Autophagy is required for necrotic cell death in *Caenorhabditis elegans*

Chrysanthi Samara, Popi Syntichaki & Nektarios Tavernarakis

Supplementary Information

Supplementary Methods

PCR oligonucleotides. Gene fragments for RNAi and GFP constructs were amplified directly from genomic DNA, using the oligonucleotide primers incorporating appropriate restriction enzyme sites for subcloning purposes. The following oligonucleotides were used for generating gene-specific RNAi constructs:

For *bec-1*: 5'GCTCTAGAGTTATCACAGAAGCTCTG3' and
5'CGGGATCCGTCCATACAATGCGTACG3', for *lgg-1*:
5'GGAATTCAAGTGGGCTTACAAGGAG3' and
5'GGAATTCGTCTTCTTCGTTTATTCATG3', for *lgg-3*:
5'GGAATTCAGAAACCGCCACAACTCCA3' and
5'GGAATTCCGGTGTAATGCTGTACTGA3', for *vha-2*:
5'CGGGATCCTAAATCGTCTTCTTATCTTCCA3' and
5'CCCAAGCTTCAACGGCGAATGATTTATTCTC3', for *atgr-18* (F41E6.13):
5'AACTGCAGAGAATCCCGACTCGATCAAC3' and
5'GCTCTAGAGGTGTGGCTCATTGGTGGG 3', for *unc-51*:
5'CATGCCATGGTAAGCCCGAATCAAGCCCTT3' and

5’AACTGCAGGGACCAAAGGACCTAAGGAA3’, for *atgr-5* (Y71G12B.12):

5’GCTCTAGAAATAATCGTCGTTCCCCGT3’ and

5’CGGGATCCAGTCAGAAAACTCCCATAC3’, for *atgr-7* (M7.5):

5’ATGGCCACGTTTGTTCCCT3’ and 5’TCACAGTGCACTGTTGGTCCA3’,

and for *atgr-9*: 5’GCTCTAGAAGTTTTTGTCTCCGGTAACG3’ and

5’CGGGATCCGATTCCAGTTTTACCTTCCAA3’

The following oligonucleotides were used for generating GFP reporter constructs:

For the p_{lgg-1}dsRed::LGG-1 reporter fusion: primers

5’GGAATTCGAAGTGGGCTTACAAGGAGGA3’ and

5’GGAATTCGTCTTCTTCGTTTATTCATG3’ for *lgg-1* coding sequences and primers

5’ACATGCATGCGCACTTTCAAGGCGACAGTA3’ and

5’GGGGTACCTCCTCCTTGTAAGCCCACTT3 for the *lgg-1* promoter. For p_{mec-4}GFP:

5’CCCAAGCTTCTTCTCACGTCATAACC3’ and

5’AACTGCAGTCTATAACTTGATAGCGAT3’. For p_{mec-17}BEC-1::GFP:

5’CGGGATCCATGACGACCCAACGAAG3’ and

5’CGGGATCCAATAGGCGATCTGAGAGC3’.

Autophagosome collocalization analysis

Worms expressing the DsRED::LGG-1 and mitoGFP or LMP-1::GFP chimeric proteins were harvested at the L1-L2 developmental stages and observed under a confocal microscope using a 40x objective lens (Plan-Neofluar, NA 0.75; Carl Zeiss, Jena, Germany). To acquire images from individual neurons, animals were scanned simultaneously with a 488nm and a 543nm laser beam to excite GFP and DsRED molecules respectively. Emitted light was gathered using 515±15 and 600±25 band-pass filters.

Statistical analysis

Statistical analyses were carried out using the Prism software package (GraphPad Software Inc., San Diego, USA) and the Microsoft Office 2003 Excel software package (Microsoft Corporation, Redmond Washington USA). Mean values were compared using unpaired t tests. For multiple comparisons, we used the one-factor (ANOVA) variance analysis corrected by the posthoc Bonferroni test.

Legends to supplementary figures

Supplementary Figure 1. Distribution of DsRed::LGG-1-labelled autophagosomes (red), in both wild type and *mec-4(d)* mutant animals. Touch receptor neurons express GFP under the control of the *mec-4* promoter. Dying neurons in *mec-4(d)* mutants are examined at three time points during the course of cell death (early, mid, late).

Supplementary Figure 2. Autophagosome and sub cellular organelle labelling. A fluorescent DsRed::LGG-1 fusion, driven by the *lgg-1* promoter, is utilized to label autophagosomes (red), in both wild type and *mec-4(d)* mutant animals. **a**, Lysosomes are labelled using LMP-1::GFP (green), expressed specifically in touch receptor neurons under the control of the *mec-17* promoter. **b**, Mitochondria are labelled using mitoGFP (green), expressed specifically in touch receptor neurons under the control of the *mec-7* promoter.

Supplementary Figure 3. Touch receptor neuron distension during necrosis. White arrows indicate PLM touch receptors. These neurons undergo dramatic swelling and degeneration in *mec-4(d)* mutant animals, reaching a size that almost spans the width of the tail. White bar denotes 20 μm.

Supplementary Figure 4. a, Quantification of surface expression of full-length, GFP-tagged MEC-4 in animals carrying the *unc-51(1189)* mutant allele, which suppresses necrotic cell death. UNC-51 depletion does not alter surface expression of the tagged MEC-4. Error bars denote S.E.M. values (n>50). **b**, Quantification of surviving touch receptor neurons. GFP expression during adulthood was used to score survival as shown in Figure 1d and also in Supplementary Figure 7. Error bars denote S.E.M. values (n>200; $P<0.001$, unpaired t-test)

Supplementary Figure 5. Quantification of RNAi efficacy for *bec-1* and *lgg-1*. Animals carrying full-length GFP fusions with either BEC-1 (**a**) or LGG-1 (**b**) were subjected to RNAi for *bec-1* or *lgg-1* respectively and silencing of GFP expression was measured in touch receptor neurons of otherwise wild type animals. Error bars denote S.E.M. values (n>50 in both experiments; P<0.001, compared to the corresponding control populations, unpaired t-test).

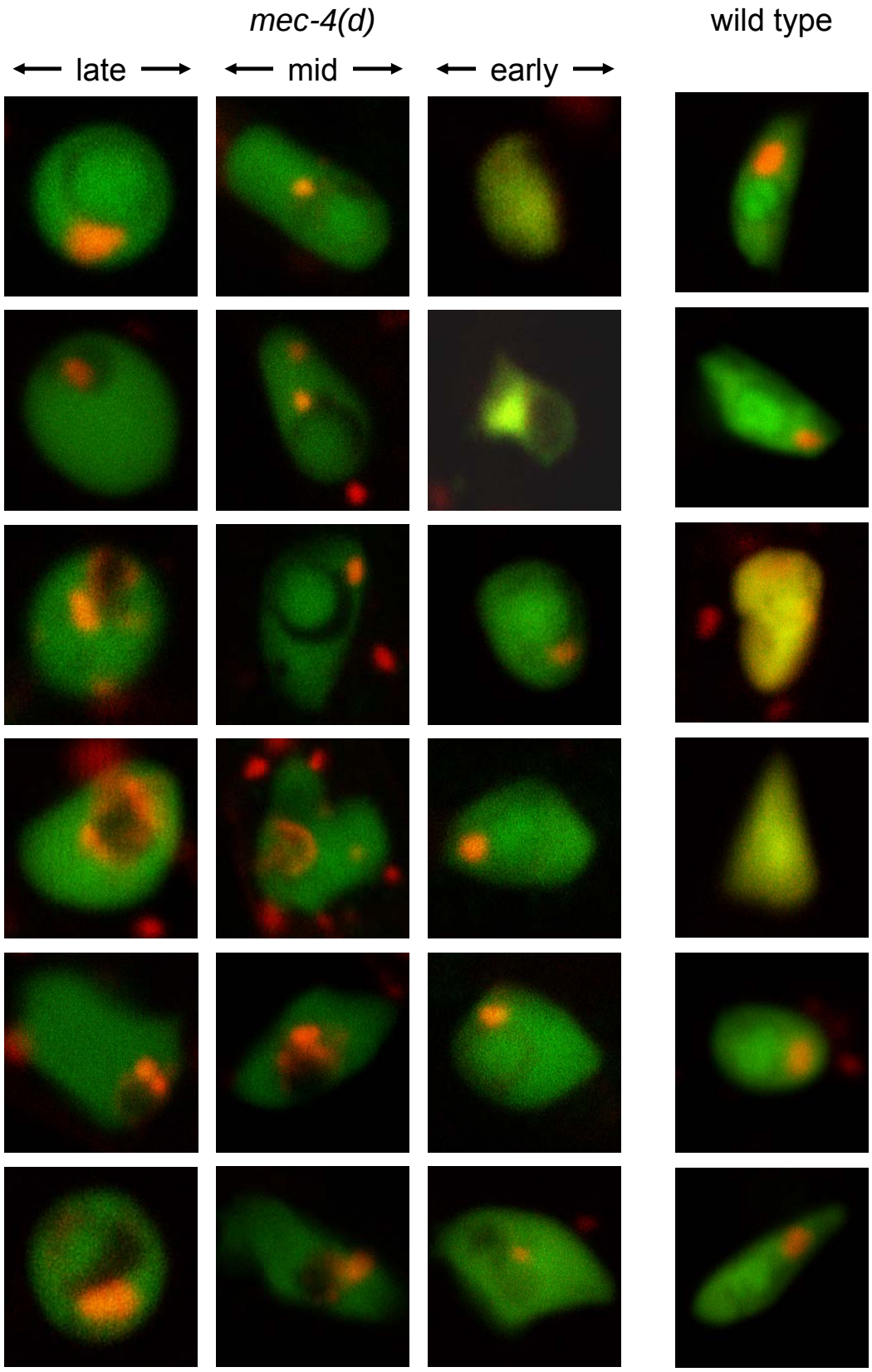
Supplementary Figure 6. Requirement for additional autophagy genes in necrosis. Knock down of *atgr-5*, *atgr-7*, *atgr-9* and *lgg-3* encoding the homologues of ATG5, ATG7, ATG9 and ATG12 respectively ameliorates neurodegeneration induced by *mec-4(d)*. Error bars denote S.E.M. values (n>250 for all populations examined; P<0.001, compared to *mec-4(d)* control animals, unpaired t-test).

Supplementary Figure 7. Surviving neurons in *unc-51(e369);mec-4(u231)* double mutant animals differentiate and fasciculate normally and they express GFP and DsRed reporter fusions driven by different promoters (GFP driven by the *mec-4* promoter is shown).

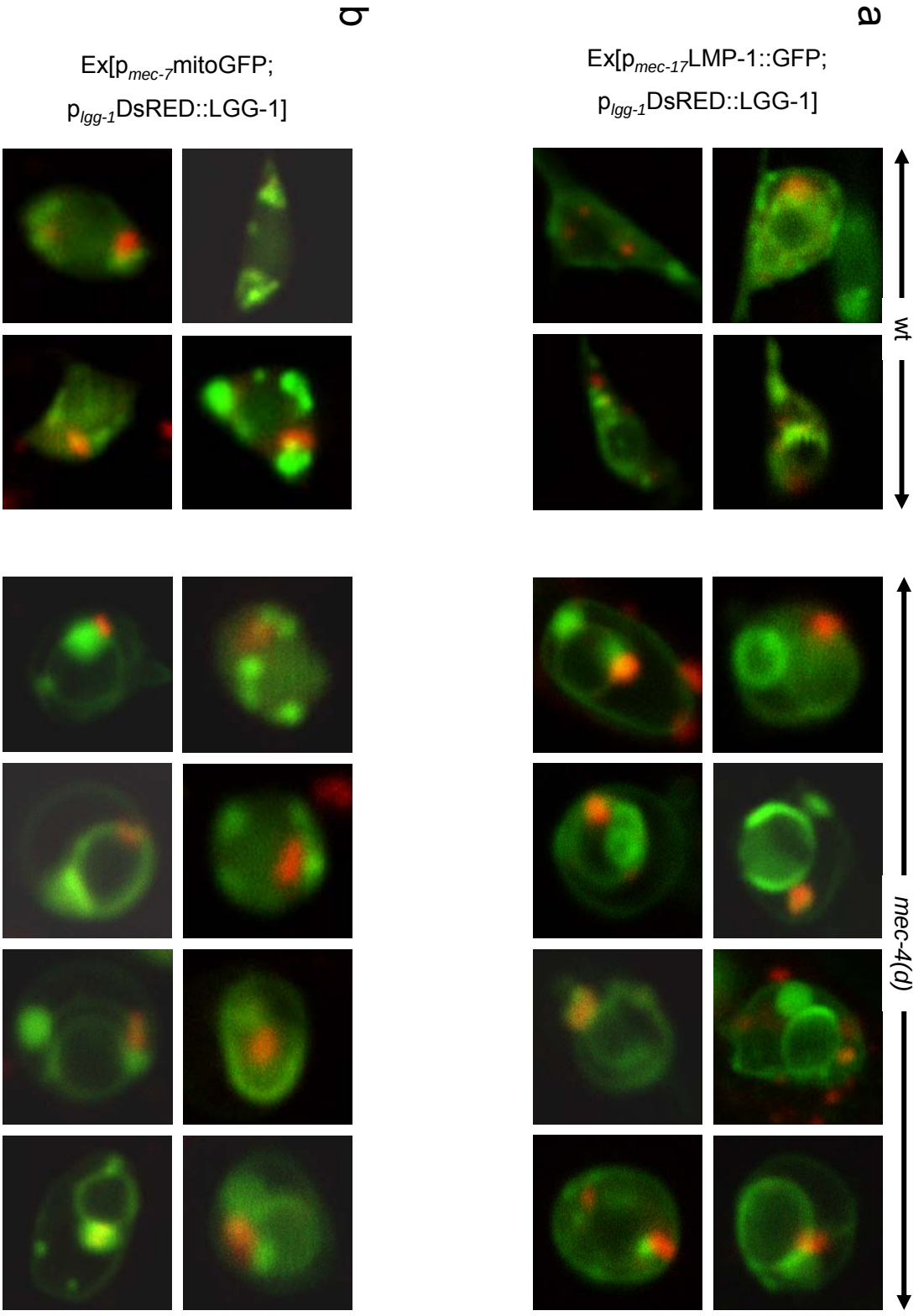
Supplementary Figure 8. Suppression of *mec-4(d)*-induced necrosis by impaired autophagy in animals depleted for UNC-51 or LGG-1 is enhanced by knock down of the aspartyl protease gene *asp-4*.Error bars denote S.E.M. values (n>250 for all populations examined; P<0.001, compared to *mec-4(d)* control animals, unpaired t-test).

Supplementary Figure 9. Behavioral assays for gentle body touch response. This behavior is mediated solely by the six touch receptor neurons expressing the *mec-4* degenerin ion channel gene. These neurons degenerate in *mec-4(d)* mutant animals. The percentage of animals responding to mechanical stimulation delivered to the body by an eyelash hair is plotted. Error bars denote S.E.M. values (n>50 for all genetic backgrounds examined).

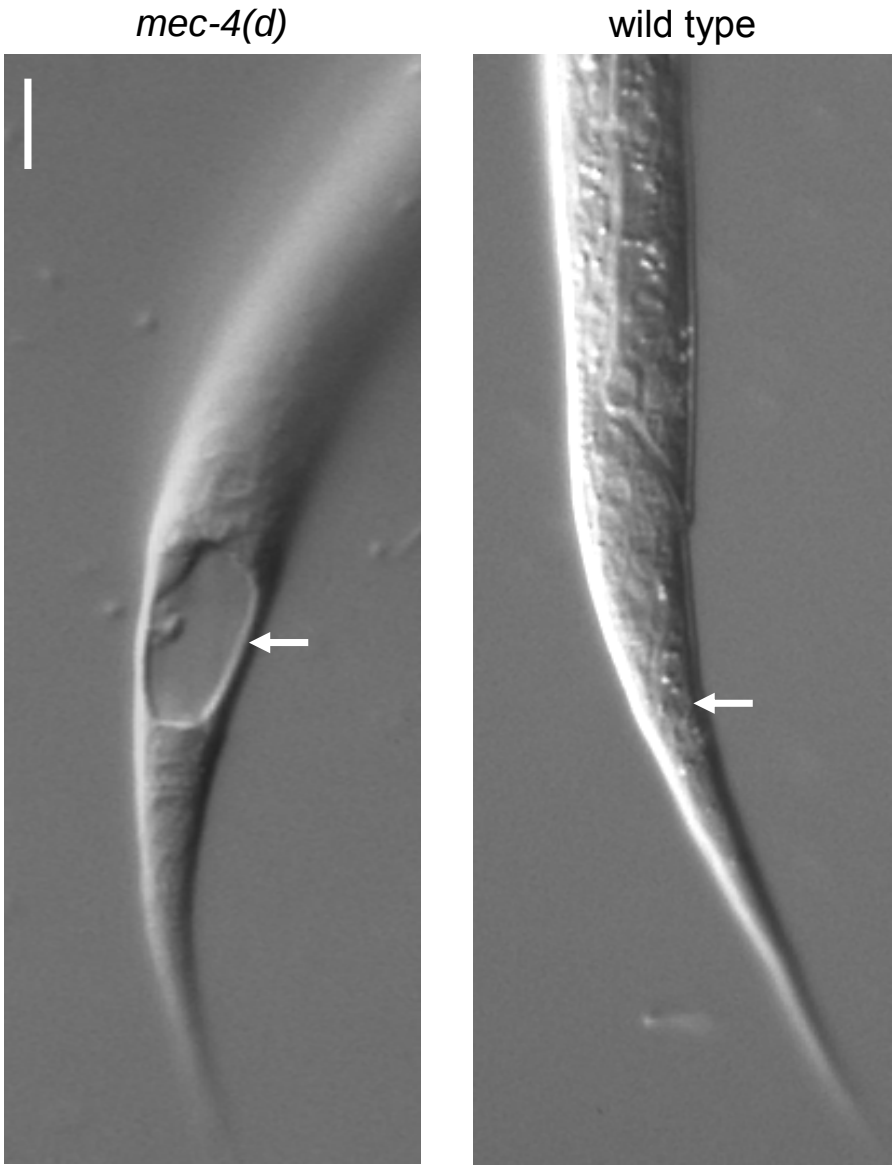
Supplementary Figure 10. A working model of necrotic cell death in *C. elegans*. Autophagy is induced upon induction of necrosis directly and/or via calpain activation and synergises with lysosomal cathepsin proteases to mediate cell death.



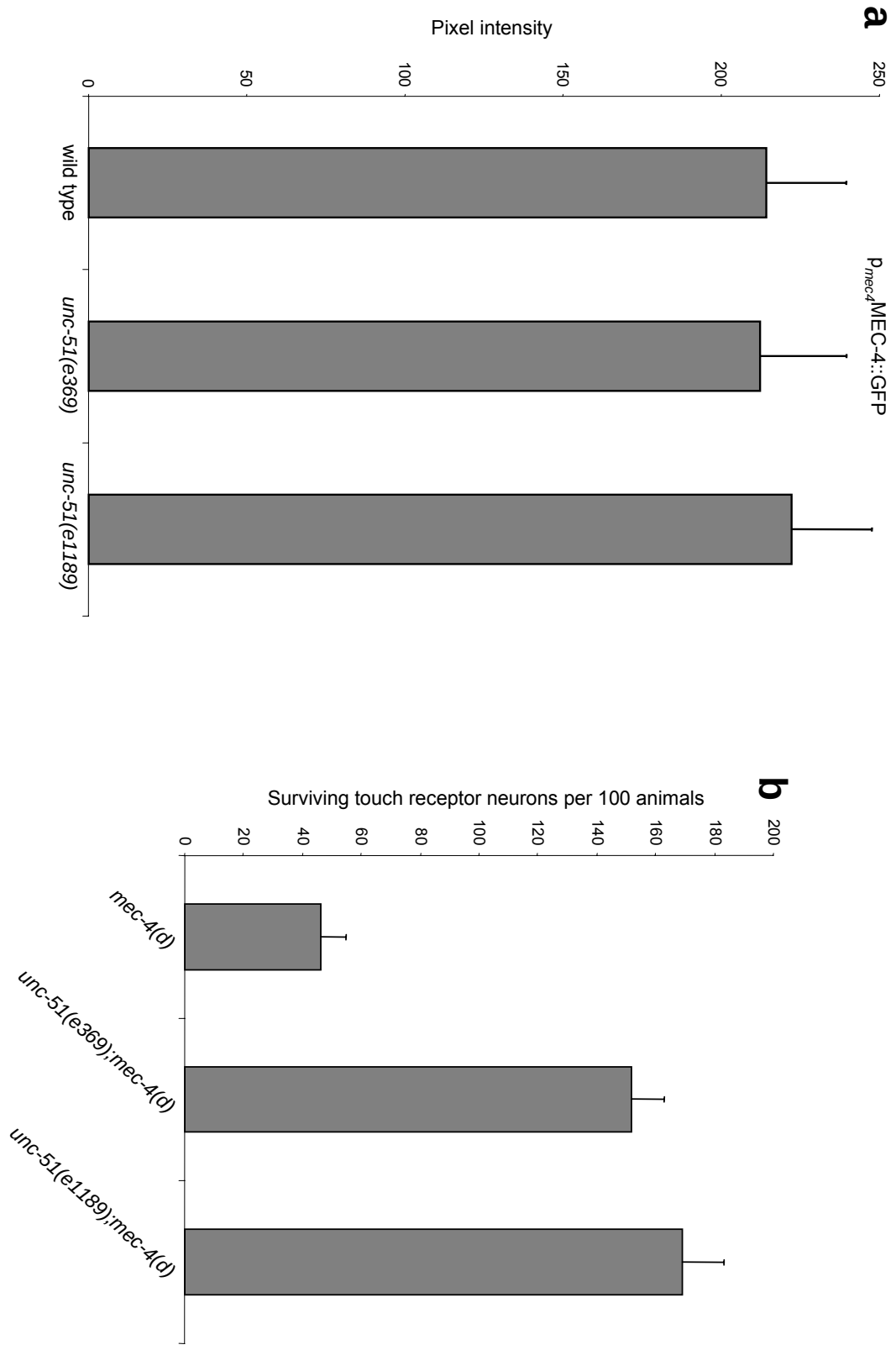
Supplementary Figure 1
Samara, Syntichaki & Tavernarakis



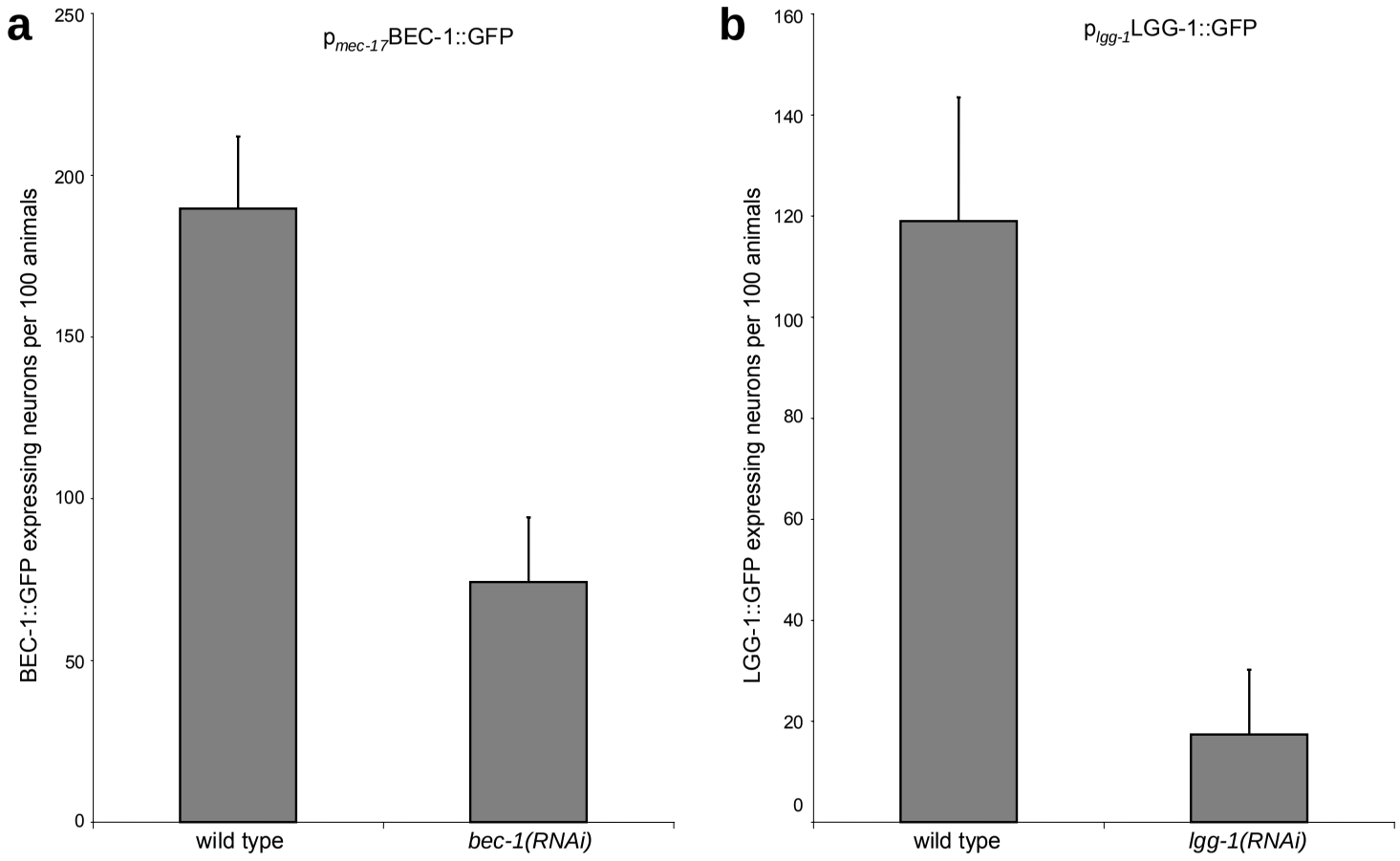
Supplementary Figure 2
Samara, Syntichaki & Tavernarakis



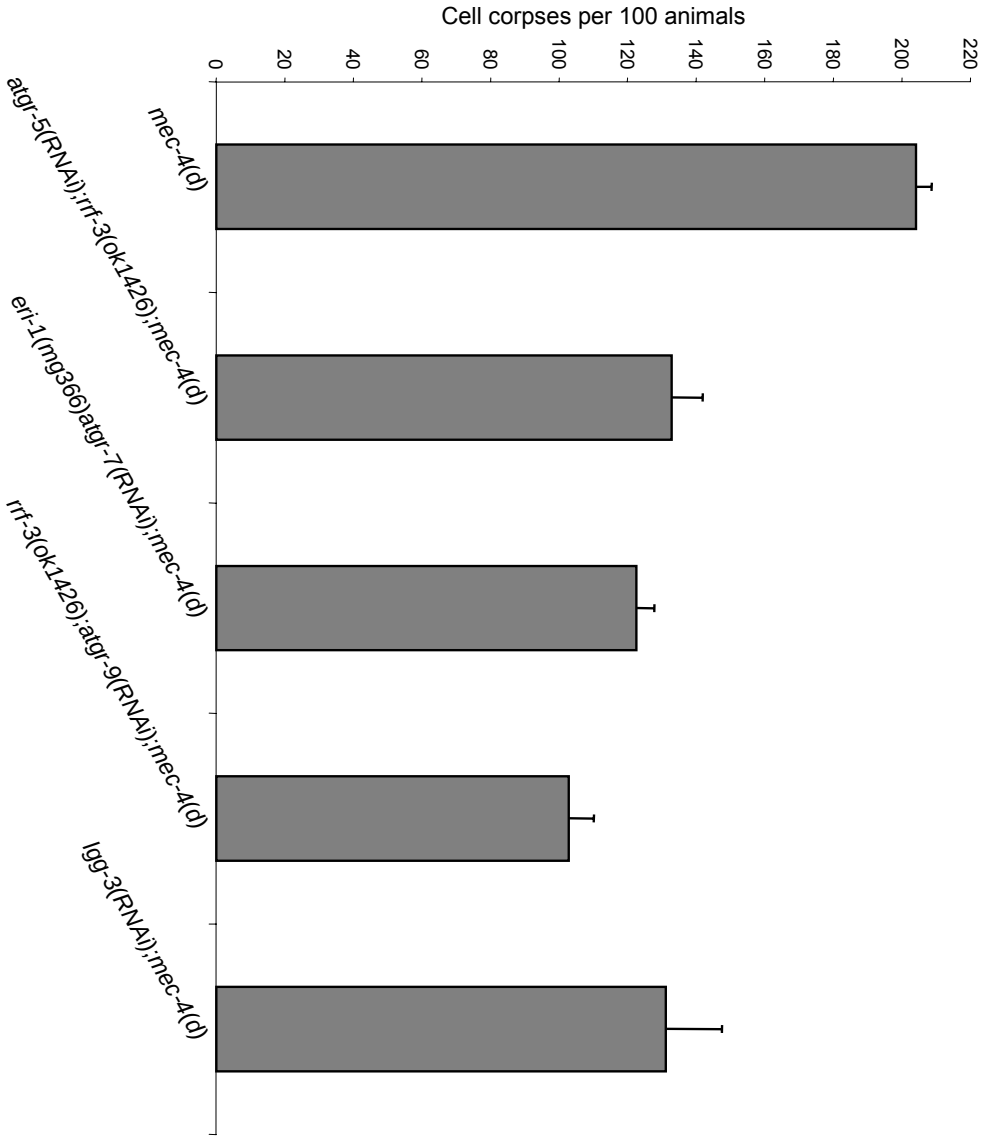
Supplementary Figure 3
Samara, Syntichaki & Tavernarakis



Supplementary Figure 4
Samara, Syntichaki & Tavernarakis

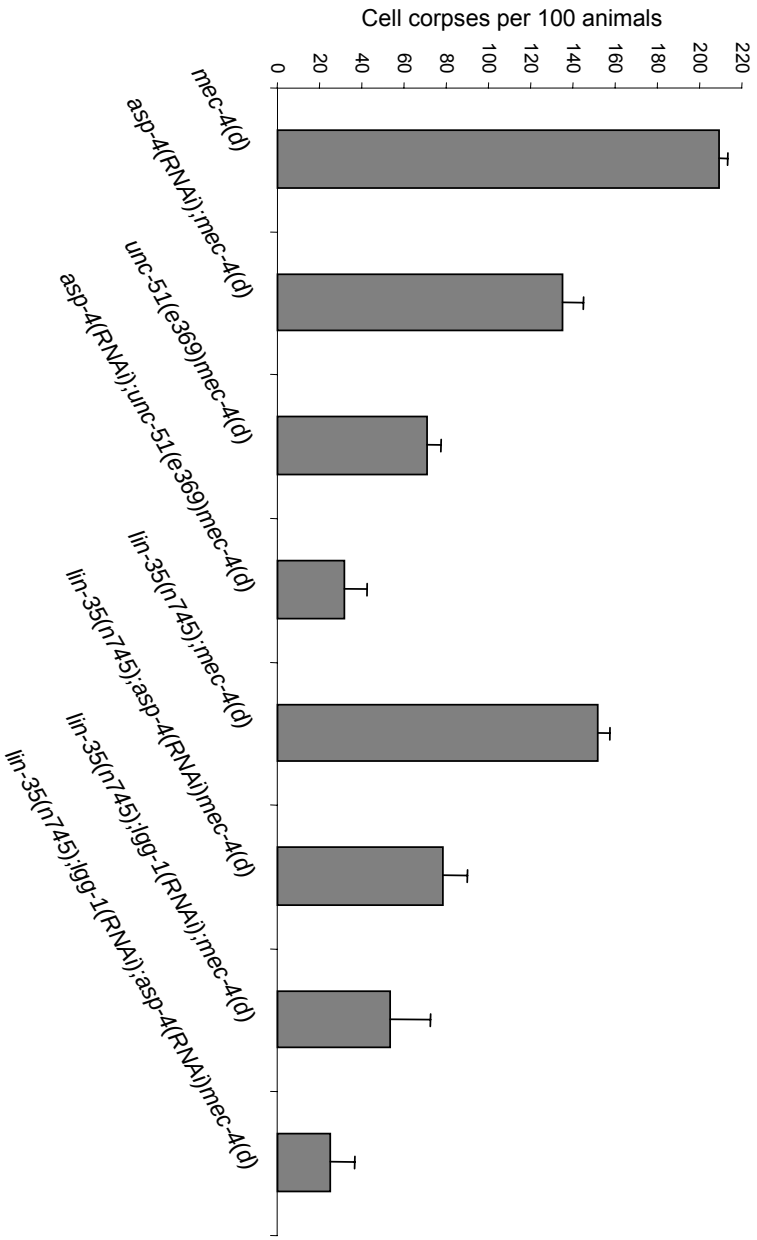


Supplementary Figure 5
Samara, Syntichaki & Tavernarakis

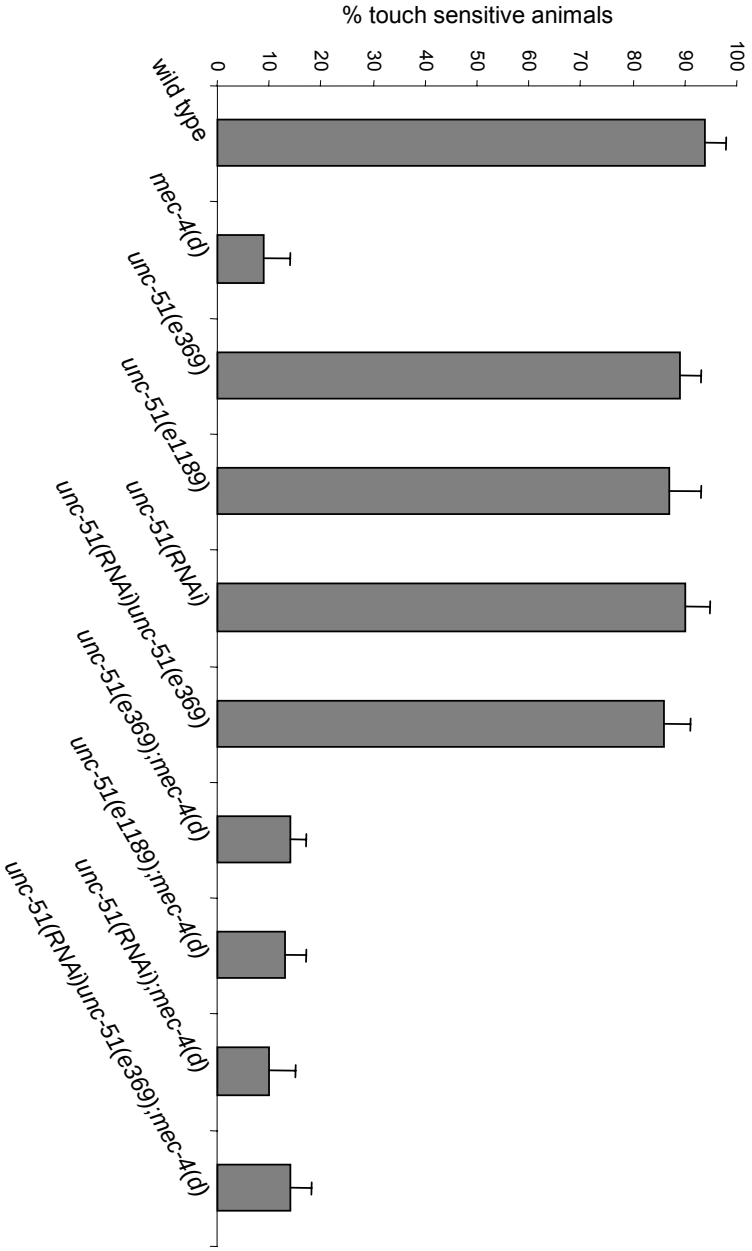


Supplementary Figure 6
Samara, Syntichaki & Tavernarakis

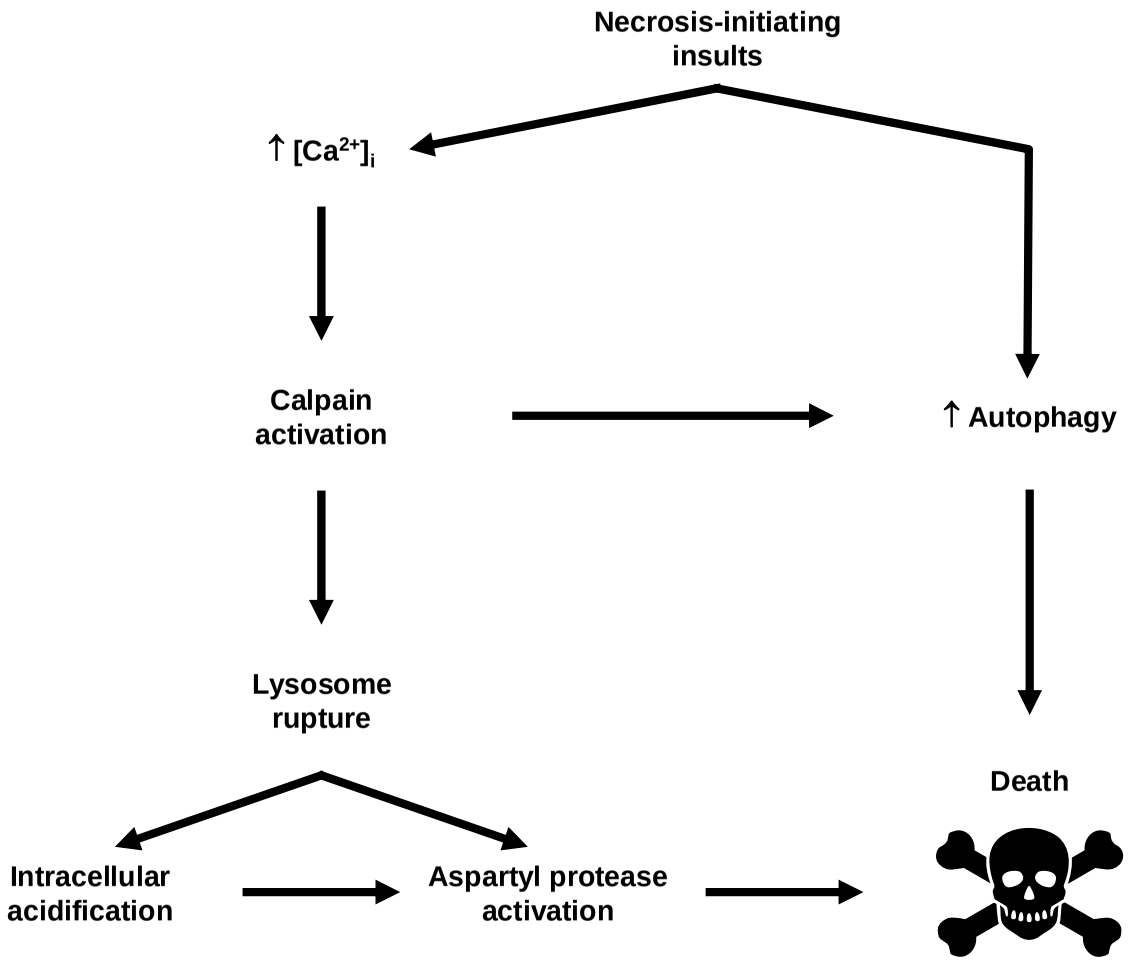
Supplementary Figure 7
Samara, Syntichaki & Tavernarakis



Supplementary Figure 8
Samara, Syntichaki & Tavernarakis



Supplementary Figure 9
Samara, Syntichaki & Tavernarakis



Supplementary Figure 10
Samara, Syntichaki & Tavernarakis