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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.

Keywords

JunB; *Xenopus* tail regeneration; cell proliferation; tissue differentiation; TGF-β signaling

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 epithelium 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, 5, 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, 8, 9, 10], and by other factors,

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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°C 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 1h 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°C 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 3h, and incubated at 4°C overnight with primary antibody (anti-phosphorylated histone H3, Upstate Biotechnology, 1 μg/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 2h at 24°C. 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°C overnight. Total RNA was purified by phenol-chloroform extraction and ethanol precipitation, and treated with DNaseI (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 μg/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 (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 whole-mount *in situ* hybridization (WISH) of tadpoles that had undergone tail amputation (Fig. 1). Expression of *junb* was widely detected near the amputation site from 0.25 hpa (hours post amputation). At 1 hpa, *junb* was expressed in the tail, including the wound epithelium. 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. 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 Figure 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 T7E1 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. 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. 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. 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. 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. 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. 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. 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 phosphorylated histone H3 (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. 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. 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. 4B). Moreover, *junb* transcripts were considerably reduced at 2 and 6 hpa by the TGF-β receptor inhibitor treatment (Fig. 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, 5, 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 *cyclinA* 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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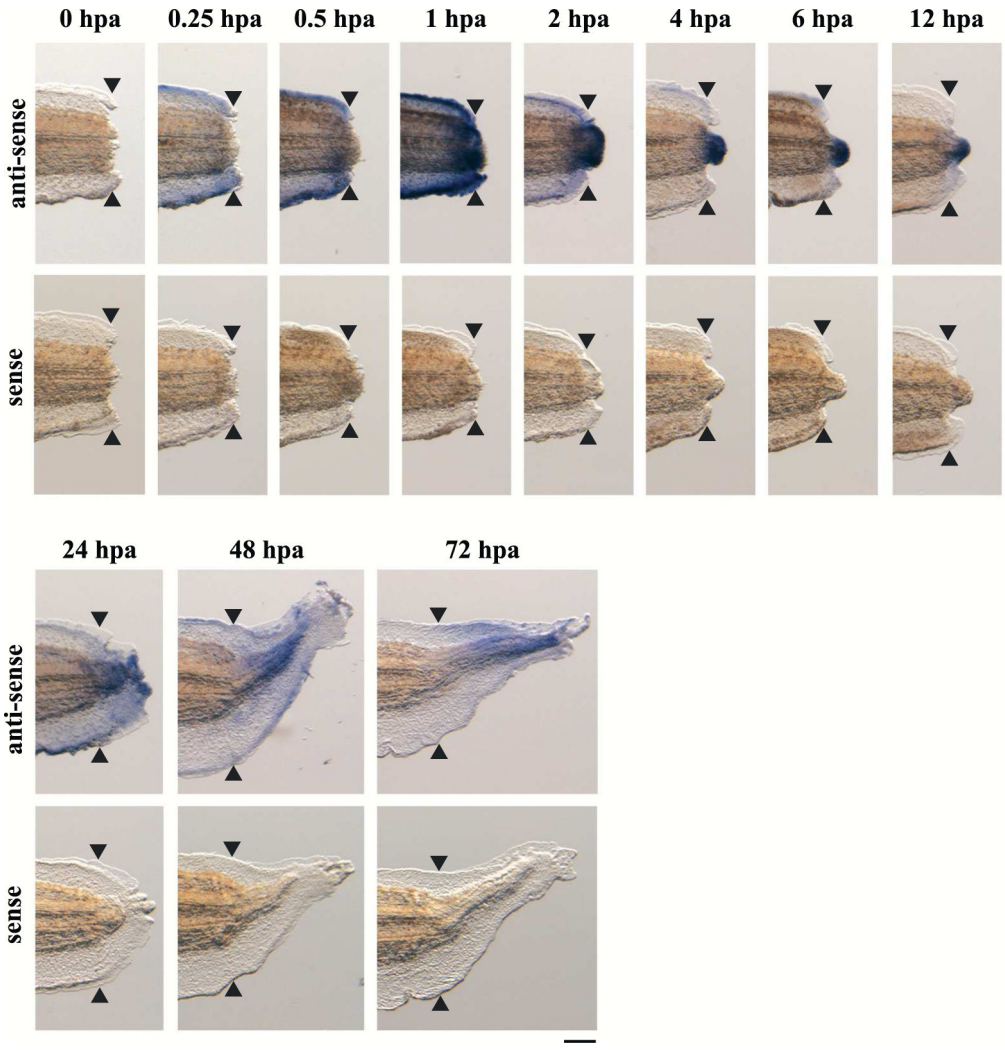

Figure 1. *junb* is expressed during tail regeneration of *X. tropicalis* tadpoles.

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.

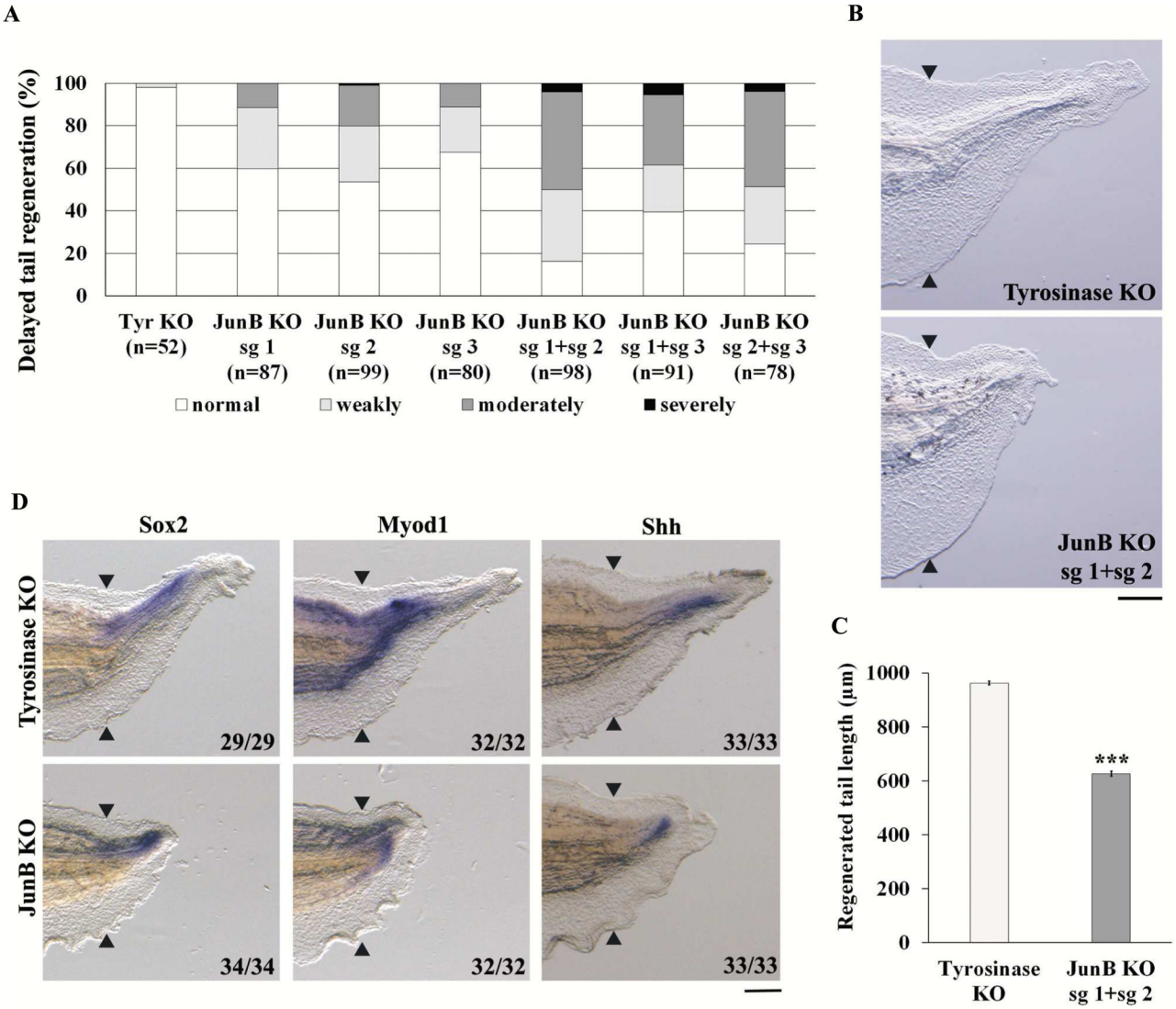

Figure 2. Knockout of *junb* prevents tail regeneration.

(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.

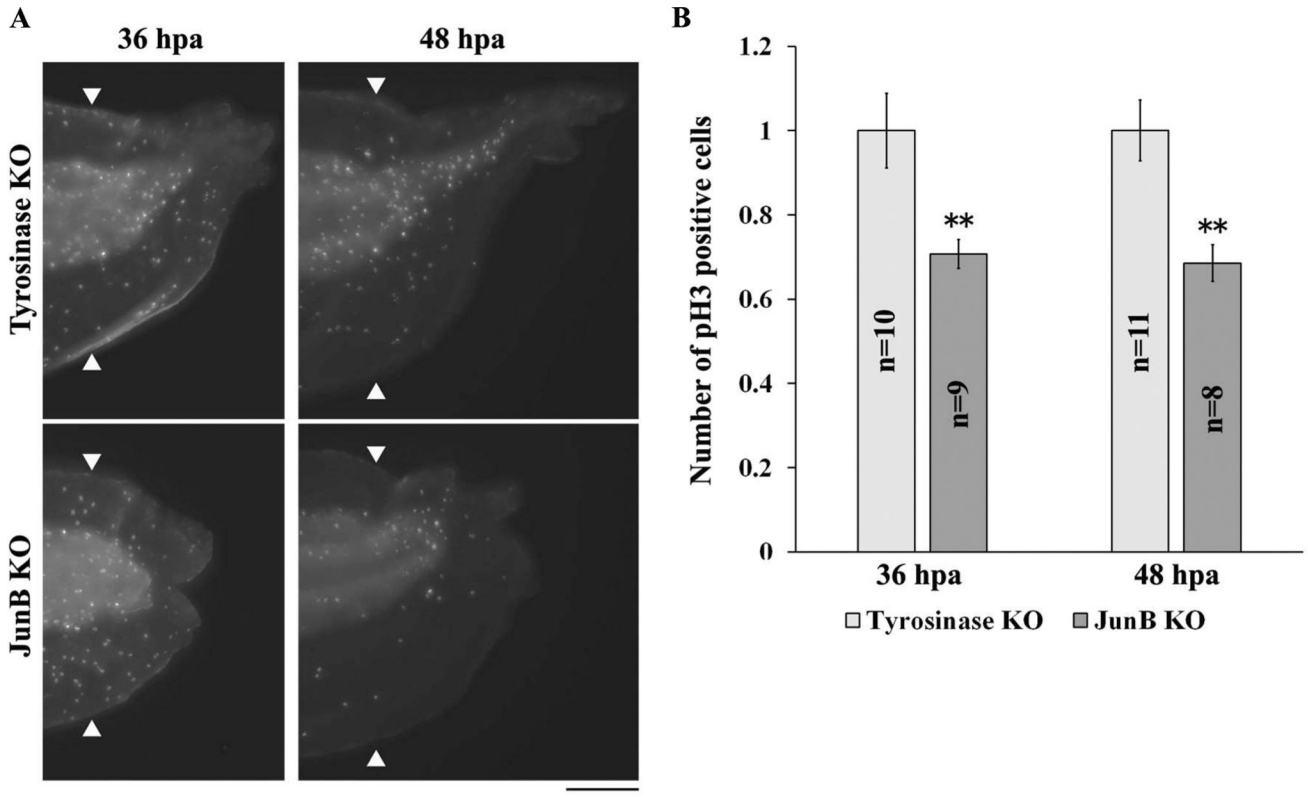

Figure 3. Knockout of *junb* reduces cell proliferation.

(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.

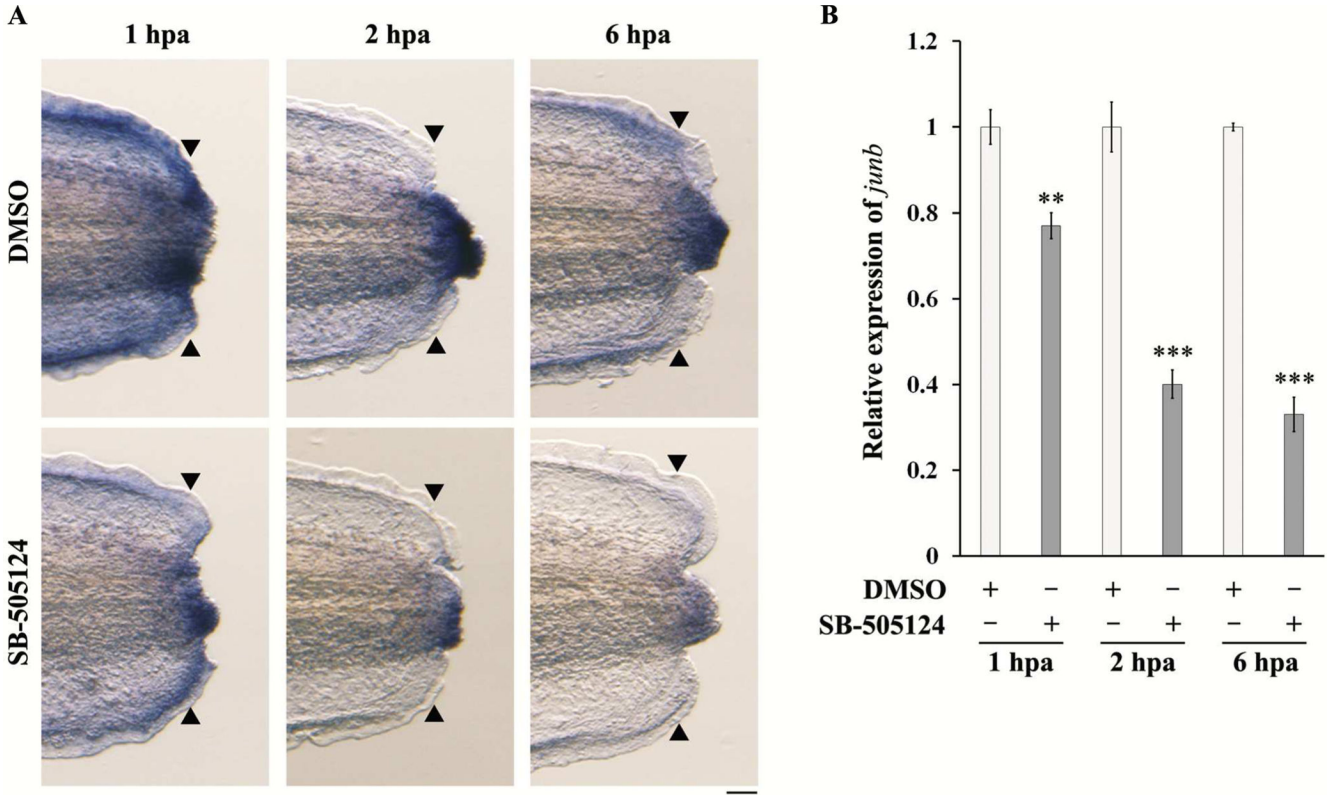

Figure 4. The expression of *junb* is downregulated by TGF-β signaling inhibition.

Tadpoles were treated with a medium containing 12.5 μM SB-505124 or DMSO (Control) from 1 h 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, *** < 0.001, Student's *t*-test.