Derlin-dependent accumulation of integral membrane proteins at cell surfaces

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Summary
Quality-control mechanisms of protein folding of transmembrane and secreted proteins is mediated by endoplasmic-reticulum-associated degradation (ERAD), which is used to detect and to degrade misfolded proteins in the ER. The ERAD machinery consists of chaperones, transmembrane proteins and ubiquitin-associated enzymes that detect, modify, and retro-translocate the misfolded proteins to the cytoplasm for degradation by the proteasome. In contrast to ERAD, little is known about the fates of integral membrane and secreted proteins that become misfolded at the plasma membrane or in the extracellular space. Derlin proteins are a family of proteins that are conserved in all eukaryotes, where they function in ERAD. Here, we show that loss of Derlin function in Caenorhabditis elegans and in mouse macrophages results in the accumulation of integral membrane proteins at the plasma membrane. Induction of LDL receptor misfolding at the plasma membrane results in a sharp decrease in its half-life, which can be rescued by proteasomal inhibitors or by reduction of Derlin-1 levels. We also show that Derlin proteins localize to endosomes and/or in endosomes.

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Introduction
The degradation of misfolded proteins is essential for cellular and organism viability. Quality control mechanisms of protein folding involve multi-component systems, which include chaperones, ubiquitylation enzymes, and ultimately degradation by the proteasome. So far, quality control mechanisms have been described in the cytoplasm, the nucleus and endoplasmic reticulum (ER) (Bader et al., 2007; Goldberg, 2003; Hampton, 2002; Jarosch et al., 2003; Meusser et al., 2005; von M Ikez, 2006).

The recognition and degradation of misfolded proteins in the ER is called ER-associated degradation (ERAD) (Hampton, 2002; Jarosch et al., 2003; McCracken and Brodsky, 2003; Meusser et al., 2005; Richly et al., 2005; Sitia and Braakman, 2003). Membrane-spanning and secretory proteins are first transported into the ER in an unfolded state through the Sec61p complex (Matlack et al., 1998). Folding of these nascent polypeptides is assisted by a number of ER-resident chaperones. Translocated proteins also undergo modifications to support folding; these include N-terminal glycosylation and disulphide bond formation (Meusser et al., 2005; Schroder and Kaufman, 2005; Sitia and Braakman, 2003). In the ER, proteins that do not fold properly are retro-translocated to the cytoplasm. During retro-translocation, these misfolded proteins are ubiquitylated by several ER-specific E3 ubiquitin ligase complexes. A cytoplasmic ubiquitin-binding and multi-ubiquitylation enzyme complex further modifies these proteins and finally transports them to the proteasome for degradation.

The accumulation of misfolded proteins in the ER activates the unfolded protein response (UPR), which is required for cells to survive conditions of stress. The UPR is mediated by three ER transmembrane proteins, IRE1, PERK and ATF6, which get activated at least in part because of the dissociation of the ER chaperone BiP, to which they are normally bound and also because of their sequestration by misfolded proteins (Bertolotti et al., 2000; Cox et al., 1993; Harding et al., 1999; Haze et al., 1999; Iwawaki et al., 2001; Kimata et al., 2004; Lee et al., 2002; Mori et al., 1993; Okamura et al., 2000). IRE1, PERK and ATF6 function to decrease the load on the ER by reducing translation rate and activating the transcription of chaperones, ERAD proteins and other enzymes. UPR activation also results in increased biosynthesis of some lipids, the elaboration of the ER and increased secretion (Sato et al., 2002; Shaffer et al., 2004; Sriburi et al., 2004). A major downstream regulator of UPR is XBP1/HAC1. Upon activation of IRE1, the XBP1 mRNA is directly spliced by an endonuclease activity in the C-terminus of IRE-1; this splice variant of XBP1 functions as a potent transcriptional activator of several genes (Calfon et al., 2002; Cox and Walter, 1996; Sidrauski and Walter, 1997; Yoshida et al., 2001).

Derlin proteins are conserved family that function in ERAD (Schekman, 2004). They have four transmembrane domains and are conserved in all eukaryotes. There are two members in Saccharomyces cerevisiae, Der1p and Dfm1p, two in C. elegans, F25D7.1 and R151.6, and three in humans, Derlin-1, Derlin-2 and Derlin-3 (DERL1-DERL3) (Lilley and Ploegh, 2004; Ye et al., 2004). Der1p in S. cerevisiae is required for the ERAD-mediated degradation of soluble, but not of membrane-spanning misfolded proteins (Hitt and Wolf, 2004; Knop et al., 1996; Taxis et al., 2003). By contrast, the HCMV-encoded US11 protein recruits major histocompatibility complex class I (MHCI) molecules to human Derlin-1, leading to retro-translocation and degradation of MHCI (Lilley and Ploegh, 2004; Ye et al., 2004). Derlin-1 and Derlin-2
function as part of a complex that includes other components of the ERAD machinery: VIMP/p97 and ubiquitylation-associated proteins such as SEL1, HRD1 and HERP (Lilley and Ploegh, 2004; Lilley and Ploegh, 2005; Schulze et al., 2005; Ye et al., 2005; Ye et al., 2004). Derlin-1, Derlin-2 and Derlin-3 form homo- and hetero-oligomers (Lilley and Ploegh, 2005; Ye et al., 2005) and have therefore been proposed to contribute to the channels through which ERAD substrates might translocate (Scheekm, 2004).

In contrast to the ER, less is known about quality control mechanisms that monitor the structure of membrane proteins at the plasma membrane and endosomes. In yeast, misfolded Pma1p at the plasma membrane is targeted to the endosome or vacuole for degradation following ubiquitylation (Gong and Chang, 2001; Pizzirusso and Chang, 2004). In mammalian cells, several transmembrane proteins that are misfolded (or perceived as such) at the plasma membrane are degraded more rapidly than their properly folded counterparts; these misfolded proteins at the plasma membrane include unliganded major histocompatibility complex (MHC) Type I, and mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) and the α2-adrenergic receptor (Benharouga et al., 2001; Ljunggren et al., 1990; Sharma et al., 2004; Wilson et al., 2001).

Our results suggest that Derlin proteins function in the recognition and/or the degradation of proteins that are misfolded at the plasma membrane and/or within the endosomal system.

**Results**

**Identification of CUP-2 and mutant phenotypes**

The *cup-2* gene was identified on the basis of the *ar506* mutation that results in decreased endocytosis in scavenger cells called coelomocytes in *Caenorhabditis elegans* (Fares and Greenwald, 2001a). *cup-2(ar506)* worms have a temperature-sensitive defect in endocytosis by coelomocytes (Fig. 1A,B). *pmyo-3::ssGFP* transgenic worms express GFP fused to a signal sequence and expressed in body wall muscles: this GFP is secreted into the body cavity and is endocytosed, and subsequently degraded, primarily by the coelomocytes (Fares and Greenwald, 2001a). Mutations that decrease endocytosis by coelomocytes result in the accumulation of GFP in the body cavity and a decrease in the size of the GFP-filled compartments in coelomocytes (Dang et al., 2004; Fares and Greenwald, 2001a; Patton et al., 2005). In addition to this temperature-sensitive endocytosis defect, *cup-2(ar506)* worms are sick or nonviable at 25°C, such that 90-95% of L4 larvae shifted...
from 20°C to 25°C die; the rest of the worms survive and lay eggs, most of which are nonviable.

We determined that CUP-2 corresponds to open reading frame F25D7.1 based on phenocopy by RNA-mediated interference (RNAi), sequence analysis of the ar506 allele and transgenic rescue of the mutant phenotypes (Fig. 1C; supplementary material Fig. S1A; Fig. 2). The ar506 allele is a nucleotide change in the first exon that results in an early stop codon and therefore represents a predicted null mutant of cup-2 (Fig. 1C; supplementary material Fig. S1B). CUP-2 is one of two C. elegans Derlin proteins and shows the highest identity to human Derlin-1 (Fig. 1D; supplementary material Fig. S1B). Derlin proteins are conserved proteins that function in ERAD and have four predicted transmembrane domains, with both the N- and the C-termini being cytoplasmic (Fig. 1D; supplementary material Fig. S1B) (Hitt and Wolf, 2004; Knop et al., 1996; Lilley and Ploegh, 2004; Lilley and Ploegh, 2005; Ye et al., 2004). Consistent with this basic cell biological function, fusion of GFP to the CUP-2 promoter resulted in ubiquitous expression of GFP in all tissues, including coelomocytes (Fig. 1E).

We confirmed that CUP-2 functions in ERAD based on two criteria. First, cup-2(ar506) was synthetically lethal at 20°C with a null mutation in ire-1, the worm orthologue of the UPR sensor Ire1 (Fig. 1F) (Shen et al., 2001). Similarly, a yeast ΔIre1 Δire1 strain was nonviable at 37°C (Hitt and Wolf, 2004). cup-2; pek-1 and cup-2; atf-6 double mutants only showed slight increases in lethality (Fig. 1F). Second, in the cup-2(ar506) mutant, or after reducing cup-2 levels by RNAi, the UPR was activated in most cells, including coelomocytes, as determined by the induction of GFP expression from an hsp-4::GFP reporter (Fig. 1G,H; supplementary material Fig. S1A). A similar effect of cup-2 RNAi on induction of hsp-4::GFP expression in intestinal cells has been reported previously (Ye et al., 2004). Unlike the endocytosis defect, the UPR induction due to cup-2(ar506) is not temperature sensitive. Furthermore, the activation of the UPR response is absent in an!xbp-1 null mutant, indicating that it is dependent on the site-specific cleavage and activation of!xbp-1 mRNA by Ire-1 (supplementary material Fig. S1A) (Calfon et al., 2002).

RNAi of R151.6, the second worm Derlin protein, did not result in detectable UPR activation in any cells of wild-type animals; it also did not affect endocytosis by coelomocytes (supplementary material Fig. S1A). However, CUP-2 and R151.6 exhibited partially redundant function in activating the UPR, because RNAi of R151.6 in cup-2(ar506) resulted in a dramatic increase in XBP-1-dependent hsp-4::GFP expression (supplementary material Fig. S1A).

Rescue of UPR activation and endocytosis defects by Derlin proteins

To determine whether CUP-2 function is conserved, we expressed several Derlin proteins from other species in cup-2 mutant worms. The UPR-2 promoter drove Derlin homologue expression in these transgenic animals. We observed essentially two patterns of rescue (Fig. 2; supplementary material Fig. S2). C. elegans CUP-2 and R151.6, and human Derlin-1 and Derlin-3 rescued both endocytosis and UPR activation phenotypes. These results indicate that both activities of CUP-2 are also found in mammalian Derlin proteins. Yeast Der1p and Dfm1p, and human Derlin-2 did not rescue either defect. Furthermore, yeast Dfm1p exacerbated the UPR activation, but not the endocytosis defect, suggesting that it might function as a dominant-negative protein that interferes with R151.6 activity in the ER.

Analysis of MCA-3 levels at the plasma membrane

To determine whether the loss of CUP-2 also affects the endocytic trafficking of transmembrane proteins, we analyzed the levels of the Ca²⁺ pump CUP-7/MCA-3. MCA-3 was originally identified on the basis of mutations that disrupt endocytosis (Fares and Greenwald, 2001a). A functional GFP::MCA-3 fusion localizes to the plasma membrane (supplementary material Fig. S3A) (Bednarek et al., 2007). GFP::MCA-3 does not localize to the large vacuoles in a cup-5 mutant that is defective in lysosome-mediated degradation, indicating that it is not normally transported to the lysosome before or after endocytosis (supplementary material Fig. S3A) (Fares and Greenwald, 2001b; Treusch et al., 2004). By contrast, GFP::MCA-3 accumulates to the membranes of expanded mCherry::RAB-11-positive recycling endosomes in an rme-1 mutant, indicating that MCA-3 continuously cycles between the plasma membrane and endosomes (supplementary material Fig. S3A,B) (Grant et al., 2001; Lin et al., 2001). RME-1 is an EH-domain-containing protein that is required for the exit from recycling endosomes; loss of RME-1 results in expanded recycling endosomes (Grant et al., 2001; Lin et al., 2001).

cup-2(ar506) (or cup-2 RNAi) results in increased levels of GFP::MCA-3 at the plasma membrane as measured by quantitative microscopy (Fig. 3A,B; and data not shown). Western blot analysis of immunoprecipitated GFP::MCA-3 indicated that the increased GFP::MCA-3 signal is due to increased levels of GFP::MCA-3 protein in cup-2 mutant worms (Fig. 3C,D). From three independent immunoprecipitation experiments, there was a 1.73±0.06 increase in the levels of GFP::MCA-3 in the coelomocytes of cup-2 mutant
worms relative to the wild type; this is in agreement with the measurements from microscopy (Fig. 3A,B). By contrast, GFP::MCA-3 does not accumulate at the plasma membrane of *cup-5* mutants with strongly reduced lysosome function nor in a *cup-4* mutant, which displays an even more severe general endocytosis defect than the *cup-2* mutant (supplementary material Fig. S3A,C) (Patton et al., 2005; Treusch et al., 2004). Neither *cup-2* nor *cup-4* mutations completely block endocytosis in coelomocytes. This indicates that the accumulation of GFP::MCA-3 at the plasma membrane is not a general consequence of decreasing internalization rates. Furthermore the increase of GFP::MCA-3 at the plasma membrane is probably independent of the UPR activation defect of *cup-2(ar506)* because this increase is evident at 20°C and not at 15°C, which correlates with the endocytosis phenotype and not the UPR activation defect (Fig. 3A,B). Finally, we checked the levels of two other integral membrane proteins that are expressed under the control of the same coelomocyte promoter used to express GFP::MCA-3. Mannosidase II::GFP localizes to the Golgi complex whereas GFP::CUP-5 localizes to lysosomes in coelomocytes (Treusch et al., 2004). Neither protein showed increased levels in *cup-2* mutants, indicating that the increase in MCA-3 levels is not due to an increase in general secretion or promoter activity (supplementary material Fig. S4A-D).

Therefore, at least one transmembrane protein, MCA-3, accumulates at the surface of coelomocytes in the absence of CUP-2. Given this result and the endocytosis defect, we assayed other markers of the endocytic pathway in coelomocytes.

**Analysis of endocytosis markers in *cup-2* mutant coelomocytes**

To better understand the nature of the endocytosis defect in *cup-2* mutants we analyzed the morphologies of all major membrane bound organelles of the endocytic and secretory pathways in *cup-2(ar506)* coelomocytes using a set of GFP-tagged markers that we
developed (Fig. 3D). There was no change in the localization of GFP-RME-1, a recycling endosome and plasma-membrane-localized EH-domain-containing protein required for endocytic traffic in coelomocytes (Fares and Greenwald, 2001a; Grant et al., 2001). By contrast, there was a significant increase in the levels of GFP-tagged clathrin heavy chain (CHC) at the surface of cup-2 (ar506) coelomocytes (Greener et al., 2001). This increase in membrane CHC localization was not accompanied by a change in the absolute levels of CHC in cup-2 (ar506) worms, indicating that the increased clathrin signal probably indicates increased clathrin assembly on membranes (Fig. 3E).

RAB-7, a marker for late endosomes, and CUP-5, a marker for lysosomes, both showed normal staining patterns, albeit of compartments that were reduced in size; this reduction in the size of endosomes or lysosomes is a general defect seen in mutations that block coelomocyte endocytosis (Grant and Hirsh, 1999; Patton et al., 2005; Treusch et al., 2004) (Fig. 3D). There was an elaboration and a dispersal of the ER in cup-2 (ar506) coelomocytes (Fig. 3D). Both of these phenotypes are seen during hyperactivation of UPR in mammalian plasma cells and in other mutations that block coelomocyte endocytosis (Patton et al., 2005; Shaffer et al., 2004; Sriburi et al., 2004). Finally, there was no obvious change in the localization of mannosidase II, a transmembrane Golgi marker (Fig. 3F).

We also determined whether the loss of CUP-2 results in a delay in transport of endocytosed BSA-Rhodamine to lysosomes. This experiment was feasible because although the rate of endocytosis of soluble molecules was reduced in the cup-2 mutant, internalization was not blocked. Wild-type and cup-2 mutant worms that express the endosomal marker RME-8::GFP or the lysosomal marker GFP::CUP-5 were injected with BSA-Rhodamine in their pseudocoeloms. In wild-type and cup-2 mutant worms, all of the BSA-Rhodamine was found in RME-8-positive, CUP-5-negative endosomes after 5 minutes of uptake, as has been previously shown (Dang et al., 2004; Treusch et al., 2004). In both wild-type and cup-2 mutant worms, we first detected BSA-Rhodamine in RME-8-negative, CUP-5-positive lysosomes 10 minutes into the time course (arrowheads in Fig. 3F). Therefore, the loss of CUP-2 does not affect the progress of endocytosed solutes and membrane through the endo-lysosomal pathway.

Therefore, the only defect that we detect in the absence of CUP-2, so far, is an accumulation of CHC at the surface of coelomocytes. This accumulation has not been observed in other mutants that disrupt coelomocyte endocytosis and is therefore not due to a general reduction in internalization (Bednarek et al., 2007; Grant et al., 2001; Patton et al., 2005; Sato et al., 2005; Xue et al., 2003; Zhang et al., 2001).

Given the roles of Derlin proteins in degrading misfolded proteins in the ER, we hypothesized that misfolded protein accumulation could explain the increased MCA-3 levels at the surface of cup-2 mutant coelomocytes. We therefore did studies in cultured cells where this idea could be tested.

Mammalian Derl1 RNAI phenotypes

The rescue of cup-2 mutant phenotypes by mammalian Derlin proteins suggests functional conservation. We therefore initiated studies in mammalian cells to allow for pulse-chase and biochemical manipulations that are not currently feasible in worms. We chose murine RAW264.7 macrophages because they are analogous to coelomocytes and therefore allow for a smooth transition for comparative analysis between the worm and the mammalian work.

We made a stable Derl1 RNAi clone in RAW264.7 cells. In these cells, Derlin-1 levels were 8.2±0.9% and Derlin-2 levels were 133.5±14.8% of that in the wild type (Fig. 4A). Derlin-3 was not detectable in the wild type or Derlin-1 RNAi clone (Fig. 4A). The increase in Derlin-2 levels is consistent with previous results showing that both Derlin-2 and Derlin-3 are upregulated by the UPR, which has probably been activated because of the reduced Derlin-1 levels (Oda et al., 2006).

Previous studies have shown that at least some of the low-density lipoprotein receptor (LDLr) at the plasma membrane is degraded by the proteasome (Martin de Llano et al., 2006; Miura et al., 1996). We therefore checked the levels of the LDLr at the plasma membrane by incubating live cells with antibodies at 4°C, followed by fixation and imaging. We detected a sevenfold increase in the levels of LDLr at the plasma membrane of the Derl1 RNAI clones relative to RAW264.7 cells or to Mcoln1 RNAi stable clones that were used as a control (Fig. 4B,C). Mcoln1 encodes mucolipin 1, which is an endosomal/lysosomal-localized protein required for efficient lysosomal trafficking and lysosomal degradation of endocytosed proteins in RAW264.7 cells (Thompson et al., 2007). We also checked the levels of Fcgamma receptors (FcR) using an antibody that recognizes CD16, CD32 and CD64 (Unkeless, 1979). Although the trafficking itinerary from the plasma membrane of these proteins is not clear, we also detected a fourfold increase in the levels of FcR at the plasma membrane of the Derl1 RNAi clone relative to RAW264.7 cells or to an Mcoln1 RNAi stable clone that was used as a control (Fig. 4B,C). Therefore, similarly to wild-type cup-2 mutants, reduced Derlin-1 levels result in an increase in the plasma membrane levels of at least two integral membrane proteins.

The increase in LDLr levels at the plasma membrane of the Derl1 RNAI clone did not result in a decrease in LDL binding to the plasma membrane. We incubated cells with fluorescent LDL at 4°C before washing and fixing the cells at 4°C. We detected similar levels of LDL binding to the surfaces of RAW264.7, Derl1 RNAI, and Mcoln1 RNAi cells (Fig. 4D,E). This suggests that the substantial number of LDLRs at the plasma membrane of Derl1 RNAI cells that are unable to bind to LDL probably represent LDLR molecules whose extracellular ligand-binding domain is misfolded.

We wanted to directly assay the fates of transmembrane proteins at the plasma membrane that become misfolded. We first tested a high-salt and low-pH buffer (741 mM citric acid, 258.7 mM sodium citrate, pH 3.5) that is predicted to affect the conformation of the extracellular domains of many receptors. Treatment of cells with this misfolding buffer for 15 minutes at 4°C resulted in approximately one-fifth of the LDL binding to the cell surfaces relative to D-PBS-treated samples (Fig. 4D,E). This indicates that this buffer, although not lethal to cells (data not shown), causes conformational changes in the extracellular domains of receptors.

We then used this misfolding buffer to determine the parameters of the degradation of misfolded LDLR at the plasma membrane. We pre-incubated cells for 2 hours in cycloheximide to block translation and treated them with D-PBS (control) or with misfolding buffer. These cells were placed back in medium containing cycloheximide alone, with the proteasomal inhibitor MG132, or with the lysosomal inhibitor leupeptin. Samples were taken every 2 hours for western blot assays to measure the rates of disappearance of the plasma-membrane-localized mature form of the LDLR (confirmed using surface biotinylation assays, data not shown). There are three main conclusions from this analysis (Fig. 4F,G). First, induction of misfolding of surface LDLR results in a sharp increase in the rate of its degradation in normal cells (red vs black solid lines in Fig. 4G).
Second, misfolded LDLR is degraded primarily by the proteasome (orange solid line in Fig. 4G) and is less dependent on lysosomal function (green solid line in Fig. 4G). This is consistent with previous studies of plasma-membrane-localized LDLR turnover in CHO cells under normal conditions, where a proteasomal pathway degrades 70% of these receptors and the rest are degraded in lysosomes (Martin de Llano et al., 2006). Third, reducing Derlin-1 levels stabilizes plasma membrane LDLR under normal conditions (black dashed lines in Fig. 4G) and after misfolding (red dashed lines in Fig. 4G). Therefore, misfolding of LDLR at the plasma membrane results in its rapid turnover in a proteasome and Derlin-1-dependent manner.

Loss of CUP-2 results in MCA-3 accumulation at the surfaces of cells. Reduced Derlin-1 levels result in an accumulation of misfolded LDLR at the plasma membrane and in a reduction in its rate of degradation. One possible explanation for this data is that some Derlin molecules localize to the plasma membrane and/or endosomes where they regulate the fates of misfolded proteins at the cell surface. We therefore assayed the localization of Derlin proteins in more detail.

Subcellular localization of CUP-2 and Derlin proteins

Derlp and Dfnlp in yeast, and Derlin-1 and Derlin-2 in humans, localize to the ER (Hitt and Wolf, 2004; Knop et al., 1996; Lilley and Ploegh, 2004; Lilley and Ploegh, 2005; Ye et al., 2004). To confirm the ER localization of CUP-2, we fused GFP to its C-terminus. Expression of this CUP-2::GFP fusion under the control of the coelomocyte promoter rescues both the endocytosis and the UPR activation defects of cup-2(ar506) in coelomocytes, indicating that the fusion protein is functional and that CUP-2 acts cell autonomously (Fig. 5A-C). In wild-type coelomocytes, CUP-

Fig. 4. Mammalian Derl1 RNAi phenotypes. (A) Western blots of proteins isolated from RAW264.7 cells or Derl1 shRNA-stable clones and probed with anti-Derlin-1, anti-Derlin-2, or anti-Derlin-3 antibodies. Anti-actin antibodies were used as loading control. (B) Confocal images of RAW264.7, Derl1 RNAi clones, or Mcoln1 RNAi clones stained with polyclonal antibodies that detect the extracellular domains of LDLR or Fc gamma receptors (FcR) and with FITC-labeled secondary antibodies. For each antibody, images were taken using the same exposure and magnification. (C) Quantification of the plasma membrane fluorescence staining of LDLR and FcR shown in B. (D) Confocal images of RAW264.7, Derl1 RNAi clones, or Mcoln1 RNAi clones stained with Bodipy FL-LDL after treating the cells with D-PBS or with a high-salt, low-pH misfolding buffer. All images were taken using the same exposure and magnification. (E) Quantification of the plasma membrane fluorescence staining of LDLR and FcR shown in B. (F) Representative western blots of the kinetics of degradation of mature LDLR. MF refers to treatment of cells with misfolding buffer. (G) Quantification of the mature LDLR levels over time from three kinetics of degradation experiments. RAW, RAW264.7 cells; Derlin1KD, Derlin-1 shRNA clone; MCOLN1KD, MCOLN1 shRNA clone; PBS, D-PBS treatment; MF, misfolding buffer treatment.
2::GFP colocalizes extensively with the smooth ER marker cytochrome b5 and the rough ER marker TRAM, indicating that at steady state, the majority of CUP-2 molecules reside in the ER (Fig. 5D) (Rolls et al., 2002). However, in addition to the ER staining, we consistently detected CUP-2::GFP, but not the other ER markers, in peripheral organelles, suggesting that CUP-2 localizes to other compartments besides the ER (Fig. 5D, arrows). This peripheral staining was not of the Golgi complex that we visualized as discrete centralized puncta in coelomocytes (see Fig. 3D) (Bednarek et al., 2007; Dang et al., 2004; Patton et al., 2005; Treusch et al., 2004). At least a portion of the peripheral CUP-2 staining was endosomal because it appeared to colocalize with RAB-5, although the elaborate nature of the ER in coelomocytes precludes an unambiguous determination (Fig. 5D, arrows).

We determined the subcellular localization of endogenous mammalian Derlin proteins to confirm this extra-ER localization. We first used immunofluorescence comparing the localization of Derlin proteins to the ER-marker calreticulin. We observed significant colocalization of Derlin-1 with calreticulin and of Derlin-2 with calreticulin in RAW264.7 macrophages (Fig. 6A). However, there were vesicular structures that labeled for endogenous Derlin proteins but that did not contain calreticulin (arrows in Fig. 6A). We then transfected RAW264.7 cells with GFP- or YFP-tagged Rab5 (early endosomes), Rab7 (late endosomes) and Rab11a (recycling endosomes), that had been fixed, and immuno-stained to detect endogenous Derlin proteins and GFP or YFP. We saw significant colocalization between Derlin proteins and Rab5 and Rab7, indicating Derlin proteins are also found on endosomes (arrows in Fig. 6B). The Derlin proteins and Rab11a showed very limited, if any, colocalization in the perinuclear region (Fig. 6B). The colocalization of Derlin proteins with the Rab proteins is not an indirect consequence of Rab overexpression affecting ER integrity because we did not observe any colocalization between the Rab proteins and calreticulin in the same Rab-transfected cells (supplementary material Fig. S5).

We used immuno-electron microscopy to determine Derlin protein localization at a higher resolution. We first allowed cells to endocytose BSA-gold (15 nm) for 10 minutes to unambiguously label endosomal compartments (Fig. 6C). We then added anti-Derlin-1, anti-Derlin-2, or PBS (control) to cells before visualization using 6-nm-gold-conjugated secondary antibodies. RAW264.7 endosomes containing the 15-nm-gold particles showed peripheral staining for Derlin-1 (25/27 endosomes) and for Derlin-2 (24/25 endosomes). Only 1 out of 16 endosomes had 6-nm-gold particles in the control. Similar staining of the Derl1 RNAi cells revealed reduced staining for Derlin-1 (6/17 endosomes) but not for Derlin-2 (24/25 endosomes) (data not shown). Although we detected specific Derlin-1 and Derlin-2 labeling near the plasma membrane, we could not ascertain whether these represented actual plasma membrane labeling or labeling of subcortical organellar (ER, endosomes) membranes.

Finally, we fractionated the post-nuclear membrane fraction of murine RAW264.7 macrophages on a continuous iodixanol gradient to biochemically probe the presence of Derlin proteins in compartments besides the ER. Endogenous Derlin-1 and Derlin-2 was separated in two pools (boxed in Fig. 6D). Pool I (fractions 9-16) included ER membranes. Pool II (fractions 2-5) did not include ER membrane but did include Golgi complex, endosomes, and
plasma membrane. Derlin-3 was not expressed in these cells (see Fig. 4A). These results indicate that Derlin proteins have a conserved localization to endosomal compartments in addition to the ER.

Discussion
We describe the identification of a null mutation in cup-2, the C. elegans orthologue of Derlin proteins, on the basis of an endocytosis defect in specialized scavenger cells called coelomocytes. We show that in cup-2 mutant worms, the UPR is activated indicating that CUP-2 is required for ERAD. Rescue experiments indicate that human Derlin-1 and Derlin-3, but not human Derlin-2, yeast Derlp1, or Dfm1p are able to substitute for CUP-2 in vivo. R151.6 and CUP-2 are functionally redundant, because reducing R151.6 activity further exacerbates the ERAD defect of cup-2(ar506) and overexpressing R151.6 rescues both the endocytosis and the ERAD defects of cup-2(ar506).
There is an increase in the levels of GFP::MCA-3 at the plasma membrane of cup-2 mutant C. elegans coelomocytes and in the levels of LDLR at the plasma membrane of Derl1 RNAi RAW264.7 macrophages. We propose two models for this observation. In the first model, reductions in Derlin protein levels lead to the accumulation of misfolded GFP::MCA-3 or LDLR in the ER and therefore an increase in the secretion of these misfolded proteins to the plasma membrane. In this model, the ERAD and/or the UPR activation defects lead to this observed accumulation of transmembrane proteins at the plasma membrane. In the second model, Derlin proteins have a second function in the endosomes of macrophages. We propose two models for this observation. In the first model, reductions in Derlin protein levels lead to the accumulation of misfolded transmembrane proteins for degradation. In this model, transmembrane proteins at the plasma membrane that have become misfolded are endocytosed into early endosomes where they are recognized by a Derlin-dependent quality control system and targeted for proteasomal degradation. In both models, the accumulation of misfolded proteins at the plasma membrane and in endosomes would lead to defects in cellular functions, for example, a reduction in the rate of endocytosis in cup-2 mutant coelomocytes because of the hyper-recruitment or sequestration of clathrin heavy chain to membranes and a corresponding decrease of available soluble clathrin. This phenotype, which we have so far only seen in cup-2 and not in other cup mutants, might reduce rates of internalization and/or intracellular transport of integral membrane proteins, leading to further increases in their levels at the surfaces of cells. We note that this increased recruitment of clathrin heavy chain to membranes was the only defect that we observed in the endo-lysosomal pathway in coelomocytes.

There are several observations that are more consistent with the second model. First, there was no correspondence between the GFP::MCA-3 accumulation defects and the UPR activation defects of the cup-2 null mutant grown at 15°C or at 20°C. Second, we saw a sharp decrease in the half-lives of LDLR that are misfolded at the plasma membranes of cells: this can be rescued more efficiently using proteasomal than lysosomal inhibitors. Third, although the majority of Derlin proteins localize to the ER, Derlin proteins are also found in endosomes.

There are several transmembrane proteins at the plasma membrane that are thought to be degraded primarily via the proteasome and not by lysosomes. For example, although some CFTR mutants at the plasma membrane seem to be degraded primarily by lysosomes, other CFTR mutants are degraded primarily via the proteasome and do not require lysosomal function (Benharouga et al., 2001; Sharma et al., 2004). Under normal conditions, the degradation of some integral membrane proteins at the plasma membrane, for example LDLR and the IFN-γ receptor 2 (IFNFR-2), requires their endocytosis followed by their proteasome-dependent degradation (Curry et al., 2004; Martin de Llano et al., 2006; Miura et al., 1996). There is also substantial evidence that one pathway for the degradation of gap junctional connexins involves the proteasome following the phosphorylation and the ubiquitylation of connexins, although it is not clear where this proteasome-mediated degradation takes place (Berthoud et al., 2004). Our results are consistent with the model that Derlin proteins might recognize these, and other, proteins at the plasma membrane or endosomes to target them for degradation.

There are physiological arguments for the presence of a quality control mechanism at the plasma membrane or endosomes. Most integral membrane proteins have long half-lives and are efficiently recycled back to the plasma membrane as a result of signals in their transmembrane and/or cytoplasmic domains (Maxfield and McGraw, 2004; Zalioaukiene et al., 2000). Without a specific quality control mechanism that monitors the folding state of these surface proteins, the misfolding of the extracellular domains of these proteins would probably not affect their recycling back to the plasma membrane following their endocytosis. This would result in their accumulation at the plasma membrane and in endosomes. Therefore, the relative importance of such a proposed quality control mechanism in endosomes would depend on how efficiently a transmembrane protein is recycled to the plasma membrane compared with being targeted to lysosomes.

Our results are consistent with a model where Derlin proteins are part of a quality control mechanism that recognizes misfolded plasma membrane or endosomal proteins and targets them for degradation. Future studies will focus on elucidating the transport steps and mechanism of this novel cellular process.

Materials and Methods

Strains and genetic methods

Standard methods were used for growth and genetic analysis of worms (Brenner, 1974). Integration of plasmid DNA was done by microparticle bombardment into unc-119(eds) worms as previously described, except that we used plasmids pH1334 or pH1337 as co-bombardment markers (Prattis et al., 2001). Markers used: cup-2(ar506) I (Fares and Greenwald, 2001a); at-6(k551) X; pek-1(k275) X (Shen et al., 2001); scrib-1(c33 II) (Shen et al., 2001); cdm-16(1)[III, rme-16(h1045) V (Grant et al., 2001), zcs4[ksp-4::GFP] V integrants express GFP under the control of the ksp-4 promoter (Calfon et al., 2002); the arIs37[pmyo-3::ssGFP] I line used to visualize uptake by coelomocytes was previously described (Fares and Greenwald, 2001a), cdIs70[pcc1::GFP::MCA-3a::unc-119(+)::pmyo-2::GFP] expresses GFP::MCA-3 in coelomocytes and GFP in the pharynx.

Percentage embryonic lethality is a determination of the number of eggs that hatched after being laid by an adult hermaphrodite. Five different hermaphrodites were assayed for each strain. The results are represented as means ± s.d. cup-2; ire-1 double mutants were identified from cup-2(ar506); ire-1(c33)/dpy-10(e128) unc-12(e120) balanced hermaphrodites: two-thirds of the progeny laid by nonDpy-nonUnc hermaphrodites were done using the Lipofectamine LTX Reagent (Invitrogen, Carlsbad, CA).

Molecular methods

Standard methods were used for the manipulation of recombinant DNA (Sambrook et al., 1989). Polymerase chain reaction (PCR) was done using the Expand Long Template PCR System (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer’s instructions. All other enzymes were from New England Biolabs (Ipswich, MA), unless otherwise indicated.

cDNA sequencing

We sequenced the cDNA clones yk1619h02 and yk1406f08 in full. This confirmed the predicted splicing pattern of cup-2. We sequenced the cDNA clones yk1619h02 and yk1406f08 in full. This confirmed the predicted splicing pattern of cup-2.

Plasmids

The plasmid pH137 carries both wild type unc-19 and pmyo-2::GFP and was made by subcloning the 2.7 kb Sphl-Apal fragment from pH118.33 into the Xhol-Apal sites of plasmid MM106B after blunt ending the ends with T4 DNA polymerase. The plasmid pH138 carries both wild type unc-19 and thp-1::GFP and was made by subcloning the 2.7 kb HindIII-Apal fragment from pH95.75-prom3 into the same sites of plasmid MM106B (Altun-Gultekin et al., 2001; Prattis et al., 2001). Plasmid pH43 contains a minimal coelomocyte-specific promoter and was made by PCR amplification using primers: 5'CGCACCGATCTGGTTGGAAGAGATTTAAGAAATTTCC-3' and 5'GCC3CAAGGAATTTGGATTGACCACAATG-3' (template: worm genomic DNA),
restriction digested with KpnI and ligated into the same site of pHD43. To make pHD224, a transcriptional fusion of the CUP-2 promoter to GFP, we PCR amplified a 3.3 kb fragment upstream of the CUP-2 ATG (primers: 5′-CACACAGCT-GCCTGACACTGCCTGACATC-3′ and 5′-CACACAGGTACCGCAGTGATG-3′; template: worm genomic DNA), restriction digested with KpnI and ligated in the 700 bp KpnI-XbaI fragment of pHD43. To make pHD226, a translational fusion of CUP-2 to GFP under the control of the CUP-2 promoter, the 1 kb KpnI fragment from pHD79 was ligated into the same site of pHD213.

To make pHD257, DER1 cDNA under the control of the histone H1 promoter and cDNA under the control of the CUP-2 promoter, we first PCR amplified a 1026 bp fragment (primers: 5′-CACACAGGTACTGGACATTC-3′ and 5′-CACACAGGTACCGCAGTGATG-3′; template: worm cDNA library), restriction digested with KpnI and Nhel and replaced the 900 bp KpnI-Nhel GFP-containing fragment of pHD213. To make pHD258, pHD257 KpnI and replaced the 900 bp KpnI-Nhel GFP-containing fragment of pHD213. To make pHD259, DER1 cDNA under the control of the CUP-2 promoter, we PCR amplified a 645 bp fragment (primers: 5′-CACACAGGTACTGGACATTC-3′ and 5′-CACACAGGTACCGCAGTGATG-3′; template: yeast cDNA library), restriction digested with KpnI and Nhel and replaced the 900 bp KpnI-Nhel GFP-containing fragment of pHD213. To make pHD260, DER1 cDNA under the control of the CUP-2 promoter, we PCR amplified a 728 bp fragment (primers: 5′-CACACAGGTACTGGACATTC-3′ and 5′-CACACAGGTACCGCAGTGATG-3′; template: worm cDNA library), restriction digested with KpnI and Nhel and replaced the 900 bp KpnI-Nhel GFP-containing fragment of pHD213. To make pHD261, DER1 cDNA under the control of the CUP-2 promoter, we PCR amplified a 765 bp fragment (primers: 5′-CACACAGGTACTGGACATTC-3′ and 5′-CACACAGGTACCGCAGTGATG-3′; template: human cDNA clone), restriction digested with KpnI and Nhel and replaced the 900 bp KpnI-Nhel GFP-containing fragment of pHD213. To make pHD262, DER1 cDNA under the control of the CUP-2 promoter, we PCR amplified a 822 bp fragment (primers: 5′-CACACAGGTACTGGACATTC-3′ and 5′-CACACAGGTACCGCAGTGATG-3′; template: human cDNA clone), restriction digested with KpnI and Nhel and replaced the 900 bp KpnI-Nhel GFP-containing fragment of pHD213. To make pHD263, DER1 cDNA under the control of the CUP-2 promoter, we PCR amplified a 1026 bp fragment (primers: 5′-CACACAGGTACTGGACATTC-3′ and 5′-CACACAGGTACCGCAGTGATG-3′; template: yeast cDNA library), restriction digested with KpnI and Nhel and replaced the 900 bp KpnI-Nhel GFP-containing fragment of pHD213. To make pHD264, DER1 cDNA under the control of the CUP-2 promoter, we PCR amplified a 728 bp fragment (primers: 5′-CACACAGGTACTGGACATTC-3′ and 5′-CACACAGGTACCGCAGTGATG-3′; template: worm cDNA library), restriction digested with KpnI and Nhel and replaced the 900 bp KpnI-Nhel GFP-containing fragment of pHD213. To make pHD265, DER1 cDNA under the control of the CUP-2 promoter, we PCR amplified a 765 bp fragment (primers: 5′-CACACAGGTACTGGACATTC-3′ and 5′-CACACAGGTACCGCAGTGATG-3′; template: human cDNA clone), restriction digested with KpnI and Nhel and replaced the 900 bp KpnI-Nhel GFP-containing fragment of pHD213. To make pHD266, DER1 cDNA under the control of the CUP-2 promoter, we PCR amplified a 822 bp fragment (primers: 5′-CACACAGGTACTGGACATTC-3′ and 5′-CACACAGGTACCGCAGTGATG-3′; template: human cDNA clone), restriction digested with KpnI and Nhel and replaced the 900 bp KpnI-Nhel GFP-containing fragment of pHD213. To make pHD267, a C31E10.7 (cytochrome b5):mCherry fusion protein under the coelomocyte-specific promoter, we first PCR amplified a 1 kb fragment (primers: 5′-TACGTTATTCCCTTCCTTTCG-3′; template: human genomic DNA), restriction digested with KpnI and Nhel and replaced the 900 bp KpnI-Nhel GFP-containing fragment of pHD213. To make pHD268, a C31E10.7:GFP fusion protein under the coelomocyte-specific promoter, we first PCR amplified a 1 kb fragment (primers: 5′-TACGTTATTCCCTTCCTTTCG-3′; template: human genomic DNA), restriction digested with KpnI and Nhel and replaced the 900 bp KpnI-Nhel GFP-containing fragment of pHD213. To make pHD269, a C31E10.7:GFP fusion protein under the coelomocyte-specific promoter, we first PCR amplified a 2 kb fragment (primers: 5′-TACGTTATTCCCTTCCTTTCG-3′; template: human genomic DNA), restriction digested with KpnI and Nhel and replaced the 900 bp KpnI-Nhel GFP-containing fragment of pHD213. To make pHD270, a C31E10.7:GFP fusion protein under the coelomocyte-specific promoter, we first PCR amplified a 2 kb fragment (primers: 5′-TACGTTATTCCCTTCCTTTCG-3′; template: human genomic DNA), restriction digested with KpnI and Nhel and replaced the 900 bp KpnI-Nhel GFP-containing fragment of pHD213. To make pHD271, a C31E10.7:GFP fusion protein under the coelomocyte-specific promoter, we first PCR amplified a 1 kb fragment (primers: 5′-TACGTTATTCCCTTCCTTTCG-3′; template: human genomic DNA), restriction digested with KpnI and Nhel and replaced the 900 bp KpnI-Nhel GFP-containing fragment of pHD213.
acquired using the same exposure and magnification. The intensity of the cell surface staining was determined using Adobe Photoshop.

**Light microscopy**

Confocal images were taken with a Nikon PCM 2000, using HeNe 543 excitation for the red dye and argon 488 for the green dye. Epifluorescence images were taken with a Nikon Eclipse E800 microscope. Deconvolution Images were acquired (1000, 1.4 NA objective, 0.2 mm 2-z-sections) on a DeltaVision RT system (Applied Precision, LLC, Issaquah, WA) using a Series 300 CCD camera (Photometrics, Tucson, AZ) and deconvoluted using DeltaVision software. For worm imaging, young adult hermaphrodites were paralyzed in 10 mM levamisole.

**Antibodies and western detection**

Antibodies used were goat anti-GFP polyclonal conjugated to HRP for Western blots (Research Diagnostics, Concord, MA), rabbit anti-GFP polyclonal for immunofluorescence (Abcam, Cambridge, UK), rabbit anti-PRM-1 polyclonal (Grant et al., 2001), rabbit anti-Derlin-1 (MBL International, Woburn, MA), rabbit anti-Derlin-2 (MBL International), goat anti-Derlin-3 (Santa Cruz Biotechnology, Santa Cruz, CA), rat anti-mouse CD16/CD32 clone 2.4G2 (BD Biosciences, San Jose, CA), rabbit anti-LDL receptor (Zhang et al., 2007) for surface staining, rabbit anti-LDL receptor (Abcam) for western blotting, rabbit-anti-EEA1 (Abcam), rabbit-anti-tegocidas-shi (ABR, Golden, CO), rabbit-anti-GM130 (Abcam), rabbit-anti-IFNGR-2 (Santa Cruz Biotechnology), and chicken anti-Calreticulin (CIT) (Genway, San Diego, CA).

For antigen detection, we used Goat anti-Rabbit IgG, Rabbit anti-Chicken IgY, and Swine anti-Goose IgG secondary antibodies conjugated to HRP (1:50,000) and the SuperSignal West Dura Extended Duration Substrate (Pierce).

**Statistical methods**

The Student’s t-test was used to compare average measurements from two samples using a two-tailed distribution (Tail=2) and a two-sample unequal variance (Type=2).

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Supplementary Figure S1. CUP-2 RNAi and sequence. (A) Epifluorescence micrographs of the indicated strains after RNAi of the indicated genes at 20°C. All of the hsp-4::GFP images were taken using the same exposure. All of the pmyo-3::ssGFP images were taken using the same exposure. (B) CUP-2 homology to C. elegans R151.6 and to human Derlin-1, Derlin-2, and Derlin-3. Amino acids that are conserved in CUP-2 and in other members are highlighted in black (identical) and in grey (similar). Amino acids that are conserved in other members but not in CUP-2 are highlighted in red or in green (identical) and in blue (similar). Similar amino acids: E/D, N/Q, I/L/V, R/K, S/T. The predicted transmembrane domains are indicated. The ar506 mutation results in a stop codon instead of the indicated Trp amino acid.
**Supplementary Figure S2.** Rescue of *cup-2*(ar506) endocytosis and ERAD defects by homologues. Confocal images of *cup-2*(ar506); *pmyo-3::ssGFP* adult hermaphrodites (left) and coelomocytes (right) and confocal images of coelomocytes in *cup-2*(ar506); *hsp-4::GFP* worms expressing the indicated proteins in front of a *cup-2* promoter. All transgenes also express GFP in the pharynx.
**Supplementary Figure S3.** GFP::MCA-3 is a recycling protein. (A) Confocal micrographs of coelomocytes in adult wild type, *cup-4(ok837)*, *cup-5(ar465)*, or *rme-1(b1045)* hermaphrodites expressing GFP::MCA-3 in coelomocytes and grown at 20°C. (B) Confocal micrographs of coelomocytes in *rme-1(b1045)* adult hermaphrodites co-expressing GFP::MCA-3 and mCherry::RAB-11 or GFP::MCA-3 and mCherry::RAB-5. (C) Quantitation of the plasma membrane staining of the cells shown in panel A.
Supplementary Figure S4. GFP::CUP-5 and Mannosidase II (ManII)::GFP levels are not altered in coelomocytes of cup-2 mutants. (A) Western blot probed with anti-GFP antibodies of the indicated strains carrying the same GFP::CUP-5 transgene. The indicated bands are GFP::CUP-5 (42 kD, the processed form) and GFP (27 kD). The GFP::CUP-5 transgene also includes DNA that expresses GFP in the pharynx of worms. (B) Ratio of the measurements of the GFP::CUP-5 band divided by the GFP band from Western blots as shown in panel A and from three independent experiments. (C) Western blot probed with anti-GFP antibodies of the indicated strains carrying the same ManII::GFP transgene. The indicated bands are ManII::GFP (42 kD) and GFP (27 kD). The ManII::GFP transgene also includes DNA that expresses GFP in the pharynx of worms. (D) Ratio of the measurements of the ManII::GFP band divided by the GFP band from Western blots as shown in panel C and from three independent experiments.
Supplementary Figure S5. Calreticulin (CRT)-Rab immunofluorescence. Confocal images of RAW264.7 cells transfected with GFP-Rab5 (canine), YFP-Rab7a (canine), or GFP-Rab11a (canine) and then methanol-fixed and stained for CRT and GFP/YFP.