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ABSTRACT

Mutations in *MCOLN1*, which encodes the cation channel protein TRPML1, result in the neurodegenerative lysosomal storage disorder Mucolipidosis type IV. Mucolipidosis type IV patients show lysosomal dysfunction in many tissues and neuronal cell death. The orthologue of TRPML1 in *Caenorhabditis elegans* is CUP-5; loss of CUP-5 results in lysosomal dysfunction in many tissues and death of developing intestinal cells that results in embryonic lethality. We previously showed that a null mutation in the ATP-Binding Cassette transporter MRP-4 rescues the lysosomal defect and embryonic lethality of *cup-5(null)* worms. Here we show that reducing levels of the Endosomal Sorting Complex Required for Transport (ESCRT)-associated proteins DID-2, PHI-33, and ALX-1/EGO-2, which mediate the final de-ubiquitination step of integral membrane proteins being sequestered into late endosomes, also almost fully suppress *cup-5(null)* mutant lysosomal defects and embryonic lethality. Indeed, we show that MRP-4 protein is hypo-ubiquitinated in the absence of CUP-5 and that reducing levels of ESCRT-associated proteins suppresses this hypo-ubiquitination. Thus, increased ESCRT-associated de-ubiquitinating activity mediates the lysosomal defects and corresponding cell death phenotypes in the absence of CUP-5.
INTRODUCTION

Mucolipidosis type IV (MLIV) is a neurodegenerative lysosomal storage disorder characterized by corneal clouding, achlorhydria, and psychomotor defects (BACH 2001; ALTARESCU et al. 2002). In MLIV patients, large lipid-rich vacuoles are found in many tissues, while psychomotor defects are thought to be due to neuronal cell death. MLIV is caused by mutations in MCOLN1, which encodes the protein human mucolipin-1/TRPML1; this protein belongs to the transient receptor potential cation channel family and is a non-selective cation channel (BARGAL et al. 2000; BASSI et al. 2000; SUN et al. 2000; LAPLANTE et al. 2002; RAYCHOWDHURY et al. 2004; DONG et al. 2008).

*Caenorhabditis elegans* protein CUP-5 is the orthologue of human TRPML1 (FARES and GREENWALD 2001b; HERSH et al. 2002). The phenotypes resulting from mutations in *cup-5(null)* mimic those found in MLIV patients: defective lysosomal degradation and the appearance of large vacuoles in most tissues (FARES and GREENWALD 2001b; SCHAHEEN et al. 2006a). In addition, this lysosomal dysfunction in the absence of CUP-5 leads primarily to the death of developing intestinal cells that results in embryonic lethality (SCHAHEEN et al. 2006a). It is not known why developing intestinal cells die in *C. elegans* or why neurons die in MLIV patients. In *C. elegans* *cup-5(null)* mutants, the embryonic lethality is not solely due to cells undergoing apoptosis from starvation; when ATP levels are restored or when apoptosis is blocked, embryonic lethality is only partially rescued (~14% of embryos hatch and arrest at the L1 larval stage) (HERSH et al. 2002; SCHAHEEN et al. 2006a).

We have shown that *C. elegans* CUP-5 and mammalian TRPML1 likely function in lysosome formation, which involves the budding of nascent lysosomes from endosomes and scission to release the nascent lysosomes (TREUSCH et al. 2004; MILLER et al. 2015). In contrast to this outward budding event, Endosomal Sorting Complex Required for Transport (ESCRT) proteins are required for the sequestration of integral membrane proteins in intraluminal vesicles through an inward budding and scission event in late endosomes/multivesicular bodies (HENNE et al. 2011). This ESCRT-dependent targeting of integral membrane proteins includes an early ubiquitination of the cargo and a late de-ubiquitination required for completion of the scission reaction for internalization into an intraluminal vesicle. This de-ubiquitination is carried out by a complex of ESCRT-associated proteins which include Did2p (*Saccharomyces cerevisiae*)/CHMP1b (human), Bro1p (*S. cerevisiae*)/Alix and HD-PTP (human), and Doa4p (*S. cerevisiae*)/USP8 [UBPY] (human) (BOWERS et al. 2004; REID et al. 2005; MAHUL-MELLIER et al. 2006; NICKERSON et al. 2006; RICHTER et al. 2007; ROW et al. 2007; ALI et al. 2013). Sequestered cargo gets transported to lysosomes for degradation. However, the mechanisms coordinating the sequestration of cargo inside late endosomes and their subsequent transport to lysosomes is unknown.

We have previously shown that mutations in *mrp-4* suppress the *cup-5(null)* lysosomal defect and embryonic lethality (SCHAHEEN et al. 2006g). MRP-4 is a member of the ATP-binding cassette (ABC) transporter superfamily found in prokaryotes and eukaryotes that use ATP energy for transport of various molecules across membranes (BAUER et al. 1999; DEAN et al. 2001; SHEPS et al. 2004). Here, we show that reducing levels of worm ESCRT-associated proteins almost completely suppress the lysosomal defect and embryonic lethality due to the loss of CUP-5. Indeed, we show that in the absence of CUP-5, misregulated ESCRT-associated protein activity results in altered ubiquitination of MRP-4, which leads to embryonic lethality. Our results implicate ESCRT-associated protein defects in cell death and tissue degeneration in this *C. elegans* model of MLIV.
RESULTS

Identification of an ESCRT-associated protein suppressor of *cup-5(null)* lethality

To decipher pathways that lead from loss of CUP-5 to developing intestinal cell death, we began a genome-wide RNAi feeding screen for suppressors of *cup-5(null)* embryonic lethality (KAMATH AND AHRRINGER 2003). We identified *did-2/phi-24/F23C8.6* as a strong suppressor of *cup-5(null)*. While 0% of eggs laid by *cup-5(null)* worms hatched after control RNAi, 79+/- 6% of eggs laid by *cup-5(null)* worms hatched after *did-2* RNAi; viable worms grew to adulthood within 2-3 days of hatching, similar to wild type worms (RNAi 1 in Figure 1A, B). This strong suppression of *cup-5(null)* lethality by *did-2* RNAi is similar to the suppression of *cup-5(null)* lethality by the *mrp-4* null allele or *mrp-4* RNAi (Figure 1B) (SCHAHEEN et al. 2006g). *DID-2* is the *C. elegans* homologue of *S. cerevisiae* Did2p (37.6% identical) and human CHMP1b (53.2% identical) (Figure S1A).

We could not use the *did-2(ok3325)* null allele to test for suppression of *cup-5(null)* lethality because homozygous *did-2(ok3325)* is embryonically lethal (MOERMANN AND BARSTEAD 2008). Although the genomic *did-2* RNAi feeding clone included an insert that does not have predicted secondary targets, we designed two other RNAi feeding constructs to confirm that the suppression was due to reduction of *did-2* levels. These two RNAi clones also suppressed *cup-5(null)* lethality, albeit with different efficiencies as has been seen in many other genes (Figure 1A, B). We used the *did-2* RNAi 1 clone for all of the future experiments.

Reducing levels of *did-2* rescues the lysosomal degradation defects of *cup-5(null)*

*cup-5(null)* mutants have dysfunctional lysosomes which lead to embryonic lethality and tissue degeneration (SCHAHEEN et al. 2006a; CAMPBELL AND FARES 2010). To determine whether DID-2 exerts its effects upstream or downstream of the lysosomal defects found in *cup-5(null)* mutants, we looked at lysosomal degradation of the yolk protein VIT-2. VIT-2::GFP is endocytosed by oocytes and subsequently degraded primarily by intestinal cells in developing embryos (GRANT AND HIRSH 1999).

As we had previously shown, VIT-2::GFP accumulates in larger vacuoles in *cup-5(null)* mutants compared to wild type (Figure 1C-E) (SCHAHEEN et al. 2006a). While RNAi of *did-2* had no significant effect on lysosome size and degradation of VIT-2::GFP in wild type embryos, RNAi of *did-2* in *cup-5(null)* mutants rescued both the expanded lysosome sizes and the defective degradation of VIT-2::GFP (Figure 1C-E). This suppression of *cup-5(null)* lysosomal defects after reducing levels of DID-2 indicates that DID-2 protein function is necessary for the development of lysosomal defects in *cup-5(null)* embryonic intestinal cells.

DID-2 protein levels are increased in *cup-5(null)* mutants

We determined whether alterations to DID-2 levels accounted for the requirement of DID-2 for the lysosomal defects in the absence of CUP-5. We first made transgenic worms that express a functional DID-2::GFP fusion protein under the control of its own *did-2* promoter (Figure S1B). DID-2::GFP localizes to the cytoplasm and to endosomal membranes. Analysis of confocal images of developing intestinal cells in embryos showed that total (membrane bound and cytoplasmic) DID-2::GFP levels were increased in *cup-5(null)* embryos relative to wild type embryos (Figure 2, A and B). We got similar results using Western
analysis on total proteins from mixed-stage embryos (Figure 2C). In these Western blots of total embryonic proteins, DID-2::GFP often appeared as a doublet with a lower band at its expected size of ~49 kD and an upper band that is 7-10 kD larger; the levels of both the lower and the upper bands are proportionally increased in the absence of CUP-5 (Figure 2C). RME-1 was used to normalize loading since RME-1 protein levels are the same in wild type and cup-5(null) embryos (Figure S2, A-C).

We then asked whether there was more DID-2::GFP being recruited and bound to endosomal membranes in cup-5(null). Quantitation of the confocal images showed that individually, both cytoplasmic and membrane bound DID-2::GFP levels are increased in cup-5(null) (Figure 2, A, D and E). However, there was not a significant difference in the ratio of cytoplasmic DID-2::GFP to membrane bound DID-2::GFP in wild type (0.62 +/- 0.14) compared to cup-5(null) (0.62 +/- 0.13) (Figure 2, A and F). This result was confirmed by membrane fractionation where the ratio of DID-2::GFP found in the supernatant (cytoplasmic) to DID-2::GFP in the pellet (membrane bound) was similar in wild type and cup-5(null) embryos (Figure 2G). In addition, the larger sized DID-2::GFP band appeared to be the predominant membrane-bound form of DID-2::GFP in both wild type and cup-5(null) embryos (Figure 2G). C. elegans LMP-1 is homologous to human Lysosomal-Associated Membrane Protein 1 (LAMP1) and was used to test for proper partitioning of membrane and cytosolic fractions (Figure 2G) (KOSTICH et al. 2000).

To determine whether increased DID-2::GFP levels in the absence of CUP-5 is due to changes in DID-2 protein stability, we expressed DID-2::GFP under the control of the intestine-specific elt-2 promoter (FUKUSHIGE et al. 1998); elt-2 promoter activity is unaffected by the loss of CUP-5 (Figure S2, D-F). If the increase in DID-2::GFP (expressed from the did-2 promoter) levels in the absence of CUP-5 is due to a defect in DID-2::GFP degradation, we would expect to see the same increase in DID-2::GFP (expressed from the elt-2 promoter) levels. However, we saw that expression of DID-2::GFP from the elt-2 promoter resulted in similar expression levels between wild type and cup-5(null) embryos using confocal microscopy and Western analysis (Figure 2, A, H and I). This indicates that increased DID-2 levels in the absence of CUP-5 is not due to altered degradation of DID-2 protein.

This increase in DID-2 levels is consistent with previous in vitro studies that showed that MLIV fibroblasts have altered gene expression, including differential regulation of genes functioning in endosome/lysosome trafficking and lysosome biogenesis (BOZZATO et al. 2008). The transcription factor EB was found as a key regulator of this differential expression; under conditions of lysosomal dysfunction, transcription factor EB translocates to the nucleus and changes the gene expression profile of lysosomal genes, including MCOLN1 (SARDIELLO et al. 2009). We used HLH-30, the C. elegans orthologue of TFEB, to determine whether the increase in DID-2 protein levels was due to TFEB-mediated transcriptional activation (LaPIERRE et al. 2013). RNAi of hlh-30 did not affect the increase of DID-2 levels in cup-5(null) embryos, indicating that the increase in DID-2 protein levels is not due to a TFEB-mediated defect in transcription, but is rather due to defects in mRNA processing, degradation, or translation (Figure S3, A and B).

**Increased DID-2 levels in cup-5(null) are not sufficient or necessary for cup-5(null) embryonic lethality**

To examine the relevance of DID-2 increased levels to cup-5(null) lethality, we asked whether this phenotype was sufficient and/or necessary. The DID-2::GFP expressed from the elt-2 promoter in wild type embryos is significantly higher
than the DID-2::GFP expressed from the did-2 promoter in cup-5(null) embryos (Figure 2, A and B). However, unlike the 100% embryonic lethality of cup-5(null) mutants, embryos laid by Pelt-2::DID-2::GFP wild type worms are fully viable. This suggests that increased DID-2::GFP levels are not sufficient to cause the embryonic lethality found in cup-5(null). However, the increase in DID-2 levels might still be necessary for cup-5(null) lethality.

We identified the RNAi condition that reduced DID-2 levels in cup-5(null) mutants to those seen in wild type embryos; this still resulted in 0% viability in cup-5(null) embryos (Figure 2, J-L). Therefore, while increased DID-2 levels is a phenotype seen in the absence of CUP-5, this increase is neither sufficient nor necessary for lethality; the mere presence of DID-2 protein in cells causes cell death in the absence of CUP-5.

**Increased MRP-4 levels in cup-5(null) is not due to a defect in MRP-4 degradation rate**

DID-2 is a member of the ESCRT complex that targets integral membrane proteins for lysosomal degradation; one of these candidate proteins is the ABC Transporter MRP-4. We had previously shown that reducing levels of MRP-4 in cup-5(null) rescues the lysosomal defects in developing intestinal cells and embryonic lethality (Figure 1, C-F) (Schaheen et al. 2006g). We had also shown, using a partially functional MRP-4::GFP construct, that MRP-4 levels are elevated in cup-5(null) embryos, which we confirmed with a similar partially functional construct using confocal microscopy and western analysis (Figure 3, A-C) (Schaheen et al. 2006g).

We had expected that the increased MRP-4 levels in cup-5(null) was caused by a defect in the rate of MRP-4 degradation because of the identification of DID-2 and its ESCRT functions. However, when we assayed for changes in the stability of MRP-4 protein by expressing MRP-4::GFP under the control of the intestine-specific elt-2 promoter that is unaffected by the loss of CUP-5, we saw that the levels of MRP-4::GFP in wild type and cup-5(null) embryos did not differ using confocal microscopy and Western analysis (Figure 3, D-F). This indicates that the increased levels of MRP-4 protein in cup-5(null) is not due to a defect in degradation. In addition, although reducing the levels of the C. elegans TFEB orthologue HLH-30 did not suppress the increase in DID-2 protein levels in cup-5(null) embryos, we found that hlh-30 RNAi suppressed the increase in MRP-4 protein levels in cup-5(null) embryos (Figure S3, C and D). This suggests that this increase in MRP-4 levels in the absence of CUP-5 is due to HLH-30 mediated transcription. We next asked whether the MRP-4 increased levels were sufficient or necessary for embryonic lethality in cup-5(null) worms.

**MRP-4 protein increased levels in cup-5(null) is not sufficient or necessary for embryonic lethality**

While wild type Pelt-2::MRP-4::GFP embryos have almost 7 times increased MRP-4::GFP relative to cup-5(null); Pmrp-4::MRP-4::GFP embryos, we could not use this Pelt-2::MRP-4::GFP strains to assay sufficiency of MRP-4 overexpression to cause embryonic lethality because the MRP-4::GFP is partially functional. Therefore, we made another transgenic strain carrying a Pelt-2::MRP-4 transgene that expresses a functional MRP-4 protein because it reverses the suppression of cup-5(null) embryonic lethality by the null allele mrp-4(cde8) (Figure 3G) [Schaheen, 2006 #35]. In three independent experiments, quantitative reverse transcription PCR (qRT-PCR) showed that mrp-4 mRNA levels in the Pelt-2::MRP-4 strain are 3, 10, and 21 times higher than non-transgenic strains (Figure 3H). Note that we do not see increased mrp-4 RNA levels in cup-5(null) compared to wild type since a 1.3 to 1.7 fold increase seen in
MRP-4::GFP protein levels between these two strains is below the sensitivity of RT-qPCR (Figure 3A-C, H). Embryos laid from Petl-2::MRP-4 hermaphrodites are 100% viable (Figure 3G). This indicates that increased levels of MRP-4 are not sufficient to cause cup-5(null) lethality.

We then assessed the pertinence of increased MRP-4 protein levels for cup-5(null) embryonic lethality using the approach we used to study DID-2 necessity. We identified the RNAi conditions that would reduce MRP-4 levels in cup-5(null) mutants to the levels found in wild type embryos (Figure 3I, J). Reduction of MRP-4 levels down to the levels seen in wild type worms still resulted in 0% viability in cup-5(null) embryos (Figure 3, I-K). Indeed, even reducing cup-5(null) MRP-4 levels down to half the amount in wild type gives only a very slight rescue of lethality (Figure 3, I-K). Thus, the increased levels of MRP-4 is not necessary for the lethality; meaning, the presence of wild type (or lower) levels of MRP-4 protein in cup-5(null) embryos is sufficient to cause embryonic lethality. Since the levels of MRP-4 protein in cup-5(null) is not necessary for lethality, we tested whether did-2 RNAi suppression is mediated by altering the localization of MRP-4 protein.

did-2 RNAi suppression of cup-5(null) defects is not caused by altered localization of MRP-4 protein
Immunofluorescence of embryos expressing a functional DID-2::RFP and MRP-4::GFP showed that while the staining patterns of these two proteins were fairly different, there was still extensive overlap (Figure 4A, B). MRP-4::GFP stains whole membranes of compartments, while DID-2::RFP appears to stain punctate micro-domains on the same membranes or adjacent compartments (Figure 4A, B). These results indicate that MRP-4 and DID-2 localize close to each other on endosomes/lysosomes, consistent with MRP-4 protein being a substrate for DID-2/ESCRT functions (see below).

Reducing levels of did-2 might rescue cup-5(null) lethality by shuttling MRP-4 protein away from late endosomes/lysosomes where MRP-4’s increased transport activity is damaging to these compartments. We therefore first assayed MRP-4 protein localization in the endo-lysosomal pathway in wild type and cup-5(null) embryos. MRP-4::GFP co-localizes with LMP-1::RFP, a lysosomal membrane marker in wild type and cup-5(null), with no difference in percent co-localization of the two proteins between wild type and cup-5(null) (Figure 4C, D) (Campbell and Fares 2010). Thus, MRP-4 protein trafficks through the endo-lysosomal system. We then assayed MRP-4 protein trafficking in the recycling pathway by looking at its localization with RAB-11::RFP, a marker for recycling endosomes. We saw no MRP-4 protein co-localized with the RAB-11 recycling endosomes in wild type and cup-5(null) embryos, indicating that MRP-4 does not typically get trafficked through the recycling pathway (Figure 4E, F).

Given MRP-4 protein’s presence in the endo-lysosomal pathway and absence in the recycling pathway, we asked whether RNAi of did-2 rescued cup-5(null) lethality by trafficking MRP-4 to the recycling pathway, or at the least away from lysosomes where its activity is damaging. RNAi of did-2 still resulted in no co-localization between MRP-4::GFP and RAB-11::RFP in wild type and cup-5(null), indicating that did-2 RNAi rescue was not due to moving MRP-4 protein to the recycling pathway (Figure 4G, H). Additionally, RNAi of did-2 did not result in less MRP-4 protein trafficking through the endo-lysosomal system since we did not see lower co-localization between MRP-4::GFP and LMP-1::RFP in cup-5(null) after RNAi of did-2; in fact, there was a slight increase in co-localization between the proteins (Figure 4G, H). This indicates that did-2 RNAi is not rescuing cup-5(null) defects by altering the endo-lysosomal localization of MRP-4
protein. We then assayed whether cup-5(null) lethality and did-2 RNAi rescue was due to alterations in MRP-4 protein.

**MRP-4 protein is hypo-ubiquitinated in cup-5(null)**

DID-2 is a member of the ESCRT-associated complex that is required for the de-ubiquitination of integral membrane proteins prior to the completion of the internalization of these integral membrane proteins in intraluminal vesicles. We therefore assayed whether the loss of CUP-5 resulted in perturbations to MRP-4 ubiquitination. We immunoprecipitated MRP-4::GFP out of total protein isolates from wild type and cup-5(null) embryos and determined the fraction of both mono- and poly-ubiquitinated MRP-4::GFP in Western blots using the P4D1 antibody. We found that MRP-4 protein is hypo-ubiquitinated in cup-5(null) embryos; at steady state, MRP-4 in cup-5(null) shows a significant decrease in ubiquitination to approximately 35-57% of the levels seen in wild type embryos (Figure 5A, B).

Mono-ubiquitination is the signal for cargo entry into the ESCRT pathway (Katzmann et al. 2001; Reggiori and Pelham 2001). We used the FK1 antibody, which only detects poly-ubiquitin to assess whether MRP-4 protein is mono- or poly-ubiquitinated. Similar to the P4D1 antibody, the FK1 antibody also detects many proteins that are ubiquitinated in total embryonic lysates (Figure 5B). However, unlike P4D1, FK1 did not detect poly-ubiquitinated immunoprecipitated MRP-4::GFP in wild type or cup-5(null) embryos (Figure 5B). This suggests that MRP-4 protein is mono-ubiquitinated at a lower level in cup-5(null) embryos. Therefore, we assayed whether the cup-5(null) hypo-ubiquitination was dependent on the presence of DID-2 protein.

**Hypo-ubiquitination in cup-5(null) is rescued by reducing levels of did-2**

We assayed MRP-4 ubiquitination in wild type and cup-5(null) embryos after RNAi of did-2. We saw a five-fold increase in MRP-4 ubiquitination at steady state after did-2 RNAi in wild type embryos (Figure 5C). This is consistent with DID-2’s function: recruitment of the de-ubiquitinating enzyme to endosomal membranes where it can act on MRP-4 (Row et al. 2007). Similarly, there was an increase in MRP-4 ubiquitination at steady state in cup-5(null) embryos after RNAi of did-2 compared to cup-5(null) embryos after the control RNAi (Figure 5D). This increase indicates that the hypo-ubiquitination of MRP-4 in cup-5(null) developing intestinal cells in embryos is dependent on DID-2 protein and suggests the involvement of other ESCRT-associated proteins in this MRP-4 ubiquitination defect.

**Reducing levels of other ESCRT-associated proteins also suppress the embryonic lethality, lysosomal defect, and MRP-4 hypo-ubiquitination in cup-5(null)**

We tested members of the ESCRT complex for RNAi suppression of cup-5(null) embryonic lethality. With the exception of vps-32.1/C56C10.3, RNAi of ESCRT genes did not cause significant lethality of wild type embryos (Figure 6A). RNAi of hgrs-1, an ESCRT-0 protein gave 38.4+/− 14.48% rescue of cup-5(null) embryonic lethality, although RNAi of the other ESCRT-0 gene stam-1 gave no rescue (Figure 6A). In addition, RNAi of vps-2 (ESCRT-II) and vps-4 (ATPase complex) gave weak rescue of cup-5(null) lethality (Figure 6A).

We saw the strongest rescue of cup-5(null) lethality after RNAi of the ESCRT-associated genes phi-33, ego-2 and alx-1 (Figure 6A). PHI-33 is the C. elegans homologue of S. cerevisiae Doa4p and human USP8/UBPY and EGO-2 and its homologue ALX-1 are the C. elegans homologues of human HD-PTP and Alix, respectively, and of S. cerevisiae Bro1p. While 0% of eggs laid by cup-
5(null) hermaphrodites hatch, RNAi of phi-33 gave 76+/−15.6% viability (Figure 6A). RNAi of ego-2 alone in cup-5(null) gave 26+/−5.14% viability while RNAi of alx-1 gave no rescue of lethality (Figure 6A). RNAi of ego-2+alx-1 in cup-5(null) gave 16+/−11.1% viability (which was not significantly different from the viability after RNAi of ego-2 alone in cup-5(null), P>0.05) (Figure 6A). However, because combination RNAis are not as effective as single RNAis, and since EGO-2 and ALX-1 may have redundant ESCRT functions, we tested the ego-2+alx-1 RNAi in the presence of an ego-2(om33) hypomorphic allele that reduces EGO-2 activity. Indeed, RNAi of ego-2+alx-1 results in 81.17+/−11.05% viability in the cup-5(null); ego-2(om33) background (Figure 6B).

Because of their strong suppression of cup-5(null) lethality, we focused our analysis on these ESCRT-associated proteins. We first assayed the degradation of Vit-2::GFP after reducing levels of these ESCRT-associated proteins in embryos laid by cup-5(null) hermaphrodites. Reducing levels of PHI-33 suppresses both the cup-5(null) degradation defect and expanded lysosome size (Figure 6, C-E). Similarly, reducing levels of EGO-2+ALX-1 also suppresses the degradation defect and the expanded lysosome size in the cup-5(null) background (Figure 6, C-E). Similar to what we observed with DID-2 and MRP-4 proteins, these results indicate that the presence of PHI-33 and ALX-1+EGO-2 are required for the appearance of the lysosomal defect in the absence of CUP-5.

The rescue of cup-5(null) lethality and the lysosomal defect by reducing levels of PHI-33 and EGO-2+ALX-1 is not due to altering the endo-lysosomal localization of MRP-4 protein. Reducing levels of PHI-33 in the cup-5(null) background resulted in an increase, rather than a decrease, in co-localization between MRP-4::GFP and LMP-1::RFP, while RNAi of alx-1+ego-2 resulted in no significant change in co-localization between the two proteins (Figure S4, A and B). This indicates that RNAi of these genes does not rescue cup-5(null) defects by trafficking MRP-4 protein away from the endo-lysosomal system where it is doing its damage.

We then assayed hypo-ubiquitination of MRP-4 protein in cup-5(null) to assess whether it was dependent on the presence of PHI-33 or EGO-2+ALX-1. RNAi of phi-33 increases MRP-4 protein ubiquitination 7.7-fold at steady state in wild type embryos when compared with control RNAi, consistent with its de-ubiquitinating activity (Figure 5C). This increase in MRP-4 ubiquitination is also seen after RNAi of phi-33 in cup-5(null) embryos at steady state with a 3.7-fold increase from control RNAi, establishing that cup-5(null) hypo-ubiquitination is dependent on PHI-33 protein activity (Figure 5D). While RNAi of alx-1+ego-2 increases MRP-4 ubiquitination at steady state in wild type embryos compared to control RNAi, we did not see an increase in MRP-4 ubiquitination in cup-5(null) embryos when compared to control RNAi (Figure 5C, D). However, in the presence of the ego-2(om33) hypomorphic allele, reducing levels of alx-1+ego-2 increases MRP-4 protein ubiquitination at steady state in wild type (2.4-fold) and cup-5(null) (1.5-fold) embryos when compared with control RNAi (Figure 5E, F).

ESCRT-associated/MRP-4 RNAi rescue of cup-5(null) lysosomal defects shows tissue and developmental specificity

Is this genetic pathway linking ESCRT-associated proteins and MRP-4 with CUP-5 seen in developing intestinal cells in embryos also present in other tissues and at other stages of development? We first assayed GFP that is secreted by muscle cells and that is then endocytosed by coelomocytes; the GFP accumulates in terminal compartments (lysosomes) of the coelomocytes (TREUSCH et al. 2004). We measured GFP-filled compartment sizes because the intensity of endocytosed GFP in cup-5(null) coelomocytes was very bright
compared to wild type and was saturated at the microscopy settings used to visualize wild type coelomocytes. As we had previously shown, the GFP-filled compartments are larger in adult cup-5(null) compared to adult wild type coelomocytes, indicating a lysosomal defect in the absence of CUP-5 (Figure S5, A-C) (FARES AND GREENWALD 2001b). RNAi of did-2, phi-33, alx-1+ego-2, or mrp-4 does not rescue this increased compartment size in adult cup-5(null) coelomocytes (Figure S5, A-C). Indeed, while RNAi of the ESCRT-associated proteins did not significantly alter the GFP-filled compartment sizes of adult wild type coelomocytes, RNAi of did-2 and alx-1+ego-2 significantly increased GFP-filled compartment sizes of adult cup-5(null) coelomocytes (Figure S5, A-C). Thus, the suppression of cup-5(null) lysosomal defects by RNAi of ESCRT-associated proteins or MRP-4 in developing intestinal cells in embryos does not occur in adult coelomocytes.

We then assayed the adult intestine to determine whether RNAi of ESCRT-associated proteins or MRP-4 rescues lysosomal defects in intestinal cells of adults cup-5(null) worms. The levels of the lysosomal marker, LMP-1::GFP is increased in the adult intestine of cup-5(null) hermaphrodites compared to wild type, indicating a lysosomal defect in the absence of CUP-5 (Figure 7, A-C). RNAi of did-2, phi-33, alx-1+ego-2, and mrp-4 cause an increase in LMP-1::GFP levels in wild type adult intestines, consistent with their functioning in this tissue. However, RNAi of these ESCRT-associated proteins or MRP-4 did not rescue the increased LMP-1::GFP levels in cup-5(null) adults (Figure 7, A-C). Thus, this genetic pathway that is evident in embryonic intestinal cells is absent in adult intestinal cells.

Taken together, the data suggest that cup-5(null) suppression by RNAi of the ESCRT-associated proteins and MRP-4 exhibits tissue and developmental specificity.

DISCUSSION

In this study, we uncover a novel genetic link between CUP-5 and ESCRT-associated proteins. Our model is that the absence of CUP-5 results in increased activity of the de-ubiquitinating enzyme PHI-33, as evidenced by the suppression of cup-5(null) defects after reducing the levels of the ESCRT-associated proteins DID-2, PHI-33, or ALX-1+EGO-2. Our results also suggest that this ESCRT-associated defect impacts lysosomal functions and embryonic lethality in the absence of CUP-5 at least in part through affecting the activity of the ABC transporter MRP-4 and possibly by altering the levels and/or activities of other proteins.

We found that the loss of CUP-5 resulted in increases in levels of DID-2 and MRP-4 proteins. The increase in DID-2 levels is not due to altered degradation or TFEB-mediated transcriptional activation in the absence of CUP-5. In contrast, our studies suggest that the increase in MRP-4 levels is due to altered transcription that is mediated by HLH-30/TFEB. However, other studies have shown that under lysosomal stress conditions, Ca^{2+} released from lysosomes by TRPML1 causes the translocation of TFEB to the nucleus where it activates transcription (MEDINA et al. 2015). Therefore, how is HLH-30 translocating to the nucleus to increase transcription of mrp-4 when CUP-5 is not present to release Ca^{2+} from lysosomes? Our model is that the lysosomal defect in the absence of CUP-5 leads to lysosome rupture or leakiness thus releasing Ca^{2+} into the cytoplasm. Indeed, the lysosomal protease Cathepsin B has been found to leak into the cytoplasm after severe knockdown of TRPML1 in HeLA cells (COLLETTI et al. 2012).
Although we detect changes in the levels of DID-2 and MRP-4 in the absence of CUP-5, our results show that the increased levels of these proteins does not contribute to cup-5(null) lethality. Therefore, while these gene expression changes are phenotypes of cup-5(null) tissues, they do not impact lysosomal functions or tissue viability. Thus, which phenotypes are relevant?

We propose that the hypo-ubiquitination of MRP-4 protein in cup-5(null) is a major contributor to the lysosomal and viability defects since both are rescued by reducing the levels of ESCRT-associated proteins, including the catalytic enzyme PHI-33. Surprisingly, given the established functions of ESCRT proteins in shuttling receptors for degradation, this hypo-ubiquitination of MRP-4 in the absence of CUP-5 had no effect on the levels of MRP-4 protein. Our model is that the mono-ubiquitin state of MRP-4 protein modulates its activity, where hypo-ubiquitination of MRP-4 results in increased MRP-4 transporter activity leading to lysosomal defects and ultimately tissue death. In addition, since overexpression of MRP-4 is not sufficient to cause embryonic lethality, this suggests that that the mono-ubiquitination of MRP-4 is used to regulate the total transporter activity of MRP-4 in late endosomes/lysosomes, irrespective of how much MRP-4 protein is present in these compartments. Our results show that CUP-5 directly or indirectly regulates the activity of the ESCRT-associated proteins such that loss of CUP-5 results in their mis-regulation. The question then becomes, is there a biochemical link between CUP-5 and the ESCRT-associated proteins?

Studies in other systems have uncovered a potential link between TRPML1 and ESCRT-associated proteins. Human TRPML1 binds the penta-EF-hand protein ALG2 (VERGARAJAUREGUI et al. 2009). In other studies, ALG2 was found to interact with Alix and HD-PTP, the human homologues of ALX-1 and EGO-2 (MISSOTTEN et al. 1999; VITO et al. 1999; ICHIOKA et al. 2007). Our future studies will probe the functionality of this potential link between the Ca\(^{2+}\) channel CUP-5, the Ca\(^{2+}\)-binding protein M04F3.4 (worm homologue of ALG2), and ALX-1/EGO-2, whose yeast homologue Bro1p binds and activates the de-ubiquitinase Dna4p and where human HD-PTP (worm EGO-2) was found to bind UBPY (worm PHI-33) as a part of EGFR’s ESCRT pathway (RICHTER et al. 2007; ALI et al. 2013). These studies would delineate how CUP-5 regulates ESCRT-associated proteins and how these ESCRT-associated proteins are misregulated in the absence of CUP-5.

We have uncovered a novel link between CUP-5 and ESCRT-associated proteins that suggests coordinate regulation of lysosome formation and function via CUP-5 with the de-ubiquitination and intraluminal sequestration of cargo in late endosomes via ESCRT-associated proteins. This genetic link seems to only be present in certain tissues and developmental stages. Thus, CUP-5 may have functions that are unique to some tissues, which may be one of the reasons why MLIV patients and mouse models of the disease show developmental, neurological defects that are more severe than in other tissues (ALTARESCU et al. 2002; GRISHCHUK et al. 2015).
MATERIALS AND METHODS

C. elegans strains and methods:
Standard methods were used for genetic analysis (BRENNER 1974). RNAi was
done by the feeding method; control RNAi was done using bacteria expressing
the double-stranded RNA generating vector L4440/pPD129.36 (TIMMONS AND
FIRE 1998). The following markers were used in this study: arls37/Pmyo-
3::ssGFP; dpy-20] I (FARES AND GREENWALD 2001a); did-2(ok3325) I (MOERMAN
AND BARSTEAD 2008); ego-2(om33) I (QIAO et al. 1995; LIU AND MAINE 2007);
bls1[vit-2::GFP; rol-6(su1006)] I (GRANT AND HIRSH 1999); unc-36(e251) III
(BRENNER 1974); cup-5(zu223) III (HERSH et al. 2002); qC1 (GRAHAM AND KIMBLE
1993); mrr-4(cd8) (SCHAHEEN et al. 2006g); pwls50[lmp-1::GFP, unc-119(+)]
(TREUSCH et al. 2004). cup-5(zu223) unc-36(e251) worms bearing various
genes were isolated from qC1-balanced parent heterozygotes; the eggs
from these cup-5(zu223) unc-36(e251) homozygous progeny were analyzed in
the various assays. The strains used in this study are listed in Table 1.

The following transgenes were made by the bombardment method
(PRATIS et al. 2001):
cdls146[MRP-4::GFP; unc-119-ttx-3::GFP]; cdls194[LMP-1::
LINKER(PGGAGAGGAGGAG)::TagRFP(S158T); unc-119-ttx-3::GFP];
cdls197[DID-2::LINKER(PGGAGAGGAGGAG)::TagRFP(S158T); unc-119-ttx-
3::GFP]; cdls212[cdIs262[Pelt-2::MRP-4::LINKER(PGGAGAGGAGGAG)::GFP;
unc-119];
cdls214[DID-2::LINKER(PGGAGAGGAGGAG)::GFP; unc-119];
cdls220[cdIs212[Pelt-2::MRP-4::GFP; cdIs243[cdIs243[Pelt-2::MRP-4; cdIs262
Pelt-2::GFP] cdEx181[Pelt-2::GFP::TagRFP(S158T):RAB-11; HYGR]

Molecular Methods
Standard methods were used for the manipulation of recombinant DNA
(SAMBROOK AND RUSSELL 2001). Polymerase chain reaction (PCR) was done
using the Expand Long Template PCR System (Boehringer Manheim, Manheim,
Germany), according to the manufacturer’s instructions. All other enzymes were
from New England Biolabs (Beverly, MA).

Plasmids
Inserts of all plasmids were sequenced to confirm that only the desired changes
were introduced. The following plasmids were made and used in this study:

GFP/TagRFP fusion plasmids:

pHD233: MRP-4 fused to GFP(S65T) expressed under the control of the mrr-4
promoter
pHD499: LMP-1 fused to TagRFP(S158T) with an unstructured linker between
the two genes and expressed under the control of the lmp-1 promoter.
pHD502: DID-2 fused to TagRFP(S158T) with an unstructured linker between
the two genes and expressed under the control of the did-2 promoter.
pHD543: GFP expressed under the control of the elt-2 promoter.
pHD763: RAB-11 fused to TagRFP(S158T) expressed under the control of the
e1t-2 promoter.
pHD774: DID-2 fused to GFP(S65T) with an unstructured linker between the two
genes and expressed under the control of the did-2 promoter.
pHD782: MRP-4 fused to GFP(S65T) with an unstructured linker between the
two genes and expressed under the control of the elt-2 promoter.
pHD823: DID-2 fused to GFP(S65T) expressed under the control of the elt-2
promoter.
RNAi plasmids used or made with the L4440/pPD129.36 vector:

pHD554: makes double-stranded RNA corresponding to the last 325 bases of did-2 cDNA.
pHD555: makes double-stranded RNA corresponding to the full 618 bases of did-2 cDNA.
pHD556: makes double-stranded RNA corresponding to the first 316 bases of did-2 cDNA.
pHD687: makes double-stranded RNA corresponding to full length R10E12.1b/alx-1b cDNA.
pHD758: makes double-stranded RNA corresponding to amino acids 710-1154 of ego-2 and full length cDNA of alx-1b.
pHD863: MRP-4 expressed under the control of the elt-2 promoter.

Plasmid sequences and details on how these plasmids were constructed are available upon request.

Sequence comparison
The DID-2/Did2p/CHMP1b sequence comparison was generated using Clustal Omega (GOUJON et al. 2010; SIEVERS et al. 2011).

Measuring embryonic viability
Adult worms were allowed to lay eggs overnight on NGM plates at 20 °C [Brenner, 1974 #1031]. The adults were then removed and the percentage of eggs that hatched and developed normally was calculated. Each viability measurement consists of at least three experiments and the results show the average of these experiments.

Confocal Microscopy
Embryos and adults were placed on a 2.2% agarose pad with 1 mM Levamisole or 9 mM Levamisole, respectively, and images were taken using a Zeiss LSM 510 Meta confocal microscope or a Zeiss 510 Meta confocal microscope, using a 63x lens, an argon 488 nm laser for excitation of the GFP and a helium neon 543 nm laser for excitation of the RFP. For intensity and compartment size measurements, exposure settings for each assay were set using the wild type or wild type control RNAi strain and were kept the same for all embryos in the assay. Control worms were always included to ensure signals did not correspond to autofluorescence or to bleed-through from the other fluorophore. Images were taken of embryos that were between the “comma” and the “1.5 fold” stages of embryonic development because these are the first embryonic stages that show the lysosomal defect in the absence of CUP-5 (SCHAHEEN et al. 2006a).

Measurement of intensity and compartment size in embryos
For RNAi, experiments, the adult hermaphrodites were placed on the RNAi bacteria and their laid embryos were analyzed. Measurements of the intensities of GFP and sizes of compartments were done using Metamorph (Sunnyvale, CA) on images that were not modified.

Measurements were made of the sizes and intensities of all VIT-2::GFP-containing compartments within a selected area (213.42 +/- 3.28 μm²) at the base of the intestine. At least 120 discrete intracellular structures from 4-8 embryos for each strain and RNAi were used in the measurements.

For Pdid-2::DID-2::GFP intensity measurements (total, membrane, cytoplasmic) were taken from a selected area (148.19 +/- 2.57 μm²) at the base of the intestine. 20-25 embryos were imaged for each strain. The integrated density (density divided by area) of DID-2::GFP punctate (membrane) and diffuse (cytoplasmic) regions was determined. At least 100 discrete intracellular structures from each strain were used in the measurements. Four randomly chosen diffuse spots were chosen from each area for the DID-2::GFP cytoplasmic measurements.

For Pelts-2::DID-2::GFP intensity measurements, 13-14 embryos were imaged for each strain and the integrated density (density divided by intestine size) was determined for each embryo.

For Pmrp-4::MRP-4::GFP intensity measurements, a z-stack was taken of each embryo (14 embryos imaged for each strain) and the average of the integrated values of all MRP-4::GFP compartments in the intestine from eight layers was determined for each embryo.

For Pelts-2::MRP-4::GFP intensity measurements, 20-25 embryos were imaged for each strain and the average of the integrated values of all MRP-4::GFP compartments in the intestine was determined for each embryo.

For the HLH-30 suppression intensity measurements (Pdid-2::DID-2::GFP and Pmrp-4::MRP-4::GFP), 7-8 embryos were imaged for each strain and the average level of each DID-2::GFP/MRP-4::GFP compartment was determined. At least 35 discrete intracellular structures from each strain and RNAi were used in the measurements.

Measurement of lysosomal size and degradation in adults
Hermaphrodites were allowed to lay eggs at 20°C. Eggs and worms were collected off the plates in M9 buffer (22 mM KH₂PO₄, 43 mM Na₂HPO₄, 86 mM NaCl, and 1 mM MgSO₄) and treated with bleach solution (1% NaClO, 1 M NaOH) to dissolve bacteria and worms and isolate the eggs. The embryos were then washed with M9 buffer and placed on NGM plates lacking OP50 bacteria. The next day, the arrested L1 larvae were transferred to plates with RNAi bacteria and the worms were imaged when they became adults: cup-5(u223) unc-36(e251) worms were imaged from the cup-5(u223) unc-36(e251)/qC1 population. To distinguish between LMP-1::GFP compartments and gut granules found in the intestine, both the argon 488 nm laser for GFP excitation and the helium neon 543 nm laser for RFP excitation were used. The autofluorescent gut granules are excited by both lasers while the LMP-1::GFP compartments are only excited by the argon 488 nm laser. Around four adults were imaged for each strain and the average level of ten LMP-1::GFP compartments was determined for each strain. A total of at least 30 discrete LMP-1::GFP compartments was determined from each strain and RNAi.

Immunofluorescence
Embryos laid at 20°C and expressing both fusion proteins were fixed on slides for 10 minutes at -20°C with 100% methanol then for 10 minutes at -20°C with 100% acetone. Samples were then washed twice with PBSTw buffer (1XPBS

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Measurement of percent co-localization/overlap

The percent overlap for MRP::GFP and DID-2::TagRFP in the embryo intestine was calculated using "Just Another Colocalization Plugin" from ImageJ (JACoP; National Institute of Health, Bethesda, MD) (SCHNEIDER et al. 2012). The values for the Manders' overlap coefficient M1 were recorded and converted to percentages for the percent overlap measurement. 8-10 embryos were analyzed for each strain.

For measurement of the percent co-localization of MRP-4::GFP and LMP-1::TagRFP in the embryo intestine, a z-stack was taken of each embryo (8-9 embryos for each strain) and the average of the M1 coefficients (calculated by JACoP) from four layers was taken for each embryo. These values were recorded and converted to percentages for the percent co-localization.

For measurement of the percent co-localization of MRP-4::GFP and LMP-1::TagRFP in the embryo intestine after RNAi of various genes, a z-stack was taken of each embryo (4-7 embryos for each strain and RNAi) and the average of the M1 coefficients (calculated by JACoP) from four layers was taken for each embryo. These values were recorded and converted to percentages for the percent co-localization.

For measurement of the percent co-localization of MRP-4::GFP and RAB-11::TagRFP in the embryo intestine, the number of MRP-4::GFP compartments that also had RAB-11::TagRFP were divided by the total number of MRP-4::GFP compartments. 7-8 embryos were analyzed for each strain.

For measurement of the percent co-localization of MRP-4::GFP and RAB-11::TagRFP in the embryo intestine after RNAi of various genes, the number of MRP-4::GFP compartments that also had RAB-11::TagRFP were divided by the total number of MRP-4::GFP compartments. 4-8 embryos were analyzed for each strain.

gRT-PCR

Hermaphrodites were allowed to lay eggs at 20°C. Eggs and worms were collected off the plates in M9 buffer and treated with bleach solution to dissolve bacteria and worms and isolate the viable eggs. The embryos were then washed three times with M9 buffer and lysed in 0.35 ml of ice-cold Qiagen RNeasy buffer RLT (Qiagen, Germantown, MD) using the Bioruptor Standard sonicator (high power, 15 seconds ON/60 seconds OFF, 8 cycles) (Diagenode, Denville, NJ). Total RNA was isolated using the Qiagen RNeasy kit followed by DNase I digestion (ThermoFisher Scientific, Rockford, IL). RNA concentration was determined using a NanoDrop UV Spectrophotometer (NanoDrop Technologies, Wilmington, DE) before and after DNase I digestion. 500 nanograms of RNA was used to perform oligo-dT cDNA synthesis with Power SYBR Green PCR Master Mix (ThermoFisher Scientific). Quantitative PCR was conducted using SYBR Green PCR Master Mix and an ABI 7300 Real Time PCR System (ThermoFisher Scientific). Raw CT values for mrp-4 were normalized to elt-2. Fold changes were calculated using standard ΔCT methods [Pfaffl, 2001 #1273].
Primers for mrp-4 and elt-2 were designed to span exon/exon junctions to further reduce the chances of genomic DNA amplification. The following primers were used for qRT-PCR. mrp-4 forward and reverse: GACCATTCCGAGGAGTTTGC and GCTGAGAAGATTGGCAGGAC. elt-2 forward and reverse: TGCCGACTTGTATCCCGTTTC and ACTTGGATGTTATCGGCAGGTC. RNA isolation from embryos and qRT-PCR was independently done three times for each stain.

DID-2 and MRP-4 necessity assays
RNAi conditions that reduced DID-2::GFP/MPR-4::GFP levels in cup-5(zu223) to wild type levels were determined by making dilutions of control/pPD129.36 RNAi with varying amounts of did-2 RNAi or mrp-4 RNAi feeding bacteria. To measure the DID-2::GFP/MPR-4::GFP levels corresponding to each dilution, 7-9 embryos were imaged for each strain and dilution. The average level of each DID-2::GFP/MPR-4::GFP compartment was determined. At least 25 discrete intracellular structures from each strain and RNAi were used in the measurements. The dilution where the average level of all the DID-2::GFP/MPR-4::GFP compartments measured in cup-5(zu223) was not statistically different from the average level of DID-2::GFP/MPR4::GFP compartments in wild type was chosen. Viability tests to measure embryonic viability were concurrently done with the imaging such that RNAis done for the imaging were done side-by-side with the RNAis for viability tests.

Western analysis
Adult wild type and cup-5(zu223) hermaphrodites were allowed to lay eggs for 14 hours at 20 °C to minimize the accumulation of dead embryos laid by cup-5(zu223) homozygous parents. Eggs and worms were collected off the plates in M9 buffer and treated with bleach solution to dissolve bacteria and worms and isolate the viable eggs. The embryos were then washed with ddH2O and lysed for Western analysis.

Pelt-2::GFP western: After isolation, embryos were suspended in 1x Western Sample Buffer (50 mM Tris pH 6.8, 10% glycerol, 4% SDS, 10 mM DTT; 1 tablet of complete inhibitor [Life Sciences, Indianapolis, IN] per 1 ml of buffer). The suspended embryos were freeze-thawed twice (freeze in liquid nitrogen and thawed at 68 °C) and then boiled for eight minutes. Following determination of sample concentrations using Spectra Max 250 (Molecular Devices, Sunnyvale, CA), bromophenol blue was added and same amounts of total protein of the wild type and cup-5(zu223) samples were used for the Western blot.

Pmrp-4::MRP-4::GFP and Pdid-2::DID-2::GFP western: After isolation, embryos were suspended in Urea/SDS/NP-40 buffer (4% SDS, 1% NP-40, 5% 2-mercaptoethanol, 6 M Urea, 10 mM DTT, 10% glycerol, 0.002% bromophenol blue, 0.05 M Tris HCl; 1 tablet of complete inhibitor per 1 ml of buffer). The suspended embryos were frozen in liquid nitrogen, thawed and sonicated using the Bioruptor Standard sonicator (high power for 15 minutes, 30 seconds ON/30 seconds OFF). The samples were then left at 37 °C for five minutes and spun down at 16,000g for ten minutes. 10 μl of each sample were loaded for Western analysis.

Pelt-2::DID-2::GFP western and Pelt-2::MRP-4::GFP western: After isolation, embryos were suspended in 1XRIPA Buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS, 0.42 mg/ml Sodium Fluoride, 0.368 mg/ml Sodium OrthoVanadate, 12.1 μg/ml Ammonium Molybdate, 2.4 mM MG132, 2.5 mg/ml N-Ethylmaleimide; 1 tablet of complete inhibitor per 1 ml of buffer). The suspended embryos were frozen in liquid nitrogen, thawed on ice, and sonicated using the Bioruptor Standard
sonicator (high power for 5 minutes, 30 seconds ON/30 seconds OFF). Following determination of sample concentrations using Spectra Max 250, 5x Western Sample Buffer was added to the samples. The samples were then put at 70 °C for ten minutes and spun down at 16,000g for ten minutes. The same amounts of total protein of the wild type and cup-5(zu223) samples were used for the Western blot.

The intensities of the bands were determined using ImageJ. RME-1 was used as a loading control and to normalize values between wild type and cup-5(zu223) samples.

**DID-2::GFP membrane fractionation**

Adult wild type and cup-5(zu223) hermaphrodites were allowed to lay eggs for 14 hours at 20 °C. Eggs and worms were treated with bleach and following washes with M9 buffer, the isolated eggs were resuspended in 0.3 ml of membrane lysis buffer (0.2 M Sorbitol, 50 mM KOAc, 2 mM EDTA, 20 mM HEPES-KOH, 1 mM DTT, pH 6.8; 1 tablet of complete inhibitor per 1 ml of buffer). The embryos were then lysed using the Bioruptor Standard sonicator (one second at high power followed by five seconds at low power). The sample was spun down at 3400g for 15 minutes at 4 °C to remove intact embryos that had not lysed and the supernatant was preserved for membrane fractionation.

The supernatant was extracted and spun down at 200,000g for 60 minutes at 4 °C. The pellet was kept as the membrane fraction and was resuspended in 200 µl of 1X Laemmlı Buffer-Urea (8% SDS, 2% NP-40, 10% 2-mercaptoethanol, 6 M Urea, 20 mM DTT, 20% glycerol, 0.004% bromophenol blue, 0.1 M Tris HCl; 1 tablet of complete inhibitor per 1 ml of buffer) and left at 37 °C for 40 minutes with occasional mixing by pipetting. The supernatant from the 200,000g centrifugation represents the cytosolic fraction and was concentrated using a MWCO 3,000 microcon (Millipore, Billerica, MA) at 4 °C. The final volume of the supernatant sample was adjusted to 200 µl in 1X Laemmlı Buffer-Urea and the sample was incubated at 37 °C for ten minutes. Equal volumes of supernatant and pellet were used for western analysis.

**MRP-4::GFP ubiquitination assay +/- RNAi**

1000 adult wild type, unc-75(e950) ego-2(om33) arls37[Pymyo-3::ssGFP; dpy-20], cup-5(zu223), or cup-5(zu223); unc-75(e950) ego-2(om33) arls37[Pymyo-3::ssGFP; dpy-20] hermaphrodites carrying the Pelt-2::MRP-4::GFP transgene were allowed to lay eggs for 14 hours at 20 °C. Eggs and worms were treated with bleach solution and following washes with M9 buffer, the isolate eggs were resuspended in 0.3 ml of ice-cold 1XRIPA Buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS, 0.42 mg/ml Sodium Fluoride, 0.368 mg/ml Sodium OrthoVanadate, 12.1 µg/ml Ammonium Molybdate, 2.4 mM MG132, 2.5 mg/ml N-Ethylmaleimide; 1 tablet of complete inhibitor per 1 ml of buffer). The embryos were lysed using the Bioruptor Standard sonicator (high power for five minutes, 30 seconds ON/30 seconds OFF). The sample was spun down to remove intact eggs and the supernatant was transferred to a new tube. 30 µl of the supernatant was added to 30 µl of 2X Western Sample Buffer (100 mM Tris pH 6.8, 20% glycerol, 8 %SDS, 20 mM DTT, 20% bromophenol blue; 1 tablet of complete inhibitor per 1 ml of buffer); this was the total protein sample. The remainder of the sample was used for immunoprecipitation.

Immunoprecipitation was done using 20 µl of Sepharose Conjugated anti-GFP beads (Abcam, Cambridge, MA) in Pierce Centrifuge Columns (Rockford, IL). The Sepharose Conjugated anti-GFP beads in 700 µl of 1XRIPA Buffer were added to the columns, then centrifuged at 2875g for one minute. The columns
were washed two more times with 1XRIPA Buffer. Following the last spin, the supernatant was added to the column and left to mix overnight at 4 °C. The beads were washed three times with 700 µl of 1XRIPA Buffer (using 1 minute 2875g centrifugation). Immunoprecipitates were eluted using 100 µl of 2XWestern Buffer without DTT preheated to 95 °C. Following elution, 2 µl of 1 M DTT was added to samples that were incubated at 70 °C for 30 minutes.

**Antibodies and western detection**

Antibodies used in this study were:
- Chicken anti-GFP (Abcam)
- Mouse anti-Ubiquitin P4D1 (Santa Cruz Biotechnology, Dallas, TX)
- Mouse anti-Ubiquitin FK1 (Enzo Life Sciences, Farmingdale, NY)
- Mouse anti-RME-1 (Developmental Studies Hybridoma Bank, Iowa City, IA) (HADWIGER et al. 2010)
- Mouse anti-LMP-1 (Developmental Studies Hybridoma Bank) (HADWIGER et al. 2010)
- Rabbit anti-TagRFP (Abcam)
- Donkey Anti-Rabbit-IgG-Cy3 (Jackson ImunoResearch Laboratories, West Grove, PA)
- Donkey Anti-Chicken-IgY-FITC (Jackson ImmunoResearch Laboratories)
- Donkey Anti-Chicken IgG-HRP (Jackson ImmunoResearch Laboratories)
- Goat Anti-Mouse IgG-HRP (Jackson ImmunoResearch Laboratories)

Western detection was done using the SuperSignal West Dura kit (ThermoFisher Scientific)

**Statistical methods**

The Student’s t-test was used to compare average measurements from two samples using a two-tailed distribution (Tails=2) and a two-sample unequal variance (Type=2). Unless otherwise specified, all error bars represent the standard deviation.

All data and reagents are available upon request.

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REFERENCES


FIGURE 1: Suppression of *cup-5*(null) defects by RNAi of *did-2*. (A) Structure of *did-2* mRNA (Filled black boxes indicate translated regions, solid lines are 5' and 3' untranslated regions and dashed lines are introns) and targeted areas by *did-2* RNAi constructs (RNAi 1-3). (B) Percent of viable worms laid by wild type or *cup-5*(null) hermaphrodites after the indicated RNAis. (C) Confocal images of “comma” to “1.5-fold” stage embryos laid by wild type or *cup-5*(null) hermaphrodites carrying the VIT-2::GFP transgene after the indicated RNAis. All images were taken at the same exposure and magnification. Arrows indicate intestinal cells. The boxed images are autofluorescence RNAi bacterial seeding clone with the control RNAi bacterial see *elt-2* protein defects. (D) Quantitation of surface area of VIT-2::GFP compartments in intestinal cells shown in C. (E) Quantitation of the intensity of the VIT-2::GFP compartments in intestinal cells shown in C. Three asterisks indicates P<0.0005. Measurements were normalized to 1 in D and E using the wild type control RNAi values. Scale bars in whole embryo images represent 10 µm.

FIGURE 2: Characterization of *did-2* protein defects in *cup-5*(null). (A) Confocal images of “1.5-fold” stage embryos laid by wild type (wt) and *cup-5*(null) (*zu223* allele) hermaphrodites carrying the *did-2::GFP* transgene expressed from the *did-2* promoter or the *elt-2* promoter. All images in Set 1 were taken using the same exposure and magnification. Under both of these microscopy conditions, embryos lacking this transgene showed no background fluorescence. Arrows indicate intestinal cells. (B) Quantitation of total *did-2::GFP* levels in intestinal cells of wild type embryos and *cup-5*(null) embryos expressing the indicated fusion protein shown in A, Set 1. *did-2::GFP* levels expressed from the *elt-2* promoter in wild type embryos from Set 1 is likely higher due to the saturation of the images. (C) Western blot showing *did-2::GFP* and RME-1 levels in embryos laid by P*did-2::DID-2::GFP* wild type or *cup-5*(null) hermaphrodites. RME-1 was used for normalization. The total of both *did-2::GFP* bands were used for the quantitation. (D) Quantitation of membrane bound *did-2::GFP* levels in intestinal cells of P*did-2::DID-2::GFP* wild type and *cup-5*(null) embryos hermaphrodites shown in A. (E) Quantitation of cytoplasmic *did-2::GFP* in intestinal cells of P*did-2::DID-2::GFP* wild type and *cup-5*(null) embryos laid by hermaphrodites shown in A. (F) Quantitation of the ratio of cytoplasmic *did-2::GFP* to membrane bound *did-2::GFP* in intestinal cells of P*did-2::DID-2::GFP* wild type and *cup-5*(null) embryos laid by hermaphrodites shown in A. (G) Membrane bound *did-2::GFP* (pellet) was separated from cytoplasmic *did-2::GFP* (supernatant) in embryos laid by P*did-2::DID-2::GFP*, wild type or *cup-5*(null) hermaphrodites. *did-2::GFP* and LMP-1 protein were then detected by Western analysis. *did-2::GFP* S/P represents the ratio of cytoplasmic *did-2::GFP* to membrane bound *did-2::GFP*; the total of both *did-2::GFP* bands was used for the quantitation. LMP-1 protein was used as a control of fractionation. (H) Quantitation of *did-2::GFP* levels in intestinal cells of wild type and *cup-5*(null) embryos laid by Pelt-2::*did-2::DID-2::GFP* hermaphrodites shown in A, Set 2. (I) Western blot showing *did-2::GFP* and RME-1 levels in embryos laid by Pelt-2::*did-2::DID-2::GFP* wild type or *cup-5*(null) hermaphrodites. Equal amounts of total protein levels were loaded. RME-1 was used for normalization. The total of both *did-2::GFP* bands were used for the quantitation. (J) Confocal images of “comma” to “1.5-fold” stage embryos laid by P*did-2::DID-2::GFP* wild type or *cup-5*(null) hermaphrodites after the indicated RNAis. The ratios of control RNAi to *did-2* RNAi represent two dilutions of the *did-2* RNAi bacterial seeding clone with the control RNAi bacterial seeding clone. All images were taken using the same exposure and magnification. Arrows indicate intestinal cells. The boxed images are autofluorescence controls of embryos that do not carry the P*did-2::DID-2::GFP* transgene. (K) Quantitation of *did-2::GFP* intensity in intestinal cells after the indicated RNAis of embryos shown in J. (L) Percent of embryos laid by P*did-2::DID-2::GFP* wild type or *cup-5*(null) hermaphrodites that hatched after the indicated RNAis shown in J. Two asterisks indicates P<0.0005; three asterisks indicates P<0.0005. Measurements were
normalized to 1 in B, D, E, H and K using the wild type or wild type control RNAi values. Scale bars represent 10 µm in all images.

FIGURE 3: Characterization of MRP-4 protein defects in cup-5(null). (A) Confocal images of “1.5-fold” stage embryos laid by wild type (wt) and cup-5(null) (zu223 allele) hermaphrodites carrying the MRP-4::GFP transgene expressed from the native mrp-4 promoter. All images were taken using the same exposure and magnification. Arrows indicate intestinal cells. (B) Quantitation of MRP-4::GFP levels in intestinal cells shown in A. Background immunofluorescence values from wild type and cup-5(null) not carrying the MRP-4::GFP transgene were subtracted. (C) Western blot showing MRP-4::GFP and RME-1 levels in embryos laid by P<sub>mrp-4::MRP-4::GFP</sub> wild type or cup-5(null) hermaphrodites. RME-1 was used for normalization. (D) Confocal images of “1.5-fold” stage embryos laid by wild type and cup-5(null) hermaphrodites carrying the MRP-4::GFP transgene expressed from the elt-2 promoter. All images were taken using the same exposure and magnification. Arrows indicate intestinal cells. Under these microscopy conditions, embryos lacking this transgene showed no background fluorescence. (E) Quantitation of MRP-4::GFP levels in intestinal cells shown in D. (F) Western blot showing MRP-4::GFP and RME-1 levels in embryos laid by P<sub>elt-2::MRP-4::GFP</sub> wild type or cup-5(null) hermaphrodites. Equal amounts of total protein levels were loaded. RME-1 was used for normalization. (G) Percent of viable worms laid by hermaphrodites with the indicated genotypes. (H) Fold-change of mrp-4 mRNA levels in P<sub>elt-2::MRP-4</sub> and cup-5(null) relative to wild type embryos as determined by qRT-PCR. Error bars represent the standard error of the mean. (I) Confocal images of “1.5-fold” stage embryos laid by P<sub>mrp-4::MRP-4::GFP</sub> wild type or cup-5(null) hermaphrodites after the indicated RNAis. The ratios of control RNAi to mrp-4 RNAi represent two dilutions of the mrp-4 RNAi bacterial seeding clone with the control RNAi bacterial seeding clone. All images were taken using the same exposure and magnification. Arrows indicate intestinal cells. (J) Quantitation of MRP-4::GFP puncta intensity in intestinal cells shown in G. (K) Percent of embryos laid by P<sub>mrp-4::MRP-4::GFP</sub> wild type or cup-5(null) hermaphrodites that hatched after the indicated RNAis shown in G. Three asterisks indicates P<0.0005. Measurements were normalized to 1 in B, E, and H using the wild type or wild type control RNAi values. Scale bars represent 10 µm in all images.

FIGURE 4: MRP-4 protein localization in wild type and cup-5(null). (A) Confocal images of “comma” to “1.5-fold” stage embryos laid by wild type (wt) or cup-5(null) (zu223 allele) hermaphrodites that were immunostained to detect DID-2::RFP and MRP-4::GFP. Arrows indicate intestinal cells. Arrowheads indicate overlap between DID-2::RFP and MRP-4::GFP. Images to the right of the embryos are magnified images of the regions indicated by the dashed white boxes. (B) Quantitation of DID-2::RFP and MRP-4::GFP percent overlap in intestinal cells shown in A. (C) Confocal images of “1.5-fold” stage embryos laid by wild type or cup-5(null) hermaphrodites expressing MRP-4::GFP and LMP-1::RFP. Arrows indicate intestinal cells. Arrowheads indicate co-localization between MRP-4::GFP and LMP-1::RFP. Images to the right of the embryos are magnified images of the regions indicated by the dashed white boxes. (D) Quantitation of MRP-4::GFP and LMP-1::RFP percent co-localization in intestinal cells shown in C. (E) Confocal images of “1.5-fold” embryos laid by wild type or cup-5(null) hermaphrodites expressing MRP-4::GFP and RAB-11::RFP. Arrows indicate intestinal cells. Images to the right of the embryos are magnified images of the regions indicated by the dashed white boxes. (F) Quantitation of MRP-4::GFP and RAB-11::RFP percent co-localization in intestinal cells shown in E. (G) Confocal images of “comma” to “1.5-fold” stage embryos laid by wild type and cup-5(null) hermaphrodites expressing MRP-4::GFP and LMP-1::RFP or RAB-11::RFP after the indicated RNAis. Only the magnified
images are shown. Arrowheads indicate co-localization. (H) Quantitation of MRP-4::GFP and LMP-1::RFP percent co-localization and MRP-4::GFP and RAB-11::RFP percent co-localization in intestinal cells shown in G. Three asterisks indicates P<0.0005. Scale bars in whole embryo images represent 10 μm.

FIGURE 5: MRP-4 protein ubiquitination in wild type and cup-5(null). (A) Western blots probing for MRP-4::GFP, ubiquitin and RME-1 protein in embryos laid by Pelt-2::MRP-4::GFP, wild type or cup-5(null) hermaphrodites. Left panels show total protein isolates and right panels are after immunoprecipitation of MRP-4::GFP. The P4D1 antibody was used to detect ubiquitin; this antibody detects both mono- and poly-ubiquitination. Ub/MRP-4::GFP represents the fraction of MRP-4 that is ubiquitinated. The + indicates control wild type worms that do not carry the Pelt-2::MRP-4::GFP transgene. (B) Western blots probing for MRP-4::GFP, ubiquitin and RME-1 protein in embryos laid by Pelt-2::MRP-4::GFP, wild type or cup-5(null) hermaphrodites. Left panels show total protein isolates and right panels are after immunoprecipitation of MRP-4::GFP. In addition to the P4D1 antibody, the FK1 antibody was used to specifically detect poly-ubiquitin. Ub/MRP-4::GFP represents the fraction of MRP-4 that is ubiquitinated. The + indicates control wild type worms that do not carry the Pelt-2::MRP-4::GFP transgene. (C) Western blots probing for MRP-4::GFP, ubiquitin and RME-1 protein in embryos laid by cup-5(null); Pelt-2::MRP-4::GFP hermaphrodites after the indicated RNAis. Left panels show total protein isolates and right panels are after immunoprecipitation of MRP-4::GFP. Ub/MRP-4::GFP represents the fraction of MRP-4::GFP that is ubiquitinated. (D) Western blots probing for MRP-4::GFP, ubiquitin and RME-1 protein in embryos laid by cup-5(null); Pelt-2::MRP-4::GFP hermaphrodites after the indicated RNAis. Left panels show total protein isolates and right panels are after immunoprecipitation of MRP-4::GFP. Ub/MRP-4::GFP represents the fraction of MRP-4::GFP that is ubiquitinated. (E) Western blots probing for MRP-4::GFP and ubiquitin in embryos laid by ego-2(om33); Pelt-2::MRP-4::GFP hermaphrodites after the indicated RNAis. Left panels show total protein isolates and right panels are after immunoprecipitation of MRP-4::GFP. Ub/MRP-4::GFP represents the fraction of MRP-4::GFP that is ubiquitinated. Measurements were normalized to 1 in A-F using the wild type, wild type control RNAi, or cup-5(null) control RNAi Ub/MRP-4::GFP values. RME-1 was used to confirm MRP-4::GFP enrichment following immunoprecipitation.

FIGURE 6: ESCRT RNAi suppression of cup-5(null) defects. (A) Percent of viable worms laid by wild type or cup-5(null) hermaphrodites after the indicated RNAis. (B) Percent of viable worms laid by ego-2(om33) or cup-5(null); ego-2(om33) hermaphrodites after the indicated RNAis. (C) Confocal images of “comma” to “1.5-fold” stage embryos laid by wild type or cup-5(null) hermaphrodites carrying the VIT-2::GFP transgene after the indicated RNAis. All images were taken at the same exposure and magnification. Arrows indicate intestinal cells. Scale bar represents 10 μm. (D) Quantitation of surface area of VIT-2::GFP compartments in intestinal cells shown in C. (E) Quantitation of the intensity of the VIT-2::GFP compartments in intestinal cells shown in C. An asterisk indicates P<0.05; two asterisks indicates P<0.005, three asterisks indicates P<0.0005. Measurements were normalized to 1 in D and E using the wild type control RNAi values.

FIGURE 7: ESCRT RNAi suppression in adult intestine. (A) Confocal images of adult wild type (wt) and cup-5(null) (zu223 allele) intestine expressing LMP-1::GFP after the
indicated RNAis. All images were taken using the same exposure and magnification. Arrows indicate LMP-1::GFP compartments. Arrowheads indicate autofluorescent gut granules. Scale bar represents 10 μm. (B) Quantitation of the intensity of LMP-1::GFP in compartments shown in A. Measurements were normalized to 1 using the wild type control RNAi value. (C) Table of $P$ values for all pairwise tests in B.
Table 1. *C. elegans* strains used in this study

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A

CONTROL RNAi  did-2 RNAi  phi-33 RNAi  alx-1+ego-2 RNAi  mrp-4 RNAi  gfp RNAi

wt

cup-5(null)

LMP-1::GFP Intensity

0.5  1.5  2.5

B

C

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P < 0.0005

Color Key

Control RNAi

RNAi

did-2

phi-33

alx-1+ego-2

mrp-4

gfp

Huynh et al., Figure 7
SUPPLEMENTAL FIGURE S1: Worm DID-2 sequence, similarity to yeast and human proteins, and DID-2::GFP/TagRFP functionality. (A) Shown is the amino acid sequence alignment of DID-2 (C. elegans), Did2p (S. cerevisiae), and CHMP1b (H. sapiens). Identical amino acids are shaded in black while similar amino acids are shaded in grey. Amino acids considered similar were I/L/V, S/T, D/E, and K/R. (B) Percent of laid embryos that hatched and grew to adults from did-2(ok3325) (null allele) hermaphrodites carrying a DID-2::GFP or DID-2::Tag RFP transgene. This partial rescue is expected because transgenes are not expressed in the germine; therefore, they cannot rescue early defects in embryogenesis.
SUPPLEMENTAL FIGURE S2: RME-1 levels and Pelt-2 activity in wild type and cup-5(null). (A) Confocal images of “1.5-fold” stage embryos laid by wild type (wt) or cup-5(null) (zu223 allele) hermaphrodites immunostained for RME-1. All images were taken at the same exposure and magnification. Arrows indicate intestinal cell staining. (B) Quantitation of the intensity of RME-1 in intestinal cells shown in A. (C) Western blot showing RME-1 levels in embryos laid by wild type or cup-5(null) hermaphrodites. Equal amount of total protein levels were loaded. The numbers are the average of three independent trials. (D) Confocal images of “1.5-fold” stage embryos laid by wild type or cup-5(null) hermaphrodites expressing GFP under the control of the elt-2 promoter. All images were taken at the same exposure and magnification. Arrows indicate intestinal cell staining. (E) Quantitation of the intensity of GFP in intestinal cells shown in D. (F) Western blot showing GFP levels in embryos laid by wild type or cup-5(null) hermaphrodites. Equal amount of total protein levels were loaded. The numbers are the average of three independent trials. The indicated P values are from Student’s t-test. Scale bars represent 10 µm in all images.
SUPPLEMENTAL FIGURE S3: Effect of TFEB homologue HLH-30 RNAi on MRP-4::GFP and DID-2::GFP. (A) Confocal images of “1.5-fold” stage embryos laid by Pdid-2::DID-2::GFP wild type or cup-5(zu223) hermaphrodites after the indicated RNAis. All images were taken using the same exposure and magnification. Arrows indicate intestinal cells. Under these microscopy conditions, embryos lacking this transgene showed no background fluorescence. (B) Quantitation of DID-2::GFP levels in intestinal cells shown in A. (C) Confocal images of “1.5-fold” stage embryos laid by Pmrp-4::MRP-4::GFP wild type or cup-5(zu223) hermaphrodites after the indicated RNAis. All images were taken using the same exposure and magnification. Arrows indicate intestinal cells. (D) Quantitation of MRP-4::GFP levels in intestinal cells shown in C. Background immunofluorescence values from wild type and cup-5(zu223) embryos not carrying the MRP-4::GFP transgene were subtracted. Two asterisks indicates $P<0.005$. Three asterisks indicates $P<0.0005$. Measurements were normalized to 1 in B and D using the wild type control RNAi values. Scale bars represent 10 µm in all images.
SUPPLEMENTAL FIGURE S4: MRP-4::GFP protein localization with LMP-1::RFP in wild type and cup-5(null) after ESCRT-associated protein RNAi. (A) Confocal images of “1.5-fold” stage embryos laid by wild type or cup-5(null) hermaphrodites expressing MRP-4::GFP and LMP-1::RFP after the indicated RNAis. Only the magnified images are shown. Arrowheads indicate co-localization. (B) Quantitation of MRP-4::GFP and LMP-1::RFP percent co-localization in intestinal cells shown in A. Three asterisks indicates $P<0.0005$. 
SUPPLEMENTAL FIGURE S5: ESCRT RNAi suppression in adult coelomocytes. (A) Confocal images of endocytosed GFP in adult wild type (wt) and cup-5(null) (zu223 allele) coelomocytes after the indicated RNAi. Scale bar represents 5 µm. (B) Quantitation of the GFP compartment sizes shown in A. Measurements were normalized to 1 using the wild type control RNAi value. (C) Table of P values for all pairwise tests in B.