The role of the ubiquitin system in human cytomegalovirus-mediated degradation of MHC class I heavy chains

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CHAPTER 9 SUMMARY & DISCUSSION

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One of the mechanisms used by HCMV to downregulate cell surface expression of the MHC class I complex involves the dislocation of newly synthesized class I heavy chains into the cytosol, where they are degraded by the proteasome [1,2]. Misfolded ER proteins have been found to be degraded via the same route that HCMV uses to dispose of MHC class I molecules (reviewed in [3,4]). The ubiquitin system plays an important role in this process [5-10]. In this thesis, the role of ubiquitin in the US2- and US11-dependent dislocation of MHC class I heavy chains has been studied. In this chapter, the results of this exploration are summarized and discussed.

MHC class I is only one of many immune evasion targets in CMV infection

Different species of CMV have developed different strategies to elude the immune system. This is exemplified by the diversity of immune evasion strategies identified for human and murine CMV [11,12], and recently, also for rat CMV [13] (**Chapter 2**). All these CMV species downmodulate MHC class I expression at the cell surface. As mentioned in the introduction (**Chapter 1**), HCMV downregulates MHC class I cell surface expression through multiple mechanisms, including retention in the ER [14-17], dislocation to the cytosol [1,2,18,19] and hindrance of class I maturation by the inhibition of peptide translocation by TAP [20-23]. MCMV, on the other hand, downregulates class I at the cell surface by preventing the export of class I complexes from the post-ER/early Golgi [24,25] and by lysosomal degradation [26]. RCMV adopts yet another strategy in that it only delays MHC class I maturation without any obvious degradation, resulting in a temporary downregulation at the cell surface (**Chapter 2**).

The downregulation of MHC class I molecules at the cell surface of RCMV-infected cells is observed during the first 24 hours of infection (**Chapter 2**). Immune escape during the early stage of infection could be important for the survival and replication of cytomegaloviruses in mammalian cells [27-29]. In cells infected with cytomegaloviruses, it has been shown that the first immune evasion genes are expressed as early as 4 hours after infection [30,31]. At later time points, MHC class I presentation is restored by the action of INFγ and TNF [32,33]. Direct targeting of MHC class I molecules may, therefore, be the preferred first line of defense of the herpes virus at this early time point but it is not ideal in the long run, since downregulation of MHC class I at the cell surface is an unsubtle way of immune evasion and may attract the attention of NK cells [34-36].

The role of E3 ligases in the dislocation and degradation of ER (glyco)proteins

In the introduction it has been described how ubiquitin plays a role in

the degradation of ER proteins, in particular, US2- and US11-mediated degradation of class I heavy chains (**Chapter 1**). Not only the proteasomal degradation of class I molecules, but also their retrograde transport to the cytosol is dependent on ubiquitination. This is illustrated by experiments in which ubiquitination is blocked by a temperature-sensitive mutation in the ubiquitin activating enzyme (E1). At the restrictive temperature, the class I heavy chains are retained in the ER membrane. Other substrates, including mutated ribophorin I and TCRα chains, are retained in the ER when ubiquitination is inhibited [5,37]. Shamu and colleagues have shown that ubiquitinated MHC class I species could be found attached to the ER of cells transfected with US11 [8]. By expressing US11 and class I in these cells we were able to prove that US11-mediated dislocation also depended on a functional ubiquitin system (**Chapter 3**) [7].

Several E3s were screened for their potential involvement in US11-dependent degradation. A RING mutant of the mammalian HRD1 (**Chapter 4**) did not influence US11-mediated degradation, despite the fact that we were able to show inhibition of degradation of two other ER degradation substrates, TCR-α CD3-δ, with this RING mutant (**Chapter 4**)[38]. Besides HRD1, we also screened TEB4, the mammalian homologue of doa10p, for involvement in US11-dependent degradation (**Chapter 5**). In a search for the genes responsible for the degradation of the cytosolic yeast mating factor-α2, Doa10 was identified as a novel S. cerevisiae E3 ubiquitin ligase [39]. Doa10 is a multi membrane-spanning RING finger-containing ubiquitin ligase that resides in the ER and the nuclear envelope [39]. It promotes the ubiquitination of proteins with a degradation signal denoted Deg1, which is also present within the N-terminal 62 residues of yeast mating factor-α2. Doa10 acts in conjunction with the E2 enzymes Ubc6 and Ubc7 [39].

Doa10 harbors an unusual RING-finger configuration [39]. Proteins containing this RING-CH motif have earlier been associated with transcriptional regulation and DNA binding [40-43], and designated as PHD- or LAP- domain containing proteins[44] [41]. These proteins do not function as E3 ubiquitin ligases. Aravind and colleagues [45], however, were able to discern structural differences, apart from cystein and histidine composition, making it possible to discriminate between RING-HC-containing proteins that act as ubiquitin ligases and PHD/LAP domain-containing proteins with other functions. This refinement places Doa10 in the family of E3 ligases and not in the PHD/LAP domain-containing group of proteins.

Since Doa10 is localized in the ER membrane, it was sensible to test whether Doa10 is involved in the degradation of ER proteins. Indeed, the degradation of the short-lived ER-resident E2 enzyme Ubc6 was markedly inhibited in a Doa10delta yeast mutant [39]. Degradation of Pma1-D378N and

Ste6-166, both misfolded forms of yeast plasma-membrane proteins, takes place from the ER and also depends on Doa10p [46,47]. It was found that the degradation of either of these proteins is independent of Hrd1p. The degradation of CPY*, which has been shown to depend on Hrd1p, was not influenced by Doa10 [46-48]. These results suggest that Hrd1p and Doa10 cooperate in yeast ER protein degradation, each serving a distinct subset of ER-substrates. However, when human CFTR was ectopically expressed in yeast, its degradation depended on both Hrd1p and Doa10. This was illustrated by the strong effect of deleting both E3s, whereas deleting either of them separately yielded only modest effects on the degradation of CFTR [49]. These data implicate that Hrd1p and Doa10 are capable of complementing each other in the degradation of a single substrate. When Hrd1p and Doa10 are both deleted, yeast cells become extremely sensitive to ER stress, as well as to cadmium treatment. Deletion of just one of the two genes only has modest effects [39]. Hrd1p and Doa10p are also linked to the Cdc48p-Npl4p-Ufd1p complex. A temperature-sensitive mutation in Npl4p causes the malfunctioning of the Cdc48p-Npl4p-Ufd1p complex. The resulting accumulation of ubiquitinated proteins in the (ER-) membrane can be suppressed by deleting both Doa10 and Hrd1p [50]. Together, these findings illustrate that both proteins have a complementary role in the degradation of ER proteins and the neutralization of ER stress in yeast.

We identified TEB4 as the mammalian homologue of yeast Doa10p (**Chapter 5**) [51]. It had originally been characterized as a transcript of the Cri-du-chat critical region on chromosome 5. It appears to be well-conserved, as genes with a high degree of homology to TEB4 occur in many species. TEB4 contains 13 predicted transmembrane regions and has a RING-CH domain near its N-terminus. It exhibits UBC7-dependent E3 ligase activity *in vitro,* which is also ubiquitin lysine 48-specific (**Chapter 5**) [51]. While it promotes its own degradation in a RING-finger and proteasome-dependent fashion (**Chapter 5**) [51], other substrates for TEB4 have not been found as yet. We tested the effect of over-expression of TEB4 and its RING-finger mutant on US11-dependent dislocation of MHC class I molecules, and on the degradation of UBC6. No effect on the degradation of either of these substrates (Hassink et al. unpublished) was observed. The putative role for TEB4 in ER protein degradation is, however, supported by its homology with S. cerevisiae Doa10p [39], its ER localization, the large number of transmembrane regions, the involvement of lysine 48 of ubiquitin in its E3 ligase activity, the *in vitro* dependence on UBC7, and its RING domain-dependent degradation by the proteasome (**Chapter 5**) [51].

Membrane-associated RING-CH (MARCH) proteins [52], such as murine gammaherpesvirus 68 mK3 and Kaposi sarcoma herpesvirus encoded

kK3 and kK5, inhibit the expression of MHC class I complexes and the co-stimulatory molecules ICAM-1 and B7.2 on the cell surface [52-59]. These E3 ligases mediate ubiquitin-dependent internalization of receptor molecules and their degradation in an endolysosomal compartment. Neither TEB4 nor its RING-finger mutant affected the surface expression of such immuno-modulatory molecules as MHC class I, Fas, TfR, CD4, and B7.2 (Hassink et al. unpublished), suggesting that TEB4 does not share this function with some of the other MARCH proteins.

The role of ubiquitin in protein dislocation

The observation that the process of retrograde transport of ER proteins to the cytosol is dependent on ubiquitination [8](**Chapter 3**) [5,7], not only raises questions concerning the E3 responsible for this process but also with regard to the specific role of ubiquitin-conjugation in this process. It is reasonable to assume that the degradation substrates become poly-ubiquitinated themselves [6]. However, ubiquitination of substrates before their dislocation is difficult to envisage for ER-lumenal substrates or proteins that lack lysines in their cytosolic domains. Yet ubiquitination is essential for the retrotranslocation of ER-lumenal substrates like CPY* [60] and mutated ribophorin I [5]. Two observations may provide hints for the explanation of this apparent paradox. First, it has been suggested that the dislocation may be divided into two distinct steps [61]. Substrates that were initially lumenal could thus be ubiquitinated while associated to the cytosolic side of the ER membrane. This then may be essential for their actual release into the cytosol, which is thought to be directed by the p97-Ufd1-Npl4 complex [61,62,131].

The second observation that may explain the role of ubiquitin in dislocation of ER proteins involves TCRα and MHC class I heavy chains, well-known ER degradation substrates, each containing a number of lysines. However, removal of the lysines from the cytosolic tail of MHC class I heavy chains does not influence their dislocation or degradation [6]. Moreover, removal of all the lysines from the TCRα chain results in dislocation and proteasomal degradation with kinetics indistinguishable from that of wild-type TCRα [63]. These data indicate that although these proteins possess lysines accessible to the ubiquitination machinery, these are not important for the removal of the proteins to the cytosol. Ubiquitination may still take place at the N-terminus of the lysine-less TCRα chains to facilitate their release from the ER membrane. This would require their relocation to the cytosolic side of the ER membrane prior to ubiquitination. Yet, we have recently provided evidence that the N-terminus is also dispensable for ubiquitin-dependent dislocation. An MHC class I heavy chain with all its lysines mutated to arginines and its N-terminus blocked by the fusion of lysine-less ubiquitin

molecule to this N-terminus [64,65], was dislocated to the cytosol as the wild-type molecule in the presence of HCMV US11 (**Chapter 6**). As a result of the lack of ubiquitination sites, however, proteasomal degradation of the mutant heavy chains was much slower, resulting in the appearance of a deglycosylated intermediate in the absence of proteasomal inhibition (**Chapter 6**). In US2-expressing cells, these lysine-less heavy chains were not dislocated but remained stable in the membrane despite the fact that they could be found associated with US2 molecules (**Chapter 6**). In this respect, US2-mediated dislocation resembles HIV Vpu-induced dislocation of CD4 [66]. These experiments established that the ubiquitin machinery uses different target NH_2 groups to discriminate between the dislocation of ER substrates and the degradation of dislocated substrates by proteasomes, thus illustrating that dislocation and degradation are separate events. Furthermore, these data indicate that fundamental differences may exist between US2- and US11-mediated dislocation. But most importantly, the experiments suggest that dislocation of ER proteins to the cytosol does not necessarily involve ubiquitination of the substrate itself. Instead, ubiquitination of an adaptor molecule *in trans* may be an essential step in the dislocation reaction. This compels us to form a new hypothesis about the process of dislocation.

Differences between US2 and US11 dependent dislocation

Several publications suggest that US2 and US11 differ in their mechanisms to dislocate heavy chains to the cytosol. To begin with, US2 works at much lower concentrations than US11 [67]. This would suggest that US2 merely induces dislocation for newly synthesized class I molecules in general, were it not for the fact that US2 has rather specific substrate-binding requirements, which argues in favor of active participation of US2 in the dislocation of specific MHC class I haplotypes [68].

Unlike US2-mediated dislocation, US11-mediated dislocation of class I heavy chains depends on the small multi-spanning ER membrane protein Derlin-1, which can be found in complex with US11 but not US2 [69,70]. Remarkably, the degradation of US2 itself does depend on Derlin-1 [70], which indicates that heavy chain dislocation and US2 dislocation are separate events and renders an earlier hypothesis that US2 functions by dislocating in complex with the heavy chain highly unlikely.

US2 and US11 also differ in their recognition patterns. US2 has a broader specificity in that it also targets MHC class II molecules for degradation [71-74]. It would be interesting to ascertain whether US2 uses the same binding surface for the recognition for both class I and II [68].

Finally, US2 and US11 differ in the requirements concerning cytosolic domains. The cytosolic tail of the heavy chain may be used as a recognition

beacon for cytosolic factors like the proteasome or the p97-Ufd1-Npl4 complex. Whereas the cytosolic tail of the heavy chain is required for US11-dependent dislocation [75,76], depending on the experimental circumstances, the heavy chain tail can be omitted in US2-mediated dislocation [68,76]. Looking at the cytosolic domains of US2 and US11 themselves, on the other hand, the situation is reversed. The tail of US2 is required for heavy chain dislocation, but the tail of US11 is not [73,77]. It could be the case, therefore, that in US11-dependent dislocation the tail of the heavy chain is used to interact with proteasomes or p97 complexes, whereas in US2-mediated dislocation this function is performed by US2 itself.

Communication between the lumen of the ER and the cytosol

It is clear that US2 and US11-dependent dislocation depend on a functional ubiquitin system, the proteasome and the p97 complex (**Chapter 3**, 5) [62,78]. How these three cytosolic components work together with the luminal side of the ER is not clear. The dependence on functional proteasomes, for instance, varies with the circumstances on the luminal side of the membrane, which is exemplified by experiments in β_2m-negative cells. β_2m binding is one of the prerequisites for MHC class I molecules to egress to the Golgi and beyond [79-81]. The folding of heavy chains occurs in β_2m negative cells, but, in contrast to maturation in β_2m positive cells, a significant portion of the newly synthesized heavy chains in β_2m negative cells is found in a reduced state (**Chapter 7**) [82]. In U373 astrocytoma cells in which β_2m expression was knocked down by RNAi, it has been shown that US2 requires β_2m for the dislocation of heavy chains when the proteasome is inhibited [78]. In β_2m negative FO-1 melanoma cells without proteasome inhibitor, however, both US2 and US11 are able to target heavy chains for dislocation, suggesting that, in principle, lack of β_2m is not sufficient to inhibit US2- and US11-mediated dislocation (**Chapter 7**). Yet again, a difference was found between US2 and US11 when the proteasome was inhibited. US11-dependent dislocation of human heavy chains and normal ER-associated degradation of human heavy chains due to lack of β_2m were much more sensitive to proteasomal inhibition than US2-dependent dislocation (**Chapter 7**) [82]. The different outcomes of the two experiments suggest that β_2m positively influences the degradation of heavy chains by US11. In either case, the proteasome has a strong influence on the efficiency of US2- and US11-mediated dislocation, as has been observed for many other cases of ER-associated degradation. These data indicate that the dislocation of ER proteins is not driven by a single component, but that the efficiency of the dislocation process is influenced by several components.

Dislocation in progress

The fact that the lumenal portion of the heavy chain does not need to be ubiquitinated in order to be dislocated by US11 also raises the question whether heavy chains are extracted via their N-termini or their C-termini (**Chapter 6**). Several studies hypothesized that US11-dependent dislocation occurs from the N-terminus to the C-terminus, but this was based on the finding that ubiquitination of the heavy chain is required for its dislocation and that lysines in the cytosolic tail of the heavy chain are not required, suggesting that ER luminal-positioned lysines are used [3,8,69]. Extraction via the N-terminus, however, is more complicated than C-terminal extraction as it involves a second contact with the membrane. Furthermore, the molecule has to bend and probably even unfold to make this possible. By determining the relative amounts of differently situated epitopes within dislocating heavy chains that were exposed to the cytosol during pulse labeling in the presence of US11, we observed that TM-proximal regions appeared in the cytosol prior to the N-terminus (**Chapter 8**). This suggests that, in the case of US11-mediated dislocation of heavy chains, extraction starts at the C-terminal end of the class I molecule.

In β_2m-negative cells, in which heavy chains are prone to ER-associated degradation, a relatively high number of heavy chains were observed to be in a reduced state (**Chapter 7**). Conversion of heavy chains to a reduced state may be a prerequisite for efficient dislocation. Co-precipitation of Sec61β with dislocated MHC class I heavy chains in the presence of US2, as well as studies with Sec61α mutants, suggest that the Sec61 complex acts as a dislocation channel [1,83-85]. In this model, TRAM could facilitate the re-entry of multi-spanning membrane proteins into the translocon. The translocon has a diameter of only 40-60 Å [86], implying that substrates using this channel will probably have to be unfolded by ER resident-chaperones prior to dislocation. However, a number of studies suggest that this is not the case. MHC class I molecules with an N-terminally fused GFP can be detected fluorescently active in cytosol in the presence of US2, suggesting that the fusion protein was dislocated in a folded state [87]. Another study shows that fusion of a dihydrofolate reductase (DHFR) domain to the N-terminus of MHC class I does not inhibit dislocation in the presence of metotrexate [88], which induces rigid folding in the DHFR domain. Both these studies point to dislocation in a folded state and require a more flexible diameter of the pore, as has already been suggested by others [89-92]. We attempted to establish whether the ER-dislocation mechanism was also able to facilitate the dislocation of larger substrates by generating large degradation-prone complexes in the lumen of the ER, consisting of an antibody that recognized a tag within an MHC class I heavy chain (**Chapter 8**). The expression of HA tag-containing MHC class I

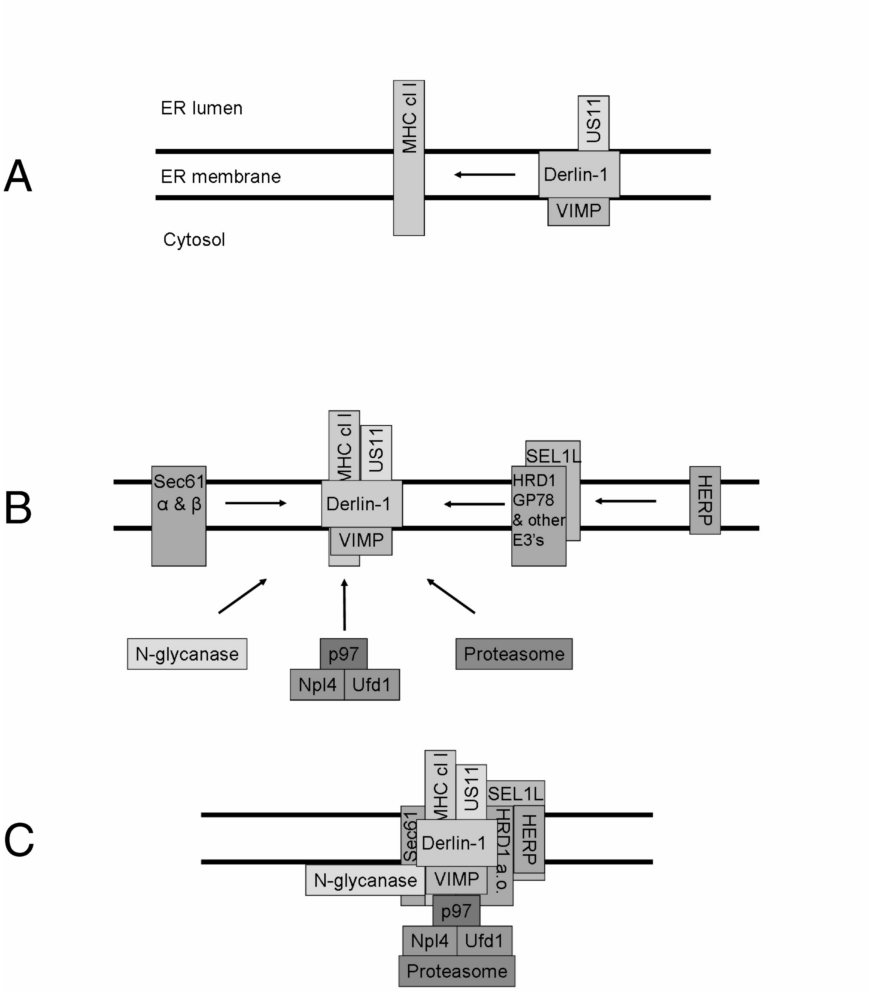

Figure 1. formation of the US11 dislocation complex. See text for explanation.

heavy chains in anti-HA antibody-producing hybridoma's resulted in the formation of class I-antibody complexes. We have been unable to obtain stable transfectants of these constructs in these hybridoma's, which may be related to toxicity of the class I heavy chain-antibody complexes for the cell (**Chapter 8**). Our transient experiments revealed, that the IgG heavy chains were more unstable in cells expressing HA-containing MHC class I heavy chains than in wild-type class I heavy chain transfectants (**Chapter 8**). This suggests that the IgG-MHC heavy chain interaction induced dislocation of

both molecules simultaneously and might imply that the IgG- and MHC-heavy chains are dislocated.

A model for US11-dependent dislocation

As mentioned earlier, the observation that US11-mediated dislocation is not dependent on ubiquitination of the MHC class I molecules combined with the observation that the dislocation process itself is dependent on ubiquitination, suggests that ubiquitination of an adaptor molecule *in trans* may be required. MHC class I-US11 complexes do not only co-precipitated with Derlin-1 and p97 [69,70], but also with HRD1 and HERP [93-95] (van Voorden unpublished).

Based on these results, the following model for US11-dependent dislocation can be envisaged (Figure 1). The first step is the recognition of MHC class I heavy chains by US11, which is in complex with Derlin-1 and the small membrane spanning molecule VIMP [69,70]. The US11-Derlin-1-VIMP complex attracts the HERP-HRD1 complex (**Chapter 6**) which may be initiated by the ubiquitination of HERP or VIMP or other still unidentified components of the dislocation machinery [69]. It is not certain whether the ubiquitination event required for dislocation is driven by HRD1, since a RING mutant of HRD1 is not able to inhibit US11-dependent dislocation [38]. It is, however, possible that the transmembrane regions of Derlin-1 and the multi-membrane spanning HRD1 form a conduit around the heavy chain as was hypothesized for the twin-arginine translocons used by peroxisomes and plant thylakoid membranes [96,97]. In addition, gp78, TEB4 and other ubiquitin ligases with multispanning membrane domains may be recruited into the dislocation complex. Furthermore, the translocon components Sec61α and β have been suggested to be part of the "dislocon" [1,85,127-130]. The advantage of forming a dislocon after the substrate has been defined is that it may be adapted to the nature of the substrate, e.g. the size of the substrate [87,88]. Such an *ad hoc* arrangement would allow easy embrace of transmembrane regions of misfolded protein substrates CFTRdelta508 [98,99] and apolipoprotein B100 [100,101]. An additional advantage would be that the membrane integrity would stay intact since, before and after dislocation, there is no pore to keep closed in the absence of dislocation substrates [102-105]. Both the p97-Ufd1-Npl4 complex and the proteasome might be attracted by the dislocon, or even stabilize it [62]. Using either the ATPase activity of p97 or that of the proteasome, the dislocation substrate is extracted from the membrane and simultaneously unfolded and degraded by the proteasome. Just before proteasomal degradation, the N-glycanase may remove any N-linked glycans from the substrate [106,107]. In the absence of proteasomal activity, the p97 complex extracts the substrate from the membrane [61]. In the absence of

ATPase acitivity in the p97 complex, the proteasome may facilitate both extraction and degradation [108,109].

The future

Taken together, this thesis contributes to the partial characterization of an ER-route of degradation co-opted by HCMV to dispose of MHC class I molecules. For the future, it will be interesting to determine the exact definition of the dislocon and to ascertain whether there is only one type of dislocon in the ER or whether several different dislocon compositions for different groups of substrates exist. As to the value of the research described in this thesis in the quest for treatment of HCMV-related problems, it could be interesting to develop inhibitors of US2- and US11-class I interactions [110]. This may contribute to the eradication of HCMV in HIV patients and patients receiving immunosuppressive drugs.

Besides CMV immune escape, this thesis deals with the role of ubiquitin in ER-associated degradation. After the discovery, in 1980, that ubiquitin was involved in protein turnover [111,112], research on ubiquitin has expanded significantly and ubiquitin-dependent degradation is now an important issue in contemporary science. This is emphasized by the fact that Aaron Ciechanover, Avram Hershko, and Irwin Rose were awarded the Nobel Prize for Chemistry in 2004 for their work on ubiquitin. Defective ubiquitination of ER proteins forms the basis of such diseases as Alzheimers disease [113-116], autosomal-recessive juvenile parkinsonism [117-120], type 2 diabetes mellitus [121], and rheumatoid arthritis [122,123]. In addition, there are indications that ER stress [124-126] could be involved in the development of type 1 diabetes mellitus. These examples clearly illustrate the crucial importance of gaining fundamental insight into such cell biological issues as the role of the ubiquitin system in relation to the degradation of ER proteins described in this thesis.

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