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Figures and figure supplements

A *mex3* homolog is required for differentiation during planarian stem cell lineage development

Shu Jun Zhu, et al.

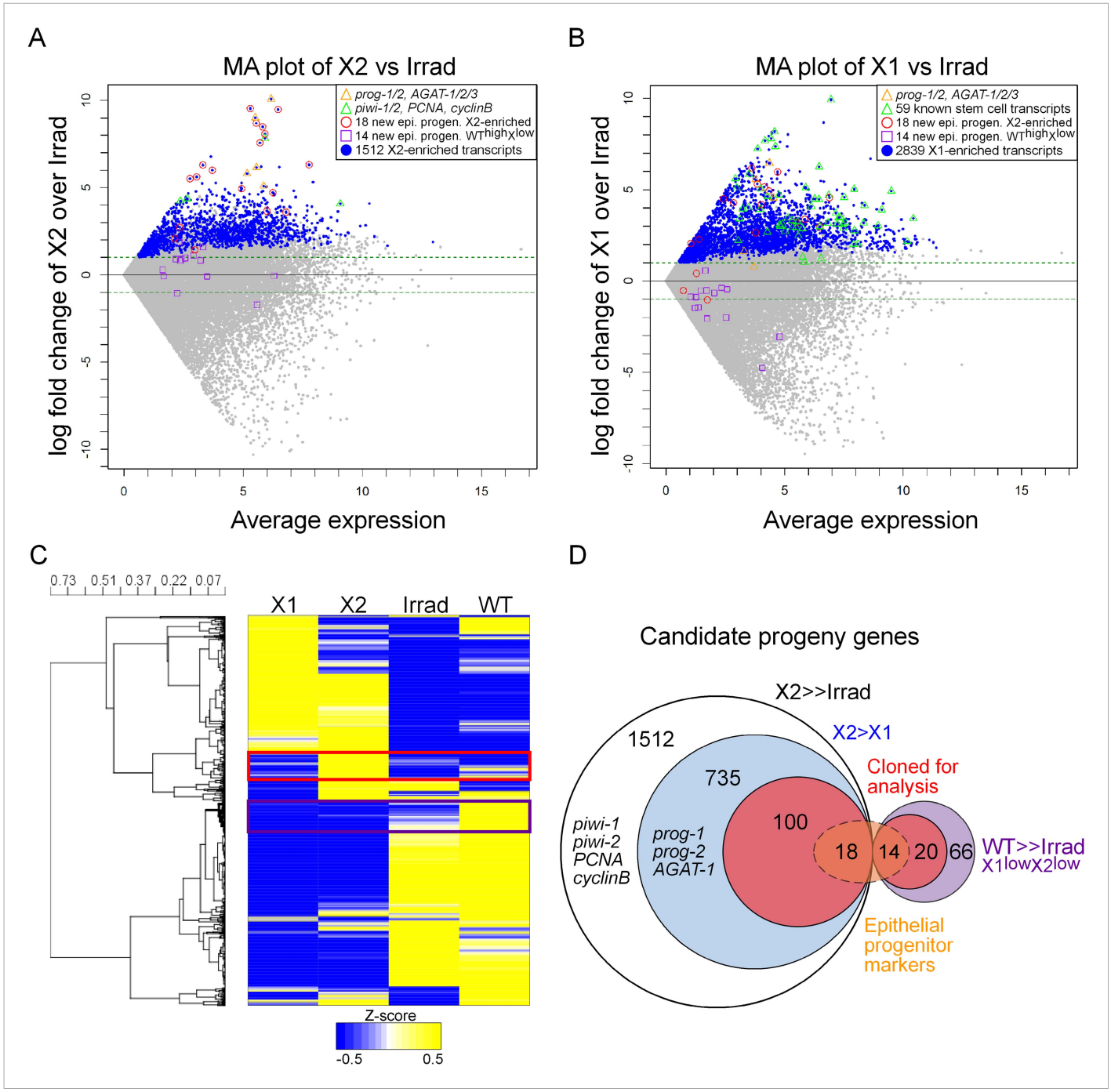


Figure 1. Transcriptional analysis of irradiation-sensitive cell populations in *Schmidtea mediterranea*. Irradiation-sensitive cell populations (X1, X2) isolated by fluorescence-activated cell sorting (FACS), lethally irradiated whole worms (Irrad), and intact control worms (WT) were analyzed by RNA-deep sequencing (RNAseq). (**A** and **B**) MA plots comparing enrichment in the X2 (**A**) or X1 (**B**) cell populations over Irrad, with average expression level. Each gray dot represents one transcript. Blue dots represent transcripts found to be significantly enriched in the respective cell population through DESeq analysis (X2, $p < 0.01$; X1, $p < 0.001$). Previously established postmitotic lineage markers are indicated by orange triangles. Previously established stem cell-specific transcripts are indicated as green triangles. Candidate genes identified in this study as markers of epithelial progenitors are indicated as red circles (X2-enriched) or purple boxes ($WT^{high}X^{low}$). (**C**) Hierarchical clustering of RNAseq data identifies transcripts enriched in the X2 population (red box), as well as a group of irradiation-sensitive transcripts, which have high expression in intact control worms but low expression in X1, X2, and Irrad populations (purple box, $WT^{high}X^{low}$). To improve visualization, the heatmap depicts z-scores scaled to the range of −0.5 to +0.5. (**D**) Selection of candidate progeny genes for analysis was determined by enriched expression in X2 fraction compared to both Irrad and X1 fractions. The pool of candidate genes validated in this study to be expressed in epithelial progenitors is indicated in orange. >> denotes more than fivefold enrichment.

DOI: 10.7554/eLife.07025.003

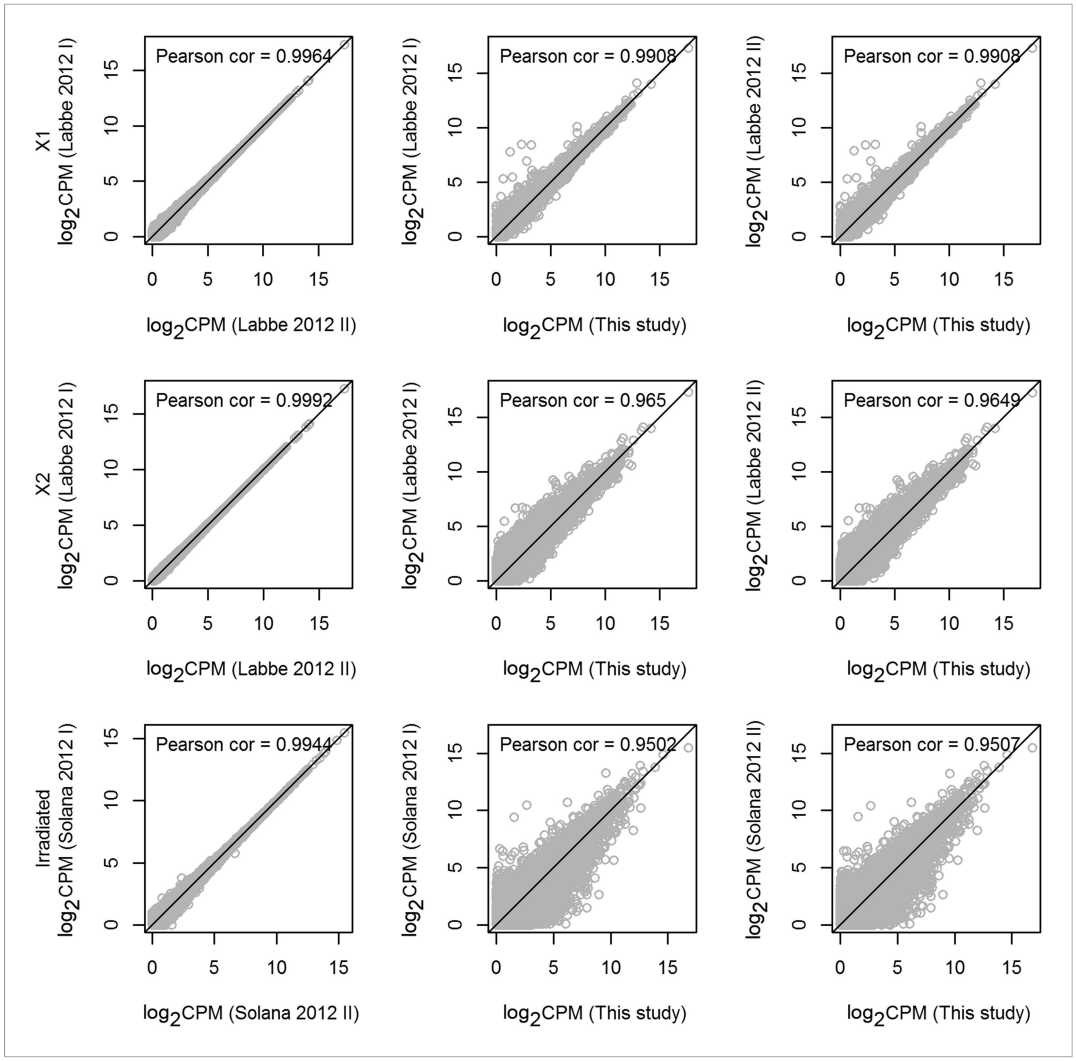


Figure 1—figure supplement 1. Statistical correlations between new and published data sets. Scatter plots of transcripts (gray circles) with reads per million values of 1000 or less, displayed as log-transformed values. The black diagonal line represents a Pearson correlation coefficient of 1. The corresponding Pearson correlation coefficients are shown at the top of each panel. All correlation-test p-values were found to be equal to 0 (Student's *t*-test). As expected, the replicates in each data are highly correlated, and the Pearson correlation coefficients between the replicates in X1 or X2 are greater than 0.95. Furthermore, sequencing from whole irradiated planarians is highly correlated.

DOI: 10.7554/eLife.07025.004

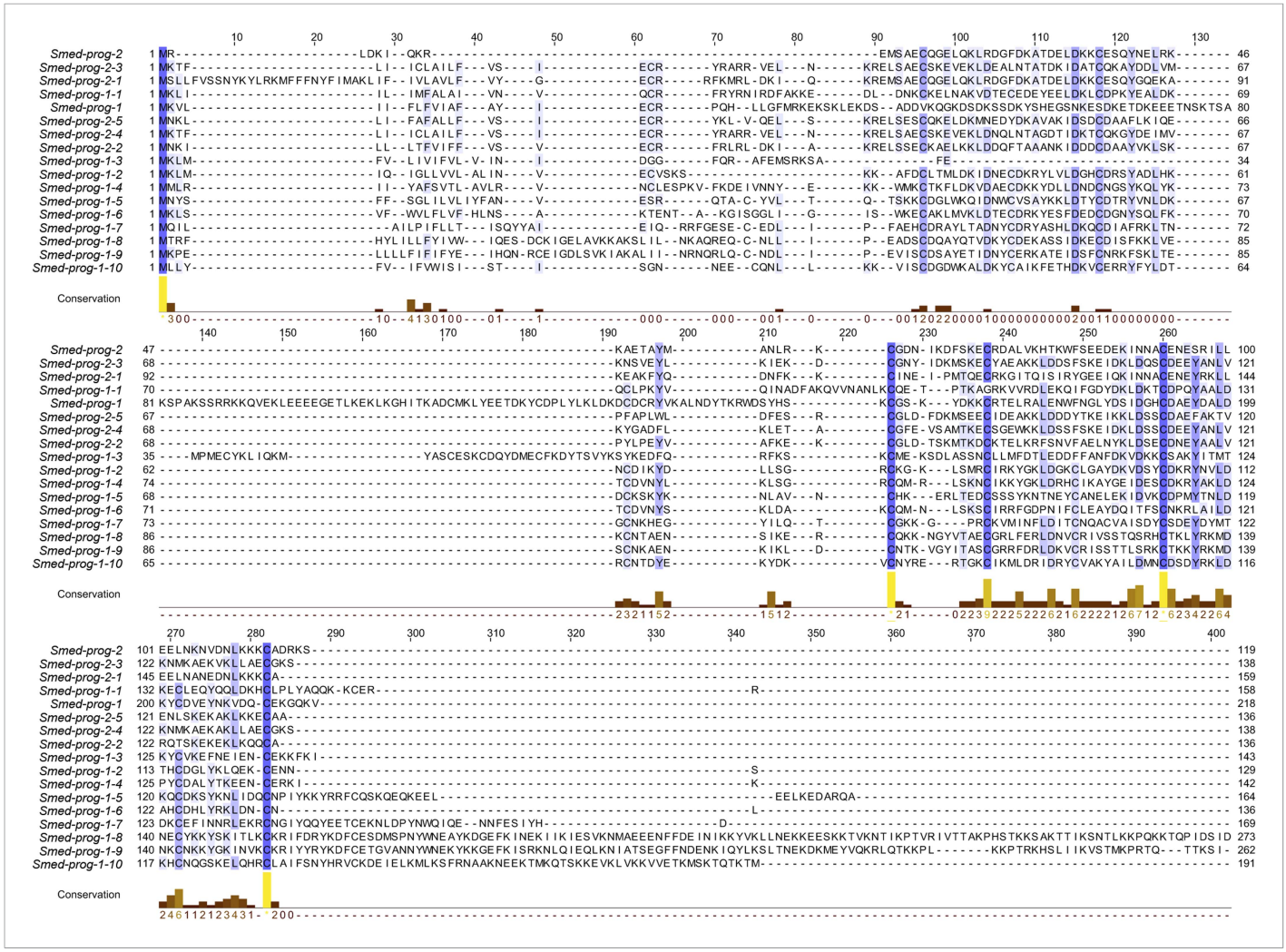


Figure 1—figure supplement 2. Predicted protein alignments of the PROG family that are irradiation-sensitive. An alignment of the 17 PROG family genes used in this study is shown using the tool MUSCLE. Blue shading of residues reflects conservation, which is also plotted below the alignment in the 'conservation' plot.

DOI: 10.7554/eLife.07025.005

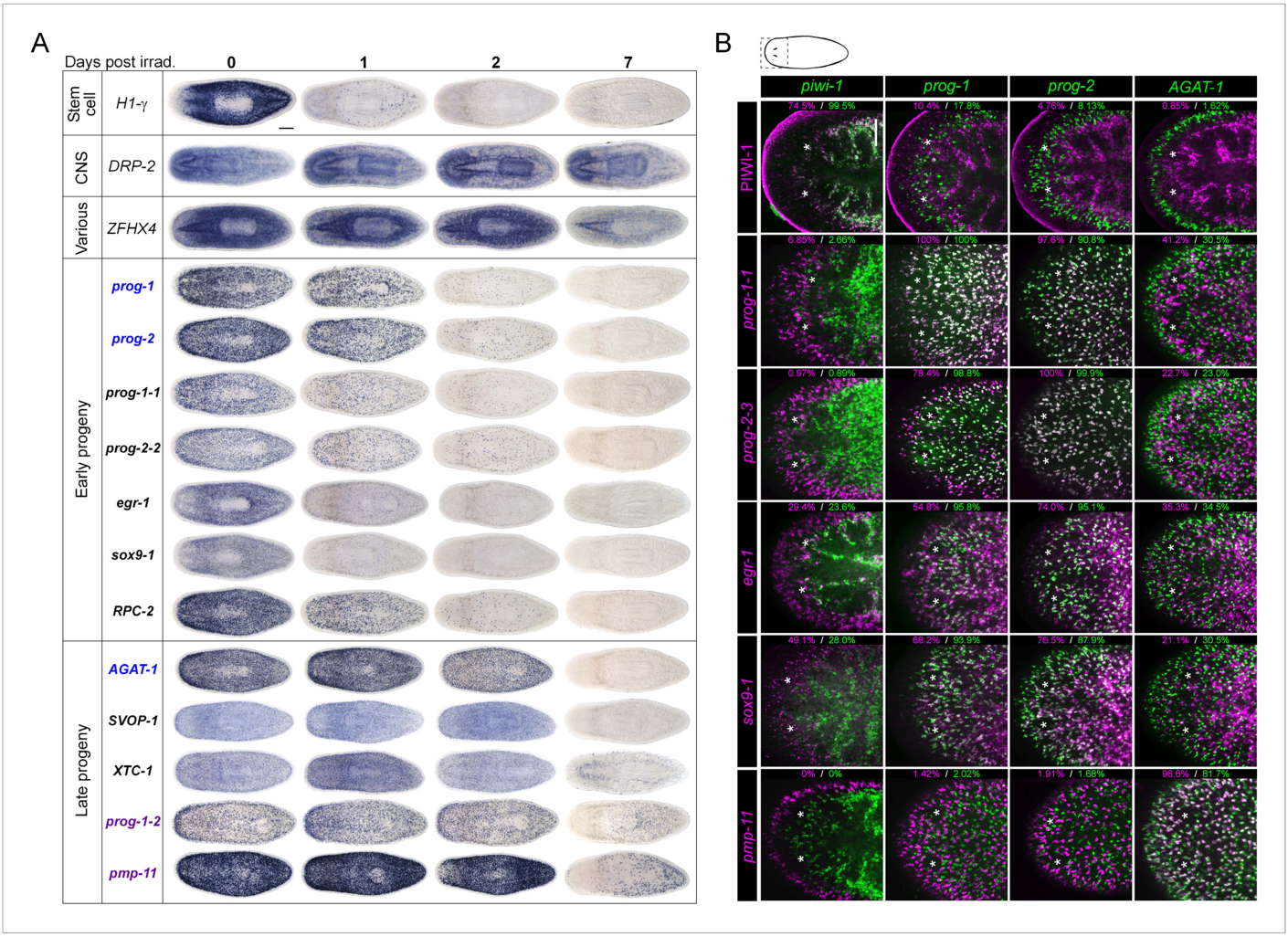


Figure 2. Expression analyses of candidate progeny genes. (**A**) Whole-mount in situ hybridization (WISH) analysis of X2-enriched and $WT^{high}X^{low}$ candidate progeny genes in control and lethally irradiated worms. Examples of genes expressed in a stem cell-like pattern, strong in the bi-lobed brain, distinct and unique patterns, or in a *prog*-like sub-epithelial pattern are shown. *prog*-like genes were categorized as early progeny markers when they displayed similar post-irradiation down-regulation kinetics as *prog-1* and *prog-2*, or late progeny markers when they displayed similar loss kinetics as *AGAT-1*. Established lineage markers *prog-1*, *prog-2*, and *AGAT-1* are included as comparisons and highlighted in blue text. Candidate epithelial progenitor genes with $WT^{high}X^{low}$ expression are indicated in purple text. Anterior, left; scale bar, 200 μm. (**B**) Combinatorial double fluorescent WISH (dFISH) between lineage markers and identified X2-enriched and $WT^{high}X^{low}$ postmitotic progeny markers was performed to assess co-localization with stem cell, early progeny, and late progeny cell populations. Percent co-localization is shown at the top of each panel and is averaged from 3 to 4 animals. *Magenta*, percent co-expression in [*row gene*]$^{+}$ cells; *green*, percent co-expression in [*column gene*]$^{+}$ cells. Images from the head, trunk, and tail regions were used for all cell counts, with a minimum of 300 cells counted. Dispersions of data are in ***Supplementary file 3***. Representative confocal projections spanning 4–6 μm of the head region are shown. Eyespots are marked by asterisks. Anterior, left; scale bar, 100 μm.

DOI: 10.7554/eLife.07025.006

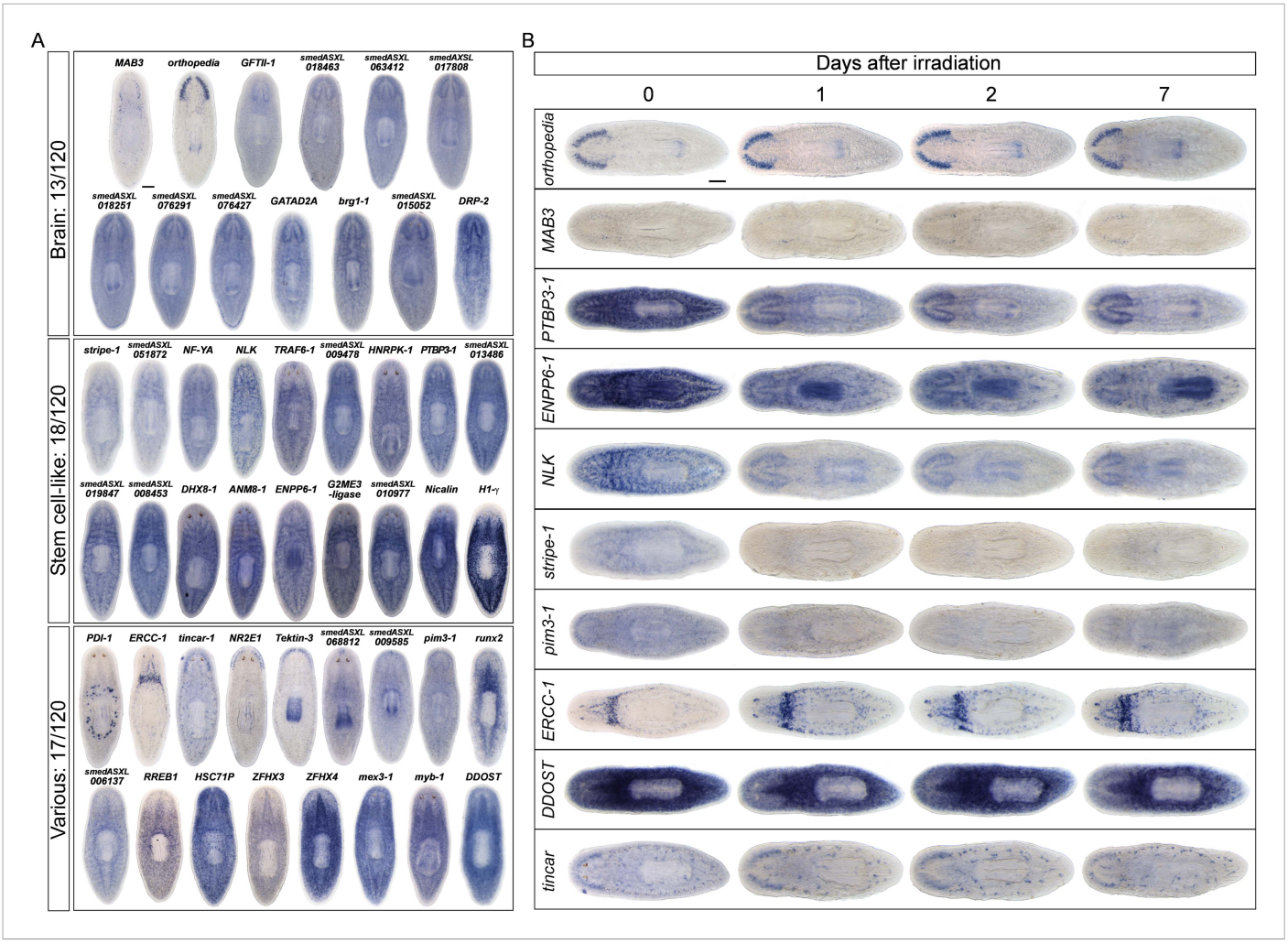


Figure 2—figure supplement 1. Gene expression analysis of candidate progeny genes. (**A**) WISH was used to determine the gene expression patterns of X2-enriched and $WT^{high}X^{low}$ genes in intact control worms. Genes for which a reliable and discrete pattern could be obtained are shown. Genes were named based on the best BLASTx result in mouse (when Expect value < $1 \times e^{-5}$) or based on transcript identity if no homology is found. Genes were categorized as either predominant in the brain (*Brain*), predominant in a stem cell pattern with or without expression elsewhere (*Stem cell-like*), or possessing unique patterns (*Various*). (**B**) A subset of candidate progeny genes was assessed by whole-mount ISH after irradiation with 60 Gys, up to 7 days after exposure. Scale bars, 200 μm.

DOI: 10.7554/eLife.07025.007

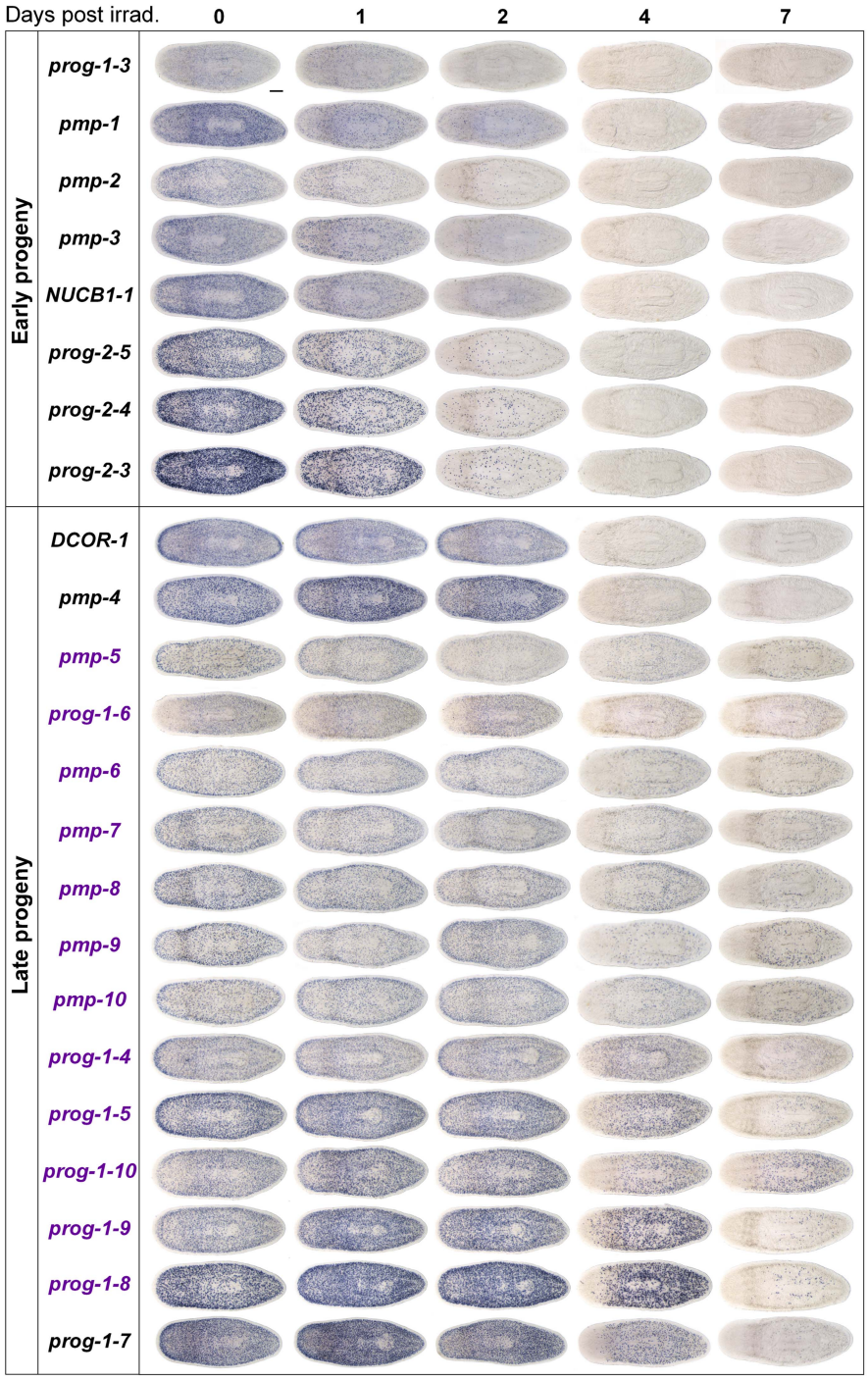


Figure 2—figure supplement 2. Gene expression analysis of candidate progeny genes with a *prog*-like expression pattern. Candidate progeny genes, which exhibited a *prog*-like expression pattern, were all assessed by WISH in irradiated worms (60 Gy) at the indicated time points. Genes were categorized as either early progeny or late progeny depending on whether they exhibited similar kinetics of down-regulation to *prog-1/2* or *AGAT-1/2/3*, respectively. Genes, which are $WT^{high}X^{low}$, are indicated in purple text. Scale bar, 200 μm.

DOI: 10.7554/eLife.07025.008

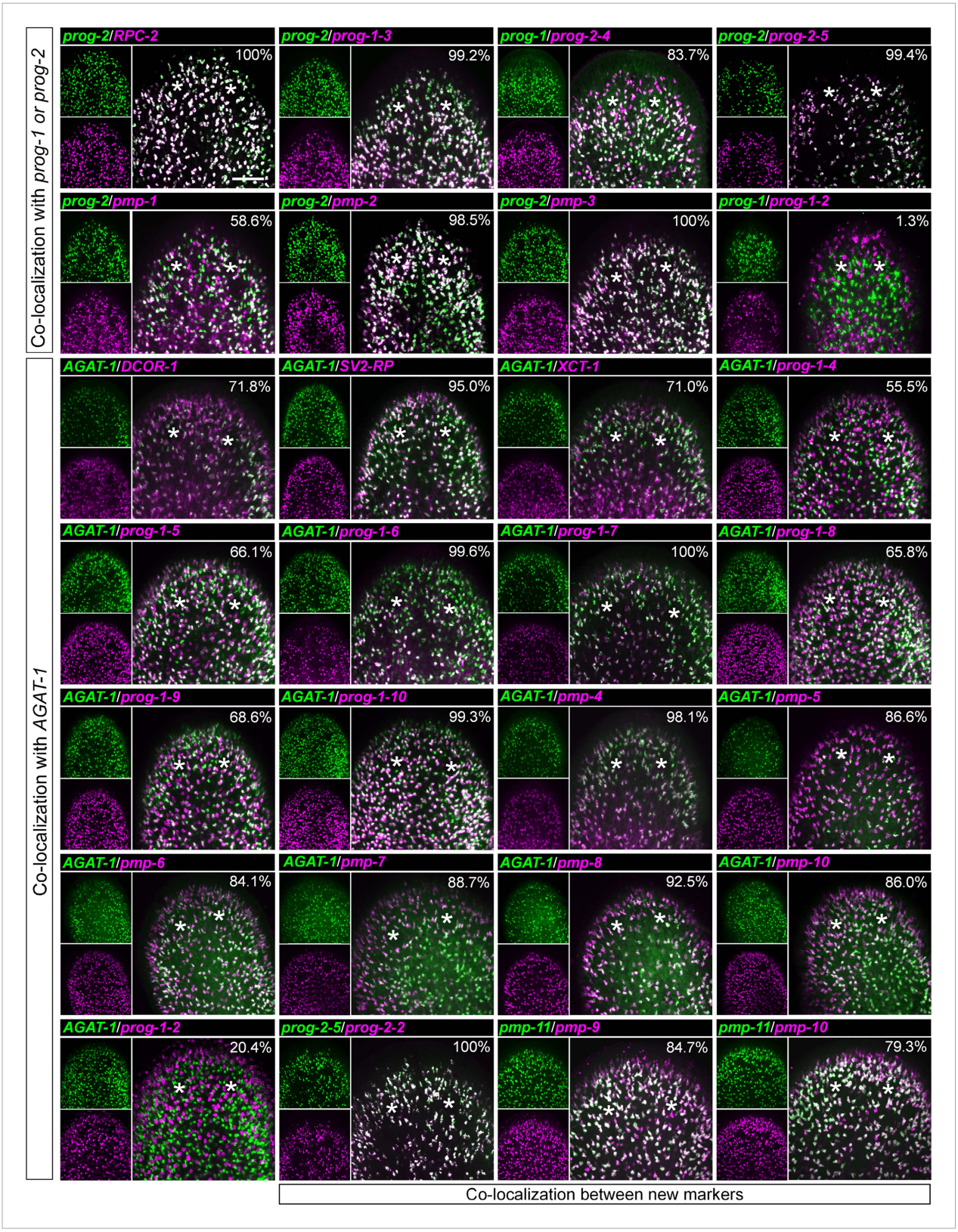


Figure 2—figure supplement 3. Co-localization of progeny markers with dFISH. Co-expression of X2-enriched and $WT^{high}X^{low}$ early and late progeny markers with established lineage markers (*prog-1*, *prog-2*, and *AGAT-1*) or with each other was determined using dFISH. Confocal projections spanning

Figure 2—figure supplement 3. continued on next page

Figure 2—figure supplement 3. Continued

6–10 μm are shown. Smaller panels on the left show individual channels with expression of one gene; larger panels on the right show merged channels. Numbers indicate the percent of magenta-labeled cells, which co-express the green-labeled transcript. 200–2000 cells were counted for each dFISH combination. Location of eyespots is marked by asterisks; anterior, up.

DOI: 10.7554/eLife.07025.009

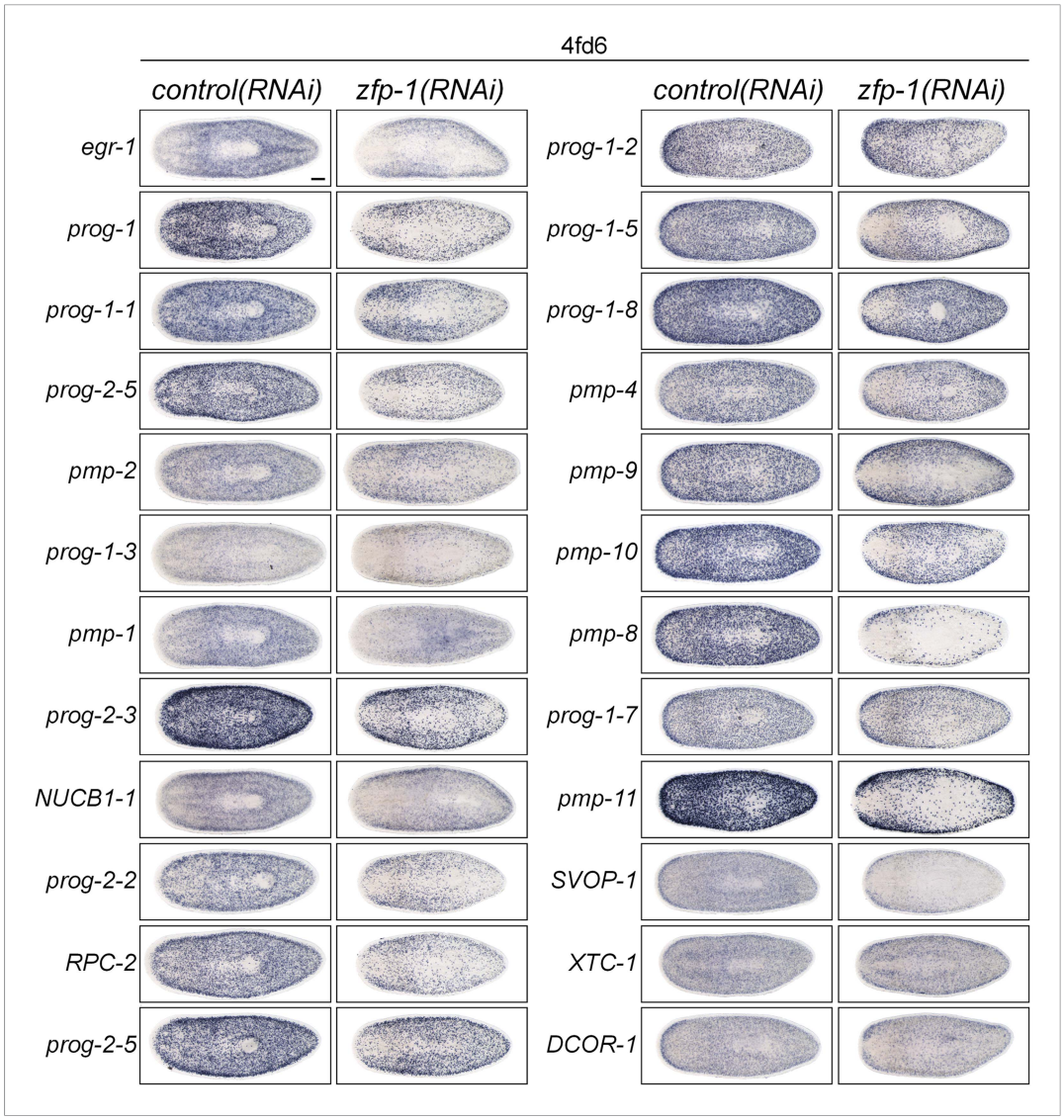


Figure 2—figure supplement 4. Analysis of new progeny markers in *zfp-1(RNAi)* animals. X2-enriched and WT^{-} ^{high}X^{low} early and late progeny transcripts identified as potential markers of epithelial progenitors were assessed by WISH in *zfp-1(RNAi)* animals. Knockdown of *zfp-1* has previously been shown to selectively ablate zeta-neoblasts and output of epithelial progenitors, and down-regulate *prog-1* and *prog-2*.

DOI: 10.7554/eLife.07025.010

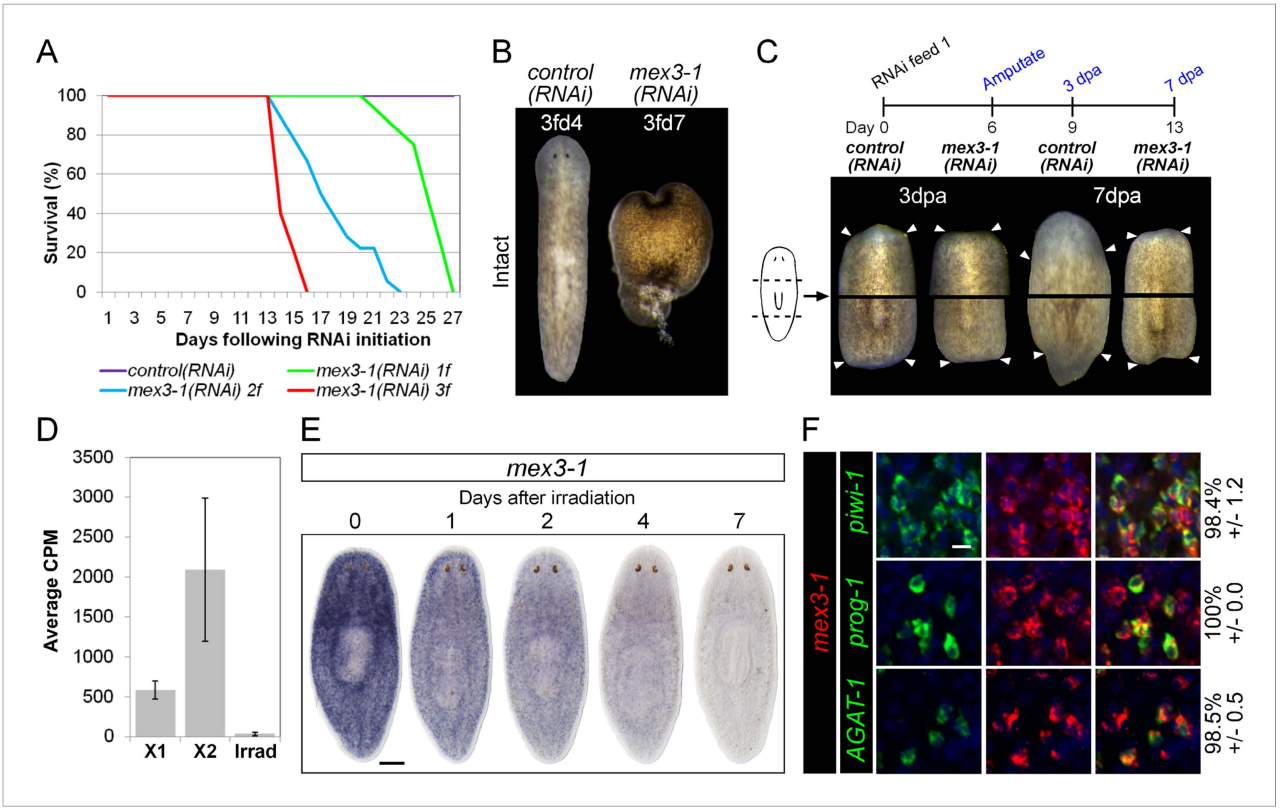


Figure 3. *mex3-1* is required for tissue homeostasis and regeneration. (**A**) Survival curves of *mex3-1(RNAi)* animals to determine the optimal RNAi dosage. One RNAi feed was sufficient to produce completely penetrant lethality by 27 days. (**B**) RNAi worms were observed for homeostatic abnormalities after 1–3 feeds. Time point at which animals were imaged is indicated as *x* days after *z* feeds (*z*fd*x*). (**C**) Regenerative ability after injury was tested according to the experimental timeline shown. Trunk fragments after pre- and post-pharyngeal amputations are shown. (**D**) Expression levels of *mex3-1* in different FACS populations by RNAseq. Error bars show standard deviation. (**E**) WISH analysis of *mex3-1* in intact worms after 60 Gray (Gy) irradiation. Scale bar, 200 μm. (**F**) dFISH was performed to examine *mex3-1* expression in stem cells and postmitotic progeny. Numbers indicate the percentage of stem cells, early progeny, or late progeny co-expressing *mex3-1* (n > 400 cells per dFISH, ± standard error). Scale bar, 10 μm.

DOI: 10.7554/eLife.07025.011

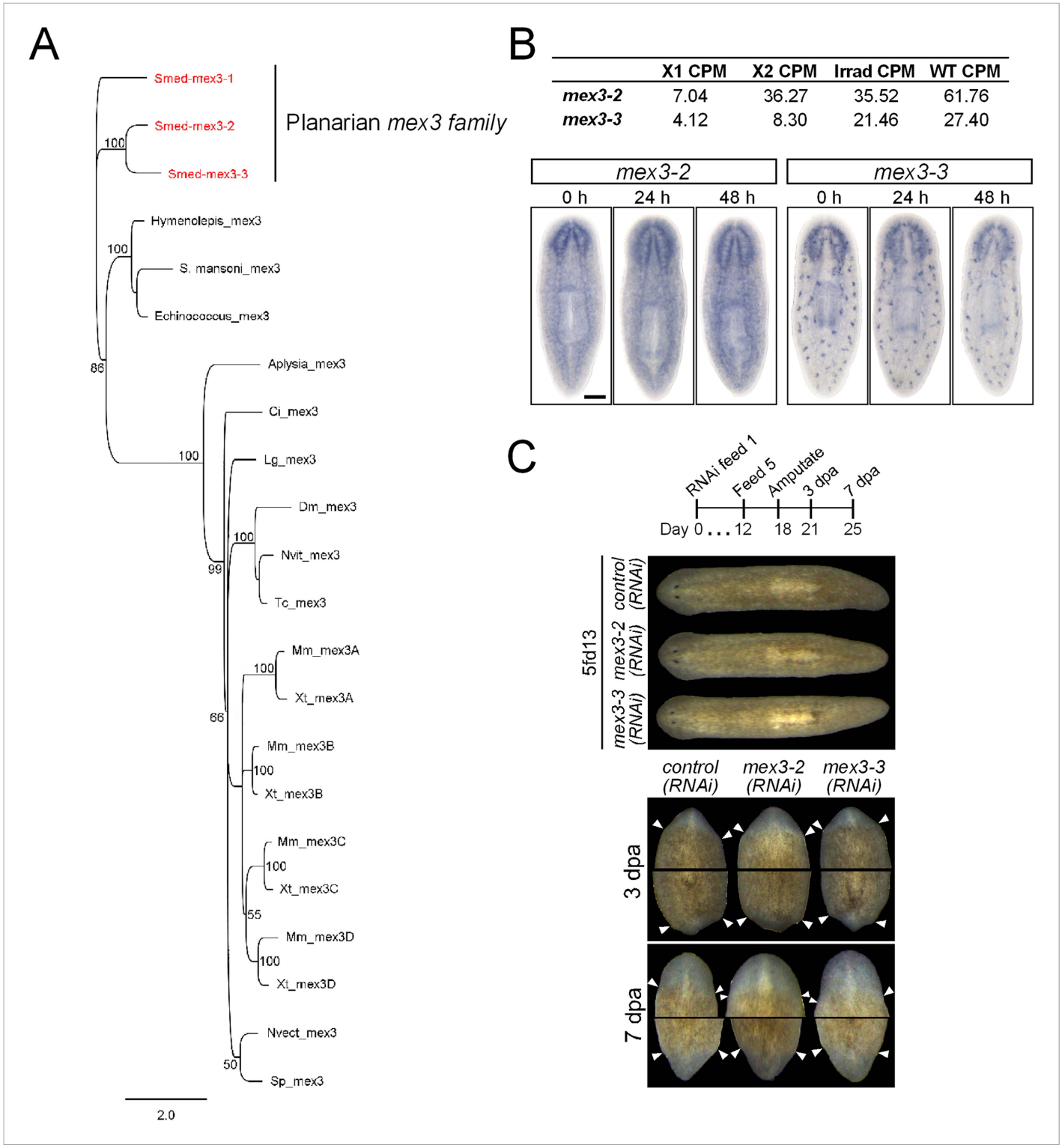


Figure 3—figure supplement 1. Identification and analysis of MEX3 homologs in *S. mediterranea*. (**A**) Phylogenetic analysis of the *mex3* gene family in planarians shows that they are likely the results of planarian-specific duplications. A Bayesian phylogeny was run as outlined in the 'Materials and methods'. Only posterior probabilities 50% and above are shown. *S. mediterranea* sequences are in red. Multiple *mex3* homologs could not be found in individual species of other flatworms. (**B**) Expression levels of *mex3-2* and *mex3-3* in RNAseq of FACS-isolated populations and control intact worms. Expression of *mex3* homologs by WISH after lethal irradiation (60 Gy) is shown below. (**C**) Phenotypes of *mex3-2* and *mex3-3* RNAi animals during

Figure 3—figure supplement 1. continued on next page

Figure 3—figure supplement 1. Continued

homeostasis (upper panel) and during regeneration after amputation (lower panel). Species sequences used in the phylogeny: *Smed* = *Schmidtea mediterranea*; *S. mansoni* = *Schistosoma mansoni*; *Echinococcus* = *Echinococcus multilocularis*; *Aplysia* = *Aplysia californica*; *Ci* = *Ciona intestinalis*; *Lg* = *Lottia gigantea*; *Dm* = *Drosophila melanogaster*; *Tc* = *Tribolium castaneum*; *Nvit* = *Nasonia vitripennis*; *Mm* = *Mus musculus*; *Xt* = *Xenopus tropicalis*; *Nvect* = *Nematostella vectensis*; *Sp* = *Strongylocentrotus purpuratus*.

DOI: 10.7554/eLife.07025.012

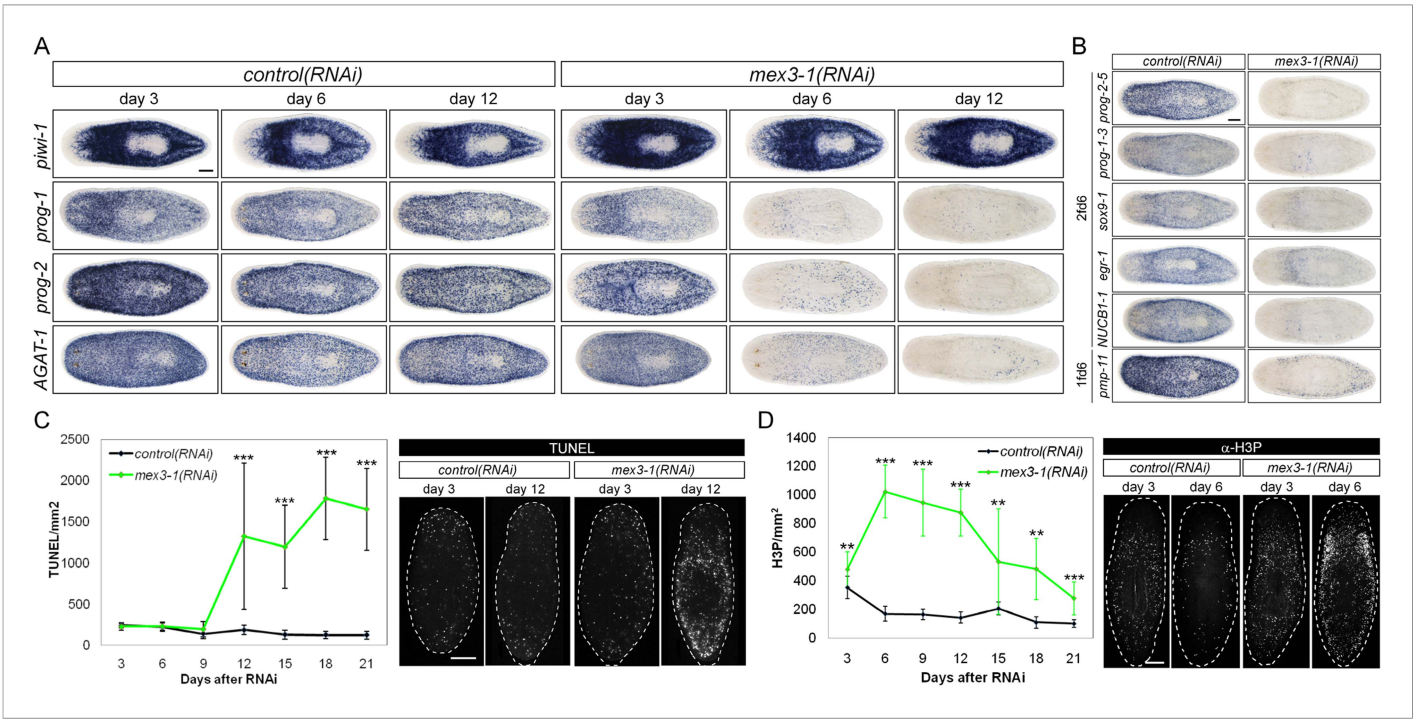
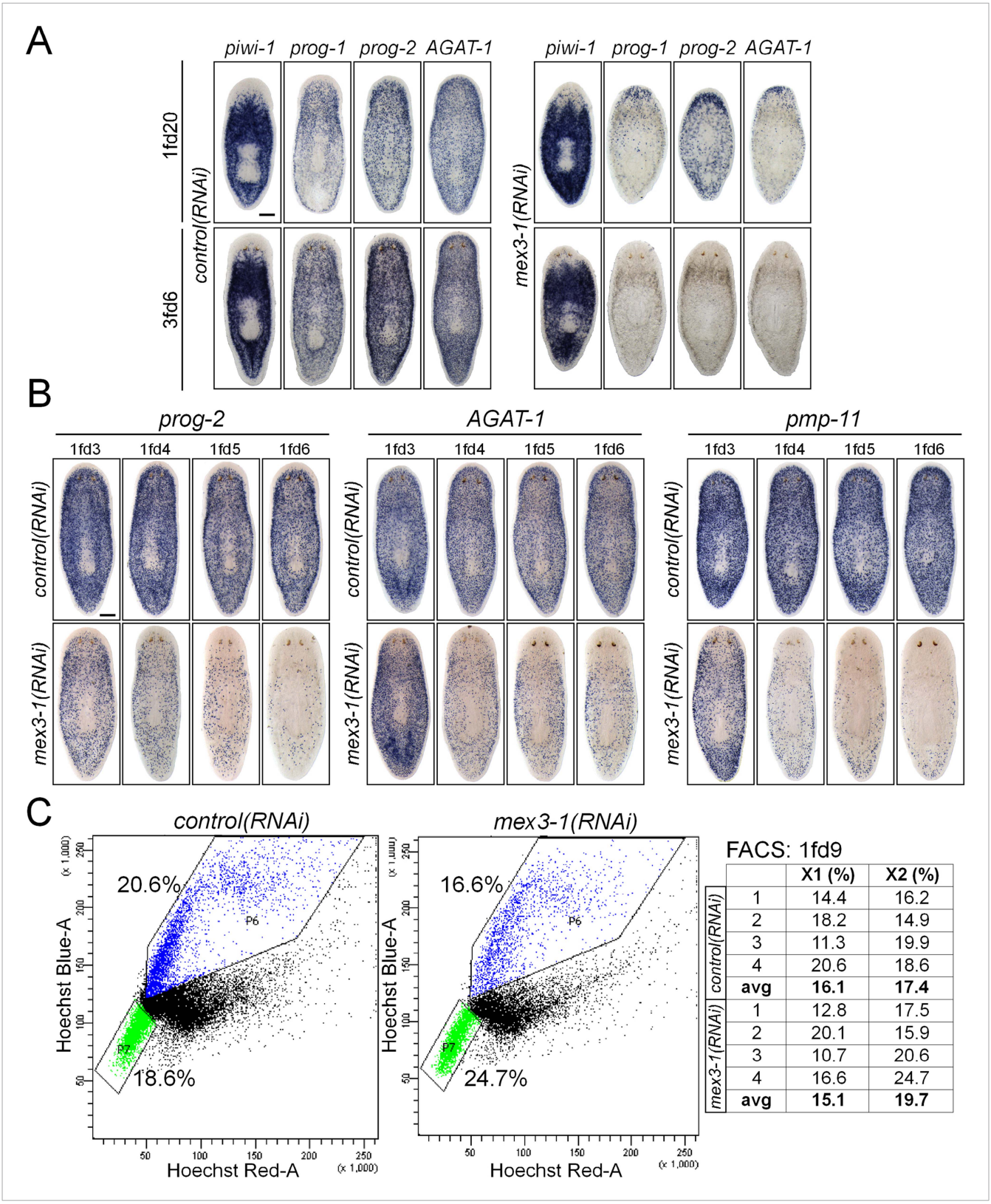


Figure 4. RNAi against *mex3-1* selectively affects progeny markers and causes hyper-proliferation. (**A**) Lineage markers labeling stem cells (*piwi-1*), early (*prog-1*, *prog-2*), and late (*AGAT-1*) progeny were assessed by WISH after RNAi. The day 12 time point reflects the maximal perturbation to progeny markers prior to obvious health decline in the animal. (**B**) Representative newly identified early and late progeny transcripts were assessed by WISH after RNAi. (**C**) Whole-animal quantification of TUNEL was performed to measure cell death in RNAi animals. Representative stains and time points are shown to the right. (**D**) Whole-animal quantification of H3P immunolabeling was performed to assess cell proliferation after RNAi. Representative stains and time points are shown to the right. Unless otherwise noted, all experimental time points are indicated as after a single RNAi feeding. Scale bars, 200 μm. Error bars are standard deviations. **p < 0.01, ***p < 0.001 (Student's *t*-test).

DOI: 10.7554/eLife.07025.013



		X1 (%)	X2 (%)
control(RNAi)	1	14.4	16.2
	2	18.2	14.9
	3	11.3	19.9
	4	20.6	18.6
	avg	**16.1**	**17.4**
mex3-1(RNAi)	1	12.8	17.5
	2	20.1	15.9
	3	10.7	20.6
	4	16.6	24.7
	avg	**15.1**	**19.7**

Figure 4—figure supplement 1. *mex3-1* RNAi depletes progeny without impairing stem cell proliferation. (**A**) Lineage markers labeling stem cells (*piwi-1*), early (*prog-1*, *prog-2*), and late (*AGAT*-1) progeny were assessed by WISH after RNAi. Shown are a late time point after one RNAi feeding and a late

Figure 4—figure supplement 1. continued on next page

Figure 4—figure supplement 1. Continued

time point with multiple RNAi feeds, when health decline is evident. (**B**) Detailed time course analysis of early (*prog-2*) and late (*AGAT-1*, *pmp-11*) progeny marker down-regulation after *mex3-1* RNAi. Scale bars, 200 μm. (**C**) FACS analysis with Hoechst staining of *mex3-1(RNAi)* animals 9 days after RNAi. FACS plots from one run are shown on the left; right table indicates proportion of X1 and X2 populations of all runs.

DOI: 10.7554/eLife.07025.014

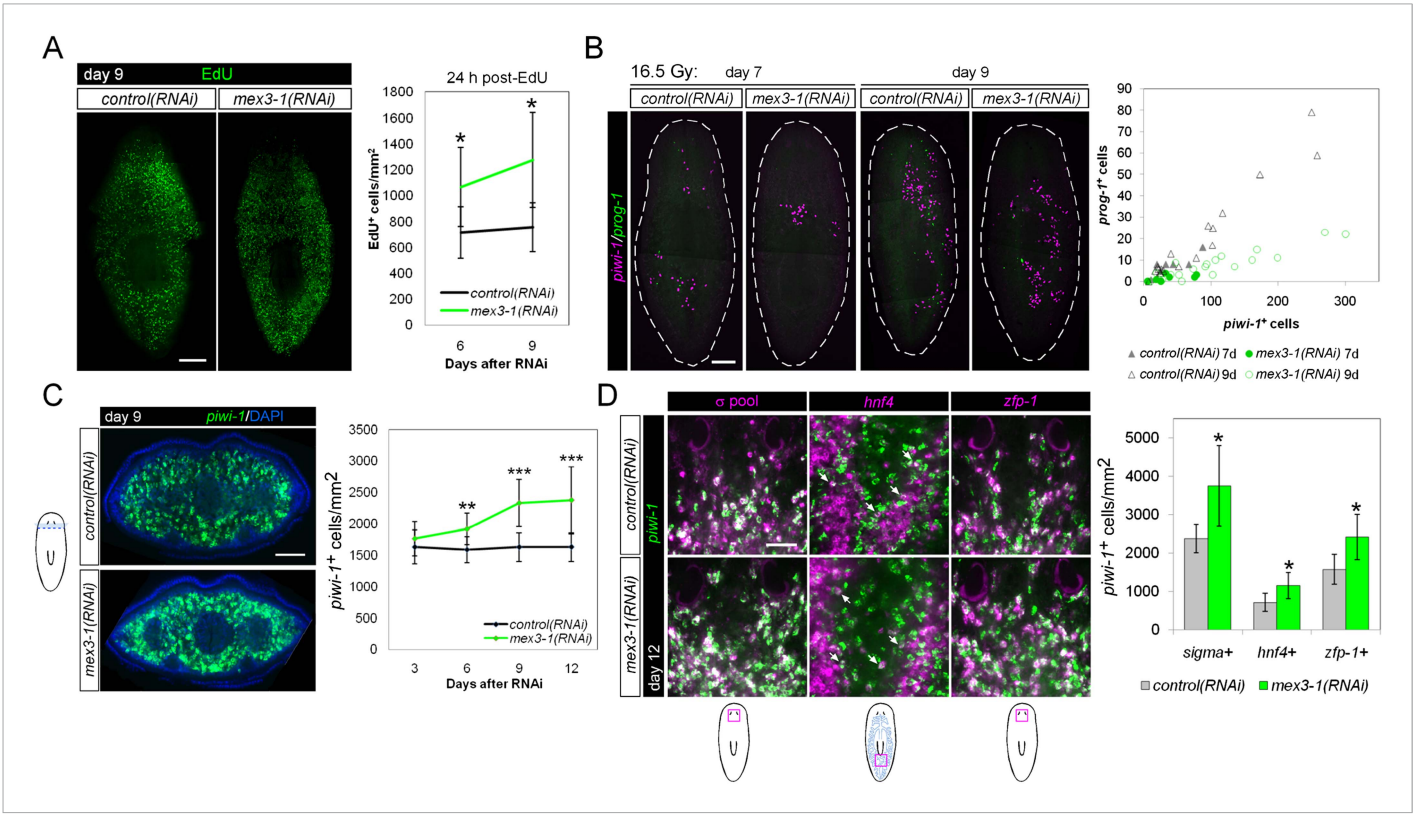


Figure 5. *mex3-1(RNAi)* animals exhibit expansion of the stem cell compartment. (**A**) RNAi worms were administered EdU at 6 or 9 days after RNAi and quantified after a 24-hr period. Counts were performed on whole-animal single confocal planes. (**B**) *mex3-1(RNAi)* animals were irradiated with a sublethal dose (16.5 Gy), and stem cells (*piwi-1*) and progeny (*prog-1*) were quantified by dFISH at 7 and 9 days after irradiation. The proportion of progeny to stem cells between *mex3-1(RNAi)* and control worms differed significantly ($p < 0.001$, analysis of covariance). Whole-animal confocal projections are shown. Each point on the graph represents one animal. (**C**) Stem cells were quantified in pre-pharyngeal cross sections of intact worms after RNAi by *piwi-1* fluorescent WISH (FISH) during phenotypic progression. Single confocal planes at day 9 after RNAi are shown; dorsal, top. (**D**) Quantification of stem cell subclasses in intact worms 12 days after RNAi. Stem cell subclass was determined by *piwi-1* labeling and expression of *soxP-1* pooled with *soxP-2* (sigma subclass), *hnf4* (gamma), or *zfp-1* (zeta). Diagrams indicate areas of worms quantified, and arrows indicate example double-positive cells. Scale bars, 200 μm in (**A**–**C**) and 50 μm in (**D**). Error bars represent standard deviation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's *t*-test).

DOI: 10.7554/eLife.07025.015

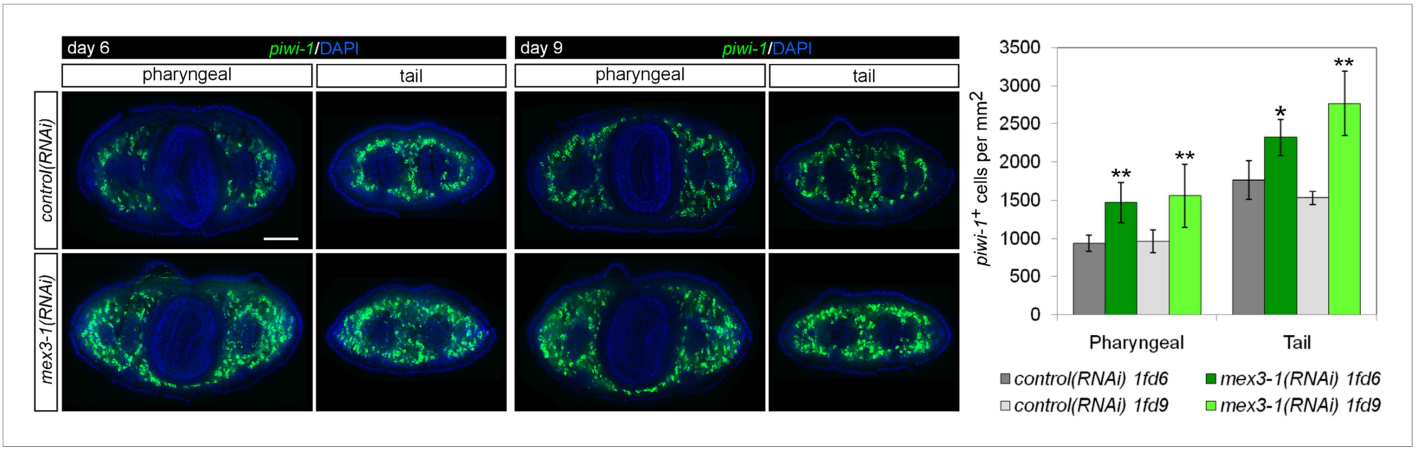


Figure 5—figure supplement 1. Quantification of stem cells in *mex3-1(RNAi)* animals. Stem cells were quantified in mid-pharyngeal and tail cross sections of intact worms at 6 and 9 days after RNAi by *piwi-1* FISH. Single confocal planes are shown; dorsal, top. Scale bar, 200 μm. *p < 0.05; **p < 0.01 (Student's *t*-test). 10 animals were counted per RNAi treatment.

DOI: 10.7554/eLife.07025.016

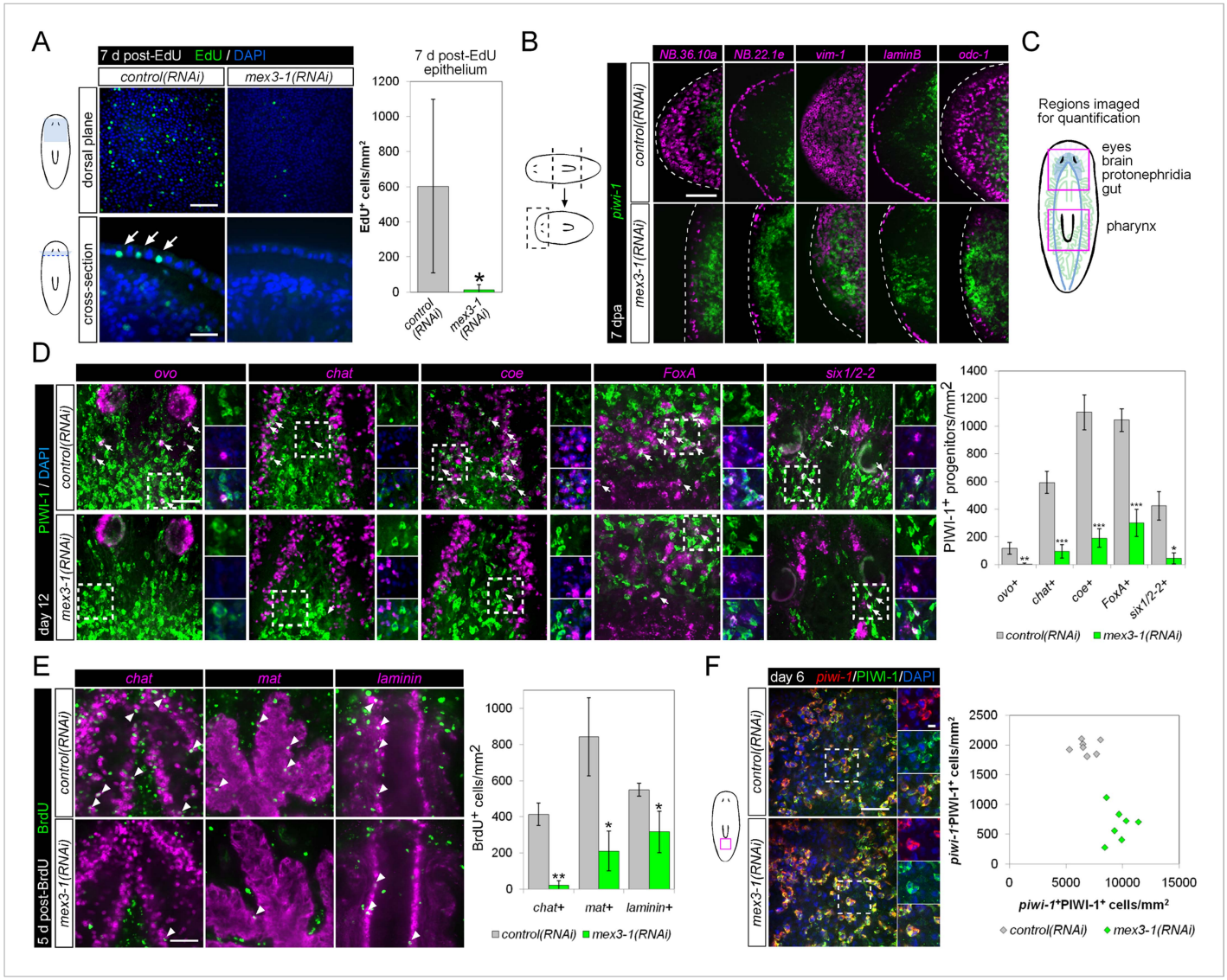


Figure 6. *mex3-1* is required for epithelial turnover and regeneration as well as differentiation toward multiple tissues. (**A**) RNAi animals were administered EdU 6 days after RNAi, and EdU$^+$ labeling in the epithelium was quantified after a 7-day period. Single confocal planes are shown, with EdU$^+$ cells in the epithelium indicated by arrows. Top panel scale bar, 100 μm; bottom panel scale bar, 50 μm. (**B**) RNAi animals amputated 7 days after RNAi were analyzed after 7 days regeneration by dFISH for stem cells and epithelial-associated genes. Single confocal planes or projections of head blastemas are shown, with anterior to the left. Dotted lines indicate animal boundary. Scale bar, 100 μm. (**C**) Diagram indicating regions of animal imaged and examined for quantification of lineage-restricted neoblast progeny. (**D**) Lineage-restricted progeny populations were quantified in intact worms 12 days after RNAi, using PIWI-1 immunolabeling and FISH for *ovo* (eyes), *chat* or *coe* (brain), *FoxA* (pharynx), and *six1/2-2* (protonephridia). Single confocal planes are shown. Arrows indicate example double-positive cells. Magnified areas are indicated by dashed boxes and inset to the right of each image. Scale bar, 50 μm. (**E**) RNAi animals were administered BrdU 6 days after RNAi, and BrdU$^+$ labeling in differentiated tissues (*chat*, brain; *mat*, gut; *laminin*, pharynx) was examined after a 5-day period. Single confocal planes are shown. Arrowheads indicate example double-positive cells. Scale bar, 50 μm. Error bars represent standard deviation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's *t*-test). (**F**) Quantification of stem cells (*piwi-1*$^+$PIWI-1$^+$) and immediate postmitotic stem cell descendants (*piwi-1*$^-$PIWI-1$^+$) were performed 6 days after RNAi, in head, pre-pharyngeal, and tail regions. Shown are single confocal planes from the tail region. Magnified areas are indicated by dashed boxes. The proportion of postmitotic descendants to stem cells between *mex3-1 (RNAi)* and control worms differed significantly ($p < 0.001$, non-linear regression analysis). Scale bar, 50 μm on left panels, 10 μm on right panels. All counts were performed in 5–10 animals.

DOI: 10.7554/eLife.07025.017

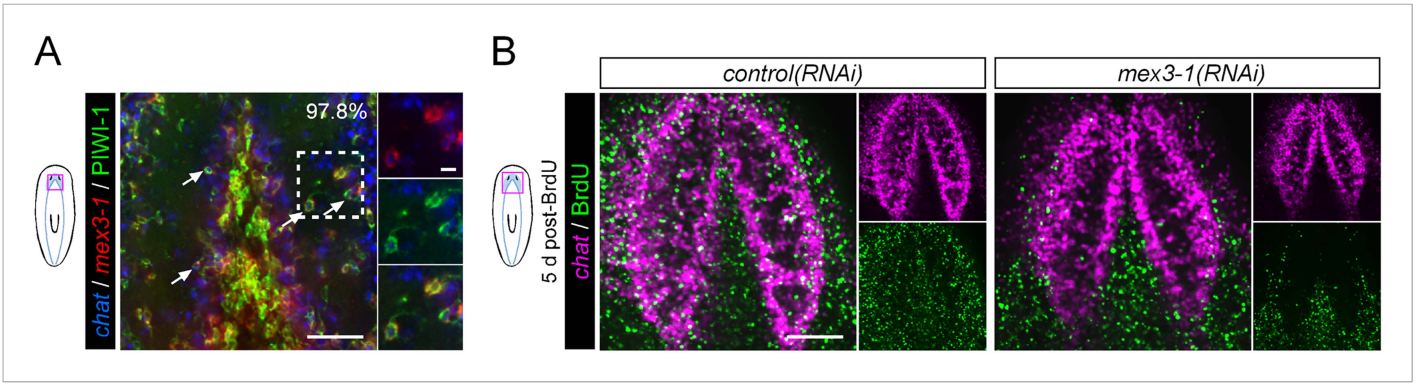


Figure 6—figure supplement 1. *mex3-1* RNAi impairs brain differentiation. (**A**) Expression of *mex3-1* in neural-restricted neoblast progeny by *mex3-1* and *chat* dFISH with PIWI-1 immunolabeling. Percentage of *chat*$^{+}$PIWI-1^{+} cells which express *mex3-1* is indicated at top-right of panel. Arrows indicate example triple-labeled cells. Dashed box indicates area of magnified panels. Left scale bar, 50 μm; right scale bar, 10 μm. (**B**) Animals were administered BrdU at 6 days after RNAi and assessed at 5 days post-BrdU. Scale bar, 100 μm. All images shown are single confocal planes.

DOI: 10.7554/eLife.07025.018

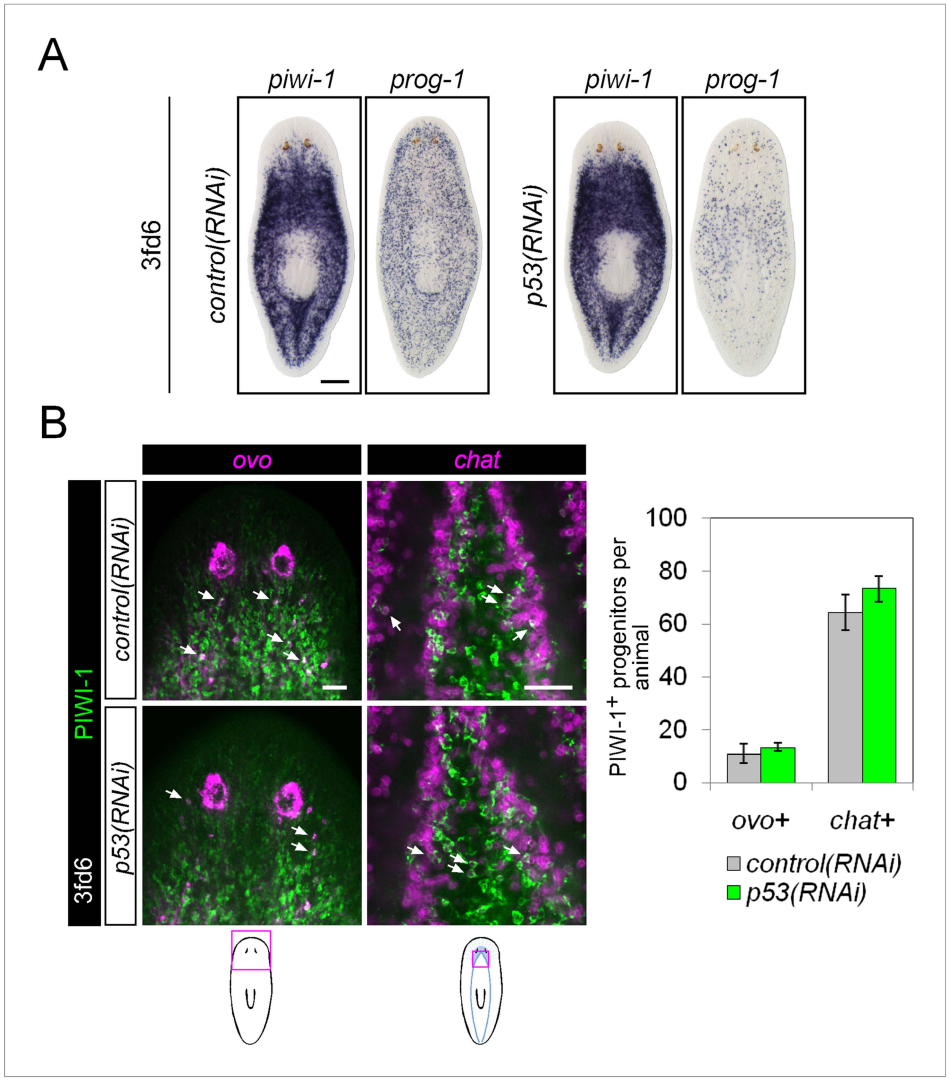


Figure 6—figure supplement 2. Differentiation in *p53(RNAi)* animals. (**A**) Stem cell (*piwi-1*) and epithelial progenitor (*prog-1*) populations were examined by WISH in RNAi animals. Scale bar, 200 μm. (**B**) Eye and neural lineage-restricted neoblast progeny were quantified in RNAi animals by FISH (*ovo*, eye; *chat*, brain) with PIWI-1 immunolabeling. Arrows indicate example double-positive cells. Scale bars, 50 μm.

DOI: 10.7554/eLife.07025.019

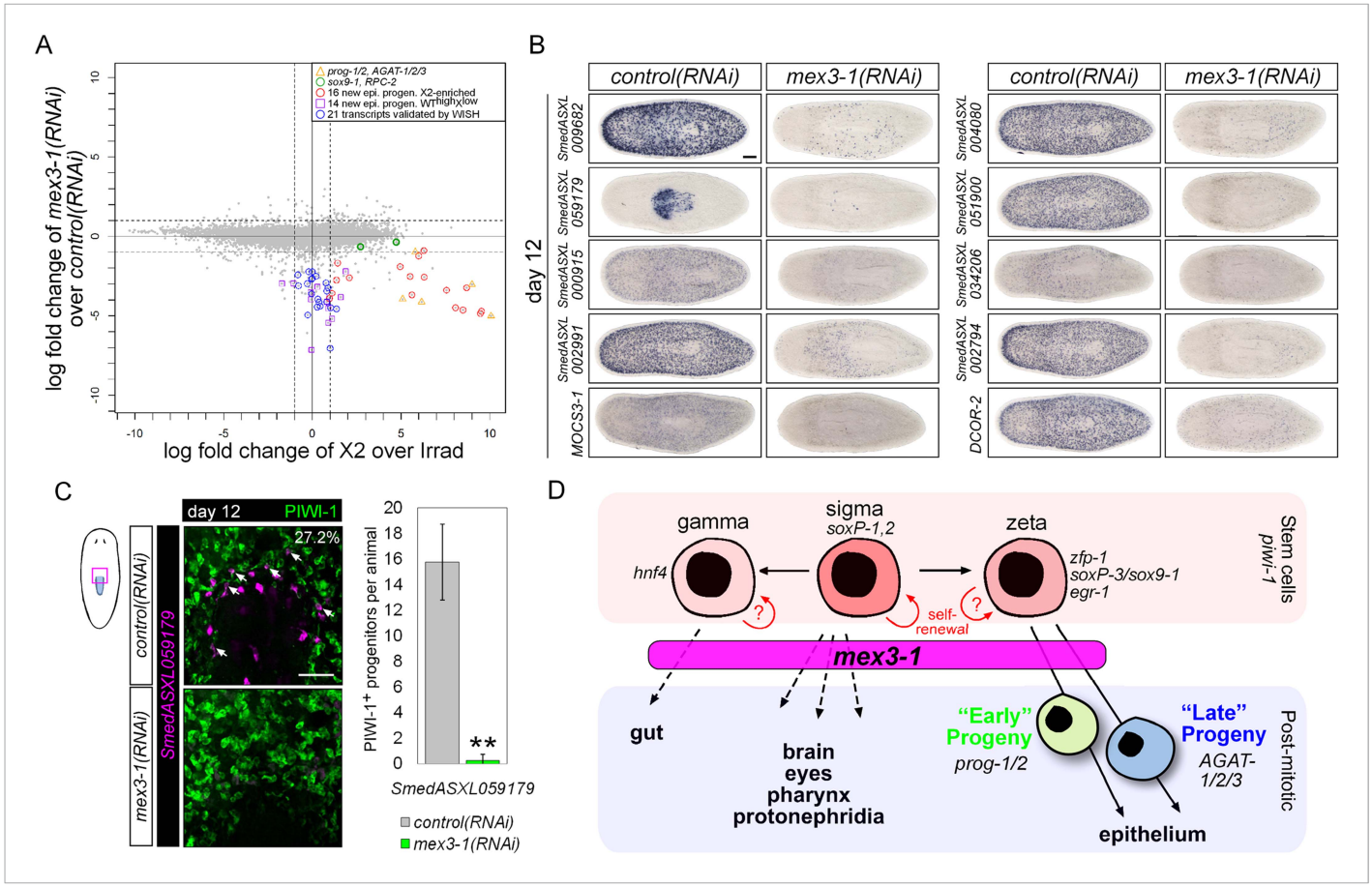


Figure 7. Transcriptional analysis after *mex3-1* RNAi identifies novel progenitor transcripts. (**A**) RNAseq was performed on *mex3-1(RNAi)* animals 12 days after RNAi. Each gray dot represents one transcript. Established and newly identified progeny markers are indicated. Top *mex3-1* down-regulated transcripts cloned out for validation by WISH are indicated as well. (**B**) The blue-circled transcripts from (**A**) all require *mex3-1* for expression. Scale bar, 200 μm. (**C**) Assessment of the *mex3-1(RNAi)*-down-regulated transcript *Smed_ASXL059179* as a marker for a novel pharynx progenitor cell type using FISH and PIWI-1 immunolabeling. Confocal projections in control and *mex3-1* RNAi animals are shown. Error bars represent standard deviation. Scale bar, 50 μm. **$p < 0.01$ (Student's *t*-test). (**D**) Model of lineage specification in planarian stem cells. *mex3-1* is a key determinant in balancing stem cell self-renewal and differentiation, acting as a promoter of postmitotic cell fates and commitment toward multiple lineages.

DOI: 10.7554/eLife.07025.020

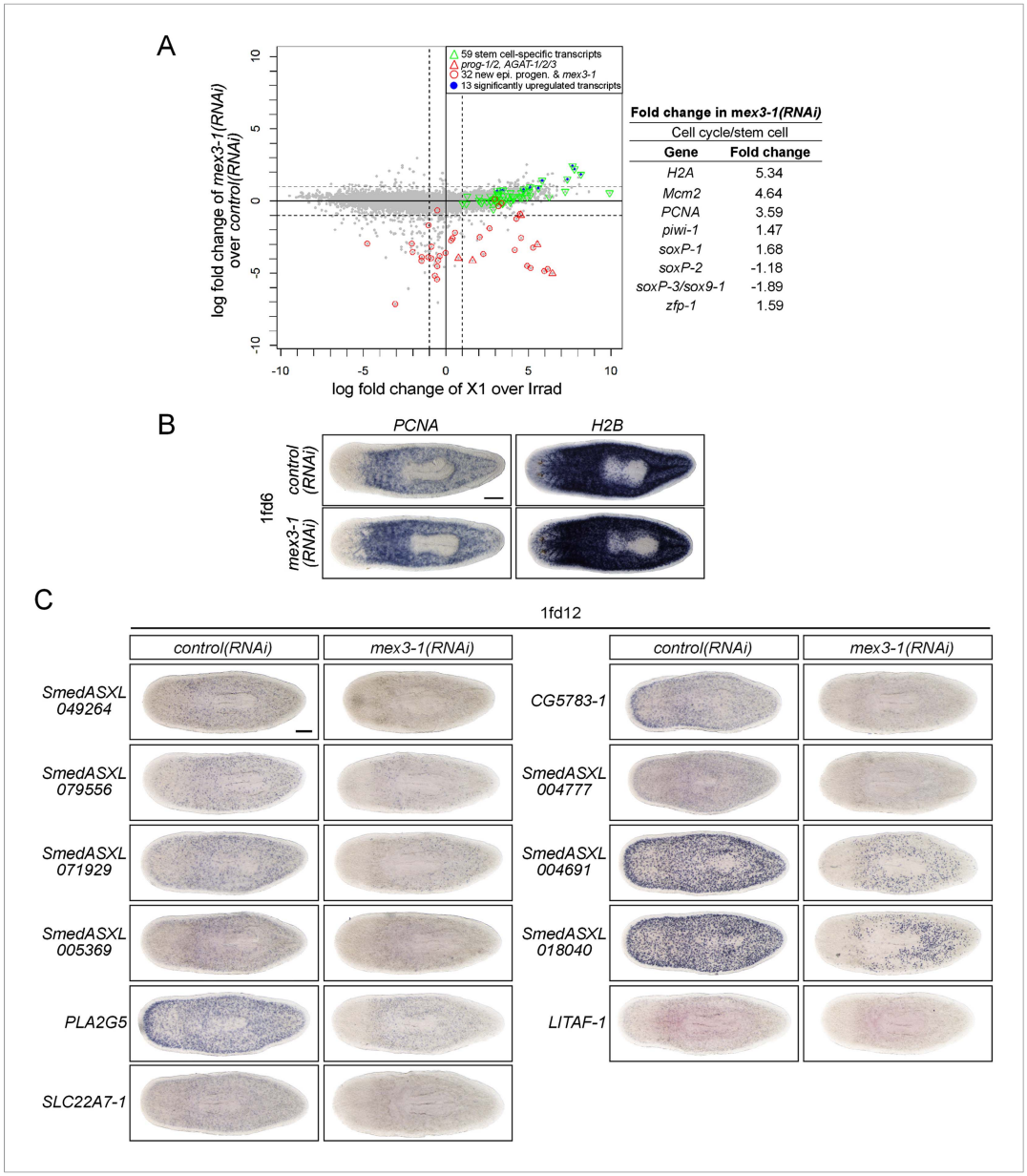


Figure 7—figure supplement 1. RNAseq analysis of *mex3-1(RNAi)* animals. (**A**) RNAseq was performed on *mex3-1 (RNAi)* animals 12 days after RNAi, and X1 enrichment is compared to fold change after *mex3-1* knockdown. Each gray dot represents one transcript. Established stem cell genes (from ***Figure 1B***), which were found to be significantly upregulated in *mex3-1(RNAi)*, are highlighted in blue ($p < 0.01$). The fold changes of a subset of known stem cell genes (involved in proliferation, pan-stem cell gene, or subclass identity) are shown on the right. (**B**) Upregulation of the cell cycle genes *PCNA* and *H2B* in RNAseq analysis was confirmed by WISH of *mex3-1(RNAi)* worms 6 days after RNAi. (**C**) From the top down-regulated genes after *mex3-1* knockdown, 21 uncharacterized genes were chosen for cloning and expression analysis by WISH (11/21 genes are shown). *mex3-1(RNAi)* animals were stained 12 days after RNAi to confirm that target genes were down-regulated. Genes were named based on the best BLASTx result to mouse when the Expect value $<1 \times e^{-5}$, or based on transcript number if no homology was found. Scale bars, 200 μm.

DOI: 10.7554/eLife.07025.021

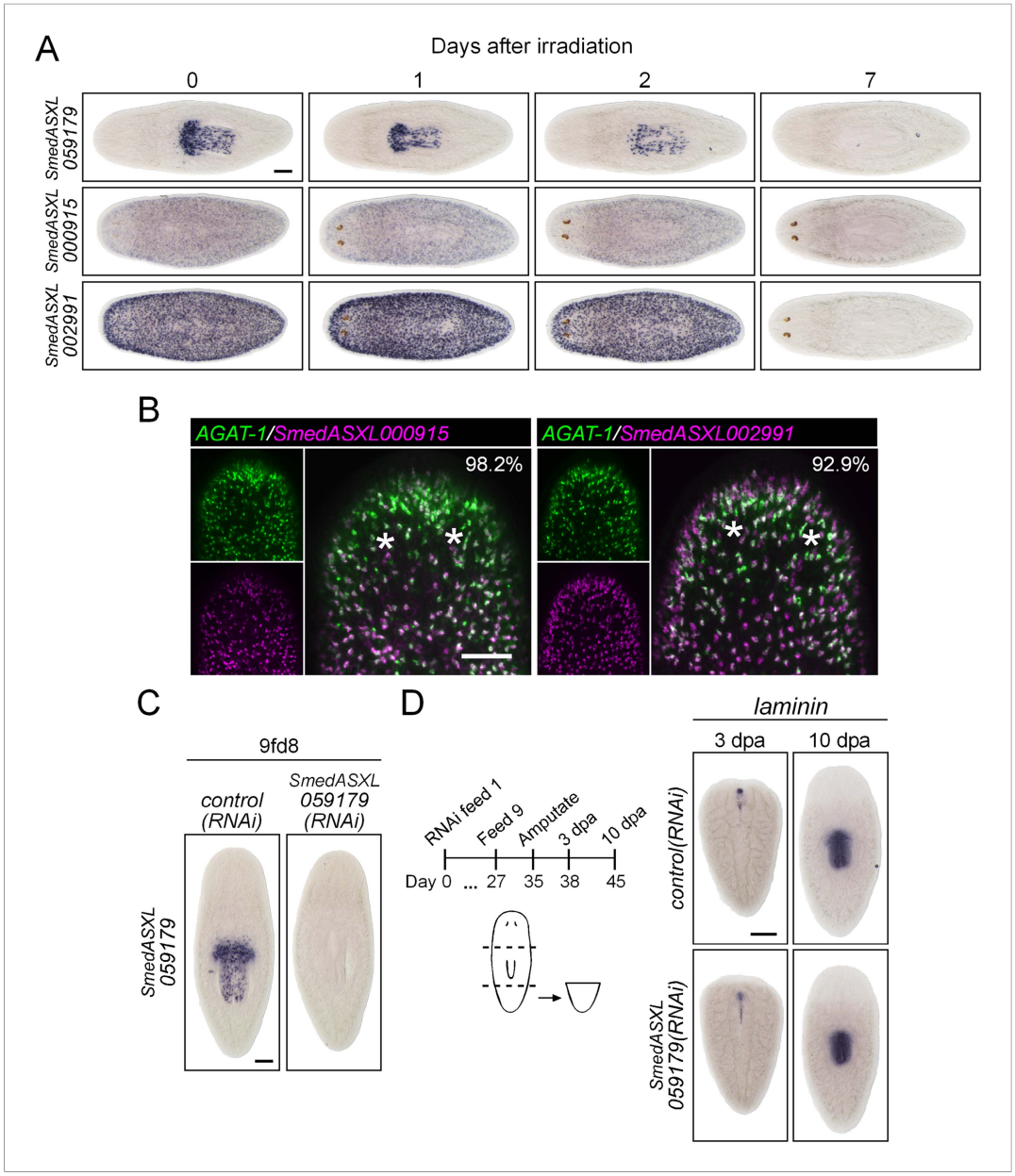


Figure 7—figure supplement 2. Characterization of down-regulated genes in *mex3-1(RNAi)* animals. (**A**) Expression of three genes down-regulated after *mex3-1* RNAi was assessed in lethally irradiated worms by WISH. Scale bar, 200 μm. (**B**) Expression of two *mex3-1(RNAi)*-down-regulated genes in late progeny was examined by dFISH with *AGAT-1*. Percentages of cells that express *AGAT-1* are indicated in the top-right of each panel. Images shown are confocal projections spanning 4 μm in depth. Scale bar, 100 μm. (**C**) Functional analysis by RNAi knockdown of the new pharyngeal progenitor marker *SmedASXL_059179*. Expression of *SmedASXL_059179* after knockdown was examined by WISH. Scale bar, 200 μm. (**D**) Diagram outlining schedule of RNAi feeds and amputation for assessment of pharynx regeneration. Shown is WISH of the pharynx marker *laminin* in regenerating tail fragments. Scale bar, 200 μm.

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