Supplementary Information for

hoxc12/*c13* as key regulators for rebooting the developmental program in *Xenopus* limb regeneration

Aiko Kawasumi-Kita, Sangwoo Lee, Daisuke Ohtsuka, Kaori Niimi, Yoshifumi Asakura, Keiichi Kitajima, Yuto Sakane, Koji Tamura, Haruki Ochi, Ken-ichi T Suzuki, and Yoshihiro Morishita*

*Corresponding author. Email: yoshihiro.morishita@riken.jp

This PDF file includes:

Supplementary Figures 1 to 16
Supplementary Table 1

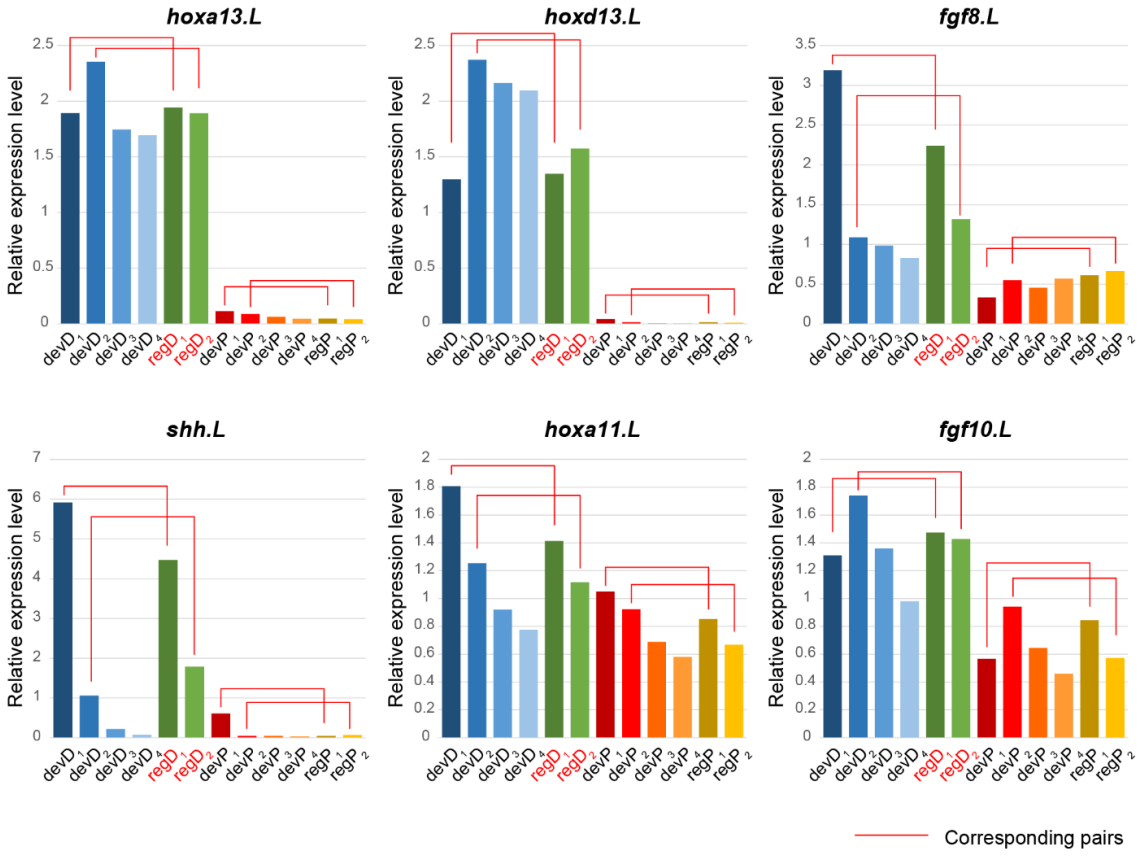

Fig. S1. Results of transcriptomic analysis for typical patterning genes essential for normal limb development

Relative expression levels of the patterning genes are comparable for each corresponding pair (the means of triplicate data). Source data are provided as a Source Data file.

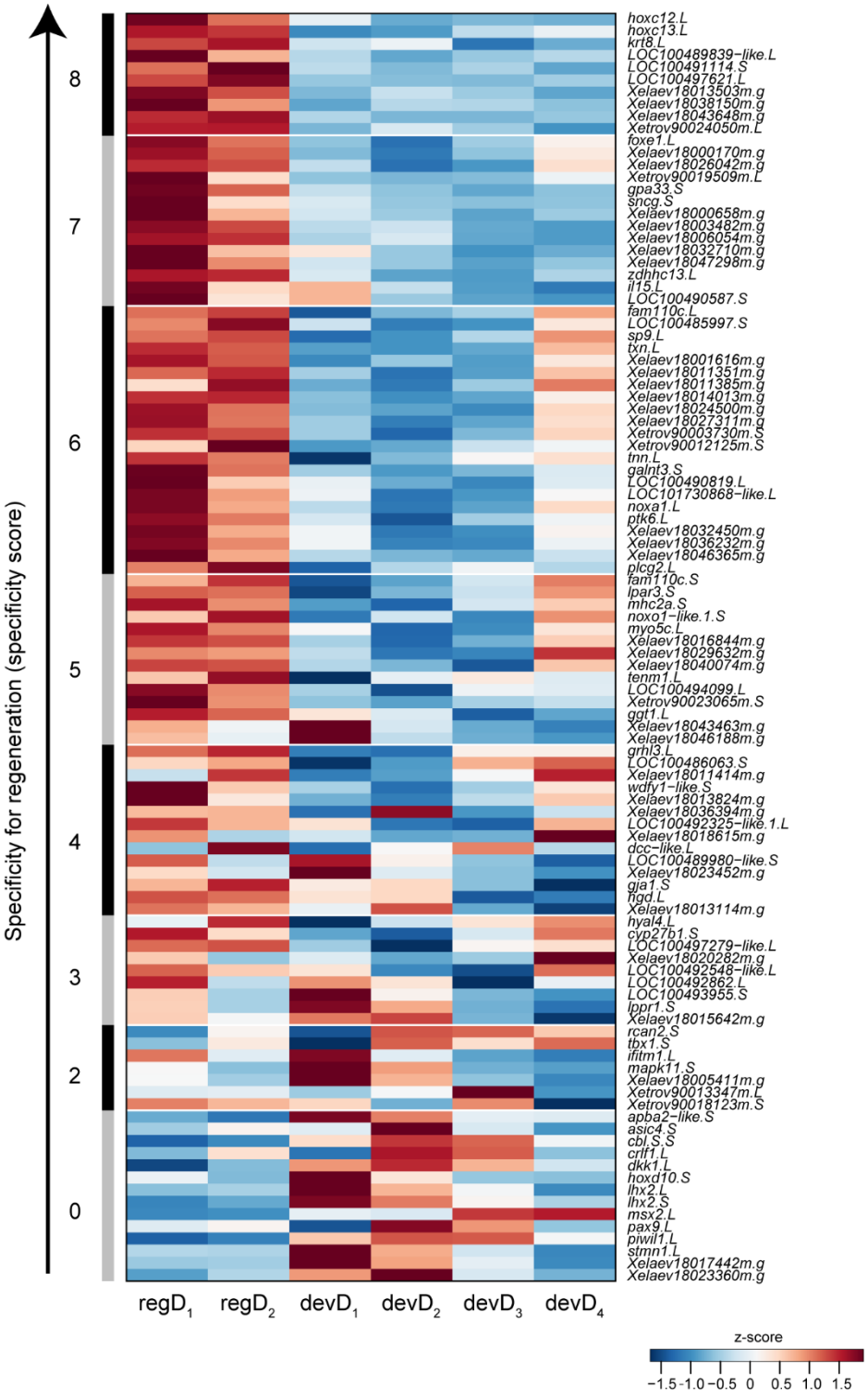

Fig. S2. List of genes remaining after the second screen in the transcriptomic analysis

The genes remaining after the second screen are arranged in order of their specificity scores (see also Figs. 1**e-f**).

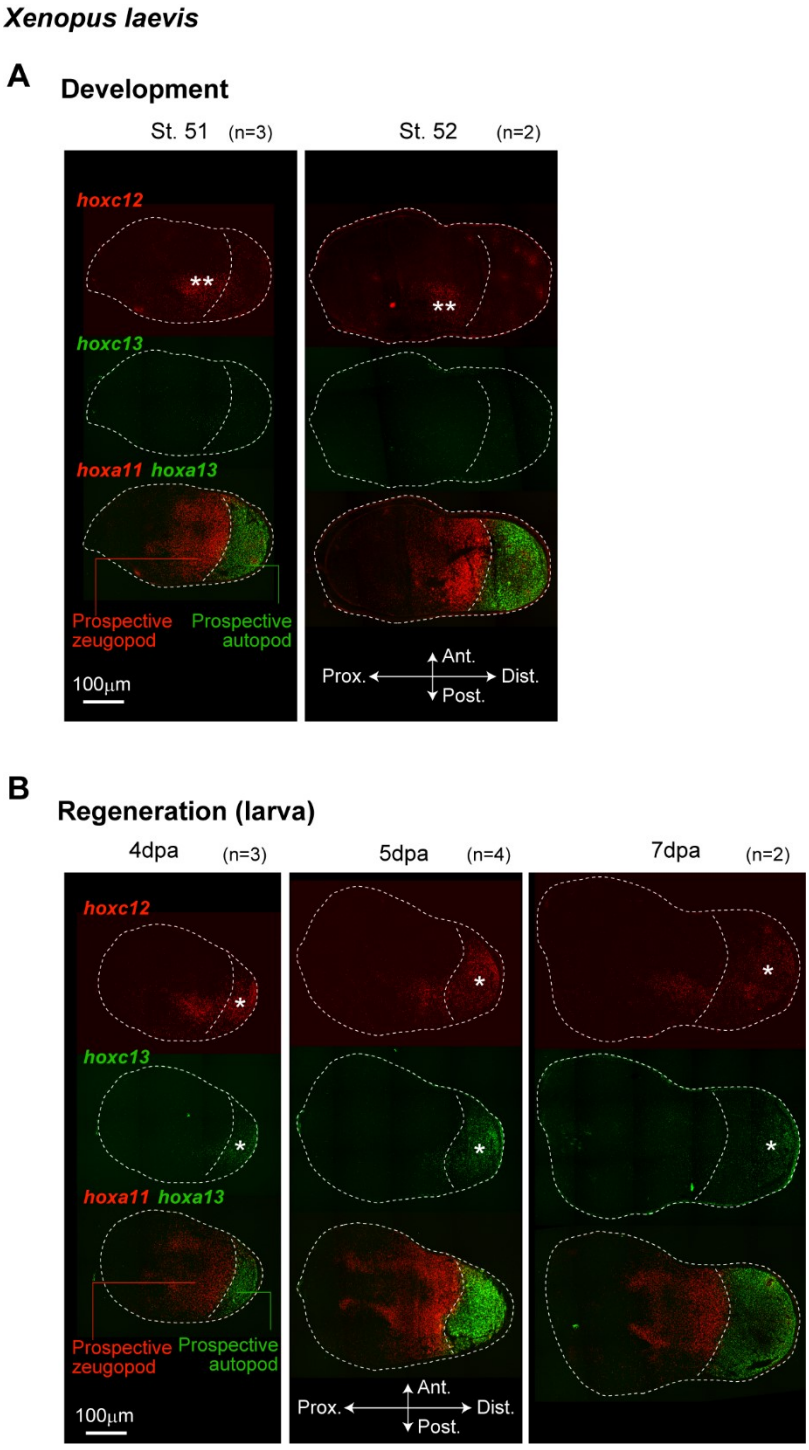

Fig. S3. *hoxc12/c13* expression patterns during limb development and regeneration

(**A**) *hoxc12/c13* expression patterns in the developing limb buds at St. 51 and 52, and (**B**) in the larval regenerating limb blastema at 4, 5, and 7 dpa. Each experiment was independently repeated for each gene with similar results.

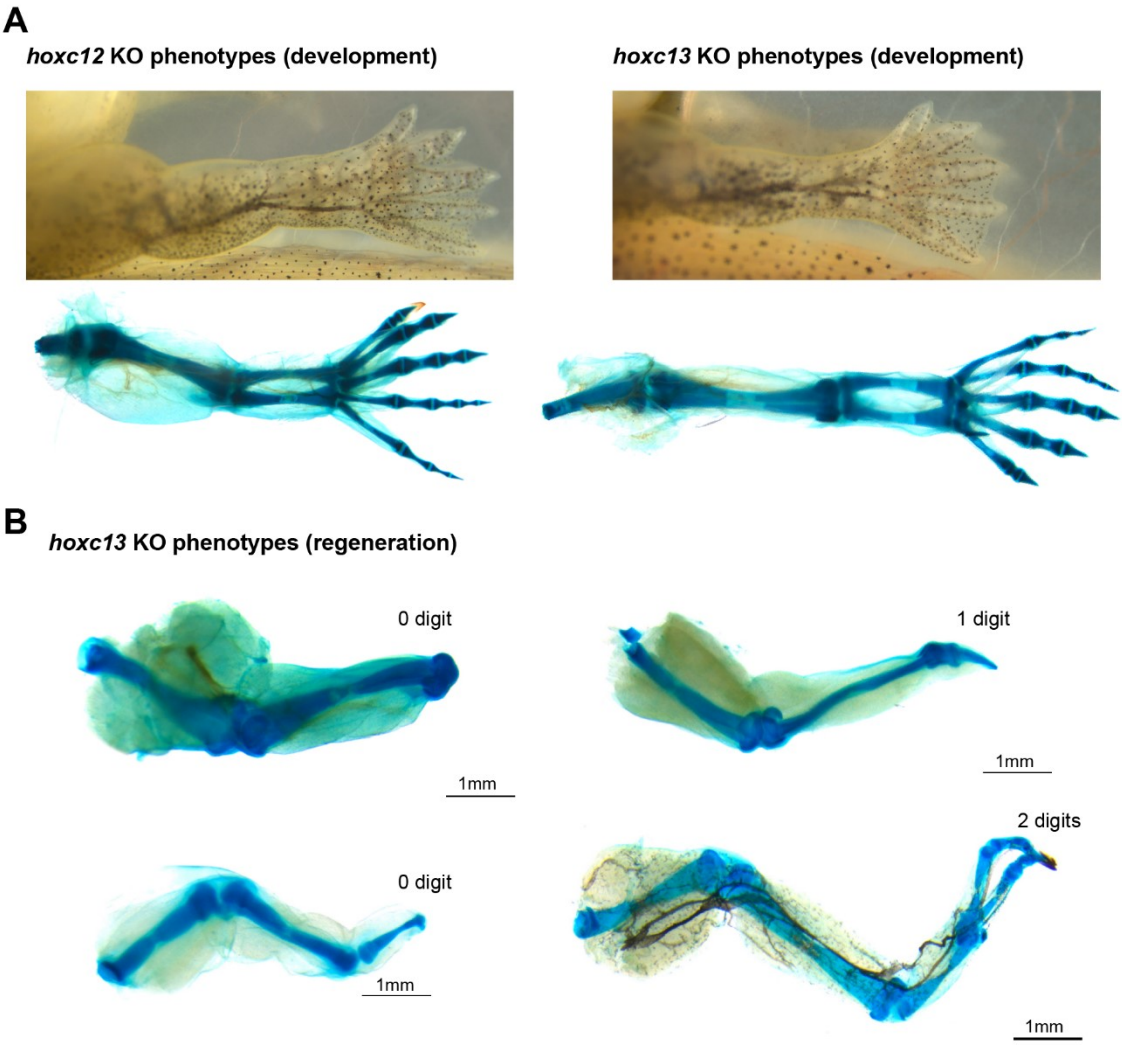

Fig. S4. Effects of *hoxc12*/*c13* knockout on larval limb development and regeneration
Examples of limb morphologies/cartilage patterns in development **(A)** and in regeneration **(B).** Cartilage was stained with Alcian blue. *hoxc12*-KO: *hoxc12*$^{-/-}$; *hoxc13*-KO: *hoxc13*$^{-/-}$.

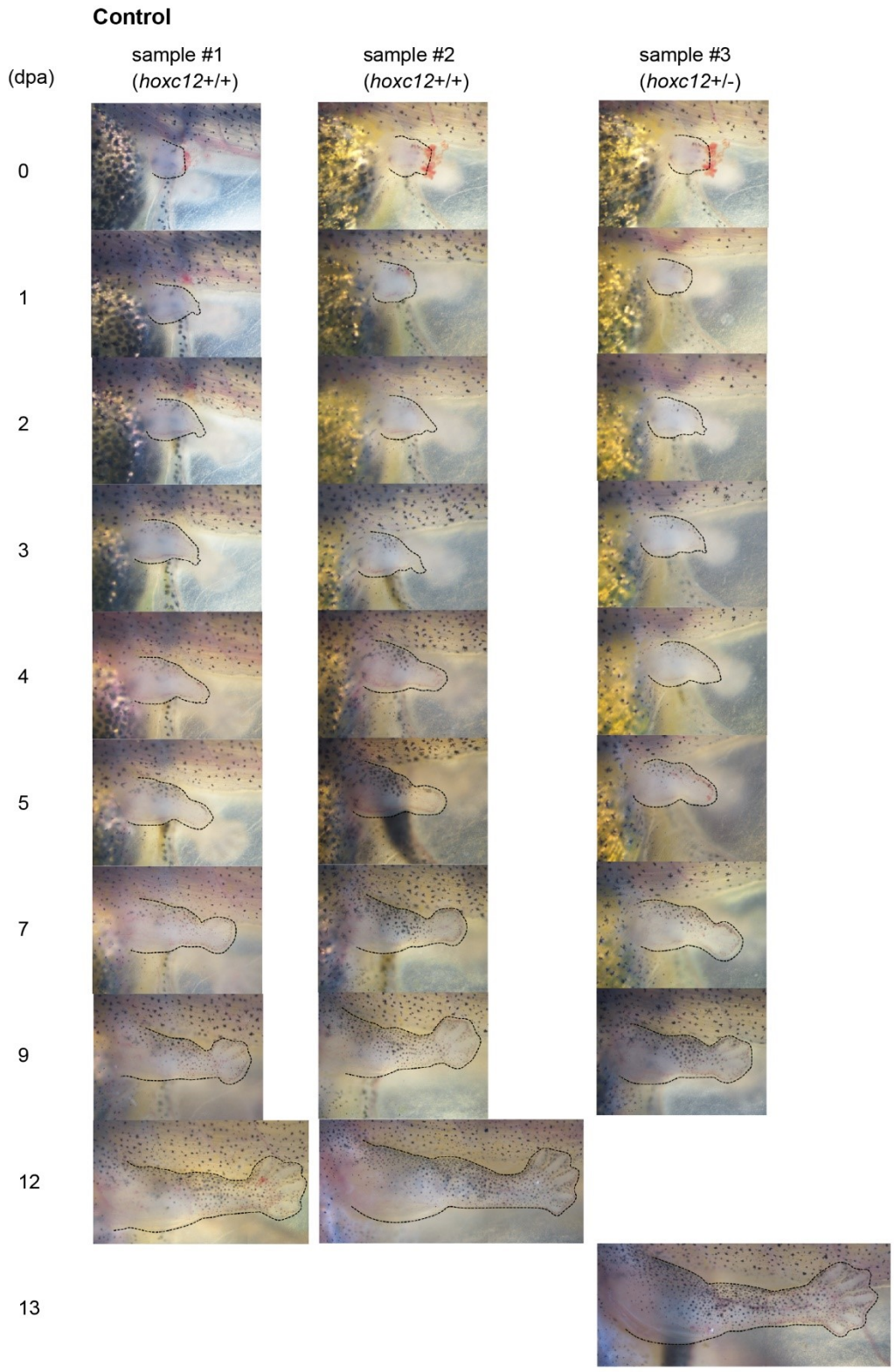

Fig. S5. Time course of regenerating limb blastema in wild type individuals

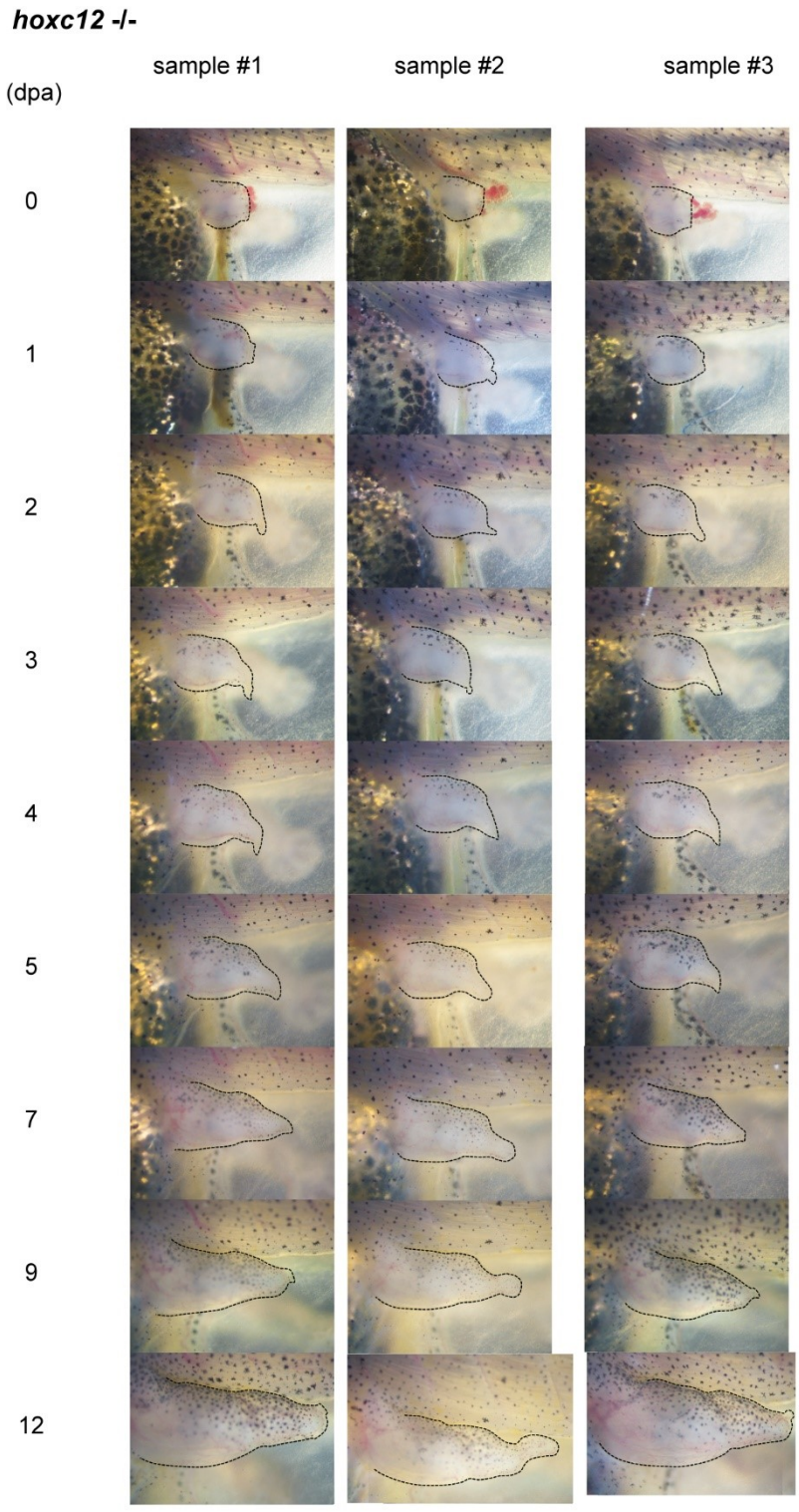

Fig. S6. Time course of regenerating limb blastema in *hoxc12* $^{-/-}$ individuals

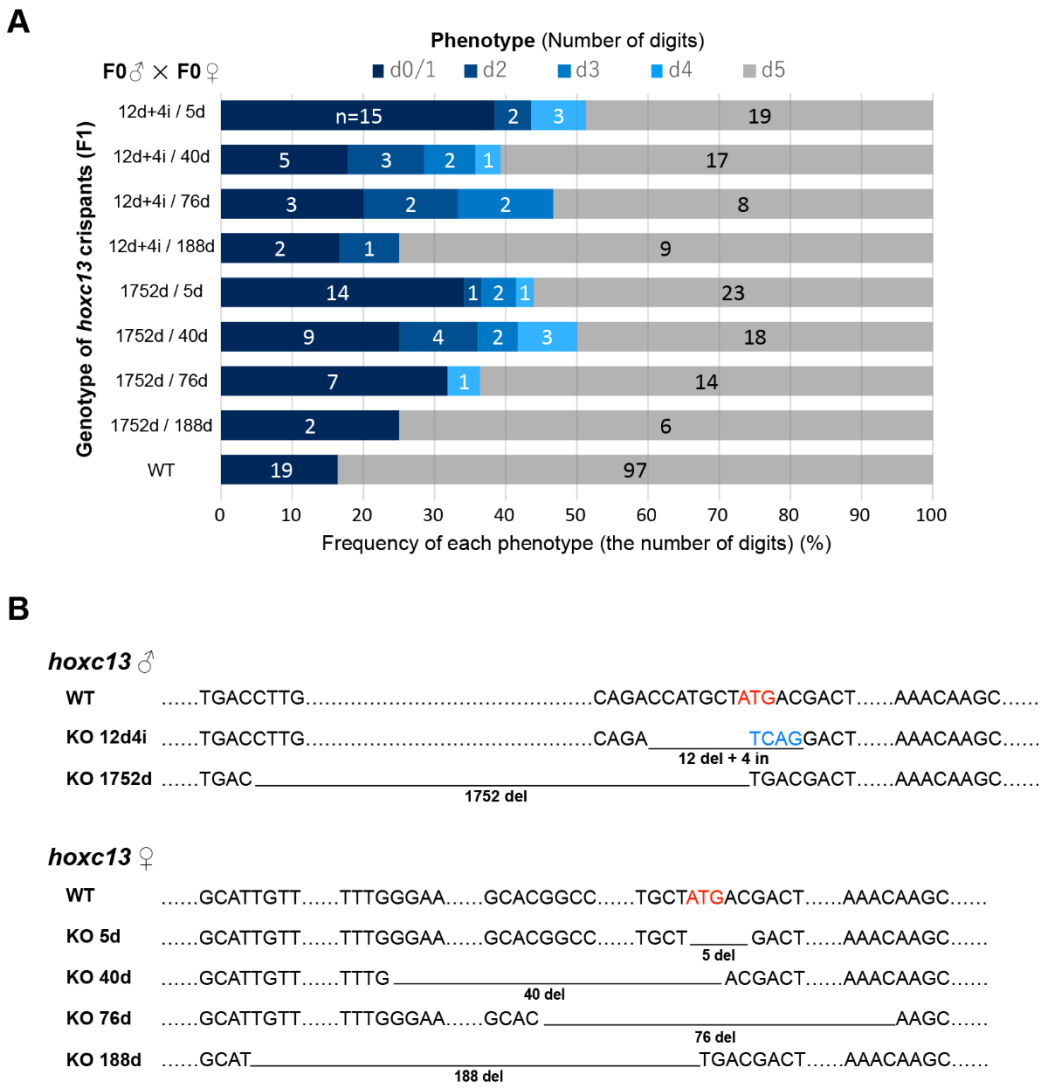

Fig. S7. Effects of *hoxc13* knockout by genome editing in larval limb regeneration

(**A**) Frequency distribution of phenotypes defined by the number of digits for individuals with deletions or insertions of different sub-sequences by genome editing. (**B**) Information about the deletion or insertion of sub-sequences for each parent.

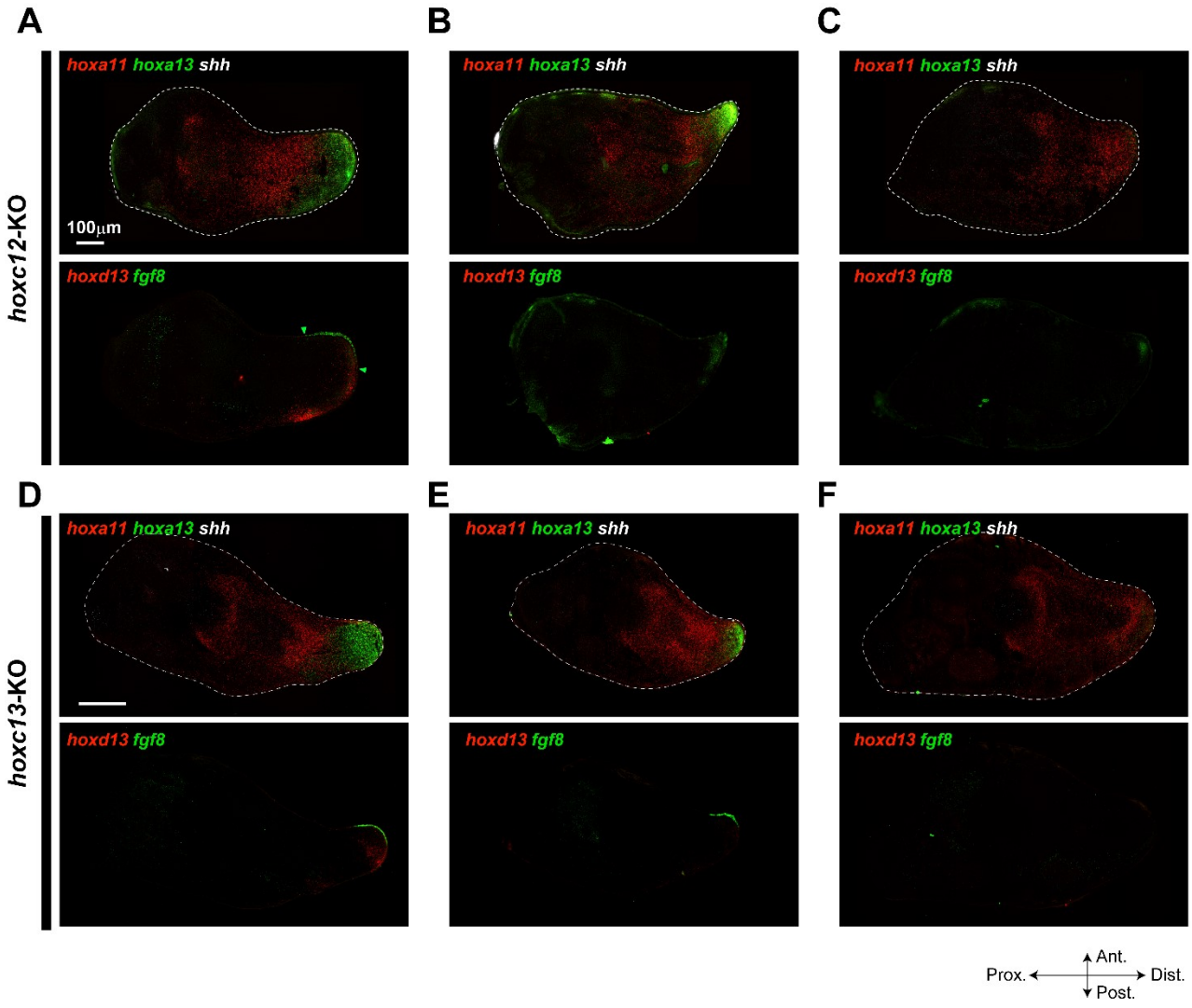

Fig. S8. RNAscope results for pattering genes (6 examples in addition to those shown in Fig.3a)

(**A**, **D**) Mild morphological defects. *hoxa11* and *hoxa13* were exclusively expressed along the PD axis. shh expression was not detected. *fgf8* expression was observed in the anterior half of the distal epithelium. The expression range of *hoxd13* was smaller than during normal development (Fig. 3**a**). (**B**, **E**) Blastema growth was severely reduced. *hoxa13* was expressed at the distalmost region, but no exclusive expression with *hoxa11* was observed. *shh*, *hoxd13* expression was almost undetectable. *fgf8* expression was almost undetectable in (**B**) and was observed in the anterior half of the distal epithelium in (**E**). (**C**, **F**) Blastema growth was also severely reduced. Expression of genes other than *hoxa11* was almost undetectable.

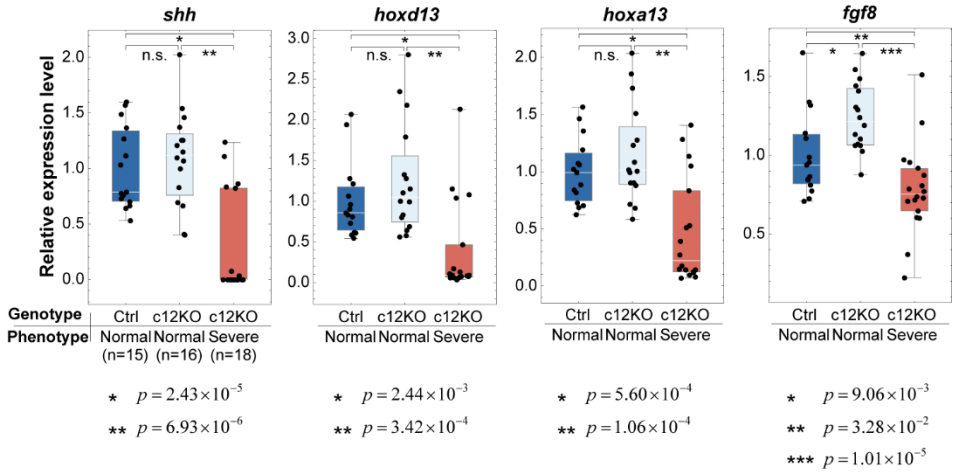

Fig. S9. Result of qPCR assay for some patterning genes. Comparisons were made among control (*hoxc12*+/-, with normal phenotype) and *hoxc12*-KO (with normal or severe phenotype). Box-plot elements: center line, median; box limits, upper and lower quartiles; whiskers, max/min. Statistical test (Student's t-test, two-sided). Source data are provided as a Source Data file.

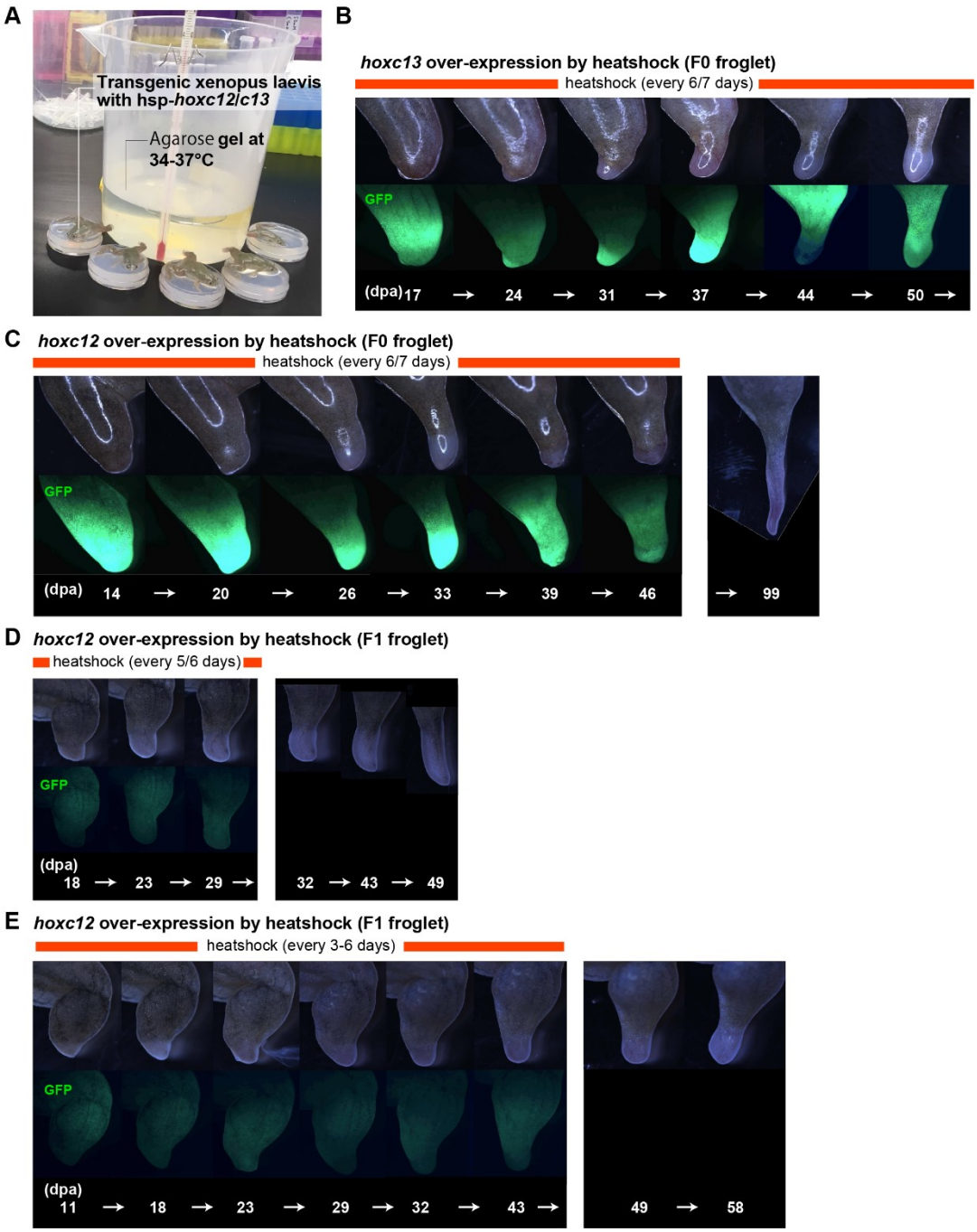

Fig. S10. Heat-shock induction of *hoxc12* or *c13* expression and phenotypes

(**A**) Photo showing the experimental set up for the heat-shock induction of *hoxc12* or *hoxc13* expression by embedding amputated hindlimbs in agarose gel at 34-37°C (see Methods). (**B**-**E**) Morphological changes in blastema with *hoxc12* or *hoxc13* overexpression induced by heat-shock at varying durations (4 examples in addition to that shown in Fig. 4**b**). It should be noted that the induced GFP level by heat-shock in F1 individuals are weaker than that in F0, but still stronger than that by amputation stress only, for which microscopic detection was difficult.

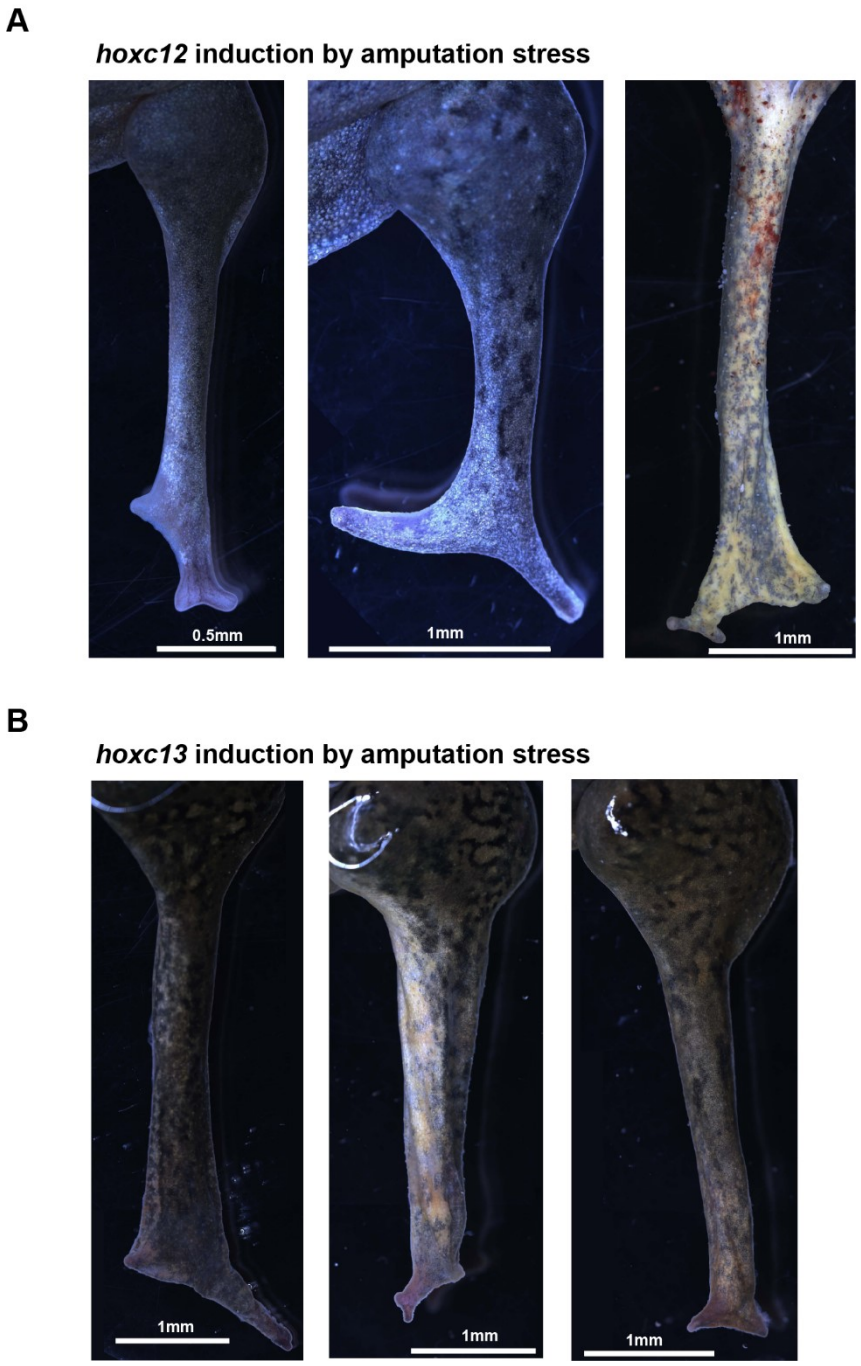

Fig. S11. Regenerated limbs with multifurcated distal cartilage

Phenotypes generated by (**A**) *hoxc12* or (**B**) *hoxc13* induction by amputation stress (without heat-shock).

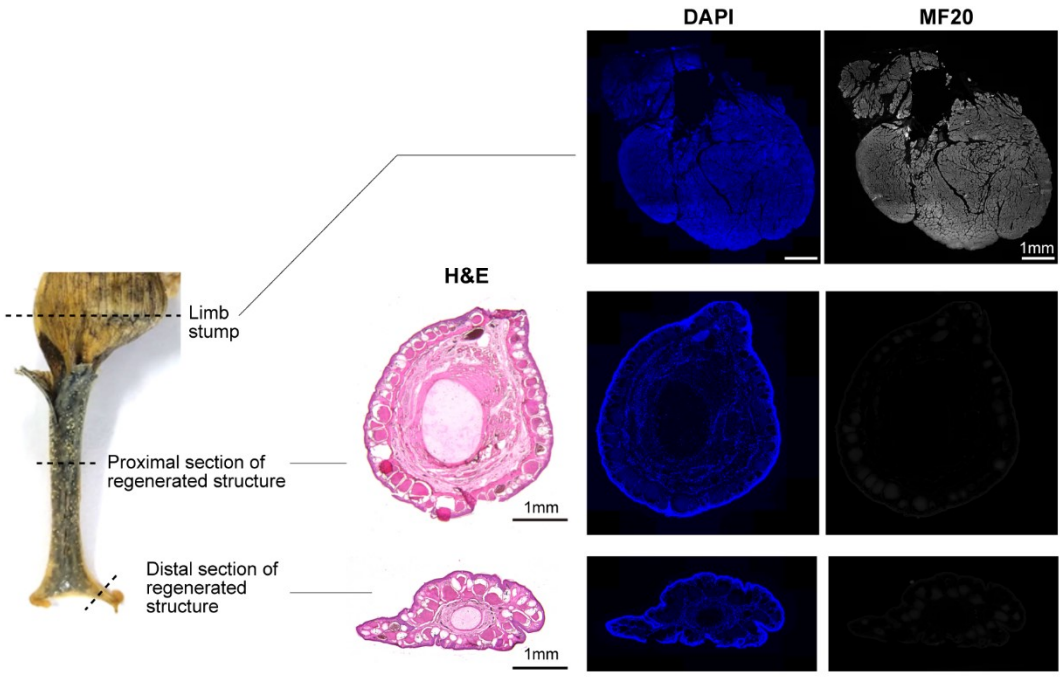

Fig. S12. Muscle staining using MF20. No muscle was observed within the regenerated limb structures of Tg individuals.

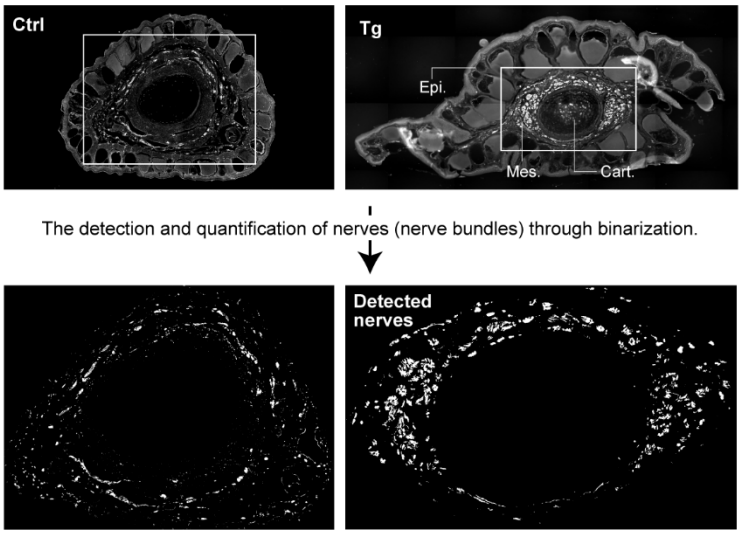

Fig. S13. Image analysis for detecting nerves (acetylated tubulin signal).

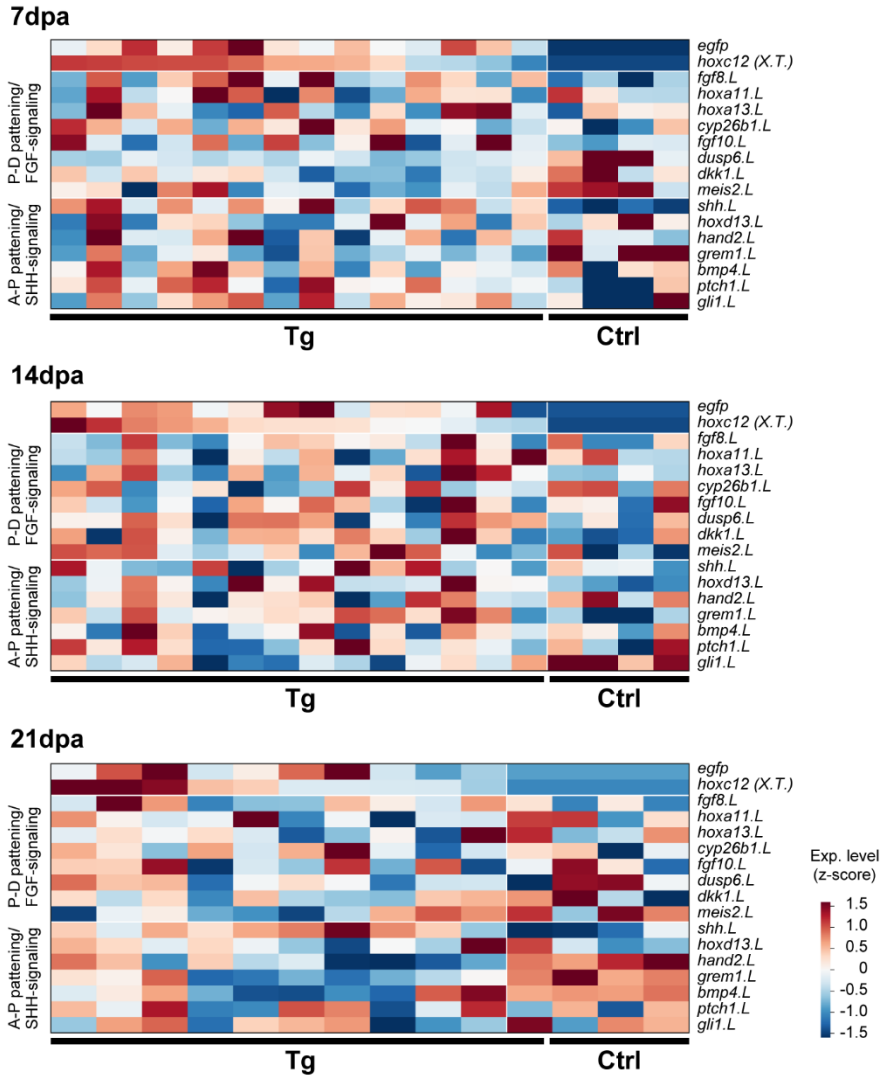

Fig. S14. Comparative analysis of transcriptome data (froglet regeneration at 7, 14, 21 dpa) for typical patterning genes that function during limb development

Source data are provided as a Source Data file.

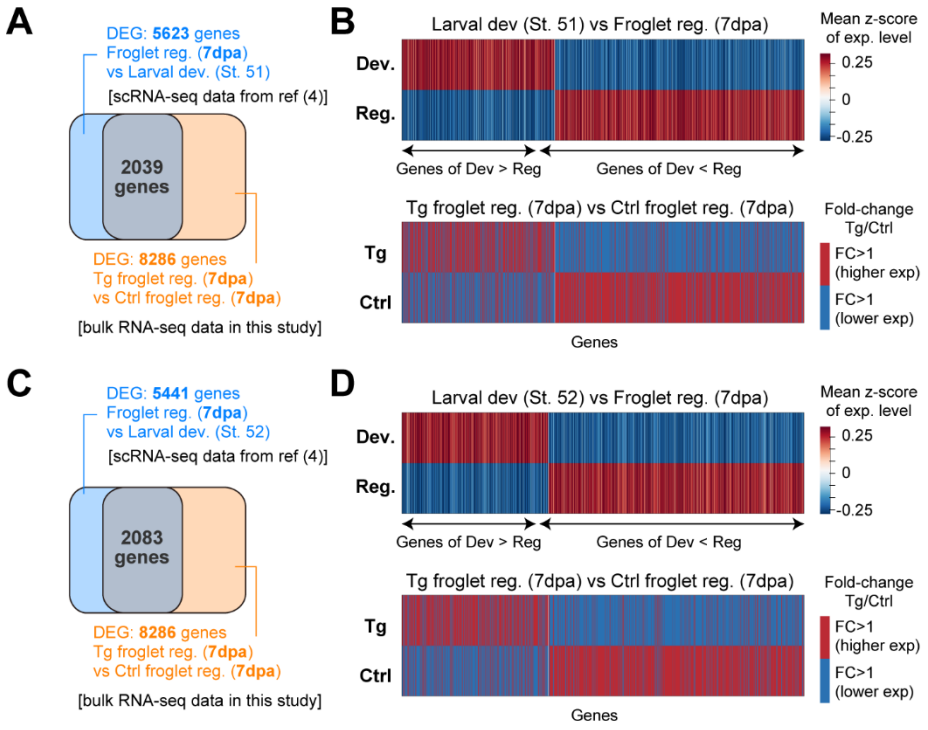

Fig. S15. Comparative transcriptome analysis. (**A**, **C**) Extraction of differentially expressed genes (DEGs) between developing limb buds (**A**: St. 51; **C**: St.52) and froglet blastema (7dpa) based on single cell transcriptome data from Lin et al (2021) ($DEG_{bl\text{-}dev}$), and DEGs between *hoxc12*Tg/control froglet blastema (7dpa) based on bulk-transcriptome data obtained in this study (DEG_{Tg}). (**B**, **D**) Comparison of expression levels for genes that are common in both $DEG_{bl\text{-}dev}$ and DEG_{Tg}, which are identified in **A** and **C**, respectively; (top) developing limb bud vs froglet blastema and (bottom) Tg vs control froglet blastema. Source data are provided as a Source Data file.

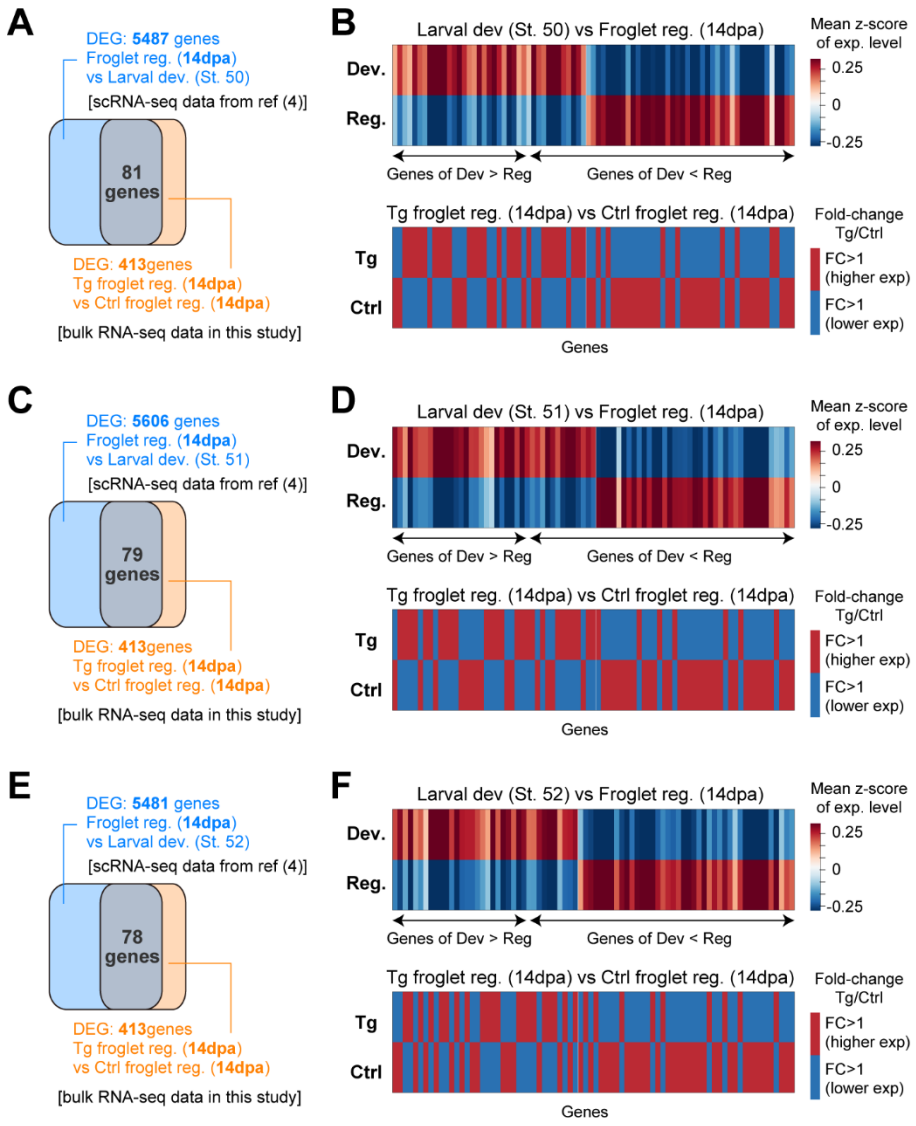

Fig. S16. Comparative transcriptome analysis. (**A**, **C, E**) Extraction of differentially expressed genes (DEGs) between developing limb buds (**A**: St. 50; **C**: St. 51, **E**: St. 52) and froglet blastema (14dpa) based on single cell transcriptome data from Lin et al (2021) ($DEG_{bl\text{-}dev}$), and DEGs between *hoxc12*Tg/control froglet blastema (14dpa) based on bulk-transcriptome data obtained in this study (DEG_{Tg}). (**B**, **D, F**) Comparison of expression levels for genes that are common in both $DEG_{bl\text{-}dev}$ and DEG_{Tg}, which are identified in **A**, **C**, and **E**, respectively; (top) developing limb bud vs froglet blastema and (bottom) Tg vs control froglet blastema. Source data are provided as a Source Data file.

Gene	Forward Primer Sequence	Reverse Primer Sequence	Reporter 1 Sequence
hoxa11 (***X.trop***)	CGGCAACAATGAGGACAAAGC	CTGGTATTTGGTATAAGGGCACCTT	CTGCCCACTGCTGCTG
hoxa13 (***X.trop***)	CAACCCACCTCTGGAAATCGT	CGAGCTGCTGTCTGACTGATG	CCTGCCGGATGTCGTG
hoxc12.L (***X.laev***)	CCGACTAATCAAGGCAACAACAGT	TCGGGACCTTGTGTGAATTGG	AAGGAGCCCCACTGGCA
hoxc13.L (***X.laev***)	CGGTCACTTATGGAAAGCACCTT	CCCCTGCGGTAGTTGCT	CCCAGATGTGGTCCCC
hoxd13 (***X.trop***)	CAAGCCCCACACTTCTGGAA	ACATGTCCGCCTGGTTTAGC	CCTTCCCAGGAGATGTAG
fgf8 (***X.trop***)	GATTAACGCCATGGCAGAAGAC	AACTCTGCTTCCAAACGTATCTGTT	ACCCACATGCCAAGTTA
Shh.L (***X.laev***)	CGAGTCCAAAGCTCATATTCACTGT	AGCACCCGCCAGACTTG	ACTGAGTTCTCTGCTTTGAC

Supplementary Table 1: The primers and fluorogenic probes for all genes in real-time PCR analysis.