Supplementary Information

A synaptic DEG/ENaC ion channel mediates learning in *C. elegans* by facilitating dopamine signalling

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Supplementary Materials and Methods

Molecular Biology

To generate the p_{asic-1}GFP and p_{asic-1}ASIC-1N::GFP reporter constructs, DNA fragments derived from the *asic-1* locus were PCR-amplified using appropriate oligonucleotide primers and fused to GFP. For the p_{asic-1}GFP reporter, oligonucleotide primers 5'CCCAAGCTTCAATCAGTCACCCAGGTGTAC3' and 5'CGCGGTACCTGCTCCGCAAAGTCAACGAC3' were used to amplify a 2 Kb DNA fragment encompassing the promoter of *asic-1*, which was digested with *Hind*III and *Kpn*I and inserted into the pPD95.77 plasmid vector (Fire et al, 1990). For p_{asic-1}ASIC-1N::GFP, a 2.2 Kb DNA fragment including the *asic-1* promoter plus the intracellular amino-terminus of ASIC-1 was PCR-amplified using primers 5'CCCAAGCTTCAATCAGTCACCCAGGTGTAC3' and 5'CGCGGATCCCATATACCTCCTCCATCCAGTTG3', digested with *Hind*III and *Bam*HI and inserted into the pPD95.77. To generate the full-length p_{asic-1}ASIC-1::GFP reporter, the complete coding region of the *asic-1* gene were PCR-amplified using the

oligonucleotide primers 5'TCCCCCGGGGGAAAGAACAGCTTAAAACG3' and 5'GAACCGGTTTATCAAGATTAAACCCGTC3', which include *Sma*I and *Age*I restriction sites respectively. The resulting 4.2 Kb DNA fragment was subcloned into the pCRII-Topo plasmid vector (Invitrogen, Carlsbad, USA). The fragment was then fused to GFP by transferring to the pPD95.77 plasmid vector (Fire et al, 1990). The *asic-1* promoter was amplified separately using primers 5'ACGCGTCGACTTAGGGTCGGTATTTTGAGT3' and 5'TCCCCCGGGCATTTGGTGGCCTGAAATTT3', which include *Sal*I and *Sma*I restriction sites respectively. The resulting 2 Kb fragment was first, inserted into the pCRII-Topo vector and subsequently transferred to the pPD95.77-ASIC-1::GFP plasmid to generate pPD95.77-p_{asic-1}ASIC-1::GFP. To label synaptic vesicles in *asic-1*-expressing neurons, a p_{asic-1}DsRED::SNB-1, reporter fusion was constructed in multiple steps, as follows. First, plasmid pDsRED2-C1 (Clontech Laboratories, Inc, Mountain View, USA) was digested with *Age*I and *Eco*RI to excise a 0.7 Kb DNA fragment, containing the DsRED coding sequence, which was subsequently inserted between the *Age*I and *Eco*RI sites on pPD95.77 to generate pPD95.77dsRED. Second, the SNB-1 coding region was PCR-amplified directly form genomic DNA with primers 5'CGG GGTACCGAATTCGGACGCTCAAGGAGATGCCGGC3' and 5'CGGGGTACCGAATTCTTTTCCTCCAGCCCATAAAACG3', digested with *Eco*RI and inserted at the *Eco*RI site of pPD95.77DsRED to generate pPD95.77DsRED::SNB-1. Third, the *asic-1* promoter region was PCR-amplified directly from genomic DNA with primers 5'CGCGGATCCTTCGACACTAATTTACAATTAGGT3'and 5'CGCGGTACCATTTGGTGGCCTGAAATTTGATC3', digested with *Bam*HI and *Kpn*I

and inserted between the *Bam*HI and *Kpn*I sites on pPD95.77DsRED::SNB-1 to generate p_{asic-1}DsRED::SNB-1. To monitor neurotransmitter exocytocis, super-ecliptic pHluorin was fused at the carboxy-terminus of *C. elegans* synaptobrevin and driven by the *asic-1* promoter (p_{asic-1}SNB-1::SEpHluorin). The p_{asic-1}SNB-1::SEpHluorin reporter fusion was constructed as follows: First, the super-ecliptic pHluorin coding region was PCR-amplified form plasmid pGEX-2T-SEpHluorin (Miesenbock et al, 1998) with primers 5'CGGGGTACCGGATCCACCGGTGGAAGT3'and 5'CCGGAATTCACCGGTTTTGTATAGTTC3', digested with *Kpn*I and *Eco*RI and inserted between the *Kpn*I and *Eco*RI sites on pPD95.77 to generate pPD95.77SEpHluorin. Second, the *asic-1* promoter region was PCR-amplified directly from genomic DNA with primers 5'CGCGGATCCTTCGACACTAATTTACAATTAGGT3'and 5'CGCGGTACCATTTGGTGGCCTGAAATTTGATC3', digested with *Bam*HI and *Kpn*I and inserted between the *Bam*HI and *Kpn*I sites on pPD95.77SEpHluorin to generate p_{asic-1}SEpHluorin. Finally, the SNB-1 coding region was PCR-amplified directly form genomic DNA with primers 5'CGG GGTACCGAATTCGGACGCTCAAGGAGATGCCGGC3' and 5'CGGGGTACCGAATTCTTTTCCTCCAGCCCATAAAACG3', digested with *Kpn*I and inserted at the *Kpn*I site of p_{asic-1}SEpHluorin to generate p_{asic-1}SNB-1::SEpHluorin. Reporter constructs were injected into the gonads of wild type animals together with pRF4, a plasmid that carries the *rol-6 (su1006),* dominant transformation marker. At least three independent, transgenic lines were obtained for each plasmid construct and roller hermaphrodites were examined for reporter fusion expression. For transgene complementation of *asic-1(ok415)*, the wild-type *asic-1* locus was PCR- amplified with primers 5'AGTCTGCCTACTACTTCTGACT3' and

5’TTCGCATCACCGATTCTACCACA3’ from cosmid ZK770 (Coulson et al, 1995). The resulting 5.8 Kb DNA fragment was injected into the gonads of *asic-1(ok415)* together with the p_{myo-2}GFP transformation marker. Five independent transgenic lines were isolated and examined in behavioural experiments. For genetic ablation of *asic-1*-expressing cells, wild type animals were injected with p_{asic-1}*mec-4(u231)*, a plasmid carrying the toxic, gain-of-function *mec-4(u231)* allele (Driscoll & Chalfie, 1991) under the control of the *asic-1* promoter, together with the p_{myo-2}GFP transformation marker. To generate the p_{asic-1}*mec-4(u231)* genetic ablation construct, the promoter and part of the first exon of the *asic-1* gene were PCR-amplified from genomic DNA with primers 5'ACGCGTCGACTTCGACACTAATTTACAATTAGGTC3' and 5'CGGGGTACCGACTCGTGTCGCCGCAAAGTCAACGAC3'. The resulting 2.4 Kb DNA fragment was digested with *Sal*I and *Kpn*I, and inserted into p_{mec-7}*mec-4(u231)* (Harbinder et al, 1997). For RNAi experiments, we constructed a plasmid that directs the synthesis of dsRNA corresponding to *asic-1*, in *E. coli* bacteria, which were then fed to animals, according to a previously described methodology (Kamath et al, 2001). A 1.5 Kb fragment of genomic DNA, encompassing 0.6 Kb of coding sequence, was amplified from an exon-rich region of the *asic-1* gene, using primers 5’TCAAGTATCCACGTGTAGTAA3’ and 5’ATCTGGAAATGGTTGCTCGTC3’. The fragment was initially inserted into the pCRII-Topo vector (Invitrogen, Carlsbad, USA), excised with *Eco*RI, and subcloned to the pL4440 plasmid vector (Timmons et al, 2001). The resulting construct was transformed into HT115 (DE3) *E. coli* bacteria, deficient for RNase-E (Kamath et al, 2001). To augment RNAi, animals were reared for two generations on dsRNA-producing *E. coli* bacteria before examination. The following primers, which

amplify specifically *asic-1* mRNA, were used for RT-PCR:

5'GTTTCGGATGAGGTTACGAAAC3', 5'AATTCTATCTTTCACACCCGTA3' and

5'AGGTATTCCACGAAATCATGTT3'.

Supplementary References

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Driscoll M, Chalfie M (1991) The *mec-4* gene is a member of a family of *Caenorhabditis elegans* genes that can mutate to induce neuronal degeneration. *Nature* **349:** 588-593

Fire A, Harrison SW, Dixon D (1990) A modular set of lacZ fusion vectors for studying gene expression in *Caenorhabditis elegans*. *Gene* **93:** 189-198

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Kamath RS, Martinez-Campos M, Zipperlen P, Fraser AG, Ahringer J (2001) Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biol* **2:** RESEARCH0002

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Supplementary Figure Legends

Supplementary Figure 1. Phylogenetic Relations Among DEG/ENaC Proteins

The genetically characterized *C. elegans* proteins are shown in red (ASIC-1, DEG-1, DEL-1, FLR-1, MEC-4, MEC-10, UNC-8, and UNC-105). Other nematode proteins are shown with blue lines. Representative DEG/ENaC proteins from a variety of organisms, ranging from snails to humans, are also included (mammalian: red lines; fly: green lines; snail: orange line, zebrafish: purple line). Branch lengths represent relative evolutionary distance (calculated number of amino acid substitutions per site; 0.1 for the scale bar).

Supplementary Figure 2. Phylogenetic Relationships Among *C. elegans*, Zebrafish, Mouse and Human ASIC Family Members

ASIC-1, ASIC-2, C24G7.4, F23B2.3 and T28B8.5 are *C. elegans* proteins. Branch lengths represent relative evolutionary distance (calculated number of amino acid substitutions per site, 0.1 for the scale bar; z: zebrafish; m: mouse; h: human).

Supplementary Figure 3. M-Coffee-Generated Multiple Sequence Alignment of Nematode ASIC-1, Mouse and Human ASIC Isoforms

The ASIC-1 amino acid residues deleted in the *asic-1(ok415)* mutant are indicated by the red box. (z: zebrafish; m: mouse; h: human)

Supplementary Figure 4. The *asic-1* Locus

(A) *asic-1* intron-exon structure. mRNA-specific primers used for reverse transcription PCR (RT-PCR) are shown (a, b, c). The region deleted in the *asic-1(ok415)* mutant is indicated. By removing exons 6-8 and parts of the fifth and ninth asic-1 exon, the *ok415* deletion eliminates the first cysteine-rich domain (CRDI) of the extracellular ASIC-1 region.

(B) Tarnsmembrane domain and topology predictions for ASIC-1. The probability of cytoplasmic and non-cytoplasmic localization is plotted against amino acid sequence. The amino terminus (amino acids 1-37) and the carboxy terminus (amino acids 811-844) are predicted to be intracellular, while the central domain (amino acids 60-782) is predicted to be extracellular. Amino acids 38-59 and 783-810 form the hydrophobic transmembrane segments. The plot was generated using the Phobius algorithm (http://phobius.cgb.ki.se/).

(C) RT-PCR amplification of *asic-1* mRNA from wild type and *asic-1(ok415)* mutant animals using two different primer sets. The *ama-1* gene (RNA polymerase II, large subunit) is used as control.

Supplementary Figure 5. Sinusoidal Locomotion Characteristics.

Wild type and *asic-1(ok415)* mutant animals inscribe sinusoidal tracks with similar wavelength and amplitude on bacterial laws (see also Table 1).

Supplementary Figure 6. Genetic Ablation of *asic-1*-Expressing Neurons.

The cytotoxic *mec-4(d)* allele is expressed in dopaminergic neurons under the control of the *asic-1* promoter. Images show vacuolated dying neurons in L1 larvae.

Supplementary Figure 7. Differential Requirement for Dopamine in Basal Slowing and Conditioning Behaviours

(A) Basal slowing behaviour of *cat-2* mutants, defective in dopamine biosynthesis, after treatment with increasing concentrations of exogenously supplied dopamine (DA). Error bars denote S.E.M. values (n=15 in 3 experiments).

(B) Exogenous addition of dopamine restores the capacity for associative learning in *cat-2* mutants at concentrations lower than those required for normal basal slowing behaviour. Bars depict chemotaxis indices towards isoamyl alcohol, calculated for either naïve (white bars) or conditioned animals (black bars), after treatment with increasing concentrations of exogenously supplied dopamine (DA). Error bars denote S.E.M. values (n=200 in 4 experiments).

Supplementary Figure 8. ASIC-1 is not Required for Adaptation.

asic-1(ok415) mutant animals show normal chemosensory adaptation behaviour. Bars depict chemotaxis indices towards isoamyl alcohol calculated for animals that were either naïve or conditioned in the absence of food for the indicated time intervals. White bars denote wild type animals, black bars denote *asic-1(ok415)* mutants. Error bars denote S.E.M. values (n=150 in 3 experiments; ***: P<0.001, unpaired t-test).

Supplementary Figure 9. Subcellular Localization of ASIC-1 in Animals With UNC-104 Deficiency.

Images of *unc-104(e1265)* mutant transgenic animals carrying a full-length p_{asic-1}ASIC-1::GFP reporter fusion. An image of a wt animal carrying the same reporter is included as control (top). The punctate distribution of GFP in dopaminergic neuron processes is not affected in the mutants. White bars denote 10 microns.

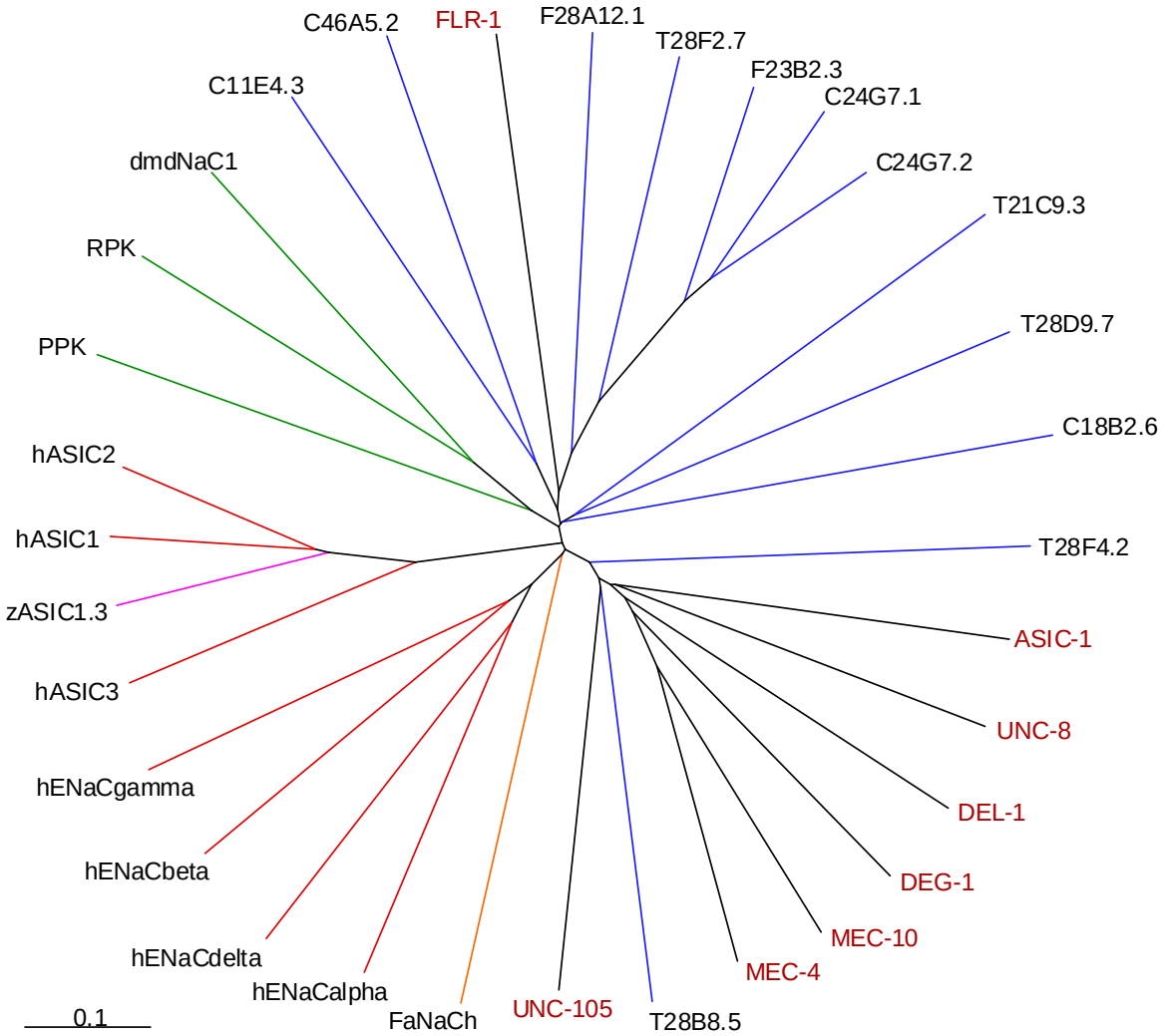
Supplementary Table

Supplementary Table 1. Genetic ablation of *asic-1*-expressing neurons. Mosaic analysis of N2*Ex*[p$_{asic-1}$*mec-4(u231)*] *Ex*[p$_{asic-1}$GFP] double transgenic animals.

Ablated neurons*	**Chemotaxis index after conditioning**†
All 12 *asic-1*-expressing neurons	56.8±5.1
All 8 dopaminergic neurons	51.8±6.3
4 non-dopaminergic tail neurons	-4.3±4.1
2 ADE dopaminergic neurons	19.4±5.2
4 CEP dopaminergic neurons	39.8±5.6
2 PDE dopaminergic neurons	21.4±7.0

* Mosaic N2*Ex*[p$_{asic-1}$*mec-4(u231)*] *Ex*[p$_{asic-1}$GFP], double transgenic animals lacking the indicated neurons were grouped and assayed.

† Chemotaxis index (±S.E.M.) towards isoamyl alcohol after conditioning to isoamyl alcohol in the absence of food (n=50 for each mosaic subtype, 3 independent experiments).



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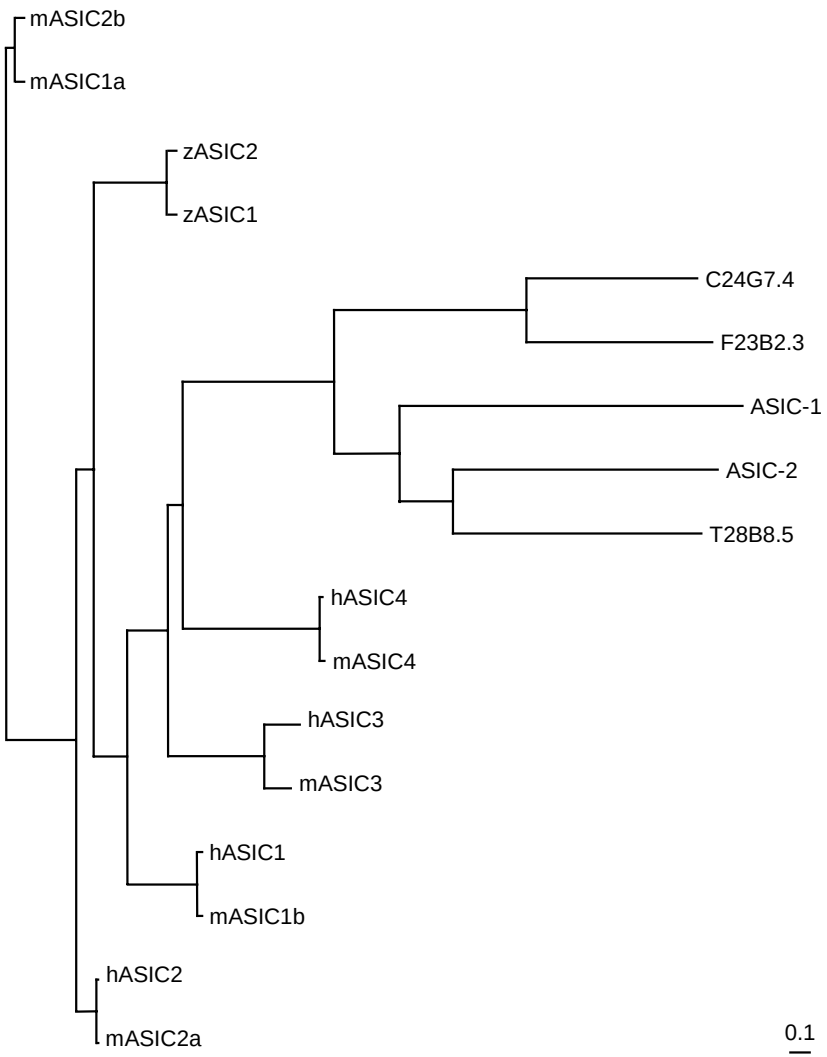
Supplemental Figure 1



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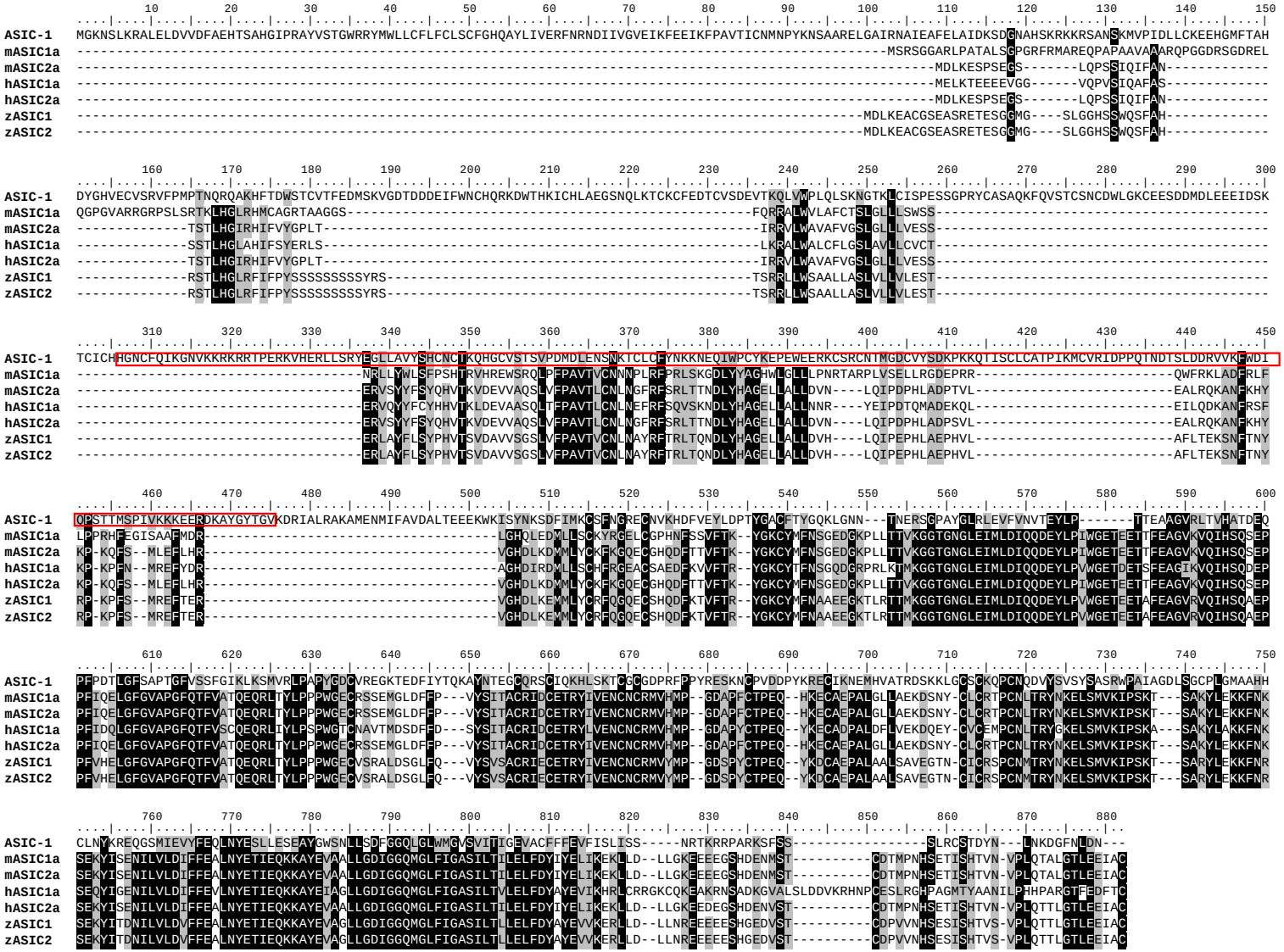

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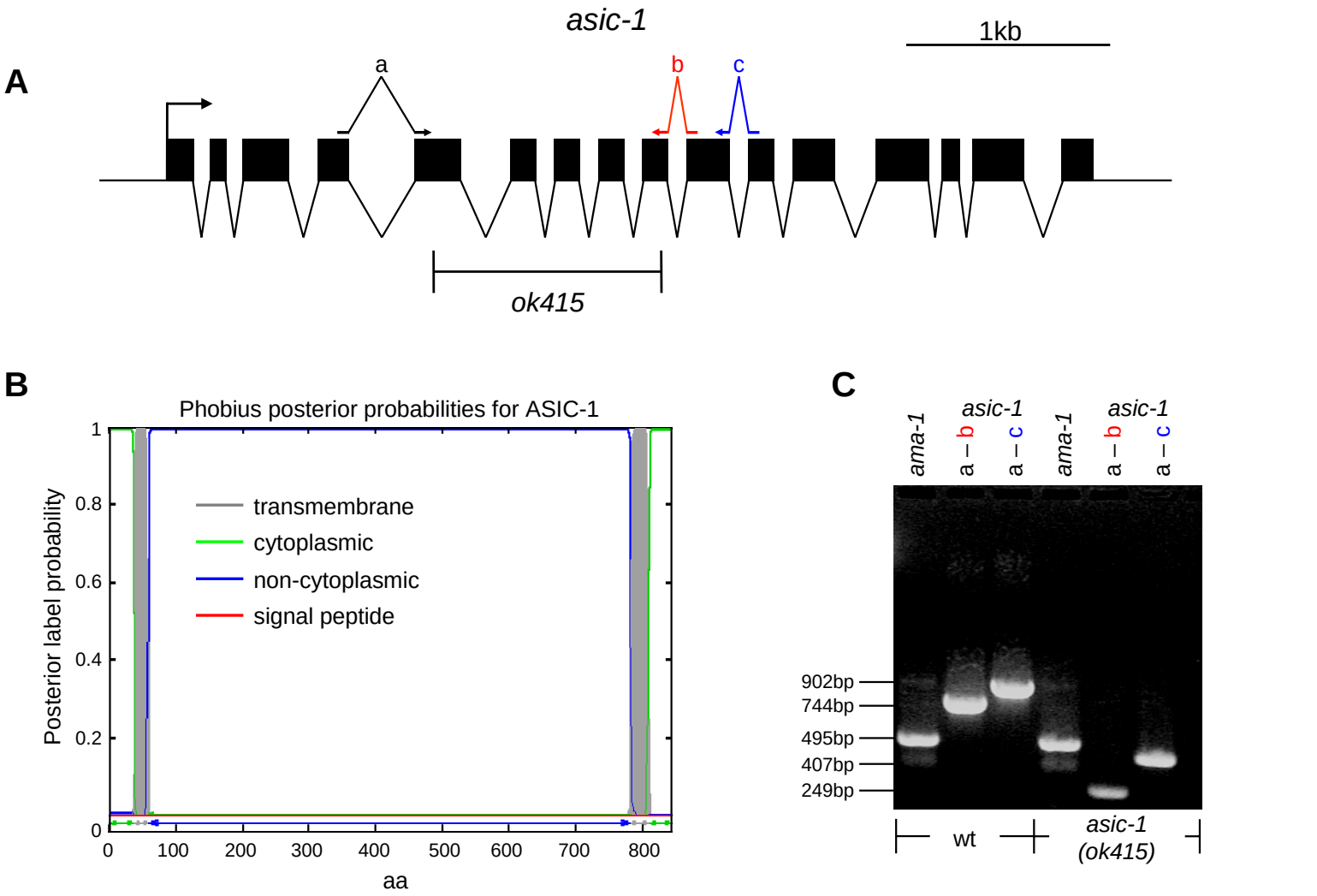

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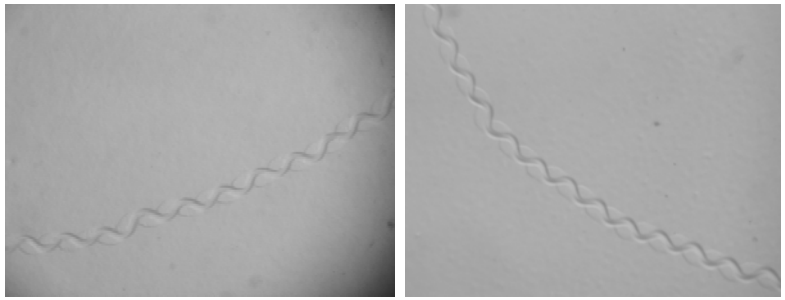
Supplemental Figure 4



wt *asic-1(ok415)*

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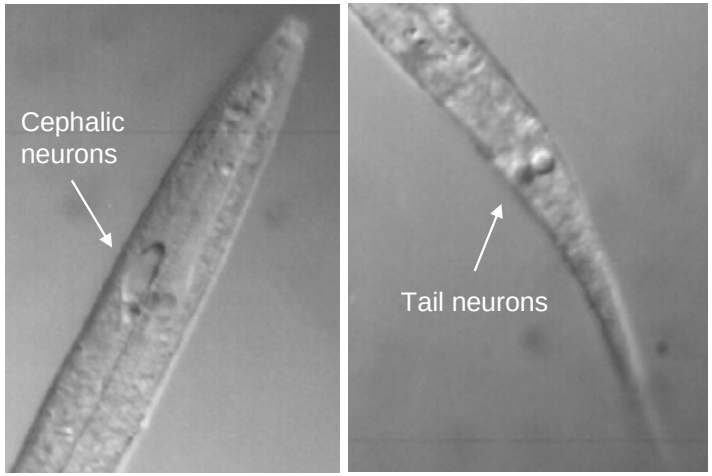
Supplemental Figure 5

$p_{asic\text{-}1}$*mec-4(d)*



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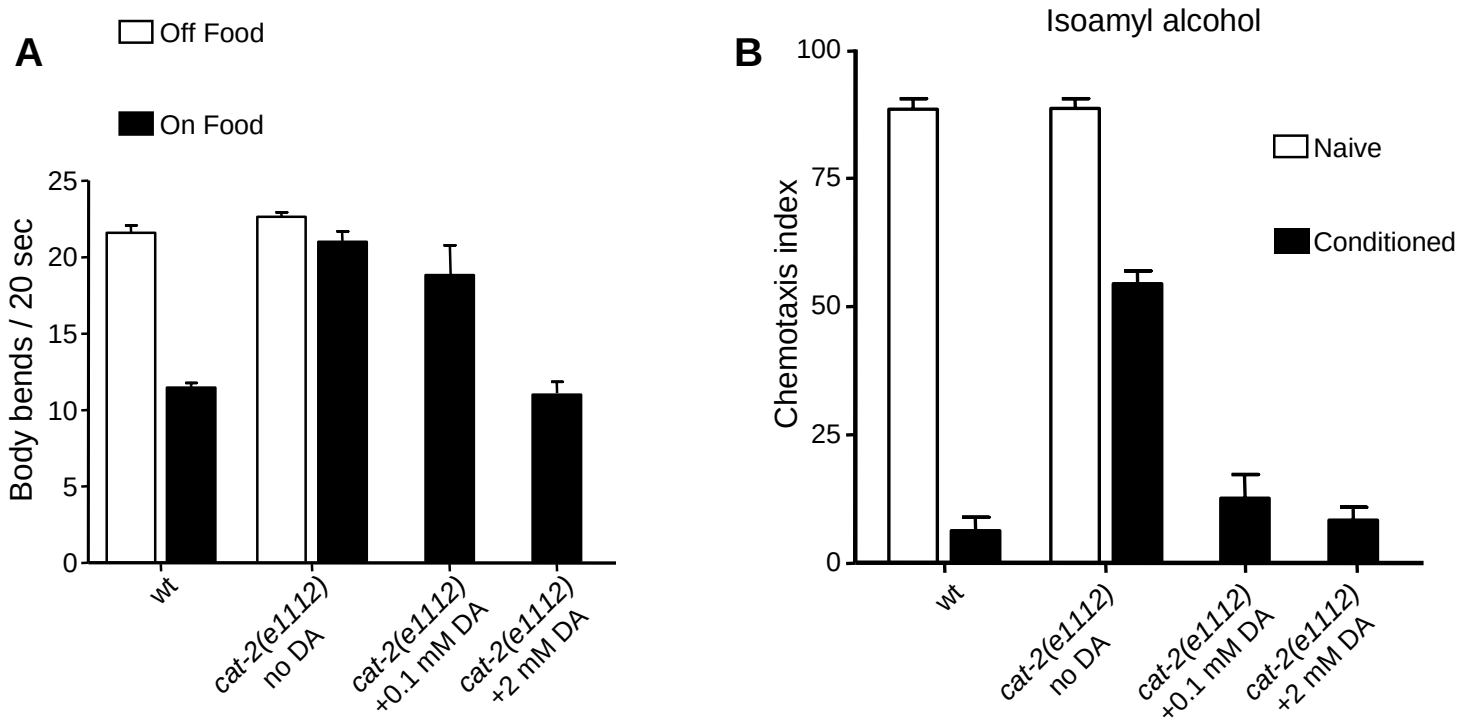
Supplemental Figure 6



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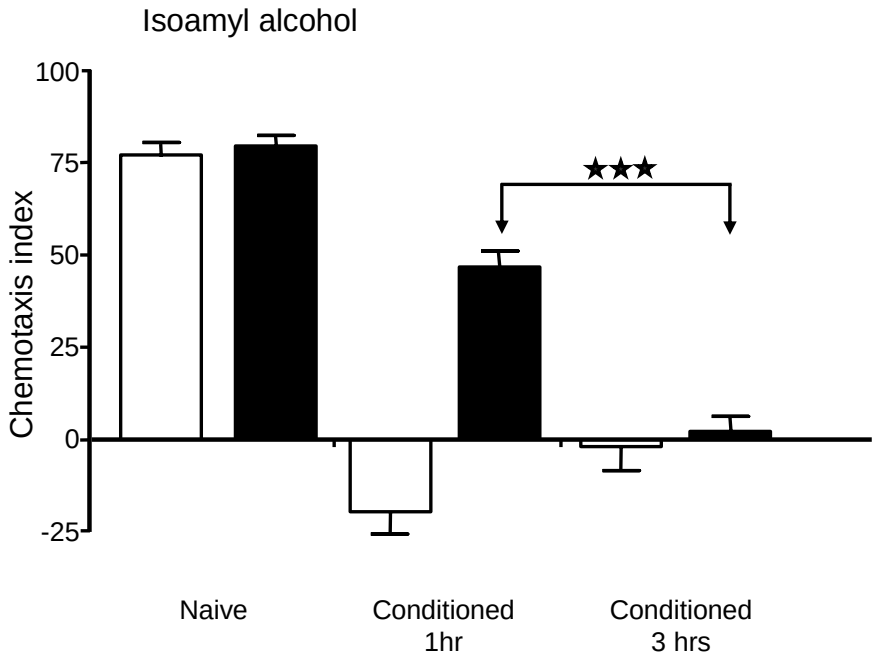
Supplemental Figure 7



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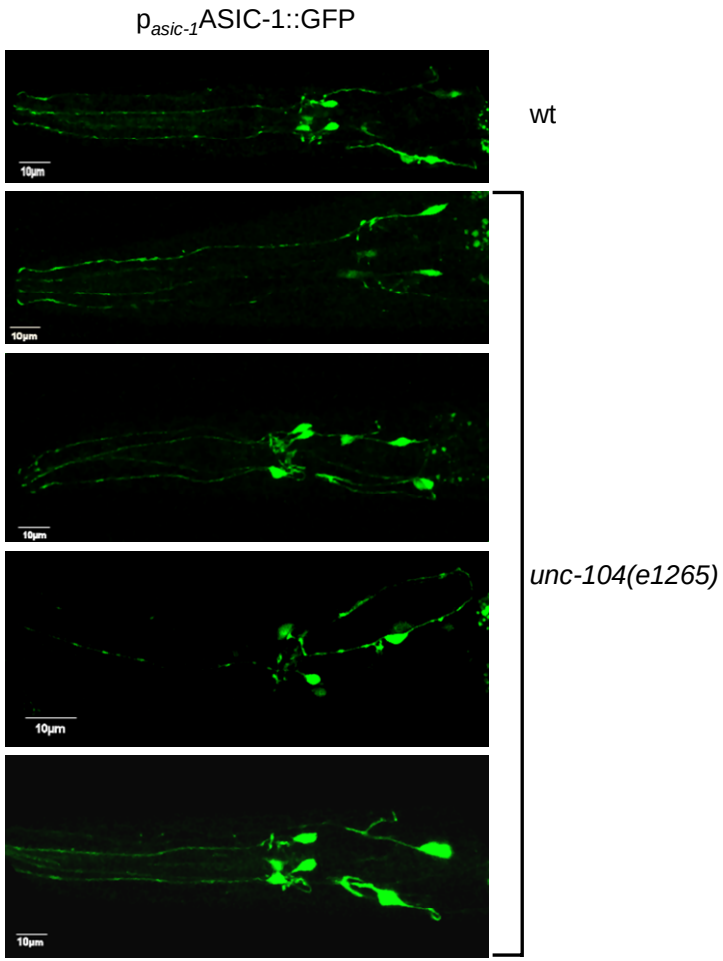
Supplemental Figure 8



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Supplemental Figure 9