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Retinoic acid signaling controls the formation, proliferation and survival of the blastema during adult zebrafish fin regeneration

Nicola Blum and Gerrit Begemann*

SUMMARY

Adult teleosts rebuild amputated fins through a proliferation-dependent process called epimorphic regeneration, in which a blastema of cycling progenitor cells replaces the lost fin tissue. The genetic networks that control formation of blastema cells from formerly quiescent stump tissue and subsequent blastema function are still poorly understood. Here, we investigated the cellular and molecular consequences of genetically interfering with retinoic acid (RA) signaling for the formation of the zebrafish blastema. We show that RA signaling is upregulated within the first few hours after fin amputation in the stump mesenchyme, where it controls Fgf, Wnt/β-catenin and Igf signaling. Genetic inhibition of the RA pathway at this stage blocks blastema formation by inhibiting cell cycle entry of stump cells and impairs the formation of the basal epidermal layer, a signaling center in the wound epidermis. In the established blastema, RA signaling remains active to ensure the survival of the highly proliferative blastemal population by controlling expression of the anti-apoptotic factor *bcl2.* In addition, RA signaling maintains blastema proliferation through the activation of growth-stimulatory signals mediated by Fgf and Wnt/β-catenin signaling, as well as by reducing signaling through the growth-inhibitory non-canonical Wnt pathway. The endogenous roles of RA in adult vertebrate appendage regeneration are uncovered here for the first time. They provide a mechanistic framework to understand previous observations in salamanders that link endogenous sources of RA to the regeneration process itself and support the hypothesis that the RA signaling pathway is an essential component of vertebrate tissue regeneration.

KEY WORDS: Proliferation, Caudal fin, Cell death, Gene network, *aldh1a2* (*raldh2*), Zebrafish

INTRODUCTION

Fish and amphibians have the ability to regenerate appendages that are lost or injured. Following amputation, the lost appendage regrows through a proliferation-dependent process known as epimorphic regeneration that involves three successive stages: wound healing, blastema formation, and regenerative outgrowth and repatterning. Despite great progress in recent years (Brockes and Kumar, 2008; Tanaka and Reddien, 2011), the underlying molecular mechanisms are still insufficiently understood. The retinoic acid (RA) signaling pathway (Theodosiou et al., 2010) has a long history in the study of vertebrate appendage regeneration. Treatment of regenerating amphibian limbs with excess RA causes patterning defects and a respecification of positional information (Maden and Hind, 2003; Maden, 1982; Maden, 1983; Niazi and Saxena, 1978). A role for RA has therefore been invoked in proximal-distal patterning. However, reliable loss-of-function experiments to verify the endogenous role(s) of RA in limb regeneration have never been performed.

Owing to the advantages offered by genetic screens, transgenesis and chemical genetics, fin regeneration in adult zebrafish has received exceptional attention (Iovine, 2007). The adult caudal fin consists of bony fin rays that are connected to each other by soft interray tissue (Akimenko et al., 2003; Becerra et al., 1983). Each fin ray is composed of two facing, concave hemirays that surround a core of fibroblasts, osteoblasts, pigment cells, nerves and blood vessels. The RA receptor *rarga* has been found to be strongly expressed in the adult fin blastema (White et al., 1994) and gene expression profiles of regenerating larval and adult fins have identified *aldh1a2* (*raldh2*), which encodes the major enzyme for embryonic RA synthesis, as highly expressed (Mathew et al., 2009). In zebrafish larvae, repair of the caudal fin fold after amputation has been shown to depend on RA signaling (Mathew et al., 2009). However, larval fin folds are different from adult fins in many respects and it is unclear whether the signaling mechanisms driving larval regeneration apply to the adult blastema. Taken together, despite more than three decades of research into the effects of exogenous RA on regenerating amphibian limbs, evidence for a functional involvement of RA signaling in regenerating appendages of adult vertebrates is still missing. In this study, we demonstrate that RA signaling is essential for adult fin regeneration and provide mechanistic insights into a function for RA signaling in wound epidermis formation and in the generation and maintenance of the blastema.

MATERIALS AND METHODS

Zebrafish husbandry and fin amputation

Zebrafish strains of Konstanz wild types and the transgenic lines [*Tg(hsp70I:dn-fgfr1)*]pd1 (Lee et al., 2005), [*Tg(hsp70l:dn-zrar-egfp)*]pd18 (Kikuchi et al., 2011) and [*Tg(hsp70I:cyp26a1)*]kn1 were reared and staged at 28.5°C according to Kimmel et al. (Kimmel et al., 1995). Transgenic strains were analyzed as heterozygotes; wild-type siblings served as controls. Fish that were 3-14 months old were used for regeneration experiments. Caudal fins were amputated along the dorsoventral axis, intersecting the median rays approximately halfway. Fish were allowed to regenerate for various times at 27-28°C.

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DEVELOPMENT

Construction of *hsp70l:cyp26a1*

To construct the *hsp70l:cyp26a1* transgene (Kikuchi et al., 2011), *egfp* from p*hsp70l:egfp* (Halloran et al., 2000) was replaced by zebrafish *cyp26a1* (NM_131146) and the entire cassette was inserted into the I-SceI backbone vector (Thermes et al., 2002). Plasmid DNA was injected together with I-SceI meganuclease (NEB) into one-cell stage embryos to create germline transgenic founder fish.

Heat-shock experiments

Embryos were heat shocked at 38°C for 1 hour. Heat shock of adult fish was performed once daily by transferring fish from 27-28°C water to 33-34°C water for 30 minutes and subsequently to 38°C water for 1 hour.

BrdU and RA treatments

For bromodeoxyuridine (BrdU, Sigma) and all-trans RA (Sigma) treatments, fish were injected intraperitoneally with 30 μl solution. BrdU, at 2.5 mg/ml in PBS, was injected 6 hours (during blastema formation) or 30 minutes (during regenerative outgrowth) prior to fixation. RA was injected at 1 mM in 1% DMSO/PBS. Control fish were injected with an equivalent concentration of DMSO/PBS. Fins of BrdU-treated fish were fixed in 4% PFA in PBS, washed in 0.3% Triton X-100 in PBS (PBTx) and DNA was denatured with 2M HCl for 20 minutes at 37°C. Fins were washed, then incubated with mouse anti-BrdU antibody (1:50, Sigma) and subsequently with goat anti-mouse Alexa Fluor 568 antibody (1:800, Molecular Probes). Cryosections were counterstained with DAPI.

Cryosectioning

For cryosectioning, fins were embedded in 1.5% agar/5% sucrose in PBS. Embedded fins were saturated in 30% sucrose and subsequently frozen in Tissue-Tek O.C.T. Compound (Sakura) in liquid nitrogen. Longitudinal sections were cut at 18 μm.

In situ hybridization

Digoxigenin-labeled RNA antisense probes were synthesized from cDNA templates: *egr2a* (*krox20*) (Oxtoby and Jowett, 1993), *myoD* (*myod1* – Zebrafish Information Network) (Weinberg et al., 1996), *aldh1a2* (Grandel et al., 2002), *rarga* (Joore et al., 1994), *rdh10a* (ImaGenes, IRAKp961E15293Q) and *rdh10b* (ImaGenes, IRBOp991E024D). In situ hybridization of whole fins and embryos was performed as previously described (Poss et al., 2000a) with minor modifications. For in situ hybridization on cryosections, proteinase K treatment was replaced by permeabilization in PBTx for 30 minutes prior to prehybridization. Stained whole-mounts and sections were cleared in ethanol. Whole-mounts were transferred into 75% glycerol in PBS for documentation.

TUNEL staining

For TUNEL staining on cryosections, fins were fixed and processed as for in situ hybridization on sections. Sections were permeabilized in PBTx and equilibrated with terminal deoxynucleotidyl transferase (TdT) buffer [200 mM potassium cacodylate, 25 mM Tris, 0.05% (v/v) Triton X-100, 1 mM $CoCl_2$, pH 7.2]. The buffer was subsequently replaced with TdT buffer containing 0.5 μM fluorescein-12-dUTP, 40 μM dTTP and 0.02 units/μl TdT (all Fermentas). Slides were incubated at 37°C for 3 hours and washed in PBT. Sections were blocked in 0.5% Blocking Reagent (Roche) in PBT and incubated with sheep anti-fluorescein-AP antibody (1:2000, Roche). The staining reaction was carried out as for in situ hybridization.

Analysis of cell proliferation, cell death and regenerative growth

For quantification of BrdU-labeled and TUNEL-labeled cells, two to six representative sections per fin from 5-11 fish per group were used. Labeled cells were counted within 100 μm proximal to the amputation plane in the epidermis and inside an area of 50 × 100 μm in the mesenchyme at 32 hours post-amputation (hpa). For quantification at 3 and 4 days post-amputation (dpa), cells were counted distal to the amputation plane and calculated per 500 μm regenerate length. To determine growth in regenerating fins of heat-shocked RA-injected *hsp70:dn-fgr1* fish, fins were photographed at 70 hpa (before the first heat shock and first RA injection) and 5 dpa. The length of the regenerate (from the amputation plane to the distal tip) was measured using AxioVision software (Carl Zeiss). Growth between the two time points was calculated for each fish (*n*=10-12 fish per group). Statistical significance was calculated using Student's *t*-test.

Hematoxylin staining

Fins were fixed in 4% PFA in PBS, transferred to methanol and stored at –20°C. Fins were rehydrated prior to cryosectioning. Sections were stained in Mayer's Hematoxylin Solution (Sigma) for 3-5 minutes, washed in water and cleared in 0.37% HCl in 70% ethanol for 5-10 seconds.

Quantitative real-time PCR

For RNA extraction at 0, 6 and 10 hpa, tissue within 1 mm proximal to the amputation plane was harvested. At 73 hpa, tissue distal to the amputation plane was harvested. Each sample was prepared from 4-11 fins. Total RNA was extracted with Trizol reagent (Invitrogen) and treated with DNase I (Fermentas). Equal amounts of total RNA from each sample were reverse transcribed with SuperScript III reverse transcriptase (Invitrogen) using oligo(dT) primers. For all samples, reverse transcriptase-negative controls were included to verify the purity of the samples. Quantitative real-time PCR (qPCR) was performed using a C1000 thermal cycler combined with a CFX96 real-time PCR detection system (Bio-Rad) and Maxima SYBR Green qPCR Master Mix (Fermentas). Primers are listed in supplementary material Table S1. qPCR reactions for each sample and each target gene were performed in triplicate. Three samples were used for each gene at 0, 6 and 10 hpa; two to three samples were used for each gene at 73 hpa. qPCR data were analyzed using CFX Manager software (Bio-Rad). Expression levels at 0 and 6 hpa in wild-type fins were normalized to *ef1a* levels (normalization to *gapdh* levels produced very similar results). Expression levels at 73 hpa were normalized to *actb1* levels (normalization to *ef1a* levels produced very similar results). Expression levels at 0 and 10 hpa under altered RA levels were normalized to the input RNA amount by performing a RiboGreen assay (Invitrogen) for exact RNA quantification. This technique was used because normalization to different reference genes gave conflicting results. Statistical significance was calculated using Student's *t*-test.

RESULTS

Blastema formation requires upregulation of RA synthesis

A previous microarray analysis has shown that expression of the RA-synthesizing enzyme *aldh1a2* is upregulated in regenerating caudal fins of adult zebrafish at 24 hours post-amputation (hpa) (Mathew et al., 2009). In order to better understand the spatial and temporal expression of *aldh1a2* and other RA pathway components during blastema formation we performed gene expression studies and found that *aldh1a2* expression is upregulated within 6 hpa (Fig. 1A). *aldh1a2* transcripts were detected within approximately one segment length proximal to the amputation plane in the ray and interray mesenchyme (Fig. 1B,C; data not shown), whereas the most distal mesenchyme is initially (at 18 hpa) devoid of *aldh1a2.* RA synthesis through *aldh1a2* requires a reliable source of retinaldehyde. Accordingly, we found that expression of *retinol dehydrogenase 10b* (*rdh10b*) is induced after amputation (Fig. 1A). *rarga* has been shown to be expressed in the mature blastema (White et al., 1994). We investigated *rarga* expression in the fin stump and detected 1.7-fold higher expression of *rarga* at 6 hpa as compared with 0 hpa (Fig. 1A). Together, our expression analyses shows that fin amputation induces upregulation of essential components of the RA pathway.

During blastema formation, which occurs at ~12-48 hpa, cells of the ray mesenchyme within one to two segment lengths proximal to the amputation plane start to proliferate and migrate distally to form the blastema. Expression of *aldh1a2* in this region suggests that blastema formation requires high levels of RA at the local origin of blastema cells. To investigate the consequences of impaired RA signaling for fin regeneration we developed a

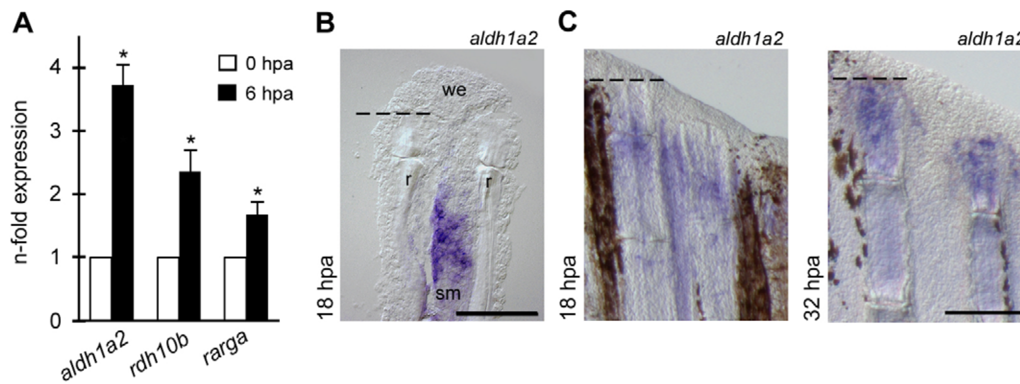

Fig. 1. Fin amputation induces RA synthesis in the stump tissue. (**A**) qPCR determination of *aldh1a2*, *rdh10b* and *rarga* transcript levels at 6 hpa relative to uncut (0 hpa) fins. Error bars, s.e.m. *, $P<0.01$. (**B**,**C**) In situ hybridization on longitudinal section (B) and whole fins (C) demonstrates *aldh1a2* expression in the stump mesenchyme. Note the absence of *aldh1a2* transcripts in the most distal mesenchyme at 18 hpa. sm, stump mesenchyme; r, hemiray; we, wound epidermis. Dashed lines indicate amputation plane. Scale bars: 100 μm in B; 200 μm in C.

transgenic zebrafish line that allows heat shock-inducible degradation of endogenous RA. The *Tg(hsp70I:cyp26a1)*^{kn1} strain, referred to hereafter as *hsp70:cyp26a1*, harbors zebrafish *cyp26a1*, which encodes an RA-degrading enzyme, driven by the heat-inducible zebrafish *hsp70* promoter (Halloran et al., 2000). We found that induction of the transgene during embryogenesis results in strong, ubiquitous *cyp26a1* expression (not shown) and phenocopies the effects of complete loss of RA signaling. A brief heat shock at 6 hours post-fertilization (hpf) caused specific and organ-wide developmental defects that are known hallmarks of impaired RA signaling (supplementary material Fig. S1) (Begemann et al., 2001; Begemann et al., 2004; Gibert et al., 2006; Grandel et al., 2002). When adult *hsp70:cyp26a1* fish were exposed to a heat shock during fin regeneration, strong *cyp26a1* expression could be detected in the whole fin (not shown). Thus, the *hsp70:cyp26a1* line is a reliable tool to interfere with RA signaling in embryos and adult fish.

To test whether RA signaling is required for blastema formation, we applied daily heat shocks to adult *hsp70:cyp26a1* fish starting with the first heat shock 2 hours before fin amputation. This treatment caused a complete and early block to fin regeneration (22/34 fish), whereas regeneration was unperturbed in heat-shocked wild-type fish (39/39 fish) (Fig. 2A). In addition, this effect is reversible: removing the heat-shock treatment resulted in normal blastema formation and complete fin regeneration. To determine the cellular nature of regenerative failure, we examined Hematoxylin-stained fin sections of heat-shocked wild-type and *hsp70:cyp26a1* fish at 45 hpa. Whereas wild-type regenerates displayed a well-developed blastema between the amputation plane and a multilayered wound epidermis, *hsp70:cyp26a1* fins exhibited a complete absence of blastema cells (Fig. 2B). Several layers of epithelial cells sealed the wound in *hsp70:cyp26a1* fish, indicating normal re-epithelialization of the stump surface. However, cells of the basal epidermal layer did not adopt their typical cuboidal shape (Fig. 2C). Extracellular matrix remodeling and disorganization of the stump mesenchyme adjacent to the amputation site are an early response prior to blastema formation. Interestingly, disorganized stump mesenchyme proximal to the wound site was also observed in *hsp70:cyp26a1* regenerates (Fig. 2D).

To confirm the absence of blastema cells in *hsp70:cyp26a1* fish we examined the expression of *fgf20a* and *msxb*, two markers that are strongly expressed in blastema cells (Akimenko et al., 1995; Whitehead et al., 2005). Neither gene could be detected in heat-shocked *hsp70:cyp26a1* fins at 32 hpa (*fgf20a*, 6/7 fins; *msxb*, 3/5 fins) and 48 hpa (*msxb*, 6/8 fins) (supplementary material Fig. S2). The absence of a distinct basal epidermal layer in *hsp70:cyp26a1* fish suggests that the initial specification of the wound epidermis is affected. *lef1*, which marks the basal epidermal layer and the distal blastema (Poss et al., 2000b), could not be detected in *hsp70:cyp26a1* fins at 46 hpa (3/4 fins) (supplementary material Fig. S2). Furthermore, *lef1* expression was also absent at an earlier time point (at 32 hpa; 5/8 fins) demonstrating that *lef1* expression is not initiated in the absence of RA signaling.

The failure of blastema formation might be a consequence of defects in wound healing and in the formation of a proper wound epidermis. We showed that this is not the case by inhibiting RA signaling after wound healing had taken place, applying the first heat shock at 24 hpa. Regeneration was completely blocked in 13 out of 21 *hsp70:cyp26a1* fish (supplementary material Fig. S3). In summary, neither blastema cells nor the basal epidermal layer is formed or specified correctly in the absence of RA signaling.

RA signaling controls cell cycle entry at the onset of blastema formation

During blastema formation, formerly quiescent cells of the ray mesenchyme start to proliferate and migrate towards the amputation plane. To understand why blastema formation fails in the absence of RA signaling, we assayed cell proliferation in heat-shocked *hsp70:cyp26a1* fish at 32 hpa. We found a dramatic decrease in proliferating stump cells (Fig. 3A,B). Similar results were obtained for the transgenic strain *hsp70:dn-zrar*, in which heat-shock treatment induces expression of a dominant-negative zebrafish *retinoic acid receptor alpha* (*rara*) (Kikuchi et al., 2011) (Fig. 3C), providing independent evidence for the requirement of RA signaling for blastema formation. The lack of proliferating cells suggested that proliferation was either not induced or that cycling cells underwent cell death. To discriminate between these possibilities, we compared the number of dying cells between wild-type and *hsp70:cyp26a1* stumps at 32 hpa. Since we did not observe enhanced cell death in *hsp70:cyp26a1* stumps, neither in the mesenchyme nor in the epidermis (supplementary material Fig. S4), we conclude that induction of cell proliferation in the ray mesenchyme fails in the absence of RA signaling.

RA signaling might be sufficient to induce cell cycle entry of blastema progenitor cells, as cells of the ray mesenchyme become exposed to high levels of RA as a consequence of fin

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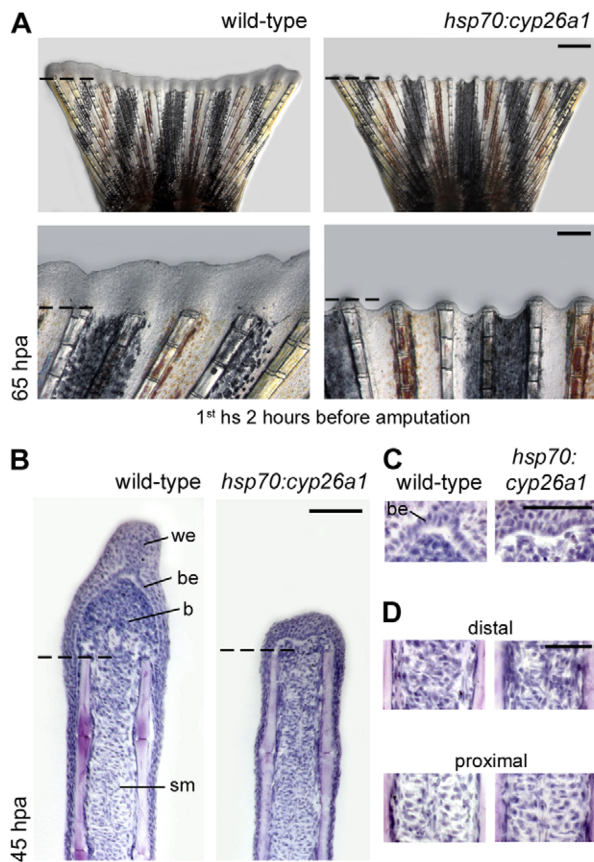

Fig. 2. RA signaling is necessary for blastema formation. (**A**) Inhibition of RA signaling in *hsp70:cyp26a1* fish by applying daily heat shocks (commencing 2 hours before fin amputation) results in an early and complete block to fin regeneration. (**B-D**) Hematoxylin-stained longitudinal sections indicate absence of blastema cells in *hsp70:cyp26a1* fins at 45 hpa and lack of a distinctive basal epidermal layer. Several layers of epithelial cells seal the amputation plane, indicating normal initial wound healing. Remodeling of the stump mesenchyme adjacent to the amputation site is apparent in both wild-type and *hsp70:cyp26a1* fish. (B) Overviews of stained sections. (C,D) Magnified view of the wound epidermis-mesenchyme boundary (C) and the stump mesenchyme (D). Dashed lines indicate amputation plane. hs, heat shock; b, blastema; be, basal epidermal layer; sm, stump mesenchyme; we, wound epidermis. Scale bars: 500 μm in A upper panels; 200 μm in A lower panels; 100 μm in B; 50 μm in C,D.

amputation. Previous studies have shown that RA treatment can cause mispatterning in regenerating fins, slow down regeneration or even block blastema formation (Géraudie et al., 1995; White et al., 1994). However, because these effects might have been caused by increased cell death, especially in the wound epidermis (Géraudie and Ferretti, 1997), we developed an RA treatment regime that efficiently enhances RA signaling in the regenerating fin, but does not induce cell death. We found that intraperitoneal (IP) injection of 1 mM RA dissolved in a low concentration of DMSO does not induce cell death during blastema formation and regenerative outgrowth, even if injected every 12 hours for several days (supplementary material Fig. S5B; data not shown). Increased RA signaling in the regenerate should result in decreased *aldh1a2* and *rdh10a* transcript levels and enhanced *cyp26a1* levels, as has been shown for embryonic development (Dobbs-McAuliffe et al., 2004; Hu et al., 2008). Accordingly, we detected an autoregulatory component of RA signaling in the regenerating caudal fin 4 hours after IP injection of 1 mM RA (supplementary material Fig. S5A).

We tested the effect of exogenous RA on proliferation of the ray mesenchyme during blastema formation. We injected RA every 12 hours, with the first injection directly after fin amputation, and assayed cell proliferation at 32 hpa. Mesenchymal proliferation was significantly increased in RA-treated fish (Fig. 3D), demonstrating that RA signaling is not only required for cell cycle entry but is also sufficient to increase the proliferation of stump cells. These findings clearly show that the previously reported negative effects of RA on fin regeneration were secondary effects caused by enhanced cell death.

Key pathways involved early in blastema and wound epidermis formation are regulated by RA signaling

To understand the molecular consequences of early upregulation of RA levels in the stump, we examined the effects of altered RA signaling on key pathways involved in blastema and wound epidermis formation. Fgf and Wnt/β-catenin signaling have been shown to be required for blastema formation and subsequent blastema proliferation. Within the first few hours after amputation, expression of *fgf20a* and *wnt10a* is strongly upregulated. Moreover, homozygous mutants in *fgf20a* (*dob*) fail to form a blastema and show an abnormal wound epidermis (Lee et al., 2005; Poss et al., 2000a; Stoick-Cooper et al., 2007; Whitehead et al., 2005). By contrast, the ligand responsible for activation of the Wnt/β-catenin signaling pathway during fin regeneration remains a matter of speculation. We examined whether induction of *fgf20a* and Wnt ligand expression in the fin stump is regulated by RA signaling by performing loss- and gain-of-function experiments. Amputation-induced *fgf20a* upregulation was clearly diminished in heat-shocked *hsp70:cyp26a1* fish at 10 hpa, whereas upregulation of *wnt10a* and *wnt10b* was unaffected (Fig. 4A). Because not all *hsp70:cyp26a1* fish respond equally well to the heat-shock treatment, the reduction in *fgf20a* expression observed might be underrepresentative. Furthermore, overexpression of *cyp26a1* results in strong downregulation of baseline *fgf20a* expression in unamputated fins (Fig. 4A), suggesting that reduced upregulation of *fgf20a* in *hsp70:cyp26a1* fins is not a secondary effect of impaired fin regeneration, but reflects a more direct requirement for RA signaling for *fgf20a* expression. Exogenous RA does not induce upregulation of *fgf20a* expression in unamputated fins and we did not detect increased upregulation of *fgf20a* in RA-treated fins at 10 hpa (Fig. 4B). Interestingly, RA treatment resulted in decreased upregulation of *wnt10a* at 10 hpa, but further increased regeneration-induced *wnt10b* upregulation. Strikingly, we found that exogenous RA is sufficient to induce *wnt10b* upregulation in unamputated fins.

Although re-epithelialization of the stump surface does not require RA signaling, formation of the basal epidermal layer depends on RA signaling. Chablais and Jazwinska (Chablais and Jazwinska, 2010) reported a fundamental role for Igf signaling in the formation of the basal epidermal layer via paracrine activation of Igfr in the wound epidermis. Igf2b is produced and secreted from cells of the stump mesenchyme, demonstrating the importance of interactions between the mesenchyme and the forming wound epidermis. The lack of the basal epidermal layer in RA-deficient fish might therefore be caused by a reduction in paracrine signals derived from the stump mesenchyme. To test this, we examined the effects of altered RA signaling on early *igf2b*

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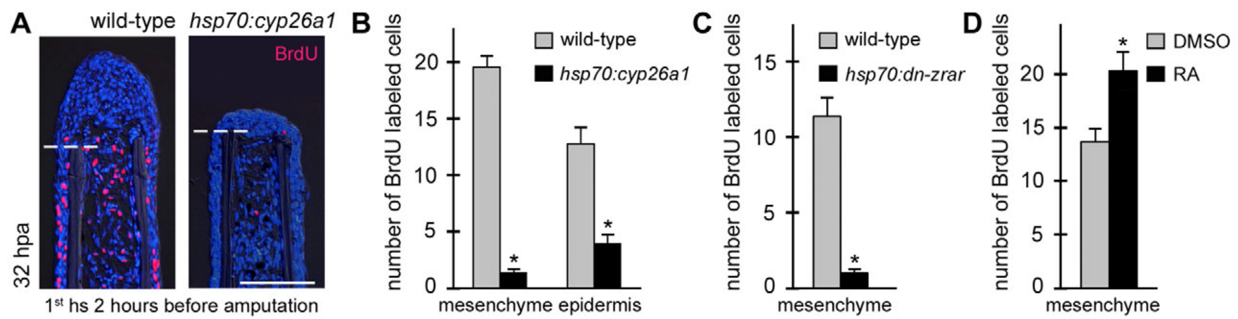

Fig. 3. Induction of cell proliferation in the fin stump requires RA signaling. (**A-C**) Inhibition of RA signaling in *hsp70:cyp26a1* and *hsp70:dn-zrar* fish results in a significant decrease in proliferating cells in the fin stump (daily heat shocks, commencing 2 hours before amputation). (A) Longitudinal sections stained for BrdU and with DAPI demonstrate a near absence of BrdU-positive cells in *hsp70:cyp26a1* fins at 32 hpa. (B,C) Quantification of BrdU-labeled cells within a defined area at 32 hpa in *hsp70:cyp26a1* (wild type, *n*=39 sections; *hsp70:cyp26a1*, *n*=44) (B) or *hsp70:dn-zrar* stumps (wild type, *n*=20; *hsp70:dn-zrar*, *n*=31) (C). (**D**) Exogenous RA promotes mesenchymal proliferation in the stump [RA intraperitoneal injection (IP) every 12 hours, first IP at 0 hpa]. Quantification of BrdU-labeled cells at 32 hpa in RA-treated stumps (DMSO vehicle, *n*=16; RA, *n*=18). Error bars, s.e.m. *, $P<0.0001$ in B,C; *, $P<0.005$ in D. Dashed lines indicate the amputation plane. hs, heat shock. Scale bar: 100 μm.

expression in the fin stump. Loss of RA in unamputated fins had no effect on baseline *igf2b* expression, whereas regeneration-induced *igf2b* upregulation was reduced in heat-shocked *hsp70:cyp26a1* fins (Fig. 4A). RA treatment further increased *igf2b* expression at 10 hpa and even induced *igf2b* upregulation in unamputated fins (Fig. 4B). Thus, RA is required for the strong induction of *igf2b* during regeneration and is sufficient to induce *igf2b* in the unamputated fin, indicating that impaired wound epidermis formation in *hsp70:cyp26a1* fish is caused by impaired *igf2b* induction. Together, these findings demonstrate that RA signaling stimulates *wnt10b* expression and is crucial for amputation-induced *fgf20a* and *igf2b* expression in the fin stump.

RA signaling is essential for proliferation and survival of the mature blastema

Following the formation of a blastema *aldh1a2*, *rarga* and *rdh10a* are strongly expressed in the blastema (supplementary material Fig. S6), whereas *rdh10b* transcripts could not be detected. Since RA signaling is necessary for cell proliferation during blastema formation, we tested the effect of inhibiting RA signaling on proliferation of the mature blastema. We applied a single heat shock at 72 hpa and examined proliferation 8 hours later. Our analysis revealed a significant reduction of BrdU-positive cells in *hsp70:cyp26a1* regenerates, both in the mesenchyme and epidermis (Fig. 5A,B). *msxb* is strongly expressed in blastema cells and has been shown to be required for blastema proliferation (Akimenko et al., 1995; Nechiporuk and Keating, 2002; Thummel et al., 2006). To test whether the observed decrease in proliferation is reflected in altered *msxb* expression, and to detect early expression changes, we examined *msxb* expression by qPCR at 73 hpa immediately after a single heat shock. We found that *msxb* transcripts were reduced to half their normal levels in *hsp70:cyp26a1* regenerates (Fig. 5C). Thus, RA signaling is not only required for blastema formation but also for subsequent blastema proliferation.

Daily heat shocks starting at 72 hpa resulted in a reversible and robust block to regenerative outgrowth in *hsp70:cyp26a1* fish (10/10 fish) and a loss of already regenerated tissue (Fig. 6A,C). Remarkably, within the first 10 hours after heat shock, the blastema

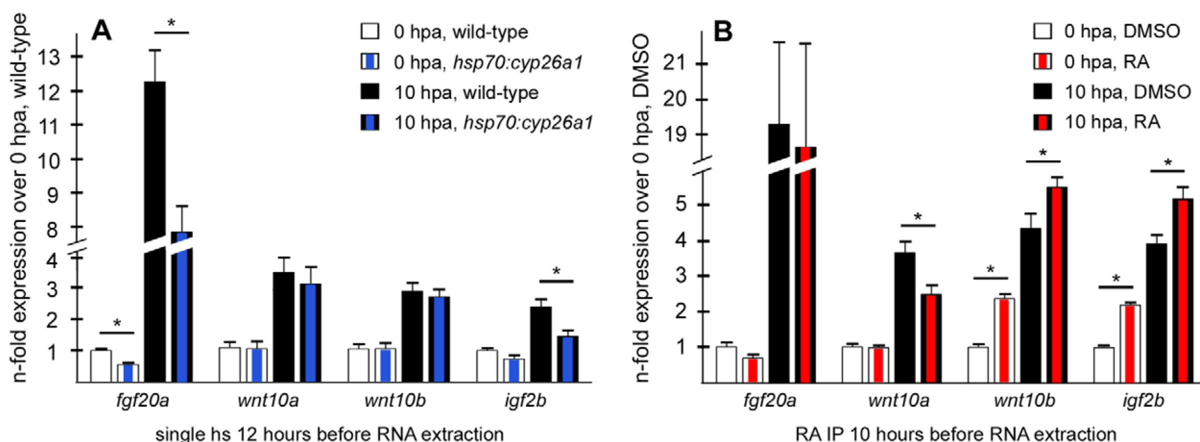

Fig. 4. RA signaling regulates Fgf, Wnt/β-catenin and Igf signaling in the fin stump. qPCR determination of *fgf20a*, *wnt10a*, *wnt10b* and *igf2b* expression levels under altered RA signaling. (**A**) Expression levels in *hsp70:cyp26a1* fins at 0 and 10 hpa relative to wild-type control at 0 hpa (single heat shock 12 hours before RNA extraction). (**B**) Expression levels in RA-treated fins at 0 and 10 hpa relative to DMSO control at 0 hpa (single RA IP 10 hours before RNA extraction). Expression of all genes was significantly higher at 10 hpa relative to 0 hpa. Error bars, s.e.m. *, $P\leq 0.01$. hs, heat shock.

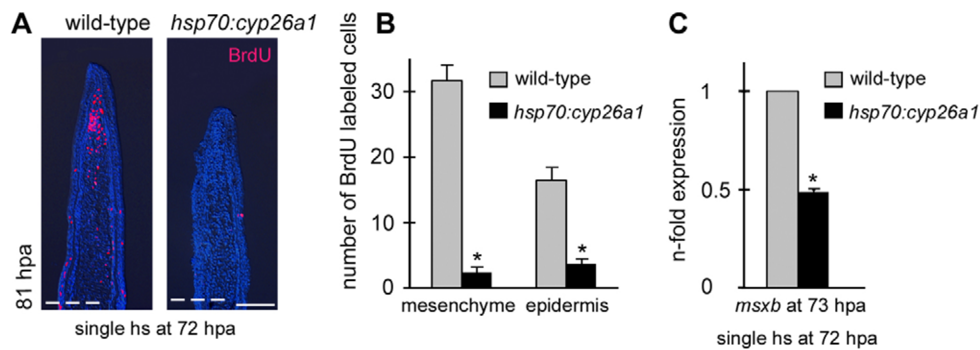

Fig. 5. RA signaling is required for blastema proliferation. Inhibition of RA signaling in *hsp70:cyp26a1* fish, when instigated during regenerative outgrowth, results in downregulation of *msxb* expression and loss of blastema proliferation. (**A**) Longitudinal sections stained for BrdU and with DAPI demonstrate absence of BrdU-positive cells in *hsp70:cyp26a1* regenerates at 81 hpa after a single heat shock at 72 hpa. (**B**) Quantification of BrdU-labeled cells (wild type, n=17 sections; *hsp70:cyp26a1*, n=16). (**C**) qPCR determination of *msxb* transcript levels in *hsp70:cyp26a1* regenerates relative to wild-type regenerates at 73 hpa. Error bars, s.e.m. *, P<0.0001. Dashed lines indicate amputation plane. Scale bar: 100 μm.

in *hsp70:cyp26a1* regenerates turned opaque (10/10 fish) (Fig. 6B), suggesting extensive cell death. In support of this, we found a high number of TUNEL-labeled mesenchymal cells in *hsp70:cyp26a1* regenerates 8 hours after heat shock (Fig. 7A,B). Importantly, most TUNEL-positive cells were restricted to the distal half of the mesenchyme, indicating that undifferentiated blastema cells are unable to survive in the absence of RA signaling. Very similar results were obtained with the *hsp70:dn-zrar* line (supplementary material Fig. S7), demonstrating that the massive cell death in *hsp70:cyp26a1* fish is caused by impaired RA signaling, rather than by non-specific effects of *cyp26a1* overexpression.

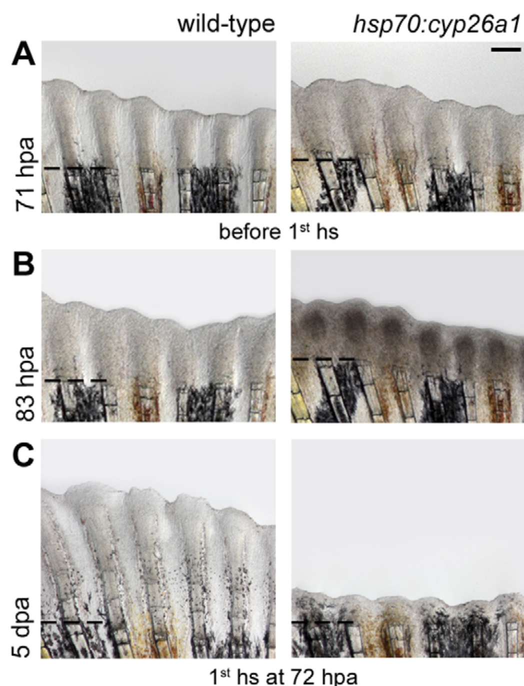

Fig. 6. RA signaling is essential for regenerative outgrowth and maintenance of the regenerate. Inhibition of RA signaling in *hsp70:cyp26a1* fish during regenerative outgrowth blocks further regeneration and abolishes maintenance of the regenerate (daily heat shocks, first heat shock at 72 hpa). (**A**) Before the first heat shock, regeneration in *hsp70:cyp26a1* fish is indistinguishable from that of wild-type fish. (**B**) Ten hours after the first heat shock, the blastema of *hsp70:cyp26a1* fish regenerates appears dark. (**C**) Two days later, regenerative outgrowth is blocked and already regenerated tissue is lost. Note the decrease in tissue distal to the amputation plane in *hsp70:cyp26a1* regenerates between A and C. Dashed lines indicate amputation plane. hs, heat shock. Scale bar: 200 μm.

Fgf, Wnt/β-catenin and Activin-βA, among other pathways, have been shown to positively regulate blastema proliferation (Jazwinska et al., 2007; Poss et al., 2000a; Stoick-Cooper et al., 2007). Cell death caused by inhibition of these pathways has not been reported. Thus, RA might be an essential part of the mechanism promoting survival of the blastema. Bcl2 family proteins are essential regulators of various cell death mechanisms, including apoptosis, necrosis and autophagy (Yip and Reed, 2008). *bcl2* overexpression has been shown to inhibit cell death induced by many stimuli, including growth factor deprivation. Bcl2 is therefore a major candidate for RA-mediated protection from cell death in blastema cells and possibly other stress-induced states. Because *bcl2* expression in the regenerating fin had not been investigated, we analyzed *bcl2* transcript levels. qPCR analysis revealed 2.5-fold higher levels of *bcl2* transcripts at 6 hpa compared with uninjured fins (0 hpa) (Fig. 7C), indicating that protection against cell death is enhanced in the regenerating fin. To test whether *bcl2* expression is regulated by RA signaling, we performed loss- and gain-of-function experiments. We applied a single heat shock to wild-type and *hsp70:cyp26a1* fish at 72 hpa and compared expression immediately following the heat shock. Transcript levels were significantly reduced in *hsp70:cyp26a1* regenerates (Fig. 7D). We next increased RA signaling by IP injection of RA and examined *bcl2* expression 4 hours later. Expression was 1.75-fold higher in RA-injected than in vehicle-injected fish (Fig. 7D). These results reveal a strong RA-mediated pro-survival mechanism in blastema cells that is mediated by upregulation of *bcl2* expression.

RA, Fgf, Wnt/β-catenin and non-canonical Wnt signaling cooperate to regulate blastema proliferation

To gain insights into the gene network underlying blastema proliferation we investigated regulatory interactions between RA, Fgf and Wnt/β-catenin at 73 hpa using a qPCR approach. Expression of the Fgf target *mkp3* (*dusp6* – Zebrafish Information Network) and the Wnt/β-catenin target *axin2* was strongly

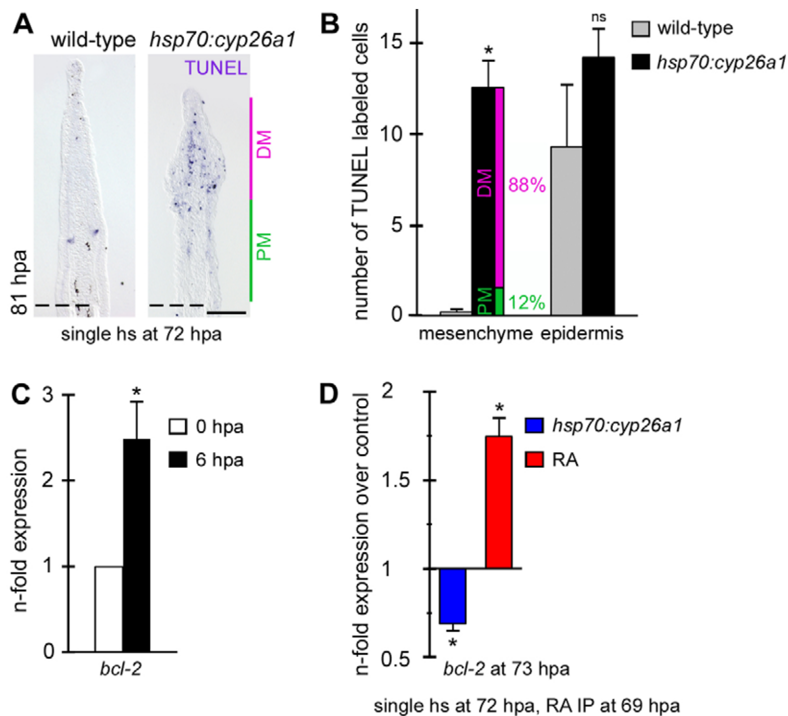

Fig. 7. Blastema cells possess a strong RA-mediated pro-survival mechanism. (**A**,**B**) Loss of RA signaling during regenerative outgrowth causes cell death in the regenerate after a single heat shock at 72 hpa. (A) TUNEL staining on longitudinal sections at 81 hpa reveals massive cell death in the distal mesenchyme in *hsp70:cyp26a1* fish. (B) Quantification of TUNEL-labeled cells in *hsp70:cyp26a1* regenerates at 81 hpa (wild type, *n*=35 sections; *hsp70:cyp26a1*, *n*=31). Pink and green bars in B show the ratio of labeled cells between the distal and proximal mesenchyme of *hsp70:cyp26a1* fish as a percentage of the total number of labeled cells in the mesenchyme. (**C**) qPCR determination of *bcl2* transcript levels at 6 hpa relative to uncut (0 hpa) fins. (**D**) *bcl2* transcript levels, determined by qPCR, in heat-shocked *hsp70:cyp26a1* fish (control is heat-shocked wild-type fish) and RA-treated fish (control is vehicle-treated fish) relative to control fish at 73 hpa (single heat shock at 72 hpa, single RA IP at 69 hpa). Error bars, s.e.m. *, $P<0.0001$ in B; *, $P<0.01$ in C; *, $P<0.001$ in D; ns, not significant. Dashed lines indicate amputation plane. DM, distal mesenchyme; PM, proximal mesenchyme; hs, heat shock. Scale bar: 100 μm.

diminished at the end of a single heat shock in *hsp70:cyp26a1* regenerates (Fig. 8A). Conversely, treating fish for 4 hours with RA caused upregulation of *axin2*. Expression of the Fgf target *mkp3* remained unchanged under these conditions, most likely owing to the short duration of the treatment.

We then investigated which Fgf and Wnt ligands might mediate the positive effect of RA. *fgf20a* showed increased expression in RA-injected fish and decreased following *cyp26a1* overexpression. Expression of both *wnt10* paralogs was unchanged in *hsp70:cyp26a1* fins. However, RA treatment resulted in downregulation of *wnt10a* expression and in a striking upregulation of *wnt10b*. Thus, it is likely that *wnt10b*, either alone or together with other Wnts, mediates the positive effect of RA signaling on the Wnt/β-catenin pathway in the mature blastema. Wnt/β-catenin signaling has previously been shown to be required for Fgf signaling during fin regeneration and has therefore been suggested to act upstream of Fgf activation (Stoick-Cooper et al., 2007). To test this, we made use of the transgenic *hsp70:dn-fgfr1* strain (Lee et al., 2005), which allows heat shock-induced inhibition of Fgf signaling. We applied a single heat shock and compared expression levels directly at the end of the heat shock. Expression of *mkp3* was strongly reduced in *hsp70:dn-fgfr1* regenerates, demonstrating the efficiency of Fgf signaling inhibition. Remarkably, both *axin2* and *aldh1a2* transcript levels were reduced in *hsp70:dn-fgfr1* regenerates (Fig. 8A). Thus, Wnt/β-catenin signaling and RA synthesis are positively regulated by Fgf signaling. Together, these findings demonstrate that RA, Fgf and Wnt/β-catenin signaling regulate each other in a positive reciprocal manner, rather than following an epistatic hierarchy during fin regeneration (Fig. 8B).

If this model is correct, enhancing RA signaling in heat-shocked *hsp70:dn-fgfr1* fish should not rescue blastema proliferation and regenerative outgrowth. To examine this, we blocked Fgf signaling in *hsp70:dn-fgfr1* fish for 2 days during regenerative outgrowth by applying the first heat shock at 72 hpa and simultaneously enhanced RA signaling through daily RA IP injections. We found an increase in tissue distal to the amputation plane by ~400 μm in vehicle- and RA-injected heat-shocked wild-type fish (supplementary material Fig. S8). By contrast, RA injection into *hsp70:dn-fgfr1* fish failed to rescue regenerative outgrowth. Moreover, there was a decrease in already regenerated tissue in *hsp70:dn-fgfr1* fish that suggests that the interactions we identified between the RA and Fgf pathways are also employed to promote cell survival.

The non-canonical Wnt pathway has been shown to act as a negative modulator of fin regeneration (Stoick-Cooper et al., 2007). Overexpression of *wnt5b* inhibits fin regeneration, whereas *wnt5b* loss-of-function accelerates regeneration. Interestingly, *wnt5b* expression is positively regulated by Fgf, Igf and Wnt/β-catenin signaling (Fig. 8A) (Chablais and Jazwinska, 2010; Lee et al., 2009; Stoick-Cooper et al., 2007), indicating that a negative-feedback mechanism modulates the overall rate of regeneration through non-canonical Wnt signaling. Because of the observed positive regulation between RA and the Fgf and Wnt/β-catenin pathways, we expected reduced *wnt5b* expression in *hsp70:cyp26a1* regenerates. However, expression was mainly unaffected (Fig. 8A). Moreover, we found a decrease in *wnt5b* expression in RA-injected fish. Our findings show that the non-canonical Wnt pathway is negatively regulated by RA, indicating that RA signaling counteracts the negative-feedback loop that is activated by Fgf and Wnt/β-catenin signaling. Moreover, the interactions between RA, Fgf and Wnt/β-catenin in the mature blastema correlate with those observed for blastema formation.

DISCUSSION

In this study we have identified fundamental roles of RA signaling in adult fin regeneration, findings that contribute to a more thorough understanding of the molecular events underlying the development and maintenance of the regeneration blastema. Amputation of the caudal fin results in the activation of as yet unidentified signals that initiate blastema formation in the stump tissue. Strong *aldh1a2* expression is rapidly induced in the stump mesenchyme, indicating that cells that will give rise to the blastema become exposed to high RA levels within the first few hours after

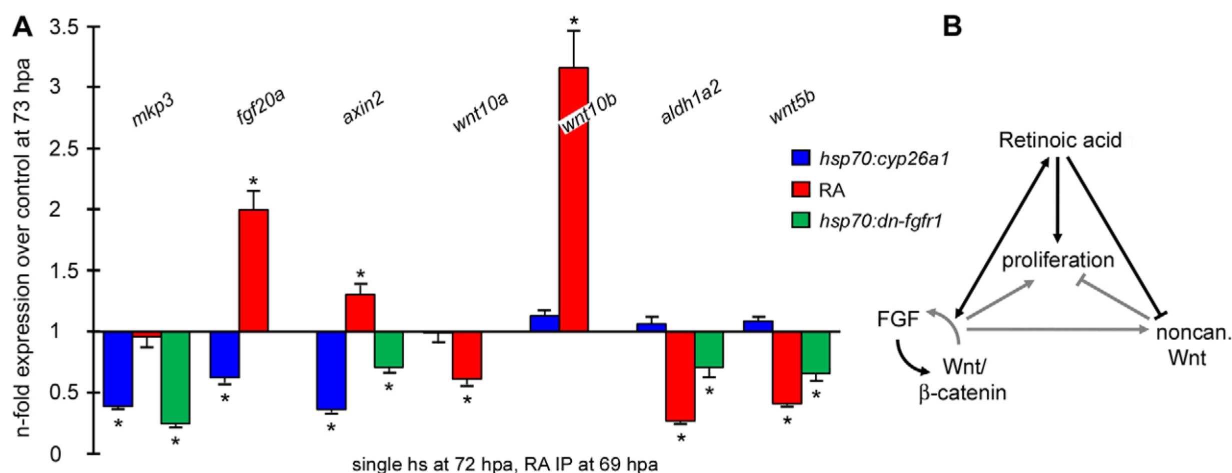

Fig. 8. A complex molecular regulatory network underlies blastema proliferation. (**A**) qPCR determination of RA, Wnt and Fgf pathway components and targets during regenerative outgrowth in RA-treated (control is vehicle-treated fish), *hsp70:cyp26a1* and *hsp70:dn-fgfr1* (control is heat-shocked wild-type fish) relative to control fish at 73 hpa (single heat shock at 72 hpa, single RA IP at 69 hpa). Error bars, s.e.m. *, $P<0.01$. (**B**) Simplified model of regulatory interactions underlying blastema proliferation. RA, Wnt/β-catenin and Fgf signaling regulate each other in a positive reciprocal manner and act as positive regulators of blastema proliferation. The non-canonical Wnt pathway inhibits proliferation and is positively modulated by Wnt/β-catenin and Fgf signaling. RA signaling negatively regulates non-canonical Wnt signaling. Black arrows indicate newly identified interactions (this study), whereas gray arrows indicate previously identified interactions (Lee et al., 2009; Stoick-Cooper et al., 2007). hs, heat shock.

amputation. Rapid induction of *aldh1a2* is also a hallmark of zebrafish heart regeneration, where it is expressed in both the epicardium and endocardium, highlighting the importance of RA signaling in promoting cell division during the injury response of a variety of tissues (Kikuchi et al., 2011; Lepilina et al., 2006).

Activation of RA signaling in the fin stump is required for the strong amputation-induced *fgf20a* expression and promotes induction of *wnt10b* expression. Thus, RA, Fgf and Wnt/β-catenin signaling constitute parts of a signaling network that controls blastema formation. After re-epithelialization of the wound, mesenchymal cells in the stump start to proliferate and migrate distally to form the blastema. Loss of RA signaling blocks entry of the blastema precursors into the cell cycle. Conversely, exogenous RA is sufficient to promote mesenchymal proliferation, suggesting that RA signaling confers mitogenic activity during blastema formation. In addition, it might control an earlier step in blastema formation that is preconditional for cell cycle entry.

Another early and essential step in regeneration is the formation of a specialized wound epidermis. Although the initial sealing of the stump surface does not require RA signaling, specification of the basal epidermal layer fails in the absence of RA. Igf signaling has been shown to regulate proper wound epidermis formation via paracrine activation of Igf receptors in the forming wound epidermis (Chablais and Jazwinska, 2010). We show that RA signaling is required and probably sufficient to induce *igf2b* expression in mesenchymal cells underlying the wound epidermis. The lack of the basal epidermal layer in RA-deficient regenerates might therefore be caused by a reduction in paracrine signals derived from the stump mesenchyme.

Once the blastema has formed, RA signaling remains highly active in the mature blastema to ensure proliferation and survival. Besides a possible, more direct mitogenic function, RA signaling indirectly regulates blastema proliferation through integrating signals that either stimulate or inhibit proliferation (Fig. 8B). RA antagonizes the inhibitory effect of non-canonical Wnt signaling and, in parallel, promotes this same pathway via stimulation of Fgf and Wnt/β-catenin signaling. We propose that this complex regulatory network ensures tight control over blastema proliferation. Moreover, our findings demonstrate that RA, Fgf and Wnt/β-catenin signaling cooperate through mutually stimulatory interactions to regulate blastema proliferation, rather than acting in a fixed epistatic hierarchy. It remains to be shown whether this also holds true for the initiation of regeneration in the fin stump.

Mature blastema cells are in a fast-cycling state. It is an open and interesting question how such a highly proliferative cell population can be maintained in an adult animal. We found that expression of the pro-survival gene *bcl2* is upregulated within the first few hours after fin amputation. Importantly, blocking RA signaling by overexpression of *cyp26a1* or *dn-zrar* in the mature blastema results in downregulation of *bcl2* expression, followed by massive cell death. Our data indicate that blastema cells receive protection against cell death through increased levels of the anti-apoptotic factor Bcl2, the expression of which is positively regulated by RA signaling. We therefore suggest that blastema cells possess a strong RA-mediated pro-survival mechanism that allows maintenance of the blastema in the adult fish and provides robustness to environmental perturbation. Interestingly, RA signaling inhibition during blastema formation does not cause cell death in the stump mesenchyme, nor does manipulation of RA signaling during the first few hours after amputation affect upregulation of *bcl2* expression (data not shown). These findings indicate that only mature blastema cells require RA signaling for enhanced *bcl2* expression and survival.

Exogenous RA has previously been shown to adversely affect fin regeneration and even block blastema formation (Géraudie et al., 1995; White et al., 1994), but the underlying cellular mechanisms have not been analyzed. A follow-up study by Géraudie and Ferretti found that incubation of zebrafish in high RA concentrations or IP injection of RA, when diluted in high DMSO concentrations, causes enhanced cell death in the wound epidermis and blastema (Géraudie and Ferretti, 1997). We have developed an RA treatment regime that does not induce cell death; rather, it

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demonstrably enhances RA signaling and positively influences fin regeneration. Thus, the reported negative effects of RA treatment (Géraudie et al., 1995; White et al., 1994) are very likely secondary effects caused by enhanced cell death.

An important question is whether this spectrum of specific functions of RA in zebrafish blastema development is at work in other regeneration-competent vertebrates. In support of this view, endogenous RA has been detected in salamander blastemas (Brockes, 1992; Scadding and Maden, 1994; Viviano et al., 1995). Furthermore, axolotl limb regeneration can be blocked by applying aldehyde dehydrogenase inhibitors with broad target specificity (Maden, 1998), suggesting that RA signaling fulfils similar roles in fin and limb regeneration.

In regenerating salamander limbs, RA is thought to be synthesized in the wound epidermis (Viviano et al., 1995); however, the expression of genes encoding RA-synthesizing enzymes has not been investigated so far. Although we cannot exclude the possibility that RA might also be produced by epidermal cells in the regenerating fin, the zebrafish *aldh1a2* and *rdh10a* expression patterns strongly suggest that RA production is restricted to the stump mesenchyme and blastema. Interestingly, a study by McEwan et al. (McEwan et al., 2011) has shown that expression of the *Xenopus aldh1a2* ortholog is not upregulated in regenerating tadpole hindlimbs. However, expression is retained from development in proximal cells bordering the body wall, indicating that an RA source would be available for regeneration. Investigating the sources of RA in regenerating limbs of adult urodele amphibians might reveal important differences in RA distribution between the regenerating limbs of adult and larval amphibians as well as between regenerating limbs and fins. Thus, although the roles of RA in regeneration might be conserved, its sources might have diverged during evolution.

In amphibians, treatment of regenerating limbs with RA results in a phenomenon known as ‘super-regeneration’, in which additional limb structures are regenerated that would normally be found proximal to the amputation plane (Maden and Hind, 2003; Maden, 1982; Maden, 1983; Niazi and Saxena, 1978). RA has therefore been proposed to act as a morphogen responsible for a gradient of positional information along the proximal-distal (P-D) axis during limb regeneration. It has been demonstrated that the P-D pattern of limb segments in mouse and chicken is specified during embryonic development through a balance between proximal RA and distal Fgf activity (Cooper et al., 2011; Roselló-Díez et al., 2011). These studies support a patterning function of RA signaling in vertebrate limbs. Provided that loss-of-function experiments can show that this also holds true for limb regeneration, super-regeneration would be the expected outcome, reflecting the earlier patterning role of RA in limb development. So far, investigations in teleosts into a putative role for RA signaling in P-D patterning of the regenerating caudal fin have proved extremely difficult owing to the lack of reliable readouts of P-D patterning and insufficient knowledge concerning the processes that set up the caudal fin P-D axis. Although fin rays branch dichotomously, the mechanisms controlling branching are unknown and might not be appropriate readouts of a hypothetical P-D patterning gradient. The findings reported here are the first to be supported by loss- and gain-of-function experiments that propagate the idea that RA signaling is an essential component of vertebrate appendage regeneration. The mechanistic framework provided here should inform and advance future research to help uncover the function of RA during the repatterning phase of regeneration and to understand its roles from an evolutionary perspective.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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