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Early requirement of Hyaluronan for tail regeneration in *Xenopus* tadpoles

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Tail regeneration in *Xenopus* tadpoles is a favorable model system to understand the molecular and cellular basis of tissue regeneration. Although turnover of the extracellular matrix (ECM) is a key event during tissue injury and repair, no functional studies to evaluate its role in appendage regeneration have been performed. Studying the role of Hyaluronan (HA), an ECM component, is particularly attractive because it can activate intracellular signaling cascades after tissue injury. Here we studied the function of HA and components of the HA pathway in *Xenopus* tadpole tail regeneration. We found that transcripts for components of this pathway, including *Hyaluronan synthase2* (*HAS2*), *Hyaluronidase2* and its receptors *CD44* and *RHAMM*, were transiently upregulated in the regenerative bud after tail amputation. Concomitantly, an increase in HA levels was observed. Functional experiments using 4-methylumbelliferone, a specific HAS inhibitor that blocked the increase in HA levels after tail amputation, and transgenesis demonstrated that the HA pathway is required during the early phases of tail regeneration. Proper levels of HA are required to sustain proliferation of mesenchymal cells in the regenerative bud. Pharmacological and genetic inhibition of GSK3β was sufficient to rescue proliferation and tail regeneration when HA synthesis was blocked, suggesting that GSK3β is downstream of the HA pathway. We have demonstrated that HA is an early component of the regenerative pathway and is required for cell proliferation during the early phases of *Xenopus* tail regeneration. In addition, a crosstalk between HA and GSK3β signaling during tail regeneration was demonstrated.

KEY WORDS: *Xenopus*, Appendage regeneration, Hyaluronan, GSK3β

INTRODUCTION

Studying the genetic mechanisms and the signaling pathways that control tissue regeneration in animal models is particularly valuable, as it provides novel insights into potential applications for regenerative medicine (Slack et al., 2008; Stoick-Cooper et al., 2007a). Amphibians such as urodeles (e.g. newts) and anurans larvae (e.g. *Xenopus* tadpoles) are very efficient in appendage regeneration, including tail and limbs. Amputation of the posterior half of the tail activates regeneration of spinal cord (neural tissue), muscle, notochord, vasculature, skin, neural crest derivatives and connective tissue (Sánchez-Alvarado and Tsonis, 2006; Slack et al., 2008).

Tail regeneration in *Xenopus* tadpoles begins with the migration of epidermal cells from the edge of the wound, covering the amputation site and thereby allowing epithelialization (0-12 hours post-amputation, hpa). During the next 2 days extensive cell proliferation can be detected and a regenerative bud is formed (Beck et al., 2006). This structure contains an apical ampulla that corresponds to the terminal vesicle of the spinal cord ependymal layer, notochord precursor cells and undifferentiated mesenchymal cells. The group of mesenchymal cells surrounding these structures might be called 'blastema' (Slack et al., 2008). Regenerative bud formation is followed by appendage outgrowth, patterning and cell differentiation (Mochii et al., 2007; Slack et al., 2004; Stoick-Cooper et al., 2007a). Cell-lineage experiments demonstrated that, in *Xenopus*, the spinal cord and notochord regenerate from the corresponding tissues on the tail stump (Gargioli and Slack, 2004). Likewise, skeletal muscle is regenerated through activation of Pax7-satellite cells resembling muscle regeneration in mammals (Chen et al., 2006).

The signaling pathways involved in *Xenopus* tail regeneration are beginning to be unraveled. Importantly, it turns out that many of the signaling pathways involved in tail development are reactivated and required for tail regeneration (Beck et al., 2003; Sugiura et al., 2004). In addition, similar pathways are involved in regeneration of appendages as diverse as the tail and limb of *Xenopus* tadpoles and zebrafish adult caudal fin (Lin and Slack, 2008; Poss et al., 2003; Sánchez-Alvarado and Tsonis, 2006; Stoick-Cooper et al., 2007a). These include components of the TGFβ, BMP, Wnt, FGF, Shh and Notch signaling pathways as well as their downstream effectors (Beck et al., 2003; Beck et al., 2006; Ho and Whitman, 2008; Kawakami et al., 2006; Lin and Slack, 2008; Stocik-Cooper et al., 2007b; Yokoyama et al., 2007). Based on their expression pattern, temporal requirement and epistatic experiments, the different signaling cascades involved in regeneration could be organized in a general regenerative pathway (Lin and Slack, 2008).

Although turnover of the extracellular matrix (ECM) is a key event during tissue injury and repair, no functional studies to evaluate its role in tail regeneration have been performed. Hyaluronan (HA), a main ECM component, is particularly interesting because it is enriched in matrices undergoing remodeling processes such as morphogenesis, wound repair and tumor growth. HA regulates cell migration, proliferation and differentiation and activates intracellular signaling cascades in tissue injury and repair (Jiang et al., 2007; Noble, 2002). HA is a non-sulfated glycosaminoglycan composed of repeating disaccharides of D-glucuronic acid and *N*-acetyl-D-glucosamine and can form polymers consisting of 2000-25,000 disaccharides with a molecular weight of approximately 10^6 Da. It is synthesized on the inner side of the plasma membrane by membrane-bound hyaluronan synthases (HAS) and is extruded onto the cell surface while is being synthesized. Following injury, activation of extra- and intracellular

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hyaluronidases degrade high-molecular-weight HA (native HA, n-HA) and generate smaller oligosaccharides (o-HA) that have different signaling activities (Deed et al., 1997; Jiang et al., 2007; Slevin et al., 2007; Toole, 2004).

HA can signal through different cell-surface receptors, including CD44, receptor for HA-mediated motility (RHAMM) and Toll-like receptors, activating a variety of intracellular signaling pathways including: ERK1/2, Src kinase, PKC, FAK, PI3K, NF-kB, RhoA and Rac1 (Turley et al., 2002). CD44 plays an important role in regulation of cell proliferation; in particular it mediates contact inhibition of growth and modulates the activity of the tumor suppressor NF2 (Morrison et al., 2001; Ponta et al., 2003). Moreover, HA and CD44 can activate nuclear translocation of β-catenin (Bourguignon et al., 2007) and are required for BMP to activate Smad1 phosphorylation (Peterson et al., 2004), showing that HA-CD44 interaction can modulate Wnt and BMP signaling pathways.

Classic biochemical studies have evaluated HA synthesis through isotope incorporation and hyaluronidase activity during regeneration of the newt limb. These analyses showed a peak of HA synthesis concomitant with blastema formation followed by an increase of hyaluronidase activity (Mescher and Munaim, 1986; Toole and Gross, 1971). No function for this early synthesis of HA has been ascribed. In this work, we studied the function of the HA pathway during tail regeneration in *Xenopus* tadpoles. We found that components of this pathway such as *HAS2*, *Hyal2*, *CD44* and *RHAMM* were transiently upregulated in the mesenchymal cells of the regenerative bud. Concomitantly, an increase in HA levels occurred in the epidermis and also, but at lower levels, in the mesenchymal cells. Functional experiments using 4-methylumbelliferone, a specific inhibitor of HA synthesis, and transgenesis showed that HA synthesis and proper levels of HAS2 are necessary for successful tail regeneration. Early inhibition of HAS activity reduced cell proliferation of mesenchymal cells in the regenerative bud. Pharmacological and genetic inhibition of GSK3β restored cell proliferation and tail regeneration, suggesting that GSK3β signaling is downstream the HA pathway. In summary, we have demonstrated that the HA pathway is an upstream component of the regenerative cascade that is required for proliferation of the mesenchymal cells in the regenerative bud to allow tail regeneration.

MATERIALS AND METHODS

Manipulation of *Xenopus* tadpoles

Regeneration experiments were performed on *Xenopus laevis* tadpoles at stages 41/42 or 48/50 as described (Beck et al., 2003). Freshly amputated tadpoles were denominated as 0 days post-amputation (dpa). Regeneration was evaluated at 6-7 dpa as percentage of regeneration (tails regenerated/total number of tails). In order to address the quality of regeneration, 10 points were given to total, 5 to partial and 0 to no tail regeneration (Beck et al., 2003). Then a mean 'score of regeneration' was calculated. Variation in the effect of the different treatments was observed among different tadpole batches. 4-Mu, UDP-Glu, LiCl, BrdU and BIO were added to the tadpole's culture medium. For statistical analysis, percentages of regeneration were analyzed using the χ^2 test, whereas scores of regeneration were analyzed using Kruskal-Wallis test and Dunn's multiple comparison post-test. Statistical analyses were processed in GraphPad Prism 4.

Histology, in situ hybridization and immunofluorescence

For histology, tadpoles at stage 41/42 were fixed in Bouin's solution for 2 days at 4°C, embedded in Paraplast, sectioned and stained with Hematoxylin and Safranin. Whole-mount in situ hybridizations and immunohistochemistry were performed as described. Tails after whole-mount in situ hybridizations were fixed overnight at 4°C in Bouin's solution, embedded in Epon and sectioned in ultramicrotome at 3 μm.

For immunofluorescence against pH3 tails were fixed overnight at 4°C in Bouin's solution, dehydrated and embedded in paraffin and 10 μm sections were cut. Sections were permeabilized for 10 minutes in PBS containing 0.1% Triton X-100 and blocked for 2 hours in PBS containing 2 mg/ml BSA, 0.1% Triton X-100 and 10% goat serum. To measure BrdU incorporation tails were fixed in MEMFA for 16 hours at 4°C, permeabilized for 30 minutes in PBS with 0.5% Triton X-100 followed by incubation in trypsin 0.25% for 12 minutes on ice, treated with 4 M HCl for 20 minutes, washed in PBS containing 0.5% Triton X-100 and blocked in PBS containing 10% goat serum, 1% DMSO, 0.5% TX-100. First and secondary antibodies were diluted in their respective blocking solutions and incubated overnight at 4°C. Antibodies against BrdU (SIGMA, B 2531) and pH3 (Upstate, #05-598) were used at 1:500 and 1:100, respectively. Secondary antibody conjugated to Alexa Fluor 488 was used at 1:500. DNA was stained with TOTO3 (Molecular Probes #T3604). Samples were analyzed by confocal microscopy (FV-1000 Olympus confocal laser scanning microscope). For the percentage of positive nuclei co-localization pH3 with nuclei staining was counted. Approximately 100-280 total nuclei were considered from each tail (6-9 tails). For proliferation experiments ANOVA and Tukey post-hoc test were used.

Table 1. Primers for RT-PCR

Gene	Forward primer 5′-3′	Reverse primer 5′-3′	Cycles	References
Axin	AAGGCCAGACCTAGACTTGG	CGAACTGTCTAACGCAAGC	32	Hedgepeth et al., 1999
Bra	GCTGGAAGTATGTGAATGGAG	TTAAGTGCTGTAATCTCTTCA	32	Agius et al., 2000
CD44	CAAGCGCGTACAGTTATCAGC	TACAGAGTCTCCCTGGTATCC	32	Ori et al., 2006
Coll2	AGGCTTGGCTGGTCCTCAAGGT	TGTAACGCATAGGGTCGGGTCC	30	Sasai et al., 1996
Delta-1	AAGCCCAGGTACCCTTCTGT	GGCAGAGTCTGGTCGTCTTC	30	Abe et al., 2004
EF1α	CCTGAACCACCCAGGCCAGATTGGTG	GAGGGTAGTCAGAGAAGCTCTCCACG	20-22	Agius et al., 2000
FGF8	CTGCGTCTTCTCGGAAATTGTC	GTTTTTATGAAGTCTGTGGAACG	32	Lin and Slack, 2008
Has1	CATGAAGCCATTCTCTTCTC	ACACGTCTTTGAACATCTCC	30	Unpublished
Has2	GTAACGCAGATGGTTCTCTC	GTTAAACGCCATCCAGTATC	28-30	Unpublished
Has3	GGGACAAAAATTACCATGAA	CAGAAATGAGATCCACGATT	35	Unpublished
Hyal2	TGCAGAGGACATTGCTGTTC	TAAGAGGTGATGGGGTCAGC	32	Vigetti et al., 2003
Msx1	GCTAAAAATGGCTGCTAA	AGGTGGGCTGTGTAAAGT	30	Unpublished*
Msx2	CACCTTCATTTAGGGATCAG	GTTTTGTGCTTCCTCAAAGT	30	Unpublished
RHAMM	CAAACAAGTGGCGCACCC	AGTCATTGCCCAGTCAGC	32	Groen et al., 2004
Shh	CTTCGCTCGGACGAGATGCTGG	CCTTCGTATCTGCCGCTGGCC	30	Sigiura et al., 2004
Syn-1	TACATCTGCGTTGCCTTCTG	TGGATTTCACAGTGGTTGGA	30	Olivares et al., 2009
Wnt5a	AAGGACTCTCCCATCTTGTT	AGAAAAGGCACTTCACACAG	30	Sigiura et al., 2004

*http://www.hhmi.ucla.edu/derobertis/.

Hyaluronan detection

Experiments were performed as described (Toole et al., 2001). Briefly, tadpoles were fixed in PFA 4%, embedded in paraffin, sectioned at 10 μm and incubated for 1 hour with biotinylated HABP 2 μg/ml (Sigma, H9910) in 10% goat serum. Then samples were washed in PBS and incubated for 1 hour with streptavidin-Alexa Fluor 488 (Molecular Probes, S32354).

Transgene constructs

A fragment of 585 bp of the *Xenopus laevis* Hsp70 promoter was amplified using the following primers: Fw 5′-CCCGTTTAGCAGGAAATAGC-CTTG-3′, Rv 5′-ATTTGCGCTCCTTACAGTTTGCTTTTCG-3′. The Hsp70 promoter was cloned into BS ISceI II KS (Pan et al., 2006) to generate the ISceI-Hsp70 vector. Downstream of the Hsp70 promoter EGFP was cloned to make ISceI-HS-EGFP. HAS2 was excised from pGEM-HAS2 (Nardini et al., 2004) and cloned into ISceI-Hsp70. dnCD44 was amplified from the pGEM-XCD44 (Ori et al., 2006) using the following primers: Fw 5′-GGTATCCTCGAGACAGTTATCAGCTGC-CGC-3′ and Rv 5′-GGTATCTCTAGATCAGACAGACAATATCAGC-CC-3′, subcloned into pCS2-NChdFlag vector and then introduced downstream of the Hsp70 to generate ISceI-Hsp70-dnCD44.

Transgenesis

Transgenesis was performed using the ISceI meganuclease protocol (Pan et al., 2006). Embryos at the one-cell stage were injected with 50 pg of HS-HAS2, HS-CD44 or HS-dnGSK3β construct together with 50 pg of HS-EGFP and ISceI meganuclease. For control, 50 pg of HS-EGFP and 50 pg of an empty vector were used. After injection, embryos were allowed to develop at 12°C until stage 3 and then at 16°C until stage 41/42. For heat shock, tadpoles were incubated in water at 34°C for 30 minutes at the times indicated. Transgenic tadpoles showing expression of EGFP were selected under the fluorescent scope and then amputated to perform regeneration studies.

RT-PCR

Details of primers and cycles for RT-PCR are listed in Table 1.

RESULTS

Early and transient expression of components of the HA pathway during tail regeneration in *Xenopus* tadpoles

The role of HA in tissue injury and repair (Jiang et al., 2007; Noble, 2002) prompted us to evaluate the expression of components of the HA pathway during tail regeneration in *Xenopus laevis* tadpoles. In order to do this, the posterior half of the tail from tadpoles at stage 48/50 and 41/42 were amputated and RNA isolated from the distal tip (regenerative bud) immediately or after different dpa. Levels of mRNA were analyzed by RT-PCR. A transient increase in *HAS2*, *CD44*, *Hyal2* and *RHAMM* was observed at 2-3 dpa in tadpoles amputated at stage 48/50 (Fig. 1A). A similar upregulation was observed in tadpoles amputated at stage 41/42, except that an increase of *HAS2* and *Hyal2* mRNA was already detected at 1 dpa (Fig. 1B). Other members of the HAS family, such as *HAS1* and *HAS3*, were not detected at any time during tail regeneration (see Fig. S1 in the supplementary material). The transient increase in *HAS2*, *Hyal2*, *CD44* and *RHAMM* expression overlaps in time with

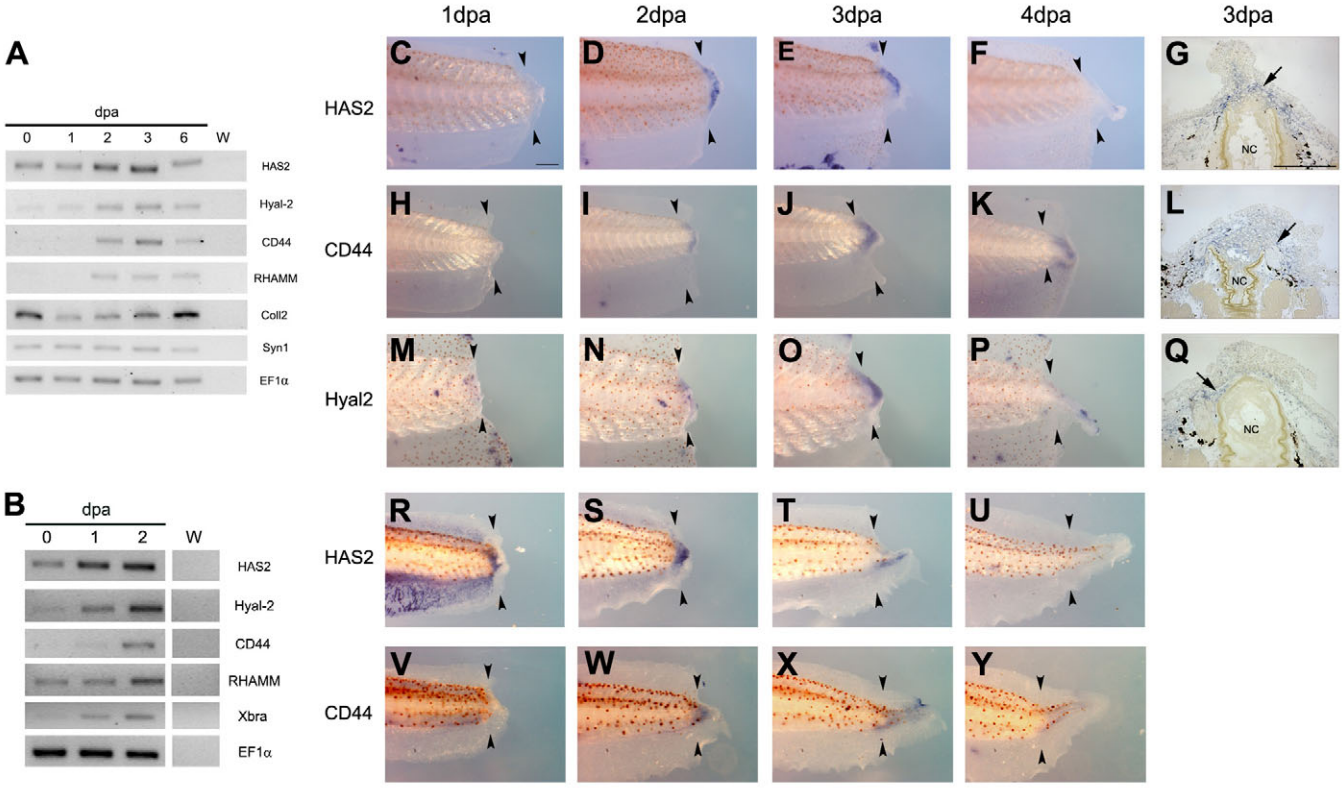

Fig. 1. Transient expression of components of the HA pathway during tail regeneration in *Xenopus* tadpoles. (**A**,**B**) Semi-quantitative RT-PCR analysis of the expression level of HA pathway components during tail regeneration of tadpoles amputated at stage (A) 48/50 and (B) 41/42. The levels of mRNA for *HAS2*, *Hyal-2*, *CD44*, *RHAMM*, *collagen type II* (*Coll II*) and *syndecan1* (*Syn1*) were evaluated using specific primers. *EF1α* was used as loading control. (**C-Y**) In situ hybridization analyses of HA pathway components from tadpoles amputated at stage (C-Q) 48/50 and (R-Y) 41/42. Tails from 1 (C,H,M,R,V), 2 (D,I,N,S,W), 3 (E,J,O,T,X) and 4 (F,K,P,U,Y) dpa were processed for in situ hybridization with the indicated riboprobes. (G,L,Q) Sagittal sections of the most posterior part of regenerating tails at 3 dpa. Arrowheads, amputation plane; arrow, mesenchymal cells; NC, notochord. Scale bar: 200 μm.

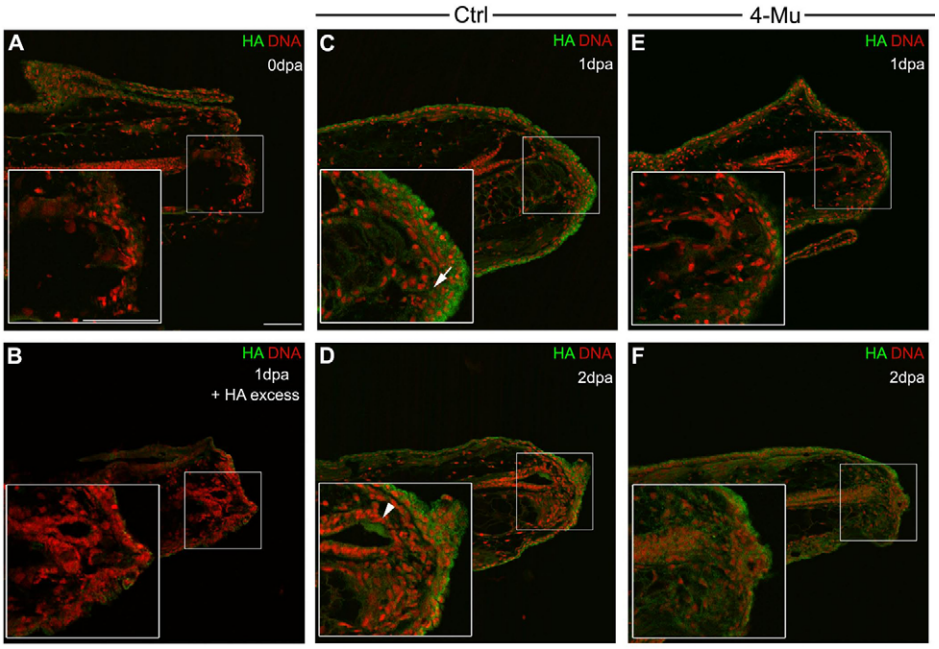

Fig. 2. HA levels increase during the first day of tail regeneration. (**A-D**) Tadpoles at stage 41/42 were amputated and fixed immediately (A), at 1 (B,C,E) or 2 (D,F) dpa. Sections were stained for bHABP (green) and nuclei (red) and analyzed by confocal microscopy. Insets show a higher magnification of the framed area. The arrow and arrowhead point to the presence of HA in the mesenchymal cells and the terminal ampulla, respectively. For experiment in panel B sections were also incubated with an excess of HA to demonstrate specificity of HA staining. (**E**,**F**) Effect of 4-Mu in HA levels. Tadpoles at stage 41/42 were amputated, incubated with 4-Mu 150 μM for 24 h and fixed at 1 (E) and 2 (F) dpa and stained for HA as above. Note the reduction in HA levels at 1 dpa. Scale bars: 50 μm.

formation of the regenerative bud, a period of intense cell proliferation that precedes tissue differentiation. In support of this, the expression of *collagen type II* mRNA, a marker of notochord differentiation, was initially downregulated and started to increase at 6 dpa (Fig. 1A).

To determine the localization of *HAS2*, *CD44* and *Hyal2* mRNAs, in situ hybridizations on tails at different dpa were performed. We found that *HAS2* and *CD44* mRNAs were also transiently upregulated on the distal tip of the regenerating tail at 2 and 3 dpa (Fig. 1C-F,H-K,R-Y). *Hyal2* mRNA was also detected in the distal tip of the regenerating tail but its appearance was delayed until 3 dpa (Fig. 1M-P). Sections showed that the mRNAs for components of the HA pathway were mainly localized in the mesenchymal cells (Fig. 1G,L,Q).

In summary, we have found that mRNA levels for components of the HA pathway are transiently upregulated during the first stages of tail regeneration. The timing of expression and the localization of mRNA for the HA pathway components suggest a role for this pathway in the early phases of tail regeneration. Of note, this transient expression of HA components is in agreement with biochemical studies in newt limb regeneration showing that HA synthesis peaks during blastema formation and is followed by increasing levels of hyaluronidase activity (Mescher and Munaim, 1986; Toole and Gross, 1971).

Increased HA levels after tail amputation

The expression analysis of mRNAs for HA components suggested that probably an increase on HA synthesis/accumulation occurs after tail amputation. In order to directly test this, we evaluated the presence of HA during tail regeneration in *Xenopus* tadpoles amputated at stage 41/42. To measure HA deposition, we stained tadpole sections with a commercially available biotinylated HA-binding protein (bHABP) (Toole et al., 2001). In tails fixed immediately after amputation, almost no signal for HA was observed (Fig. 2A). Importantly, in tails at 1 and 2 dpa, a strong signal for HA was detected mainly in the epidermis (Fig. 2C,D). Lower levels of HA deposition were detected in the sub-epidermal mesenchymal cells and the terminal ampulla (see arrow and arrowhead in Fig. 2C,D). No signal was detected in the absence of bHABP, and the staining was competed with an excess of HA (Fig. 2B; data not shown), indicating that HA detection was specific. In summary, we have found that tail amputation results in an early accumulation of HA in different tissues of the regenerative bud.

Early inhibition of HA synthesis impairs tail regeneration

In order to investigate the role of HA synthesis in tail regeneration we performed functional experiments. The use of specific chemical inhibitors have been of great help in demonstrating the importance of a variety of signaling pathways during tail regeneration in *Xenopus* tadpoles (Beck et al., 2003; Ho and Whitman, 2008; Lin and Slack, 2008). 4-Methylumbelliferone (4-Mu) depletes the intracellular pool of UDP-Glucuronic Acid (UDP-GlcUA), one of the substrates required for HAS enzymatic activity (Kakizaki et al., 2004), resulting in specific inhibition of HA synthesis (Nakamura et al., 1995).

First, we tested the effect of 4-Mu in HA synthesis. For this, tadpoles at stage 41/42 were amputated, incubated with 4-Mu 150 μM during the first 24 hpa, fixed immediately (1 dpa) or 1 day later (2 dpa), and HA was detected as indicated above. We found that at 1 dpa incubation with 4-Mu resulted in a strong inhibition of HA levels (Fig. 2, compare C with E). 4-Mu had no effect on the levels of HA observed at 2 dpa (Fig. 2D,F) suggesting that inhibition of HA synthesis is transient and reversible.

To quantitate tail regeneration, the percentage (Fig. 3A, white bars) and a score of regeneration (Fig. 3A, black bars) was obtained through evaluation of tail external appearance. Addition of 4-Mu during the first 24 hpa resulted in concentration-dependent inhibition of tail regeneration in tadpoles amputated at stage 41/42 (Fig. 3A). The effect of 4-Mu is very reproducible and robust. An average of 30 independent experiments with tadpoles at stage 41/42 showed that only 34.1% of the amputated tadpoles incubated during the first 24 hours with 150 μM 4-Mu were able to regenerate (score=2.77; n=956), a very penetrant effect when compared to wild-type tadpoles (regeneration=88%, score=8.26, n=975). 4-Mu also inhibited tail regeneration, although less

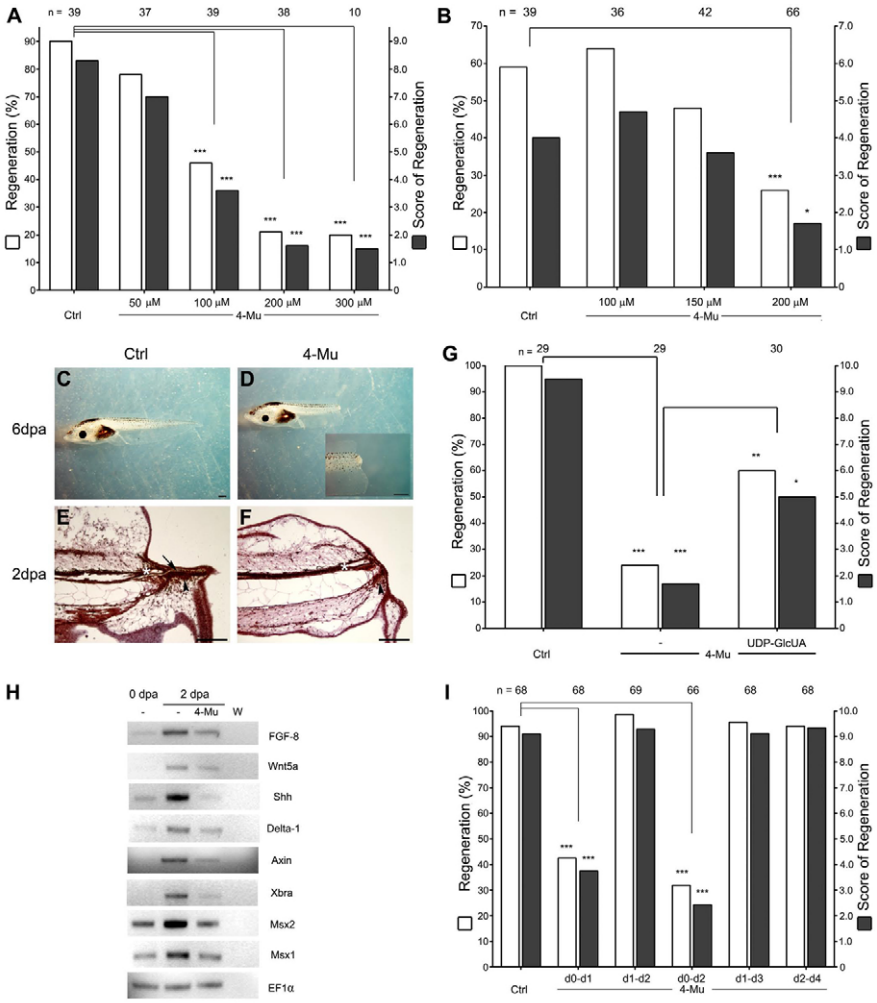

Fig. 3. 4-Methylumbelliferone blocks tail regeneration in a specific and time-dependent manner. (**A**,**B**) Tadpoles at stage 41/42 (A) or 49/50 (B) were amputated and incubated during the first 24 or 48 hpa with 4-Mu, respectively. After removal of the chemical tails were allowed to regenerate and regeneration assessed by tail external appearance under the stereoscope. Regeneration percentage (white bars) and score of regeneration (black bars) are depicted. (**C-F**) Morphological appearance of regenerating tadpoles incubated with 4-Mu. (C) Control and (D) 4-Mu treated tadpoles were allowed to regenerate for 6 days and their external appearance was evaluated. (E) Control and (F) 4-Mu treated regenerating tails were fixed at 2 dpa and stained with Hematoxylin/Safranin. The terminal ampulla (asterisk), immature notochord (arrow) and mesenchymal cells (arrowhead) are indicated. (**G**) 4-Mu effect is specific. Amputated tails were incubated during the first day with 200 μM 4-Mu and equimolar amounts of UDP-GlcUA. (**H**) Semi-quantitative RT-PCR analysis of the expression levels of signaling pathway components at 2 dpa. Amputated tadpoles were incubated during the first 24 hours with or without 200 μM 4-Mu. (**I**) Time-dependent effect of 4-Mu. Tails from stage 41/42 tadpoles were amputated, incubated during different time windows with 150 μM 4-Mu and allowed to regenerate up to 6 dpa. The numbers on top of each bar correspond to the number of tadpoles analyzed for each experimental point. Scale bars: 500 μm in C,D; 200 μm in E,F. **P*<0.05; ***P*<0.01; ****P*<0.001.

efficiently in tadpoles amputated at stage 48/50 (Fig. 3B). Importantly, HAS inhibition had no toxic effects in tadpole development (Fig. 3C,D), and the inhibitory effect of 4-Mu could be partially reverted when equimolar amounts of UDP-GlcUA were added (Fig. 3G), indicating that 4-Mu blocks tail regeneration through specific inhibition of HAS activity.

Histological analysis of sagittal sections of the regenerative bud showed that in the presence of 4-Mu wound healing occurred normally and that a terminal ampulla was still formed (Fig. 3E,F, asterisk). Conversely, no immature notochord (Fig. 3E,F, arrow) and reduced amounts of mesenchymal cells (Fig. 3E,F, arrowhead) were observed in 4-Mu treated tadpoles. In addition, we evaluated the effects of HAS inhibition in the activation of the different signaling cascades involved in tail regeneration. Tail amputation resulted in increased mRNA levels for ligands such as *FGF8*, *Wnt5a*, *Shh* and *delta-1*, the BMP and FGF target genes *Msx-1* and *-2*, and the Wnt-target genes *axin* and *Xbra*. This upregulation was reduced when HAS activity was inhibited (Fig. 3H).

A relevant question when studying the role of signaling pathways on complex biological processes, such as tail regeneration, is to establish the temporal requirement of each signaling pathway. To determine when HAS activity is required, we added 4-Mu at different time windows after tail amputation and quantified its effect on tail regeneration at 6 dpa. Addition of 4-Mu during the first 24 or 48 hpa significantly inhibited tail regeneration (Fig. 3I). By contrast, when amputated tadpoles were incubated with 4-Mu between 1 and 2 dpa, no effect on tail regeneration was observed (98% of tails regenerate, 9.3 for the regeneration score). Similarly, no significant effects were observed when the chemical was added between 1 and 3 or 2 and 4 dpa (Fig. 3I). The finding that 4-Mu affected tail regeneration only when added during the first 24 hpa was in agreement with the observation that *HAS2* mRNA levels were already increased in the distal tip of the regenerating tail at 1 dpa in experiments performed in tadpoles at stage 41/42 (Fig. 1B) and that 4-Mu inhibited the accumulation of HA at 1 dpa (Fig. 2C,E).

It is important to note that the effect of 4-Mu on tail regeneration was very specific. No toxic effect on tadpole development was appreciated (Fig. 3C,D), it was displaced by UDP-GlcUA (Fig. 3G), it was time-dependent (Fig. 3I), and as we will show below, it could be rescued by modulation of other signaling pathways (see Fig. 6). In summary, incubation with 4-Mu is a very reliable tool to study the role of HAS activity in tail regeneration. Together, these experiments implicate that proper levels of HA synthesis are required during the early stages of the regenerative process for tail regeneration.

Overexpression of *HAS2* and dominant-negative *CD44* reduces tail regeneration

To give further support to the role of the HA pathway in tail regeneration we studied the effects of overexpressing HAS2 and disrupting HA signaling using a *Xenopus* dominant-negative

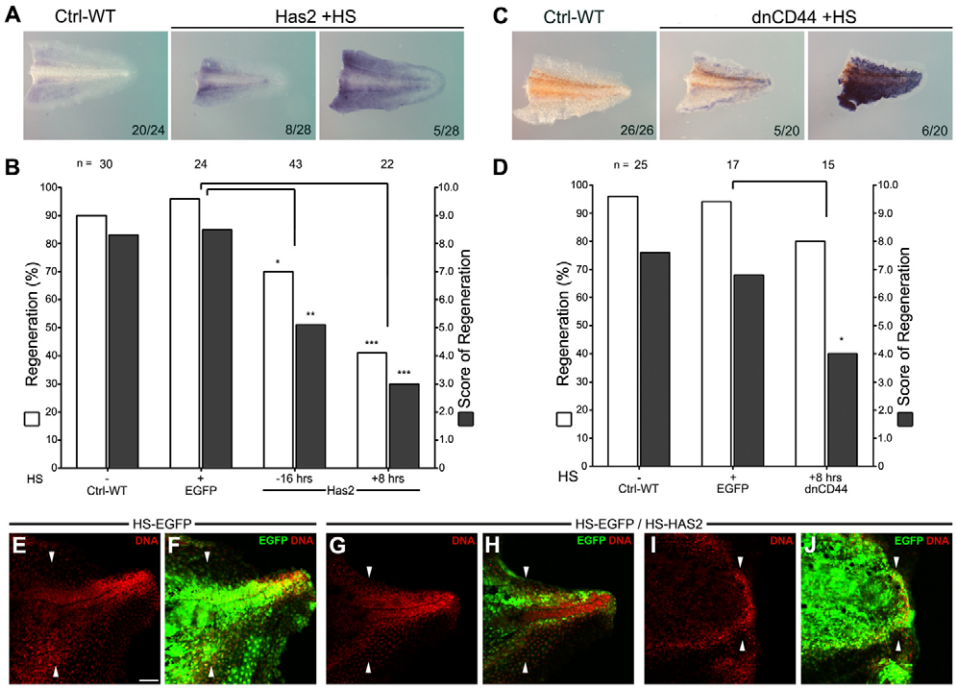

Fig. 4. HAS2 and dnCD44 F0 transgenic tadpoles have reduced levels of tail regeneration. (**A-D**) HAS2 (A,B) and dnCD44 F0 (C,D) transgenic tadpoles were prepared. (A,C) Levels of transgene expression were evaluated by in situ hybridization in EGFP-positive tails from tadpoles amputated and fixed 16 hours after heat shock. (B,D) Efficiency of regeneration was evaluated and compared to EGFP F0 transgenics that were also heat shocked. White bars, percentage of regeneration; black bars, score of regeneration). The numbers on top of each bar correspond to the number of tadpoles analyzed for each experimental point. All EGFP-positive tadpoles were considered for these experiments. The results shown are representative of three independent experiments. **P*<0.05; ***P*<0.01; ****P*<0.001. (**E-J**) HS-EGFP (E,F) or HS-EGFP/HS-HAS2 (G-J) F0 transgenic tadpoles were prepared and heat shocked 16 hours before amputation; EGFP-positive tadpoles were selected, heat shocked again at 8 hpa and fixed at 2 dpa. Nuclear DNA was stained with TOTO3 (red) and analyzed by confocal microscopy. Panels E,G and I show the red channel (nuclei) and panels F, H, J merge image (green: EGFP). Scale bar: 50 μm.

CD44 (dnCD44) construct (Jiang et al., 2007; Peterson et al., 2004). To overexpress HAS2 and dnCD44, we used the *I-SceI* meganuclease (Ogino et al., 2006; Pan et al., 2006). To allow temporal control of transgene expression, heat-shock (HS) inducible vectors were constructed. To select for positive F0 transgenic tadpoles, one-cell embryos were co-injected with a mixture of HS-HAS2 or HS-dnCD44 together with HS-EGFP, raised until stage 41/42 and heat shocked, and after 16 hours EGFP-positive tadpoles were selected. In situ hybridization showed detectable levels of HAS2 and CD44 in only half of the selected EGFP-positive tadpoles (Fig. 4A,C). The fact that not all the EGFP-positive tadpoles showed increased levels of HAS2 or dnCD44 expression could be explained because this method is known to produce mosaic transgenics (Pan et al., 2006). Mosaicism was directly demonstrated when a more detailed observation of EGFP localization was performed (Fig. 4H,J).

To analyze the effect of HAS2 overexpression in regeneration, one-cell embryos were injected with a mixture of HS-HAS2 and HS-EGFP, at stage 42 tadpoles were heat shocked 16 hours before or 8 hours after amputation, EGFP-positive tadpoles were selected and the efficiency of regeneration was determined. Significantly reduced levels of regeneration were observed in comparison to heat-shocked EGFP transgenic tadpoles (Fig. 4B). The strongest effect was obtained when tadpoles were heat shocked at 8 hours post-amputation (regeneration=40% and regenerative score=3.0), an effect similar to those obtained with 4-Mu treatment (Fig. 3A). The fact that HAS2 overexpression was not completely penetrant could be explained because not all EGFP-positive tadpoles had detectable levels of HAS2 (Fig. 4A) and because of the mosaic expression of the transgene (Fig. 4G,H; note that tail regeneration was blocked only when EGFP was expressed in all the tailbud tissues). Importantly, in F0 HS-EGFP transgenic tadpoles regeneration proceeded normally, even when EGFP was expressed in all the tissues of the regenerative bud (Fig. 4B,E,F). This experiment indicates that overexpression of HAS2 blocks tail regeneration very efficiently, and the fact that just one single heat shock applied before or soon after amputation is sufficient to disrupt tail regeneration provides genetic evidence that HAS2 is required during the early steps of regeneration.

Similarly, dnCD44 F0 tadpoles heat shocked 8 hours after amputation showed a significant reduction in the score of regeneration, but no effect was seen on the percentage of regeneration when compared to EGFP transgenic animals (Fig. 4D). dnCD44 overexpression resulted in a less penetrant effect compared with HAS2 overexpression (Fig. 4, compare B with D). This lower penetrance could be explained because of the cell-autonomous effect of dnCD44, the possibility that dnCD44 has no effect on all the multiple alternative forms of CD44 (Jiang et al., 2007; Turley et al., 2002) and the likely role of other HA-signaling receptors, such as RHAMM, which is also expressed during tail regeneration (Fig. 1A,B).

In summary, the upregulation of *HAS2* and *CD44* mRNA during the first days after amputation (Fig. 1), the ability of 4-Mu to block HA synthesis and tail regeneration when added during

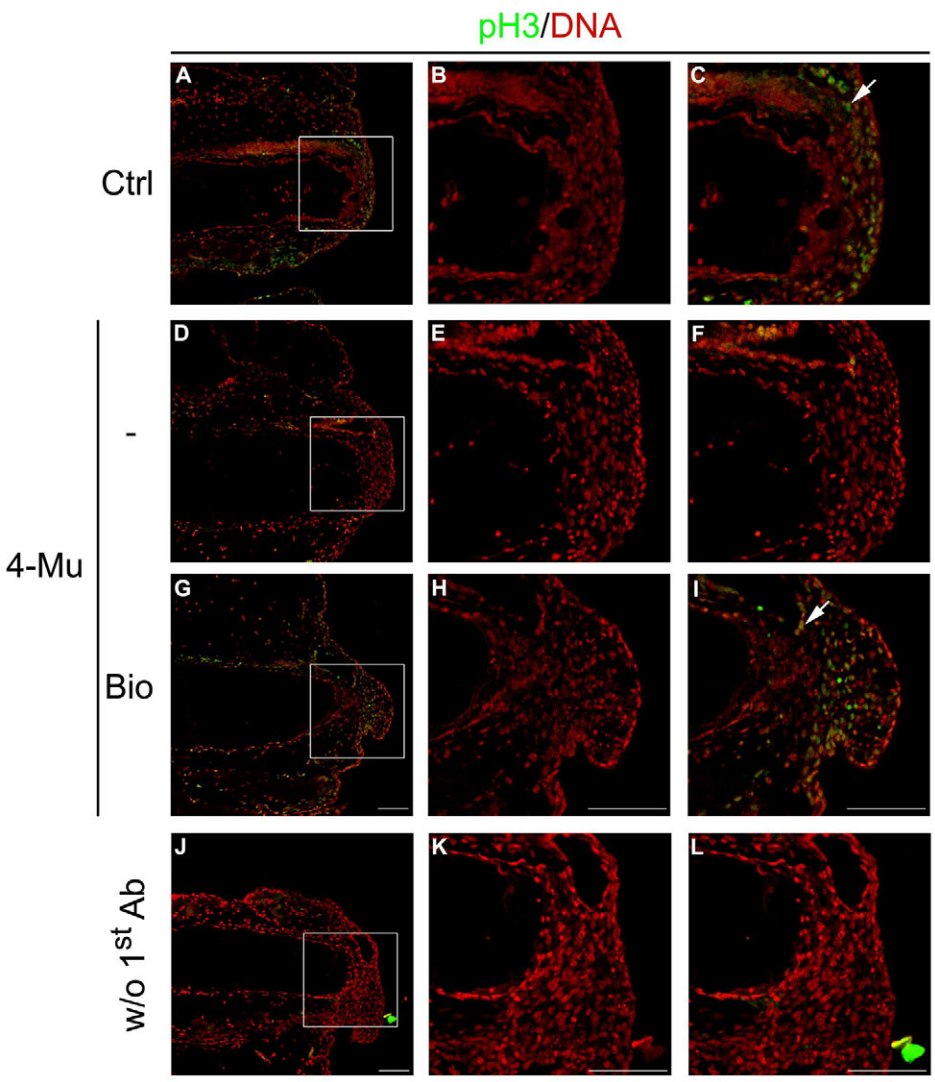

Fig. 5. HA synthesis is required for cell proliferation during tail regeneration. (**A-I**) Tadpoles at stage 48/50 were amputated and received no treatment (A-C) or were incubated during the first 24 hours with 150 μM 4-Mu (D-F) or 4-Mu during 24 hpa and 200 nM BIO during 48 hpa (G-I). (**J-L**) Tails were developed by immunohistochemistry in the absence of anti-pH3 antibody. Panels B, E, H and K show only the red channel and C, F I and L the merge of green and red channels from magnifications of the areas framed in panels A, D, G and J. Arrows indicate pH3 staining in cells lining the spinal cord ampulla. Scale bar: 50 μm.

the first 24 hours (Fig. 2C,E; Fig. 3I) and the inhibitory effect of early overexpression of HAS2 or dnCD44 (Fig. 4) indicate that, for successful tail regeneration to occur, correct levels of HA synthesis and CD44 are required during the early phases of regeneration.

Role of HA in cell proliferation during tail regeneration

To understand the mechanisms triggered by HA to regulate tail regeneration, we evaluated the effect of early inhibition of HA synthesis on cell proliferation. To estimate cell proliferation, the levels of phospho-Histone 3 (pH3) and BrdU incorporation were measured. pH3 is an excellent marker for cell-cycle activation; first it can be detected in pericentromeric heterochromatin at interphase even before the appearance of an obvious prophase chromosomal organization (approximately 10% of interphase nuclei are stained in different cell lines), then spreads throughout the condensing chromatin and labels chromosomes just before the formation of the prophase chromosomes until the beginning of telophase (Hendzel et al., 1997). Regenerating tails from tadpoles amputated at stage 48/50 were incubated with 4-Mu, fixed at 2 dpa, sectioned and stained with anti-pH3. We found that 12% (n=2141) of the nuclei in the regenerative bud showed a positive signal for pH3, including staining that probably corresponded to different cell-cycle phases such as late G2, prophase and metaphase (Fig. 5A-C; see Fig. S2 in the supplementary material). No signal was detected in the absence of the primary antibody, indicating that the staining is specific (Fig. 5J-L). After treatment of amputated tails with 4-Mu during the first 24 hpa, only 5.4% (n=1552) of pH3-positive nuclei were observed (Fig. 5D-F). Of note, pH3 staining was also detected in cells lining the spinal cord ampulla (Fig. 5C, arrow), a signal that was inhibited by 4-Mu (Fig. 5F). Furthermore, BrdU incorporation in the regenerative bud was strongly inhibited when amputated tadpoles were incubated with 4-Mu (see Fig. S3 in the supplementary material). We concluded from these experiments that HA synthesis is required during the early phases of regeneration for proliferation of mesenchymal cells on the regenerative bud.

Inhibition of GSK3β is sufficient to restore cell proliferation and tail regeneration when HA synthesis is blocked

Wnt/β-catenin signaling is essential for regeneration across different species including zebrafish, *Xenopus*, axolotl and planarian (Gurley et al., 2008; Kawakami et al., 2006; Lin and Slack, 2008; Yokoyama et al., 2007) and modulates stem-cell proliferation (Reya and Clevers, 2005). This, together with our observation that 4-Mu blocks

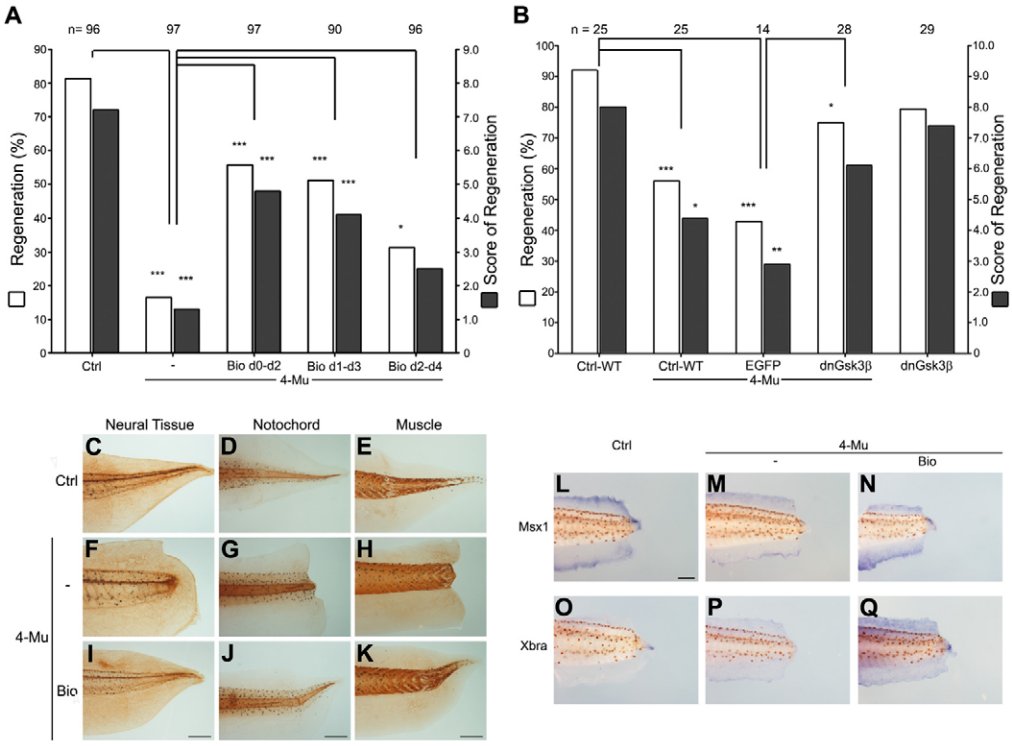

Fig. 6. Inhibition of GSK3β is sufficient to rescue tail regeneration in the presence of 4-Mu. (**A**) BIO could rescue tail regeneration when HAS activity was inhibited. White bars, percentage of regeneration; black bars, score of regeneration. The numbers on top of each bar correspond to the number of tadpoles analyzed for each experimental point. The results shown are the average of three experiments in different frog batches. (**B**) HS-dnGSK3β and HS-EGFP F0 transgenic tadpoles were prepared. Tadpoles were heat shocked 16 hours before amputation and 8 and 24 hpa. When indicated tadpoles were incubated with 100 μM 4-Mu during the first 24 hpa and then tails allowed to regenerate for 6 days and the efficiency of regeneration quantitated. **P*<0.05; ***P*<0.01; ****P*<0.001. (**C-K**) Immunohistochemical analysis of regenerated tails. Stage 41/42 tadpoles were amputated and received no treatment (C-E) or incubation with (F-H) 4-Mu during the first 24 hpa or (I-K) 4-Mu during the first 24 hpa and BIO during the first 2 dpa. Tails were allowed to regenerate, fixed at 6 dpa and developed by immunohistochemistry using antibodies against spinal cord (acetylated tubulin) (C,F,I), notochord (MZ15) (D,G,J) and muscle (E,H,K) (12/101). Five to eleven tails were analyzed for each experimental point. Scale bars: 200 μm. (**L-Q**) Rescue of molecular markers with BIO. Stage 41/42 tadpoles were amputated and received no treatment (L,O) or were incubated with 4-Mu (M,P) or 4-Mu and BIO (N,Q). Tails regenerate until 2 dpa and Msx1 (L-N) or Xbra (O-Q) were detected by in situ hybridization. Scale bars: 500 μm.

induction of the Wnt downstream effectors *Axin*, *Xbra* and *FGF8* (Fig. 3H), prompted us to study the relationship between the HA and Wnt pathways.

For this, we evaluated the effect of Wnt pathway activation in tail regeneration when HA synthesis was inhibited. For Wnt activation, GSK3β was inhibited using BIO and LiCl, two molecularly unrelated chemical inhibitors. BIO restored tail regeneration in the presence of 4-Mu when added between 0 and 2 dpa or 1 and 3 dpa, but when added from 2 to 4 dpa, regeneration was less efficient (Fig. 6A). Similar results were obtained with LiCl treatment (see Fig. S4 in the supplementary material). Immunohistochemical analysis using notochord, muscle and spinal-cord specific markers show that tails rescued by BIO in the presence of 4-Mu contained a complete set of axial structures (Fig. 6C-K).

To probe the specificity of the pharmacological inhibition of GSK3β we prepared F0 transgenic tadpoles by injection of a dominant-negative GSK3β construct (dnGSK3β) that was under the control of the heat-shock promoter. Incubation with 4-Mu of wild-type and heat-shocked EGFP transgenic tadpoles resulted in significant inhibition of tail regeneration, but heat-shocked transgenic dnGSK3β tadpoles were able to partially rescue tail regeneration (Fig. 6B, compare dnGSK3β/4-Mu with EGFP/4-Mu). In addition, 4-Mu was not able to inhibit tail regeneration in dnGSK3β transgenic (Fig. 6B, compare dnGSK3β with dnGSK3β/4-Mu). These results indicate that pharmacological and genetic inhibition of GSK3β is sufficient to restore complete tail regeneration when HA synthesis is blocked.

Furthermore, we tested if GSK3β inhibition was sufficient to restore cell proliferation when early HA synthesis was inhibited. For this, tadpoles were amputated, incubated with 4-Mu and BIO during the first 24 and 48 hpa, respectively, and tails were fixed and stained for pH3 as above. Interestingly, BIO was able to restore proliferation in the regenerating bud (Fig. 5G-I), even to levels higher to those observed in control tails (21% pH3-positive cells, *n*=951). To evaluate the restoration of molecular marker expression, in situ hybridization for *Msx1* and *Xbra* were performed. We found that at 2 dpa most of the control regenerating tails expressed *Msx1* (70%, *n*=10) and *Xbra* (80%, *n*=10) on the tip of the tail (Fig. 6L,O). This expression was efficiently blocked when the tails were incubated during the first 24 hours with 4-Mu (*Msx1*, 0%, *n*=10; *Xbra*, 25%, *n*=8) (Fig. 6M,P). Importantly, incubation with BIO during the first 48 hours was able to partially bring back *Msx1* (60%, *n*=10) and *Xbra* (60%, *n*=10) expression (Fig. 6N,Q). In conclusion, GSK3β inhibition is able to restore cell proliferation, molecular markers and tail regeneration when early HA synthesis is blocked.

DISCUSSION

This work contributes to the understanding of the molecular and cellular mechanism involved in tail regeneration. We have demonstrated for the first time that HA synthesis and components of the HA pathway are required for proper tail regeneration through a mechanism that modulates proliferation of mesenchymal cells in the regenerative bud. Our findings are based on the following observations: (1) components of the HA pathway, including the mRNAs for *HAS2*, *Hyal2*, *CD44* and *RHAMM*, are transiently upregulated in the mesenchymal cells of the regenerative bud during the early phases of tail regeneration; (2) peak accumulation of HA in the epidermis and at lower levels in the mesenchymal cells is detected at 1 dpa; (3) early inhibition of HA synthesis and overexpression of *HAS2* and dnCD44 mRNAs block tail regeneration; (4) HA synthesis is required for proliferation of mesenchymal cells in the regenerating bud; (5) inhibition of GSK3β is sufficient to rescue cell proliferation and tail regeneration when HA synthesis is blocked, suggesting that GSK3β acts downstream or in parallel to the HA pathway.

It has been proposed that regeneration takes place in part through recapitulation of development (Stocum, 2004). Different signaling pathways required for tail development are activated during tail regeneration, among others BMP, Wnt, FGF and Notch signaling (Beck et al., 2003; Beck et al., 2006; Lin and Slack, 2008). Even though its function in tail development has not been addressed, the fact that *HAS2* is expressed at the tip of the extending tail at stage 30 (Koprunner et al., 2000) suggests that *HAS2* could be another example of a gene involved in tail development that is reactivated during regeneration.

At a first glance, our experiments to determine the function of HAS seem to be in opposition, as both inhibition of HAS activity (Fig. 3) and overexpression of HAS2 (Fig. 4A,B) block tail regeneration. Interestingly, high molecular weight or n-HA and small o-HA, generated by the action of hyaluronidases, have opposite activities in cell signaling. The ability of o-HA to induce early gene expression in endothelial cells leading to angiogenesis is inhibited by n-HA through competition for its cognate signaling receptor CD44 (Deed et al., 1997). Furthermore, binding of n-HA results in rapid downregulation of cell-surface CD44, decreasing signaling activation by o-HA (Deed et al., 1997). Among the HAS family, HAS2 generates HA with extremely large size (Jiang et al., 2007), something that has been confirmed for *Xenopus* HAS2 (Koprunner et al., 2000). From our results we propose a model whereby o-HA is produced by the coordinated action of HAS2 and Hyal2 during the early phases of regeneration, resulting in activation of cell proliferation and intracellular signaling through CD44 and RHAMM receptors. Addition of 4-Mu blocks synthesis of n-HA with a consequent decrease in o-HA production and signaling. Conversely, HAS2 overexpression could result in biosynthesis of high levels of n-HA that probably are not efficiently degraded to o-HA by endogenous hyaluronidases. Excessive accumulation of n-HA could compete with o-HA for the binding to its cognate receptor CD44 and/or RHAMM, resulting in a reduction in HA signaling and the consequent failure in tail regeneration.

Based on its time of expression, temporal requirement and epistatic experiments, we could place the HA pathway and HA synthesis in relation to other pathways involved in tail regeneration. Activation of TGF-β signaling measured by pSmad2 levels occurs during the first hpa (Ho and Whitman, 2008), probably before increasing levels of *HAS2* mRNA can be detected. Interestingly, members of the TGF-β family rapidly induce expression of *HAS2* mRNA in *Xenopus* animal caps (Ori et al., 2006) and mouse skin cells (Sugiyama et al., 1998). Altogether, these observations suggest that activation of HAS2 expression and perhaps other components of the HA pathway are downstream the initial burst of Smad2 signaling. Epistatic experiments using pharmacological inhibitors of GSK3β rescued cell proliferation, molecular markers and tail regeneration when HA accumulation was blocked, suggesting that HA acts either upstream or in parallel to GSK3β. The ability of HA and CD44 to activate nuclear translocation of β-catenin and TOP-flash reporter (Bourguignon et al., 2007) supports regulation of Wnt signaling as a plausible mechanism of action for HA during tail regeneration. Considering that GSK3β impinges on different signaling pathways, it is not possible to conclude that HA functions exclusively through Wnt signaling during tail regeneration. The interaction between HA and Wnt/GSK3β is particularly intriguing in view of their roles in stem-cell biology. A key role for both pathways in proliferation and cell renewal of hematopoietic (Khaldoyanidi et al., 1999; Nilsson et al., 2003; Reya et al., 2003; Trowbridge et al., 2006) and neural (Lie et al., 2003; Sherman and Feistel, 2008) stem cells has been described. The crosstalk between these two pathways in proliferation of mesenchymal cells in the regenerative bud could be a mechanism to regulate the amount of these cells required for tail regeneration.

A role for HA signaling in tissue regeneration seems to be part of a conserved response to tissue injury. Upregulated expression of HA pathway components (*HAS2*, *Hyal2*, *CD44* and *RHAMM*) followed by accumulation of HA (n-HA and o-HA) has been observed in response to a variety of tissue injuries, including radiation-induced lung injury, epidermal trauma caused by tape stripping and ischemic stroke (Al'Qteishat et al., 2006; Jiang et al., 2007; Slevin et al., 2007). Our findings are the first demonstration that HA synthesis is required for regeneration of a complex structure containing a spinal cord, muscle, vasculature and cartilage. In addition, our results on the relationship between HA and GSK3β pathways suggest that a crosstalk between these pathways occurs during regeneration. We envision that manipulation of the HA pathway should lead to a better understanding of how to control tissue and appendage regeneration.

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Competing interests statement

The authors declare that they have no competing interests.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/17/2987/DC1

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