Figure S1. Mutations in *mtm-1, 5, 6, and 9* do not affect degradation of autophagic cargo.

(A) The gene structures of *C. elegans* myotubularin phosphatases are shown with filled boxes representing exons and thin lines indicating the introns. The arrows show the direction of transcription. The positions of genetic deletions are shown underneath.

(B-P) Confocal fluorescent images of wild-type and *mtm-1, 5, 6, 9* mutant embryos at the 1.5-fold stage stained by DAPI and anti-SQST-1 (B-F), anti-SEPA-1 (G-K) or anti-LGG-1 (L-P) antibodies are shown. No abnormal accumulation of autophagic cargo or structures was observed. Scale bars: 5 µm.
Figure S2. *mtm-3* mutants accumulate autophagic cargo.

DIC and confocal fluorescent images of wild-type and *mtm-3* embryos expressing SQST-1::GFP (A-B’), SEPA-1::GFP (C-D’), C17E4.2::GFP (E-F’) or C33D9.6::GFP (G-H’) are shown. Autophagic substrates accumulate in *mtm-3* but not wild-type embryos. Scale bars: 5 µm.
Figure S3. Expression of wild-type but not catalytically inactive MTM-3 rescues the autophagy phenotype in mtm-3 mutants.

(A-H) Confocal fluorescent images of wild-type (F, G) and mtm-3(tm4475)(A-E) embryos without transgene (A, A’, F) or expressing GFP::MTM-3 (B), GFP::MTM-3(C459S) (C), human MTMR3 (D) or MTM-3 driven by the heat-shock promoter (E, G) and stained by anti-LGG-1 antibody. The expression of human MTMR3 (D) and wild-type but not
catalytically inactive MTM-3 [MTM-3(C459S)] significantly reduced LGG-1 accumulation in *mtm-3* mutants (H). At least 10 embryos were quantified in each strain and data are shown as mean ± SD. **P<0.0001. N.S: not statistically different. Scale bars: 5 µm.


(J) Western blot analysis of GFP::MTM-3 and GFP::MTM-3(C459S) in wild-type worms.

(K-P) GFP::MTM-3 is widely distributed and diffuse in the cytoplasm in embryos (K-L’), larvae [pharynx (M), cells in the tail region (N)] and adults [intestine (O) and vulva muscle (P)].

(Q-T) The expression of GFP::MTM-3 is not affected in autophagy mutants.
Figure S4. Loss of \textit{mtm-3} does not affect endocytic transport.

(A-B’) GFP secreted from body wall muscle cells is endocytosed from the body cavity by coelomocytes and transported to lysosomes (arrows; arrowheads indicate endosomes) in both wild type (A-A’) and \textit{mtm-3}(tm4475) mutants (B-B’).

(C-H’) In both wild type and \textit{mtm-3}(tm4475) mutants, VIT-2::GFP secreted from the intestine is efficiently endocytosed by oocytes (C-D’; arrows indicate spermatheca). VIT-2::GFP is observed in embryos (E-F’) but is completely degraded in L1 larvae (G-H’). Scale bars: 5 \textmu m.
**Figure S5.** Genetic interaction of *mtm-3* with other autophagy mutants.

Confocal fluorescent images of 200-cell-stage embryos from the indicated strains stained by DAPI, anti-SEPA-1 and anti-LGG-1 antibodies. Insets show magnified views and arrows indicate overlapping SEPA-1 and LGG-1 puncta. Scale bars: 5 µm.
**Figure S6.** MTM-3 acts at a similar step to EPG-5 in the aggrephagy pathway.

Confocal fluorescent images of *atg-2, mtm-3;atg-2, epg-5* and *epg-5;mtm-3* mutant embryos at the 200-cell stage stained by DAPI, anti-SEPA-1 and anti-LGG-1 antibodies. Insets show magnified views and arrows indicate overlapping SEPA-1 and LGG-1 puncta. Scale bars: 5 µm.
**Figure S7.** Autolysosome formation is impaired in *mtm-3* mutants.

(A-E) Confocal fluorescent images of 1.5-fold embryos from the indicated strains expressing both GFP::LGG-1 and NUC-1::mCHERRY are shown. Insets show magnified views and arrows indicate overlapping or closely associated GFP and mCHERRY puncta. Scale bars: 5 µm.

(F, G) The percentage of GFP::LGG-1 puncta that co-localize(F) or closely associate(G) with NUC-1::mCHERRY was quantified in the indicated strains. At least 10 embryos were scored in each strain and data are shown as mean ± SD. **P<0.0001. N.S: not statistically different.
Figure S8. ATG-18::GFP is diffuse in autophagy mutants *atg-2* and *epg-5*.

(A-D’) DIC and fluorescent images of 1.5-fold embryos expressing ATG-18::GFP in wild type (A, A’), *mtm-3* (B, B’), *atg-2* (C, C’) and *epg-5* (D, D’). ATG-18::GFP is diffuse in the cytoplasm in wild-type, *atg-2* and *epg-5* embryos but forms puncta in *mtm-3* mutants (arrows). Scale bars: 5 µm.
(E) Western blot analysis of ATG-18::GFP in wild type and *mtm-3* mutants.

(F) Proposed model of the role of MTM-3 in autophagosome maturation into autolysosome (top). For comparison, models of the autophagic functions of yeast Ymr1 (middle) and mammalian Jumpy and MTMR3 (bottom) are also included.