Retinoic acid signaling in zebrafish fin regeneration

Dissertation

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Table of contents

Summary

Teleost fish and urodele amphibians are unique among vertebrates in that they possess the ability to replace limbs and fins with the exact replicates of the original throughout life by epimorphic regeneration. Following appendage amputation, a pool of lineage-restricted highly-proliferative mesenchymal cells (the so called blastema) forms at the site of regeneration, from which the lost appendage faithfully regrow.

For this thesis, I have investigated the functions of retinoic acid (RA) signaling in zebrafish fin regeneration. I demonstrate that RA signaling is an essential component of the genetic network underlying blastema formation and subsequent proliferation and survival of blastema cells. Synthesis of RA becomes upregulated within the first hours after fin amputation close to the amputation site, where it is required to mobilize cell division in post-mitotic stump cells that will give rise to the blastema. Genetic inhibition of RA signaling upon fin amputation causes suppression of blastema formation and failure of fin regeneration. RA synthesis remains high in the mature blastema and drives regenerative outgrowth by promoting blastema proliferation. The blastema is a mass of fast-cycling cells in an adult animal, raising the question of why blastema cells are not eliminated due to anti-cancer mechanisms. My findings indicate that blastema cells evade cell death by elevated levels of the anti-apoptotic factor Bcl2, the expression of which is positively regulated by RA signaling.

The zebrafish caudal fin is supported by several bony fin rays, derived from intramembranous ossification, which run from proximal to distal and are separated by soft interray tissue. Rapid replacement of lost bone during fin regeneration is achieved via dedifferentiation of osteoblasts from a post-mitotic, matrix-producing state to a cycling, immature preosteoblastic state, and vice versa, redifferentiation to a mature state. Here, I demonstrate that RA signaling inhibits switching between the mature and immature state while promoting osteoblast proliferation and bone matrix synthesis and unravel how the osteoblast regenerative program is achieved against continued RA synthesis during fin regeneration. Stump osteoblasts that participate in blastema formation transiently produce Cyp26b1, an enzyme that inactivates RA. This elegant mechanism allows the establishment of an osteoblast progenitor pool despite raising RA levels in the fin stump. Preosteoblasts pass through a number of cell divisions in the distal blastema where RA synthesis is high, whereas more proximal cells redifferentiate to form new bone. Fibroblasts-like blastema cells in those areas of redifferentiation lower local RA concentrations via Cyp26b1 activity, thereby ensuring redifferentiation of osteoblasts. This allows two processes to run in parallel: Proliferation for the continuous supply of osteoblasts in the distal part and redifferentiation of osteoblasts more proximally where the fin rays re-emerge.

During fin regeneration, osteoblasts have to respect fin ray-interray borders in order to faithfully reestablish the original fin pattern. However, why preosteoblast remain restricted to ray regions and do not invade the regenerating interray tissue has so far been unresolved. Here, I show that epidermal niches of low RA levels are established in regions where new rays are to form. This allows the spatially restricted production of a signal that pilots preosteoblasts to target regions. Interestingly, it emerged

that osteoblasts themselves exert a piloting function for other cell types that also have to be directed to appropriate regions.

Zusammenfassung

Knochenfische und Schwanzlurche können ihr ganzes Leben hindurch ihre Gliedmaßen vollständig nachwachsen lassen. Diese Fähigkeit ist einzigartig unter Wirbeltieren und beruht auf dem Prozess der epimorphen Regeneration. Nach Amputation einer Gliedmaße wird ein sogenanntes Blastem, das aus sich schnell-teilenden, gewebetreuen Zellen besteht, an der Amputationswunde gebildet. Aus dem Blastem wird der verlorene Teil der Gliedmaße regeneriert.

Im Rahmen dieser Dissertation wurden die Funktionen des Retinsäure Signalweges während der Flossenregeneration im Zebrafisch untersucht. Hierbei konnte gezeigt werden, dass der Retinsäure Signalweg eine entscheidende Rolle während der Blastembildung spielt und anschließend für die Proliferation und das Überleben der Blastemzellen benötigt wird. Innerhalb weniger Stunden nach der Amputation wird die Synthese von Retinsäure im Stumpfgewebe hochreguliert. Postmitotische mesenchymale Blastem-Vorläuferzellen benötigen die erhöhte Retinsäure-Konzentration, um in den Zellzyklus einzutreten. Wird der Retinsäure Signalweg blockiert, so kann sich kein Blastem bilden, und die Flosse wächst nicht nach. Nachdem sich das Blastem gebildet hat, wird Retinsäure weiterhin benötigt, um die Proliferation der Blastemzellen sicherzustellen und dadurch das regenerative Auswachsen voranzutreiben.

Das Blastem ist eine Ansammlung von sich schnell-teilenden Zellen. Es stellt sich deshalb die Frage, warum Blastemzellen in einem erwachsenen Tier überhaupt überleben können und nicht aufgrund von Mechanismen, welche normalerweise die Entstehung von Tumoren verhindern, eliminiert werden. In diesem Zusammenhang konnte ich zeigen, dass Blastemzellen aufgrund einer erhöhten Konzentration des anti-Apoptose-Faktor Bcl2 überleben können. Die Expression von *bcl2* wird durch Retinsäure gefördert.

Die Schwanzflosse des Zebrafisches wird durch knöcherne Flossenstrahlen verstärkt. Flossenstrahlen entstehen durch desmale Ossifikation und verlaufen parallel zur proximal-distalen Flossenachse. Zwischen den einzelnen Flossenstrahlen befindet sich weiches Zwischenstrahlgewebe. Die rasche Regeneration der Flossenstrahlen nach der Amputation wird durch eine temporäre Dedifferenzierung von ausdifferenzierten Osteoblasten sichergestellt. Diesbezüglich konnte ich zeigen, dass Retinsäure sowohl die Dedifferenzierung als auch die anschließende Rückdifferenzierung der Osteoblasten verhindert. Die Proliferation der Osteoblasten und die Produktion von Knochenmatrix wiederum werden von Retinsäure gefördert. Osteoblasten im Stumpf müssen sich vor Retinsäure schützen, um dedifferenzieren zu können. Hierfür produzieren Osteoblasten Cyp26b1, ein Retinsäure-abbauendes Enzym. Mit Hilfe von Cyp26b1 können Osteoblasten im Stumpf dedifferenzieren und zu Blastemzellen werden. Nach mehreren Zellteilungen im distalen Bereich des Regenerats differenzieren Osteoblasten wieder zu Matrix-produzierenden Knochenzellen. Eine erhöhte Retinsäure-Konzentration im distalen Regenerat fördert die Proliferation der dedifferenzierten Osteoblasten. In proximalen Regionen wird Retinsäure hingegen durch Cyp26b1 in Fibroblasten abgebaut. Durch diesen Mechanismus kann sichergestellt werden, dass sich Osteoblasten in proximalen Bereichen nicht weiter teilen und stattdessen differenzieren.

Zusammenfassung

Osteoblasten müssen während der Flossenregeneration die Grenzen der Flossenstrahlen respektieren und dürfen nicht in das Zwischenstrahlgewebe einwandern. Nur so kann das ursprüngliche alternierende Muster von Knochenstrahlen und Zwischenstrahlgewebe wieder hergestellt werden. Wie dies erreicht wird, war bisher völlig unklar. Hier konnte ich zeigen, dass bestimmte Regionen in der Epidermis Retinsäure abbauen und dadurch die Produktion eines Signals ermöglichen, welches die Osteoblasten an die richtigen Stellen im Regenerat lotst. Andere Zelltypen wiederum orientieren sich an den Osteoblasten.

Abbreviations

Adh	alcohol dehydrogenase
Aldh	aldehyde dehydrogenase
Bcl2	B-cell lymphoma
Bmp	Bone morphogenetic protein
Crabp	cellular retinoic acid-binding protein
Crbp	cellular retinol-binding protein
Cyp26	cytochrome P450 subfamily 26
Fgf	Fibroblast growth factor
hpa	hours post amputation
Igf	Insulin growth factor
P-D	proximo-distal
RA	retinoic acid
RAR	retinoic acid receptor
RARE	retinoic acid response element
Rbp	retinol-binding protein
RXR	retinoic X receptor
Shh	Sonic hedgehog
Stra6	stimulated by RA gene 6

Introduction

Regeneration in animals

Regeneration refers to the morphological and functional restoration of lost or damaged tissues or body parts. By contrast, although often misleadingly called homeostatic regeneration, homeostasis refers to the maintenance of tissues.

Regeneration has always fascinated the human imagination. The study of regeneration holds the potential of impacting human life by providing medical strategies to repair and replace injured tissues and organs. The idea of regrowing a lost or damaged body part can be traced back to the beginnings of civilization, and testimonies to that account can be found from the texts of acient Egypt to Greek mythology to Middle Age writings (Sánchez Alvarado, 2000; Tsonis, 2000). In the Greek mythology, Promethus regenerated its liver after each attack of a ravenous eagle and the many-headed hydra responded to Heracles` attacks by growing two new heads for every one that was cut off.

The ability for regeneration is widely but non-uniformly represented among all animal phyla (Brockes and Kumar, 2008; Sánchez Alvarado and Tsonis, 2006). Some invertebrate species are able to restore their entire body from a few remaining cells. Planarians are extreme in that they are capable to replace their whole body by a single so called c-neoblast (Wagner et al., 2011). The ability to restore all tissues and organs is restricted to few invertebrate species, but remarkable regenerative capacities can also be found among vertebrate species (Sánchez Alvarado and Tsonis, 2006). Teleost fish and amphibians can regenerate a variety of complex structures, e.g. their limbs and fins and the heart. Conversely, mammals have very limited capacities for regeneration. In general, regenerative abilities are higher in vertebrate embryos and larvae than in adults. For instance, anuran amphibians can regenerate their limbs as tadpoles, but are unable to do so after metamorphosis. Young mammals have some capacity to regenerate their digit tips, while this ability is largely lost in adults (Douglas, 1972; Masaki and Ide, 2007).

Despite the field of regenerative biology has made remarkable progress in identifying the underlying cellular and molecular mechanisms of regeneration, many fundamental aspects are still poorly understood und it has remained unclear why mammals are largely incapable of regenerating damaged body parts.

Basic mechanisms of regeneration

Regeneration of tissues and organs requires dramatic changes in cellular behavior. Different regenerative strategies are used in different scenarios, all resulting in the reestablishment of appropriate tissue structure and function and integration of polarity and positional identity cues with preexisting body structures (Sánchez Alvarado and Tsonis, 2006). Of note, the same tissue in different animals does not always regenerate in the same way. For instance, regeneration of the newt lens occurs via transdifferentiation from pigment epithelial cells at the tip of the dorsal iris, whereas during lens regeneration in *Xenopus* species, a new lens regenerate from the inner layer of the outer cornea (Henry and Tsonis, 2010). A further example is the restoration of muscle during amphibian limb regeneration. Myofiber dedifferentiation takes place during newt limb regeneration, while axolotls activate muscle stem cells (Sandoval-Guzmán et al., 2014).

Regeneration can occur by three major ways, that are not mutually exclusive (Gilbert, 2000; Sánchez Alvarado and Tsonis, 2006). The first mechanism, termed morphallaxis, proceeds without cell proliferation. During morphallaxis lost body parts are replaced by remodeling of the remaining part. Hydra head regeneration provides a typical example for morphallaxis. After decapitation, positional values are reset along the remaining body axis resulting in a properly patterned but smaller hydra. The second mechanism, termed epimorphic regeneration, involves formation of a mass of undifferentiated proliferating cells (the so-called blastema), that give rise to the different cell types and will reconstitute the lost part. Blastema cells can arise by two different mechanisms: activation of stem cells or dedifferentiation of mature cells. Epimorphic regeneration is characteristic of regenerating vertebrate appendages. A third type of regeneration, termed compensatory regeneration, is an intermediate type. Here, cells proliferate, but maintain their differentiated function. This type of regeneration is characteristic of the mammalian liver.

Appendage regeneration in vertebrates

Regeneration of lost limbs and fins in vertebrate is the most dramatic and prominent example for epimorphic regeneration. While the cellular and molecular mechanisms involved in limb and fin development are highly conserved among vertebrates, the responses upon amputation varies greatly (Sánchez Alvarado and Tsonis, 2006; Stoick-Cooper et al., 2007). Urodele amphibians can regenerate limbs throughout life and from any level along the proximo-distal (P-D) axis. During tadpole stages before metamorphosis, anuran amphibians can regrow their limbs perfectly (Slack et al., 2008; Suzuki et al., 2006). However, this ability gradually declines in

the term of pattern formation, resulting in incomplete regenerates. After metamorphosis, limb regeneration in anuran amphibians results in a cartilaginous protrusion referred to as spike. Birds cannot regenerate their limbs and wings, while some ability for limb regeneration has been reported in mammals. Regeneration of the digit tips occurs in neonatal mice as well as in embryos after amputation through the distal phalanx (Masaki and Ide, 2007; Reginelli et al., 1995), and cases of digit tip regeneration has also been reported in children (Douglas, 1972). Teleost fish regenerate their paired and unpaired fin throughout life, but they cannot regenerate internal skeletal elements at the base of their fins (Akimenko et al., 2003).

Appendage regeneration is a local response of the stump and in its most successful form generates a near-perfect copy of the lost appendage. Central questions in understanding appendage regeneration are: How does the limb or fin discriminate between a normal wound and an amputation? What are the cellular sources of the new tissues? What are the similarities and differences between blastema formation and malignant transformation and how does the evolution of central cellular growth and tumor suppressor mechanisms impacts regenerative capacity? How are the new tissues functionally integrated with the scale and size of the pre-existing tissues?

Zebrafish fin regeneration

Owing to its accessibility for genetic manipulation and simple anatomical structure, the zebrafish (*Danio rerio*) fin has emerged as a powerful model for unravelling the underlying cellular and molecular mechanisms of vertebrate appendage regeneration (Akimenko et al., 2003; Gemberling et al., 2013; Poss et al., 2003). Most studies have been performed on the caudal fin because it is easily accessible for manipulations and surgery and provides a large amount of tissues to examine compared to the other fins.

The zebrafish caudal fin consists of endoskeletal and exoskeletal elements, but only the exoskeletal elements can regenerate (Akimenko et al., 2003). The endoskeleton, located at the base of the fin, is made of endochondral bone and supports the exoskeleton. The exoskeletal is composed of 16-18 bony fin rays (called lepidotrichia) that run from proximal to distal and are separated by soft interray tissue. Each fin ray consists of two concave and opposed hemirays of accelular bone, which are made up of successive segments and held in place by nonmineralized ligaments (Fig. 1). Hemirays form via intramembranous ossification and surround a soft core of fibroblasts, osteoblasts, pigment cells, arterial blood vessels and nerves. The bone matrix is laid down by osteoblasts that cover the inner and outer bone surface. Fin rays are separated by boneless tissue, composed of fibroblasts, venous blood vessels, pigment cells and nerves.

The zebrafish caudal fin grows throughout life via the sequential, distal addition of new segments to each fin ray (Hall, 2010). Of note, hemiray thickness increases over time by adding new matrix along the entire fin length. With the exception of the most lateral, fin rays bifurcate at specific positions along the P-D axis. The most proximal segment of the fin ray is connected to muscles of the fin base by tendons. The external part of the fin is devoid of muscles.

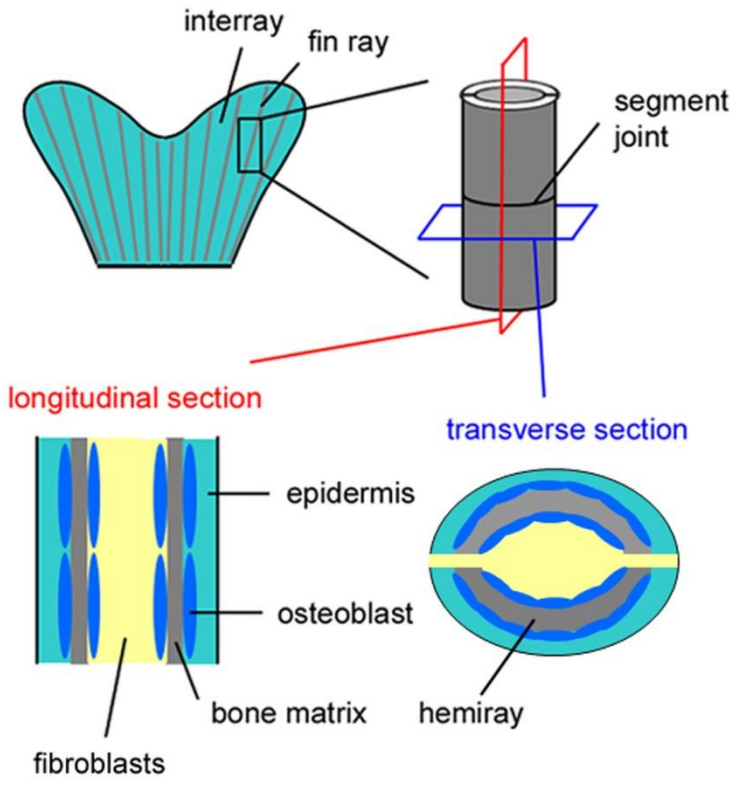

Fig.1. Overview of relevant structures and cell types of a fin ray.

Fin regeneration is a rapid, temperature dependent process (Akimenko et al., 2003; Gemberling et al., 2013; Poss et al., 2003). The zebrafish caudal fin regenerates faster at higher temperatures. Moreover, the more tissue removed, the faster is the regrow. This is clearly demonstrated when a "staircase" amputation is performed on a single fin. In such fins, regeneration proceeds fastest from the most proximal amputation site.

After amputation, the fin regrows within approximately two weeks through epimorphic regeneration. Due to the fin ray-interray structure of the fin, the regenerate consists of a succession of two types of structures: the dense ray blastema forming at the level of each fin ray surrounded by a loose blastema arising from the boneless interray tissue (Fig. 2). While ray blastema formation and restoration of the ray regions has been studied in much detail, regeneration of the interray regions is poorly understood. To avoid confusion, in this thesis, blastema only refers to the ray blastema.

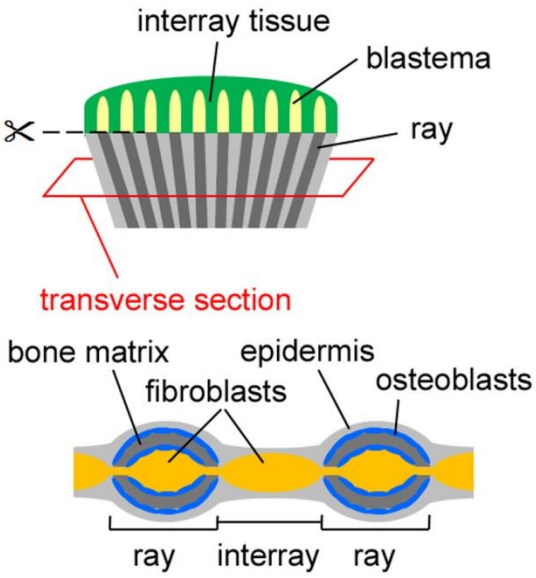

Fig.2. Overview of a regenerating fin.

Fin regeneration can be divided into three successive events very similar to those known from amphibian limb regeneration (Akimenko et al., 2003; Gemberling et al., 2013; Pfefferli and Jaźwińska, 2015a): (1) Wound healing and formation of a wound epidermis, (2) blastema formation and (3) regenerative outgrowth and repatterning (Fig. 3). In the following section each step will shortly be discussed.

(1) Wound healing and formation of a wound epidermis: Within the first 1-3 hours after amputation (hpa), wound healing is achieved by rapid apical migration of epidermal cells located lateral and proximal to the amputation site. This early response is independent of cell proliferation (Poleo et al., 2001). After amputation, there is very little bleeding and injured blood vessels have healed within 24 hours (Bayliss et al., 2006; Huang et al., 2003). Following wound closure, epidermal cells accumulate at the wound site to form a multilayered epidermal layer called wound epidermis. Wound epidermis formation is also dominated by cell migration (Poleo et al., 2001). Successful fin regeneration crucially depends on reciprocal interactions between the mature wound epidermis and blastema cells (Chablais and Jazwinska, 2010).

(2) Blastema formation (18-48 hpa): As the wound epidermis is forming, blastema formation is initiated in the stump mesenchyme. The fin blastema is a mass of lineage-restricted highly proliferating progenitor cells (Gemberling et al., 2013; Knopf et al., 2011; Stewart and Stankunas, 2012). During blastema formation, cells of the ray mesenchyme within one-two segment lengths two the amputation site start to proliferate and migrate towards the wound site where they accumulate into a blastema beneath the wound epidermis. The blastema forms essentially from fibroblasts of the stump connective tissue and from stump osteoblasts via dedifferentiation of mature cells. In addition, a small population of pigment cell precursors that

arise from stem cells contribute to the blastema. Finally, blood vessels and nerves grow into the mature blastema.

(3) Regenerative outgrowth and repatterning (48 hpa to approximately two weeks after amputation): Following blastema formation, regenerative outgrowth is marked by changes in proliferation, morphological, and molecular profiles (Akimenko et al., 2003; Pfefferli and Jaźwińska, 2015b). During regenerative outgrowth, the regenerate comprises a mature blastema in its distal portion and a differentiating region in its proximal portion. Thus, differentiation progresses in a distal-to-proximal direction, so that fast cycling blastema cells in the distal region become slow-cycling cells in more proximal regions, which subsequently mature to pattern the new fin tissue. As during fin growth, fin rays regenerates by the successive distal addition of new segments.

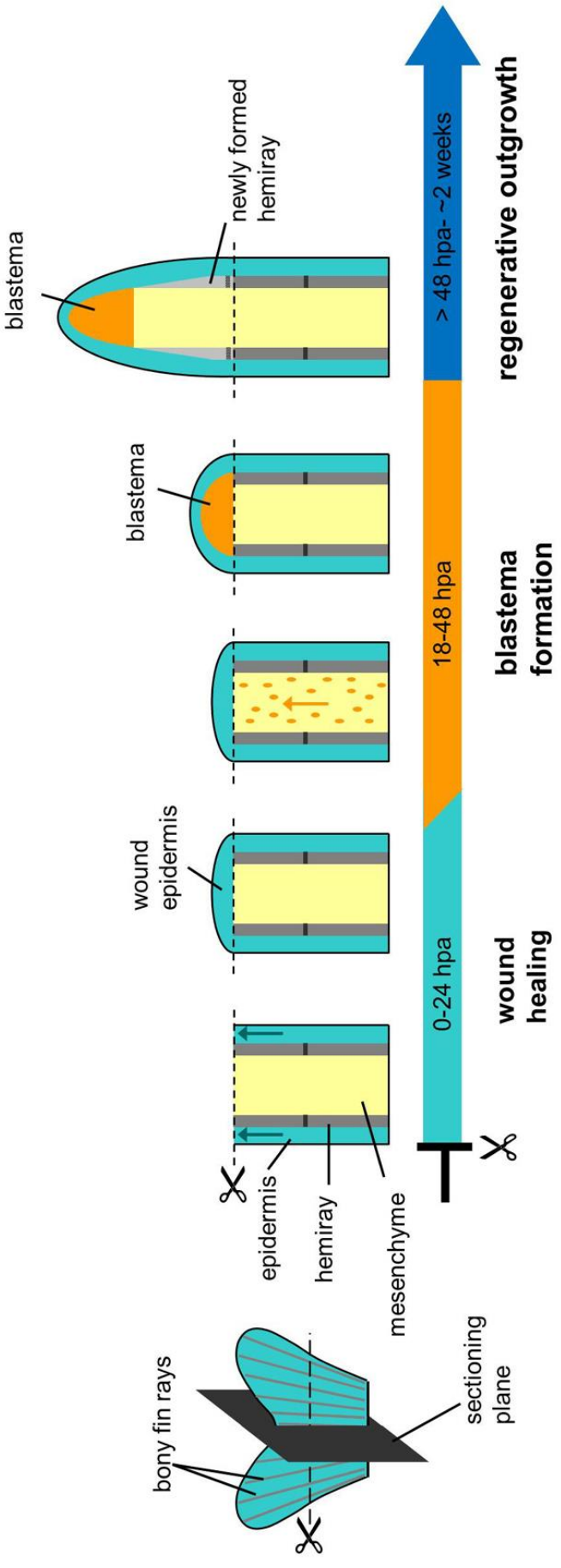

Fig.3. Stages of fin regeneration depicted as longitudinal section through a regenerating fin ray.

The retinoic acid signaling pathway

Retinoic acid (RA) is a low molecular weight, lipophilic signaling molecule derived from retinol (vitamin A). Vitamin A is best known for its requirement for vision, as isomerization of its derivative retinaldehyde triggers the phototransduction process in photoreceptors of the retina (Parker and Crouch, 2010). Vitamin A deficiency in vertebrate embryos leads to wide and complex spectrum of developmental abnormalities (Niederreither and Dolle, 2008; Rhinn and Dollé, 2012). The molecular basis of vitamin A action during development was clarified when it was demonstrated, that its metabolite RA, activates target gene expression by acting as a ligand for RA receptors (RARs). Owing to its ability to modulate transcription, RA regulates a variety of processes during embryogenesis and in adults such as organogenesis and embryonic pattern formation, tissue homeostasis and immunity (Duester, 2008; Kam et al., 2012; Niederreither and Dolle, 2008; Rhinn and Dollé, 2012).

Animals cannot synthesize vitamin A de novo. β-carotene, a vitamin A precursor, or vitamin A has to be taken up by dietary sources. Since vitamin A is lipophilic, distribution via the circulatory system requires binding to retinol binding proteins (Rbp) and complex formation with transthyretin (Ttr) (Duester, 2008; Theodosiou et al., 2010). The transmembrane protein Stra6 (stimulated by retinoic acid gene 6) mediates uptake of the retinol-RBP-Ttr complex (Kawaguchi et al., 2007). Intracellular retinol is bound to cellular retinol-binding proteins (Crbp) (Theodosiou et al., 2010). RA is synthesized from retinol via two consecutive oxidations (Fig. 4). During the first step, retinol is oxidized to retinaldehyde either by microsomal retinol dehydrogenases (Rdhs) or by cytosolic alcohol dehydrogenases (Adhs). Alternatively, retinaldehyde can be synthesized by cleavage of β-carotene, a reaction carried out by β-carotene oxygenases. Subsequently, retinaldehyde is further oxidized to RA by up to three retinaldehyde dehydrogenases, which are commonly referred to Raldh1-3. Under the new nomenclature for aldehyde dehydrogenases (Aldhs), these proteins have been renamed to Aldh1as (Vasiliou et al., 1999). Zebrafish possess only two Aldh1a enzymes (Aldh1a2 and Aldh1a3) (Pittlik et al., 2008). Whereas, retinaldehyde can be reduced to retinol by members of the dehydrogenase/reductase SDR family, the oxidation of retinaldehyde to RA is an irreversible process (Duester, 2008; Theodosiou et al., 2010). Although, there are two biologically active isomers of RA, all-*trans* RA and 9-*cis* RA, 9-*cis* RA is not detected during mouse development and a putative biological function has been discussed controversially (Kane, 2012; Mic et al., 2003). all-*trans* RA acts as a ligand for RARs. Mammals have three RARs (RARα, RARβ, and RARγ), whereas zebrafish possess four genes (*raraa*, *rarab*, *rarga*, and *rargb*) (Waxman and Yelon, 2007). RARs are steroid receptor and form heterodimers with the retinoid X receptor (RXRs) (Rhinn and Dollé, 2012). The RAR/RXR heterodimer binds to RA response elements (RAREs) in regulatory regions upstream of target genes. In the absence of RA, RAR/RXRs act as transcriptional repressors by recruiting a complex of corepressor proteins.

Binding of RA to RAR/RXR leads to dissociation of the corepressors complex and recruitment of coactivators resulting in target gene expression.
RA can be degraded into more polar metabolites such as 4-*oxo*-RA and 4-OH-RA by enzymes of the cytochrome P450 subfamily 26 (Cyp26a1, Cyp26b1, and Cyp26c1). Cellular RA-binding proteins (Crabp-I and -II) are thought to provide robustness to RA signaling as they are capable to either sequester RA and transfer it to Cyp26s (Crabp-I) or to deliver it to RARs (Crabp-II).

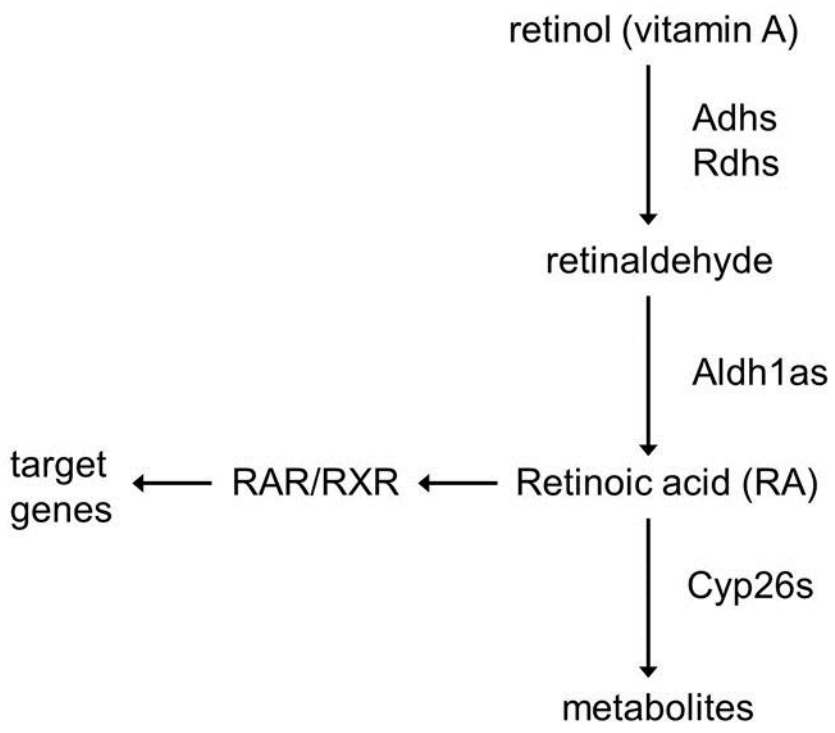

Fig. 4. Simplified overview of RA synthesis, degradation and signaling.

Retinoic acid signaling in regeneration

One major goal in regeneration research is to gain a thorough knowledge of the underlying signaling networks of regeneration. RA has long been associated with regeneration due to the ability of exogenous RA treatment to induce "super-regeneration" in amphibian limbs, the formation of supernumerary elements along the P-D limb axis (Maden, 1982; Maden, 1983; Maden and Hind, 2003). However, it has remained unresolved whether RA has an endogenous function during vertebrate appendage regeneration. For detailed information about the phenomenon of "super-regeneration" and our current understanding on RA signaling functions in regeneration, the reader is referred to chapter 2.

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Synopsis

Thesis topic

By using the regenerating caudal fin of zebrafish as model system, I proved a putative involvement of RA signaling in vertebrate appendage regeneration and explored its specific functions during fin regeneration.
The findings of this thesis are presented in the form of a cumulative thesis, consisting of three published research articles (chaper 1,3 and 4) and one published review article (chapter 2), and are summarized below.

Chapter 1

In chapter 1, I demonstrate that RA signaling is indispensable for adult zebrafish fin regeneration, thereby providing for the first time evidence that RA signaling has an endogenous function in adult vertebrate appendage regeneration.
By genetic and pharmacological manipulations, I investigated the cellular and molecular consequences of loss- and gain of RA signaling for fin regeneration. I show that fin amputation induces upregulation of RA synthesis in the stump mesenchyme where it controls the expression of ligands of the Fgf, Wnt/β-catenin and Igf signaling pathway, which have previously been identified as crucial regulators of blastema formation and wound epidermis maturation. Inhibition of RA signaling upon fin amputation causes suppression of blastema formation by preventing cell cycle entry of postmitotic stump cells and interferes with formation of a proper wound epidermis. In the established blastema, RA signaling remains high and drives regenerative outgrowth by supporting blastema proliferation. Besides a putative, more direct mitogenic function, RA signaling indirectly regulates 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.
Cell cycle reentry of postmitotic cells and dedifferentiation are characteristics of malignant transformation, raising the question of why blastema cells are not eliminated through tumor suppressor mechanisms. My findings indicate that blastema cells evade cell death by elevated levels of the anti-apoptotic factor Bcl2, the expression of which is positively regulated by RA signaling.

Together, this study support the hypothesis that RA signaling is an essential component of vertebrate appendage regeneration and provides mechanistic insights of RA signaling in wound epidermis formation, blastema formation and blastema maintenance in the regenerating fin.

Chapter 2

In chapter 2, I integrate the gained insights of RA signaling in fin regeneration with what is known more generally about RA signaling in animal regeneration and discuss a putative involvement of RA signaling in amphibian limb regeneration.

Chapter 3

RA is a diffusible signaling factor, raising the question in which cell types RA signaling acts during fin regeneration and how cell lineage-specific programs are protected from regenerative crosstalk between neighboring fin tissues. In chapter 3, I show how bone regeneration is achieved against a background of massive RA synthesis during fin regeneration.
Osteoblasts revert from a non-cycling, mature to a cycling, immature preosteoblastic state during blastema formation. During regenerative outgrowth, preosteoblasts finally redifferentiate into mature bone matrix producing osteoblasts. My findings demonstrate that RA signaling promotes bone matrix synthesis and osteoblast proliferation while inhibiting switching between the mature and immature state. Upon fin amputation, stump osteoblasts that will participate in blastema formation, counteract raising RA levels by upregulation of the RA degrading enzyme *cyp26b1*. This elegant mechanism allows the establishment of an osteoblast progenitor pool in a high RA environment that is required for blastema formation. RA dependent proliferation of preosteoblasts is ensured by downregulation of *cyp26b1* upon dedifferentiation. Redifferentiation of preosteoblasts is controlled by a presumptive RA gradient, in which high RA levels towards the distal tip of the regenerate inhibit differentiation and promote proliferation. This might be achieved through repression of Bmp signaling and promotion of Wnt/β-catenin signaling. Fibroblasts-like blastema cells in more proximal regions lower local RA concentrations via Cyp26b1 activity, thereby ensuring redifferentiation of osteoblasts. This allows two processes to run in parallel: Proliferation for the continuous supply of osteoblasts in the distal part and redifferentiation of osteoblasts more proximally where the fin rays re-emerge. In addition, my findings indicate that proper hemiray regeneration requires the interplay between bone matrix-producing osteoblasts and bone-resorbing osteoclasts and

suggest that RA signaling controls formation of new bone matrix at two levels, by ensuring matrix synthesis by osteoblasts and by preventing resorption by osteoclasts.
In summary, this study reveals how RA signaling orchestrates osteoblast behavior throughout all stages of fin regeneration and unravel a so far unnoticed important role of bone resorption by osteoclasts in fin regeneration.

Chapter 4

Fin rays are separated by soft interray tissue. This pattern has to be re-established during regeneration. However, the mechanisms that confine osteoblasts to only extend the existing rays have remained unresolved. Having shown in chapter 3 that the osteoblast regenerative program depends on the tight regulation of RA levels, chapter 4 addresses a putative involvement of RA signaling in the spatial regulation of bone regeneration and ray-interray patterning.
Upon dedifferentiation, preosteoblasts migrate into the nascent blastema, where they remain restricted to proximal lateral positons. My findings show that epidermal niches of low RA levels, established by Cyp26a1, allow the spatially restricted production of a signal that pilots preosteoblasts to target regions. Disruption of these niches causes preosteoblasts to ignore ray-interray boundaries and to invade interrays where they form ectopic bone. Moreover, it emerged that osteoblasts themselves exert a piloting function for non-osteoblastic blastema cells and blood vessels. During regenerative outgrowth, the *cyp26a1*-expressing niches remain required for the production of Shh which in turn promotes osteoblast proliferation. Finally, my data indicate that *cyp26a1* expression is spatially confined by Fgf signaling.
In summary, this study uncovers the mechanism that compels osteoblasts to respect ray-interray boundaries and explain how the alternating pattern of rays and interray tissue becomes re-established during fin regeneration.

Own contributions

Chapter 1:

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

Nicola Blum and Gerrit Begemann

Published research article, *Development* (2012) 139,107-116.

I conceived the study, designed and performed the experiments, analyzed and interpreted the data, prepared the figures and wrote the manuscript. G.B. edited the manuscript.

Chapter 2:

The roles of endogenous retinoid signaling in organ and appendage regeneration

Nicola Blum and Gerrit Begemann

Published review article. *Cellular and Molecular Life Sciences.* (2013) 70, 3907–3927.

I wrote the manuscript jointly with G.B. G.B. prepared the figures.

Chapter 3:

Osteoblast de- and redifferentiation are controlled by a dynamic response to retinoic acid during zebrafish fin regeneration

Nicola Blum and Gerrit Begemann

Published research article, *Development* (2015) 142, 2894-2903.

I conceived the study, designed and performed the experiments, analyzed and interpreted the data, prepared the figures and wrote the manuscript. G.B. edited the manuscript.

Chapter 4:

Retinoic acid signaling spatially restricts osteoblasts and controls ray-interray organization during zebrafish fin regeneration

Nicola Blum and Gerrit Begemann

Published research article, *Development* (2015) 142, 2888-2893.

I conceived the study, designed and performed the experiments, analyzed and interpreted the data, prepared the figures and wrote the manuscript. G.B. edited the manuscript.

Chapter 1

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

Nicola Blum and Gerrit Begemann

Published research article, *Development.* 139, 107-116 (2012)

Abstract

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 blastemal 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 blastemal 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.

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; Becerr 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.

Material and Methods

Zebrafish husbandry and fin amputation

Zebrafish strains of Konstanz wild types and the transgenic lines [*Tg(hsp70l:dn-fgfr1)*]^{pd1} (Lee et al., 2005), [*Tg(hsp70l:dn-zrar-egfp)*]^{pd18} (Kikuchi et al., 2011) and [*Tg(hsp70l: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.

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 CoCl2, pH 7.2]. The buffer was subsequently replaced with TdT buffer containing 0.5 □M 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 50x100 µm in the mesenchyme at 32 hours post-amputation (hpa). For quantification at 3 and 4 days postamputation (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 blastemal 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 blastemal (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.

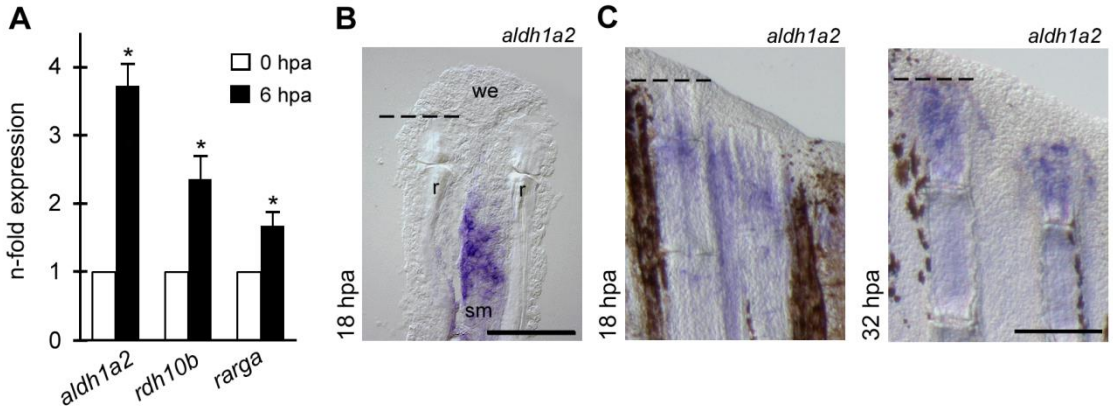

Fig. 1. Fin amputation induces RA synthesis in the stump tissue. (A) qPCR determination of *aldh1a2*, *rdh10b* and *rarga* transcript levels at 6 hpa relative to uncut (0 hpa) fins. Error bars, s.e.m. *, *P*<0.01. (B,C) In situ hybridization on longitudinal section (B) and whole fins (C) demonstrates *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: 100µm in B; 200mm in C.

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 transgenic zebrafish line that allows heat shock-inducible degradation of endogenous RA. The

Tg(hsp70l: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.

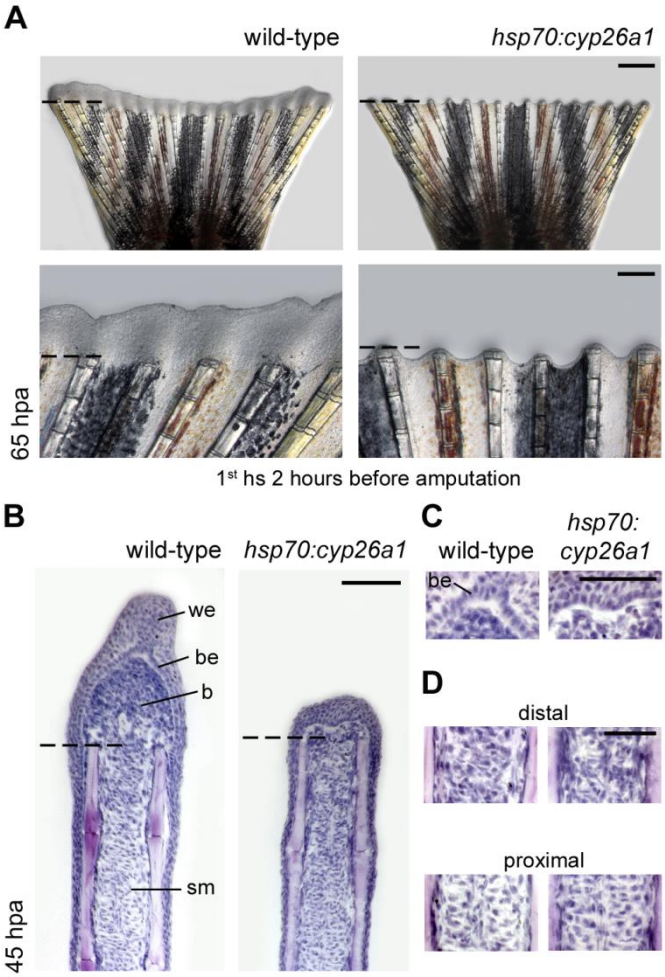

Fig. 2. RA signaling is necessary for blastema formation. (A) Inhibition of RA signaling in *hsp70:cyp26a1* fish by applying daily heat shocks (commencing 2 hours before fin amputation) results in an early and complete block to fin regeneration. (**B-D**) Hematoxylin-stained longitudinal sections indicate 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 wildtype 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.

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 blastemal 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 wildtype 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 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.

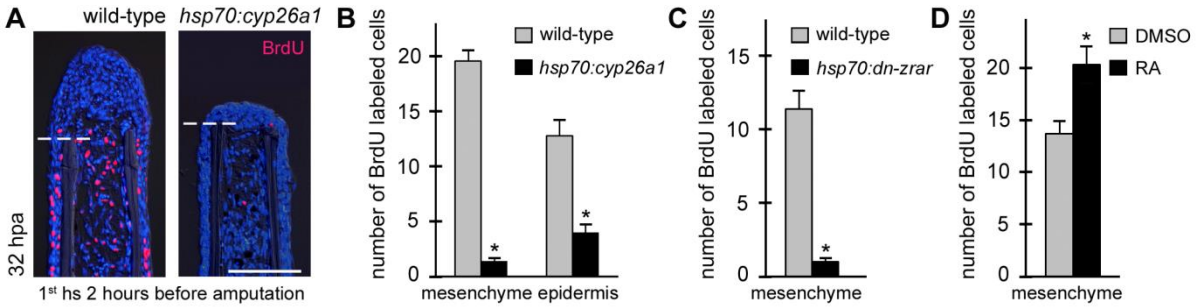

Fig. 3. Induction of cell proliferation in the fin stump requires RA signaling. (A-C) Inhibition of RA signaling in *hsp70:cyp26a1* and *hsp70:dnzrar* fish results in a significant decrease in proliferating cells in the fin stump (daily heat shocks, commencing 2 hours before amputation). (A) Longitudinal 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.

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* expression in the fin stump. Loss of RA in unamputated fins had no effect on baseline *igf2b* expression, whereas regenerationinduced *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.

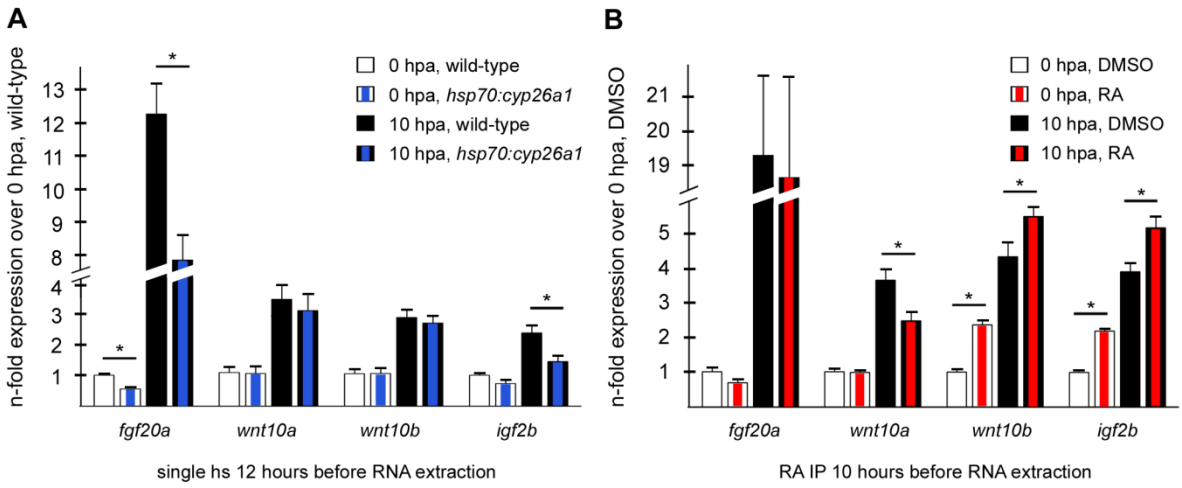

Fig. 4. RA signaling regulates Fgf, Wnt/β-catenin and Igf signaling in the fin stump. qPCR determination of *fgf20a*, *wnt10a*, *wnt10b* and *igf2b* expression levels under altered RA signaling. (**A**) Expression levels in *hsp70:cyp26a1* fins at 0 and 10 hpa relative to wild-type control at 0 hpa (single 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.

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 blastemal 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 blastemal 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 blastemal 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. 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 vehicleinjected fish (Fig. 7D). These results reveal a strong RA-mediated pro-survival mechanism in blastema cells that is mediated by upregulation of *bcl2* expression.

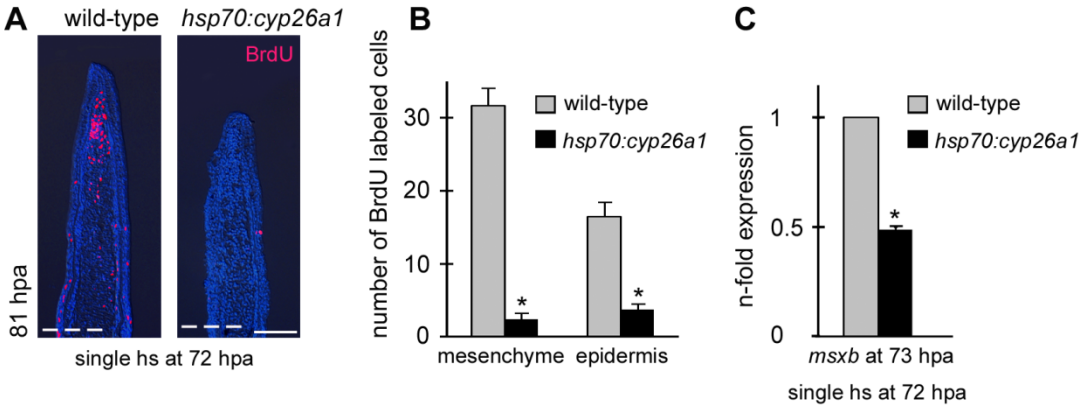

Fig. 5. RA signaling is required for blastema proliferation. Inhibition of RA signaling in *hsp70:cyp26a1* fish, when instigated during regenerative outgrowth, results in downregulation of *msxb* expression and loss of blastema proliferation. (**A**) Longitudinal sections stained for BrdU and with DAPI 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.

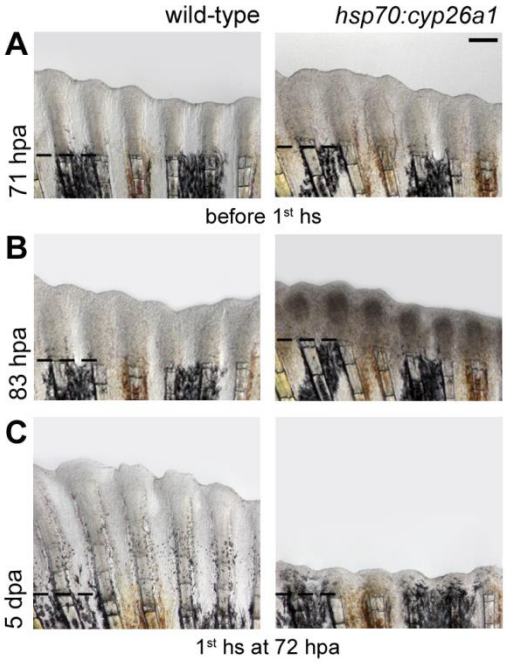

Fig. 6. RA signaling is essential for regenerative outgrowth and maintenance of the regenerate. Inhibition of RA signaling in *hsp70:cyp26a1* fish during regenerative outgrowth blocks further regeneration and abolishes maintenance of the regenerate (daily heat shocks, first heat shock at 72 hpa). (A) 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.

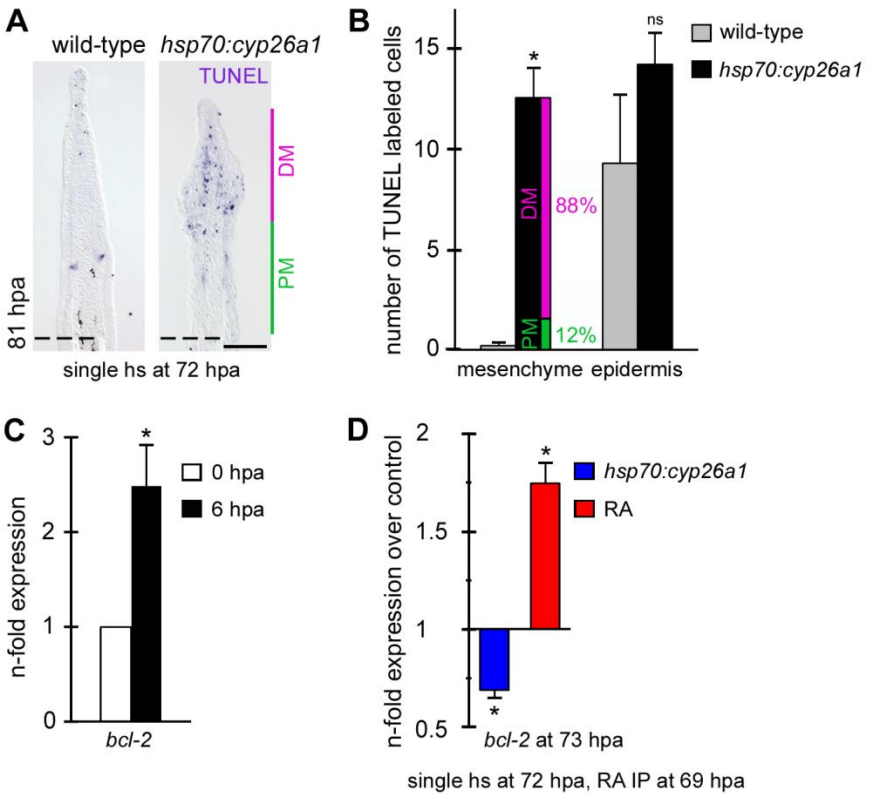

Fig. 7. Blastema cells possess a strong RA-mediated pro-survival mechanism. (A,B) Loss of RA signaling during regenerative outgrowth causes cell death in the regenerate after a single heat shock at 72 hpa. (A) TUNEL staining on longitudinal sections at 81 hpa reveals massive cell death in the distal 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.

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

To gain insights into the gene network underlying blastemal 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 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 b 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 noncanonical 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.

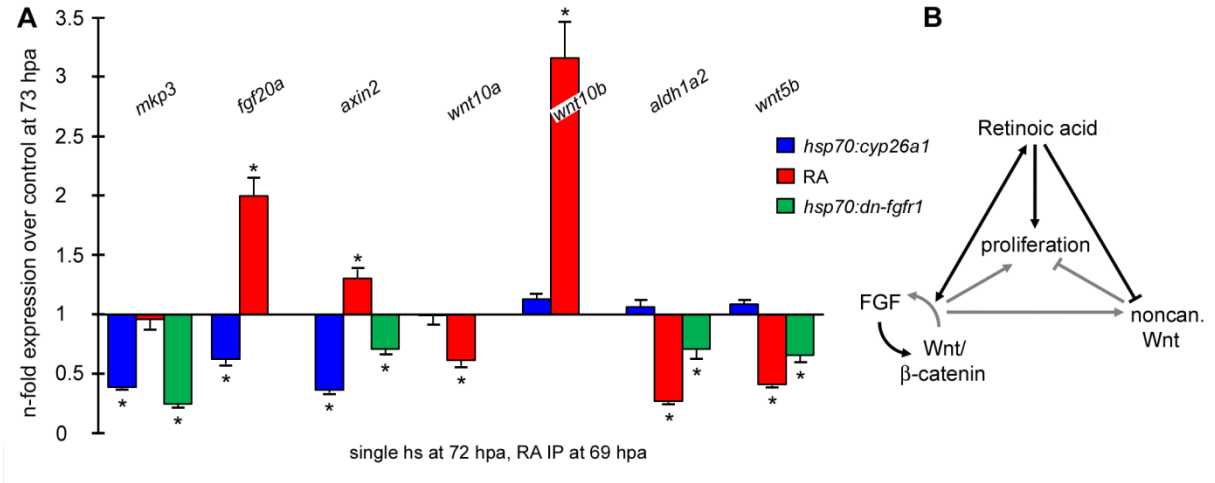

Fig. 8. A complex molecular regulatory network underlies blastema proliferation. (A) qPCR determination of RA, Wnt and Fgf pathway components and targets during regenerative outgrowth in RA-treated (control is vehicle-treated fish), *hsp70:cyp26a1* and *hsp70:dn-fgfr1* (control is heat-shocked wild-type 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 blastemal 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.

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 blastemal formation in the stump tissue. Strong *aldh1a2* expression is rapidly induced in the stump mesenchyme, indicating that cells that will give rise to the blastemal become exposed to high RA levels within the first few hours after 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 blastemal precursors into the cell cycle. Conversely, exogenous RA is sufficient to promote mesenchymal proliferation, suggesting that RA signaling confers mitogenic activity during blastemal formation. In addition, it might control an earlier step in blastemal 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 blastemal 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 blastemal 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 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 PD 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.

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Supplementary material

Table S1. qPCR primers

Gene	Forward primer	Reverse primer
aldh1a2	GAGAGAGACAGTGCTTACCTTGC	CACAAAGAAGCAGGGGAGG
axin2	GCAGCACAGTTGATAGCCAG	GTCTTGGCTGGCACATATCC
bactin1	TTGCTCCTTCCACCATGAAG	CTTGCTTGCTGATCCACATC
bcl-2	AAGCGAGGATATGTGTGG	CAGTCAAAAGTGTGGCAG
cyp26a1	GATGGGAGCTGATAATGTG	CCTGAACCTCCTCTCTGACC
ef1a	TACGCCTGGGTGTTGGACAAA	TCTTCTTGATGTATCCGCTGAC
fgf20a	AAAAGCTGTCAGCCGAGTGT	TGGACGTCCCATCTTTGTTG
gapdh	GTGGAGTCTACTGGTGTCTTC	GTGCAGGAGGCATTGCTTACA
igf2b	GCAGGTCATTCCAGTGATGC	TCTGAGCAGCCTTTCTTTGC
mkp3	GACTTGTGGAGCGGAGGAC	CCTCTCTTCTCTCGTCGTCG
msxb	CATCTTTCACATCTCCTCCTCG	CTTTCGCCCTCCTGTTCTG
rarga	CAAGAGCAAAAGCAGCAGG	GAATACTGCGACGGAAGAAAC
rdh10a	CAACCCGATGTCAAAAGAGG	CCAACCTCCCTACGCACTTTC
rdh10b	GGGACATCAACCGACAGAG	GTCTCCCACCTCACTGCG
wnt10a	ATTCACTCCAGGATGAGACTTCATA	GTTTCTGTTGTGGGCTTTGATTAG
wnt10b	TGAGCAGCACACCTTCATC	TGGAGAGAAACGGATAAACAGAC
wnt5b	GCCGCCTATGCAACAAGAC	GCACACAAACTGGTCTACGAG

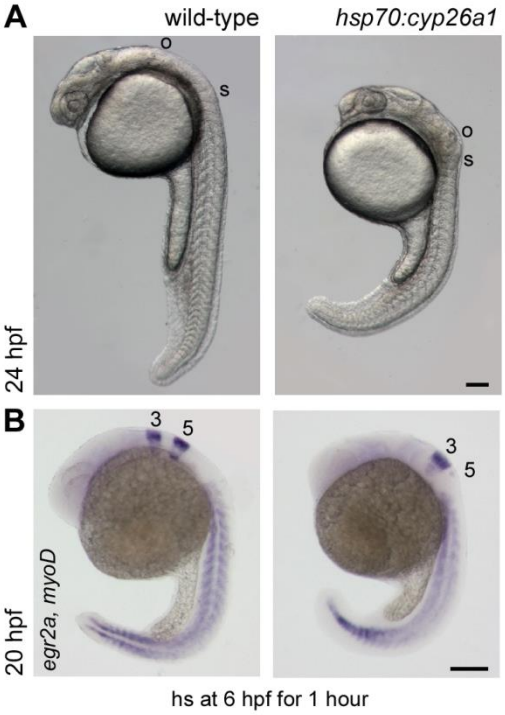

Fig. S1. The *hsp70:cyp26a1* line efficiently inhibits RA signaling. (A) Heat shock of *hsp70:cyp26a1* embryos for 1 hour starting at 6 hpf results in close proximity of the first somite to the otic vesicle and a kink at the head-trunk boundary at 24 hpf, representative of complete loss of RA signaling. (B) Whole mount in situ hybridization with *egr2a*, a marker of hindbrain rhombomeres 3 and 5, illustrates absence of rhombomere 5 in *hsp70:cyp26a1* embryos, caused by posterior expansion of more anterior rhombomeres. hs, heat shock; o, otic vesicle; s, first somite; 3, rhombomere 3; 5, rhombomere 5. Scale bars: 100 µm.

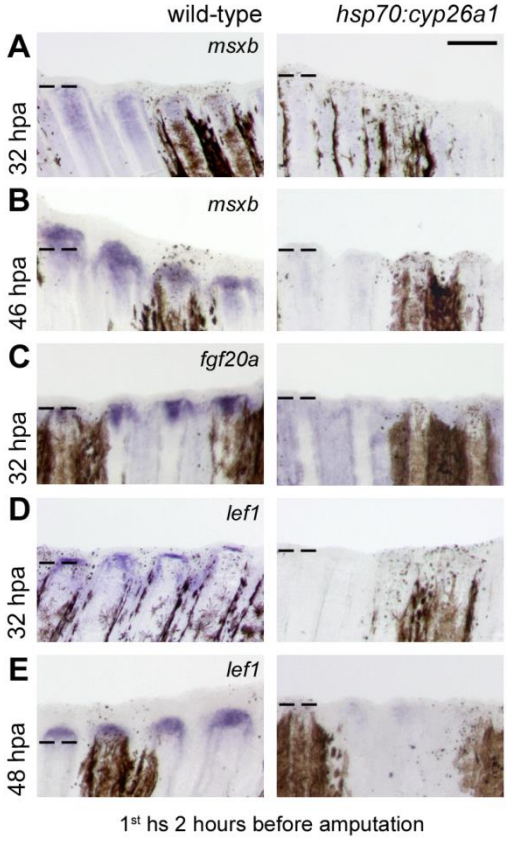

Fig. S2. Expression of markers for the blastema and the basal epidermal layer are impaired in the absence of RA signaling. Whole mount in situ hybridization demonstrates that neither the blastema nor the basal epidermal layer are formed or specified correctly in heat-shocked *hsp70:cyp26a1* fish (daily heat shocks, 1st heat shock 2 hours before amputation). (A-C) In wild-type regenerates, *msxb* and *fgf20a* are strongly expressed in cells of the forming blastema at 32 or 46 hpa. Expression of both genes is undetectable in regenerates of *hsp70:cyp26a1* fish. (D-E) Expression of *lef1*, which marks the basal epidermal layer and distal blastema cells in wild-type regenerates, is absent in *hsp70:cyp26a1* fins at 32 and 48 hpa. Dashed lines indicate the amputation plane. hs, heat shock; Scale bar: 200 µm.

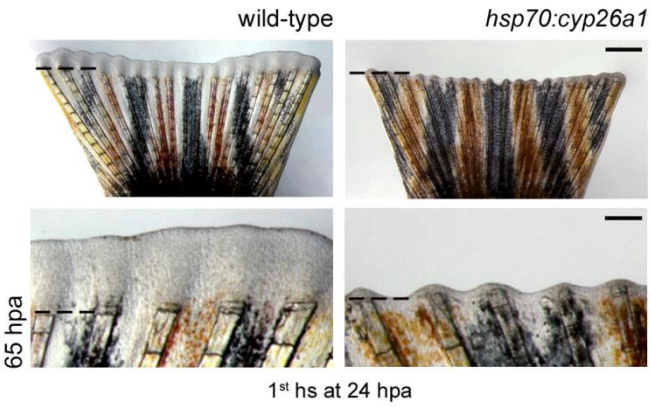

Fig. S3. Impaired blastema formation in RA-deficient regenerates is independent of the wound healing process. Inhibition of RA signaling in *hsp70:cyp26a1* fish after wound healing has taken place results in a complete block of fin regeneration (daily heat shocks, 1st heat shock at 24 hpa). Dashed lines indicate the amputation plane. hs, heat shock; Scale bars: 500 µm upper panel; 200 µm lower panel.

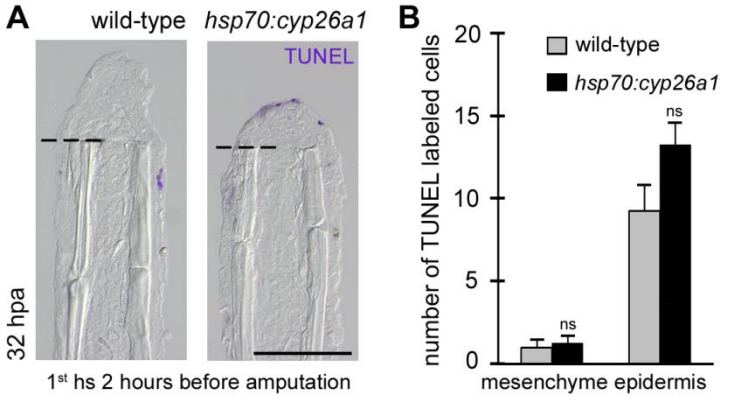

Fig. S4. Inhibition of RA signaling does not enhance cell death during blastema formation. Inhibition of RA signaling in *hsp70:cyp26a1* fish does not enhance the number of TUNEL positive cells in the fin stump (daily heat shocks, 1st heat shock 2 hours before amputation). (A) Longitudinal sections stained for TUNEL show few TUNEL positive cells in the epidermis of wild-type and *hsp70:cyp26a1* fins at 32 hpa. (B) Quantification of TUNEL-labeled cells within a defined area at 32 hpa (wild-type: n=32; *hsp70:cyp26a1*: n=25). Error bars, SEM. ns, not significant. Dashed lines indicate the amputation plane. hs, heat shock; Scale bar: 100 µm.

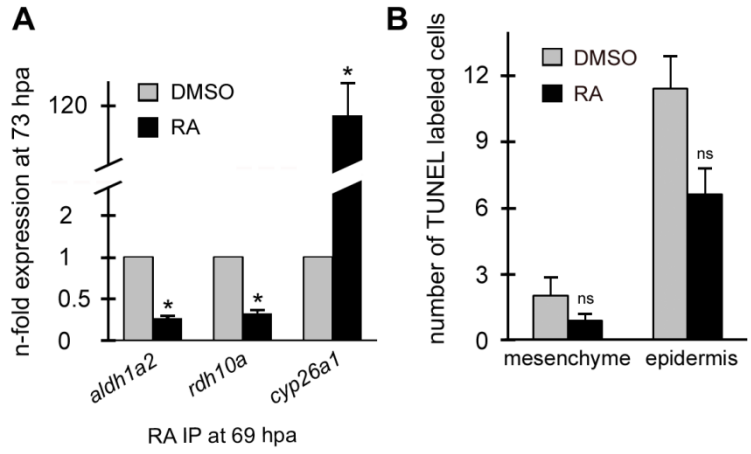

Fig. S5. Intraperitoneal injection of RA efficiently enhances RA signaling in the regenerating fin but does not enhance cell death. (A) Increased RA signaling after intraperitoneal (IP) injection of RA causes decreased expression of RA synthesizing enzymes and enhanced expression of *cyp26a1*. qPCR determination of *aldh1a2, rdh10a and cyp26a1* transcript levels in regenerates of RA-treated fish relative to control (DMSO injected) fish at 73 hpa (single RA IP at 69 hpa). (B) IP injection of 1 mM RA does not enhance cell death (RA IP every 12 hours, 1st IP at 3 dpa). Quantification of TUNEL labeled cells at 4 dpa (DMSO: n=13; RA: n=12). Error bars, SEM. *P<0.0001.

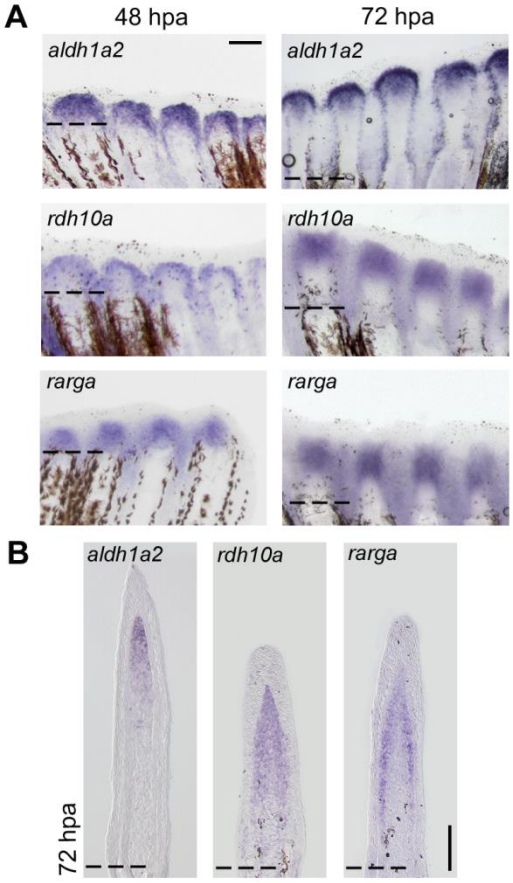

Fig. S6. RA pathway components are highly expressed during regenerative outgrowth. Whole mount in situ hybridization at 48 and 72 hpa (A) and in situ hybridization on longitudinal sections at 72 hpa (B) reveals expression of *aldh1a2*, *rdh10a* and *rarga* in the blastema. *rarga* and *rdh10a* transcripts can be detected in the whole blastema at 72 hpa, whereas *aldh1a2* expression is more restricted to distal blastema cells. Dashed lines indicate amputation plane. Scale bars: 200 µm in A; 100 µm in B.

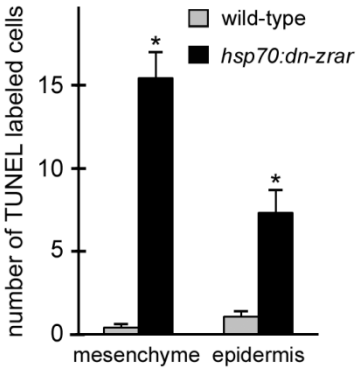

Fig. S7. Blastema cells rapidly die after a single heat shock in *hsp70:dn-zrar* fish. Quantification of TUNEL labeled cells in *hsp70:dn-zrar* regenerates at 79 hpa (single heat shock at 72 hpa; wild-type: n=25; *hsp70:dn-zrar*: n= 19) Error bars, SEM. *P<0.0001.

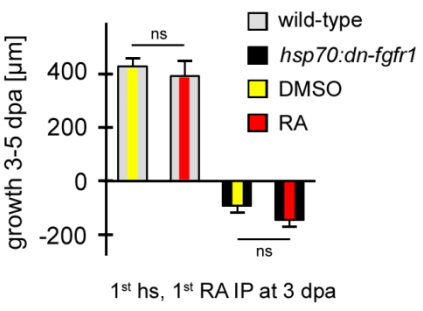

Fig. S8. RA treatment fails to rescue regenerative outgrowth in heat-shocked *hsp:dn-fgfr1* fins. Enhancing RA signaling in heat-shocked *hsp70:dn-fgfr1* fins does not rescue morphological growth. Determination of increase/decrease of fin tissue distal to the amputation plane between 3 and 5 dpa in RA and/or vehicle injected, heat-shocked wild-type and *hsp70:dn-fgfr1* fish. The first heat shock was applied at 72 hpa, injections were given daily starting with the first at 70 hpa. Error bars, SEM. ns, not significant. hs, heat-shock; IP, intraperitoneal.

Chapter 2

The roles of endogenous retinoid signaling in organ and appendage regeneration

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Abstract

The ability to regenerate injured or lost body parts has been an age-old ambition of medical science. In contrast to humans, teleost fish and urodele amphibians can regrow almost any part of the body with seeming effortlessness. Retinoic acid is a molecule that has long been associated with these impressive regenerative capacities. The discovery 30 years ago that addition of retinoic acid to regenerating amphibian limbs causes “super-regeneration” initiated investigations into the presumptive roles of retinoic acid in regeneration of appendages and other organs. However, the evidence favoring or dismissing a role for endogenous retinoids in regeneration processes remained sparse and ambiguous. Now, the availability of genetic tools to manipulate and visualize the retinoic acid signaling pathway has opened up new routes to dissect its roles in regeneration. Here, we review the current understanding on endogenous functions of retinoic acid in regeneration and discuss key questions to be addressed in future research.

Introduction

The ability of some animals to regenerate whole organs has always fascinated and inspired artists and scientists alike. Even though the ability to regrow lost tissues is widely distributed within the animal kingdom [1, 2], examples of organ regeneration in amniotes in the wild remain sparse.

Among vertebrates, urodele amphibians and teleost fish are the true champions of regeneration, being able to regrow whole organs including appendages, the brain and spinal cord, the liver and the heart throughout life. Anuran tadpoles possess similar regenerative capacities that are, however, absent in adult frogs. Autotomy of the tail is widespread among lizards, along with the ability to regenerate parts of the severed tail [3, 4]. While birds seem to be incapable of regenerating any body parts, adult mammals are capable of liver regeneration. Furthermore, the mammalian heart is able to partially regenerate before and during a short time period after birth [5] and digits can regenerate postnatally and to some extent even in adults [6]. The ability to shed and regenerate patches of skin may have evolved to escape predation in African spiny mice [7].

One of the hopes of regeneration research is to uncover ways to improve regeneration of damaged tissues and organs or even induce regeneration of non-regenerating body parts in humans. If this is to become a feasible goal, a thorough knowledge of the underlying molecular mechanisms in animals capable of this remarkable feat is essential and should eventually lead to an understanding why it fails in humans.

The vitamin A metabolite retinoic acid (RA) is an important regulator of vertebrate development [8–10]. Thirty years ago, it was first discovered that the addition of retinoids to the amputated amphibian limb generates duplications of proximal skeletal elements [11, 12]. Since then, the ability of RA to respecify positional identity during limb regeneration has been the focus of intense investigation and prompted research into its effects on other regenerating organs and cell types [13, 14]. Despite progress in understanding the effects of increased retinoid signaling during regeneration, it had long been neglected to investigate whether retinoids are truly required for regeneration.

The identification of the genes involved in retinoid and particularly RA metabolism and the possibility to examine the consequences of manipulating and visualizing RA signaling in genetically accessible organisms have now led to a better understanding of the roles of endogenous RA during regeneration. In this review, we highlight recent findings on the roles of endogenous retinoids during regeneration. Our main focus will be on organ and appendage regeneration in vertebrates, while a look at invertebrate regeneration will add to the picture of endogenous retinoid signaling in regeneration. We would like to point the reader towards a number of topical review articles that to varying extents touch upon retinoid signaling in tissues and organs that could not be covered in this review. These

include the roles of RA during lung regeneration [15, 16] and regeneration in the central nervous system [17–22].

A short guide to the retinoic acid signaling machinery

RA is a small lipophilic molecule derived from retinol (vitamin A). The acidic vitamin A metabolite RA acts as a ligand for transcription factors that regulate a variety of processes, including organogenesis, embryonic pattern formation, cell proliferation and differentiation, cell death, immunity and tissue homeostasis [23, 24]. Since animals cannot synthesize vitamin A de novo, retinol or its precursor β-carotene has to be taken up through dietary sources. Typically, retinol is distributed via the circulatory system bound to retinol-binding protein (Rbp), and this complex is associated with another protein, transthyretin (Ttr) [9, 25] (Fig. 1). Binding of Ttr to Rbp prevents the elimination of retinol in the kidney. Retinol-Rbp-Ttr is taken up by target cells via the multitransmembrane protein Stra6 (stimulated by *retinoic acid gene 6*) [26]. Intracellular retinol is bound to cellular retinol-binding proteins (Crbp) [23]. Synthesis of RA from retinol requires two consecutive enzymatic reactions: Initially, retinol is oxidized to retinaldehyde, a step carried out either by cytosolic alcohol dehydrogenases (Adhs) or by retinol dehydrogenases (Rdhs). Conversely, retinaldehyde can be reduced to retinol by members of the dehydrogenase/ reductase SDR family (Dhrs) [27–29]. Retinaldehyde can be further oxidized to RA by up to three retinaldehyde dehydrogenases that still are commonly referred to as Raldh1-3. Here we use the new ALDH nomenclature, which is based upon that used for P450 enzymes, under which these proteins have been renamed to Aldh1a1-3 [30].

There are two biologically active isomers of retinoic acid, all-*trans* RA and 9-*cis* RA. Little is known about their specific synthesis and isomerization [31], but all-*trans* RA is the abundant form with described biological functions. An alternative route to RA synthesis is the cleavage of β-carotene into two molecules of retinaldehyde, followed by oxidation to RA. Cleavage of β-carotene is carried out by β-carotene oxygenase I (Bco-I) (Fig. 1). A tight control over expression levels of RA synthesizing enzymes, most importantly the Aldh1a-class proteins, is an important mechanism in controlling spatial and temporal availability of RA during developmental processes.

RA acts as a positive regulator of transcriptional activation by binding to retinoic acid receptors (RARs). RARs are highly conserved among vertebrates and primarily bind all-*trans* RA. Mammals possess three RARs (RARα, RARβ, and RARγ), whereas zebrafish possess four genes (*raraa, rarab, rarga*, and *rargb*). RARs are steroid receptors that act as heterodimers with retinoid X receptors (RXRs). RXRs can bind 9-*cis* RA, however, 9-*cis* RA cannot be detected endogenously in mouse embryos [32] and its biological function has been a matter of debate [33], suggesting that RXRs mainly act as heterodimeric partners for RARs and other

steroid receptors. In the nucleus, RAR/RXR heterodimers bind to retinoic acid response elements (RAREs) in regulatory regions upstream of target genes (Fig. 1). Binding to RAREs is independent of RA availability, so that RAR/RXRs recruit a complex of corepressor proteins to the promoter region in the absence of RA, resulting in repression of downstream genes. Binding of RA to RAR/RXR leads to dissociation of corepressors and recruitment of coactivators resulting in target gene expression [8, 9]. Beside the canonical mode of RA action, mediated by RAR/RXR heterodimers, several alternative mechanisms have been suggested [34–37] that we will not revisit here.

The interplay between cells expressing enzymes that either synthesize or degrade RA produces local sources and sinks of RA that can result in the establishment of RA gradients [38]. Tight control of the distribution and levels of RA is therefore essential. Enzymes of the cytochrome P450 subfamily 26 (Cyp26a1, Cyp26b1, and Cyp26c1) degrade RA into more polar metabolites [23]. Intracellular RA can be bound by cellular RA-binding proteins (Crabp-I and -II). Crabps transport RA both to RARs and to Cyp26s. Whereas Crabp-II favors delivery of RA to RARs, Crabp-I sequesters available RA and transfers it to Cyp26s. Crabps are therefore thought to play an important function in signaling robustness as they are able to compensate for changes in RA production [39]. For more detailed information about RA signaling, the reader is referred to recent reviews on the topic [8, 23, 24].

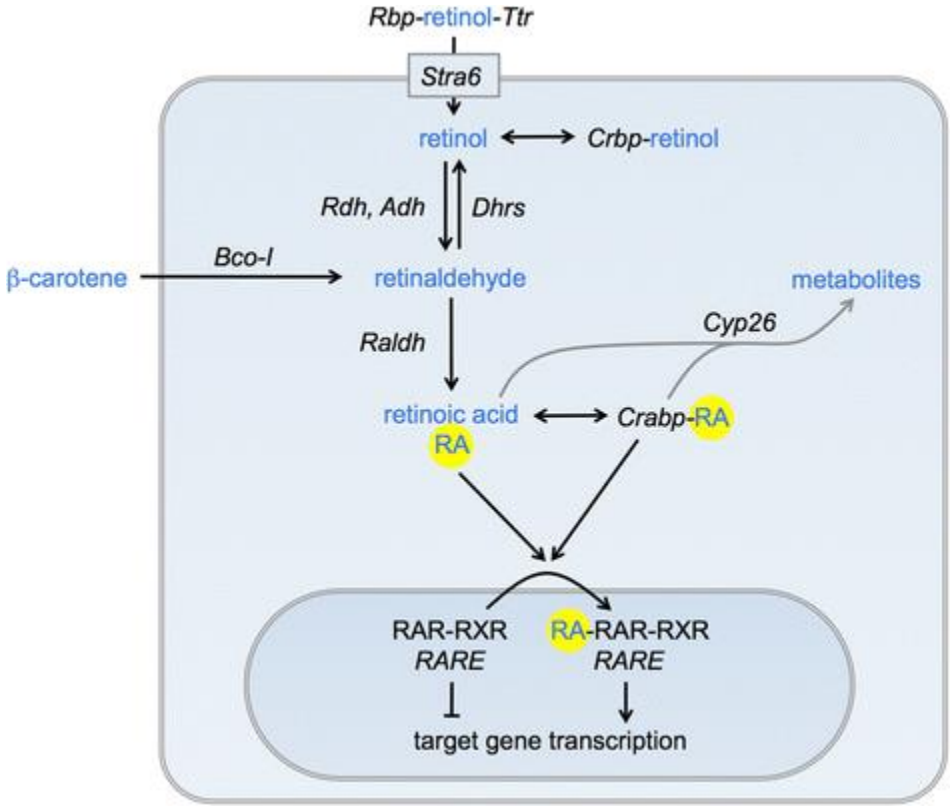

Fig.1. Simplified scheme of retinoid metabolism and retinoic acid signaling in vertebrates. Oviparous eggs of fish, amphibians, and reptiles already contain stores of retinaldehyde in the yolk that are processed during embryonic development. Adult vertebrates obtain retinoids through the diet in the form of carotenes, e.g., as β-carotene, which are converted to retinaldehyde. Alternatively, retinol circulates the blood stream, enters the cell, and is successively oxidized to retinaldehyde and to retinoic acid (*RA*). In the absence of RA, dimers of RA- and retinoid X-receptors (RAR-RXR) suppress the transcription of target genes. In the presence of RA, co-repressors are released and replaced with co-activators, resulting in target gene activation. RA is degraded by Cyp26, which oxidizes RA into biologically inactive metabolites. See main text for further details.

Exogenous RA and "super-regeneration" in the limb

Amphibians and teleost fish are able to regenerate lost appendages through the formation of a blastema at the amputation site. The blastema is a mass of highly proliferative, undifferentiated progenitor cells that provide a source of new cells to replace the lost structures. For a comprehensive overview of regeneration in the limbs and tails of amphibians and the zebrafish caudal fin we refer the reader to recent reviews on these topics [40–47].

RA has a long history in the study of vertebrate appendage regeneration. When administered during the stage of initial blastema formation, RA proximalizes, posteriorizes, and ventralizes the positional identities of blastema cells in a concentration-dependent manner [48]. The work of Stocum and Cameron [49] provides an account on the history of research into the roles of RA in determining positional identity, with a focus on the urodele

amphibians. The effect that has received most attention is that of proximalization, in which the regenerate contains additional proximal limb structures, a phenomenon referred to as “super-regeneration” [11, 12, 14, 50] that has been described for limb regeneration in several urodele and anuran species. Furthermore, several studies demonstrated spectacular effects of RA treatment on regenerating amphibian tails, in which additional hindlimbs formed from the amputation site [14, 51–53].

Super-regeneration has a clear dose–response, as increasing the RA concentration added to the regenerate leads to the regeneration of ever more proximal structures of the limb. This observation led to a search for the molecules that mediate this effect and culminated in the identification of Prod 1, a cell-surface molecule of the Ly6 family, whose expression is upregulated by RA [54]. Prod 1 is expressed in dermal fibroblasts and in a gradient with high proximal expression and low distal expression during development and regeneration [55]. Several assays showed that Prod 1 expression on the cell surface is crucial for converting a distal to a proximal cell identity. In search for a Prod 1 ligand, nAG (newt anterior gradient protein) was identified as a secreted growth factor for blastema cells that binds to Prod 1 in a yeast two-hybrid screen [55]. After limb amputation and retraction of severed axons, nAG is expressed in Schwann cells of the distal nerve sheath when regenerating axons repopulate the distal stump and later is required for its own expression in gland cells of the wound epithelium [55]. nAG is required for the continued proliferation of blastema cells, and the shift in nAG expression from Schwann cell to wound epithelium reflects a corresponding shift in the dependency of blastema proliferation from nerve to wound epithelium [56]. Notably, Prod1, and thereby its role in encoding P-D identity, is considered to be salamander-specific [57]. Thus, the failure of limb regeneration in other vertebrates could be correlated with a lack of positional identity in adult limb cells due to the absence of Prod1.

In the regenerating zebrafish caudal fin, treatment with high RA concentrations was shown to result in fin patterning defects, and regeneration was slowed down or blocked [58, 59]. In a follow-up study, Géraudie and Ferretti [60] showed that as a consequence of RA treatment there was a strong increase in cell death in the wound epidermis and blastema. Thus, the reported phenotypes were most likely due to secondary effects caused by enhanced cell death.

RA in regeneration of the zebrafish fin

RA in larval caudal fin regeneration

The caudal fin of adult zebrafish forms via a larval precursor, the caudal part of the median fin fold. The larval fin has a very simple layout, being composed of an epithelial sheet that is filled with mesenchymal cells [61, 62]. Its structure is based upon radially extending

unmineralized fiber bundles called actinotrichia. During larval-to-adult transition the task of skeletal support is taken over by the bony fin rays (lepidotrichia), which form through intramembranous ossification. Actinotrichia remain only at the distal growing ends of each fin ray [44].

Regeneration of the larval caudal fin occurs very quickly, within 3–4 days [63]. Within a few hours after amputation the wound is sealed by epithelial cells that form the wound epidermis. Next, proliferation is induced in mesenchymal cells throughout the caudal fin. In contrast to fin regeneration in adult fish, however, formation of a distinct blastema, i.e., accumulation of proliferating cells beneath the wound epithelium, is not observed [64].

aldh1a2, the gene that encodes the major RA-producing enzyme in the embryo, is among those genes that are strongly upregulated during zebrafish larval and adult caudal fin regeneration [65]. Within 4 h after amputation, *aldh1a2* becomes upregulated, which suggests that RA signaling may be required for regeneration. As a first approach towards testing a potential role for RA in larval fin regeneration, caudal fins of zebrafish larvae were amputated and then treated with an antagonist of RA synthesis (4-diethylaminobenzaldehyde, DEAB). Fin regeneration in DEAB treated larvae was blocked probably due to reduced cell proliferation in the mesenchyme. In addition, molecular markers specific to the wound epidermis and the proliferating mesenchyme were not induced [65]. Since injection of morpholinos designed to block the translation of *aldh1a2* mRNA resulted in the same phenotypes, the observed effects could be attributed to the loss of Aldh1a2-mediated RA synthesis. Although this study has demonstrated an essential function for RA signaling in larval fin regeneration, the exact functions remain to be resolved. An interesting question is whether RA regulates mesenchymal proliferation in a direct or indirect way. Transgenic zebrafish lines that report activation of RARs [66, 67] should be helpful in identifying cell populations that are direct targets of RA. Notably, *aldh1a2* is not expressed in the larval caudal fin and is neither required for its initial development [68, 69] nor for its later outgrowth (Blum and Begemann, unpublished), suggesting that its up-regulation is a regeneration-specific response.

Fgf and Wnt/β-catenin signaling have been shown to be required for larval fin regeneration and it has been proposed that both signaling pathways act upstream of RA signaling [65]. However, this model is not sufficiently supported and is partly inconsistent with a previous study. Inhibition of Wnt/β-catenin signaling results in failure of *aldh1a2* upregulation after amputation, but it remains to be shown whether activation of Wnt/β-catenin is independent of RA signaling. Although expression of *wnt10a* is not affected in the regenerating larval fin upon DEAB treatment, expression of Wnt/β-catenin target genes have not been investi-gated. Importantly, the ligand responsible for Wnt/β-catenin activation in the regenerating fin has not been identified and inhibition of RA signaling during adult fin regeneration results in downregulation of Wnt/β-catenin signaling that is independent of Wnt10a [70].

RA signaling is required for wound epidermis formation in the larval caudal fin and was placed upstream of Fgf signaling [65]. If this proves true, then inhibition of Fgf signaling should cause wound epidermis defects. This is in conflict with an earlier study by Kawakami et al. [63], which suggests that Fgf signaling is not involved in wound epidermis formation. Further investigations are therefore required to clarify this discrepancy and unravel putative epistatic relationships.

Regeneration of the adult caudal fin

The adult zebrafish caudal fin consists of 16–18 parallel bony fin rays that are separated by soft interray tissue [44, 71]. Each fin ray possesses two facing segmented hemirays of dermal bone (Fig. 2). The fin harbors a relatively small number of cell types: three types of pigment cells, osteoblasts that line up along the bone matrix that they secrete, dermal fibroblasts, endothelial cells, neuronal axon tracts and associated glial cells, resident blood cells, and cells of the epidermis. Importantly, striated muscle and cartilage are absent.

Adult fin regeneration passes through several steps: first, epidermal cells surrounding the wound become migratory and close the wound with a thin epithelial sheet [44, 72]. Subsequently, the epithelial covering thickens by accumulation of additional epithelial layers and forms the wound epidermis, which is characterized by a basal epithelium of cuboidal cells (Fig. 2). Both processes, initial covering of the wound and subsequent thickening of the covering, are dominated by cell migration and do not require cell proliferation. Next, formerly quiescent non-epidermal cells of the stump enter the cell cycle and migrate towards the amputation plane. They form the blastema, an outwardly homogeneous mass of proliferating progenitor cells that provide a source of new cells to replace the amputated structures. Several studies have shown that blastema cells in the regenerating fin are highly restricted with respect to developmental identity [73–75]. However, Singh et al. [76] could show that osteoblasts are dispensable for regeneration of bony fin rays and suggest that fibroblasts may have the potential to transdifferentiate into skeletal tissue. With the exception of melanocyte stem cells [75, 77] it is dedifferentiated cells of the distinct cell lineages that contribute to the blastema instead of undifferentiated precursors [73, 74, 78]. Eventually, cells of the proximal blastema drop out of the cell cycle and enter a differentiation program that restores the lost tissue types [44, 72, 79].

RA in blastema formation and proliferation

aldh1a2 is expressed during adult caudal fin regeneration [65] and our own work has helped to uncover the endogenous roles of RA signaling in this process [70]. Within 6 h after caudal fin amputation, *aldh1a2* as well as *rdh10b*, encoding a retinol dehydrogenase [80, 81], and

the RA receptor *rarga* are upregulated in the stump tissue next to the amputation site. Expression of *aldh1a2* extends up to two fin ray segments proximal to the wound in the ray mesenchyme, a region from which cells are recruited that contribute to the blastema [82, 83]. Thus, blastema precursor cells become exposed to high RA levels upon fin amputation. Several genes in the RA signaling pathway are also transcribed in proliferating cells of the blastema, suggesting that RA is essential for blastema cells at all stages of regeneration [70].

Two possible approaches are currently in use to genetically block RA signaling in adult zebrafish. One is to overexpress a dominant negative form of zebrafish *raraa* (*dn-zrar*) [84] that has lost its RA-dependent transcriptional activation activity while retaining the ability to form heterodimers with RXR. This construct blocks the activation of RA target genes despite the presence of RA [85]. Another method is to induce expression of *cyp26a1* with the goal of breaking down endogenously available RA to interfere with target gene activation [70]. Both strategies accomplish complete and organ-wide inactivation of RA signaling by putting them under control of a *heat shock promoter* (*hsp*) and activating strong overexpression by timed heat-shocks in *hsp:cyp26a1* and *hsp:dn-zrar* transgenic animals. When RA signaling is inactivated during fin regeneration, early steps of blastema development are initiated, as indicated by the remodeling of extracellular matrix and disorganization of the stump mesenchyme adjacent to the amputation site [70]. However, blastema precursor cells do not start to enter the cell cycle or express molecular markers of the proliferating blastema. Both *hsp:cyp26a1* and *hsp:dn-zrar* produced identical phenotypes, thus providing independent evidence for an endogenous function of RA in blastema formation (Fig. 2).

Failure of cell-cycle entry seems to be an inherent trait of the mesenchyme itself and not a secondary effect caused by defects in the wound epidermis (which without RA does not form properly, as delineated further below), because applying a single heat-shock once a proper wound epidermis has formed does not rescue proliferation. Accordingly, *rarga* is strongly expressed in the blastema once it has formed, but a low-level expression of other RA receptor genes in the wound epidermis is likely. Injection of RA into the blood stream during blastema formation is sufficient to increase the proliferation of mesenchymal blastema precursors. Once a blastema has formed and regeneration is well under way, suppression of RA signaling leads to a severe reduction in cell proliferation not only in the blastema but also in the epidermis. Therefore, RA signaling is required for cell-cycle entry of cells that are destined to contribute to the blastema and to provide a progenitor-niche for proliferating blastema cells (Fig. 2b, c). It is not yet known whether RA is also required for the dedifferentiation process of blastema precursors, nor whether all blastema precursors synthesize RA and also receive the signal. Another open question is whether RA directly regulates proliferation in blastema cells or indirectly influences the proliferation via other factors. Since RA promotes Fgf and Wnt/β-catenin signaling [70], both of which are required

for blastema proliferation [86–88], RA might act through Fgf and/or Wnt/β-catenin as a pro-proliferative signal.

It has been a long-standing question to what extent limb and fin regeneration recapitulates development. While wound healing and blastema formation are regeneration-specific events, the underlying cellular and molecular mechanisms of growth and patterning are very similar in regenerating and developing appendages [40, 43]. The adult caudal fin grows throughout life by adding new bone segments to the distal end of each fin ray. Accordingly, strong expression of *aldh1a2* can be found in the growing fin ray tips in the uncut fin (Blum and Begemann, unpublished), suggesting that RA is also required for proliferation during normal fin outgrowth. An interesting question is whether RA signaling has similar functions in larval and adult fin regeneration. RA signaling is required for cell proliferation in both models. However, larval fin regeneration is different from adult regeneration in many respects. There is no evidence for a proliferating blastema, the larval fin lacks tissue types, e.g., bone and blood vessels, which are prominent in the adult fin and the wound epidermis is only a very thin structure without a distinct basal layer [63]. Importantly, adult fin regeneration requires cell dedifferentiation and re-entry into the cell cycle within the otherwise quiescent stump tissue, whereas larval fin regeneration is rather accomplished by an increase in proliferation in an already growing organ. Thus, direct comparisons are difficult and it remains to be shown whether RA signaling regulates cell proliferation in a similar or different way (e.g., directly or indirectly) in the larval and adult fin.

RA and the formation and function of the adult wound epidermis

The wound epidermis serves as a physical barrier and provides important signals for the underlying mesenchyme. Reciprocal interactions between the wound epidermis and blastema cells are essential for successful regeneration [89, 90]. Although the wound surface is covered with several epithelial layers, the wound epidermis lacks a distinct basal layer in the absence of RA signaling [70]. Chablais and Jazwinska [90] found that Igf signaling plays a crucial role in the formation of the basal epidermal layer. The ligand Igf2b is synthesized and secreted from the stump mesenchyme and activates Igfr in the wound epidermis in a paracrine fashion. Notably, RA signaling is required for induction of *igf2b* expression after fin amputation and is even able to induce *igf2b* expression in unamputated fins [70]. Thus, RA might control wound epidermis formation through induction of *igf2b* in the stump mesenchyme (Fig. 2b). It is currently unknown if activation of *igf2b* by RA is a direct process, which would require the isolation and functional characterization of RAREs in the *igf2b* promoter. However, an in silico analysis identified DR5 RAREs in the promoter region of human and mouse Igf2 [91], suggesting that *igf2b* is directly regulated by RA in the

regenerating fin. Activation of Igf signaling in the wound epidermis is essential for proliferation in the underlying mesenchyme [90]. Interestingly, initiation of cell proliferation in the stump mesenchyme during blastema formation does not require Igf signaling, thereby excluding the possibility that RA acts exclusively through Igf in the regenerating fin.

Wound epidermis formation is initially independent of proliferation, but regenerative outgrowth requires a constant supply of new epidermal cells. At the same time, RA production in the blastema is necessary for epidermal proliferation [70] (Fig. 2b, c). Whether epidermal cells receive RA signals in a paracrine manner, or whether RA signals indirectly to the epidermis through other pathways has not yet been resolved. Igf signaling in the wound epidermis is not required for its proliferation [90], thus an involvement of Igf signaling in mediating the pro-proliferative effect of RA on epidermal cells can be ruled out.

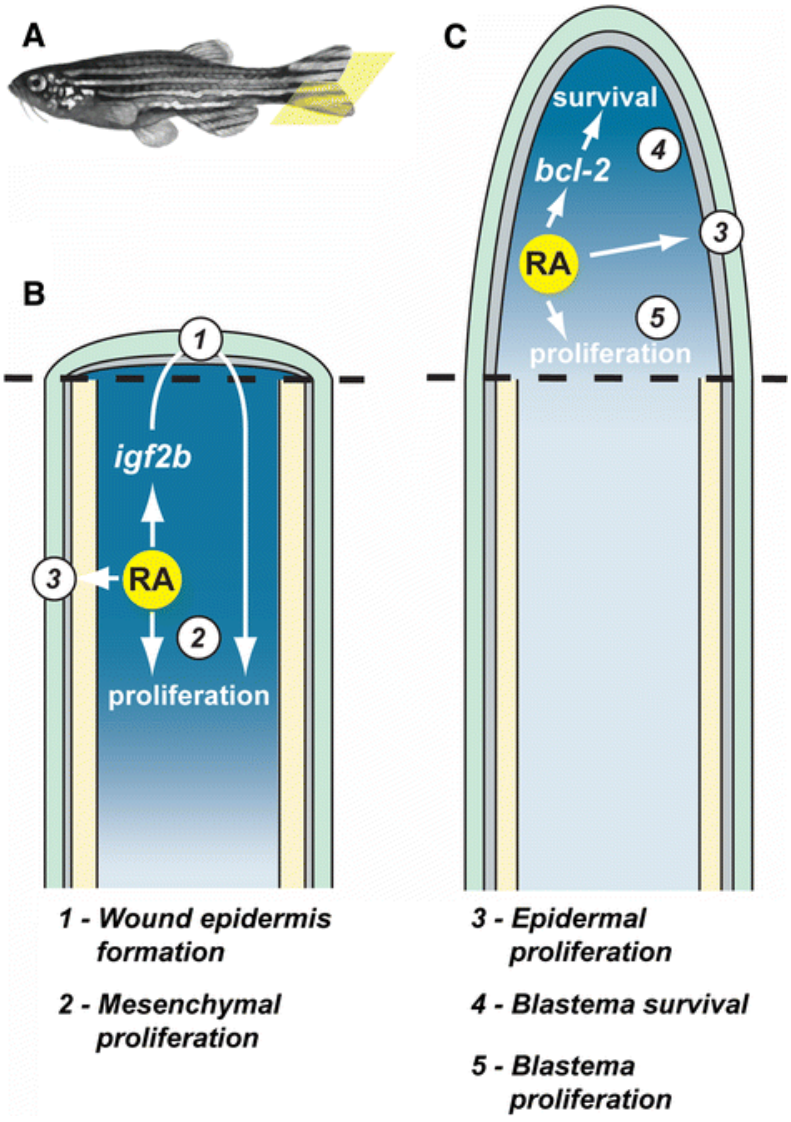

Fig. 2 Zebrafish fin regeneration. a *Longitudinal section* through a regenerating zebrafish caudal fin, indicated by *yellow parallelogram*. b Formation of the wound epidermis and the blastema. c Regenerative outgrowth. The fin is covered by a multilayered epidermis (*green* and *grey*) that is characterized by a basal epidermal layer (*grey*). Underneath, hemirays are formed by bone matrix (*yellow*), areas of RA synthesis (inferred from *aldh1a2* expression; *dark blue*) are found in distal stump tissue and in proliferating blastema. *Circled numbers* indicate biological roles of RA signaling during both stages of regeneration.

RA in blastema survival of the adult fin

Blastema formation involves the transformation of formerly quiescent differentiated stump cells into migratory and less differentiated cells that accumulate to form a highly proliferative cell mass. Typically, such behavior triggers apoptosis to prevent tumor formation. It is an open and interesting question how blastema cells escape apoptosis. Blastema cells are exposed to high concentrations of RA in the regenerating fin. On the other hand, RA is well known for its anticarcinogenic and pro-apoptotic activities and is used in therapeutic agents to treat several human cancers [92]. Therefore, it is somewhat surprising that down-regulation of RA signaling in hsp:cyp26a1 and hsp:dn-zrar fish results in rapid death of blastema cells [70]. The exact mechanisms underlying this phenomenon are currently not understood.

During cancer formation, cells avoid apoptosis through the upregulation of anti-apoptotic proteins and/or downregulation of proapoptotic signals [93]. Apoptosis is mediated through two general routes, the intrinsic and extrinsic pathways, both of which ultimately activate caspases. The intrinsic pathway is triggered by intracellular events such as DNA damage, growth factor deprivation and oxidative stress, and is controlled through mitochondria and the endoplasmic reticulum [94]. The intrinsic apoptotic pathways are regulated by members of the Bcl-2 family of proteins [95]. The family contains anti-apoptotic proteins like Bcl-2 itself that promote survival, as well as the pro-apoptotic members that promote cell death. The anti-apoptotic proteins sequester pro-apoptotic ones, therefore their relative expression levels are important and an increase in anti-apoptotic proteins protects a cell from death. Enhanced levels of Bcl-2 can be found in a variety of human cancers, where they are responsible for resistance to radiation and to chemotherapeutic drugs that counteract uncontrolled proliferation by inducing apoptosis [93]. Bcl-2 has therefore been an attractive target for anticancer drug design. Besides being an anti-apoptotic protein, Bcl-2 is involved in other forms of cell death. An inhibitory function of Bcl-2 has been reported for autophagy and programmed necrosis [96, 97].

In the regenerating fin increased RA levels upregulate bcl-2 expression and a heat shock in the *hsp:cyp26a1* and *hsp:dn-zrar* lines rapidly reduces expression [70]. These findings indicate that blastema cells avoid cell death through RA-dependent upregulation of *bcl-2* thereby allowing them to survive in an adult organism (Fig. 2c). However, it remains to be shown which mode(s) of cell death are inhibited by Bcl-2 and RA in the regenerating fin. In human and mouse DR5 RAREs have been identified in the Bcl-2 promoter [91], thus, *bcl-2* might be a direct target of RA signaling in the regenerating fin. Interestingly, protection from cell death is also inferred from proteomic analysis in the regenerating axolotl limb. Here, metabolism in the blastema is reduced and protective mechanisms are at work at various levels, including the upregulation of antimicrobial and antioxidant proteins, the differential

regulation of proapoptotic and antiapoptotic proteins, and responses targeted at reducing cell stress caused by the accumulation of unfolded proteins [98].

The ability of RA to promote proliferation and prevent death in blastema cells may appear curious, since RA is well known for its ability to cause cell-cycle arrest, differentiation and apoptosis [92, 99]. However, growth inhibition is cell type-specific and in the context of other cell types RA may even enhance proliferation and survival [100]. Schug et al. [34] proposed that the opposing effects of RA on cell growth emanate from the preferred activation of either of two different nuclear receptors: RA acts as a signal that inhibits proliferation and induces apoptosis via activation of RARs. In contrast, binding to peroxisome proliferator-activated receptor (PPAR) β/δ, a nuclear receptor which also forms heterodimers with RXRs, promotes proliferation and survival. Although, this might be an elegant explanation, another study clearly demonstrated that RA is not able to activate PPARβ/δ [101]. Further investigations will be necessary to determine how RA promotes cell growth in some models and inhibits cell growth in others.

Signaling pathways interacting with RA in adult fin regeneration

The identification of a signal that is sufficient to induce regeneration in an amputated mammalian limb would be an attractive goal of regeneration research. However, it is not yet clear whether one signal alone is sufficient to activate the full spectrum of regenerative responses or whether several signals have to act simultaneously. An important step towards this goal is to clarify how distinct pathways communicate with each other. Several interaction partners of RA signaling in the regenerating fin have been identified [70]. The regulatory relationship between RA and Igf signaling has already been discussed in the context of wound epidermis formation. Apart from RA signaling two other pathways that are required for blastema formation are Fgf and Wnt/β- catenin signaling [86–88, 102]. *fgf20a*, alone or in cooperation with other Fgf ligands, is responsible for Fgf activation in the regenerating fin [102], whereas the ligand responsible for Wnt/β-catenin activation has not been identified. We could show that activation of RA signaling in the fin stump is required for the expression of *fgf20a* and of *wnt10b* [70]. However, even though RA treatment induces upregulation of *wnt10b*, it is not sufficient to induce *fgf20a* expression in an unamputated fin.

Once a blastema has formed, RA ensures proliferation and survival of blastema cells. Here, RA, Fgf and Wnt/β- catenin signaling cooperate through reciprocal stimulatory interactions. Interestingly, the non-canonical Wnt pathway, which has been shown to act as a negative modulator of fin regeneration [86], is repressed by RA signaling. Possible interactions with other pathways remain to be shown.

Even though first insights into how RA signaling interacts with other pathways have already been gained, a more thorough understanding is currently hampered by our limited knowledge of the function of each pathway. More precisely, it needs to be clarified in which cell lineage(s) each pathway acts and which cellular mechanism(s) it regulates. As tools for tissue-specific genetic manipulations in adult zebrafish are emerging and systems that reliably report activity of distinct pathways in adult tissues are developed [74, 103–105], a new level of understanding should be reached concerning tissue- and pathway-specific questions that provide important new insights into the molecular regulation of the distinct cell lineages during adult fin regeneration.

Endogenous RA signaling in amphibian limb regeneration

RA has traditionally been studied for the teratogenic effects following exposure to exogenous RA in regenerating amphibian limbs (as described above). The ability of RA to cause dramatic effects in limb regeneration raises the question whether these effects are indicative of a corresponding biological role of RA. Only few studies have focused on a putative endogenous function of RA in regenerating amphibian limbs. Maden [106] showed that limb regeneration in axolotl is inhibited by treatment with disulfiram, a broad range inhibitor of aldehyde dehydrogenases. Disulfiram also blocks the activity of DNA methyltransferases [107] and the 26S proteasome [108], and can elicit pro-apoptotic and anti-proliferative effects. Thus treating regenerating limbs with this compound might efficiently block RA synthesis, but most likely also interferes with other processes. Del Rincon and Scadding [109] demonstrated that implanted beads soaked with RAR antagonists can modify patterning in the regenerating limb. However, the underlying mechanisms have not been investigated.

To better understand the roles of RA in amphibian limb regeneration, it was important to determine which tissues express RA pathway components, and to show which cells respond to RA. By using high performance liquid chromatography (HPLC) RA could be detected in both the epidermis and blastema of regenerating axolotl limbs [110, 111]. Several RARs are expressed in the newt blastema [112–116] and at least RARδ1, an ortholog of mammalian RARγ, is expressed in both the blastema and epidermis [117].

Monaghan et al. [118] showed that *aldh1a3* and *rdh10* are expressed in regenerating limbs of adult axolotl. Whereas the spatial expression pattern of *rdh10* has not been investigated, *aldh1a3* was detected exclusively in a subset of cells within peripheral nerve bundles. Although *aldh1a3*-positive cells might provide enough RA for RAR activation in their direct environment, it is less likely that they produce sufficient amounts of RA for the blastema or overlying wound epidermis.

As in the regenerating zebrafish fin, *aldh1a2* might also provide a major source for RA in the regenerating amphibian limb. A study by McEwan et al. [119] has shown that there is no de novo expression of *aldh1a1-3* in regenerating hindlimbs of *Xenopus* tadpoles. However, *aldh1a2* expression is retained from development in a patch of anterior/proximal stump cells and in a patch of proximal limb cells. It has not been tested how far RA can penetrate into neighboring tissue, but blastema cells in amputated tadpole limbs are close to the proximal limb. The generation of transgenic *Xenopus* reporting RA activity could help to answer this question. After limb amputation RA might diffuse from the proximal limb to the wound site, providing a source of RA required for regeneration. Such a mechanism could operate in anuran amphibians, as they are only able to regenerate prior to metamorphosis when limbs are still small and RA from proximal limb cells is still available. In contrast, urodele amphibians can regenerate limbs also during adulthood, suggesting that an amputation-induced new source of RA would be required. Unfortunately, expression of *aldh1a2* has not been investigated in adult regenerating urodele limbs. Thus, it remains an open question whether, and how, sufficient amounts of RA are provided in the regenerating limb of adult amphibians. In favor of a role for RA in urodele limb regeneration, *rdh10* is expressed in the regenerating axolotl limb, where it provides a reliable source of retinaldehyde [118]. Investigating the spatial expression pattern of both *aldh1a2* and *rdh10* in regenerating limbs should therefore be an important next step.

A transgenic axolotl line that harbors a reporter construct (RARE:GFP) containing eight DR5-type retinoic acid response elements from the mouse *RAR*β promoter region allows visualization of RAR activation in living animals throughout life [120]. RARE:GFP transgenic axolotl do report RA signaling activity in the regenerating limb, but in the wound epidermis rather than in the mesenchyme. The few structures that report RA activity within the blastema could be identified as axons and cells in close proximity of axons. These findings are in accordance with the expression of *aldh1a3* in a subset of cells associated with nerve bundles in the blastema (as mentioned above). Notably, upregulation of RA pathway components has been shown for regenerating mammalian neurons [121–123] and RA treatment induces axon outgrowth where it does not normally occur [124]. Furthermore, an antagonist specific for RARβ inhibits neurite outgrowth from spinal cord cultures of newt, suggesting that RA has a conserved function in neuron regeneration.

Given the important mesenchymal function of RA in the regenerating zebrafish fin it is curious that RARE:GFP activity is absent from the majority of mesenchyme in transgenic axolotl. Transgenic zebrafish lines that harbor constructs very similar to that used for the RARE:GFP axolotl line do not reliably report RA activity and report only weakly post-development [66, 67]. A similar explanation for the absence of reporter activity in axolotl blastema cells is rather unlikely, since RARE:GFP activity is clearly detected in some mesenchymal cells. Support for the idea that RA does not directly act in the axolotl limb

mesenchyme is provided by the exclusive expression of *crabp1* in the mesenchyme [118], as Crabp1 is thought to sequester RA and facilitate its degradation by Cyp26s [125–127]. It therefore should be important to determine whether RA signaling in the regenerating zebrafish fin mainly acts in the blastema or in the wound epidermis.

RA might be a critical element of limb regeneration by regulating wound epidermis function rather than directly controlling processes in the mesenchyme. Appendage regeneration depends on mutual interactions via paracrine signals between wound epidermis and the underlying blastema [89, 90]. It is therefore possible that RA signaling in the axolotl wound epidermis results in secretion of growth factors from epidermal cells that control blastema proliferation in a paracrine manner. The wound epidermis in newt has previously been shown to produce RA [111]. Curiously, the main stereoisomer detected was 9-*cis* RA. While it is generally accepted that all-*trans* RA is the main biologically active stereoisomer, a biological role for 9-*cis* RA is still a matter of debate [33].

Of particular interest is a better understanding of the involvement of RA in proximal–distal patterning. Interestingly, when RARE:GFP axolotls are treated with RA, *gfp* expression is induced only in a blastema cell population likely to be fibroblasts [120]. The positional memory of the limb is thought to reside in connective tissue fibroblasts [128–130], therefore this distinct cell population might be responsible for the proximalizing effect of RA. Thus, if it were possible to single out RARE-GFP-positive fibroblasts and determine their expression profiles, new insights might be gained into the long-known phenomenon of super-regeneration. Notably, reporter activity is absent in the blastema in animals that did not receive RA treatment. This finding raises the question whether RA has an endogenous function in proximo-distal identity. Unfortunately, reporter activity in RARE:GFP animals has only been examined in limbs that were amputated just proximal to the elbow. If RA signaling directly controls positional identity in connective tissue fibroblasts, then amputation at different positions along the proximal–distal axis should result in differences in RARE:GFP activity in these cells. An alternative explanation might be that RA activity in the wound epidermis regulates the release of yet unknown factors that determine positional identity in fibroblasts. This model might explain why reporter activity is absent from the mesenchyme. In chicken and mouse, it has been demonstrated that proximal– distal identity of limb segments is specified during development through a balance between proximal RA and distal Fgf activity [131, 132]. If positional information in amphibian limb cells is also established during development by RA activity, de novo positional information during regeneration would be unnecessary. However, high RA concentrations might allow respecification during regeneration.

Overall, although there is strong support for an endogenous function of RA in amphibian limb regeneration, conclusive evidence is still not available.

Endogenous RA in amphibian tail regeneration

Tail amputation in adult urodele amphibians results in the complete replacement of all tail structures. This ability is intriguing because tail regeneration involves the restoration of the spinal cord. Although the tail regenerates via similar steps and mechanisms as the limb, there are important differences: whereas a blastema-like structure forms from cartilage, muscle and fibroblasts, the spinal cord forms the so-called ependymal tube [47]. The ependymal tube is a pseudostratified neuroepithelium that forms from ependymal cells that line the central canal of the spinal cord and is very similar to the neural tube of an embryo. As regeneration proceeds, the ependymal tube elongates and neurons and glia cells differentiate.

In urodele amphibians RA signaling has been investigated in adult newt. Carter et al. [116] could show that following tail amputation the levels of RARβ2 mRNA and protein in the stump increase in ependymal cells and in meningeal cells that ensheath the spinal cord, and also increase in the ependymal tube. Three weeks after amputation RARβ2 expression can also be detected in a population of blastema cells. Notably, tail regeneration can be inhibited by treatment with a RARβ-specific antagonist. Interestingly, antagonist treatment starting at 6 dpa does not impair tail regeneration. This finding demonstrates that RARβ2 has an early function in tail regeneration, but is most likely not required for the proliferation of blastema and ependymal tube cells. The expression pattern of RARβ2 suggests an involvement of RARβ2-mediated signaling in the formation of the ependymal tube rather than in blastema formation. Using the same antagonist, it has previously been shown that RARβ activity is crucial for neurite outgrowth from newt spinal cord cultures [133], supporting the idea that RARβ2 activity controls regeneration of the spinal cord in the urodele tail. After tail amputation ependymal cells start to proliferate and migrate towards the wound site where they form the ependymal tube. It will therefore be interesting to investigate whether RARβ2 activity plays a role within the ependymal cells during ependymal tube formation. Also, the source of RA is yet to be determined. Interestingly, *aldh1a2* is upregulated as a consequence of spinal cord lesion also in the rat [134]. Besides a possible function of RA signaling in ependymal tube formation, RA might fulfill additional roles in tail regeneration. Examining RA activity in the regenerating tail of transgenic RARE:GFP axolotl might be a useful next step. Overall, RARβ-mediated RA signaling is required for tail regeneration in adult newt, most likely for the formation of the ependymal tube.

Aldh1a2 has been shown to be up-regulated in the neural ampulla, notochord cells and mesenchyme during *Xenopus* tail regeneration [119]. Moreover, several *Rar*s are expressed in the regenerating tail of another anuran species [135]. Disulfiram treatment has been shown to inhibit tail regeneration in anuran tadpoles, but as discussed above in the context

of limb regeneration, the inhibitory effect might be caused by interfering with mechanisms other than RA signaling.
It should be noted that the regenerated anuran tadpole tail is only an imperfect copy of the amputated part [45]. Importantly, the neural tissue does not seem to regenerate properly. The segmented pattern of the spinal nerves is not re-established and dorsal root ganglia fail to regrow. Furthermore, tail regeneration in anurans involves replacement of the notochord, which is not present in the adult urodele tail and fails to regenerate in urodele tadpoles. Thus, caution is warranted when generalizing findings on tail regeneration.

RA in zebrafish heart regeneration

In humans, cardiac infarction results in the local disruption of the blood supply to the heart. Deprived of oxygen, heart muscle cells undergo cell death and are subsequently removed by immune cells. A scar remains that disturbs the fine-tuned interplay of muscle contraction and the hydrodynamics of blood flow through the chambers. Further complications and heart failure are the likely consequences. While cardiac muscle cells in humans do not regenerate after myocardial infarction, zebrafish are very efficient at regenerating cardiac muscle. A common procedure to study heart regeneration has been the amputation of the tip of the ventricular muscle, which is readily accessible through the pericardium [136]. However, amputation does not produce the same kind of damage as cardiac infarction, which leaves behind the debris of dead cells. Cryocauterization, in which ventricular cardiomyocytes undergo massive necrosis after contact with a frozen object, overcomes this limitation, resembles the effects of reduced blood supply and results in many of the same cellular responses as amputation [137–139]. The molecular mechanisms underlying zebrafish heart regeneration, which we discuss below, have so far been examined in the amputation model.

The cellular mechanisms underlying zebrafish heart regeneration

The vertebrate heart consists of three layers: The epicardium on the outside, which provides precursors for the coronary vasculature, the muscular myocardium, and the endocardium, a vasculogenic mesothelial layer that faces the cardiac lumen. Upon injury, a clot of erythrocytes forms within minutes after amputation, which within a week is replaced by an assembly of fibrin fibers and collagen deposits. Next, the clot is surrounded, invaded and eventually replaced by myofibers in a slow process that takes up to 60 days [136, 140]. The outer region of the myocardium is formed by two layers that are comprised of compact myocytes on the outside and myocytes that are organized into trabecular arrangements

more internally. Uninjured adult hearts have a low rate of proliferation in compact myocytes and little proliferation in trabecular myocytes. Rather than by proliferation (hyperplasia), adult hearts mainly gain volume through an increase in cell size (hypertrophic growth).
Upon injury, a blastema of proliferating cells forms within the myocardium that gradually replaces the amputated heart muscle. The source of regenerated cardiomyocytes has been identified using an inducible recombination system that allowed cardiomyocytes to be genetically marked early in development. Most, if not all regenerated cardiomyocytes were shown to arise from differentiated labeled cardiomyocytes that re-entered the cell cycle [141, 142]. As cardiomyocytes detach from each other, they disassemble their sarcomeres and upregulate markers of cell-cycle progression [136, 141, 143], but they do not upregulate cardiac progenitor markers, suggesting that dedifferentiation does not turn back the clock to the earliest stages of cell type specification.

RA signaling during cardiomyocyte regeneration

Microarray analyses aimed at finding genes that respond to regeneration in the zebrafish adult and larval caudal fin as well as the adult heart have shown that despite radical differences in tissue composition between these organs, common expression changes exist that might reflect conserved molecular signaling during the initiation of regeneration [65, 144, 145]. A significant observation resulting from these studies was that strong upregulation of *aldh1a2* expression was conserved and common to all three regenerating organs.
Myocardial regeneration involves all three layers of the heart. A particularly interesting idea was that the epicardium might be involved in the regeneration process, similar to the multilayered wound epidermis [146, 147]. Intact hearts show little epicardial *aldh1a2* expression, yet within 24 hpf upon injury *aldh1a2* is strongly upregulated throughout the entire epicardium, including the atrium and outflow tract. By 3 dpa most epicardial *aldh1a2*-positive cells enter S-phase and by 7 dpa this response localizes to the wound. Thus, the entire epicardium expands in an organ-wide fashion to create a new epithelial cover for the exposed myocardium. By 7–14 dpa, at a time when myocyte proliferation is also maximal, epicardial proliferation homes in on the wound to close the epithelial cover [146].
Soon afterwards, subsets of epicardial cells migrate and integrate into the wound. Instead of contributing to the pool of cardiomyocytes, these cells are fibroblast-like cells, some of which are later found surrounding the vascular network [147, 148]. While *aldh1a2* expression subsides in the epicardium during this phase, integrating epicardial cells maintain high expression while regeneration continues. A surge in *Aldh1a2* expression in the atrial and ventricular epicardium is also observed in the developing mouse heart, where at the same time both the epicardium and myocardium respond to RA as demonstrated by

expression of a RARE-driven reporter [149]. Upregulation of epicardial RA synthesis thus seems to be a response that is reactivated from an earlier developmental program. Several other genes required for zebrafish heart regeneration also regulate embryonic heart development, leading to the proposal that adult hypertrophic cardiomyocytes dedifferentiate back to the embryonic hyperplastic stage so that they can proliferate once more [150, 151].

The epicardium is not the only cardiac layer that upregulates *aldh1a2* expression upon injury. All three layers start to proliferate in response to injury, including endocardial cells close to the injured site [139]. Immediately following ventricle amputation, endocardial cells round up in shape and detach from the myocardium, initially independent of their proximity to the injury site, but within 1 day after injury they are confined to positions close to the wound. *aldh1a2* is upregulated within 3 hpa in the endocardial endothelium of both chambers. As early as 1 dpa *aldh1a2* localizes to the injury site and remains there throughout 7–14 dpa [84]. Endocardial cells near the wound site migrate into the wound where they continue to express *aldh1a2*.

The co-occurrence of both epicardial and myocardial proliferation and epithelial migration conforms with possible roles for RA synthesis in controlling the proliferation of dedifferentiated cardiomyocytes in the blastema as well as regulating the emigration of epithelial cells into the myocardium. Direct proof that RA synthesis is required for the regenerative proliferation of cardiomyocytes comes from induced transgenic expression of Cyp26a1 (*hsp*:*cyp26a1*), and a dominant negative form of RARα (*hsp70*:*dn-zrar).* When RA signaling is blocked at 6 dpa and analyzed 1 day later, proliferation in the myocardium is reduced dramatically [84]. Thus, RA synthesis in epicardial and endocardial cells controls replacement of the underlying myocardium. Interestingly, exogenously supplied RA or a synthetic agonist that stimulates RARs fail to increase myocardial proliferation rates. This finding suggests that RA is a permissive rather than an instructive signal for myocardial regeneration. It remains to be shown whether RA acts directly in myocardial cells in a paracrine fashion or indirectly through other signals produced by epithelial cells in response to RA. Another open question is which cellular mechanism(s) are regulated by RA. The requirement for proliferation suggests that RA either controls the cell cycle and/or dedifferentiation of cardiomyocytes as a precondition to proliferation. It has been shown in the *Drosophila* eye and in Schwann cells that dedifferentiation is possible without the necessity to also proliferate [151]. This suggests that also cardiomyocyte dedifferentiation and proliferation might be separable steps that require distinct molecular triggers.

Several studies into the function of RA signaling in the developing heart support the idea that RA is required for cardiomyocyte proliferation rather than regulating the number of proliferating cells indirectly by controlling de-differentiation. In mouse embryos at around 12.5 days post-coitum (dpc), there is a major surge in RA synthesis in the ventricular

epicardium. At the same time, myocardial cells in the compact zone, which is closely attached to the epicardium, expand through proliferation to thicken the ventricular wall [152]. Together, this indicates that RA from the epicardium may induce cell proliferation in the compact cells through a paracrine mechanism. In support of this, RXRα knockout mice suffer from a hypoplastic myocardial wall and multiple ventricular septal defects. In fact, RXRα knockout mice as well as vitamin A-deficient rats show precocious sarcomeric organization in the compact layer, suggesting that RA is normally required to induce proliferation and prevent premature differentiation [149, 152–154].

It was further shown that epicardial proliferation during regeneration is not affected by the absence of RA signaling [84], however, an involvement of RA signaling in regulating invasion of epicardial- and/or endocardial cells into the wound has not been tested.

The question whether RA signaling is associated with the ability to regenerate the heart from an evolutionary perspective was addressed in *Polypterus*, a fish whose lineage branches off at a basal position of the ray-finned fish (actinopterygia). The events following ventricular amputation are very similar to those in zebrafish, including fast and organ-wide *aldh1a2* upregulation in the epicardium, and sustained epicardial and endocardial *aldh1a2* expression in regions of injury and cardiomyocyte proliferation. The parsimonious interpretation is that RA-mediated heart regeneration is a basic trait of ray-finned fish [84]. The situation is different in mice, though. When myocardial infarction was induced by ligation of the coronary artery, no immediate *Aldh1a2* expression was detectable in any cardiac tissue, only the epicardium showed limited sustained expression. Thus, the inability of the mouse heart to regenerate may be connected to the failure to express *Aldh1a2* in non-myocardial heart tissues [84].

RA in lens regeneration

Some teleost and amphibian species are able to regenerate the lens following surgical removal (lentectomy). While lens regeneration has only been poorly investigated in teleosts, the cellular mechanisms underlying lens regeneration in amphibians are well known. In urodeles the lens regenerates through a process called Wolffian lens regeneration, in which cells of the dorsal iris transdifferentiate into lens fibers [155]. After lentectomy, pigmented epithelial cells (PECs) of the iris lose their pigment, dedifferentiate and re-enter the cell cycle. Several days later, a lens vesicle forms from de-differentiated PECs and cells in its posterior part start to elongate and differentiate into lens fibers. Cells in the anterior part contribute to the lens epithelium, a simple cuboidal epithelium that regulates most homeostatic functions of the lens. The size of the regenerating lens continuously increases through the addition of new lens fibers that differentiate from the lens epithelium. In anuran

amphibians, lens regeneration has been observed in tadpoles of some *Xenopus* species. In contrast to Wolffian lens regeneration, lens cells in anurans transdifferentiate from the central region of the inner layer of the outer cornea [45]. We refer the reader to Henry and Tsonis [155] for a more comprehensive overview of amphibian lens regeneration.
Although gene expression profiles identified RXRγ as being upregulated during *Xenopus* lens regeneration [156], the expression of RA pathway components has not been systematically investigated and studies of the putative roles of endogenous RA signaling in anuran lens regeneration are missing. In contrast, it was shown that RA has an endogenous function during lens regeneration in adult newts. After lentectomy expression of RARδ and RARα is upregulated in dedifferentiating PECs and remains high during lens fiber differentiation [157, 158]. Remarkably, treatment with antagonists that either block the activity of all RARs (pan-antagonist) or antagonists specifically targeted to RARα inhibits lens regeneration [157, 158], but the mechanisms underlying the failure of lens vesicle formation have not been investigated. It will be interesting to examine whether RA signaling is required for de-differentiation and/or proliferation of PECs and therefore has a similar function in the recruitment of progenitors in the newt lens as it has in zebrafish fin regeneration. In some cases treatment with the pan-antagonist resulted in the formation of an ectopic lens from the ventral iris or even from the cornea. These findings are surely very interesting, however, with only very limited knowledge about the action of RA signaling during lens regeneration, it is not possible to interpret these results. Investigations into the temporal and spatial expression of RA metabolizing enzymes during lens regeneration might unravel putative RA sinks and sources and therefore could help to understand why inhibition of RAR activity blocks regeneration dorsally, but induces ectopic regeneration ventrally.

Regeneration in mammals: RA and bone development in cervid antlers

There are a number of examples of appendage regeneration in mammals, as in the closure of punch-hole wounds in the ears of rabbits and the MRL mouse strain, the regeneration of amputated digit tips in newborn rodents and humans, and the annual regeneration of antlers in deer [159], but so far an involvement of RA signaling has only been shown in antler regeneration. Deer antlers are a rare example of full appendage regeneration in mammals. Rather than a response to wounding by amputation, antler regeneration can be viewed as a regularly recurring phase of bone development (often referred to as physiological regeneration) that does not depend on tissue loss or wounding. This becomes evident when testosterone levels are artificially kept high: as a consequence, the preceding year's antlers are not cast and a new set develops at the base of the old ones [160].

Seasonal regeneration of antlers

Antlers originally develop and are regenerated from pedicles, bilateral bony protrusions of the frontal bone. After antlers have been cast, the epidermis closes over the pedicle wound and forms a wound epithelium. This process is accompanied by the formation of the dermis that is separated from the wound epithelium by a basal lamina. Full-thickness skin inhibits regeneration in amphibians when placed on the amputation stump, but antler regeneration does not require contact between the wound epithelium and the proliferating mesenchyme.
Underneath the skin the surface of the pedicle bone is formed by the periosteum, which provides a niche for resident stem cells that give rise to a mesenchymal growth zone. The growth zone has been compared to a blastema, because it consists of proliferating and undifferentiated cells that will develop into a new antler [161, 162]. Up until recently, the formation of the growth zone was thought to involve cellular dedifferentation processes. However, it has been shown that blastema cells express a marker for mesenchymal stem cells and can be differentiated into more than one cell type in vitro, suggesting a stem cell contribution to the blastema [163]. The mesenchymal growth zone is localized at the apex of the outgrowth, underneath the future perichondrium, and will give rise to the new antler by a continuous process of differentiation that generates chondroblasts. Chondroblasts mature into chondrocytes, which further differentiate into hypertrophic chondrocytes in a distal to proximal sequence (Fig. 3). Osteoblast progenitors from the perivascular tissue invade the hypertrophic cartilage, differentiate and secrete bone matrix. Thus, antler regeneration follows a mode of endochondral ossification to elongate the antler skeleton. In contrast, the simultaneous increase in antler width is brought about by intramembranous ossification.

RA signaling in antler regeneration

RA signaling is involved in the regulation of chondrogenesis and osteogenesis during antler regeneration. In the course of endochondral ossification during embryonic development, RA limits, and therefore controls, the timing of condensation and chondroblast differentiation when the skeletal cast is laid down, and it positively regulates the maturation and replacement by bone at later stages [164]. In other words, loss in RA signaling induces differentiation of chondroprogenitors early on but at later stages prevents hypertrophic differentiation of chondrocytes. The overall gene expression patterns of RA pathway components in the regenerating antler support what is known about its roles controlling cellular transitions during chondrogenesis in the embryo.
The perichondrium contains a distal fibrous layer and a proximal blastema, which shows extensive proliferation and is, at least partially, if not wholly, derived from a stem cell niche in the periosteum. The expression of Aldh1a2 and the detection of all-*trans* RA confirm that the perichondrium is a source of RA. As during embryonic development, RARα is expressed

in the perichondrium, but is downregulated in the chondroprogenitors [165]. Experiments on primary mouse limb mesenchyme have confirmed that downregulation of RARα is required to ensure the differentiation of chondroblasts [166, 167]. As would be predicted for chondrogenesis, the chondroprogenitor layer does not expresses Aldh1a2, because RA signaling is a negative regulator of the chondroblast marker *Col2a1* [166]. Finally, the cartilage layer is the site of chondrocyte hypertrophy and posthypertrophic chondrocyte mineralization. RA is a major regulator of these processes [164, 168] and its effects may be mediated by RXRβ, whose expression in chondrocytes continually increases as cells differentiate from chondroprogenitors to mature chondrocytes. Replacement of cartilage by bone originates in the cartilage layer, accordingly both Aldh1a2 and RARα are expressed in osteoblast progenitors located in the cartilage region and in the lateral pedicle, where they promote the increase in antler width through intramembranous bone formation. Treatment of cultured mesenchymal antler cells with RA promotes osteoblast differentiation and mature osteoblasts express Aldh1a2 in antler bone [165]. Taken together, the regenerating antler passes through the stages of endochondral ossification, in which RA regulates the transition between cell differentiation stages similar to the processes in embryonic bone development.

In contrast, the mechanisms of antler blastema formation are inadequately understood and further examination is required to reveal whether RA could serve an earlier role in the periosteum stem cell niche and the mesenchymal growth zone. Both Rarα and Aldh1a2 are expressed in the blastema (prechondrogenic mesenchyme) and this tissue, in analogy to the situation in the zebrafish caudal fin blastema, might require RA to control growth and other aspects of the earliest regenerative events. Another lead in favor of this possibility comes from experiments in which RA was injected into the incipient pedicle anlage, which emerges from within a larger “antler regeneration area” of the frontal periosteum. The resulting shape changes in the injected pedicle and the increase in first antler size was examined histologically and thought to be caused by increased proliferation rates of cells presumed to reside in the perichondrium [169, 170]. Such a growth-promoting effect would be comparable to the known roles of RA in zebrafish fin and heart regeneration.

Finally, in addition to bone tissue, antler regeneration involves the regeneration of epidermis, dermis, and vasculature and it needs to be investigated as to which extent RA signaling is involved in these processes.

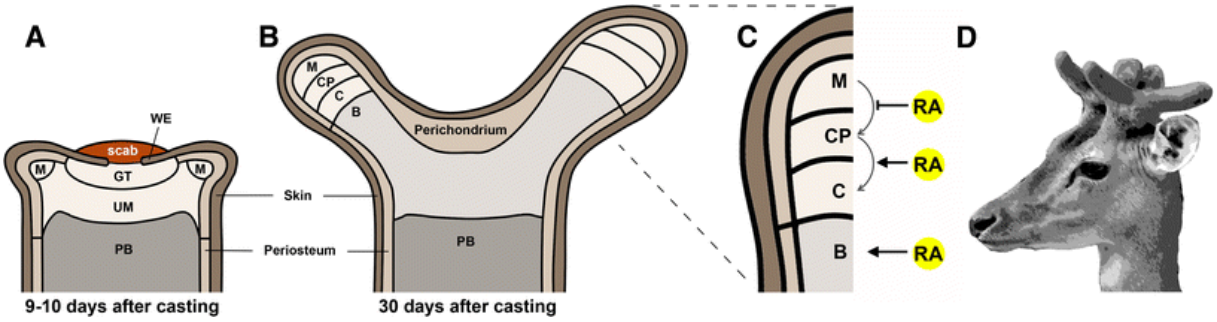

Fig. 3 Antler regeneration. a Model of antler bud 9–10 days after casting. A wound epithelium (*WE*) closes over the wound, protected by a scab. In the center of the pedicle bone (*PB*), skin and wound epithelium overlay a granulation tissue (*GT*), its proximal part appears as undifferentiated mesenchymal tissue (*UM*). Mesenchymal growth zones (*M*) form at sites of antler branch formation. A new perichondrium envelopes the regenerating antler and is coherent with the pedicle periosteum. b Thirty days after casting, branches grow longitudinally at the distal tip, in which mesenchymal growth zones (*M*) form the inner layer of the perichondrium. Chondroprogenitors (*CP*) are situated more proximally, followed by cartilage (*C*). Osteoblasts replace cartilage (endochondral ossification) and form the new antler bone (*B*) (a, b modified after Price et al. [205]). c RA signaling acts at different levels of antler regeneration: RA is synthesized in the perichondrium and keeps the mesenchymal growth zone in a prechondrogenic state. Chondroblast differentiation is inhibited as long as RA signaling is high. Chondroprogenitors (chondroblasts) require RA for maturation, which might be provided by osteoblasts invading perivascular spaces in the cartilage zone. Osteoblasts produce RA and differentiate under the influence of RA signaling. Growth in width occurs by intramembranous bone formation, which also requires RA (not shown). d Fallow deer with “young” antlers (as in b).

Invertebrate regeneration and the retinoid signaling machinery

The RA signaling pathway, including the genetic machinery it employs, is not specific for vertebrates, in fact several of its physiological functions have been shown to be conserved in all chordates, which also include the invertebrate groups of the cephalochordates and the urochordates, also known as tunicates.

The cephalochordate *Amphioxus* may play an important role in uncovering the origins and evolution of chordate regeneration mechanisms. *Amphioxus* shows extensive regenerative abilities and can regrow several organ types following amputations, both anterior and posterior to the pharynx. Tail regeneration has been shown to be an epimorphic process that employs stem cells as well as dedifferentiation of existing cells, the formation of a blastema, and the upregulation of genes in the blastema that are not expressed during embryonic development of the regenerating organ [171]. Cephalochordates evolved at the base of the chordates and therefore may possess regenerative mechanisms inherited and further modified by vertebrates. At the same time, various developmental processes that are RA-regulated in vertebrates, like the control of antero-posterior pattern formation and neuronal

specification in a Hox-dependent manner [172] are also under the control of RA in *Amphioxus*. Accordingly, the *Amphioxus* genome possesses orthologs of most of the vertebrate components of the RA signaling pathway, including the metabolic enzymes and single copies of RA and RX receptors [173–175] (reviewed in: [176–178]). Whether the metabolic enzymes are comparable in their substrate specificities, however, remains to be explored [179, 180]. Like its vertebrate counterparts, RA binds to and activates the *Amphioxus* RXR/RAR heterodimer [175]. On the other hand, orthologs of vertebrate Crabp, lecithin-retinol acyltransferase (Lrat), Rbp4, Ttr, and Stra6 are absent from the *Amphioxus* genome [179, 181]. In vertebrates retinol is esterified to retinyl esters by Lrat and stored in the liver. Rbp4 and Ttr transport retinol in the blood stream. The cellular uptake is subsequently mediated by Stra6 [25]. Taken together, at the base of the chordate lineage the basic components of the RA signaling machinery were most likely already present, whereas the system for retinoid storage, transport and cellular uptake might be functional innovations of vertebrates [179].

Among the invertebrate animals, ascidians (the most representative class of the tunicates) possess the remarkable ability to regenerate the whole body [182]. Ascidians reproduce both sexually and asexually, forming colonies of clonal individuals. Regeneration has much in common with asexual reproduction by vegetative outgrowth, as both require local mitosis at the site of budding and regeneration, respectively [183]. RA treatment induces repatterning of the body in buds that sprout from the thoracic body wall of the tunicate *Polyandrocarpa misakiensis.* [184, 185]. The process is thought to be mediated by RAR-RXR heterodimers that are characterized by an affinity to vertebrate-type RAREs and the ability to act as RA-dependent transcriptional activators [186]. The effects of RA are likely to be indirect, since coelomic cells treated with RA become competent to induce dedifferentiation of a multipotent cell type, the atrial epithelial cells constituting the inner vesicle of the bud [184]. In the colonial ascidian *Botrylloides leachi*, entire functional individuals can be regenerated from adult tissues, a process that requires RA synthesis and signaling through a RA receptor that is expressed in regeneration niches contained in fragments of blood vessels [187]. Although urochordates are thought to represent the sister group to vertebrates, compared to cephalochordates their derived anatomy is less well suited to unravel the roles of RA in regeneration from the perspective of an animal at the base of the phylum chordata. Moreover, RA signaling in tunicates has secondarily been modified, leading to diverged functions and loss of key components of the RA signaling machinery [177, 178]. The basic components for RA signaling (a single RAR/RXR heterodimer, Aldh1a and Cyp26) are present in the genome of the ascidian tunicate *Ciona intestinalis*. However, the larvacean tunicate *Oikopleura dioica* has lost key components of the RA signaling machinery, including RAR, Aldh1a and Cyp26 [177, 188]. Accordingly, the functions of RA signaling in tunicate

development seem to be limited. For instance, RA signaling probably only plays a minor role in the regulation of Hox genes [189–191].

The ecdysozoans (among them insects, crustaceans and nematodes) differ from annelids and molluscs in their sister group, the lophotrochozoans, in that they appear to have secondarily lost both the RA receptor (but not RXR-like sequences) and the ability to regulate RA degradation through CYP26 enzymes [8, 177, 192]. Curiously, these specific losses also appear to have occurred in platyhelminths (flatworms), a phylum within the lophotrochozoans that exhibits exceptionally remarkable regenerative abilities [192, 193] and in which exogenous RA affects regeneration of anterior but not of posterior structures [194]. Thus, in these groups RA activity may occur independently of DNA binding through non-genomic signaling, yet influences the progress of regeneration.

In *Drosophila*, a checkpoint mechanism operates in the third larval instar that delays entry into pupariation following tissue damage. When whole larvae are irradiated or when the apoptotic program is genetically activated in the imaginal discs, a neuroendocrine pathway that regulates the increase in ecdysone production that is required for progression from larval to pupal stages is temporarily inhibited [195]. Interestingly, a genetic screen for genes that take part in the checkpoint mechanism and thus diminish the developmental delay in response to damage, turned up several members of the retinoid signaling machinery. Among these were the genes for β-carotene 15,15'-monooxygenase (BCO), which controls the initial step in the release of retinaldehyde from stored carotenoids (Fig. 1), a gene with homology to mammalian type III alcohol dehydrogenase, which oxidizes vitamin A to retinaldehyde, a scavenger receptor that transports β-carotene into cells, and the aldehyde dehydrogenase gene that might act as a source of RA in *Drosophila*. In support of the interpretation that retinoid signaling is an important part of the checkpoint mechanism, larvae reared on carotenoid-deficient food exhibit a substantially attenuated delay before progressing into pupariation upon damage, but feeding β-carotene rescues the checkpoint mechanism. The retinoid signaling machinery was shown to be one part of the mechanism that suppresses the activation of the neuroendocrine pathway that stimulates ecdysone synthesis, although retinoid-independent mechanisms must exist as well [195]. Thus, retinoid biosynthesis in *Drosophila* is important for the maintenance of a condition that is permissive for regenerative growth.

Another case of ecdysozoan regeneration that is influenced by RA signaling is exemplified by the fiddler crab (*Uca pugilator)*, which is able to reflexively discard limbs as a response to predation (autotomy). Amputated and autotomized limbs can be regenerated completely during a single molt cycle (for a review see [196]), which is regulated by ecdysteroids that circulate in the hemolymph. During this first phase of limb regeneration, termed basal growth, a blastema forms under the wound and develops a fully segmented albeit small limb. Ecdysteroid signaling may play a role in the deposition of a flexible cuticle during basal

growth. This is followed by proecdysial growth during which tissue size increases due to protein synthesis and water uptake, and which is completed as the crab molts. Endogenous retinoic acids have been isolated from proecdysial growth phase limb blastemas and retinaldehyde enzymatic activity was detected in the regenerate [196, 197]. Interestingly, proecdysial growth is affected by artificially increased RA signaling: When RA is added to the seawater of newly autotomized fiddler crabs during the first 5 days of blastema formation, limb regeneration proceeds normally. However, exposure to RA during later stages results in segmentation defects that have been attributed to a failure of the epidermis to fold into the nascent blastema [198]. It has not been determined yet whether these effects originate in the epidermis, but RXR protein localizes to the epidermis. Another problem is that because both ecdysteroids and RA act through RXRs, ligand-bound RA receptors may compete with ecdysteroid receptors for the availability of RXRs. Because molting is regulated by ecdysteroids and is highly sensitive to changes in receptor activation, high concentrations of RA may interfere with regenerative steps that require low levels of ecdysteroid signaling [198]. In fact, RA has been shown to increase the expression of *U. pugilator* RXR in relation to the ecdysone receptor during the proecdysial growth phase of regeneration [199]. Finally, upon limb autotomy, immunostaining with heterologous vertebrate antibodies detects the presence of FGF 2-like proteins in the area distal to the severed nerve of the fiddler crab [200]. It remains to be shown whether limb regeneration in this arthropod is regulated by an endogenous retinoid pathway and analogously to the vertebrate limb, with regulatory functions embedded in the regeneration epidermis and regulatory interactions between the RA and FGF signaling pathways.

Conclusions

Evidence confirming activation of endogenous retinoid signaling pathways during animal regeneration has been accumulating over the last decade. At the same time, pharmacological as well as genetic inhibition of RA signaling has shown that an activated RA pathway may be indispensable for regeneration. The necessity for RA is independent of the injured organ, as exemplified by zebrafish heart and fin regeneration, and there are indications from research into limb, tail and lens regeneration in amphibians that RA might be essential in controlling these processes. In the future rigorous testing of these hypotheses will be required. The zebrafish is perfectly suited to investigate putative functions of RA signaling in other regenerating organs (i.e., liver, kidney and brain) due to its ability to regenerate many organs and its accessibility to genetic and pharmacological manipulations. Investigations into RA signaling in kidney regeneration might be of particular interest. RA signaling is crucial during kidney organogenesis [201] and RA activity has been detected in the kidney of adult mice [202]. Notably, kidney tubulogenesis in vitro can be induced by RA

[203, 204]. Thus, RA has been proposed to promote kidney regeneration. Much needed thorough analyses of the distribution of sources and sinks of RA in regenerating organs should reveal similarities and differences across the regeneration models that may prove informative from a functional as well as evolutionary point of view. Finally, it will be absolutely essential to identify which signals activate RA signaling in response to injury. Probably even more important are investigations into the direct targets of RA signaling during regeneration, which should help to understand central issues that have emerged in the field so far: is RA directly required for blastema proliferation or does it play an exclusive or additional role in dedifferentiation and redifferentiation? If either is true in some animals, do others manage to regenerate without it and if so, how? What is the molecular basis of RA-mediated patterning during super-regeneration? Due to our extensive knowledge concerning RA pathway regulation and a growing array of drugs that target specific components, an in-depth understanding of the roles of endogenous RA signaling in regeneration holds great promise for regenerative therapies in human patients.

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Chapter 3

Osteoblast de- and redifferentiation are controlled by a dynamic response to retinoic acid during zebrafish fin regeneration

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Abstract

Zebrafish restore amputated fins by forming tissue-specific blastema cells that coordinately regenerate the lost structures. Fin amputation triggers the synthesis of several diffusible signaling factors that are required for regeneration, raising the question of how cell lineage-specific programs are protected from regenerative crosstalk between neighboring fin tissues. During fin regeneration, osteoblasts revert from a non-cycling, mature state to a cycling, preosteoblastic state to establish a pool of progenitors within the blastema. After several rounds of proliferation, preosteoblasts redifferentiate to produce new bone. Blastema formation and proliferation are driven by the continued synthesis of retinoic acid (RA). Here, we find that osteoblast dedifferentiation and redifferentiation are inhibited by RA signaling, and we uncover how the bone regenerative program is achieved against a background of massive RA synthesis. Stump osteoblasts manage to contribute to the blastema by upregulating expression of the RA-degrading enzyme *cyp26b1*. Redifferentiation is controlled by a presumptive gradient of RA, in which high RA levels towards the distal tip of the blastema suppress redifferentiation. We show that this might be achieved through a mechanism involving repression of Bmp signaling and promotion of Wnt/β-catenin signaling. In turn, *cyp26b1*$^{+}$ fibroblast-derived blastema cells in the more proximal regenerate serve as a sink to reduce RA levels, thereby allowing differentiation of neighboring preosteoblasts. Our findings reveal a mechanism explaining how the osteoblast regenerative program is protected from adverse crosstalk with neighboring fibroblasts that advances our understanding of the regulation of bone repair by RA.

Introduction

Regeneration of amphibian and fish appendages proceeds through epimorphic regeneration, in which a blastema of cycling progenitor cells forms at the wound site. The blastema harbors a mixture of cells with distinct origins and fate-restricted potential that coordinately regenerate the lost appendage (Gemberling et al., 2013; Tanaka and Reddien, 2011). The zebrafish caudal fin, which completely regrows within two weeks upon amputation, has emerged as a powerful model to study the underlying cellular and molecular mechanisms of appendage regeneration. Amputation triggers successive steps (wound healing, blastema formation and regenerative outgrowth) that finally restore the original tissues, including bone, connective tissue, blood vessels, nerves, epidermis and pigment cells (Akimenko et al., 2003; Poss et al., 2003). Several signaling pathways have been implicated in fin regeneration (e.g. FGF, Igf, RA and Wnt/β-catenin signaling) (Blum and Begemann, 2012; Chablais and Jazwinska, 2010; Poss et al., 2000; Stewart et al., 2014; Stoick-Cooper et al., 2007; Wehner et al., 2014; Whitehead et al., 2005). However, our understanding of their tissue-specific functions is very limited. Identifying the signals that act on the distinct cell lineages is therefore a crucial next step towards a thorough understanding of fin regeneration. Another major unresolved question is how regenerative programs specific for a particular cell lineage are protected from regenerative crosstalk between neighboring fin tissues. For example, high concentrations of diffusible signals required by many cells might interfere with a subset that must avoid them.

The skeletal elements of the fin, the fin rays, run from proximal to distal and are separated by soft interray tissue (Akimenko et al., 2003). Each fin ray is made up of two opposed and segmented hemirays, derived from intramembranous ossification that surround a soft core of fibroblasts, blood vessels, nerves and pigment cells (Fig. 1A). The bone matrix is laid down by osteoblasts that line up along the bone surface. During fin regeneration osteoblasts switch from a non-cycling, matrix-producing (mature) state to a cycling, less differentiated (preosteoblastic) state, and vice versa (supplementary material Fig. S1; Knopf et al., 2011; Sousa et al., 2011; Stewart and Stankunas, 2012). This remarkable behavior allows rapid replacement of lost bone. Upon amputation, mature osteoblasts next to the amputation site become proliferative and migrate towards the wound site. In the nascent blastema, preosteoblasts align at proximal lateral positions, thereby forming a spatially restricted subpopulation of the blastema. After several rounds of division, preosteoblasts redifferentiate to form new bone tissue. Differentiation progresses in a distal-to-proximal direction, so that fast cycling preosteoblasts at the distal leading edge of aligned osteoblasts become slow-cycling differentiating cells, which subsequently mature into non-cycling matrix-producing cells (Stewart et al., 2014). Despite their dedifferentiation, osteoblasts remain lineage restricted (Knopf et al., 2011; Stewart and Stankunas, 2012). To achieve proper reconstitution of lost bone, appropriate ratios between osteoblast dedifferentiation, proliferation and redifferentiation must be tightly controlled.

However, the molecular mechanisms that control the transition between the distinct differentiation states within the osteoblast lineage have only recently begun to be addressed. Wnt/β-catenin and BMP signaling have been shown to regulate preosteoblast proliferation and differentiation via opposing activities (Stewart et al., 2014). The signals that control osteoblast dedifferentiation in the stump are unknown so far.

Retinoic acid (RA) has been shown to play multiple roles in bone development and repair by exerting pleiotropic effects on cells of the chondroblast, osteoblast and osteoclast lineages (Adams et al., 2007; Allen et al., 2002; Conaway et al., 2013; Dranse et al., 2011; Koyama et al., 1999; Laue et al., 2008, 2011; Li et al., 2010; Lie and Moren, 2012; Lind et al., 2013; Nallamshetty et al., 2013; Song et al., 2005; Spoorendonk et al., 2008; Weston et al., 2003; Williams et al., 2009). Although partially conflicting results were reported, the general consensus for osteoblastogenesis is that RA signaling restricts osteoblast differentiation but promotes subsequent bone matrix synthesis by mature osteoblasts.

Fin amputation triggers massive RA synthesis that is indispensable for blastema formation and maintenance (Blum and Begemann, 2012, 2013), raising the question of how the osteoblast lineage deals with the massive background of RA signaling. Here, we demonstrate that two key steps of the osteoblast regenerative program, osteoblast dedifferentiation and subsequent redifferentiation, are adversely affected by RA from neighboring tissues. Moreover, we provide a conceptual framework for understanding how bone regeneration is achieved during fin regeneration.

Results

Aldh1a2 and Cyp26b1 cooperate to control osteoblast activity in the uninjured fin

The zebrafish caudal fin grows throughout life. Fin growth is achieved via the sequential, distal addition of new segments to each fin ray (Haas, 1962). At the same time, hemiray thickness increases over time by adding new bone matrix along the entire fin length, meaning that hemirays are thicker in older fish and at more proximal positions (Fig. 1B; supplementary material Fig. S2A). Thus, osteoblasts in the uninjured fin continuously synthesize basal levels of matrix. In larval zebrafish, RA promotes bone matrix synthesis (Laue et al., 2008; Li et al., 2010; Spoorendonk et al., 2008). We therefore expected a comparable role for RA signaling in osteoblasts in the adult fin. We detected expression of the RA synthesizing-enzyme *aldh1a2* in proximity to hemirays (supplementary material Fig. S2B). Using *aldh1a2:gfp* fish (Pittlik and Begemann, 2012), *aldh1a2*-expressing cells could be identified as fibroblasts adjacent to osteoblasts (Fig. 1C). Osteoblasts were visualized by immunohistochemistry (IHC) for ZNS-5, an uncharacterized cell surface antigen specifically present on osteoblasts, irrespective of their differentiation status (Johnson and Weston, 1995; Knopf et al., 2011). This finding suggests that fin osteoblasts receive RA from neighboring fibroblasts. We have previously shown that intraperitoneal (IP) injection of RA efficiently enhances RA levels in the adult fin (Blum and Begemann, 2012). A single injection of RA resulted in enhanced expression of the bone matrix genes *osteocalcin* (*osc*) (also known as *bglap*), *osteonectin* (*osn*), *collagen* (*col*) *1a1a* and *col10a1* in the uninjured fin 10 h after injection (Fig. 1D). Conversely, breakdown of endogenous RA levels via overexpression of the RA-degrading enzyme *cyp26a1* in *hsp70l:cyp26a1* fish (Blum and Begemann, 2012) caused downregulation within 12 h after a single heat shock (Fig. 1D). Moreover, the hemiray surface was fully covered by osteoblasts after 10 days of RA inhibition in *hsp70l:cyp26a1* fish (supplementary material Fig. S2C), indicating that downregulation of bone matrix genes was not due to osteoblast death. Together, these data show that RA signaling positively regulates bone matrix synthesis in the adult uninjured fin.

In order to maintain the right balance between stiffness and flexibility of hemirays, matrix synthesis has to be tightly controlled. The RA-degrading enzyme Cyp26b1 attenuates osteoblast activity in larval zebrafish (Laue et al., 2008; Spoorendonk et al., 2008). *In situ* hybridization (ISH) on uninjured fins revealed expression of *cyp26b1* in fin osteoblasts (Fig. 1E). By contrast, expression of the two other RA-degrading enzymes, *cyp26a1* and cyp26*c1*, could only be detected in a few single cells scattered throughout the mesenchyme and epidermis (supplementary material Fig. S2D,E), although we cannot completely rule out expression in few osteoblasts. These findings suggest that osteoblasts in the uninjured fin counteract excessive RA through Cyp26b1 activity. We tested this idea by blocking Cyp26 activity with the R115866 compound, a selective antagonist of Cyp26 enzymes (Hernandez et al., 2007; Stoppie et al.,

2000), and examined expression levels of *osc*, *osn*, *col1a1a* and *col10a1*. Expression was upregulated 12 h after IP injection (Fig. 1D). We conclude that Aldh1a2 in fibroblasts and Cyp26b1 in osteoblasts cooperate to control RA levels, and thus osteoblast activity in the adult fin (Fig. 1F).

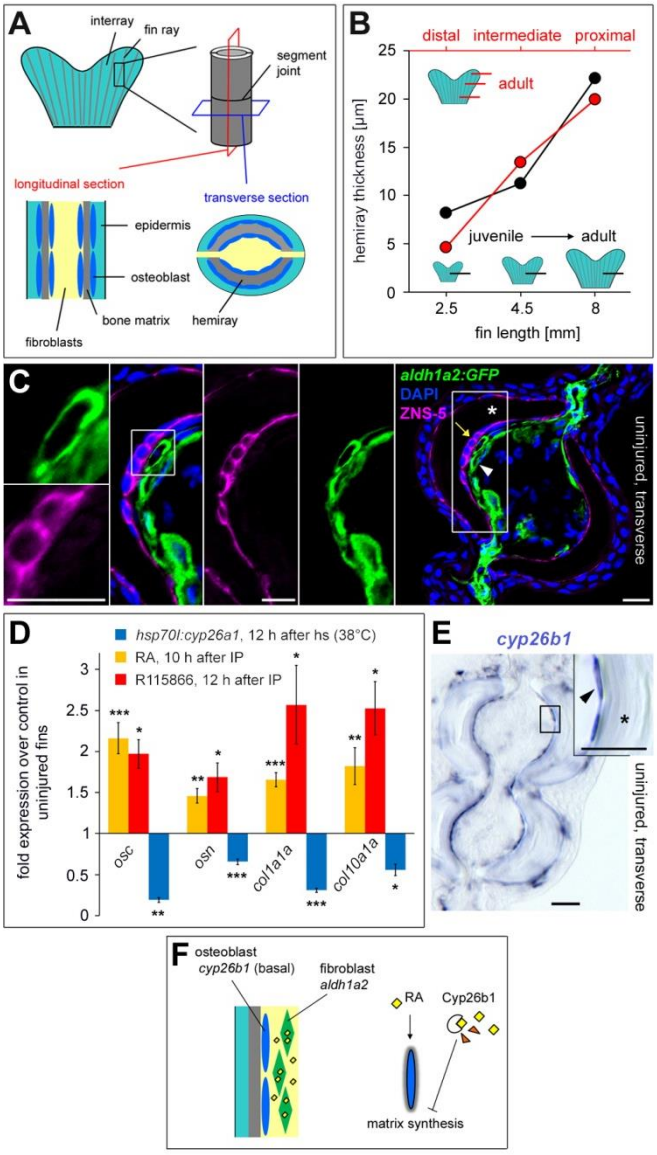

Fig. 1. Bone matrix synthesis in the uninjured fin is controlled by RA production and degradation. (A) Overview of relevant structures and tissue types of a fin ray. (B) Mean hemiray thickness at different positions along the proximodistal axis in adult fish (red) and at an intermediate position in fins of different lengths (black) show that hemirays are thicker in older fish (longer fin) and at more proximal positions. (C) IHC for ZNS-5 and GFP in *aldh1a2:gfp* fish in the uninjured fin reveals *aldh1a2* expression in fibroblasts (arrowhead) adjacent to osteoblasts (arrow). Asterisk indicates bone matrix. (D) RA and R115866 injections upregulate expression of bone matrix genes. Conversely, inhibition of RA signaling in *hsp70l:cyp26a1* fish downregulates expression (qPCR analysis). (E) ISH demonstrates *cyp26b1* expression in osteoblasts (arrowhead). Asterisk indicates bone matrix. (F) Model of bone matrix synthesis regulation by RA synthesis and degradation in the uninjured fin. Data are represented as mean±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001. Scale bars: 10 µm in C; 20 µm in E. h, hours; hs, heat shock.

Stump osteoblasts upregulate *cyp26b1* prior to dedifferentiation

Fin amputation has previously been shown to induce upregulation of *aldh1a2* in the stump mesenchyme in proximity to the wound site, and the resulting increase in RA levels is indispensable for blastema formation (Blum and Begemann, 2012) (Fig. 2A). Of note, analyses of stump tissue at 24 hours post amputation (hpa) in *aldh1a2:gfp* fish revealed that *aldh1a2*

expression remains excluded from osteoblasts upon amputation (Fig. 2B). The increase in RA synthesis in stump fibroblasts should enhance matrix synthesis in adjacent osteoblasts. However, expression of *osc* (Knopf et al., 2011; Sousa et al., 2011), *osn*, *col1a1a* and *col10a1* (supplementary material Fig. S3A) decreases upon amputation, suggesting that enhancing RA levels in osteoblasts upon fin amputation are counterproductive for their dedifferentiation. We therefore hypothesized that stump osteoblasts have to counteract rising intracellular RA levels in order to switch to a preosteoblastic state. Indeed, we detected increased expression of *cyp26b1* at 5 hpa, followed by further upregulation during the next hours (Fig. 2A). Expression levels of *cyp26a1* and *cyp26c1* were unchanged or downregulated (supplementary material Fig. S3B). We noticed that whole-mount ISH (WISH) is not sensitive enough to detect *cyp26b1* expression in the uninjured fin, but allows visualization of enhanced *cyp26b1* expression in the fin stump. Upregulated expression of *cyp26b1* could be detected in single osteoblasts within one segment length proximal to the amputation plane (Fig. 2C) as early as 12 hpa. RA signaling employs a number of autoregulatory feedback mechanisms in order to obtain appropriate RA levels. For instance, enhanced RA levels usually cause upregulation of *cyp26* expression (Dobbs-McAuliffe et al., 2004; Hu et al., 2008; Lee et al., 2012). Accordingly, we detected increased *cyp26b1* levels in uninjured fins 10 h after RA injection and decreased levels within 12 h after a single heat shock in *hsp70l:cyp26a1* fish (supplementary material Fig. S3C). We therefore examined whether upregulation of *cyp26b1* expression in stump osteoblasts also occurs when endogenous RA is removed. Interestingly, *cyp26b1* expression was upregulated at 24 hpa despite heat shock-induced *cyp26a1* expression in *hsp70l:cyp26a1* fish (supplementary material Fig. S3D), suggesting that an RA-independent regeneration-specific program underlies upregulation of *cyp26b1.*

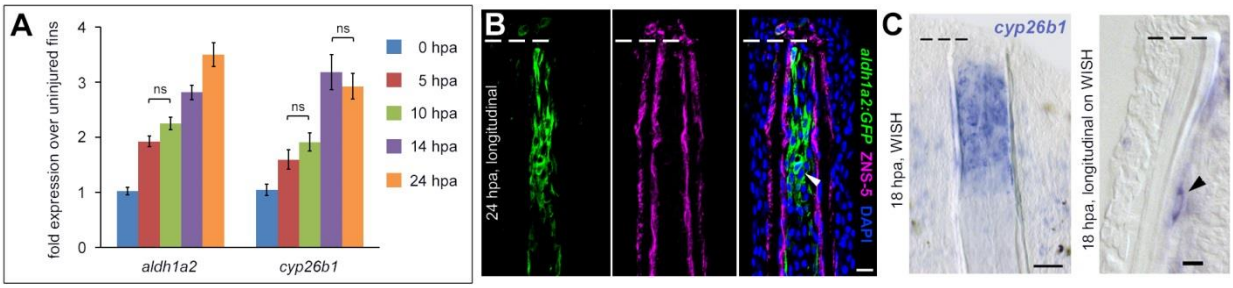

Fig. 2. Stump osteoblasts upregulate *cyp26b1* expression. (A) Fin amputation induces upregulation of *aldh1a2* and *cyp26b1* expression. qPCR analysis. *P*<0.05 unless noted otherwise. (B) IHC for ZNS-5 and GFP in *aldh1a2:gfp* fish at 24 hpa reveals *aldh1a2* expression in fibroblasts (arrowhead). (C) WISH indicates upregulated *cyp26b1* expression within one segment length proximal to the amputation plane at 18 hpa. Longitudinal section on WISH shows upregulated *cyp26b1* expression in single osteoblasts (arrowhead). Scale bars: 20 µm in B and C (section); 50 µm in C WISH. Dashed lines indicate amputation planes.

Osteoblast dedifferentiation requires inactivation of RA by Cyp26b1

Osteoblast dedifferentiation is accompanied by downregulation of *osc* and upregulation of *runx2b*, a marker of immature osteoblasts (Knopf et al., 2011; Sousa et al., 2011). We found a similar upregulation of *runx2a* (not shown). In order to determine the requirement of Cyp26b1 activity for osteoblast dedifferentiation we injected fish with either R115866 or RA and examined *runx2* and *osc* expression at 10 or 24 hpa, respectively. Treatments were started 10 h before amputation (first IP at −10 hpa) to ensure sufficient drug concentrations in fin osteoblasts at the onset of dedifferentiation. Expression of *runx2b* was lower and *osc* expression was higher in RA- and R115866-treated fish than in control fish (Fig. 3A). In addition, *runx2a* expression was lower in RA-treated fish (Fig. 3A). Conversely, inhibition of RA signaling in *hsp70l:cyp26a1* fish caused enhanced *runx2* and decreased *osc* levels (Fig. 3A). These data reveal that stump osteoblasts fail to adopt characteristics of an immature state if RA levels are too high. Upregulation of *fgf20a* and *igf2b*, two positive regulators of blastema formation and proliferation (Chablais and Jazwinska, 2010; Whitehead et al., 2005), was enhanced in RA-treated fish at 10 hpa (first IP at −10 hpa) (supplementary material Fig. S4A), indicating that our treatment regime did not generally impair initiation of fin regeneration. Furthermore, in treatments starting with the first injection at 0 hpa or later, *runx2* upregulation and *osc* downregulation were largely unaffected (not shown), showing that Cyp26b1 activity is required very early in the stump. We next tested whether stump osteoblasts are able to enter the cell cycle in the absence of Cyp26b1 activity or during exogenously enhanced RA levels by assaying for 5-ethynyl-2′-deoxyuridine (EdU) incorporation at 24 hpa. The number of $EdU^+/ZNS\text{-}5^+$ cells was strongly reduced in R115866- and RA-injected fish (first IP at −10 hpa) (Fig. 3B,C). We only rarely detected dying osteoblasts in control, RA- and R115866-treated fish (data not shown), demonstrating that the decrease in proliferating osteoblasts was not due to enhanced cell death. Thus, stump osteoblasts do not start to proliferate if RA inactivation fails. An alternative explanation might be that RA signaling interferes more directly with osteoblast proliferation. However, injection of RA at 22 hpa, after many osteoblasts probably had already dedifferentiated, did not result in reduced osteoblast proliferation at 30 hpa (supplementary material Fig. S4B).

Among the three cyp26 genes, only *cyp26b1* is significantly expressed in the fin and becomes upregulated in response to fin amputation, implying that R115866 mainly acts in stump osteoblasts. Interestingly, in both RA- and R115866-treated fish (first IP at −10 hpa), regenerate length was slightly shorter at 2 dpa (supplementary material Fig. S4C,D), and the number of proliferating fibroblasts and epidermal cells was reduced at 24 hpa (supplementary material Fig. S4E). Cell death was not enhanced in fibroblasts at 24 hpa, and was only weakly enhanced in the epidermis of R115866-treated fish but not in RA-treated fish (supplementary material Fig. S4F). Notably, exogenous RA increases, rather than inhibits, fibroblast proliferation at 30

hpa, when RA treatment starts after osteoblasts have dedifferentiated (first IP at 22 hpa) (supplementary material Fig. S4G).

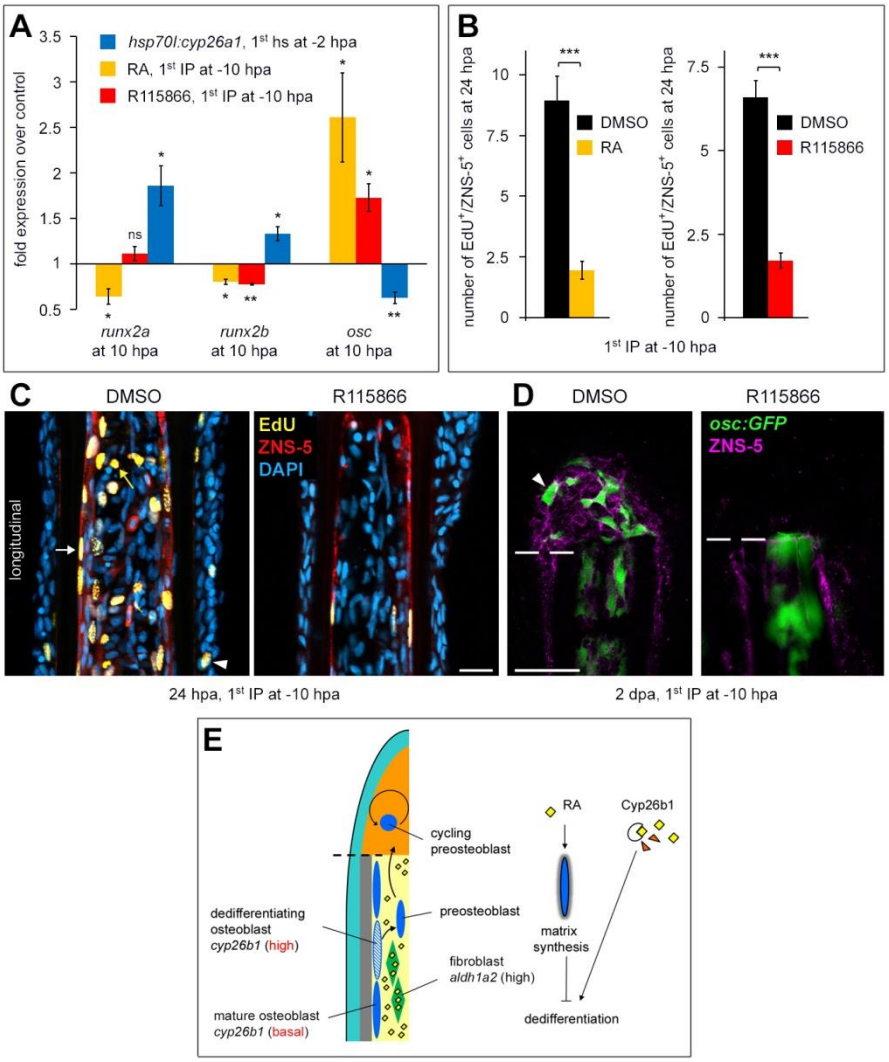

Fig. 3. Osteoblast dedifferentiation requires RA inactivation by Cyp26b1. RA and R115866 injections starting at −10 hpa block osteoblast dedifferentiation. Inhibition of RA signaling in *hsp70l:cyp26a1* fish positively affects dedifferentiation. (A) qPCR analysis of osteoblast markers at 10 and/or 24 hpa. (B) EdU$^+$/ZNS-5$^+$ cells per section at 24 hpa. (C) IHC for ZNS-5 combined with EdU labeling. Images show the stump region adjacent to the amputation plane. White arrow indicates EdU$^+$/ZNS-5$^+$ cell, yellow arrow denotes EdU$^+$ fibroblast, arrowhead indicates EdU$^+$ epidermal cell. (D) IHC for ZNS-5 and GFP in *osc:gfp* fish demonstrates absence of preosteoblasts in the blastema of R115866-treated fish at 2 dpa. Arrowhead indicates GFP$^+$ preosteoblast in control fish. (E) Model for Cyp26b1 function in osteoblast dedifferentiation. Data are represented as mean±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001. ns, not significant. Scale bars: 20 µm in C; 100 µm in D. Dashed lines indicate amputation planes. hs, heat shock.

Dedifferentiating osteoblasts downregulate the mature osteoblast marker *osc*, and redifferentiated osteoblasts upregulate its expression between 5 and 6 dpa (Knopf et al., 2011). Owing to the persistence of GFP protein, the transgenic *osc:gfp* line can therefore be used to detect dedifferentiated osteoblasts in the early blastema (Knopf et al., 2011; Sousa et al., 2011).

We assayed for the presence of GFP^+ preosteoblasts in the blastema of RA- and R115866-injected *osc:gfp* fish (first IP at −10 hpa). Whereas a population of GFP^+ cells had accumulated in the blastemas of control fish at 2 dpa, the majority of blastemas in RA- and R115866-treated fish were largely devoid (<6 cells) of GFP^+ cells (Fig. 3D; data not shown). Notably, RA or R115866 treatment does not interfere with preosteoblast migration (Blum and Begemann, 2015), strongly suggesting that the absence of preosteoblasts in the blastema of RA- or R115866-treated fish is due to dedifferentiation failure. Together, our data reveal that RA signaling inhibits the switch from mature osteoblasts to proliferating preosteoblasts and demonstrate that Cyp26b1 activity is crucially required for osteoblast dedifferentiation (Fig. 3E). By contrast, subsequent proliferation does not require RA inactivation. Of note, the early blastema of RA- and R115866-treated fish (first IP at −10 hpa) did not harbor ZNS-5^+ cells (Fig. 3D; data not shown), showing that missing dedifferentiated osteoblast were not replaced by cells that had differentiated *de novo* to osteoblasts.

RA signaling positively controls preosteoblast proliferation

Preosteoblasts and other stump cells migrate towards the wound site, where they form a blastema within the first two days after amputation. Subsequently, a high proliferation rate in the distal blastema cells ensures regenerative outgrowth, whereas more proximal cells differentiate to rebuild the lost fin structures. Preosteoblasts form a subpopulation at proximal lateral blastema positions (Knopf et al., 2011; Sousa et al., 2011). *aldh1a2* is not expressed in preosteoblasts and redifferentiated osteoblasts, but in adjacent fibroblast-derived blastema cells at 3 dpa (Fig. 4A,B). Intriguingly, although *cyp26b1* becomes upregulated in fibroblast-derived cells in the proximal medial blastema, we could not detect *cyp26b1* expression in osteoblasts at 2 or 3 dpa (Fig. 4C; data not shown). This finding indicates that, in contrast to dedifferentiating osteoblasts, preosteoblasts have ceased to remove RA. Having shown previously that RA signaling positively regulates blastema proliferation (Blum and Begemann, 2012), we next examined whether preosteoblasts also proliferate in an RA-dependent manner. As complete inhibition of RA signaling causes death of blastema cells (Blum and Begemann, 2012), we chose a mild inhibition of RA signaling, as achieved by heat-shocking *hsp70l:cyp26a1* fish at 37°C instead of 38°C, to investigate the effect of decreased RA levels on osteoblast proliferation during regenerative outgrowth. To estimate the reduction in RA levels upon heat-shocking fish at 37°C in comparison to heat-shocking at 38°C, we examined expression levels of *axin2* and *cyp26b1* at 3 dpa. Wnt/β-catenin signaling in the regenerating fin is positively regulated by RA signaling, and, accordingly, *axin2* expression becomes rapidly downregulated upon heat shock treatment in *hsp70l:cyp26a1* fish (Blum and Begemann, 2012). Similar to the uninjured fin, *cyp26b1* is a sensitive RA target during regenerative outgrowth (N.B. and G.B., unpublished). Downregulation of both genes was diminished and took longer in fish that

received a 37°C heat shock at 3 dpa in comparison to fish that received a 38°C heat shock (supplementary material Fig. S5A). The number of EdU$^+$/ZNS-5$^+$ cells was strongly reduced in *hsp70l:cyp26a1* fish 3 h after a single 37°C heat shock at 3 dpa (Fig. 3D). Cell death was neither enhanced in osteoblasts nor in other cell types (data not shown). We next tested whether RA signaling is already required for preosteoblast proliferation in the stump. In contrast to blastema cells, stump cells survive well upon RA depletion (Blum and Begemann, 2012). We therefore examined the number of EdU$^+$/ZNS-5$^+$ stump cells at 30 hpa upon a 38°C heat shock at 24 hpa in *hsp70l:cyp26a1* fish. We found a strong reduction in proliferating stump cells in *hsp70l:cyp26a1 fish* (supplementary material Fig. S5B). Injection of RA at 3 dpa caused a strong increase in the number of EdU$^+$/ZNS-5$^+$ cells within 6 h after injection (Fig. 4D,E). As mentioned before, elevated RA levels between 22 and 30 hpa did not affect proliferation of stump osteoblasts (supplementary material Fig. S4B). However, this could be explained by the opposing effects of RA on dedifferentiation and proliferation. Taken together, our data demonstrate that the proliferation of preosteoblasts is positively controlled by RA.

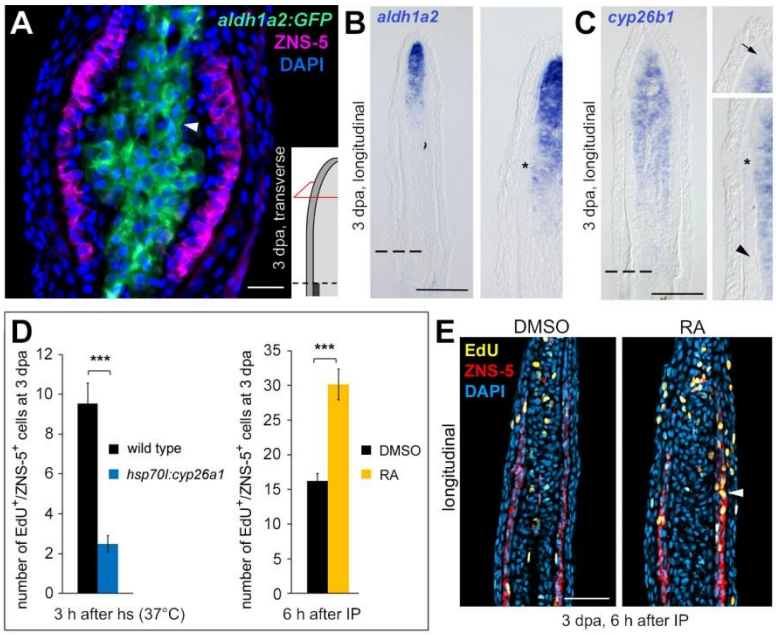

Fig. 4. RA signaling promotes osteoblast proliferation. (A) IHC for ZNS-5 and GFP in *aldh1a2:gfp* fish at 3 dpa reveals *aldh1a2* expression in fibroblast-derived blastema cells (arrowhead). (B) ISH for *aldh1a2* at 3 dpa shows that expression is strongest in fibroblast-derived cells in the distal blastema but weak expression reaches far proximally. Asterisk indicates distal leading edge of preosteoblasts. (C) ISH for *cyp26b1* at 3 dpa demonstrates expression in fibroblast-derived cells of the proximal medial blastema. Note absence of expression in the distal blastema (arrow) and in osteoblasts. Asterisk indicates distal leading edge of preosteoblasts, arrowhead denotes redifferentiated osteoblasts. (D,E) Inhibition of RA signaling in *hsp70l:cyp26a1* fish at 3 dpa downregulates osteoblast proliferation. RA injection causes an increase in osteoblast proliferation at 6 h after IP. (D) EdU$^+$/ZNS-5$^+$ cells per section at 3 dpa. (E) IHC for ZNS-5 combined with EdU labeling. Arrowhead indicates EdU$^+$/ZNS-5$^+$ cell. Data are represented as mean±s.e.m. ***P<0.001. Dashed lines indicate amputation planes. Scale bars: 20 µm in A; 100 µm in B,C,E. h, hours; hs, heat shock.

RA signaling prevents preosteoblast redifferentiation

After several rounds of proliferation, preosteoblasts become slow-cycling, differentiating cells in more proximal parts of the regenerate and eventually mature to matrix-producing osteoblasts (Stewart et al., 2014). The expression patterns of *aldh1a2* and *cyp26b1* at 3 dpa imply that RA concentrations within the regenerate decrease from distally high to proximally low levels (Fig. 5A and Fig. 4B,C). *aldh1a2* expression is high in fibroblast-derived cells in the distal blastema, but rapidly decreases in more proximal cells. By contrast, *cyp26b1* expression in fibroblast-derived blastema cells extends far proximally, but is absent in more distal cells, indicating that proximal fibroblast-derived blastema cells probably act as a sink for extracellular RA. Hence, osteoblasts experience different RA concentrations along the proximodistal axis. Given the positive role of RA in preosteoblast proliferation, we speculated that RA might keep preosteoblasts in a proliferative state and prevent differentiation towards the distal tip. This notion is also supported by the negative effect of RA signaling on early osteoblastogenesis during zebrafish larval development (Li et al., 2010). To test this hypothesis, we examined preosteoblast differentiation upon RA injection (first IP at 3 dpa). Because preosteoblast differentiation is accompanied by upregulation of *osx*, we used *gfp* expression in *osx:gfp* fish (*Olsp7:nlsgfp*; Spoorendonk et al., 2008) as a readout. The *osx*-free distal domain was expanded proximally in RA-treated fins 6 h after injection at 3 dpa (data not shown) and more pronounced 24 h after injection (Fig. 5B,C), indicating that preosteoblast differentiation was blocked. Expression of *cyp26b1* in proximal fibroblast-derived blastema cells suggests that RA degradation is important to lower RA levels in proximal regions, which, in turn, should allow preosteoblast differentiation. In fact, differentiation was inhibited at 4 dpa in R115866-injected fish (IP at 3 dpa) (Fig. 5C). We therefore propose that, as soon as RA levels fall below a certain threshold in more proximal parts, preosteoblasts reduce their proliferation rate and differentiate. Thus, changes in RA levels along the proximodistal blastema axis should result in a shift of the distal limit of differentiating preosteoblasts. To test this model, we took advantage of the finding that *aldh1a2* expression is expanded proximally, and that the distal limit of *cyp26b1* expression is shifted proximally in fins that have been amputated at a more proximal position (=proximal cut), relative to the situation in fins that have been amputated at a more distal position (=distal cut) (Fig. 5D-G). In such regenerates, preosteoblast differentiation would be expected to be shifted proximally due to enhanced RA synthesis and delayed RA degradation. In agreement with this view, the *osx*-free distal domain extended further proximally (Fig. 5H).

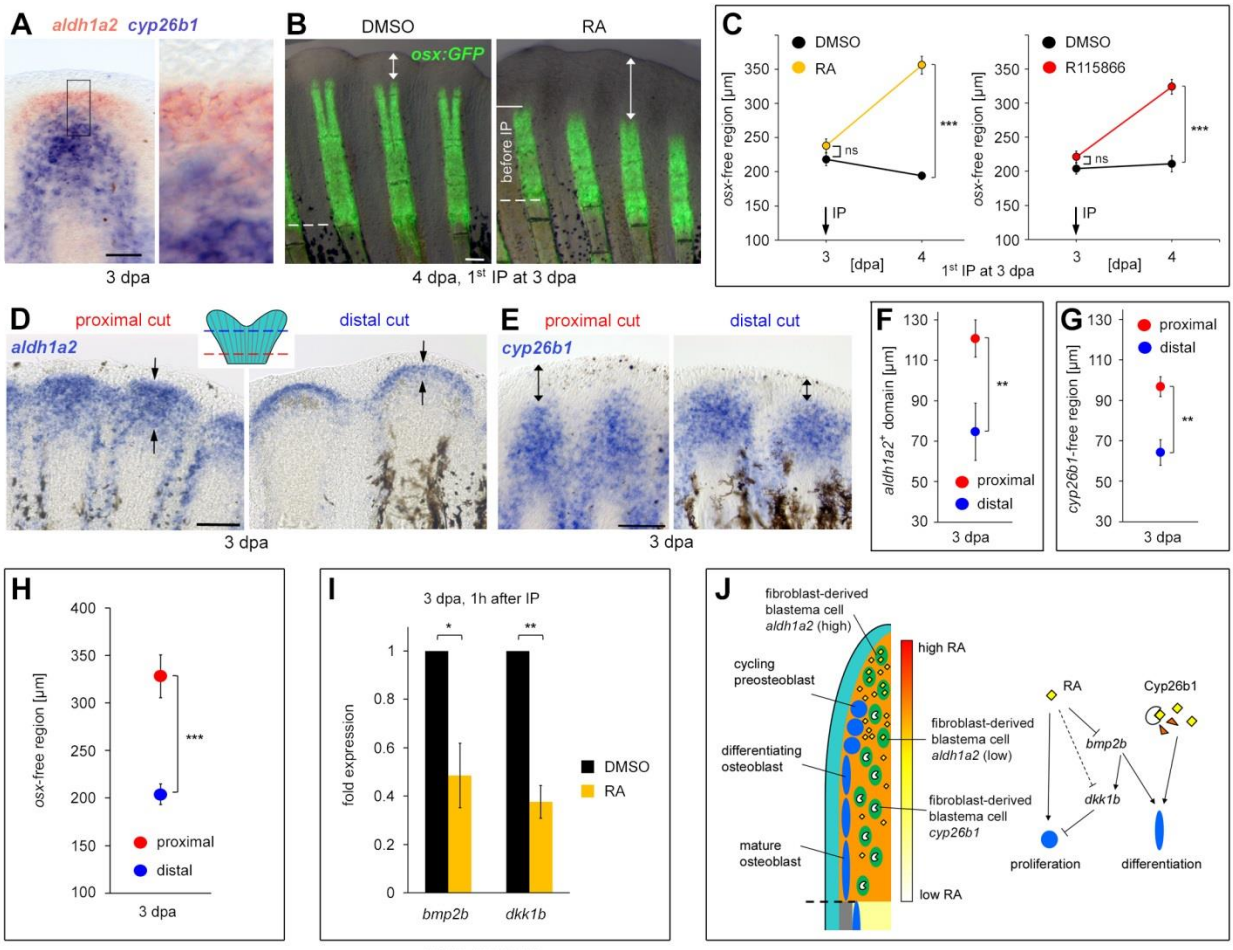

Fig. 5. RA signaling keeps preosteoblasts in an undifferentiated state. (A) Distal blastema cells express *aldh1a2* but not *cyp26b1*. Double WISH at 3 dpa. (B,C) RA and R115866 injections at 3 dpa block preosteoblast differentiation. (B) Live images of *osx:gfp* fish at 4 dpa demonstrate an expanded distal *osx*-free region (doubled-headed arrow) one day after RA injection. (C) Length of the *osx*-free domain. (D-H) *aldh1a2* expression (D,F), the distal *cyp26b1*-free domain (E,G) and the *osx*-free domain (H) extend further proximally in regenerates that had been amputated at a more proximal level. (D) WISH for *aldh1a2* at 3 dpa. Arrows indicate expression boundaries. (E) WISH for *cyp26b1*. Double-headed arrows indicate length of the *cyp26b1*-free region. (F-H) Length of the expression domain or of the distal expression-free region. (I) Injection of RA at 3 dpa downregulates *bmp2b* and *dkk1b* expression (qPCR analysis). (J) Model for regulation of preosteoblast differentiation by RA signaling. Data are represented as mean±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001. Dashed lines indicate amputation planes. Scale bars: 100 µm. h, hours.

We next examined possible mechanisms that could explain how RA signaling interferes with preosteoblast differentiation. Wnt/β-catenin and BMP signaling have been shown to coordinate preosteoblast proliferation and differentiation (Stewart et al., 2014). Wnt/β-catenin signaling promotes proliferation towards the distal tip of the regenerate, whereas BMP signaling positively regulates differentiation by activating expression of *osx* and of the *dickkopf WNT signaling pathway inhibitor 1b* (*dkk1b*). *bmp2b* is expressed in differentiating preosteoblasts and is therefore probably the ligand responsible for activation of BMP signaling during differentiation. Intriguingly, we found that RA treatment downregulates expression of *dkk1b*

and *bmp2b* within one hour after IP injection at 3 dpa (Fig. 5I). These data imply that RA signaling might inhibit preosteoblast differentiation by negatively interfering with BMP and promoting Wnt/β-catenin signaling. In summary, our findings suggest a model in which preosteoblast differentiation is controlled by differences in RA concentrations along the proximodistal axis (Fig. 5J). High RA levels in the distal part promote proliferation and prevent premature differentiation. Conversely, non-cell-autonomous reduction of RA levels in more proximal regions through Cyp26b1 result in differentiation. Thus, both the switch from a mature to a preosteoblastic state in the stump and, vice versa, the switch back from a preosteoblastic to a mature state during regenerative outgrowth require inactivation of RA by Cyp26b1.

RA signaling is required for bone matrix regeneration and prevents its degradation

Redifferentiated osteoblasts start to secrete bone matrix to replace the lost bone. Given the positive effect of RA signaling on bone matrix production in the uninjured fin, we assumed a similar function during regenerative outgrowth. To test this we examined transcript levels of bone matrix genes under altered RA signaling conditions at 4 and 8 dpa. Because increased RA-levels prevent preosteoblasts from differentiating, we restricted our analyses to GFP^+ proximal tissue in *osx:gfp* (or *osx:gfp*, *hsp70l:cyp26a1* double transgenic) fish and excluded the GFP-free distal tissue to ensure similar numbers of differentiated osteoblasts. Similar to the uninjured fin, expression of bone matrix genes was strongly downregulated after a single heat shock in *hsp70l:cyp26a1* fish at 4 (0 h after heat shock) or 8 dpa (6 h after heat shock) (Fig. 6A; supplementary material Fig. S6). By contrast, expression of bone matrix genes was largely unaffected after RA injection (Fig. 6A; supplementary material Fig. S6). Very similar results were obtained by restricting our analyses to GFP^+ proximal tissue in *osc:gfp* (or *osc:GFP*, *hsp70l:cyp26a1* double-transgenic) fish to ensure similar numbers of mature osteoblasts at 8 dpa (data not shown). As fin regeneration requires rapid formation of hemirays to support the new fin tissue, these results suggest that bone matrix genes are expressed at very high levels during regenerative outgrowth and cannot be further upregulated by exogenous RA. We next examined regenerated bone matrix by Alizarin Red staining in *hsp70l:cyp26a1* fish that received a mild heat shock treatment (37°C), starting with the first heat shock at 3 dpa. As expected for mild inhibition of RA signaling, regenerative outgrowth was only slightly slowed down in *hsp70l:cyp26a1* fish at 5 dpa (data not shown). However, the calcified fraction of the regenerate was reduced in *hsp70l:cyp26a1* fish compared with wild-type fish (Fig. 6B). Thus, RA signaling inhibition during regenerative outgrowth interferes with bone calcification, which further demonstrates that bone matrix formation depends on RA signaling. Remarkably, RA and R115866 treatment for 3 days (first IP at 3 dpa) resulted in regeneration of irregular-shaped hemirays (Fig. 6C; data not shown). This phenotype was rather unexpected because enhanced

RA signaling barely affects expression of bone matrix genes. Resorption of bone matrix by osteoclasts is an obligatory process during bone growth, remodeling and fracture healing in mammals (Väänänen et al., 2000). Moreover, osteoclasts have been shown to be involved in healing of fin ray fractures in Medaka (Takeyama et al., 2014). Thus far, osteoclasts have not been implicated in fin regeneration. We assayed for osteoclasts using tartrate-resistant acid phosphatase (TRAP) staining to analyze the presence of the osteoclast-specific TRAP. $TRAP^+$ cells were absent in the uninjured fin but could be detected after fin amputation at the wound site and within 1-2 segment lengths proximal to the amputation plane as early as 24 hpa (Fig. 6D). During regenerative outgrowth $TRAP^+$ cells were still present at the wound site. Additionally, many $TRAP^+$ cells were found at the inner and outer surface of new bone matrix (Fig. 6D,E). Whereas osteoclasts at the wound site are probably involved in healing of bone fractures that are occasionally induced during amputation, the presence of osteoclasts at newly regenerated rays suggest that hemiray regeneration requires the participation of osteoclasts.

RA signaling has been demonstrated to inhibit osteoclast differentiation *in vitro* (Conaway et al., 2009; Hu et al., 2010). We thus speculated that excess of bone matrix in RA- and R115866-treated fins was caused by defects in bone resorption. Indeed, the regenerate of RA- and R115866-treated fish were largely devoid of $TRAP^+$ cells at 6 dpa upon 3 days of treatment (Fig. 6F; data not shown). Lack of $TRAP^+$ cells suggests that enhanced RA levels either prevent osteoclast differentiation or induce osteoclast death. Alternatively, treatment might simply interfere with TRAP expression or activity. Interestingly, RA and R115866 treatment did not affect $TRAP^+$ cells at the wound site (arrowhead in Fig. 6F). Given that osteoclasts at the wound site were already present at 3 dpa when treatment was initiated, this result indicates that enhanced RA levels are likely to interfere with osteoclast differentiation. Consistently, $TRAP^+$ cells were absent from the wound site at 2 dpa in fish that received the first RA injection directly after fin amputation (Fig. 6G). These findings suggest that RA signaling controls formation of new bone matrix at two levels, by ensuring matrix synthesis by osteoblasts and by preventing degradation by osteoclasts (Fig. 6H).

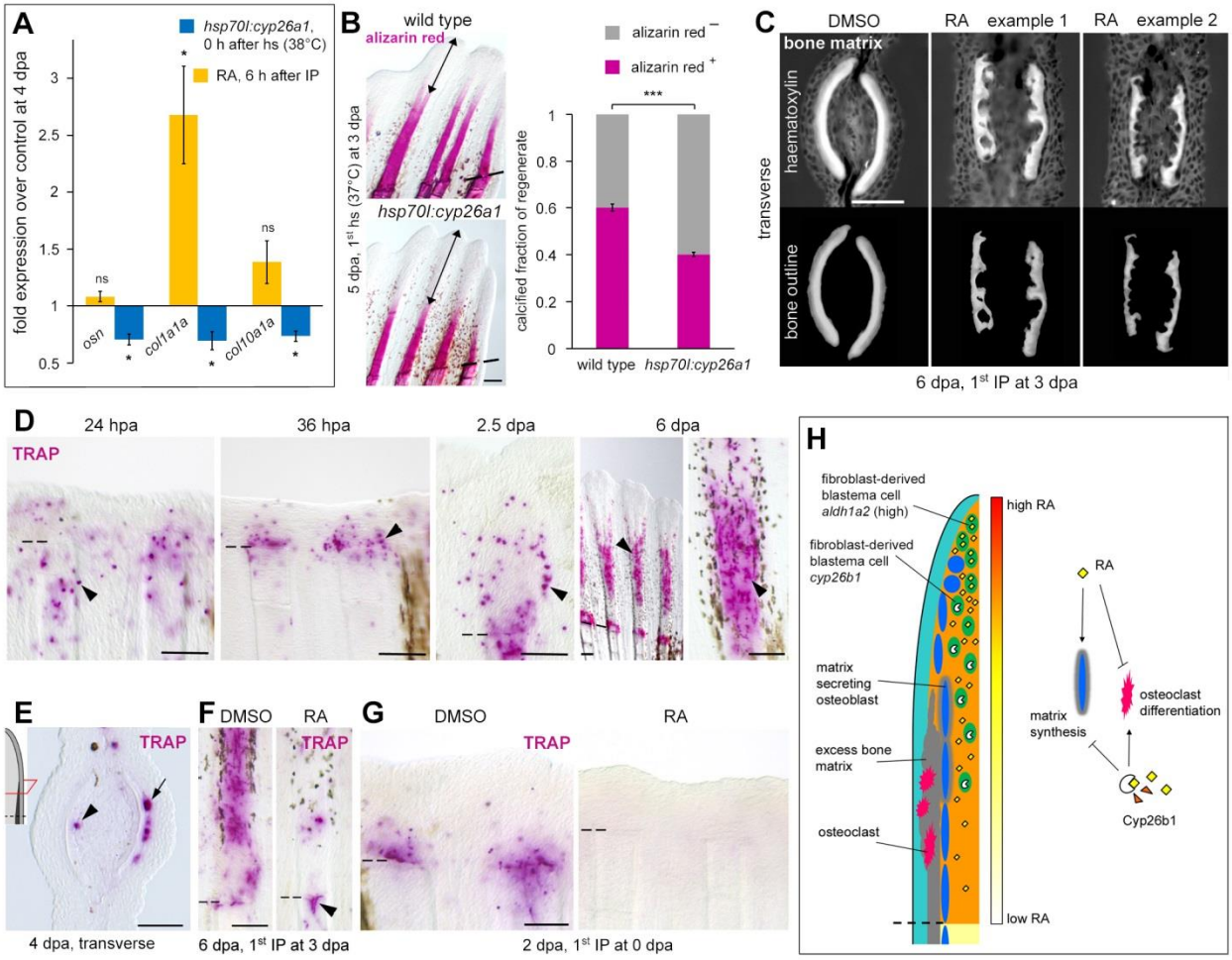

Fig. 6. RA signaling promotes bone matrix synthesis while inhibiting degradation. (A) qPCR analysis. Inhibition of RA signaling in *hsp70l:cyp26a1* fish at 4 dpa causes downregulation of bone matrix genes. Conversely, RA injections barely affect expression. (B) Downregulation of RA signaling in *hsp70l:cyp26a1* fish between 3 and 5 dpa results in an enlarged noncalcified (Alizarin Red^{-}) distal region (double-headed arrow) at 5 dpa. (C) RA injections starting at 3 dpa result in regeneration of irregularly shaped hemirays at 6 dpa. Hematoxylin staining. Lower panel shows hemiray outlines. (D,E) TRAP staining demonstrates presence of osteoclasts (arrowheads in D) at the wound site, in the early blastema and at the inner (arrowhead in E) and outer surface (arrow in E) of newly formed bone during regenerative outgrowth. (F) RA injections starting at 3 dpa result in fewer TRAP^{+} cells in the regenerate but not at the wound site (arrowhead) at 6 dpa. (G) RA injections starting at 0 hpa result in absence of TRAP^{+} cells at the wound site and in the early blastema. (H) Model for RA signaling function in bone matrix synthesis and degradation. Data are represented as mean±s.e.m. **P*<0.05, ****P*<0.01. ns, not significant. Dashed lines indicate amputation planes. Scale bars: 100 µm.

Discussion

The fin blastema is a heterogeneous mixture of fate-restricted progenitor cells that are exposed to high levels of a variety of diffusible signaling molecules. Our work reveals that the amputation-induced burst of RA synthesis, which is indispensable for blastema formation and function (Blum and Begemann, 2012), is in conflict with the osteoblast regenerative program at two crucial stages, the switch from a mature to a preosteoblastic state and, vice versa, the switch back to a mature state. Specifically, we show that, although osteoblast proliferation and bone matrix synthesis requires RA from neighboring fibroblasts, dedifferentiating and redifferentiating osteoblasts have to avoid it. These findings provide novel insights into the molecular mechanisms that guide the regenerative program of osteoblasts during fin regeneration and shed light on the significant, but so far largely overlooked, issue of adverse regenerative crosstalk between neighboring fin tissues.

Mature osteoblasts in the uninjured fin continuously produce low amounts of bone matrix. Here, RA signaling positively regulates expression of bone matrix genes. Notably, RA levels in osteoblasts are determined by RA synthesis in fibroblasts and cell-autonomous RA degradation by Cyp26b1 (Fig. 1F). This cooperation between RA synthesis and degradation in adjacent tissues might help to ensure tightly controlled RA levels in osteoblasts.

Fin amputation results in enhanced RA synthesis in stump fibroblasts. The simultaneous upregulation of *cyp26b1* in osteoblasts counteracts increasing levels of RA and thus facilitates their dedifferentiation (Fig. 3E). In the absence of Cyp26b1 activity, stump osteoblasts fail to adopt an immature state. Interestingly, enhanced *cyp26b1* expression was only detected in a limited number of stump osteoblasts at a given time point, which might reflect either a short-term requirement and/or is an indication of potential asynchronous dedifferentiation of osteoblasts. Alternatively, only a small number of osteoblasts might dedifferentiate in order to establish the preosteoblast progenitor pool. It would be of interest in the future to test whether short-term upregulation of *cyp26b1* in single stump osteoblasts is sufficient to allow their dedifferentiation.

Our previous work has demonstrated that blastema formation and mesenchymal proliferation in the stump are positively regulated by RA signaling (Blum and Begemann, 2012). Unexpectedly, in this study, we found that both RA treatment and inhibition of Cyp26 activity slow down proliferation of fibroblasts and epidermal cells if treatment is initiated sufficiently early to block osteoblast dedifferentiation. Importantly, our treatment regime did not generally impair initiation of blastema formation, as shown by enhanced upregulation of *fgf20a* and *igf2b* expression. Moreover, RA treatment even increases the proliferation rate of fibroblasts when RA treatment starts too late to interfere with osteoblast dedifferentiation. Although RA treatment most likely enhances RA levels in all fin tissues, inhibition of Cyp26 activity is thought to primarily enhance RA levels in cells that express at least one of the three cyp26 genes. Thus,

the spatial and temporal expression patterns of the three cyp26 genes in the fin stump imply that R115866 mainly acts in stump osteoblasts. Moreover, due to the rather small number of osteoblasts within the stump cell population, it is unlikely that *cyp26b1*-expressing osteoblasts act as a sink for extracellular RA and lower the concentration of RA within neighboring cells. Interestingly, ablation of osteoblasts during regenerative outgrowth dramatically slows down regeneration (Singh et al., 2012), suggesting that signals from osteoblasts promote proliferation of other blastema cells. We therefore propose that dedifferentiated osteoblasts might have a positive impact on other cell types already in the stump, and argue that reduced epidermal and fibroblast proliferation in the stump of RA- and R115866-treated fish might be due to disturbed osteoblast dedifferentiation.

Singh et al. (2012) have shown that, following ablation of osteoblasts in the uninjured fin, bone can regenerate normally, suggesting the presence of a ZNS-5$^-$ osteoblast progenitor population that have yet to be identified, or that other cell types can transdifferentiate into osteoblasts. Although this alternative mechanism probably does not play a role in normal fin regeneration, it has been debated whether impaired osteoblast dedifferentiation can be compensated by activation of an alternative bone regeneration program. The absence of ZNS-5$^+$ cells in the blastema of RA- and R115866-treated fish indicates that missing preosteoblasts were not replaced. This finding shows that a putative alternative mechanism either does not become activated in the presence of stump osteoblasts or also requires protection from RA signaling. In contrast to dedifferentiating osteoblasts, cycling preosteoblasts do not express *cyp26b1* and require RA signaling to proliferate, suggesting that dedifferentiation and subsequent proliferation are regulated by distinct signaling mechanisms. In agreement with this notion, Fgf signaling is required for preosteoblast proliferation, but not for dedifferentiation (Knopf et al., 2011). Because *aldh1a2* expression is high in stump fibroblasts, downregulation of *cyp26b1* should allow a rapid increase in RA levels in preosteoblasts and thus might be sufficient to ensure preosteoblast proliferation upon dedifferentiation. The transition back to a differentiated state during regenerative outgrowth is prevented and proliferation is promoted by high RA levels towards the distal tip of the blastema. Moreover, the rapid downregulation of *bmp2* and *dkk1b* expression in RA-treated fish suggests that RA signaling controls preosteoblast differentiation through repression of Bmp signaling and promotion of Wnt/β-catenin signaling (Fig. 5J). Of note, differences in RA concentration along the proximodistal axis are established by opposing patterns of *aldh1a2* and *cyp26b1* expression in fibroblast-derived blastema cells. In contrast to the cell-autonomous function of Cyp26b1 in dedifferentiating stump osteoblasts, Cyp26b1 in proximal fibroblast-derived blastema cells acts non-cell-autonomously and thereby lowers extracellular RA that may diffuse into neighboring osteoblasts. Osteoblasts themselves neither produce RA nor inactivate it, indicating that RA levels in osteoblasts are determined by neighboring cells. In this model, inactivation of RA has a crucial function in controlling the proliferation rate and the position along the proximodistal axis where preosteoblasts

differentiate. *shha*, a member of the Hedgehog (Hh) family, is expressed in the basal epidermal layer adjacent to preosteoblasts and has been proposed to induce their differentiation (Laforest et al., 1998). Moreover, *shha* expression was reported to become downregulated upon immersion of fish in RA (Laforest et al., 1998), suggesting that the inhibitory effect of RA on preosteoblast differentiation might be at least partly due to interfering with Hh signaling. However, we found that, although expression of *shha* requires an RA-free epidermal niche established by Cyp26a1, inhibition of Hh signaling does not interfere with preosteoblast differentiation but rather blocks proliferation (Blum and Begemann, 2015).

During regenerative outgrowth RA from fibroblast-derived blastema cells is indispensable for the expression of bone matrix genes. However, redifferentiated osteoblasts start to produce bone matrix at a position along the proximodistal axis where RA levels are already decreased, suggesting that low levels of RA are sufficient for matrix synthesis. New bone forms very rapidly during regenerative outgrowth. This requires a high rate of bone matrix secretion, raising the question of how the formation of thin, regularly shaped hemirays is ensured. The high number of osteoclasts in the regenerating fin and the striking bone phenotype in the absence of osteoclasts in RA- and R115866-treated fish is an indication for a thus far unnoticed important role of bone resorption in fin regeneration. We therefore propose that removal of excess matrix by osteoclasts is required to generate the final hemiray shape. The inhibitory effect of RA signaling on osteoclast development suggests that osteoclasts can only differentiate at positions along the proximodistal axis where RA levels are already low, a constituting regulatory mechanism that might serve to prevent premature bone resorption.

During bone development and repair, RA inhibits osteoblast differentiation and drives subsequent matrix synthesis (Laue et al., 2008; Li et al., 2010; Lie and Moren, 2012; Spoorendonk et al., 2008), whereas in the osteoclast lineage, RA signaling has been shown to promote proliferation of precursors and bone matrix resorption but blocks differentiation (Conaway et al., 2009, 2013; Hu et al., 2010). Our findings on the function of RA signaling in the formation of osteoblasts and osteoclasts during fin regeneration are consistent with those found for bone development and remodeling. Importantly, we demonstrate that RA signaling orchestrates osteoblast behavior throughout all stages of fin regeneration.

Material and Methods

Zebrafish husbandry and fin amputations

Zebrafish were raised under standard conditions at 27-28°C. Experiments were performed with 3- to 18-month-old fish. Size-matched siblings were used in all experiments. The following zebrafish lines were used: Konstanz wild type, *hsp70l:cyp26a1*^{kn1} (Blum and Begemann, 2012), *aldh1a2:aldh1a2-gfp*^{kn2} (Pittlik and Begemann, 2012), *Ola.bglap:egfp*^{hu4008} (Knopf et al., 2011), *Ola.sp7:nls-gfp*^{zf132} (Spoorendonk et al., 2008). *hsp70l:cyp26a1* fish were analyzed as heterozygotes; wild-type siblings served as controls. Reporter lines were analyzed as hetero- or homozygotes. Caudal fins were amputated along the dorsoventral axis, intersecting the median rays halfway for normal cuts, at ~30% ray length for proximal cuts and at ~70% ray length for distal cuts. Fish were allowed to regenerate for various lengths of time at 26-28°C. All animal experiments were approved by the state of Baden-Württemberg, Germany.

Heat shock and drug treatment conditions

Heat shocks were performed once daily by transferring fish to 33-34°C for 30 min and subsequently to 38°C (or 37°C) for 1 h. IP injections were performed every 12 h for experiments until 2 dpa, and every 24 h during regenerative outgrowth. Approximately 20 µl RA or R115866 were injected. The following concentrations were used: 1 mM RA (*all-trans* RA, Sigma) in 1% DMSO/PBS; 0.67 mM R115866 (a gift from Janssen Pharmaceutica) in 10% DMSO/PBS. Control fish were injected with an equivalent concentration of DMSO/PBS. For IP injection, anesthetized fish were placed belly up in a slit in an agar plate, and the injection needle was inserted at a low angle with the tip pointing cranially close to the pelvic girdle. 1-ml tuberculin syringes (Omnifix, Braun) and 30-G hypodermic-needles (Sterican, Braun) were used.

qPCR, analysis of cell proliferation and cell death, cryosectioning

Gene expression levels were analyzed by qPCR (for primers, see supplementary material Table S1), cell death by TUNEL staining and cell proliferation by EdU labeling. Cryosectioning was used to produce longitudinal and transverse fin sections. Further information concerning these methods, as well as descriptions of imaging and length measurements, immunohistochemistry, *in situ* hybridization, TRAP staining, Hematoxylin staining and Alizarin Red staining can be found in the supplementary material methods.

Statistics

For quantified data, significance of differences was tested using Student's *t*-test. The numbers of specimens used for quantitative and nonquantitative experiments, and the number of specimens that showed the phenotype (for nonquantitative data) are given in supplementary material Tables S2 and S3.

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Competing interests

The authors declare no competing or financial interests.

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Supplementary material

Supplementary Material and Methods

qPCR analysis

For RNA extraction from uninjured fins, a 1 mm wide tissue stripe from the middle of the fin was harvested. For RNA extraction from 0-24 hpa, tissue within 1 mm proximal to the amputation plane was harvested. At 3, 4 and 8 dpa, tissue distal to the amputation plane was used. To analyze expression levels of bone matrix genes at 4 or 8 dpa, *osx:gfp* and *osc:gfp* (or *hsp70l:cyp26a1*, *osx:gfp* (*osc:gfp*) double transgenic) fish were used and the distal GFP-free tissue was carefully removed prior to RNA extraction by using an injection needle. Tissues from 4-10 fins were pooled for each RNA sample. Total RNA was extracted with Trizol reagent (Invitrogen) or TriPure (Roche) and treated with DNase I. Equal amounts of total RNA from each sample were reverse transcribed with SuperScript III reverse transcriptase (Invitrogen) or Maxima Reverse Transcriptase (Thermo Scientific) using anchored oligo(dT) primers. 3-4 RNA samples were reverse transcribed per Experiment (Table S2 and S3). 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 (Thermo Scientific). Primers are listed in Table S1. qPCR reactions for each cDNA pool and each target gene were performed in triplicate. qPCR data were analyzed using the CFX Manager software (Bio-Rad). *ef1a*, *tbp* and *actb1* were used as reference genes. Expression levels were normalized to expression levels of two reference genes (expression stability: Mean M <0.5) and expression ratios were calculated relative to control samples. Uninjured fins (= 0 hpa) were used as control for comparisons of expression levels between different time points. Regenerates of heat-shocked non-transgenic siblings were used as control if expression levels were examined in *hsp70l:cyp26a1* fish and regenerates of vehicle treated fish were used as control if expression levels were examined in RA- or R115866 treated fish. Reference genes were used in different combinations, depending on the treatment condition and regeneration stage. If normalization to different reference genes gave conflicting results (expression stability: Mean M ≥0.5), results were verified by normalization to the input RNA amount by performing RiboGreen or Qubit assays (Invitrogen).

Imaging and measurements

Images were captured with the Zeiss AxioVision or Zeiss ZEN software on a Zeiss Stemi 2000-C stereomicroscope equipped with an AxioCam ERc5s, a Leica MZ10F stereomicroscope equipped with an AxioCam MRc or a Zeiss Axio Imager.M2 equipped with a AxioCam MRc or a AxioCam MRm. For fluorescent microscopy of IHC or EdU stained sections and whole mounts, structured illumination microscopy were used (Zeiss ApoTome.2, Zeiss Axio Imager.M2). Zeiss ZEN and

Adobe Photoshop were used for image processing and length measurements. All length measurements were performed on the third dorsal and the third ventral fin ray. Measurements of hemiray thickness were performed on the third and fourth dorsal and the third and fourth ventral fin rays. Fin length was determined by measuring the length of the third ventral ray (peduncle to distal tip).

Quantification of cell proliferation and cell death

For quantification of cell proliferation and cell death at 24 and 30 hpa, EdU- or TUNEL-labeled epidermal cells and osteoblasts were counted within one segment length proximal to the amputation plane. Labeled stump fibroblasts were counted inside a defined area of 50 x 200 µm adjacent to the amputation plane. For quantifications at 3 dpa, labelled cells were counted in the tissue distal to the amputation plane and normalized to the regenerate length.

Cryosectioning

For cryosectioning, fixed fins were decalcified in 10 mM EDTA in PBT (phosphate buffered saline (PBS) containing 0.1 % Tween20) and embedded in 1.5% agar, 5% sucrose in PBS. Embedded fins were saturated in 30% sucrose in PBS and subsequently snap-frozen in Tissue-Tek O.C.T. Compound (Sakura) in liquid nitrogen. Sections were cut at 16 µm.

Immunohistochemistry

For IHC, fins were fixed in 4% Paraformaldehyde (PFA) in PBS for 3 hours at room temperature or over night at 4°C, transferred to MeOH and stored at –20°C. Fins were rehydrated and cryosectioned or directly subjected to whole mount IHC. Sections or fins were washed in PBT, permeabilized in PBTx (PBS containing 0.3% Triton X-100) (30 min for sections and 1 hour for whole mounts), blocked in 2% blocking reagent (Roche) in PBT and subsequently incubated with primary antibodies diluted in 2% blocking reagent over night at 4°C. After several washes in PBT, tissue was incubated with secondary antibodies diluted in PBT for 3-4 hours at room temperature or over night at 4°C. Whole mounts were transferred to 70% glycerol in PBS for imaging. Sections were counterstained with DAPI and mounted using Mowiol containing DABCO. The following antibodies and dilutions were used: mouse ZNS-5 (Zebrafish International Resource Center) 1:1500, rabbit anti-GFP (Invitrogen, A6455) 1:500. Alexa- (Invitrogen) or Atto- (Sigma) labeled secondary antibodies were used. INT/BCIP (Roche) was used to detect AP-coupled antibodies.

TUNEL labeling

TUNEL labeling was performed in combination with IHC for ZNS-5. Fins were fixed in 4% PFA in PBS for 3 hours at room temperature or over night at 4°C, transferred to MeOH and stored at –20°C. Fins were rehydrated and cryosectioned. Sections were washed in PBS, permeabilized for 30 min in PBTx and equilibrated in terminal deoxynucleotidyl transferase (TdT) buffer (200 mM potassium cacodylate, 25 mM Tris, 0.05% Triton X-100, 1 mM CoCl2, 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 Thermo Scientific). Slides were incubated at 37°C for 1-2 hours and washed in PBS. Sections were blocked in 2% Blocking Reagent in PBS and incubated with anti-fluorescein-AP-coupled antibody (1:2000, Roche) and ZNS-5 antibody (1:1500) in 2% blocking reagent at 4°C over night. After several washes in PBT, TUNEL labeled cells were detected with NBT/BCIP. AP activity was subsequently quenched with 100 mM glycin (pH 2.2). Sections were washed in PBS, blocked in 2% blocking reagent and incubated with anti-mouse-AP-coupled antibody (1:500, Sigma). ZNS-5 labeled cells were detected with INT/BCIP (Roche). Sections were mounted using Mowiol.

EdU labeling

For EdU labelling, fish were IP injected with approximately 20 µl of 2.5 mg/ml EdU (Jena Bioscience) in PBS 1 hour (for analyses at 24 or 30 hpa) or 30 min (for analyses at 3 dpa) prior to fixation. Fins were fixed in 4% PFA in PBS for 3 hours at room temperature or over night at 4°C, transferred to MeOH and stored at –20°C. Fins were rehydrated, washed in PBT and permeabilized in PBTx for 30 min. Subsequently, fins were equilibrated in 100 mM Tris/HCl pH 8 and EdU was detected using a copper-catalyzed azide-alkyne click chemistry reaction (0.6 µM Cy3- or Fluor488- labeled azides (Jena Bioscience), 100 mM Tris, 1 mM CuSO4, 100 mM ascorbic acid, pH 8) with 20 min incubation time. Labeled fins were cryosectioned. For EdU/IHC double staining, EdU labeling was performed on whole mounts and IHC was subsequently performed on sections.

In situ hybridization

Digoxigenin (DIG)- or fluorescein labeled RNA antisense probes were synthesized from cDNA templates: *aldh1a2* (Grandel et al., 2002), *cyp26a1* (Kudoh et al., 2002), *cyp26b1* (Hernandez et al., 2007), *cyp26c1* (Gu et al., 2005). WISH or ISH on sections was performed as previously described (Blum and Begemann, 2012). For double WISH DIG- and fluorescein-labeled probes were hybridized simultaneously. Fins were first incubated with anti-DIG-AP coupled antibody and color reaction was performed with BCIP/NBT. AP activity was quenched with 100 mM glycin (pH 2.2) and fluorescein was detected by using anti-fluorescein-AP coupled antibody and INT/BCIP as substrate.

TRAP staining

For TRAP staining, fins were fixed in 4% PFA in PBS for 3 hours at room temperature or over night at 4°C, washed in PBT and permeabilized in PBTx for 30 min. Subsequently, fins were equilibrated in TRAP Puffer (0.1M NaAcetate, 0.1M acetic acid, 50mM NaTartrate) and color reaction was performed in TRAP buffer containing 0.1 mg/ml Naphtol AS-MX phosphate (Sigma) and 0.3 mg/ml Fast Red Violet LB (Sigma). Labeled fins were transferred to 70% glycerol in PBS for imaging or were cryosectioned.

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.

Alizarin Red staining

For Alizarin Red staining, fins were fixed in 4% PFA in PBS, transferred to MeOH and stored at -20°C. Fins were rehydrated, washed in PBT and stained in 0.1% Alizarin Red in 0.5% KOH overnight. Excess dye was removed by several washes in 0.5% KOH. Stained fins were transferred to 70% glycerol in 0.5% KOH for imaging.

Supplementary References

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Table S1. Primer sequences for qPCR experiments.

Gene	Forward primer	Reverse primer
aldh1a2	GAGAGAGACAGTGCTTACCTTGC	CACAAAGAAGCAGGGGAGG
axin2	GCAGCACAGTTGATAGCCAG	GTCTTGGCTGGCACATATCC
bactin1	TTGCTCCTTCCACCATGAAG	CTTGCTTGCTGATCCACATC
bmp2b	CTGCTGACCACAAGTTTTCG	CAAAGACAGCAGCAATCCC
col10a1a	GCATTCTTCTTCTCCTGGTG	CCTGAACCCCAACCCCC
col1a1a	CAAAACAACGAAAACATCCC	GCATTTGGTTTCGCTCTTTC
cyp26a1	GATGGGAGCTGATAATGTG	CCTGAACCTCCTCTCTGACC
cyp26b1	GCTGGCTGCGTGTTTAGTG	GCCGTCCCAGTAGATGAGTC
cyp26c1	GCAGGAGACAAGGAGGAGG	GCTTCTGCCGTCTCGTGTG
dkk1b	ATGCCAGAGACACTAAATGAACA	TATGAAGGAAACCAGTTGAAAAA
ef1a	TACGCCTGGGTGTTGGACAAA	TCTTCTTGATGTATCCGCTGAC
fgf20a	AAAAGCTGTCAGCCGAGTGT	TGGACGTCCCATCTTTGTTG
igf2b	GCAGGTCATTCCAGTGATGC	TCTGAGCAGCCTTTCTTTGC
osc	CCTGATGACTGTGTGTCTGAG	CGCTTCACAAACACACCTTC
osn	GTGGAGGATGTTATTGCTGAG	GGGGCAGGTCAAAGGGTC
runx2a	GATTTGTGCTCCCGCTTTAG	CTGCTGGACGGCGGACTG
runx2b	GGAGTGGAGGGAGATGGAAG	TAGCGAGTGGAAGAGTACAGATTG
tbp	CGGTGGATCCTGCGAATTA	TGACAGGTTATGAAGCAAAACAACA

Table S2. Number of specimens used in quantitative and nonquantitative experiments (Figs. 1-6). Numbers for corresponding experiments, which are not shown in the figure, are included. For nonquantitative experiments: the first number indicates the number of specimens showing the phenotype, the second number the total number.

Figure	n=
1B	33-46 rays per position along the proximodistal axis in adult fish; 27-39 rays per fin length class
1D	3 cDNA samples per condition
2A	3 cDNA samples per time point
3A	3 cDNA samples per condition
3B	RA: DMSO= 15 sections (4 fins), RA= 35 sections (5 fins); R115866: DMSO= 35 sections (6 fins), R115866=53 sections (6 fins)
3D	RA: DMSO= 36/175 rays, RA= 181/237 rays; R115866: DMSO= 35/211 rays, RA= 122/223 rays
4D	wild type= 24 sections (6 fins), *hsp70l:cyp26a1*= 32 sections (10 fins); DMSO= 31 sections (8 fins), RA=29 sections (6 fins)
5C	RA: DMSO= 12 rays, RA= 12 rays; R115866: DMSO= 12 rays, R115866= 12 rays
5F	proximal= 7 rays, distal=7 rays
5G	proximal= 7 rays, distal=8 rays
5H	proximal= 13 rays, distal=12 rays
5I	3 cDNA samples per condition
6A	3-4 cDNA samples per condition
6B	wild type= 16 rays (8 fins), *hsp70l:cyp26a1*= 16 rays (8 fins);
6C	RA: DMSO= 0/5 fins , RA= 6/6 fins; R115866: DMSO= 0/6 fins, R115866= 6/6 fins
6F	RA: DMSO= 0/6 fins , RA= 5/6 fins; R115866: DMSO= 0/5 fins, R115866= 4/5 fins
6G	DMSO= 0/5 fins , RA= 6/6 fins

Table S3. Number of specimens used in quantitative and nonquantitative experiments (Figs. S1-4). Numbers for corresponding experiments, which are not shown in the figure, are included. For nonquantitative experiments: the first number indicates the number of specimens showing the phenotype, the second number the total number.

Figure	n=
S2C	wild type= 5/5 fins, *hsp70l:cyp26a1*= 5/5 fins
S3A	3 cDNA samples per time point
S3B	3 cDNA samples per time point
S3C	3 cDNA samples per condition
S3D	3 cDNA samples per condition
S4A	3 cDNA samples per condition
S4B	DMSO= 25 sections (5 fins), RA= 25 sections (4 fins)
S4D	RA: DMSO= 30 rays, RA= 35 rays; R115866: DMSO= 24 rays, R115866= 26 rays
S4E	RA: DMSO= 15 sections (4 fins), RA= 35 sections (5 fins); R115866: DMSO= 35 sections (6 fins), R115866=53 sections (6 fins)
S4F	RA: DMSO= 16 sections (5 fins), RA= 25 sections (6 fins); R115866: DMSO= 27 sections (7 fins), R115866=30 sections (7 fins)
S4G	DMSO= 25 sections (5 fins), RA= 25 sections (4 fins)
S5A	3 cDNA samples per condition
S5B	wild type= 17 sections (6 fins), *hsp70l:cyp26a1*= 17 sections (6 fins)
S6	3 cDNA samples per condition

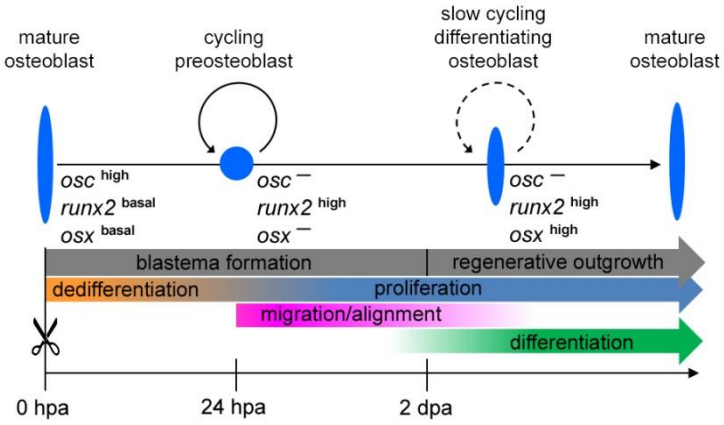

Fig. S1. Overview of osteoblast differentiation states during fin regeneration. Wound epidermis formation, which overlaps with blastema formation, is not shown.

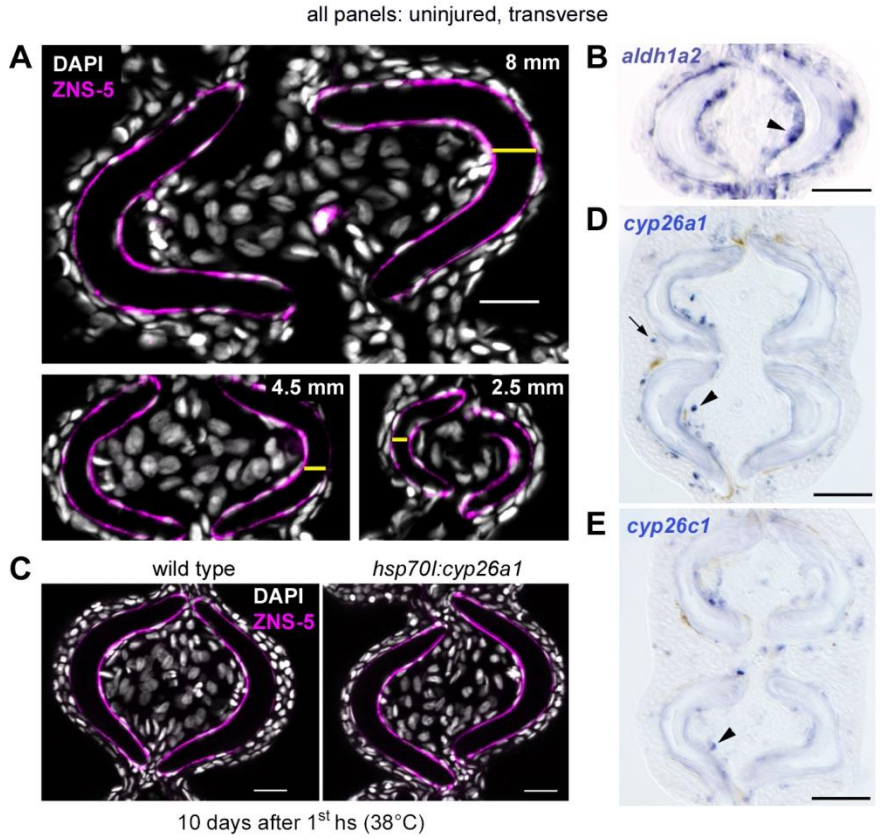

Fig. S2. Hemiray thickness increases during fin growth; RA signaling is not required for osteoblast survival. (A) Sections of uninjured fins of different fin lengths reveal correlation between fin length and hemiray thickness. Fin lengths: 8, 4.5 and 2.5 mm. Yellow bars: Hemiray thickness. (B) ISH demonstrates expression of *aldh1a2* in proximity to hemirays (arrowhead). (C) Inhibition of RA signaling does not interfere with survival of mature osteoblasts. IHC for ZNS-5 demonstrates a similar number of osteoblasts in wild type and *hsp70l:cyp26a1* fish upon 10 days of heat-shock treatment. (D and E) ISH demonstrates expression of *cyp26a1* (D) and *cyp26c1* (E) in single epidermal (arrow) and mesenchymal (arrowhead) cells in uninjured fins. Scale bars: 20 µm in A, C; 50 µm in B, D and E. hs, heat-shock.

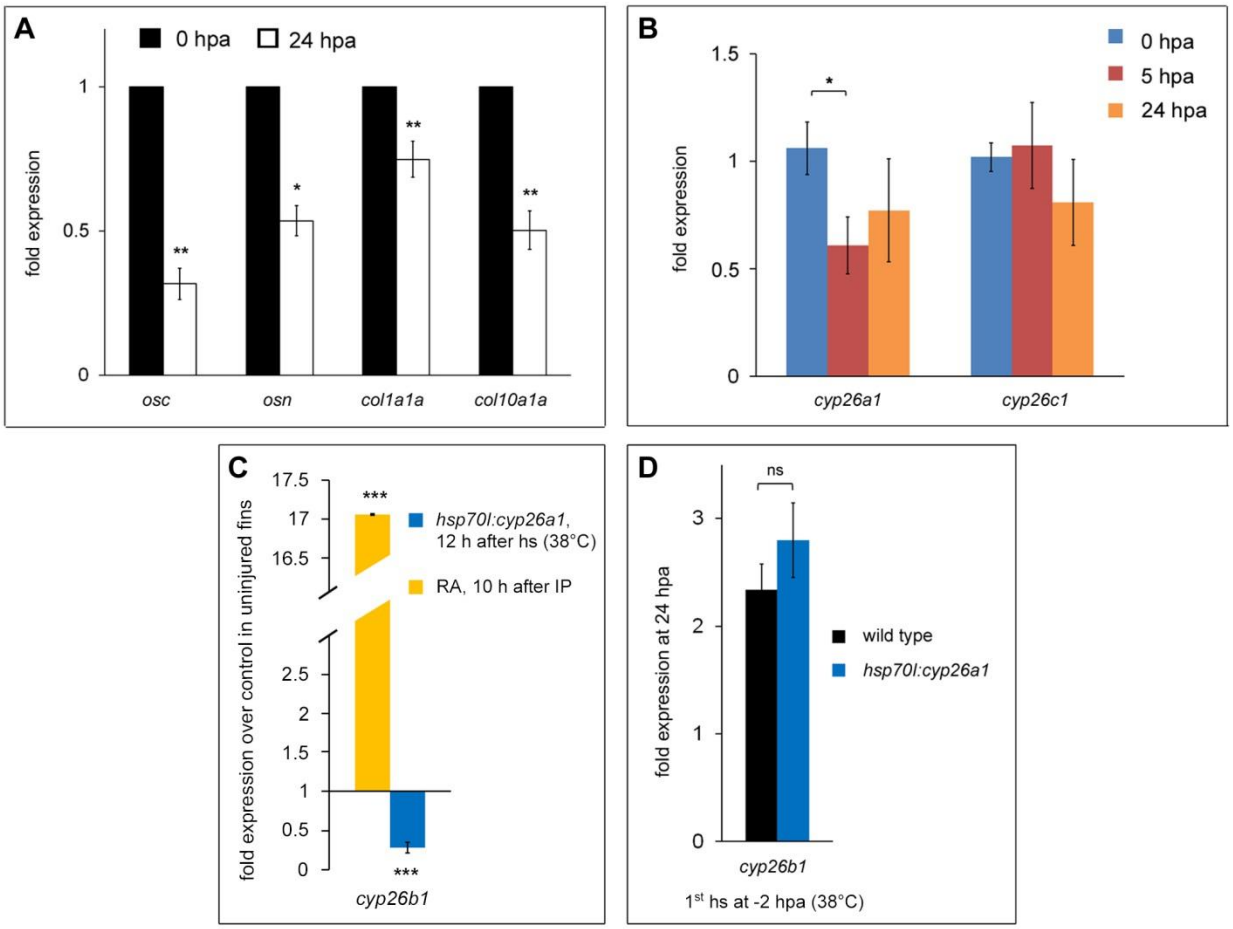

Fig. S3. Stump osteoblasts downregulate bone matrix genes; upregulation of *cyp26b1* does not require RA signaling. (A) Fin amputation causes downregulation of bone matrix genes. qPCR analysis at 0 and 24 hpa. (B) Expression of *cyp26a1* and *cyp26c1* is unchanged or temporarily downregulated upon fin amputation. qPCR analysis at different time points after amputation. All not significant unless noted otherwise. (C) RA injection upregulates expression of *cyp26b1* in uninjured fins. Inhibition of RA signaling in *hsp70l:cyp26a1* fish downregulates expression. qPCR analysis. (D) Inhibition of RA signaling in *hsp70l:cyp26a1* fish does not prevent *cyp26b1* upregulation upon fin amputation. qPCR analysis at 24 hpa. Data are represented as mean±s.e.m. *p < 0.05, **p < 0.01, ***p < 0.001. ns, not significant. h, hours. hs, heat-shock.

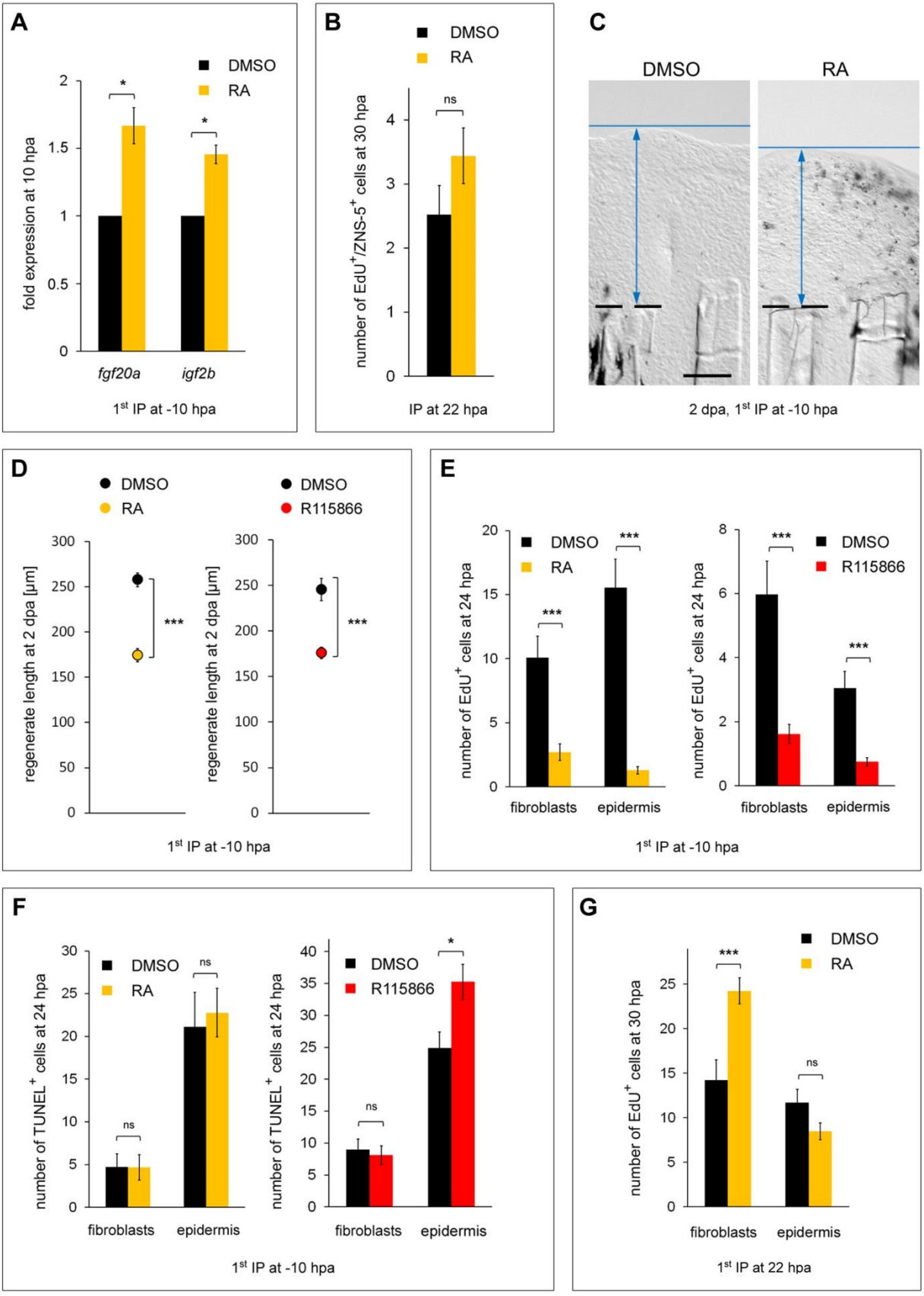
A
DMSO
RA
fold expression at 10 hpa
fgf20a
igf2b
1st IP at -10 hpa
B
DMSO
RA
number of EdU+/ZNS-5+ cells at 30 hpa
ns
IP at 22 hpa
C
DMSO
RA
2 dpa, 1st IP at -10 hpa
D
DMSO
RA
DMSO
R115866
regenerate length at 2 dpa [µm]
***
1st IP at -10 hpa
E
DMSO
RA
number of EdU+ cells at 24 hpa
fibroblasts
epidermis
DMSO
R115866
1st IP at -10 hpa
F
DMSO
RA
number of TUNEL+ cells at 24 hpa
ns
fibroblasts
epidermis
DMSO
R115866
1st IP at -10 hpa
G
DMSO
RA
number of EdU+ cells at 30 hpa
1st IP at 22 hpa

Fig. S4. Stump osteoblasts require Cyp26b1 activity for dedifferentiation but not for subsequent proliferation. (A) RA injections starting at -10 hpa do not prevent upregulation of *fgf20a* and *igf2b* expression in the fin stump. qPCR analysis at 10 hpa. (B) Proliferation of stump osteoblasts is unaffected upon injection of RA at 22 hpa. $EdU^+/ZNS\text{-}5^+$ cells per section at 30 hpa. (C-F) Both RA and R115866 injections starting at -10 hpa slow down regeneration (C and D) and negatively impact proliferation of fibroblasts and epidermal cells (E), but do not increase cell death (F). (C) Fixed regenerates of RA-injected and control fish at 2 dpa. (D) Regenerate length of RA- and R115866-injected fish. (E and F) EdU^+ (E) or $TUNEL^+$ (F) cells per section at 24 hpa. (G) RA injection at 22 hpa promotes proliferation of fibroblasts. EdU^+ cells per section at 30 hpa. Data are represented as mean±s.e.m. *p < 0.05, ***p < 0.001. ns, not significant. Dashed lines indicate amputation plane.

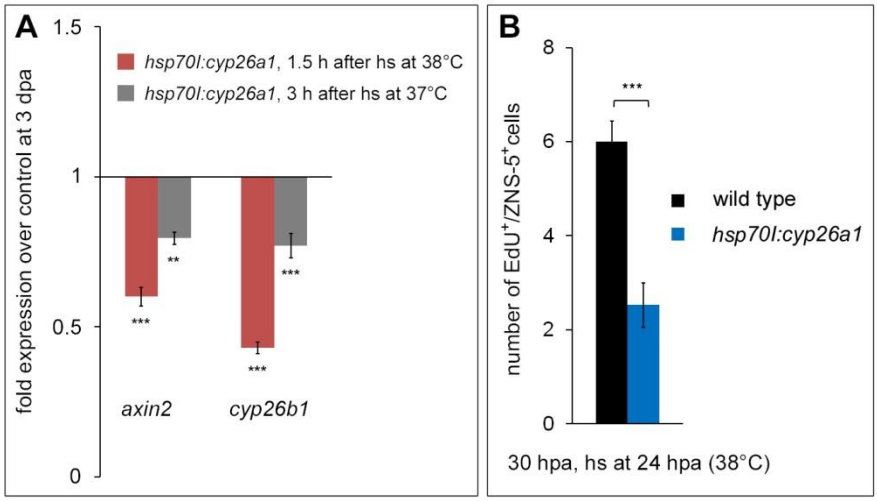

Fig. S5. Osteoblast proliferation in the stump requires RA signaling. (A) Comparison of *axin2* and *cyp26b1* downregulation during regenerative outgrowth between *hsp70l:cyp26a1* fish that received a heat-shock at 37°C and *hsp70l:cyp26a1* fish that received a 38°C heat-shock. qPCR analysis at 3 dpa. (B) Inhibition of RA signaling in *hsp70l:cyp26a1* fish causes downregulation of osteoblast proliferation in the stump. $EdU^+/ZNS\text{-}5^+$ cells per section at 30 hpa. Data are represented as mean±s.e.m. **p < 0.01, ***p < 0.001. hs, heat-shock.

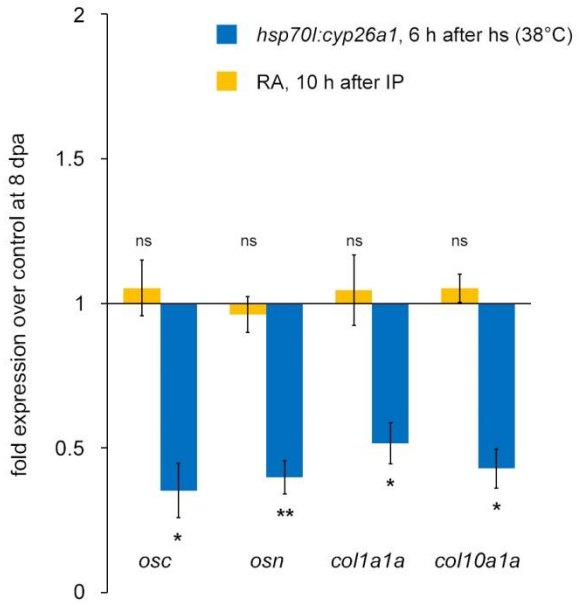

Fig. S6. Expression of bone matrix genes at 8 dpa requires RA signaling. Inhibition of RA signaling in *hsp70l:cyp26a1* fish at 8 dpa causes downregulation of *osc*, *osn*, *col1a1a* and *col10a1a* expression. Conversely expression levels are unchanged in RA injected fish. qPCR analysis. Data are represented as mean±s.e.m. *p < 0.05, **p < 0.01. ns, not significant.

Chapter 4

Retinoic acid signaling spatially restricts osteoblasts and controls ray-interray organization during zebrafish fin regeneration

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Abstract

The zebrafish caudal fin consists of repeated units of bony rays separated by soft interray tissue, an organization that must be faithfully re-established during fin regeneration. How and why regenerating rays respect ray-interray boundaries, thus extending only the existing bone, has remained unresolved. Here, we demonstrate that a retinoic acid (RA)-degrading niche is established by Cyp26a1 in the proximal basal epidermal layer that orchestrates ray-interray organization by spatially restricting osteoblasts. Disruption of this niche causes preosteoblasts to ignore ray-interray boundaries and to invade neighboring interrays where they form ectopic bone. Concomitantly, non-osteoblastic blastema cells and regenerating blood vessels spread into the interrays, resulting in overall disruption of ray-interray organization and irreversible inhibition of fin regeneration. The *cyp26a1*-expressing niche plays another important role during subsequent regenerative outgrowth, where it facilitates the Shha-promoted proliferation of osteoblasts. Finally, we show that the previously observed distal shift of ray bifurcations in regenerating fins upon RA treatment or amputation close to the bifurcation can be explained by inappropriate preosteoblast alignment and does not necessarily require putative changes in proximodistal information. Our findings uncover a mechanism regulating preosteoblast alignment and maintenance of ray-interray boundaries during fin regeneration.

Introduction

Zebrafish regenerate amputated fins by establishing lineage-restricted blastema cells (Gemberling et al., 2013; Tanaka and Reddien, 2011). The zebrafish caudal fin possesses 16-18 fin rays, each consisting of two segmented and opposing hemirays of acellular bone that surround a core of fibroblasts, osteoblasts, pigment cells, arterial blood vessels and nerves (Fig. 1A) (Akimenko et al., 2003). Fin rays are separated by boneless interray tissue, composed of fibroblasts, venous blood vessels, pigment cells and nerves. The principles that compel regenerating fin rays to respect ray-interray boundaries, therefore confining regenerating bone to extend the existing rays, are still unknown. Upon fin amputation, osteoblasts that cover the hemiray surfaces dedifferentiate into proliferating preosteoblasts and migrate into the nascent blastema, where they align at proximal lateral positions (Knopf et al., 2011; Sousa et al., 2011; Stewart and Stankunas, 2012). Thus, preosteoblasts form a layer between the basal epidermal layer and fibroblast-derived blastema cells. The distal blastema remains devoid of preosteoblasts. During subsequent regenerative outgrowth, proliferating preosteoblasts at the distal leading edge become differentiating osteoblasts in more proximal parts (Stewart et al., 2014). Notably, neither osteoblasts nor non-osteoblastic blastema cells mix with adjacent interray cells (Stewart and Stankunas, 2012). Thus, the regenerating fin consists of repeating blastema units dedicated to each fin ray that are separated by regenerating interrays (Fig. 1A).

Results and Discussion

cyp26a1-expressing epidermal niches control preosteoblast alignment and ray-interray organization

During fin regeneration, fin rays respect ray-interray boundaries. An interesting exception occurs after amputation close to a bifurcation site (short cut, Fig. 1B). In such regenerates, sister rays ignore ray-interray boundaries and fuse (Fig. 1C) (Laforest et al., 1998), whereby the probability for ray fusion increases as the distance between them decreases (Fig. 1D). Using the *osc:gfp* line, which allows detection of preosteoblasts in the early blastema (Knopf et al., 2011; Sousa et al., 2011), we found ectopic preosteoblasts in the interray separating the two sister rays at 2 dpa (Fig. 1E). This finding reveals that sister ray fusion is due to preosteoblasts spreading into the interray. We were interested in the mechanisms that cause preosteoblasts to respect ray-interray boundaries. *shha* is expressed within the basal epidermal layer adjacent to pre- and differentiating osteoblasts (supplementary material Fig. S1) (Laforest et al., 1998; Lee et al., 2009; Quint et al., 2002; Zhang et al., 2012). We found a similar expression pattern for the RA-degrading enzyme *cyp26a1* (Fig. 1F,G). By contrast, the RA-synthesizing enzyme *aldh1a2* is expressed in fibroblast-derived blastema cells (Fig. 1G) (Blum and Begemann, 2012). Although proximal fibroblast-derived blastema cells express *cyp26b1* (Blum and Begemann, 2015), it is unlikely that RA diffusion into adjacent epidermal cells is efficiently prevented. We thus suspected that *cyp26a1*-expressing cells provide niches of low RA levels that might facilitate expression of RA-sensitive genes.

It has been shown that immersion of fish in RA reduces *shha* expression (Laforest et al., 1998). However, RA administration via immersion induces epidermal cell death (Ferretti and Géraudie, 1995; Géraudie and Ferretti, 1997); therefore, it has remained unclear whether this was a specific effect. We used intraperitoneal (IP) injection of RA, which efficiently enhances RA levels in the adult fin without causing cell death (Blum and Begemann, 2012), and found decreased expression of *shha* and of the Hh receptor and target *ptch2* 6 h after injection at 4 dpa (Fig. 1H). Injection of R115866, a selective antagonist of Cyp26 enzymes (Hernandez et al., 2007; Stoppie et al., 2000), also caused downregulation of *shha* and *ptch2* (Fig. 1H,I), indicating that *shha* expression requires RA degradation. Accordingly, overexpression of *cyp26a1* in *hsp70l:cyp26a1* fish (Blum and Begemann, 2012) at 4 dpa resulted in enhanced and laterally extended *shha* expression within the basal epidermal layer directly at the end of a single heat shock (Fig. 1J,K). We did not observe *shha* expression in interrays. We propose that this was due to the requirement for Fgfs (and putative other blastema-derived signals) for *shha* expression (Lee et al., 2009).

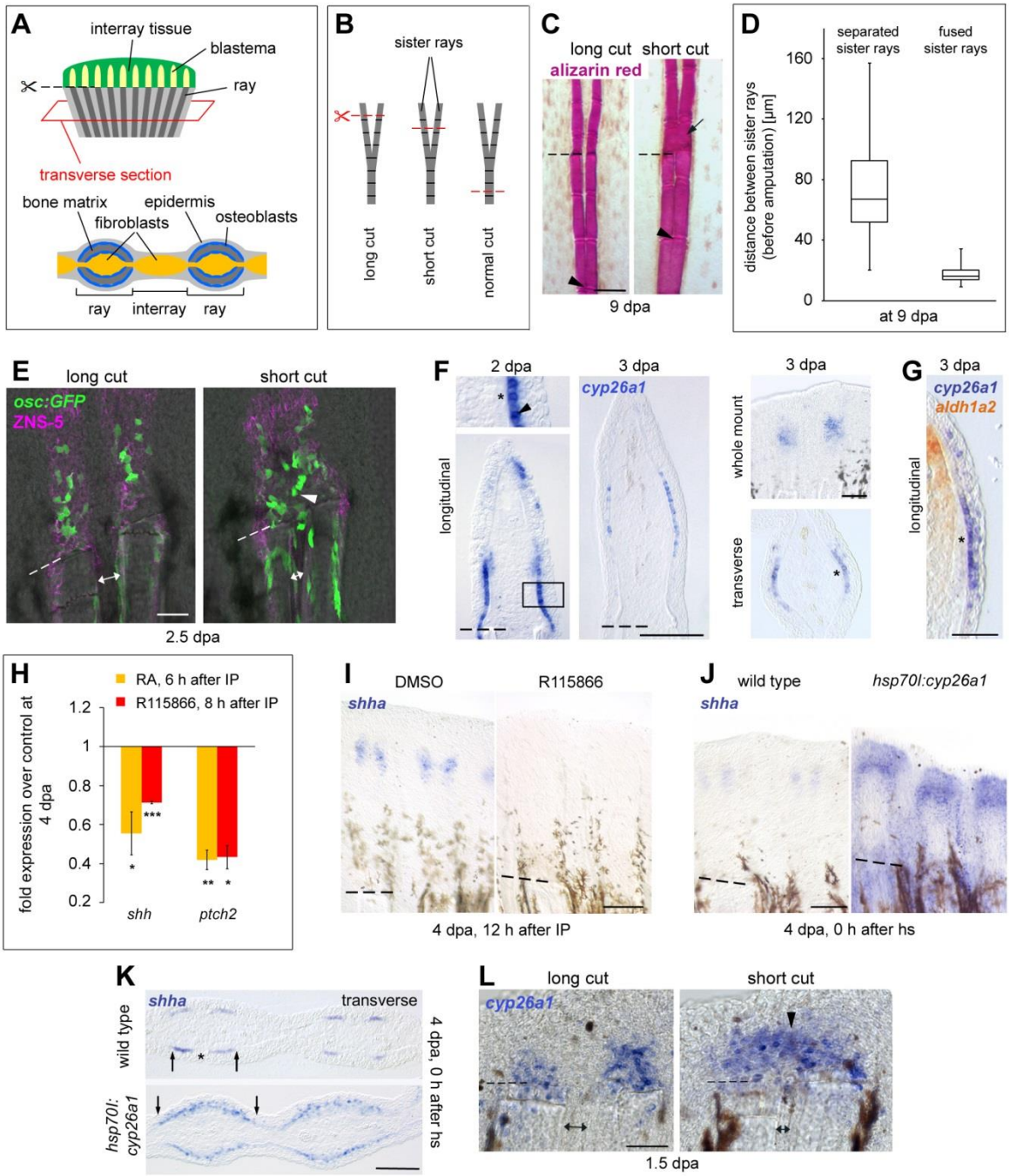

Fig. 1. *shha* expression is controlled by *cyp26a1*-expressing epidermal niches. Lateral expansion of the *cyp26a1*-expressing niches and misguidance of preosteoblasts precede fusion of sister rays. (A) Overview of a regenerating fin. (B) Schematized representation of the amputation levels. (C-E,L) Amputations in proximity to bifurcations cause fusion of *cyp26a1* expression domains (arrowhead in L), followed by spreading of preosteoblasts into the interray separating the two sister rays (arrowhead in E) and fusion of sister rays (arrow in C). (C) Alizarin Red staining at 9 dpa. Arrowheads indicate branching sites. (D) Distance range between sister rays for separated and fused rays. (E) IHC for ZNS-5 and GFP in *osc:gfp* fish at 2.5 dpa. (F,G) WISH and ISH on sections for *cyp26a1* reveals expression in the proximal basal epidermal layer (arrowhead) adjacent to preosteoblasts (asterisks) at 2 and 3 dpa. (G) *aldh1a2* is expressed in fibroblast-derived blastema cells, but not in preosteoblasts adjacent to *cyp26a1*-expressing cells. (H,I) RA and R115866 injection downregulates *shha* and *ptch2*. Transcript levels 6 or 8 hours after injection at 4 dpa measured by qPCR (H); WISH for *shha* 12 hours after R115866 injection at 4 dpa (I). (J,K) Overexpression of *cyp26a1* in *hsp70l:cyp26a1* fish at 4 dpa causes upregulation of *shha* expression and lateral

expansion of the expression domains. Arrows mark the lateral expression limits. Of note, *shha* in wild-type controls is already expressed in subdomains due to the upcoming bifurcation event. (L) WISH for *cyp26a1* at 1.5 dpa. Double-headed arrows in E and L: distance between sister rays. Asterisk marks the gap between the two subdomains. Data are represented as mean±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001. Dashed lines indicate amputation plane. Scale bars: 100 µm. h, hours; hs, heat shock.

Short-cut amputations resulted in shared *cyp26a1* expression domains between sister rays at 1.5 dpa (Fig. 1L), indicating that fusion of the RA-degrading niches precedes spreading of preosteoblasts into interrays. To examine how preosteoblasts behave if efficient lowering of RA levels in the *cyp26a1*-niches fails, we injected fish with RA or R115866, starting with the first IP directly after amputation (normal cut). We observed spreading of preosteoblasts into interrays in RA- and R115866-injected *osc:gfp* fish at 2.5 dpa (Fig. 2A; data not shown), indicating that preosteoblasts failed to align at proximolateral parts of the blastema and disregarded ray-interray boundaries. Of note, formation of a distinct basal epidermal layer was not inhibited (supplementary material Fig. S2A). As R115866 treatment also reduced Cyp26b1 activity in fibroblast-derived blastema cells, we cannot exclude that misguidance of preosteoblasts in R115866- or RA-treated fish was at least partially due to enhanced RA levels in the mesenchyme. However, the expression pattern of *cyp26a1* strongly supports a model in which alignment, and thereby spatial restriction of preosteoblasts, is controlled by signals from *cyp26a1*-expressing epidermal cells.

Interestingly, treatment with RA or R115866 also caused expansion of the blastema marker *msxb* (Akimenko et al., 1995) into interrays (Fig. 2B; supplementary material Fig. S2B). 5-ethynyl-2'-deoxyuridine (EdU) labeling further suggested that these were cycling blastema cells (Fig. 2C). We did not observe an enlarged preosteoblast or *msxb*$^{+}$ cell population in RA- or R115866-treated fish (Fig. 2A,B; supplementary material Fig. S2B), thus making it unlikely that spreading into interrays was simply due to increased cell numbers.

Blood vessels regenerate into the blastema accompanied by anastomosis between the injured arteries and veins of the same ray (Huang et al., 2003). Intriguingly, wounded arteries and veins had formed connections with vessels of neighboring rays in RA- and R115866-treated *fli:gfp* fish (Lawson and Weinstein, 2002) at 2.5 dpa (Fig. 2D; data not shown). Together, these data show that also non-osteoblastic cell types ignore ray-interray boundaries if efficient RA degradation in the proximal basal epidermal layer fails.

Expansion of *msxb* expression into interrays was also observed in regenerates that lacked preosteoblasts, but showed expression of *cyp26a1* (supplementary material Fig. S3). This was achieved by treating fish with the Hh inhibitor cyclopamine from 24 hpa onwards, a treatment condition that is expected to inhibit preosteoblast proliferation in the stump. We thus conclude that, rather than signals from *cyp26a1*-expressing cells, aligning preosteoblasts themselves provide spatial orientation for non-osteoblastic blastema cells.

To test how disrespect of ray-interray boundaries by blastema cells affects the overall organization of the regenerating fin, we gave the last RA or R115866 injection at 1.5 dpa and examined the long-term consequences for regeneration. Of note, owing to autoregulatory feedback mechanisms of RA signaling, *cyp26a1* expression became upregulated in the entire regenerate during RA or R115866 treatment (data not shown). This upregulation can only counterbalance small fluctuations in RA levels and can therefore be neglected during the treatment period. However, ectopic expression persisted for some days after termination of treatment (supplementary material Fig. S4A), and was therefore expected to allow for *shha* expression in the entire regenerate. Consistently, we detected *shha* expression in both rays and interrays in R115866-treated *shh:gfp* fish (Ertzer et al., 2007) 2 days after treatment was stopped at 4 dpa (supplementary data Fig. S4B). As heat shock-induced overexpression of *cyp26a1* at 4 dpa is insufficient to induce *shha* expression in interrays (Fig. 1J,K), this result suggests that *shha* expression in interrays in R115866-treated fish was due to ectopic blastema cells in the interray region. Thus, this finding supports a model in which *shha* expression is spatially confined by RA-degrading epidermal cells and signals from underlying blastema cells.

In fins of R115866-treated fish, regeneration was subsequently irreversibly blocked and ectopic bone had formed in interrays at 11 dpa (Fig. 2E). However, bone matrix did not seal the wound (supplementary material Fig. S5), indicating that inhibition of regeneration was not due to a mechanical block but rather due to mispatterning of the early regenerate. In RA-treated fins, neighboring rays were occasionally connected by bony bridges at the amputation site (Fig. 2F), but otherwise, regeneration proceeded normally. This weaker phenotype was probably due to rapid clearance of excess RA after treatment had stopped. When fins were amputated within the third segment distal to the first branching point (long cut), RA treatment caused fusions of sister rays (supplementary material Fig. S6A). This is consistent with results obtained previously by RA treatment via immersion (Géraudie and Ferretti, 1997; Géraudie et al., 1995; White et al., 1994). We next determined the distance range at which formation of ectopic bone occurs, and found a much greater range in RA-treated fish (supplementary material Fig. S6B). Fusion of sister rays had sometimes been interpreted as a proximalization of the regenerate (White et al., 1994). However, our data show that sister rays fuse if preosteoblasts spread into interrays, which can be caused either by insufficient lowering of RA levels in the proximal basal epidermal layer or by induction of two RA-degrading domains in close proximity. Thus, our findings provide a more parsimonious explanation for sister ray fusion that is not based on putative changes in proximodistal information.

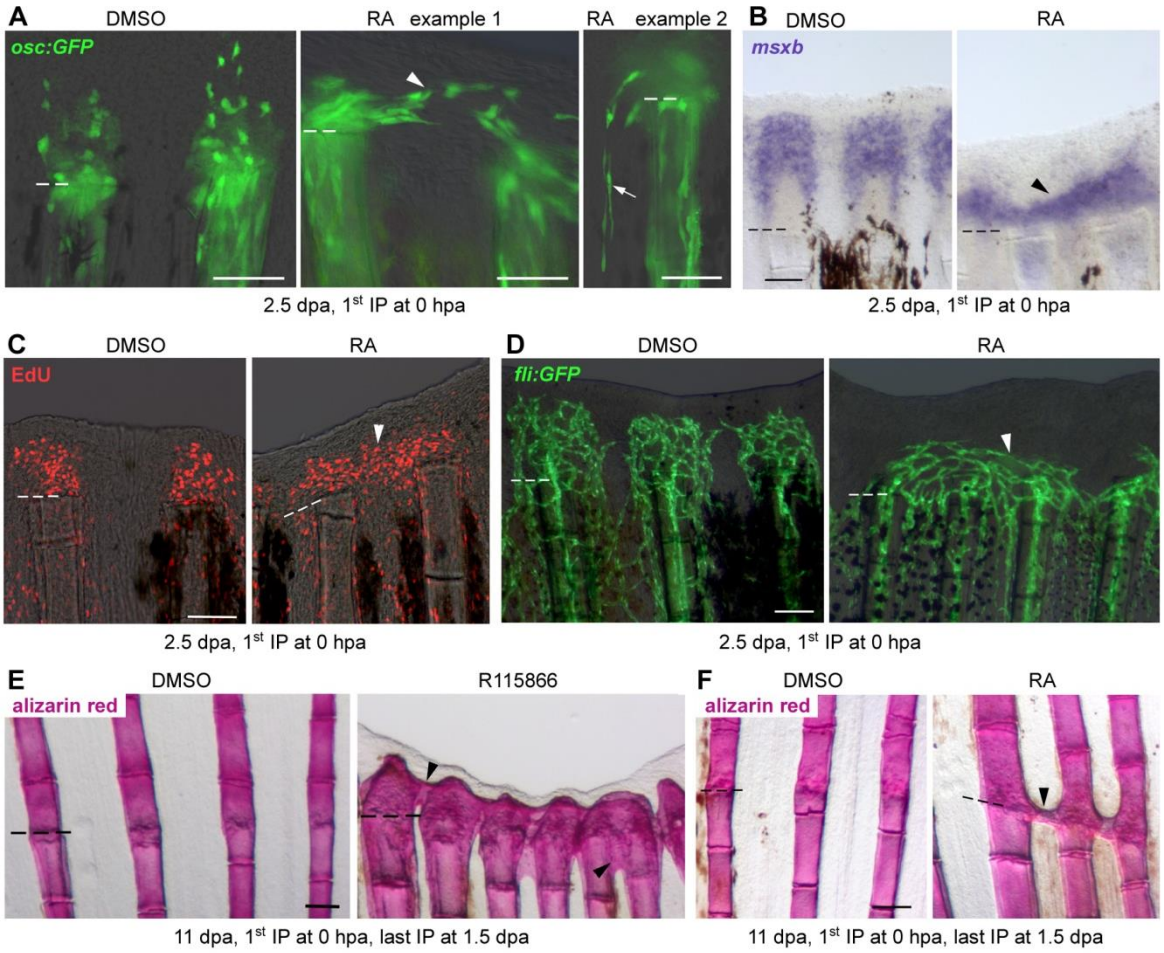

Fig. 2. Preosteoblast alignment and maintenance of the ray-interray organization requires RA-degrading epidermal niches. (A) IHC for GFP in *osc:gfp* fish reveals ectopic GFP^{+} preosteoblasts in interrays in RA-treated fish at 2.5 dpa. Arrowhead indicates preosteoblast in interray tissue, arrow indicates proximally migrating preosteoblast in interray tissue. (B,C) WISH for *msxb* (B) and EdU labeling (C) at 2.5 dpa demonstrate expansion of blastema cells into interrays in RA-treated fish. Arrowheads indicate ectopic blastema cells. (D) *fli:gfp* fish show misconnected blood vessels (arrowhead) in RA-treated fish at 2.5 dpa. (E,F) RA and R115866 treatment during blastema formation result in formation of ectopic bone at the wound site and in R115866-treated fish in an irreversible regeneration block. Arrowheads indicate ectopic bone, dashed lines indicate amputation plane. Scale bars: 100 µm.

Cyp26a1 activity facilitates osteoblast proliferation through *shha* expression

Proliferation of preosteoblasts has been suggested to be controlled by Shha (Laforest et al., 1998; Lee et al., 2009; Quint et al., 2002; Zhang et al., 2012). We found reduced osteoblast proliferation within 12 h of cyclopamine treatment (Fig. 3A). Osteoblast differentiation was unaffected (supplementary data Fig. S7). Moreover, we did not detect TUNEL^{+} osteoblasts in control or cyclopamine-treated fish (data not shown). Hence, even though RA signaling promotes osteoblast proliferation (Blum and Begemann, 2015), prolonged experimental RA exposure should cause downregulation of *shha* and consequently lead to a reduction of osteoblast proliferation. Indeed, osteoblast proliferation was reduced 12 h after RA injection at 3 dpa (Fig. 3B). Neither osteoblasts nor other cells undergo cell death after RA treatment via IP

injections (Blum and Begemann, 2012). Concomitant activation of Hh signaling, using the Smoothened agonist SAG, rescued osteoblast proliferation (Fig. 3C), thus confirming that decreased proliferation upon RA treatment was due to impaired Hh signaling. Together, these findings indicate that Shha from *cyp26a1*-expressing epidermal cells promotes proliferation of adjacent osteoblasts. Interestingly, cyclopamine treatment has been reported to block proliferation of fibroblast-derived blastema cells (Lee et al., 2009), for which *ptch* expression has not been demonstrated. Accordingly, prolonged RA treatment downregulated proliferation of fibroblast-derived blastema cells, and concomitant SAG treatment could rescue this effect (supplementary material Fig. S8). Although we cannot exclude a direct effect, Hh signaling might indirectly promote proliferation of other cell types via osteoblasts.

Besides a requirement for *shha* expression, Fgf signaling has been shown to exclude *shha* from distal regions (Lee et al., 2009), suggesting that Fgf signaling restricts *shha* to the proximal basal epidermal layer by repressing *cyp26a1*. We manipulated Fgf signaling at 3 dpa by either overexpressing a dominant negative Fgfr1 (*hsp70l:dn-fgfr1*; Lee et al., 2005) or a constitutively active Ras (*hsp70l:v-ras*; Lee et al., 2009) and quantified *cyp26a1* expression. *cyp26a1* was downregulated in *hsp70l:v-ras* fish 2 h after a single heat shock and upregulated in *hsp70l:dn-fgfr1* fish at the end of a single heat shock (Fig. 3D), demonstrating that Fgf signaling inhibits *cyp26a1* expression. Thus, proximal expansion of Fgf signaling should result in proximal regression of *cyp26a1* expression. We took advantage of the finding that Fgf activity expands more proximally in fins that had been amputated at a more proximal position and retracts distally as regeneration proceeds (Lee et al., 2005, 2009). We found that amputation at a proximal position results in a proximal shift of the *cyp26a1* expression domain (Fig. 3F,G). In return, *cyp26a1* expression shifted distally during the course of regeneration (Fig. 3E). Notably, also the distal limit of aligned preosteoblasts was always adjacent to the distal limit of *cyp26a1* expression (Fig. 3E-H), suggesting that *cyp26a1*-expressing cells spatially confine preosteoblasts also along the proximodistal axis.

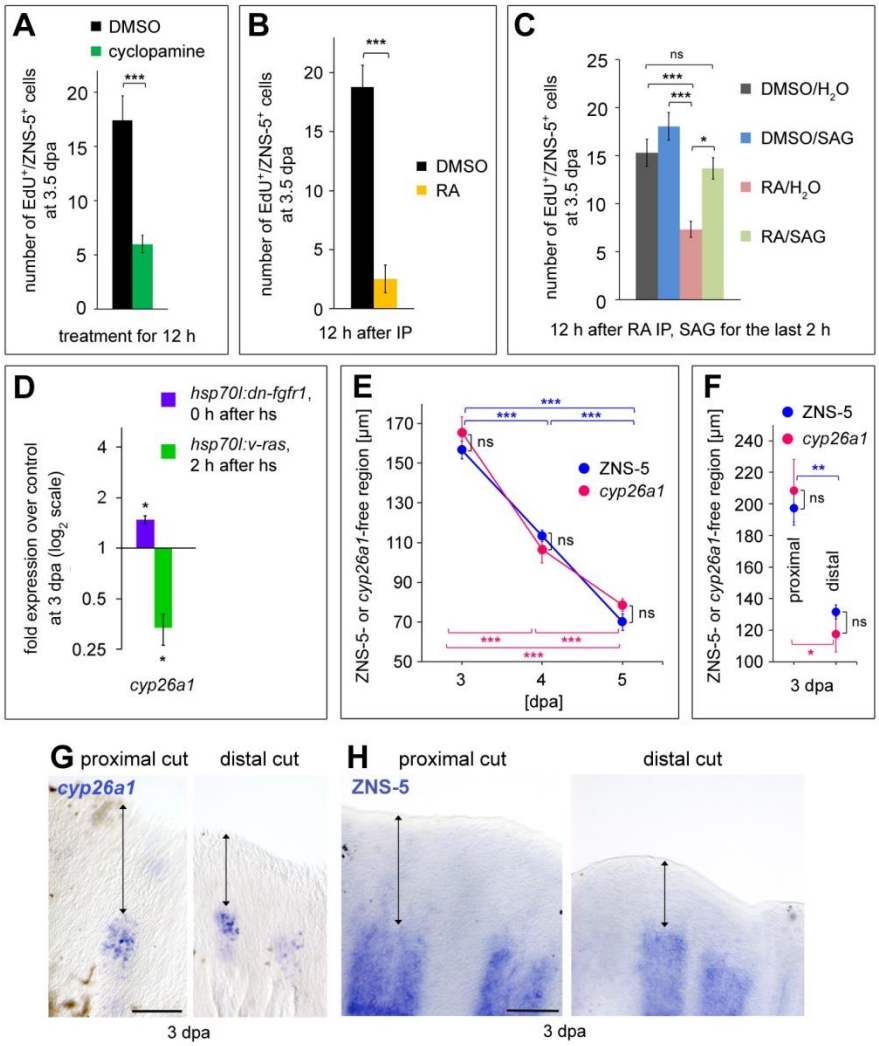

Fig. 3. Shha-promoted osteoblast proliferation requires *cyp26a1* expression that is restricted to proximal regions by Fgf activity. (A,B) Cyclopamine (A) or prolonged RA (B) treatment downregulates osteoblast proliferation. $EdU^+/ZNS\text{-}5^+$ cells per section at 3.5 dpa. (C) Inhibition of osteoblast proliferation 12 h after RA injection can be rescued by concomitant SAG treatment. $EdU^+/ZNS\text{-}5^+$ cells per section at 3.5 dpa. (D) Inhibition of Fgf signaling in *hsp70l:dn-fgfr1* fish results in upregulation of *cyp26a1* expression. Conversely, activation of Fgf signaling in *hsp70l:v-ras* fish results in the downregulation of *cyp26a1*. Transcript levels at 3 dpa measured by qPCR. (E-H) ZNS-5- and *cyp26a1*-free distal domains (double-headed arrows) extend further proximally in regenerates that had been amputated at a more proximal level at 3 dpa (F-H), and retracts distally as regeneration proceeds (E). (G) WISH for *cyp26a1*. (H) IHC for ZNS-5. (E,F) Length of the *cyp26a1*- or ZNS-5-free distal domain. Data are represented as mean±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001. ns, not significant; hs, heat shock. Scale bars: 100 µm.

Conclusions

During fin regeneration the ray-interray organization has to be faithfully re-established in order to rebuild an exact copy of the lost fin parts and to ensure proper function of the regenerated fin. Here, we show that disrespect of the ray-interray boundaries by preosteoblasts and other blastema cells in the nascent blastema has adverse consequences for subsequent fin patterning and may disrupt the whole regeneration process. Our findings support a model in which signals from RA-degrading niches established by Cyp26a1 in the basal epidermal layer ensure the appropriate initial alignment of preosteoblasts in the nascent blastema (Fig. 4) and compel blastema cells to respect ray-interray boundaries. Furthermore, during regenerative outgrowth, Cyp26a1 activity remains important to facilitate Shha-promoted proliferation in adjacent preosteoblasts (Fig. 4).

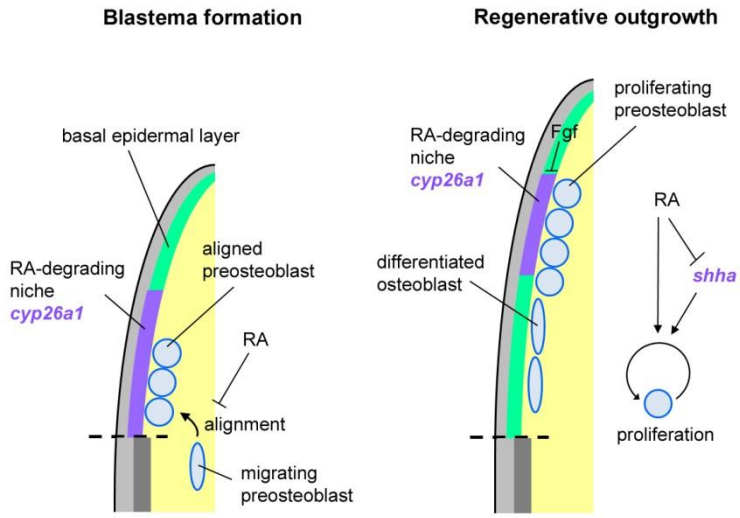

Fig. 4. Model for Cyp26a1 functions during fin regeneration. Schematic summary of Cyp26a1-mediated preosteoblast alignment and proliferation.

Material and Methods

Zebrafish husbandry, fin amputations, heat shock and drug treatment conditions

Zebrafish were raised under standard conditions at 27-28°C. Caudal fins of 3- to 18-month-old fish were amputated along the dorsoventral axis, intersecting the median rays halfway for normal cuts, at ~30% ray length for proximal cuts, at ~70% for distal cuts, within 1-2 segments distal to the first branching point for short cuts and within the third segment away for long cuts. Heat shocks were performed at 38°C for 1 h. Approximately 20 µl RA (all-trans RA, Sigma) or R115866 (Janssen Pharmaceutica) were intraperitoneally injected into size-matched siblings every 12 h. RA: 1 mM in 1% DMSO/PBS. R115866: 0.67 mM 10% DMSO/PBS. For cyclopamine treatment, fish were incubated in 5 µM cyclopamine (Sigma), 0.1% EtOH, 0.1% DMSO in E3-medium (HEPES-buffered at pH 7.4). Cyclopamine was exchanged daily. SAG (Calbiochem) treatment was performed using 5 µM SAG in E3-medium. SAG stock solution was prepared in water. Control fish were always treated with an equivalent concentration of the drug solvent. All animal experiments were approved by the state of Baden-Württemberg, Germany.

Osteoblast differentiation, qPCR, TUNEL staining, EdU labeling and cryosectioning

Osteoblast differentiation was examined by measuring the GFP-free distal region in *osx:gfp* fish (*Olsp7:nlsgfp*; Spoorendonk et al., 2008). Gene expression levels were analyzed by qPCR (for primers see supplementary material Table S1), cell death by TUNEL staining and cell proliferation by EdU labeling. Cryosectioning was used to produce longitudinal and transverse fin sections. Further information concerning these methods, as well as descriptions of length measurements and cell number quantifications, imaging, immunohistochemistry, *in situ* hybridization and Alizarin Red staining can be found in the supplementary material methods.

Statistics

Student's *t*-test was used to test significance of differences. The numbers of specimens used are given in supplementary material Table S2.

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Competing interests

The authors declare no competing or financial interests.

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Supplementary material

Supplementary Material and Methods

Zebrafish lines

Konstanz wild type, *Ola.bglap:egfp*^{hu4008} (Knopf et al., 2011), *Ola.sp7:nls-gfp*^{zf132} (Spoorendonk et al., 2008), *hsp70l:dnfgfr1-egfp*^{pd1} (Lee et al., 2005), *hsp70l:v-ras*^{pd9} (Lee et al., 2009), *2.2shh:gfp*:ABC#15^{sb15} (Ertzer et al., 2007) and *fli1a:egfp*^{y1} (Lawson and Weinstein, 2002) fish were used. Heat-shock lines were analyzed as heterozygotes; wild type siblings served as controls.

Examination of osteoblast differentiation

Because osteoblast differentiation is accompanied by upregulation of *osx*, we used *gfp* expression in *osx:gfp* fish (*Ola.sp7:nlsgfp*) as marker for differentiated osteoblasts and determined the length of the *osx*-free distal region to detect putative effects on osteoblast differentiation.

Length measurements and quantification of cell numbers

Length measurements were performed on the third dorsal and/or the third ventral fin ray. EdU- or TUNEL-labeled cells were counted in the tissue distal to the amputation plane in longitudinal sections. The distance between (sister) rays were measured at the amputation site.

qPCR analysis

Tissue distal to the amputation plane was used for RNA extraction. Tissues from 4-10 fish were pooled for each RNA sample. Trizol reagent (Invitrogen) or TriPure (Roche) was used for RNA extraction. RNA was treated with DNase I and equal amounts of total RNA from each sample were reverse transcribed with SuperScript III reverse transcriptase (Invitrogen) or Maxima Reverse Transcriptase (Thermo Scientific) using anchored oligo(dT) primers. A C1000 thermal cycler combined with a CFX96 real-time PCR detection system (Bio-Rad) was used for quantitative real-time PCR (qPCR). PCR reactions were performed by using the Maxima SYBR Green qPCR Master Mix (Thermo Scientific). qPCR reactions for each cDNA pool and each target gene were performed in triplicates. Data were analyzed with the CFX Manager software (Bio-Rad). *ef1a*, *tbp* and *actb1* expression levels were used for normalization. Expression levels were normalized to expression levels of two reference genes (expression stability: Mean M <0.5) and expression ratios were calculated relative to control samples. Regenerates of heat-shocked

non-transgenic siblings were used as control if expression levels were examined in *hsp70I:dn-fgfr1* or *hsp70I:v-ras* fish and regenerates of vehicle treated fish were used as control if expression levels were examined in RA- or R115866 treated fish. Reference genes were used in different combinations, depending on the treatment condition. If normalization to different reference genes gave conflicting results (expression stability: Mean M ≥0.5), results were verified by normalization to the input RNA amount by performing RiboGreen or Qubit assays (Invitrogen). Primers are listed in Table S1.

Imaging

Images were captured with the Zeiss AxioVision or Zeiss ZEN software on a Zeiss Stemi 2000-C stereomicroscope equipped with a AxioCam ERc5s, a Leica MZ10F stereomicroscope equipped with a AxioCam MRc or a Zeiss Axio Imager.M2 equipped with a AxioCam MRc or a AxioCam MRm. For fluorescent microscopy of IHC or EdU stained fins structured illumination microscopy was used (Zeiss ApoTome.2, Zeiss Axio Imager.M2). Zeiss ZEN and Adobe Photoshop were used for image processing and length measurements.

Cryosectioning

Fixed (and stained) fins were decalcified in 10 mM EDTA in PBT (PBS containing 0.1% Tween20), embedded in 1.5% agar, 5% sucrose in PBS and soaked in 30% sucrose in PBS. Embedded fins were snap-frozen in Tissue-Tek O.C.T. Compound (Sakura) in liquid nitrogen and sections were cut at 16 µm.

Immunohistochemistry

For IHC, fins were fixed in 4% Paraformaldehyde (PFA) in PBS and stored in MeOH at –20°C. IHC was performed on cryosections or whole mounts. Sections or fins were permeabilized in PBTx (PBS containing 0.3% Triton X-100) (30 min for sections and 1 hour for whole mounts), blocked in 2% blocking reagent (Roche) in PBT and subsequently incubated with primary antibodies over night at 4°C. Tissue was washed and incubated with secondary antibodies for 3-4 hours at room temperature or over night at 4°C. Whole mounts were transferred to 70% glycerol in PBS for imaging. Sections were counterstained with DAPI and mounted using Mowiol containing DABCO. The following antibodies and dilutions were used: mouse ZNS-5 (an uncharacterized cell surface antigen that is specifically present on osteoblasts irrespective of their differentiation status) (Zebrafish International Resource Center) 1:1500, rabbit anti-GFP (Invitrogen, A6455) 1:500. Alexa Fluor®- (Invitrogen), Atto- (Sigma) or alkaline phosphatase (AP) - (Sigma) conjugated secondary antibodies were used. NBT/BCIP (Roche) was used as substrate for alkaline phosphatase.

TUNEL labeling

TUNEL labeling was performed in combination with IHC for ZNS-5 on cryosections. Fins were fixed in 4% PFA and stored in MeOH at –20°C. Sections were permeabilized for 30 min in PBTx and equilibrated in terminal deoxynucleotidyl transferase (TdT) buffer (200 mM potassium cacodylate, 25 mM Tris, 0.05% Triton X-100, 1 mM CoCl2, pH 7.2). Subsequently sections were incubated with TdT buffer containing 0.5 µM fluorescein-12-dUTP, 40 µM dTTP and 0.02 units/µl TdT (all Thermo Scientific) for 1-2 hours at 37°C. Sections were washed in PBT, blocked in 2% Blocking Reagent in PBS and incubated with anti-fluorescein-alkaline phosphatase coupled antibody (1:2000, Roche) and ZNS-5 antibody (1:1500) at 4°C over night. TUNEL labeled cells were detected with NBT/BCIP, AP activity was quenched with 100 mM glycin (pH 2.2) and ZNS-5 labeled cells were detected with anti-mouse-AP-coupled antibody (Sigma) using INT/BCIP (Roche) as substrate. Sections were mounted using Mowiol.

EdU labeling

Fish were IP injected with approximately 20 µl of 2.5 mg EdU (Jena Bioscience) in PBS 30 minutes prior to fixation. Fins were fixed in 4% PFA and stored in MeOH at –20°C. EdU labeling was performed on whole mounts and cryosectioning was performed afterwards. For EdU detection, fins were permeabilized in PBTx for 30 min, equilibrated in 100 mM Tris/HCl pH 8 and EdU was detected using a copper-catalyzed azide-alkyne click chemistry reaction (0.6 µM Cy3- or Fluor488- labeled azides (Jena Bioscience), 100 mM Tris, 1 mM CuSO4, 100 mM ascorbic acid, pH 8) with 20 min incubation time. IHC for ZNS-5 was subsequently performed on sections. Sections were counterstained with DAPI and mounted using Mowiol containing DABCO. Whole mounts were transferred to 70% glycerol/PBS for imaging.

In situ hybridization

Digoxigenin labeled RNA antisense probes were synthesized from cDNA templates: *aldh1a2* (Grandel et al., 2002), *cyp26a1* (Kudoh et al., 2002), *msxb* (Akimenko et al., 1995), *shha* (Quint et al., 2002). WISH and ISH on sections was performed as previously described (Blum and Begemann, 2012). For double WISH DIG- and fluorescein-labeled probes were hybridized simultaneously. Fins were first incubated with anti-DIG-AP coupled antibody and color reaction was performed with BCIP/NBT. AP activity was quenched with 100 mM glycin (pH 2.2) and fluorescein was detected by using anti-fluorescein-AP coupled antibody and INT/BCIP as substrate.

Alizarin Red staining

For Alizarin Red staining, fins were fixed in 4% PFA in PBS, transferred to MeOH and stored at -20°C. Fins were rehydrated, washed in PBT and stained in 0.1% Alizarin Red in 0.5% KOH overnight. Excess dye was removed by several washes in 0.5% KOH. Stained fins were transferred to 70% glycerol in 0.5% KOH for imaging.

Supplementary References

Grandel, H., Lun, K., Rauch, G.-J., Rhinn, M., Piotrowski, T., Houart, C., Sordino, P., Küchler, A. M., Schulte-Merker, S., Geisler, R., et al. (2002). Retinoic acid signalling in the zebrafish embryo is necessary during pre-segmentation stages to pattern the anterior-posterior axis of the CNS and to induce a pectoral fin bud. *Development* 129, 2851–65.

Kudoh, T., Wilson, S. W. and Dawid, I. B. (2002). Distinct roles for Fgf, Wnt and retinoic acid in posteriorizing the neural ectoderm. *Development* 129, 4335–46.

Table S1. Primer sequences for qPCR experiments.

Gene	Forward primer	Reverse primer
bactin1	TTGCTCCTTCCACCATGAAG	CTTGCTTGCTGATCCACATC
cyp26a1	GATGGGAGCTGATAATGTG	CCTGAACCTCCTCTCTGACC
ef1a	TACGCCTGGGTGTTGGACAAA	TCTTCTTGATGTATCCGCTGAC
ptch2	GGAGATTTACCCCCAAGTTAC	CCAACAGACAGGGCTCCG
shha	CGGCAGAAGAAGACATCC	GAGCAATGAATGTGGGCTTT
tbp	CGGTGGATCCTGCGAATTA	TGACAGGTTATGAAGCAAAACAACA

Table S2. Number of specimens used in quantitative and nonquantitative experiments (Fig. 1-3). Numbers for corresponding experiments, which are not shown in the figure, are included. For nonquantitative experiments: the first number indicates the number of specimens showing the phenotype, the second number the total number.

Figure	n =
1C	36/39 rays (5 fins)
1D	fused rays = 37, separated rays = 138
1E	19/25 rays (10 fins)
1H	3 cDNA pools per condition
1I	DMSO = 0/5 fins, R115866 = 5/5 fins
1J,K	wild type = 0/11 fins, *hsp70l:cyp26a1* = 10/10 fins
1L	10/10 rays (4 fins)
2A	RA: DMSO = 0/30 fins, RA 30/31 fins; R115866: DMSO = 0/8 fins, R115866 = 9/9 fins
2B	DMSO = 0/23 fins, RA 21/21 fins
2C	DMSO = 0/8 fins, RA 9/9 fins
2D	RA: DMSO = 0/6 fins, RA 6/6 fins; R115866: DMSO = 0/6 fins, R115866 = 6/6 fins
2E	DMSO = 0/15 fins, R115866 19/19 fins
2F	DMSO = 0/21 fins, RA 4/22 fins
3A	DMSO = 20 sections (4 fins), cyclopamine = 35 sections (5 fins)
3B	DMSO = 22 sections (4 fins), RA = 24 sections (4 fins)
3C	24 sections (6 fins) per condition
3D	3 cDNA pools per condition
3E	*cyp26a1*: 3 dpa = 11 rays, 4 dpa = 6 rays, 5 dpa = 5 rays; ZNS-5: 3 dpa = 6 rays, 4 dpa = 9 rays, 5 dpa = 9 rays
3F	*cyp26a1*: proximal = 5 rays, distal = 5 rays; ZNS-5: proximal = 6 rays, distal = 7 rays

Table S3. Number of specimens used in quantitative and nonquantitative experiments (Fig. S1-8). Numbers for corresponding experiments, which are not shown in the figure, are included. For nonquantitative experiments: the first number indicates the number of specimens showing the phenotype, the second number the total number.

Figure	n =
S2A	RA: DMSO = 5/5 fins, RA = 5/5 fins; R115866: DMSO = 6/6 fins, R115866 = 5/5 fins
S2B	DMSO = 0/12 fins, R115866 = 12/12 fins
S3A	DMSO = 0/8 fins, cylopamine = 9/9 fins
S3B	DMSO = 4/4 fins, cylopamine = 4/5 fins
S3C	DMSO = 0/10 fins, cylopamine = 11/11 fins
S4A	DMSO = 0/5 fins, R115866 = 4/4 fins
S4B	DMSO = 0/5 fins, R115866 = 5/6 fins
S6A	DMSO = 3/17 rays (5 fins), RA = 15/18 rays (6 fins)
S6B	DMSO = 32 rays, RA = 150 rays
S7	DMSO = 10 rays, cyclopamine = 12 rays
S8	24 sections (6 fins) per condition

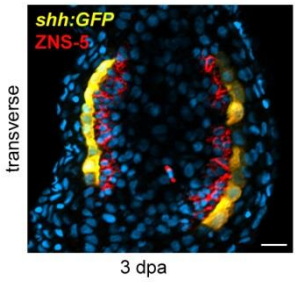

Fig. S1. *shha* is expressed within the basal epidermal layer adjacent to aligned osteoblasts. IHC for ZNS-5 and GFP in *shh:gfp* fish at 3 dpa reveals *shha* expression in the basal epidermal layer adjacent to osteoblasts (ZNS-5^{+}). Scale bar: 50 µm.

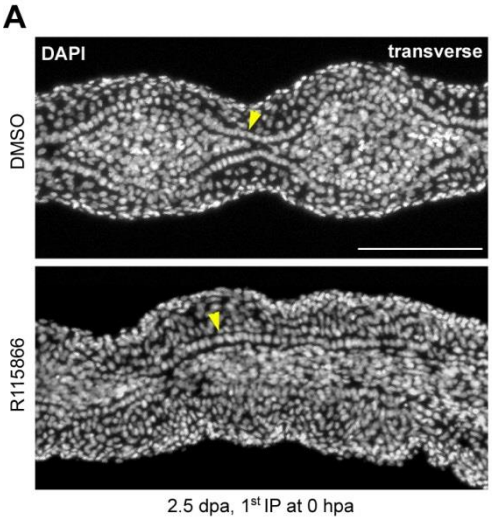

Fig. S2. R115866 treatment does not interfere with basal epidermal layer formation, but cause lateral expansion of *msxb*$^{+}$ cells into neighbouring interrays. (A) DAPI-stained transverse sections through the regenerate of DMSO- and R115866-treated fish reveal presence of a distinct basal epidermal layer. Arrowheads: Basal epidermal layer. (B) WISH for *msxb* at 2.5 dpa demonstrates expansion of blastema cells into interrays in R115866-treated fish. Arrowheads: Ectopic blastema cells. Dashed lines indicate amputation plane. Scale bars: 50 µm in A and 100 µm in B.

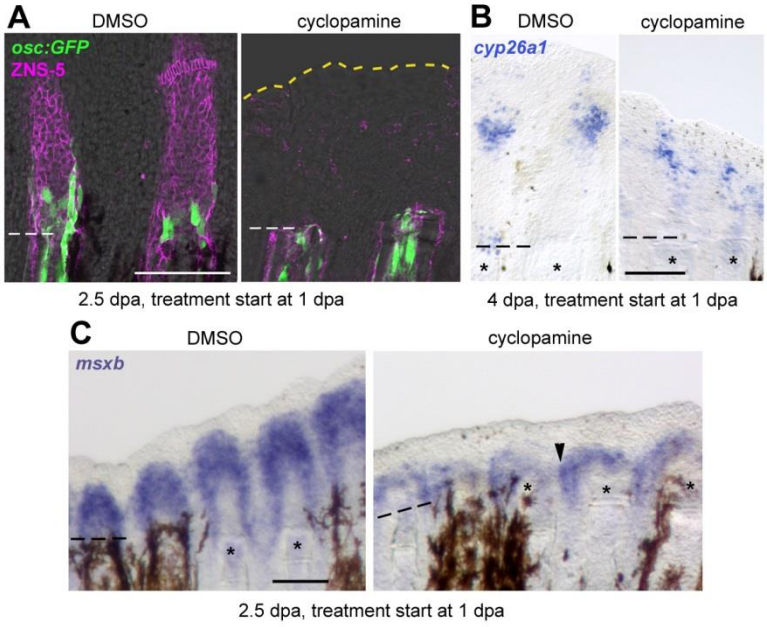

Fig. S3. Absence of preosteoblasts causes blastema expansion. Cyclopamine treatment starting at 1 dpa results in preosteoblast-free blastema and lateral expansion of blastema cells, but normal *cyp26a1* expression. (A) IHC for ZNS-5 and GFP in *osc:gfp* fish demonstrates lack of GFP^{+} preosteoblasts in the blastema of cyclopamine treated fish at 2.5 dpa. Yellow dashed line indicates the distal blastema edge in cyclopamine treated fish. (B) WISH for *cyp26a1* at 4 dpa reveals unaltered *cyp26a1* expression in cyclopamine treated fish (C) WISH for *msxb* demonstrates lateral expansion of blastema cells into the interray tissue (arrowhead) in cyclopamine treated fish. Dashed lines indicate amputation plane. Scale bars: 100 µm.

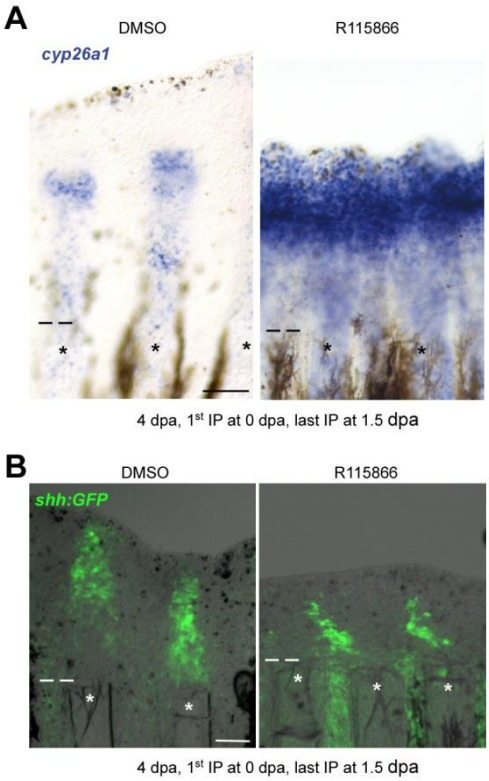

Fig. S4. *shha* expression is ectopically expressed in interrays upon recovery from R115866 treatment. (A) WISH for *cyp26a1* 2 days after termination of R115866 treatment at 4 dpa reveals *cyp26a1* expression in the entire epidermis of ray and interray tissue. (B) IHC for GFP in *shh:gfp* fish 2 days after termination of R115866 treatment at 4 dpa reveals ectopic *shha* expression in interrays. Asterisks indicate rays. Dashed lines indicate amputation plane. Scale bars: 100 µm.

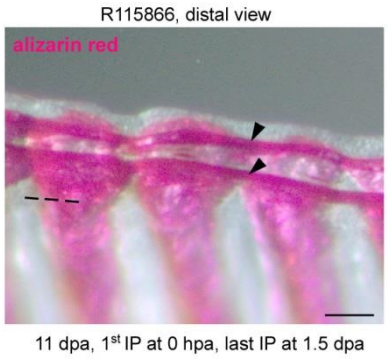

Fig. S5. Ectopic bone does not seal the wound in regenerates of R115966 treated fish. Distal view of an Alizarin Red stained regenerate of R115866-treated fish reveals that ectopic bone is restricted to lateral regions and does not seal the blastema. Arrowheads: Ectopic bone in the interray region. Dashed line indicate amputation plane. Scale bars: 100 µm.

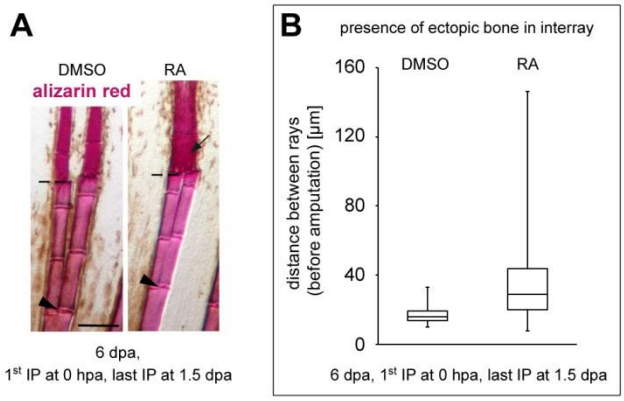

Fig. S6. RA treatment induces ectopic bone in interrays and fusion of sister rays. (A) RA injections upon amputation within 3-4 segments distal to the first branching point (long cut) result in fusion of sister rays. Alizarin Red staining. Arrowheads: Branching site. Dashed lines indicate amputation plane. Scale bar: 100 µm. (B) The distance range between rays where formation of ectopic bone occurs is much greater in RA-treated fish. Box plot of distances between rays.

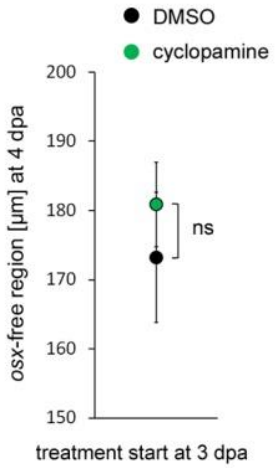

Fig. S7. Cyclopamine treatment does not impair osteoblast differentiation. Length measurement of the *osx*-free distal domain (tissue that does not contain differentiated osteoblasts) in *osx:gfp* fish at 4 dpa reveals that the osteoblast differentiation is unaffected in cyclopamine-treated fish. Data are represented as mean±s.e.m. ns, non significant.

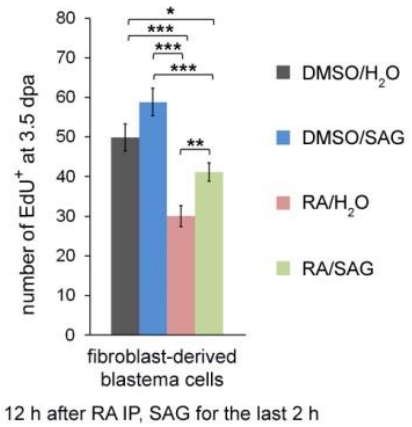

Fig. S8. Prolonged RA treatment impairs proliferation of fibroblast-derived blastemal cells through repression of Hh signaling. Inhibition of proliferation of fibroblast-derived blastemal cells 12 h after RA injection can be rescued by concomitant SAG treatment. EdU^+ cells per section at 3.5 dpa. Data are represented as mean±s.e.m. *p < 0.05, **p < 0.01, ***p < 0.001.

Publications

Blum N., Begemann G. 2015. Retinoic acid signaling spatially restricts osteoblasts and controls ray-interray organization during zebrafish fin regeneration. *Development*. 142: 2888-2893.

Blum N., Begemann G. 2015. Osteoblast de- and redifferentiation is controlled by a dynamic response to retinoic acid during zebrafish fin regeneration. *Development*. 142: 2894-2903.

Blum N., Begemann G. 2013. The roles of endogenous retinoid signaling in organ and appendage regeneration. *Cell Mol Life Sci*. 70:3907–3927. (Review article)

Blum N., Begemann G. 2012. Retinoic acid signaling controls the formation, proliferation and survival of the blastema during adult zebrafish fin regeneration. *Development*. 139:107-116.

Kikuchi K., Holdway J.E., Major R.J., **Blum N.**, Dahn R.D., Begemann G., Poss K.D. 2011. Retinoic acid production by endocardium and epicardium is an injury response essential for zebrafish heart regeneration. *Developmental Cell*. 20:397-404.

Offen N., **Blum N**., Meyer A., Begemann G. 2008. Fgfr1 signalling in the development of a sexually selected trait in vertebrates, the sword of swordtail fish. *BMC Developmental Biology*. 8:98.

Manuscript in preparation:

Blum N., Begemann G. Retinoic acid signaling restricts proliferation of the lens epithelium and prevents cataract formation in zebrafish.

Conference contributions

Parts of this thesis were presented at the following international conferences:

Blum N., Begemann G. Dynamic responsiveness to retinoic acid controls osteoblast de- and redifferentiation during fin regeneration. Joint meeting of the german and french societies of developmental biologists. Nuremberg, Germany, 2015. (**Oral presentation**)

Blum N., Begemann G., Restriction of retinoic acid activity by Cyp26a1 and Cyp26b1 regulates osteoblast behavior in the regenerating fin. 8th European Zebrafish Meeting. 2013. Barcelona, Spain. **(Poster presentation)**

Blum N., Begemann G. Retinoic acid signaling controls osteoblast behavior during bone regeneration. International Joint Meeting of the German Society for Cell Biology and Society for Developmental Biology. Heidelberg, Germany, 2013. (**Poster presentation**)

Blum N., Begemann G. Retinoic acid signaling controls osteoblast behavior during bone regeneration. EMBO Conference on the molecular and cellular basis of regeneration and tissue repair, Oxford, 2012. (**Oral presentation**)

Blum N., Begemann G. Retinoic acid signaling controls blastema formation, maintenance and differentiation of blastema cells during adult zebrafish fin regeneration. 7th European Zebrafish Meeting. 2011. Edinburgh, UK. (**Poster presentation**)

Blum N., Begemann G. Retinoic acid signaling controls blastema formation, maintenance and differentiation of blastema cells during adult zebrafish fin regeneration. Joint meeting of the German and Japanese Societies of Developmental Biologists. 2011. Dresden, Germany. (**Oral presentation**)

Blum N., Begemann G. Retinoic acid signaling plays essential roles in vertebrate appendage regeneration. Molecular and cellular basis of regeneration and tissue repair. EMBO conference series. 2010. Sesimbra, Portugal. (**Poster presentation**)

Blum N., Begemann G. Retinoic acid signaling in vertebrate appendage regeneration. 16th International Society of Developmental Biologists Congress. 2009. Edinburgh, UK. (**Poster presentation**)

Blum N., Begemann G. Retinoic acid signaling in adult and larval fin regeneration. 18th Meeting of the German Society of Developmental Biologists. 2009. Hannover, Germany. (**Oral presentation**)

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(Eidesstattliche) Versicherungen und Erklärungen

(§ 5 Nr. 4 PromO)

Hiermit erkläre ich, dass keine Tatsachen vorliegen, die mich nach den gesetzlichen Bestimmungen über die Führung akademischer Grade zur Führung eines Doktorgrades unwürdig erscheinen lassen.

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