広島大学学位請求論文

Analysis of the molecular mechanisms that activate cell proliferation in *Xenopus tropicalis* tadpole tail regeneration

（ネッタイツメガエル幼生尾の再生過程における細胞分裂活性化機構の解析）

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基礎生物学プログラム

中村　誠

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Analysis of the molecular mechanisms that activate cell proliferation in *Xenopus tropicalis* tadpole tail regeneration

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

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Chapter 1.

General introduction

General introduction

Amphibians can regenerate their lost tissues in response to injury, while mammals have limited regenerative capacity [1, 2]. The *Xenopus tropicalis* tadpole is a useful animal model for tissue regeneration research [3]. The tail of the tadpole is composed of several types of tissues, such as spinal cord, muscle, and notochord (Fig. I-1). Upon tail amputation, epithelial cells migrate to the amputation plane and cover the wound with multilayered cells, which is called the wound epidermis. After the wound is completely sealed, the regeneration bud is constructed by accumulating tissue progenitors in the tip of the regenerating tail. Subsequently, the damaged tail is restored through the activation of cell proliferation and tissue differentiation.

Adult mice cannot stimulate mitotic activation upon cardiac injury and are unable to regenerate their heart [4, 5]. However, the overexpression of multiple cell cycle regulators (CDK1, CDK4, Cyclin B, and Cyclin D) induces cardiomyocyte proliferation and significantly improves heart function after myocardial infarction, indicating that cell proliferation is a critical process for tissue regeneration [6]. The importance of mitotic activation is conserved in regenerative animals as the treatment of cell cycle inhibitors severely prevents tissue regeneration in *Xenopus* tails, zebrafish fins and amphioxus heads and tails [7, 8]. Therefore, the elucidation of the molecular mechanisms that activate cell proliferation is essential for understanding the regenerative capacity in these animals.

During *Xenopus* tail regeneration, multiple signaling pathways (BMP, Wnt, FGF, and TGF-β) are involved in the activation of cell proliferation. Overexpression of *noggin* (BMP antagonist) or *dkk1* (extracellular Wnt inhibitor) reduces regenerative proliferation, and the pharmacological perturbation of the FGF signal retards tail regeneration [9, 10]. Moreover, the TGF-β signal is involved in both mitotic activation and wound healing [7]. Smad2/3 transcription factors are downstream mediators of the TGF-β signal. They are activated immediately after *Xenopus* tail amputation and expressed in the wound epidermis and regeneration bud. When the TGF-β signal is prevented by TGF-β receptor inhibitors, phosphorylated Smad2/3 are downregulated, and tadpoles fail to form the wound epidermis and regeneration bud, resulting in defects in cell proliferation and tissue

differentiation. Therefore, the TGF-β signal is critical for the formation of wound epidermis and promotion of regenerative proliferation. However, the TGF-β family ligands that activate the TGF-β signal upon tail amputation, and the TGF-β target genes that regulate mitotic activity during *Xenopus* tadpole tail regeneration are still unknown.

The transcription factor activator protein-1 (AP-1) modulates multiple biological processes, including proliferation, apoptosis, and inflammation [11, 12]. AP-1 is a dimeric complex which is composed of four subfamily proteins (Jun, Fos, Atf, and Maf) and regulates the transcription of downstream target genes. In a previous study, we showed that JunB, one of the Jun subfamily, is involved in tail elongation during *Xenopus* embryogenesis [13]. Since tail regeneration is accomplished by the reconstruction of the tail structure, I speculated that JunB may function in *Xenopus* tadpole tail regeneration. In this paper, I investigated the function of JunB in tissue regeneration by analyzing the expression dynamics of *junb*, requirement of JunB in mitotic activation and tissue differentiation, and the regulation of *junb* expression upon tail amputation. I found *junb* transcripts in regenerating tissues throughout tail regeneration. CRISPR-mediated knock-out experiments showed that JunB is required for the activation of cell proliferation and tissue regeneration. In addition, the TGF-β signal regulates the expression of *junb* immediately after tail amputation. These suggest that JunB, which is a downstream target of the TGF-β signal, functions as a positive regulator of cell proliferation during *Xenopus* tail regeneration. I examined the molecular mechanisms of TGF-β signal activation upon injury by trying to identify the TGF-β ligand that works immediately after injury. I demonstrated that *tgfb1* transcripts are in the tail before amputation and continue to be expressed in the regenerating tail. Moreover, TGF-β1 is required for the activation of Smad2/3, cell proliferation, and tissue differentiation, suggesting that TGF-β1 initiates the regeneration processes upon tail amputation. Therefore, JunB and TGF-β1 work as positive regulators of cell proliferation in response to injury in the promotion of *Xenopus* tail regeneration.

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Figure

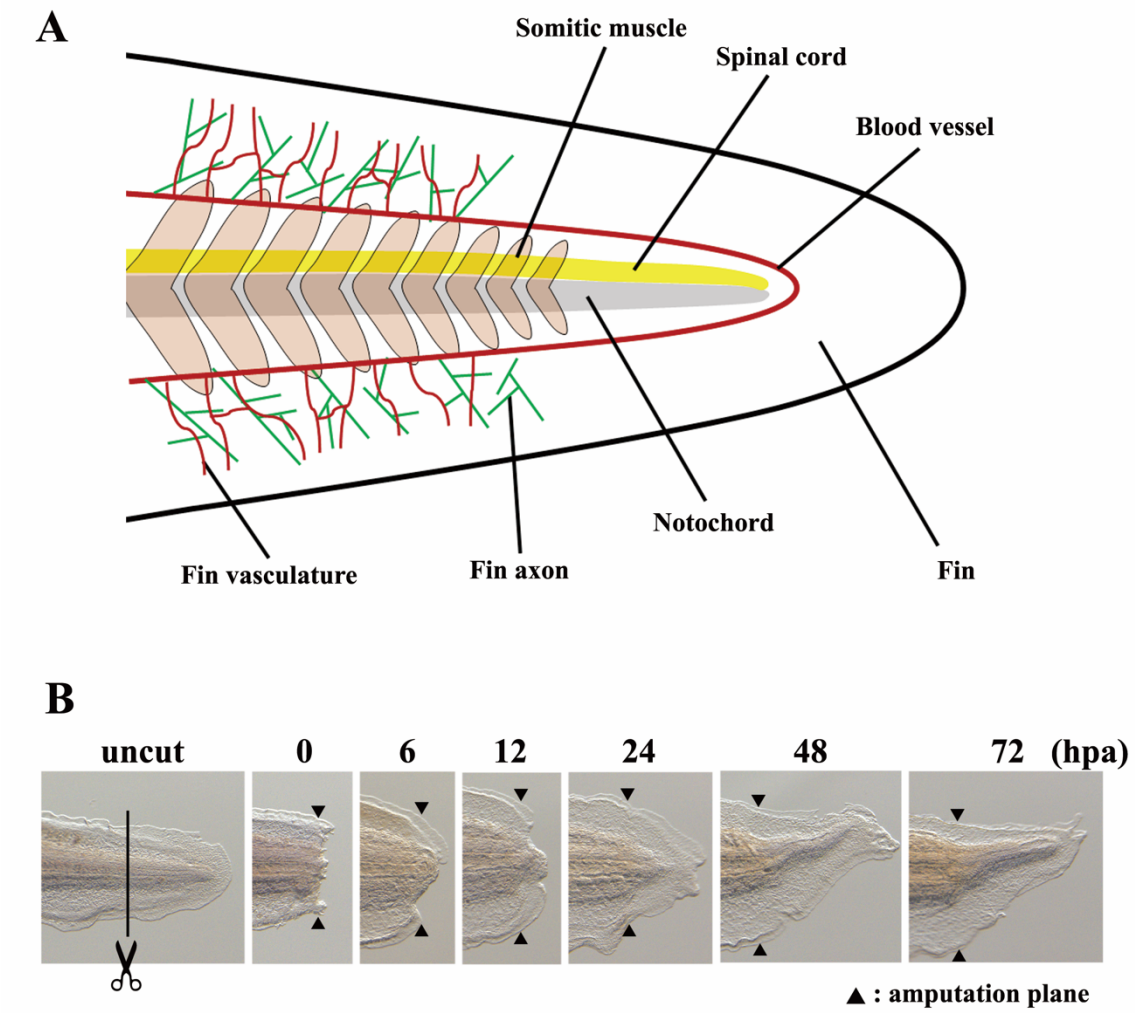

Figure I-1. Schematic of *Xenopus* tadpole tail, and time course of tail regeneration

(A) The *Xenopus tropicalis* tadpole tail is composed of multiple tissues, including somitic muscle, spinal cord, blood vessel, fin axon, fin vasculature, notochord, and fin. (B) After tail amputation, the wound epidermis covers the tip of the amputated tail at 6 hpa (hours post amputation). The regeneration bud is constructed by 24 hpa and the tail is restored through the activation of cell proliferation and tissue differentiation from 24 to 72 hpa.

Chapter 2.

The AP-1 transcription factor JunB functions in *Xenopus* tail regeneration by positively regulating cell proliferation

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Abstract

Xenopus tropicalis tadpoles can regenerate an amputated tail, including spinal cord, muscle and notochord, through cell proliferation and differentiation. However, the molecular mechanisms that regulate cell proliferation during tail regeneration are largely unknown. Here we show that JunB plays an important role in tail regeneration by regulating cell proliferation. The expression of *junb* is rapidly activated and sustained during tail regeneration. Knockout (KO) of *junb* causes a delay in tail regeneration and tissue differentiation. In *junb* KO tadpoles, cell proliferation is prevented before tissue differentiation. Furthermore, TGF-β signaling, which is activated just after tail amputation, regulates the induction and maintenance of *junb* expression. These findings demonstrate that JunB, a downstream component of TGF-β signaling, works as a positive regulator of cell proliferation during *Xenopus* tail regeneration.

Introduction

After traumatic injury, mammals such as humans and adult mice can heal wounds but not regenerate most of the damaged tissues [1]. In contrast, other vertebrates, such as *Xenopus* tadpoles, can regenerate lost appendages [2, 3]. Following amputation of the tail of a *Xenopus* tadpole, the amputation plane is covered by wound epidermis to prevent the leakage of cellular material. After wound healing, a regeneration bud is formed at the amputated site. Subsequently, the tail begins to regrow due to cell proliferation and is completely restored through the differentiation of spinal cord, muscle and notochord [4-6]. It has been reported that cell proliferation during tail regeneration is regulated by several signaling pathways, such as BMP, Wnt, TGF-β and Hippo [7-10], and by other factors, such as V-ATPase and small GTPase [11, 12]. However, the detailed molecular mechanisms that regulate cell proliferation during tail regeneration are not well understood. AP-1 family members (e.g., JunB and c-Jun) are involved in diverse biological processes such as cell proliferation and differentiation [13, 14]. It has been shown that c-Jun is important for mesoderm induction by FGF signaling during *Xenopus* development [15]. Moreover, we previously reported that over-expression of JunB led to ectopic tail-like structures in *Xenopus* and induced the expression of differentiation-related genes [16]. Since tail regeneration is caused by the reconstruction of tail structure, in the present study we examined the role of JunB during tail regeneration. We found that immediately after tail amputation *junb* was expressed in the regenerating tail. Tail regeneration was retarded by knockout (KO) of *junb* due to a reduction in the number of mitotic cells prior to tissue differentiation. Moreover, we also found that inhibition of TGF-β signaling decreased *junb* expression during tail regeneration. These results suggest that, downstream of TGF-β signaling, JunB is essential for tail regeneration by regulating cell proliferation.

Materials and methods

Design and synthesis of sgRNAs

CRISPR direct was used to design sgRNAs against *junb* (http:// crispr.dbcls.jp/). The following primers were used: *tyrosinase* sgRNA forward 5′-ATT TAG GTG ACA CTA TAG GAA CTG GCC CCT GCA AAC AGT TTT AGA GCT AGA AAT AGC AAG-3′ [17]; *junb* sgRNA forward [Target 1, 5′-ATT TAG GTG ACA CTA TAG GGG CTG TCG GTA GCA GCT TGT TTT AGA GCT AGA ATA GCA AG-3′; Target 2, 5′-ATT TAG GTG ACA CTA TAG GCA TAA GTG GTC CGA GCG GGT TTT AGA GCT AGA AAT AGC AAG-3′; Target 3, 5′-ATT TAG GTG ACA CTA TAG GAC TGT GCC CGA TAC CGC CGT TTT AGA GCT AGA AAT AGC AAG-3′; Target 4, 5′-ATT TAG GTG ACA CTA TAG GGT GGC AAT GGC ATA ACG GGT TTT AGA GCT AGA AAT AGC AAG-3′; Target 5, 5′-ATT TAG GTG ACA CTA TAG GAA GCT GGA GAG AAT CGC CGT TTT AGA GCT AGA AAT AGC AAG-3′]; reverse 5′-AAA AGC ACC GAC TCG GTG CCA CTT TTT CAA GTT GAT AAC GGA CTA GCC TTA TTT TAA CTT GCT ATT TCT AGC TCT AAA AC-3′. Synthesis of sgRNA was carried out following a previously described protocol [18]. The sgRNA templates were generated by a PCR-based method and *in vitro* transcription using the MEGA script® SP6 Transcription Kit (Thermo Fisher Scientific).

Growth, manipulation and microinjection of *X. tropicalis*

Animal experiments were carried out in accordance with the guidelines of the Animal Experimentation Ethics Committee of Hiroshima University and conformed with generally agreed international regulations. *X. tropicalis* tadpoles were obtained by *in vitro* fertilization and were cultured in 0.1X Marc's Modified Ringer's (MMR) solution containing 50 μg/ml gentamycin on 1% agarose coated dishes. For the KO experiment, 1000 pg (single injection) or 500 pg (combinatorial injection) of sgRNAs and 1 ng of Cas9 protein (Integrated DNA Technologies) were injected into the animal pole at the 1-cell stage in 0.1X MMR containing 6% Ficoll, 50 μg/ml gentamycin and 0.1% BSA within 40 min of fertilization. For the rescue experiment, *FLAG-junb* mRNA was added

to the sgRNAs and Cas9 protein. Tadpoles at stage 41/42 [19] were anesthetized in 0.01% MS-222/0.1X MMR and the tail was surgically amputated at the mid-point. After amputation, tadpoles were kept at 24℃ in tap water. The TGF-β receptor inhibitor SB-505124 (Cayman Chemical) was prepared in DMSO (Nacalai) and used at 12.5 μM. Tadpoles were treated with inhibitor-containing medium from 1 hour before tail amputation and the medium was changed every day until 72 hours post amputation (hpa). F0 *junb* KO frogs (sg 4 + sg 5 injected males and sg 5 injected females) were confirmed to have germline transmission by direct sequencing of PCR amplicons [20]. These F0 frogs were intercrossed to obtain compound heterozygous F1 *junb* mutants.

Plasmids

Probes were synthesized using the following plasmids: *pDH105-junb* [16], *pCS-sox2*, *pCS-myod1*, and *pCS-shh* (gifts from Dr. R. Harland). For the rescue experiment, *pDH105-FLAG-junb* was generated by subcloning a SalI/XbaI fragment from *pDH105-junb* into a *pDH105-FLAG* vector.

Whole-mount immunostaining

Tadpoles were fixed with MEMFA for 30 min at room temperature and stored at 20℃ in 100% methanol. After rehydration with 1X PBS, samples were treated with bleaching solution (1% H_2O_2, 5% formamide, 0.5X SSC). These samples were blocked with 10% normal goat serum (NGS) in PBST (1X PBS, 0.1% Triton X-100, 0.2% BSA) for 3 hours, and incubated at 4℃ overnight with primary antibody (anti-phosphorylated histone H3 (pH3), Upstate Biotechnology, 1 mg/ml) diluted at 1:500 in PBST plus 10% NGS. A secondary antibody conjugated to Alexa-488 (Molecular Probe, 2 mg/ml) was diluted at 1:500. pH3 positive cells were manually counted in the regenerated tail, and the area of regenerating tissues was measured using Image J software (National Institutes of Health, USA). The number of pH3 positive cells was divided by the individual area (pixels), and normalized by the average of control samples at respective time points.

Whole-mount *in situ* hybridization

X. tropicalis embryos were fixed in MEMFA for 2 hours at 24℃. Whole-mount *in situ* hybridization (WISH) was performed following standard methods [21] with a minor modification as described in Takebayashi-Suzuki *et al*. [22].

Quantitative RT-PCR (qPCR)

Regenerating tails were digested in lysis solution (0.25 mg/ml proteinase K, 50 mM Tris-HCl (pH7.5), 5 mM EDTA, 50 mM NaCl, 0.5% SDS) and incubated at 42℃ overnight. Total RNA was purified by phenol-chloroform extraction and ethanol precipitation, and treated with DNase I (Roche). cDNA synthesis and qPCR were carried out following previously described protocols [22]. The following primer sequences were used for qPCR analysis: *junb*, forward 5′ -CAT GGA GGA TCA GGA GAG GA-3′ and reverse 5′ -CTC TCA CCC TCA CCT TCA GC-3′; *rps18*, forward 5′-TTC AGC ACA TTT TGC GTG TT-3′ and reverse 5′-GTT CAC CAG CAC GCT TTG TA-3′.

Genotyping

At 72 hpa, individual tadpoles were digested in lysis solution (0.1 mg/ml proteinase K, 10 mM Tris-HCl (pH8.0), 100 mM EDTA, 0.5% SDS) and treated with RNase A (50 mg/ml) to extract genomic DNA. Genomic DNA was purified by phenol-chloroform extraction and ethanol precipitation or using a GenElute mammalian genomic DNA miniprep kit (Sigma-Aldrich). The genomic region of *junb* was amplified using Q5 High Fidelity DNA polymerase (New England Biolabs). The following primer sequences were used for genotyping of *junb* KO tadpoles: forward 5′-CCA GCA CCC ACC TAC AAC TT-3′ and reverse 5′ -TTC CGA ATG CCT TGG AGT AG-3′. The amplicons were annealed and incubated with T7 Endonuclease I (T7EI, New England Biolabs). The digested PCR products were separated on a 1.5% agarose gel. For TA cloning, the PCR products were cloned into TOPO vector (Invitrogen). Single colonies were analyzed for each tadpole by Sanger sequencing to determine the mutation types. Sequences of F1 tadpoles were analyzed using poly peak parser software (http://yosttools.genetics.utah.edu/PolyPeakParser/). Compound heterozygous mutants were selected using the genotyping results.

Microscopy and statistics

Fluorescence images were acquired with Axio Zoom V-16 (Zeiss). Tail lengths in regenerating tadpoles were measured using CellSens standard software (Olympus). *P* values were determined using Student's *t*-test. Error bars indicate the standard error of the mean.

Results

junb is expressed during tail regeneration in *Xenopus* tadpoles

The expression pattern of *junb* during tail regeneration was analyzed using WISH of tadpoles that had undergone tail amputation (Fig. II-1). Expression of *junb* was widely detected near the amputation site from 0.25 hpa. At 1 hpa, *junb* was expressed in the tail, including the wound epidermis. The expression of *junb* intensified in the tip of the regenerating tail at 2-12 hpa and was observed throughout the regenerating tissues at 24-72 hpa. WISH using a *junb* sense-probe did not show any signals in the regenerating tails. These results demonstrate that *junb* is expressed during tail regeneration, and that JunB may play an important role in tail regeneration.

JunB is required for tail regeneration

To confirm the role of JunB during tail regeneration, we carried out a KO experiment using the CRISPR-Cas9 system. Three sgRNAs were designed against the genomic region corresponding to the transactivation domain of JunB (Supplementary Fig. II-1A); each sgRNA was co-injected with Cas9 protein into 1-cell stage eggs. We amputated the tails of F0 tadpoles at stage 41/42 and graded the degree of tail regeneration at 72 hpa. As shown in Fig. II-2A, *junb* KO tadpoles showed a delay in tail regeneration (sg 1, 40%; sg 2, 46%; sg 3, 33%) compared to control tadpoles (*tyrosinase* KO, 2%). To confirm the induction of mutations in the *junb* locus, we performed a T7EI assay of *junb* KO tadpoles and identified mutations induced by each *junb* sgRNA, but not by *tyrosinase* sgRNA (data not shown). Next, we injected combinations of sgRNAs (sg 1 + sg 2, sg 1 + sg 3, sg 2 + sg 3) to increase the mutation rate and induce frame-shift mutations at two sites in *junb*. The *junb* KO tadpoles injected with two sgRNAs showed considerable delay in tail regeneration compared to those injected with a single sgRNA, especially for the combination sg 1 + sg 2 (84%, Fig. II-2A). In addition, we found that the tail length regenerated in *junb* KO tadpoles injected with sg 1 + sg 2 was significantly less than in controls (Fig. II-2B and C). A sequencing analysis of tadpoles injected with sg 1 + sg 2 showed that all alleles were mutated at both sg 1 and sg 2 target sites (Supplementary Fig.

II-1B-D). The region of the regenerating tail expressing *sox2* (spinal cord marker) [23], *myod1* (muscle marker) [23] and *sonic hedgehog* (*shh*, notochord marker) [24] was reduced in *junb* KO tadpoles (100%, Fig. II-2D), demonstrating that tissue differentiation was prevented in *junb* KO at 72 hpa. To assess the specificity of the *junb* KO, we injected *junb* mRNA with *junb* sg 1 + sg 2 into fertilized eggs. The delay in tail regeneration caused by *junb* KO was partially but significantly rescued by co-injection of *junb* mRNA, showing that this phenotype results from inactivation of the *junb* gene (Supplementary Fig. II-2A and B). To further explore the phenotype of *junb* KO, we generated F0 *junb* KO tadpoles by injecting with a different set of sgRNAs (sg 4 and sg 5, Supplementary Fig. II-3A). These F0 tadpoles were raised to adulthood until sexual maturation, and intercrossed to create compound heterozygous F1 *junb* mutant tadpoles. We identified tadpoles carrying compound heterozygous mutations (Mut/Mut) and found that these tadpoles exhibited a delay in tail regeneration similar to *junb* KO sg 1 + sg 2 tadpoles (Supplementary Fig. II-3B and C). These data strongly indicate that JunB is essential for tail regeneration.

junb KO causes the downregulation of cell proliferation

Based on the observations described above and the difficulty of obtaining sufficient numbers of compound heterozygous F1 mutants, we used F0 tadpoles injected with *junb* sg 1 + sg 2 in the following experiments. Since *junb* KO tadpoles exhibited a delay in tail regeneration at 72 hpa and because activation of cell proliferation is important for tail regrowth during regeneration, we examined cell proliferation using whole-mount immunostaining with an antibody against pH3. Cell proliferation has been reported to be activated at 24-36 hpa in *X. tropicalis* regenerating tails [4, 5]. We counted the number of pH3 positive cells in regenerating tails, excluding the fin, at 36 and 48 hpa and found that the number of mitotic cells was significantly reduced at both time points (Fig. II-3A and B). Cell proliferation in the fin was not affected by *junb* KO (data not shown). These results indicate that JunB is important for the regulation of cell proliferation during tail regeneration.

The expression of *junb* is regulated by TGF-β signaling

As JunB is important for tail regeneration and its expression is induced just after amputation, we addressed the mechanisms by which the expression of *junb* is regulated during tail regeneration. Phosphorylated Smad2, a TGF-β signal transducer, is expressed from 0.25 hpa during *Xenopus* tail regeneration [9]. In addition, Smads bind to the *junb* promotor in mouse NIH3T3 embryonic fibroblast cells [25]. Therefore, we inhibited TGF-β signaling using the TGF-β receptor inhibitor SB-505124 and examined the expression of *junb* at 1, 2 and 6 hpa. When TGF-β signaling was blocked by SB-505124, *junb* expression was downregulated in the regenerating tail (Fig. II-4A). qPCR analysis showed that the inhibition of the TGF-β signaling caused a reduction in the amputation-induced level of *junb* expression at 1 hpa (Fig. II-4B). Moreover, *junb* transcripts were considerably reduced at 2 and 6 hpa by the TGF-β receptor inhibitor treatment (Fig. II-4B). These results suggest that the induction and maintenance of *junb* expression is regulated by TGF-β signaling during tail regeneration.

Discussion

In this study, we showed that *junb* was widely expressed in the regenerating tail at 0.25-12 hpa and that expression was sustained at 24-72 hpa except in the fin. In addition, *junb* KO tadpoles showed a delay in tail regeneration at 72 hpa and a reduction in the number of mitotic cells at 36 and 48 hpa. Cell proliferation is a crucial aspect of tail regeneration after amputation: the number of mitotic cells begins to increase around 24-36 hpa during *Xenopus* tail regeneration before the activation of tissue differentiation [4-6]. Our results suggest that the delay in tail regeneration in *junb* KO tadpoles is caused by the downregulation of cell proliferation, which precedes tissue differentiation. It has been reported that JunB participates in the promotion of *cyclin A* transcription by binding to its promoter and functions as a positive regulator of cell proliferation [26]. In lymphoma cell lines, JunB knock-down reduces cell proliferation due to a prolongation of the G_0/G_1 phase [27]. A previous study also showed that JunB may be involved in cell proliferation during fin and finfold regeneration in zebrafish [28]. Therefore, one of the main functions of JunB is the control of cell proliferation, and this function may be conserved among animal species that can undertake regeneration. We also showed here that axial tissues, including the spinal cord, muscle and notochord, could not properly extend in *junb* KO tadpoles. As these differentiated tissues grow after the activation of cell proliferation at 48-72 hpa during *Xenopus* tail regeneration [5], it is possible that JunB indirectly contributes to tissue differentiation by regulating cell proliferation.

We found that TGF-β signaling regulates the expression of *junb* during *Xenopus* tail regeneration. This observation is consistent with previous reports in cultured cell lines [25, 29]. However, *junb* expression was not completely eliminated by treatment with a TGF-β receptor inhibitor. These results suggest that, in addition to TGF-β, other factors may regulate the expression of *junb* during tail regeneration. In our study, we found that cell proliferation, which contributes to tissue differentiation, was perturbed by *junb* KO. As cell proliferation and differentiation in the regeneration bud are prevented by TGF-β receptor inhibition during *Xenopus* tail regeneration [9], JunB may be partly involved in events that are promoted by TGF-β signaling.

It is well-known that JunB forms a homodimer or heterodimer with other AP-1 family proteins and regulates the expression of downstream genes. In addition, the binding affinity of JunB to AP-1 elements is higher as a heterodimer with Fos proteins than as a JunB homodimer [30]. Since most AP-1 family genes are expressed during *Xenopus* tail regeneration ([31]; data not shown), the relationships between JunB and other AP-1 family proteins and the roles of the AP-1 heterodimer in regeneration will be investigated in future studies.

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Figures

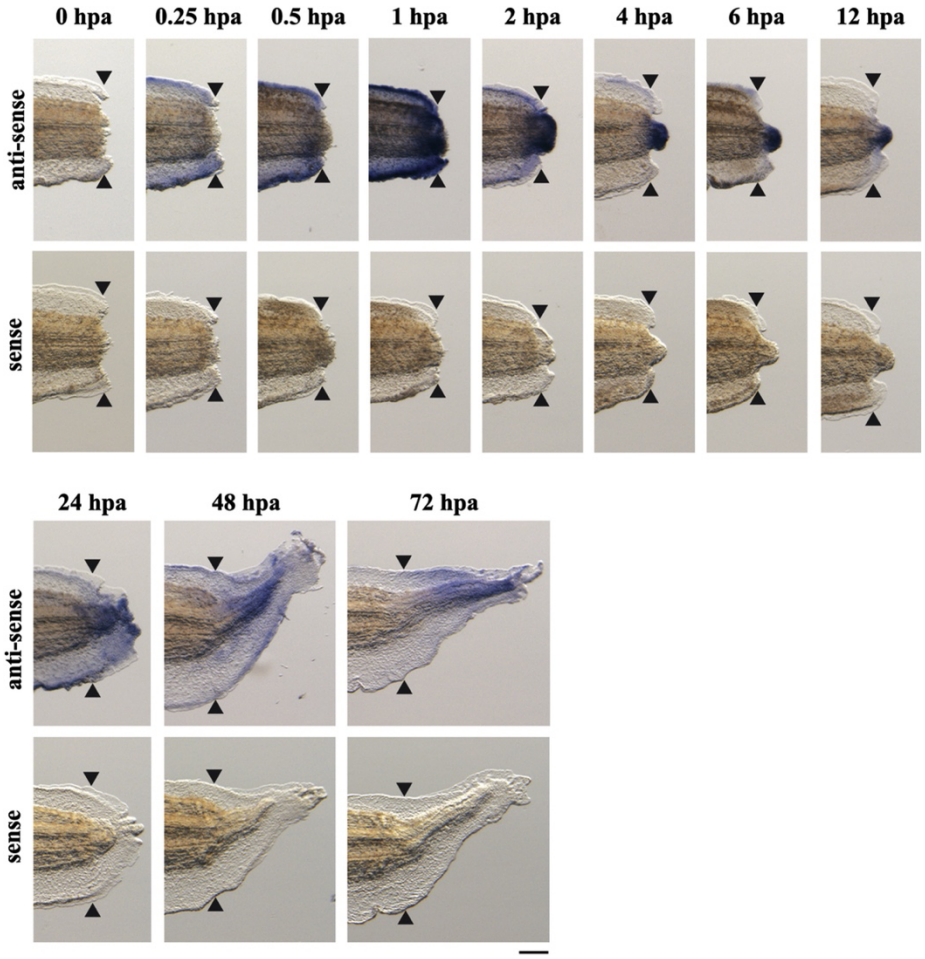


Figure II-1. *junb* is expressed during tail regeneration of *X. tropicalis* tadpoles

WISH analysis was performed using regenerating tadpoles at 0, 0.25, 0.5, 1, 2, 4, 6, 12, 24, 48 and 72 hpa. The expression of *junb* is shown in blue/purple. Black arrowheads indicate the amputation plane. Scale bar: 200 μm.

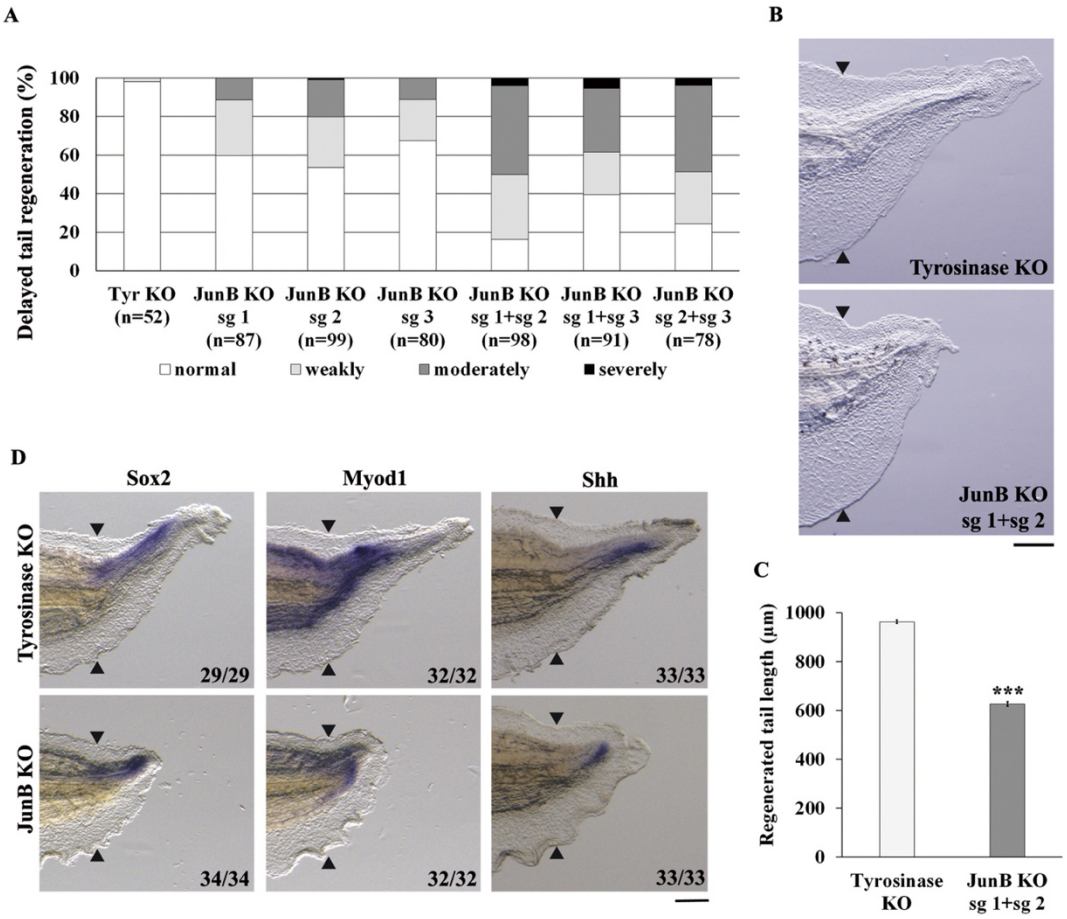


Figure II-2. Knockout of *junb* prevents tail regeneration

(A) Summary of phenotypes in regenerating tadpoles at 72 hpa. The tadpoles were classified into 4 types: normal tail regeneration; weakly delayed tail regeneration; moderately delayed tail regeneration; and severely delayed tail regeneration. (B) *junb* KO sg 1 + sg 2 tadpoles show considerably delayed tail regeneration. *tyrosinase* KO was used as the control. (C) Lengths of regenerating tails. (D) WISH analysis of *sox2*, *myod1* and *shh* in *tyrosinase* KO and *junb* KO tadpoles. Black arrowheads indicate amputation plane. Scale bar: 200 μm. ***$P < 0.001$, Student's *t*-test.

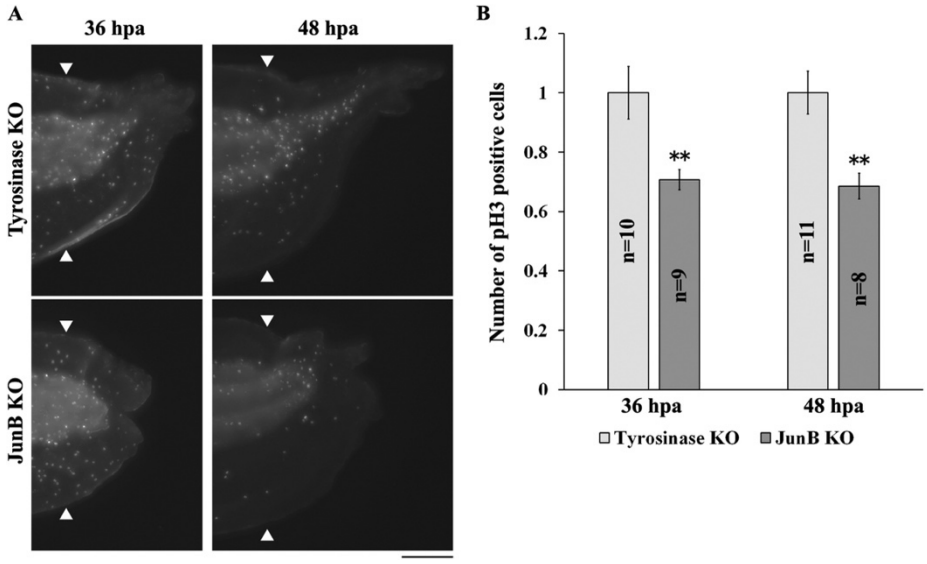


Figure II-3. Knockout of *junb* reduces cell proliferation

(A) Whole-mount immunostaining of *junb* KO tadpoles with pH3 antibody at 36 and 48 hpa. (B) Quantification of pH3 positive cells in the region of regenerating tail, excluding the fin. The number of pH3 positive cells was divided by individual area, and normalized against control samples. White arrowheads indicate the amputation plane. Scale bar: 200 μm. $^{**}P < 0.01$, Student's *t*-test.

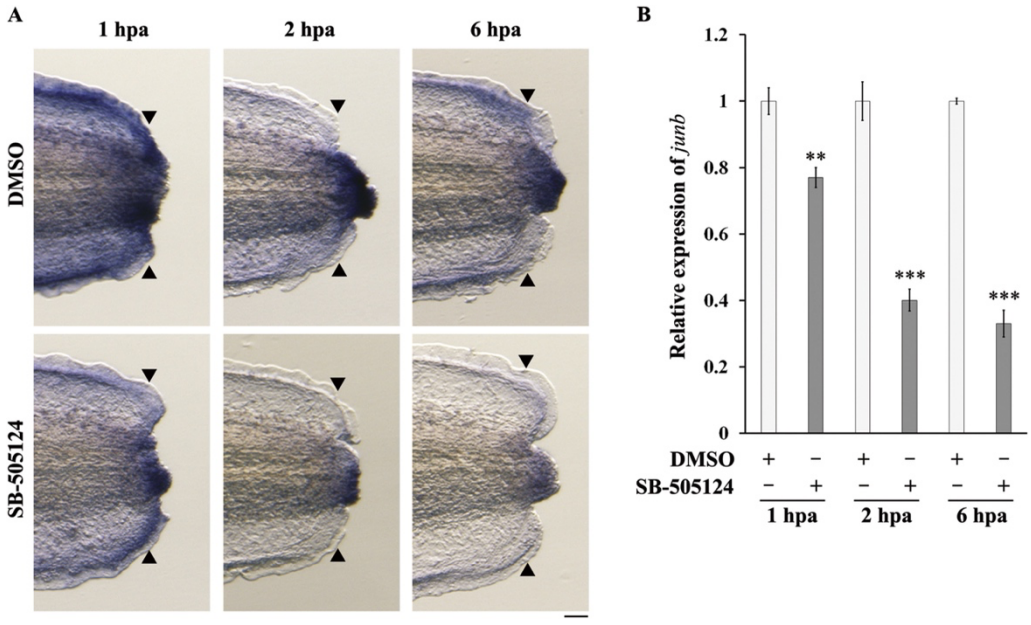
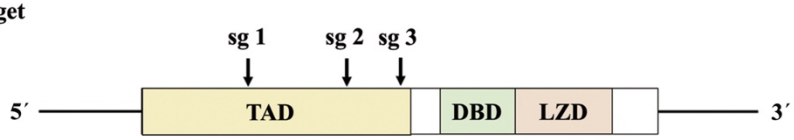


Figure II-4. The expression of *junb* is downregulated by TGF-β signaling inhibition

Tadpoles were treated with a medium containing 12.5 μM SB-505124 or DMSO (Control) from 1 hour before tail amputation and cultured until 1, 2 and 6 hpa. (A) WISH analysis of *junb* in SB-treated tadpoles. N = 29 for each sample. (B) qPCR analysis of *junb* in SB-treated tadpoles. The expression of *junb* was normalized against *rps18* expression, and then against control samples. Scale bar: 50 μm. $^{**}P < 0.01$, $^{***}P<0.001$, Student's *t*-test.

A

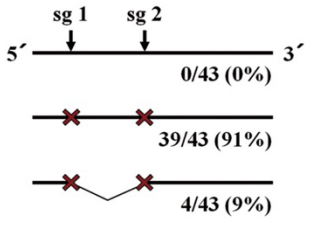


B

Version 9.1 : scaffold_185:139110..142823

X. tropicalis Reference	sgRNA 1 TCACAGTGGCCCGGGGGTCATAAGTGGTCCGAGCGGTGGCCAACTCTACTACTCG	sgRNA 2 ACCTGGCCAATTACCACCCAAGCTGCTACCGACAGCCCTCTGCCACCATTAACTA	Freq.	Mutation types
***junb* indel mutation in Tyrosinase KO**				
#1 tadpole	TCACAGTGGCCCGGGGGTCATAAGTGGTCCGAGCGGTGGCCAACTCTACTACTCG	ACCTGGCCAATTACCACCCAAGCTGCTACCGACAGCCCTCTGCCACCATTAACTA	9/9	Wild-type
#2 tadpole	TCACAGTGGCCCGGGGGTCATAAGTGGTCCGAGCGGTGGCCAACTCTACTACTCG	ACCTGGCCAATTACCACCCAAGCTGCTACCGACAGCCCTCTGCCACCATTAACTA	10/10	Wild-type
#3 tadpole	TCACAGTGGCCCGGGGGTCATAAGTGGTCCGAGCGGTGGCCAACTCTACTACTCG	ACCTGGCCAATTACCACCCAAGCTGCTACCGACAGCCCTCTGCCACCATTAACTA	10/10	Wild-type
#4 tadpole	TCACAGTGGCCCGGGGGTCATAAGTGGTCCGAGCGGTGGCCAACTCTACTACTCG	ACCTGGCCAATTACCACCCAAGCTGCTACCGACAGCCCTCTGCCACCATTAACTA	8/8	Wild-type
#5 tadpole	TCACAGTGGCCCGGGGGTCATAAGTGGTCCGAGCGGTGGCCAACTCTACTACTCG	ACCTGGCCAATTACCACCCAAGCTGCTACCGACAGCCCTCTGCCACCATTAACTA	9/9	Wild-type
***junb* indel mutation in JunB KO (sg 1 + 2)**				
#1 tadpole	TCACAGTGGCCCGGGGGTCATAAGTGGTCCGA----------CTCTACTACTCG	ACCTGGCCAATTACCACCC---CTGCTACCGACAGCCCTCTGCCACCATTAACTA	1/9	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGG----------	----------CCACCATTAACTA	1/9	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTCCGA----------CTCTACTACTCG	----------	1/9	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTCCGATGACCTGCACAACTCTACTACTC	ACCTGGCCAATTACCACCCAAGCT---ACCGACAGCCCTCTGCCACCATTAACTA	1/9	Out-of-frame
	TCACAGTGGCCCGGGGGTCATA----------AGCGGTGGCCAACTCTACTACTCG	ACCTGGCCAATTACCACCCAAGCT---ACCGACAGCCCTCTGCCACCATTAACTA	1/9	In-frame
	TCACAGTGGCCCGG----------TGGCCAACTCTACTACTCG	ACCTGGCCA-TTACCA------CTGCTACCGACAGCCCTCTGCCACCATTAACTA	1/9	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTC--ATAAGTGGCCAACTCTACTACTCG	ACCTGGCCAATTACCACC----CTGCTACCGACAGCCCTCTGCCACCATTAACTA	1/9	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTCCGA----------CTCTACTACTCG	ACCTGGCCAATTACCACC----------ATTAACTA	1/9	Out-of-frame
	TCACAGTGGCCCGGGGGTCATA----------GGTGGCCAACTCTACTACTCG	ACCTGGCCAATTACC----------GACAGCCCTCTGCCACCATTAACTA	1/9	In-frame
#2 tadpole	TCACAGTGGCCCGGGGGTCATAAGTGGCCAG----GTGGCCTACTCTACTACTCG	ACCTGGCCAATTACCACCC----------GACAGCCCTCTGCCACCATTAACTA	3/8	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTCCGAGGG----------	----------CTACCGACAGCCCTCTGCCACCATTAACTA	2/8	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTCCG----GTGGCCTACTCTACTACTCG	ACCTGGCCAATTACCACCCAAGCT---ACCGACAGCCCTCTGCCACCATTAACTA	1/8	Out-of-frame
	TCACAGGGGCCCGGGGGTCATAAGTGGTCCG----GTGGCCTACTCTACTACTCG	ACCTGGCCAATTACCACC----------GACAGCCCTCTGCCACCATTAACTA	1/8	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTCCG----GTGGCCTACTCTACTACTCG	ACCTGGCCAATTACCACC----------GACAGCCCTCTGCCACCATTAACTA	1/8	Out-of-frame
#3 tadpole	TCACAGTGGCCCGGGGGTCATAAGTGGTCCGAG---TGGCCAACTCTACTACTCG	ACCTGG----------TACCGACAGCCCTCTGCCACCATTAACTA	2/10	Out-of-frame
	TCACAGTGGCCCGG----------TGGCCAACTCTACTACTCG	ACCTGGCCAATTACCACC----CTGCTACCGACAGCCCTCTGCCACCATTAACTA	1/10	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTCCG----GTGGCCAACTCTACTACTCG	ACCTGGCCAATTACCAC-----CTGCTACCGACAGCCCTCTGCCACCATTAACTA	1/10	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTCCGATCGG---CCACCTCTACTACTCG	ACTTGG----------TACCGACCTCCCTCTGCCACCATTATCTA	1/10	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTCCGAG---TGGCCAACTCTACTACTCG	ACCTGGCCAATTACCACC----------GACAGCCCTCTGCCACCATTAACTA	1/10	In-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTCCGACACACAGAGTGGCCAACTCTACT	ACCTGGCCAATTACCACCCA----GCTACCGACAGCCCTCTGCCACCATTAACTA	1/10	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTCCGAG-----GCCAACTCTACTACTCG	ACCTGGCCAATTACCACC----------GACAGCCCTCTGCCACCATTAACTA	1/10	Out-of-frame
	----------CGGTTGCCAACTCTACTACTCG	ACCTG----------CTACCGACAGCCCTCTGCCACCATTAACTA	1/10	Out-of-frame
	----------CGGTGGCCAACTCTACTACTCG	ACCTGGCCAATTACCACC----------GACAGCCCTCTGCCACCATTAACTA	1/10	
#4 tadpole	TCACAGTGGCCCGGGGGTCATAAGTGGTCCG----GTGGCCAACTCTACTACTCG	ACCTGGCCAATTACCA----------CAGCCCTCTGCCACCATTAACTA	2/10	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGG----------CCAACTCTACTACTCG	ACCTGGCCAATTACCACCCAACAGAGGGCTACCGACAGCCCTCTGCCACCATTAA	2/10	In-frame
	TCACAA----------	ACCTGGCCAATTACCAC-----CTGCTACCGACAGCCCTCTGCCACCATTAACTA	1/10	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTCCGAGTGG----CCACTCTACTACTCG	ACCTGGCCAATTACCACC----CTGCTACCGACAGCCCTCTGCCACCATTAACTA	1/10	Out-of-frame
	TCACAGTGGCCCGGAGGTCATAAGTGGTCCG----GTGGCCAACTCTACTACTCG	ACCTGGCCAATTACCA----------CAGCCCTCTGCCACCATTAACTA	1/10	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTCCA---CGGTGGCCAACTCTACTACTCG	ACCTGGCCAATTACCAC-----CTGCTACCGACAGCCCTCTGCCACCATTAACTA	1/10	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTCCGAG---TGGCCAACTCTACTACTCG	ACCTGGCCAATTACCACCC----------GACAGCCCTCTGCCACCATTAACTA	1/10	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTCCG----GTGGCCAACTCTACTACTCG	ACCTGGCCAATTACCACCCTACCACCCTACTGCTACCGACAGCCCTCTGCCACCA	1/10	Out-of-frame
#5 tadpole	TCACAGTGGCCCGGGGGTCATAAGTGGTC--ATAAGTGGCCAACTCTACTACTCG	ACCTGGCCAATTACCACCGACAGCCCTCTGCCACCATTAACTAGGGCTGCTACCG	2/6	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTC--ATAAGTGGCCAACTCTACTACTCG	ACCTGGCCAATTACCACC----------ATTAACTA	1/6	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTC--ATAAGTGGCCAACTCTACTACTCG	ACCTGGCCAATTACCACCCA----------CCACCATTAACTA	1/6	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTC--ATAAGTGGCCAACTCTACTACTCG	ACCTGGCCAATTACCA----------CAGCCCTCTGCCACCATTAACTA	1/6	Out-of-frame
	TCACAGTGGCCCGGGGGTCATAAGTGGTAC----------AACTCTACTACTCG	ACCTGGCCAATTACC----------GACAGCCCTCTGCCACCATTAACTA	1/6	Out-of-frame

C



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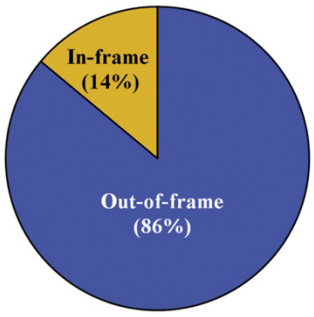
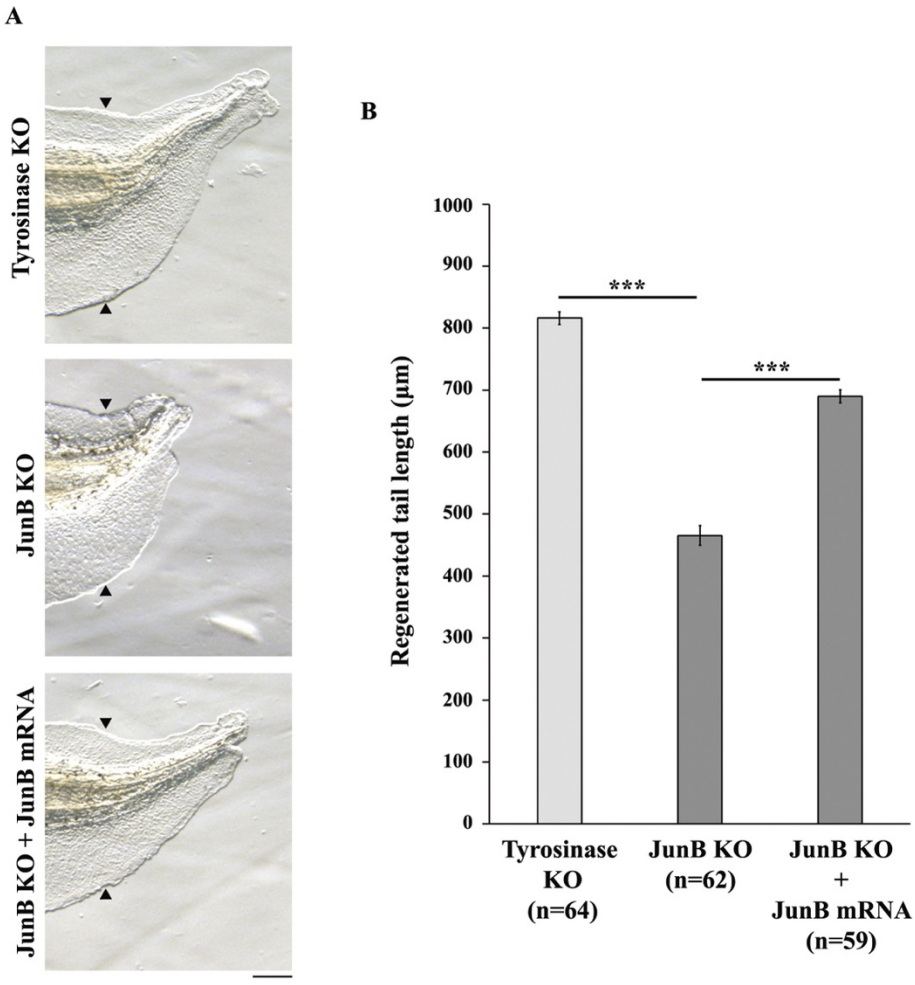


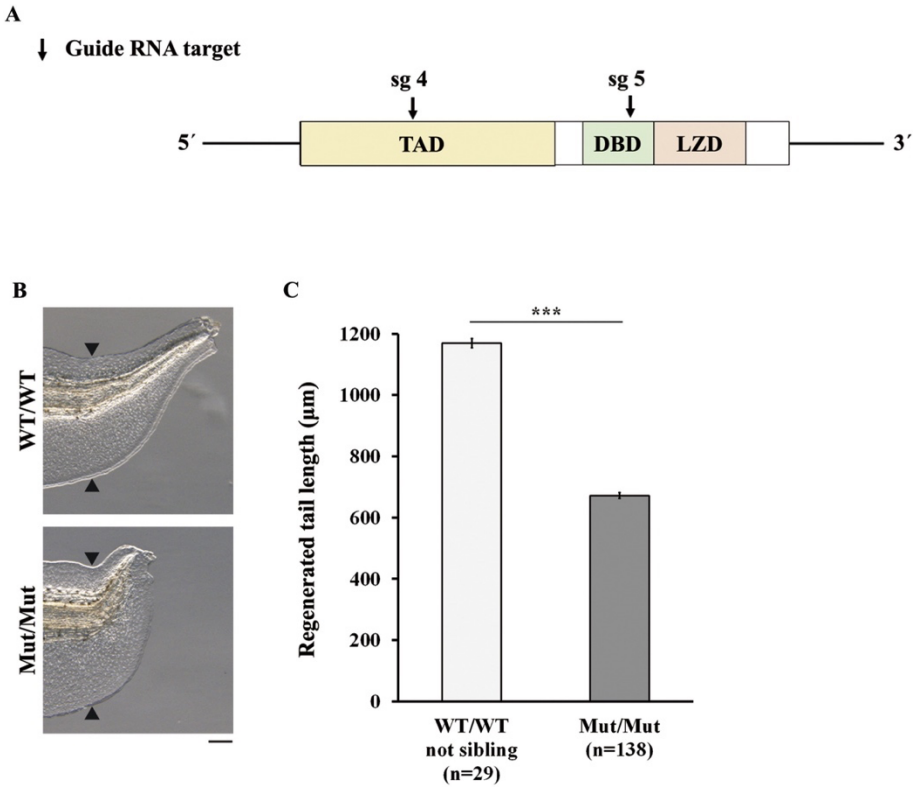
Supplementary Figure II-1. Genotyping of *junb* KO sg 1 + sg 2 tadpoles

(A) Schematic drawing of *junb* structure and *junb* sgRNA target sites. Bars, untranslated region; box, coding region; TAD, transactivation domain; DBD, DNA -binding domain; LZD, leucine zipper domain. (B) Sequencing analysis of *junb* mutations in *tyrosinase* KO (n = 5) and *junb* KO (n = 5) tadpoles. Target sites of sg 1 and sg 2 are highlighted in green, and blue boxes indicate the PAM sequence. Deleted sequences are highlighted with red dashes. Insertions and substitutions are shown in blue and red letters, respectively. Mutation types were categorized as wild-type, in-frame, and out-of-frame. (C) Summary of mutation types as in panel B. Sequencing analysis of TA cloning demonstrates that all of alleles contain mutations in both sg 1 and sg 2 target sites. (D) Percentage of mutation types of *junb* KO sg 1 + sg 2 as in panel B.



Supplementary Figure II-2. The delay in tail regeneration in *junb* KO is rescued by *junb* mRNA

(A) Co-injection of *junb* mRNA (250 pg) with *junb* sg 1 + sg 2 rescues the *junb* KO phenotype. (B) Lengths of regenerating tails. Black arrowheads indicate the amputation plane. Scale bar: 200 μm. ***$P < 0.001$, Student's *t*-test.



Supplementary Figure II-3. Compound heterozygous *junb* mutants show a delay in tail regeneration

(A) Schematic drawing of *junb* structure and *junb* sgRNA target sites. (B) Compound heterozygous *junb* mutants (Mut/Mut) show considerable delay in tail regeneration compared to wild-type (WT/WT, not sibling) tadpoles. WT/WT tadpoles were used as the control. (C) Lengths of regenerating tails. Black arrowheads indicate the amputation plane. Scale bar: 200 μm. ***$P < 0.001$, Student's *t*-test.

Chapter 3.

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

[published in *Biochemical and Biophysical Research Communications* 565: 91-96 (2021)]

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*.

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 [4-6]. 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 TGF-βs, 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 InhibinβA 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 antibodieswere 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 non-specific fluorescence signal measured in SB-505124 treated tadpoles was subtracted from the mean value of the fluorescence signal in *tyrosinase* KO and *tgfb1* KO tadpoles.

Results

tgfb1 is expressed throughout *Xenopus* tail regeneration

Expression of *tgfb1* has previously been described during *X. laevis* tadpole tail regeneration [4]. Consistently, RNA-seq 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. III-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. III-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. III-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. III-1). Furthermore, *tgfb1* KO tadpoles (sg 1 + sg 2 + sg 3) showed a clear delay in tail regeneration (66.6%; Fig. III-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. III-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. III-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. III-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. III-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. III-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 TGF-β1 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. III-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 epidermis 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 epidermis [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.

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Figures

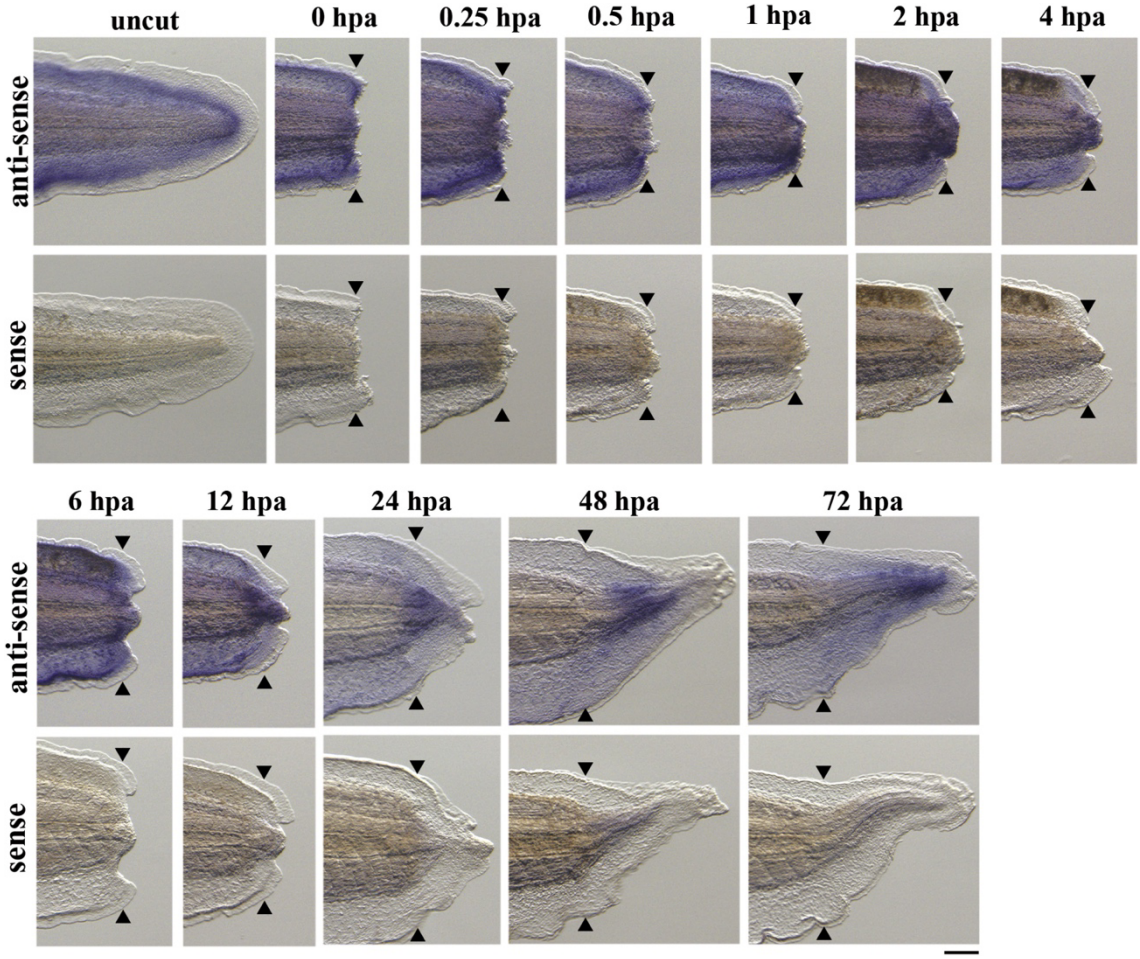


Figure III-1. Expression of *tgfb1* in *X. tropicalis* tadpoles before and after tail amputation

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.

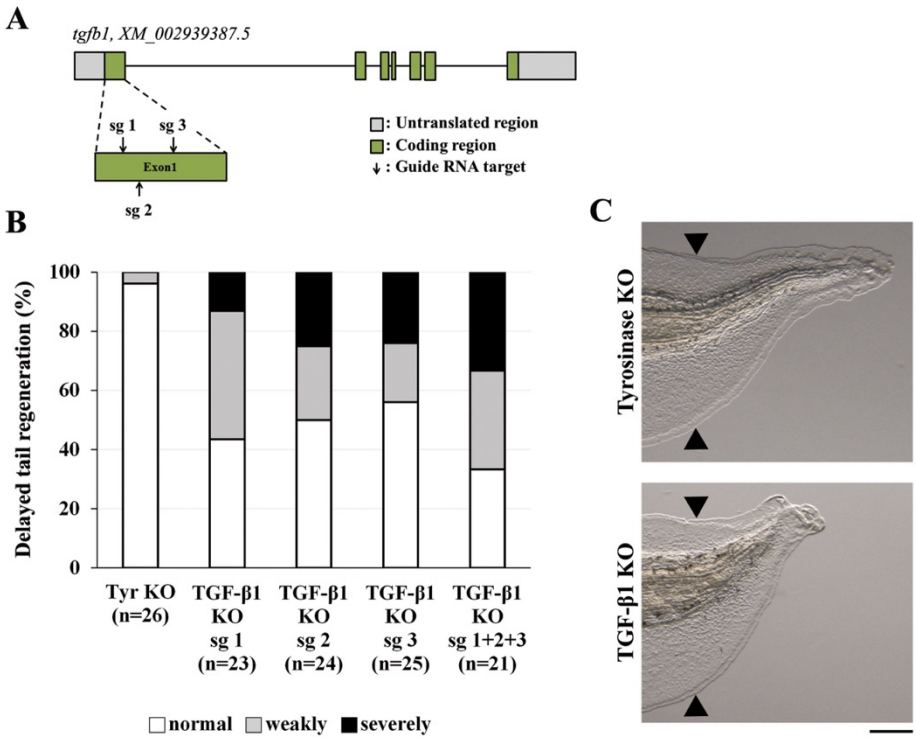


Figure III-2. TGF-β1 is required for *Xenopus* tail regeneration

(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.

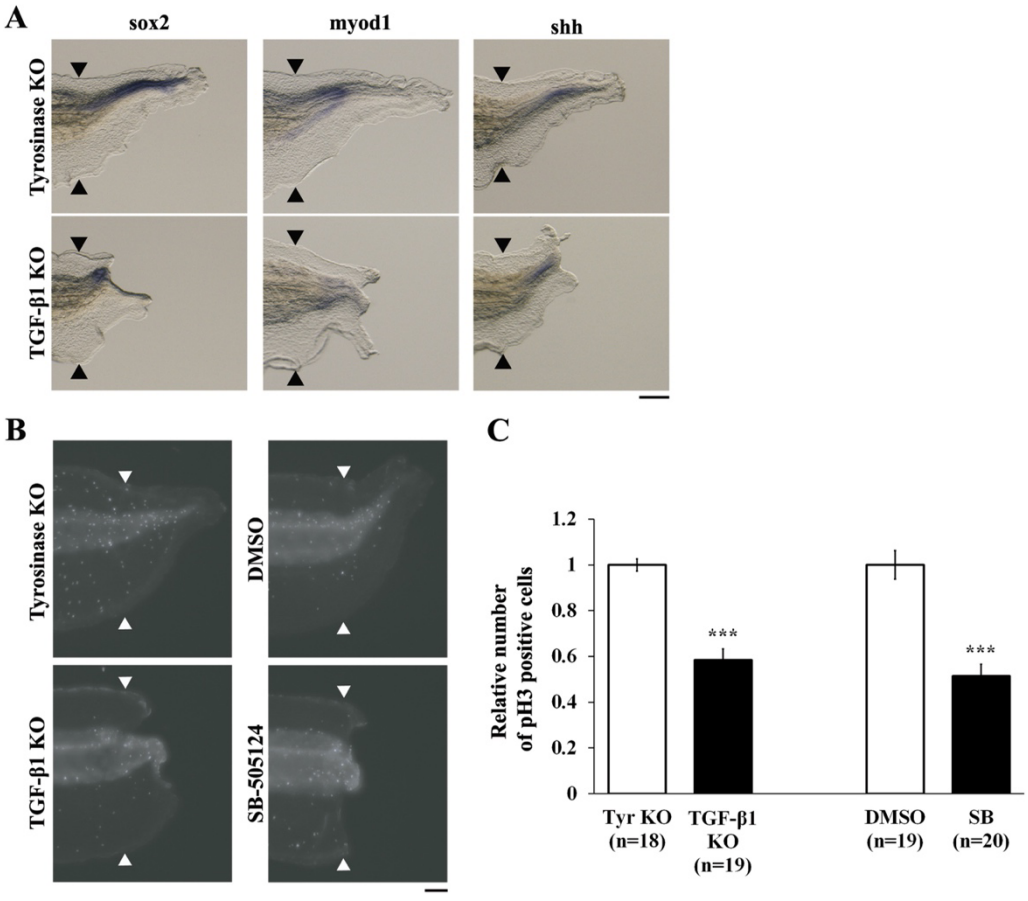


Figure III-3. TGF-β1 regulates tissue differentiation and cell proliferation

(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. ***$P < 0.001$.

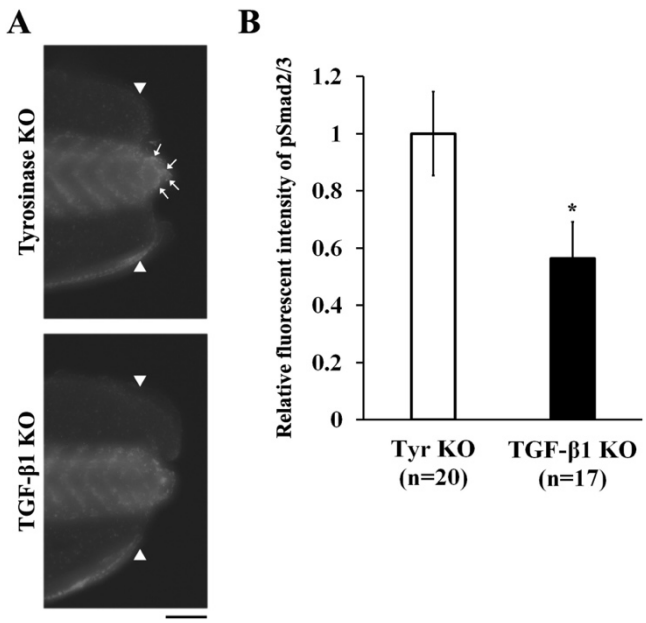
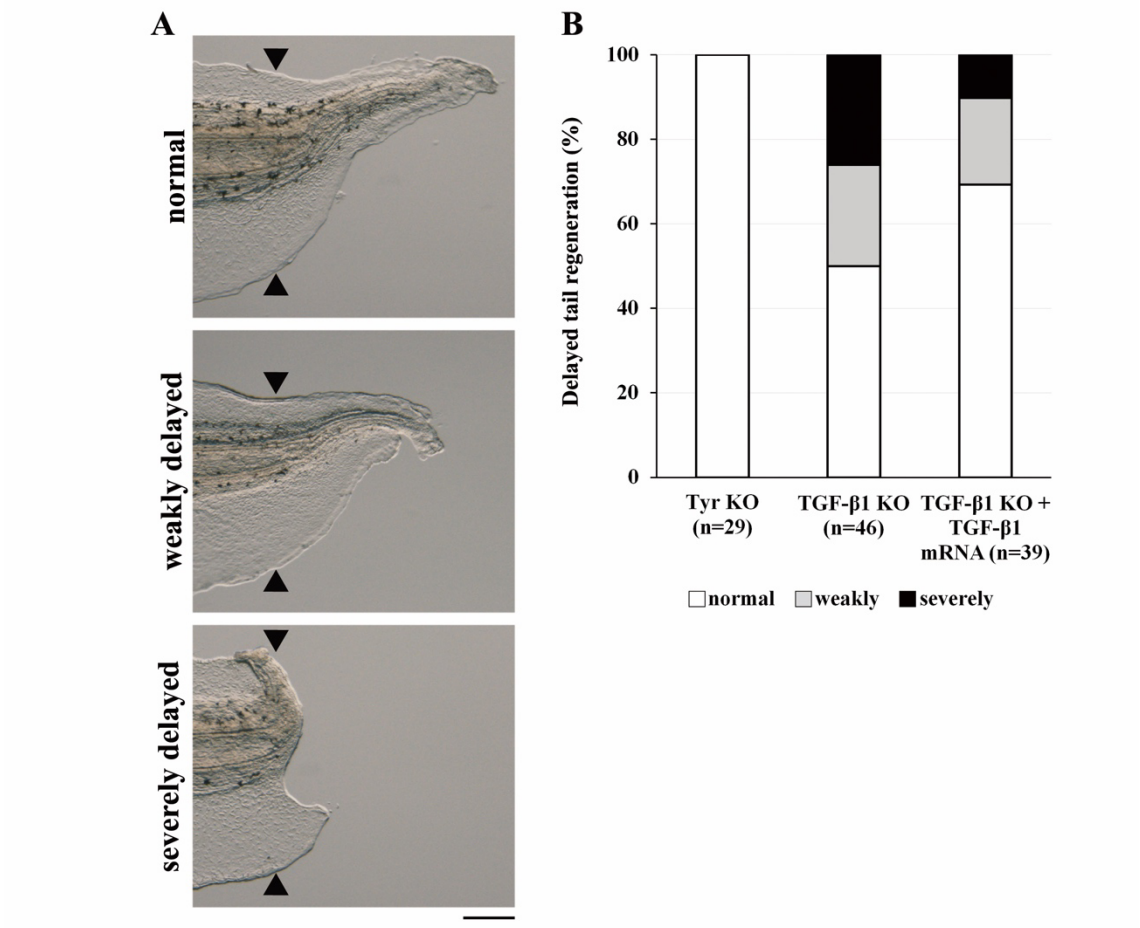


Figure III-4. TGF-β1 regulates the activation of Smad2/3

(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. $*P < 0.05$.

Supplementary Figure III-1. Genotyping of *tgfb1* KO (sg 1 + sg 2 + sg 3) tadpoles

Sequencing analysis was performed using *tyrosinase* KO (n=5) and *tgfb1* KO (n=6) tadpoles. At the top, three sgRNA target sites and the PAM sequence are highlighted in blue and green, respectively. Deleted sequences are shown as black dashes, and insertions and substitutions are shown in blue and red font, respectively.



Supplementary Figure III-2. Rescue experiment of the *tgfb1* KO phenotype by injection of *tgfb1* mRNA

(A) Representative phenotypes observed in this experiment. Tadpoles were categorized into three types: normal tail regeneration, weakly delayed tail regeneration, and severely delayed tail regeneration. (B) Rescue of delayed tail regeneration in the *tgfb1* KO tadpoles by injection of *tgfb1* mRNA. The extent of tail regeneration was classified at 72 hpa. Black arrowheads show amputation sites. Scale bar, 200 μm.

Chapter 4.

General discussion

General discussion

The function of JunB in tissue regeneration

I showed that JunB is required for mitotic activation in *Xenopus* tadpole tail regeneration. Cell proliferation is controlled by multiple cell cycle regulators, such as cyclins and CDKs. In fibroblast cells, JunB activates Cyclin A/CDK2 and Cyclin B/CDC2 to drive the transition from S phase to G2/M phases, while JunB extends the G1 phase by inducing the expression of *p16*, a negative regulator of CDK, suggesting that JunB functions as both positive and negative regulators of the cell cycle [1, 2]. It has been reported that JunB regulates the expression of target genes by forming a homodimer or heterodimer with other AP-1 family proteins [3]. In a published RNA-seq data and our preliminary data, Jun, Fos, Atf, and Maf subfamily genes were highly expressed in *Xenopus* tail regeneration [4]. In addition, JunB dimerizes with c-Fos during axolotl spinal cord regeneration, and JunB and Fosl1 cooperatively regulate the expression of cell cycle-related genes in *Xenopus* heart regeneration [5, 6]. These observations suggest that JunB may stimulate cell proliferation by binding with other AP-1 family proteins in the regenerating *Xenopus* tail. We found that *fosl1* and *fosl2* were expressed throughout tail regeneration, while *c-fos* was transiently induced immediately after tail amputation (unpublished observation). In future studies, it will be important to examine which AP-1 family proteins form a dimer with JunB in *Xenopus* tail regeneration.

JunB promotes spinal cord regeneration by facilitating glial bridging through the activation of *twist1a* expression, which is crucial for epithelial-mesenchymal transition (EMT) and ependymal cell proliferation [7]. Given this finding, JunB might stimulate cell proliferation and tail regeneration by increasing the transcription of EMT-related genes. In axolotl, EMT-like processes are also known to be important for wound healing upon limb amputation [8]. As *junb* transcripts were detected in the wound epidermis at 1 hpa (Fig. II-1), JunB may contribute to the formation of the wound epidermis in tail regeneration by promoting EMT-like processes. However, *junb* KO did not cause severe defects in wound healing, whereas I found a significant reduction in mitotic cells and delay of tail regeneration in *junb* KO tadpoles. Thus, I hypothesized that JunB is less important in wound closing than mitotic activation during tail regeneration. It is necessary

to investigate cell movement during wound closing in *junb* null mutants using live-imaging systems in the future to assess the requirement of JunB in wound healing.

The function of TGF-β1 in tissue regeneration

In this study, *tgfb1* was expressed in the regeneration bud, which is composed of tissue progenitor cells, and *tgfb1* KO caused a delay in tissue differentiation. It has been reported that the TGF-β signal is required for the accumulation of progenitor cells, including immature notochord and muscle satellite cells, in the tip of the regeneration bud [9]. Therefore, TGF-β1 might contribute to the formation of the regeneration bud by recruiting tissue progenitors to promote differentiation. I demonstrated that TGF-β1 is required for the activation of Smad2/3 in response to injury, but the pSmad2/3 signal is not completely reduced by *tgfb1* KO. In *Xenopus* tadpoles, the expression of both *tgfb1* and *tgfb2* was detected in intact and regenerating tail [9]. Additionally, during tail regeneration, *inhba* expression was induced during wound healing and *gdf11* transcripts were detected from the proliferation phase. I speculate that Smad2/3 are activated by multiple TGF-β family ligands during tail regeneration. In addition to TGF-β1, TGF-β2 may contribute to the phosphorylation of Smad2/3 immediately after tail amputation, and other TGF-β family ligands, such as InhibinβA and GDF11, possibly maintain Smad2/3 activity after wound healing. Furthermore, this sequential induction of TGF-β family ligands may be essential for the TGF-β signal to activate multiple regeneration processes throughout tail regeneration.

I showed that *tgfb1* is expressed in the tadpole tail before amputation. Moreover, it is well known that TGF-β1 plays essential roles in early development [10, 11]. These raise the question as to whether the effect of *tgfb1* KO on tissue regeneration is due to the regeneration-specific function of TGF-β1. In my observations, *tgfb1* KO tadpoles did not exhibit defects in embryogenesis before tail amputation and until 24-36 hpa, when a delay in tail regeneration by *tgfb1* KO became apparent. Hence, *tgfb1* KO seems to mainly affect tail regeneration. According to single cell analysis during *Xenopus* tail regeneration, *tgfb1* is specifically expressed in several cell types including lymphoid cells that are essential for the activation of cell proliferation during tissue regeneration in zebrafish [12, 13]. In addition, both intact and regenerating tails show *tgfb1* expression in the same cell

types, such as the epidermis, myotome, and mesenchyme. Therefore, TGF-β1 may be essential for embryogenesis in *Xenopus* tadpoles in addition to tissue regeneration. Future studies should aim to clearly define the necessity and function of TGF-β1 during tail regeneration. It is important to perform a conditional knock-down/knock-out experiment of TGF-β1 using a vivo-morpholino oligo or Cre-loxP system.

The functional relationship between JunB and TGF-β1

In Fig. IV-1, I proposed that TGF-β/Smad signaling initiated by TGF-β1 stimulates the expression of *junb* upon tail amputation. However, to prove this model, it is important to analyze the mechanisms of how TGF-β1 regulates *junb* expression at the single cell level. Single cell analysis of the regenerating *Xenopus* tail showed that *junb* transcripts are in most of cell types (45 out of 46 cell types), while *tgfb1* is expressed in 17 out of the 45 cell types that have *junb* expression [12]. This suggests that the expression of *tgfb1* partially overlaps with that of *junb* in the regenerating tail. Moreover, *tgfb receptor 1* (*tgfbr1*), which activates Smad2/3 upon TGF-β1 binding, was detected in various cell types, not only those expressing *tgfb1*. Based on these observations, in cells that express both *tgfb1* and *tgfbr1*, TGF-β1 may regulate the expression of *junb* by an autocrine mechanism. In addition, in cell types expressing only *tgfbr1*, the transcription of *junb* may be activated by TGF-β1 secreted from surrounding cells. Since *junb* expression is observed in cell types which do not show either *tgfb1* or *tgfbr1* expression, it is possible that other signaling pathways, in addition to the TGF-β1/Smad pathway, are involved in the regulation of *junb* expression. Recent reports showed that the MEK/ERK and ROS pathways are also activated upon *Xenopus* tail amputation, so I hypothesized that these injury-induced signaling pathways stimulate the expression of *junb* in tail regeneration [14, 15]. Future studies should investigate the functional relationship between *junb* transcription and these pathways by using their chemical inhibitors in tissue regeneration.

Overall, I demonstrated that TGF-β1 activates the expression of *junb*, which promotes cell proliferation and tissue regeneration in response to injury. Thus, this study provided a new insight into the molecular mechanisms that activate cell proliferation upon tail amputation in *Xenopus* tail regeneration (Fig. IV-1).

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Figure

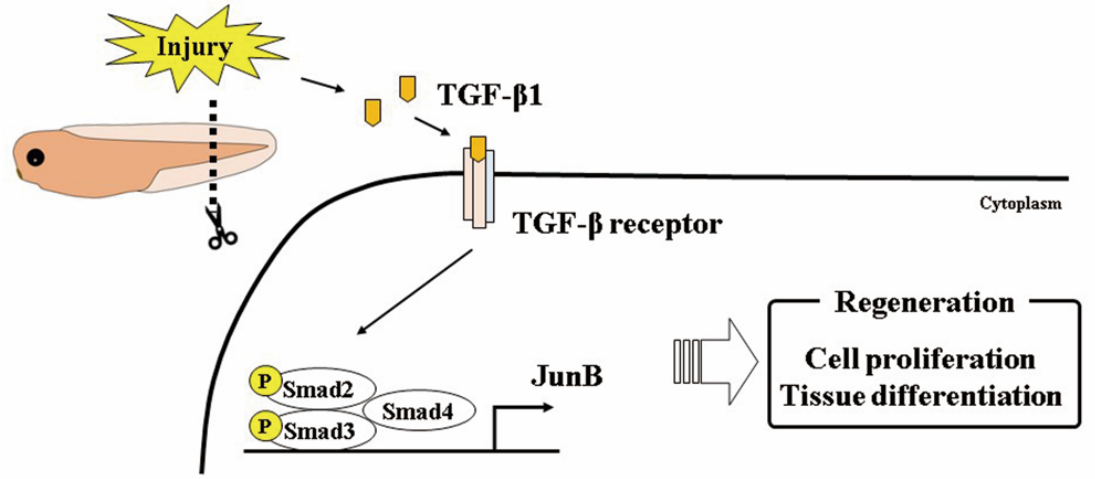

Figure IV-1. Schematic of the mechanism activating cell proliferation by JunB and TGF-β1 in *Xenopus* tail regeneration

Upon tail amputation, TGF-β1 activates Smad2/3 by binding to its receptors. Smads stimulate the expression of *junb*, and JunB activates cell proliferation and tissue differentiation, thereby promoting *Xenopus* tail regeneration.

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2. 学位要件論文 1

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The AP-1 transcription factor JunB functions in *Xenopus* tail regeneration by positively regulating cell proliferation

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3. 学位要件論文 2

学位要件論文 2

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

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