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TGF-β1 signaling is essential for tissue regeneration in the *Xenopus* tadpole tail

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Abstract

Amphibians such as *Xenopus tropicalis* exhibit a remarkable capacity for tissue regeneration after traumatic injury. Although transforming growth factor-β (TGF-β) receptor signaling is known to be essential for tissue regeneration in fish and amphibians, the role of TGF-β ligands in this process is not well understood. Here, we show that inhibition of TGF-β1 function prevents tail regeneration in *Xenopus tropicalis* tadpoles. We found that expression of *tgfb1* is present before tail amputation and is sustained throughout the regeneration process. CRISPR-mediated knock-out (KO) of *tgfb1* retards tail regeneration; the phenotype of *tgfb1* KO tadpoles can be rescued by injection of *tgfb1* mRNA. Cell proliferation, a critical event for the success of tissue regeneration, is downregulated in *tgfb1* KO tadpoles. In addition, *tgfb1* KO reduces the expression of phosphorylated Smad2/3 (pSmad2/3) which is important for TGF-β signal-mediated cell proliferation. Collectively, our results show that TGF-β1 regulates cell proliferation through the activation of Smad2/3. We therefore propose that TGF-β1 plays a critical role in TGF-β receptor-dependent tadpole tail regeneration in *Xenopus*.

Keywords

TGF-β1; *Xenopus* tail regeneration; cell proliferation; Smad2/3

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Introduction

Xenopus tropicalis tadpoles are able to regenerate appendages, including tails and limbs, through the activation of cell proliferation [1]. By contrast, mammals have a limited capacity for regeneration of damaged tissues due in part to the inability to reactivate cell proliferation after traumatic injury, indicating that cell proliferation is an important component of tissue regeneration [2, 3]. TGF-β signaling regulates several aspects of regeneration such as wound healing, cell proliferation, and tissue differentiation in the *Xenopus* tadpole tail and also in the zebrafish heart and axolotl limbs [4, 5, 6]. Thus, the function of TGF-β signaling is conserved among animal species that can accomplish tissue regeneration. However, it is not clear whether TGF-β ligands are required for tissue regeneration.

The TGF-β superfamily of ligands includes TGFβs, activins, bone morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs) [7]. TGF-βs are produced in an inactive form that associates with the latency associated peptide (LAP) and are stored in the extracellular matrix (ECM). The latent TGF-βs are activated following the cleavage of LAP by protease digestion and are released from the ECM [7, 8]. TGF-β signaling is initiated upon ligand binding to the receptor, which phosphorylates downstream Smad2/3 transcription factors. Following phosphorylation, the pSmad2/3 form a complex with Smad4 and translocate into the nucleus to regulate the transcription of target genes [7]. During *Xenopus* tadpole tail regeneration, inhibition of TGF-β signaling causes a reduction of pSmad2 expression and of the number of mitotically dividing cells [4]. Several TGF-β superfamily ligands (*tgfb1*, *tgfb2*, *inhba* and *gdf11*) are expressed in the regenerating *Xenopus* tail. In zebrafish, it has been shown that morpholino-mediated knock-down of *inhba* impairs fin regeneration [9]. These studies suggest that multiple TGF-β superfamily ligands might be involved in the activation of Smad2/3 and cell proliferation for tissue regeneration. Therefore, determining the function of TGF-β superfamily ligands is essential for a better understanding of how TGF-β signaling regulates tissue regeneration.

In this study, we investigated *X. tropicalis* tadpole tail regeneration and show that *tgfb1* is strongly expressed throughout the regeneration processes. Using the CRISPR/Cas9 technique, we found that TGF-β1 is required for tail regeneration and for activation of Smad2/3 that is crucial for cell proliferation. Furthermore, TGF-β1 was found to positively regulate cell proliferation and tissue differentiation during tail regeneration. These results suggest that TGF-β1 is a key regulator of *Xenopus* tadpole tail regeneration; our findings also contribute to understanding the regulatory mechanisms of tissue regeneration.

Materials and Methods

Animals, microinjection, and amputation

X. tropicalis tadpoles were obtained and maintained as described previously [10]. CRISPR/Cas9 mutagenesis was carried out with minor modifications of previously described protocol [10]. In brief, 1000 pg of sgRNAs were injected (for combinatorial injection, 333 pg each of three sgRNAs) with Cas9 protein (1 ng, Integrated DNA Technologies) into fertilized eggs in 6% Ficoll or 0.2% methylcellulose solution containing 0.1% BSA, 0.5X MMR and 50 μg/ml gentamycin. Tails were amputated from tadpoles at stage 41/42 and the tadpoles were

maintained for 72 hours post amputation (hpa). To inhibit TGF-β signaling, tadpoles were treated with 12.5 μM of SB-505124 (Cayman Chemical) and DMSO (Nacalai) from 1 hour before tail amputation. Animal experiments followed the guidelines of the Animal Experimentation Ethics Committee of Hiroshima University and international regulations.

Phenotyping of *tgfb1* KO tadpoles

We carried out CRISPR/Cas9-mediated mutagenesis of *tgfb1* in *X. tropicalis* embryos following a published F0 mutagenesis strategy [11]. To ensure phenotypic consistency among *tgfb1* KO tadpoles, we generated three sgRNAs targeting different sites of the *tgfb1* gene. We monitored tadpoles daily following injection of the sgRNAs until 72 hpa to evaluate the effect of *tgfb1* KO on tail regeneration. Tadpoles were graded for the extent of tail regeneration based on tail length at 72 hpa as follows: normal tail regeneration, weakly delayed tail regeneration, and severely delayed tail regeneration. The efficiency of *tgfb1* KO was determined using a T7E1 assay and by TA cloning-based genotyping. A rescue experiment using *tgfb1* mRNA was performed to examine the specificity of *tgfb1* KO.

Cloning of the *tgfb1* gene

Full-length *tgfb1* was amplified from *X. tropicalis* embryo (stage 29/30) cDNAs using the following primers: forward 5'- AAG GCC TCA ACC AGG ATC TCC CAC ACT −3' and reverse 5'- GCT CTA GAT GTG GGT TGC GTT GTT TCT A −3'. The amplified product was digested with StuI and XbaI and subcloned into *pDH105* (*pDH105-tgfb1*).

Whole-mount immunostaining and *in situ* hybridization

Immunostaining of phosphorylated Histone H3 (pH3) and pSmad2/3 was performed as previously described with a minor modification [10]. For pSmad2/3 staining, tadpoles were treated with a permeabilization solution (1% NP-40, 1X PBST) for 30 min after bleaching. The following primary and secondary antibodies were used: anti-pH3 antibody at 1:500 dilution (Upstate Biotechnology); anti-pSmad2/3 antibody at 1:500 dilution (Cell Signaling Technology); Alexa Fluor 488 goat anti-rabbit antibody at 1:500 dilution (Molecular Probe); Alexa Fluor 488 goat anti-mouse antibody at 1:500 dilution (Molecular Probe). Whole-mount *in situ* hybridization (WISH) and probe synthesis were carried out as described previously [12, 13]. The *tgfb1* antisense and sense probes were generated from *pDH105-tgfb1*.

Preparation of sgRNAs and mRNA synthesis

sgRNAs were designed and produced as previously described [10, 14]. The following forward primers for sgRNAs targeting *tgfb1* locus were used: sg 1, 5'- ATT TAG GTG ACA CTA TAG GTG TCT ACC TGT AAG ACT GGT TTT AGA GCT AGA AAT AGC AAG −3'; sg 2, 5'- ATT TAG GTG ACA CTA TAG GAG AAT TGA AGC CAT CAG GGT TTT AGA GCT AGA AAT AGC AAG −3'; sg 3, 5'- ATT TAG GTG ACA CTA TAG GTT TAC AAT AGC ACC TTG GGT TTT AGA GCT AGA AAT AGC AAG −3'. Capped *tgfb1* mRNA was generated by *in vitro* transcription of *pDH105-tgfb1* using an SP6 transcription kit (Invitrogen).

Genotyping

Lysis, PCR, T7E1 assay, and TA cloning were performed as described previously [10]. To confirm the genotype of *tgfb1* KO tadpoles, the following primers were used for PCR amplification of the *tgfb1* locus: forward 5'- AAG ACG GGA CAG CAA CTT TC −3' and reverse 5'- TGG CAC ACA TGC AGA ACT ATC −3'.

Quantification and statistical analysis

Fluorescent images were captured with a Zeiss Axio Zoom V-16 system. CellSens standard software (Olympus) was used to measure tail lengths. Statistical comparisons were performed using Student's *t*-tests (*$P < 0.05$, ***$P < 0.001$). The fluorescent intensity of pSmad2/3 immunostaining was quantified using Zen Blue software (Zeiss). A previous report [4] and the present analysis observed a non-specific signal of pSmad2/3 staining in regenerating tails that was not reduced after TGF-β receptor inhibition. To measure the specific fluorescent intensity of pSmad2/3, the mean value of the fluorescence signal in *tyrosinase* KO and *tgfb1* KO tadpoles was subtracted by that of the non-specific signal in SB-505124-treated tadpoles.

Results

tgfb1 is expressed throughout *Xenopus* tail regeneration

Expression of *tgfb1* has previously been described during *X. laevis* tadpole tail regeneration [4]. Consistently, RNAseq analysis of regenerating *X. tropicalis* tails has indicated that *tgfb1* is strongly expressed compared to other TGF-β superfamily ligands [15]. Therefore, we performed a detailed examination of *tgfb1* expression in regenerating *X. tropicalis* tails using WISH (Fig. 1). Before amputation, *tgfb1* expression was detectable throughout the entire tail, especially in the inner fin region. Following amputation, *tgfb1* expression was observed in the amputation plane at 0–1 hpa. At 2–12 hpa, *tgfb1* expression commenced in the regenerating tail tip. Subsequently, at 24–72 hpa, *tgfb1* transcripts were widely present in the regenerating tissues. No signals were detected in regenerating tail tissues using a sense probe as a negative control. These results suggest that *tgfb1* was expressed before and after tail amputation, and that TGF-β1 might be involved in *Xenopus* tadpole tail regeneration.

TGF-β1 is required for *Xenopus* tail regeneration

To determine whether TGF-β1 is required for *Xenopus* tail regeneration, we conducted a CRISPR-mediated loss-of-function experiment. First, we generated *tgfb1* KO tadpoles using single sgRNAs (sg 1, sg 2, or sg 3) that target the latency associated peptide of TGF-β1; the sgRNA was injected into fertilized eggs with Cas9 protein (Fig. 2A). *tgfb1* KO tadpoles injected with single sgRNAs showed a delay in tail regeneration (sg 1, 56.5%; sg 2, 50%; sg 3, 44%; when weakly and severely delayed phenotypes were combined), while *tyrosinase* KO tadpoles did not show this delay (*tyrosinase* KO, 3.8%; Fig. 2B). This phenotypic consistency suggested that the regeneration defect of *tgfb1* KO tadpoles was caused by loss of TGF-β1 function. As single sgRNAs induced a moderate rate of *tgfb1* mutations as determined by a T7E1 assay (data not shown), we injected a combination of all three sgRNAs (sg 1 + sg 2 + sg 3) into fertilized eggs to increase the rate of mutations.

Sequencing analysis of *tgfb1* KO tadpoles produced by the combination of the three sgRNAs showed that all had mutations in the *tgfb1* locus: in-frame mutations, 13.7%; out-of-frame mutations, 86.3% (Supplementary Fig. 1). Furthermore, *tgfb1* KO tadpoles (sg 1 + sg 2 + sg 3) showed a clear delay in tail regeneration (66.6%; Fig. 2B and C) compared to tadpoles injected with a single sgRNA. Therefore, we used *tgfb1* KO tadpoles (sg 1 + sg 2 + sg 3) in the following experiments. We performed a rescue experiment of *tgfb1* KO tadpoles to explore the specificity of the knockout. Overexpression of *tgfb1* partially but significantly rescued the knockout phenotype, indicating that the delay in tail regeneration in *tgfb1* KO tadpoles resulted from inactivation of *tgfb1* (Supplementary Fig. 2).

TGF-β1 is important for tissue differentiation and cell proliferation

Next, we analyzed the effects of *tgfb1* KO on tissue differentiation and cell proliferation in *Xenopus* tail regeneration. The tadpole tail is composed of several types of tissue including spinal cord, muscle, and notochord. We evaluated tissue differentiation at 72 hpa using WISH with *sox2*, *myod1*, and *shh* probes [10, 16, 17]. Differentiation of tail tissues was greatly reduced in *tgfb1* KO tadpoles but was present in *tyrosinase* KO tadpoles, demonstrating that TGF-β1 is necessary for proper tissue differentiation (Fig. 3A). Since blastema cell proliferation precedes tissue differentiation [18], we performed whole-mount immunostaining for pH3 in regenerating tails at 48 hpa to examine cell proliferation in *tgfb1* KO tadpoles. It has been shown that the numbers of mitotic cells in regenerating tail in *Xenopus* tadpoles are reduced by treatment with a TGF-β receptor inhibitor [4]. In agreement with this report, we found that cell proliferation in the regenerating tail excluding the fin was significantly downregulated by treatment with the TGF-β receptor inhibitor SB-505124. Moreover, the numbers of mitotic cells in *tgfb1* KO tadpoles were significantly decreased at 48 hpa compared to *tyrosinase* KO tadpoles (Fig. 3B and C). These results suggest that TGF-β1 is required for cell proliferation and subsequent tissue differentiation in *Xenopus* tail regeneration.

TGF-β1 activates Smad2/3, which is important for cell proliferation

After tail amputation, several mediators of the early responses to injury (reactive oxygen species, mitogen-activated protein kinase, and Smad2/3) are activated in the amputation plane [4, 19, 20]. Among these mediators, pSmad2/3, a downstream signal transducer of the TGF-β receptor, is known to be essential for TGF-β-mediated wound healing and cell proliferation [4]. To investigate whether TGF-β1 is involved in the activation of Smad2/3 in *Xenopus* tail regeneration, we performed a whole-mount immunostaining analysis of pSmad2/3 in the regenerating tails of *tgfb1* KO tadpoles. We found that pSmad2/3 expression was reduced in *tgfb1* KO tadpoles at 6 hpa (Fig. 4). Collectively, the results of these analyses demonstrate that TGF-β1 controls cell proliferation through the activation of Smad2/3 in *Xenopus* tail regeneration.

Discussion

We demonstrate here that TGF-β1 is required for tissue regeneration in the *X. tropicalis* tadpole tail. Previous studies investigated the role of TGF-β signaling in tissue regeneration using TGF-β receptor inhibitors in regenerating tissues of several animal species [4, 5, 6, 21,

22]. However, this approach did not enable a detailed analysis of the role of TGF-β ligands in TGF-β receptor-dependent tissue regeneration. As multiple TGF-βs are expressed during tissue regeneration [4, 5, 22], it has been difficult to identify which TGF-βs are critical for regeneration. Here, we show that knockout of *tgfb1* prevents cell proliferation and Smad2/3 activation, and impairs tail regeneration. Our observations clearly show that TGF-β1 is an essential TGF-β ligand for TGF-β receptor-dependent tissue regeneration in *Xenopus* tadpoles.

Interestingly, treatment of tadpoles with a TGF-β receptor inhibitor caused a more severe delay in tail regeneration than the *tgfb1* KO (data not shown) [4]. The TGF-β receptor inhibitors SB-505124 and SB-431542 are potent inhibitors of ALK4, 5 and 7 that interact with multiple TGF-β superfamily ligands (TGF-βs, activins, and GDFs) [23, 24, 25]. It has also been reported that *inhba* and *gdf11* are upregulated at 4 and 48 hpa, respectively, during *X. laevis* tail regeneration [4]. This suggests that in addition to TGF-β1, other TGF-β superfamily ligands might function in *Xenopus* tail regeneration.

Similar functional redundancy may also occur among TGF-βs. Both *tgfb1* and *tgfb2* are expressed during *X. tropicalis* tail regeneration (Fig. 1; data not shown) [15]. In zebrafish, expression of *tgfb2* during Müller glia-mediated retinal regeneration is activated at a similar time-course as *tgfb1* expression. Additionally, although administration of TGF-β1 increases the numbers of mitotic cells after retinal injury, knock-down of *tgfb1* did not significantly affect glial proliferation [21, 22]. These studies and our results suggest that both TGF-β1 and TGF-β2 might contribute to tissue regeneration. Further detailed analyses will be necessary to resolve the likely redundancy of TGF-βs in tissue regeneration.

Expression of *tgfb1* has been shown to gradually increase after tail amputation in *X. laevis* tadpoles [4]. However, the pattern of *tgfb1* expression in undamaged tadpole tails is not well documented. In the present study, we showed that *tgfb1* expression was present in undamaged tails (prior to tail amputation at stage 41/42) of *X. tropicalis* tadpoles (Fig. 1). This suggests that in addition to injury-induced expression of *tgfb1*, TGF-β1 protein might be stored in the ECM of undamaged tails and might contribute to the activation of Smad2/3 immediately after tail amputation. The rapid mobilization of TGF-β1 protein from the ECM may be crucial to the restoration of damaged tissues and for protection against infection, as TGF-β signaling is important not only for cell proliferation but also for the formation of wound epithelium that occurs at an early stage of tail regeneration processes [4]. Consistently, after amputation of *X. laevis* tails or limbs, *tgfb1* is expressed in the apical epithelial cap (AEC) which is located at the distal part of the wound epithelium [26]. As described above, *tgfb1* is the first of the TGF-β superfamily ligands to be expressed during *Xenopus* tadpole tail regeneration and thus it may function as a master regulator to orchestrate the initial responses to injury that eventually result in cell proliferation and differentiation. In addition to *Xenopus* tails and limbs, *tgfb1* expression has been observed during regeneration of other tissues and organs (e.g., fin, heart, retina, and spinal cord) in zebrafish [5, 9, 22, 27]. Therefore, TGF-β1 may be widely involved in the regeneration of lost appendages and of damaged tissues/organs.

Until now, the contribution of TGF-β1 to tissue regeneration had not been completely revealed. Our results clearly demonstrate the essential role of TGF-β1 in the promotion of tissue regeneration through the regulation of cell proliferation activated by pSmad2/3. Thus, this study provides new insights into the molecular mechanisms of TGF-β signal-dependent tissue regeneration upon injury in animals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- *tgfb1* is expressed in *Xenopus* tadpole tail before and after tail amputation
- TGF-β1 is essential for tail regeneration
- TGF-β1 regulates cell proliferation during regeneration by activation of Smad2/3

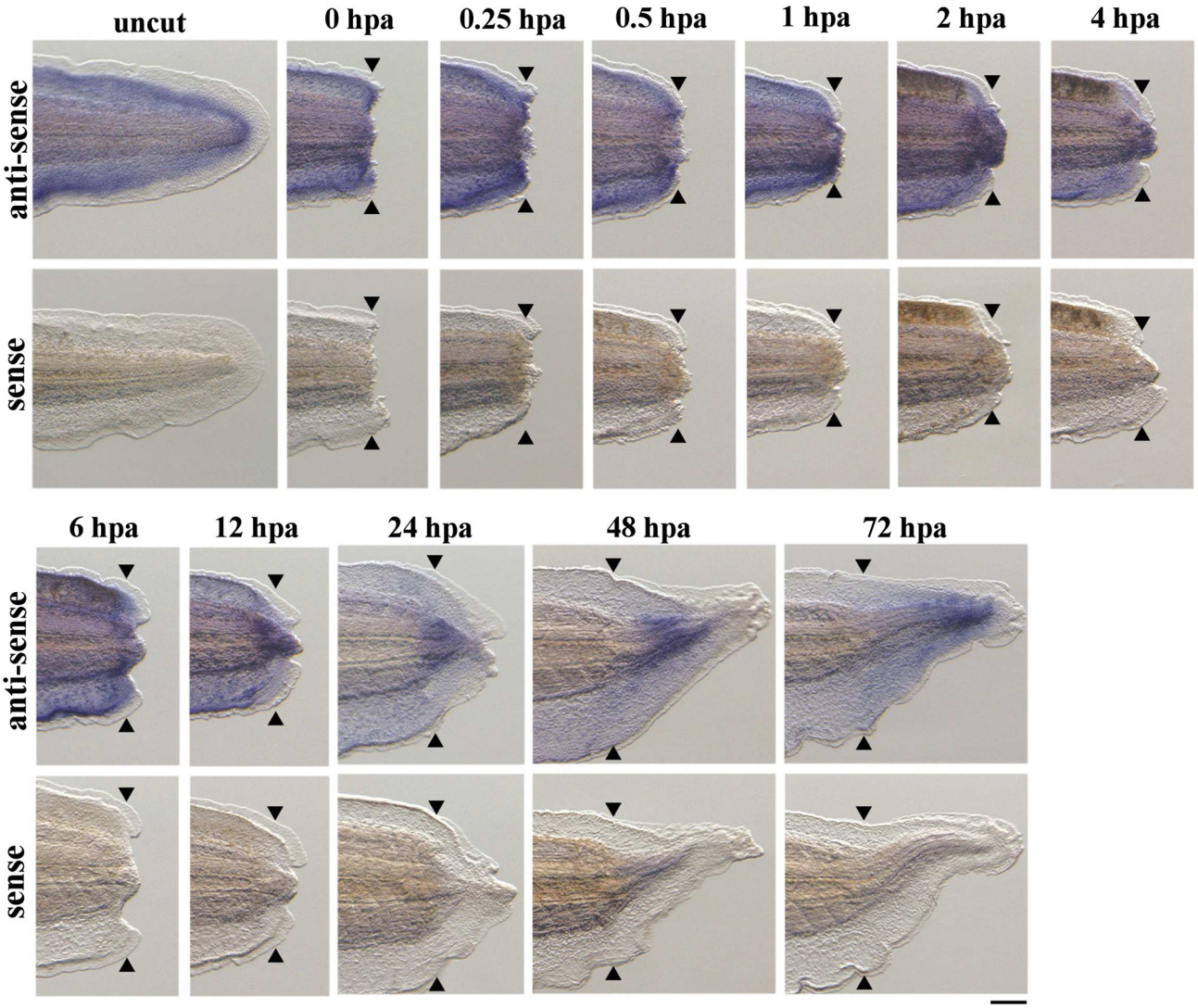

Figure 1. Expression of *tgfb1* in *X. tropicalis* tadpoles before and after tail amputation.

Lateral views of uncut tadpole tails and amputated tails at 0, 0.25, 0.5, 1, 2, 4, 6, 12, 24, 48 and 72 hours post amputation (hpa) after whole-mount *in situ* hybridization using *tgfb1* antisense and sense RNA probes. Black arrowheads show amputation sites. Scale bar, 200 μm.

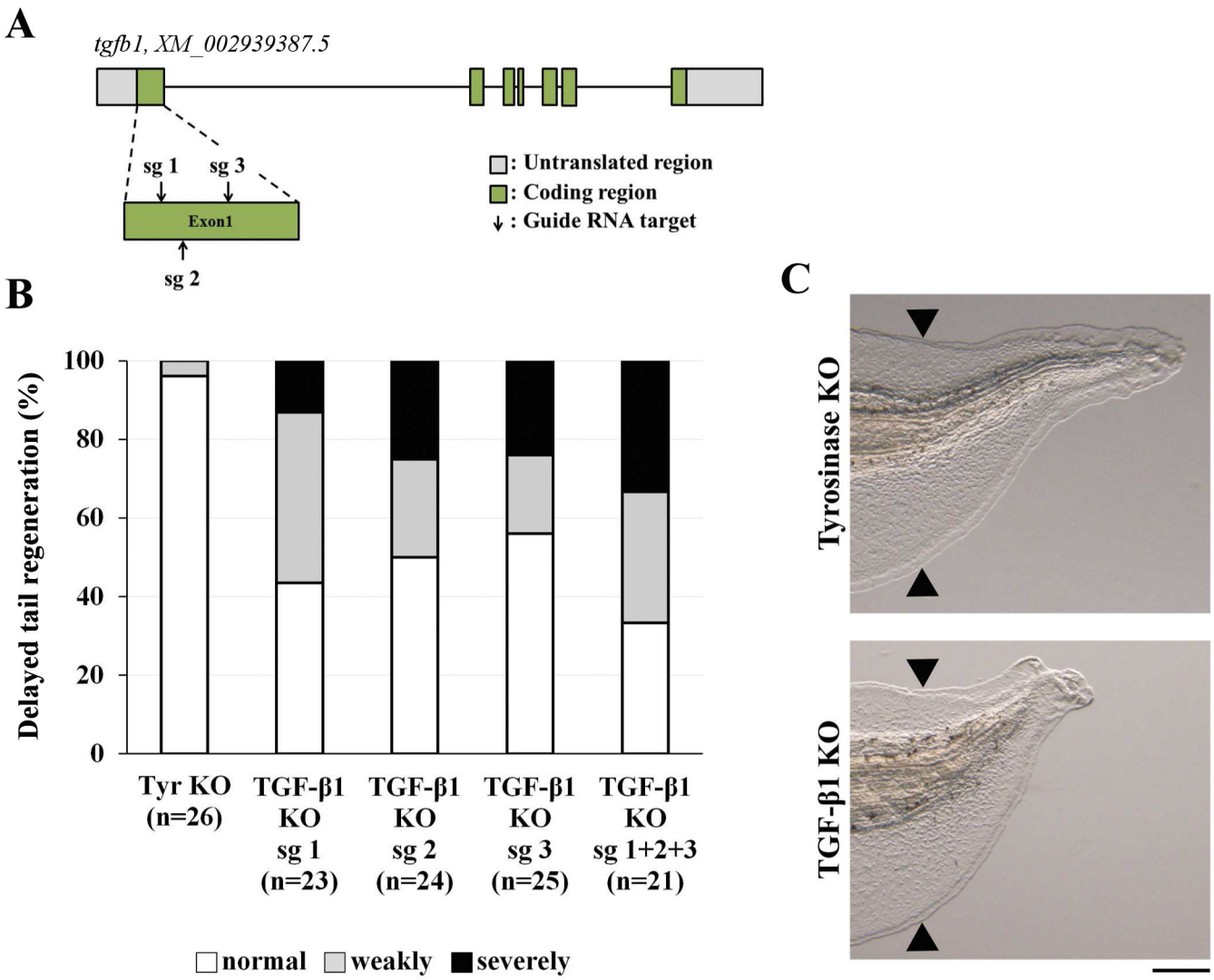

Figure 2. TGF-β1 is required for *Xenopus* tail regeneration.

(A) Schematic drawing of sgRNA target sites (sg 1, sg 2 and sg 3) in the *tgfb1* locus. Grey boxes, untranslated region; green boxes, coding region; arrows, sgRNA target sites; bars, intron regions. (B) Delayed tail regeneration in *tgfb1* KO tadpoles. The extent of tail regeneration was classified at 72 hpa as normal tail regeneration, weakly delayed tail regeneration, and severely delayed tail regeneration. (C) The phenotypes of *tyrosinase* KO (control) and *tgfb1* KO tadpoles (sg 1 + 2 + 3) at 72 hpa. Black arrowheads show amputation sites. Scale bar, 200 μm.

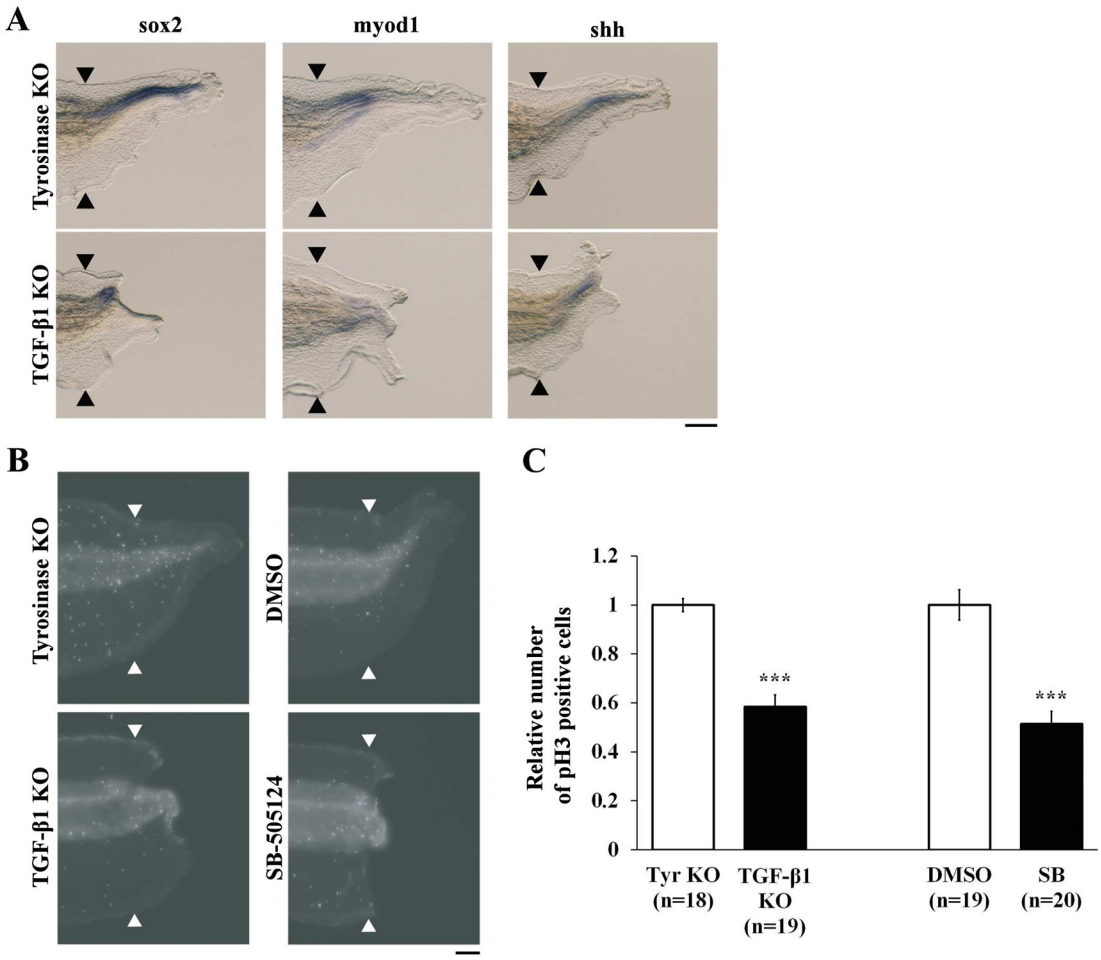

Figure 3. TGF-β1 regulates tissue differentiation and cell proliferation.

(A) Lateral views of WISH performed with *sox2* (spinal cord), *myod1* (muscle) and *shh* (notochord) antisense RNA probes at 72 hpa. Scale bar, 200 μm. (B) Whole-mount immunostaining of phosphorylated Histone H3 (pH3) at 48 hpa. Scale bar, 100 μm. (C) Quantification of mitotic cells in the regenerating tail. The number of pH3 positive cells in *tgfb1* KO and SB-505124-treated tadpoles was normalized against *tyrosinase* KO and DMSO-treated control tadpoles, respectively. Black and white arrowheads indicate amputation sites. ***$P < 0.001$.

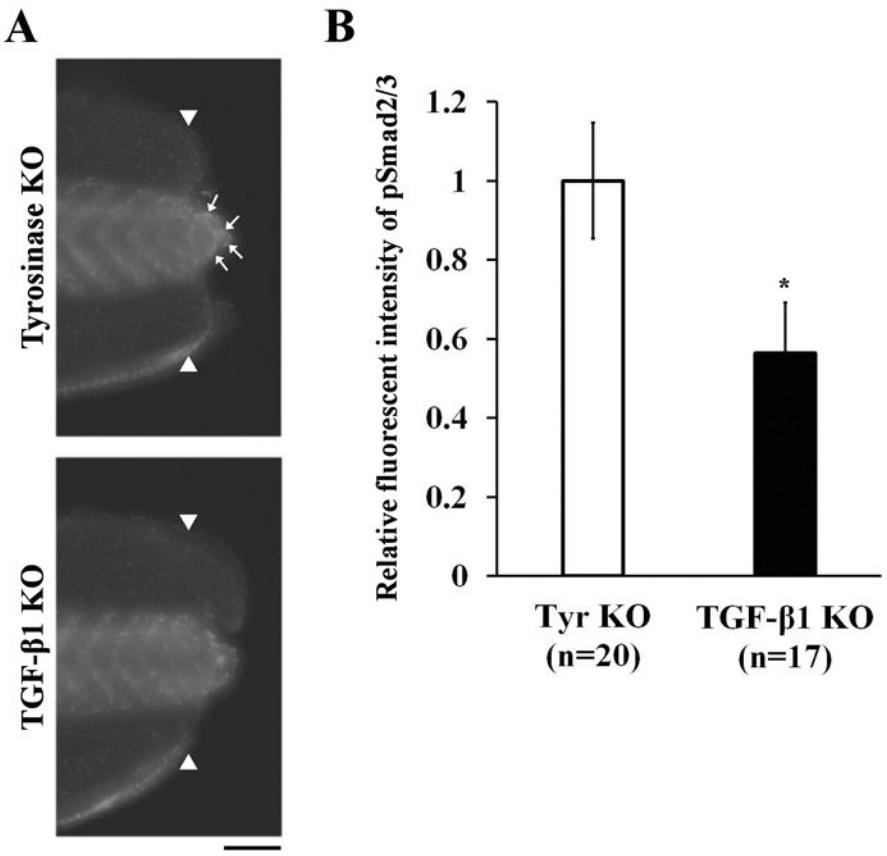

Figure 4. TGF-β1 regulates the activation of Smad2/3

(A) Whole-mount immunostaining of phosphorylated Smad2/3 (pSmad2/3) at 6 hpa. Scale bar, 100 μm. (B) Quantitative fluorescence intensities of pSmad2/3 immunostaining in regenerating tails. The vertical axis indicates the average fluorescence intensity in regenerating tails of *tgfb1* KO tadpoles normalized against *tyrosinase* KO control tadpoles. White arrowheads indicate amputation sites. White arrows indicate the localization of fluorescence signals in the regenerating tail tip. $*P < 0.05$.