# Supporting Information

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### SI Materials and Methods

**Animals.** *Ambystoma maculatum* embryos were obtained from Charles D. Sullivan Company. The embryos were stored at 10 °C in sterile tap water and the developmental progress was followed according to Harrison's staging method (1). The experiments described here were performed over 3 y during February–March, which is the egg-laying season of spotted salamanders. The embryos were dejellied from their clutch by immersion in 1% KOH for 30 s followed by several washes in sterile tap water. The embryos were separated from the mass by using a pair of no. 5 biological tweezers. Individual embryos were transferred to 1× Steinberg solution containing 50 μg/mL gentamicin and were maintained in an incubator at 10 °C with a 12 h:12 h light:dark cycle. *Xenopus laevis* tadpoles were obtained from the European *Xenopus* Resource Centre and raised in the laboratory. The animals were staged according to Nieuwkoop and Faber (2).

**Parabiosis.** Parabiosis was performed in embryos between stages 24 and 26 (1, 3) with modifications. The embryos in identical developmental stages were matched and selected for parabiosis. To hold the embryo for parabiosis, a chamber was made using soft modeling clay (FIMO; Eberhard Faber) (4). The modeling clay was flattened to about 5-mm thickness and affixed onto a 10-cm sterile Petri dish. A convex groove was created by punching the clay with a round-bottom Falcon tube. The embryo pairs in 1× Steinberg solution were placed in each well. A rectangular piece of flank epidermis was removed from each embryo and the pair was fused together at the wound site. The pair was held together by adjusting the size of the holding well using a fine palette knife, so that molding clay touched the embryo to minimize ciliary movement. The embryos were kept at room temperature (18 °C) overnight to allow the fusion of the pairs. Thereafter, the fused pairs were gently removed from the holding groove and transferred to a sterile 12-well microplate containing 1× Steinberg solution. The parabiotic pairs were allowed to develop at 12 °C. The efficiency of the parabiosis was 68% ($n$ = 22) and 64% ($n$ = 84) survival in years 1 and 2, respectively.

**Aneurogenic Larvae.** Aneurogenic larvae were created by removing the entire neural tube from the embryo as described previously (1, 3, 5). Two routines were used for neural tube removal: ($i$) removal of neural tube from individual *A. maculatum* embryos and ($ii$) removal of neural tube from one of the parabiotic pairs.

***Removal of the neural tube from an embryo.*** The surgical manipulation was performed in embryos between stages 29 and 32. The embryos were held in an agarose plate (2.5%) with a groove, and filled with 1× Steinberg solution to avoid collapse of the body during manipulation. The entire length of the neural tube was removed from the base of the hind brain to the last somite, reaching the base of the tail bud using a pair of tungsten needles of 2- to 5-μm tip diameter. After microsurgery, the embryos were placed individually in a 12-well microplate containing 1× Steinberg solution and transferred to an incubator maintained at 7 °C. The embryos were maintained at this temperature for 5–7 d to complete wound healing and transferred to another incubator maintained at 12 °C for growth. The survival from this procedure was 91% ($n$ = 325) from various batches of embryos.

***Removal of neural tube from parabiotic pair.*** The procedure is essentially as described above, except that the neural tube was removed from one member of the pair only.

**Transplantation Experiment.** The aneurogenic limb was orthotopically transplanted to the normal host limb. Aneurogenic and host larvae were anesthetized in 0.04% Tricaine. The larvae were placed in holding wells on an agarose dish, and the left forelimb from the normal host was removed from its proximal base and discarded. Thereafter, the left forelimb from the aneurogenic larva was removed and placed on the normal host, precisely on the groove where the forelimb had been removed. The transplant was held in place by applying a fine drop of Histoacryl glue (B. Braun, Aesculap AG, Germany). The larvae were returned to 1× Steinberg solution for recovery. The right forelimbs from the aneurogenic larvae were removed and processed for immunohistochemistry. A total of 40 transplantations were performed in various batches of experiments, and 22 of the transplants were integrated into the host limb. Pairwise analysis of the transplanted limb with its respective aneurogenic counterpart was carried out in nine samples.

**Cloning of *A. maculatum* Anterior Gradient Protein.** Total RNA was extracted from whole *A. maculatum* larvae using TRI reagent (Sigma) and poly(A)$^+$ RNA was purified on Dynabeads oligo dT (Invitrogen) and used for the preparation of RACE-ready cDNA (Clontech). 5′ RACE with oligo 5′-AGCTTGAGAGTGTGGRCAGTCRTCTCTGTGGTT-3′ produced a 400-bp fragment and 3′ RACE with oligo 5′-AACCACAGAGAYGACTGYCCACACTCTCAAGCT-3′ gave a 600-bp fragment. Together these fragments comprised the whole of the *A. maculatum* Anterior Gradient (mAG) protein-coding region. A full-length expression construct was made using PCR with oligos 5′-CGCCTCGAGTAATCGCTCATCATGGCA-3′ and 5′-CGCCTCGAGTAATCGCTCATCATGGCA-3′ on *A. maculatum* cDNA. This gave a 550-bp fragment that was cloned into the XhoI/XbaI sites of pCI-neo vector (Promega). The accession number for the mAG gene is HQ676606.

**Transmission Electron Microscopy.** Whole larval animals were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 3 h at room temperature. The fixed samples were washed in cacodylate buffer and the limb tissues were dissected under a stereomicroscope. The samples were postfixed in 1% $OsO_4$ in cacodylate buffer and dehydrated in an ethanol series. The tissues were en bloc stained in saturated uranyl acetate in 70% ethanol overnight, dehydrated in ethanol, and embedded in resin. Thin sections were cut and mounted on a formvar-coated single-slot grid to allow uninterrupted views of the entire epithelial tissue. The sections were stained with uranyl acetate and lead citrate and observed under a JEOL 1200 EXII microscope operating at 80 kV. Images of several successive adjacent areas across the tissue section were collected using a Gatan digital camera and montages were assembled using the Photomerge tool in Adobe Photoshop CS4. The number of cells with glandular morphology was counted from entire sections mounted on single-slot grids from aneurogenic and normal larval skin.

**Immunogold Labeling.** Tissue processing and immunogold labeling were as described previously (6). The samples were fixed in 4% paraformaldehyde containing 0.025% glutaraldehyde. The tissues were dehydrated to 100% ethanol in a freeze-substitution device maintained at −30 °C. The samples were embedded in Lowicryl K4M (Agar Scientific, UK) and polymerized under UV light at −30 °C. Thin sections were mounted on nickel grids. The sections were blocked in 10% goat serum containing 0.15%

Tween-20 in PBS. The sections were reacted with the primary antibody (peptide antibody 224) at 1:50 dilution in 100 mM lysine in PBS with 0.15% Tween-20 overnight at 4 °C. After extensive washes, the sections were incubated in goat anti-rabbit antibody conjugated to 10-nm gold particles at a dilution of 1:50. They were then exposed briefly to 0.1% glutaraldehyde in PBS followed by washes in distilled water. The sections were counterstained with uranyl acetate and lead citrate. For negative controls, the primary antibody was omitted or the sections were reacted with concentration-matched nonspecific rabbit antibody.

**Antibodies and Immunofluorescence.** The tissues were fixed in 4% paraformaldehyde overnight at 4 °C. Fixed tissues were washed in PBS and embedded in Shandon Cryochrome (Thermo Fisher Scientific). Serial longitudinal sections were cut at 12-μm thickness and collected on Superfrost plus slides (Fisher Scientific, UK). The slides were air-dried at room temperature and processed for immunohistochemistry. The peptide antibody 224 was used at a dilution of 1:200 to localize the reactivity of mAG protein. Innervation to the tissues was visualized using anti-acetylated tubulin antibody (clone 6-11B-1; Sigma) or anti-neuron-specific β-III tubulin antibody (R&D Systems). The secondary antibodies were Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 596 goat anti-mouse at 1:1,000 dilution. The slides were observed under a Zeiss Axioplan 2 microscope and images were acquired using an Orca ER (Hamamatsu Photonics) digital camera driven through Openlab (PerkinElmer). For comparative analysis of tissue samples, the images were acquired with identical camera settings and illumination and contrast enhancement was applied to the entire images in Openlab. The images were resized and labeled using Adobe Creative Suite 5.

**BrdU Labeling and TUNEL Assay.** The larvae were injected with 10 mM BrdU solution using a Picospritzer II pressure injector (Intracel, UK) mounted on a stereomicroscope. After 8 h, the larvae were fixed in 4% paraformaldehyde and limb samples were processed for BrdU immunohistochemistry (7). For the TUNEL assay, tissue sections were reacted with fluorescein-dUTP using the in situ cell death detection kit (Roche) following the manufacturer's protocol with minor modifications. The sections were incubated for 4 h at room temperature with fluorescein-dUTP reagent.

**Assay for Cultured Blastemal Cells.** Blastemal cells were isolated from newt limb blastema as described (8). The cells were cultivated on a 96-well microplate in serum-free medium. Cos 7 cells were transfected with mAG- or GFP-plasmid and cultured without serum for 72 h. The medium was collected and concentrated using an Amicon ultracentrifugal filter (Millipore). The concentrated medium was added to blastemal cells with 0.05% FCS and 10 μM BrdU and incubated for 72 h. Parallel cultures were maintained in 0.05% serum with BrdU as control. The cultures were fixed and processed as described (9). Briefly, the cells were fixed in 1% paraformaldehyde for 2 min, washed in PBS, and postfixed in ice-cold methanol for 5 min. The cultures were air-dried, rehydrated in PBS, and reacted with anti-mouse BrdU monoclonal antibody. Alexa Fluor 488 goat-anti mouse antibody was used as secondary antibody. The cultures were washed and counterstained with Hoechst 33258. The microwells were scanned on a Zeiss Axiovert 200 microscope equipped with a scanning stage using Axiovision (Carl Zeiss) software. Quantitative analysis of the cells was carried out using Image-Pro Plus (Media Cybernetics).


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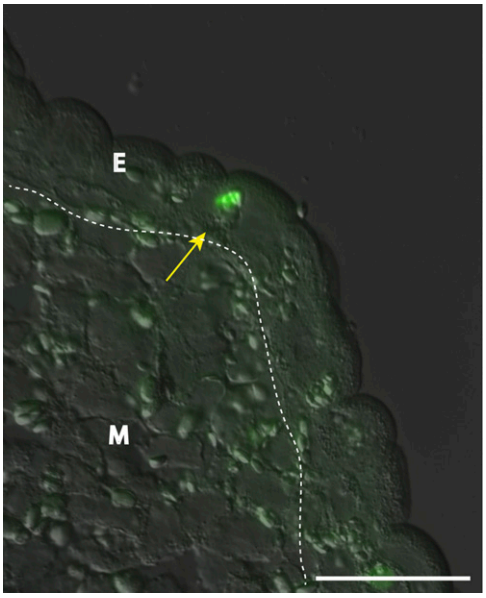


**Fig. S1.** Apoptosis in larval limbs during development. Larval limb sections were analyzed at stage 40 by TUNEL assay for the detection of apoptotic cells. A gland cell shows apoptosis within the larval epidermis (arrow). The boundary of the larval epidermis is demarcated with white dotted lines. The image is an overlay of fluorescence with differential interference contrast to show the morphology. E, epidermis; M, mesenchyme. (Scale bar, 50 μm.)

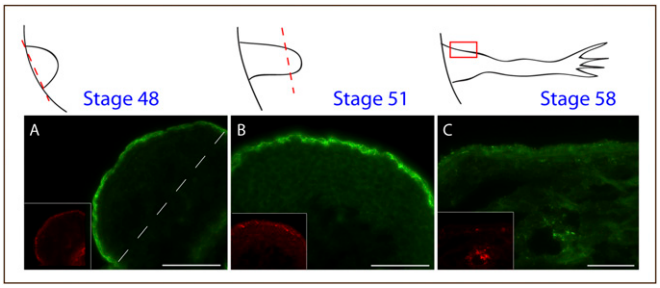


**Fig. S2.** Expression of *Xenopus* anterior gradient protein (XAG) during limb development. (*Upper*) Diagrammatic illustration of the developing limb at corresponding developmental stages. XAG reactivity in the tissues was determined by cross-reactivity with the peptide antibody 224. (*A*) A limb bud at stage 48 shows moderate expression throughout the epithelium. (*Inset*) AAT staining to show the innervation to the epithelial cells. (*B*) Limb bud at stage 51. Note that the distal tip of the limb bud is showing strong reactivity with XAG, and innervation to the developing epidermis is extensive (*Inset*). (*C*) Proximal area (boxed) from a stage 58 limb shows complete down-regulation of XAG during prometamorphic stage. The innervation to the skin is relatively sparse (*Inset*). E, epidermis; M, mesenchyme. [Scale bars, 100 μm (*A* and *B*); and 50 μm (*C*).]

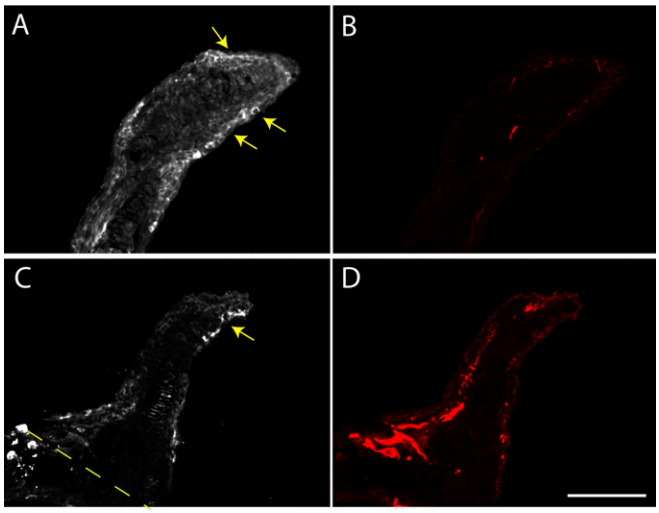


**Fig. S3.** Regulation of mAG protein in aneurogenic larval skin by innervation following transplantation of the limb. The experimental design is as described in Fig. 4. The aneurogenic limb was collected and analyzed after 30 d of transplantation into the normal host limb. (*A*) The reference aneurogenic limb showing expression of mAG protein throughout the larval skin layers (arrows). (*B*) AAT staining, which shows lack of innervation. (*C*) Transplanted aneurogenic limb showing down-regulation of mAG protein after invasion of nerve elements from the stump tissue of the host. Extensive down-regulation is visible throughout the dorsal larval skin. (*D*) Innervation of the transplanted limb from the host tissue. The dorsal aspect of the limb is strongly innervated, which is correlated with the down-regulation of mAG protein. On the ventral side of the limb, the innervation is not extensive and a patch of the larval skin (arrow in *C*) shows a level of mAG comparable to the aneurogenic state of the reference limb. [Scale bar, 200 μm (*A*–*D*).]

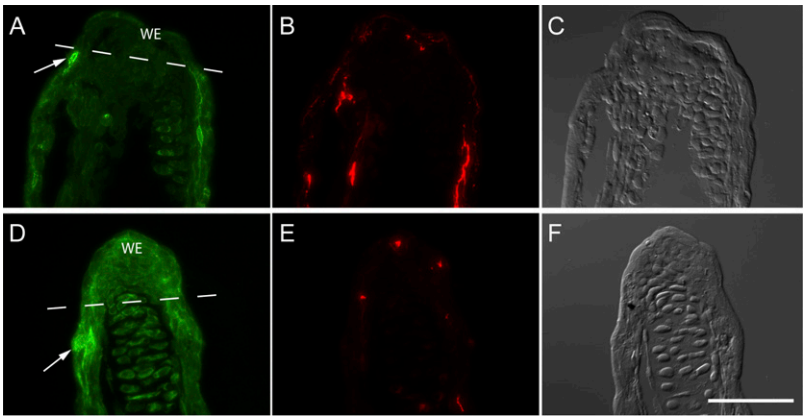


**Fig. S4.** Expression of mAG protein during limb regeneration in normal and aneurogenic larval *A. maculatum*. The limb blastemas from a parabiotic pair were analyzed at 3 d after amputation of the forelimb. The amputation plane is shown with a white dotted line. (*A*) Normal limb blastema from the host larva showing the wound epithelium. Gland cells proximal to the amputation plane up-regulate mAG (arrow) after amputation of the limb, but the wound epithelium is negative. (*B*) AAT staining shows the nerve branches in the tissue. (*D*) Limb blastema from the aneurogenic limb stained with antibody to mAG protein. The wound epithelium in the aneurogenic limb blastema is at an advanced stage, with multilayered wound epithelium showing strong reactivity with mAG protein. The arrow indicates a gland cell. (*E*) Reactivity with AAT shows the absence of nerve elements in the tissue. (*C* and *F*) Differential interference contrast images showing the morphology of the corresponding limb tissue. [Scale bar, 100 μm (*A*–*F*).]