Figures and figure supplements

LIN-12/Notch signaling instructs postsynaptic muscle arm development by regulating UNC-40/DCC and MADD-2 in *Caenorhabditis elegans*
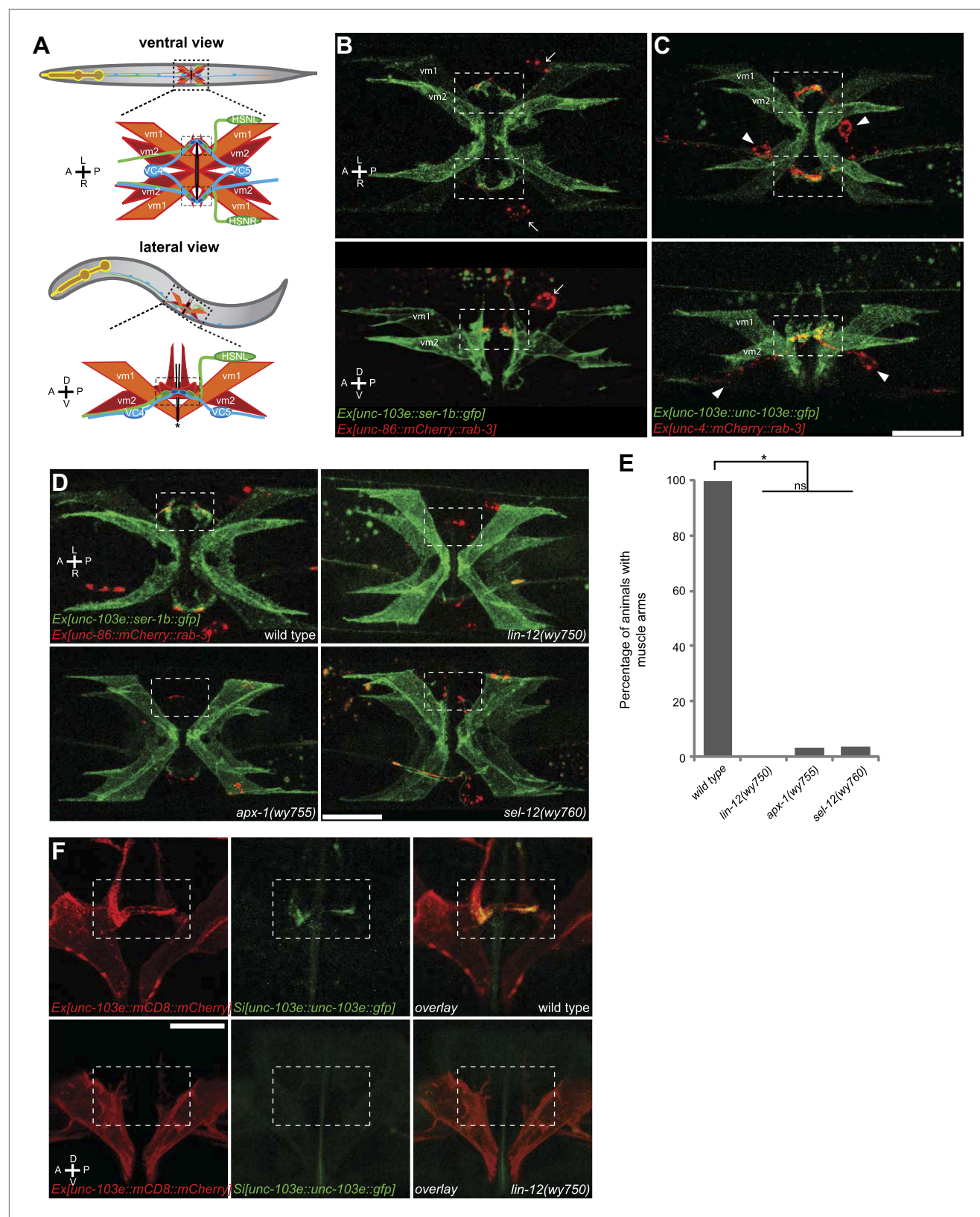
Pengpeng Li, et al.

Figure 1. The muscle arms of type 2 vulval muscles (vm2) are missing in *lin-12(wy750)*, *apx-1(wy755)* and *sel-12(wy760)* mutants. (**A**) An illustration showing ventral (top) and lateral (bottom) views of *C. elegans* egg-laying circuit. vm1 (orange) and vm2 (red) are vulval muscles. HSN (green) and VC4/5

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(blue) are presynaptic motoneurons. Each of the four vm2 cells extends a dendritic spine-like muscle arm laterally, on which it receives synapses with HSN and VC neurons (boxed areas). Top row of (**A**–**C**) are ventral views; bottom row of (**A**–**C**) are left lateral views. Anterior (A), posterior (P), left (L), right (R), dorsal (D), ventral (V). (**B**) Ventral (top) and lateral (bottom) views of vulval muscles labeled by SER-1B::GFP transgene in a young adult. HSN neurons are labeled by a mCherry::RAB-3 transgene. The boxed areas indicate synaptic regions. Arrows indicate the HSN cell bodies. Note that the laterally extended muscle arms on vm2 cells are colocalized with the HSN presynaptic specializations. (**C**) Ventral (top) and lateral (bottom) views of vulval muscles labeled by a UNC-103E::GFP transgene and VC4/5 neurons labeled by a mCherry::RAB-3 transgene. The boxed areas indicate synaptic regions. Arrowheads indicate cell bodies of VC4 and VC5. Note that the laterally extended muscle arms on vm2 cells are colocalized with the VC4/5 presynaptic specializations. Scale bar is 20 µm. (**D**) Ventral views of the vulval muscles and presynaptic regions of HSN neurons in wild-type, *lin-12(wy750)*, *apx-1(wy755)* and *sel-12(wy760)* animals. The boxed areas indicate synaptic regions. Note the absence of vm2 muscle arms in mutants. Scale bar is 20 µm. (**E**) Quantification of the vm2 muscle arm defects in wild-type, *lin-12(wy750)*, *apx-1(wy755)* and *sel-12(wy760)* animals. * p<0.0001, n.s. no significant difference, chi-squared test, n = 90–114 animals. (**F**) High magnification lateral views of the synaptic region in wild type and *lin-12(wy750)*. vm2 muscle arms are visualized by a single-copy UNC-103E::GFP transgene. Vulval muscle morphology is labeled by mCD8::mCherry. The boxed areas indicate synaptic regions. Scale bar is 10 µm.
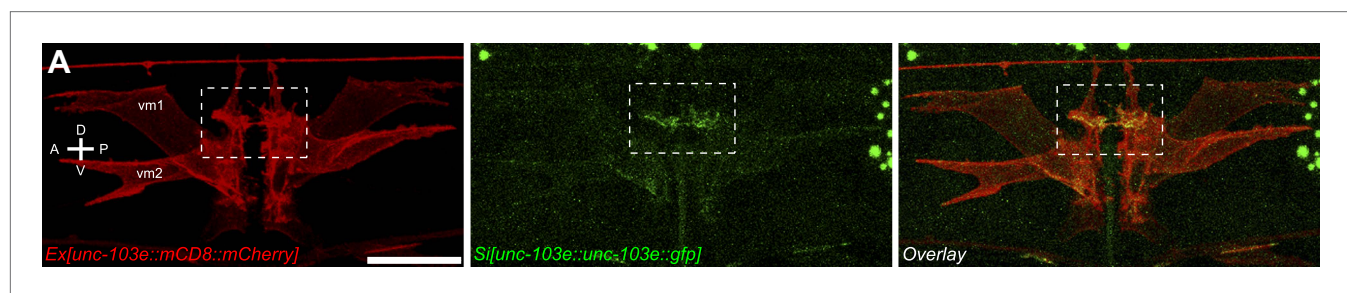
Figure 1—figure supplement 1. vm2 muscle arms are the postsynaptic specializations. (**A**) Lateral views of the vulval muscles. The vulval muscles are labeled by mCD8::mCherry (top). vm2 muscles arms are labeled by a single-copy *unc-103e::gfp* transgene (middle). Note that the UNC-103E::GFP is enriched on the muscle arms. Boxed areas indicate synaptic regions. Anterior (A), posterior (P), dorsal (D), ventral (V). Scale bar is 20 µm.
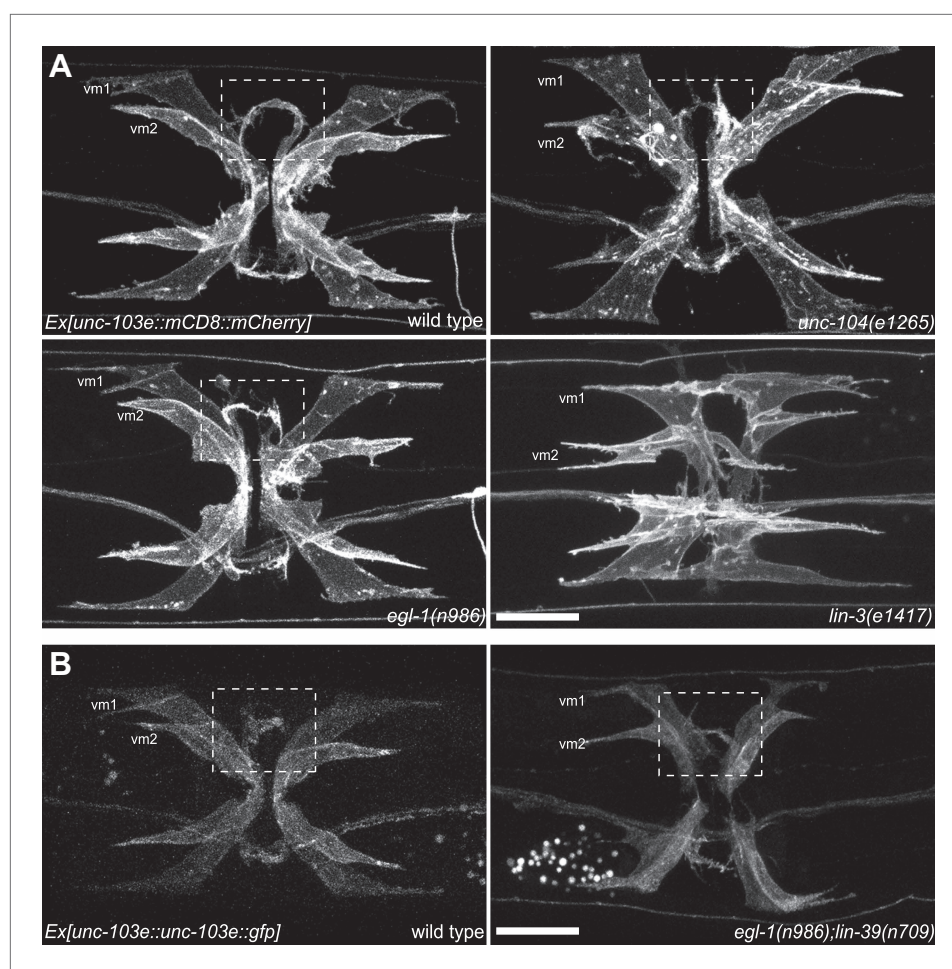
Figure 1—figure supplement 2. vm2 muscle arm development is a guidance event independent of presynaptic specialization. (**A**) Ventral views of the vulval muscle morphology in wild-type, *unc-104(e1265)*, *egl-1(n986)* and *lin-3(e1417)* mutant animals. Boxed areas indicate synaptic regions. Scale bar is 20 µm. (**B**) Ventral views of the vulval muscle morphology in wild-type (left) and *egl-1(n986); lin-39(n709)* (right) mutant animals. Boxed areas indicate synaptic regions. Scale bar is 20 µm.
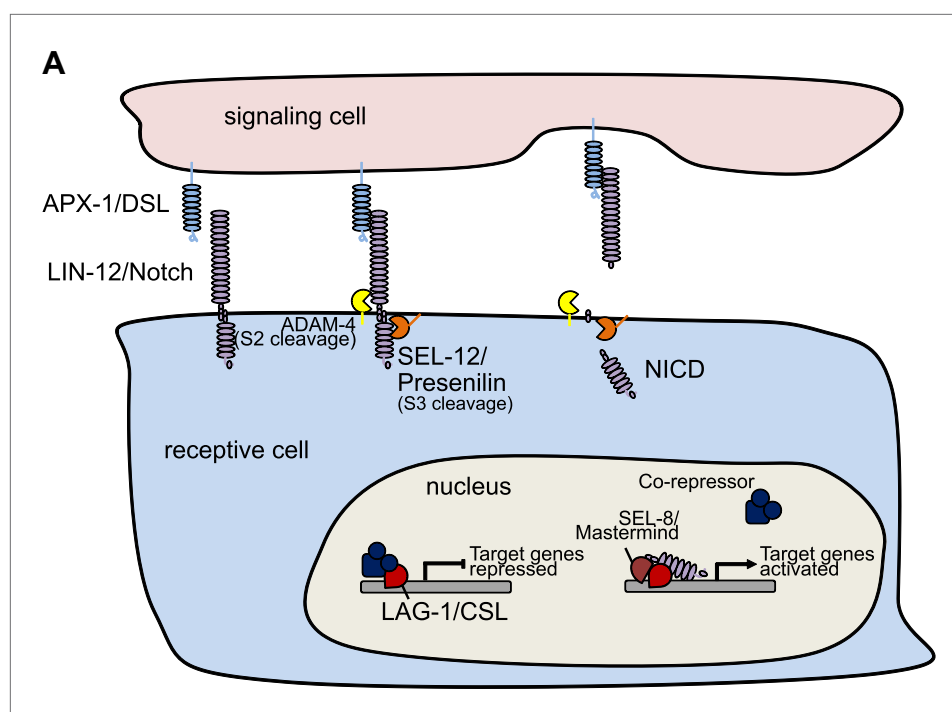DOI: 10.7554/eLife.00378.005

Figure 1—figure supplement 3. Schematic model of LIN-12/Notch signaling pathway. (**A**) Schematic model showing the core LIN-12/Notch signaling pathway. The binding of the ligand APX-1/DSL to the receptor LIN-12/Notch triggers proteolytic cleavages. SEL-12/Presenilin releases the Notch intracellular domain (NICD), which enters the cell nucleus and interacts with LAG-1/CSL and other transcription factors to activate target gene transcription.
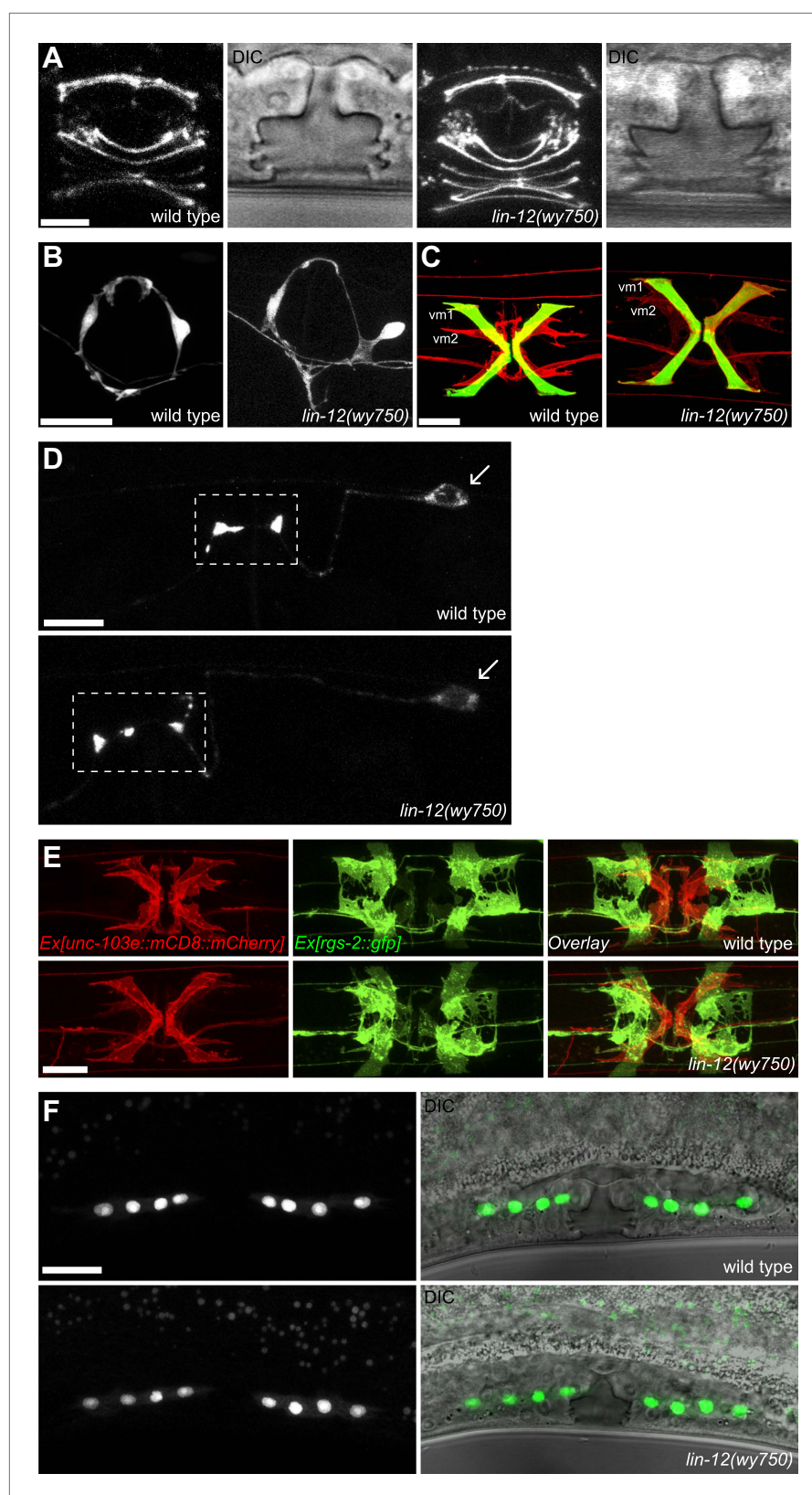
DOI: 10.7554/eLife.00378.006

Figure 1—figure supplement 4. *lin-12(wy750)* has specific vm2 muscle arm defects. (**A**) Vulval morphology in wild-type (left two panels) and *lin-12(wy750)* (right two panels) animals. Vulval morphogenesis is examined by visualizing the apical boundaries of vulval epithelial cells with AJM-1::GFP or by DIC microscopy. Scale bar is 5 μm. *Figure 1—figure supplement 4. Continued on next page*

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(**B**) VC4 and VC5 axons in wild-type (left) and *lin-12(wy750)* (right) animals. VC axons are labeled with CAT-1::GFP. Scale bar is 20 μm. (**C**) Visualization of vm1 and vm2 in wild-type (left) and *lin-12(wy750)* (right) animals. Vulval muscles (both vm1 and vm2) are labeled with mCD8::mCherry. *egl-15*::GFP labels vm1 but not vm2 in both wild type and *lin-12(wy750)* animals. Scale bar is 20 μm. (**D**) HSN presynapstic regions in wild-type (top) and *lin-12(wy750)* (bottom) animals. HSN presynapsic regions are labeled by SNB-1::YFP. Boxed areas indicate synaptic regions. Arrows indicate the HSN cell bodies. Scale bar is 10 μm. (**E**) Visualizations of undifferentiated M lineage cells in wild-type (top) and *lin-12(wy750)* (bottom) mutants by *hlh-8*::GFP in mid-L4 animals (lateral view). Note that the pattern of the M lineage cells do not change in the *lin-12(wy750)* mutant. Scale bar is 20 μm. (**F**) Ventral view of vulval muscles and uterine muscles in wild-type (up) and *lin-12(wy750)* (down) animals. Vulval muscles (both vm1 and vm2) are labeled with mCD8::mCherry. *rgs-2*::GFP labels uterine muscles in both wild type and *lin-12(wy750)* animals. Note that the uterine muscle differentiation and morphology do not change in *lin-12(wy750)* mutant. Scale bar is 10 μm.
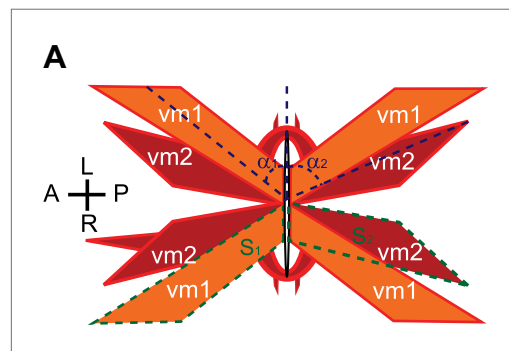
Figure 1—figure supplement 5. Schematic demonstrations of the quantitative measurements of vulval muscle morphology. (**A**) Schematic demonstration of vulval muscles. The intersection angles between vulval opening and vulval muscles are shown with dashed blue lines. vm1 and vm2 are delineated by dashed green lines.
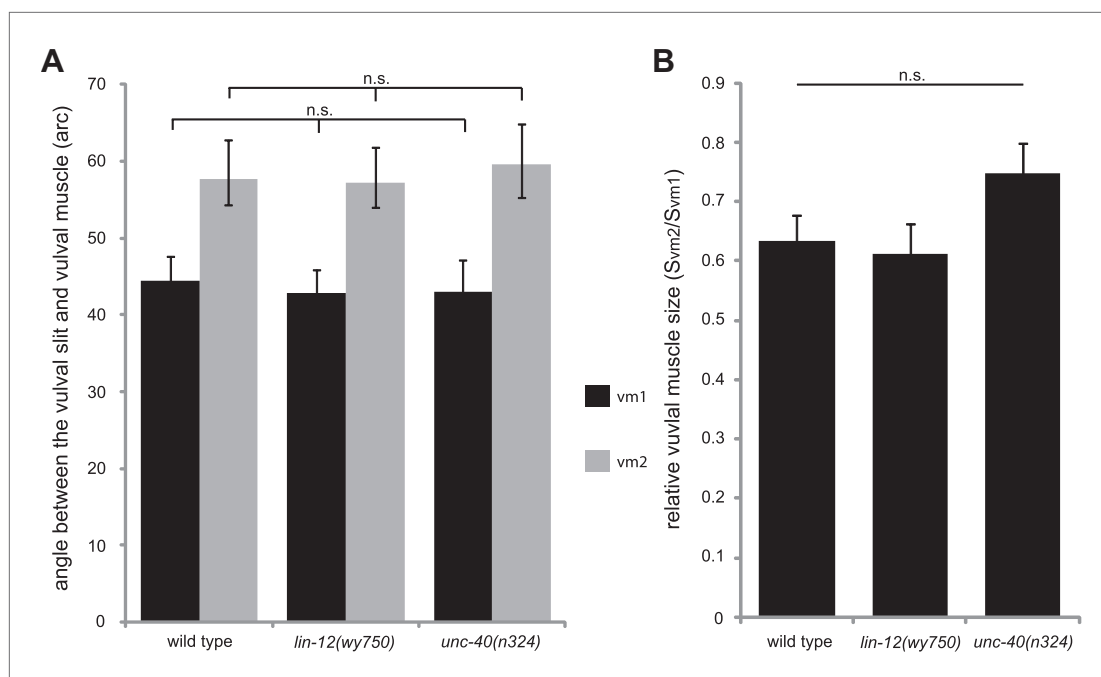
Figure 1—figure supplement 6. Quantifications of the vulval muscle morphology. (**A**) Quantification of the intersection angles between vulval opening and vulval muscles with different genotypes indicated on the X-axis. n.s., no significant difference, t test, n = 48–80 pieces of vulval muscles.
(**B**) Quantification of the relative size of vm2 to vm1 (S_{vm2}/S_{vm1}) with different genotypes indicated on the X-axis. n.s., no significant difference, t test, n = 48–80 pairs of vulval muscles.
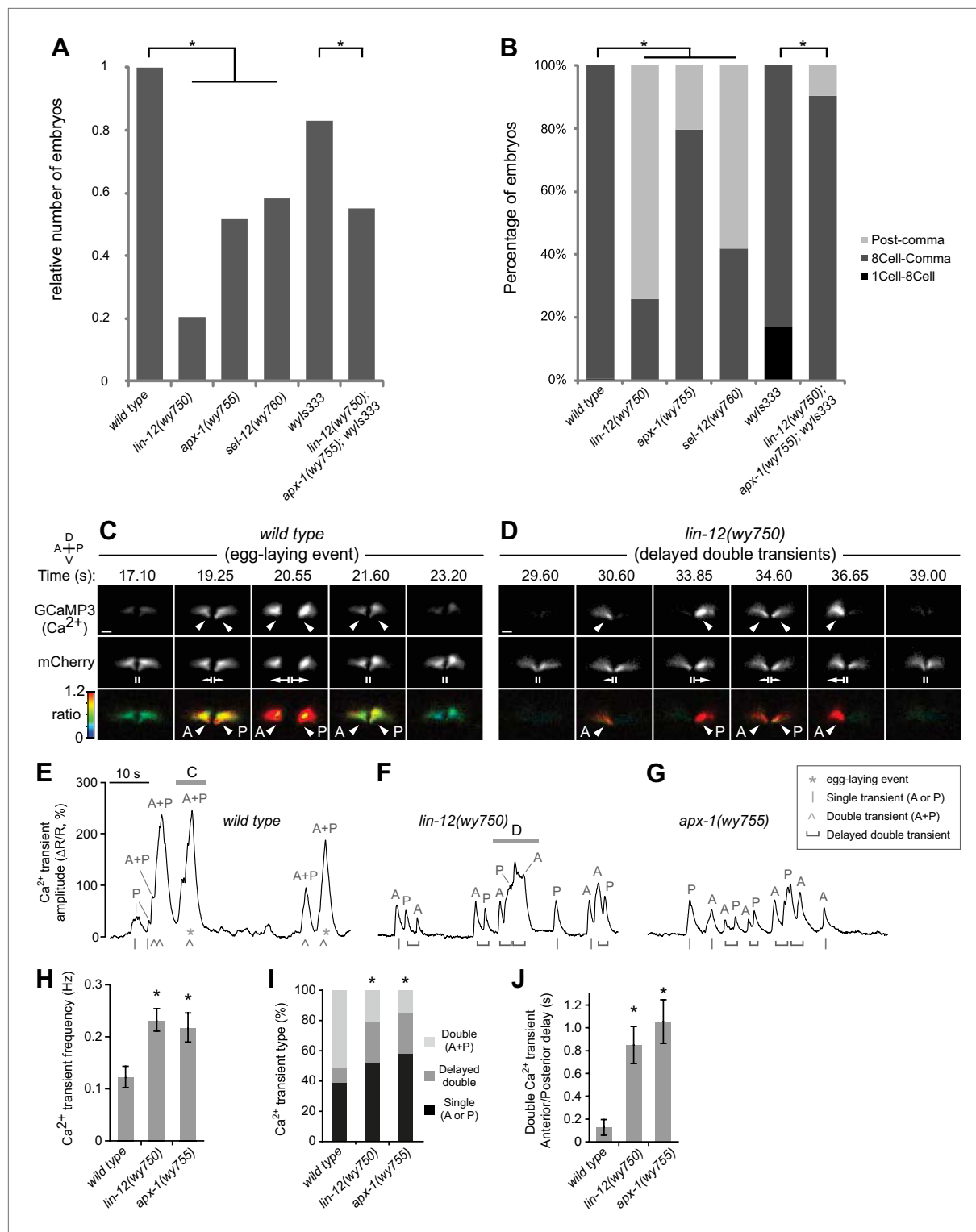DOI: 10.7554/eLife.00378.009

Figure 2. Muscle arm defective mutants have egg-laying behavioral and physiological defects. (**A**) Relative numbers of embryos freshly-laid by wild-type and mutant animals. *wyIs333* is the marker that double-labels the HSN presynaptic specializations and vulval muscles. *p<0.0001, Fisher's exact test, n = 62–303 embryos. (**B**) Percentage of different stages of embryos freshly-laid by wild-type and mutants animals. *p<0.0001, Fisher's exact test, n = 62–303 embryos. (**C**) and (**D**) Ratiometric Ca²⁺ imaging in the vulval muscles in behaving wild-type (**C**) and *lin-12(wy750)* (**D**) animals. GCaMP3 (top) and mCherry (middle) were co-expressed in the vulval muscles and the GCaMP3/mCherry fluorescence ratio (bottom) was used to record Ca²⁺ transients (arrowheads). Time points are shown from ***Video 1*** (Wild type) and ***Video 2*** (*lin-12(wy750)* mutant). Vertical lines (II) indicate the vulval muscles at rest, and arrows

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indicate vulval muscle twitches (small) and egg-laying (large) contractions. Anterior (A), posterior (P), left (L), right (R), dorsal (D), ventral (V). Scale bars are 10 μm. (**E–G**) traces of vulval muscle GCaMP3/mCherry ratio change (ΔR/R) from the same wild-type (**E**) and *lin-12(wy750)* mutant (**F**) animals shown above (horizontal bars) and in the *apx-1(wy755)* mutant (**G**). Also indicated are egg-laying events (*), single transients limited to the anterior or posterior (A or P) vulval muscles (l), double transients occurring simultaneously in both anterior and posterior (A + P) vulval muscles (^), or delayed double transient where anterior and posterior transients within a body bend are separated by a visually discernible interval (horizontal bracket). (**H–J**) Quantitations of vulval muscle Ca^{2+} signaling (6-min recording per animal, 11 or 12 animals per genotype; error bars indicate 95% confidence intervals). (**H**) *lin-12(wy750)* and *apx-1(wy755)* mutants have more frequent Ca^{2+} transients than does the wild type. *p<0.0001, one-way ANOVA, n = 215–488. (**I**) Fewer synchronous double (A + P) Ca^{2+} transients in *lin-12(wy750)* and *apx-1(wy755)* mutants. *p<0.0001, chi-squared test, n = 200–305. (**J**) Consecutive anterior and posterior Ca^{2+} transients are delayed in *lin-12(wy750)* and *apx-1(wy755)* mutants. *p<0.0001; one-way ANOVA, n = 123–190.
DOI: 10.7554/eLife.00378.011

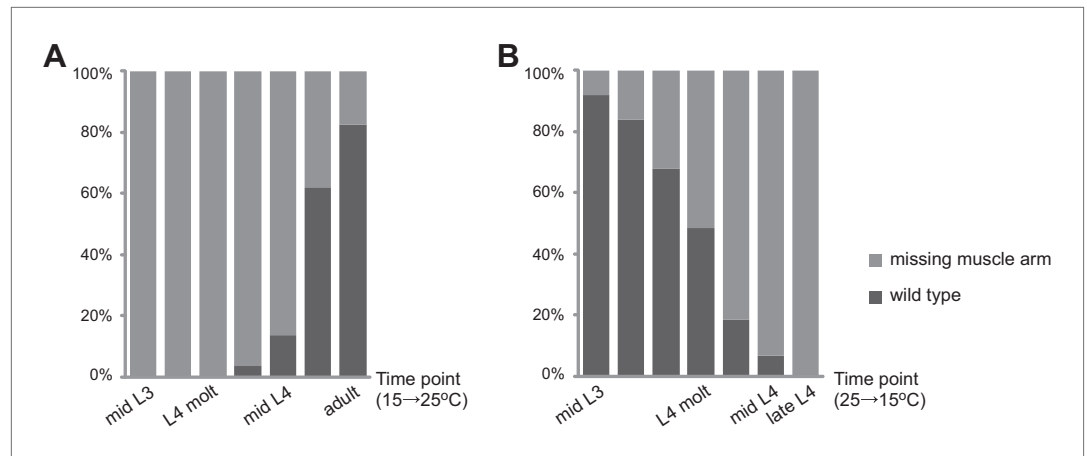**Figure 3**. Temporal requirement of LIN-12 in vm2 muscle arm formation. (**A**) and (**B**) Temperature up-shift (**A**) and down-shift (**B**) experiments of *lin-12(n676n930)*. The percentages of animals with normal or defective muscle arm phenotype are indicated by dark or light gray bars, respectively. The time points when animals are shifted are shown on X-axis.
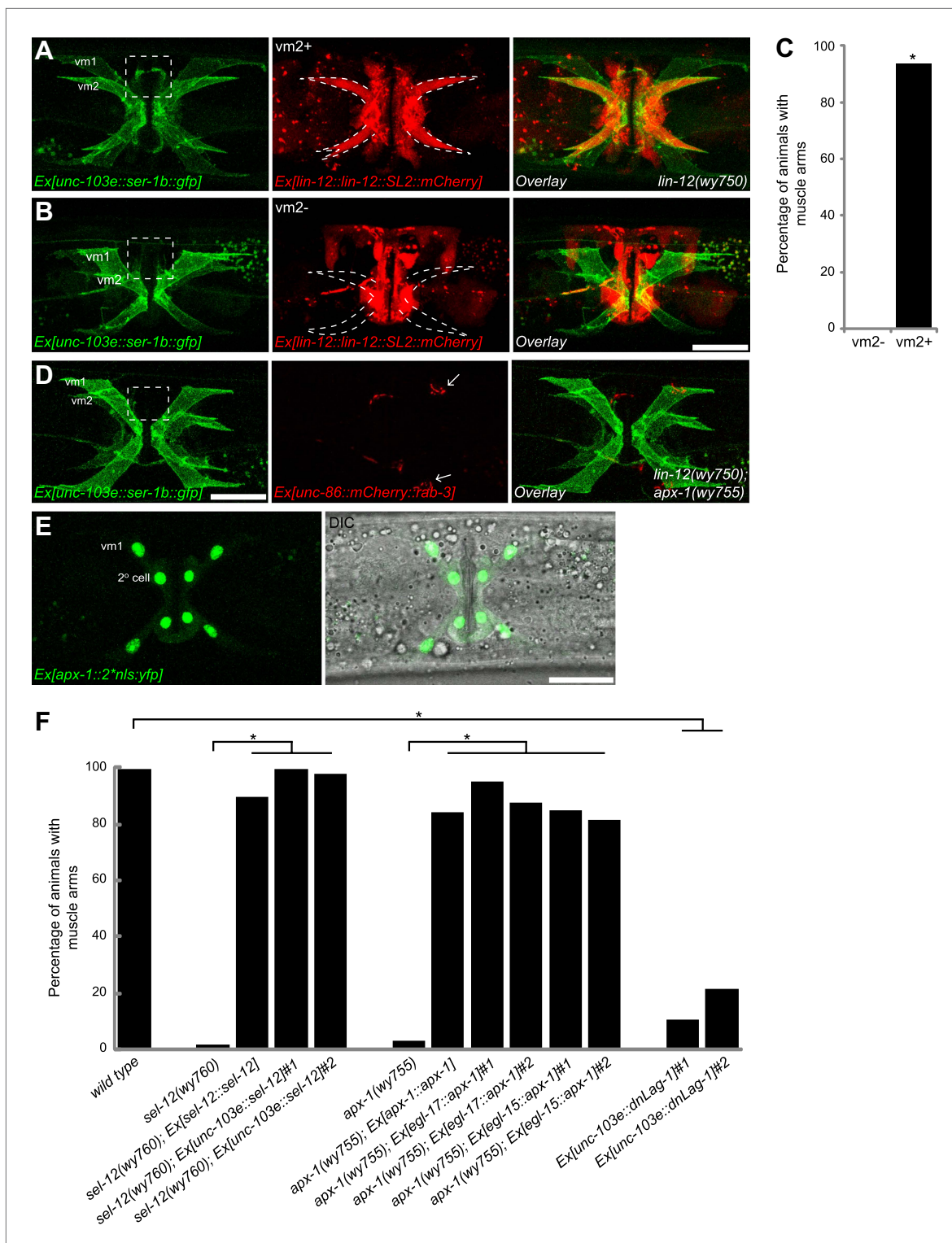DOI: 10.7554/eLife.00378.015

Figure 4. Cell-autonomous requirement of LIN-12 in vm2. (**A**) and (**B**) Representative images of *lin-12(wy750)* mutant animals with non-mosaic (**A**) and mosaic (**B**) expression of *lin-12::SL2::mCherry*. Left column shows the vulval muscles labeled by SER-1B::GFP. Middle column shows the *lin-12* expression patterns. Right column shows the overlaid images. Note that the complete *lin-12* expression (vm2+) rescues muscle arm defects of *lin-12(wy750)* (**A**). Lacking of *lin-12* expression in vm2 (vm2-) abolishes its capacity to rescue muscle arm defects (**B**). Boxed areas indicate synaptic regions. Dashed lines indicate the morphology of vm2. Scale bar is 20 μm. (**C**) Quantification of the vm2 muscle arm phenotypes in the mosaic transgenic animals. *p<0.0001, Fisher's exact test, n = 48–50. (**D**) Vulval muscle morphology in the *apx-1(wy755); lin-12(w750)* mutant animals. Boxed area indicates synaptic region. *Figure 4. Continued on next page*

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Arrows indicate HSN cell bodies. Scale bar is 20 μm. (**E**) Epifluorescence and DIC images showing the *apx-1* expression pattern in young adult. The four cells in the center of the image are 2° vulval epithelial cells. The four cells at the periphery are vm1 cells. Scale bar is 20 μm. (**F**) Cell-autonomous requirement of *apx-1*, *sel-12* and *lag-1*. Quantification of muscle arm phenotypes in animals with different genotypes indicated on the X-axis. *, P<0.0001, Fisher's exact test, n = 40–57.
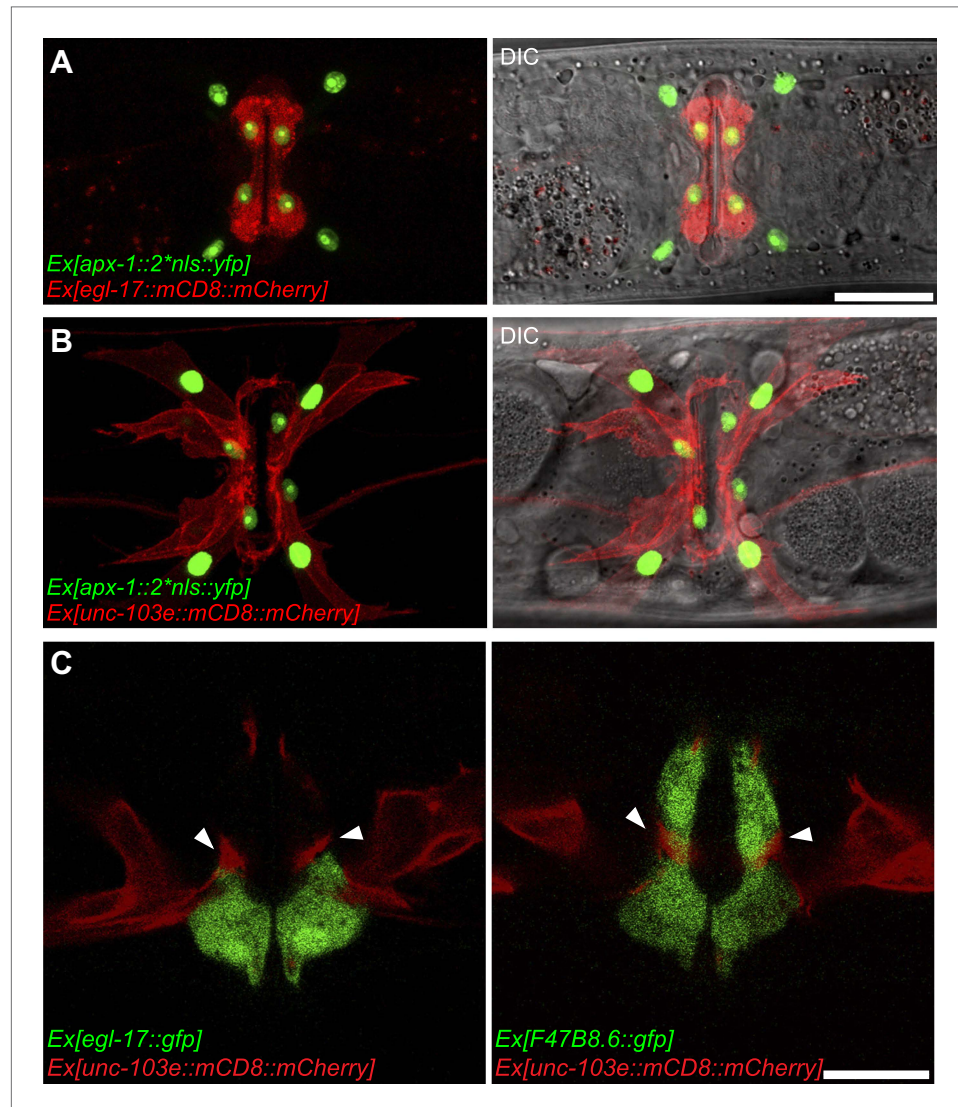
Figure 4—figure supplement 1. APX-1 expression pattern and the relative position of vm2 muscle arms. (**A**) Overlaid epifluorescence and DIC images showing the identity of the *apx-1* expressing cells. The *egl-17::mCD8::mCherry* transgene labels the 2° vulval epithelial cells in young adult. Scale bar is 20 μm. (**B**) Overlaid epifluorescence and DIC images showing the identity of the *apx-1* expressing cells. The *unc-103e::mCD8::mCherry* transgene labels the vm1 cells in young adult . Scale bar is 20 μm. (**C**) Single confocal slices showing the position of muscle arms and the vulval epithelial cells. Vulval muscles are labeled by mCD8::mCherry. Secondary vulval epithelial cells are labeled by *egl-17::gfp* transgene (left). Both primary and secondary vulval epithelial cells are labeled by *F47B8.6::gfp* transgene (right). Note that the muscle arms are located between the 1o and the 2o vulval epithelial cells and directly contact these cells. Arrowheads indicate muscle arms. Scale bar is 10 μm.
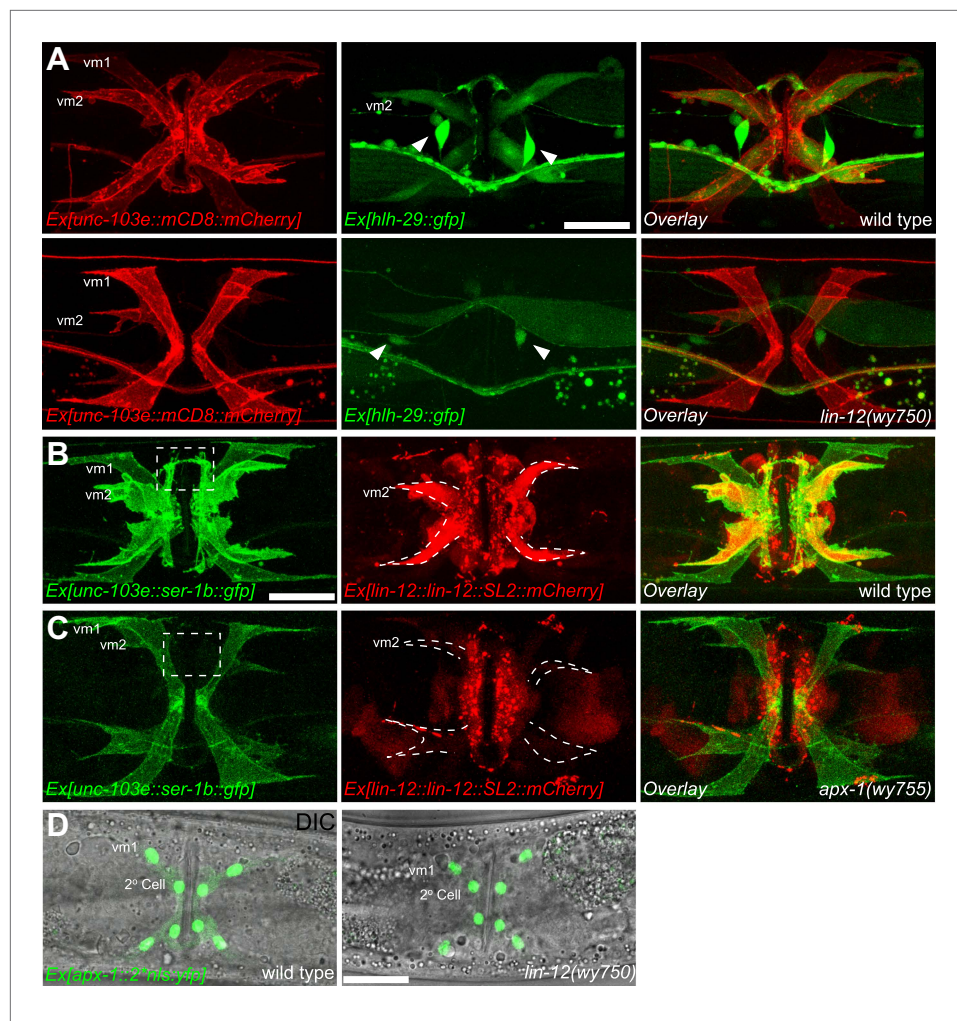
Figure 5. vm2 muscle arm development requires APX-1-induced LIN-12/Notch signaling activity. (**A**) *hlh-29*::GFP expression in wild-type (top) and *lin-12(wy750)* (bottom) animals. Left column shows the vulval muscles labeled by mCD8::mCherry. Middle column shows the *hlh-29*::GFP expression patterns. Right column shows the overlaid images. Arrowheads indicate VC4/5 cell bodies. Note that *hlh-29*::GFP is specifically expressed in vm2 but not vm1 in the wild type, and is down-regulated in the *lin-12(wy750)* mutant. Scale bar is 20 μm. (**B**) and (**C**) *lin-12::SL2::mCherry* expression in wild-type (**B**) and *apx-1(wy755)* (**C**) animals. Left column shows the vulval muscles labeled by SER-1::GFP. Middle column shows the *lin-12* expression patterns. Right column shows the overlaid images. Boxed areas indicate synaptic regions. Dashed lines delineate the morphology of vm2. Note that *lin-12* transcription is specifically down-regulated in vm2 cells in *apx-1(wy755)* mutant animals. Scale bar is 20 μm. (**D**) *apx-1* expression in wild-type (left) and *lin-12(wy750)* (right) animals. The four cells in the center of the image are 2° vulval epithelial cells. The four cells at the periphery are vm1 cells. Note that the *apx-1* expression pattern does not change in the *lin-12(wy750)* mutant. Scale bar is 20 μm.
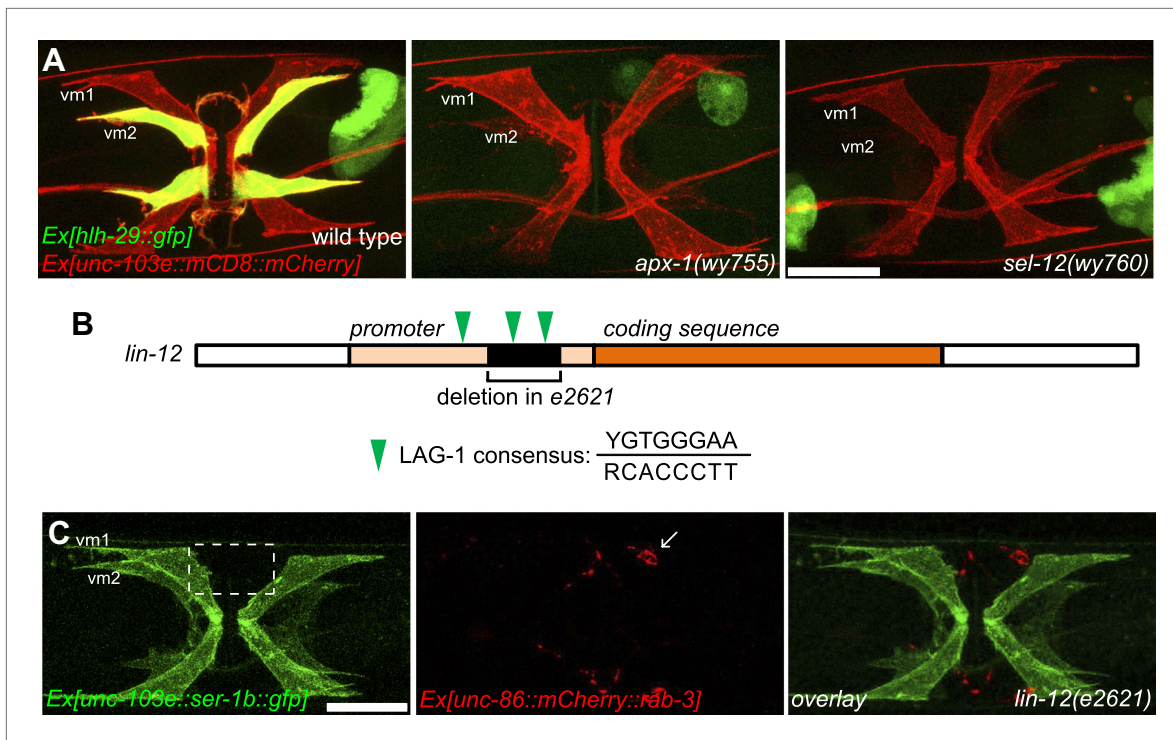DOI: 10.7554/eLife.00378.018

Figure 5—figure supplement 1. vm2 muscle arm development requires high LIN-12/Notch signaling activity. (**A**) *hlh-29::gfp* expression in wild-type (left), *apx-1(wy755)* (middle) and *sel-12(wy760)* (right) mutant animals. Vulval muscles are labeled by mCD8::mCherry. Note that *hlh-29::gfp* is specifically expressed in vm2 but not vm1 in the wild type, and is down-regulated in the *apx-1(wy755)* and *sel-12(wy760)* mutants. Scale bar is 20 μm. (**B**) Schematic illustration of the molecular lesion (top) and muscle arm phenotype (bottom) of *lin-12(e2621)*. *lin-12* promoter and coding sequence are indicated by yellow and orange colors. Black box and bracket show the deletion in *e2621*. LAG-1 consensus binding sites are indicated by green arrowheads. Note that there are two LAG-1 consensus binding sites located in the deletion region. Boxed area indicates synaptic region. Arrow indicates HSN cell body. Scale bar is 20 μm.
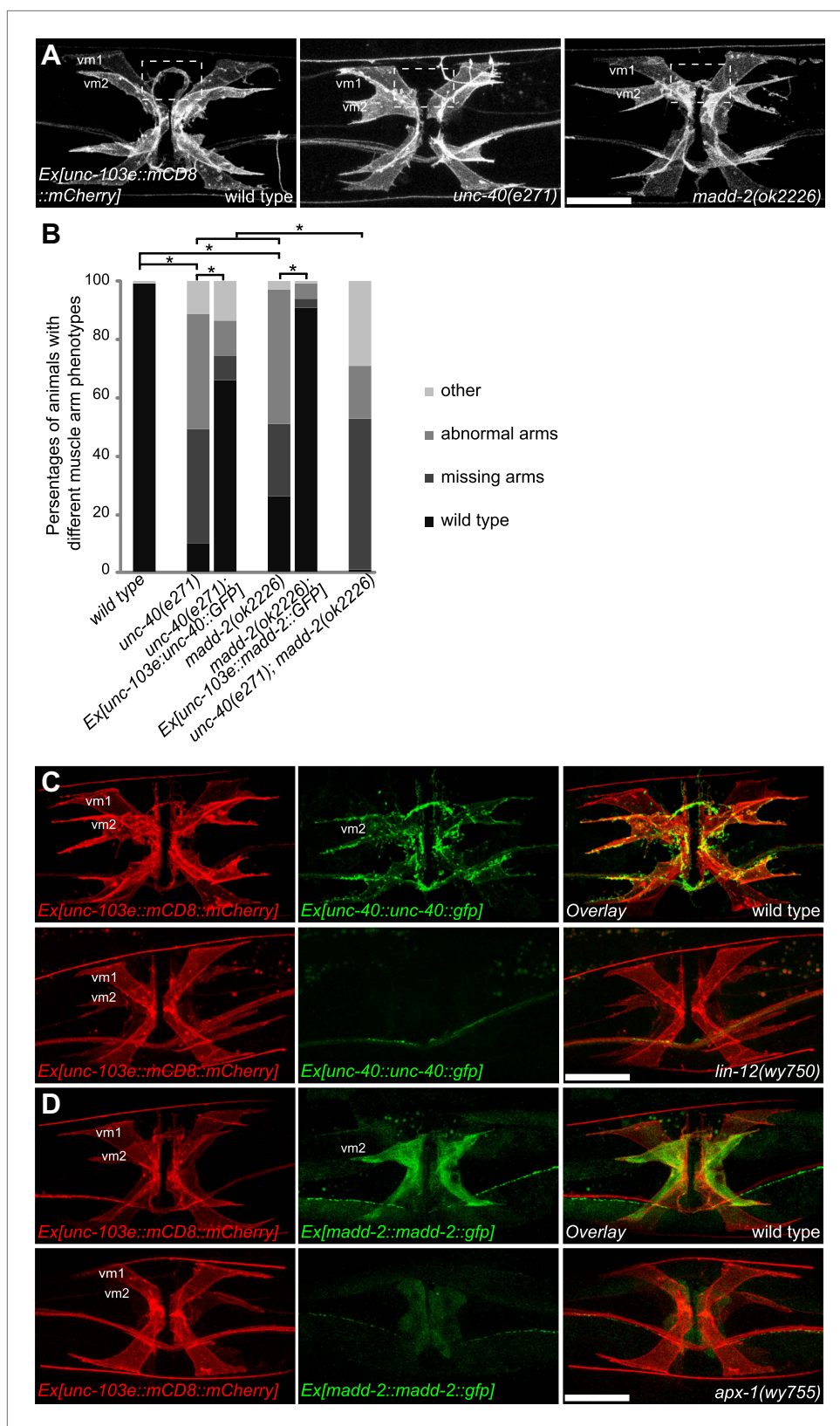
Figure 6. LIN-12/Notch signaling instructs vm2 muscle arm by regulating *unc-40/DCC* and *madd-2*.
(**A**) Representative images showing the vulval muscle morphology in wild type (left), *unc-40(e271)* (middle) and
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madd-2(ok2226) (right) animals. Boxed areas indicate synaptic regions. Note that *unc-40(e271)* and *madd-2(ok2226)* show missing (middle) or abnormal (right) muscle arm phenotypes. Scale bar is 20 μm. (**B**) Quantification of muscle arm phenotypes in animals with different genotypes indicated on the X-axis. 'Wild type' indicates the animals with normal muscle arms. 'Missing arms' indicates the animals with missing muscle arms. 'Abnormal arms' indicates the animals with flimsy muscle arms or abnormal muscle arms extending from incorrect positions. 'Other' indicates animals with severe vulval muscle morphology phenotype that the muscle arms could not be scored. *p<0.0001, chi-squared test, n = 73–194. (**C**) Double-labeling of the expression of UNC-40 and vulval muscle morphology in wild type (top) and *lin-12(wy750)* animals (bottom). Left column shows the vulval muscles labeled by mCD8::mCherry. Middle column shows the UNC-40::GFP expression patterns. Right column shows the overlaid images. Note that UNC-40::GFP is preferentially expressed in vm2 and enriched on the muscle arms in the wild type, and is down-regulated in the *lin-12(wy750)* mutant. Scale bar is 20 μm. (**D**) Double-labeling of the expression of MADD-2 and vulval muscle morphology in wild type (top) and *apx-1(wy755)* animals (bottom). Left column shows the vulval muscles labeled by mCD8::mCherry. Middle column shows the MADD-2::GFP expression patterns. Right column shows the overlaid images. Note that MADD-2::GFP is preferentially expressed in vm2 in the wild type, and is down-regulated in the *apx-1 (wy755)* mutant. Scale bar is 20 μm.
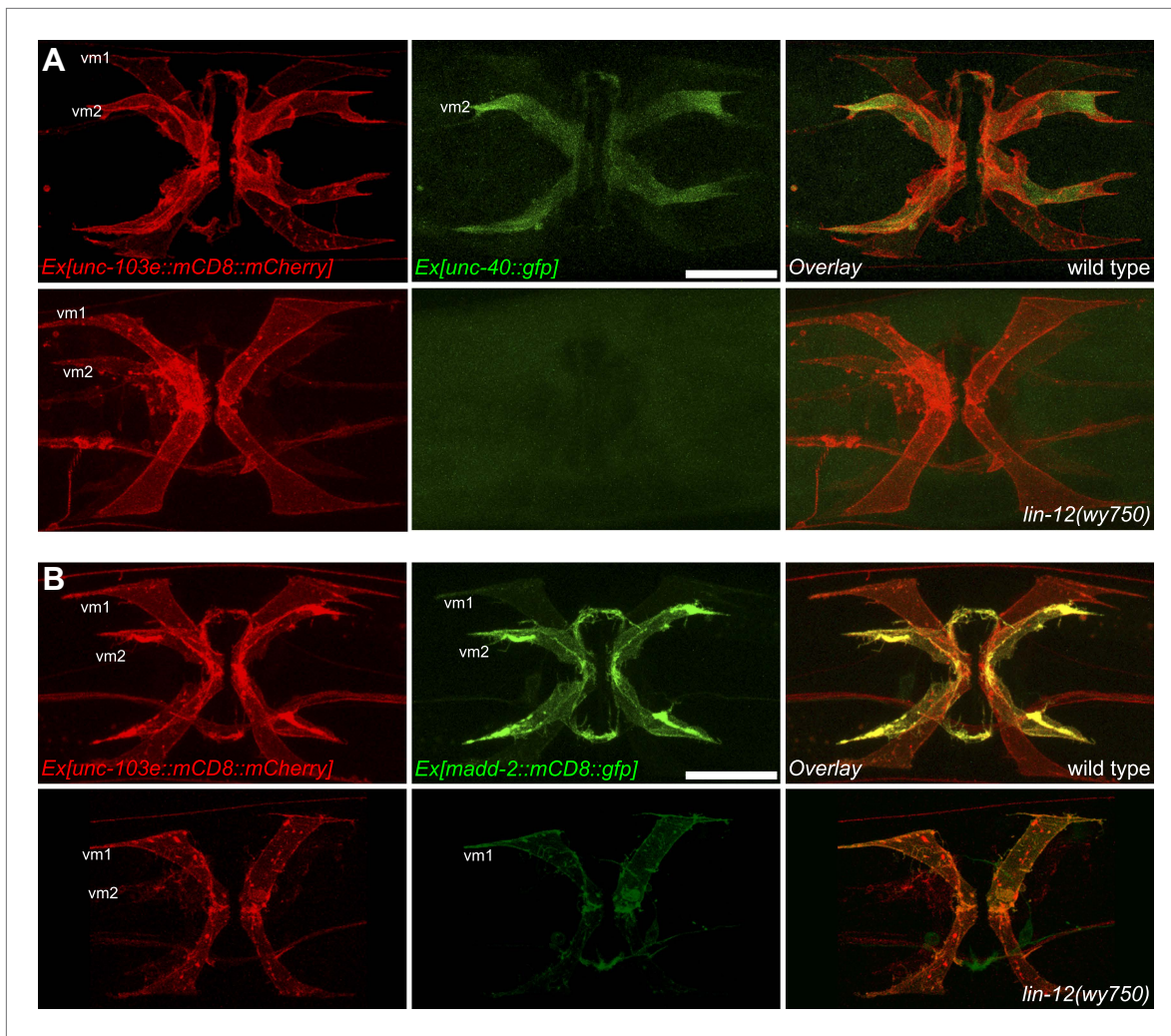
DOI: 10.7554/eLife.00378.020

Figure 6—figure supplement 1. LIN-12/Notch signaling instructs vm2 muscle arm by regulating *unc-40*/DCC and *madd-2*. (**A**) Ventral views of the double-labeling of the transcription of *unc-40* and vulval muscle morphology in wild type (top) and *lin-12(wy750)* animals (bottom). Left column shows the vulval muscles labeled by mCD8::mCherry. Middle column shows the *unc-40* transcription patterns. Right column shows the overlaid images. Note that *unc-40* is preferentially transcribed in vm2 in wild type, and down-regulated in *lin-12(wy750)* mutant. Scale bar is 20 μm. (**B**) Ventral views of the double-labeling of the transcription of *madd-2* and vulval muscle morphology in wild type (top) and *lin-12(wy750)* animals (bottom). Left column shows the vulval muscles labeled by mCD8::mCherry. Middle column shows the *madd-2* transcription patterns. Right column shows the overlaid images. Note that *madd-2* is preferentially transcribed in vm2 in wild type, and down-regulated in *lin-12(wy750)* mutant. Scale bar is 20 μm.
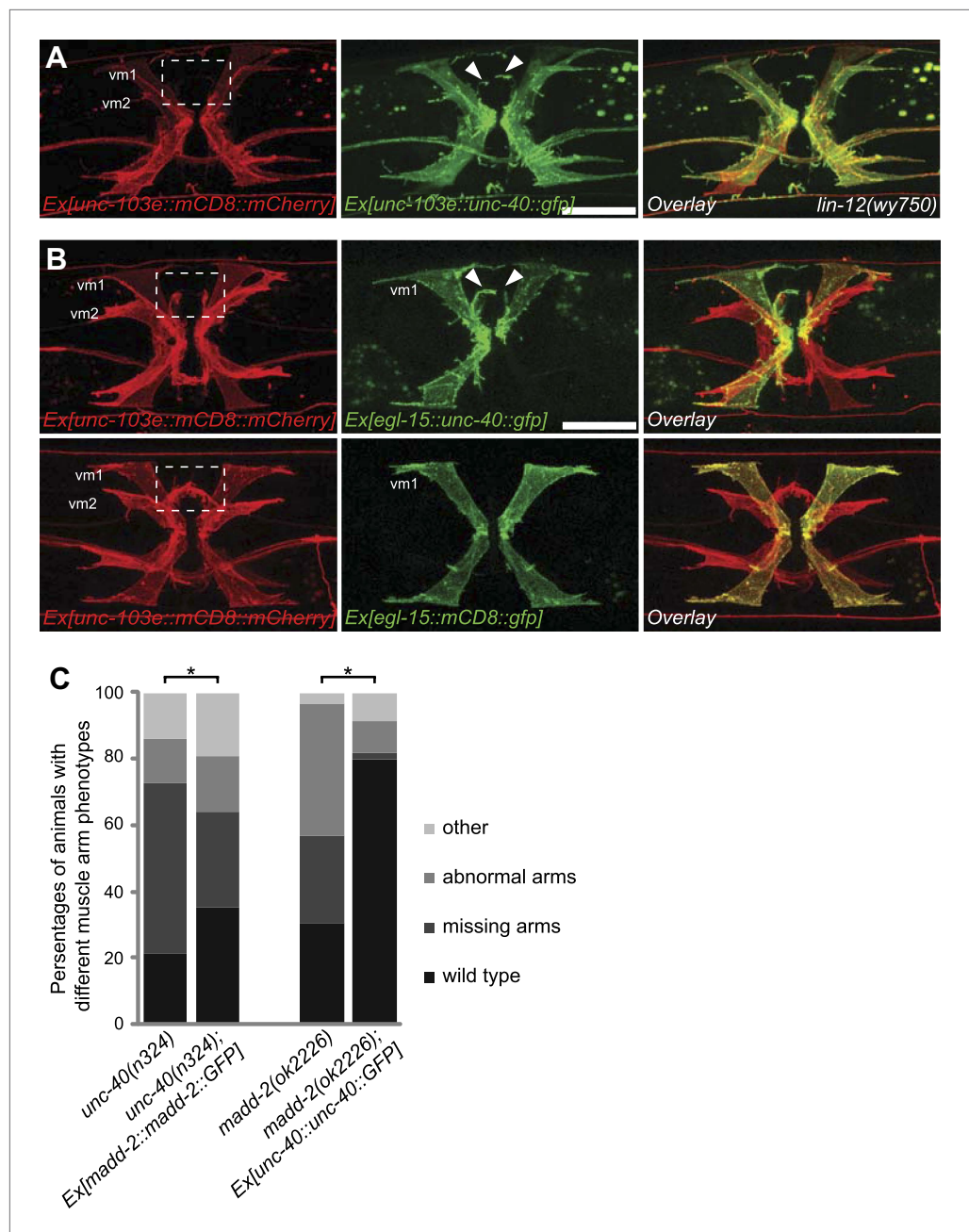DOI: 10.7554/eLife.00378.021

Figure 7. *unc-40* and *madd-2* function partly in parallel in regulating vm2 muscle arm development. (**A**) Expression of UNC-40::GFP (middle) in vulval muscles in the *lin-12(wy750)* animals. Vulval muscles are labeled by mCD8::mCherry (left). Boxed area indicates synaptic region. Arrowheads indicate rescued muscle arms. Note that the forced expression of UNC-40 in vm2 from the *unc-103e* promoter induces the formation of muscle arms in the *lin-12(wy750)* mutant. Scale bar is 20 μm. (**B**) Ectopic expression of *unc-40::gfp* (top) or *mCD8::gfp* (bottom) transgene in vm1. Left column shows the vulval muscles labeled by mCD8::mCherry. Middle column shows forced transgene expression patterns. Right column shows the overlaid images. Boxed areas indicate synaptic regions. Arrowheads indicate ectopic muscle arm-like structures. Note that the ectopic expression of UNC-40 in vm1 induces the muscle arm-like membrane extensions. Scale bar is 20 μm. (**C**) Quantification of muscle arm phenotype in animals with different genotypes indicated on the X-axis. 'Wild type' indicates the animals with normal muscle arms. 'Missing arms' indicates the animals with missing muscle arms. 'Abnormal arms' indicates the animals with flimsy muscle arms or abnormal muscle arms extending from incorrect positions. 'Other' indicates animals with severe vulval muscle morphology phenotype that the muscle arms could not be scored. *p<0.0001, chi-squared test, n = 105–311.
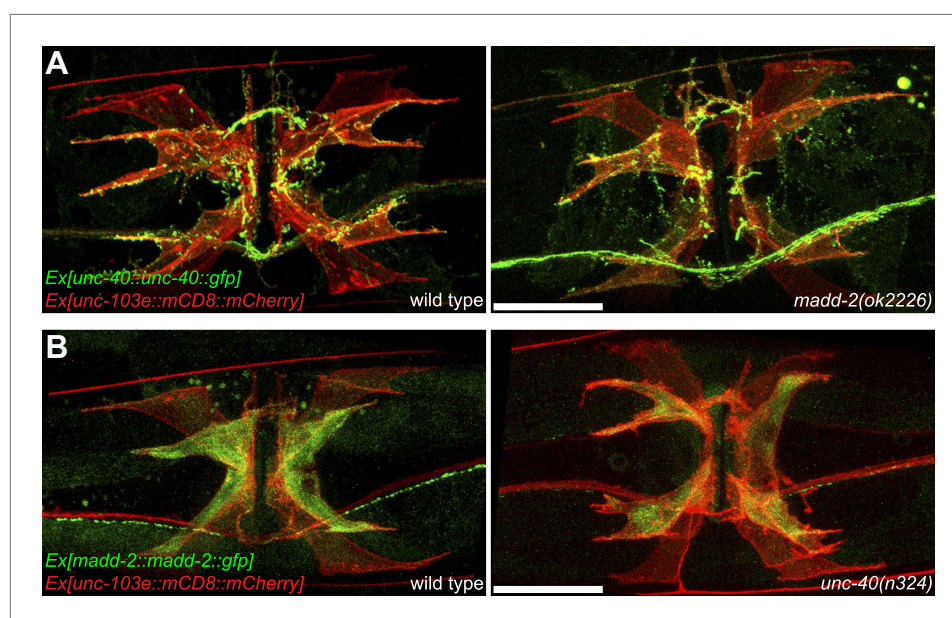
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Figure 7—figure supplement 1. Ectopic expression of UNC-40 in vm1 induces muscle arm-like structures. (**A**) UNC-40 expression in the wild-type (left) and *madd-2(ok2226)* (right) animals. Vulval muscles are labeled by mCD8::mCherry. Note that in both wild-type and *madd-2(ok2226)* mutant animals, UNC-40 are preferentially expressed in vm2 cells. Scale bar is 20 μm. (**B**) MADD-2 expression in the wild-type (left) and *unc-40(n324)* (right) animals. Vulval muscles are labeled by mCD8::mCherry. Note that in both wild-type and *unc-40(n324)* mutant animals, MADD-2 are preferentially expressed in vm2 cells. Scale bar is 20 μm.
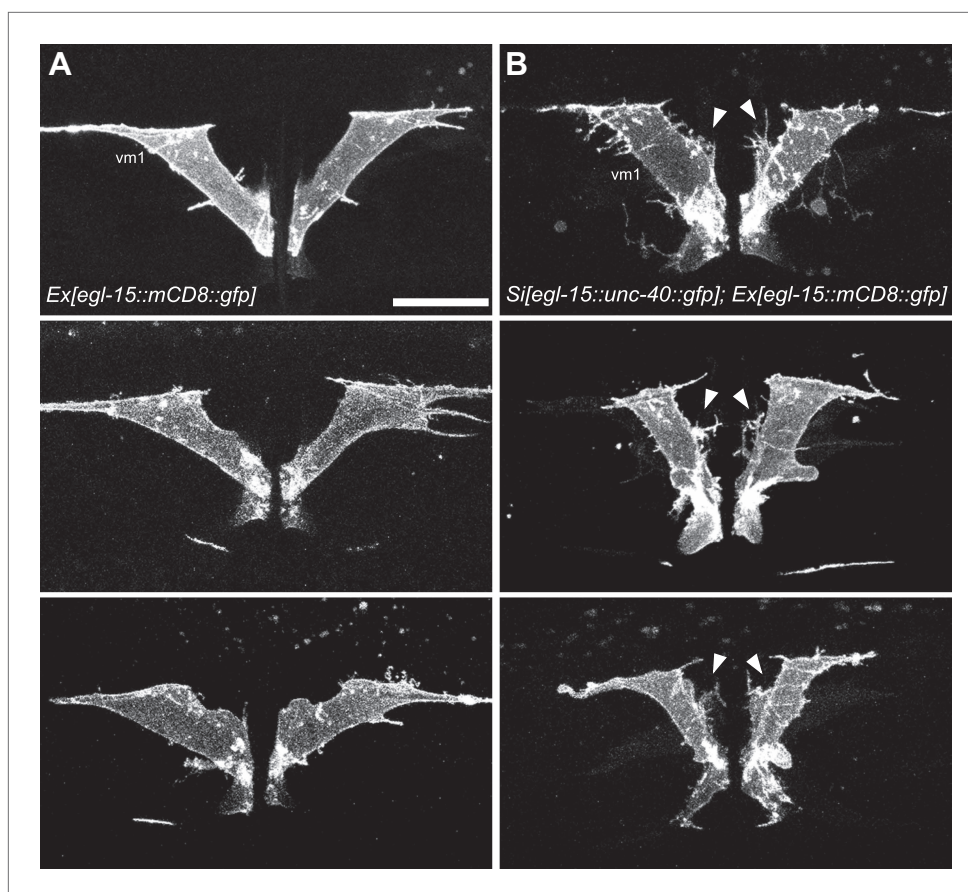DOI: 10.7554/eLife.00378.023

Figure 7—figure supplement 2. *unc-40* and *madd-2* are not mutually regulated. (**A**) Three examples showing the vm1 morphology in animals with the expression of a single-copy transgene *egl-15::unc-40::gfp*. vm1 is visualized by *egl-15::mCD8::gfp*. Arrowheads indicate the induced muscle arm-like membrane protrusions. Scale bar is 20 μm.
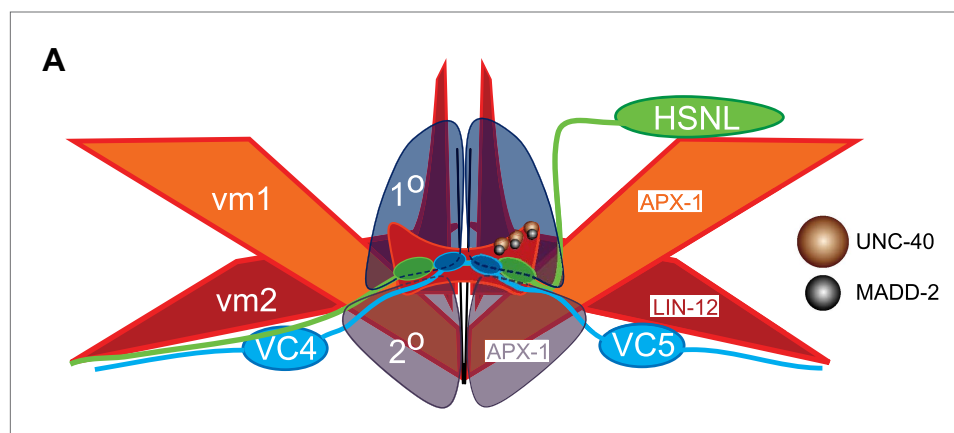
Figure 7—figure supplement 3. Schematic model of muscle arm development and vm2 postsynaptic specification. (**A**) Schematic model showing the physical organization of the vulva organ and the mechanism of vm2 postsynaptic target selection. Note that vm2 muscle arms (red) directly contact the primary (1°, dark blue) and secondary (2°, dark gray) vulval epithelial cells. APX-1/DSL is expressed in vm1 cells (orange) and the secondary vulval epithelial cells. LIN-12/NOTCH is expressed in vm2 cells (red). UNC-40/DCC (brown circles) and MADD-2 (black circles) interact with each other and are localized on vm2 muscle arms.