CHARACTERIZING VARIOUS CUP PROTEINS INVOLVED IN LYSOSOME FORMATION AND TRANSPORT

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ABSTRACT

Lysosomes are the main degradative organelles in eukaryotic cells and are essential for many cellular processes, including endocytosis, apoptosis, and autophagy. However, little is known about how they form. Endocytosed material is trafficked from early endosomes to late endosomes; materials destined for degradation are concentrated in structures that bud and separate from late endosomes, forming lysosomes. Acid hydrolases and other enzymes responsible for degradation are trafficked to these lysosomes to effect degradation. There are only two genes known to be essential for lysosome formation, *rab*-2 and *cup*-5 in *Caenorhabditis elegans*. Due to the complexity of lysosome formation, which includes budding, extension, and fission, we hypothesize that there are other genes involved in lysosome formation. A mutagenesis screen was conducted to identify different genes involved in lysosome formation. We used transgenic wild type *C. elegans* that secrete green fluorescent protein (GFP) from the body wall muscles into the body cavity of the worm; this GFP is endocytosed and degraded in lysosomes of scavenger cells called coelomocytes. We mutagenized these worms and identified mutants that had bright green coelomocytes indicative of defects in lysosome formation and/or function. We report our analysis of these new *cup* mutants and their possible functions in lysosomes.

INTRODUCTION

Mucolipidosis Type IV (MLIV) is a rare, autosomal recessive lysosomal storage disorder characterized by neuropathological and opthalmological degradation (Altarescu et al., 2002). Symptoms of MLIV manifest within the first year of life, and include psychomotor retardation, retinal degeneration, and corneal clouding (Bach, 2001). Phenotypically, the histology of patients with MLIV is characterized by abnormal lipid accumulation, neuronal cell death, and greatly enlarged vacuoles. MLIV is caused by loss of the protein TRPML1, encoded by the human gene *MCOLN1*. TRPML1 is a non-selective cation channel in the Transient Receptor Potential protein family (LaPlante et al., 2002).

Lysosomes are ubiquitous organelles known to have several functions within cells, including waste degradation, wound repair (Klumperman et al., 2015), and cellular death in the forms of apoptosis and necrosis (Guicciardi et al., 2004). Lysosomes are membrane-bound and contain acid hydrolase enzymes, which are suited to bind to and degrade numerous cellular substrates. Lysosomes fuse with late endosomes to degrade materials that cells have endocytosed. The endocytic pathway is composed of several distinct membrane compartments. Molecules from the extracellular surface are internalized via the plasma membrane and trafficked to early endosomes, then to late endosomes, and finally to lysosomes, with each subsequent compartment increasing in acidity (Grant et al., 2002).

In *Caenorhabditis elegans*, the orthologue to human TRPML1 is CUP-5 (Fares et al., 2001). Deletion of *cup-5* leads to lysosomal defects, including large vacuoles and intestinal cell death, similar to the large vacuoles and neuronal cell death seen

in humans with MLIV. Expression of human TRPML1 (*MCOLN1)* in *C. elegans* rescues all lysosomal defects (Treusch et al., 2004), further proving the efficacy of using the *cup-5* mutant as a model for MLIV.

In this study, we examine several proteins in *C. elegans* that were discovered to play a role in lysosomal formation and development through a mutagenesis screen. Proteins were determined to be involved in lysosomal formation if, mutations led to a similar phenotype as that of *cup-5* mutants, shown in Fig. 1 below.

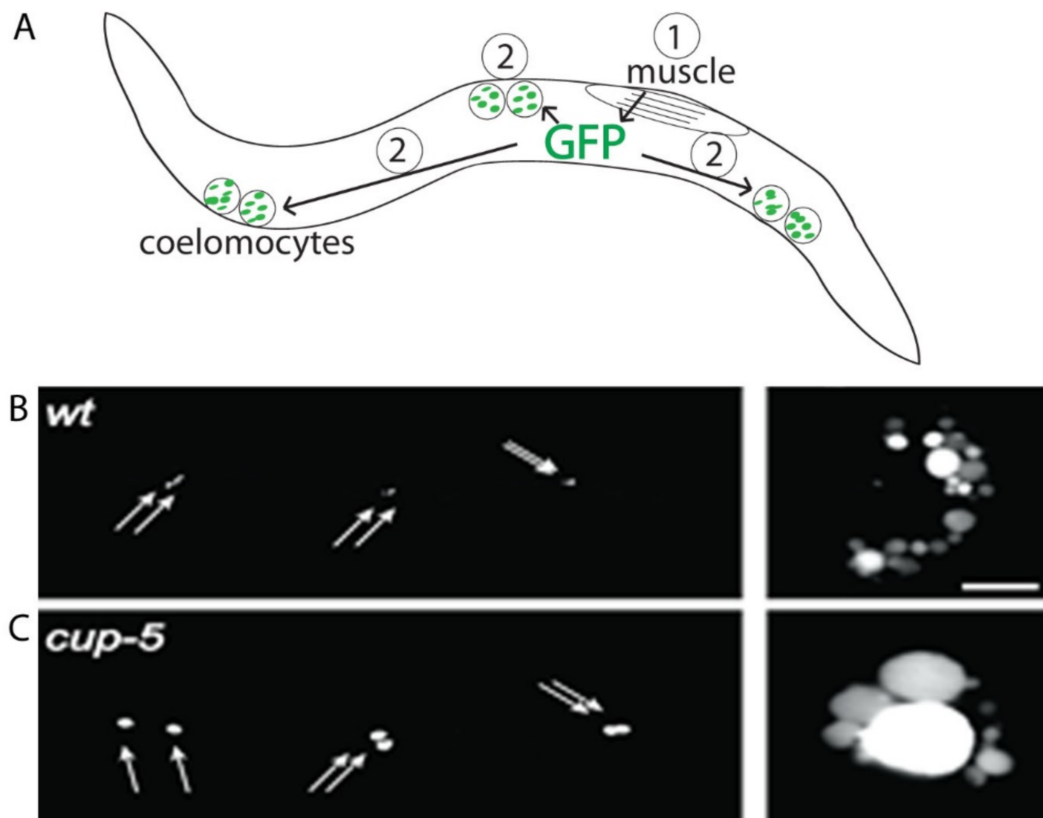


Figure 1. The coelomocyte uptake assay was used to identify worms with lysosomal degradation defects. (A) Schematic of coelomocyte uptake assay. 1. GFP is secreted from muscle cells into the pseudocoelom. 2. GFP is endocytosed by coelomocytes. (B) Wild-type worms endocytose GFP in coelomocytes (left) where it localizes to lysosomes at steady state (right). (C) *cup-5* mutant worms accumulate GFP in coelomocytes (left) but are unable to efficiently degrade it (right), giving rise to a bright coelomocyte phenotype. Scale bar represents 5 µm.

Coelomocytes are specialized scavenger cells found in the body cavity of *C. elegans*. Each nematode has a total of six coelomocytes. As a result of their specialized role, coelomocytes have a high concentration of lysosomes and make an ideal target cell for lysosomal study. The worm strains used in this study express the transgene *arIs37*, which causes green fluorescent protein (GFP) to be secreted into the body cavity. In a healthy worm, GFP is then taken up by the coelomocytes and degraded.

In this study, we report the results of our analysis of these new *cup* mutants and their possible functions in lysosomes, along with the potential impact on humans.

MATERIALS and METHODS

Strains

GS1912: *dpy-20(e1282); arIs37*

NP1397: *cup-5(cd12); arIs37*

NP1345: *cup-12 (cd7); dpy-20 (e1282); arIs37*

NP1346: *cup-13(cd17); dpy-20(e1282); arIs37*

NP1378: *cup-14(cd32); arIs37*

NP1379: *cup-15(cd33); arIs37*

NP1639: *cup-16(cd50); arIs37*

NP1531: *cup-17(cd49); arIs37*

Confocal Imaging Protocol

Day 1: 50 L4 worms were picked and placed on a 60 mm NGM+OP50 plate and left at 20°.

Day 2: Adult hermaphrodites were placed in a drop of 1xPBS/9 mM levamisole to take pictures. Pictures taken using Zeiss confocal microscope (in the MCB department). Two magnifications were taken:

1) 10X Objective (+DIC if possible): All images were taken using the same exposure that was set for GS1912. GFP intensity was measured in whole coelomocytes.

2) 100 X objective: Images were not taken using the same exposure. Sizes of GFP compartments in each coelomocyte were measured.

Metamorph was used to make the measurements. The results were entered in Excel to determine averages and standard deviations,.

Microinjection Protocol

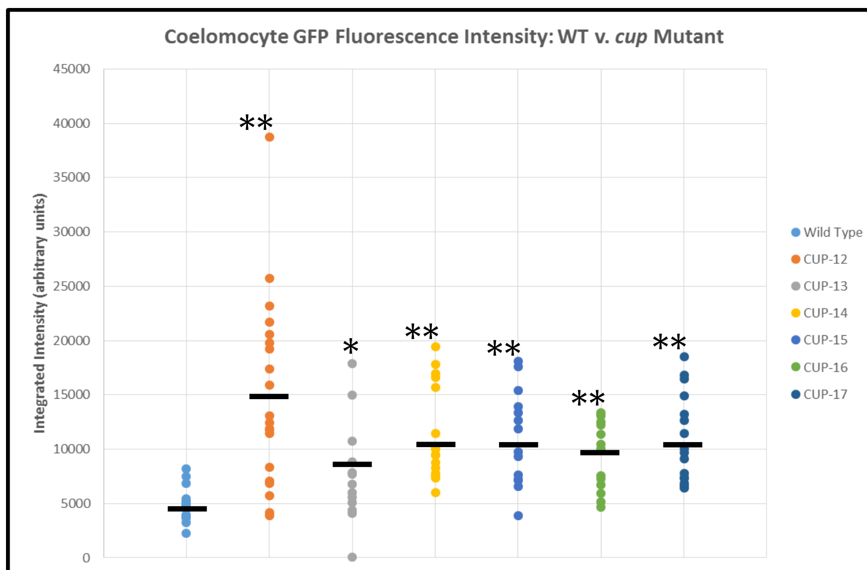
Two worms were placed on an agarose pad in a drop of paraffin oil. Worms were injected using BSA-Alexa594. Injected worms were placed on an NGM+OP50 60mm plate for fifteen minutes post injection. After fifteen minutes, the worms were placed on a slide for imaging. Images were taken on Zeiss confocal microscope using a 100x objective. The results were entered in Excel to determine averages and standard deviations.

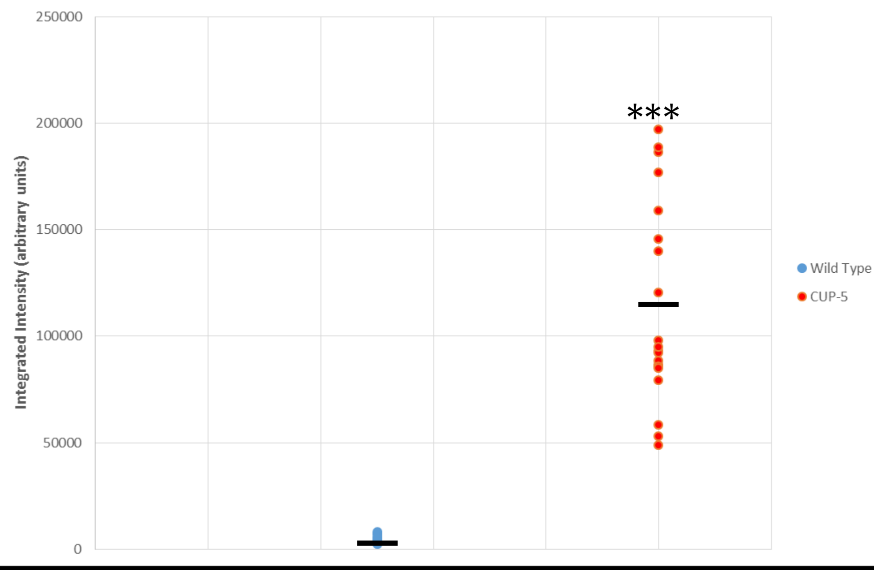
RESULTS

The *cup-5* phenotype is two-fold: 1) significantly enlarged compartments and 2) intensely bright green coelomocytes. We sought to examine whether the new *cup* mutants—*cup-12, cup-13, cup-14, cup-15, cup-16, and cup-17*—displayed a similar phenotype.

First, we looked at the intensity of the GFP fluorescence within the coelomocytes of the various mutants. Since each of these mutants express the transgene *arIs37*, GFP is secreted into their body cavity constantly. In a healthy worm, the coelomocytes endocytose this GFP and degrade it relatively quickly, leading to low levels of GFP within the coelomocytes. This leads to a dim green phenotype. Therefore, if the coelomocytes appear to be a brighter green than the wild type, then this is suggestive of some defect in lysosomal formation. Indeed, each of the *cup* mutants had coelomocytes with a greater fluorescence intensity, relative to the wild type (Fig. 2).

2A. Coelomocyte GFP Intensity of WT v. *cup* Mutant





Coelomocyte GFP Fluorescence Intensity: WT v. cup-5
Integrated Intensity (arbitrary units)
250000
200000
150000
100000
50000
0
***
Wild Type
CUP-5

2B. Coelomocyte GFP Intensity of WT v. *cup* Mutant Images

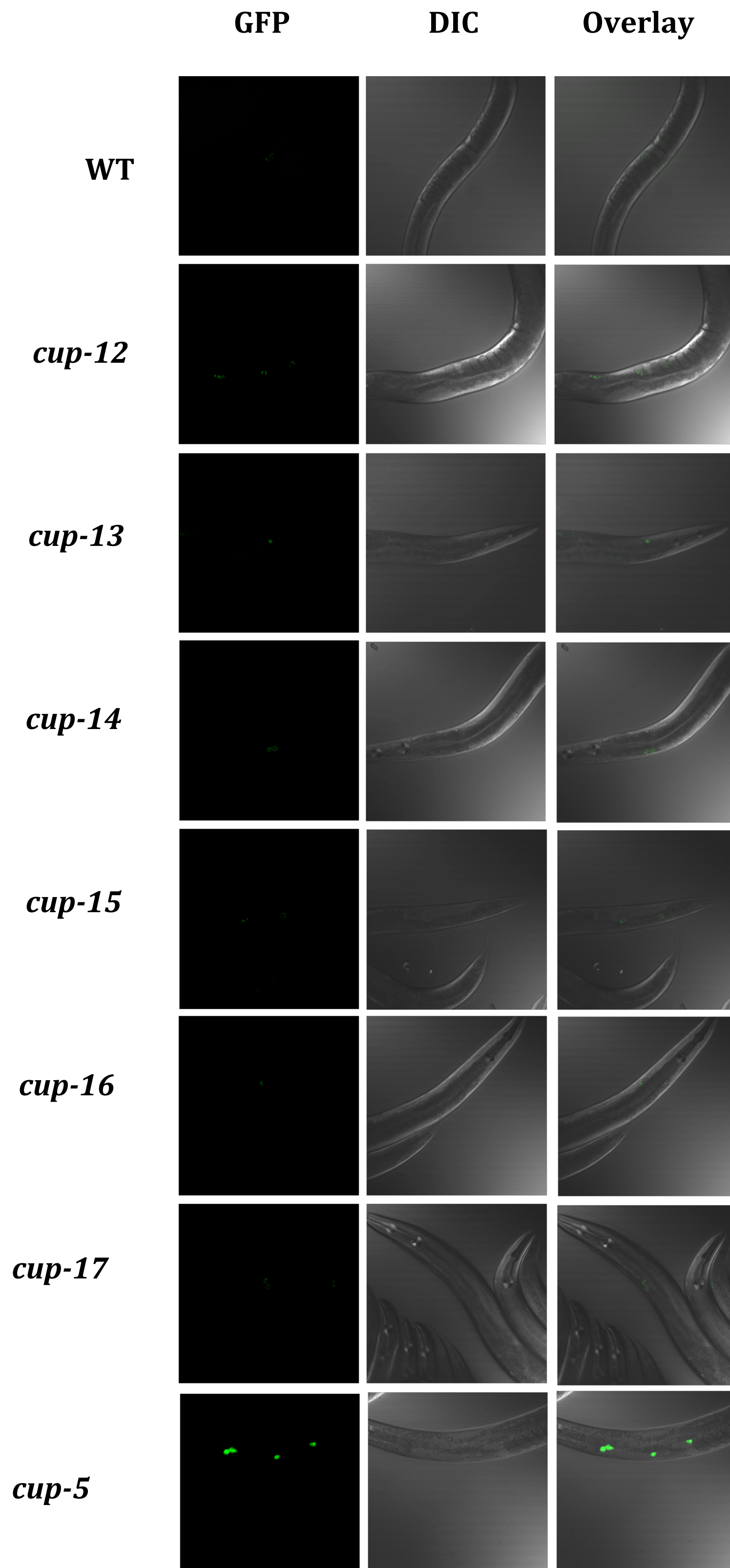
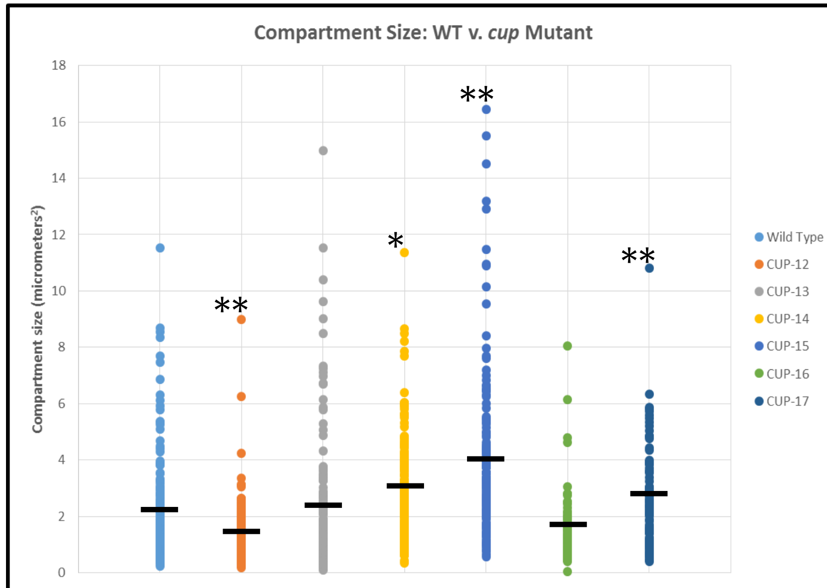
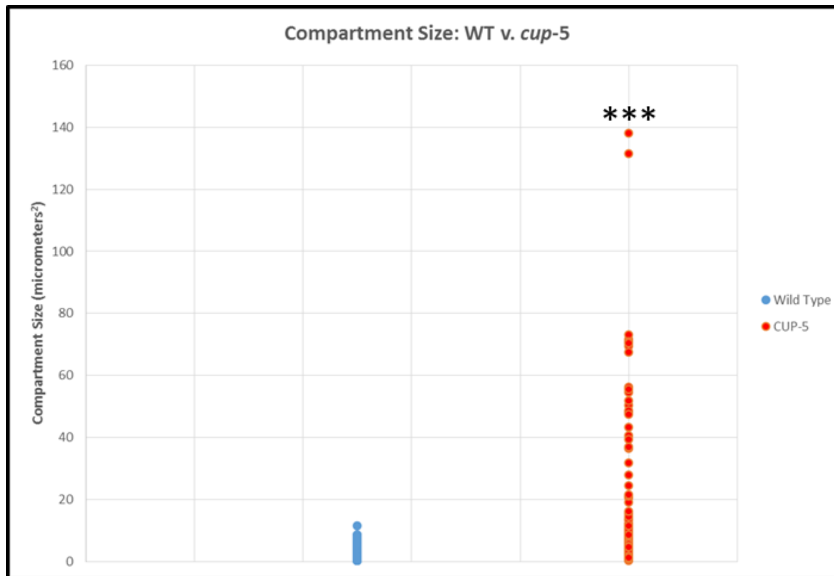


Figure 2. *cup* mutants display increased fluorescence intensity compared to wild-type worms. A. Coelomocyte GFP fluorescence intensity measured using confocal microscopy. * = $p < .05$, ** = $p < .005$, *** = $p < 0.0005$, relative to wild-type (WT) strain. Black bar represents average of data set for each strain. B. Left panel represents GFP image, middle panel represents DIC image, and right panel represents GFP and DIC overlay. All *cup* mutants have significantly brighter compartments relative to wild type.

After assessing the fluorescence levels, we then examined the other phenotype—compartment size within the coelomocytes. We measure the sizes of GFP-filled compartment in each of the mutants. The differences in compartment size can be attributed to many different factors and can provide significant insight into the potential functions of each protein. Lysosomes form by budding off of a late endosome. After budding, scission occurs and a nascent lysosome forms. This lysosome can then fuse with other lysosomes or even fuse with another late endosome. If we see larger compartment sizes in a particular *cup* mutant, then it's possible that the protein is involved in scission or fusion with the late endosome. Meaning, not enough scission is occurring or too much fusion is occurring, leading to enlarged compartment sizes. Relative to the wild type, *cup-14* and *cup-15* show increased compartment sizes, *cup-12* and *cup-16* show smaller compartment sizes, and there was no significant difference in the *cup-13* and *cup-17* mutants relative to wild type (Fig. 3).

3A. Compartment Size in Coelomocytes of WT v. *cup* Mutant





3B. Compartment Size in Coelomocytes of WT v. *cup* Mutant Images

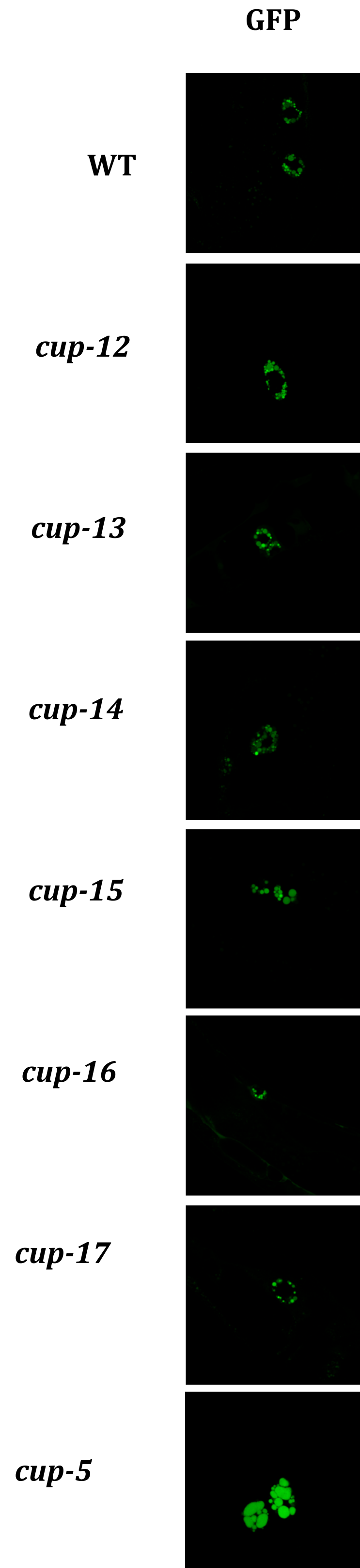
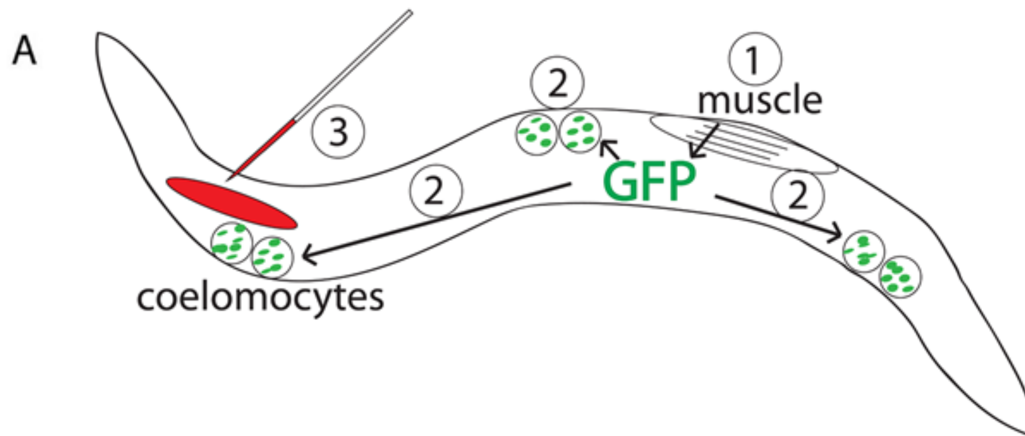


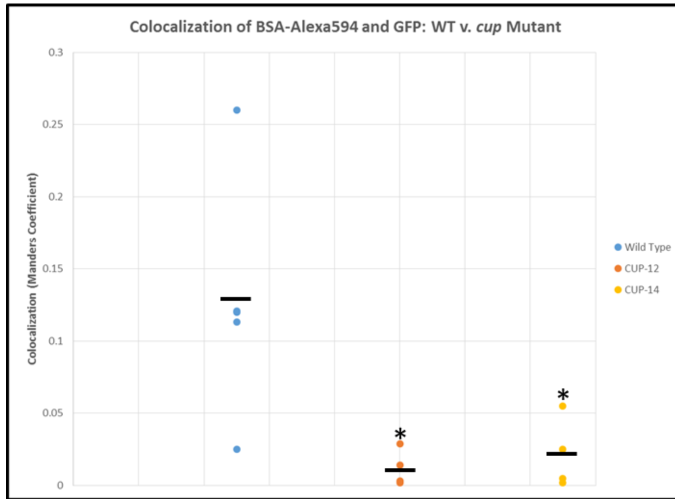
Figure 3. *cup* mutants have significantly different compartment sizes than wild-type.
A. Compartment size (area) measured using confocal microscopy. * = p < .05, ** = p < .005, *** = p < 0.0005, relative to wild-type (WT) strain. Black bar represents average of data set for each strain. B. Images of each strain show GFP in coelomocytes. Compartment (vesicle) area measured using Metamorph. Relative to the wild type, *cup-14* and *cup-15* show increased compartment sizes, *cup-12* and *cup-16* show smaller compartment sizes, and there was no significant difference in the *cup-13* and *cup-17* mutants.

The new *cup* mutants have, to this point, only been examined for phenotypic differences. We have yet to examine trafficking to coelomocytes and lysosomes in real time. To do this, we injected BSA-Alexa594 into the body cavity of two of the *cup* mutants—*cup-12* and *cup-14*. After injection, we waited fifteen minutes and then imaged the worms to see where the dye was relative to the GFP in the lysosomes of the coelomocytes. In both mutants, there was significantly less of the dye in the coelomocytes, suggesting both mutants are defective in new lysosome formation due to the delay in trafficking to mature lysosomes (Fig. 4).

4A. Schematic Representation of Microinjection into Body Cavity



4B.



4C.

	GFP	**BSA-Alexa594**	**Overlay**
WT			
***cup*-12**			
***cup*-14**			

Figure 4. *cup-12* and *cup-14* mutants display delayed trafficking of proteins to lysosomes
A. Schematic of microinjection procedure. B. Localization of BSA-Alexa594 relative to GFP in coelomocytes 15 minutes post-microinjection. * = $p < .05$. Black bar represents average.
C. Left panel represents GFP, middle panel represents BSA-Alexa594, and right panel represents overlay of GFP and BSA-Alexa594. *cup-12 and cup-14* mutants show delayed trafficking to lysosomes relative to the wild type.

DISCUSSION

Our analysis of these various *cup* mutants begins to paint a picture of where in the endocytic pathway the CUP mutants may function. Since little to nothing is known about these proteins, the data presented here reveals different characteristics of their respective mutants.

Between the wild type and each of the *cup* mutants, there lies a statistically significant difference in the fluorescence intensity of their respective coelomocytes (Figure 2), with the mutants showing a marked increase. These findings suggest that perturbation of these proteins leads to defects in lysosomal degradation, as the GFP is being degraded more slowly than in the wild type.

While the differences are all trending in a specific direction, the differences in compartment sizes (figure 3) show more variance. *cup*-14 and *cup*-15 mutants have significantly larger compartments relative to wild type. *cup*-12 and *cup*-16 have smaller compartment sizes relative to wild type. *cup*-13 and *cup*-17 did not have statistically significant different compartment sizes than wild type. This suggests that each of these mutants affects different steps of lysosome biology, including lysosome formation, fission, fusion, and degradation.

When examining the *cup-12* and *cup-14* mutants for any trafficking delays, we found that, relative to the wild type, both of the mutants showed a decrease in the amount of BSA-Alexa594 that was localized to lysosomes (Figure 4), suggesting that these proteins are involved in lysosome formation.

Ultimately, these proteins are studied in *C. elegans* to provide insight into the function of their human counterparts and how they affect disease. We identified *cup-17* as the *ctns-1* gene in *C. elegans*, which is the orthologue to human *CTNS*. *CTNS*, or cystinosin, is mutated in the lysosomal storage disorder cystinosis (Town et al., 1998). By studying *ctns-1* in a more readily available model organism, we can gain insight into how *CTNS* functions in humans. This suggests a functional link between cystinosis and MLIV and can provide translational researchers with the opportunity to develop targeted therapies for these diseases.

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