Overlapping functions of microRNAs in control of apoptosis during Drosophila embryogenesis

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Regulation of apoptosis is crucial for tissue homeostasis under normal development and environmental stress. In Drosophila, cell death occurs in different developmental processes including embryogenesis. Here, we report that two members of the miR-2 seed family of microRNAs, miR-6 and miR-11, function together to limit the level of apoptosis during Drosophila embryonic development. Mutants lacking both miR-6 and miR-11 show embryonic lethality and defects in the central nervous system (CNS). We provide evidence that miR-6/11 functions through regulation of the proapoptotic genes, reaper (rpr), head involution defective (hid), grim and sickle (skl). Upregulation of these proapoptotic genes is responsible for the elevated apoptosis and the CNS defects in the mutants. These findings demonstrate that the activity of the proapoptotic genes is kept in check by miR-6/11 to ensure normal development.

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Apoptosis is an important regulatory mechanism during growth development of animal embryos, and in disease. Cell proliferation is intimately linked with cell death. Cues that drive cell growth and division also induce apoptosis. Overcoming the apoptosis barrier is a critical step in the ability of cancers to grow in vivo. Many of the signaling pathways implicated in the normal control of tissue growth during animal development have been found to coordinate cell proliferation and apoptosis. For example, the Hippo signaling pathway has an evolutionarily conserved role in controlling tissue growth rates and organ size during animal development and mutations that cause a net gain of function can lead to cancer.

In Drosophila, apoptotic inputs converge to a common death program through the activation of the proapoptotic genes: reaper (rpr), grim, head involution defective (hid) and sickle (skl). The proapoptotic activity of the protein products of these four genes results from their ability to bind and inactivate the Drosophila Inhibitor of Apoptosis (DIAP), which in turn inhibits Caspsases. Activity of the proapoptotic genes has been identified as a key target of many signaling pathways that regulate growth and patterning in Drosophila. Targets of the Hippo signaling pathway include regulators of cell proliferation, including cyclE and myc as well as regulators of apoptosis, including DIAP and the antiapoptotic bantam miRNA. Signaling via the MAPK pathway also controls hid expression and activity. In addition, environmental stress such as, UV and X-ray can activate the DNA damage p53 pathway to regulate expression of rpr. Wingless signaling activates the expression of hid, rpr and grim to induce apoptosis in eye development. The steroid hormone ecdysone signaling is required for the induction of rpr and hid during metamorphosis.

Emerging evidence has shown that miRNAs have a key role in controlling apoptosis to maintain the balance of cell life and death by targeting proapoptotic or antiapoptotic genes. For instance, miR-21 functions as an antiapoptotic factor in many different cancer cells. The miR-34 microRNA family can induce apoptosis and its expression is upregulated in many tumor types.

Results

Generation and characterization of miR-11 mutant alleles. To explore the functions of the members of the miR-2 family we have generated mutants that remove miRNA function. The miR-2 seed family contains 13 precursor miRNAs, which generate 8 different mature miRNAs (Figure 1a). The 8 mature miRNAs could fall into two subgroups according to 3’ portion similarity: the miR-2/13 group and the miR-6/11 subgroup of the miR-2 seed family. These studies provide evidence for partially redundant functions in control of apoptosis for miR-6 and miR-11 during development of the embryonic central nervous system (CNS).

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Abbreviations: rpr, reaper; hid, head involution defective; skl, sickle; DIAP, Drosophila Inhibitor of Apoptosis

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the three genomic copies of miR-6, along with 5 other miRNAs, referred to here as the miR-6-cluster. Figure 1c illustrates the targeting strategy used to knockout the closely related miR-11 gene.

miR-11 is located in an intron of the E2F gene, which encodes a cell cycle transcription factor. The targeting strategy involved replacing miR-11 sequences with a mini-white reporter gene flanked by loxP sites and by inverted attP sites to allow recombinase mediated cassette exchange (RMCE) at the targeted locus (as described in Weng et al.30). Introns in the mini-white gene are expected to disrupt splicing of the host gene E2F. We confirmed that this was the case by crossing the miR-11 targeted allele miR-11KO (w+/C0) to a null allele of E2F. The combination was semi-lethal, indicating disruption of E2F function. To generate an allele of miR-11 that did not impair E2F function, the mini-white cassette was excised by expression of Cre recombinase. The resulting allele lacked the mini-white marker, but retains a single LoxP site in the intron, as well as the inverted attP sites (Figure 1c, third diagram).

Quantitative RT-PCR analysis showed that the miR-11 miRNA was absent in RNA samples from the homozygous miR-11KO (w/C0) mutant (Figure 1d). We made use of the inverted attP sites to prepare a genetic rescue using RMCE to replace the miR-11 hairpin back into the targeted locus (Figure 1c, fourth diagram). The rescue construct restored miR-11 RNA to ~90% of the control levels (Figure 1d).

As the miR-11 locus is located in the intron of E2F we wanted to determine whether the effects of the mutant alleles could be cleanly attributed to loss of miR-11. Quantitative RT-PCR, using exon specific primers showed that mature spliced E2F mRNA levels were near normal in the homozygous miR-11KO (w–) mutant (Figure 1d). When crossed to a null allele of E2F, the miR-11KO (w–)/E2F trans-heterozygous combination was viable. Thus the miR-11KO (w–) allele does not meaningfully impair function of the E2F gene.

Overlapping roles of miR-11 and miR-6. As reported previously, a deletion removing the miR-6 cluster, which contains
the three miR-6 genes, showed a modest reduction in viability to adulthood (confirmed in Figure 1e). miR-11 mutants were normally viable. However, the miR-6, miR-11 double mutant combination showed a strong reduction in survival to adulthood (Figure 1e). This suggests that miR-11 and the miR-6 cluster may have partially overlapping functions during Drosophila development. To explore these functions in more detail we examined combinations of these mutants for their ability to complete embryogenesis.

miR-6 expression is undetectable during oogenesis, but is transcriptionally upregulated at the onset of zygotic transcription in the blastoderm-stage embryo and continues to be expressed throughout embryonic development.32 As a consequence we do not expect maternal contribution of miR-6 to support its function in the embryo. In contrast, miR-11 is the thirteenth most abundant miRNA in ovaries,33 allowing for the possibility that maternally provided miR-11 could suffice for embryonic development. Homozygous miR-11 mutants showed no significant reduction in the completion of embryogenesis compared with controls (Figure 2a). Similarly, homozygous miR-6 cluster deletion mutants showed little or no effect. We noted that removing one copy of miR-11 in the miR-6 cluster mutant background resulted in a modest but statistically significant decrease in survival (Figure 2a). The reciprocal combination, removing one copy of miR-6 cluster in the miR-11 homozygous mutant background did not significantly affect survival.

The miR-6/11 double mutant combination showed strong embryonic lethality (Figure 2a). This lethality could be rescued by restoring expression of miR-11 in the double mutant
background using the miR-11R allele. The miR-11f allele was made by reintroducing miR-11 sequences back into the targeted miR-11KO mutant chromosome, using RMCE. Reconstructing a functional miR-11 locus in the mutant chromosome provides a consistent genetic background for the comparison. Unlike the original miR-11KO mutant chromosome, introducing one copy of the miR-11R allele/miR-11f allele does not affect survival of the miR-6 mutant (Figure 2a; there was no significant difference between miR-6 mutants with two normal alleles of miR-11 versus one normal allele/miR-11f). Restoring miR-6 activity in the double mutant combination by introduction of a rescue transgene expressing miR-6 under control of its endogenous promoter was also sufficient to rescue the lethality of the double mutant (Figure 2a). The transgene expressed miR-6, but not the other members of the cluster: miR-3, miR-4, miR-5, miR-286 and miR-309. This indicates that the absence of the other 5 miRNAs is not responsible for the lethality of the double mutant. These data suggest that miR-11 and miR-6 have overlapping functions during embryogenesis. Consistent with this proposal, these miRNAs are broadly coexpressed in the embryo, with miR-11 showing slightly elevated expression in the embryonic CNS (Figure 2b).

Embryonic CNS defects in miR-6/miR-11 double mutants. Having established that the miR-11 miR-6 double mutant combination shows reduced survival during embryonic development, we examined the mutants for the evidence of defects that could be associated with lethality. There was no indication for reproducible defects in the patterning or differentiation of epidermal cuticle or of the head skeleton, suggesting that major processes of morphogenesis proceeded more or less normally in the mutants. However, a reproducible defect was observed in the organization of the embryonic CNS, using BP102 antibody to label the axonal scaffold of the CNS (Figures 2c and d). Longitudinal connectives were thin and showed occasional gaps between segments, typically interrupting the nerve cord in the mid-abdominal region. Commissures were also abnormal in spacing and thickness. In all 100% of double mutant embryos showed abnormal CNS morphology in at least one segment (Figure 2e). On average 2 segments per embryo lacked longitudinal connectives (Figure 2f). These defects were not observed in double mutant embryos rescued by inclusion of the miR-6 rescue transgene, and were considerably less frequent in double mutant embryos rescued by inclusion of the rescue allele miR-11R (Figures 2e and f).

To examine the CNS axonal scaffold phenotype in more detail, we asked if the midline glial cells were affected in the double mutant embryos. Midline glia has a key role in organization of the axon scaffold.34 Embryos were labeled with antibody to the midline glia-specific marker Wrapper, a member of the immunoglobulin superfamily.35 Wrapper-expressing cells were irregular in shape and spacing, and in some cases were lost in the affected region of the double mutant embryos (Figure 2g).

miR-6/miR-11 double mutant phenotypes due to elevated apoptosis. The observed defects in the CNS development would be consistent with elevated apoptosis. Antibody labeling to visualize the activated form of Caspase 3 showed elevated Caspase activity in the double mutant embryos (Figure 3a). Previous reports have suggested that members of the miR-2 seed family can regulate the expression of proapoptotic genes, including rpr, grim and skl.25–27 We therefore sought to measure the expression of the proapoptotic genes in miR-6/miR-11 double mutant embryos. Quantitative RT-PCR showed that rpr, grim, skl and hid were upregulated by 2–4 fold in RNA samples from miR-6/miR-11 double mutant embryos (Figure 3b).

**Figure 3** Regulation of proapoptotic genes by miR-6/miR-11. (a) Lateral views of embryos labeled with antibody to the activated form of Caspase 3 (red) and with DAPI (blue). Scale bar: 50 μm. Stacks of confocal sections were used to create 3D-reconstructions with the ‘isosurface’ module of Imaris software and the total enclosed volume was measured as a surrogate to quantify apoptosis (arbitrary units in the histogram). Error bars represent standard deviation from analysis of four embryos of each genotype. * indicates that the difference was statistically significant, *P < 0.05 (Student’s t-test). Scale bar: 100 μm. (b) Histogram showing the levels of rpr, grim, hid and skl mRNAs measured by quantitative RT-PCR. RNA was extracted from w1118 control embryos and embryos doubly mutant for miR-6-cluster and miR-11f. Error bars represent standard deviation from three independent experiments.
To ask whether the elevated expression of proapoptotic genes was sufficient to explain the embryonic lethality observed in the double mutant embryos, we made use of a set of chromosomal deletions that remove hid, rpr, grim and skl. Df(3L)H99 removes rpr, grim and hid. Df(3L)X38 removes rpr and skl. Df(3L)X14 removes hid. Each of these deletions was recombined onto the miR-11 mutant chromosome, and the recombinant chromosome was introduced into the miR-11/miR-6 double mutant background. The mir-6 miR-11 double mutant embryos carrying Df(3L)X38 have only one copy of the rpr and skl genes, which should limit their ability to overexpress rpr and skl. This combination showed a statistically significant partial suppression of the lethality of the mir-6 miR-11 double mutant embryos (Figure 4a). Removing one copy of hid in the mir-6 miR-11 double mutant background using the Df(3L)X14 recombinant produced strong suppression of the lethality (Figure 4a). Removing one copy each of rpr, grim and hid in the miR-6 miR-11 double mutant background using the Df(3L)H99 recombinant also produced strong suppression (Figure 4a). The Df(3L)H99 recombinant also showed suppression of the milder lethal phenotype in the mutant combination lacking miR-6 but having one copy of miR-11 (Figure 4a).

We made use of the strong suppression provided by introducing the Df(3L)H99 to examine the suppressed phenotype in more detail. The axonal scaffolding defects were suppressed in the embryonic CNS of the miR-6 miR-11 double-mutant embryos carrying Df(3L)H99 (Figure 4b). The percentage of double mutant embryos showing defects in the patterning of the embryonic CNS decreased from 100 to ~ 15% (Figure 4c), and the number of affected segments per embryo decreased from an average of 2 to ~ 0.2 (Figure 4d). These experiments provide evidence that the lethality observed in the miR-6 miR-11 double mutants was caused by elevated proapoptotic gene expression, and show a correlation between the morphological defects in the CNS and lethality. They provide evidence that multiple proapoptotic genes can contribute to the lethality, but suggest that hid has a significant role.

Misregulation of proapoptotic genes in miR-6/miR-11 double mutant embryos. The 3’UTRs of the proapoptotic genes, rpr, grim, skl and hid contain predicted binding sites for miR-6 and miR-11. Depletion of miR-6 in the embryo using antisense oligonucleotides has been reported to affect the expression of co-injected 3’UTR reporters for hid, skl and rpr, whereas depletion of miR-11 affected rpr, grim and skl 3’UTR reporters. 27 To ask whether the effects of miR-6 and miR-11 were direct, we prepared luciferase reporters in which their predicted target sites were mutated. Coexpression of miR-6 or miR-11 with the intact grim reporter transgenes in S2 cells led to downregulation of luciferase activity (Figure 5a). In both cases mutation of the predicted site abrogated this downregulation. Coexpression of miR-6 or miR-11 with the intact rpr reporter transgenes in S2 cells led to downregulation of luciferase activity (Figure 5b). Again, mutation of the predicted site abrogated downregulation. The skl reporter has two predicted sites. This reporter was also downregulated by coexpression of miR-6 or miR-11 (Figure 5c). Mutation analysis showed that site 2 was functionally important, whereas mutation of site 1 had no effect. Comparable experiments using the hid 3’UTR reporter in S2 cells showed an unanticipated upregulation by both miR-6 and miR-11. This was observed for the intact and the target-site mutant versions of the hid 3’UTR (data not shown). This suggests that the miRNAs indirectly alleviated repression of the hid 3’UTR reporters, perhaps as a consequence of regulating another target in the S2 cells. Using the full length 3’UTR, we cannot determine whether they directly affect hid regulation via the predicted sites. However, a recent report has provided independent evidence that miR-11 can function via smaller fragments of the hid 3’UTR containing the predicted sites. 36 These observations suggest that that miR-6 and miR-11 can each function via the identified sites in the 3’UTRs of grim, rpr and skl to regulate their expression.

Discussion

Multiple members of a miRNA family target the proapoptotic pathway. In this study, we have presented evidence that miR-6 and miR-11 control apoptosis in vivo, through regulation of genes of the proapoptotic pathway. Limiting the capacity of the miR-6/miR-11 mutants to overexpress the proapoptotic genes was sufficient to rescue the mutants to viability and to restore normal patterning of the CNS. This suggests that the proapoptotic genes rpr, grim, skl and hid...
are biologically significant targets through which miR-6/11 functions in vivo. It is noteworthy that the two miRNAs appear to have overlapping functions. They are coexpressed in the embryo, and removal of both is required to elicit a strong phenotype. Removing one copy of miR-11 in the miR-6 mutant background showed a slight effect, but the reciprocal combination did not. There are three copies of miR-6 in the miR-309 cluster and based on miRNA sequence data, miR-6 is considerably more abundant than miR-11 at most stages of embryogenesis. Consistent with this, expression from one copy of the miR-6 locus is sufficient, whereas expression from one copy of miR-11 is not quite sufficient to provide normal function. However, the observed 5–10 fold disparity in their relative copy number in total embryonic RNA might be misleading. It is possible that their activity levels are more

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**Figure 5** miRNA sites in the proapoptotic genes. (a) Regulation of the grim 3’UTR reporter by miR-6 (left panel) and miR-11 (right panel). Diagram illustrating the predicted pairing between the miRNAs and the predicted site in the grim UTR is shown above the histograms. Changes introduced into the seed region to destroy the binding site are shown in red. Histograms show the effect of coexpressed miR-6 or miR-11 on the luciferase activity from the reporter transgene with the intact (grim-3’UTR) or mutated target sites (grim-mut-3’UTR). Control samples were cotransfected with the empty miRNA vector and did not express the miRNA. Error bars represent standard deviation from three independent experiments. * indicates that the difference between the indicated pair was statistically significant, P < 0.05 (Student’s t-test). (b) As in a, depicting the rpr 3’UTR and the corresponding luciferase assays. (c) As in a, depicting two sites in the skl 3’UTR and the corresponding luciferase assays.
comparable in the developing embryonic CNS, where miR-11 appears to be relatively more abundant. Tissue specific data on miRNA sequence levels would be needed to address this. The miR-2 seed miRNA family has six other members, which might contribute to regulation of the proapoptotic genes. The ability of the miR-2 and miR-13 groups of miRNAs to regulate 3'UTR reporters for the proapoptotic genes has been demonstrated. Injection of antimirs directed against oligonucleotides (antimirs) to deplete miRNA function in the embryo was reported to cause severe apoptosis in embryos, with milder effects following injection of antimirs to miR-11. However, as reported here, mutants removing miR-11 or removing miR-6 miRNAs individually did not cause embryonic lethality (this work and Bushati et al26). Instead, we found that embryonic lethality and elevated apoptosis resulted in the double mutant combination that simultaneously removes both miR-11 and miR-6. This lethality could be rescued by restoring expression of miRNA genetically. Assessment of the evidence suggesting that depletion of miR-2 and miR-13 group miRNAs results in embryonic apoptosis,27 should await functional characterization of loss-of-function mutants that remove their functions in vivo.

Roles of miRNAs in embryonic CNS development. Apoptosis has a key role in pattern formation during development of the nervous system. Pruning of excess cells by apoptosis is central to the developmental of the CNS. In the developing retina, EGFR-mediated signaling selects cells to form ommatidia,37 whereas excess cells are pruned through apoptosis. Genetic analysis has shown that rpr is involved in the pruning of neuroblasts.13 Regulated apoptosis of glia is also important in CNS patterning, as these cells contribute to the correct organization of the axon scaffold. A subset of CNS midline glia cells undergoes apoptosis, resulting in selection of 3 cells per segment from initial pool of 10 cells. Cell interactions may provide trophic signals that support survival of these glia. Intriguingly, previous studies have shown that overexpression of rpr and hid, or grim in the midline glia leads to axon scaffold defects38,39 that resemble those we have observed in the miR-6/11 double-mutant embryo. The abnormal pattern of the axonal scaffold in miR-6/11 double-mutant embryo might be due to loss of midline glia as a consequence of the failure to limit proapoptotic gene expression. These miRNAs may contribute to maintaining the balance between cell survival and apoptosis during patterning of the CNS in the embryo. It is possible that the other members of the miR-2 family of miRNAs have similar roles in other aspects of the CNS development, or in other tissues.

Materials and Methods
Fly strains and genetics. D;3L;UAS-PRD, D;3L;X83 and D;3L;X14 flies were provided by Kristin White. The miR-6 genomic rescue transgene was made by cloning genomic fragments containing the promoter and miR-6 hairpin into site-specific integration vector pB3.5. Promoters for the promoter were 5'-CTGGT AAGAAGCTGAGCCCGCTACCAAACAAAGAATGCTGATTG-3' and 5'-GATTTGCTGACTTTAACATCTGTGGAGGAGAGACACAC-3'. Measurement of loss-of-function mutants that remove their functions following injection of antimirs to have been examined using injection of 2-O-methyl antisense oligonucleotides (antimirs) to deplete miRNA function in the embryo. Injection of antimirs directed against miR-6 was reported to cause severe apoptosis in embryos, with milder effects following injection of antimirs to miR-11. However, as reported here, mutants removing miR-11 or removing miR-6 miRNAs individually did not cause embryonic lethality (this work and Bushati et al26). Instead, we found that embryonic lethality and elevated apoptosis resulted in the double mutant combination that simultaneously removes both miR-11 and miR-6. This lethality could be rescued by restoring expression of miRNA genetically. Assessment of the evidence suggesting that depletion of miR-2 and miR-13 group miRNAs results in embryonic apoptosis,27 should await functional characterization of loss-of-function mutants that remove their functions in vivo.

Generation of microRNA knockout mutants. miRNA mutants were generated using homologous recombination-based ends-out gene targeting,53 using vectors as described.54 Briefly, homology regions flanking miR-11 were amplified and cloned into the targeting vector pW25-RMCE. Details of the targeting protocol are available on request (or see Chen et al55). Two pairs of primers were used to amplify upstream and downstream flanking sequences:

upstream flank: 5'-GGCGCGCGCAGAAATCAGATGAGCAGGAGCC-3' and 5'-GGCGCGCGCAGAAATCAGATGAGCAGGAGCC-3'. Measurements of loss-of-function mutants that remove their functions following injection of antimirs to have been examined using injection of 2-O-methyl antisense oligonucleotides (antimirs) to deplete miRNA function in the embryo. Injection of antimirs directed against miR-6 was reported to cause severe apoptosis in embryos, with milder effects following injection of antimirs to miR-11. However, as reported here, mutants removing miR-11 or removing miR-6 miRNAs individually did not cause embryonic lethality (this work and Bushati et al26). Instead, we found that embryonic lethality and elevated apoptosis resulted in the double mutant combination that simultaneously removes both miR-11 and miR-6. This lethality could be rescued by restoring expression of miRNA genetically. Assessment of the evidence suggesting that depletion of miR-2 and miR-13 group miRNAs results in embryonic apoptosis,27 should await functional characterization of loss-of-function mutants that remove their functions in vivo.

Examination of embryonic development phenotype. 0–6 h embryos were collected, and either plated on a fresh apple juice plate immediately or aged overnight before plating. GFP balance was used for identifying mutant chromosome. Mutant embryos were selected by separating GFP-expressing embryos from GFP-negative embryos under a fluorescence microscope. Percentage of completion of embryogenesis was determined by counting the number of hatchng embryos that gave rise to first instar larva after 48 h.

Cell culture and luciferase assays. S2 cells were grown at 25°C in Express five SFM (Invitrogen) supplemented with L-glutamine. Rpr, grim, hid and sir 3'UTR luciferase reporters and miR-6 or miR-11 expression plasmids were expressed under the control of the tubulin promoter. Details of the plasmids are available on request. S2 cells were transfected using Cellfectin (Invitrogen, Carlsbad, CA, USA) in 96-well plates with 80 ng of miR-6 or miR-11 expression plasmid or empty vector, 10 ng of firefly luciferase reporter plasmid and 10 ng of Renilla luciferase DNA as a control. Transfections were done with triplicate technical replicates in three independent experiments. Dual luciferase assays (Promega, Madison, WI, USA) were performed on the transfected cell at 60 h post transfection.

qRT-PCR. Total RNA was extracted from samples with TRIzol reagent (Invitrogen). Quantitative real-time PCR was performed using an ABI7500 fast real-time PCR machine (Applied Biosystems, Foster City, CA, USA). Taqman miRNA assay was used for miRNA qRT-PCR. Primer sets were obtained from Applied Biosystems. Reverse transcription was done on 10 ng of total RNA and miRNA levels was normalized to u27. For miRNA qRT-PCR, total RNA was treated with RNAase-free DNasel. cDNA was synthesized by using oligo-dT primers and Superscript RT-III (Invitrogen). Samples were then treated with RNAseH and used for miRNA qRT-PCR with the ABI SYBR green system. Measurements of transcript level were normalized to rp49. The following primers were used for qRT-PCR:

rpr: 5'-GCTAAAAGCGCTGACAAAATA-3' and 5'-TCCGGTCCAAGCTGAGC-3':52; 5'-ACAGCAAGACAGAGCTGTGC-3' and 5'-TGATGGGACACAAAGCC-3'.

skl: 5'-ACCAGGAGAAAGCACAGC-3' and 5'-GTTGCGCTTTAGTTGCTGAC-3'.

grim: 5'-ACAGCAACATCGCAGCAGC-3' and 5'-CAGAAAGTCTGGGCCAA-3'.

tat: 5'-GGACAGGAAGGACGACATG-3' and 5'-GGACTTTCTCCGGTCTGAC-3'.

híd: 5'-CCTTACAGTGCTGAGGC-3' and 5'-CGTCGGGAAAGACACACACGAG-3'.

Antibody labeling and in situ hybridization. Mouse monoclonal antibody BP102 (Developmental Studies Hybridoma Bank, DSHB, Iowa City, IA, USA) was used at 1:50. Mouse monoclonal antibody anti-Wrap (DSHB) was used at 1:10. HRP-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA, USA) was used at 1:500. For immunohistochemical detection, a glucose–oxidase–DAB–nickel method56 was used for visualization. Rabbit anti-Caspase3 antibody (Cell Signalling, Beverly, MA, USA) was used at 1:50. Chicken anti-GFP antibody (Abcam, Cambridge, UK) was used at 1:2000. Alexa Fluor 488 or 555-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA) were used at 1:250 or 1:500. Samples were imaged using a Zeiss LSM700 confocal microscope (Carl Zeiss, Jena, Germany), and images were taken and analyzed with Cell Death and Differentiation.

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Conflict of Interest
The authors declare no conflict of interest.

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