Molecular functions of the ubiquitin domain protein Herp in Synoviolin mediated endoplasmic reticulum associated protein degradation (ERAD)

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Abstract

The accumulation of aberrant proteins in the endoplasmic reticulum (ER) induces the unfolded protein response (UPR) pathway for surmounting this cellular stress situation. One of the strongly UPR-induced genes in mammalia encodes the ubiquitin domain protein Herp. Herp interacts with the E3 ligase Synoviolin, a central component of ER associated protein degradation (ERAD) mediating multiprotein complexes. Dependent on its ubiquitin-like (UBL) domain, Herp is required for the efficient degradation of Synoviolin substrates. The molecular mechanism underlying this function of Herp is poorly understood.

In the present study, it was shown that Herp is continuously exchanged at Synoviolin based complexes. However, Herp did not serve as a Synoviolin substrate. Since both stabilisation and depletion of Herp resulted in the impaired degradation of Synoviolin substrates, the continuous turnover of Herp seems to be decisive for ERAD.

Herp was also shown to regulate the composition of Synoviolin based complexes. The deubiquitinating enzyme Usp7 was linked to Synoviolin via its interaction with Herp. However, Usp7 did not influence the stability of Herp or ERAD substrates. In addition, Herp improved the association of the CUE domain protein AUP1 with Synoviolin. AUP1 triggered the ERAD process in a CUE domain dependent manner.

Also Herp2, a homologue of Herp, was found to associate with Synoviolin based complexes. However, in contrast to Herp, Herp2 was not induced by the UPR, was stable, and did not bind Usp7 supporting the idea of Herp having a unique function in ERAD.

In conclusion, Herp is a dynamic ERAD component recruiting accessory proteins to Synoviolin thus enabling Synoviolin dependent ubiquitination of substrates. These findings point out the crucial role of Herp for the elimination of misfolded proteins, which is important for cell survival.

keywords: Herp, ERAD, UPR, Synoviolin, Usp7, AUP1, Herp2

Zusammenfassung

Die Akkumulation fehlerhafter Proteine im Endoplasmatischen Retikulum (ER) induziert den „unfolded protein response“ (UPR) - Signalweg zur Überwindung dieser zellulären Stress-Situation. Ein in Säugern stark UPR-induziertes Gen kodiert für das Ubiquitin-Domäne-Protein Herp. Herp interagiert mit der E3-Ligase Synoviolin, einer zentralen Komponente von Multiproteinkomplexen, welche die ER assoziierte Protein-Degradation (ERAD) vermitteln. Abhängig von seiner Ubiquitin-ähnlichen (‘UBL’) Domäne wird Herp für den effizienten Abbau von Synoviolin-Substraten benötigt. Der zugrundeliegende molekulare Mechanismus dieser Funktion von Herp ist kaum bekannt.

In der vorliegenden Studie wurde gezeigt, dass Herp kontinuierlich an Synoviolin-basierten Komplexen umgesetzt wird, aber kein Substrat ist. Da sowohl Depletion als auch Stabilisierung von Herp zum verminderten Abbau von Synoviolin-Substraten führt, lässt sich schlussfolgern, dass der kontinuierliche Umsatz von Herp entscheidend ist für ERAD.

Weiterhin regulierte Herp die Zusammensetzung Synoviolin-basierter Komplexe. Das deubiquitinierende Enzym Usp7 ist über seine Bindung an Herp mit Synoviolin assoziiert. Usp7 beeinflusste aber nicht die Stabilität von Herp oder ERAD-Substraten.

Zusätzlich verstärkte Herp die Interaktion zwischen dem CUE-Domäne-Protein AUP1 und Synoviolin. In Abhängigkeit von der CUE-Domäne steigerte AUP1 den ERAD-Prozess. Auch das Herp-Homolog Herp2 war mit Synoviolin-basierten Komplexen assoziiert. Im Gegensatz zu Herp wurde Herp2 nicht durch den UPR-Signalweg induziert, war stabil und interagierte nicht Usp7. Diese Daten unterstreichen die einzigartige Funktion von Herp im ERAD-Prozess.

Schlussfolgernd ist Herp eine dynamische ERAD-Komponente, welche die Rekrutierung akzessorischer Proteine an Synoviolin vermittelt und damit die Ubiquitinierung von Synoviolin-Substraten ermöglicht. Diese Daten zeigen die kritische Rolle von Herp für die Beseitigung fehlerhafter Proteine und das Überleben der Zelle.

Schlagwörter: Herp, ERAD, UPR, Synoviolin, Usp7, AUP1, Herp2

1 Introduction

1.1 The ubiquitin proteasome system (UPS)

Cellular protein homeostasis is essential for cell survival. To sustain a certain level of intracellular proteins, continuous synthesis and degradation takes place. The major pathway for the regulated degradation of intracellular proteins is the ubiquitin proteasome system (UPS). The small protein modifier ubiquitin and the 26S proteasome, a multi protease complex, have key positions within this system. These two components of the UPS are abundantly present in the cytoplasm and the nucleus of all eukaryotic cells. Regulatory proteins such as cyclins or p53, which are involved in essential cellular processes such as cell-cycle control or apoptosis, respectively, are processed by the UPS. Therefore, this pathway is essential for cell survival (Hershko and Ciechanover, 1998). In addition, the UPS is involved in the generation of antigenic epitopes which are presented by major histocompatibility complex (MHC) class I molecules, a central process of the cellular immune response (Kloetzel, 2004).

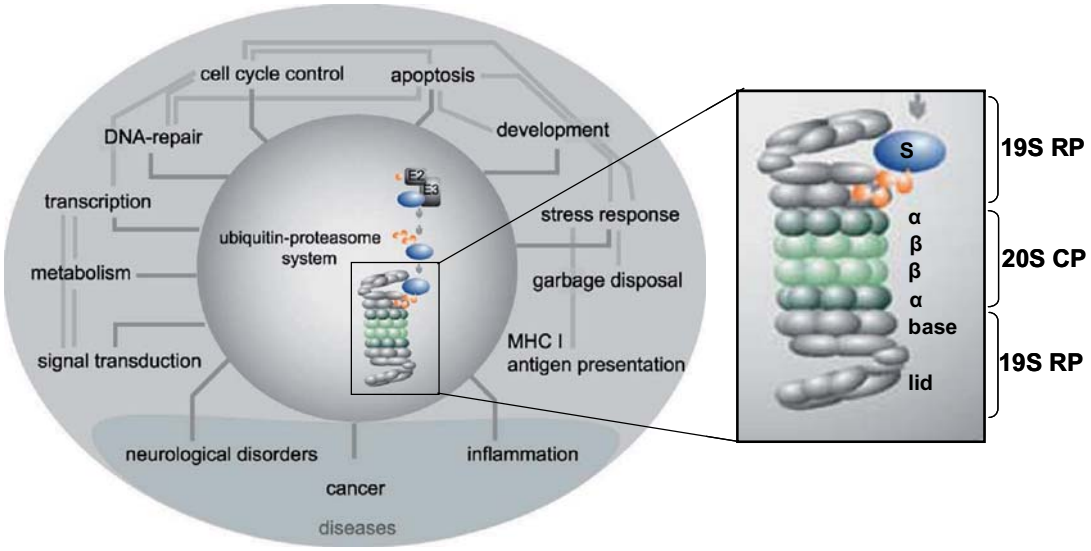

Figure 1: Schematic overview of the ubiquitin proteasome system (UPS) and its connections to various cellular processes. Degradation of regulatory proteins by the UPS is essential for a variety of highly interconnected cellular processes such as DNA-repair and therefore maintains cell survival. Disturbances within the UPS may lead to severe diseases such as inflammation. Protein degradation is mediated by the 26S proteasome (highlighted right hand) which consists of the 20S core particle (CP) and one or two 19S regulatory particles (RP). The CP is composed of four heptameric rings formed by the inner β- and the outer α-subunits. The RP is composed of base and lid substructures. S indicates a ubiquitinated substrate, E2 and E3 indicate the ubiquitination machinery (adapted from (Wolf and Hilt, 2004).

As part of the protein quality control, the UPS is the primary intracellular mechanism which is responsible for the degradation of defective proteins.

This includes incomplete, misfolded, denatured, oxidised or else damaged proteins which otherwise accumulate and have a tendency to form cytotoxic aggregates. Degradation of UPS substrates includes two major sequential steps: first, ubiquitination and second, degradation by the proteasome. These processes are subject to stringent regulation at the steps of substrate selection, substrate processing and product generation. Dysregulation of the UPS can lead to a variety of diseases such as cancer, inflammation or neurodegenerative disorders (Figure 1, Hershko and Ciechanover, 1998).

1.1.1 Composition of the 26S proteasome

Protein degradation within the UPS is mediated by the 26S proteasome, a large cytoplasmatic protein complex, consisting of the proteolytically active 20S core particle and one or two terminal 19S regulatory particles as reviewed in (Tanaka, 2009). The 20S core particle, which has a molecular mass of about 750 kDa, consists of four stacked heptameric rings each containing evolutionary related proteins. These rings shape a cylindrical structure (Groll et al., 1997). The constituents of the core particle are subdivided into α- and β-subunits according to their homologies. In eukaryotes the outer rings are composed of seven different α-subunits and the inner rings of seven different β-subunits. Three of the β-subunits display proteolytic activity. The 19S regulatory particle comprises approximately 20 different subunits forming two subcomplexes which are termed base and lid with the base associating with the α-rings of the 20S core particle (Glickman et al., 1998). The 19S regulatory particle is important for the recruitment, deubiquitination and entry of substrates into the 20S chamber.

1.1.2 The role of ubiquitin

Ubiquitin is a heat-stable polypeptide of 7.6 kDa, highly conserved and ubiquitously expressed in eukaryotes (Ciehanover et al., 1978) (Wilkinson et al., 1980). During a process termed ‘ubiquitination’ ubiquitin is covalently attached to target proteins in an ATP dependent manner resulting in the formation of a mono- or polyubiquitinated protein (Ciechanover et al., 1980). Within this polyubiquitin chain the single molecules are connected through isopeptide bonds, formed between the C-terminal glycine 76 of one molecule and a lysine residue of the other ubiquitin molecule. Ubiquitin possesses seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) of which K11, K29, K48 and K63 can form ubiquitin-ubiquitin linkages *in vivo* (Dubiel and Gordon, 1999). Polyubiquitin, consisting of at least four molecules connected to a protein, serves as a signal for the recruitment of the ubiquitinated protein to the 26S proteasome. Predominantly, G76-K48 linked ubiquitin chains signal proteasomal degradation (Chau et al., 1989; Thrower et al., 2000).

Apart from ubiquitin, a number of ubiquitin-like proteins such as SUMO, Nedd8 or ISG15 function as protein modifiers in a similar way as ubiquitin (Kerscher et al., 2006). Moreover, ubiquitin-like (UBL) domains have been described to be integral parts of ubiquitin domain proteins (UDPs). These UBL domains are, in contrast to ubiquitin-like modifiers, neither processed nor conjugated with other proteins. A variety of proteins have been reported to harbour ubiquitin interacting motifs. Such motifs are for example ubiquitin associated (UBA) domains binding to polyubiquitin (Wilkinson et al., 2001) and coupling of ubiquitin conjugation to ER degradation (CUE) domains described to bind poly- and monoubiquitin (Hurley et al., 2006).

1.1.3 The process of ubiquitination

The ligation of ubiquitin to a protein is a reversible process and requires the sequential action of three classes of enzymes: ubiquitin-activating, ubiquitin-conjugating and ubiquitin-protein ligating enzymes, called E1, E2 and E3 enzymes, respectively (Hershko et al., 1983). In humans, two E1, approximately 40 E2 and several hundred E3 enzymes are known. The ATP dependent attachment of ubiquitin to a target protein follows a distinct reaction cascade (Pickart, 2001).

In the first step, the C-terminal glycine 76 of ubiquitin is activated in an ATP-consuming manner by an E1 enzyme. An intermediate ubiquitin adenylate is formed and pyrophosphate (PP_i) is released, followed by the binding of ubiquitin to a reactive cysteine residue of the E1 enzyme in a thioester linkage, with the release of AMP. In the second step, catalysed by an E2 enzyme, ubiquitin is transferred onto an active site cysteine of the E2 protein whereby a thioester is formed. Finally, an E3 ligase associates with the E2 enzyme and catalyses the binding of ubiquitin to the substrate. Thereby the carboxy terminus of ubiquitin binds to an ε-amino group of a lysine residue of the substrate leading to the formation of an amide isopeptide linkage (Hershko et al., 1983).

The ubiquitin conjugation system can act on one substrate several times, resulting in ubiquitin chain formation. Thereby, the isopeptide bond formation takes place between one of the lysine residues of the proximal ubiquitin and the C-terminal glycine (G76) residue of the distal ubiquitin molecule. Recently it was shown that ubiquitin chains can already preassemble at the E2 enzyme from which they are transferred onto the substrate (Li et al., 2007). Substrate selectivity is thought to be mediated by the collaboration of E2 and E3 enzymes. In some cases the elongation of a ubiquitin-polymer requires an additional enzyme, referred to as E4. This enzyme recognises oligoubiquitinated substrates and with the help of E1, E2 and E3 catalyses the elongation of the ubiquitin chain. Some E4 enzymes such as Hsc70-interacting protein (CHIP) can also execute E3 activity (Hoppe, 2005).

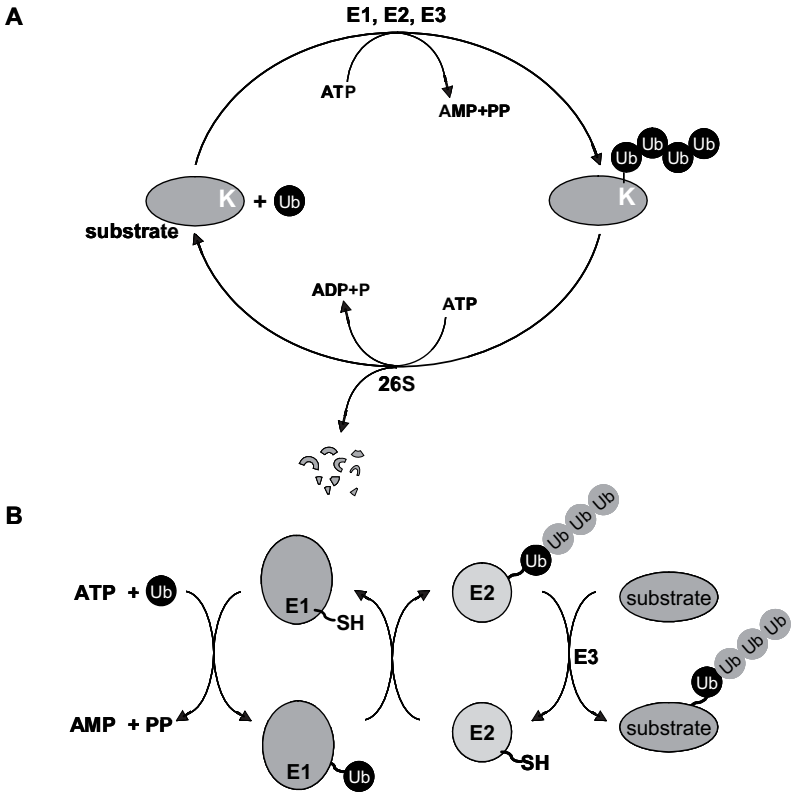

Figure 2: Components and mechanisms in the ubiquitin proteasome system. (**A**) Overview of the pathway showing the consumption of ATP in the conjugative (top) and degradative (bottom) phases. E1, E2 and E3 represent the ubiquitin-activating, -conjugating and -ligating enzymes, respectively. K indicates a lysine residue; the black circle with 'Ub' indicates a ubiquitin molecule. (**B**) Cascade of ubiquitination. Ubiquitin is activated by E1, transferred to E2, where a preassembly of ubiquitin chains may occur, and finally attached to a substrate, a process mediated by E3. A row of light grey circles with 'Ub' indicates a preformed polyubiquitin chain (Pickart, 2004).

Originally, ubiquitination was thought to mainly target proteins for proteasomal degradation. However, it was gradually assessed that this posttranslational protein modification serves diverse functions within the cell aside from proteolysis. The fate of a protein depends on the amount of ubiquitin, which may be attached as a single molecule or as a chain. In addition, the kind of linkage within polyubiquitin chains is decisive as monoubiquitination provides a signal for receptor internalisation or transcription regulation (Hicke, 2001; Strous et al., 1996), whereas K48-linked polyubiquitin predominantly leads to proteasomal degradation. Moreover, K63-linked polyubiquitin plays a key role in the activation of kinases within NF*k*B signalling. The IL-1 receptor associated kinase (IRAK1), *e.g.*, is activated through modification with K63-linked polyubiquitin (Keating and Bowie, 2009; Windheim et al., 2008).

1.1.4 E3 ubiquitin protein ligases

The human genome encodes hundreds of E3 ubiquitin ligases (E3s) which are responsible for the final step of ubiquitination. E3 enzymes are classified as 'really interesting new gene' (RING) E3s and 'homologous to E6 associated protein carboxy terminus' (HECT) E3s. Acting as single proteins or within multiprotein complexes, E3s promote substrate recognition, E2-ubiquitin recruitment and transfer of ubiquitin onto the target protein (Pickart, 2001). E6-associated protein (E6-AP) was the first described HECT E3 enzyme, identified in cells which had been transfected with the human papilloma virus oncoprotein E6. In the presence of the E6 protein E6-AP ubiquitinates p53, which leads to the rapid proteasomal degradation of p53 (Scheffner et al., 1993). The C-terminal HECT domain of E6-AP is evolutionarily conserved and present in all HECT E3s. A cysteine residue within this domain serves as an acceptor of the activated ubiquitin from the E2 enzyme (Huibregtse et al., 1995; Scheffner et al., 1995). Thus, a thioester intermediate between ubiquitin and the HECT E3 is formed. Finally, ubiquitin is transferred to the substrate (Figure 3, A).

The large family of the RING E3 enzymes contains a characteristic conserved motif, which is rich in cysteine and histidine residues and termed RING domain (Freemont et al., 1991). The RING domain coordinates zinc ions and determines the activity of the protein (Lorick et al., 1999). In contrast to HECT-E3s, RING E3s do not form a thioester with ubiquitin but bring the ubiquitin-loaded E2s and the substrate in close proximity and promote ubiquitin transfer onto the substrates. Therefore, this class of E3s acts as scaffold (Deshaies and Joazeiro, 2009). RING E3 ligases are either cytoplasmatic such as CHIP or are membrane associated such as the mammalian Hrd1p orthologue Synoviolin (Hrd1) integrated in the ER membrane (Ballinger et al., 1999; Kaneko et al., 2002). In addition, RING E3s either exist as a single molecule or as a component of multi protein complexes. Single subunit E3s such as parkin harbour a substrate recognition element and the RING domain on the same molecule (Figure 3, B-1; (Imai et al., 2001). Multi subunit RING E3s such as the SCF complex contain a member of the cullin protein family as backbone, a RING domain protein harbouring enzyme activity and other proteins which are adaptors involved in substrate recognition and E2 binding (Deshaies and Joazeiro, 2009).

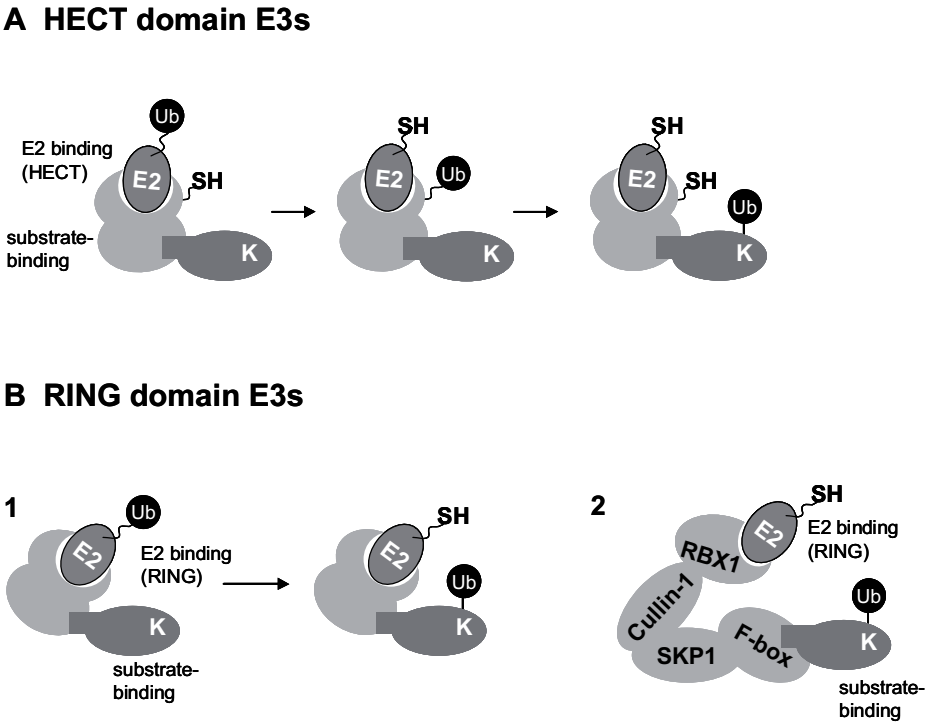

Figure 3: Schematic overview of the major E3 classes. (**A**) HECT domain E3 mediated ubiquitination. The E2 is bound by the HECT domain and ubiquitin is transiently transferred to a conserved cysteine within the same region. The substrate binds to another domain within the same protein. (**B**) RING domain E3 mediated ubiquitination. (**1**) Single subunit RING E3. The E2 is bound by the RING domain and the substrate by a different domain within the E3. Ubiquitin is transferred from the E2 to the substrate. (**2**) Multi subunit RING E3 (SCF complex). E2 and substrate binding are mediated by domains of different subunits (adapted from Pickart, 2004).

1.1.5 Deubiquitinating enzymes (DUBs)

Ubiquitination is a reversible protein modification. Regulated ubiquitin removal from a substrate is performed by deubiquitinating enzymes (DUBs). These proteases cleave ubiquitin or ubiquitin-like proteins from precursor proteins as well as from conjugates of target proteins (Reyes-Turcu et al., 2009). Within the UPS, DUBs have diverse functions including the processing of ubiquitin precursors for their activation, recycling of ubiquitin, generation of monoubiquitin from polyubiquitin and reversal of ubiquitination. Antagonising the action of E3 ligases, DUBs are potent regulators of ubiquitin mediated cellular processes.

Approximately one hundred human DUBs have been identified which are organised in five different gene families (Nijman et al., 2005). They are classified either as JAB1/MPN/Mov34 metalloproteases (JAMM) or as cysteine proteases. The cysteine proteases are further subdivided into ubiquitin-specific hydrolases (USPs, the largest family with more than 50 members in humans), ubiquitin C-terminal hydrolases (UCHs), otubain proteases (OTUs) and Machado-Joseph disease proteases (MJDs). Deubiquitination, as well as ubiquitination, is a highly regulated process which is involved in various functions of the cell such as gene expression, DNA repair, kinase activation and lysosomal as well as proteasomal protein degradation.

Ubiquitin specific processing protease 7 (Usp7)

The ubiquitin specific processing protease 7 (Usp7) was originally found as an intracellular deubiquitinating enzyme which associates with the Herpes virus protein ICP0 and was therefore referred to as Herpes virus associated ubiquitin specific protease (HAUSP) (Everett et al., 1997; Meredith et al., 1994). Later, Usp7 was also found to be implicated in the regulation of the DNA damage response by deubiquitination of the tumor-suppressor protein p53 (Li et al., 2002). Additionally, Usp7 interacts with the E3 ligase Mdm2 and its homologoue Mdmx (human orthologues: Hdm2 and Hdmx, respectively), which specifically ubiquitinate p53. The binding of Usp7 to Mdm2/Mdmx leads to the deubiquitination and stabilization of Mdm2/Mdmx (Cummins and Vogelstein, 2004; Li et al., 2004; Meulmeester et al., 2005). This process depends on the death domain-associated protein (Daxx) linking Usp7 and Mdm2/Mdmx (Tang et al., 2006). These mechanisms together ensure a tight regulation of the cellular p53 level.

Usp7 resides predominantly in the nucleoplasm. However, upon binding to viral ICP0, this DUB was reported to translocate to the cytoplasm (Daubeuf et al., 2009). Usp7 reveals an N-terminal meprin and TRAF homology (MATH) domain which is responsible for the direct interaction of Mdm2 and p53 in a mutually exclusive manner (Hu et al., 2006; Saridakis et al., 2005). The viral proteins ICP0 or EBNA1 bind to the C-terminus or the N-terminus, respectively. Moreover, Usp7 seems to harbour four UBL domains near its C-terminus (Zhu et al., 2007). A schematic structure of Usp7 is depicted in Figure 4.

Figure 4: Schematic structure of Usp7. Domains of Usp7 are depicted as boxes. MATH indicates the Meprin and Traf homology domain, ICP0 the Herpes virus E3 ligase, Poly Q a stretch of six glutamines. EBNA1=Epstein-Barr virus protein, mdm2=mouse double minute protein 2. The numbers of amino acid residues are given in parentheses. Aa = amino acid (Cheon and Baek, 2006).

1.2 Endoplasmic reticulum (ER) protein quality control

1.2.1 Protein synthesis at the ER

The endoplasmic reticulum (ER) is an intracellular membrane network, which consists of tubules, vesicles and cavities, and achieves various functions. These include the synthesis, folding and modification of proteins destined for cellular membranes or for secretion. Thus, the ER is the entry port for proteins to the secretory pathway. Ribosomes which synthesise proteins bearing an N-terminal hydrophobic signal sequence are recognised by the signal recognition particle (SRP) and translation comes to a transient halt. The ribosome-nascent protein-SRP complex is then targeted to the ER membrane, where it binds to the SRP receptor. Then, the ribosome is transferred to the Sec61 protein conducting channel, where protein synthesis proceeds. Nascent polypeptides are cotranslationally inserted into the ER membrane or if soluble, released into the ER lumen (Rapoport, 2007).
The ER lumen provides an environment that enables protein modifications which are virtually impossible in the cytoplasm. The oxidizing surrounding in the ER lumen allows, *e.g.,* disulfide bond formation within proteins. Additionally, the ER represents the primary calcium-storage organelle of the cell. The availability of intraluminal calcium is essential for protein folding and chaperone functionality. Further protein modifications, which are achieved in the ER lumen, are, *e.g.*, glycophosphatidylinositol (GPI)-anchor addition and N-linked glycosylation (Malhotra and Kaufman, 2007).

1.2.2 ER quality control, ER stress and the unfolded protein response (UPR)

During translocation and processing, nascent proteins bind to ER chaperones. Once correctly folded, assembled and modified, these proteins are released for transfer to their final destination. The complex system which monitors protein synthesis and their transport from the ER to the Golgi can be divided into a primary quality control (primary QC) and a secondary quality control (secondary QC). The primary QC applies to all secretory proteins and the secondary QC refers to various selective mechanisms regulating the export of distinct subsets of proteins.
Three groups of ER integral molecular chaperones are known to establish the primary QC. The most important one is that of the heat shock protein family including the glucose regulated protein 78 (Grp78, also referred to as BiP, an Hsp70), its cochaperones, *e.g.*, Hsp40 = ERdj 1-5 and the Hsp90 family member Grp94.

These chaperones bind to nascent proteins, assist their folding and are involved in stress signalling and degradation processes. Also lectins such as calnexin and calreticulin as well as thiol-disulfide oxidoreductases such as protein disulfide isomerase (PDI) belong to the primary QC system. Calnexin together with Calreticulin interacts with monoglycosylated N-linked glycans and determines the revision or degradation of the glycoprotein. PDIs catalyse the oxidation, reduction and isomerisation of disulfide bonds (Ellgaard and Helenius, 2003; Nishikawa et al., 2005).

Despite the existence of this complex ER quality control system, the process of protein synthesis, modification and targeting is error prone due to mutations, transcription-, translation- or folding deficiencies. Estimated 30% of newly synthesised proteins are defective (Schubert et al., 2000). Furthermore, mature proteins might also be damaged, *e.g.,* by environmental noxes such as high energy radiation or chemical insults. In addition, disorders of the ER such as perturbation of calcium homeostasis, disturbance of the ER luminal redox state, increased protein cargo or altered protein glycosylation occur. All of these situations may lead to the accumulation of misfolded proteins within the ER. Since the accumulation of irreparable proteins is a threat for cell viability, this ER stress induces multiple signalling pathways to avoid cell toxic protein aggregation. These pathways are designated as the unfolded protein response (UPR). The UPR is an adaptive mechanism leading to the induction of genes which enhance the ER folding capacity and the degradation of unfolded proteins while general protein synthesis is stopped. Beyond that, prolonged ER stress leads to the activation of programmed cell death (Schroder and Kaufman, 2005).

1.2.3 ER stress signalling

Three major ER-localised transmembrane UPR-signal transducers, independently initiating adaptive responses, are activated upon ER stress, the inositol-requiring protein 1 (Ire1), the PRKR-like endoplasmic reticulum kinase (Perk) and the cAMP-dependent transcription factor 6 (ATF6). With their ER luminal domains all three stress sensors bind the ER chaperone Grp78 (BiP). The binding of misfolded proteins to Grp78 leads to its dissociation from these UPR signalling proteins thereby activating them (Figure 5; (Kohno, 2007).

Ire1 is evolutionary conserved from yeast to human with paralogues in mammalia, Ire1-α and Ire1-β, displaying different tissue distributions. Their cytosolic domain comprises a serine-threonine kinase and an endoribonuclease activity. Sensing accumulated proteins, presumably also independent of Grp78, Ire1 dimer- or oligomerises, becomes autophosphorylated and activates its own ribonuclease activity. Ire1 activation results in spliceosome-independent splicing of the precursor of X-box-binding protein 1 (Xbp1)–mRNA (Sidrauski et al., 1996).

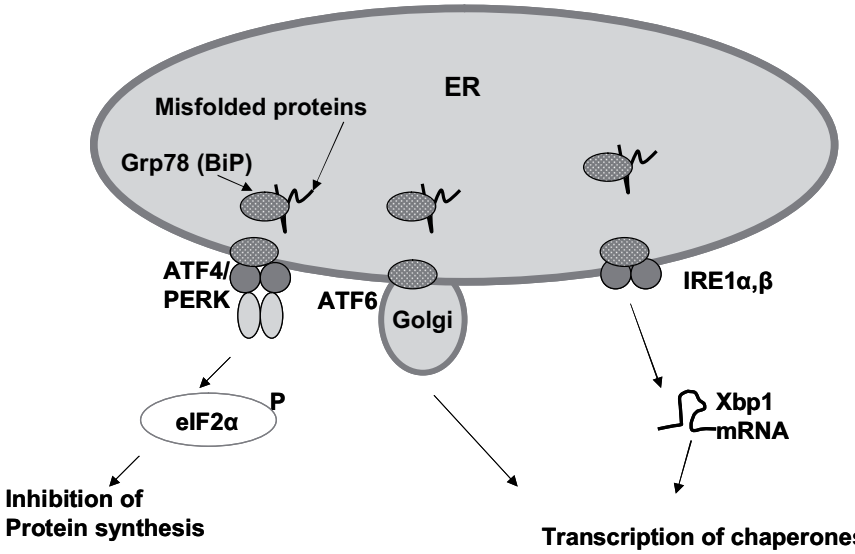

Figure 5: Schematic overview of the unfolded protein response (UPR) signalling pathways. Misfolded proteins within the ER lumen bind to the ER chaperone Grp78 (BiP) which leads to the induction of three independent signalling pathways. Activated Perk phosphorylates eIF2α, which results in cell cycle arrest and stop of translation. ATF4 mRNA is translated in this situation and induces UPR genes. Activated ATF6 translocates to the Golgi, where it is cleaved and then acts as a transcription factor, which results in the induction of UPR target genes. Activated Ire1 splices the Xbp1-mRNA which encodes a transcription factor that induces UPR target genes (Hayden et al., 2005).

Xbp1 pre-mRNA has a DNA-binding domain (DBD) and a transcription activation domain (AD) in different open reading frames and thus is inactive. The unconventional splicing removes the intron and xbp1 is converted to mature mRNA, from which the active transcription factor is translated. Targets of Xbp1 include genes encoding ER chaperones and proteins involved in ER degradation, transcription factors (Xbp1 itself) and components of the secretory pathway such as Sec61 (Yoshida, 2007).

Perk, a type 1 transmembrane protein, binds Grp78 in its inactive state and has a cytosolic domain with kinase activity. Upon ER stress, Perk autophosphorylation and oligomerisation occurs analogous to Ire1. Active Perk phosphorylates eIF2-α, resulting in the attenuation of protein synthesis. However, the translation of some mRNAs is induced in this situation such as that of the transcription factor ATF4, which in turn induces UPR target genes (Harding et al., 1999).

ATF6 is a basic leucine zipper transcription factor that binds to ER stress response elements (ERSE), thereby inducing ER chaperone genes such as human Grp78 and Grp94. During ATF6 activation, two luminal Golgi localisation sites are responsible for the transfer of ATF6 to the Golgi. There, a proteolytic intramembrane cleavage of ATF6 leads to the release of its cytosolic domain which represents the active transcription factor, harbouring DNA binding and transcription activation sites (Yoshida et al., 1998).

1.2.4 ER associated protein degradation (ERAD)

Proteins which are damaged or cannot reach their native conformation or fail to get post-translationally modified are selected by the ER quality control system, which sorts them for ER associated protein degradation (ERAD). Unassembled subunits of multimeric protein complexes are selected for this pathway as well (Vembar and Brodsky, 2008). Initially, proteolysis was thought to occur in the ER lumen. However, orphan subunits of the heptameric T-cell receptor complex (TCR), which were not assembled in heterooligomeric complexes before ER export, were found to be degraded in a pre-Golgi compartment but not in the lysosomes (Lippincott-Schwartz et al., 1988). Later, it was found that defective proteins such as mutated Sec61, mutated cystic fibrosis transmembrane conductance regulator (CFTR) and mutated yeast Carboxypeptidase Y (CYP*) are transported from the ER back to the cytoplasm to be degraded by the 26S proteasome (Hiller et al., 1996; Sommer and Jentsch, 1993; Ward et al., 1995). Thus, with exceptions, the majority of misfolded proteins is translocated back to the cytosol for degradation (Schmitz and Herzog, 2004).

Substrates traverse this ERAD pathway in four consecutive steps (Figure 6):
(1) Recognition, selection and targeting to the retrotranslocation machinery
(2) Retrotranslocation and ubiquitination
(3) Extraction from the ER membrane
(4) Transfer to the 26S proteasome and degradation

Recognition and selection of ERAD substrates

Molecular chaperones, which recognise ERAD substrates, can differentiate between misfolded and nascent proteins. Although a common biophysical property of ERAD substrates has not been identified, some features were found to make proteins prone to degradation.

An example for that are hydrophobic patches which are normally buried within the protein. If a protein is unfolded, these patches might be exposed to the ER lumen. There, Hsp70 chaperones such as Grp78 bind to these potential substrates to maintain their solubility, which is a prerequisite for later retrotranslocation (Nishikawa et al., 2001). Accordingly, membrane proteins, harbouring defects in their cytoplasmatic domains, are recognised by cytoplasmatic Hsp70 or Hsp90 chaperones, as shown for CFTR which binds to Hdj-2/Hsc70 (Meacham et al., 1999). As the Grp78 orthologue in yeast, Kar2p, is associated with ubiquitination mediating complexes, this chaperone was suggested to have a role in the recruitment of ERAD substrates to these complexes (Denic et al., 2006).

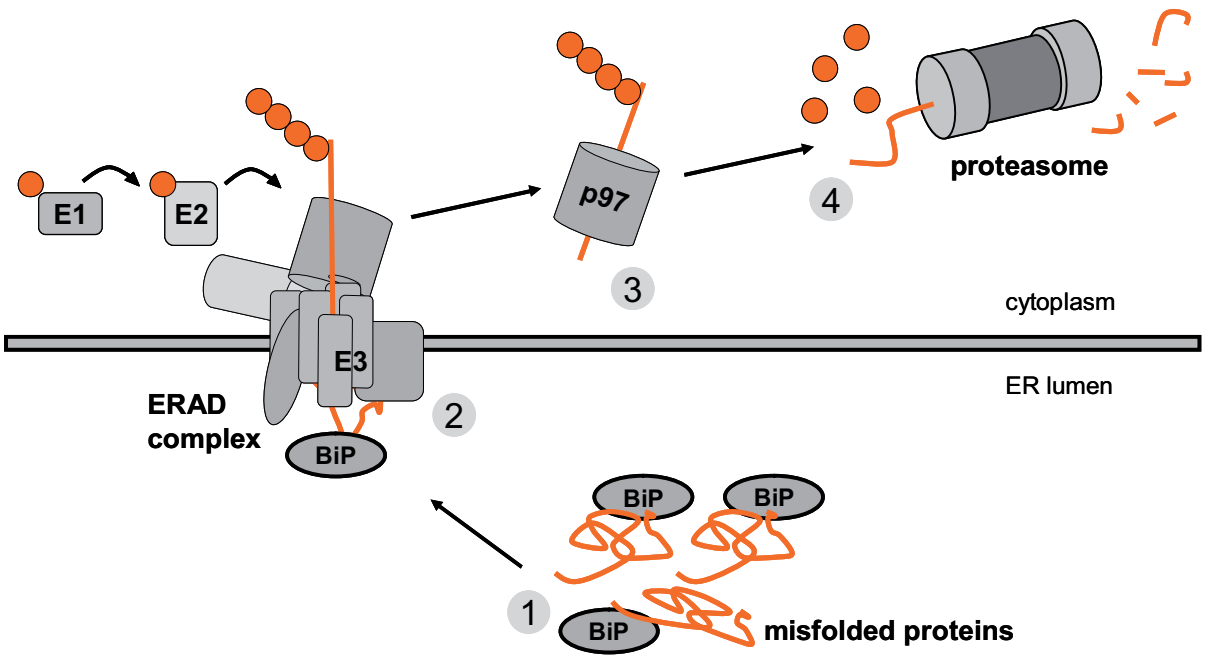

Figure 6: Illustration of the single steps of the ER associated protein degradation (ERAD). (**1**) Misfolded proteins are recognised with the help of Grp78 (=BiP) and transferred to the ER membrane. (**2**) E3 ligase containing multiprotein complexes mediate ubiquitination and retrotranslocation of these substrates. (**3**) The p97 complex extracts polyubiquitinated substrates from the ER membrane. (**4**) Finally, the substrates are transferred to the 26S proteasome, possibly with the help of a shuttle protein, where they are finally degraded.

During synthesis, nearly all secretory proteins are modified by a preformed N-linked oligosaccharide structure. Trimming of this sugar moiety by glucosidases and folding facilitated by the lectin-like chaperones calnexin and calreticulin results in a glycoprotein which is ready for secretion. Defects in glycoproteins lead to their re-glycosylation by UDP-glucose:glycoprotein-glucosyltransferase (UGGT) so that they can re-enter the calnexin cycle for their correction (Caramelo and Parodi, 2008). However, irreparably misfolded glycoproteins cannot cycle endlessly between calnexin and the UGGT. Therefore, an ER mannosidase acts as a kind of timer and cleaves the mannose residues off the N-glycans. This cleavage leads to a reduced calnexin/calreticulin re-binding and an enhanced binding to ER degradation enhancing alpha-mannosidase-like protein (EDEM) which results in the degradation of these substrates (Hosokawa et al., 2003; Oda et al., 2003).

Another type of chaperones, protein disulfide isomerases (PDIs), involved in redox state dependent protein maturation, have been shown to interact with Grp78 and to be important for ERAD substrate recognition (Molinari and Helenius, 2000). Recently, the yeast E3 ligase Hrd1p has been shown to directly recognise misfolded membranous proteins, dependent on its own transmembrane regions (Sato et al., 2009).

Retrotranslocation and ubiquitination

Since degradation of ERAD substrates takes place in the cytoplasm, ER derived proteins have to pass the ER membrane in the opposite direction as protein synthesis occurs. This process is termed retrotranslocation. In addition, the process of ubiquitination is also located at the cytoplasmatic site of the ER. This means that for their degradation, ERAD substrates at least have to gain access to this compartment. Starting retrotranslocation is a prerequisite for ubiquitination. All ER lumen and most ER membrane derived proteins are thought to be retrotranslocated presumably by a protein-conducting channel.

A few proteins have been suggested to form this retrotranslocation pore. The mammalian ER transmembrane protein Derlin-1 was found in a complex with cytoplasmatic and membrane-resident ERAD mediating proteins as well as with ERAD substrates (Katiyar et al., 2005; Lilley and Ploegh, 2004; Schulze et al., 2005). A depletion of Derlin1 results in the induction of the UPR and in retarded degradation of selected substrates (Ye et al., 2005). Also E3 ligases are assumed to mediate retrotranslocation and ubiquitination. In yeast, the RING E3 ligases Hrd1p and Doa10 are integral components of distinct multiprotein complexes consisting of ER luminal, ER membrane and cytoplasmatic proteins. While Hrd1p mediates the degradation of substrates that harbour misfolded domains within the ER lumen (ERAD-L), Doa10 is required for proteins with a folding error in their cytosolic domain (ERAD-C) (Carvalho et al., 2006; Vashist and Ng, 2004).

In mammalia, the membrane-multispanning E3 ligase Gp78, an orthologue of yeast Hrd1p, was suggested to mediate retrotranslocation (Zhong et al., 2004). A different hypothesis implies that retrotranslocation of protein complexes or of large proteins requires the formation of so called lipid droplets from the ER membrane (Ploegh, 2007).

ERAD substrates have to be ubiquitinated prior to extraction and proteasomal targeting and ubiquitination requires the sequential action of E1, E2 and E3 enzymes (see 1.1.3). The mammalian ER-resident E3 ligases Gp78 and Hrd1 (also called Synoviolin), for instance, together with the E2 enzyme Ube2g2 mediate the degradation of CD3-δ and TCR-α, respectively (Fang et al., 2001; Kikkert et al., 2004).

Cytosolic ubiquitin ligases may also be implicated in the ERAD process, as this was reported for the RING E3 parkin which ubiquitinates the parkin-associated endothelin receptor-like receptor (Pael-R) (Imai et al., 2001). Since Synoviolin was described to also ubiquitinate Pael-R, the efficient turnover of substrates may depend on more than one E3 ligase (Omura et al., 2006). This was also shown for the ERAD substrate CFTR which is processed by the cooperation of two E3 ligases, Gp78 and RMA1 (Morito et al., 2008).

Membrane extraction of ERAD substrates

Polyubiquitination of ERAD substrates is not only a signal for proteasomal degradation but also for the extraction of substrates from the ER membrane (Kikkert et al., 2001). To start this process, a minimal length of the polyubiquitin chain bound to the substrate is decisive (Jarosch et al., 2002). The polyubiquitin moiety is recognised by a cytosolic molecular chaperone complex, consisting of the AAA-ATPase p97 (also called VCP for Valosin containing protein or Cdc48 in yeast), which forms a homohexamer, and the associated proteins Ufd1 and Npl4. In yeast, the Cdc48-Ufd1-Npl4 complex was shown to bind ubiquitin conjugates and to be required for the ATP dependent extraction of various substrates such as MHC class I molecules (Dai and Li, 2001; Rape et al., 2001; Ye et al., 2001).

P97 was suggested to act as a motor, actively pulling the substrate out of the ER membrane in an ATP-consuming process and dependent on the binding of p97 to the ER membrane (Ye et al., 2003). The recruitment of the p97 complex to the ER membrane was proposed to be mediated by ER-resident proteins. Among these proteins are the VCP interacting membrane protein (VIMP) and ubiquitin regulator-X (UBX) domain proteins such as Ubxd2 (Liang et al., 2006; Ye et al., 2004). Also Ubxd8 was suggested as a p97 recruitment factor in mammalia (Mueller et al., 2008).

Transfer of ERAD substrates to the 26S proteasome

Since it has been found to interact with 26S subunits, p97 was initially assumed to escort the extracted ERAD substrate to the 26S proteasome for degradation (Dai et al., 1998). Later, a variety of proteins exhibiting a UBA as well as a UBL domain were described to execute this shuttle function. Harbouring both domains allows these proteins to interact with ubiquitinated substrates and the 26 proteasome concomitantly. The most prominent UBL and UBA domain containing proteins are Rad23 and Dsk2 required for ERAD in yeast (Medicherla et al., 2004). In humans, two homologues of Rad23, hHR23A and hHR23B interact with the proteasomal 19S subunit Rpn10 (S5a) via their UBL domain (Hiyama et al., 1999).

1.2.5 Synoviolin based ERAD complexes

Synoviolin based ERAD complexes consist of a variety of proteins which are introduced below and schematically depicted in Figure 7, B. Mammalian Synoviolin, also referred to as Hrd1, is an ER-resident E3 ligase of the RING type (see 1.1.4) and involved in the ubiquitination and retrotranslocation of ERAD substrates. This enzyme of 617 amino acids is one of two mammalian orthologues of yeast Hrd1p which was first described in connection with the turnover of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and therefore named Hrd1 for HMGR degradation (Hampton et al., 1996).

The other Hrd1p orthologue is Gp78, also known as autocrine motility factor receptor (AMFR) (Fang et al., 2001; Nadav et al., 2003). Gp78 harbours a CUE domain and a specific Ube2g2 binding site, which are both essential for its function in protein degradation (Chen et al., 2006). Furthermore, Gp78 interacts with various other proteins involved in ERAD such as the AAA ATPase p97. This interaction is essential for the degradation of the ERAD substrates CD3-δ and the Z variant of α1-antitrypsin (Ballar et al., 2006; Zhong et al., 2004). Synoviolin is ER stress induced, dependent on Ire1 and ATF6 activity. It reveals six transmembrane domains and a ubiquitin ligase activity harbouring RING domain near the C-terminus (Kaneko and Nomura, 2003; Nadav et al., 2003). Importantly, Synoviolin is highly expressed in synovial cells of patients with rheumatoid arthritis and was shown there to protect cells from ER stress induced apoptosis (Amano et al., 2003). Apoptosis inhibition leads to synovial hyperplasia which is causative for the progression of the disease. In mammalia, Synoviolin mediates the basal degradation of HMGR, whereas the turnover of the sterol-regulated isoform of HMGR is mediated by Gp78 (Kikkert et al., 2004; Song et al., 2005). In addition, degradation of a variety of substrates such as CD3-δ, TCR-α, misfolded insulin and Ig-μ chains is mediated by Synoviolin (Allen et al., 2004; Cattaneo et al., 2008; Kikkert et al., 2004). The direct interaction of Synoviolin with the ERAD components Homocysteine inducible endoplasmic reticulum-resident protein (Herp), Derlin-1 and p97 was described. Furthermore, *in vitro* interaction studies gave rise to the assumption that Synoviolin is able to homooligomerise (Schulze et al., 2005). Additionally, Sel1L and Derlin-2 as well as the lectins OS-9 and XTP3-B were found to be associated with this E3 ligase (Christianson et al., 2008; Lilley and Ploegh, 2005). The association of these proteins results in the formation of multiprotein complexes located at the ER membrane. Synoviolin is thought to be the major component of these multimeric structures. However, the mode of Synoviolin complex assembly or the exact composition of the functional ERAD complexes is not known. Since the study presented here deals with this Synoviolin based ERAD complexes, major components of these complexes are introduced below.

In humans, three members of the Derlin family, ER-resident proteins of about 240 amino acids in size, UPR inducible and harbouring four transmembrane domains, have been shown to be associated with Synoviolin. Derlin-1 was found to be essential for the human cytomegaloviral US11 protein triggered MHC class I dislocation and to be a central component of complexes containing VIMP, p97 and Synoviolin (Lilley and Ploegh, 2004; Schulze et al., 2005; Ye et al., 2004). Furthermore, Derlin-1 forms homooligomeric structures (Ye et al., 2005). In addition to the degradation of ERAD substrates, Derlin-1 has also been reported to be implicated in the processing of pathogenic proteins such as cholera toxin (Bernardi et al., 2008). Derlin-2 and Derlin-3 were also reported to be components of ERAD complexes. Derlin-2 and Derlin-3 heterooligomerise with the other Derlins.

Moreover, Derlin-2 was found to homooligomerise (Lilley and Ploegh, 2005). Derlin-2 and Derlin-3 provide a link between EDEM and p97 and are involved in the degradation of misfolded glycoproteins (Oda et al., 2006).

The Suppressor of lin-12-like protein1, Sel-1L, mammalian homologue of Hrd3p, is another ERAD complex component. Sel1L is an ER luminal glycoprotein of 794 amino acids with a potential transmembrane domain at the C-terminus. Sel1L is induced by ER stress and was reported to be associated with Synoviolin, Derlin-1, Derlin-2 and the AAA-ATPase p97 as well as with Ubxd8, Ube2j1 and OS9 (Kaneko and Nomura, 2003; Lilley and Ploegh, 2005; Mueller et al., 2008). Since Sel1L is involved in glycoprotein degradation, it was suggested to play a role in the selection of misfolded proteins or to stabilise Synoviolin, comparable to its yeast orthologue Hrd3p (Mueller et al., 2006). Recently, two additional soluble Sel1L isoforms were identified which are secreted and involved in sorting and export of Ig-μ chains (Cattaneo et al., 2009).

Ancient ubiquitous protein 1, AUP1, is an ER-resident protein of 476 amino acids. Harbouring an N-terminal membrane anchor, the majority of the protein is cytoplasmatic. Near its C-terminus, AUP1 possesses a CUE domain, which is thought to be involved in ubiquitin binding or the recruitment of E2 enzymes and shows similarities to the UBA domain (Hurley et al., 2006). Initially, AUP1 was found to be associated with platelet integrins and thought to be involved in integrin signalling. However, this CUE domain protein is associated with Sel1L and Sel1L interacting ERAD proteins, such as Ube2j1 and OS9. The elevated expression of a GFP-tagged AUP1 inhibited viral US11 protein mediated dislocation of ER resident MHC class I molecules, which implicates a function of AUP1 in ERAD (Mueller et al., 2008).

Various other proteins such as lectins, selenoproteins, UBX domain proteins, E2 enzymes and ubiquilins are associated with Synoviolin. The ER-luminal lectins OS9 and XTP3-B represent the human orthologues of yeast Yos9p which recognises N-glycans in yeast and promotes their degradation. Two isoforms of OS9, OS9.1 and OS9.2 and XTP3-B interact with Sel1L and bind the ERAD substrate NHK (Christianson et al., 2008). The selenoprotein VIMP binds p97 and Derlin-1 concomitantly (Ye et al., 2004). UBX domain proteins such as Ubxd8 also interact with p97 and Sel1L (Mueller et al., 2008). VIMP and Ubxd8 seem to recruit p97 to the site of dislocation. In addition, p97 (see 1.2.4), also directly interacts with Synoviolin (Schulze et al., 2005). The E2 enzyme Ube2j1 (Ubc6e) was shown to be associated with Sel1L and thus suggested to work in concert with Synoviolin (Mueller et al., 2008). Ubiquilin 1 and 2, human orthologues of yeast Dsk2, were recently found to interact with Herp, a component of Synoviolin based complexes (Kim et al., 2008). Ubiquilins (also referred to as hPlic) belong to the family of ubiquitin domain proteins (UDPs) and bind the 26S proteasome and polyubiquitin moieties concomitantly (Kleijnen et al., 2000).

1.2.6 Homocysteine inducible endoplasmic reticulum - resident protein (Herp)

The Homocysteine inducible endoplasmic reticulum resident protein Herp (Swiss-Prot entry: Q15011) is strongly induced by UPR inducing agents such as homocysteine and β-mercaptoethanol as well as the N-glycosylation inhibitor tunicamycin or the calcium-ATPase inhibitor thapsigargin (Hori et al., 2004; Kokame et al., 2000; Kokame et al., 1996). Apart from ER stress, the Herp encoding gene, herpud1, is also induced in response to osmotic stress and by DNA damaging agents such as methyl methanesulfonate (MMS). Therefore, Herp was also termed MMS inducible factor1 (Mif1). The herpud1 promoter contains the 'ER stress response elements' (ERSE I and II) which are binding sites for the UPR dependent transcription factors ATF6 and Xbp1 (Kokame et al., 2000; van Laar et al., 2000). Structural analysis revealed that Herp harbours a UBL domain at the very N-terminus thus belonging to the family of the UDPs. Herp is an integral ER-membrane protein of 391 amino acids in size with both termini facing the cytoplasm (Kokame et al., 2000). Biochemical studies showed that Herp is associated with Synoviolin based ERAD complexes through direct binding of the E3 ligase Synoviolin (Schulze et al., 2005).

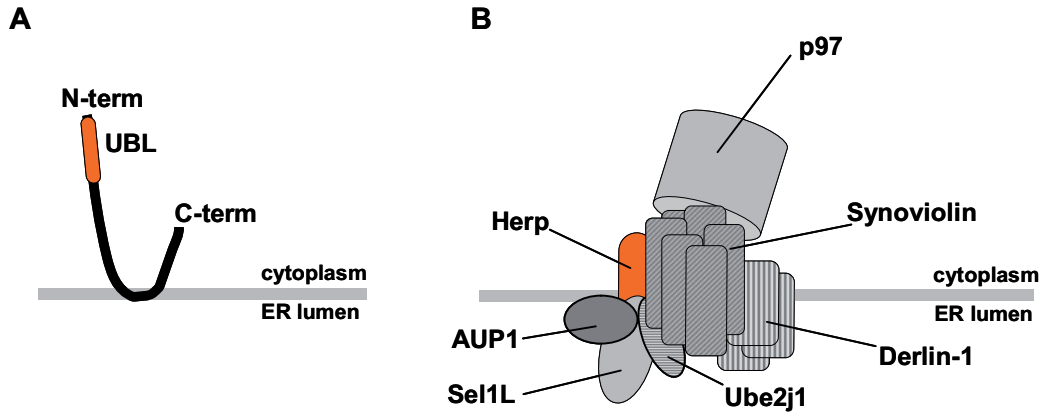

Figure 7: Illustration of the homocysteine inducible ER stress protein (Herp). (A) Membrane topology of Herp. The UDP faces the cytoplasm with both termini**. (B)** Components of Synoviolin based ERAD complexes. Herp directly interacts with the potentially oligomeric E3 ligase Synoviolin and thus is part of Synoviolin based complexes. Thereby, Herp is also associated with other Synoviolin interacting proteins such as p97 or Derlin-1 (Schulze et al., 2005). Recently, the E2 enzyme Ube2j1 and the AUP1-protein have been found in association with Sel1-L which is a direct interaction partner of Synoviolin (Mueller et al., 2008).

Herp is essential for the effective degradation of ERAD substrates such as Connexin 43, CD3-δ and a nonsecreted Igκ light chain (Hori et al., 2004; Okuda-Shimizu and Hendershot, 2007; Schulze et al., 2005). These findings support the functional role of Herp in ERAD. Interestingly, the UBL domain of Herp seems to mediate the effect of Herp in the ERAD process. Herp-dependent substrates were stabilised, if Herp lacking the UBL domain was overexpressed (Schulze, 2006). UBL domains within UDPs display diverse binding specificities which are important for their molecular function (Madsen et al., 2007). Since proteasome binding had been demonstrated for the UBL domains of a subset of UDPs such as BAG-1 (Luders et al., 2000), Herp was proposed to recruit the 26S proteasome to the ER upon ER stress (van Laar et al., 2001). However, the UBL domain of Herp does not interact with the 26S proteasome and is also dispensable for the interaction of Herp with Synoviolin (Schulze et al., 2005). Herp and the 26S proteasome might nevertheless be found associated, since the UDP is a substrate of the UPS (Sai et al., 2003). A yeast two hybrid screen for target proteins of the Herp N-terminus (including the UBL domain) identified Usp7 as binding partner (Schulze, 2006). The functional importance of this deubiquitinating enzyme for ERAD has not been tested. Moreover, the UBL domain of Herp seems to determine its half-life of about three hours (Kokame et al., 2000; Sai et al., 2003). Beyond, the lysine 61 residue within the Herp UBL domain seems to be the crucial ubiquitination site, as shown by *in vitro* ubiquitination experiments (Li et al., 2007).

Although Herp was shown to be ubiquitinated and degraded by the proteasome, E2 or E3 enzymes involved in this process have not been identified (Sai et al., 2003). Only the soluble E3 ligase ‘plenty of SH3 domains’ (POSH) was reported to ubiquitinate Herp, but with K63 linked polyubiquitin which does not lead to degradation but to the redistribution of Herp from the Trans Golgi network to the ER. Upon calcium perturbation, Herp, dependent on its UBL domain, induces the oligomerisation and activation of POSH (Tuvia et al., 2007).

A few data on the cellular function of Herp have been reported. The induction of Herp by ER stress is connected with the improvement of the folding capacity of the ER. With this, Herp is involved in the protection of cells against ER stress (Hori et al., 2004). In neurons, the elevated expression of Herp promotes cell survival by stabilisation of calcium homeostasis and maintaining mitochondrial function. However, prolonged ER stress leads to the cleavage of Herp by caspases and to apoptosis (Chan et al., 2004). Concerning ERAD, Herp was shown to associate with Ubiquilin1 and 2 and suggested to thereby enhance the degradation of CD3-δ (Kim et al., 2008). However, a general molecular function of Herp has not been shown.

In yeast, the UDP U1 SNP1-associating protein 1 (Usa1p) associates with Hrd1p, Hrd3p and Der1p and is required for the efficient ERAD of the model substrate CPY*. Usa1p and Herp reveal similar domain architectures. Thus, both UDPs seem to be structurally related. Usa1p links Der1p to the Hrd1p/Hrd3p complex and was suggested to be the functional equivalent of Herp, since the mammalian UDP was able to partly rescue the Usa1p deletion in yeast (Carvalho et al., 2006). Most recently, it was shown that Usa1p, dependent on its N-terminus which harbours a UBL domain, functions as a scaffold protein enabling the oligomerisation of Hrd1p. This process was postulated to be a prerequisite for the degradation of membrane derived ERAD substrates (Horn et al., 2009). However, a general role of the Usa1p UBL domain in yeast ERAD is not given, since this domain was shown to be essential only for the degradation of 6myc-Hmg2 but not of Hmg2-GFP or CPY* (Carroll and Hampton, 2010; Horn et al., 2009; Kim et al., 2009). Comparable to Herp, the UBL domain of Usa1p is not associated with the 26S proteasome (Kim et al., 2009).

A database search using the SMART program was performed to find human proteins harbouring a UBL and transmembrane domains comparable to Herp. With this approach four candidates were found and designated as transmembrane-associated protein containing a ubiquitin-like domain (mubl). One member of this family is Herp2 (mubl2, Swiss-Prot entry: Q9BSE4) which is most similar to Herp (Schulze, 2006). The amino acid sequence of Herp2 (406 amino acids) is 40% identical with the Herp sequence. The sequences of the UBL domains of both UDPs are even 50% identical. Both proteins display similar domain architectures with a UBL domain at the N-terminus and a transmembrane domain close to the C-terminus. Herp2 additionally contains a serine-rich region downstream its UBL domain. A Herp2 splice variant lacking amino acids 49-70 was also identified. So far, there are no further data reported on Herp2 in the literature.

1.3 Aim of this study

The UPR-induced ER-resident protein Herp, which is associated with Synoviolin based complexes, plays an important role in the degradation of the Synoviolin substrate CD3-δ. Depletion of Herp or disturbance of the Herp UBL domain leads to the stabilisation of this substrate. However, the mechanisms underlying the function of Herp are poorly understood.

Thus, the aim of this study was to characterise the molecular function of Herp within Synoviolin mediated ERAD.

Compared to Synoviolin, ER stress caused induction and degradation of Herp occurs more rapidly. Therefore, it was investigated whether these dynamics of Herp are important for its function in ERAD. In this context, the turnover of Herp at Synoviolin based complexes was analysed and with regard to this aspect it was tested whether Herp is a substrate of Synoviolin or its associated proteins. Moreover, it was analysed whether the degradation of Herp is required for the process of ERAD.
The UBL domain of Herp, crucial for the function of this UDP, specifically binds the deubiquitinating enzyme Usp7. Therefore, it was analysed whether Usp7 is a component of Synoviolin based complexes playing a role in determining the stability of Herp or interfering with the ERAD process.
In yeast, the ubiquitin domain protein Usa1p, required for the efficient ERAD process, was shown to maintain the integrity of ERAD complexes. Therefore, it was tested whether Herp had an analogous function in mammalia; Herp was tested for influence on the oligomerisation of Synoviolin or the interaction of accessory proteins with Synoviolin based complexes.
Herp2, a homologue of Herp, was hypothesised to be capable of taking over the cellular functions of Herp. Therefore, it was tested whether Herp2 revealed the same properties as Herp such as UPR inducibility, binding of Usp7, instability and association with Synoviolin.

2 Material and Methods

2.1 Instruments, consumables and chemicals

Table 1: Instruments used in this study

Instrument	Supplier
Phosphoimager FLA3000 Fujifilm	Raytest
Freezing container Cryo 1°C	Nalgene
G-Box gel documentation	Syngene
mini-Protean 3 electrophoresis system	Biorad
Scintillation counter Wallace 1410	Pharmacia
semidry blotting apparatus	Peqlab
SW 40 swing out rotor	Beckman Coulter
Table top centrifuge	Eppendorf
Thermocycler Uno Thermoblock	Biometra
Ultracentrifuge OptimaTM-L	Beckman Coulter
UV VIS photometer Ultrospec 2100 pro	Amersham Biosciences
G:BOX gel documentation system	Syngene

Table 2: Consumables used in this study

Consumable	Supplier
BioMAX film	Kodak
Cell culture flasks, dishes and multiwell plates	Greiner
Cryoconservation vials	Corning
Nitrocellulose membranes	Whatman
Whatman paper	Schleicher und Schuell
XOmat-UV film	Kodak

General chemicals were purchased from VWR International, Roth, Sigma or Applichem, if not otherwise noted. They all were of at least 99% purity. All solutions in this study are aqueous, unless otherwise stated.

Table 3: Chemicals used in this study

Chemical	Supplier
1 Kb Marker	New England Biolabs
1 KB Plus DNA Ladder	Invitrogen
^{35}S-S-L-methionine / ^{35}S-L-cysteine (Tran^{35}S-Label™, metabolic labelling reagent)	MP Biomedicals
Ampicillin	Roth
Blasticidin S	Sigma
Complete™ protease inhibitor	Roche
Cycloheximide	Sigma
Dharmafect	Dharmacon
DMSO	Sigma
dNTP mix	New England Biolabs
Doxicycline	Clontech
Fetal calf serum (and dialysed fetal calf serum)	Biochrom
Glutathione sepharose	Amersham / GE
Iscove's or DMEM medium	Biochrom
L-glutamin	PAA
Lipofectamin 2000	Invitrogen
MG132	Calbiochem
OptiMEM	Biochrom
Penicillin	PAA
Polynucleotide kinase	Roche
Protein A – HRP	Biorad
Protein A- and Protein G-sepharose	Amersham / GE
Puromycin	Sigma
RPMI 1640 medium (also methionine free)	Biochrom
shrimp alkaline phosphatase	Roche
Streptavidin agarose	Novagen
Streptomycin	PAA
T4 DNA ligase and accordant buffer	NEB
Thapsigargin	Sigma
Trypsin/EDTA (1x)	PAA
Tunicamycin	Sigma
Zeocin™	Invitrogen

2.2 Molecular biology

2.2.1 Cultivation and storage of *Escherichia coli* (*E.coli*)

- Luria-Bertani (LB) medium: 1% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl

E.coli cells were cultured in LB medium. Liquid cultures were incubated on a rotating shaker at 37°C, for protein expression likewise at room temperature (RT). For growing bacteria on plates 1.5% agar (w/v) was added to LB medium and the incubation was performed at 37°C over night. To select transformed cells, 100 µg/mL ampicillin or 50 µg/mL kanamycin was added to liquid medium or plates, referred to as LB-amp or LB-kan, respectively. The optical density of liquid cultures was determined by measuring the absorbance at a wavelength of 600 nm (OD_{600}). For long-term storages, cultures in stationary phase were frozen at -80°C in LB medium containing 15% (v/v) glycerol.

2.2.2 Isolation of plasmid DNA from *E.coli*

Preparation of DNA using alkaline lysis

- P1: 50 mM Tris-HCl, pH adjusted to 8.0 with HCl ,10 mM EDTA, 10 µg/mL RNaseA
- P2: 200 mM NaOH, 1% (w/v) SDS
- P3: 3 M potassium acetate, pH 5.5

To test potential positive clones from a transformation (2.2.6), a single bacteria colony from the agar plate was taken to inoculate five mL of LB medium containing the accordant antibiotic, which were then shaken over night at 37°C. From the resulting culture two mL were centrifuged in a reaction tube at 10,000 g for three min to sediment the cells. The sediment was resuspended in 300 µL of buffer P1. Then 300 µL of buffer P2 were added and the suspension was carefully inverted several times. Then 300 µL of buffer P3 were added and the suspension was vigorously mixed and then centrifuged for 10 min at 13,000 g. The supernatant was transferred into a new tube and the DNA was precipitated by addition of 500 µL isopropanol. To pellet the DNA, a centrifugation step for 20 min at 13,000 g was performed and sedimented DNA was subsequently washed with 70% ethanol, dried at RT and resuspended in 50 µL of sterile water.

Preparation of DNA using ion exchange chromatography ("Mini-, Maxi-Prep")

Plasmid DNA for transformations or sequencing reactions was isolated from three mL of a bacteria culture with the Plasmid Mini-Prep Kit (Qiagen). For the preparation of plasmid DNA from a 200 mL bacteria culture, needed for the transfection of mammalian cells, the Plasmid Maxi-Prep Kit (Qiagen) was used. The isolation was done according to the supplier's instructions.

2.2.3 Separation of DNA fragments by agarose gel electrophoresis

- TAE buffer: 40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA
- DNA loading buffer: 40% (w/v) sucrose, 0.5% (w/v) SDS and 0.25% (w/v) bromophenol blue (both: sodium salt)

DNA fragments, generated by restriction endonucleases or cDNA fragments were electrophoretically separated on 1 - 2% agarose gels containing 0.1 µg/mL ethidium bromide. For production of gels, the agarose was dissolved in TAE buffer by heating and ethidium bromide was added after cooling to 60°C. TAE buffer was also used as running buffer. One µL DNA loading buffer was added to nine µL of each DNA solution and the samples were electrophoretically separated on the gels at ten V/cm. DNA fragments were visualised via the intercalated ethidium bromide using a UV transluminator. The size of the fragments was estimated by standard size markers such as the 1 KB Plus DNA ladder (Invitrogen), run on the identical gels.

2.2.4 Determination of DNA and RNA concentration in solution

DNA and RNA concentrations were determined by measuring the absorbance of a nucleic acid solution at a wavelength of 260 nm (A_{260}). An A_{260} of one equals a concentration of 50 µg/mL double stranded DNA, 40 µg/mL single stranded DNA and RNA or 33 µg/mL desoxyribonucleotides.

2.2.5 Preparation of competent *E.coli* cells

- φa-medium: 0.5% (w/v) yeast extract, 2% (w/v) bacto tryptone, 40 mM $MgSO_4$, pH adjusted to 7.6 with 1M KOH, sterile filtered
- TFB-I buffer: 30 mM potassium acetate, 50 mM $MnCl_2$, 100 mM RbCl, 10 mM $CaCl_2$, 15% (v/v) glycerol, pH adjusted to 5.8 with acetic acid, sterile filtered
- TFB-II solution: 10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% (v/v) glycerol, pH adjusted to 7.0 with 1 M KOH, sterile filtered

A single *E.coli* colony from a LB agar plate was used to inoculate five mL of φa-medium which then were shaken over night at 37°C. Two mL of this culture were diluted in 100 mL fresh medium and grown to an OD_{600} of 0.5 at 37°C. After chilling the culture on ice for five min the cells were sedimented by centrifugation (10 min, 5,000 g, 4°C). All following steps were performed on ice with pre-chilled solutions. The sediment was resuspended in 40 mL TFB-I buffer and incubated on ice for five minutes. Then cells were sedimented again by centrifugation (10 min, 5,000 g, 4°C), resuspended in four mL TFB-II solution and further incubated on ice for 30 minutes. Then the cells were transferred into reaction tubes in 100 µL aliquots and quickly frozen in liquid nitrogen and stored at -80°C.

2.2.6 Transformation of *E.coli* with plasmid DNA

- SOC medium: 2% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 2.0 mM glucose

For transformation of *E.coli* with plasmid DNA 100 µL of competent cells (XL1 blue or One Shot®TOP10 cells (Invitrogen)) were thawed on ice. 50 ng of plasmid DNA were added to the cells and incubation was continued for 30 min. A brief heat shock (42°C, 45 s) was followed by incubation on ice for ten min. 500 µL of SOC medium were added and cells were kept at 37°C for one h while shaking. This suspension was streaked out onto agar plates containing the accordant antibiotics and incubated over night at 37°C.

2.2.7 Isolation of total RNA from HeLa cells

For the analysis of the transcription of RNA, coding for proteins of interest, total RNA from HeLa cells was isolated and used for reverse transcriptase PCR analysis. For that purpose HeLa cells were left untreated or treated with 10 µg/mL tunicamycin or 2 µM thapsigargin for up to ten h. Then 10^6 cells were subjected to RNA isolation using the High Pure RNA isolation kit (Roche) according to the manufacturer's instructions.

2.2.8 Amplification of DNA by polymerase chain reaction (PCR)

Amplification of DNA was done using a PCR protocol (Table 4) adjusted either for the generation of plasmid constructs or for reverse transcriptase PCR (RT-PCR). The reaction volume of 50 µL contained 10 ng of plasmid DNA, 10 pmol of the forward and the reverse primers (Tables 5, 6), 1 µL dNTP mix (10 mM each) and 2-4 U DNA polymerase.

For DNA analysis *Taq*-DNA polymerase (NEB) and for downstream applications Fast Start High-Fidelity *Pfu* polymerase (Roche) was used in the corresponding PCR buffer supplied by the manufacturer. PCR was carried out in a thermocycler. The Standard PCR reaction profile (Table 4) was adjusted according to the quantity and quality of template DNA, the length and G/C content of the oligonucleotides, the length of the amplified sequences (Tables 4,5,6).

Table 4: Standard PCR reaction profile

PCR program	Temperature [°C]	Time [min]	Cycles
Initial denaturation	95	3-5	1
Denaturation	95	1	30
Hybridisation	50-70	1	
Elongation	72	1-3	
Terminal elongation	72	5	1

2.2.9 Semiquantitative analysis of mRNA levels by reverse transcriptase (RT) PCR

cDNA was synthesised from RNA via a reverse transcriptase reaction using the cDNA synthesis kit (Roche). For this purpose two µg of total RNA were utilised and the reaction was carried out according to the manufacturer's protocol. Briefly, total RNA and 100 pmol of oligo-(Tang et al.)-15 nucleotides were incubated at 65°C for 10 min. Then 20 U protector RNase inhibitor (RNAsin), 10 U transcriptor reverse transcriptase and 1 mM dNTPs were added and synthesis was done at 55°C for 30 min. The enzymes were finally inactivated by incubation at 85°C for five min. cDNA was either stored at -20°C or directly used as a template for the PCR reaction. The polymerase chain reaction was carried out as described (2.2.8) using the specific primers (Table 5) for the sequences of interest. In parallel, amplification of GAPDH with the specific primers in another PCR allowed a semiquantitative analysis of the original amount of transcript in the RNA sample, which is based on the fact that GAPDH mRNA is equally abundant in the samples. The PCR products were visualised by agarose gel electrophoresis.

Table 5: Synthetic oligonucleotides used for RT-PCR. The length of the PCR products is given by the size in base pairs with the size for spice variants given in square brackets. Bp = base pairs, fw = forward, rev = reverse, t_m=melting temperature

Name	Gene	Sequence (5' – 3')	Size / size of splice variant [bp]	t_m [°C]
GAPDH fw	gapdh	ATT TGG CCG TAT TGG GCG CCT G	761	66
GAPDH rev	gapdh	GCT TCA CCA CCT TCT TGA TGT CAT CA		
Herp fw	herpud1	CTG GGA AGC TGT TGT TGG AT	337	58
Herp rev	herpud1	GAA AGC TGA AGC CAC CCA TA		
Herp2 fw	herpud2	GGC ACC ATG GAC CAA AGT GG	497 / 546	63
Herp2 rev	herpud2	GAA TCC AGG TGG AGC AGC TTG		
XBP1 fw	xbp1	CCT TGT AGT TGA GAA CCA GG	442 / 416	58
XBP1 rev	xbp1	CCT GGT TCT CAA CTA CAA GG		

2.2.10 *In vitro* recombination of DNA

Cleavage of DNA using restriction endonucleases

Restriction endonucleases (NEB, Fermentas) were used for the cleavage of DNA as described in the supplier's protocols. In general, 1 µg of DNA was incubated with 1 U enzyme in the appropriate buffer for 1 h at 37°C. The cleaved DNA was analysed by agarose gel electrophoresis (2.2.3).

Isolation of DNA from agarose gels

DNA fragments were separated on an agarose gel by electrophoresis. Afterwards DNA bands were visualised using an UV transilluminator and desired bands isolated by excising the respective piece of agarose. The DNA was extracted from the agarose slice using the GFX™ Extraction Kit (GE healthcare) according to the manufacturer's instructions.

Dephosphorylation of linearised plasmid DNA

Dephosphorylation of the linearised plasmid DNA was done to avoid recircularisation after restriction digestion and isolation from agarose gels. The DNA was incubated with 1 U shrimp alkaline phosphatase and the corresponding buffer for 1 h at 37°C. The reaction was stopped by incubation at 70°C for 20 min for inactivation the enzyme.

Annealing of DNA oligonucleotides

- Annealing buffer: 10 mM Tris-HCl adjusted to pH 8.0 with HCl, 100 mM NaCl, 0,05 mM EDTA

For the generation of shRNA encoding pSuper constructs containing Sel1L-specific sequences, 1 µL of sense and antisense oligonucleotide (10 µg/µL) were diluted in 18 µL annealing buffer, heated in a water bath to 95°C for ten min and cooled down to room temperature and finally to 4°C at a cooling rate of 0,02 °C/s. The resulting annealed oligonucleotides were utilised for the ligation reaction (2.2.10).

Table 6: Synthetic oligonucleotides used for expression vector construction. Fw=forward, rev=reverse, mut stands for mutagenesis primer, t_m=melting temperature, sh=short hairpin.

Name	Sequence (5' – 3')	t_m [°C]	Comment
Sel1-shRNA-fw	GATCCCCGGCTATACTGTGGCTAGAATTCAA-GAGATTCTAGCCACAGTATAGCCTTTTTGGAAA	84	primer: Sel1L-shRNA cloning in pSuper= M 480
Sel1-shRNA-rev	AGCTTTTCCAAAAAGGCTATACTGTGGCTAGAATCT-CTTGAATTCTAGCCACAGTATAGCCGGG	84	
K61R-mut-fw	CAGAGGTTAATTTATTCTGGGA-GGCTGTTGTTGGATCACCAATGT	78	primer: Herp mutagenesis in pSG5 and pSG5-HTB = M 472, 473
K61R-mut-rev	ACATTGGTGATCCAACAACAGC-CTCCCAGAATAAATTAACCTCTG	78	
AUP1-fw	GCGCTCAGATCTATGGAGCTTCCCTCAGGGCCG	80	primer: AUP1 cloning in pQE = M 496
AUP1-rev	CCCAAGCTTGTCAGCCTCCTGGGCTCGTCT	77	
AUP1-C-fw	GGCGCCGCGGATCCCACGTCTTCCTGGTCAGCTGC	85	primer: AUP1-C cloning in pQE = M 495
AUP1-C-rev	CCCAAGCTTGTCAGCCTCCTGGGCTCGTCT	77	
AUP1-mut-fw	CTTGACTATCACTAATGCGGCTGAGGGGGCCGTAGC	80	primer: AUP1 mutagenesis in pcDNA3.1= M 497
AUP1-mut-rev	GCTACGGCCCCCTCAGCCGCATTAGTGATAGTCAAG	80	

Phosphorylation of isolated DNA inserts

The annealed double stranded oligonucleotides were phosphorylated at the 5' ends by polynucleotide kinase. Seven µL of oligonucleotide solution were mixed with one µL ATP (10 mM), one µL of ten-fold concentrated reaction buffer and one µL of the kinase and incubated at 37°C for one h. Then the reaction was stopped by incubation at 65°C for 10 min. The sample was subjected to the ligation reaction.

Ligation of linearised vector and insert DNA fragments

To test the DNA quality and estimate the quantity before a ligation reaction, isolated DNA fragments (inserts) and linearised vectors were subjected to agarose gel electrophoresis. For the ligation reaction 100 ng of dephosphorylated vector, 500 – 1000 ng of phophorylated insert and 10 U of T4 DNA ligase were mixed with the accordant buffer to a volume of 20 µL. The samples were incubated at 16°C for 14 h. Resulting plasmids were subjected to transformation reactions.

2.2.11 Site-directed mutagenesis

For the insertion of site specific mutations into double-stranded DNA the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) was used. Two complementary oligonucleotide primers were used, carrying the codon to be mutated in the middle flanked by 15 additional base pairs on each side that correspond to the target sequence. The experiment was performed according to the supplier's manual. Briefly, 50 ng of template DNA were mixed with 125 ng of each primer, 1 µL of desoxyribonucleotide mix (10 µM), 5 µL of 10 times concentrated reaction buffer and 1 µL DNA polymerase in a volume of 50 µL. Then a PCR reaction was carried out (Table 4) and the resulting products were digested with *DpnI* to eliminate parental DNA. *E.coli* XL1 blue cells or One Shot®TOP10 cells (Invitrogen) were transformed with the resulting DNA (see 2.2.6).

Common remarks on molecular biology:

- All desoxyribonucleotide sequences were confirmed by

Agowa sequencing services / Berlin.

- All DNA oligonucleotides were synthesised by Biotez Berlin.
- siRNA was purchased from Dharmacon RNAi Technologies (Thermo Scientific).

2.3 Tissue culture

2.3.1 Cell lines and media used in this study

- cell culture medium: basal medium (Iscove's, RPMI, DMEM), 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin

In this study HeLa human cervix carcinoma cells and Tet repressor expressing LS 174T human colon carcinoma cells (derived from LS 180) were used predominantly. HeLa cells were maintained in Iscove's based, LS 174T cells in RPMI based medium. The medium for LS 174T cells was additionally supplemented with 10 µg/mL Blasticidin S for Tet repressor selection. For the cultivation of HeLa cells, stably transfected to express Hexahistidin Biotin-tagged Synoviolin or Herp, puromycin was added to a final concentration of 2 µg/mL. Herp siRNA inducible LS22 cells were cultured with additional 400 µg/mL Zeocin^{TM}. Cells were cultivated at 37°C and 5% CO_2. All used cell lines are listed in Table 7.

Table 7: Human cell lines used in this study. All cells were maintained with 10% fetal bovine serum, 2mM L-glutamine and 100 U/mL penicillin/streptomycin in basal medium ('Medium'). For selection of stable transfectants the corresponding antibiotics were added to the medium ('Selection'). HTB=Hexahistidin-Biotin-tag.

Cell line	Cell Type	Medium	Selection	Origin / Remarks
HeLa	cervix adenocarcinoma	Iscove's	-	-
HeLa-Synoviolin-HTB	cervix adenocarcinoma	Iscove's	2 µg/mL puromycin	M.Seeger – AG Kloetzel / usage of clones with different expression efficiency (clone 6 low, clone 38 high expression level)
HeLa-Herp-HTB	cervix adenocarcinoma	Iscove's	2 µg/mL puromycin	M.Seeger – AG Kloetzel
LS 174T	colorectal adenocarcinoma	RPMI – 1640	10 µg/mL blasticidinS	kind gift of M. Maurice, expression of Tet repressor (van de Wetering et al., 2003)
LS 88	colorectal adenocarcinoma	RPMI - 1640	10 µg/mL blasticidinS 400 µg/mL zeocin	kind gift of M. Maurice, derived from LS 174T, Doxicycline-inducible USP7-siRNA (Meulmeester et al., 2005)
LS 89	colorectal adenocarcinoma	RPMI - 1640	10 µg/mL blasticidinS 400 µg/mL zeocin	kind gift of M. Maurice, derived from LS 174T, Doxicycline-inducible USP7 (Meulmeester et al., 2005)
LS 22	colorectal adenocarcinoma	RPMI - 1640	10 µg/mL blasticidinS 400 µg/mL zeocin	derived from LS 174T, Doxicycline-inducible Herp-siRNA expression
RKO	colon carcinoma	DMEM	-	-

2.3.2 Culture of cells

- Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4

Cells were generally grown in sterile culture dishes or flasks and passaged when they reached 80% confluence, depending on the generation time. For the passage of the cells, the culture medium was removed and the cells were washed with PBS and incubated with a trypsin/EDTA solution for 2-5 min. After the detachment, serum containing culture medium was added to the cells and they were transferred to a new tube and sedimented by short centrifugation at 250 g. Cells were resuspended in fresh medium containing all necessary additives and plated in the appropriate density.

Counting of cells

- trypan blue solution: 0,1% (w/v) trypan blue, 0,3% (w/v) NaCl in PBS

10 µL of a single cell suspension were inspected using a Neubauer counting chamber under a microscope. Cells in four selected large squares were counted. The volume of one large square is 0.1 µL. The mean value of four large squares was multiplied with 10^4, resulting in the number of cells per 1 mL cell suspension.
To evaluate only living cells, the cell suspension was mixed 1:2 with trypan blue solution. Living cells do not incorporate the dye, whereas dead cells or cell debris get stained.

2.3.3 Cryoconservation and thawing of cells

- freezing medium: 20% FCS, 10% (v/v) DMSO in culture medium (RPMI or Iscove's)

For long term storage of human cell lines 10^6 cells were resuspended in 1 mL of freezing medium and immediately transferred into a cryoconservation vial and placed in a freezing container, filled with pre-cooled isopropanol. The container was kept at -80°C over night to ensure a 1°C/min cooling rate. The next day the tubes were transferred into a liquid nitrogen (-196°C) tank for the final storage.
Thawing of the cells was performed in a water bath heated to 37°C. The suspension of thawed cells was carefully mixed with 10 mL of culture medium and sedimented at 1,000 g for 5 min. The culture medium was removed and the cells were resuspended in fresh medium and afterwards plated in 10 mL culture medium on a 10 cm culture dish.

2.3.4 Transfection of mammalian cells

Transfection of cells with DNA by calcium phosphate precipitation

- HEPES buffered saline (HBS): 280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM dextrose, 50 mM HEPES adjusted to pH 7.05 with NaOH

HeLa cells were plated at 5 x 10^5 cells per 10 cm dish in Iscove's medium and incubated over night at 37°C. On the next day 500 µl of HBS was pipetted into a 5 mL polypropylene vial. Then, 62 µL 2 M $CaCl_2$ and 10 µg DNA were diluted in 440 µL of sterile water. This solution was slowly dripped into the vial containing the HBS buffer while slowly mixing. The resulting mixture was incubated at room temperature for 30 min to gain precipitation and finally added dropwise to the cells. The plate was gently shaken to distribute the DNA and incubated at 37°C. After 16 hours the medium was exchanged and the cells were harvested after a total incubation time of 24 to 72 hours.

Transfection of cells with DNA by lipofection

HeLa cells were plated at 10^5 cells per well in a 24-well-plate in 500 µL medium without antibiotics and incubated over night. For each well 0.8 µg DNA and 0.8 µL Lipofectamin 2000 were diluted in 50 µL OptiMEM, mixed, incubated at room temperature for 20 min and then transferred to the well containing the cells. The plate was gently shaken to distribute the DNA and then further incubated at 37°C. After a total incubation of 24 to 48 h after transfection the cells were harvested.

Transfection of cells with siRNA by lipofection

HeLa cells were plated at 5 x 10^5 cells per 10 cm dish in 5 mL medium without antibiotics and incubated over night at 37°C. 25 µL of siRNA (20 µM) were mixed with 25 µL OptiMEM transfection medium. In parallel, 10 µL of Dharmafect lipofection reagent were diluted in 490 µL Optimem. Both solutions were combined, mixed and incubated at room temperature for 20 min. The whole mixture was then added dropwise to the cells. For cycloheximide chase experiments (see 2.2.7) one day after transfection the cells were detached and seeded onto a 24 well plate in an appropriate dilution.

Table 8: Plasmids for expression in mammalian cells used in this study. The plasmids pSG5 (Stratagene), pSuper (Oligoengine) and pTER (van de Wetering et al., 2003) were primarily used in this study. AUP1-mut* stands for the mutation of the CUE domain with base exchanges C997G, T998C, C1000G, T1001C. MCS indicates the multiple cloning sites with the particular restriction sites. If not otherwise noted the plasmids were generated in the group of M. Seeger / AG Kloetzel (= M.S.). A.f. = adapted from, aa = amino acid.

Name	Insert	Vector	Tag	MCS	Source/ Comment
M31	ZZ	pSG5	-	BamHI	M.S.
M444	Herp	pSG5	-	EcoRI/BamHI	M.S.
M401	Herp	pSG5	HTB	EcoRI/BamHI	M.S.
M472	Herp-K61R	pSG5	-	EcoRI/BamHI	M.S.
M473	Herp-K61R	pSG5	HTB	EcoRI/BamHI	M.S.
M445	Herp-d-UBL	pSG5	-	EcoRI/BamHI	M.S.
M129	Herp2	pSG5	ZZ	EcoRI/BamHI	M.S.
M411	Herp2	pSG5	HTB	EcoRI/BamHI	M.S.
M412	Herp2-splice	pSG5	HTB	EcoRI/BamHI	M.S.
M470	Synoviolin	pSG5	-	EcoRI/BamHI	M.S.
M229	Synoviolin	pSG5	myc	EcoRI/BamHI	M.S.
M230	Synoviolin-C329S	pSG5	myc	EcoRI/BamHI	M.S.
M400	Synoviolin	pSG5	HTB	EcoRI/BamHI	M.S.
M69	Synoviolin-N-term	pSG5	HTB	EcoRI/BamHI	M.S.
M71	Synoviolin-C-term	pSG5	HTB	EcoRI/BamHI	M.S.
M72	Synoviolin-C-term wo RING	pSG5	HTB	EcoRI/BamHI	M.S.
M158	myc	pCMV-tag3B	-	-	M.S.
M312	Ube2J1	pCMV-PLD	myc	-	kind gift of T.Sommer (Sommer and Jentsch, 1993)
M313	Ube2J1-C91S	pCMV-PLD	myc	-	
M279	his/myc	pcDNA3.1	myc / his	-	M.S.
M384	Usp7	pcDNA3.1	myc / his	EcoRI/NotI	kind gift of M. Maurice (a.f.(Canning et al., 2004)
M468	Usp7-C223S	pcDNA3.1	myc / his	EcoRI/NotI	M.S.
M19	AUP1	pcDNA3.1	myc / his	HindIII/EcoRI	M.S.
M497	AUP1-mut*	pcDNA3.1	myc / his	HindIII/EcoRI	aa exchange: L333A, L334A
M175	EGFP shRNA	pSuper	-	EcoRI/HindIII	M.S.
M124	Herp shRNA	pTER	-	EcoRI/HindIII	M.S.
M336	Synoviolin shRNA	pSuper	-	EcoRI/HindIII	M.S.
M480	Sel1L shRNA	pSuper	-	EcoRI/HindIII	M.S.
M457	CD3-δ-YFP	based on EGFP-N1	YFP	-	M.S.
M452	NHK (Null Hong Kong mutant of α1-antitrypsin)	PBR322 based IG-lambda	-	-	kindly provided by Paolo Paganetti (Novartis)

Table 9: Sequences of pre-designed siRNA (Dharmacon). The sequence of the non targeting control oligonucleotide and the sequences of the smart pools of targeting siRNAs (set of four oligonucleotides (1.-4.)) are given.

Name of siRNA	Sequences of RNA oligonucleotides	
control	5'-UGGUUUACAUGUCGACUAA-3'	
Synoviolin	1. UCAUCAAGGUUCUGCUGUAUU	2. GAGAAGAGAUGGUGACUGGUU
	3. CAACAUGAACACCCUGUAUUU	4. GGAAAGGCCUCCAGCUCCUUU
Herp	1. CGACAGUACUACAUGCAAUUU	2. GGGCCACCGUUGUUAUGUAUU
	3. GGCUUCAGCUUUCCUGGUUUU	4. GCGGAUGAAUGCACAAGGUUU
Usp7	1. AAGCGUCCCUUUAGCAUUAUU	2. GCAUAGUGAUAAACCUGUAUU
	3. UAAGGACCCUGCAAAUUAUUU	4. AUAAAGAAGUAGACUAUCGUU
AUP1	1. GCACUAUAUGAAUACGCAA	2. GCAGAUUCGUAGUGCGGAC
	3. GAGCACAUGAAGCGACAAA	4. CGACCACAACAUAGUCAAU

2.3.5 Generation of a stable inducible cell line expressing Herp specific shRNA

LS 174T cells, expressing the Tet repressor protein (van de Wetering et al., 2003) were utilised to generate a cell line, stably expressing an inducible vector coding for Herp shRNA. For that purpose, the accordant silencing DNA oligonucleotides had been inserted into a pTER plasmid, with which the cells were subsequently transfected using the calcium phosphate method (2.2.5). Two days after transfection the cells were replated in different dilutions from 5 x 10^2 to 10^4 cells per well in a round bottom 96 well plate. The culture medium contained 10 µg/mL Blasticidin S for the selection of Tet repressor expression and 400 µg/mL Zeocin^{TM} for selection of the pTER plasmid. After two to three weeks single cells formed colonies which were expanded and tested for Herp shRNA expression.

Tet repressor molecules inhibit the expression of the pTER plasmid. Upon addition of 1 µg/mL doxicycline (an analogue of tetracycline) to the culture medium, the expression is induced by the direct inhibition of the repressor protein. Positive clones were identified by Western blot analysis using a Herp specific antibody (Figure 18). Each positive clone was cryoconservated (2.2.3).

2.3.6 Cycloheximide chase analysis

For the estimation of the half-life of proteins, 10^5 cells expressing the respective protein were seeded per well on a 24 well plate and incubated at 37°C over night. Then cycloheximide (chx) was added to a final concentration of 50 µg/mL. In order to analyse proteasome dependent degradation, 10 µM MG132, an inhibitor of the proteasome, dissolved in DMSO, was added to one chx containing well. In this case the equivalent volume of the solvent DMSO was added to the other samples. Cells were harvested at various time points and frozen in liquid nitrogen. For further analysis, cells of each sample were lysed in 100 µL of RIPA buffer (2.4.1). 10 µL of each lysate were subjected SDS-PAGE (2.4.4) and Western blot analysis (2.4.5).

2.3.7 Metabolic labelling using [^{35}S]-methionine/-cysteine and pulse chase analysis

- starving medium: methionine free RPMI, 1%(v/v) dialysed fetal calf serum

For the determination of the half-life of a protein or the analysis of intrinsic protein interactions, 4 x 10^5 cells per well on a 6 well plate or 10^6 cells on a 10 cm dish, expressing the protein of interest, were seeded and incubated at 37°C over night. Then cells were left untreated or treated with 2 µM thapsigargin for up to 8 h to induce ER stress. After washing the cells twice with 3 mL (5 mL for the dish) PBS, 1 mL (2.5 mL for the dish) of starving medium was added and cells were incubated for 1 h at 37°C.
Then, [^{35}S]-S-L-methionine / [^{35}S]-L-cysteine (Tran^{35}S-Label^{TM}) was directly added to a final concentration of 0.1 mCi/mL and cells were labelled for 1 h. Then the label medium was removed and two washing steps followed with 2 mL (5 mL for the dish) of PBS. Then the cells were harvested directly or medium, containing all additives, was added and they were further incubated for various periods of time. The collected cells were either lysed in 500 µL of RIPA buffer (2.4.1) and subjected to immunoprecipitation for pulse chase analysis or in 500 µL of DBC containing buffer (2.4.1) to precipitate ERAD complexes for the analysis of physiological protein interactions (2.4.7). An aliquot of each lysate was subjected to scintillation counting and samples were normalised for ^{35}S-methionin incorporation.

2.4 Protein biochemistry

2.4.1 Lysis of mammalian cells

- RIPA lysis buffer: 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA (sodium salt), 1% (v/v) Nonidet P40, 0.5% (w/v) Sodium-deoxycholate, 0.1% (w/v) SDS and Complete^{TM}, adjusted to pH 8.0 with NaOH
- DBC lysis buffer: 1% (w/v) DBC (Applichem), 33 mM HEPES, 150 mM Kalium-acetate, 4 mM Magnesium-acetate, 10% (w/v) glycerol, Complete^{TM}, adjusted to pH 7.3 with KOH

Mammalian cells were lysed either in RIPA lysis buffer for analysis of whole cell extracts or in deoxy-big-chap (DBC) lysis buffer for analysis of ERAD complexes. For RIPA lysis cells were resuspended in ice cold RIPA lysis buffer and incubated on ice for 20 min and then centrifuged for 15 min at 4°C and 20,000 g. The supernatant was subjected to immunoprecipitation or SDS-PAGE. For DBC lysis cells were resuspended in DBC lysis buffer and gently agitated for 30 min at 4°C. After that, two centrifugation steps of 5 and 10 min at 10,000 g and 4°C were performed. The final supernatant was either subjected to immunoprecipitation, precipitation experiments or SDS-PAGE.

2.4.2 Determination of protein concentration in solution

Protein concentrations in solution were determined by measuring the absorbance at 280 nm in an UV-VIS photometer. An absorbance of one at the wavelength of 280 nm equals a concentration of 1 mg protein/mL solution.

2.4.3 Precipitation of proteins using trichloro acetic acid (TCA)

For the precipitation of protein complexes, separated on a glycerol gradient, TCA was added to the sample to a final concentration of 10% (w/v). This mixture was kept on ice for 20 min, followed by centrifugation at 20,000 g and 4°C for 15 min. The sediment was washed 3 times with 500 µL of ice cold acetone (-20°C). After another centrifugation step (5 min, 4°C, 14,000 g) the sediment was air dried and resuspended in 60 µL SDS sample buffer (see 2.4.4).

2.4.4 Sodium dodecyl sulfate polyacrylamid gel electrophoresis (SDS-PAGE)

- Concentrated sodium dodecyl sulfate (SDS)-sample buffer (ten-fold): 500 mM Tris-HCl, pH 6.8, 20% (w/v) SDS, 60% (v/v) glycerol, 1 M DTT, 1% (w/v) bromophenol blue
- Electrophoresis running buffer: 25 mM Tris, 200 mM glycine, 0.1% (w/v) SDS
- 4 x separating gel buffer:1.5 M Tris-HCl, 0.4 % (w/v) SDS, adjusted to pH 8.8 with HCl
- 4 x stacking gel buffer: 0.5 M Tris-HCl, 0.4% (w/v) SDS, adjusted to pH 6.8 with HCl

For the separation of proteins under denaturing conditions SDS polyacrylamid gel electrophoresis (SDS-PAGE) was performed using a discontinuous gel buffer system (Laemmli, 1970) with the vertical mini-Protean 3 electrophoresis system (Biorad). The concentration of acrylamide in the separating gels varied from 10-15%, depending on the desired resolution range. The stacking gel contained 5% acrylamide. The precise composition of the gels is given in Table 10.
Sedimented proteins or protein solutions were mixed with SDS sample buffer, incubated for 5 to 10 min at 95°C, while shaking and shortly centrifuged and before loading onto gels. Protein separation was carried out in electrophoresis running buffer at 80 to 120 Volts. The molecular weight of the protein bands was estimated by comparison with a prestained standard size marker, separated on the same gel.

Table 10: Composition of SDS polyacrylamide gels. Indicated volumes (Vol.) of the solutions (components) were mixed for the given final concentrations of acrylamide. AA = acrylamide

Components	**Vol.**	**Separating gel**			**Stacking gel**
		10% AA	**12% AA**	**15% AA**	**5% AA**
Acrylamide (30:0,8)	mL	6,7	8	10	3
H_2O	mL	8,3	7	5	12
4 x separating gel buffer	mL	5	5	5	-
4 x stacking gel buffer	mL	-	-	-	5
TEMED	µL	53	53	53	80
10% APS	µL	53	53	53	80

2.4.5 Western blot analysis

- cathode buffer: 40 mM ε-amino caproic acid, 0,01% (w/v) SDS, 20% (v/v) methanol
- anode buffer 2: 25 mM Tris, 20% (v/v) methanol
- anode buffer 1: 300 mM Tris, 20% (v/v) methanol

After separation of proteins by SDS-PAGE they were transferred from the gels to nitrocellulose membranes in a semi dry blotting procedure using a three buffer system. Four Whatman 3 MM papers were soaked in the cathode buffer and put on the bottom (cathode) of a semidry blotting apparatus. First the gel and then the membrane were piled upon it. then a layer of two Whatman 3 MM paper sheets, soaked in anode buffer 2 and finally two Whatman 3 MM paper sheets, soaked in anode buffer 1 were put on top of it. The transfer was carried out at 320 mA for 1 h at room temperature. Immunodetection was performed as described in 2.4.6.

2.4.6 Protein visualisation

Ponceau staining of proteins on nitrocellulose membranes

- Ponceau staining solution: 1% (w/v) Ponceau S in 5% (w/v) TCA-solution

After the protein transfer onto nitrocellulose membranes these were incubated in Ponceau staining solution for 1 min and thereafter destained in deionised water until the desired contrast of protein bands was achieved.

Coomassie Blue staining of proteins in gels

- Coomassie Blue staining solution: 0.1% (w/v) Coomassie Brilliant Blue R250, 40% (v/v) methanol, 10% (v/v) acetic acid, filtered before use
- destaining solution: 40% (v/v) methanol, 10% (v/v) acetic acid

Mini gels were incubated in Coomassie Blue staining solution for 30 min at room temperature while being agitated. For destaining the gels were incubated in destaining solution for 30 min, exchanging the solution several times.

Immunodetection of proteins

- PBS-T: PBS, 0.1% (v/v) Tween 20
- blocking solution: PBS-T, 5% (w/v) milk powder

For the specific detection of proteins on nitrocellulose membranes, these were incubated for one hour in blocking solution followed by the incubation with the primary antibody in PBS-T buffer for 15 h at 4°C. The membrane was then washed four times with PBS-T for at least ten minutes and subsequently incubated for one h at room temperature with the secondary antibody coupled to horseradish peroxidase diluted in PBS-T. The membrane was washed four times with PBS-T and detection was carried out with the ECL Plus™ Substrate kit as described in the supplier's manual (GE Healthcare). Proteins were visualised by exposure of the nitrocellulose membrane to a XOmat-UV film for one second to five minutes depending on the protein to detect. Resulting pictures are referred to as immunoblots (IB).

Table 11: Primary and secondary antibodies used for protein detection in Western blot analysis. (m)=monoclonal**.**

Name / Specificity	Species	Dilution	Supplier / Source
primary antibodies			
anti-β-Tubulin	mouse (m)	1:10,000	Covance
anti-GAPDH	rabbit	1:20,000	Santa Cruz
anti-Herp	rabbit	1:50,000	M. Seeger / Pineda
anti-Synoviolin	rabbit	1:1,000	M.Kikkert
anti-p97 (Cdc48)	rabbit	1:20,000	R.Hartmann-Petersen
anti-Grp78	rabbit	1:1,000	abcam
anti-His (RGS)	mouse (m)	1:1,000	Qiagen
anti-Sel1L	mouse (m)	1:500	Alexis
anti-Ube2j1	mouse (m)	1:500	Abnova
anti-α1-antitrypsin-HRP	goat	1:50,000	Bethyl laboratories
anti-CD3-δ	rabbit	1:50,000	M. Seeger / Pineda
anti-Usp7	rabbit	1:10,000	Bethyl laboratories
anti-Derlin1	rabbit	1:10,000	MBL
anti-Herp2	rabbit	1:50,000	M. Seeger / Pineda
anti-Gp78	mouse (m)	1:1,000	Abnova
anti-p53 (DO7)	mouse (m)	1:500	Novocastra
anti-AUP1	rabbit	1:50,000	Sigma
secondary antibodies			
anti- rabbit-IgG HRP	sheep	1:10,000	Seramun
anti- mouse- HRP	sheep	1:10,000	Seramun

Detection of ^{35}S-methionine metabolically labelled proteins

Metabolically labelled proteins were separated on SDS-PAGE. The resulting gels were dried on a vacuum gel drier and subjected to film exposure using BioMAX films and cassettes with intensifying screens. Exposure was done at -80°C for three to seven days. Alternatively, the dried gels were analysed by exposure to imaging plates of the Bio Imaging Analyser (BAS-MS, Fujifilm) at room temperature over night. Signals were detected with the accordant phosphoimager system (FLA 3000 Fujifilm).

2.4.7 Affinity precipitation of proteins

Immunoprecipitation (IP) of intrinsic proteins using specific antibodies

- DBC washing buffer: 0.2% (w/v) DBC (Applichem), 33 mM HEPES adjusted to pH 7.3 with KHO, 150 mM Kalium-acetate, 4 mM Magnesium-acetate, 1 mg/mL BSA

HeLa or LS cells of one well of a six-well plate were lysed in 500 µL of DBC containing buffer as described (2.4.1) and supernatants were used for immunoprecipitation. Specific antibodies were added to the lysates in an appropriate dilution (Table 12). These were gently agitated at 4°C for 4 h. The immunocomplexes were precipitated using a mixture of 20 µL packed Protein A-Protein:Protein-G sepharose (1:2) equilibrated in the lysis buffer. Further incubation was performed at 4°C over night. Then the sepharose beads were washed four times with one mL of DBC washing buffer for 5 min. Precipitated proteins were eluted by adding 50 µL SDS sample buffer and visualised by SDS-PAGE and Western blot analysis using specific antibodies (Table 11). In order to reduce disturbing chemoluminescence signals of IgG heavy and light chains that stem of the IP, Protein A-HRP (Biorad), recognizing only native IgG, was applied in a dilution of 1:2,000 instead of the secondary antibody.

Table 12. Antibodies used for the immunoprecipitation of proteins. (m)=monoclonal

Name	Origin	Dilution	Supplier / Source
anti-p97 (VCP)	mouse (m)	1:200	Affinity Bio Reagents
anti-Herp	rabbit	1:100	M. Seeger / Pineda
anti-Synoviolin	rabbit	1:100	M.Kikkert
anti-Sel1L	mouse (m)	1:50	Alexis
anti-Usp7	rabbit	1:100	Bethyl laboratories
anti-Herp2	rabbit	1:100	M. Seeger / Pineda
anti-p53 (DO-7)	mouse (m)	1:100	Novocastra
anti-α1-antitrypsin (NHK)	rabbit	1:50	Biozol
anti-AUP1	rabbit	1:1000	Sigma

Precipitation of Hexahistidin-Biotin (HTB)-tagged proteins

The Hexahistidin-Biotin-tag (HTB) as depicted in Figure 8 was originally designed for tandem affinity purification and contains a RGS-6-His and an *in vivo* biotinylation site, separated by a TEV protease cleavage site. For the precipitation of C-terminal HTB-tagged proteins streptavidin agarose beads were utilised. Therefore HeLa cells (of a 10 cm culture dish at a density of about 80%) expressing HTB-constructs were lysed in 500 µL of DBC containing buffer (2.4.1) and beads, equilibrated in the same buffer, were added to the lysates in a volume of 20 µL packed beads per 500 µL of lysate. The suspension was incubated at 4°C for 2 h while being gently agitated. Unspecifically bound proteins were removed by washing the beads four times with 1 mL of DBC washing buffer (2.4.7). Finally the beads were resuspended in SDS sample buffer (2.4.4).

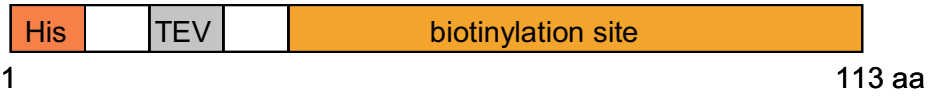

Figure 8: Histidin-Biotin-Tag (HTB). His=Hexahistidin; TEV=TEV protease cleavage site.

Co-precipitation of [^{35}S]-methionine metabolically labelled proteins

For the analysis of endogenous protein interactions HeLa cells of a 10 cm dish were metabolically labelled as described (2.3.8) and lysed in 500 µL DBC containing buffer (2.4.1). The lysates were subjected to a first IP (2.4.7) using the accordant specific antibodies (Table 12). DBC washed Protein A/G beads then were resuspended in 500 µL RIPA buffer and incubated for 1 h in order to achieve the dissociation of the precipitated complexes. During this process the bait protein remained bound to the beads. These were washed four times with 1 mL RIPA buffer and resuspended in 50 µL of SDS sample buffer (2.4.4). The supernatants, containing the dissociated proteins, were subjected to a second IP, using the specific antibodies to detect the desired proteins, originally bound to the first bait. The second IP was done as described before and beads were resuspended in 50 µL SDS sample buffer. 25 µL of each sample were subjected to SDS-PAGE and visualised by autoradiography (2.4.6).

2.4.8 Separation of proteins by glycerol gradient centrifugation

10^7 HeLa cells were lysed in 500 µL of DBC containing lysis buffer (2.4.1). The lysates were put on top of a glycerol gradient ranging from 10-50% glycerol based on the lysis buffer. Then centrifugation was performed using an ultracentrifuge for 16 h at 260,000 g and 4°C with an SW40 rotor. Beginning from the top, gradient fractions of 1 mL were collected and subjected to TCA precipitation (2.4.3). Resulting samples were analysed via SDS-PAGE and immunodetection.

2.4.9 *In vitro* binding studies

Expression of proteins in *E.coli*

Glutathione-S-transferase (GST) - tagged recombinant fusion proteins (Table 13) were expressed in the XL1 blue *E.coli* strain. Therefore the bacteria were transformed with the respective expression vectors and streaked out on LB-amp agar plates and incubated over night at 37°C. Then a single colony was picked and added to 10 mL liquid LB-amp medium and shaken over night at 37°C. At the next day 200 mL LB-amp were inoculated with the 10 mL culture and grown at 37°C until an OD_{600} of 0.5 was reached. Then 0.4 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to the culture to induce protein expression and incubation was continued at RT for four h followed by cell sedimentation. Aliquots were taken before and after IPTG induction to analyse the protein expression via SDS-PAGE and Coomassie staining.

Table 13. Plasmids used for expression in *E.coli*. For the *in vitro* precipitation assays the plasmids pGEX-KG (Guan and Dixon, 1991), pQE30 (Qiagen), pRSET (Invitrogen) were used. The inserts code for proteins of which the names and the range of amino acids is given. MCS = multiple cloning site, GST = glutathione S transferase, His = Hexahistidin. All constructs were generated in the laboratory of M. Seeger.

Name	Insert	Vector	Tag	MCS
M5	-	pGEX-KG	GST	
M99	Herp1 1-240	pGEX-KG	GST	BamHI/EcoRI
M123	Herp1 1-240 A39T	pGEX-KG	GST	BamHI/EcoRI
M100	Herp2 1-240	pGEX-KG	GST	BamHI/EcoRI
M122	Herp2 1-240 T39A	pGEX-KG	GST	BamHI/EcoRI
M111	Herp 304-391	pGEX-KG	GST	BamHI/EcoRI
M110	Herp 1-260	pGEX-KG	GST	BamHI/EcoRI
M360	Synoviolin 236-616	pGEX-KG	GST	BamHI/EcoRI
M496	AUP1 1-410	pQE30	His	BamHI/HindIII
M495	AUP1 24-410	pQE30	His	BamHI/HindIII
M400	Usp7 1-208	pRSET	His	BamHI/EcoRI

Lysis of bacteria and binding of GST fusion proteins to glutathione sepharose

- GST lysis buffer: 1%(w/v) Triton X-100, 50 mM Tris-HCl adjusted to pH 7.3 with NaOH, 150 mM NaCl, 10% (w/v) glycerol, 1 mM PMSF, Complete™, 1 mg/mL lysozyme
- GST washing buffer: 1%(w/v) Triton X-100, 50 mM Tris-HCl adjusted to pH 7.3 with NaOH, 150 mM NaCl, 10% (w/v) glycerol, 1 mM PMSF, Complete™

Bacteria cells of a 200 mL liquid culture, containing the protein of interest were sedimented by centrifugation (5,000 g, 4°C, 30 min) and lysed in 5 mL GST lysis buffer, sonicated four times for 30 s and gently agitated for 1 h at 4°C. Lysates were centrifuged for 30 min at 20,000 g and supernatants were transferred into a fresh Falcon tube. Precipitation of GST fusion proteins was performed by incubating 5 mL of the lysate with 300 µL of glutathione sepharose, equilibrated in lysis buffer for at least 2 h on a rotor at 4°C. Glutathione sepharose beads with bound GST fusion proteins were washed four times with GST washing buffer, solubilised in 1 mL of the same buffer containing 0.01% (w/v) sodium azide for storage at 4°C.

Preparation of *E.coli* cell lysates containing His-tagged fusion proteins

- Tris buffer: 50 mM Tris-HCl adjusted to pH 7.3 with NaOH, 150 mM NaCl, 10% (w/v) glycerol, 1 mM PMSF, Complete™

Expression of His-tagged proteins was performed with pQE30 vectors (Qiagen) and the *E.coli* strain XL1 blue. Cells of a 200 mL culture were lysed in 5 mL Tris buffer, sonicated and incubated for 1 h at 4°C during gentle agitation. Lysates were centrifuged for 30 min at 20,000 g and the supernatants were used for *in vitro* interaction studies. For the incubation of immobilised GST fusion proteins with cell lysates from human cells, $5x10^6$ HeLa cells were lysed in 1 mL Tris buffer, incubated for 30 min at 4°C and centrifuged for 10 min at 20,000 g and 4°C. Supernatants were utilised for the *in vitro* interaction studies.

***In vitro* interaction studies using HeLa- or *E.coli*- cell lysates**

For the *in vitro* precipitation experiment 20 µL of GST fusion protein bound to glutathione sepharose were incubated with 500 µL of *E.coli* lysate or 1 mL of human cell lysate for 2 h or over night with gentle agitation. After extensive washing with Tris buffer (2.4.9) containing 0.2% (w/v) DBC and 1 mg/mL BSA the beads were taken up in 60 µL of two times concentrated SDS sample buffer. Precipitated proteins were analysed with SDS-PAGE and immunodetection.

2.5 Bioinformatics and databases

The bioinformatics tools and databases, used in this study, are listed in Table 14.

Table 14. Online databases and tools used in this study.

URL	Program / Database / Version
http://www.ebi.ac.uk/Tools/clustalw/index.htmL	Clustal W2 alignment program (EMBL-EBI)
http://www.basic.northwestern.edu/biotools/oligocalc.htmL	Oligonucleotide properties calculator 3.2.3
http://rna.lundber.gu.se/cutter2/	Webcutter 2.0
http://tools.neb.com/NEBcutter2/index.php	NEB cutter V2.0
http://www.ensembl.org	Ensembl genome database – release 54
http://www.addgene.org/pqvec1	Addgene Plasmid database 2003-2009
http://gentle.magnusmanske.de	GENtle DNA and AA editing ©2004-2008
http://www.expasy.org	ExPASy proteomics server / UniProt 15.5
http://smart.embl-heidelberg.de	SMART proteomics server version 6
http://www.ihop-net.org/UniPub/iHOP	information hyperlinked Over Proteins (iHOP)
http://rsbweb.nih.gov/ij	densitometry software / version 1.41

3 Results

3.1 The importance of the dynamics of Herp for ERAD

The ubiquitin domain protein Herp is a component of Synoviolin based complexes, which mediate ER associated protein degradation. Compared to other proteins taking part in the formation of these complexes, Herp reveals the special feature of an enhanced turnover. In contrast to Synoviolin revealing a half-life of 15 hours, Herp is a rather short lived protein with a half-life of three hours (Kikkert et al., 2004; Sai et al., 2003). In addition, Herp protein expression is strongly induced within four hours upon cell exposure to ER stress, whereas an increased expression of Synoviolin, which is also a target of the unfolded protein response, is not observed before six hours after ER stress induction (Donati et al., 2006). With regard to protein stability and UPR inducibility Herp also differs from other components of Synoviolin based complexes. Grp78 for example is as fast induced as Herp upon ER stress but reveals a half-life of at least 48 hours (see Table 16 / Appendix). Thus, synthesis and degradation rates of Herp, in this work referred to as 'dynamics of Herp', are increased compared to the dynamics of Synoviolin or other proteins of these ERAD complexes. This fact suggests that the Herp dynamics have a special role in Synoviolin mediated ERAD, a point which was focused on in this work. It was tested whether the dynamics of Herp influence the assembly of Synoviolin based complexes. Furthermore, it was assessed whether the stability of Herp is determined by these ERAD complexes or if the special dynamics are crucial for the function of Herp in the process of Synoviolin mediated ERAD.

3.1.1 Herp is exchanged at Synoviolin based complexes

Upon ER stress, the expression of Herp is induced prior to other components of Synoviolin based ERAD complexes. Therefore, Herp was suggested to be a prerequisite for the formation of Synoviolin based complexes. These complexes are necessary for the elimination of misfolded proteins occurring in these ER stress situations. Hence, the process of Synoviolin oligomerisation or the recruitment of accessory proteins to Synoviolin may depend on the presence of Herp. Being entirely involved in the process of Synoviolin complex assembly, Herp would most likely be associated exclusively with *de novo* synthesised Synoviolin and not with functional Synoviolin complexes. To test this hypothesis, pulse chase experiments followed by co-immunoprecipitation studies were performed. HeLa cells were treated with 2 µM thapsigargin for six hours to induce ER stress and the enhanced expression of UPR target proteins such as Herp and Synoviolin. Then, these cells were metabolically labelled with ^{35}S-methionine/-cysteine for one hour (pulse) and lysed

immediately or after a further incubation for three and six hours with medium lacking radioactively labelled amino acids (chase). Before the chase cell cultures were split into halfes of which one half was additionally incubated with cycloheximide to stop translation. Herp and p97 were immunoprecipitated from the lysates using antibodies specific for these proteins (first IP). Due to mild lysis conditions whole ERAD complexes were co-precipitated with p97 or Herp. These complexes were dissociated by incubation in SDS containing buffer and the supernatants including the single ERAD components were subjected to a second immunoprecipitation (second IP) using an antibody specific for Synoviolin. Immunoprecipitates were subjected to SDS-PAGE and analysed by autoradiography. This experiment enables to monitor the degradation of Herp and p97, which is displayed by the decrease of the labelled protein population over time. In addition, the association of Synoviolin with these proteins can be evaluated. As shown in Figure 9, the expression of Herp protein was increased upon ER stress treatment by thapsigargin whereas the expression of p97 did not change (Figure 9, b and c, upper panel). Radioactively labelled Herp was completely degraded within six hours of chase, irrespective of cycloheximide addition. In contrast, p97 remained stable for the whole chase period.

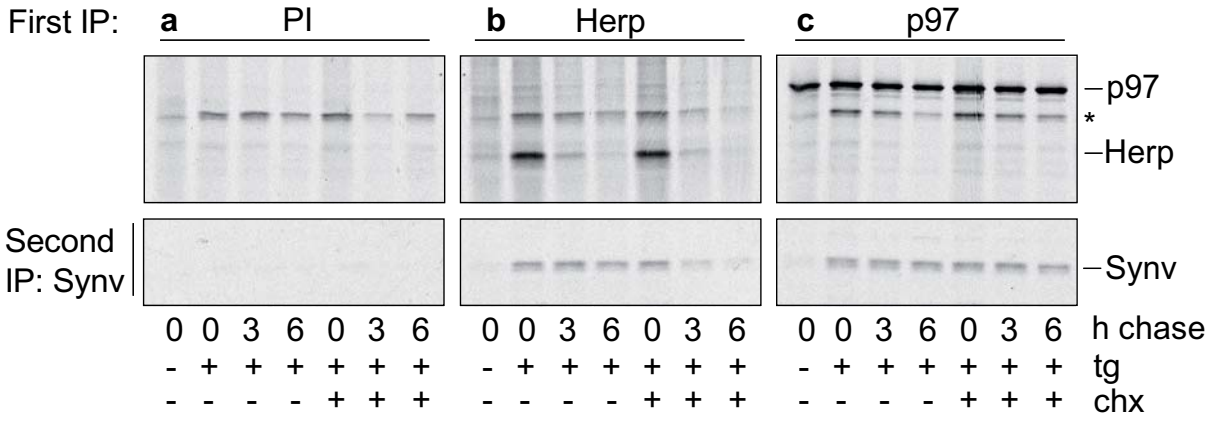

Figure 9: Dynamics of Herp at Synoviolin based complexes. HeLa cells were left untreated or treated with 2 µM thapsigargin for six h. Then, cells were metabolically labelled with ^{35}S-methionine and, to stop translation, 50 µg/mL cycloheximide were added where indicated. Where indicated, thapsigargin was present during incubation. Cells were lysed at the indicated time points (chase) in DBC containing buffer. Lysates were equalised for overall radioactivity after scintillation counting. For the first immunoprecipitation (IP) either pre-immune serum (PI) or the specific antibodies as indicated were utilised (a-c). Dissociation of co-precipitated proteins was achieved by the incubation in RIPA buffer. The supernatant was subjected to a second IP with an antibody specific for Synoviolin. Precipitated proteins were separated on SDS-PAGE and visualised by autoradiography. An unspecific cross reaction, occurring in thapsigargin treated samples, is marked with an asterisk. Synv=Synoviolin; h=hour; tg=thapsigargin; chx=cycloheximide.

Meeting the expectations, the expression of the E3 ligase Synoviolin was induced by ER stress treatment, since a Synoviolin specific signal was only observed in the autoradiography upon thapsigargin treatment (Figure 9, b and c, lower panel). Independent of cycloheximide addition, the amount of Synoviolin, which was co-precipitated with stable p97, remained constant during the chase.

On the other hand, the amount of Synoviolin, which was co-precipitated with Herp, decreased in correlation with the degradation of Herp when treated with cycloheximide. In the absence of cycloheximide the amount of Synoviolin, co-precipitated with Herp, remained constant, although the population of labelled Herp was degraded (Figure 9, b). This observation can be explained by the fact that newly synthesised and therefore not labelled Herp substitutes the older population of labelled Herp occurring at already existing Synoviolin based complexes. Therefore, it appears that Herp is not exclusively bound to *de novo* synthesised Synoviolin but is exchanged at already existing Synoviolin based complexes. This exchange has not been shown for any of the other proteins associated with Synoviolin.

3.1.2 Synoviolin complex components are not essential for the degradation of Herp

Herp is a substrate of the ubiquitin proteasome system and is associated with Synoviolin (Sai et al., 2003; Schulze et al., 2005). As shown in 3.1.1, Herp is subject to constant degradation and reassociation with established Synoviolin based complexes suggesting that Herp is a substrate of Synoviolin. Initial experiments which focused on Synoviolin potentially mediating Herp degradation showed that the elevated expression of Synoviolin did not enhance its degradation but led to the stabilisation of Herp, irrespective of the E3 ligase activity (Schulze, 2006). Thus, Synoviolin seems not to be required for the degradation of Herp. To confirm this result and to test whether further components of Synoviolin based complexes are involved in the regulation of Herp stability, degradation of Herp was monitored after depletion of Synoviolin and additionally after depletion of Sel1L, a Synoviolin associated protein that was described to be required for ERAD (Mueller et al., 2008). Depletion of Synoviolin and Sel1L protein in HeLa cells was performed by the expression of shRNA specific for these proteins. ShRNA expressing cells were subjected to a pulse chase experiment for six hours and proteins of interest were immunoprecipitated and analysed by autoradiography.
Compared to the expression of shRNA targeting GFP as control, the expression of shRNA specific either for Synoviolin or for Sel1L led to the reduced expression of these proteins by about 50% (Figure 10, A). Depletion of either of these proteins did not result in an altered degradation of Herp (Figure 10, B), although the reduction of the cellular level of Sel1L by 50% was reported to impair degradation of NHK (Christianson et al., 2008). Therefore, the results presented here indicate that Synoviolin and Sel1L are not involved in the degradation of Herp.

The E2 enzyme Ube2j1 is another component of Synoviolin based complexes which was suggested to work in concert with Synoviolin for the degradation of ERAD substrates (Mueller et al., 2008). Here, Ube2j1 was tested for its impact on the degradation of Herp. HeLa cells expressing Ube2j1-myc or the inactive Ube2j1-C91S-myc were utilised as published (Lenk et al., 2002). Cells were treated with thapsigargin for four hours to induce Herp expression. For degradation analysis, cycloheximide was added to the cells to inhibit translation. Degradation of Herp was monitored for six hours by lysing the cells at distinct time points after cycloheximide addition. To test whether protein degradation was dependent on the proteasome, MG132 was added to an additional sample for the whole chase period. Cell lysates were subjected to SDS-PAGE and Western blot analysis.

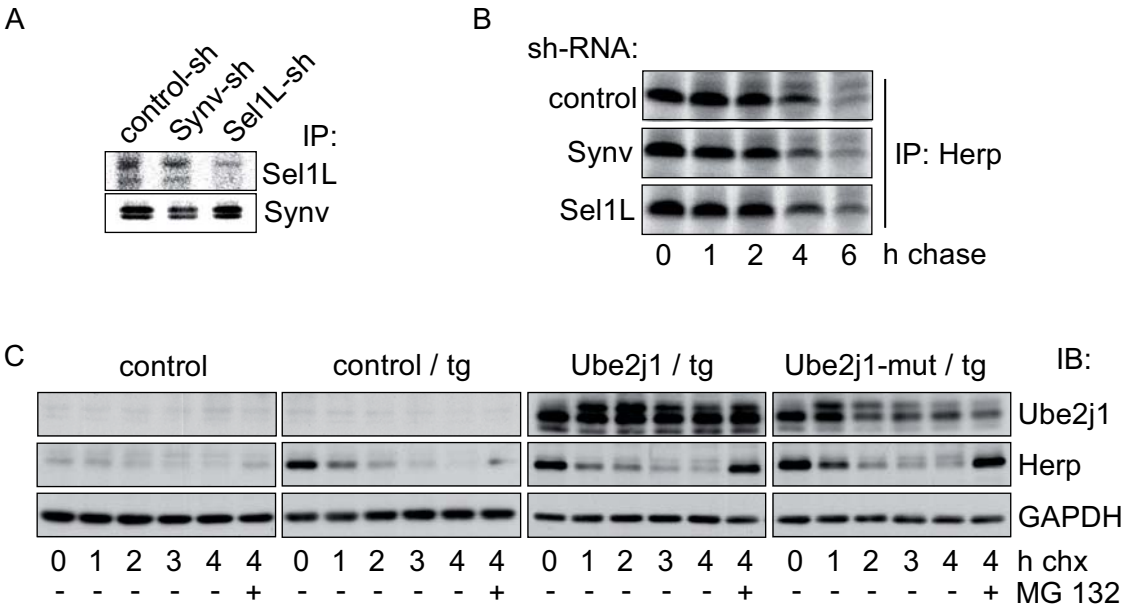

Figure 10: Regulation of Herp by components of Synoviolin based complexes. HeLa cells were transfected with plasmids encoding shRNA either specific for Synoviolin or Sel1L or with an empty plasmid as control. At 48 h after transfection cells were metabolically labelled using ^{35}S-methionine. Cells were lysed at indicated time points in RIPA buffer. (**A**) Herp was immunoprecipitated with the specific Herp antibody (IP). Precipitated proteins were separated on SDS-PAGE and visualised by autoradiography. (**B**) To analyse protein depletion, Sel1L and Synoviolin were precipitated from the '0 h' lysates using the corresponding specific antibodies. (**C**) HeLa cells were transfected with plasmids encoding Ube2J1-myc, the inactive mutant Ube2J1-C91S-myc or with the empty pCMV-tag3B (control). At 48 h after transfection cells were left untreated or treated with 2 µM thapsigargin (tg) for four h before 50 µg/mL cycloheximide (chx) were added to all samples and MG132 to a final concentration of 10 µM where indicated. After further incubation for the indicated time points cells were lysed in RIPA buffer and proteins were separated on SDS-PAGE and visualised by Western blot analysis using the indicated specific antibodies. h=hour; IB=immunoblot; Synv=Synoviolin

The elevated expression of myc-tagged wild type or myc-tagged mutated Ube2j1 resulted in the detection of three bands in the Western blot using an antibody specific for Ube2j1 (Figure 10, C, right hand panels). As described before the upper band represents phosphorylated Ube2j1 (Oh et al., 2006). This modified E2 appeared after cycloheximide addition and was gradually degraded within the chase period (Figure 10, C).

Ube2j1 phosphorylation was reported to be ER stress related but not to alter the stability, binding properties or localisation of Ube2j1 and thus is of no importance for substrate ubiquitination (Oh et al., 2006). Concerning the degradation of Herp, the induction of ER stress led to the elevated expression of Herp, but did not alter the rate of Herp degradation, as it was observed before (Figure 10, C, left panels; (Schulze, 2006). Compared to the control, only a slight delay of the degradation of Herp was observed in case of E2 enzyme overexpression, irrespective of E2 enzyme activity (Figure 10, C, right hand panels). Therefore, the elevated expression of myc-tagged Ube2j1 and Ube2j1-C91S did not significantly change the stability of Herp. These data demonstrate that the ERAD mediating proteins Synoviolin, Sel1L and Ube2j1 are not required for the degradation of Herp.

3.1.3 Herp-K61R is stabilised and impairs the degradation of NHK

The experiments described above strongly suggest that the turnover of Herp is not mediated by Synoviolin based complexes. So far, only the UBL domain of Herp was demonstrated to determine its stability, as Herp lacking this domain was shown to be stable (Sai et al., 2003). In more detail, the expression of a recombinant Herp-version (aa 1-259) together with the E2 enzyme Ube2g2 and the active C-terminus of the E3 enzyme Gp78 in *E.coli* revealed that lysine 61, located within the UBL domain, is the crucial polyubiquitination site of this Herp-version (Li et al., 2007). In fact, the UBL domain of Herp was demonstrated to be essential for the effective ERAD of CD3-δ (Schulze, 2006). Collectively, these data suggest a correlation between the stability of Herp, determined by the UBL domain, and the function of Herp in Synoviolin mediated ERAD processes. It was hypothesised that an enhanced stability of Herp is beneficial for ERAD. To check this hypothesis, cellular Herp levels were increased but the elevated expression of Herp had no effect on the stability of ERAD substrates, which is presumably due to an enhanced degradation of Herp in this situation (M.Seeger, personal communication). To circumvent this difficulty, lysine 61 was substituted by an arginine using site-directed mutagenesis in order to obtain a stabilised Herp (Herp-K61R). To test whether the resulting Herp mutant was more stable than wild type Herp, HeLa cells, expressing wild type Herp or Herp-K61R were subjected to cycloheximide chase analyses. Additionally, HeLa cells expressing Herp or Herp-K61R as fusion proteins with a C-terminal hexa-histidine-biotin (HTB)-tag were included in the assay. Degradation was assessed by Western blot analysis using an antibody specific for Herp.

Compared to the wild type Herp, Herp-K61R was shown to be stabilised (Figure 11, A). The same result was observed for the Herp-versions which were C-terminally fused with the HTB-tag. Therefore, the single amino acid substitution K61R within the UBL domain was sufficient to stabilise Herp.

Next, this stabilised Herp mutant was tested for having an impact on the degradation of an ERAD substrate. For this purpose, the Null Hong Kong mutant of α1-antitrypsin (NHK) was chosen as a substrate, because its degradation was shown before to be dependent on Synoviolin as sole E3 ligase (Christianson et al., 2008). Associated with Synoviolin, Herp was suggested to also influence the degradation of NHK. Considering that Herp positively affects the turnover of NHK, stabilisation of Herp was hypothesised to improve this process. To test this hypothesis, degradation of NHK was investigated in HeLa cells co-expressing NHK and either the wild type Herp or Herp-K61R. In parallel, NHK-expressing HeLa cells were transfected with siRNA specific for Herp. Cycloheximide chases were performed for degradation analysis.

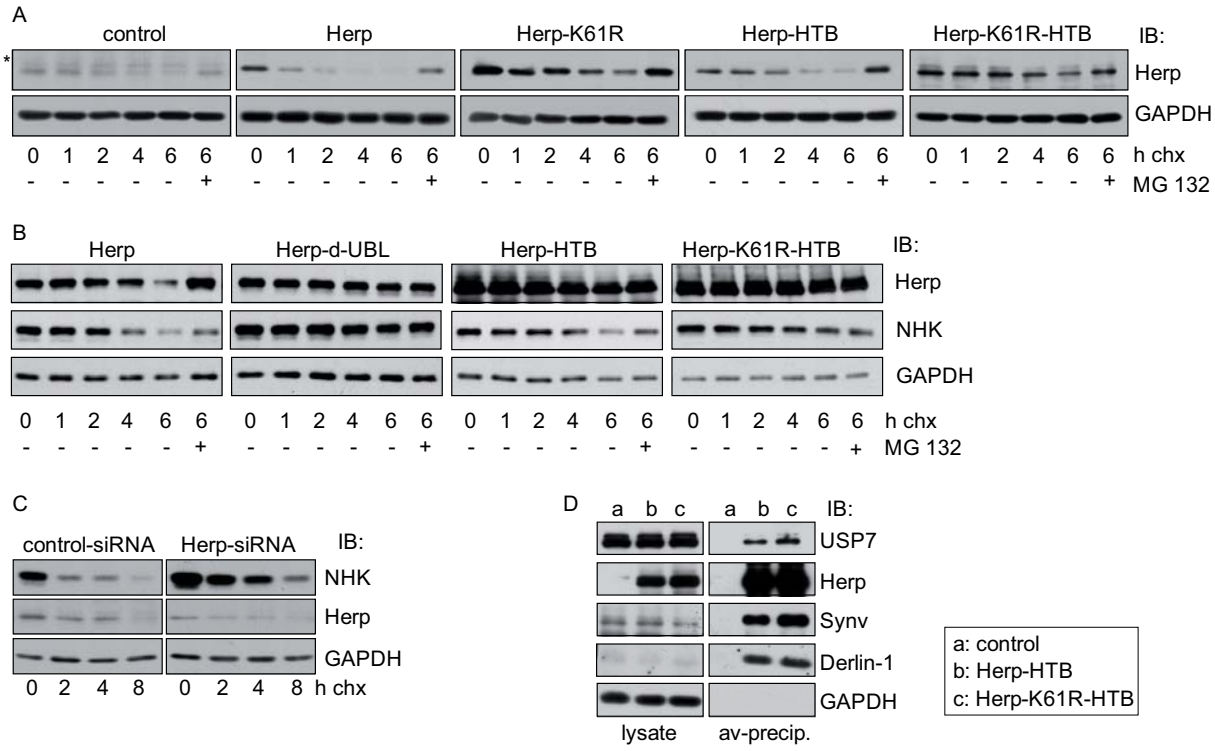

Figure 11: Analysis of the Herp-K61R mutant. (**A**) HeLa cells were transfected with plasmids encoding Herp or Hexahistidin-Biotin (HTB)-tagged Herp with or without the K61R mutation. At 48 h after transfection 50 µg/mL cycloheximide (chx) were added to all samples and MG132 to a final concentration of 10 µM where indicated. After further incubation for the indicated times (chase), cells were lysed in RIPA buffer. Proteins were separated on SDS-PAGE and visualised by Western blot analysis using the indicated specific antibodies. (**B**) HeLa cells were cotransfected with plasmids encoding the Null Hong Kong mutant of α1-Antitrypsin (NHK) and the indicated Herp constructs. The experiment was proceeded as described in A. (**C**) HeLa cells were co-transfected with either a control siRNA or a siRNA specific for Herp and 24 h later with a plasmid encoding NHK. At 48 h after transfection 50 µg/mL cycloheximide were added and cells were further incubated as indicated. Further analysis was performed as described in A. (**D**) HeLa cells were transfected with plasmids encoding HTB-tagged Herp or Herp-K61R. At 48 h after transfection cells were lysed in DBC containing buffer. HTB-tagged proteins were precipitated using streptavidin agarose. Proteins were separated on SDS-PAGE and visualised by Western blot analysis using the specific antibodies as indicated. IB=immunoblot; av-precip.=streptavidin agarose precipitation, an unspecific cross reaction is marked by an asterisk.

Degradation of NHK occurred within six hours of chase and was dependent on the proteasome, since the incubation with MG132 impaired NHK degradation (Figure 11, B). Utilisation of siRNA specific for Herp led to the depletion of the Herp protein by 50% and resulted in the impaired degradation of NHK indicating that the substrate's degradation is Herp dependent (Figure 11, C). In addition, compared to the co-expression of wild type Herp with NHK, the co-expression of a dominant negative Herp-version lacking the UBL domain also resulted in the stabilisation of this ERAD substrate (Figure 11, B, left panels). These results showed for the first time that effective degradation of NHK depends on the presence of Herp. The UBL-domain of Herp was indispensable for its ERAD promoting function.
Regarding the correlation between the stability of Herp and the stability of the substrate NHK, a surprising observation was made: the stabilisation of Herp did not improve but rather impair the degradation of NHK as shown by the co-expression of HerpK61R-HTB in comparison with Herp-HTB (Figure 11, B, right hand panel). These results demonstrate that the effective degradation of the Herp and Synoviolin dependent ERAD substrate NHK depends on the presence of the Herp-UBL domain and is positively correlated to the degradation rate of Herp, whereas stabilisation of Herp seems to have a negative effect on the ERAD process. According to its enhanced stability, Herp-K61R might be associated with Synoviolin for a longer time than wild type Herp. To ensure that Herp-K61R associates with Synoviolin at all, Herp-K61R-HTB and Herp-HTB were expressed in HeLa cells and precipitated using streptavidin agarose. Co-precipitated proteins were analysed by Western blotting. Herp- and Herp-K61R-HTB were found to be expressed at equal levels and comparable amounts of these proteins were precipitated (Figure 11, D, Herp). Endogenous Synoviolin and Derlin-1 were co-precipitated with both Herp versions. In addition, also Usp7 was co-precipitated with Herp-HTB and Herp-K61R-HTB (Figure 11, D, av-precip.). This result indicates that Herp-K61R as well as wild type Herp is associated with Synoviolin based complexes.

Taken together, this first part of the study shows that Herp reveals distinctive dynamics, when compared to other components of Synoviolin based complexes. Herp is induced earlier upon ER stress and degraded faster. Interestingly, Synoviolin associated Herp is constantly substituted by newly synthesised Herp molecules without being a substrate of this E3 ligase or the associated proteins Sel1L and Ube2j1. Degradation and reassociation with Synoviolin seems to be important for the function of Herp at the ERAD complexes, since the depletion as well as the stabilisation of Herp led to an impaired degradation of the ERAD substrate NHK.

3.2 The role of Herp in maintaining the integrity of Synoviolin based complexes

Dynamically acting at Synoviolin based complexes, Herp may be vital to organise the composition and integrity of these multimeric structures. Having this role, Herp would be involved in determining the stochiometry of the proteins within these complexes, regulate the oligomeric status of single components or enable the association of accessory proteins with the protein multimer. Therefore, it was tested whether Herp regulates the process of Synoviolin oligomerisation or the recruitment of accessory proteins to the ERAD complex.

3.2.1 Herp does not alter the formation of Synoviolin oligomers

The E3 ligase Synoviolin was described to be able to oligomerise and, together with a couple of proteins, to form multimeric ERAD complexes in mammalia (Schulze et al., 2005). However, the oligomer stochiometry or the regulation of the process of Synoviolin oligomerisation has not been investigated. In yeast, Hrd1p, the orthologue of Synoviolin, was also shown to oligomerise, and this process was suggested to be regulated by a ubiquitin domain protein called Usa1p. Carvalho and colleagues postulated Usa1p to be the functional equivalent of Herp, since the heterologous expression of Herp in a yeast Usa1p-deletion mutant rescued the phenotype of impaired ERAD (Carvalho et al., 2006). Furthermore, Usa1p was shown to establish Hrd1p oligomerisation (Horn et al., 2009). These observations suggested that Herp, similar to Usa1p, stabilises the Synoviolin oligomers in mammalia. To test this hypothesis, HeLa cells expressing either HTB-tagged Synoviolin only or together with untagged Synoviolin at a ratio of 1:6 were transfected with plasmids encoding Herp or HerpΔUBL. Utilisation of different amounts of the Synoviolin fusion protein allowed studying Synoviolin homooligomerisation. Considering that Herp-Δ-UBL significantly impairs the degradation of NHK, this Herp-version was chosen to detect a potential effect on Synoviolin oligomerisation. The HTB-fusion proteins were precipitated and further analysed.

Immunoblotting of the precipitation samples revealed two Synoviolin-specific bands. The lower band migrated at approximately 70 kDa as expected for endogenous Synoviolin. Synoviolin-HTB contains additional 119 amino acids due to the HTB-tag and is represented by the upper band (Figure 12, av-precip.). Endogenous as well as co-expressed wild type Synoviolin were co-precipitated with Synoviolin-HTB, which demonstrates oligomerisation.

Correlating with the ratios of transfected plasmids, the expression of Synoviolin-HTB alone resulted in oligomers containing predominantly HTB-tagged Synoviolin (Figure 12, left panel), whereas the co-expression of Synoviolin led to oligomers containing mainly untagged Synoviolin (Figure 12, right hand panel).

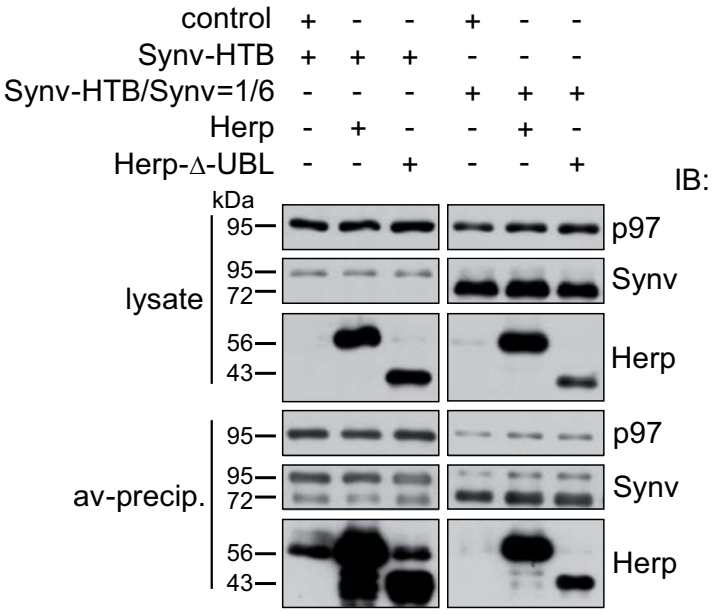

Figure 12: Role of Herp in Synoviolin oligomerisation. HeLa cells were transfected with plasmids encoding Hexahistidin-Biotin (HTB)-tagged Synoviolin alone or together with not tagged Synoviolin at a ratio of 1:6. Herp, HerpΔUBL or plasmid without insert as control were cotransfected. At 20 h after transfection cells were lysed in DBC containing buffer. HTB-tagged Synoviolin was precipitated using streptavidin agarose. Proteins were separated on SDS-PAGE and visualised by Western blot analysis using the specific antibodies as indicated. Protein standard sizes are given in kDa (kilodalton). His=Histidin; IB=immunoblot; av-precip.=streptavidin agarose precipitation, Synv=Synoviolin

Co-expression of Herp and dominant negative HerpΔUBL did not alter the oligomerisation status of Synoviolin. The ratio of tagged to untagged Synoviolin remained the same for all chosen conditions. Herp and HerpΔUBL were co-precipitated with Synoviolin-HTB in positive correlation to the amount of expressed Synoviolin-HTB (lysate). In addition, the amount of p97 co-precipitated with Synoviolin did not change upon the co-expression of Herp or HerpΔUBL, but correlated positively with the amount of precipitated Synoviolin. This observation shows that the UBL domain of Herp is not required for maintaining the oligomeric status of the E3 ligase or for the association of p97 with the ERAD complex. It remains open whether Herp maintains the integrity of Synoviolin based ERAD complexes by recruiting accessory proteins besides p97 to already established Synoviolin oligomers.

3.2.2 Usp7 is a target of the Herp UBL domain

The UBL domain of Herp has a decisive function in ERAD, as shown for CD3-δ and NHK degradation (Schulze, 2006); Figure 11,B). To elucidate the function of this important domain of Herp, a yeast two hybrid screen was performed to identify interaction partners. With this approach the deubiquitinating enzyme Usp7 was found and in the course of analyses verified as specifically UBL domain interacting protein (Schulze, 2006). Therefore, in this study it was investigated whether Usp7 is also associated with Synoviolin or is a component of Synoviolin based complexes possessing functional importance for the ERAD process.

3.2.2.1 The AXXS motif contributes to an efficient binding of Usp7 to Herp

Since an interaction of Usp7 with other UDPs such as Ubiquilin-1 was not observed, Herp was suggested be the only UDP binding Usp7 via its UBL domain. However, it is also feasible that Usp7 interacts with other UBL domain containing proteins of the mubl family to which Herp belongs (Schulze, 2006). Herp2, harbouring a UBL and potential transmembrane domains is a member of this family and was assumed to be a candidate to bind Usp7. The UBL domains of Herp and Herp2 reveal more than 50% amino acid sequence identity. Therefore, it was tested whether Usp7 interacts with Herp2 using a streptavidin agarose precipitation assay. Precipitation was followed by a Western blot analysis using HeLa cells expressing HTB-fusion proteins of Herp, Herp2 or the splice variant of Herp2 (Herp2 lacking amino acids 49-70).

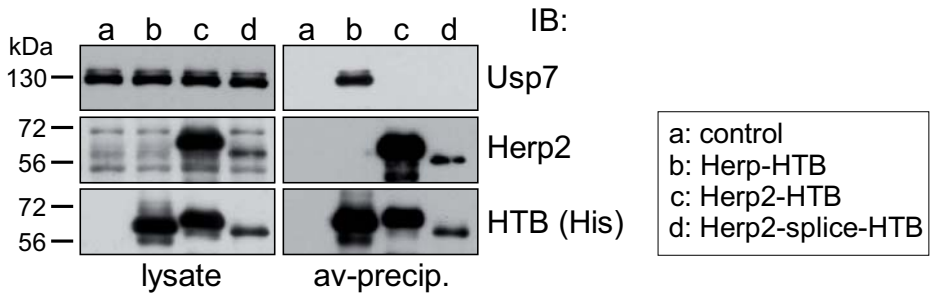

Figure 13: Interaction of Usp7 with Herp and Herp2. HeLa cells were transfected with either an empty pSG5-plasmid (control) or plasmids encoding Hexahistidin-Biotin (HTB)-tagged versions of Herp, Herp2 or the splice variant of Herp2. At 24 h after transfection cells were lysed in DBC containing buffer and the HTB-tagged proteins were precipitated using streptavidin agarose. Proteins of the lysates and the precipitations were separated on SDS-PAGE and visualised with the indicated specific antibodies by Western blot analysis. Protein standard sizes are given in kDa (kilodalton). Av-precip.=streptavidin agarose precipitation; His=Hexahistidin; IB=immunoblot.

Endogenous Usp7 was equally expressed in all tested cells. The hydrolase is depicted as a double band in the Western blot occurring at an apparent molecular weight of about 130 kDa, which represents Usp7 and a recently described isoform of Usp7, Usp7-β (Figure 13, lysate). Herp-HTB and Herp2-HTB were expressed at equal levels, only Herp2-splice-HTB was expressed at a lower level. Co-precipitation of endogenous Usp7 was only observed with Herp-HTB and not with Herp2-HTB indicating that the binding of Usp7 to the UBL domain of Herp is specific (Figure 13, av-precip.).

In the literature, the P/AXXS-motif, with X indicating any amino acid, was described to mediate the direct interaction of Usp7 with p53 and Mdm2 both harbouring this motif (Sheng et al., 2006). It therefore appeared that this motif played a role for the interaction of Usp7 and Herp as well. To address this assumption, the UBL domains of Herp and Herp2 were screened for this short amino acid sequence.

It was observed that Herp harbours an AHLS sequence starting at position 39 within the UBL domain whereas Herp2 has a THLS motif at the same position. Considering that Herp2, lacking the AXXS motif, does not bind Usp7 and that Herp harbouring this motif binds Usp7 it appeared possible that the AXXS motif of Herp is sufficient to bind Usp7. To test this hypothesis, a site directed mutagenesis was performed to substitute alanine 39 by threonine (A39T) within Herp to obtain a potential loss of function and threonine 39 by alanine (T39A) within Herp2 for a gain of function concerning Usp7 binding. For *in vitro* interaction studies, the Herp- and Herp2-variants were expressed as glutathione S transferase (GST) fusion proteins in *E.coli*, immobilised on glutathione sepharose and incubated with a HeLa cell lysate or with an *E.coli* extract containing the N-terminus of Usp7 (aa 1-210).

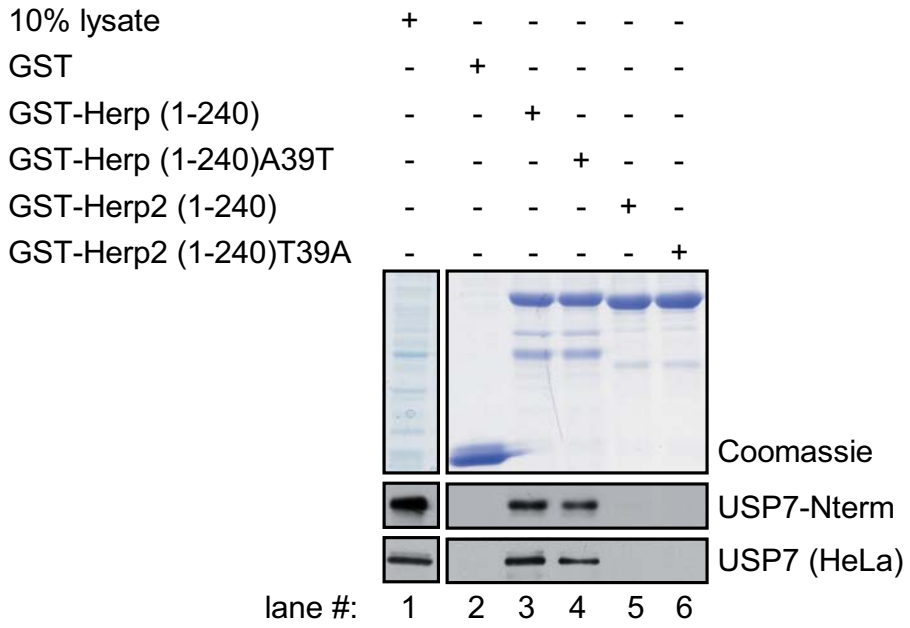

Figure 14: Impact of the AHLS motif of Herp on Usp7 binding. GST and GST fusion constructs of Herp (1-240), Herp(1-240)A39T and Herp2 (1-240), Herp2(1-240)A39T were expressed in *E.coli*, immobilised on glutathione sepharose and incubated with HeLa cell lysate or with a lysate from *E.coli* containing the N-terminus of Usp7 (1-210). The upper panel depicts the Coomassie stained precipitated proteins after SDS-PAGE and semidry blot to show equal loading of the glutathione beads. Usp7 was visualised by Western blot analysis using the specific Usp7-antibody, shown in the lower panels.

As expected, Western blot analysis revealed that endogenous Usp7 from HeLa cell lysates as well as the soluble N-terminus of Usp7 from *E.coli* extracts interacted with immobilised Herp-GST (Figure 14, lane 3). This interaction was not observed for Usp7 and Herp2-GST (Figure 14, lane 5). The A39T exchange within Herp resulted in a decrease of the amount of interacting Usp7 (Figure 14, lane 4). However, the T39A exchange within Herp2 did not result in an interaction of Usp7 with Herp2 (Figure 14, lane 6). Thus, the AXXS motif within the UBL domain of Herp contributes to an efficient binding of this UDP to Usp7 but is not sufficient to mediate this interaction.

3.2.2.2 Herp recruits Usp7 to Synoviolin

Since Usp7 and Synoviolin both directly interact with Herp, an association of Usp7 with Synoviolin is likely. Recent experiments revealed that only upon elevated expression of Herp, but not HerpΔUBL, Usp7 is associated with Synoviolin (M.Seeger, personal communication). Therefore, it was suggested that the induction of Herp protein expression in case of ER stress leads to an association of Usp7 and Synoviolin. To test this hypothesis, HeLa cells expressing Synoviolin-HTB were treated with tunicamycin for up to six hours to induce ER stress. For Western blot analysis, HTB-tagged Synoviolin was precipitated and tested for co-precipitated proteins.

Within six hours of continuous ER stress the expression of Herp was increased, whereas the expression of Synoviolin and Usp7 remained even (Figure 15, A, lysate). Constant amounts of Synoviolin-HTB were precipitated at all tested time points, whereas the amount of co-precipitated Herp increased over time (Figure 15, A, av.precip.). At six hours of ER stress treatment a small subpopulation of endogenous Usp7 was also co-precipitated with Synoviolin-HTB indicating that the association of Usp7 and Synoviolin is mediated by Herp (Figure 15, A, av-precip.). An inverse observation was made for Derlin-1, another Synoviolin associated protein. Although Derlin-1 protein expression was marginally induced by ER stress, the amount of Derlin-1 which co-precipitated with Synoviolin declined during the period of stress treatment indicating that ER stress on the one hand leads to the increased association of proteins with Synoviolin and on the other hand to the dissociation of distinct proteins from these ERAD complexes.

To evaluate the stability of the association of Usp7 with Synoviolin, ER stress exposed HeLa cells expressing Synoviolin-HTB were treated with cycloheximide for up to six hours before streptavidin agarose precipitation and Western blot analysis was performed as before.

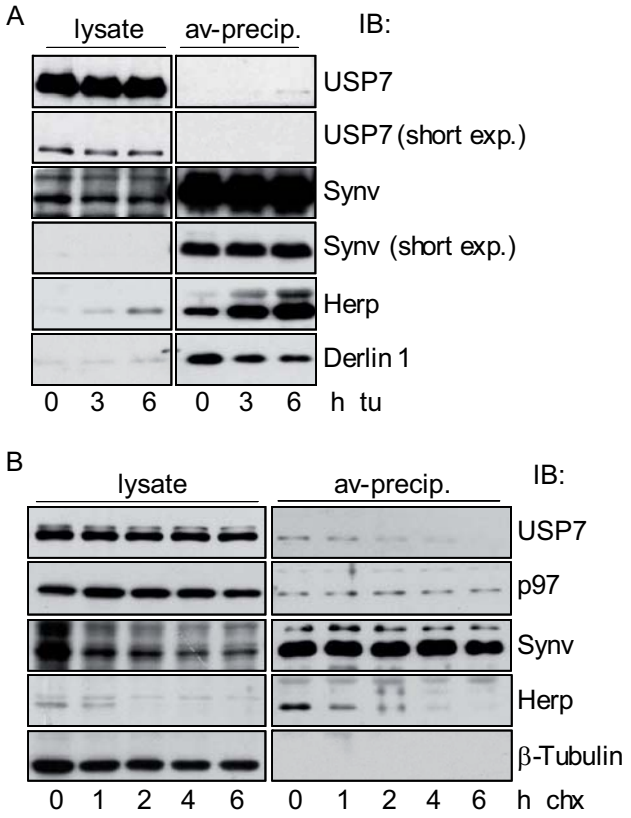

Figure 15: Association of Usp7 and Synoviolin. (**A**) HeLa cells, expressing hexahistidin-biotin (HTB)-tagged Synoviolin (clone 6, low expression level) were treated with 10 µg/mL tunicamycin (tu) for the indicated times. Then the cells were lysed in DBC containing buffer and Synoviolin-HTB was precipitated with streptavidin agarose. Proteins of the lysates and the precipitations were separated on SDS-PAGE and visualised by Western blot analysis using the specific antibodies as indicated. (**B**) HeLa cells, stably expressing Synoviolin-HTB (clone 6) were treated with 10 µg/mL tunicamycin for four h. After that 50 µg/mL cycloheximide was added and cells were lysed in DBC containing buffer at the indicated time points. The experiment was continued as described in (A). Exp=exposure; IB=immunoblot; h=; chx=cycloheximide; av-precip.=streptavidin agarose precipitation.

Usp7 and p97 remained stable for six hours of the cycloheximide chase, whereas Herp was completely degraded within this time period (Figure 15, B, lysate). The amount of Usp7 that was co-precipitated with Synoviolin decreased over time, which went along with a decrease of the amount of Synoviolin co-precipitated Herp (Figure 15, B, av-precip.). In contrast, the amount of p97 that was co-precipitated with Synoviolin remained constant. Taken together, these data demonstrate that Usp7 is associated with Synoviolin. Furthermore, the interaction of Usp7 and Synoviolin is positively correlated to the interaction of Herp with Synoviolin indicating that the association of Usp7 and Synoviolin depends on Herp.

Usp7 binds to the UBL domain of Herp, whereas Synoviolin directly interacts with a region distal from the UBL domain (Schulze, 2006; Schulze et al., 2005). Thus, Usp7 and Synoviolin are likely to interact with Herp simultaneously. Particularly in an ER stress situation, Herp may function as a linker of these proteins. To test this assumption, endogenous Herp, Usp7 and Synoviolin were tested for their mutual interactions. For this purpose HeLa cells were metabolically labelled and Herp or Usp7 were immunoprecipitated.

Before the labelling process, cells were subjected to ER stress for four hours to induce Herp but not Synoviolin protein expression in order to investigate Herp dependent interactions. The amount of co-precipitated Synoviolin or Herp was assessed by a second immunoprecipitation. Proteins were analysed by SDS-PAGE and autoradiography.

ER stress treatment led to the elevated expression of Herp, whereas the expression of Usp7 was not affected, which is in agreement with the previous results (Figure 16, left panel). When Herp was increased through ER stress treatment, also an increased amount of Synoviolin was co-precipitated with Herp (Figure 16, lane 7). In the absence of ER stress Herp or Synoviolin were not co-precipitated with Usp7 (Figure 16, -Tg, lanes 6, 8). In contrast, induction of ER stress led to the co-precipitation of Herp and Synoviolin with Usp7 (Figure 16, +Tg, lanes 6, 8). These data revealed that Usp7 is associated with Synoviolin predominantly in case of ER stress. This finding strongly suggests that Herp mediates the interaction of both enzymes.

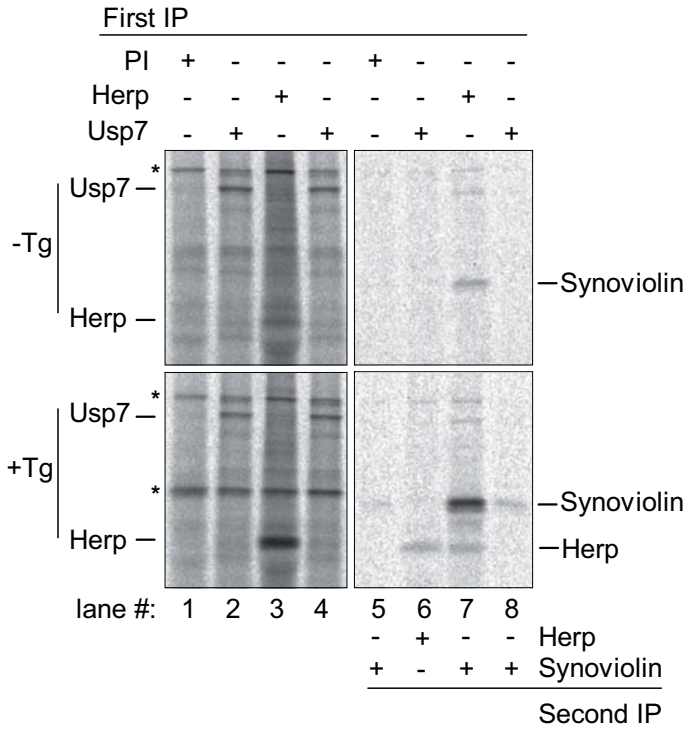

Figure 16: Herp dependent association of Usp7 and Synoviolin. HeLa cells were left untreated or treated with 2 µM thapsigargin for four h. Then metabolic labelling with ^{35}S-methionine was followed by the lysis of the cells in DBC containing buffer. For the first immunoprecipitation (IP) either pre-immune serum (PI) or specific antibodies as indicated were utilised. The dissociation of the co-precipitated proteins was accomplished by incubation in RIPA buffer. The resulting supernatants were subjected to a second immunoprecipitation using the indicated specific antibodies. Precipitated proteins were separated on SDS-PAGE and visualised by autoradiography. First IPs are depicted in the left, second IPs in the right hand panels. Nonspecific cross reactions are marked by an asterisk. tg=thapsigargin; Synv=Synoviolin.

3.2.2.3 Usp7 does not affect the stability of Herp or NHK

Usp7 binds and deubiquitinates its substrates thereby stabilising them, as shown for the tumor-suppressor protein p53 (Li et al., 2002). The direct interaction of Usp7 with Herp led to the assumption that Usp7 also deubiquitinates and stabilises Herp.

In fact, ubiquitination and degradation assays indicated that Usp7 is able to deubiquitinate and stabilise Herp, if both proteins are expressed at elevated levels in cells (M.Seeger, personal communication). To confirm these data in the study presented here, the impact of Usp7 on the stability of endogenous Herp was analysed using doxicycline inducible Usp7-shRNA expressing LS88 cells or Usp7 expressing LS89 cells and LS174T control cells according to (Meulmeester et al., 2005). For degradation analysis, cycloheximide was added to untreated or doxicycline treated cells.

Doxicycline treatment had no effect on the degradation of Herp as shown for the LS174T control cells (Figure 17, LS 174T). Surprisingly, despite an efficient depletion of Usp7 in doxicycline treated LS88 cells of more than 50% and an elevated expression of Usp7 of about three fold in doxicycline treated LS89 cells, the stability of endogenous Herp was not altered (Figure 17). Although the expression levels of Herp varied between the different cell lines, the degradation rates of Herp were unchanged in all examined samples indicating that Usp7 does not regulate the stability of Herp.

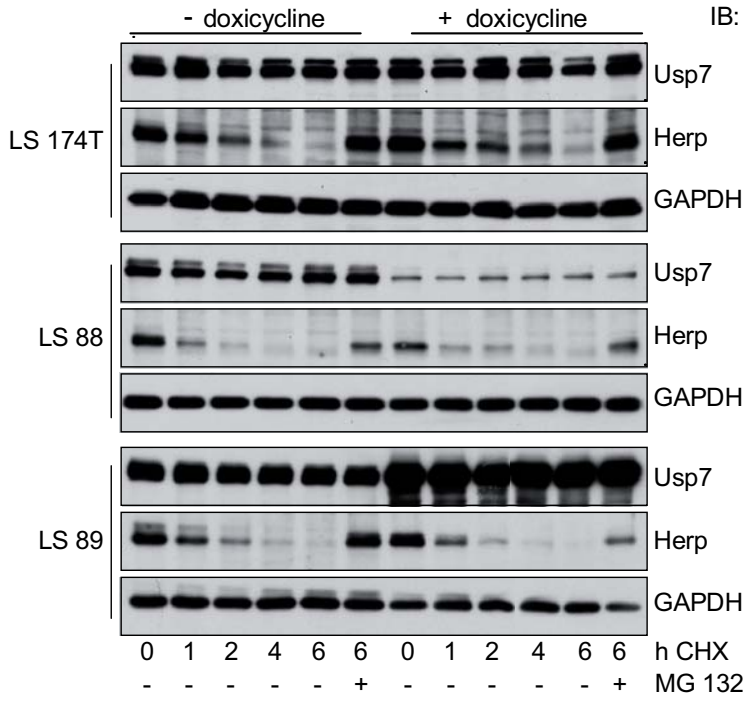

Figure 17: Impact of Usp7 on the stability of Herp. Doxicycline inducible Usp7-shRNA expressing LS88, doxicycline inducible Usp7 expressing LS89 or LS174T control cells were left untreated or treated with 1 µg/mL doxicycline for 72 h. Then 50 µg/mL cycloheximide (chx) were added to all samples and MG132 to a final concentration of 10 µM where indicated and cells were lysed at the indicated time points. Proteins were separated on SDS-PAGE and visualised by Western blot analysis using the specific antibodies as indicated. h=hour; IB=immunoblot.

In an ER stress situation, Herp recruits Usp7 to Synoviolin based complexes indicating that Usp7 may have a role in ERAD. In more detail, by interacting with the UBL domain of Herp, Usp7 could mediate the effect of Herp and promote the process of Synoviolin dependent protein degradation. Based on this hypothesis, Usp7 was tested for its influence on the degradation of the ERAD substrate NHK. For this purpose, HeLa cells expressing NHK were transfected with plasmids encoding either wild type or mutated inactive Usp7 (Usp7-C223S) and protein degradation was evaluated by a pulse chase analysis.

Overexpression of Usp7 and Usp7-C223S was about three fold and, similar to the endogenous protein, these constructs were stable for eight hours (Figure 18, A, lower panel). However, proteasome dependent degradation of NHK was not altered by the co-expression of either the active or inactive dominant negative Usp7 (Figure 18, A, upper panel). This result implies that Usp7 is not involved in the turnover of NHK.

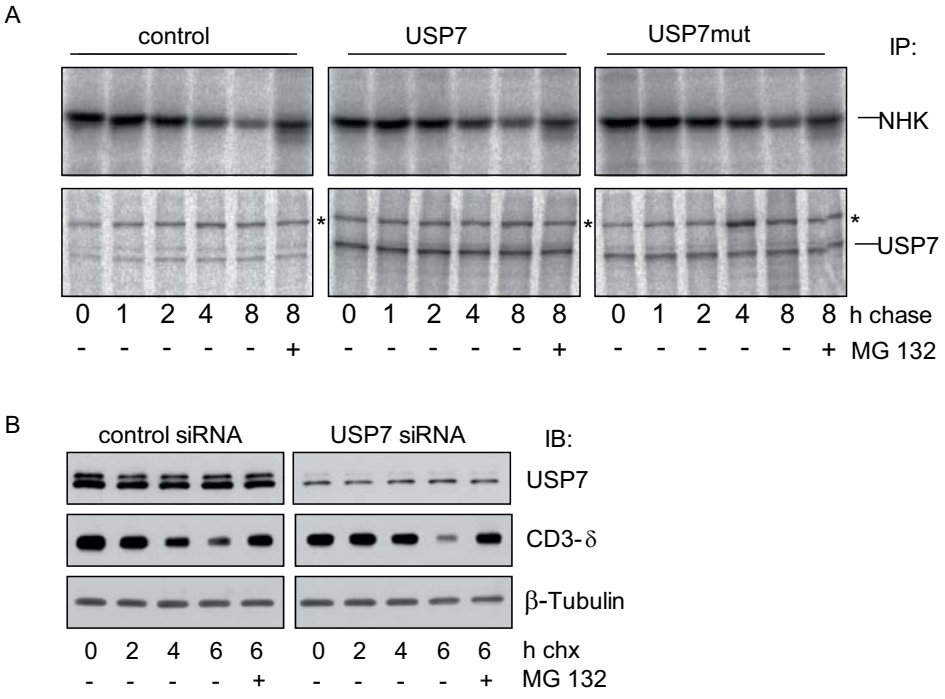

Figure 18: Usp7 effects on ERAD. (**A**) HeLa cells were cotransfected with plasmids encoding NHK and Usp7, the inactive C223S-mutant of Usp7 (Usp7mut) or the empty plasmid as control. At 48 h after transfection MG132 was added to a final concentration of 10 µM to the indicated samples and cells were metabolically labelled using ^{35}S-methionine and lysed at the indicated time points in RIPA buffer. Usp7 and NHK were immunoprecipitated (IP) from the lysates using the accordant specific antibodies. Proteins were separated on SDS-PAGE and visualised by autoradiography. An unspecific cross reaction is marked by an asterisk. (**B**) HeLa cells were co-transfected with siRNA specific for Usp7 or a control and 24 h later with a plasmid encoding CD3-δ. At 48 h after transfection 50 µg/mL cycloheximide and MG132 to a final concentration of 10 µM were added and cells were further incubated as indicated. Cells were lysed in RIPA buffer and proteins were separated on SDS-PAGE and visualised by Western blot analysis using the indicated specific antibodies. h=hour; IB=immunoblot; chx=cycloheximide.

To test whether Usp7 is plays a role in the Synoviolin mediated ERAD of an alternative substrate, its impact on the degradation of CD3-δ was analysed by cycloheximide chase analysis (Schulze et al., 2005).

For this purpose, Usp7 was depleted in CD3-δ expressing HeLa cells by the transfection of siRNA specific for Usp7. As expected, proteasome dependent degradation of CD3-δ occurred within four hours of the chase experiment (Figure 18, B). However, depletion of endogenous Usp7 protein to less than 50% of the original level did not alter the degradation rate of CD3-δ indicating that Usp7 is also not involved in CD3-δ turnover. Taken together, these results indicate that Usp7 does not influence degradation of Synoviolin dependent ERAD substrates and is unlikely to mediate the ERAD promoting action of Herp.

3.2.2.4 Herp is not involved in the regulation of p53

Although associated with Synoviolin via Herp, Usp7 seems not to be involved in the turnover of Synoviolin dependent ERAD substrates. However, Usp7 is known to bind, deubiquitinate and stabilise proteins such as p53 (Li et al., 2002). P53, in turn, is regulated by a variety of proteins besides Usp7. One of these p53 regulators is Synoviolin which was recently found to be implicated in p53 ubiquitination and degradation (Yamasaki et al., 2007). Since Herp links the two opposing p53 regulators Synoviolin and Usp7, it was assumed that Herp is also involved in the degradation of p53. To study the impact of Herp on the degradation of p53, wild type p53 protein expressing LS174T cells were chosen. Derived from these cells, stable transfectants expressing Herp specific shRNA upon induction by doxicycline were generated (see 2.2.6).

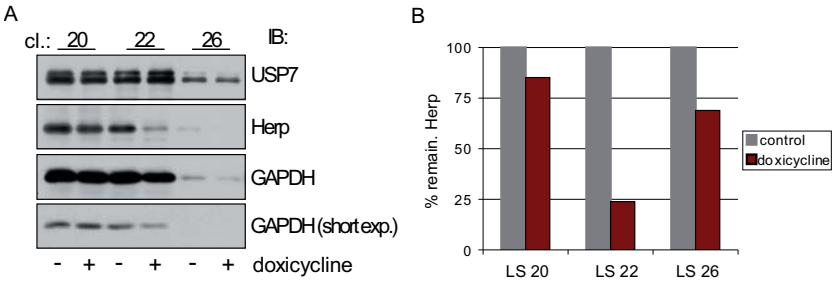

Figure 19: Generation of a cell line, expressing inducible Herp specific shRNA. (**A**) LS174T cells, expressing the Tet-repressor under the selection of blasticidine, were transfected with a zeocine-resistance carrying pTER encoding Herp specific shRNA. Blasticidine and zeocine resistant clones were selected and left untreated or treated with 1 µg/mL doxicycline for 72 h. After that 2 µM thapsigargin was added for four h. Then, cells were lysed and proteins were separated on SDS-PAGE and visualised by Western blot analysis using the specific antibodies as indicated. Here three exemplary positive clones, LS20, LS22 and LS26 are presented. (**B**) Densitometrical analysis of A. For knockdown evaluation, the signal intensities of Herp representing bands were normalised against the accordant GAPDH signals and the intensities of untreated samples were set as 100%. cl=clone; remain.=remaining; exp.=exposure; IB=immunoblot.

To evaluate the doxicycline dependent knockdown of Herp, zeocine resistant clones were analysed by Western blotting. Three of more than 20 positive cell clones are presented here, clones 20, 22 and 26 (Figure 19, A). Decreased expression of Herp upon two days of doxicycline treatment was observed in all three cell clones. However, with 75% clone 22 revealed the most effective depletion of Herp, demonstrated by the corresponding densitometrical analysis (Figure 19, B).

Thus, clone 22, designated as LS22, was chosen for the following experiments. To determine whether Herp regulates the stability of p53, untreated or doxicycline treated LS22 cells were subjected to a cycloheximide chase assay. To test whether a potential p53 regulation was dependent on ER stress, these cells were left untreated or additionally treated with thapsigargin prior to the chase experiment.

With doxicycline treatment a depletion of Herp by 75% in not stressed and by 50% in ER stressed cells was achieved (Figure 20, A). Cellular p97 levels remained constant despite Herp depletion. P53 was degraded within six hours and proteasome dependent, since the addition of MG132 inhibited p53 degradation. Depletion of Herp, independent of ER stress, did not alter the degradation rates of p53 indicating that Herp is not involved in the regulation of the turnover of p53. As Yamasaki and colleagues showed that the cellular level of p53 is increased upon depletion of Synoviolin in RKO cells, these cells were also used in the study presented here to analyse the impact of Herp on p53 steady state levels (Yamasaki et al., 2007). Transfection of Herp specific siRNA in RKO cells led to a dramatic decrease of cellular Herp protein levels (Figure 20, B). However, this depletion of Herp did not result in an alteration of the p53 steady state levels, which is in line with the data obtained from LS22 cells. This finding supports the idea that Herp does not regulate the stability of p53.

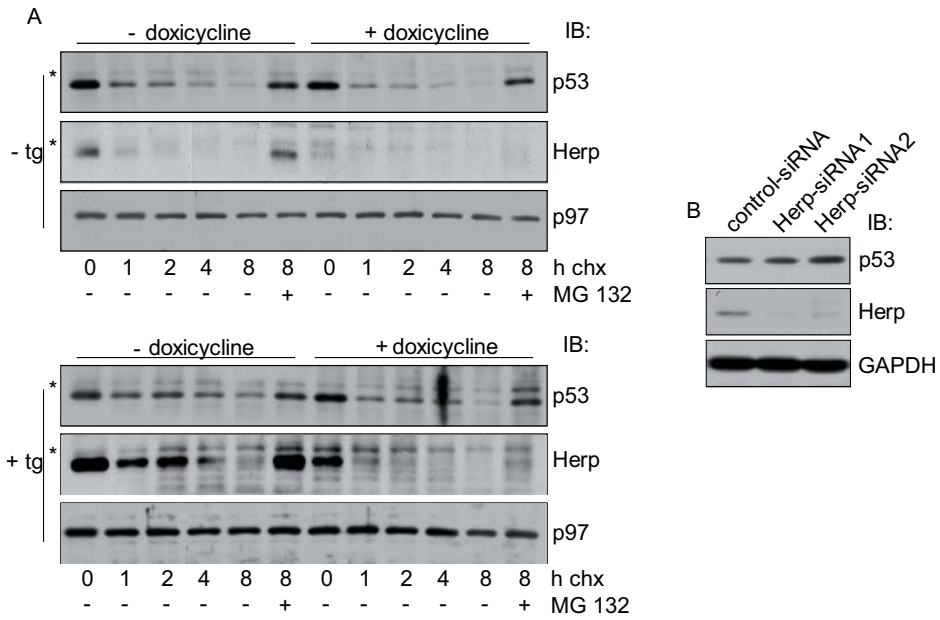

Figure 20: Role of Herp in p53 regulation. (**A**) Herp-shRNA inducible LS22 cells were left untreated or treated with 1 µg/mL doxicycline for 72h. The incubation was continued with 2 µM thapsigargin for four h where indicated before 50 µg/mL cycloheximide (chx) and MG132 to a final concentration of 10 µM were added. After the indicated times the cells were lysed and proteins were separated on SDS-PAGE and visualised by Western blot analysis using the accordant specific antibodies. (**B**) RKO cells were transfected with 50 nM (2) or 100 nM (1) of Herp specific siRNA or control siRNA. At 48 h after transfection the cells were lysed and proteins were separated on SDS-PAGE and visualised by Western blot analysis using the indicated specific antibodies. Tg=thapsigargin; h=hour; unspecific cross reactions are marked by an asterisk.

Yamasaki and colleagues not only showed the functional regulation of p53 by Synoviolin but also the interaction of both proteins (Yamasaki et al., 2007). In the study presented here, it was analysed whether the interaction of p53 and Synoviolin can also be detected in LS22 cells and whether Herp has an effect on this association. It was furthermore investigated whether p53 and Herp interact. Therefore, LS22 cells with normal versus depleted Herp expression were ER stress exposed and metabolically labelled. P53, Sel1L or Herp were immunoprecipitated (first IP) and the amount of co-precipitated Synoviolin or p53 was assessed by a second immunoprecipitation.

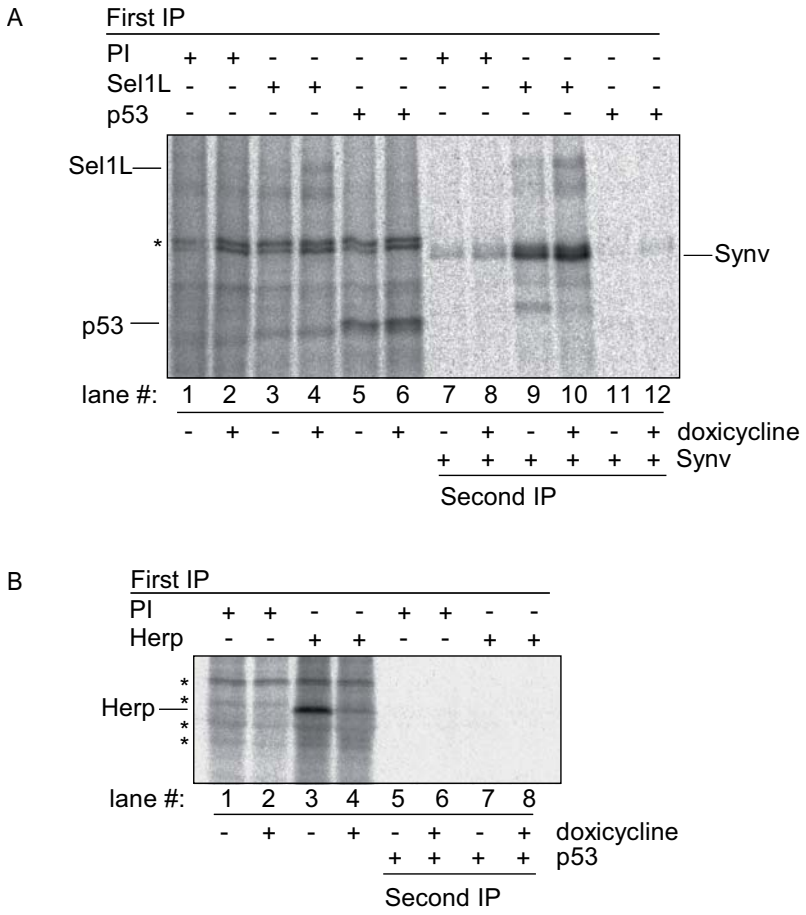

Figure 21: Interaction of p53 with Herp and Synoviolin. Herp-shRNA inducible LS22 cells were left untreated or treated with 1 µg/mL doxicycline for 72 h followed by incubation with 2 µM thapsigargin for 8 h. Afterwards the cells were metabolically labelled using ^{35}S-methionine and lysed in DBC containing buffer. The lysates were subjected to immunoprecipitation (first IP) either with pre-immune serum (PI) or specific antibodies as indicated. Co-precipitated proteins were dissociated in RIPA buffer and subjected to a second immunoprecipitation (Second IP) either with pre-immune serum or with (**A**) a Synoviolin specific antibody or (**B**) a p53 specific antibody as indicated. Precipitated proteins were separated on SDS-PAGE and visualised by autoradiography. Synv=Synoviolin; unspecific cross reactions are marked by an asterisk.

Independent of the depletion of Herp, Synoviolin was co-precipitated with Sel1L (Figure 21, A, lanes 9, 10). Surprisingly, Synoviolin was not co-precipitated with p53, irrespective of Herp depletion (Figure 21, A, lanes 11,12). In addition, p53 was also not found to interact with Herp (Figure 21, B) indicating that p53 is not associated with Synoviolin based complexes.

3.2.3 Ancient ubiquitous protein 1 (AUP1) is associated with Synoviolin

Herp is able to modulate the composition of Synoviolin based complexes by directly binding and recruiting proteins, as shown here for Usp7. Another protein which is linked to Synoviolin via Herp or its UBL-domain is not known. As Herp is dynamically acting at Synoviolin based complexes, it potentially affects the association of further proteins with the ERAD complex. A protein that had not been tested for being associated with Synoviolin dependent on Herp is the Ancient ubiquitous protein 1 (AUP1), which was recently described to be associated with Sel1L and to be involved in the dislocation of MHC class I molecules in cells expressing the viral protein US11 (Mueller et al., 2008).

3.2.3.1 Herp regulates the association of AUP1 with Synoviolin

The association of AUP1 with Sel1L, which in turn interacts with Synoviolin, indicates that AUP1 and Synoviolin are associated as well. Furthermore, it was suggested that the association of AUP1 and Synoviolin depends on Herp. To address this issue, LS22 cells with basal or depleted Herp levels and exposed to ER stress were metabolically labelled. Herp, Sel1L or AUP1 were immunoprecipitated and co-precipitated proteins were analysed.

In fact, it was observed that AUP1 is associated with Synoviolin. As depicted in the autoradiography, Synoviolin was co-precipitated with AUP1 (Figure 22, A, lanes 7, 8) and with Sel1L (Figure 22, A, lanes 5, 6). Furthermore, the association of AUP1 with Synoviolin was decreased by 25% in case of Herp depletion. This was not observed for Synoviolin which interacted with Sel1L indicating that ER stress leads to the selective Herp dependent association of proteins with Synoviolin. The relative amounts of Synoviolin, co-precipitated with Herp, Sel1L and AUP1 are given in the densitometrical analyses of the Western blots, which also demonstrate that the association of AUP1 with Synoviolin depends on Herp (Figure 22, B). To confirm these data, an analogous co-precipitation experiment was performed *vice versa*. LS22 cells were treated as described above excluding radioactive labelling and Synoviolin was immunoprecipitated. Co-precipitated proteins were analysed by Western blotting.

Doxicycline treatment of LS22 cells led to an efficient decrease of Herp protein expression (Figure 22, C, lanes 2, 4). Herp downregulation resulted in the reduced co-precipitation of Herp with Synoviolin (lanes 10, 12). Along with the depletion of Herp, also the amount of Usp7, co-precipitated with Synoviolin, was reduced. In addition, the amount of AUP1, co-precipitated with Synoviolin, was decreased under the conditions of Herp depletion (lanes 10, 12). This effect was independent of ER stress, as also shown by the densitometrical analysis (Figure 22, D).

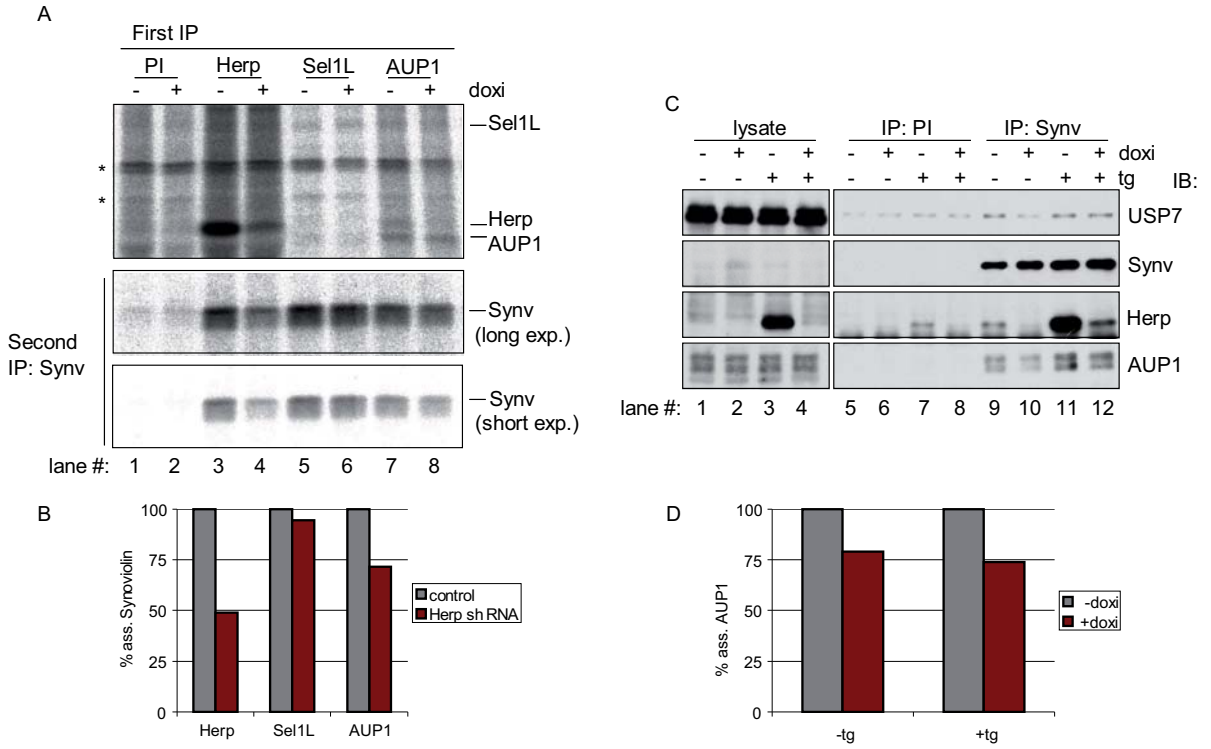

Figure 22: Effect of Herp on the association of AUP1 with Synoviolin. (**A**) Herp-shRNA inducible LS22 cells were left untreated or treated with 1 µg/mL doxicycline for 72 h. After the addition of thapsigargin to a final concentration of 2 µM, the incubation was continued for six h. Cells were metabolically labelled with ^{35}S-methionine, lysed in DBC containing buffer and subjected to immunoprecipitation (first IP) either with pre-immune serum (PI) or the indicated specific antibodies. Co-precipitated proteins were dissociated in RIPA buffer and supernatants were subjected to a second immunoprecipitation (Second IP) with the Synoviolin specific antibody. Precipitated proteins were separated on SDS-PAGE and visualised by autoradiography. Unspecific cross reactions are marked with an asterisk. (**B**) Densitometrical analysis of (A). For evaluation of protein association, the signal intensities of Synoviolin representing bands of not doxicycline treated samples were set as 100%. (**C**) LS22 cells were treated with doxicycline and thapsigargin as described in (A). After lysis in DBC containing buffer immunoprecipitation was performed using either pre-immune serum (PI) as a control or the specific Synoviolin antibody (Synv). Proteins of the lysates and the IPs were separated on SDS-PAGE and visualised by Western blot analysis using the indicated specific antibodies. (**D**) Densitometrical analysis of (C). For evaluation of protein association, the signal intensities of AUP1 bands were normalised for that of Synoviolin bands. AUP1 bands representing not doxicycline treated samples were set as 100%. Exp.=exposure; IB=immunoblot; tg=thapsigargin; doxi=doxicycline.

3.2.3.2 AUP1 binds to Synoviolin

AUP1 is likely to be a component of Synoviolin based complexes dependent on Herp. Hence, it was hypothesised that AUP1 directly interacts with Herp comparable to Usp7. To test this hypothesis, *in vitro* interaction studies were performed. Therefore, either the N- or the C-terminus of the Herp protein was expressed as GST fusion protein in *E.coli*. Additionally, GST-tagged Synoviolin was utilised. Recombinant full length AUP1 and N-terminally truncated AUP1 were generated as Hexa-histidine (His) tagged proteins. The latter was thought to reveal an improved solubility, since it lacks a predicted GPI anchor. The GST fusion proteins were bound to GSH sepharose and incubated with the AUP1 proteins *in vitro*.

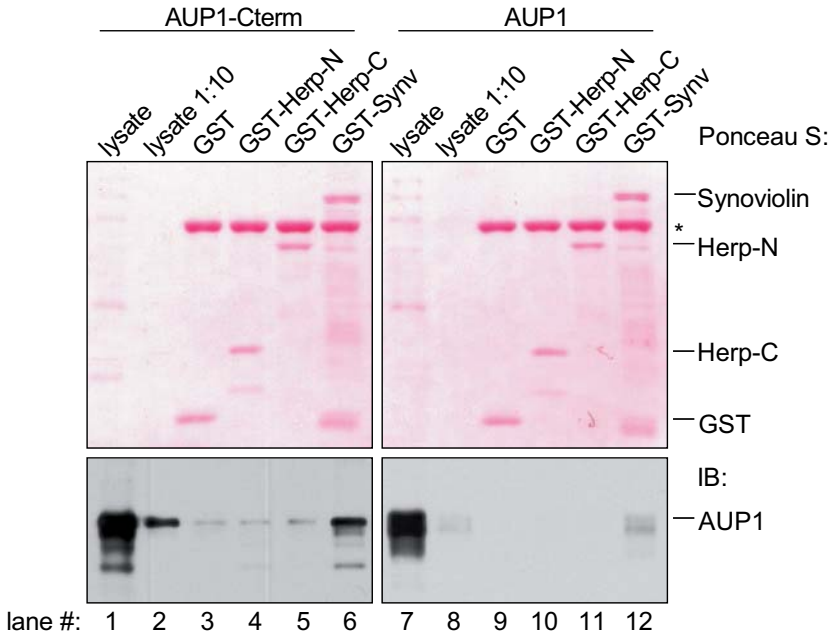

Figure 23: Direct interactions of AUP1. GST alone or GST fusion constructs of the Herp-C-terminus (Herp-C, aa 304-391), the Herp-N-terminus (Herp-N, 1-260) and Synoviolin (Synv) were expressed in *E.coli*, immobilised on glutathione sepharose and incubated with lysates from *E.coli* containing the Hexa-Histidin (His)-tagged AUP1 or the His-tagged AUP1-C-terminus. The upper panel shows the Ponceau S stained precipitated proteins after SDS-PAGE and the blotting procedure to visualise equal loading of the glutathione beads. The asterisk (*) marks BSA which was included in the washing buffer. Co-precipitated AUP1 was visualised by Western blot analysis using the specific AUP1-antibody.

Compared to the control, neither the truncated nor the full length AUP1 protein showed an interaction with any of the Herp versions. This finding applied to both utilised AUP1 proteins (Figure 23, lanes 4, 5, 10, 11). Instead, both AUP1 versions were found to directly interact with Synoviolin (lanes 6, 12). Thus, this experiment demonstrated that AUP1 directly interacts with the E3 ligase Synoviolin but not with Herp. This finding indicates that Herp also regulates the direct interaction of accessory proteins with Synoviolin.

3.2.3.3 AUP1 is required for the degradation of NHK

Mueller and colleagues reported that the elevated expression of a GFP-AUP1 fusion protein impairs the dislocation of MHC class I molecules in viral US11 protein expressing cells suggesting that AUP1 regulates the Synoviolin mediated ERAD process (Mueller et al., 2008). Thus, in this study it was tested whether AUP1 is involved in Synoviolin mediated ERAD. Therefore, degradation of the Synoviolin and Herp dependent substrate NHK was analysed after depletion of AUP1 in HeLa cells using specific siRNA.

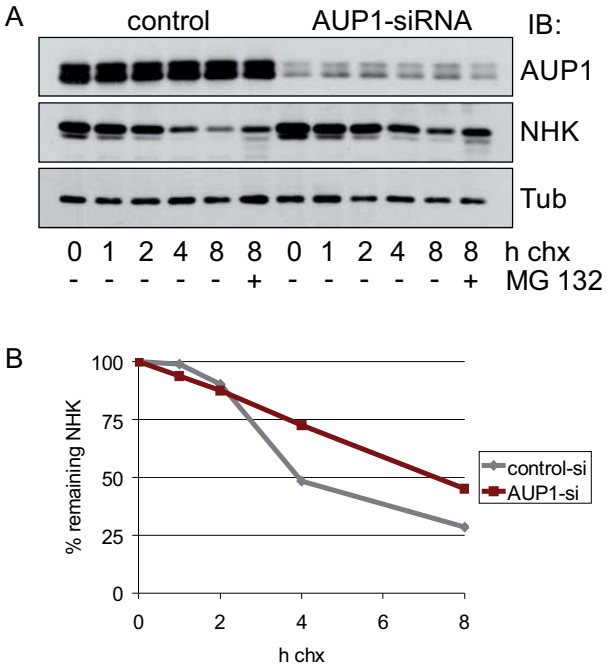

Figure 24: Impact of AUP1 on ERAD. (**A**) HeLa cells were co-transfected with siRNA specific for AUP1 and 48 h later with a plasmid encoding the substrate NHK. At 72 h after initial transfection 50 µg/mL cycloheximide (chx) and MG132 to a final concentration of 10 µM were added and further incubation was done as indicated. Cells were lysed in RIPA buffer and proteins were separated on SDS-PAGE and visualised by Western blot analysis using the indicated specific antibodies. (**B**) Densitometrical analysis of A. For evaluation of protein degradation, the signal intensities of NHK bands were normalised against the accordant β-tubulin signals and NHK signals of the '0 h' sample were set as 100%. Tub= β-tubulin

The reduction of AUP1 protein expression by more than 50% was obtained, when using specific siRNA (Figure 24, A, AUP1). AUP1 remained stable for eight hours of cycloheximide treatment. In the Western blot, AUP1 was depicted as a constant double band presumably due to the existence of stable isoforms revealing different molecular weights. Depletion of AUP1 led to the stabilisation of NHK as observed by the Western blot showing the impaired degradation of this substrate (Figure 24, A, NHK), which is also shown by the corresponding densitometrical analysis (Figure 24, B). This result demonstrates that AUP1 is required for the degradation of Synoviolin and Herp dependent NHK.

3.2.3.4 The AUP1-CUE domain is required for the efficient degradation of NHK

A search for domains within AUP1 playing a role in the ubiquitin proteasome system and potentially in the process of ERAD revealed that AUP1 contains a CUE domain, which was described to bind ubiquitin and ubiquitinated proteins (Hurley et al., 2006). Here, it was tested whether the CUE domain of AUP1 is involved in mediating the action of AUP1 within ERAD of NHK. A site directed mutagenesis was performed to substitute amino acids leucine 333 and 334 by alanine (L333A-L334A) in order to obtain a protein potentially incapable of ubiquitin binding, since leucine 333 lies within the CUE domain and was stated to bind ubiquitin (Kang et al., 2003). HeLa cells co-expressing either AUP1-myc or the CUE domain mutant AUP1-mut-myc and NHK were utilised for degradation analysis.

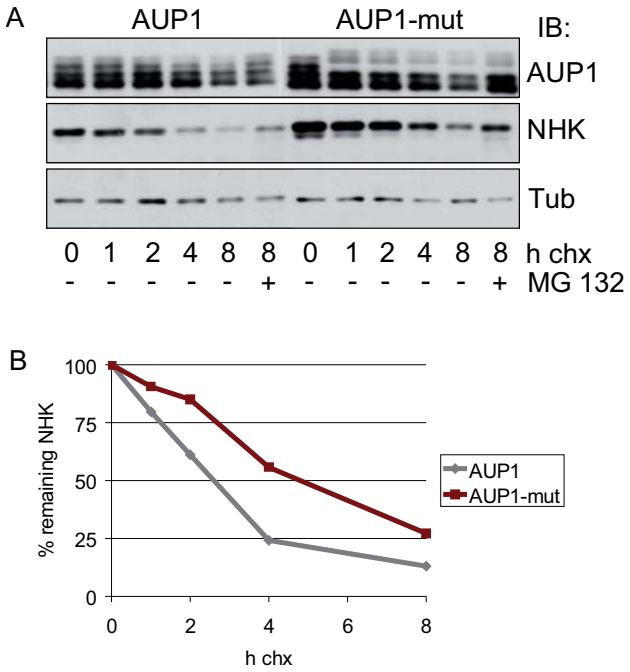

Figure 25: Impact of the AUP1-CUE domain on ERAD. (**A**) HeLa cells were co-transfected with plasmids encoding either AUP1-myc (AUP1) or the CUE domain mutant AUP1-L333A-L334A-myc (AUP1-mut) together with NHK. At 48 h after transfection 50 µg/mL cycloheximide (chx) and MG132 to a final concentration of 10 µM were added and further incubation was done as indicated. Cells were lysed in RIPA buffer and proteins were separated on SDS-PAGE and visualised by Western blot analysis using the indicated specific antibodies. (**B**) densitometrical analysis of A. For evaluation of protein degradation, the signal intensities of NHK bands were normalised against the accordant β-tubulin signals and NHK signals of the '0 h' sample were set as 100%. Tub= β-tubulin

Using an antibody specific for AUP1, both AUP1-myc and AUP1-mut-myc were detected as triple band in the Western blot due to the additional myc-tag fused to the protein (Figure 25, A, AUP1). In contrast to endogenous AUP1, these myc fusion proteins were not stable but gradually degraded within the chase period of eight hours. This unforeseen instability of AUP1 was presumably caused by the introduction of the myc-tag. AUP1-myc degradation seemed to depend on the proteasome, as it was partly inhibited in the presence of MG132. Compared to the elevated expression of wild type AUP1, the co-expression of the CUE domain mutant of AUP1 led to the stabilisation of NHK (Figure 25, A) which is also depicted in the corresponding densitometrical analysis (Figure 25, B). Thus, the CUE domain of AUP1 has an important role for the function of AUP1 promoting ERAD.

Summarising this second part of the study, it was demonstrated that Herp is capable of modulating the composition of Synoviolin based ERAD complexes. Herp does not influence Synoviolin oligomerisation but has a role in the recruitment of accessory proteins. Herp either directly binds these proteins, as shown for Usp7, or facilitates their interaction with Synoviolin, as shown for AUP1. Usp7 did not influence Synoviolin dependent ERAD, whereas AUP1, directly interacting with Synoviolin, improved ERAD dependent on its CUE domain. Most likely, further proteins associate with Synoviolin in a Herp dependent manner.

3.3 Characterisation of Herp2

Herp seems to have a unique function within ERAD. However, there is the possibility that other ubiquitin domain proteins similar to Herp might function in the same way and therefore are able to substitute Herp. A screen for such proteins revealed that apart from Herp three other UDPs contain a UBL domain and potential transmembrane domains, which were classified as one family called the mubl family (Schulze, 2006). One of these family members, Herp2, reveals 40% amino acid sequence identity with Herp. The UBL domains of both proteins are even 53% identical. Thus, Herp and Herp2 were suggested to be paralogues and share functions in the cell. To decide whether Herp2 has the ability to take over the tasks of Herp at all, Herp2 was tested for showing typical properties of Herp such as the special dynamics and the association with other proteins.

3.3.1 Herp2 reveals dynamics different from Herp

Herp protein expression is induced by ER stress. In contrast, treatment of cells with β-mercaptoethanol (β-ME), which causes ER stress by the alteration of the cellular redox status, does not lead to the induction of Herp2 expression (Schulze, 2006). To prove the latter result, the UPR triggering agents tunicamycin, preventing N-glycosylation, and thapsigargin, inhibiting the ER-resident Ca^{2+}-ATPase, were tested with regard to Herp2 induction to be able to make a general statement on the correlation of ER stress and Herp2 expression. HeLa cells were treated with tunicamycin or thapsigargin for up to ten hours and Herp2, Herp and Xbp1 mRNA expression was analysed by reverse transcriptase PCR. Xbp1 mRNA is a mediator of UPR signalling and spliced upon ER stress treatment (Sidrauski et al., 1996). Herp2, Herp and Grp78 protein expression was assessed from identical samples by SDS-PAGE and Western blotting.

Reverse transcriptase PCR analysis revealed that Xbp1 mRNA is spliced at one hour of ER stress treatment indicating the activation of the UPR (Figure 26, A). As expected, the expression of Herp mRNA was induced after four hours of ER stress treatment. In contrast, Herp2 mRNA expression (full length or spliced) was not induced by ER stress induction, shown for both utilised ER stress agents. Protein expression analysis led to the same observations. Grp78 (BiP), a marker for ER stress, was induced at four hours of treatment with thapsigargin or tunicamycin. Herp protein expression was also induced after four hours of ER stress treatment (Figure 26, B). In contrast, protein expression of Herp2 remained almost constant for ten hours of ER stress treatment.

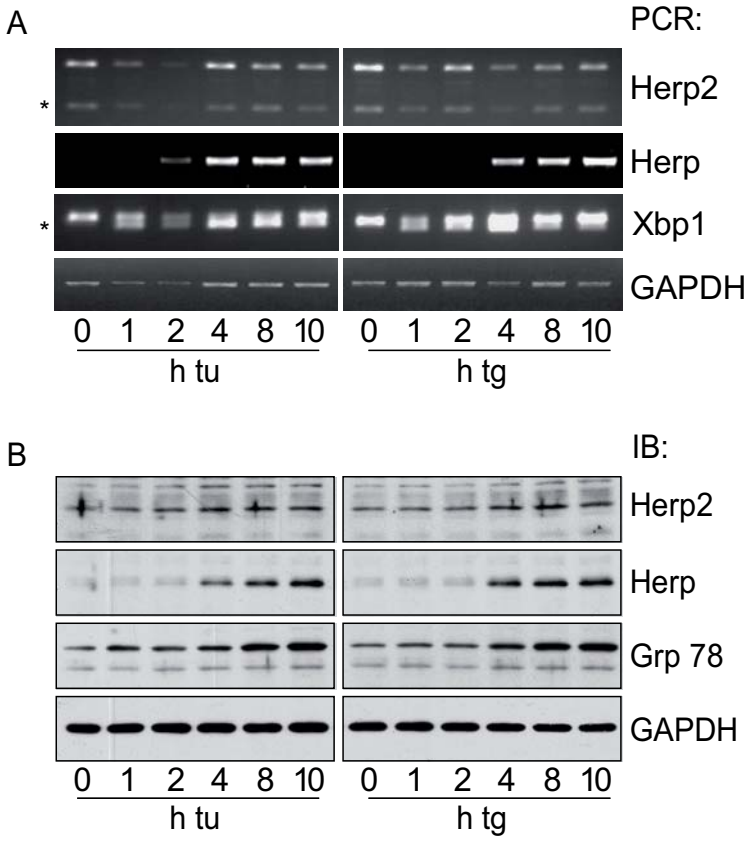

Figure 26: ER stress induction of Herp and Herp2. HeLa cells were left untreated or treated with 10 µg/mL tunicamycin or 2 µM thapsigargin for the indicated times. Cells were harvested and divided for RNA-isolation or lysis in RIPA buffer. (**A**) RNA was reversely transcribed into accordant cDNA which was subjected to PCR using the specific primers as indicated. DNA fragments were separated on agarose gels and visualised using ethidiumbromide staining. Asterices indicate the splice variants of Herp2- and Xbp1-mRNA (**B**) Proteins of the lysates were separated on SDS-PAGE and visualised by Western blot analysis using the indicated specific antibodies. h=hour; tg=thapsigargin; tu=tunicamycin; IB=immunoblot.

Next, Herp2 was tested for its turnover rates. Therefore, a pulse chase experiment was performed using HeLa cells to study endogenous protein turnover. Herp and Herp2 were immunoprecipitated and analysed for their degradation rates.

Meeting the expectations, Herp was completely degraded within eight hours of chase and its degradation was proteasome dependent (Figure 27, A). In contrast, Herp2 was only gradually degraded. After eight hours of chase, 75% of the labelled Herp2 population was still present. In addition, Herp2 degradation showed not to be proteasome dependent. Different degradation curves are depicted in the corresponding densitometrical analysis (Figure 27, B). These data indicate that Herp2 has a half-life of more than eight hours. In conclusion, Herp2, compared to Herp, is rather stable and seems not to be a substrate of the ubiquitin proteasome system.

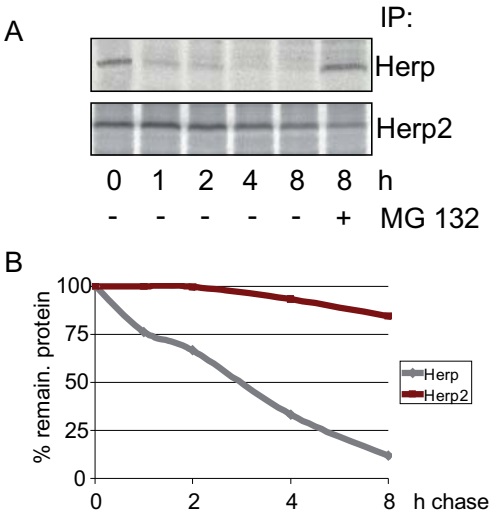

Figure 27: Degradation of Herp and Herp2. (**A**) HeLa cells were metabolically labelled using ^{35}S-methionine and lysed at the indicated time points in RIPA buffer. Herp or Herp2 were immunoprecipitated (IP) using specific antibodies. MG132 was added to a final concentration of 10 µM where indicated. Precipitated proteins were separated on SDS-PAGE and visualised by autoradiography. (**B**) Densitometrical analyis of (A). For evaluation of protein degradation, the signal intensities of Herp or Herp2 '0 h' samples were set as 100%.

Taking the data on cellular Herp2 turnover together, Herp2 is not induced by ER stress but continuously expressed. Furthermore, Herp2 is stable and not degraded by the UPS. These findings indicate that Herp2 does not reveal the same dynamics as Herp, which distinguish Herp from other proteins implicated in Synoviolin mediated ERAD.

3.3.2 Herp and Herp2 form homo- and heterooligomers

Recent experiments revealed that the precipitation of HTB-tagged Herp from cells led to the co-precipitation of endogenous Herp indicating that Herp is able to homooligomerise. Furthermore, these experiments also indicated that Herp2 binds to Herp (M. Seeger, personal communication). These findings suggested that Herp and Herp2 might be associated with Synoviolin concomitantly. Therefore, in the study presented here it was tested whether Herp and Herp2 are associated with each other and whether each of these UDPs is able to homooligomerise. Lysates from cells expressing either HTB-tagged Herp or Herp2 only or together with untagged Herp or Herp2 were subjected to streptavidin agarose precipitation. Due to the size differences of tagged and untagged proteins, oligomer formation of the UDPs could be visualised analogous to Synoviolin oligomerisation (3.2.1). Lysates and precipitates were analysed by Western blotting.

The expression levels of untagged and tagged Herp and Herp2 proteins were sufficient to evaluate putative oligomers (Figure 28, lysate). Homooligomers are represented by double bands in the accordant Western blots (Figure 28, precipitation) showing that Herp-HTB was able to interact with endogenous Herp (lane 11). This effect was even more striking when Herp was co-expressed (lane 15). Comparably, Herp2-HTB also interacted with co-expressed Herp2 (lane 16) and to a lesser extend with endogenous Herp 2 (lane 13) indicating that both UDPs are indeed able to homooligomerise.

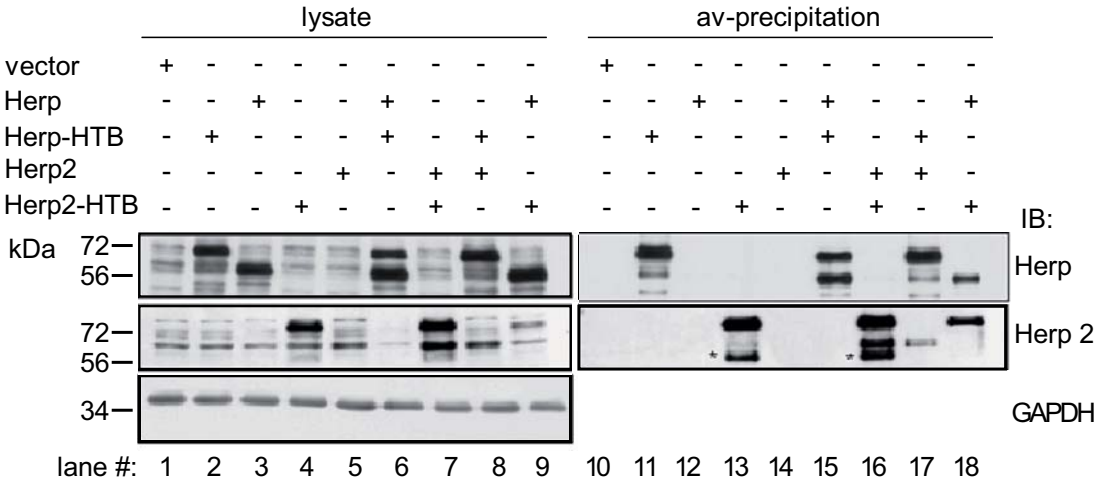

Figure 28: Homo- and heterooligomerisation of Herp and Herp2. HeLa cells were transfected with either an empty pSG5 plasmid as control or cotransfected with plasmids encoding untagged or Hexahistidin-Biotin (HTB)-tagged Herp or Herp2 as indicated. At 48 h after transfection cells were lysed in DBC containing buffer and HTB-constructs were precipitated with streptavidin agarose. Proteins of the lysates and the precipitations were separated on SDS-PAGE and visualised by Western blot analysis with the indicated specific antibodies. An unspecific cross reaction is marked by an asterisk. Protein standard sizes are given in kDa (kilodalton). av=streptavidin agarose; IB=immunoblot.

Concerning heterooligomerisation, co-expression of Herp-HTB with Herp2 resulted in the interaction of both proteins (lane 17), whereas no association of Herp-HTB with endogenous Herp2 could be observed (lane 11). Inversely, overexpression of Herp2-HTB did not lead to the co-precipitation of endogenous Herp (lane 13). Only the co-expression of Herp with Herp2-HTB resulted in a visible interaction of both proteins (lane 18). Taken together, Herp and Herp2 were able to homooligomerise, whereas heterooligomerisation was only observed upon the elevated expression of both UDPs, indicating that Herp-Herp2 heterooligomers are not likely to be physiological.

3.3.3 Herp2 is associated with Synoviolin based complexes

The association with Synoviolin based ERAD complexes is assumed to be a precondition for Herp acting as an ERAD promoting protein. Therefore, Herp2 was suggested to also be a part of these ERAD complexes in order to be able to function in the same way as Herp. Thus, Herp2 may be associated with Synoviolin. To uncover whether this notion holds true, Herp2 was tested for its co-migration with components of Synoviolin based ERAD complexes in a density gradient. Therefore, lysates of HeLa cells were loaded on top of a 15-50% glycerol gradient and subjected to ultracentrifugation. Samples from the resulting fractions were analysed by Western blotting. This experiment demonstrated that Herp2 co-migrates with the other tested components of Synoviolin based complexes such as Synoviolin itself, Herp, p97 and Derlin-1 within the identical fractions of glycerol gradients (Figure 29, A). In fact, this indicates that Herp2 is a novel component of Synoviolin based complexes.
To test whether Herp2 is associated with Synoviolin, Herp-HTB or Synoviolin-HTB, expressed in HeLa cells, was subjected to streptavidin agarose precipitation and Western blot analysis.
With this experiment it was demonstrated that endogenous Synoviolin is associated with Herp2 and Herp to a comparable extend. In contrast, the Synoviolin homologue Gp78 was not co-precipitated with Herp or Herp2 indicating that Herp2 specifically interacts with Synoviolin based ERAD complexes (Figure 29, B). The other way round, when expressing Synoviolin as HTB-fusion protein, endogenous Herp as well as Herp2 were co-precipitated. Thus, Herp2 is associated with Synoviolin.

To summarise the third part of this study, Herp2 was shown to be a novel component of Synoviolin based ERAD complexes. Like Herp, Herp2 is able to homooligomerise and interacts with Synoviolin. In contrast to Herp, Herp2 expression is not induced by ER stress, Herp2 is stable and not a substrate of the UPS indicating that Herp2 reveals dynamics different from Herp. In addition and in contrast to Herp, Herp2 does not bind Usp7. As Herp and Herp2 did not show an interaction, it is likely that both UDPs exclude each other from Synoviolin based complexes. Taken together, Herp2 most likely has a function different from Herp.

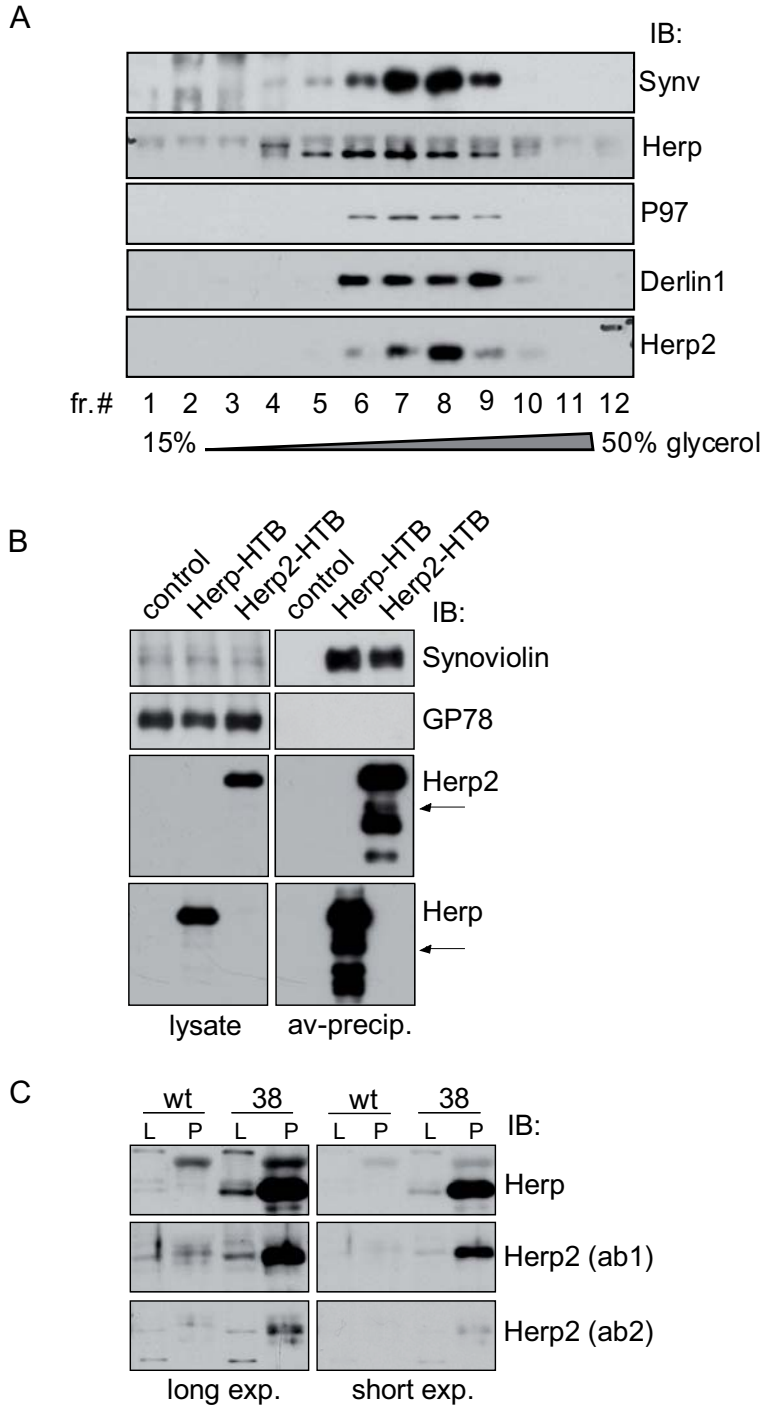

Figure 29: Association of Herp2 with Synoviolin based complexes. (**A**) HeLa cells were lysed in DBC containing buffer and proteins were separated on a 15-50% glycerol gradient by ultracentrifugation. The proteins of the fractions were precipitated with trichloro-acetic acid, solubilised in SDS sample buffer and separated on SDS-PAGE and visualised by Western blot analysis using the specific antibodies as indicated. (**B**) HeLa cells were transfected with either the empty plasmid (control) or plasmids encoding Hexahistidin-Biotin (HTB)-tagged Herp and Herp2. At 24 h after transfection cells were lysed in DBC containing buffer and HTB-tagged proteins were precipitated using streptavidin agarose. Proteins of the lysates and the precipitations were separated on SDS-PAGE and visualised by Western blot analysis using the specific antibodies as indicated. Arrows indicate the endogenous proteins. (**C**) HeLa or HeLa stably expressing hexahistidin-biotin (HTB)-tagged Synoviolin (clone 38, high expression level) were subjected to a streptavidin agarose precipitation assay and further analysed as described in b). Herp2 was detected by specific polyclonal rabbit-antibodies, which were generated in two different animals (ab1 and ab2). fr#=fraction number; IB=immunoblot; L=lysate; av=streptavidin; exp.=exposure; ab=antibody.

4 Discussion

4.1 The role of the dynamics of Herp in ERAD

The Herp protein, one of the ER stress induced components of Synoviolin based ERAD complexes, plays a crucial role for the effective degradation of misfolded proteins. Herp protein expression is induced early, whereas other Synoviolin associated ERAD proteins such as Synoviolin itself or Derlin-1 are induced at later time points upon ER stress (Donati et al., 2006; Kokame et al., 2000; Kokame et al., 1996; Ma and Hendershot, 2004; Oda et al., 2006). Some of the Synoviolin associated proteins such as Usp7 and p97 are even not induced by ER stress, as it has been shown here. Data on ER stress induction of Synoviolin associated proteins are compiled in Table 16 (Appendix). The only protein that is as fast induced by ER stress as Herp is the Hsp70-like ER chaperone Grp78 (BiP), which is also part of Synoviolin complexes (Chigurupati et al., 2009; Donati et al., 2006; Hosokawa et al., 2008; Kokame et al., 1996). Since Grp78 mediates ERAD substrate recognition and UPR pathway activation, it acts as a central player at the luminal side of the ERAD machinery (see 1.2.2). The fact that the majority of Herp is located in the cytoplasm indicates that Herp, according to Grp78, plays an important role at the cytoplasmatic side of the ERAD system (Kokame et al., 2000).

The half-life of Herp is relatively short compared to half-lives of other Synoviolin associated proteins such as Synoviolin or Usp7 (Boutell et al., 2005; Kikkert et al., 2004; Sai et al., 2003). In contrast to Herp, Grp78 is also a stable protein revealing a half-life of about 50 hours (Knittler, 1992). Comparing literature data on ER stress inducibility and stability of Synoviolin complex components indicates that Herp reveals the highest turnover rates of Synoviolin complex components (Table 16 / Appendix). The fact that Herp, compared to other components of Synoviolin based ERAD complexes, shows a different cellular turnover rate points towards a unique function of Herp in the process of ERAD.

4.1.1 The turnover of Herp at Synoviolin based ERAD complexes

As Herp protein expression is induced earlier upon ER stress than the expression of other Synoviolin associated proteins, Herp was reasoned to be a prerequisite for the assembly of Synoviolin based complexes. If Herp would act as an assembly factor, it should be found exclusively associated with assembling precursor complexes. The function as such an assembly factor has been reported for another protein, the proteasome maturation protein (POMP). POMP mediates the assembly of the 20S proteasome and is associated only with precursor complexes. Once matured, 20S proteasomes degrade POMP (Griffin et al., 2000).

However, the work presented here showed that Herp is not exclusively associated with *de novo* synthesised Synoviolin but can also be found with mature populations of this E3 ligase. Therefore, Herp acts not only as an assembly factor like POMP, but very likely also has a role for the readily assembled Synoviolin based complexes.

To evaluate whether Herp is associated with functioning ERAD complexes, its interaction with the AAA-ATPase p97 was tested in the study here. Ye and colleagues found that p97 is able to extract a ubiquitinated substrate from the ER membrane, only if this substrate enters the cytoplasmatic side through the process of retrotranslocation (Ye et al., 2003). Furthermore, Cdc48p, the yeast homologue of p97, was also demonstrated to associate exclusively with functional Hrd1p based ERAD complexes (Gauss et al., 2006). These findings lead to the conclusion that p97 is an indicator for readily assembled and functional ERAD complexes.

The study presented here in fact indicated that Herp and p97 bind to Synoviolin simultaneously. It was also shown that Herp populations are subject to exchange at Synoviolin complexes. Furthermore, co-precipitation experiments using Herp-ZZ revealed that p97 is associated with Herp (Schulze et al., 2005). The demonstrated interaction of Herp with p97 excludes Herp from being restricted to precursor complexes as in case of POMP, and suggests that Herp acts during the process of ERAD. Nevertheless, as the UDP was found to be associated with *de novo* synthesised Synoviolin, Herp might also assist Synoviolin complex assembly. In addition, the fact that Herp molecules are continuously replaced at Synoviolin indicates that the presence of Herp at the ERAD machinery is required for protein processing. Apart from Herp, no other protein being exchanged at Synoviolin complexes is known. Only ERAD substrates such as NHK that transiently interact with these complexes have been described (Hosokawa et al., 2008).

The continuous turnover of a protein at an enzymatic complex is characteristic for substrates, which associate transiently with the enzyme and after being processed are substituted by the next substrate molecules. Such a transient association of an ERAD substrate with the Synoviolin complex has been shown for NHK, which associates with hXTP3B, a component of Synoviolin based ERAD complexes (Hosokawa et al., 2008). Similar to NHK, Herp was assumed to be a substrate of Synoviolin complexes, since Herp also transiently interacts with Synoviolin. Based on this hypothesis, degradation of Herp should be impaired upon disruption of major components of the Synoviolin complex.

However, the study presented here showed that neither the reduced expression of the central ERAD complex components Sel1L and Synoviolin nor the elevated expression of the dominant negative inactive E2 enzyme Ube2j1 affected the degradation of Herp. Other studies revealed that the depletion of the central ERAD complex components Synoviolin and

Sel1L led to the impaired degradation of the ERAD substrate NHK (Christianson et al., 2008; Mueller et al., 2008). In addition, the elevated expression of the inactivated Ube2j1 inhibited the degradation of another ERAD substrate, TCR-α (Lenk et al., 2002). The present study shows that the elevated expression of both myc-tagged wild type and inactive Ube2j1 led to a modest stabilisation of Herp. However, this effect is presumably due to the overexpression of the myc-tagged proteins, as this was also demonstrated for the degradation of MHC class I molecules (Mueller *et al.*, 2008).

Taken together, the fact that depletion or disturbance of the major ERAD components Synoviolin and Sel1L or Ube2j1, respectively, affect the stability of known ERAD substrates but not the stability of Herp strongly suggests that Herp is not a substrate of Synoviolin based ERAD complexes. Furthermore, Herp is essential for the effective degradation of Synoviolin dependent ERAD substrates such as NHK, as it has been shown here. If Herp were also a substrate of Synoviolin, it would act as a potent competitor of NHK and rather impede than promote its degradation. As this is not the case, this fact also points towards Herp not being a Synoviolin substrate.

Although ER stress induction significantly enhances the expression of Herp, the degradation rates of Herp remain unaffected, as it has been shown here and by others (Schulze, 2006). To ensure this efficient turnover of Herp in an ER stress situation, more than one type of ERAD complexes might be involved in Herp degradation. In this case, Synoviolin based complexes can also play a role for the stability of Herp. To finally exclude that Herp degradation is mediated by Synoviolin or Sel1L in case of ER stress, degradation analyses after depletion of Synoviolin or Sel1L should be performed under ER stress conditions.

The present study showed that in an ER stress situation the elevated expression of Ube2j1 did not increase the degradation rate of Herp. However, data from the literature showed that overexpression of Ube2j1 improves the degradation of SGK1, a Synoviolin dependent substrate (Arteaga et al., 2006). Taken together, the findings from this study and those of the literature strongly indicate that Herp is not a substrate of Synoviolin based complexes. Therefore, it is likely that another E3 ligase besides Synoviolin is involved in the degradation of Herp. In the literature, the two E3 ligases Gp78 and POSH were reported to ubiquitinate Herp. Li and colleagues showed that recombinant Herp (aa 1-259) was ubiquitinated by the Synoviolin homologue Gp78 along with the E2 enzyme Ube2g2 *in vitro*, whereas a K61R mutant of Herp failed to undergo polyubiquitination (Li et al., 2007). This result indicates that Gp78 mediates Herp degradation. Interestingly, Gp78 was recently shown to be a substrate of Synoviolin, demonstrating that Synoviolin regulates the stability of its homologue (Shmueli et al., 2009). In addition, it was demonstrated that the elevated expression of Synoviolin leads to the stabilisation of Herp, whereas depletion of Synoviolin reduced cellular Herp steady state levels (Schulze, 2006).

Taken together, the combination of these findings indicates that Gp78 very likely mediates the ubiquitination and degradation of Herp.

However, in the study presented here, an association of Herp and Gp78 was not verified. In line with this observation, other studies also showed that Gp78 is excluded from complexes containing Sel1L and Synoviolin (Hosokawa et al., 2008). The finding that Gp78 is not associated with Herp rather argues against Herp being a substrate of Gp78 and Gp78 being a substrate of Synoviolin. However, these discrepancies of the results can be explained by the fact that the transient interaction of a substrate with an enzyme might not be detected by conventional interaction studies. The Plenty of SH3s (POSH) protein is the other E3 ligase that was reported to ubiquitinate Herp. Once bound to POSH, Herp acts concomitantly as activator and substrate of POSH. Upon calcium perturbation POSH polyubiquitinates Herp with lysine-63-type ubiquitin chains. This type of ubiquitin linkage results in the translocation of Herp from the trans-Golgi network to the ER. In turn, Herp promotes POSH oligomerisation and activation in a UBL domain dependent manner (Tuvia et al., 2007). Importantly, the fact that K63-type polyubiquitination does not affect the stability of Herp excludes POSH as a mediator of Herp degradation. It was demonstrated that, under ER stress conditions, Herp is predominantly located at the ER membrane (Kokame et al., 2000; Sai et al., 2003). Since the present study deals with the importance of Herp for ERAD, the localisation of this protein in proximity to the other ERAD components is directly connected to its function concerning ERAD. However, a fast availability of Herp through its POSH mediated redistribution to the ER could enhance its function at ERAD complexes. In summary, Herp degradation is not mediated by Synoviolin based complexes or POSH. Other E3 ligases such as Gp78 are likely to execute this function.

4.1.2 Correlation of the turnover of Herp and ERAD substrates

Herp is an instable protein being continuously exchanged at Synoviolin based complexes. Since elevated expression of the E3 ligases Synoviolin or Gp78 leads to enhanced degradation of the ERAD substrates Ire1 or CD3-δ (Gao et al., 2008; Zhong et al., 2004), it was assumed that also an increased availability of Herp within the cell is beneficial for its function in ERAD.

The work presented here showed that an elevated expression of Herp has no effect on the degradation of the substrate NHK, whereas depletion of Herp impairs ERAD of NHK. The observation that elevated Herp levels do not improve ERAD could argue for several possibilities. The endogenous Herp level could already be sufficient to promote the degradation process. In this case, Herp would not be the rate-limiting factor for ERAD. Another possibility could be that the majority of overexpressed Herp protein is not integrated into Synoviolin complexes and therefore does not provide any function in ERAD.

A third option is that, when overexpressed, the excess of Herp protein is degraded more efficiently. To address the question of a potential enhanced degradation of overexpressed Herp, a stabilised Herp was generated and utilised for degradation studies.
As shown in the study presented here, the K61R mutation was sufficient to stabilise Herp. In addition, Herp-K61R was associated with Synoviolin, Derlin1 and Usp7. Surprisingly, the elevated expression of Herp-K61R did not improve but slowed down degradation rates of the ERAD substrate NHK. Impairment of ERAD was also observed upon overexpression of a dominant negative Herp variant lacking the UBL domain. Sai and colleagues found that Herp lacking the UBL domain is stable (Sai et al., 2003). In addition, Li and colleagues showed that lysine 61 is the crucial ubiquitination site of Herp (Li et al., 2007); see 4.1.2). Therefore, the results from my study confirm that the UBL domain determines the stability of Herp and, in particular, that lysine 61 is crucial for that determination. Furthermore, the data reveal that the stability of Herp is positively correlated to the stability of the ERAD substrate. The stabilisation of Herp impairs the process of ERAD. Taken together, from these data it can be concluded that the UBL domain of Herp has a crucial role in the degradation of Herp, and that its degradation rate correlates with its function in ERAD.
The Herp-UBL domain was demonstrated to be fundamental for Herp mediated cellular processes. Dependent on this domain, Herp promotes ERAD and maintains calcium homeostasis in stressed neuronal cells. Both functions are important anti-apoptotic mechanisms (Chigurupati et al., 2009; Schulze et al., 2005). In contrast, within the pathogenesis of Alzheimer's disease, Herp promotes amyloid β-protein (Aβ) generation. Deletion of the Herp UBL domain even enhances Aβ-production, presumably due to the stabilisation of Herp (Sai et al., 2003). These data strongly suggest that different cellular functions of Herp are based on its stability, which is determined by its UBL domain. In general, this argues for the Herp-UBL domain to have a cytoprotective function. Accordingly, Herp-K61R, which is associated with Synoviolin, is exchanged at Synoviolin based complexes at lower rates, which could then lead to the impairment of ERAD. The fast exchange of Herp at Synoviolin presumably determines its proper function in ERAD.

The data presented here on the dynamics of Herp indicate that its function in the process of Synoviolin mediated ERAD is strongly dependent on its degradation rate. Herp only acts efficiently in this process, when continuously dissociating from Synoviolin, followed by its degradation and replacement by newly synthesised Herp molecules. Stabilised Herp very likely leads to blockage of processes downstream from ERAD substrate retrotranslocation and ubiquitination. Future studies should address the association dynamics of stabilised Herp with Synoviolin based complexes in greater detail. In addition, it would be of great interest to establish an instable Herp and analyse its impact on the ERAD process.

4.2 The importance of Herp for the integrity of Synoviolin based complexes

4.2.1 The impact of Herp on Synoviolin oligomerisation

In vitro interaction studies revealed that Synoviolin molecules are able to interact with each other suggesting that they are able to form homooligomers (Schulze et al., 2005). In the study presented here, Synoviolin oligomerisation was demonstrated to occur in Synoviolin-HTB expressing mammalian cells *in vivo*. Dependent on the expression level of Synoviolin-HTB, the ratio of tagged to untagged Synoviolin within an oligomer changed in a reciprocal correlation. In addition, titration of the plasmid encoding Synoviolin-HTB before transfection revealed that at least five molecules of wild type Synoviolin were co-precipitated with one molecule of Synoviolin-HTB. Therefore, at least Synoviolin homohexamers were assumed to exist physiologically (M. Seeger, personal communication). Also the Synoviolin homologue Gp78 was demonstrated to form homooligomers. These Gp78 oligomers bind several molecules of the E2 enzyme Ube2g2 enabling the process of ERAD substrate polyubiquitination (Li et al., 2009). In addition, the yeast orthologue of Synoviolin, Hrd1p, was also demonstrated to oligomerise (Horn et al., 2009). However, the oligomer stochiometry of Gp78 in mammalia or Hrd1p in yeast is not known so far. Nevertheless, the findings on ERAD E3 ligases' oligomerisation indicate that the process of homooligomerisation is a general feature of these enzymes and presumably required for the process of substrate ubiquitination and retrotranslocation. ER membrane resident oligomers consisting of more than two molecules of the E3 ligase potentially form a kind of pores that are needed for the retrotranslocation of ERAD substrates.

Although Synoviolin oligomerisation is evident, it is not known how this process is regulated. In yeast, the ubiquitin domain protein Usa1p was demonstrated to mediate Hrd1p oligomerisation (Horn et al., 2009). In addition, Usa1p was shown to link Der1p to Hrd1p, which is necessary for the formation of a functional Hrd1p based ERAD complex (Carvalho et al., 2006). Since heterologously expressed Herp partly rescued the Usa1p deletion phenotype in yeast, Herp was proposed to be the functional equivalent of Usa1p (Carvalho et al., 2006). These data point towards Herp regulating Synoviolin oligomerisation in mammalia. However, the study presented here showed that upon elevated expression of dominant negative HerpΔUBL neither the cellular amount nor the ratio of Synoviolin molecules within the oligomers was affected. Additionally, depletion of Herp had also no effect on Synoviolin oligomerisation (data not shown) suggesting that this process is Herp-independent. Horn and colleagues reported that Usa1p mediated Hrd1p oligomerisation in yeast depends on the Usa1p N-terminus, which contains a UBL domain (Horn et al., 2009).

However, a general importance of the Usa1p UBL domain for ERAD has not been demonstrated. A recent study revealed that Usa1p is involved in the ubiquitination of the soluble model substrate CPY*, independent of the Usa1p UBL domain (Kim et al., 2009). However, contrary data were reported concerning the significance of the Usa1p UBL domain for the degradation of membrane derived substrates, as 6myc-Hmg2 but not of Hmg2-GFP degradation required the Usa1p UBL domain (Carroll and Hampton, 2010; Horn et al., 2009). In contrast to Usa1p, the Herp UBL domain is dispensable for the oligomerisation of Synoviolin but required for the degradation of the different Synoviolin dependent ERAD substrates NHK and CD3- δ. Taken together, Herp and Usa1p differ in essential functions and are therefore not necessarily functional equivalents. However, their similar domain architecture could explain why Herp is able to rescue a Usa1p deletion in yeast, at least partly. Since Herp cannot function analogously to Usa1p, another UDP such as Herp2 (see 4.3) is imaginable to fulfil this function in mammalia.

Herp was indeed shown to be involved in the process of oligomerisation of the soluble E3 ligase POSH. Herp mediated oligomerisation and activation of POSH depends on the UBL domain of Herp. Following activation, POSH induces Herp translocation to the ER (Tuvia et al., 2007). Besides this function, POSH also mediates pro-apoptotic signalling by forming a scaffold for kinases such as JNK (Xu et al., 2003). In contrast, Herp and Synoviolin both promote ERAD and therefore have anti-apoptotic functions in the cell (Amano et al., 2003; Chan et al., 2004). The fact that Herp activates a pro-apoptotic protein could be connected to the hampering of cellular survival after a longer time of persisting ER stress. Subsequently, apoptotic signalling is activated, whereas cell survival mechanisms are inactivated. This cellular situation might lead to Herp mediated activation of POSH. However, the action of Herp on POSH most likely is limited, since apoptosis also leads to caspase mediated Herp cleavage (Amano et al., 2003; Chan et al., 2004).

To investigate the functional relevance of Herp in Synoviolin mediated ERAD, recent degradation analyses have been performed using the ERAD model substrate CD3-δ. Herp protein depletion significantly inhibited the turnover of CD3-δ. Therefore, this substrate is a suitable model to investigate ERAD (Schulze et al., 2005). However, besides Synoviolin also other E3 ligases such as Rfp2 and Gp78 mediate CD3-δ degradation (Lerner et al., 2007; Zhong et al., 2004). Since Synoviolin and Gp78 are homologues, this is an explanation for the specificity of both E3 ligases for the same substrate. However, as known so far, Herp is excluded from Gp78 mediated ERAD processes. Thus, an ERAD substrate exclusively dependent on Herp and Synoviolin was suggested to be inevitable to investigate this particular ERAD pathway. So far, degradation of NHK, another model ERAD substrate, was shown to be dependent only on Synoviolin (Christianson et al., 2008).

In contrast to membrane associated CD3-δ, NHK, which is bound to calreticulin, remains solubilised in the ER lumen before it is degraded (Oda et al., 2003).
The study presented here demonstrated for the first time that Herp is required for the effective degradation of NHK, which was dependent on the UBL domain of Herp. Therefore, NHK represents an appropriate substrate to investigate the Synoviolin and Herp dependent ERAD process in particular. This result seems to contradict data from the literature showing that a reduced expression of Herp does not interfere with the degradation of NHK (Okuda-Shimizu and Hendershot, 2007). The oligomerisation of Synoviolin could be an explanation for this divergent situation. It is conceivable that binding of just one molecule of Herp to a Synoviolin oligomer is sufficient to maintain the function of the Synoviolin complex. If this is the case, a reduction of the cellular Herp protein level by just 50% as shown in the publication of Okuda-Shimizu and colleagues would not be sufficient to observe any effects on the rate of substrate degradation. However, in contrast to the work of Okuda-Shimizu and colleagues, my study shows that a depletion of Herp by 50% leads to a delayed degradation of NHK, a fact that could be due to the different used cell lines. Beyond, as shown here, the lack or disturbance of the Herp UBL domain results in an almost complete stabilisation of the substrate verifying that Herp is indeed required for the degradation of ER-luminal NHK.

4.2.2 Herp dependent recruitment of Usp7 to Synoviolin

The UBL domain of Herp determines the stability of this protein (Sai et al., 2003). Furthermore, the Herp-UBL domain has a crucial role in the process of Synoviolin mediated ERAD (Schulze, 2006); Kny *et al.*, manuscript). UBL domains of different ubiquitin domain proteins vary regarding their protein binding specificity. The yeast protein Rad23, *e.g.,* binds the 26S proteasome and the E4 enzyme Ufd2 via its UBL domain in a mutual exclusive manner. Hhr23, the human orthologue of Rad23, also binds different proteins such as the deubiquitinating enzyme Ataxin-3 or the 26S proteasome via its UBL domain (Madsen et al., 2007). In contrast to Hhr23, the UBL domain of Herp does not interact with the 26S proteasome (Schulze et al., 2005). Starting with the analysis of the molecular function of Herp, its UBL domain was assumed to bind proteins that mediate the function of this UDP. As demonstrated by *in vitro* and *in vivo* interaction studies, the deubiquitinating enzyme Usp7 was identified as a target of the Herp UBL domain (Schulze, 2006).
In the work presented here, the specific interaction of endogenous Usp7 with Herp-HTB was shown. In addition, also the higher molecular weight isoform of Usp7, Usp7β, was found to bind to Herp-HTB. Initially, Usp7 was stated to only reside in the nucleus and thus, Usp7 was questionable to be able to interact with the cytoplasmatic UBL domain of Herp under *in vivo* conditions.

However, immunofluorescence experiments revealed that a certain amount of Usp7 and the isoform Usp7β is also present in the cytoplasm (Antrobus and Boutell, 2008; Meredith et al., 1994). In addition, under certain conditions such as the infection of cells with Herpes Simplex virus Usp7 can translocate from the nucleus to the cytoplasm (Daubeuf et al., 2009). Thus, an interaction of cytoplasmatic Usp7 and Herp is generally possible. Since Usp7β is stable and binds viral proteins such as ICP0, it was suggested to have the same functions as Usp7 (Antrobus and Boutell, 2008). The finding that Usp7β also binds Herp strengthens the results of Antrobus and Boutell who stated Usp7β to reveal the same binding properties as Usp7. Taken together, by demonstrating the interaction of endogenous Usp7 with Herp, the study here confirmed Usp7 to be a target of Herp.

The literature reveals that the P/AXXS motif, a short amino acid sequence that is contained, *e.g.*, in Mdm2 and p53, mediates Usp7 binding. In close proximity of the P/AXXS motif, with X indicating any amino acid, several lysines are subject to polyubiquitination, possibly inhibited by Usp7 (Sheng et al., 2006). Since Herp also directly interacts with Usp7, Herp was screened for this possible Usp7 interaction site.

Indeed, as demonstrated in the study here, Herp harbours an AHLS motif, which is located within its UBL domain. This AHLS motif of Herp was found to contribute to the efficient binding of Herp with Usp7. However, this motif alone was not sufficient to mediate the interaction of Usp7 and Herp. Mdm2 and p53 both exhibit two closely spaced P/AXXS motifs, which are involved in Usp7 binding. Beyond that, additional regions might be required to enable the interaction of Usp7 with Mdm2 or p53 (Sheng et al., 2006). Altogether, these findings show that a single P/AXXS motif is not sufficient to mediate Usp7 binding. In conclusion, apart from the AHLS motif, other regions within the Herp UBL domain must contribute to the binding of Usp7 to Herp.

Usp7 interacts with the UBL domain of Herp, while Synoviolin interacts with a region distal from the UBL domain (Schulze, 2006; Schulze et al., 2005). Therefore, Usp7 was assumed to be associated with Synoviolin through its interaction with Herp.

Indeed, the study here demonstrated that the deubiquitinating Usp7 is associated with the ubiquitin ligase Synoviolin. Furthermore, this interaction showed to be dependent on Herp, since a marked increase of Usp7 interacting with Synoviolin was observed only after an increased expression of Herp.

Simultaneous binding of two enzymes with opposing activities to one protein can enable a fine-tuning of these activities towards the common substrate. This has been shown for, *e.g.*, Hdmx, a protein involved in p53 regulation. The binding of both Hdm2, a specific ubiquitin E3 ligase, and Usp7, the ubiquitin hydrolase, to Hdmx enables a tight regulation of the ubiquitination status of the Hdmx protein. Upon DNA damage, Usp7 dissociates from Hdmx, which leads to the degradation of both Hdm2 and Hdmx.

Then, Usp7 binds and stabilises p53 (Meulmeester et al., 2005). Coupling ubiquitination and deubiquitination can positively influence the degradation of a substrate.
Since a ubiquitinating ERAD system is prone to autoubiquitination, single components of this system might be degraded by the proteasome resulting in ERAD inhibition. For example, the yeast orthologue of Synoviolin, Hrd1p, is subject to autoubiquitination (Bays et al., 2001). Then, the ERAD system benefits from a hydrolase, stabilising its components by deubiquitination. Besides Usp7, other deubiquitinating enzymes have been reported to stabilise E3 ligases. One example of such stabilising enzymes is Usp15, which antagonises polyubiquitination of the Cullin E3 ligase component Rbx1 (Hetfeld et al., 2005). Bringing together Synoviolin and Usp7 at one protein complex points to a tight regulation of the ubiquitination status of target proteins. Since Usp7 and Synoviolin are part of multiprotein complexes, their action on separate proteins is also conceivable.

The direct binding of Usp7 to, *e.g.*, p53, Mdm2 and viral ICP0 leads to their deubiquitination and stabilisation (Canning et al., 2004; Li et al., 2004; Li et al., 2002). Since it binds to Herp, Usp7 was suggested to also stabilise Herp. *In vitro* ubiquitination studies and cycloheximide degradation assays indeed revealed that Usp7 can deubiquitinate and stabilise Herp, if both proteins are expressed at elevated levels in cells (M. Seeger, personal communication).
Surprisingly, the study presented here demonstrated that neither decrease nor increase of cellular Usp7 protein affects degradation of endogenous Herp. This discrepancy of the Usp7-action on endogenous versus exogenous Herp can be explained by the fact that the majority of overexpressed Herp is not integrated into ERAD complexes but rather accumulates outside these complexes and becomes ubiquitinated. In this case, the excess of Usp7 could act on the increased amount of Herp. It is also thinkable that the elevated expression of Usp7 of three fold or the depletion of Usp7 by 50%, as observed in this study, was not sufficient to affect the turnover of endogenous Herp. However, another study showed that the depletion of Usp7 by 50% resulted in an enhanced degradation of Alix/HP95, a Usp7 interacting protein (Kessler et al., 2007). This finding indicates that, if Herp were also a substrate of Usp7, depletion of Usp7 should result in the stabilisation of Herp, which is not the case. Taken together, although directly binding to Herp, Usp7 does not stabilise endogenous Herp.

Regarding the functional role of a ubiquitin hydrolase at Synoviolin based complexes, Usp7 was suggested to regulate the degradation of ERAD substrates. Considering the deubiquitinating activity of Usp7, its binding to an ERAD complex was expected to inhibit or at least delay substrate degradation.
However, this work here demonstrated that Usp7 does not affect degradation of the ERAD substrates CD3-δ and NHK. Ubiquitin hydrolases can deubiquitinate ERAD substrates,

a process that needs not necessarily lead to their stabilisation but can also result in their improved degradation. This phenomenon has been shown for Ataxin-3, a deubiquitinating enzyme, which shortens the ubiquitin chains of the ERAD substrate TCR-α to an appropriate size enabling the recognition by downstream components such as p97. Hence, a deubiquitination step is important for TCR-α extraction from the ER membrane (Wang et al., 2006). Usp13 is another deubiquitinating enzyme, which was recently found to be associated with p97 and acts on TCR- α. In contrast to Ataxin-3, Usp13 was demonstrated to inhibit degradation of TCR-α (Sowa et al., 2009). Thus, Usp13 and Ataxin-3 act on the same substrate but with the opposite outcome. These data demonstrate that two different deubiquitinating enzymes acting in one ERAD system determine the fate of the substrate and introduce a further step of ERAD regulation. Other than Ataxin-3 or Usp13, Usp7 did not show to influence the degradation of the substrates NHK or CD3-δ indicating that Usp7 does not regulate the ERAD process. However, the example of the opposing enzymes Ataxin-3 and Usp13 both acting on TCR-α shows that these DUBs exert a very specific and not a general function towards an ERAD substrate. This finding allows concluding that also Usp7 specifically acts on the degradation of ERAD substrates besides CD3-δ and NHK.

Since Usp7 is associated with Synoviolin based ERAD complexes, it was assumed to be ER stress induced. However, the study presented here revealed that Usp7 is a stable protein and that its expression is not induced by ER stress.
Boutell and colleagues also demonstrated that Usp7 is stable, except in case of, *e.g.,* Herpes Simplex virus infection, when the viral E3 ligase ICP0 targets Usp7 for fast proteasomal degradation (Boutell et al., 2005). Furthermore, Usp7 was found to be associated with the 26S proteasome (Besche et al., 2009; Bousquet-Dubouch et al., 2009). These data demonstrate that Usp7 becomes a substrate of the UPS under certain cellular conditions such as viral infections. The observation that, upon ER stress, a small subpopulation of Usp7 is recruited to Synoviolin based ERAD complexes but not degraded shows that Usp7 does not serve as a substrate of Synoviolin. The Herp dependent association of Usp7 with Synoviolin rather points towards a specific role of Usp7 at the ERAD complexes in case of ER stress. Therefore, Usp7 still might be required for the turnover of Synoviolin dependent ERAD substrates, which have not been investigated so far. Usp7 is a specific target of the Herp-UBL domain but does not promote the degradation of the ERAD substrates CD3-δ or NHK. Therefore, it is conceivable that other target proteins of Herp fulfil this task. Herp mediated recruitment of further accessory proteins beside Usp7 to Synoviolin could decisively affect the qualitative and quantitative composition of the ERAD complexes. This function of Herp is therefore likely to determine the ERAD complexes' specificity and function.

Herp is dispensable for the regulation of p53 stability

In 2007, Yamasaki and colleagues reported that ubiquitination and degradation of the tumor-suppressor protein p53 depends on Synoviolin (Yamasaki et al., 2007). Since Herp binds Usp7, a well-known regulator of p53, and Synoviolin concomitantly, Herp was hypothesised to be involved in Synoviolin mediated p53 degradation.

However, Herp was not required for the Synoviolin mediated turnover of p53, as it was shown in the study here. This finding allows concluding that Herp is required only for the degradation of a subset of Synoviolin dependent ERAD substrates and not for all substrates in general. Data from yeast experiments suggested that different kinds of ERAD substrates are processed by distinct ubiquitination systems, dependent on the location of the misfolded domain within the substrate. Therefore, ERAD was sub-classified as 'ERAD-C' for substrates harbouring a defect in their cytosolic domain and 'ERAD-M' or 'ERAD-L' for substrates with a defect in their transmembrane or luminal domain, respectively. 'ERAD-C' substrates are processed by the E3 ligase Doa10p and associated proteins, whereas 'ERAD-M' or 'ERAD-L' substrates are ubiquitinated by the Synoviolin orthologue Hrd1p and its interacting proteins (Carvalho et al., 2006). This classification was suggested to apply also to the mammalian ERAD systems. However, a simple sub-classification of the mammalian ERAD pathways is not feasible, since an individual substrate can be processed by different ERAD systems as shown for CD3-δ, which is ubiquitinated by Synoviolin, Gp78 and Rfp2 (Fang et al., 2001; Kikkert et al., 2004; Lerner et al., 2007). Moreover, the ubiquitination of different ERAD substrates such as CD3-δ, p53 and NHK can be performed by the same E3 ligase, Synoviolin (Christianson et al., 2008; Kikkert et al., 2004; Yamasaki et al., 2007). Thus, it is conceivable that in mammalian cells rather a complex network of interacting ERAD proteins decides about processing of an ERAD subtrate. Herp, *e.g.,* could be such a protein required only for the turnover of Synoviolin substrates that have to be retrotranslocated such as membrane-associated CD3-δ and ER-luminal NHK. In contrast, Herp seems to be dispensable for the degradation of substrates that are already cytoplasmatic such as p53. The degradation of such cytoplasm derived substrates does not require ERAD complex proteins, which mediate the steps retrotranslocation or extraction. Since these central ERAD steps are dispensable for p53 degradation, this protein is not a classical ERAD substrate.

Also the existence of distinct cellular pools of p53 that are diversely regulated could be an explanation for the fact that Herp is not involved in the degradation of p53. Nuclear p53 is regulated by a couple of proteins such as the E3 ligase Hdm2, Hdmx and Usp7 (see 1.1.5) and the E3 ligases COP1 and Pirh2 (Brooks and Gu, 2004). Genotoxic stress, *e.g.*, triggers Hdm2 mediated monoubiquitination of a subpopulation of p53, which translocates to the mitochondria, where a mitochondrial Usp7 stabilises p53 and promotes the pro-apoptotic action of p53 (Marchenko et al., 2007).

Furthermore, besides Synoviolin, another E3 ligase, the Parkin like protein Parc, traps p53 and sequesters it in the cytoplasm (Nikolaev et al., 2003). Parc and Synoviolin might serve to store p53, until cellular conditions such as DNA damage lead to the redistribution of p53 to the nucleus or to the mitochondria. As p53 is regulated by many other proteins besides Synoviolin and is located primarily in the nucleus, Synoviolin based ERAD complexes most likely play a minor role for the regulation of p53 stability.

As a substrate of Synoviolin p53 has been also demonstrated to be associated with this E3 ligase (Yamasaki et al., 2007). Therefore, Herp was assumed to be associated with p53, too. However, in the study presented here, p53 was not found to interact with Synoviolin or Herp. This discrepancy between the results presented here and the published data might be due to the different cell lines used in these different studies. Another explanation for the fact that p53 was not found to be associated with Synoviolin or Herp could be that the interaction experiments here were performed after eight hours of ER stress. As prolonged ER stress induces apoptosis, p53 most likely dissociates from Synoviolin and translocates to its sites of action in the nucleus and the mitochondria (Li et al., 2006).

4.2.3 Herp dependent association of Synoviolin and AUP1

The ancient ubiquitous protein 1 (AUP1) was identified as a novel protein interacting with Sel1L (Mueller et al., 2008). Since Sel1L is bound to Synoviolin, AUP1 was assumed to be a component of Synoviolin based ERAD complexes.

In fact*, in vitro* interaction analyses of the study presented here demonstrated that AUP1 directly binds to Synoviolin. This finding strongly points towards AUP1 being a new-found component of Synoviolin based ERAD complexes. Interestingly, the interaction of AUP1 and Synoviolin was demonstrated to depend on the presence of Herp.

These data indicate that Herp mediates the recruitment of AUP1 to Synoviolin, thereby regulating the integrity of Synoviolin based ERAD complexes. However, the drastic decrease of the Herp protein resulted only in a slight decrease of AUP1 associating with Synoviolin. This effect can be explained by the presence of Synoviolin oligomers. The binding of just one Herp molecule per Synoviolin oligomer could promote the binding of several AUP1 molecules per oligomer. Therefore, even reduced Herp levels could be sufficient to enable this interaction of AUP1 and Synoviolin. Presumably, only the complete depletion of cellular Herp protein would lead to the loss of AUP1 association with the E3 ligase Synoviolin.

The finding that both, AUP1 and Usp7 are associated with Synoviolin based complexes, in a Herp dependent manner, demonstrates that Herp is involved in establishing the integrity of Synoviolin based complexes. Depletion of Herp leads to the loss of Usp7 and AUP1 proteins from these complexes.

Herp dependent recruitment of accessory proteins to Synoviolin based complexes obviously occurs in different ways. On the one hand, Herp enables direct binding of proteins with a component of the ERAD complex, as it was shown for AUP1. On the other hand, Herp directly binds to proteins and links them to the ERAD complexes, as it was demonstrated for Usp7. Besides Usp7, Herp also directly binds to Ubiquilin 2 and links this protein to Synoviolin (Kim et al., 2008). However, this interaction depends on a region between the amino acids 115 and 200 located distal from the Herp UBL domain and not on the UBL domain itself. Also ubiquilins harbour a UBL domain, capable of interacting with the proteasome, and additionally a UBA domain binding to ubiquitin or ubiquitinated substrates (Kleijnen et al., 2000). Therefore, ubiquilins are able to shuttle ubiquitinated substrates to the proteasome. In the study of Kim and colleagues, depletion of Ubiquilin 1 and Ubiquilin 2 as well as overexpression of a truncated dominant negative Herp version lacking the critical binding region for Ubiquilin 2 led to the impaired degradation of the ERAD substrate CD3-δ (Kim et al., 2008). These data on the interaction between Herp and Ubiquilin 2 support the idea that Herp represents an important Synoviolin complex component, which is inevitable for the recruitment of essential ERAD proteins. Thus, Herp regulates the integrity of ERAD complexes. To strengthen these findings, it should be further addressed whether the association of other proteins with Synoviolin can also be subject to Herp regulation. In addition, it would be important to analyse how an alteration of the stability of Herp influences its recruitment function. Recruited proteins should be tested for their impact on ERAD.

AUP1 is required for Sel1L mediated dislocation of MHC class I molecules (Mueller et al., 2008). Since Sel1L is a component of Synoviolin based ERAD complexes and AUP1 interacts with Synoviolin, AUP1 was suggested to generally improve Synoviolin mediated ERAD.

Indeed, the study presented here demonstrated that AUP1 promotes the degradation of the Synoviolin and Herp dependent ERAD substrate NHK. This was shown by depletion of the cellular AUP1 protein using siRNA. In the study of Müller and colleagues a GFP-tag was fused to AUP1 to disturb protein function. Overexpression of AUP1-GFP resulted in the decreased dislocation of MHC class I molecules from the ER to the cytoplasm (Mueller et al., 2008). Thus, both studies demonstrated that AUP1 is an important Synoviolin complex component, which is required for the effective degradation of substrates.

AUP1 possesses a C-terminal ′coupling of ubiquitin conjugation to ER degradation′ (CUE) domain, which was discussed to be responsible for the promoting effect of AUP1 on Synoviolin mediated ERAD. Indeed, the study here showed that disruption of the CUE domain impaired the function of AUP1 in ERAD.

CUE domains reveal structural homology to ubiquitin associated (UBA) domains and both bind similarly to a hydrophobic patch within ubiquitin. A number of proteins, which belong to the network of the ubiquitin proteasome system, contain CUE domains. These proteins promote their own ubiquitination, as it was shown for the E3 ligase Gp78 (Hurley et al., 2006; Kang et al., 2003). The CUE domain of Gp78 is involved in the recruitment of the E2 enzyme Ube2g2 and is therefore essential for the functionality of Gp78. The first identified CUE domain containing protein in yeast, Cue1p, also recruits the E2 enzyme Ubc7p. Degradation of the ERAD substrate CD3-δ is impaired upon the elevated expression of a dominant negative Gp78 lacking the CUE domain (Chen et al., 2006). According to the data of Kang and colleagues, leucine 333 within the CUE domain was assumed to be essential for ubiquitin binding (Kang et al., 2003). In the study here, it was shown that the substitution of this critical lysine 333 by an alanine impaired degradation of NHK. The importance of the CUE domain might base on its ability to bind ubiquitin, since an *in vitro* binding study demonstrated a diminished binding of ubiquitin upon mutation of the CUE domain (M. Seeger, personal communication). Taken together, these data indicate that AUP1 is involved in the binding or transfer of ubiquitinated substrates. A study of Morito and colleagues revealed that the CUE domain of Gp78 is sufficient to bind the substrate CFTRΔF508. Deletion of the Gp78-CUE domain inhibited its binding of CFTRΔF508, whereas the introduction of this domain into Synoviolin had the reversed effect and enabled the binding of CFTRΔF508 with Synoviolin (Morito et al., 2008). Therefore, the CUE domain of Gp78 is not only able to bind ubiquitin but also ubiquitinated ERAD substrates. In addition, the same study showed that the E3 ligase Gp78 exerts E4 activity, which is also mediated by its CUE domain. Based on its CUE domain, the functional role of AUP1 in ERAD can be diverse, as this is supported by the fact that the CUE domain of Gp78 has various functions. AUP1 might provide ubiquitin, recruit the E2 enzyme or the substrate, or could be involved in ubiquitin chain elongation, all processes that enhance ERAD.

Taken together, it was demonstrated here that the UDP Herp is essential for maintaining the integrity of Synoviolin based complexes. Although Herp is dispensable for the process of Synoviolin oligomerisation, it was shown to have a decisive role for determining the composition of Synoviolin based complexes by recruiting accessory proteins. Herp either binds directly to these proteins, as in case of Usp7, and thereby provides a link to Synoviolin or enables their binding to one of the complex components, as this has been shown for AUP1. Herp mediated regulation of the composition of Synoviolin based complexes very likely is crucial for the function of these complexes in ERAD.

4.3 Comparison of Herp and Herp2

The ubiquitin domain proteins Herp and Herp2 belong to the mubl protein family (1.2.6) and reveal 40% (their UBL domains even 50%) amino acid sequence identity. Based on these similarities, Herp2 was suggested to be a paralouge of Herp. Thus, a central question of the study presented here was whether Herp2 is able to take over the functional role of Herp within cells. Therefore, Herp2 was hypothesised to also associate with Synoviolin.

Indeed, the data presented here demonstrated that Herp2 interacts with Synoviolin. In addition, Herp2 was demonstrated to be a component of Synoviolin based ERAD complexes. Together, these findings point towards Herp2 being a component of Synoviolin based ERAD complexes. However, these data did not reveal whether Herp and Herp2 are integrated in the same Synoviolin based ERAD complex simultaneously.

The data presented here showed that Herp and Herp2 are not associated with each other and therefore are not integrated in the same complex. Only in case of the elevated expression of Herp and Herp2 both proteins interact with each other, a fact that is unlikely to represent the physiological situation. However, these interaction studies also revealed that both Herp and Herp2 have the ability to homooligomerise.

The finding that Herp does not bind to Herp2 indicates that these UDPs exclude each other from the same Synoviolin complex. This fact points towards the existence of at least two types of Synoviolin based complexes, harbouring either Herp or Herp2. These different kinds of ERAD complexes potentially exert different functions in the cell, *e.g.*, processing of selected ERAD substrates. These data also suggest that Herp and Herp2 compete for direct binding to Synoviolin. In this case, the composition of the ERAD complexes would depend on the cellular concentration and relation of these two UDPs. The analysis of protein expression of both UDPs revealed that Herp2 is predominantly expressed in brain, spleen and thymus, whereas Herp is ubiquitously expressed (Kokame et al., 2000; Schulze, 2006). Therefore, it is conceivable that in Herp2 expressing tissues the majority of Synoviolin based ERAD complexes contains Herp2 instead of Herp. It remains open whether Herp2 also directly binds to Synoviolin or to another component of these multiprotein complexes.

In contrast to heterooligomerisation, homooligomerisation of Herp or Herp2 is likely to occur at physiological conditions, since the endogenous protein was experimentally co-precipitated with a HTB-tagged protein. This finding indicates that homooligomeric substructures consisting of Herp or Herp2 can interact with Synoviolin based complexes. Very likely, the amount of Herp or Herp2 molecules bound to one Synoviolin oligomer determines the function of the whole ERAD complex. In general, oligomerisation seems to be a widespread feature of components of Synoviolin based complexes. Besides Herp, Herp2 and Synoviolin also the Derlin-proteins and p97 were shown to oligomerise.

The AAA-ATPase p97 forms homohexamers (Ogura and Wilkinson, 2001). Derlin-1 was shown to homooligomerise (Ye et al., 2005). In addition, Derlin-1, -2 and -3 were shown to form mixed Derlin-oligomers (Lilley and Ploegh, 2005). The authors therefore suggested that the Derlin proteins, by oligomerisation, fulfil the requirements to form a pore that enables retrotranslocation. Generally, association of potentially ring shaped oligomeric structures could enable the formation of a retrotranslocation pore that is suitable for substrates of even larger size. Using structural analysis by, *e.g.*, electron microscopy, the composition, arrangements, and stochiometry of these high molecular weight ERAD complexes could be analysed in detail.

Besides the strong similarities between Herp and Herp2 also considerable differences of both UDPs were found. In contrast to Herp, Herp2 expression was increased by the ER stress inducing agent β-mercaptoethanol (Schulze, 2006).
As also presented in this work here, the ER stress induction by disturbance of glycosylation or calcium homeostasis did not increase Herp2 expression. In addition, it was demonstrated that Herp2 is a stable protein, which is not subject to proteasomal degradation. Moreover, Herp2 did not bind Usp7.
As shown here, ER stress treatment of the cells led to the detection of a minor increase of the Herp2 protein level. However, as the accordant Herp2 mRNA levels were not enhanced upon ER stress treatment, the slightly increased Herp2 protein level cannot be attributed to an induction of protein expression but can rather be explained by a diminished degradation and accumulation of the protein. Herp2, in contrast to Herp, is degraded slowly and proteasome-independent indicating that the stability of these UDPs is regulated by different degradation systems. In contrast to Herp, Herp2 protein expression is not induced by ER stress and Herp2 turnover is markedly delayed. These findings point towards the different dynamics of these UDPs that distinguish Herp and Herp2 decisively. Thus, it is likely that Herp2 is not capable of taking over the function of Herp in ERAD. Although the UBL domains of Herp and Herp2 show a significant similarity, Herp2 does not bind Usp7 supporting the notion that different UBL domains have binding specificities (4.2.2).

Herp2 might be a candidate which represents the functional analogue of the yeast ubiquitin domain protein Usa1p. Usa1p was shown to link Der1p to Hrd1p and enables Hrd1p oligomerisation thereby ascertaining the ERAD complex integrity (Carvalho et al., 2006; Horn et al., 2009). Herp2 and Usa1p reveal similar domain architectures and it was recently demonstrated that Usa1p as Herp2 is stable supporting the idea that these proteins are indeed functional analogues (Kim et al., 2009). Thus, it would be interesting to address whether Herp2 has an impact on the ERAD process in mammalia.

In summary, the findings on Herp2 reveal remarkable differences of Herp and Herp2 suggesting that these UDPs are not able to substitute each other in the cell. Although both ubiquitin domain proteins are components of Synoviolin based ERAD complexes, it is evident that the role of Herp2 within these complexes differs from that of Herp, a fact that underlines the peculiarity of Herp.

4.4 Conclusion

This study reveals insight into the molecular function and characteristics of Herp within Synoviolin mediated ERAD. It was shown that Herp is dynamically exchanged at Synoviolin based complexes due to its high degradation and synthesis rates in an ER stress situation. The turnover of Herp seems to be essential for its function in ERAD since both stabilisation and knockdown of Herp led to an impaired degradation of Synoviolin substrates. Although behaving like a substrate itself, Herp was not processed by Synoviolin complex components. Additionally, Herp mediated the recruitment of accessory proteins to Synoviolin. Whereas Usp7 - directly bound to the Herp UBL domain - had no influence on ERAD of CD3-δ or NHK, AUP1 - directly interacting with Synoviolin - positively affected the degradation of NHK, dependent on its CUE domain. Since Herp directly influenced the ERAD complex composition, this UDP most likely functions at the level of ubiquitination and retrotranslocation within ERAD. Herp2 is also associated with Synoviolin based complexes, but reveals different characteristics than Herp and is therefore proposed to exert different functions in ERAD, presumably in Synoviolin complexes lacking Herp. As Herp2 is unable to substitute Herp, this supports the unique role of Herp in Synoviolin mediated ERAD. Taken together, the findings of the study presented here underline the extraordinary role of Herp in the process of Synoviolin mediated ERAD which is essential for cell survival.

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Appendix

Abbreviations

Table 15. Abbreviations

Aa	amino acid
Amp	Ampicillin
AUP1	Ancient ubiquitous protein 1
BSA	bovine serum albumin
CD-δ	cluster of differentiation
CFTR	cystic fibrosis transmembrane conductance regulator
Chx	Cycloheximide
CUE	coupling of ubiquitin conjugation to ER degradation
DBC	DeoxyBigChap
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
DTT	Dithiothreitol
DUB	deubiquitinating enzyme
E.coli	Escherichia coli
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase
ECL	enhanced chemiluminescence
e.g.	exempli gratia (lat. for example)
EDTA	ethylenediamine tetraacetic acid
ER	endoplasmic reticulum
ERAD	ER associated protein degradation
FCS	fetal calf serum
Fw	Forward
G	acceleration of gravity
GAPDH	glycerin aldehyd 3 phosphate dehydrogenase
Grp78	glucose regulated protein 78
GST	glutathione S transferase
H	Hour
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
Herp	Homocysteine inducible endoplasmic reticulum-resident protein
HMGR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
Hrd1	HMG-CoA reductase degradation
HTB	hexahistidine-TEV protease-biotinylation-tag
IB	immunoblot (identical to Western blot)
Ig	Immunoglobulin
IP	Immunoprecipitation
IPTG	isopropyl-beta-D-thiogalactopyranoside
Kan	Kanamycin
Kb	Kilobase

kDa	Kilodalton
LB	Lysogeny broth
M	mol/L
Mdm2 /Hdm2	mouse / human double minute protein 2
MHC	major histocompatibility complex
NHK	Null Hong Kong mutant of α1-antitrypsin
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
QC	quality control
Rev	Reverse
RING	really interesting new gene
RIPA	radioimmunoprecipitation assay
Rpm	revolutions per minute
RT	Room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
S	Svedberg sedimentation coefficient
SDS	sodium dodecylsulfate
Sel1L	Suppressor of lin-12-like protein1
siRNA	small interfering RNA
TAE	Tris-acetate-EDTA
TCA	trichloro-acetic acid
TCR-α	T cell receptor α
TEMED	tetramethyl ethylene diamine
TEV	Tobacco Etch Virus
Tg	Thapsigargin
Tris	Tris (hydroxylmethyl) aminomethane
Tu	Tunicamycin
U	Unit
UBA	ubiquitin-associated
UBL	ubiquitin-like
UDP	ubiquitin domain protein
UPR	unfolded protein response
UPS	ubiquitin proteasome system
Usa1p	U1 SNP1-associating protein 1
USP	ubiquitin specific protease

Table 16: ER stress induction and half-lives of components of Synoviolin based ERAD complexes. For the induction of ER stress in cells the agents thapsigargin (tg) or tunicamycin (tu) were utilised. The time points of first significant induction of mRNA or protein are given. The right column of the table shows the half-lives of the corresponding proteins. n.d.=not detected; n.i.=not induced.

Component	Time point of first ER stress induction	Half-life of protein
Herp	mRNA: 4 h (tu/tg) (Kokame et al., 1996; Ma and Hendershot, 2004) - protein: 4 h (tu/tg) (Figure 26)	~3 h (Sai *et al.*, 2003, Figure 9)
Synoviolin	mRNA: 8 h (tg) (Donati *et al.*,2006)	~15 h (Kikkert *et al.*, 2004)
Derlin1,2,3	mRNA: 8 h (tu) (Oda *et al.*, 2006)	n.d.
Ube2j1	mRNA: 8 h (tu) (Oh *et al.*, 2006)	~24 h (Oh *et al.*, 2006)
AUP1	n.i. (Figure 22)	> 6 h (Figure 24)
P97	n.i. (tu) (Lilley *et al.*, 2005; Figure 9)	> 6 h (Figures 9, 15B)
OS-9	protein: ~16 h (tg) (Alcock *et al.*, 2008)	n.d.
Usp7	n.i. (tu) (Figure 15A)	> 12 h (Boutell *et al.*, 2005, Figure 15B)
Herp2	n.i. (tu/tg) (Figure 26)	> 8 h (Figure 27)
Grp78 (BiP)	mRNA: 4 h (tg) (Donati *et al.*, 2006, Figure 26)protein: 4 h (tu) (Chigurupati *et al.,* 2009)	> 48 h (Knittler and Haas, 1992)
Sel1L	mRNA: 6 h (tu), (Kaneko and Nomura, 2003) protein: 24 h (tu, tg) (Kaneko et al., 2007)	~ 3 h (Mueller et al., 2006)

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