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Supplemental Information

Live Imaging of Axolotl Digit Regeneration Reveals Spatiotemporal Choreography of Diverse Connective Tissue Progenitor Pools

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Currie et al. Figure S1

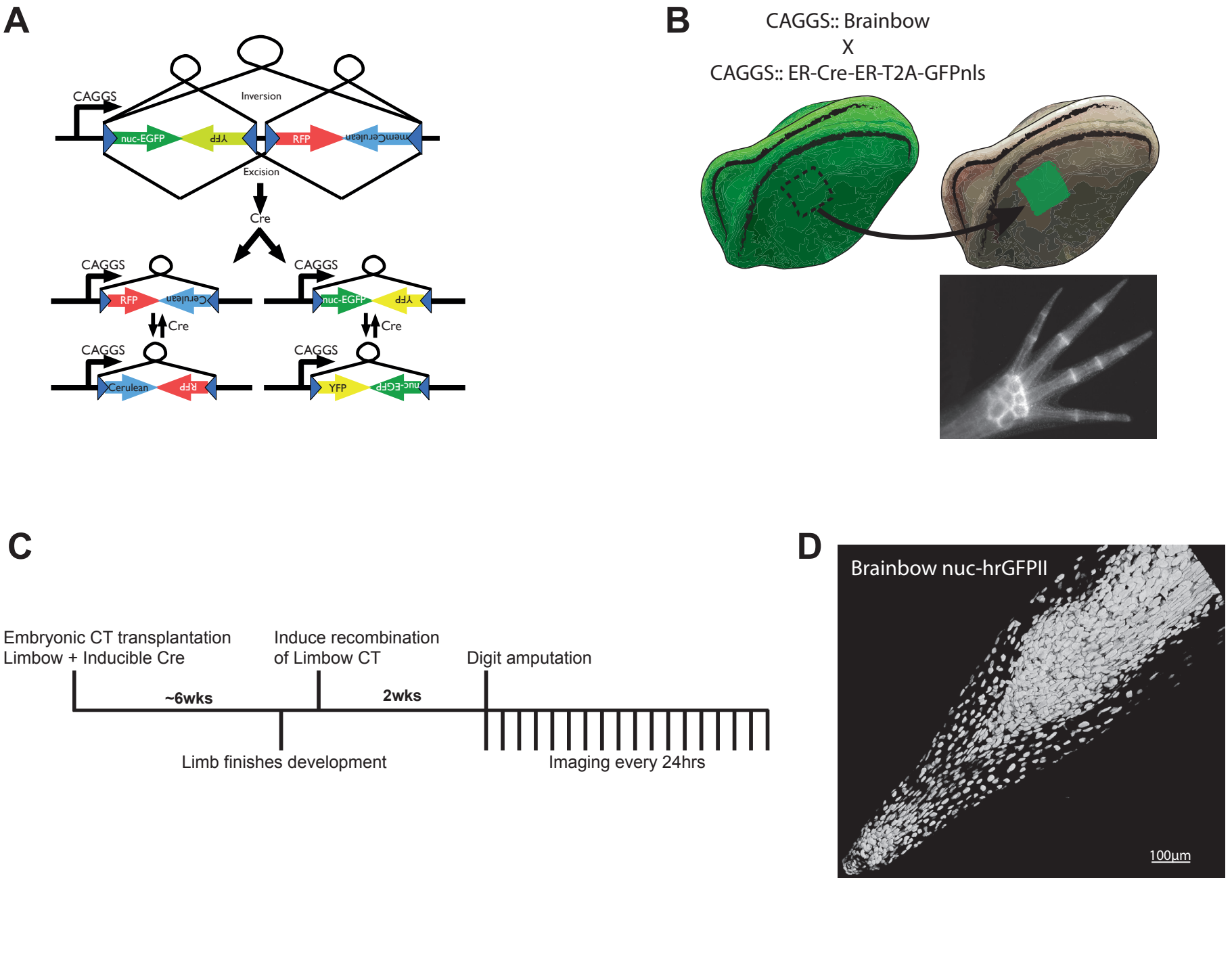
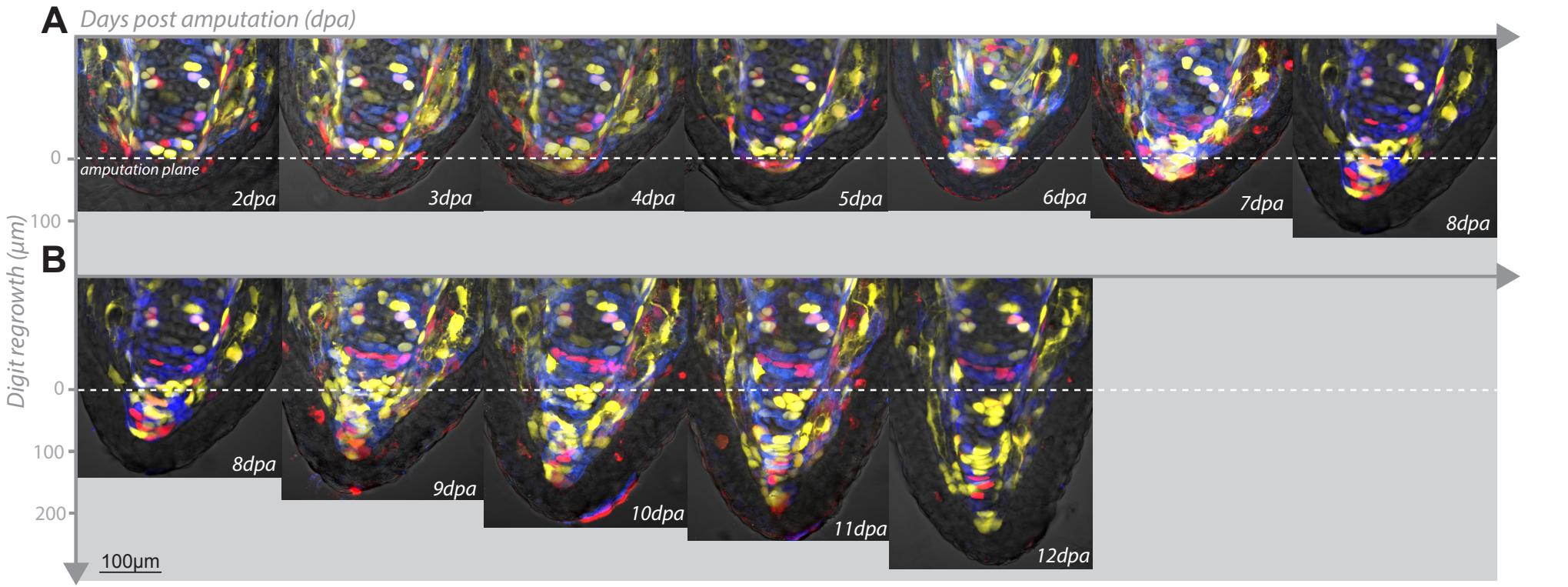
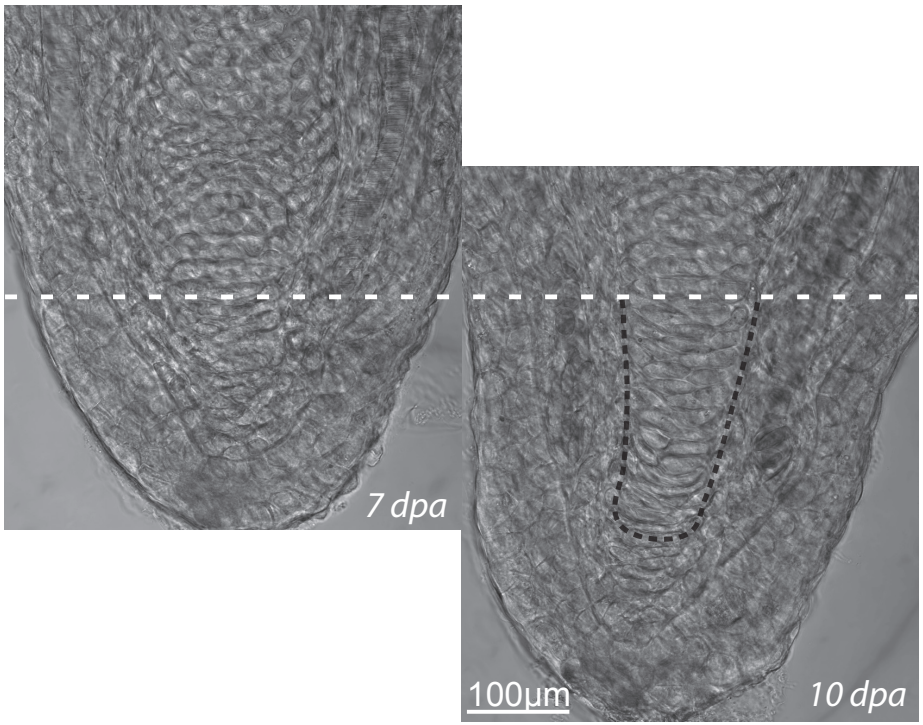


Figure S1, related to Figure 1. Creation of stable transgenic brainbow axolotls that specifically label connective tissue. (A) Schematic of the brainbow construct as modified from Livet et al. Ubiquitous CAGGs promoter drives expression of the default nuclear hrGFPII. Upon Cre recombinase activity, stochastic inversion or excisions lead to one of three other fluorescent proteins to be expressed. Multiple copies integrated into the genome can give cells unique color identities. (B) To specifically label limb connective tissue, lateral plate mesoderm (LPM) from stage 16 double transgenic embryos is transplanted onto a non-transgenic host embryo. This yields labeling of all connective tissue compartments of one limb in the host animal. (C) Experimental timeline for live imaging of Limbow digit regeneration. (D) Confocal volume rendering of Limbow digit tip nuclear-hrGFPII before recombination.

Currie et al. Figure S2





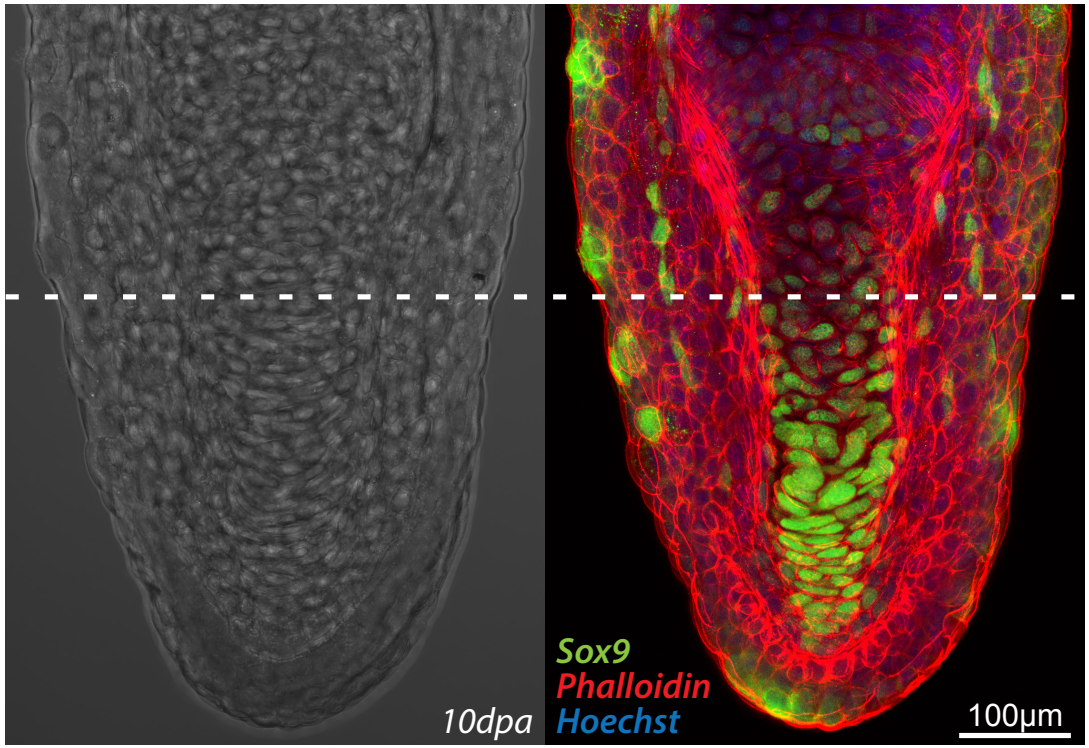


Figure S2, related to Figure 1. Migration of cells during early stages of digit regeneration and condensation of cartilage marks a transitional middle phase of digit regeneration. (A,B) Expanded panels from Figure 1B-G showing maximum intensity projections from 20μm volume of digit tip regeneration from Days 2-12 post amputation. (C) Brightfield images of regenerating digit tips prior to (left) and during (right) the condensation of nascent cartilage, marking the first signs of new tissue boundaries being formed (dashed lines). (D) Wholemount staining for cartilage progenitor marker Sox9 at 10 days post amputation.

Currie et al. Figure S3

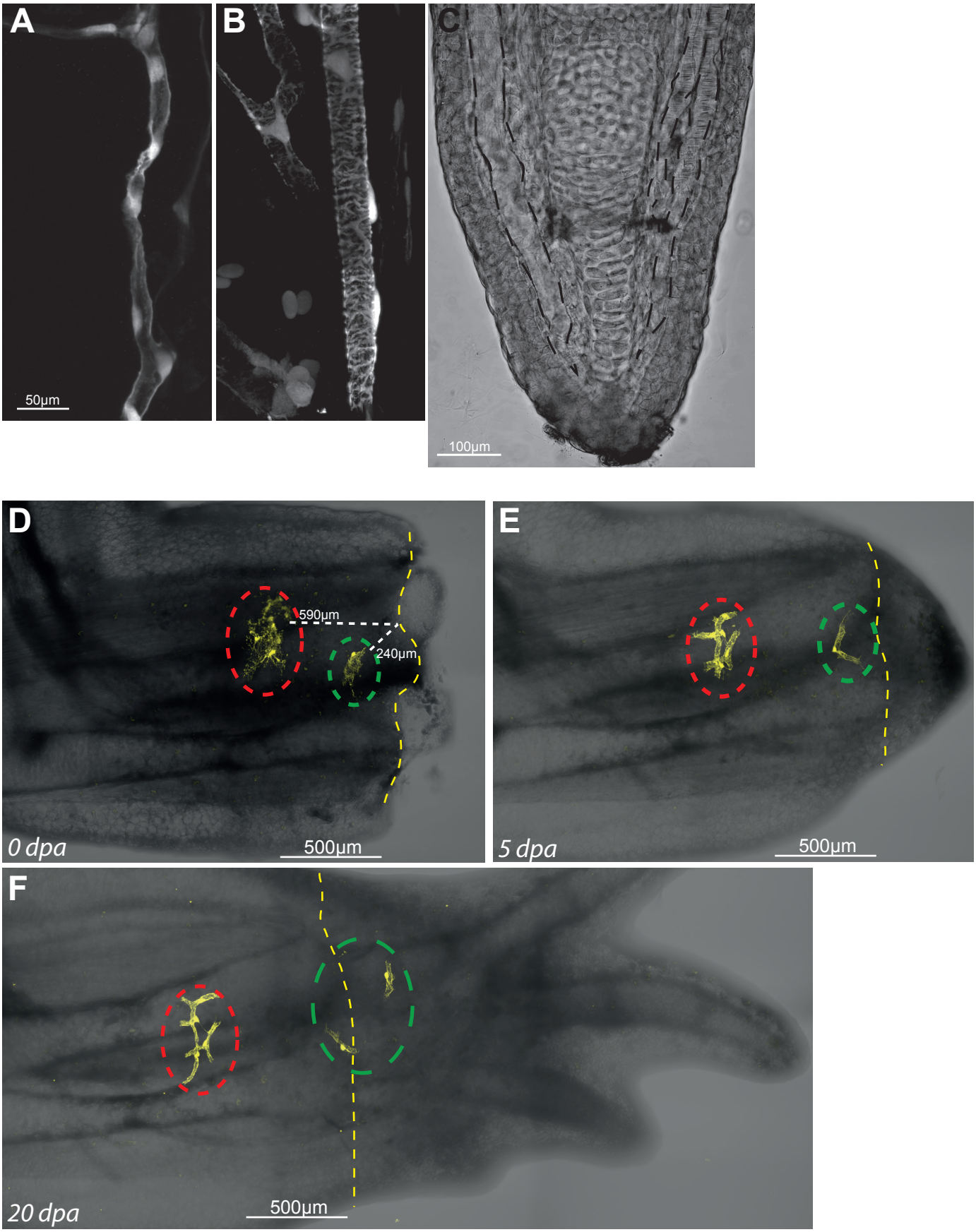


Figure S3, related to Figure 2. Morphological distinction of pericytes and associated vessels during digit regeneration and the restriction of pericytes in lower limb regeneration. (A) Endothelial cells display a smooth, continuous membrane labeling and cell bodies within the plane of the vessel. (B) Pericytes display discontinuous membrane tendrils around blood vessels and a protruding cell body. Cells from (A) and (B) were labeled through Brainbow embryonic transplantation, in which more tissue than limb connective tissue was transplanted in (A) which results in limb endothelial cell labeling (Kragl et at, 2009). Such animals were never used for connective tissue clonal tracking. (C) Brightfield image produced by maximum intensity projection of digit volume. Blood vessels can be identified by contrast of vessel walls or the blur effect caused by rapid movement of platelets through the vessel lumen. (D) Labeled perictyes from Limbow skin grafts at 0 dpa. White dashed lines indicate the shortest distance to the amputation plane (yellow dashed lines) for cells within 500μm (green circle) and beyond (red circle). Images at 5dpa (E) and 20 dpa (F) show a mobilization of the cell within the source zone, but production only of pericytes that remain bound to vessels in the regenerate.

Currie et al., Figure S4

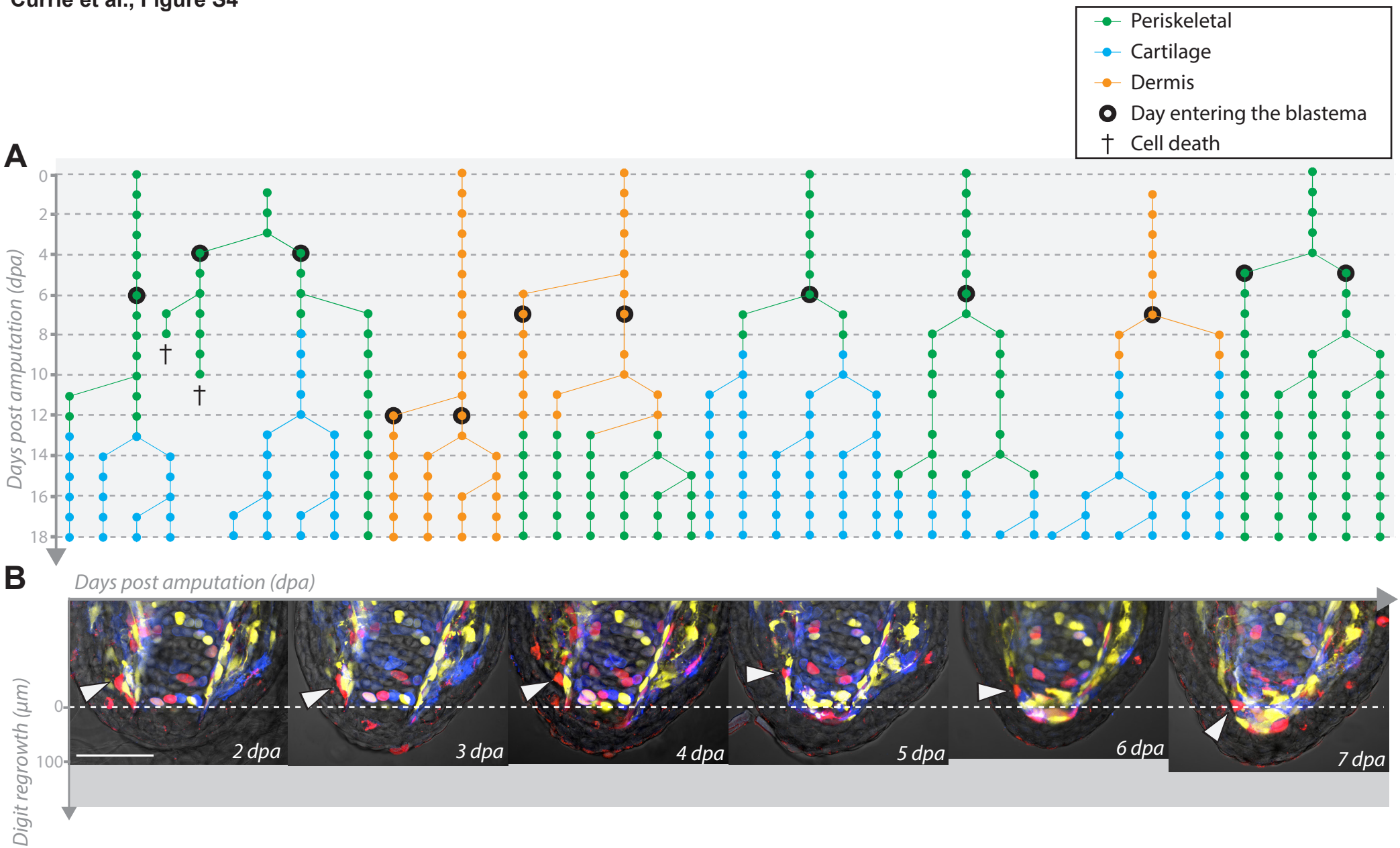


Figure S4, related to Figure 3. Migration and proliferation of dermal and periskeletal cells to form multiple tissue types in the regenerated digit tip. (A) Lineage trees of dermal (orange spots at day 0/1 post amputation) or periskeletal (green spots at day 0/1 post amputation) showing representative clones that were tracked throughout digit tip regeneration. Color changes of spots and links represent the time when a clear change of tissue compartment was observed in the regenerate. Bold outlined spots indicate when a cell had transited beyond the amputation plane. Crosses indicate cell death. (B) Expanded panels from Figure 3B of a dermal cells forming cartilage in the regenerate. Panels show maximum intensity projections from a 12μm volume of digit tip regeneration from Days 2-7 post amputation, with the cell of interest highlighted with white arrowheads.

Currie et al. Figure S5

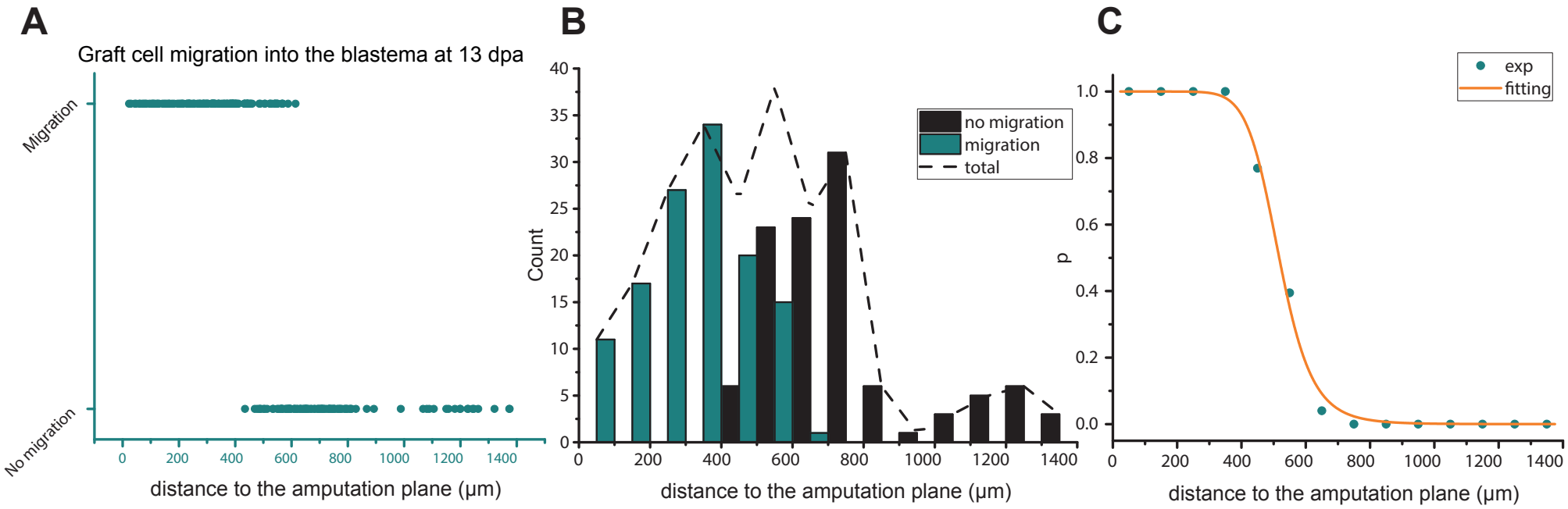
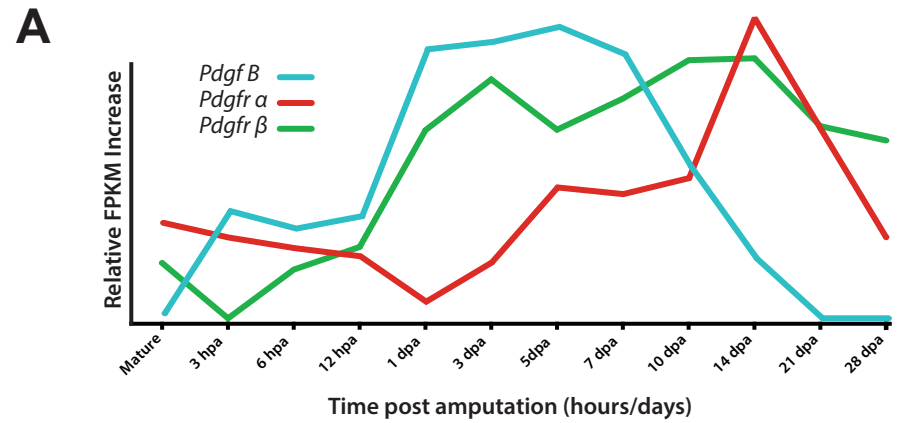
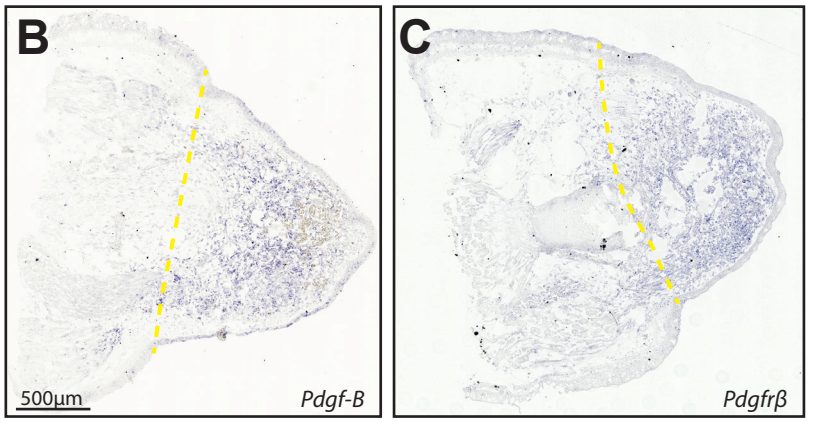
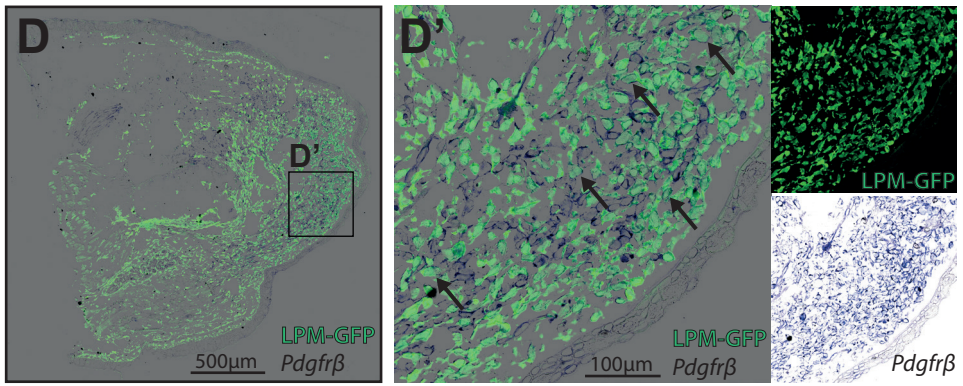


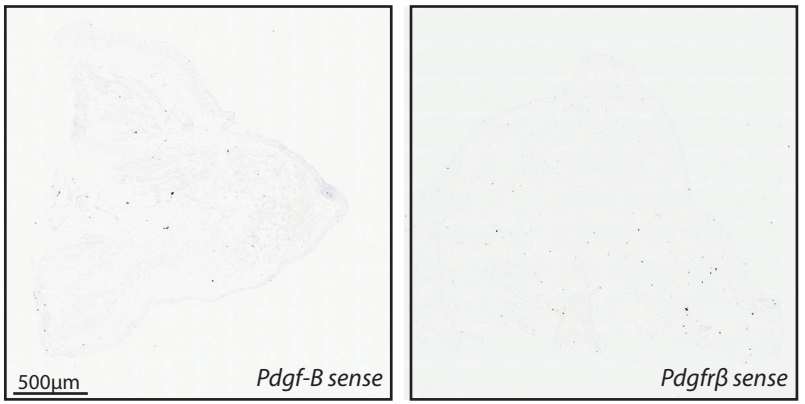
Figure S5, related to Figure 5. Calculating a 500µm source zone of dermal blastema progenitors during lower limb regeneration. (A) Grafted dermal progenitors were categorized in terms of whether they migrated into the blastema at 13 dpa or not. (B) The histogram of cell distances from the amputation plane at 0 dpa plotted for the two categories (columns). Added columns yielded a normalized quantity (dashed line). (C) Normalized quantities were used to estimate the probability *p* for each distance, x, of whether a cell would enter into the blastema at 13 dpa (green dots). This probability distribution was fitted with a Hill-like expression (see Materials and methods) to interpolate all points behind the amputation plane (orange continuous curve).

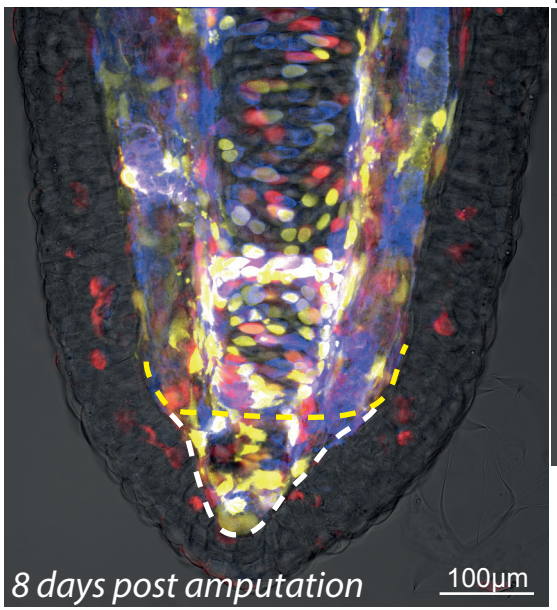
Currie et al., Figure S6











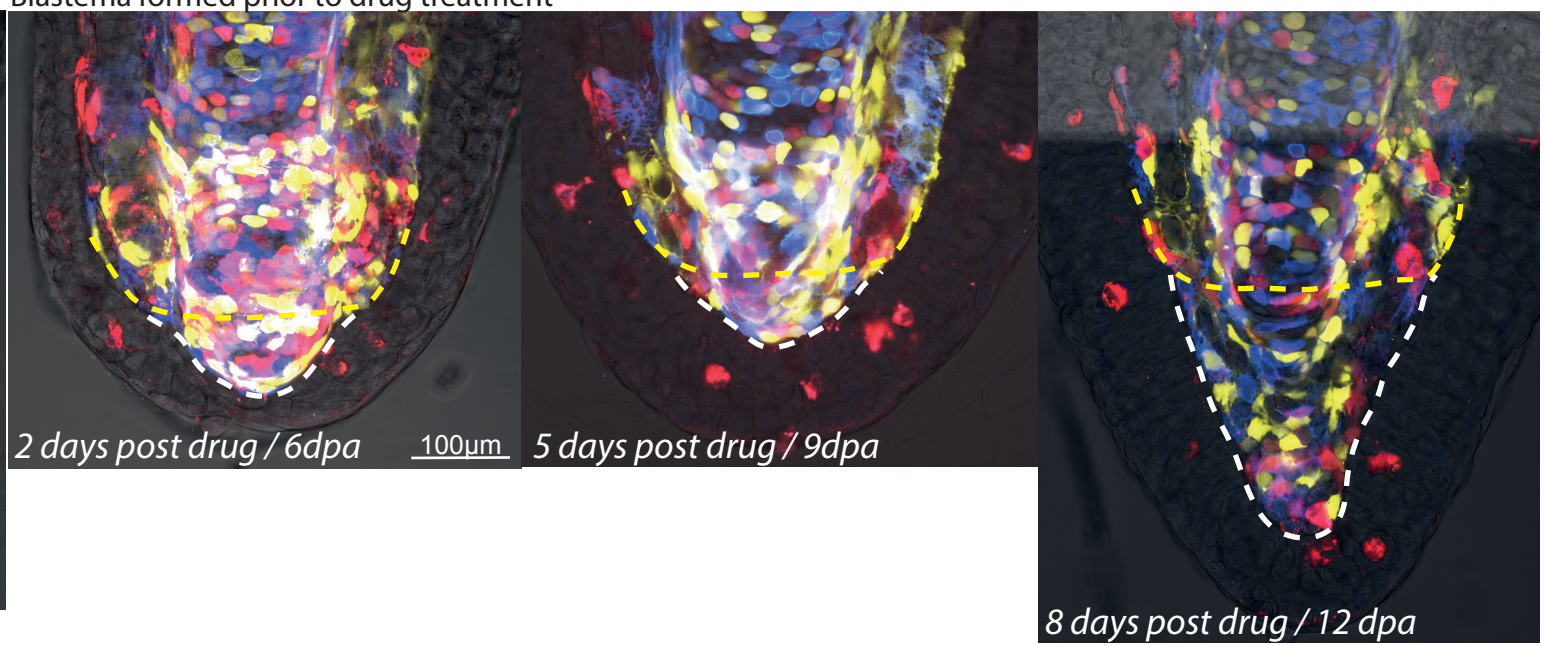


Figure S6, related to Figure 6. PDGF signaling is required for blastema formation in vivo but does not inhibit blastema growth or patterning. (A) Relative RNA abundance as measure by RNA Seq (Stewart et al., 2013) of *Pdgf-b*, *Pdgfβ*, and *Pdgfrα* across timepoints of limb regeneration. *Pdgf-b* and *Pdgfrβ* are upregulated early during limb regeneration, while *Pdgfrα* is upregulated only during late stages of regeneration. (B) In situ hybridization of *Pdgf-b* in a mid bud upper arm blastema shows signal within the mesenchymal blastema but not wound epidermis. (C) *Pdgfrβ* is present within the mesenchymal blastema but not wound epidermis of a mid-bud stage upper arm blastema (D) In situ hybridization of *Pdgfrβ* on sections of GFP lateral plate mesoderm (LPM) transplanted animal. (D') inset from D. Although GFP-LPM transplantation may incompletely label all connective tissue, there is substantial co-localization of *Pdgfrβ* in situ signal (bottom right) with GFP-labeled connective tissue (top right). (E) Sense strand in situs for *Pdgf-b* (left) and *Pdgfrβ* (right). (F) DMSO treated control animals form a robust blastema by 8 dpa. (G) Blastemas that were formed prior to PDGFR inhibitor treatment are unaffected in patterning of regenerated tissue compartments after 8 days post inhibitor treatment and 6dpa (right).

Movie S1, related to Figure 1. Timelapse of digit tip regeneration from 0 to 18 days post amputation.

Timelapse images of digit tip regeneration over 18 days after amputation. Images are maximum intensity projections of selected planes within the total image volume acquired every 24 hours.

Experimental Procedures:

Larval surgeries and drug treatment

Tissue grafts of full thickness skin were performed by removing a small patch of ventral, lower limb skin from anesthetized Limbow and age/size (5cm body length) matched non-transgenic animals. Limbow skin was placed onto the vacant space on non-transgenic hosts and allowed to heal for a period of two weeks. Host animals were screened for uptake of the graft and only animals with seamless integration of the graft were used for further experiments. Limb amputations were executed at the mid radius/ulna level of the lower limb. Digit amputations were done on the distal phalanx of digits 1-3 between the joint and calcified area in the distal segment.

For animal drug treatments, PDGFR Inhibitor V (Cas# 347155-76-4, Merck Millipore), dissolved in DMSO, was added at a final concentration of 500nM to tap water. Treated water was changed daily during imaging and animals were kept protected from light to prevent drug degradation. Drug treated or control treated water never exceeded a DMSO concentration of 0.005% total volume. All experiments were done in accordance with the Saxony Animal Ethics Committee.

Scratch assay quantification

Scratch assay images were analyzed using ImageJ. The area of the wound in each field was quantified manually using the polygon selection tool at times 0 h and 24h/ 48h, and the percent wound closure (%Closure) was subsequently calculated. The %Closure for each scratch was obtained by calculating the mean of the %Closure data for all the fields of the same scratch. Finally, the %Closure of each scratch is presented together with the global mean per condition using GraphPad Prism 6 (Graphpad Sofware). Non-parametric tests were carried out to analyze the statistical significance (Kruskal-Wallis Test and Dunn's Multicomparison Test). *P* values < 0.05 were considered statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, *ns*: non-significant).

Deriving axolotl ex vivo cultures

CAGGs::EGFP lateral plate mesoderm (LPM GFP^+) transplanted axolotls approximately 10cm in body length were anesthetized using a 0.01% benzocaine solution. Forelimbs were amputated at the level of mid-humerus and approximately three weeks later mid bud blastemas were harvested for cell explants. In a laminar flow hood, blastemas were cut and the wound epidermis was peeled away using forceps. Blastema tissue was then sterilized in 70% ethanol for 10 seconds, and placed in a Petri dish containing a small amount of cold culture amphibian base media (AmnioMax, Thermo Fisher, diluted with sterile water to 0.8x). Tissue pieces were placed on an explant tissue culture dish (Corning) pre-coated with gelatin and containing 140 µl of culture media supplemented with 1x supplements (AmnioMax C100, Thermo Fisher). Explants were given fresh media every 7 days. After 15-20 days, cells were trypsinized, filtered and seeded into a 6-well plate pre-coated with fibronectin from bovine plasma (33.3 µg/ml, SIGMA) for 1 h at RT.
LPM GFP^+ Blastema cultures were maintained in culture media (AmnioMax, Thermo Fisher, diluted with sterile water to 0.8x) with 1x supplements (AmnioMax C100, Thermo Fisher) in a humidified incubator (2% CO_2) at 25ºC. Dishes were first coated with fibronectin from bovine plasma (33.3 µg/ml, SIGMA) for 1 h at RT. Cells were given fresh media every 4 days and passaged every 7 days, according to standard amphibian tissue culture protocols and sterile technique. Primary explants were passaged up to 10 times, although we noticed a progressive loss of motility after 8 passages. For all experiments, we restricted ourselves to cells that were younger than passage 8.

In situ hybridization and Immunohistochemistry

Wholemount immunohistochemistry was performed using standard techniques for Goat anti-Sox9 (R&D Systems) and Alexa 555 Phalloidin (Life Technologies).

mRNAs for *Pdgfrβ* 5' and internal and *Pdgf-b* 3' probes were extracted from st.50 embryos using standard Isogen RNA isolation method (Nippon gene). cDNA synthesis from these mRNAs and subsequent PCR reactions were performed using the PrimeScript 1st strand cDNA Synthesis kit (Takara). Primer pairs were as follows:
for 1st PCR,

Pdgfrβ-5' region (final product size 1617 bp),
5'- AGCTGTAATACGACTCACTATAGGGAAACTTCTCCGCGAGCCAACAGCGGAAAGTGG-3' (forward) and
5'-AAGCTATTTAGGTGACACTATAGAAGCCTCGCAACGCACAGAAATGGGTTCTTCC-3' (reverse).
Nested primers were as follows:
5'-AGCTGTAATACGACTCACTATAGGGAAAAGGCTTCTTTTCCAGGCCGGGAGGAGCG-3' (forward), and the reverse primer was the same as the 1st PCR.

Pdgfrβ-internal region (final product size 1301 bp),
5'-AGCTGTAATACGACTCACTATAGGGGGAAGAACCCATTTCTGTGCGTTGCGAGGC-3' (forward) and
5'- AAGCTATTTAGGTGACACTATAGAAAGGACGGCCTGGCCTCATACTTCTCATCCC-3' (reverse).
Nested primers were as follows:
5'-AAGCTATTTAGGTGACACTATAGAAGATCTCGTAGATGTCCTCTGACGCGTGAGCGG-3' (reverse), and the forward primer was the same as the 1st PCR.

Pdgf-b-5' region (final product size 1551 bp),
5'-AGCTGTAATACGACTCACTATAGGGTTGCTTCCTGCAGGAGACCCCGCGACTCC-3' (forward) and
5'-AAGCTATTTAGGTGACACTATAGAACCTGCCCACACGTTTCAGAAAGCGCGTTCACAC-3' (reverse).
Nested primers were as follows:
5'- AGCTGTAATACGACTCACTATAGGGCGCGACTCCCTCTAGCCGGAAGTGGTACTG-3' (forward), and
5'-AAGCTATTTAGGTGACACTATAGAAGTGAAGGTAGTTGGGGTCGTTGGCCATGGCC-3' (reverse).

Pdgf-b-3' region (final product size 1217 bp),
5'-AAAATCGGGCCTGTCCTAGATGGC-3'(forward) and
5'-ACTTCCCAACAGAAGAAAATGACTCGATGTAC-3' (reverse).
Nested primers were as follows:
5'-TGTCCTAGATGGCAGCACCCTG-3'(forward) and
5'-CCTCGAGTGTTCCACAAGTTACAAAGC-3' (reverse).

PCR products were purified by PCR purification kit (QIAGEN), and products were cloned into a pGEMT-easy vector, using pGEM®-T Easy Vector Systems (Promega).

Probes were synthesized from linearized plasmids or PCR product (SP6 and T7) by using a DIG-labeled nucleotide mixture (Roche). In situ hybridization was performed as previously described (Knapp et al., 2013). Briefly, slides were washed 3 times in PBS 0.3% triton and incubated overnight at 60 °C in hybridization buffer with probes. The next day, slides were washed in salt solutions at 60 °C as follows: in 5 × SSC 0.1% tween with 50% formamide, in 2 × SSC 0.1% tween twice with 50% formamide, in 0.2 × SSC 0.1% tween once and in 0.2 × SSC 0.1% tween room temperature, 30 min each steps. The reaction was stopped by washing in 1 × PBS 0.3% triton several times.
Following termination of the in situ colorimetric reaction, slides were washed extensively in PBS and submitted to standard immunostaining using a polyclonal rabbit anti-GFP (Rockland). Images were acquired using a color camera equipped upright microscope (Olympus OVK).