Published in final edited form as:

Nat Struct Mol Biol. ; 18(10): 1147–1152. doi:10.1038/nsmb.2111.

Derlin-1 is a rhomboid pseudoprotease required for the dislocation of mutant α -1 antitrypsin from the endoplasmic reticulum

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Abstract

The degradation of misfolded secretory proteins is ultimately mediated by the ubiquitin-proteasome system in the cytoplasm, therefore endoplasmic reticulum-associated degradation (ERAD) substrates must be dislocated across the ER membrane through a process driven by the AAA ATPase p97/VCP. Derlins recruit p97/VCP and have been proposed to be part of the dislocation machinery. Here we report that Derlins are inactive members of the rhomboid family of intramembrane proteases and bind p97/VCP through C-terminal SHP boxes. Human Derlin-1 harboring mutations within the rhomboid domain stabilized mutant α -1 antitrypsin (NHK) at the cytosolic face of the ER membrane without disrupting the p97/VCP interaction. We propose that substrate interaction and p97/VCP recruitment are separate functions that are essential for dislocation and can be assigned respectively to the rhomboid domain and the C terminus of Derlin-1. These data suggest that intramembrane proteolysis and protein dislocation share unexpected mechanistic features.

Human Derlin-1 was originally identified as a US11-interacting protein required for cytomegalovirus-mediated turnover of the class I major histocompatibility complex (MHC) heavy chain¹ and as an endoplasmic reticulum (ER) membrane binding site for p97/VCP (known as Cdc48 in yeast)². In mammalian cells, degradation of both soluble and integral membrane ERAD substrates requires Derlin-1 or one of its paralogs, Derlin-2 or Derlin-3 (refs. 1,3,4). By contrast, yeast lacking the Derlin-1 ortholog Der1 are unable to degrade secreted ERAD substrates but are proficient at degrading integral membrane proteins^{5–7}. In addition, yeast contain a second Derlin homolog, Dfm1, deletion of which does not appear to confer a defect in degradation of misfolded secreted proteins^{8,9} but may stabilize some integral membrane ERAD substrates¹⁰. Derlins are integral membrane proteins proposed to span the ER membrane four times^{1,8} and have been observed to form homo-oligomers and heteromultimeric complexes^{11–13} with membrane-bound machinery essential for ERAD, including the E3 ubiquitin ligase Hrd1 and its binding partner SEL1L. Antibodies targeting

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AUTHOR CONTRIBUTIONS

E.J.G. and R.R.K. contributed to the design of all of the experiments and wrote the manuscript. E.J.G. did the experiments and analyses in **Figure 1a–d,f,g**; **Figure 2–4**; and **Supplementary Figures 1, 2, 4 and 5**. The microscopy experiments in **Figure 1e** and **Supplementary Figures 3 and 6** were conducted by J.A.O. All authors contributed to the interpretation and conclusions of the experiments.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

the cytosolic C terminus of Derlin-1 abolish the *in vitro* dislocation of mutant pro- α -factor from canine microsomes¹⁴. Whereas the precise function of Derlins is unclear, they have been proposed to function in the formation of an ER export channel through which ERAD substrates can pass^{1,2}. In this study we identified an evolutionary relationship between Derlins and rhomboid family intramembrane proteases, ubiquitous enzymes found in all kingdoms of life, whose functions extend to a broad range of biological activities^{15–18}.

The crystal structure of the bacterial rhomboid protease GlpG revealed the presence of an active site Ser-His dyad contained within an aqueous membrane-embedded cavity¹⁹, thereby allowing water to access the active site during substrate hydrolysis. A number of rhomboid family members lack the catalytic Ser-His dyad required for proteolytic activity²⁰ but are predicted to retain the overall rhomboid architecture. The function of these inactive rhomboid pseudoproteases is poorly understood. We found that Derlins also lack an active site Ser-His dyad but contain other conserved motifs that are essential for the catalytic function of homologous rhomboid proteases. Our data suggest a role for the Derlin-1 rhomboid pseudoprotease domain in facilitating the release of ERAD substrates from the ER following their transfer across the membrane.

RESULTS

Homology of Derlin-1 with rhomboid membrane proteases

Bioinformatic analysis of the Derlin-1 membrane domain using the Phyre homology server²¹ revealed an unexpected but highly significant shared homology (E value of 6.3×10^{-13}) between Derlin-1 and GlpG, a bacterial member of the rhomboid family of intramembrane proteases, whose ability to cleave membrane protein substrates has been characterized extensively *in vitro* and *in vivo*^{22–26}. In addition, the remote homology detection server HHpred²⁷ predicted Derlin-1 and GlpG to be homologs at a confidence level of 99.8%. Rhomboids are ubiquitous across evolution and function in a wide variety of biological processes, including EGFR signaling^{16,17}, quorum sensing²⁸ and parasite invasion^{29,30}. Although rhomboids are highly divergent, sharing only ~6% sequence identity across known family members within a core six-pass transmembrane region²⁴, the Phyre and HHpred analyses identified several regions that are highly conserved in rhomboids and were also found to be highly conserved among Derlins (**Fig. 1a**). In GlpG and secretase-type rhomboids, a 'WR motif' (Q/ExWRxxS/T) is conserved in loop L1 connecting TM1 and TM2 (ref. 20) of the core rhomboid domain, a motif previously noted in Derlins²². The L1 loop is a structural feature unique to rhomboid family proteins and unusual in that it is membrane-embedded and protrudes laterally into the bilayer^{19,31}. Molecular dynamics simulations of GlpG within a model lipid bilayer suggest that L1 mediates rearrangement of the surrounding lipid environment and may also be involved in regulating the positioning and dynamics of the enzyme³². A second feature, the GxxxG transmembrane dimerization motif in TM6 of the core rhomboid domain, probably enables tight packing of TM4 and TM6, thereby aligning the active site Ser-His dyad^{19,31}. Mutations within either motif substantially reduce or abolish proteolytic activity^{22,26,33,34}. A threaded homology model of Derlin-1 based on a published structure of GlpG¹⁹ predicts that conserved WR and GxxxG motifs are located in putative L1 and TM6 of Derlin-1 respectively, identical to their positions in GlpG (**Fig. 1b,c**). In addition, positively charged residues were found flanking the cytoplasmic side of each predicted transmembrane span in the homology model (**Fig. 1d**), consistent with the positive-inside topological rule³⁵. Because the rhomboid active site Ser-His dyad is not conserved within Derlins (**Fig. 1a**), Derlins are unlikely to be proteolytically active despite their homology to rhomboid proteases. Phylogenetic analysis of rhomboid family proteins grouped Derlins with two subfamilies of rhomboid proteins that contain predicted UBA domains at their C termini (**Supplementary Fig. 1**), suggesting a conserved ubiquitin-related function among this subset of rhomboids.

We validated the Derlin-1 homology model by Ramachandran plot analysis (**Supplementary Fig. 2**), which revealed that ~98% (174 of 178) of the residues in the Derlin-1 homology model were in allowed regions. This is highly comparable to a recently reported homology model of the mitochondrial rhomboid PARL³⁶, generated from structures of GlpG, in which ~97% of the modeled residues were in allowed regions. In both models, the outlying residues were located exclusively within loops or turns, which are known to be difficult to model. These data suggest that the Derlin-1 homology model is of similar quality to one constructed of PARL, an established member of the rhomboid family.

The core membrane domain of rhomboid family members contains a six-pass transmembrane topology¹⁹. Therefore, the placement of Derlins into the rhomboid family is incompatible with previous studies that support a four-pass transmembrane structure^{1,8}, which may have confounded the interpretation of previous sequence comparisons between Derlins and known rhomboid proteases²⁰. To distinguish between the four- and six-pass models, we experimentally mapped Derlin-1 topology by subcellular localization of the N- and C- termini and insertional mutagenesis of N-linked glycosylation acceptor sequences. Antibodies against hemagglutinin (HA) or S-tag were used to label the ER in cells expressing Derlin-1 tagged with these epitopes at its N- and C termini, respectively, under conditions in which the plasma membrane, but not the ER membrane, was selectively permeabilized (**Fig. 1e**). Both termini of Derlin-1 were thus exposed to the cytoplasm, consistent with previous studies localizing the Derlin-1 C terminus to the cytoplasm^{1,4}. Glycosylation acceptor sequences were inserted into the predicted loops of Derlin-1, and N-glycosylation, indicative of luminal localization, was assessed by immunoblot analysis of cell lysates treated with endoglycosidase H (Endo H). S-tagged Derlin-1 was glycosylated when acceptor sequences were inserted after residues Ala45, Leu114 and Val170, but not Ala90 or Val142, consistent with the six-pass rhomboid homology model and incompatible with the previously reported four-pass model (**Fig. 1f,g**). We found that the cytoplasmic localization of the C terminus of each of the chimeras was unaffected by the insertion of the glycosylation acceptor sequence (**Supplementary Fig. 3**), suggesting that the overall topology of the Derlin-1 chimeras was not disrupted. These data, together with the structural modeling and bioinformatic analysis, support a six-pass transmembrane topology for Derlin-1 and thus its classification as a rhomboid family member.

Derlin-1-bound p97/VCP is required for the extraction of NHK

Considerable genetic and biochemical evidence implicate the AAA ATPase p97/VCP in driving dislocation of ERAD substrates^{37–39}. We reasoned that disruption of the Derlin-1–p97/VCP interaction^{11,12} should impair the dislocation of a folding-defective secreted protein. The yeast Derlin homolog Dfm1 (but not Der1) binds Cdc48, the yeast ortholog of p97/VCP, through two SHP boxes (FxGxGQRn, where n is a non-polar residue) in its cytoplasmic domain⁹, but it is dispensable for the degradation of misfolded secreted proteins^{8,9}. We identified sequences at the C termini of Derlin-1 and Derlin-2 that fit the consensus SHP box motif present in Dfm1 and other p97/VCP/Cdc48 binding proteins⁴⁰ (**Fig. 2a,b**). Deletion of the SHP box in Derlin-1 or Derlin-2 resulted in the loss of p97/VCP association (**Fig. 2c**) but not that of another interacting protein, UBXD8 (ref. 41), demonstrating that Derlin1/2 SHP boxes are essential specifically for their ability to bind to p97/VCP.

To determine whether the Derlin-1–p97/VCP interaction is essential for dislocation of a soluble ERAD substrate, we assessed the effect of S-tagged wild-type Derlin-1 (Derlin-1_{WT}) or mutant Derlin-1_{1–240} lacking the SHP box on the degradation of coexpressed NHK, a constitutively degraded truncated variant of α -1 antitrypsin^{4,42}. Coexpression of HA-tagged NHK with Derlin-1_{1–240} or Derlin-1_{1–193} lacking the entire cytoplasmic domain led to the appearance of a nonglycosylated form of NHK that comigrated with NHK from lysates

treated with Endo H (**Fig. 2d** – and **Supplementary Fig. 4**). The abundance of this species was reduced when NHK was co-transfected with a green fluorescent protein (GFP) control or wild-type Derlin-1 expressed at similar levels (**Fig. 2d–f**). Knockdown of PNG1, an enzyme that deglycosylates ERAD substrates in the cytoplasm before degradation⁴³, largely eliminated this nonglycosylated form in cells coexpressing NHK and Derlin-1_{1–240} (**Fig. 2h**), indicating that these faster migrating species represent NHK species that had been deglycosylated by PNG1 and confirming their exposure to the cytoplasm^{43–45}. Deglycosylated forms of NHK coprecipitated with mutant Derlin-1_{1–240} and Derlin-1_{1–193} (**Fig. 2d**), demonstrating that disruption of the Derlin-1–p97/VCP interaction stabilized a fraction of NHK in a deglycosylated form at the cytoplasmic face of the ER membrane still associated with Derlin-1. In addition, the observation that NHK coprecipitated with Derlin-1_{1–193} (**Fig. 2d**) demonstrates that the Derlin-1 rhomboid domain is sufficient to bind NHK. Together, these data indicate that in mammalian cells, unlike in yeast, the Derlin-1 SHP box recruits p97/VCP to functional ERAD complexes for the extraction of misfolded secreted proteins from the cytoplasmic face of the ER membrane. Stabilization of ERAD substrates at the cytoplasmic face of the ER has also been observed in yeast strains expressing mutant Cdc48 (ref. 46) or its cofactor Ufd1 (ref. 38), in microsomes incubated with mutant p97/VCP³⁷ and in mammalian cells incubated with the proteasome inhibitor MG132 (ref. 47). Thus, complete dislocation of a luminal substrate like NHK can be divided into two normally coupled steps: the first, in which the substrate is transferred from the ER lumen to the cytoplasmic face of the ER membrane, and the second, in which the dislocated substrate is physically released from the membrane into the cytosol—or ‘handed off’ to the proteasome^{46,47}. Our data therefore suggest a role for Derlin-1-bound p97/VCP in the latter step in the dislocation of NHK.

The GxxxG motif is essential for NHK extraction from the ER

To test whether the Derlin-1 rhomboid domain is required for NHK dislocation, we compared the effect of expressing Derlin-1_{G176V}, mutated within the GxxxG motif, with the SHP box mutant Derlin-1_{1–240} on the dislocation of NHK. Coexpression of Derlin-1_{G176V} with NHK resulted in a marked stabilization of deglycosylated NHK, compared to expression of Derlin-1_{WT}, truncated Derlin-1_{1–240} or a GFP control (**Fig. 3a,b**). We observed deglycosylated NHK coprecipitating with Derlin-1_{G176V} (**Fig. 3a**), indicating that a fraction of NHK remained associated with the ER membrane. We assessed whether deglycosylated forms of NHK stabilized by Derlin-1_{G176V} were cytosolically exposed by treating membranes purified from cells expressing NHK and Derlin-1_{G176V} with proteinase K. The C-terminal HA-tag of deglycosylated NHK was completely sensitive to proteolysis, whereas a substantial fraction of fully glycosylated NHK was protected, indicating that deglycosylated NHK was accessible to the cytoplasm (**Fig. 3c,d**). By contrast, luminal proteins (BiP and GRP94) or the luminal domain of an integral membrane protein (SEL1L) were resistant to protease treatment. Identical results were obtained with NHK tagged at both its N- and C termini with 3×Flag and HA-tags respectively (**Supplementary Fig. 5**), indicating that both termini of deglycosylated NHK were exposed to the cytoplasm. However, these forms were still associated with the lipid bilayer, as they sedimented exclusively in a membrane fraction (**Supplementary Fig. 5**). This effect was specific to mutation of the GxxxG motif because expression of another GxxxG mutant, Derlin-1_{G180V}, but not Derlin-1_{G29V} or Derlin-1_{G147V}, that contains glycine mutations outside the GxxxG motif, resulted in a similar phenotype (**Fig. 3e**). Thus, expression of Derlin-1_{G176V} or Derlin-1_{G180V} severely impairs the extraction of NHK from the ER membrane. The observation that Derlin-1_{G180V} still coprecipitated p97/VCP together with endogenous Derlin-1 (**Fig. 3f**) demonstrates that the dislocation defect observed in the presence of the rhomboid domain mutant is not due to impaired p97/VCP binding or loss of the ability to form homo-oligomers. In addition, short hairpin RNA (shRNA)-mediated knockdown of

Derlin-1 resulted in comparable stabilization of deglycosylated NHK (data not shown), indicating that the dominant negative effect of the Derlin-1 GxxxG mutants is a result of a loss of Derlin-1 function. Together, these data demonstrate that the GxxxG motif within the Derlin-1 rhomboid domain is essential for the extraction of NHK from the cytoplasmic face of the ER membrane independently of Derlin-1's function as an ER membrane anchor for p97/VCP.

Expression of Derlin-1 WR motif mutants impairs NHK dislocation

To determine whether the L1 loop of the Derlin-1 rhomboid domain is essential for the dislocation of NHK, we tested the effect of expressing Derlin-1 containing mutations within the WR motif on the steady-state levels and glycosylation status of NHK. Unexpectedly, the levels of Triton X-100-soluble NHK were greatly reduced when NHK was coexpressed with Derlin-1 mutated at any of the four conserved WR motif residues (Q51A, W53A, R54A or T57A), but not with Derlin-1_{WT} or Derlin-1_{W106A}, mutated at a less conserved tryptophan residue outside the WR motif, or with a GFP control (**Fig. 4a**). This reduction in NHK levels may be explained by either reduced solubility or increased degradation of NHK. NHK was largely found in a Triton X-100-soluble fraction in cells expressing Derlin-1_{WT}, whereas the majority of NHK became Triton X-100-insoluble in cells expressing mutant Derlin-1_{R54A} (**Fig. 4b**). A substantial fraction of this insoluble material was deglycosylated (**Fig. 4b**), indicating that expression of Derlin-1_{R54A} leads to both impaired dislocation and accumulation of aggregated, insoluble NHK. The effect of disrupting the WR motif on NHK degradation was not due to impaired binding of p97/VCP, as equivalent amounts of p97/VCP were coprecipitated with Derlin-1_{WT} and Derlin-1_{R54A} (**Fig. 4c**). Furthermore, expression of Derlin-1_{W53A} or Derlin-1_{G176V}, but not Derlin-1_{WT}, resulted in a marked stabilization of NHK (**Fig. 4d,e**). In addition, we tested the stability of the Derlin mutants W53A and G176V, which appeared to be expressed at lower levels in some experiments, and found no gross differences in the stability of these variants compared to wild-type Derlin-1 (**Fig. 4f**). The effects of these mutants was not due to ectopic cellular localization, as the Derlin-1 mutants were localized normally to the ER (**Supplementary Fig. 6**). Therefore, the Derlin-1 WR motif is essential for NHK dislocation in a manner that is independent of p97/VCP recruitment; its disruption leads to stabilization of aggregated forms of NHK, suggesting a critical role for the L1 loop of Derlin-1 in maintaining the solubility of folding-defective secreted protein during dislocation.

DISCUSSION

Derlins were originally proposed to be transmembrane channels through which ERAD substrates are dislocated across the ER membrane^{1,2}. However, the structure of the homologous rhomboid protease GlpG, the only member of this protein family for which a high resolution structure exists, lacks any evident path through the membrane and is thus inconsistent with a channel-like function. Therefore, barring major structural differences between Derlins and GlpG, or induced conformational changes, it is unlikely that Derlins themselves function as dislocation channels.

The destabilization and unfolding of polypeptide substrates is a prerequisite to their hydrolysis by rhomboid proteases, as scissile peptide bonds within the membrane are normally hidden within transmembrane α -helices. The Derlin-1 rhomboid domain may, together with p97/VCP, promote unfolding of the integral membrane oligomeric complexes formed by the dislocation machinery⁴⁸, thereby facilitating disassembly of the dislocation machinery and enabling the subsequent release of substrates following their transfer across the membrane. Complete loss of Derlin function would lead to a failure to disassemble ERAD complexes for a second round of dislocation, and this may explain why pre-incubation of microsomes with antibodies against Derlin-1 was observed to block the *in*

vitro dislocation of mutant pro- α -factor only after a short lag in which the initial rate of dislocation was unaltered¹⁴.

Finally, Derlins may interact directly with substrates to enable their extraction from the ER membrane. Derlins and p97/VCP may function in an analogous fashion to the mitochondrial rhomboid Pcp1, which also cooperates closely with a AAA ATPase, *m*-AAA, to dislocate and cleave Ccp1 within the mitochondrial inner membrane⁴⁹. The irregular shape of rhomboids, due in part to the amphipathic L1 loop and their small hydrophobic thickness, has been proposed to substantially compress and deform the bilayer to facilitate the unfolding and exposure of substrate cleavage sites³³. This property may endow Derlins with the ability to facilitate the release of substrates from a membrane-associated state during dislocation.

Of the thirteen predicted rhomboid genes in the human genome, including Derlins, only five encode the conserved catalytic Ser-His dyad (**Supplementary Fig. 2**). The biological functions of inactive rhomboids are largely unknown. However, one recent report⁵⁰ demonstrates that the ER-localized inactive rhomboid iRhom functions as a negative regulator of neuronal EGFR signaling in *Drosophila melanogaster* by facilitating the degradation of EGFR ligands by the proteasome, thereby establishing a functional link between a rhomboid pseudoprotease and ERAD. We propose that Derlins represent an expansion of this function in the broader context of ER quality control.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/nsmb/>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank M. Pearce, J. Christianson and C. Richter for their insightful comments on the manuscript. We also thank H. Ploegh (Whitehead Institute of Biomedical Research) and W. Lennarz (Stony Brook University) for generously providing reagents. This work was supported by a grant from the National Institute of General Medical Science (NIGMS) to R.R.K. E.J.G. was supported by a US National Institutes of Health (NIH) predoctoral training grant and J.A.O. is the recipient of a National Research Service Award from the NIH.

ONLINE METHODS

Plasmids and constructs

The following constructs were provided: human Derlin-1 (H. Ploegh) and mouse PNG1 (W. Lennarz). Derlin-2 was cloned from a full-length human complementary (cDNA) (Open Biosystems). Derlin-1, Derlin-2 and PNG1 were appended with a C-terminal S-tag (KETAAAKFERQHMDS) and cloned into pcDNA3.1(-). For cDNA and shRNA coexpression experiments, the Δ CD4 open reading frame of pSUPERSTAR⁵¹ vectors expressing shRNAs targeting PNG1 or GFP was replaced with open reading frames encoding WT or mutant Derlin-1-S. Target sequences for silencing PNG1 (TTGTGGAGCTTGTGAATT) and GFP (GAAGCAGCAGACTTCTTC) were previously reported^{44,52}. Glycosylation acceptor sequences were introduced into Derlin-1 by insertion of AgeI and BamHI sites, followed by ligation of an oligonucleotide encoding a 35-amino acid sequence (GSGGGGDYKDDDDKIDNSTCTDYKDDDDKGGGGTG,

glycosylation acceptor sequence indicated in bold). All point mutations were made using the QuikChange Mutagenesis Kit (Stratagene) and confirmed by sequencing.

Cell culture, transfections and emetine chase experiments

HEK293 cells were maintained in DMEM containing 10% animal serum complex (Gemini Bio-Products). Cells were transiently transfected using the standard calcium phosphate precipitation method. Emetine chase experiments were conducted 2 d after transfection. Transfectants grown in six-well tissue culture plates were incubated with 75 μ M emetine for the indicated times and lysed in a 2% (w/v) SDS solution.

Affinity capture, immunoblotting and enzymatic assays

Cells were collected manually in PBS and lysed in either 2% SDS or buffer containing 150 mM NaCl, 25 mM Tris, pH 7.4, 5mM EDTA, protease inhibitor cocktail (Roche) and either 1% (v/v) Triton X-100 or 1% (w/v) digitonin as indicated. Lysates were cleared by centrifugation at 20,000g. Triton X-100-insoluble material was solubilized by sonication in a 1% SDS solution. S-tagged proteins were precipitated from 1% (w/v) digitonin cell lysates using S-protein agarose (Novagen). Samples were washed three times in lysis buffer containing 0.1% (w/v) digitonin, resuspended in loading buffer containing 100 mM dithiothreitol and treated with Endo H (New England Biosystems) where specified. Samples were run on uniform SDS-PAGE gels and transferred to PVDF membranes for immunoblotting.

For the protease protection assay, cells were homogenized in a glass Dounce homogenizer, and unbroken cells and nuclei were removed by centrifugation at 600g for 5 min. Membranes were pelleted by centrifugation at 100,000g for 30 min and resuspended in MSB buffer (150 mM potassium acetate, 5 mM magnesium acetate, 50 mM HEPES, pH 7.4, 200 mM sucrose). Proteinase K was added to membrane aliquots at the indicated concentrations in the presence or absence of 1% Triton X-100. After 30 min on ice, the reactions were inactivated by addition of 100% trichloroacetic acid to a final concentration of 15% (v/v), and samples were pelleted by centrifugation at 8,000g for 5 min. The samples were then solubilized by sonication in sample buffer and analyzed by immunoblotting.

Homology modeling and validation of the Derlin-1 rhomboid domain

Fold recognition and generation of a threaded homology model of the Derlin-1 rhomboid domain onto the structure of GlpG¹⁹ (PDB 2IC8) was conducted using the Phyre remote homology detection server²¹. The Derlin-1 homology model was validated by MolProbity⁵³ analysis. Approximately 98% of the modeled Derlin-1 residues (174 of 178) were in Ramachandran-allowed regions and ~90% (161 of 178) were in Ramachandran-favored regions (**Supplementary Fig. 2**). There were four outliers (Phe91, Leu121, Thr148 and Asn172), and each was predicted to be in a loop region connecting transmembrane spans. These residues were found in loops connecting TM2 and TM3 (Phe91), TM3 and TM4 (Leu121), TM4 and TM5 (Thr148), and TM5 and TM6 (Asn172). The figures were made using PyMOL (<http://www.pymol.org/>). Multiple sequence alignments using ClustalW were done with MacVector (<http://www.macvector.com>).

Immunofluorescence microscopic analysis of protein topology and localization

HeLa cells maintained in DMEM containing 10% animal serum complex (Gemini Bio-Products) were transfected using the FuGENE 6 transfection reagent (Roche) and plated on

poly-L-lysine-coated glass coverslips. Following 24 h of growth, cells were washed with PBS buffer and fixed in 4% paraformaldehyde. After extensive PBS washes, cells were incubated with PBS containing 20 μ M digitonin for 1.5 min or 20 μ M digitonin and 0.1% Triton X-100 for 3 min to selectively expose cytosolic or cytosolic and ER luminal epitopes, respectively. Cells were then incubated with 1% bovine serum albumin for 30 min and subsequently stained with primary antibodies for 2 h and AlexaFluor (Invitrogen) secondary antibodies for 1 h. Nuclei were stained by incubation with 10 mg ml⁻¹ bisbenzamide for 10 min. Coverslips were mounted in fluoromount G (Electron Microscopy Sciences) and cells visualized with a Zeiss Axiovert 200M outfitted with a 40 $\times$ air objective.

Antibodies

The following antibodies were used: anti-KDEL (Assay Designs), anti-Derlin-1, anti-UBXD8 and anti-SEL1L (kind gifts of H. Ploegh), anti-p97/VCP (Novus Biologicals), anti-HA.11 (Covance), anti-Flag M2 (Sigma) and anti-tubulin (Abcam). Rabbit polyclonal antibodies to S-tag were generated and affinity-purified.

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Figure 1.

Derlins belong to the rhomboid family. **(a)** ClustalW alignment (UniProtKB accession number indicated) of the transmembrane regions of Derlin-1 from *Homo sapiens* (hs, Q9BUN8), *Danio rerio* (dr, Q7ZVT9), *Caenorhabditis elegans* (ce, Q93561), Derlin-2 (hs, Q9GZP9) and Derlin-3 (hs, Q96Q80) from *H. sapiens* and GlpG from *Shewanella trabarsenatis* (st, Q0HDA1), GlpG, *Yersinia frederiksenii* (yf, C4SQG5), and *Escherichia coli* (ec, P09391). Pink, the active site dyad in GlpG, absent in Derlins. WR and GxxxG motifs are indicated by *. **(b–d)** Homology model of the Derlin-1 rhomboid domain. The positions of the WR motif **(b)**, GxxxG motif **(c)** and positively charged residues on the cytoplasmic side of each transmembrane span in the homology model **(d)** are indicated. N- and C termini of Derlin-1 are located in the cytoplasm. **(e)** Representative immunofluorescence images of cells expressing N- and C-terminally tagged HA-Derlin-1-S in which the plasma membrane was permeabilized without (digitonin) or with (digitonin + Triton X-100) ER permeabilization. Scale bars are 10 μ m. **(f)** Comparison of the four-pass topology model of Derlin-1 as inferred from the reported topology of the *Saccharomyces cerevisiae* ortholog Der1 (ref. 8) with the six-pass topology model of Derlin-1 as predicted by homology with GlpG. **(g)** Derlin-1 spans the ER membrane six times. Immunoblot of lysates treated with or without Endo H from cells expressing Derlin-1-S containing a glycosylation acceptor sequence inserted after the indicated residue.

Figure 2.

p97/VCP binding to Derlin-1 through an SHP box is required for efficient dislocation of NHK. **(a)** Domain organization of Derlin-1-S and Derlin-2-S constructs used in this study. **(b)** Alignment of SHP boxes from the p97/VCP/Cdc48 binding proteins *H. sapiens* Derlin-1 and Derlin-2, *S. cerevisiae* Dfm1, *H. Sapiens* Ufd1 and p47 and *S. cerevisiae* Shp1. **(c)** The SHP box is required for Derlin-1 and Derlin-2 interactions with p97/VCP. Immunoblot of digitonin lysates and S-protein agarose-precipitated material from untransfected (UT) cells or cells expressing wild-type (WT) or mutant Derlin-1-S or Derlin-2-S lacking the SHP box (1–240 and 1–231, respectively). **(d–g)** Overexpression of Derlin-1 lacking the SHP box results in stabilization of deglycosylated forms of NHK associated with Derlin-1. **(d)** Immunoblots of digitonin lysates or S-protein agarose-precipitated material from cells coexpressing NHK-HA with the indicated Derlin-1-S variants or a GFP-S control. Deglycosylated forms of NHK-HA are indicated by arrowheads. **(e)** Immunoblot of four biological replicates similar to those in **d**, except cells were lysed in SDS lysis buffer. **(f)** Quantification of the levels of deglycosylated NHK-HA in **e** as a percentage of total NHK-HA levels. Error bars represent s.e.m. **(g)** Immunoblot of material from **d** treated with or without Endo H. **(h)** Left panel, immunoblots of Triton X-100 lysate from cells coexpressing NHK-HA, wild-type or mutant Derlin-1 or a control protein (Δ CD4) and an shRNA targeting GFP or PNG1. Quantification of data was carried out as in **f**. n.d., not detectable. To enable detection, Derlin-1-S and mPNG1-S were enriched by S-protein agarose precipitation. Right panel, efficacy of shRNA-mediated PNG1 knockdown. CHO, carbohydrate; AP, affinity precipitation.

Figure 3.

The GxxxG motif in the Derlin-1 rhomboid domain is required for the dislocation of NHK. (a) Derlin-1_{G176V} acts a dominant negative mutant by inhibiting the dislocation of NHK. Immunoblots of digitonin lysates and S-protein agarose-precipitated material from cells coexpressing NHK-HA and the indicated Derlin-1-S variant or a GFP control. Deglycosylated forms of NHK are indicated by arrowheads. (b) Quantification of deglycosylated NHK-HA levels in a as a percentage of total NHK-HA levels. (c) Deglycosylated forms of NHK are exposed to the cytoplasm. Immunoblots of membranes obtained from homogenized cells coexpressing NHK-HA and Derlin-1-S_{G176V} treated with the indicated concentrations of proteinase K and Triton X-100. The cytoplasmic C-terminal S-tag of Derlin-1-S, or luminal (SEL1L, GRP94 and BiP) markers were used as controls. (d) Quantification of the data from c. (e) Disruption of NHK dislocation is specific to mutation of the Derlin-1 GxxxG motif. Immunoblots of SDS lysates from cells coexpressing NHK-HA with wild-type Derlin-1-S or the indicated GxxxG mutants (G176V or G180V) or mutants outside of the GxxxG motif (G29V or G147V), or a GFP control, treated with or without Endo H. (f) Mutation of the GxxxG motif does not disrupt homo-oligomerization of Derlin-1 or interaction with p97/VCP. Immunoblots of digitonin lysates and S-protein agarose-precipitated material from cells expressing wild-type or mutant Derlin-1-S or a GFP control.

Figure 4.

The Derlin-1 WR motif is essential for the dislocation of NHK. **(a)** Expression of Derlin-1 WR motif mutants results in a reduction in NHK levels in Triton X-100 lysates. Immunoblots of Triton X-100 lysates from cells coexpressing NHK-HA and Derlin-1-S_{WT}, the indicated Derlin-1-S WR motif mutants (Q51A, W53A, R54A or T57A), Derlin-1-S mutated at a nonconserved tryptophan (W106A), or a GFP control. **(b)** Overexpression of Derlin-1_{R54A} results in aggregation and accumulation of deglycosylated NHK. Immunoblots of Triton X-100 soluble and insoluble material. **(c)** Mutation of the Derlin-1 WR motif does not disrupt interaction with p97/VCP. Arrowheads indicate deglycosylated NHK. **(d-f)** Expression of Derlin-1 containing mutations in the WR or GxxxG rhomboid motifs stabilizes NHK. **(d)** Immunoblot of SDS lysates from cells coexpressing NHK-HA and wild-type or mutant Derlin-1-S or a GFP control that were treated with emetine for the indicated durations. **(e)** Quantification of four independent experiments is shown. Asterisks denotes $P \leq 0.05$. Error bars indicate s.e.m. **(f)** Derlin-1 GxxxG and WR motif mutants are stable and expressed at similar levels.