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Sall4 regulates downstream patterning genes during limb regeneration

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ABSTRACT

Many salamanders can completely regenerate a fully functional limb. Limb regeneration is a carefully coordinated process involving several defined stages. One key event during the regeneration process is the patterning of the blastema to inform cells of what they must differentiate into. Although it is known that many genes involved in the initial development of the limb are re-used during regeneration, the exact molecular circuitry involved in this process is not fully understood. Several large-scale transcriptional profiling studies of axolotl limb regeneration have identified many transcription factors that are up-regulated after limb amputation. *Sall4* is a transcription factor that has been identified to play essential roles in maintaining cells in an undifferentiated state during development and also plays a unique role in limb development. Inactivation of *Sall4* during limb bud development results in defects in anterior-posterior patterning of the limb. *Sall4* has been found to be up-regulated during limb regeneration in both *Xenopus* and salamanders, but to date it function has been untested. We confirmed that *Sall4* is up-regulated during limb regeneration in the axolotl using qRT-PCR and identified that it is present in the skin cells and also in cells within the blastema. Using CRISPR technology we microinjected gRNAs specific for *Sall4* complexed with cas9 protein into the blastema to specifically knockout *Sall4* in blastema cells only. This resulted in limb regenerate defects, including missing digits, fusion of digit elements, and defects in the radius and ulna. This suggests that during regeneration *Sall4* may play a similar role in regulating the specification of anterior-proximal skeletal elements.

1. Introduction

Limb regeneration is a phenomenon that has fascinated scientists for centuries (Bando et al., 2018; Brockes, 1987, 1991, 1994; Brockes and Kumar, 2005, 2008; Call and Tsonis, 2005; Daponte et al., 2021; Farkas and Monaghan, 2017; Gardiner and Bryant, 1996; Gardiner et al., 2002; Min and Whited, 2023; Monaghan and Maden, 2013; Raymond and McCusker, 2023; Roy and Levesque, 2006; Simon and Tanaka, 2013; Stocum, 1991, 2017; Whited and Tab lesin and 2009). This intriguing field of study delves into the intricate processes by which living organisms, ranging from amphibians to certain invertebrates, can regrow lost or damaged limbs with astonishing precision. Limb regeneration involves a complex interplay of cellular events, signaling pathways, and timing to orchestrate the recreation of the complex limb structure, which includes bones, muscles, nerves, and skin (Bando et al., 2018; Brockes, 1987, 1991, 1994; Brockes and Kumar, 2005, 2008; Call and Tsonis, 2005; Daponte et al., 2021; Farkas and Monaghan, 2017; Gardiner and Bryant, 1996; Gardiner et al., 2002; Min and Whited, 2023; Monaghan and Maden, 2013; Raymond and McCusker, 2023; Roy and Levesque, 2006; Simon and Tanaka, 2013; Stocum, 1991, 2017; Whited and Tab lesin and 2009).

To date, work from many scientists has begun to unravel the molecular and cellular mechanisms underlying the ability to regenerate a complex functional limb. It is now well-documented that many genes involved in the initial specification and patterning of the limb bud are re-expressed during limb regeneration. Many signaling pathways involved in the anterior-posterior specification on the limb axis and those that pattern the dorsal-ventral axis of the hand have been identified. The well-studied Hox genes are essential for limb development and are re-expressed during limb regeneration, as are many well-known signaling factors like *Fgf* genes and *Shh*; both are crucial for limb development and regeneration (Gardiner and Bryant, 1996; Brown and Brockes, 1991;

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Crawford and Stocum, 1988; Oliveira et al., 2022; Roensch et al., 2013; Savard et al., 1988; Stocum, 1996; Vieira et al., 2023).

Sall4 and *Gli3* have also emerged as crucial players in regulating limb development (Akiyama et al., 2015; Böhm et al., 2006; Chen et al., 2020; Kawakami et al., 2023; Neff et al., 2005). *Sall4*, a zinc finger transcription factor, plays a pivotal role in maintaining the undifferentiated state of limb progenitor cells (Akiyama et al., 2015). It exerts influence during early limb bud formation, ensuring the proper balance between self-renewal and differentiation as cells become distinct limb structures. On the other hand, *Gli3*, a member of the Hedgehog signaling pathway, is instrumental in regulating the patterning and growth of limbs (Akiyama et al., 2015; Bastida et al., 2004; Bowers et al., 2012; Büscher et al., 1997; Büscher and Rüther, 1998; Chen et al., 2004; Letelier et al., 2021; Litingtung et al., 2002; Matsubara et al., 2017; Motoyama, 2006). *Gli3* acts as a molecular switch, transducing signals that guide limb cells to assume specific fates and positions within the developing limb bud. *Gli3* acts to restrict *Shh* expression to the posterior region of the limb bud, opposing *Fgf8* in the anterior region (Aoto et al., 2002; O'Rourke et al., 2002; Sheeba et al., 2012; Sheth et al., 2013; te Welscher et al., 2002; Zakany et al., 2007). Expression domains in the limb bud must be calibrated correctly to ensure the correct patterning of the future digits is achieved (Akiyama et al., 2015; Bowers et al., 2012; Litingtung et al., 2002; O'Rourke et al., 2002; Sheth et al., 2013; Zakany et al., 2007; Barna et al., 2005; Bimonte et al., 2011; Deimling et al., 2018; Duan et al., 2022; Guéro, 2018; Kuijper et al., 2005; Panman et al., 2005; Sheeba et al., 2014; Smith et al., 2016; Zákány et al., 2004). Together, *Sall4* and *Gli3* form integral components of the intricate molecular network that directs limb development, ensuring the precise formation of correctly patterned limb.

Another well-studied gene family in limb development and regeneration are the *Wnt* genes. During limb development, *Wnt* signaling pathways play a pivotal role in establishing the limb bud's anterior-posterior and proximal-distal axes, guiding the differentiation and patterning of cells (Adamska et al., 2004; Al-Qattan, 2011; Barrow, 2011; Church and Francis-West, 2002; Collette et al., 2010; Cooper, 2015; Currier et al., 2010; Dealy et al., 1993; Geetha-Loganathan et al., 2008; Glimm et al., 2020; Glover et al., 2023). *Wnt* proteins act as morphogens, influencing cell fate determination, proliferation, and the formation of key structures within the developing limb. *Wnt* genes, key players in developmental signaling pathways, contribute significantly to the spatial and temporal coordination of limb bud growth and patterning. They guide the differentiation of limb progenitor cells along the anterior-posterior and proximal-distal axes, ensuring the proper arrangement of skeletal elements and tissues. During limb regeneration, *Wnt* signaling also takes center stage. Studies in regenerative models, such as salamanders and axolotls, have highlighted the reactivation of *Wnt* pathways as a key factor in the initiation and maintenance of the regenerative response. *Wnt* signaling promotes the formation of a blastema, a pool of undifferentiated cells capable of giving rise to the diverse tissues required for limb regeneration (Daponte et al., 2021; Glover et al., 2023; Du et al., 2018; Ghosh et al., 2008; Glotzer et al., 2022; Kawakami et al., 2006; Lovely et al., 2022; Shah et al., 2011).

Together, *Wnt* genes and *Sall4* form a synergistic partnership, directing the molecular cues that govern limb development. Work in mice has shown that *Sall4* acts upstream of *Wnt/ß-catenin* signaling, and *Sall4* promotion of *Wnt* signaling acts to maintain neuroectodermal progenitors in an undifferentiated state during axis formation (Akiyama et al., 2015; Kawakami et al., 2023; Chen et al., 2022). Several papers have shown that *Sall4* and *Wnt* genes are up-regulated during axolotl limb regeneration (Neff et al., 2005, 2011; Chen et al., 2022; Mahapatra et al., 2023; Mescher et al., 2013; Stewart et al., 2013), and inhibition of *Wnt* signaling leads to defects in the size of the regenerated limb (Lovely et al., 2022). To determine the role of *Sall4* in limb regeneration, we have knocked out *Sall4* and determined that it is necessary for the faithful patterning of the limb regenerate.

2. Materials and methods

2.1. Animal handling and limb amputations

All axolotls used in these experiments were obtained and bred at the University of Minnesota or the Marine Biological Laboratory in accordance with IACUAC regulations. Prior to all *in vivo* experiments, animals (5–8 cm) were anesthetized in 0.01% p-amino benzocaine (Sigma). Limb amputations were performed using a number 10 scalpel; the limb was amputated halfway between the elbow and the wrist. After amputation, animals were housed individually in cups and monitored for the duration of the experiments.

2.2. CRISPR and morpholino injections

Guides were designed against the axolotl *Sall4* gene using open source design tool website ChopChop: https://chopchop.cbu.uib.no/(Labun et al., 2016, 2019; Montague et al., 2014). Initially, 4 guides were tested, and then one was selected for use in all further experiments, which gave the best INDEL. To determine which guide to use, genomic DNA was extracted from individual embryos using a ThermoFisher Purelink Genomic DNA extraction kit. The samples were sent for sequencing and analysis was carried out using the TIDE program. The guides were synthesized by IDT. Prior to injection the guide was complexed with cas9 protein according to previous publications (Fei et al., 2016). The complex was injected into the mature limb prior to amputation, the limb was injected from the hand along the length of the lower limb using a World Precision Instruments pressure injector; after injection and electroporation of the limb, the limb was amputated in the mid-radius/ulna region. Animals 5–8 cm in length are used in these experiments, at this stage the animals contain cartilage in the limbs, they do not yet have ossified bones as the limbs and animals' overall are still growing. At three days post-amputation, when a blastema was visible, the complex was injected into the blastema and electroporated. To confirm an INDEL was generated the blastema was harvested 5 days post amputation, genomic DNA was extracted using a ThermoFisher Purelink Genomic DNA extraction kit and sent for sequencing. The guide used in these experiments generated a 20bp deletion in the *Sall4* gene (Fig. S2).

For morpholino experiments, the control and Ax*Sall4* morpholinos were designed and synthesized by Gene Tools, LLC. The negative control morpholino was designed to target a beta-globin that causes beta-thalassemia; this oligo has been well documented to cause little to no changes in phenotype of any known test system, including the axolotl. Prior to injection, both control and *Sall4* morpholinos were diluted to 1mMol in PBS and Fast Green. Morpholinos were injected into the mature limb prior to amputation using a World Precision Instruments pressure injector. Similar to our CRISPR experiments, immediately following injection animals were electroporated using an ECM 830 electroporation system, with 5-pulses of 50V, each for 50ms. Further, regenerating limb blastemas were injected and electroporated every 2–3 days with either the control or *Sall4* morpholino.

2.3. Alcian blue stainings

Limbs were fixed overnight in 4% paraformaldehyde, then washed 3 × 10 min in phosphate buffered saline (PBS). The samples were then dehydrated through a graded series of alcohol washes (25%, 50%, 75%, 100% EtOH) for 20 min at each step. Samples were then placed in an Alcian blue solution made up of EtOH and acetic acid (60:40). Staining was carried out at room temperature for 2 days and when staining was complete, samples were washed in EtOH/acetic acid mix for 1hr at RT. Samples were then gradually rehydrated in 90%, 70%, 50%, and 25% ETOH/PBS. Samples were imaged on a Zeiss Stereo Discovery.V8 dissecting scope.

2.4. Quantitative reverse transcriptase polymerase chain reaction

Blastemas were collected from control and *Sall4* CRISPR-injected animals, and blastemas from six animals were pooled for RNA extraction. Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. Subsequent cDNA was synthesized from 1 μg of DNaseI (NEB) treated RNA using the iScript cDNA Synthesis Kit (BioRad). The following qRt-PCR primers were used:

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
18S	CGGCTTAATTTGACTCAACACG	TTAGCATGCCAGAGTCTCGTTC
Sall4	AATCCCTCGCAAGCCC	CCAGCTATGAGGGGAACATT
Gli3	GCATGAGCATCTTGCAACTCAGC	TGGTGTGTGATGGGACATTAGCCT
Shh	GTAACCCTGGAGCATGGAGT	TCGGACTCTGCTGGCAAATA
Fgf8	TTTGTCCTCTGCATGCAAGC	GTCTCGGCTCCTTTAATGCG
Fgf10	AAACTGAAGGAGCGGATGGA	TCGATCTGCATGGGAAGGAA
Hand2	CGCACGCAGAGCATCAA	TCCATGAGGTAGGCGATGTA
Alx4	GGGTAACCTCTTTGGCACTG	TTAAGTGCCCTGTCATGTGG
Meis1	CCATCTACGGACACCCCCT	GGAAGAACACACGTCCCCG
Meis2	AGTGGAGGGCACGCTTCT	GCTTCTTGTCTTTGTCCGGGT
Hoxa13	TCTGGAAGTCCTCTCTGCCG	TCAGCTGGACCTTGGTGTACG

2.5. Western blotting

Limb blastema's were harvested at 3 days post amputation (dpa) and immediately flash frozen on liquid nitrogen. Tissues were homogenized using a handheld dounce homogenizer in lysis buffer, then centrifuged for 30 min at 4 °C. Total protein concentrations were determined using the Pierce BCA protein assay, and 20 μg of total protein was run on a NuPAGE 4–12% Bis-Tris Gel for 35 min at 200V. After transfer onto Invitrolon PVDF membrane, blots were blocked in 3% nonfat milk powder/0.1% Tween-20/PBS for 1 h. Membranes were next incubated in a SALL4 primary antibody (1:1000; 8459S Cell Signaling) overnight at 4 °C with gentle shaking. The next day, blots were washed in PBS/0.1% Tween-20 and incubated in stabilized peroxidase conjugated goat anti-rabbit antibody (1:10,000) for 1 h at room temperature. Finally, membranes were incubated in Supersignal West Pico PLUS Chemiluminescent Substrate for 5 min. Membranes were immediately imaged using an Amersham Imager 600 (General Electric).

2.6. Immunofluorescence

Limbs were harvested and immediately fixed overnight at 4 °C in 4% paraformaldehyde. The next day, tissues were decalcified in 10% EDTA (pH 7.4) overnight at room temperature. Limb tissues were next rinsed in PBS and washed for 10 min in 15% sucrose. After incubation in 30% sucrose at 4 °C overnight, samples were embedded in O.C.T. (Tissue-Tek) and cryosectioned on a Leica Cryomicrotome 1850. For immunostaining procedures, sections were incubated in PBS at 70 °C for 20min, then permeabilized for 3 × 10min in PBS/0.1% Triton-X. Samples were blocked in blocking buffer (2% bovine serum albumin/2% goat serum/0.1% Triton-X in PBS) for 1 h at room temperature, then incubated in a SALL4 antibody (1:250, 8459S Cell Signaling) overnight at 4 °C. The next day, sections were briefly washed in PBS/0.1% Tween-20, incubated in a secondary antibody (Alexa Fluor 568, Invitrogen) for 2 h, and counterstained using DAPI. Sections were finally imaged on a Leica DMI 6000 B epifluorescent microscope.

2.7. Fluorescent in situ hybridization

All RNAscope® *in situ* hybridization procedures were performed according to the manufacturer's instructions (Advanced Cell Diagnostics). In brief, cryosections were incubated in PBS for 10 min to remove the OCT, and then baked at 60 °C for 30 min. The slides were next post-fixed in 4% paraformaldehyde for 15 min at 4 °C, and then dehydrated in a graded series of ethanol dilutions before being incubated in absolute ethanol for 5 min. After briefly air-drying the slides for 5 min, sections were next treated with hydrogen peroxide to quench endogenous peroxidase activity for 10 min at room temperature. Next, samples were briefly washed in deionized water, then incubated in target retrieval buffer at 90 °C for 5 min. Following target retrieval, the slides were rinsed in deionized water for 15 s and treated with absolute ethanol for 3 min. Slides were next permeabilized in protease III for 30 min before hybridization with RNAscope® probes at 40 °C for 2 h. Following hybridization, sections were placed in 5x SSC overnight. The next day, sections were incubated in Amp1 and Amp2 at 40 °C for 30 min each, followed by Amp3 for 15 min. Next, slides were treated with HRP-C1 to detect *Fgf10*, followed by a 30-min incubation in OpaI-690 fluorescent dye. After treatment with HRP blocking buffer, samples were next incubated in HRP-C2 to detect *Wnt5a*, followed by a 30-min incubation in OpaI-570 dye. After an additional treatment with HRP blocking buffer, slides were counterstained with DAPI and imaged using a Zeiss 780 Confocal Microscope.

2.8. Statistical analyses

All results are presented as mean ± s.d. unless otherwise stated. Analyses were performed using GraphPad Prism v10.1.1. Means were compared using an Ordinary one-way ANOVA with a Tukey posthoc test for multiple comparisons. Differences between treatment groups were considered significant by a p-value of ≤ 0.05 (p-values of $^{*}\leq 0.05$, $^{**}\leq 0.01$, $^{***}\leq 0.001$, $^{****}\leq 0.0001$).

3. Results

3.1. Sall4 is re-expressed during limb regeneration

Sall4 is a transcription factor that plays a well-documented role in maintaining cells in an undifferentiated state during early embryogenesis. More recent work has also shown that *Sall4* plays a crucial role in regulating expression boundaries in the limb bud during development to ensure correct patterning of the limb digits (Akiyama et al., 2015; Kawakami et al., 2023; Koshiba-Takeuchi et al., 2006). Several studies in limb regeneration have found that *Sall4* is up-regulated during the process of axolotl limb regeneration, however its functional role is unknown. Here, using qRT-PCR we confirmed that *Sall4* is indeed upregulated by 3 days post injury, and that *Sall4* remains elevated in the blastema cells (Fig. 1A). We additionally used a SALL4 antibody to determine its localization within regenerating limbs. We first confirmed the specificity of the SALL4 antibody on axolotl limb tissue using western blotting, demonstrating a single band at the predicted SALL4 molecular weight of ~80 kDa (Fig. S1A). Using immunohistochemistry, we found that SALL4 was expressed at low levels in the cartilage and skin of uninjured limb tissues (Figs. S1B–D). In regenerating limb tissues, SALL4 protein was expressed in most cells of the blastema but was largely excluded from the wound epithelium (Fig. 1B). Interestingly, while SALL4 expression was absent from the wound epithelium, it was present in the surrounding cells of the dermal and epidermal layers. Additionally, all cells in the blastema appeared to upregulate SALL4, including cells in the zone adjacent to the injury site, especially cartilage cells.

3.2. Knock-out of Sall4 causes defects in limb regeneration

To explore the functional role of *Sall4* in the regenerating limb we initially knocked down *Sall4* levels in the blastema cells by direct injection of a morpholino against *Sall4*, which we have previously validated and used to test the function of *Sall4* in axolotl skin during regeneration (Erickson et al., 2016). We first performed immunohistochemistry on control and *Sall4* morpholino-injected limb tissues to further confirm the specificity of the morpholino. We discovered that SALL4 protein expression was dramatically reduced in *Sall4*

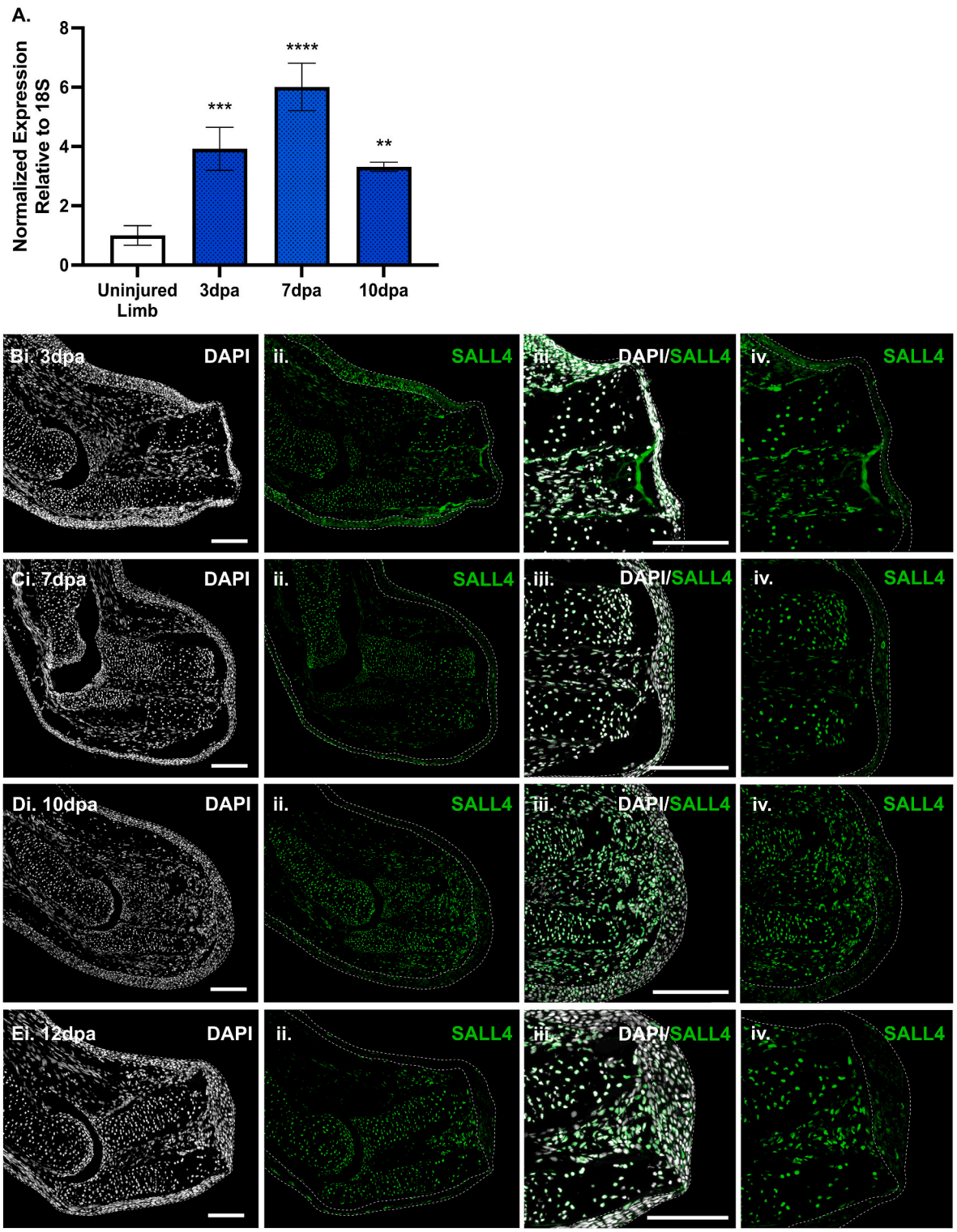

Fig. 1. Sall4 is upregulated after limb amputation. A) Quantitative RT-PCR analysis reveals that *Sall4* is significantly up-regulated during limb regeneration compared to the uninjured, mature limb. Peak levels of *Sall4* transcript are found at 7 days post-amputation (dpa), and by 10 dpa, *Sall4* levels begin to return to homeostatic levels (**p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001). **B)** SALL4 immunostaining after limb amputation demonstrates that SALL4 is expressed in cells of the regeneration blastema, but is absent from the wound epithelium. Scale bars: 500 μm.

morpholino-treated limbs in comparison to controls, specifically within the cartilage and blastema cells (Figs. S2A and B). Importantly, depletion of *Sall4* directly in the blastema cells led to defects in the patterning of cells in the hand when compared to the control (Figs. S2C–F). *Sall4* morpholino injected animals also showed defects in the number of digits (n = 5/17, Fig. S2E), patterning of the metacarpals (11/17), and in the pattern of cartilage differentiation in the regenerated radius and ulna (n = 10/17, Fig. S2F). The defects we observed in morpholino-injected animals are not observed in all animals, only around 50% of animals injected with the morpholino showed a morphological phenotype at 40 days post amputation. This is possibly due to the short half-life of the morpholino, or a result of diluting the morpholino as the cells in the

blastema divide.

To further confirm this phenotype we designed several CRISPR guides against *Sall4* and injected them into embryos and harvested the genomic DNA after 5 days. Sequencing confirmed that one of the guides created a 20bp deletion in *Sall4* (Figs. S3A and B), and we used this guide for all further studies. The guide was injected into mature cells of the limb prior to electroporation and then the limb was amputated through the mid-radius and ulna region. At three days post injury when a blastema was visible we re-injected the *Sall4* CRISPR guide. We further validated the *Sall4* CRISPR knock-out using immunohistochemistry on limb tissue at 7 days post amputation (dpa). We found that SALL4 protein expression was reduced in *Sall4* CRISPR-injected animals compared to controls, specifically within the regeneration blastema (Figs. S3C and D). After confirming the efficiency of the *Sall4* CRISPR knock-out, we fixed regenerating limb tissues at 40dpa from control and *Sall4* CRISPR animals for phenotypic analysis. Using the CRISPR knock-out of *Sall4* during limb regeneration, similar results were found to the morpholino phenotype, but with much higher penetrance. Ninety-eight percent of the animals injected with the *Sall4* CRISPR had defects in patterning of the limb, whilst control injected animals had no morphological phenotypes (n = 0/25). *Sall4* knock-out animals were missing digits (n = 11/15) and metacarpals (n = 13/15), had fused metacarpals, and also had thickened bulges in the radius and ulna (n = 11/15, Fig. 2C). Moreover, the CRISPR animals all displayed numerous patterning defects (Fig. 2D).

3.3. Loss of Sall4 leads to defects in re-expression of key patterning genes

To gain a more comprehensive understanding of the function of *Sall4* in limb regeneration, we looked at expression levels of genes involved in limb patterning and in the *Sall4* signalling pathway in CRISPR and control animals. The outgrowth of the limb blastema is driven by the re-expression of *Fgf* genes and up-regulation of *Shh* in the posterior blastema cells (Glotzer et al., 2022; Kawakami et al., 2006; Lovely et al., 2022; Brockes, 1997; Bryant et al., 2002, 2017; Campbell et al., 2011; Dwaraka and Voss, 2021; Géraudie and Ferretti, 1998; Gerber et al., 2018; Knapp et al., 2013), and is essential for the correct patterning of the regenerated limb. By qRT-PCR, we determined that *Fgf8* and *Fgf10* are significantly up-regulated after *Sall4* knockout in comparison to control limb blastema's. Work in mouse limb development has

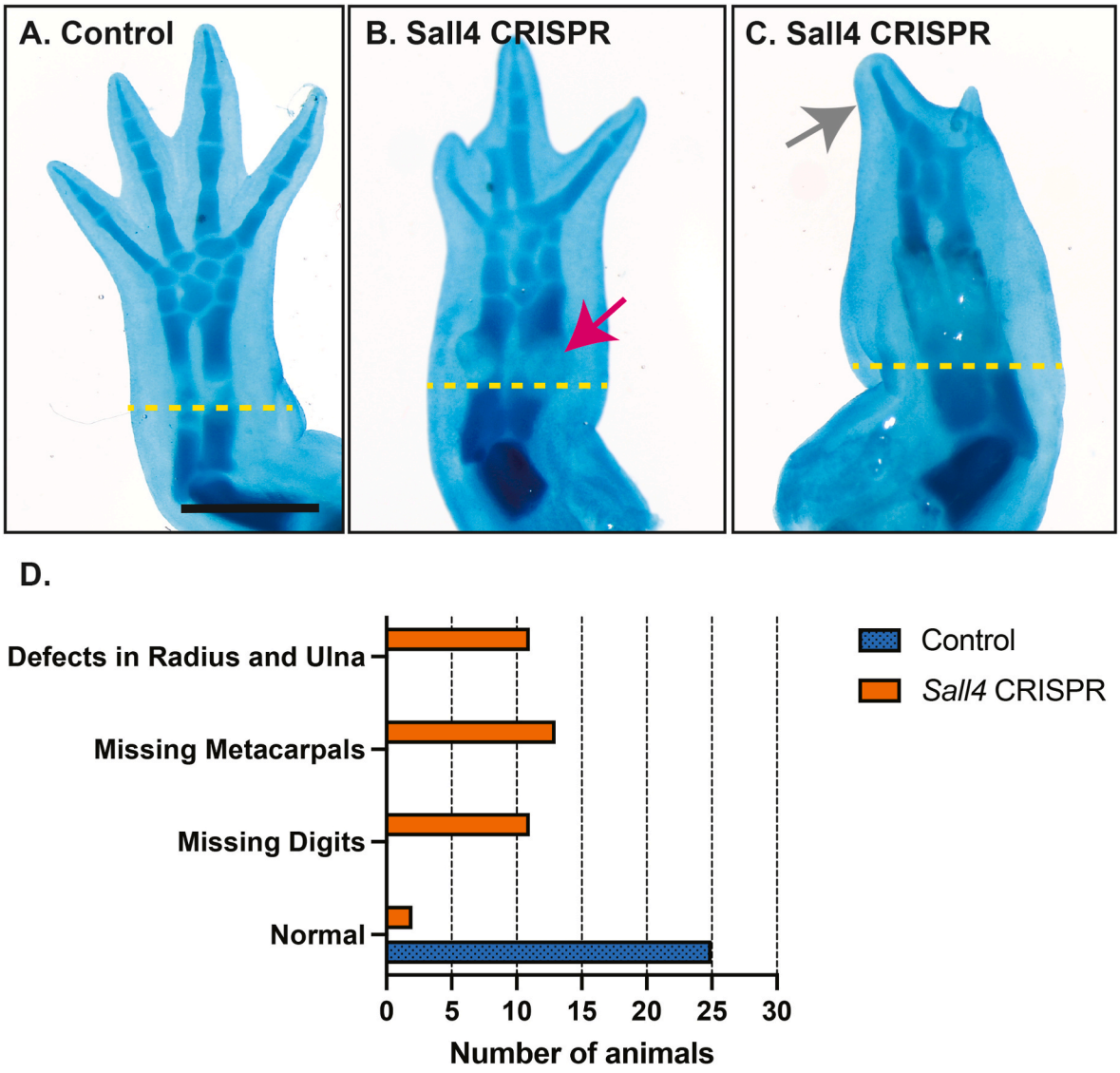

Fig. 2. Knock-out of *Sall4* in blastema cells causes defects in regeneration. (A–C) At 40 days post amputation (dpa), blastemas injected with a CRISPR guide against *Sall4* (B, C; n = 26) fail to correctly pattern the regenerated limb compared to control injected animals (A, n = 25). Animals with reduced *Sall4* expression resulted in multiple defects in the regenerate, which included bulges in the radius and ulna (B, pink arrow) and failure to reach the correct number of metacarpals and/or digits (C, grey arrow). Scale bar: 1 mm. **D)** Graph displays the number of *Sall4* CRISPR animals with limb abnormalities compared to control animals.

suggested an interaction between *Sall4* and *Gli3* upstream of *Shh* signalling (Akiyama et al., 2015), while knockout of *Gli3* results in defects in the proximal limb but no effect on the hand in mice (Barna et al., 2005). We also examined the levels of *Gli3* and *Shh* in the *Sall4* knockout limb blastemas, and we found the levels of *Gli3* and *Shh* were much higher than in control blastemas. We also quantified the expression levels of other known drivers of limb regeneration and patterning and found that *Meis1 & 2, Hand2*, and *Alx4* were all highly up-regulated in *Sall4* knockouts in comparison to control regenerating limbs. We also quantified the expression of the distally expressed *Hox* gene, *Hoxa13*, and found that these levels were not changed compared to control limb blastema, suggesting that the positional identity of the blastema cells was not changed. To determine if the expression domains of some of the patterning genes were changed, we carried out fluorescent *in situ* hybridization for *Wnt5a* and *Fgf10*. In control regenerating blastema, *Wnt5a* is restricted to a small zone of distally located blastema cells adjacent to the wound epithelium. In *Sall4* knock-out animal, we observed an expansion of the *Wnt5a* expression domain; it now extended more proximal in the blastema (Fig. 3). We also investigated the expression of *Fgf10* in the CRISPR animals, in control animals it is expressed in an overlapping domain to *Wnt5a*, in a small group of cells in the distal blastema (Fig. 3C), however in the *Sall4* knock-out animals the expression domain in significantly increased and we observed *Fgf10* to now be expressed in most cells in the blastema (Fig. 3D), in a larger domain than we see *Wnt5a* expressed in but there is still overlap in their increased expression domains (Fig. 3C and D). Taken together, these data suggest that *Sall4* acts upstream of *Wnt* signaling to control the expression levels of downstream target patterning genes.

4. Discussion

SALL4 encodes multiple Cys2His2 zinc finger (C2H2-ZF) domain-containing transcription factors that activate or repress gene transcription depending on the cell type. In mammals, expression of SALL4 has been primarily detected in embryonic stem cells (ESCs), where it acts as a core controller regulating cell "stemness" in early development. SALL4 is required for ESC pluripotency, and it is an essential component of the 'stemness' regulatory circuit involving OCT4, SOX2, NANOG and other factors that maintain ESC self-renewal and pluripotency (Chen et al., 2008; Lim et al., 2008; Tan et al., 2013; Wu et al., 2006; Yang et al., 2008, 2010; Zhang et al., 2006; Zhou et al., 2007). SALL4 is also expressed in extraembryonic endoderm cells, where it participates in cell fate decisions by simultaneously activating key pluripotency maintaining factors and silencing endoderm lineage-associated factors such as GATA6, GATA4, and SOX17 (Chen et al., 2008; Lim et al., 2008; Tan et al., 2013; Wu et al., 2006; Yang et al., 2008, 2010; Zhang et al., 2006; Zhou et al., 2007). At later developmental stages, heterozygous disruption of the *Sall4* allele leads to multi-organ malformations

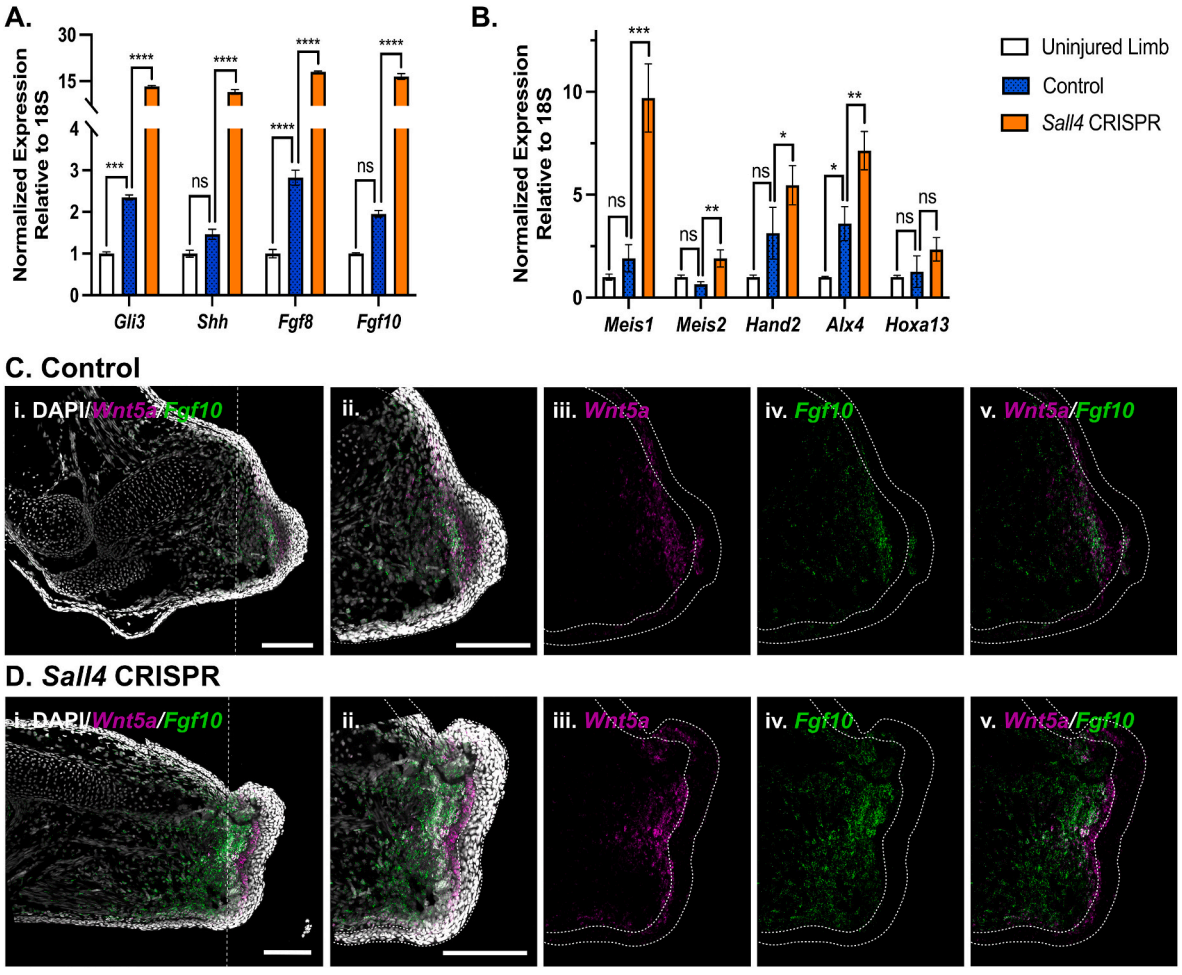

Fig. 3. *Sall4* inhibition results in the up-regulation of key genes involved in patterning the limb during regeneration. (A, B) qPCR demonstrates that after knocking out *Sall4* during limb regeneration, many genes associated with limb patterning are significantly up-regulated in comparison to controls. dpa = days post amputation, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. **C)** After *Sall4* inhibition, *in situ* hybridization demonstrates that the localization of *Wnt5a* and *Fgf10* dramatically increases in the blastema immediately adjacent to the wound epithelium. Vertical white line indicates the original plane of amputation. Scale bars: 500 μm.

including limb and heart defects (Akiyama et al., 2015; Bohm et al., 2007). Work in *Xenopus* embryos identified a novel role for *Sall4* in repressing *Pou5f3* family members to enable neural patterning and differentiation during early development (Exner et al., 2017; Young et al., 2014).

In this study, we generated *Sall4* knock-outs during limb regeneration to determine if it is necessary for faithful limb regeneration in the axolotl. Our worked has uncovered a role for *Sall4* upstream of classical genes involved in patterning. Knock-out of *Sall4* leads to clear patterning defects in the regenerate and to increased expression levels of many genes involved in limb patterning. This data suggests that genes like *Fgfs, Wnts, Hand2* and *Shh* that are spatially restricted during limb development and regeneration may expand their expression domains in *Sall4* knock-out blastemas, this we have shown for both *Wnt5a* and *Fgf10* (Fig. 3). Interestingly, in all genes we quantified except for *HoxA13*, knock-out of *Sall4* led to increased expression of the genes, suggesting that *Sall4* is not necessary for the activation of gene expression but rather is may play a role in regulating levels and timing of expression of downstream genes. *Meis* genes have been found to play an important role in proximal-distal regulation in limb regeneration. *Meis* genes are highly expressed in the blastema of a proximally amputated limb and, when overexpressed in the cells of a hand blastema, can change their identity. *Meis* is also upstream of the other known positional identity genes *Prod1* and *Tig1*. Given that we see a significant increase in the *Meis1* levels in *Sall4* knock-out blastema, we thought it may be changing the positional identity of these cells, however, we see no changes in *HoxA13* expression and no phenotypic evidence that the overall P/D positional information changes in the limb regenerates. It is more likely that changes we found in genes involved in anterior posterior and dorsal/ventral patterning like *Hand2, Shh, Gli3 and Fgf* genes are what lead to the observed patterning defects. It is also well-known that *Sall4* plays an essential role in maintaining cells in an undifferentiated state during early development (Wu et al., 2006; Yang et al., 2010; Zhang et al., 2006; Xiong, 2014; Xiong et al., 2015; Yamaguchi et al., 2015; Yuri et al., 2009). As *Sall4* is up-regulated early in blastema formation it is possible that *Sall4* plays an essential role in driving cells into a more immature state to form a blastema. *Sall4* may be necessary to maintain the undifferentiated state of the blastema, and the knock-out of *Sall4* in some blastema cells leads to premature differentiation of the cells and, in combination with changes in expression levels of key patterning genes, results in defectively patterned regenerate.

CRediT authorship contribution statement

J.R. Erickson: Writing – review & editing, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **S.E. Walker:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **C. Arenas Gomez:** Writing – review & editing, Investigation, Data curation. **K. Echeverri:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2024.07.015.

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