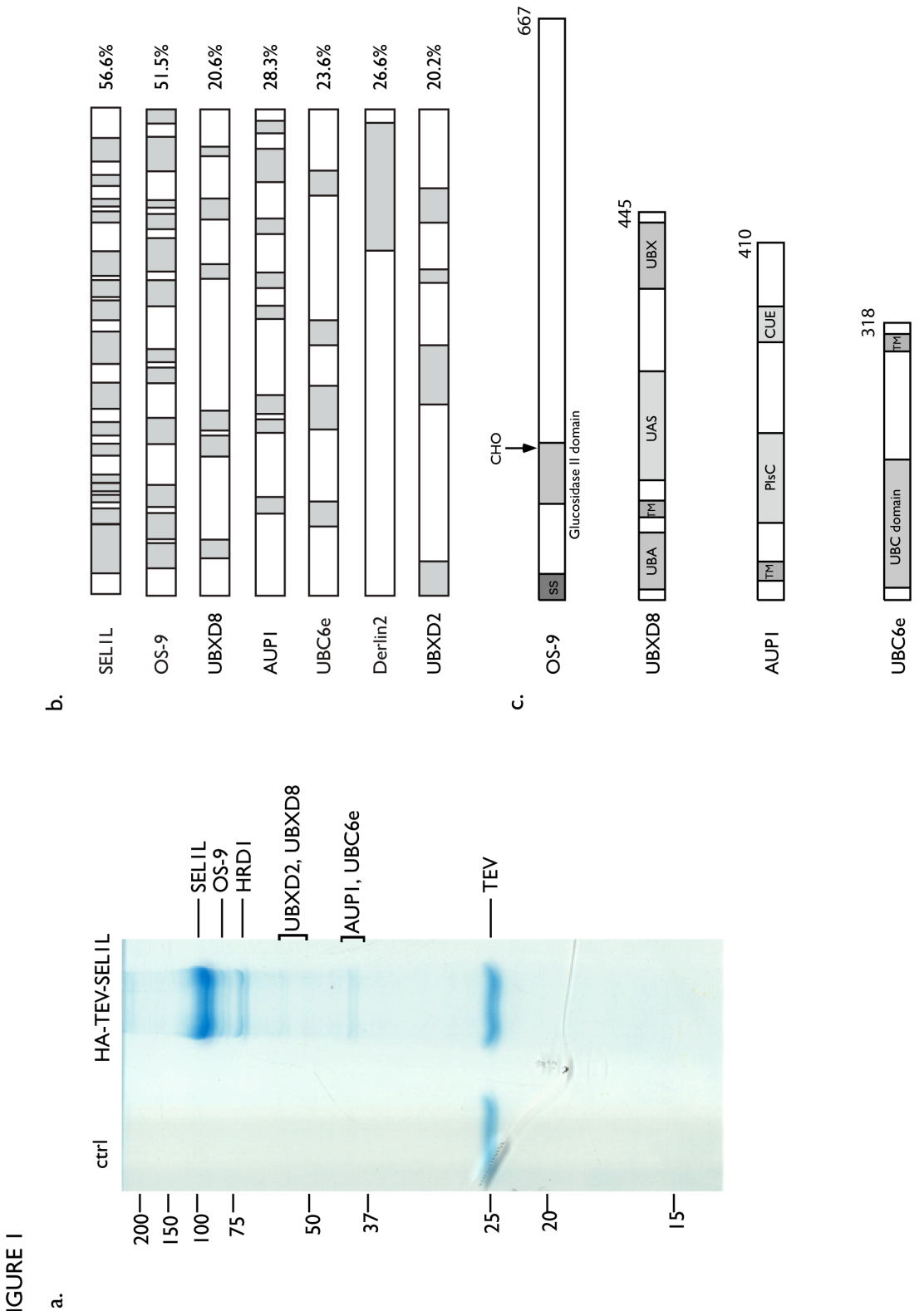
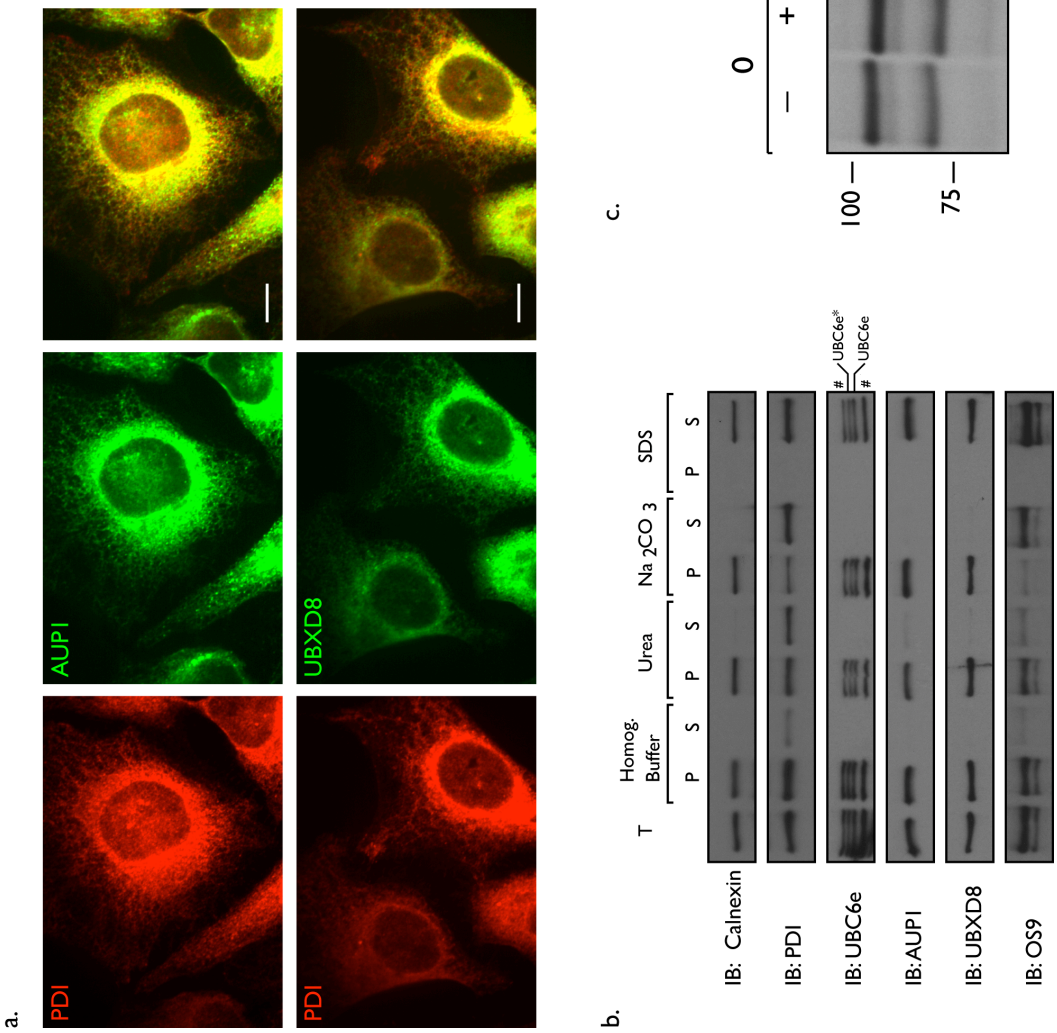

a.
ctrl
HA-TEV-SEL1L
SEL1L
OS-9
HRD1
UBXD2, UBXD8
AUP1, UBC6e
TEV
200
150
100
75
50
37
25
20
15
b.
SEL1L
56.6%
OS-9
51.5%
UBXD8
20.6%
AUP1
28.3%
UBC6e
23.6%
Derlin2
26.6%
UBXD2
20.2%
c.
CHO
667
SS
Glucosidase II domain
OS-9
445
UBA
TM
UAS
UBX
UBXD8
410
TM
PlsC
CUE
AUP1
318
UBC domain
TM
UBC6e

FIGURE 2


a.
PDI
AUP1
PDI
UBXD8
b.
T
Homog. Buffer
Urea
Na 2CO 3
SDS
P S P S P S P S
IB: Calnexin
IB: PDI
IB: UBC6e
#UBC6e*
#UBC6e
IB: AUP1
IB: UBXD8
IB: OS9
c.
min (chase)
0
90
— + — +
EndoH
OS9 Var I + CHO
OS9 Var I – CHO
OS9 Var2 + CHO
OS9 Var2 – CHO
100 —
75 —

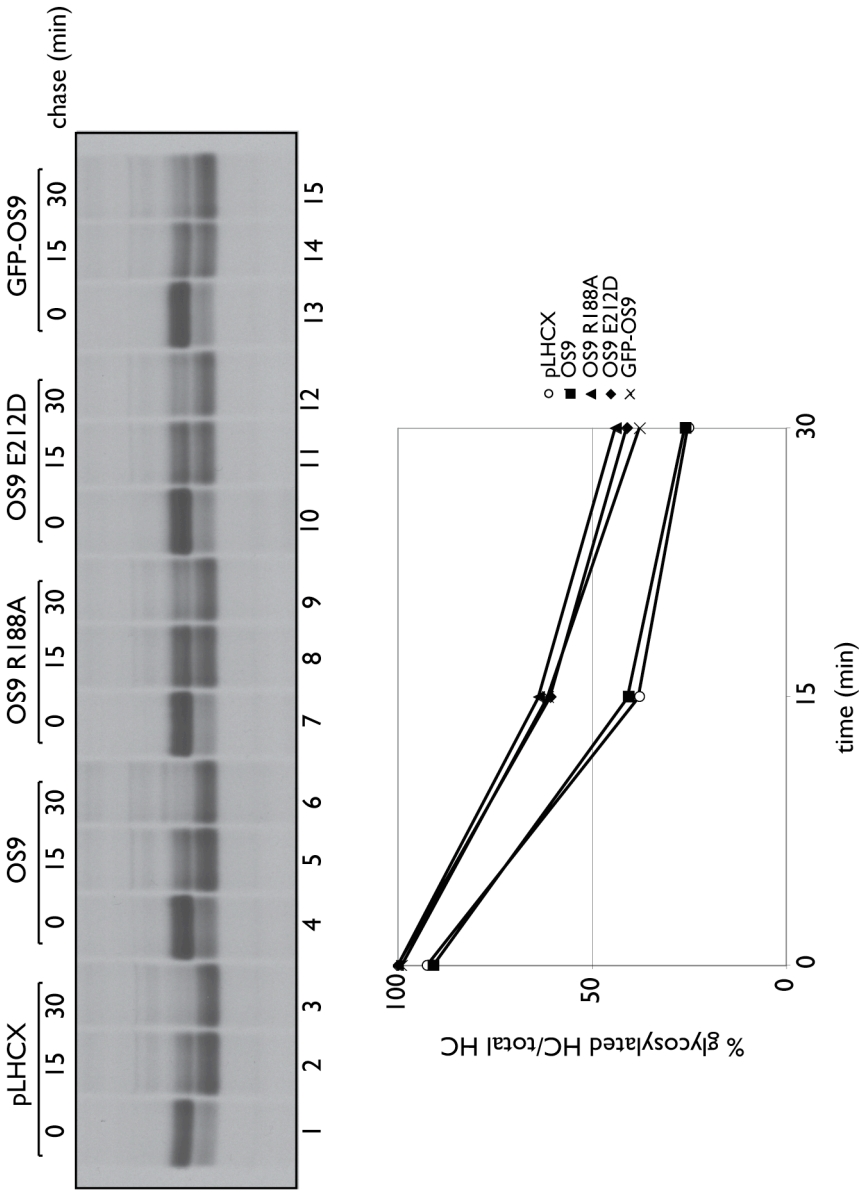

chase (min)
pLHCX
OS9
OS9 R188A
OS9 E212D
GFP-OS9
0 15 30
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
pLHCX
OS9
OS9 R188A
OS9 E212D
GFP-OS9
% glycosylated HC/total HC
100
50
0
0
15
30
time (min)

FIGURE 3

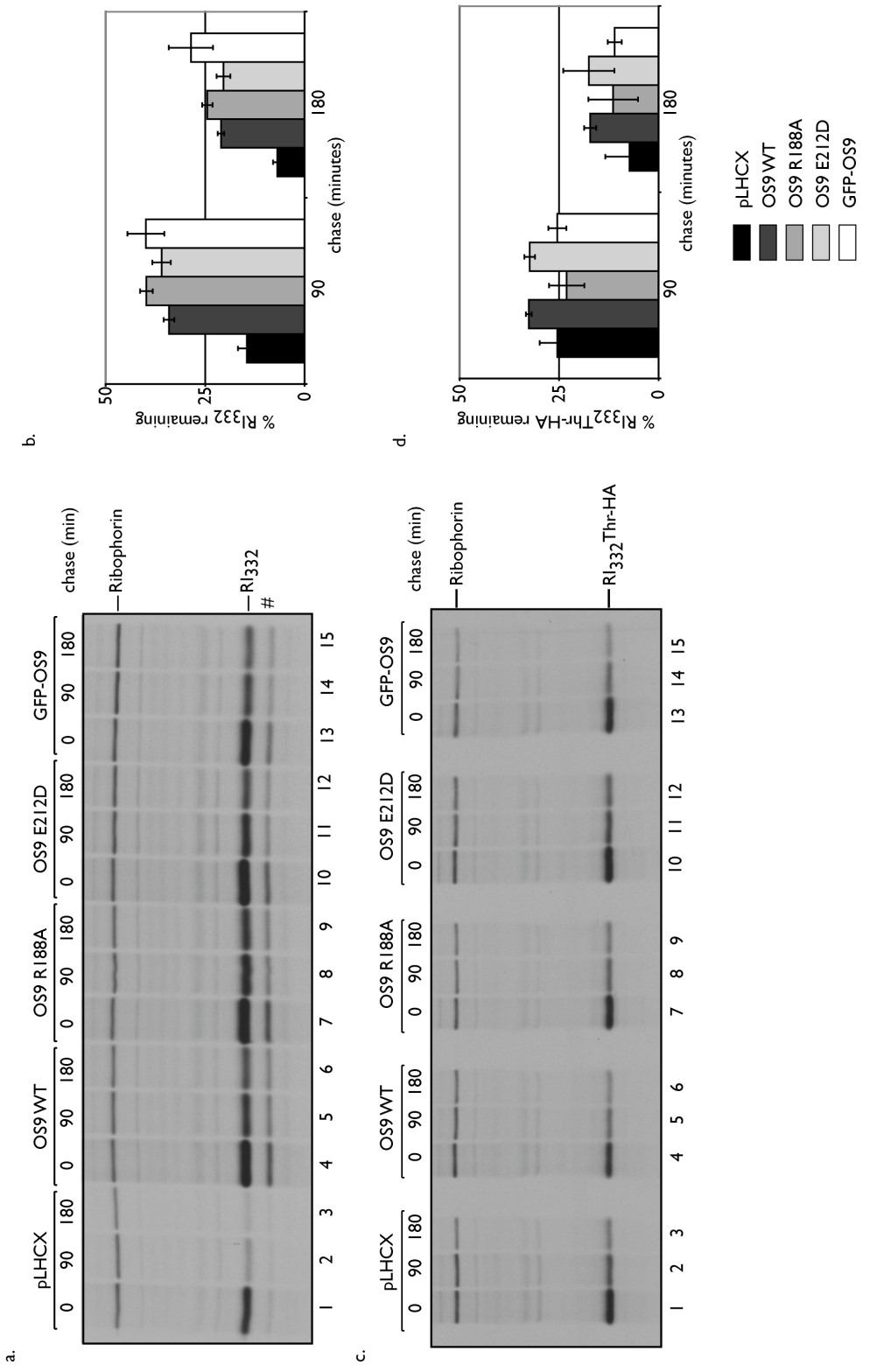

a.
pLHCX
OS9 WT
OS9 R188A
OS9 E212D
GFP-OS9
chase (min)
0 90 180 0 90 180 0 90 180 0 90 180 0 90 180
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Ribophorin
RI332
#
b.
% RI332 remaining
50
25
0
0 90 180
chase (minutes)
c.
pLHCX
OS9 WT
OS9 R188A
OS9 E212D
GFP-OS9
chase (min)
0 90 180 0 90 180 0 90 180 0 90 180 0 90 180
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Ribophorin
RI332Thr-HA
d.
% RI332Thr-HA remaining
50
25
0
0 90 180
chase (minutes)
pLHCX
OS9 WT
OS9 R188A
OS9 E212D
GFP-OS9

FIGURE 4

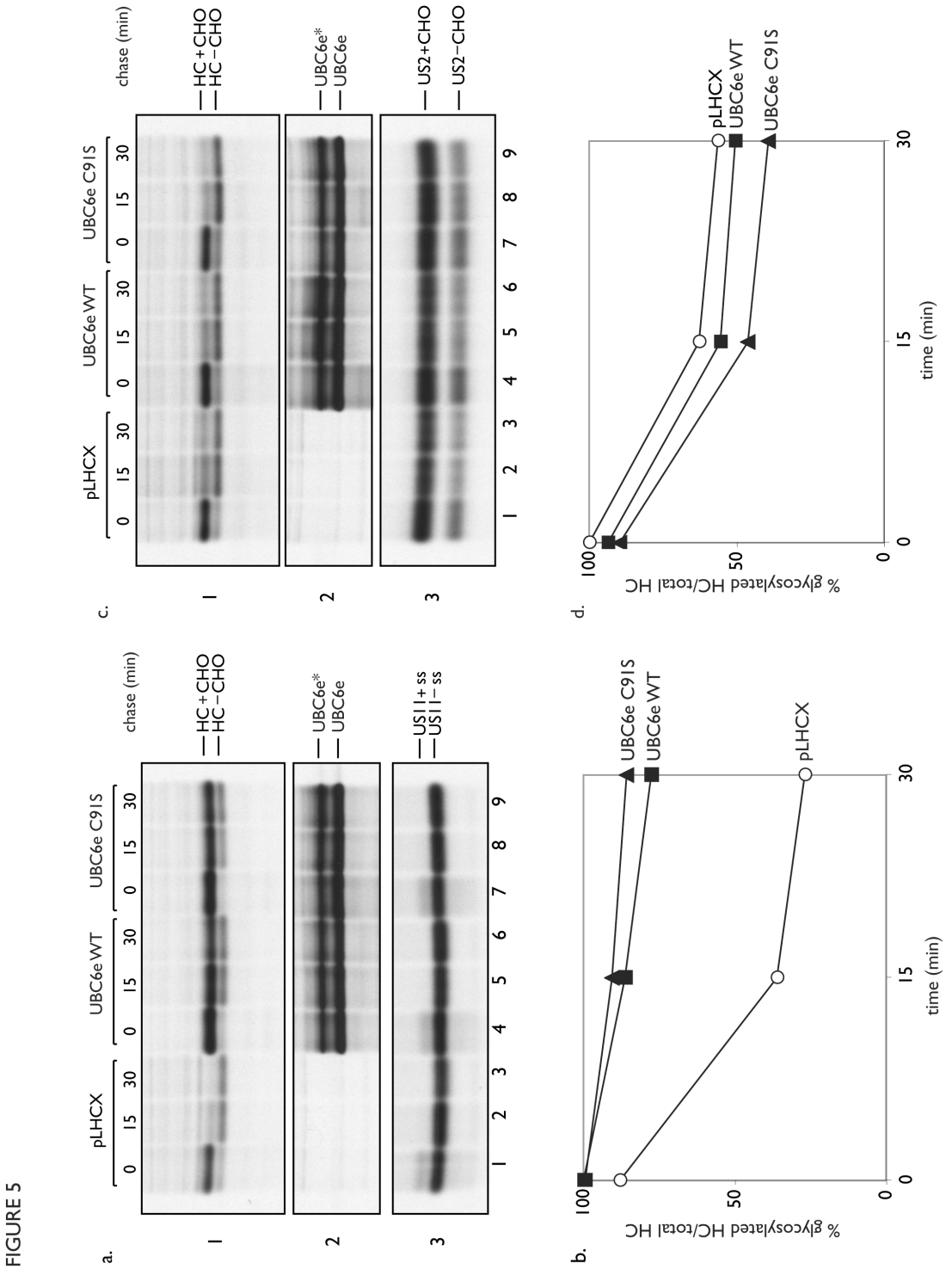

a.
pLHCX
UBC6e WT
UBC6e C91S
chase (min)
0 15 30 0 15 30 0 15 30
1 2 3 4 5 6 7 8 9
HC+CHO
HC−CHO
UBC6e*
UBC6e
US11+ss
US11−ss
1
2
3
b.
% glycosylated HC/total HC
100
50
0
0 15 30
time (min)
UBC6e C91S
UBC6e WT
pLHCX
c.
HC+CHO
HC−CHO
UBC6e*
UBC6e
US2+CHO
US2−CHO
d.
% glycosylated HC/total HC
pLHCX
UBC6e WT
UBC6e C91S

FIGURE 5

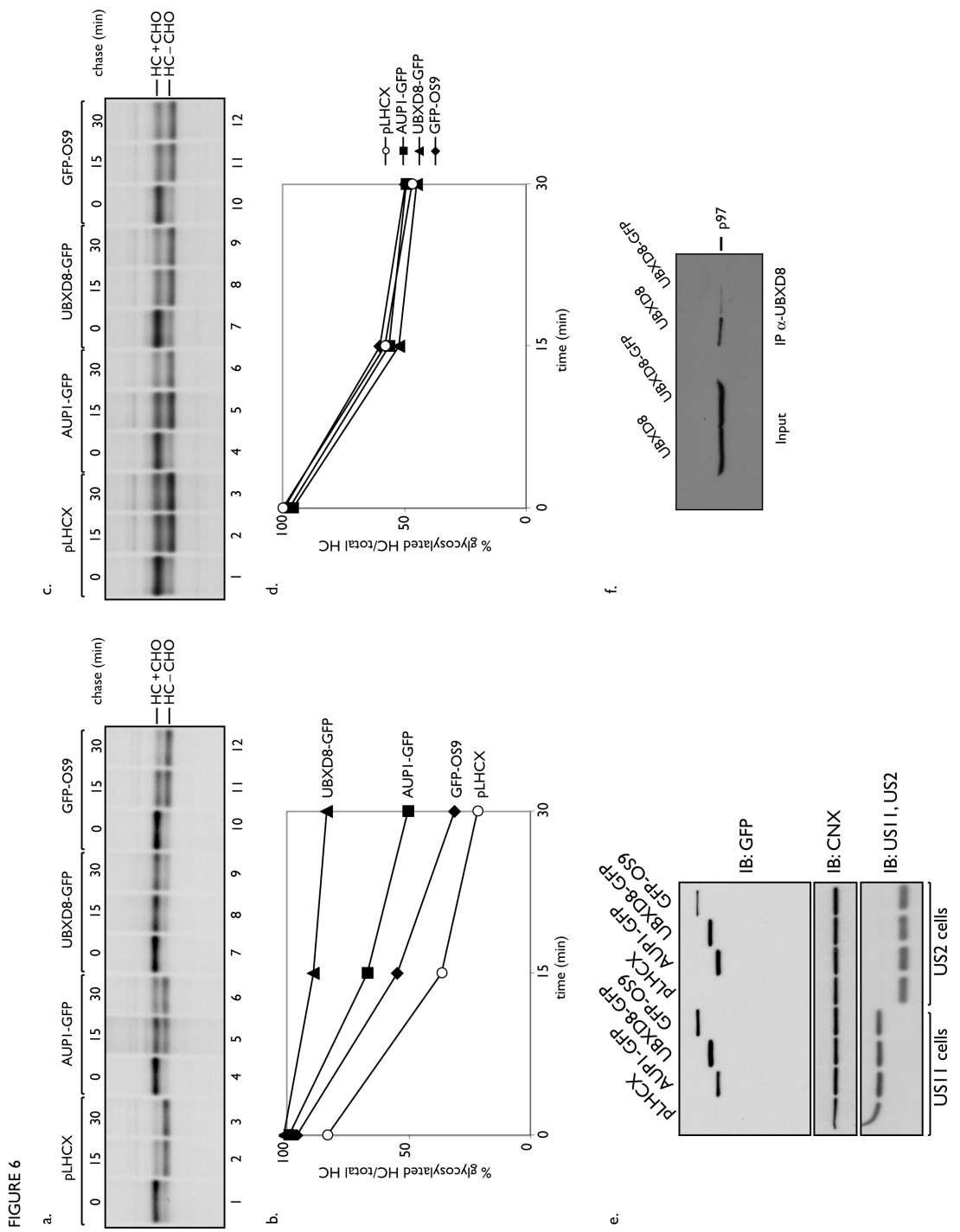


FIGURE 6
a.
pLHCX
AUP1-GFP
UBXD8-GFP
GFP-OS9
chase (min)
HC+CHO
HC−CHO
b.
% glycosylated HC/total HC
time (min)
UBXD8-GFP
AUP1-GFP
GFP-OS9
pLHCX
c.
d.
pLHCX
AUP1-GFP
UBXD8-GFP
GFP-OS9
e.
IB: GFP
IB: CNX
IB: US11, US2
US11 cells
US2 cells
f.
p97
IP α-UBXD8
Input
UBXD8
UBXD8-GFP

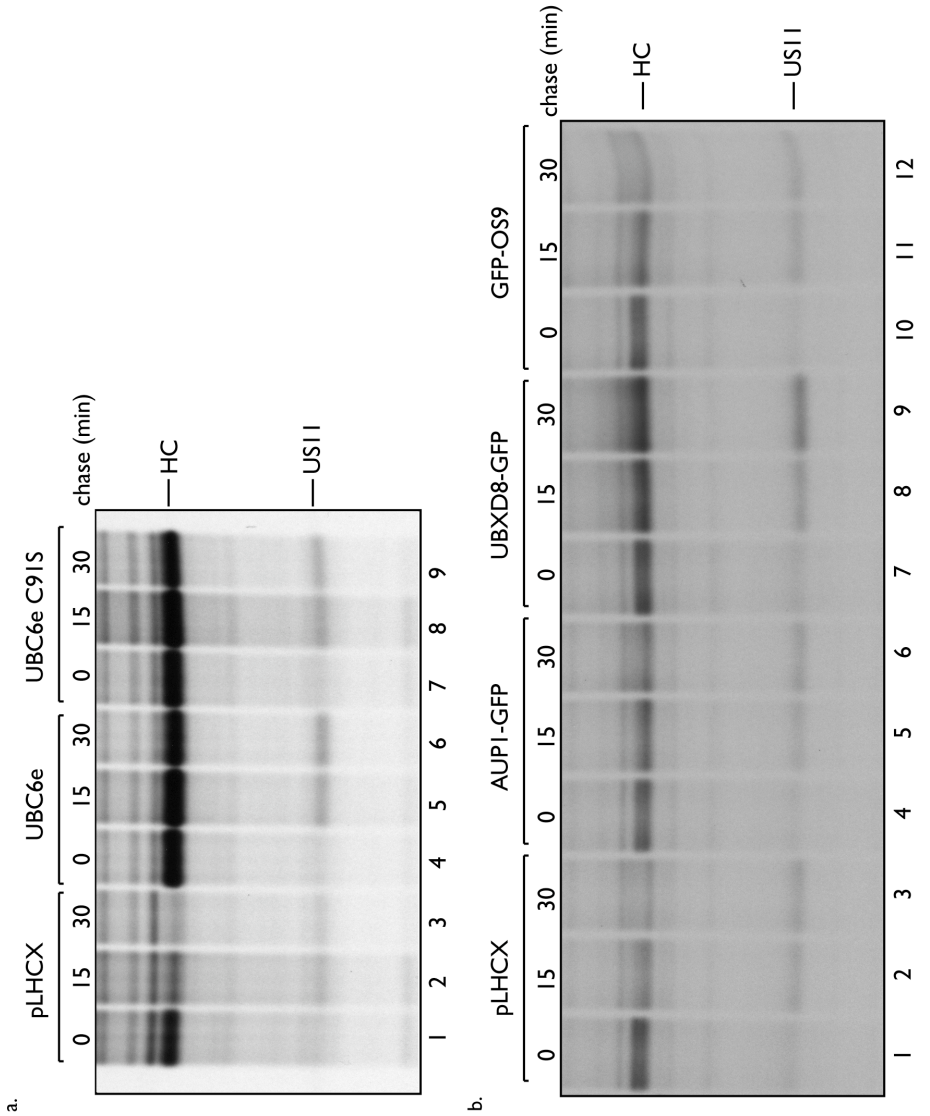
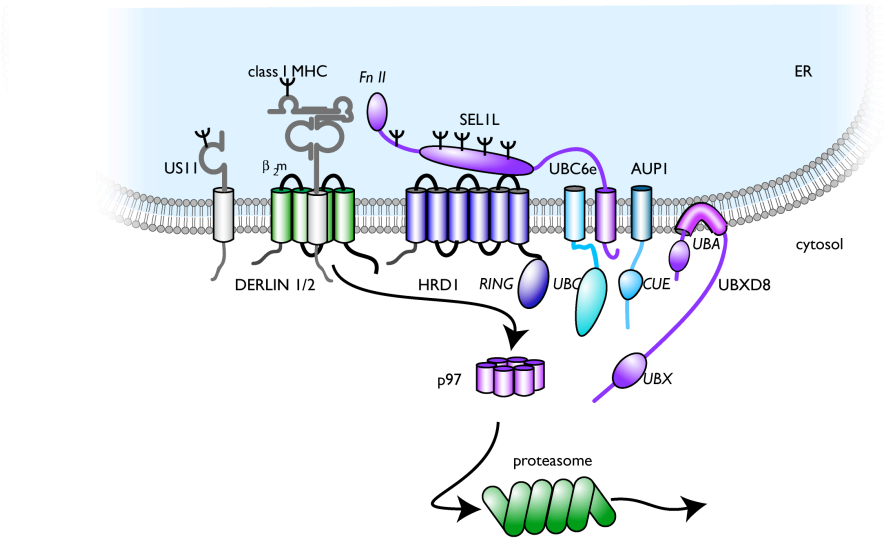

a.
pLHCX
UBC6e
UBC6e C91S
chase (min)
0 15 30 0 15 30 0 15 30
1 2 3 4 5 6 7 8 9
HC
US11
b.
pLHCX
AUP1-GFP
UBXD8-GFP
GFP-OS9
chase (min)
0 15 30 0 15 30 0 15 30 0 15 30
1 2 3 4 5 6 7 8 9 10 11 12
HC
US11

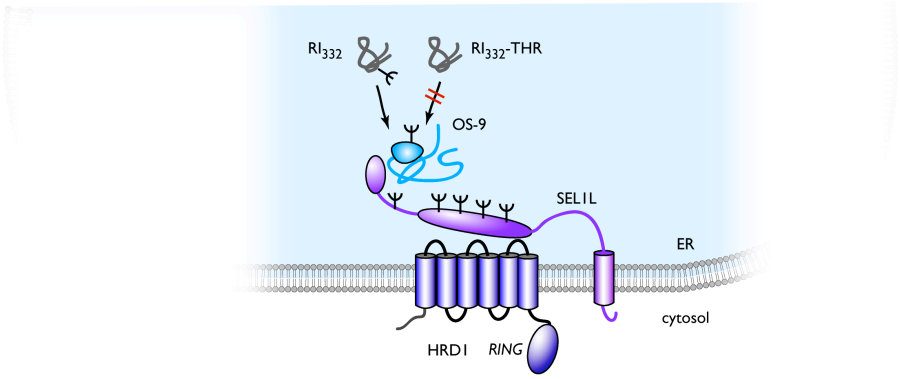
FIGURE 7

FIGURE 8

a.


class I MHC
Fn II
ER
SEL1L
US11
β 2m
UBC6e
AUP1
UBA
cytosol
DERLIN 1/2
HRD1
RING
UBC
CUE
UBXD8
p97
UBX
proteasome

b.


RI332
RI332-THR
OS-9
SEL1L
ER
cytosol
HRD1
RING

4.4 The mouse polyubiquitin gene UBC is essential for fetal liver development, cell cycle progression and stress tolerance

Ryu KY, Maehr R, Gilchrist CA, Long MA, Bouley DM, Mueller B, Ploegh HL, and Kopito R 2007 Jun 6;26(11):2693-706. Epub 2007 May 10

A functional ubiquitin-proteasome system is essential for all eukaryotic cells. Ubiquitin plays numerous roles in the cell (Hershko and Ciechanover, 1998). As ubiquitin is a very conserved molecule, antibody production against ubiquitin is difficult. Therefore, an HA-ubiquitin knock-in mouse was made. This mouse was used in a study investigating a UBC-/- knock-out mouse. There are four ubiquitin genes, UbB, UbC, UbA52, and UbA80 (Redman and Rechsteiner, 1989). UbB and UbC encode for ubiquitin polyproteins, therefore contribute more to the overall pool of ubiquitin than the UbA genes that encode for ubiquitin fusions with ribosomal subunits (Finley et al., 1989; Wiborg et al., 1985).
The question was whether the amount of knocked-in HA-ubiquitin could rescue the phenotype of the UBC-/- mice. UBC-/- mice are embryonically lethal due to liver failure on day 13.5 of gestation. A rescue, even if partial, would prove that this phenotype is attributable solely to the lack of ubiquitin. HA-Ub: UBC-/- mice lived up to day 17.5 of gestation, and interestingly did not show signs of liver malformation. Furthermore, MEFs (mouse embryonic fibroblasts) from UBC-/- mice showed reduced growth rate that was not present in MEFs from the UBC-/- HA-Ub crosses.

My task was to characterize the HA-ubiquitin knock-in mouse. We designed a PCR-based screening for the HA-Ub mice and bread them to C57BL/6J mice for germline transmission. Once we obtained a clean HA-Ub +/+ mice, we analyzed their HA-Ub expression levels by western blotting and immunoprecipitation. We showed that the HA-tagged ubiqutin is expressed in all organs. Furthermore, we made MEFs on day 13 of gestation.

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The mouse polyubiquitin gene *UbC* is essential for fetal liver development, cell-cycle progression and stress tolerance

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***UbC* is one of two stress-inducible polyubiquitin genes in mammals and is thought to supplement the constitutive *UbA* genes in maintaining cellular ubiquitin (Ub) levels during episodes of cellular stress. We have generated mice harboring a targeted disruption of the *UbC* gene. *UbC*$^{-/-}$ embryos die between embryonic days 12.5 and 14.5 *in utero*, most likely owing to a severe defect in liver cell proliferation. Mouse embryonic fibroblasts from *UbC*$^{-/-}$ embryos exhibit reduced growth rates, premature senescence, increased apoptosis and delayed cell-cycle progression, with slightly, but significantly, decreased steady-state Ub levels. *UbC*$^{-/-}$ fibroblasts are hypersensitive to proteasome inhibitors and heat shock, and unable to adequately increase Ub levels in response to these cellular stresses. Most, but not all of the *UbC*$^{-/-}$ phenotypes can be rescued by providing additional Ub from a poly hemagglutinin-tagged Ub minigene expressed from the *Hprt* locus. We propose that *UbC* is regulated by a process that senses Ub pool dynamics. These data establish that *UbC* constitutes an essential source of Ub during cell proliferation and stress that cannot be compensated by other Ub genes.**

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Subject Categories: cell cycle; differentiation & death
Keywords: cellular stress; gene expression; liver development; polyubiquitin; ubiquitin

Introduction

Ubiquitin (Ub) is a highly conserved 76 amino-acid protein that plays critical roles in the function of eukaryotic cells (Hochstrasser, 1996; Hershko and Ciechanover, 1998). Ub signaling is a covalent process initiated by formation of an isopeptide bond between the C-terminal carboxyl group of Ub and a free amino group of a substrate, and is terminated by enzymatic cleavage of this linkage (D'Andrea and Pellman, 1998). Ub can be conjugated to itself and the resulting polyubiquitin chains are likely to have distinct roles in Ub-dependent signaling processes (Pickart, 2000; Pickart and Fushman, 2004). Because of its widespread use in intracellular signaling and the large numbers of Ub moieties that may be conjugated to a single substrate, Ub is very abundant, accounting for 0.1–5% of total cellular protein (Ohtani-Kaneko *et al*, 1996; Takada *et al*, 1996; Osaka *et al*, 2003; Ryu *et al*, 2006). Maintenance of cellular Ub at levels sufficient to sustain its multiple cellular functions under all metabolic conditions is therefore of critical importance to cellular survival.

Cellular Ub is comprised of two distinct pools consisting of free Ub and Ub–substrate conjugates. Ub isopeptidases antagonize Ub conjugation and ensure that these pools are in dynamic equilibrium (Wilkinson, 1997). Although conjugated Ub is largely recycled to the monomer pool by isopeptidases associated with the 26S proteasome, steady-state levels of cellular Ub depend on adequate levels of Ub gene transcription to compensate for basal Ub turnover, which has been estimated to range from $t_{1/2}$ ~2 h in yeast (Hanna *et al*, 2003) to 31 h in mammalian cells (Haas and Bright, 1987). In mammals, Ub is encoded by four Ub genes, two of which (*UbB* and *UbC*) encode Ub polyproteins and two of which (*UbA52* and *UbA80*) encode fusions between Ub and two small ribosomal proteins (Lund *et al*, 1985; Wiborg *et al*, 1985; Baker and Board, 1987, 1991; Finley *et al*, 1989; Redman and Rechsteiner, 1989). Thus, all Ub is translated from fusion proteins that are processed to generate free Ub monomers by ubiquitously expressed Ub-specific proteases. Because Ub fusions, the primary translation products of Ub genes, are generally undetectable in cells, it is assumed that they are rapidly and efficiently processed into Ub monomers which, owing to the exquisite conservation of the Ub gene, are chemically identical.

The polyubiquitin genes in all eukaryotes that have been studied are stress-regulated genes and contain heat-shock elements in their promoter regions (Bond and Schlesinger, 1986; Fornace *et al*, 1989). In yeast, the single polyubiquitin gene (*UBI4*) is dispensable under vegetative growth conditions, suggesting that the *UbA* genes (*UBI1–3*) provide the bulk of cellular Ub (Finley *et al*, 1987). *ubi4* cells, however, are sensitive to a variety of different types of stress and cannot sporulate (Finley *et al*, 1987). Far less is known about the functional organization, regulation and expression of the mammalian polyubiquitin genes. The two polyubiquitin genes *UbB* and *UbC* differ most notably in the number of Ub coding units they contain, arranged in a spacerless, tandem, head-to-tail fashion (Wiborg *et al*, 1985). The *UbB* genes are most similar to yeast *UBI4* in that they contain 3–5 Ub units

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(*UBI4* has 5), whereas the *UbC* gene contains 9–10 Ub units. Both polyubiquitin genes are upregulated by cellular stresses and contain classical heat-shock elements in their promoters (Fornace *et al*, 1989). Despite the broad importance of Ub in virtually every aspect of eukaryotic cell function, surprisingly little is known about the relative contributions and functions of the individual Ub genes and how they are regulated to maintain an adequate supply of Ub in normal and stressed cells. In this study, we have used conventional gene targeting methodology to ablate the single Ub-coding exon of the murine *UbC* gene. While loss of a single *UbC* allele has no apparent phenotype, we find that homozygous deletion of the mouse *UbC* gene causes mouse embryos to die *in utero* in midgestation, most likely due to arrested fetal liver proliferation. Homozygous loss of *UbC* also leads to severely reduced proliferative capacity of mouse embryonic fibroblasts (MEFs), a delay in cell-cycle progression and increased susceptibility to cellular stress.

Results

UbC is essential for fetal development

The murine *UbC* locus consists of two exons spanning 3.5 kb on mouse chromosome 5. To disrupt the *UbC* gene, we constructed a targeting vector to replace the single exon containing the entire *UbC* coding sequence with sequence encoding a promoterless GFP-puromycin-resistance fusion protein (GFP-puro) flanked by *loxP* sites (Figure 1A). Homologous recombinants were generated in embryonic stem (ES) cells by positive selection with puromycin and negative selection (against non-homologous recombinants) using diphtheria toxin. Two independent ES cell clones were isolated, verified by Southern blotting (Figure 1B, upper panels) and injected into C57BL/6J blastocysts to generate six chimeric lines, all of which had identical phenotypes. Heterozygous $UbC^{GFP\text{-}puro}$ mice were phenotypically normal and were intercrossed to obtain progeny homozygous for the floxed targeted allele. Genotyping of the progeny failed to detect homozygous $UbC^{GFP\text{-}puro}$ pups implying that *UbC* null embryos die *in utero* (Table I). To establish the timing of embryonic lethality, embryos were harvested at various stages of development and genotyped. Homozygous $UbC^{GFP\text{-}puro}$ embryos were recovered at the expected Mendelian frequencies up to E11.5 but the fraction of viable homozygous embryos decreased at E12.5 and later ages (Table I). Correspondingly, the number of dead or partially resorbed embryos found *in utero* increased with embryonic age; the vast majority of these were homozygous for the $UbC^{GFP\text{-}puro}$ allele.

The gross morphological appearance of viable homozygous $UbC^{GFP\text{-}puro}$ embryos at E13.5 was generally similar to wild-type and heterozygous littermates (Figure 1C). They were pink in color and well vascularized but distinctly smaller in overall size. The only apparent morphological or histological difference was the drastically reduced size of the fetal liver mass evident at E13.5 (Figure 1C, arrow). Histological analysis (Figure 1D) confirmed that livers from E13.5 homozygous $UbC^{GFP\text{-}puro}$ embryos were severely reduced in size compared to wild-type or heterozygous littermates. These livers were distinguished by reduced hepatic parenchyma and enlarged venous sinusoids (Figure 1D). Both hepatocytes (white arrow, lower) and hematopoietic precursors (yellow arrow, upper) are evident in homozygous $UbC^{GFP\text{-}puro}$ livers, although their numbers are reduced (Figure 1D). The hepatic phenotype will be described in greater detail in a forthcoming manuscript.

In principle, the midgestation lethality and liver development phenotypes observed in embryos homozygous for the disrupted *UbC* could be due to the loss of *UbC* coding sequence or to the presence of the GFP-puro selection cassette. To discriminate between these two possibilities, mice heterozygous for the $UbC^{GFP\text{-}puro}$ allele were bred with mice expressing Zp3-Cre recombinase in order to excise the 'floxed' GFP-puro sequence and generate a true null allele. Although $UbC^{+/}$ progeny of these crosses were obtained at expected Mendelian frequencies, no viable $UbC^{-/-}$ pups were obtained. E13.5 $UbC^{-/-}$ embryos exhibited the same gross phenotypic characteristics as did those homozygous for the floxed $UbC^{GFP\text{-}puro}$ allele; notably the smaller overall size and greatly diminished fetal liver mass (Figure 1E). We conclude that the major phenotypes resulting from homozygous *UbC* disruption are due to the loss of *UbC* coding sequence and not the presence of GFP-puro. Because the floxed GFP-puro selection cassette precisely replaces the only coding exon of the *UbC* gene, the recombinant allele, $UbC^{GFP\text{-}puro}$ represents a complete null and will be referred to in this work as $UbC^{-/-}$. Moreover, the availability of GFP under the control of the murine *UbC* promoter provides a useful reporter of *UbC* transcriptional activity that will be exploited later.

Partial rescue of the $UbC^{-/-}$ embryonic phenotype by ectopic expression of hemagglutinin-tagged Ub

To verify that the embryonic lethal phenotype observed in the *UbC* null mice was due to a deficiency in the cellular availability of Ub, we sought to introduce extra copies of Ub into the mouse genome. To generate mice that express epitope-tagged Ub, we employed a gene-targeting strategy that would allow us to incorporate a known number of hemagglutinin-tagged Ub (HA-Ub) copies transcribed under the control of the human *UbC* promoter to ensure ubiquitous expression (Figure 2A). HM-1 ES cells were targeted to reconstitute their disrupted *Hprt* locus (Bronson *et al*, 1996) and allow expression of the engineered *HA-Ub* gene. Subsequent to the HAT selection, ES cell colonies were screened by Southern blotting for the appropriate recombination (Figure 2B, left panel) and two clones were used for blastocyst injection. Three resulting chimeric mice were bred to C57BL/6J mice to obtain germline transmission.

Homozygous (+/+) *HA-Ub* knock-in female mice were then bred to $UbC^{+/}$ males to generate $UbC^{+/}$; *HA-Ub* mice (Figure 2C). Although interbreeding of $UbC^{+/}$; *HA-Ub* mice did not result in any viable $UbC^{-/-}$; *HA-Ub* live pups, $UbC^{-/-}$; *HA-Ub* embryos were observed at expected Mendelian frequencies up to E15.5, and viable $UbC^{-/-}$; *HA-Ub* embryos were found even at E17.5 (Table II, Figure 2D). $UbC^{-/-}$; *HA-Ub* embryos appeared normal upon gross histological examination, although they were slightly smaller in overall size. Importantly, the $UbC^{-/-}$; *HA-Ub* embryos did not exhibit evidence of liver malformation (Figure 2E). These data indicate that both the midgestation embryonic lethality and the liver development phenotypes are partially rescued by providing extra genomic copies of Ub, suggesting that the *UbC* null phenotype is the direct consequence of a deficiency in the abundance or availability of Ub during fetal development.

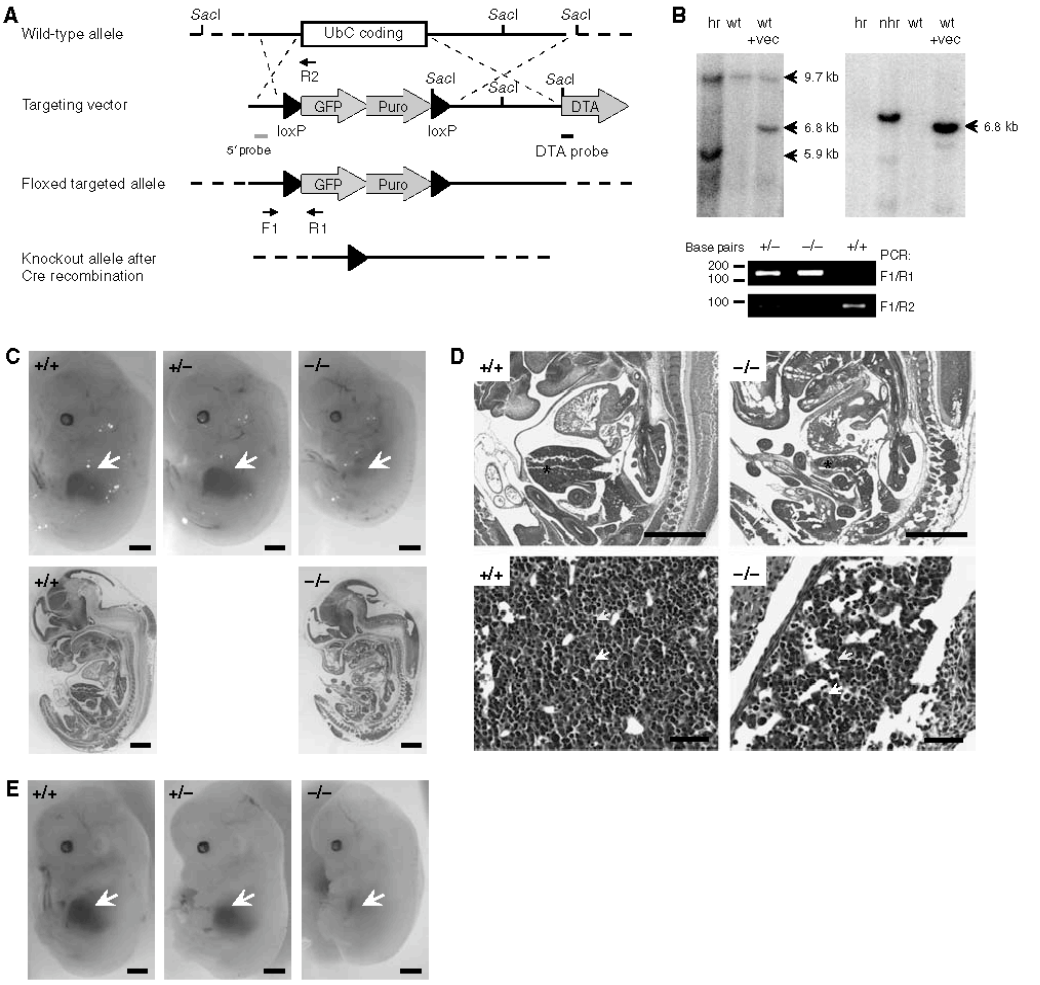


Figure 1 Targeted disruption of *UbC* locus results in impaired fetal liver development. (**A**) Schematic representation of targeting strategy. From top to bottom: partial restriction map of *UbC* locus, targeting vector, genomic structure of disrupted allele before and after Cre recombination. The position of 5′ probe and DTA probe for Southern blotting and the location of PCR primers used for screening homologous recombinants and genotyping are shown. The map is not drawn to scale. (**B**) Southern blot analysis of *SacI*-digested genomic DNA from ES cell clones. Upper left panel: 5′ probe detected 9.7 and 5.9 kb fragments from wild-type (wt) and disrupted alleles, in homologous recombinants (hr), but only 9.7 kb fragment from wt allele in non-recombinant, wt ES cells. It also hybridized to linearized 6.8 kb targeting vector (+vec). Upper right panel: DTA probe detected linearized 6.8 kb targeting vector (+vec) and >3.9 kb fragment from non-homologous recombinants (nhr), but not from homologous recombinants (hr) or wt ES cells. Lower panel; PCR results for wt (+/+), heterozygous (+/−) and homozygous (−/−) knockout embryos are displayed. (**C**) Morphology of wt (+/+), heterozygous (+/−) and homozygous (−/−) knockout embryos at E13.5 (upper panel) and histology of sagittal embryonic sections stained with H&E (lower panel). Fetal liver is indicated by white arrow. Scale bar, 1 mm. (**D**) Histology of sagittal embryonic (upper panel) and liver (lower panel) sections from E13.5 embryos. Note the reduced size of *UbC* $^{/}$ embryonic liver (indicated with asterisk). Sections were stained with H&E. Hepatocytes (white arrow, lower) are stained lightly, whereas hematopoietic precursors (yellow arrow, upper) are stained darkly. Upper panel, scale bar, 1 mm; lower panel, scale bar, 50 μm. (**E**) Morphology of wt (+/+), heterozygous (+/−) and homozygous (−/−) knockout embryos at E13.5 with floxed GFP-puro cassette removed by Zp3-Cre recombinase. Fetal liver is indicated by white arrow. Scale bar, 1 mm.

Reduced proliferation and premature senescence in homozygous UbC null MEFs

To assess the cellular consequences of loss of the *UbC* gene, MEFs were isolated from E12.5 to E13.5 embryos. Even at early passages, the growth rates of *UbC* $^{/}$ MEFs were distinctly reduced compared to MEFs from wild-type or heterozygous mice (Figure 3A). Quantification of proliferation marker Ki-67 labeling of MEFs suggests that proliferation of *UbC* $^{/}$ MEFs was significantly reduced compared to *UbC*$^{+/+}$ MEFs (Figure 3B and C). In addition, *UbC* $^{/}$ MEFs exhibited increased senescence-associated β-galactosidase (SA-β-gal) staining (Figure 3B) and enlarged and flat-

Table I Embryonic lethality in $UbC^{-/-}$ mice

Age	Litters	$UbC^{+/+}$	$UbC^{+/-}$	$UbC^{-/-}$	Dead	Total
E9.5–11.5	4	10	19	7	0	36
E12.5	17	46	66	20	21	153
E13.5	26	21	51	11	21	104
E14.5–15.5	7	18	29	2	13	62
E16.5–17.5	5	9	21	0	8	38
E18.5	2	3	9	0	1	13
Birth	52	95	196	0	0	291

Embryos were dissected at the indicated time of gestation and examined heartbeat for viability. Genotypes were determined by PCR using genomic DNA isolated from embryos. E: embryonic day.

tened morphology (data not shown) implying that homozygous loss of *UbC* leads to premature senescence (Goldstein, 1990; Dimri *et al*, 1995). Neither the proliferation defect (Figure 3A) nor the premature senescence (data not shown) phenotypes were evident in *UbC* $^{/}$; *HA-Ub* MEFs indicating that these phenotypes are the direct consequence of Ub deficiency.

To assess endogenous *UbC* transcriptional activity, we took advantage of the GFP-puro coding sequence that precisely replaces the polyubiquitin coding exon in the *UbC* locus (Figure 1A). GFP-puro fluorescence was significantly higher in $UbC^{+/}$ MEFs compared to wild-type MEFs but was roughly half that of *UbC* $^{/}$ MEFs, indicating that steady-state GFP-puro fluorescence correlates well with $UbC^{GFP\text{-}puro}$ copy number (Figure 3D). We found that GFP-puro fluorescence was slightly but not significantly decreased in *UbC* $^{/}$; *HA-Ub* compared to *UbC* $^{/}$ MEFs, suggesting that the additional HA-Ub in these cells is not able to significantly repress basal *UbC* transcription. Strikingly, we found that GFP-puro fluorescence increased about three-fold as MEFs were maintained through multiple passages in culture (Figure 3E). This increase was not mitigated by the presence of HA-Ub nor was it exacerbated by the loss of the second *UbC* allele, suggesting that transcription of the endogenous *UbC* promoter increases as cells senesce.

Delayed mitotic entry in UbC $^{/}$ *MEFs*

To understand the basis of the reduced proliferative capacity observed in *UbC* $^{/}$ MEFs, we used flow cytometry to analyze cell-cycle progression (Figure 4). Unsynchronized early-passage *UbC* $^{/}$ MEFs displayed a reduced fraction of cells with 2n and an increased fraction with 4n DNA content when compared to wild-type MEFs (Figure 4A), suggesting that *UbC* deletion reduces the length of the G1 phase of the cell cycle with a corresponding increase in the length of G2/M. Correspondingly, there was no difference in the fraction of *UbC* $^{/}$ MEFs labeled with an antibody to phospho-histone H3, a marker for M phase, compared with $UbC^{+/+}$ or $UbC^{+/}$ MEFs (data not shown), suggesting that the increased fraction of cells at G2/M is likely due to delayed mitotic entry. The fraction of cells with less than 2n DNA content, presumably apoptotic, was significantly increased in *UbC* $^{/}$ MEFs (Figure 4A), although this increase does not fully account for the reduced G1 population in *UbC* $^{/}$ MEFs (Figure 4A; 3% increase in apoptotic, but 9% reduction in G1). All of the cell-cycle progression phenotypes observed in *UbC* $^{/}$ MEFs were fully rescued by coexpression of HA-Ub, suggesting that they are likely the consequence of Ub deficiency.

Analysis of GFP-puro fluorescence in $UbC^{+/}$ and *UbC* $^{/}$ MEFs by flow cytometry permitted correlation of *UbC* transcriptional activity with cell-cycle progression (Figure 4B–D). GFP-puro levels in cells with 2n DNA content (G1) were comparable to those of the bulk population, with the fluorescence of *UbC* $^{/}$ roughly twice that of $UbC^{+/}$ MEFs (Figure 4C). GFP-puro fluorescence was elevated ~2-fold relative to the G1 level in MEFs of all genotypes with 4n DNA content, suggesting that *UbC* transcription is increased in G2 (Figure 4C). Finally, we observed that GFP-puro fluorescence was elevated in *UbC* $^{/}$ but not $UbC^{+/}$ MEFs in S phase more than two-fold above the levels in the same cell population in G1 phase (Figure 4D), suggesting that *UbC* $^{/}$ MEFs respond to reduced Ub levels by attempting to increase *UbC* transcription in S phase.

Unequal contribution of Ub genes to cellular Ub pools

To directly determine whether the phenotypes arising from homozygous loss of *UbC* are due to reduced levels of Ub, we measured the levels of cellular Ub and mRNA transcripts of the four Ub genes in MEFs (Figure 5). Cellular Ub is partitioned between pools of free Ub monomer and covalent Ub-protein conjugates. Because there are no reagents to accurately determine the concentrations of either of these two forms of Ub without interference with the other, we used a Ub-specific protease (Usp2-cc) to convert all Ub conjugates to Ub monomer and an indirect competitive ELISA method to measure the concentration of Ub monomer in MEFs of the different genotypes (Figure 5A, left panel). This approach, which provides an accurate measurement of total cellular Ub content (Ryu *et al*, 2006), reveals that steady-state Ub levels were reduced by 40% in *UbC* $^{/}$ MEFs compared to wild-type, which was fully restored to wild-type levels with coexpression of HA-Ub. This rescue can be accounted for by the contribution of HA-Ub, and is thus unlikely to be due to increased transcription of other Ub genes (Figure 5A, right panel). Thus, *UbC* contributes significantly to the maintenance of steady-state total Ub levels in cultured early-passage MEFs.

A central role of *UbC* in maintenance of steady-state Ub levels in MEFs was also supported by real-time quantitative RT–PCR analysis of Ub transcripts (Figure 5B). UbC transcript levels were reduced by approximately half in $UbC^{+/}$ MEFs and, as expected, were undetectable in *UbC* $^{/}$ cells. Although no significant changes in the levels of UbA transcripts were observed in MEFs harboring one or two disrupted *UbC* alleles, we observed a significant increase of transcripts from the *UbB* locus in *UbC* $^{/}$ cells. Evidently, this increase was not enough to compensate for the loss of *UbC*

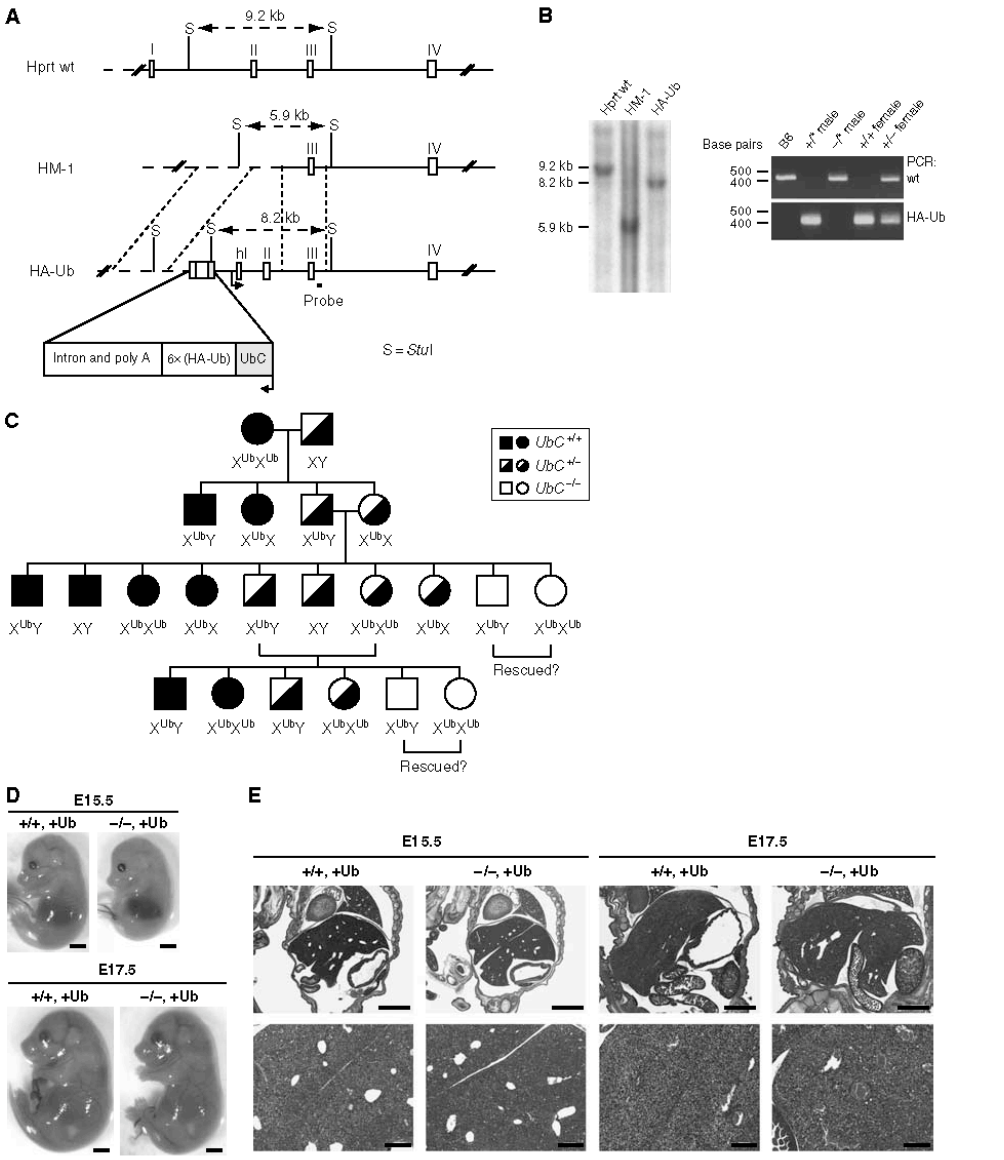


Figure 2 Partial rescue of *UbC* $^{-/-}$ phenotypes by ectopically expressed HA-Ub. (**A**) Targeting strategy. From top to bottom: wild-type mouse *Hprt* locus (Hprt wt), disrupted *Hprt* locus from HM-1 cells (HM-1) and targeted *Hprt* locus (HA-Ub). Sites recognized by the *StuI* restriction enzyme are indicated. The map is not drawn to scale. (**B**) Southern blot analysis and genotyping by PCR. Left panel: Southern blot analysis after digestion of genomic ES cell DNA with *StuI* restriction enzyme is shown. DNA was used from wild-type ES cells (Hprt wt), disrupted *Hprt* containing HM-1 ES cell (HM-1) and a targeted ES cell clone (HA-Ub) that was used for subsequent injections. Right panel: PCR results for C57BL/6J (B6), hemizygous wild-type (−/*) or *HA-Ub* knock-in (+/*) male mice, and heterozygous (+/−) or homozygous (+/+) *HA-Ub* knock-in female mice are displayed. (**C**) Breeding strategy to generate *UbC* $^{-/-}$ *; HA-Ub* mice. Owing to random inactivation of X chromosome in females, rescued phenotype is expected from *UbC* $^{-/-}$ *; HA-Ub* $^{+/*}$ male or *UbC* $^{-/-}$ *; HA-Ub* $^{+/+}$ female mice. Both genotypes will be referred to in this work simply as *UbC* $^{-/-}$ *; HA-Ub* when the gender does not need to be defined. $X^{Ub}X^{Ub}$ = *HA-Ub* $^{+/+}$ female; $X^{Ub}X$ = *HA-Ub* $^{+/-}$ female; $X^{Ub}Y$ = *HA-Ub* $^{+/*}$ male; XY = *HA-Ub* $^{-/*}$ male. (**D**) Morphology of *UbC* $^{+/+}$*; HA-Ub* (+/+, +Ub) and *UbC* $^{-/-}$*; HA-Ub* (−/−, +Ub) embryos at E15.5 and E17.5. Scale bar, 2 mm. (**E**) Histology of sagittal liver sections from E15.5 and E17.5 embryos. Liver sections were stained with H&E. Note that no developmental defects are found in *UbC* $^{-/-}$ livers with ectopic expression of HA-Ub. Upper panel, scale bar, 1 mm; lower panel, scale bar, 200 μm.

Table II Effect of HA-Ub on embryonic lethality in *UbC* $^{-/-}$ mice

Age	Litters	*UbC*$^{+/+}$; *HA-Ub*	*UbC*$^{+/-}$; *HA-Ub*	*UbC* $^{-/-}$; *HA-Ub*	Dead	Total
E12.5–13.5	2	6	10	3	0	19
E14.5–15.5	4	8	21	11	0	40
E16.5–17.5	2	2	8	4	3	17
E18.5	1	2	6	0	3	11
Birth	23	58	96	0	0	154

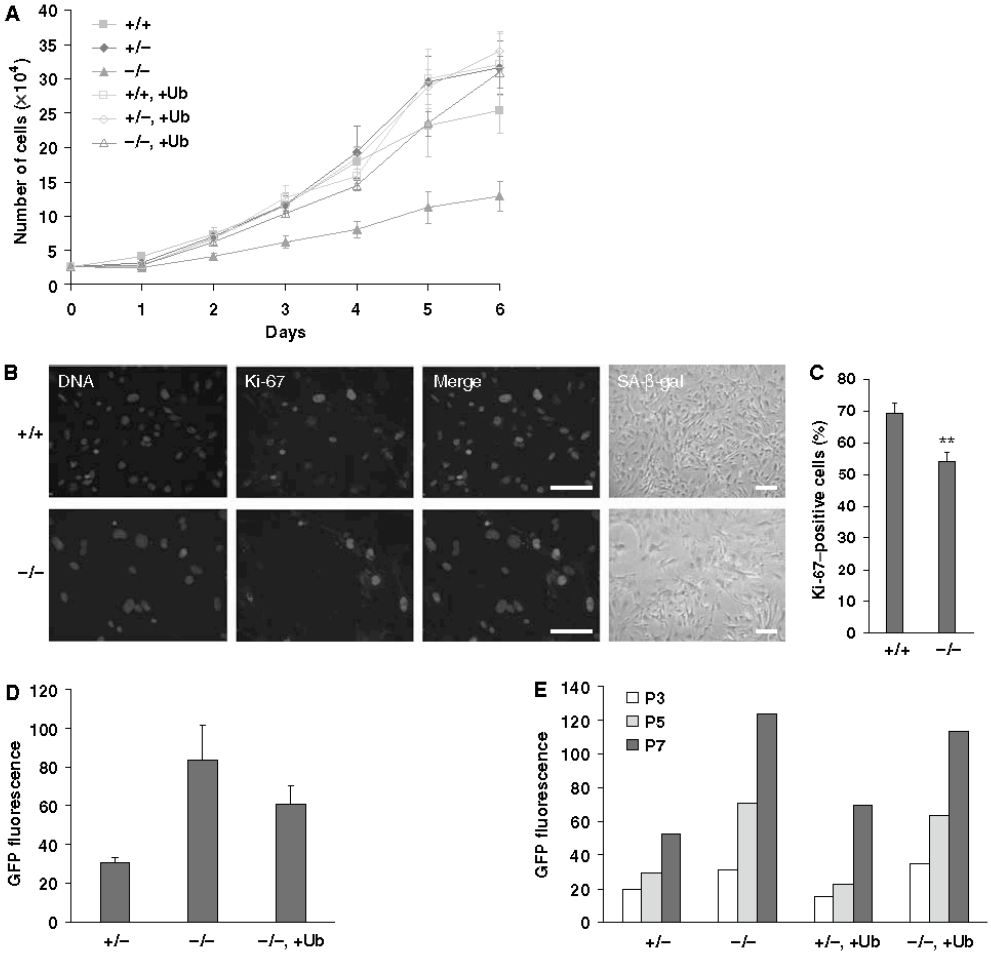
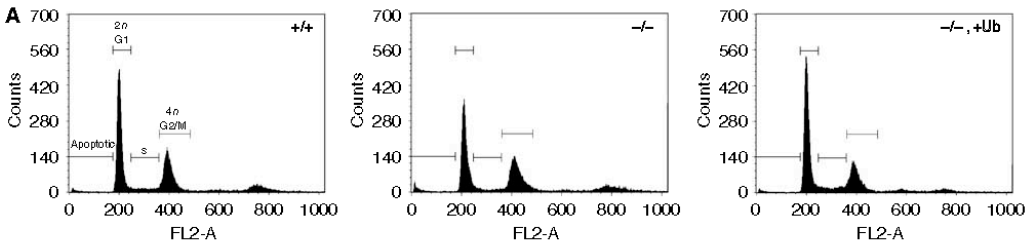


Figure 3 Reduced proliferation and premature senescence of *UbC* $^{-/-}$ MEFs. (**A**) Proliferation curve of *UbC*$^{+/+}$ (+/+), *UbC*$^{+/-}$ (+/−) and *UbC* $^{-/-}$ (−/−) MEFs with (+Ub) or without ectopic expression of HA-Ub. A total of 2.5×10^4 cells were plated on 12-well plate and counted over a 6-day period. Data are expressed as mean ± s.e.m. from 4–10 MEFs originated from different embryos per genotype. (**B, C**) *UbC*$^{+/+}$ (+/+) and *UbC* $^{-/-}$ (−/−) MEFs stained with Ki-67 and SA-β-gal. Representative data from passage = 5 are shown. Ki-67 positive cells (%) were counted in three MEFs originated from different embryos per genotype. At least four different fields were counted in each MEFs. Scale bar, 100 μm, $^{**}P < 0.01$. (**D**) GFP fluorescence as an indicator of transcriptional activity of *UbC* gene. Mean GFP fluorescence from population of *UbC*$^{+/-}$ (+/−), *UbC* $^{-/-}$ (−/−) and *UbC* $^{-/-}$; *HA-Ub* (−/−, +Ub) MEFs is shown after subtracting background fluorescence of wild-type MEFs. Data are expressed as mean ± s.e.m. from 5–8 MEFs originated from different embryos per genotype. (**E**) Increase of GFP fluorescence in MEFs with passage number. Mean GFP fluorescence from population of *UbC*$^{+/-}$ (+/−), *UbC* $^{-/-}$ (−/−), *UbC*$^{+/-}$; *HA-Ub* (+/−, +Ub) and *UbC* $^{-/-}$; *HA-Ub* (−/−, +Ub) MEFs is shown after subtracting background fluorescence of wild-type MEFs at each passage. Data are from two MEFs originated from different embryos per genotype at different passage number.

gene due to the low abundance of *UbB* gene in MEFs (Figure 5B). The central role of *UbC* as a source of Ub in MEFs is even more evident when the fact that the polyubiquitin genes *UbB* and *UbC* encode, 4 and 9 Ub moieties per transcript, respectively—as opposed to only one for *UbA* is considered (Figure 5C).



MEFs (P = 3)	*UbC*$^{+/+}$	*UbC*$^{-/-}$	*UbC*$^{-/-}$; *HA-Ub*
n	5	5	8
Apoptotic (%)	2.1±0.52	5.1±1.3$^{\#}$	2.2±0.31
G1 (%)	5.7±1.6	48±1.7**	58±0.74
S (%)	8.1±0.99	9.4±1.7	10±0.53
G2/M (%)	33±2.4	37±2.1	30±0.7

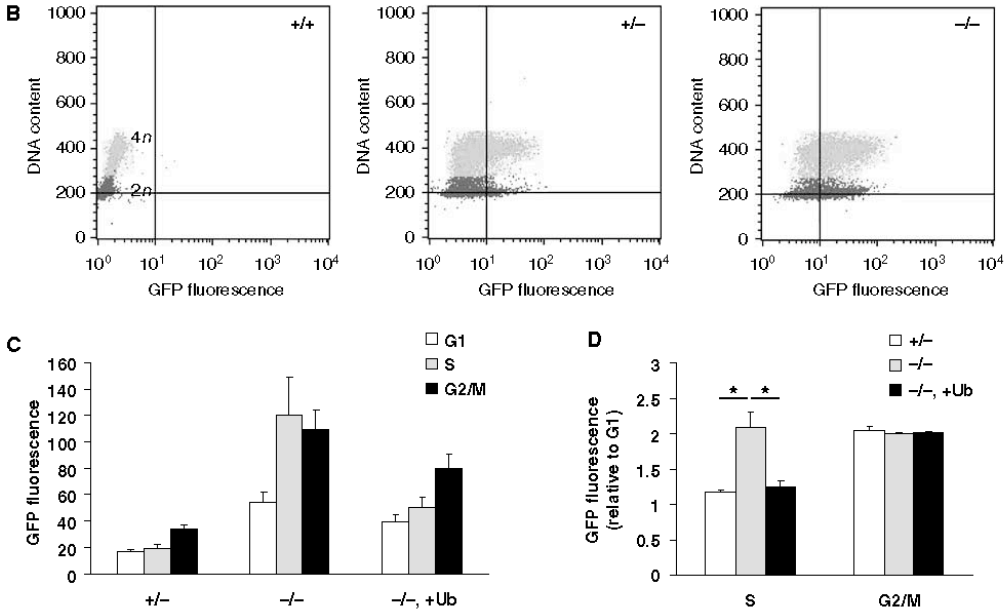


Figure 4 Cell-cycle defect in *UbC* $^{/}$ MEFs and its rescue by ectopically expressed HA-Ub. (**A**) Increased apoptosis, reduced G1 and increased G2/M boundaries in *UbC* $^{/}$ ($^{/}$) MEFs (passage = 3) that can be rescued by ectopic expression of HA-Ub in *UbC* $^{/}$; *HA-Ub* ($^{/}$, +Ub) MEFs. Representative cell-cycle profile analyzed by flow cytometry is shown. $^{\#}P<0.1$ versus wild-type (+/+) MEFs, $^{**}P<0.01$ versus wild-type (+/+) MEFs. (**B**) GFP fluorescence of *UbC*$^{+/+}$ (+/+), *UbC*$^{+/}$ (+/) and *UbC* $^{/}$ ($^{/}$) MEFs as a function of DNA content. GFP fluorescence in cells with 4n DNA content (G2/M) is higher than with 2n DNA content (G1). (**C**) GFP fluorescence in each phase of cell cycle in MEFs. Mean GFP fluorescence from population of *UbC*$^{+/}$ (+/), *UbC* $^{/}$ ($^{/}$) and *UbC* $^{/}$; *HA-Ub* ($^{/}$, +Ub) MEFs is shown after subtracting background fluorescence of wild-type MEFs. Data are expressed as mean ± s.e.m. from 5–8 MEFs originated from different embryos per genotype. (**D**) GFP fluorescence in S and G2/M phase is expressed relative to G1 phase. $^{*}P<0.05$.

Increased sensitivity of UbC $^{/}$ *MEFs to cellular stress*

Our data demonstrate that *UbC* contributes importantly to the maintenance of steady-state Ub pools and that *UbC* transcription, as monitored by GFP-puro fluorescence, strongly correlates with the senescence of MEFs, suggesting that *UbC* transcription is responsive to cellular stress. To determine the extent to which the *UbC* gene contributes to stress tolerance, we investigated whether *UbC* $^{/}$ MEFs show increased sensitivity to heat shock (Figure 6A and B). The promoter region of the mammalian *UbC* gene contains a heat-shock

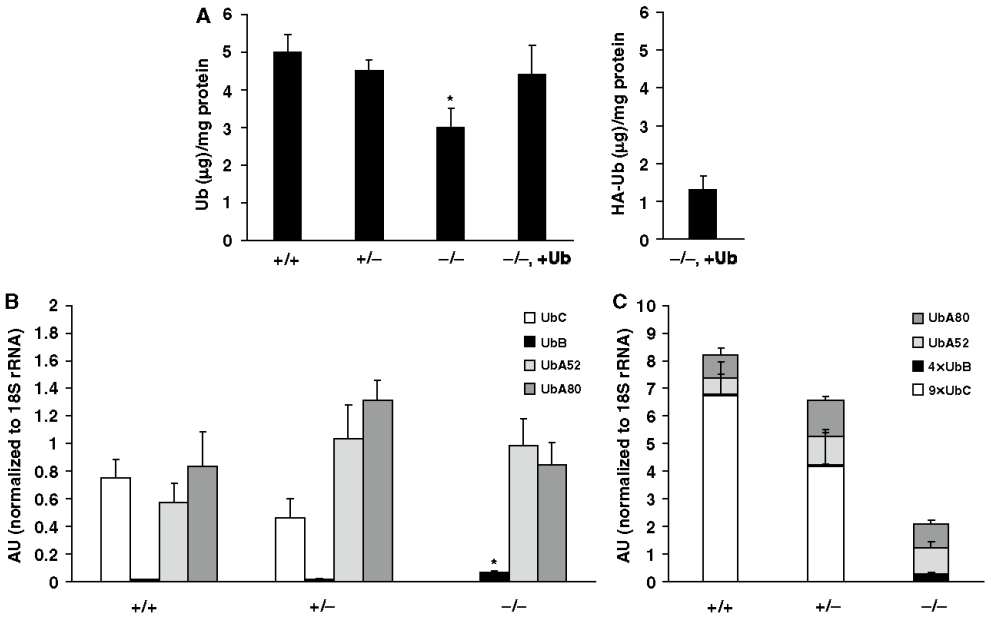


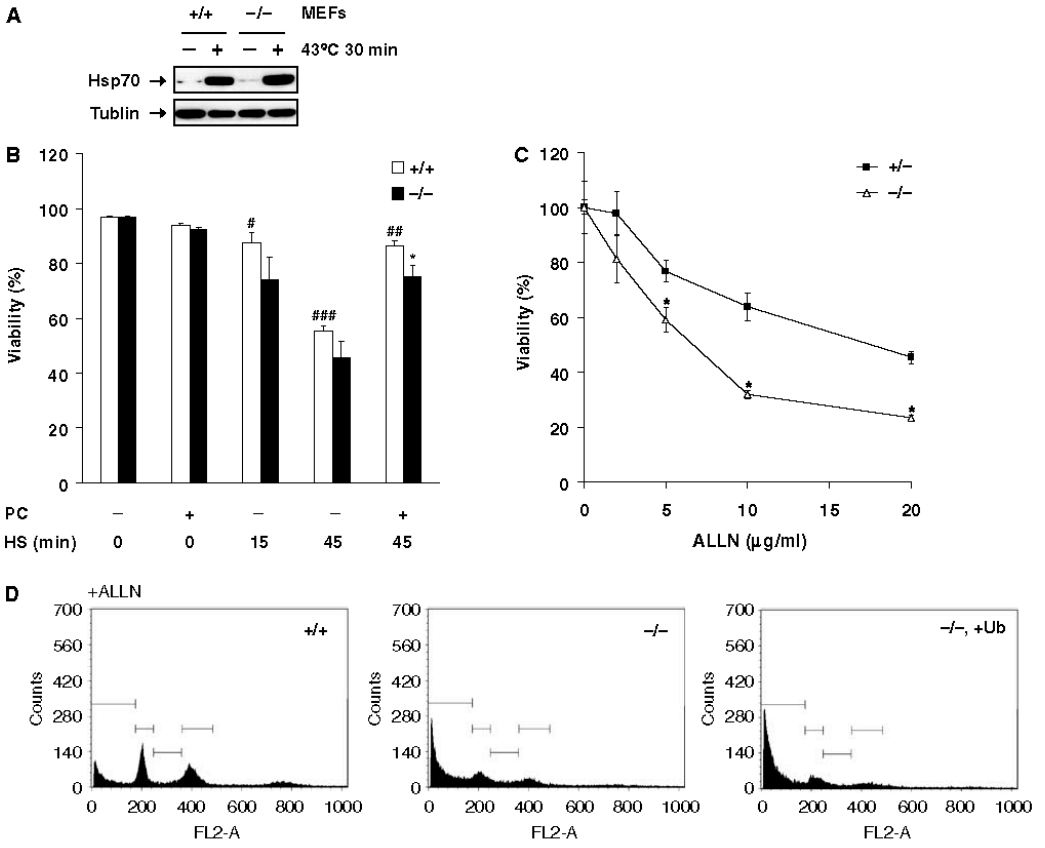
Figure 5 Disruption of *UbC* gene results in reduced cellular Ub content. (**A**) Indirect competitive ELISA for total Ub (left panel) or HA-Ub (right panel) levels in MEFs. Total cell lysates from $UbC^{+/+}$ (+/+), $UbC^{+/-}$ (+/−), $UbC^{-/-}$ (−/−) and $UbC^{-/-}$; *HA-Ub* (−/−, +Ub) MEFs were digested with Usp2-cc and subjected to indirect competitive ELISA. Data are expressed as mean±s.e.m. from four MEFs originated from different embryos per genotype with triplicate experiments. *$P<0.05$ versus wild-type (+/+) MEFs. (**B**) Various Ub transcript levels in MEFs. Total RNA was isolated from $UbC^{+/+}$ (+/+), $UbC^{+/-}$ (+/−) and $UbC^{-/-}$ (−/−) MEFs and UbC, UbB, UbA52 and UbA80 mRNA levels were measured by quantitative real-time RT–PCR and normalized to 18S rRNA levels. Data are expressed as mean±s.e.m. from five MEFs originated from different embryos per genotype. *$P<0.05$ versus the corresponding transcript levels in wild-type (+/+) MEFs. (**C**) Contribution of Ub genes to total Ub levels. Ub transcript levels in MEFs shown in (B) are normalized by the number of Ub moieties that each Ub transcript generates.

element, and previous studies have shown that UbC transcript levels are increased in response to thermal stress (Fornace *et al*, 1989; Sherlock *et al*, 2001; Murray *et al*, 2004). Exposure of MEFs to mild heat stress (43°C for 30 min) induced heat-shock protein 70 (Hsp70) in both wild-type and *UbC* $^{-/-}$ MEFs to a similar extent (Figure 6A), indicating that the *UbC* gene is not required for activating the heat-shock response. This mild stress, which preconditions cells to survive a subsequent lethal heat shock (45°C) (McMillan *et al*, 1998), resulted in a negligible reduction of viability in MEFs of both genotypes (Figure 6B). Exposure of MEFs to lethal heat shock for 15 or 45 min, without preconditioning, led to a significant reduction in viability that was more pronounced in cells lacking *UbC*. Viability of MEFs of both genotypes was dramatically improved by preconditioning, consistent with our finding (Figure 6A) that cells of both genotypes were able to induce Hsp70 levels to a similar extent following a mild heat stress. However, the extent to which *UbC* $^{-/-}$ MEFs were rescued by preconditioning was significantly reduced compared to wild-type MEFs (Figure 6B). Thus, loss of *UbC* leads to increased sensitivity to lethal heat shock and impairs the acquisition of thermotolerance.

To impose a stress more directly related to the function of the Ub proteasome system (UPS), we investigated the effects of proteasome inhibitors on *UbC* $^{-/-}$ MEFs. In contrast to thermal stress, which activates only heat-shock transcription factor 1 (HSF1) expression (Pirkkala *et al*, 2000), proteasome inhibitors activate a more extensive heat-shock response that includes activation of HSF2 (Mathew *et al*, 1998). We found that *UbC* $^{-/-}$ MEFs were killed by significantly lower concentrations of ALLN (Figure 6C) or MG132 (data not shown) than were MEFs heterozygous for the *UbC* knockout. ALLN treatment also decreased the fraction of cycling MEFs and increased the fraction of cells with sub-G1 DNA content to a far greater extent in *UbC* $^{-/-}$ MEFs than in wild-type cells (Figure 6D). This effect was not reversed in the HA-Ub background, suggesting that, although the extra copies of Ub provided by this locus were able to rescue most of the observed *UbC* $^{-/-}$ phenotypes, they were not sufficient to compensate for the stress of proteasome inhibitor treatment. Together, these data establish that the *UbC* gene contributes to the response of cells to cellular stress and to thermotolerance.

Activation of Ub gene expression by cellular stress

To assess the contribution of *UbC* to the maintenance of cellular Ub levels under stress, we exploited GFP-puro fluorescence to monitor the effect of heat shock on *UbC* gene expression (Figure 7A). GFP-puro fluorescence in viable *UbC* $^{-/-}$ MEFs was significantly increased ~1.6-fold by even a mild preconditioning heat shock and was not further activated by a lethal heat shock whether or not it was preceded by preconditioning (Figure 7A). It is currently unclear why GFP-puro fluorescence was significantly reduced in viable *UbC* $^{-/-}$ MEFs, following a 45 min lethal heat shock without precondi-



MEFs (P=3), +ALLN	*UbC*$^{+/+}$	*UbC*$^{-/-}$	*UbC*$^{-/-}$; *HA-Ub*
n	5	5	8
Apoptotic (%)	40±4.2	54±3.4*	58±7.2*
G1 (%)	25±2.1	19±1.5*	17±2.6*
S (%)	12±1.2	13±1.7	11±1.9
G2/M (%)	22±2.3	14±1.5*	14±2.8*

Figure 6 Enhanced sensitivity of *UbC* $^{/}$ MEFs to heat stress and proteasome inhibition. (**A**) Immunoblot analysis for inducible Hsp70 expression in MEFs before and after exposure to mild heat shock (HS) at 43°C for 30 min and recovery at 37°C for 6 h. Total cell lysates from *UbC*$^{+/+}$ (+/+) and *UbC* $^{/}$ (/) MEFs (20 μg) were subjected to SDS–PAGE followed by immunodetection with anti-Hsp70 antibody. α-tubulin was used as a loading control. (**B**) Viability of MEFs upon heat stress. *UbC*$^{+/+}$ (+/+) and *UbC* $^{/}$ (/) MEFs were exposed to heat stress as described in Materials and methods. Preconditioned (PC+) or non-preconditioned (PC) MEFs were exposed to lethal HS at 45°C for the time as indicated and recovered at 37°C for 24 h. After recovery, the percent of viable cell populations (both adherent and floating) was determined by propidium iodide (PI) staining. Data are expressed as mean±s.e.m. from 6–7 MEFs originated from different embryos per genotype. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ versus wild-type (+/+) MEFs with no treatment (PC , HS 0 min), $^{*}P<0.05$ versus wild-type (+/+) MEFs with the same treatment. (**C**) MTT cell viability assay for MEFs. *UbC*$^{+/}$ (+/) or *UbC* $^{/}$ (/) MEFs were treated with indicated concentration of ALLN for 24 h. At the end of incubation, viability was accessed by the percentage of MTT conversion. Data are expressed as mean±s.e.m. from three MEFs originated from different embryos per genotype. $^{*}P<0.05$ versus *UbC*$^{+/}$ (+/) MEFs. (**D**) Cell-cycle abnormality in *UbC* $^{/}$ (/) MEFs (passage=3) and failure of its rescue by ectopic expression of HA-Ub in *UbC* $^{/}$; *HA-Ub* (/ , +Ub) MEFs in the presence of proteasome inhibition. MEFs were exposed to 10 μg/ml ALLN (+ALLN) for 24 h and subjected to cell-cycle analysis by flow cytometry. Representative cell-cycle profile is shown. $^{*}P<0.05$ versus wild-type (+/+) MEFs/+ALLN.

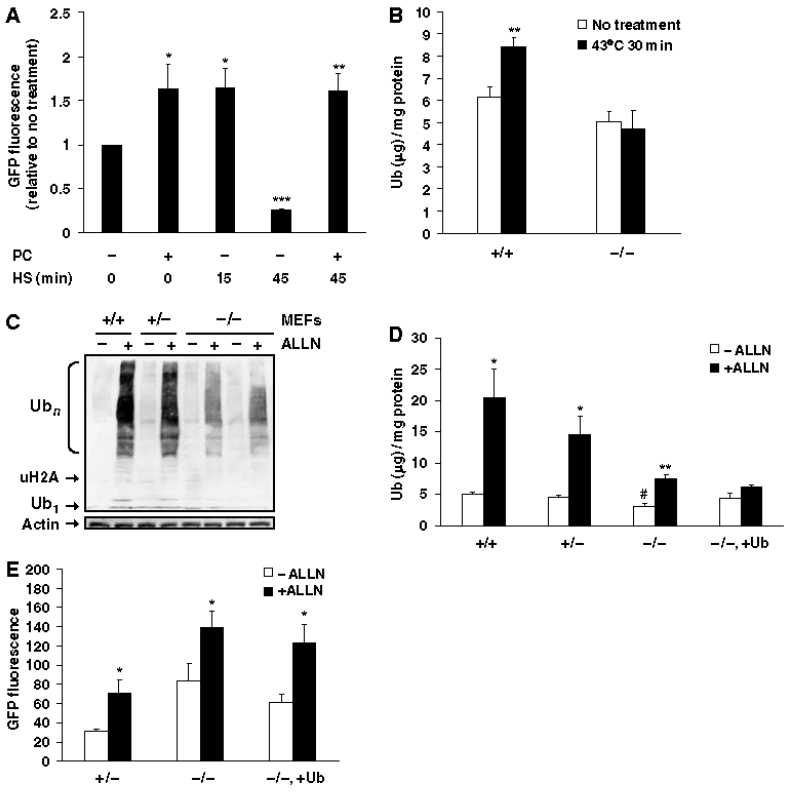


Figure 7 Effect of heat shock and proteasome inhibitor on *UbC* gene expression and cellular Ub content. (**A**) Change of GFP fluorescence in *UbC* / (/) MEFs upon heat stress. MEFs were exposed to heat stress as indicated and mean GFP fluorescence from population of viable (PI-negative) cells was measured. Change of GFP fluorescence relative to no treatment was calculated in each MEFs and expressed as mean ± s.e.m. from six MEFs originated from different embryos. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus MEFs with no treatment. (**B**) Indirect competitive ELISA for total Ub levels in MEFs before and after exposure to mild heat shock at 43°C for 30 min and recovery at 37°C for 6 h. Total cell lysates from *UbC*$^{+/+}$ (+/+) and *UbC* / (/) MEFs were digested with Usp2-cc and subjected to indirect competitive ELISA. Data are expressed as mean ± s.e.m. from 5–6 MEFs originated from different embryos per genotype with triplicate experiments. $^{**}P<0.01$ versus wild-type (+/+) MEFs/no treatment. (**C**) Immunoblot analysis of ubiquitinated proteins in MEFs exposed to DMSO (ALLN) or 10 μg/ml ALLN (+ALLN) for 24 h. Total cell lysates from *UbC*$^{+/+}$ (+/+), *UbC*$^{+/}$ (+/) and *UbC* / (/) MEFs (50 μg) were subjected to SDS–PAGE followed by immunodetection with anti-Ub antibody. β-actin was used as a loading control. Ub$_n$, ubiquitin conjugates; uH2A, mono-ubiquitinated histone 2A; Ub$_1$, ubiquitin monomer. (**D**) Indirect competitive ELISA for total Ub levels in MEFs exposed to DMSO (ALLN) or 10 μg/ml ALLN (+ALLN) for 24 h. Total cell lysates from *UbC*$^{+/+}$ (+/+), *UbC*$^{+/}$ (+/), *UbC* / (/) and *UbC* / ; *HA-Ub* (/ , +Ub) MEFs were digested with Usp2-cc and subjected to indirect competitive ELISA. Data are expressed as mean ± s.e.m. from four MEFs originated from different embryos per genotype with triplicate experiments. $^{\#}P<0.05$ versus wild-type (+/+) MEFs/ ALLN, $^{*}P<0.05$ versus ALLN, $^{**}P<0.01$ versus ALLN. (**E**) Transcriptional activation of *UbC* gene under proteasome inhibition as monitored by the increase of GFP fluorescence. GFP fluorescence of *UbC*$^{+/}$ (+/), *UbC* / (/) and *UbC* / ; *HA-Ub* (/ , +Ub) MEFs is shown after subtracting background fluorescence of wild-type MEFs. Data are expressed as mean ± s.e.m. from 5–8 MEFs originated from different embryos per genotype. $^{*}P<0.05$ versus ALLN.

tioning. Consistent with this increase in *UbC* transcriptional activity and the dominant contribution of *UbC* to the maintenance of Ub pools in MEFs (see Figure 5C), we observed that total Ub levels in wild-type MEFs were significantly increased following a preconditioning heat shock (Figure 7B). Strikingly, heat shock failed to upregulate the already-diminished basal levels of Ub in *UbC* / MEFs. Thus, *UbC* activation and the consequent ~1.4-fold increase in total Ub levels is an important contributor to thermotolerance.

By contrast to heat shock, exposure to proteasome inhibitor led to a far larger (~4-fold) increase in total Ub in wild-type MEFs (Figure 7D), which included a substantial increase in the level of Ub conjugates, as revealed by immunoblotting (Figure 7C). The response of *UbC* / MEFs to proteasome inhibitors was severely blunted compared to wild-type MEFs (Figure 7D) with a drastically diminished abundance of Ub conjugates (Figure 7C). Although coexpression of HA-Ub increased steady-state Ub levels in untreated *UbC* / MEFs, total Ub levels in *UbC* / ; *HA-Ub* MEFs were not increased significantly in response to ALLN (Figure 7D), indicating that the minimal human *UbC* promoter, which drives HA-Ub expression in this strain, is not properly regulated in this ectopic locus. This result may explain the inability of the additional HA-Ub to rescue the cell-cycle abnormality in

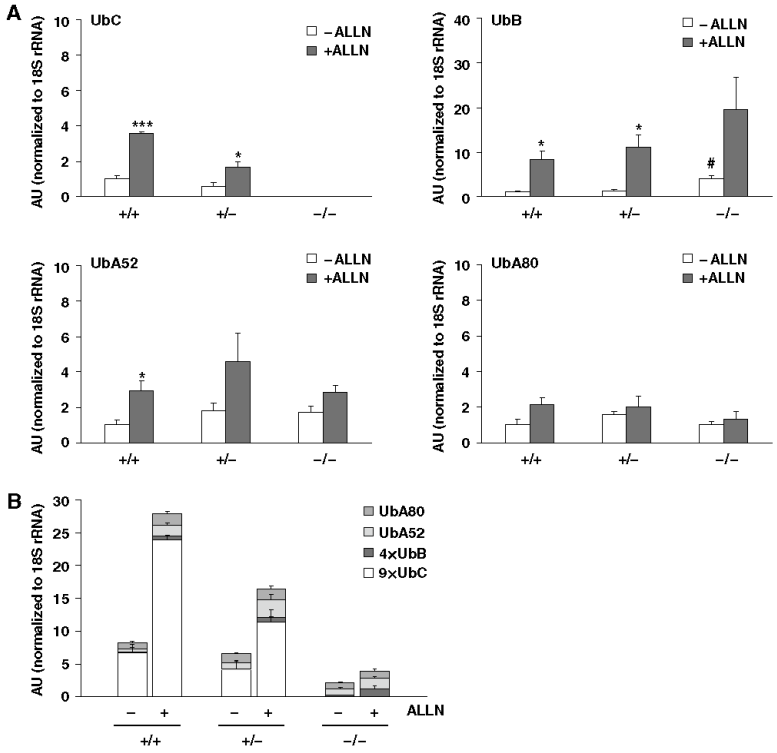


Figure 8 Effect of proteasome inhibitor on Ub gene expression. (**A**) Various Ub transcript levels in MEFs after exposure to DMSO (−ALLN) or 10 μg/ml ALLN for 24 h (+ALLN). Total RNA was isolated from $UbC^{+/+}$ (+/+), $UbC^{+/-}$ (+/−) and $UbC^{-/-}$ (−/−) MEFs with or without proteasome inhibition and UbC, UbB, UbA52 and UbA80 mRNA levels were measured by quantitative real-time RT–PCR and normalized to 18S rRNA levels. mRNA levels of wild-type (+/+) MEFs without proteasome inhibition (−ALLN) were arbitrarily assigned as 1 for each transcript. Data are expressed as mean±s.e.m. from five MEFs originated from different embryos per genotype. $^{\#}P<0.05$ versus wild-type (+/+) MEFs/−ALLN, $^{*}P<0.05$ versus −ALLN, $^{***}P<0.001$ versus −ALLN. (**B**) Contribution of Ub genes to total Ub levels with or without proteasome inhibition. Ub transcript levels in MEFs shown in (B) are normalized by the number of Ub moieties that each Ub transcript generates.

UbC $^{/}$ *; HA-Ub* MEFs exposed to proteasome inhibitor (see Figure 6D). The increase in Ub levels in wild-type and $UbC^{+/}$ MEFs exposed to ALLN correlated well with activation of the *UbC* promoter, assessed by GFP-puro fluorescence (Figure 7E). Interestingly, despite the blunted response to proteasome inhibitors of *UbC* $^{/}$ MEFs, the nearly two-fold increase in Ub levels suggests that other Ub genes, in addition to *UbC*, might be activated in response to severe stress. Indeed, the increase in total Ub in response to ALLN in wild-type MEFs can be accounted for by increased transcription of all four Ub genes (Figure 8A), although the absolute contribution of *UbC*, normalized for its 9 Ub coding units, is by far the dominant one (Figure 8B). Even though UbB transcript levels are increased dramatically in response to proteasome inhibition, *UbB*'s contribution to total Ub pools in these cells is evidently insufficient to compensate for the loss of *UbC*.

Discussion

The embryonic lethality observed in *UbC* $^{/}$ mice establishes an essential role for polyubiquitin gene expression during embryonic development. As the ultimate product of all four mammalian Ub genes is chemically identical irrespective of its genetic provenance, the simplest explanation for our findings is that all of the observed phenotypes are the consequence of Ub deficiency. This conclusion is strongly supported by the demonstration of reduced steady-state Ub levels in *UbC* $^{/}$ MEFs, and by the extensive, if incomplete, rescue of multiple *UbC* $^{/}$ phenotypes by expression of six copies of HA-Ub from the *Hprt* locus.

The dramatic impairment of hepatogenesis is the most likely explanation for the midgestation embryonic lethality observed in *UbC* $^{/}$ mice. This hypothesis is supported by the finding that HA-Ub largely, if not completely, rescues the hepatogenesis defect in *UbC* $^{/}$ embryos and delays embryonic death. The fetal liver is the primary site of definitive hematopoiesis in midgestation mouse embryos. Not surprisingly, genetic disruption of a large and diverse array of genes that control both hematopoiesis and hepatogenesis lead to fetal liver hypoplasia and embryonic death at around E12.5–E15.5 (Dzierzak and Medvinsky, 1995; Zaret, 1998). Between E12.5 and E15.5, the developing liver undergoes massive expansion, primarily to support the developing fetal blood supply (Zaret, 1998). The well-established essential role for Ub-dependent proteolysis in the eukaryotic cell cycle, and our

observation that decreased Ub levels delay mitotic progression in *UbC* $^{-/-}$ MEFs, lead us to speculate that the midgestation lethality observed in *UbC* $^{-/-}$ embryos may be due to a failure to meet the demand for Ub during this period of high mitotic activity.

There are several possible explanations for why the lethality of *UbC* $^{-/-}$ embryos is only partially rescued by HA-Ub. First, the *HA-Ub* minigene contains 30% fewer Ub moieties per transcript than endogenous *UbC* (6 in the *Hprt*-linked allele versus 9 in the endogenous allele). Therefore, even if transcriptional activity was the same as that from the wild-type locus, the amount of Ub produced from this locus will be less than from endogenous *UbC*. This decreased Ub potential is further exacerbated by the fact that only a single rescued allele is expressed from the X-linked *Hprt* locus. A second concern is that the presence of the HA-epitope tag in the ectopic allele could partially interfere with Ub function by influencing either the stability of the mRNA or protein products, or the susceptibility of conjugates to Ub isopeptidases or processing enzymes (Ellison and Hochstrasser, 1991). Third, it is possible that the strength of the minimal human *UbC* promoter used to drive this construct may be reduced when expressed ectopically. It is unlikely that this ectopically expressed, minimal promoter is able to fully recapitulate the regulatory features of the endogenous *UbC* allele. This possibility is supported by our observation that, while HA-Ub can fully suppress the growth and mitotic phenotypes of unstressed *UbC* $^{-/-}$ MEFs, it fails to completely rescue the defects in MEFs stressed by exposure to proteasome inhibitor. Although each of these possibilities could contribute to the partial rescue, there is no simple way to discriminate among them.

The genomic architecture of Ub genes is highly conserved throughout the eukaryotic domain; all organisms contain at least two genes encoding Ub fusions to small C-terminal extensions and at least one polyubiquitin gene. The *UbA*-type genes (in yeast, *UBI1–3*) encode fusions to small proteins that are components of the large and small ribosomal subunits, underscoring the profoundly conserved linkage between the protein synthesis and protein degradation systems (Finley *et al*, 1989). In yeast, these genes provide sufficient Ub for vegetative growth but the single polyubiquitin gene, *UBI4*, is essential for survival of most types of cellular stress (Finley *et al*, 1987). Regulation of the two polyubiquitin genes in mammals is less well understood. Like the single *UBI4* polyubiquitin gene in yeast, both *UbB* and *UbC* promoters contain heat-shock elements and their expression is increased in mammalian cells subjected to various types of stress including proapoptotic stimuli (Kugawa and Aoki, 2004), tumor promoters (Finch *et al*, 1992), oxidative stressors and heat shock (Fornace *et al*, 1989; Murray *et al*, 2004). The minimal *UbC* promoter has been widely used to drive transgene expression in mice because of its robust expression in most mouse tissues (Schorpp *et al*, 1996). Unless this gene is strongly repressed in its normal chromosomal locus, it is probable that *UbC*, unlike the prototypical *UBI4* gene in yeast, contributes to maintenance of Ub pools in unstressed cells as well. Indeed, we find that the *UbC* gene contributes a substantial fraction of the Ub transcriptome in most mouse tissues, with liver being notably the highest (Figure 9A). Interestingly, the tissue expression pattern of *UbC* appears to be roughly complementary to that of *UbB* suggesting, consistent with the conclusion, that these two polyubiquitin genes perform non-redundant functions (Figure 9B). Indeed, our data indicate that the loss of *UbC* cannot be compensated by increased transcription of the *UbA* or the *UbB* genes.

Our data establish that *UbC* contributes an especially large proportion of Ub in MEFs, consistent with the observation of growth defects in *UbC* $^{-/-}$ cells. The slow growth phenotype, owing to diminished levels of Ub in cells lacking *UbC*, appears to be due to impaired cell-cycle progression. The UPS plays a well-documented central role in the cell cycle, ensuring the unidirectionality of progression by degrading key modulators including cyclins and cyclin-dependent kinases (Hershko, 2005). The anomalous cell-cycle progression observed in *UbC* $^{-/-}$ MEFs, and its rescue by HA-Ub suggests that the reduced Ub capacity resulting from loss of *UbC* is insufficient for some critical Ub-dependent processes required for mitotic entry, reminiscent of the effects of

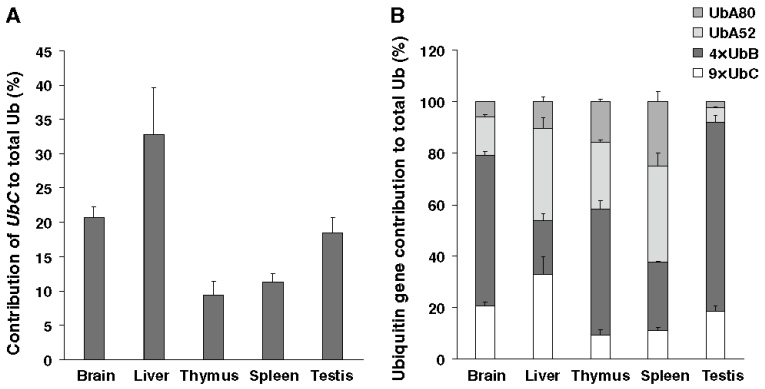


Figure 9 Relative contribution of Ub genes to total Ub levels in various mouse tissues. Total RNA was isolated from various tissues in 5-month-old wild-type mice ($n=7$ for testis; $n=3$ for all other tissues). UbC, UbB, UbA52 and UbA80 mRNA levels were measured by quantitative real-time RT–PCR and contribution of *UbC* (**A**) or four different Ub genes (**B**) to total Ub levels are shown after normalization by the number of Ub moieties that each Ub transcript generates. Data are expressed as mean ± s.e.m. from the indicated number of tissues.

proteasome inhibitors. Perhaps the principal antiproliferative effect of these drugs, which are increasingly being used as antitumor agents (Teicher *et al*, 1999; Roccaro *et al*, 2006), is through their profound ability to reduce pools of free Ub. The availability of mice defective in *UbC* gene expression and containing a *UbC*-regulated GFP reporter should be an excellent resource to facilitate our understanding of the mechanism of regulation of the Ub system.

Our data also establish that, Ub contributed by *UbC* is important to the ability of MEFs to survive acute lethal heat shock and that upregulation of this gene by the heat shock response contributes to thermotolerance. *UbC* also contributes to the ability of MEFs to survive a challenge with proteasome inhibitors, although the extent to which cellular Ub levels increase in response to the former insult is far more modest (~1.6-fold) than to the latter (~4-fold). This discrepancy cannot be accounted for simply by differences in the strength of the *UbC* transcriptional response (compare Figure 7A and E), which is activated to similar levels by heat shock and proteasome inhibition. Indeed, our data suggest the surprising conclusion that all four of the Ub genes are activated by exposure of MEFs to proteasome inhibitors.

Ub is required for a remarkably diverse set of fundamental cellular processes. Maintenance of an adequate supply of Ub in response to physiological demand is critical for cellular function and survival. Our data suggest that *UbC* constitutes an important, but not sufficient source of Ub during both stressed and unstressed conditions. Further research is needed to understand how Ub levels are sensed within the cell and how the different Ub genes are regulated in order to be able to survive the stress of protein folding.

Materials and methods

Details of construction and husbandry of mouse lines, as well as routine analytical procedures are provided in online Supplementary Information.

Cell-cycle analysis

Asynchronously growing MEFs were trypsinized and fixed in 70% ethanol for 24 h at −20°C, resuspended in PBS (1×10^6 cells/ml) and treated with RNase A (100 μg/ml) for 30 min at 37°C. Propidium iodide was added to a final concentration of 50 μg/ml and analyzed by FACSCalibur (BD).

Cell viability assay

MEFs were treated with indicated concentration of ALLN for 24 h. At the end of incubation, medium was removed and replaced with MTT working solution (500 μg/ml) and incubated for 4 h at 37°C. The converted dye was solubilized with acidic isopropanol and absorbance of the converted dye was measured at 570 nm with background subtraction at 650 nm.

Heat shock of MEFs

Heat shock of MEFs was performed essentially as described previously (McMillan *et al*, 1998). MEFs (3×10^5 cells) were plated out to 25-cm^2 flask 36 h before the heat shock experiment. Heat stress was induced by completely submerging flask in the temperature-controlled water bath. To induce thermotolerance, MEFs were preconditioned with mild heat shock at 43°C for 30 min and recovered at 37°C for 6 h. MEFs were then lethally heat-shocked at 45°C for 45 min, followed by recovery at 37°C for 24 h. Alternatively, MEFs were lethally heat-shocked at 45°C for 15 or 45 min without preconditioning and recovered at 37°C for 24 h. MEFs exposed to heat stress (both adherent and floating cells) were trypsinized and resuspended in PBS containing propidium iodide (1 μg/ml) and analyzed by FACSCalibur (BD) to determine the viability of cells.

For immunoblot analysis, preconditioned MEFs were harvested and resuspended in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, with protease inhibitor cocktail from Roche) and total cell lysates were prepared as described in Supplementary data. Total cell lysates (20 μg) were subjected to SDS–PAGE followed by immunoblot detection with monoclonal anti-Hsp70 antibody (SPA-810; Stressgen). Indirect competitive ELISA was performed as described in Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

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4.5 Work in progress: UBC6e and TLR signaling

4.5.1 Introduction

The innate immune system is the first line of defense that protects a metazoan host from the microbial pathogens that surround it. Innate immune recognition is mediated by receptors on the cell surface that recognize specific patterns that are conserved and invariant features of microorganisms (Janeway, 1989). The targets of pattern recognition receptors (PRR) are also referred to as pathogen associated molecular patterns (PAMP). PAMPs are often part of the bacterial or fungal cell wall. Viral PAMPS consist of DNA that has modifications that distinguish it from host metazoan DNA. All PAMPs have in common that they are unique to micro-organisms and are essential for microbial physiology (Medzhitov, 2007).
The best characterized class of PRRs are the toll-like receptors (TLRs). TLRs are type I transmembrane proteins that are expressed on antigen-presenting cells such as dendritic cells (DCs) or macrophages. Each of the 10 human (or 13 mouse) TLRs apparently recognizes distinct PAMPs. TLR4 recognizes lipopolysaccharides (LPS) from the outer membrane of gram-negative bacteria (Poltorak et al., 1998). TLR1, 2, 4, and 6 recognize lipids/lipopeptides (Akira, 2006). Double-stranded RNA, single-stranded RNA, and unmethylated bacterial DNA (CpG) engage TLR3, 7, and 9, respectively (Alexopoulou et al., 2001; Bauer et al., 2001; Diebold et al., 2004; Heil et al., 2004; Hemmi et al., 2000). Upon ligand interaction, TLRs form homo or hetero dimers that recruit cytoplasmic adaptor molecules. The cytosolic adaptor MyD88 (myeloid differentiation primary response gene 88) is recruited by all TLRs except TLR3, and activates MAP (mitogen activated protein) kinases and eventually NFκB, a transcription factor that activates the transcription of inflammatory cytokine genes such as TNFα, IL-6, IL-1β, and IL-12 (Akira, 2006; Akira and Takeda, 2004).

4.5.1.1 TLR signaling

TLR signaling is classified into two pathways: an MyD88 dependendent (canonical) and an MyD88 independent (non-canonical) pathway (Akira and Takeda, 2004; Hoebe and Beutler, 2004; Liew et al., 2005). MyD88 interacts with MAL/TIRAP

(myeloid adaptor like, TIR adaptor protein), thereby stimulating the recruitment of members of the IRAK (IL-1 receptor associated kinases) family, including IRAK1,2, and 4. IRAK-4 is the point of convergence of the TLR pathway (Lowe et al., 2006): IRAK4 deficiency leads to a complete block in all TLR signaling (Suzuki et al., 2002). IRAK4 phosphorylates IRAK1 which then dissociates from MyD88 to interact with the ubiquitin ligase TRAF6 (tumor necrosis receptor associated factor 6). TRAF6 autoubiquitinates itself with K63-linked ubiquitin chains with the help of the ubiquitin conjugating enzymes UBC13 and UEV1A (Deng et al., 2000). Polyubiquitinated TRAF6 then is recognized by TAB2 and TAB3 (Tak1 binding protein) that recruit TAK1 (a MAPKKK) into the complex (Wang et al., 2001; Wullaert et al., 2006). This kinase complex phosphorylates members of the IKK (inhibitor of IκB kinase) kinase complex. This kinase complex consists of the two catalytic subunits IKK1 and IKK2 with its regulatory subunit Nemo (NFκB essential modulator) and catalyzes the phosphorylation of IκBα (inhibitor of κ–B). Phosphorylated IκB is a target for the cytosolic ubiquitin ligase SCF, that in conjunction with the E2 enzymes UBC3 and UBC4 ubiquitinate IκB, thereby marking it for degradation by the proteasome (Strack et al., 2000). Once NFκB is freed from its inhibitor protein, it translocates into the nucleus where it controls the expression of cytokines (Kawai and Akira, 2007). TLR4 signaling is represented schematically in figure 7.

4.5.1.2 Non-canonical TLR signaling

MyD88 deficient mice can still produce IFNβ (interferon) after treatment with LPS (TLR4) or polyI:C (TLR3) (Kawai et al., 1999). Therefore, a TLR signaling pathway must exist that does not depend on MyD88. The latter pathway relies on the adaptor molecule TRIF (Oshiumi et al., 2003; Yamamoto et al., 2002). TLR4 signaling functions normally but with delayed kinetics in MyD88 knockout mice, suggesting that TLR4 uses both pathways: signaling through MyD88 and through TRIF (Kawai et al., 1999). TRIF also recruits TRAF6, thereby activating NFκB. For binding to TRAF6, the adaptor molecule RIP1 is necessary. RIP1 is part of a family of proteins involved in TNF receptor mediated activation of NFκB (Kawai and Akira, 2007). In addition, RIP1 is polyubiqutinated (presumably by TRAF6, but it is unknown

whether UBC13 plays a role in this reaction) and mediates recruitment of TAK1 (Cusson-Hermance et al., 2005).

4.5.1.3 TLR structure

TLRs can be subdivided into two groups: TLR 1, 2 ,4 ,5, and 6 are expressed on the plasma membrane, TLR 3, 7, and 9 localize to intracellular compartments, such as the ER and endosomes (Beutler et al., 2006). The intracellular TLRs sense nucleic acids (Beutler et al., 2006). All TLRs are type I glycosylated transmembrane proteins. Their cytoplasmic domain consists of a TIR (toll/ IL-1R resistance) domain, and their luminal or ectodomains are composed of leucine rich repeats (LRRs). TLRs build homodimers, with the exception of TLR1/2 and TLR2/6 which form heterodimers (Beutler et al., 2006). LRRs assume a horseshoe-shape. Recently the crystal structure of the TLR pair 1 and 2 in conjunction with its ligand Pam3CSK4 has been solved, and shows that Pam3CSK4 binds to the convex surface of the LRR horseshoe, thereby dimerizing the two TLRs and bringing the cytosolic TIR domains into very close contact (Brodsky and Medzhitov, 2007; Jin et al., 2007).

4.5.1.4 UNC93B

Most of the aforementioned TLR signaling proteins were uncovered through spontaneous or chemically induced mutations in mice (Beutler et al., 2006). MyD88 and TRIF adaptor molecules were found in forward genetic screens (Hoebe et al., 2003; Kawai et al., 1999), as well as some of the downstream kinases, such as IRAK-4 (Suzuki et al., 2002), TAK1 (Shim et al., 2005), and IKKα (Hoshino et al., 2006). The mutagen ENU (N-ethyl-N-nitrosurea) has been used in forward genetic screens, and led to the discovery of a mouse, the “triple D” mouse (3d), defective in three TLR signaling pathways, TLR 3, 7, and 9 (Tabeta et al., 2006). Apart from defects in the TLR pathway, the 3d mouse also displays defects in class I and II MHC restricted antigen presentation (Tabeta et al., 2006). The ENU-induced mutation was mapped to a single histidine to arginine amino acid exchange in the ER polytopic transmembrane protein UNC93B (Tabeta et al., 2006). UNC93B had not previously been implicated in the TLR pathway, and was a protein of unknown

function in mammals. UNC93B binds to TLR 3, 7, and 9, while mutant UNC93B (H412R) does not (Brinkmann et al., 2007). Furthermore, UNC93B plays a role in TLR trafficking from the ER to endosomes. DCs from the 3d mice fail to translocate TLR9 to endosomes upon stimulation with CpG DNA (Kim et al., 2008). Further experiments are necessary to understand how TLRs, especially those in the ER, sense their ligands.

4.5.1.5 Regulation of TLR signaling

TLR signaling can be modulated in numerous ways: (i) by sequestering signaling components (ii) by regulating the expression level of the components, (iii) by dominant negative versions of the components (iv) by post-translational modifications such as phosphorylation and dephosphorylation and ubiquitination and deubiquitination. In the remainder of the introduction I will focus on the role of ubiquitin in the TLR pathway (Lowe et al., 2006).

4.5.1.6 Regulation of TLR signaling by ubiquitin

Some of the signaling molecules within the TLR pathway are themselves ubiquitin ligases (E3s). These ligases usually activate the TLR pathway and have been described in chapter 4.5.11.

There are several inhibitory ubiquitination events that may fine-tune the TLR response, and that can turn it off when pathogens have been cleared (Lowe et al., 2006). TLRs themselves can be downregulated by proteasomal degradation. TRIAD3 is a ubiquitin ligase that ubiquitinates TLR9 and TLR4 with K48 linked ubiquitin chains, thereby initiating their destruction. TRIAD3 can also ubiquitinate itself (Chuang and Ulevitch, 2004). The MyD88 adaptor molecule Mal is also a target of an E3 ligase: the E3 ligase SOCS1 (suppressor of cytokine signaling) ubiquitinates Mal for proteasomal distruction (Mansell et al., 2004). A very interesting protein that plays a role in neutralizing the TLR response is A20. A20 is induced when TLR signaling is activated upon induction with LPS, poly I:C and viruses, or when the TNF pathway is activated upon induction with TNFα (Beyaert

et al., 2000). A20 can act as a deubiquitinating enzyme of the Otubain family of DUBs (Komander and Barford, 2008; Love et al., 2007) and as an E3 ligase (Wertz et al., 2004). As a DUB, A20 removes ubiquitin chains from TRAF6, thereby terminating the TLR response (Boone et al., 2004; Jensen and Whitehead, 2003). A20 knockout mice are hypersensitive to LPS-induced shock and show amplified activation of NFκB (Boone et al., 2004). As an E3 enzyme, A20 mediates K48-linked ubiquitin chain addition onto RIP, thereby targeting it for degradation. As RIP is involved not only in the TNF pathway but is also a mediator of the non-canonical TLR pathway that involves TLR3, A20 can directly fine-tune this pathway, by removing the K63 linked chain from RIP and simultaneously adding K48 linked chains to target it for degradation (Lowe et al., 2006).

Cezanne is another DUB that belongs to the A20 family. It is induced by exposure of cells to TNFα (Evans et al., 2003; Evans et al., 2001). Cezanne, just like A20, can deubiquitinate RIP, thereby inhibiting NFκB signaling (Enesa et al., 2008). Another DUB, CYLD, does not target RIP for deubiquitination but instead targets TRAF6 and IKKγ (Nemo) (Zhang et al., 2006). Nemo can be ubiquitinated with K63 linked chains by RIP2 (McCarthy et al., 1998). This reaction leads to the activation of NFκB (Bartek and Lukas, 2006).

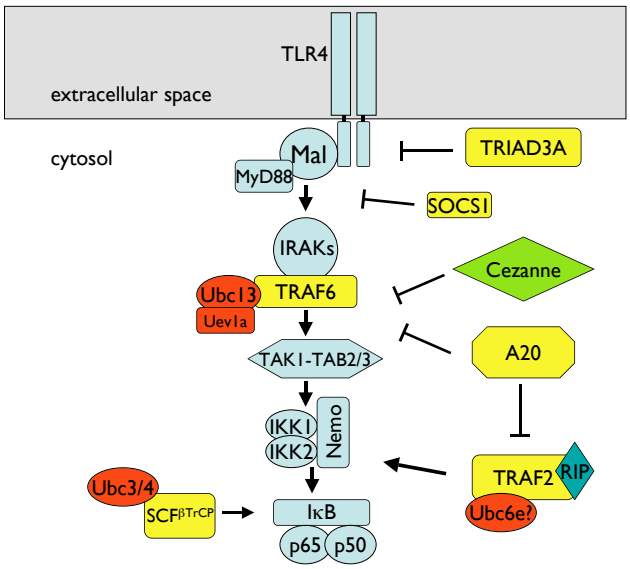

Figure 7: Schematic representation of TLR4 signaling. Interfering ubiquitin ligases and DUBs and depicted on the right. E3 ligases are yellow, E2 enzymes are red and DUBs are green.

4.5.2 Results

4.5.2.1 UBC6e is highly expressed in dendritic cells and is phosphorylated upon ER stress

We designed an antibody against the cytosolic domain of UBC6e, described in chapter 4.2. The amount of endogenous UBC6e recovered from HeLa cells was consistently very low (see chapter 4.2), so we searched for cells that would express higher levels and simplify detection with the antibody we generated. The Novartis expression database indicates that levels of UBC6e are very high in dendritic cells. UBC6e had been previously reported to be phosphorylated at Ser184 upon induction of ER stress with DTT, tunicamycin, or thapsigargin (Oh et al., 2006). We confirmed these results using thapsigargin as an ER stress inducer and primary bone marrow derived dendritic cells (BMDCs) as the cell line of choice (Figure 8). UBC6e phosphorylation is rapid and reversible.

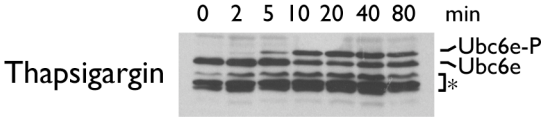

Figure 8: Thapsigargin induces phosphorylation of UBC6e. BMDCs were treated with 300nM thapsigargin and samples were removed at the indicated time points. Cells were lysed and analysed by immunoblotting with anti-UBC6e antibodies.

4.5.2.2 UBC6e is phosphorylated when TLR signaling is induced

As UBC6e is highly expressed in dendritic cells, we speculated that –apart from its role in US11- mediated dislocation of class I MHC- it may have a function specific to immune cells. We therefore treated BMDCs form wild-type mice with the different TLR agonists LPS (TLR4), CpG (TLR9), Imiquimod (TLR7), Malp2 (TLR2/6), and Pam_3CSK_4 (TLR1/2). All agonists tested led to phosphorylation of UBC6e, albeit with different kinetics. Malp2 acts the fastest and most abruptly, with fully phosphorylated UBC6e already appearing after 10min (Figure 8). The two

lipoprotein-based TLR activators both act with very fast kinetics, changing from no phosphorylation to quantitative phosphoryation within 10 – 20 minutes. In contrast, LPS and CpG induce maximal phosphorylation of UBC6e after 40 min. After 80 min of exposure to all agonists tested, most of the phosphorylated UBC6e has been dephosphorylated. To ensure that this phosphoryation event is due to functional TLR signaling pathway, we used BMDCs from the 3d mice as a control. The 3d mice display a defect in the intracellular TLR 3, 7, and 9 pathway due a mutation in the protein Unc93B that abolishes TLR binding and TLR trafficking to endosomes (Brinkmann et al., 2007; Tabeta et al., 2006, Kim et al., *in press*). TLR signaling through the TLRs that are situated on the cell surface proceeds unpurturbed. As expected, UBC6e phosphorylation is absent in the 3d DCs after stimulation with Imiquimod (TLR7) or CpG DNA (TLR9). UBC6e is phosphorylated with identical kinetics in WT and 3d BMDCs, suggesting that UBC6e phosphorylation is dependent on a functional TLR signaling pathway and specifically induced by the TLR ligands.

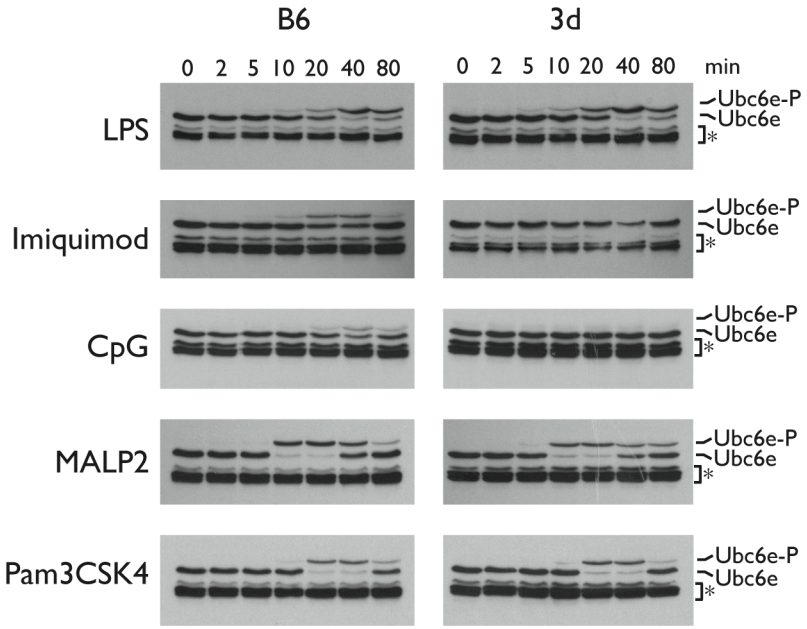

Figure 9: UBC6e is phosphorylated when TLR signaling is induced. BMDCS were treated with different TLR agonists for the indicated time points, lysed and analysed on 10%SDS PAGE. UBC6e was visualized by immunoblotting with anti-UBC6e antibodies.

To strengthen this argument, we compared DCs from WT mice with those from mice that had been knocked out for the essential TLR adaptor molecule MyD88. With the exception of TLR3 and TLR4, all TLRs depend on MyD88 for signaling. Since TLR4 can signal through both pathways, the MyD88 dependent and independent pathways, TLR4 signaling shows delayed kinetics when one of the two pathways is blocked. When we treated MyD88 knock-out (KO) DCs with LPS, a delay in UBC6e phosphorylation occurred compared to WT cells (Figure 10). Cells treated with CpG DNA did not show any UBC6e phosphorylation when MyD88 is not present.

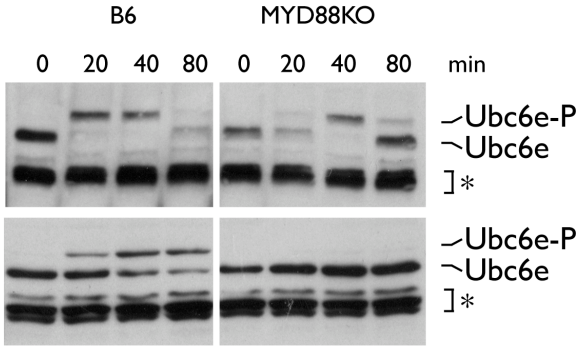

Figure 10: UBC6e phosphorylation depends on MyD88.

4.5.2.3 UBC6e phosphorylation correlates with IκBα phosphorylation

We reprobed the western blot to detect UBC6e phosphorylation for immuno-blotting with a phospho-specific antibody against IκBα. IκBα first is phosphorylated and then ubiquitinated and immediately degraded. In LPS-induced DCs, phosphorylated IκBα appears immediately prior to phosphorylation of UBC6e (Figure 11). When UBC6e is fully phosphorylated, IκBα disappears, suggesting that UBC6e might play a role in the degradation of IκBα. A second wave of phosphorylated IκBα appears once UBC6e phosphorylation declines.

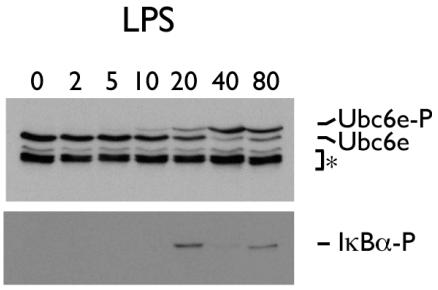

Figure 11: UBC6e phosphorylation and IκBα phosphorylation correlate.

4.5.2.4 UBC6e phosphorylation is not a secondary effect of TNFα production

TLR activation is usually measured by the amount of TNFα produced, as this is the primary cytokine evoked by all TLR activation pathways. TNFα also leads to NFκB activation through the TNF receptor pathway, similar to the TLR pathway (Wullaert et al., 2006). Although unlikely based on the rapid kinetics of the responses observed, to rule out that UBC6e phosphorylation is a response to elevated TNFα levels (or to NFκB activation), we treated BMDCs from WT mice and MyD88 knockout mice with TNFα (Figure 12). TNFα treatment does induce some UBC6e phoshorylation after 20, but already after 40 min it is reversed. TNFα enhances UBC6e phosphorylation but cannot explain the rapid and quantitative UBC6e phosphorylation that occurs for example when cells are treated with lipoproteins. Furthermore, all TLR agonists induce UBC6e phosphorylation with different kinetics, so the observed phosphorylation is unlikely to be a secondary effect of TNFα production.

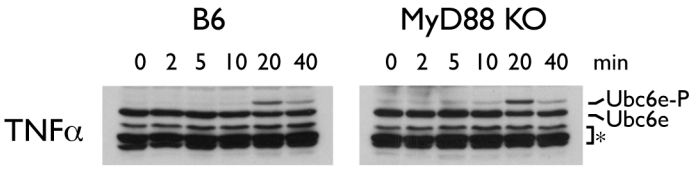

Figure 12: UBC6e is only poorly phosphorylated upon TNFα treatment.

4.5.2.5 UBC6e phosphorylation upon treatment of cells with H_2O_2

Reactive oxygen species (ROS) such as H_2O_2 play a pivotal role in innate immune response. When a pathogen enters dendritic cells or macrophages, the phagosome forms around the pathogen, and ROS are generated by the resident NADPH oxidase to attack the pathogen (Hampton et al., 1998). The role of H_2O_2 in NFκB activation remains controversial, but H_2O_2 could be a second messenger in activation of NFκB. For example, LPS activated TLR4 can activate NADPH oxidase, thereby initiating H_2O_2 release and eventually NFκB activation (Park et al., 2004). However, H_2O_2 does not activate NFκB in many cell lines (Forman and Torres, 2001; Janssen-Heininger et al., 2000). After TNFα treatment, depending on the cell line examined, H_2O_2 can stimulate or inhibit NFκB activation (Byun et al., 2002; de Oliveira-Marques et al., 2007; Kamata et al., 2002a; Kamata et al., 2002b; Lahdenpohja et al., 1998; Panopoulos et al., 2005) .

After treatment of BMDCs with H_2O_2 we saw rapid phosphorylation of UBC6e with a maximum at 20 min. There is no difference between WT BMDCs or MyD88 KO BMDCs (Figure 13).

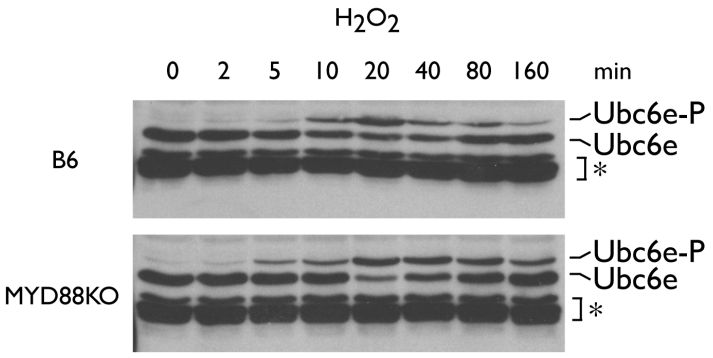

Figure 13: UBC6e is phosphorylated upon induction of oxidative stress.

4.5.2.6 Knockdown of UBC6e

We stably transduced DC2.4 cells with lentivirus encoding for shRNA against UBC6e or GFP. Cells were selected 4 days with puromycin prior to analyzing their

expression level (Figure 14). Additionally, we treated the cells for 20 min with LPS to have both versions of UBC6e present. One of the tested shRNAs results in almost 100% knockdown of UBC6e (Figure 14).

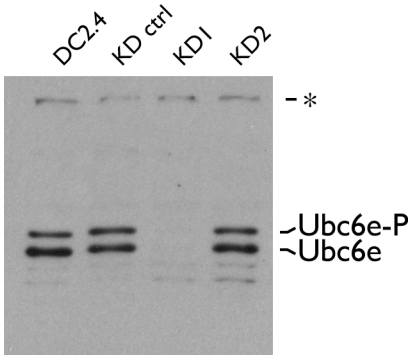

Figure 14: Lentivirally transduced UBC6e knockdown in DC2.4 dendritic cells.

4.5.3 Discussion

UBC6e is an ER bound ubiquitin conjugating enzyme involved in protein dislocation from the mammalian ER (Lenk et al., 2002; Younger et al., 2006). It is phosphorylated upon ER stress, but this phosphorylation does not obviously change its activity as an E2 (Oh et al., 2006). We report here that UBC6e plays a role in TLR signaling. It is highly expressed in dendritic cells and is phoshorylated upon stimulation with various TLR ligands. Furthermore, this phosphorylation is quantitative and reaches much higher levels than what had been previously reported for ER stress-induced phosphorylation of UBC6e.

Why would an ER transmembrane protein involved in ER dislocation have a role in modulating an innate immune response pathway? There has been some evidence that ER dislocation and cross presentation might be linked (Ackerman et al., 2006). Cross-presentation refers to Class I MHC molecules that present antigens internalized from extracellular space (otherwise presented on class II MHC molecules), and not from protein products made inside the cytosol (Rock et al., 1990). Only DCs and macrophages cross-present. It is still unclear how peptides from outside the cell can reach Class I molecules. First, DCs internalize debris from dying cells into phagosomes. The antigenic peptides must reach the cytosol, as cross-

presentation depends on the proteasome and on TAP (Ackerman and Cresswell, 2004; Huang et al., 1996; Kovacsovics-Bankowski and Rock, 1995; Norbury et al., 1995). Much research has been dedicated to understanding how peptides from the phagosome reach the cytosol. One theory involves fusion of the phagosome with the ER membrane (Gagnon et al., 2002). In this intriguing model proteins from extracellular space could be internalized by phagosomes and then reach the cytosol by utilizing the dislocation machinery. It has been shown that dominant negative forms of p97 inhibit cross-presentation from purified phagosomes (Ackerman et al., 2006), suggesting that the ER dislocation machinery, also inhibited by dominant negative versions of p97, might be used. Furthermore, phagocytosed microspheres with a polypeptide that contains the glycan attachment site are glycosylated after uptake, providing evidence for exposure to the enzymes of the ER, since N-linked glycosylation occurs exclusively in the ER (Ackerman et al., 2006; Rock, 2006). Recently, Giodini and Cresswell showed that internalized exogenous protein antigens are not only dislocated into the cytosol, but can even refold there with the help of HSP90 family members (Giodini and Cresswell, 2008). UBC6e could play a role in cross-presentation by dislocating and ubiquitinating the internalized antigens. UBC6e might fine-tune cross-presentation. Phosphorylated UBC6e presumably has different partner subunits than its unmodified counterpart, thereby providing a dynamic switch to alter cellular responses to the presence of pathogens.

4.5.4 Material and Methods

4.5.4.1 Antibodies

The anti phospho- IκBα antibody was purchased from Cell Signaling. The anti-UBC6e antibody has been described (see chapter 4.2).

4.5.4.2 Cell preparation

BMDCs were harvested by flushing out the tibia and femur of B6, 3d, and MyD88ko mice with a 25 gauge needle. The cells were plated in 12 well dishes at a concentration of 1 x 10^6 cell per well. Cells were cultured for 4 days in complete

DMEM that was supplemented with 1ng/ml IL-4 (Roche) and 10ng/ml GM-CSF (PeproTech). The medium was replenished on day 1 and on day 3.

For TLR activation or induction of the UPR, the following concentrations were used: H_2O_2 1mM, LPS 100ng/ml, CpG 1µM, Imiquimod 10uM, Pam_3CSk_4 1µg/ml, Malp2 100ng/ml, Thapsigargin 300nM.

Activation was stopped by addition of ice-cold PBS. Cell were scraped in NP-40 lysis buffer (150mM NaCl, 2mM MgCl, 0.5% NP-40, protease inhibitor tablets) for 30 min on ice, centrifuged (16000g, 10min) and the supernatant was boiled in sample buffer and loaded on a 10% SDS-PAGE. Immunoblotting was performed using standard procedures.

5 Outlook

This work contributed to the understanding of the process of ER dislocation in mammalian cells. Several new players involved in ERAD were identified and characterized. As the point of departure, a key player in mammalian dislocation, SEL1L, was used to identify new proteins involved in dislocation. In future studies, the new proteins OS9, UBXD8, AUP1, and UBC6e could be analyzed further by mutational analysis and by obtaining knock-out mice. So far, except for their role in dislocation, not much is known about these proteins.

These proteins helped shape the mammalian dislocation complex, and provided a glimpse into a new direction: when overexpressed, UBXD8 and AUP1 localize to lipid droplets (Klemm E, Mueller B, Ploegh HL, unpublished). This observation might provide the answer to one of the key questions in the field: how does a protein cross the lipid bilayer?

Equally important, future experiments with SEL1L and OS9 (a protein fulfilling US11's task of targeting substrates to the dislocation complex) and its interaction partners will explain how the cellular dislocation machinery distinguishes between terminally misfolded proteins and those that are in the process of attaining their final conformation.

UBC6e provided an interesting link between dislocation and TLR signaling. Future experiments will show whether UBC6e and with this, retrotranslocation from the phagosome to the cytosol of exogenous antigens, plays a role in cross-presentation.

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