Supplementary Materials and Methods:

Cloning of Axolotl Smad2, Smad3 and Smad7

Partial axolotl Smad2, Smad3 and Smad7 cDNA were obtained from axolotl larvae total RNA by RT-PCR. The cDNAs were amplified with primers (see sup. table1) designed from human cDNA sequences. The full length Smad2, Smad3 and partial Smad7 cDNA were subsequently obtained by screening an axolotl cDNA library (Stratagene, CA, USA), following the manufacturer's instructions and using the RT-PCR fragments as probes radioactively labeled with α^{32}P-dCTP (Perkin Elmer, MA, USA).

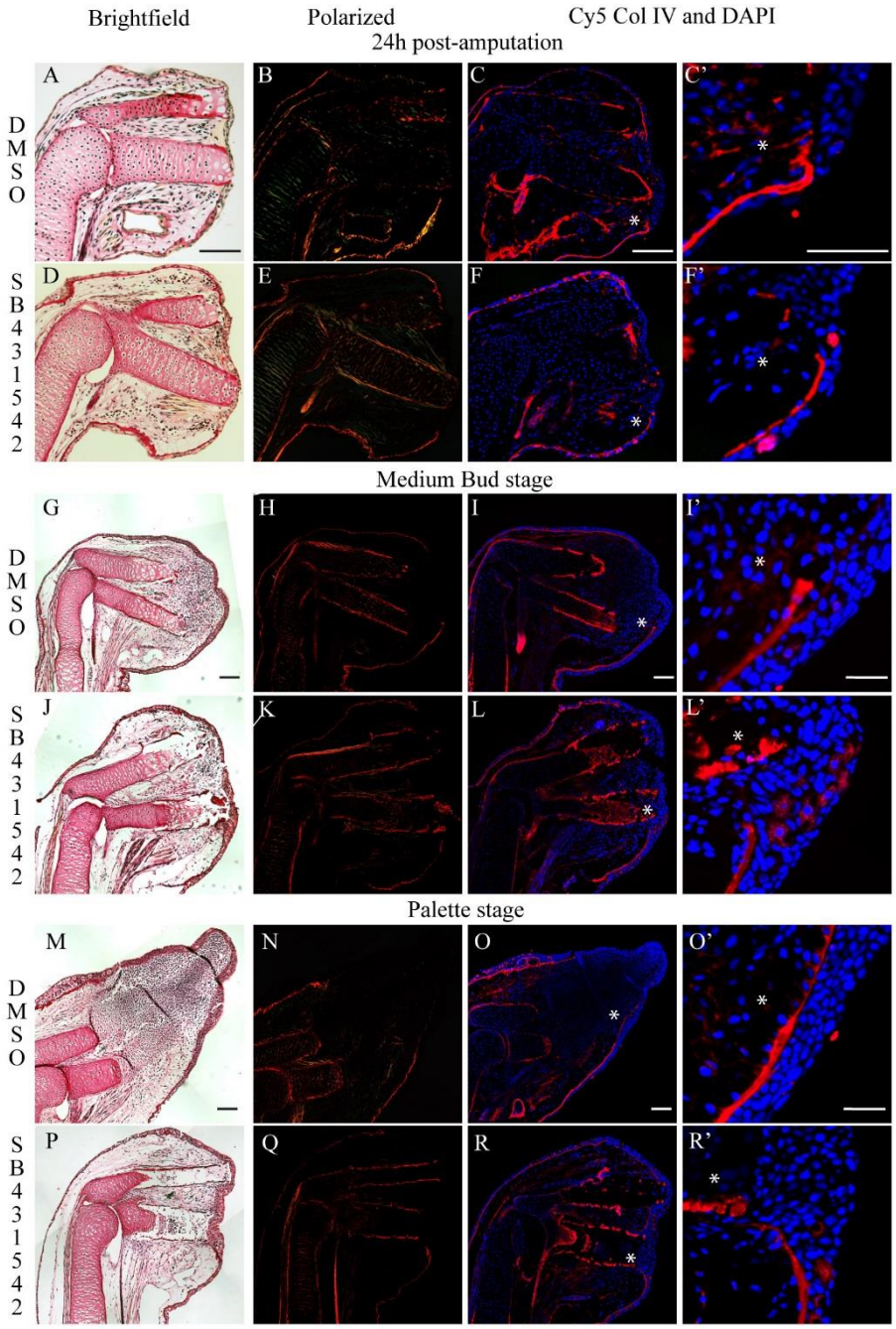
Acridine Orange / Ethidium bromide staining for apoptosis

AL-1 cells were electroporated (approx. 150k cells) with expression vectors (GFP with β-gal, axolotl Smad3 or axolotl Smad3 SΔD, see sup. table 3) and plated in a 12 well plate. Cells were harvested 48h post-electroporation and stained with Acridine Orange / Ethidium bromide as described in Ribble et al, 2005 and Kasibhatla et al, 2006 (Ribble et al., 2005; Kasibhatla et al., 2006).

TUNEL assay

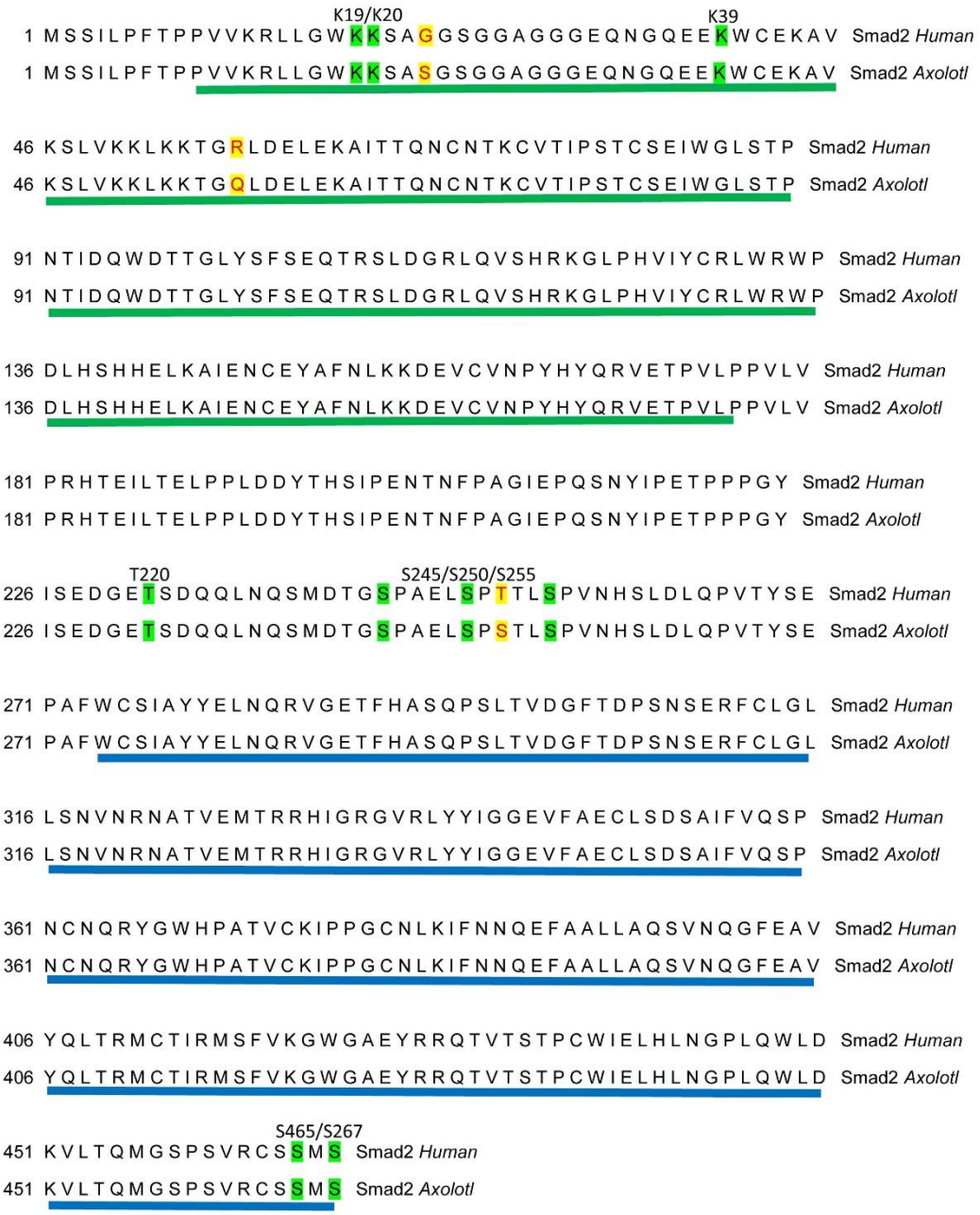
Following injection and electroporation of plasmids (details for in situ electroporation can be found in Guimond et al 2010)(Guimond et al., 2010), 10 µm sections of paraffin embedded limbs were rehydrated then treated with Proteinase K 20µg/ml (Invitrogen, Ref#25530-015) for 20min at RT. Positive control were treated with DNAse 1 1U/50µL (Invitrogen,

ref#18068-015) for 10min at RT. Sections were then rinsed in TBS then TdT buffer 1X (Invitrogen, ref#16314-015) and treated with recombinant terminal deoxynucleotidyl transferase (TdT) 3,75U/µL (Invitrogen, ref#10533-065), Digoxigenin-11-dUTP 2µM (Roche, ref#11093088910) in TdT buffer 1X for 1h at 37°C. Slides were rinsed in TBS then blocking was performed at RT for 15min with 2% sheep serum in TBS. Slides were incubated with a primary Antibody against digoxigenin 1/1500 (Roche, ref#113333062910) at 4°C overnight. Slides were rinsed (4X 15min) with PBST then incubated with a secondary antibody coupled to Alexa fluor 594 (anti-mouse 1/250) (Invitrogen, ref#A11020) in blocking solution for 2h at RT in the dark. Slides were rinsed (4X15min) with PBST and mounted with ProLong® Gold antifade regent containing DAPI (Invitrogen, ref#36931). Slides were visualized with a Zeiss Axio Imager M2 Optical Microscope and composite images were generated (Zeiss, Munich, Germany).



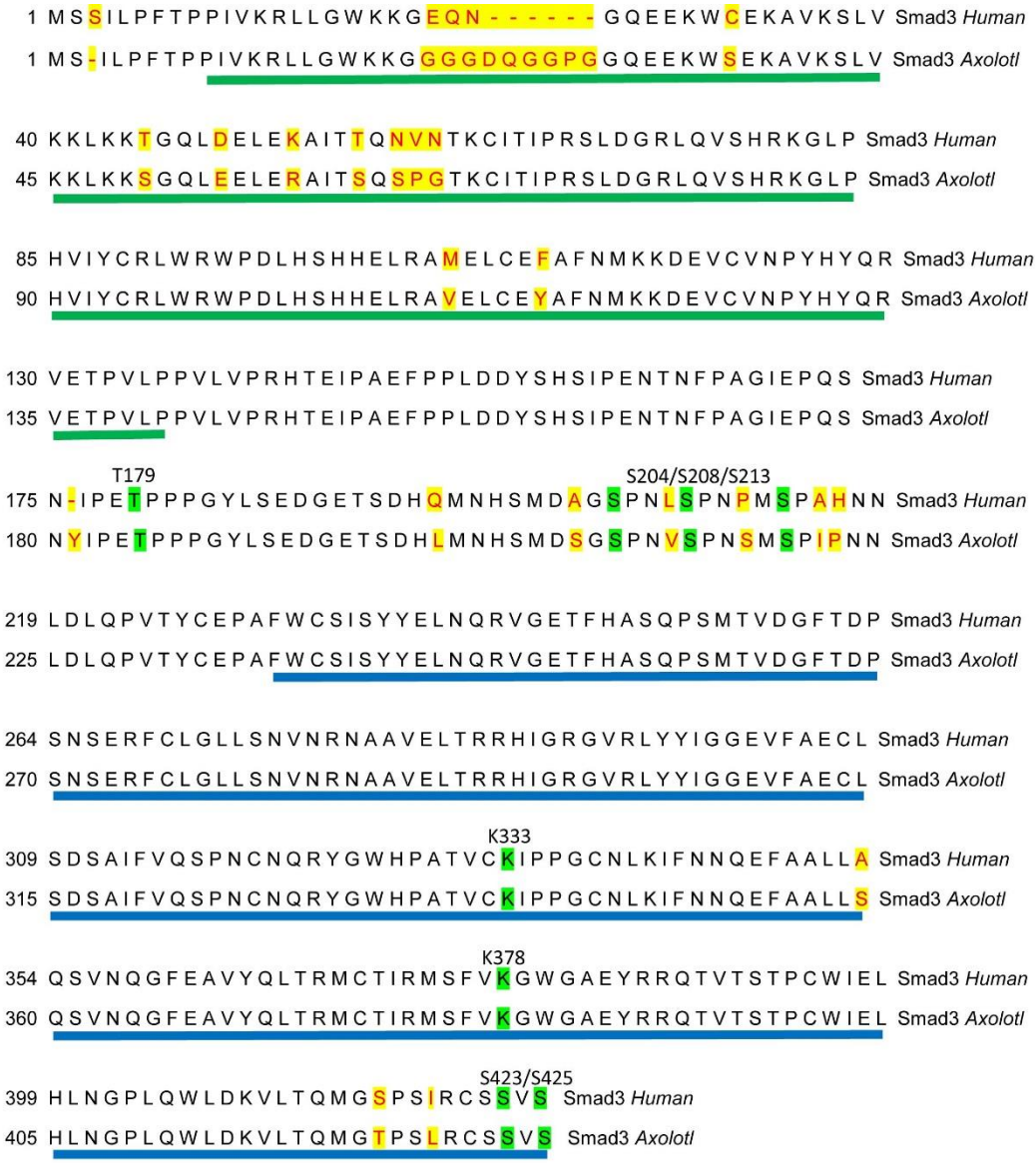
Supplementary Figure 1: **SB-431542 does not affect basement membrane reformation in regenerating limbs**

(**A-C**, **G-I**, **M-O**) Animals treated with DMSO (**A**,**G**,**M**) Brightfield view of limb indicates that a blastema is forming normally. Polarized light show some collagen deposition in Palette stage (**N**) in basement membrane region but not at 24h (**B**) or MB stage (**H**). Col IV expression (red) shows some collagen deposition in Palette stage (**O**,**O'**) in basement membrane region but not at 24h (**C**, **C'**) or MB stage (**I**,**I'**). (**D-F**, **J-L**, **P-R**) Animals treated with 25μM SB-431542 (**D**,**J**,**P**) Brightfield view of limb indicates that no blastema is forming. Polarized light show some collagen deposition in Palette stage (**Q**) in basement membrane region but not at 24h (**E**) or MB stage (**K**). Col IV expression (red) shows some collagen deposition in Palette stage (**R**,**R'**) in basement membrane region but not at 24h (**F**, **F'**) or MB stage (**L**,**L'**). Results show that basement membrane is not restored prematurely in limbs treated with SB-431542. Scale bar 200μM (**A**,**C**,**G**,**I**,**M**,**O**) and 60μM (**C'**,**I'**,**O'**). Composite images are shown and stars indicate areas of magnification.



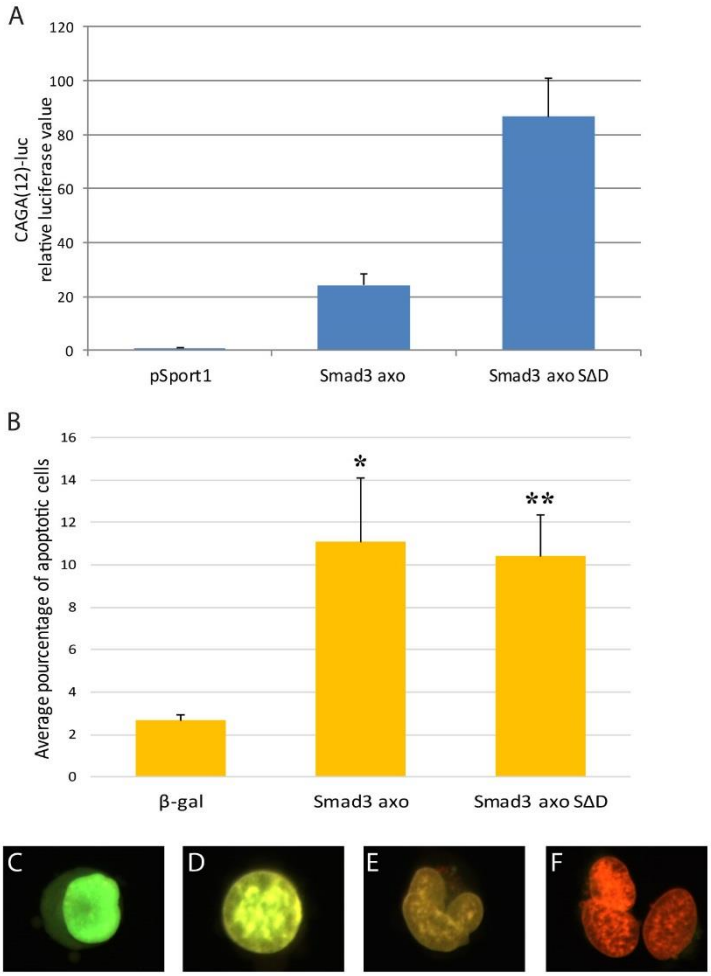
Supplementary Figure 2: **Protein alignment of Smad2**

Protein sequences for human and axolotl Smad2 were aligned using DNASTAR MegAlign. Sequences are greatly conserved between species. The axolotl Smad2 sequence has 99% identity with the human Smad2. MH1 domain is underlined in green. MH2 domain is underlined in blue. Linker domain is located between MHI and MH2 domain, with no underlining. Important post-translational modification sites are indicated on top of the aligned sequences and highlighted in green. Differences in aa are highlighted in yellow with red writing. All post-translational modification sites are conserved between both species.



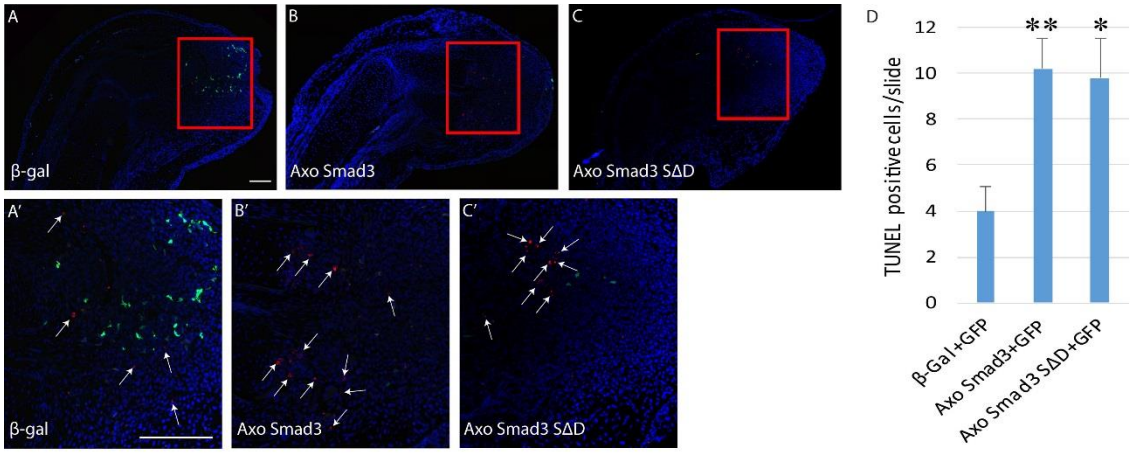
Supplementary Figure 3: **Protein alignment of Smad3**

Protein sequences for human and axolotl Smad3 were aligned using DNASTAR MegAlign. Sequences are greatly conserved between species. The axolotl Smad3 sequence has 93% identity with the human Smad3. MH1 domain is underlined in green. MH2 domain is underlined in blue. Linker domain is located between MHI and MH2 domain, with no underlining. Important post-translational modification sites are indicated on top of the aligned sequences and highlighted in green. Differences in aa are highlighted in yellow with red writing. All post-translational modification sites are conserved between both species.



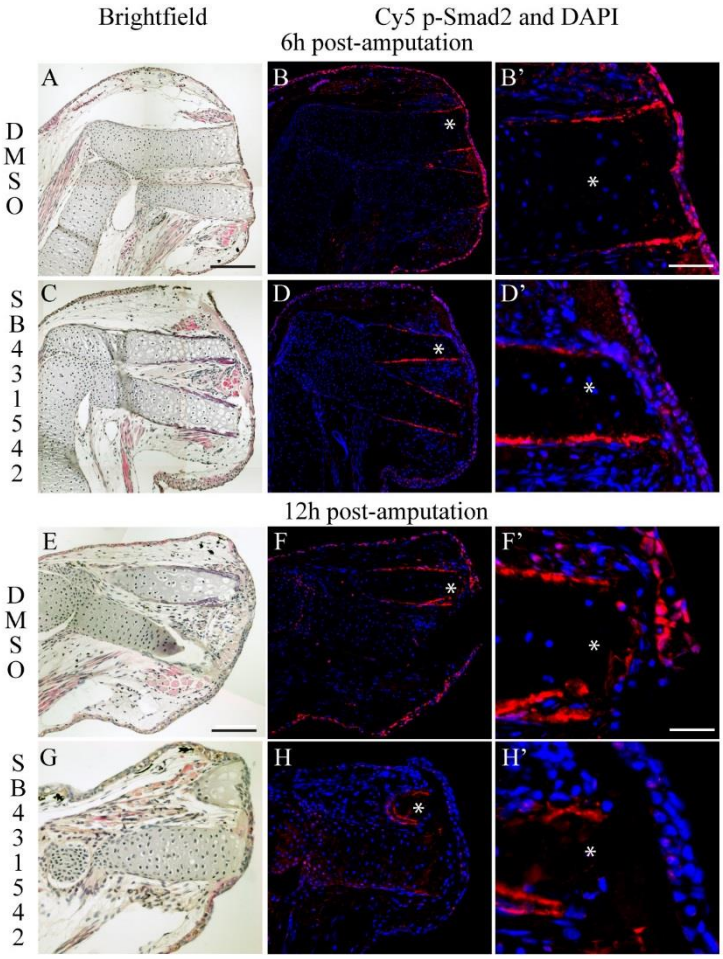
Supplementary Figure 4: **Overexpression of axolotl Smad3 leads to increased apoptosis in AL-1 cell line**

(**A**) Luciferase assay showing that axolotl Smad3 (Smad3 axo) and axolotl Smad3 SΔD (Smad3 axo SΔD, a phosphomimetic Smad3) overexpression have more activity on the CAGA promoter driving luciferase in AL-1cells than control vector (pSport1) (n=12). Data is presented as mean relative luciferase value ± SEM. (**B**) Acridine Orange/Ethidium Bromide double staining cell count for apoptosis; (**C**) viable cell (not counted), (**D**) early apoptosis (counted as apoptotic), (**E**) late apoptosis (counted as apoptotic) and (**F**) necrotic cells (not counted). Data is presented as mean percentage ± SEM. Student's t-test were performed to compare means. There is significantly more apoptosis in axolotl Smad3 and axolotl Smad3 SΔD compared to β-gal (**p< 0.01, * p < 0.05, n=3).



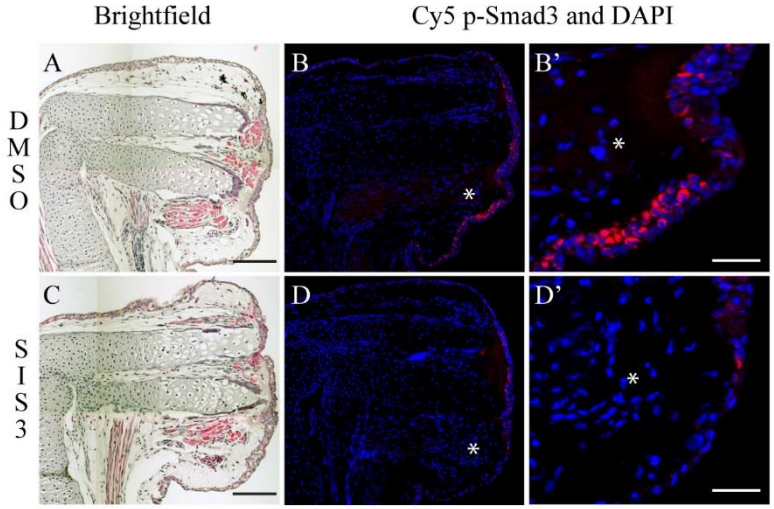
Supplementary Figure 5: **Overexpression of axolotl Smad3 in vivo leads to apoptosis**

(**A**-**A′**) Co-injection and electroporation of β-gal and GFP. Multiple fluorescent cells can be seen. Only a few cells are positive for apoptosis (TUNEL, 4 days post-transfection). (**B**-**B′**) Co-injection and electroporation of axolotl Smad3 and GFP. GFP positive cells are almost absent from slide. Multiple cells near the injection site are positive for apoptosis (TUNEL, 4 days post-transfection). (**C**-**C′**) Co-injection and electroporation of axolotl Smad3 SΔD and GFP. Similar results to what is observed for axolotl Smad3 presented in B and B′, GFP positive cells are almost absent from slide. Multiple cells near the injection site are positive for apoptosis (TUNEL, 4 days post-transfection). Regions in red square are magnified (**A′**, **B′**, **C′**). TUNEL positive cells (red) are pointed with arrows. Composite images are shown and scale bars represent 200μm. (**D**) TUNEL positive cells were counted for each condition (n=5 different animals). Data is presented as number of positive cells ± SEM. There are less apoptotic cells in β-gal and GFP co-injected blastemas compared to axolotl Smad3 and GFP or axolotl Smad3 SΔD and GFP co-injected blastemas. Welch's t-test was performed to compare apoptosis between different treatments; observed difference are statistically significant. ** $p < 0.01$, * $p < 0.05$.



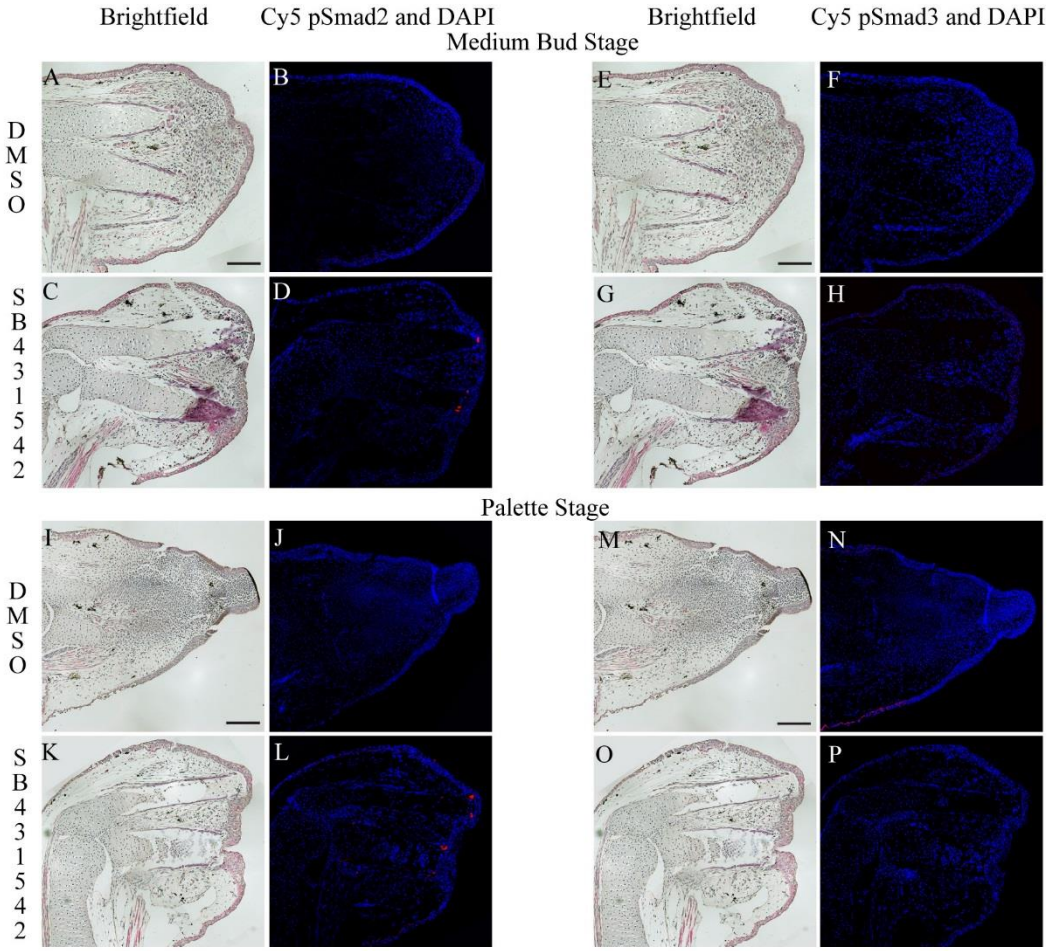
Supplementary Figure 6: **SB-431542 prevents phosphorylation of p-Smad2 in regenerating limbs**

Control animal treated with DMSO for 6h (**A**-**B**) or 12h (**E**-**F**). (**A**,**E**) Hematoxylin and Eosin staining shown in brightfield microscopy. Scale bar is 200µm. (**B**,**F**) Nuclei staining with DAPI (blue) overlaid with immunofluorescence of p-Smad2 (red) shows phosphorylation in most epithelial cells of the wound epithelium and some in the underlying mesenchymal cells. (**B'**,**F'**) Magnified view (Scale bar 50µm, composite images are shown and stars indicate area of magnification). Phosphorylated proteins are often in the nucleus. Animal treated with 25µM SB-431542 for 6h (**C**-**D**) or 12h (**G**-**H**) . (**C**,**G**) Hematoxylin and Eosin staining shown in brightfield microscopy. (**D**-**D'**, **H**-**H'**) Overlay of DAPI and immunofluorescence of p-Smad2 shows very limited positive cells for phosphorylated Smad2 protein. Composite images are shown and stars indicate area of magnification.



Supplementary Figure 7: **SIS3 treatment reduces phosphorylation of p-Smad3**

(**A**-**B**) Control animal treated for 3h with DMSO. (**A**) Hematoxylin and Eosin staining shown in brightfield microscopy. Scale bar is 300µm. (**B**) Overlay of nuclei staining with DAPI (blue) and immunofluorescence of p-Smad3 (red) shows phosphorylation in epithelial cells of the wound epithelium, especially near the plane of amputation. (**B'**) Magnified view (scale bar is 60µm, composite images are shown and stars indicate area of magnification) shows that phosphorylated proteins are often in the nucleus (pink). (**C**-**D**) Animal treated for 3h with 5µM SIS3. (**C**) Hematoxylin and Eosin staining shown in brightfield microscopy. Scale bar is 300µm. (**D**-**D'**) Overlay of nuclei staining with DAPI (blue) and immunofluorescence of p-Smad3 (red) shows reduced number of positive cells for phosphorylated Smad3 protein and (**D'**) p-Smad3 signal is not localized in nucleus. Scale bar is 60µm. Composite images are shown and stars indicate area of magnification.



Supplementary Figure 8: **p-Smad2 and p-Smad3 are not detected at Medium Bud and Palette stages**

Control animal treated with DMSO until MB (**A**-**B**, **E**-**F**) or Pal (**I**-**J**,**M**-**N**). (**A**,**E**,**I**,**M**) Hematoxylin and Eosin staining shown in brightfield microscopy. Composite images are shown and scale bars are 200µm. (**B**,**F**,**J**,**N**) Nuclei staining with DAPI (blue) overlaid with immunofluorescence of p-Smad2 (**B**,**J**) or p-Smad3 (**F**,**N**) shows no phosphorylation. **Animal treated with 25µM SB-431542** until MB (**C**-**D**, **K**-**L**) or Pal (**G**-**H**, **O**-**P**). (**C**,**G**,**K**,**O**) Hematoxylin and Eosin staining shown in brightfield microscopy. (**D**,**H**,**L**,**P**) Overlay of DAPI and immunofluorescence shows no positive cells for phosphorylated proteins.

Supplementary Table 1: List of primers

Gene Name		Primer sequence 5' to 3'
Smad3 probe primers	3RSmd3-11 (forward)	AATCAGGGTTTCGAAGCCGT
	5RSmd3-5 (reverse)	CTGATTTACAGATTGGGACAA
Axolotl Smad2 (used for probe)	aSmd2F59	ATTCAGAACCAGCGTTTTGG
	aSmd2R598	ATTGCAGAGGTCCATTCAGG
Axolotl Smad3	Smad3_axo_F515	GGAGCTCTGCGAGTATGCCT
	Smad3_axo_R997	CTCTCCCACTCGTTGATTAAGC
Axolotl Smad7 (used for probe)	aSmd7F95	GCCTTCCTCCACTGAAACTG
	aSmd7R445	GTGGCCGACTTGATGAAAAT
Axolotl MMP2	aMMP2F100	TCAGAAGGCTCTCCCTGTGT
	aMMP2R779	GCTGCATCCACATGTTTCAC
Axolotl MMP9	F485_MMP9axo	AAGGGGGCTTGCAGGATAA
	R1091_MMP9axo	AGCACAGAAGTGTGGGCTCT
Axolotl MMP13	F2381_MMP13axo	AAAACGACGCTCCAAAACAC
	R2565_MMP13axo	AAGGCACACTCTCAGCCAAA
Axolotl MMP14	F2795_MMP14axo	TGGATAACTGAATGTGCGGA
	R3046_MMP14axo	GACGCTGACACTCAACCTCA
Axolotl GAPDH	aGAPDHF709	AGCTCAATGGGAAACTCACTGGC
	aGAPDHR966	TCACAAAGTGATCGTTGAGGGCA

Supplementary Table 2: Antibodies and blotting conditions

Antibody	Manufacturer	Ref #	Dilution	Conditions	Blocking	ECL
Smad2	Cell Signaling	5339	1/500	O.N. 4°C	5% milk in PBST, 6h 4°C	GE*
p-Smad2	Cell Signaling	3108	1/500	O.N. 4°C	5% chicken serum, 0,75% BSA in TBST, 1h30 RT	LL+**
Smad3	Zymed	51-1500	1/500	O.N. 4°C	5% milk in PBST, 6h 4°C	GE*
p-Smad3	*See materials and methods*	N/A	1/2500	O.N. 4°C	5% chicken serum, 0,75% BSA in TBST, 1h30 RT	SFE***
TGF-β1	Santa Cruz	Sc-146	1/500	O.N. 4°C	5% chicken serum, 0,75% BSA in TBST, 1h30 RT	GE*
GAPDH	Sigma	G8795	1/1000	1h RT	5% chicken serum in PBST, 1h RT	GE*

*Western blotting detection reagents, GE Healthcare, ref#RPN2109, Buckinghamshire, UK

**Lumi-Light^{Plus} Western Blotting Substrate, Roche, ref#12015196001

***SignalFire^{TM} Elite ECL Reagent, Cell Signaling, ref#12757

Supplementary Table 3: Plasmids used in injection/electroporation experiment

Gene expressed by plasmid with CMV promoter	Backbone	Quantity used (µg)
GFP	Max GFP	0.5
β-gal	pSport1	1
axolotl Smad3 (wild type)	pSport1	1
Axolotl Smad3 SΔD (Serine 423-425 mutated to glutamic acids)	pSport1	1

Supplementary References:

Guimond, J. C., Levesque, M., Michaud, P. L., Berdugo, J., Finnson, K., Philip, A. and Roy, S. (2010). BMP-2 functions independently of SHH signaling and triggers cell condensation and apoptosis in regenerating axolotl limbs. *BMC Dev Biol* **10**, 15.
Kasibhatla, S., Amarante-Mendes, G. P., Finucane, D., Brunner, T., Bossy-Wetzel, E. and Green, D. R. (2006). Acridine Orange/Ethidium Bromide (AO/EB) Staining to Detect Apoptosis. *CSH Protoc* **2006**.
Ribble, D., Goldstein, N. B., Norris, D. A. and Shellman, Y. G. (2005). A simple technique for quantifying apoptosis in 96-well plates. *BMC Biotechnol* **5**, 12.