Notch signaling coordinates cellular proliferation with differentiation during zebrafish fin regeneration

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SUMMARY
Zebrafish can completely regenerate amputated fins via formation of a blastema, a proliferative mass of undifferentiated precursor cells. During regenerative growth, blastema proliferation must be tightly coordinated with cellular differentiation, but little is known about how this is achieved. Here, we show that Notch signaling is essential for maintenance of blastema cells in a proliferative undifferentiated state. We found that the Notch pathway is activated in response to fin amputation in the highly proliferative region of the blastema. Chemical interference with Notch signaling resulted in a complete block of regeneration. Notch signaling was not required for the earliest known cellular processes during blastema formation, i.e. dedifferentiation and migration of osteoblasts, but specifically interfered with proliferation of blastema cells. Interestingly, overactivation of the pathway via misexpression of the intracellular domain of the Notch receptor (NICD) likewise inhibited regenerative outgrowth. In NICD-overexpressing fins, overall blastemal cell proliferation was not enhanced, but expanded into proximal regions where cellular differentiation normally occurs. Similarly, blastemal and epidermal gene expression territories invaded proximal regions upon sustained Notch activation. Concomitantly, NICD overexpression suppressed differentiation of osteoblasts and caused an expansion of the undifferentiated blastema. Together, these data suggest that Notch signaling activity maintains blastemal cells in a proliferative state and thus coordinates proliferation with differentiation during regenerative growth.

KEY WORDS: Notch, Regeneration, Blastema, Zebrafish, Caudal fin, Osteoblasts, LY411575

INTRODUCTION
In contrast to mammals, many lower vertebrates display remarkable capacities to fully regenerate organs or appendages after injury. Regrowth of salamander limbs and fish fins, which represent some of the most spectacular examples of vertebrate regeneration, occurs via formation of a proliferative population of lineage-restricted progenitor cells, termed the blastema (Poss et al., 2003; Brookes and Kumar, 2008; Kragl et al., 2009; Knopf et al., 2011; Tu and Johnson, 2011). How blastema proliferation is coordinated with cellular differentiation and tissue patterning during appendage regeneration is an important, yet poorly understood, issue.

The zebrafish tail fin regenerates completely and rapidly, and appears to have unlimited regenerative potential (Azevedo et al., 2011). The caudal fin consists of segmented bony fin rays built of two concave bones (the lepidotrichia) that are formed by osteoblasts lining the bone. The lepidotrichia enclose fibroblast-like cells, nerves, blood vessels and pigment cells, and are covered by epidermis. Fin regeneration can be described as occurring in three phases: wound healing, blastema formation and regenerative outgrowth. When fish are kept at 28°C, epidermal cells migrate covering the wound and a multi-layered wound epidermis forms within 24 hours post amputation (hpa). Concomitantly, osteoblasts in the stump dedifferentiate, start to proliferate and – probably together with fibroblasts – migrate beyond the amputation plane to form the blastema (Pooleo et al., 2001; Knopf et al., 2011). At 28°C, the blastema has fully formed by 48 hpa and regenerative outgrowth is initiated. During this phase, the blastema organizes into a small distal zone that is barely proliferating and a proximal zone where cells proliferate rapidly (Fig. 2A) (Nechiporuk and Keating, 2002).

Osteoblast progenitors are localized in the lateral regions of the proximal, proliferative blastema. Throughout regenerative outgrowth, the organization of the blastema is maintained at the distal tip of the growing fin, while osteoblast maturation and differentiation occur sequentially along the proximal-distal axis, with distal regions containing pre-osteoblasts, and proximal regions containing committed and differentiated osteoblasts (Brown et al., 2009).

Zebrafish tail fin regeneration has emerged as a very productive model for identification of molecules that regulate vertebrate regeneration. Several signaling pathways have been found to be required for fin regeneration, including Wnt, FGF, activin, IGF, retinoic acid, sonic hedgehog and BMP pathways (for reviews, see Stoick-Cooper et al., 2007a; Poss, 2010; Tal et al., 2010). Although we are far from achieving a thorough understanding of the precise roles these pathways play in fin regeneration, all of these signals have been shown to be required for proliferation of the progenitors of the blastema. However, little is known about the signals that regulate differentiation. Sonic hedgehog (shha) is expressed in a proximal subregion of the wound epidermis that overlays a part of the underlying mesenchyme where osteoblast differentiation is thought to be induced (Laforest et al., 1998). Ectopic expression of shha induces ectopic bone formation, while inhibition of hedgehog signaling with the small molecule cyclopamine inhibits regenerative growth and blastema proliferation (Quint et al., 2002). However, whether hedgehog signaling is required for blastema cell differentiation has not been shown. Misexpression of bmp2b can also induce ectopic bone formation, while inhibition of BMP signaling has been found to repress pre-osteoblast markers and to cause reduced matrix mineralization (Quint et al., 2002; Smith et
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al., 2006). Despite these advances, much needs to be learned about how proliferation and differentiation are coordinated during the distal growth of the regenerating fin.

In many biological systems, Notch signaling regulates the decision of progenitor cells to maintain their progenitor status or to differentiate (Chiba, 2006). In neural progenitors, active Notch signaling inhibits differentiation (Kageyama et al., 2009); it is required for maintenance of progenitor status in muscle precursors during development and in satellite muscle stem cells in the adult (Vasyutina et al., 2007), and for the maintenance of intestinal stem cells (van Es et al., 2005). Notch signaling probably also inhibits differentiation of hematopoietic stem cells (Suzuki and Chiba, 2005). Thus, the Notch pathway represents a good candidate for a signal regulating blastema progenitor cell maintenance versus differentiation. Notch signaling has been shown to be required for Xenopus larval tail regeneration, which also involves formation of a blastema, and forced activation of the Notch pathway was found to be sufficient to stimulate regeneration of Xenopus tails during the refractory period, where they normally cannot regenerate (Beck et al., 2003). However, the cellular mechanisms regulated by Notch during Xenopus tail regeneration or during regeneration of any other vertebrate appendage have not been identified.

Notch signaling is primarily activated when ligands of the DSL (Delta, Serrate/Jagged, LAG-2) family, which mostly are transmembrane proteins, activate Notch receptors on adjacent cells (Fortini, 2009). This results in a series of receptor cleavage events that allow for release and nuclear localization of the Notch intracellular domain (NICD), which together with transcriptional regulators modifies expression of target genes. These often include transcription factors of the hairy-related (Her, also called Hes in mammals) and Hey families (Fischer and Gessler, 2007).

Here, we show that Notch signaling is activated in the proximal, proliferative compartment of the zebrafish fin blastema during regenerative outgrowth. Upon interference with Notch signaling, blastema proliferation is inhibited, whereas Notch gain of function results in a massive expansion of the blastema and a concomitant reduction of osteoblast differentiation. We propose that Notch signaling maintains blastema cells in a proliferative state and thus coordinates proliferation with differentiation during regenerative growth.

MATERIALS AND METHODS

Fish lines and fin amputations

The following transgenic zebrafish lines were used: her4.3:EGFP<sup>38</sup> (Yeo et al., 2007), hsp70β:Gal41.5<sup>2<sup>4<sup>6</sup> (Scheer et al., 2001), UAS:myc-Notch1a-intra<sup>33</sup> (Scheer and Campos-Ortega, 1999), OslSp7:mCherry<sup>211<sup>31</sup> (Spoorendonk et al., 2008) and Ola.Osteocalcin.1:EGFP<sup>0088</sup> (Knopf et al., 2011). About 50% of the caudal fin was amputated as previously described (Pess et al., 2000), after which fish were returned to 27-28.5°C.

DAPT and LY411575 treatment

Fish were incubated with 10 µM (≥6 hour treatment) or 50 µM (6 hour treatment) DAPT or 5 µM LY411575, dissolved in DMSO, in fish system water. Solutions were exchanged daily and fish were kept in the dark during treatment.

In situ hybridization and immunohistochemistry

Whole-mount in situ hybridization and in situ hybridization combined with immunohistochemistry were performed as described previously (Nechiporuk and Keating, 2002; Stocke-Cooper et al., 2007b). Whole-mount stained fins were cryosectioned, except for Fig. 1B, where in situ hybridization was performed on sections using the whole-mount protocol with briefer washing steps. Two-color reactions were performed as described (Prince et al., 1998) with the following modifications: fluorescein-UTP was detected with anti-fluorescein antibody (Roche, 1:3000) and staining was achieved with BCIP (Roche) and INT (Sigma) solution. Antibody staining on cryosections was carried out as previously described (Knopf et al., 2011). Primary antibodies used were: rat anti-BrdU (Serotec, 1:200), mouse anti-Zns5 (Zebrafish International Resource Center, Eugene, OR, USA, 1:200), chicken anti-GFP (Abcam, 1:500), rabbit anti-Myc (Santa Cruz, 1:300), rabbit anti-Aldh1a2 (Abmart, 1:500) and mouse anti-PCNA (Dako, 1:100).

Quantification of Ola.Osteocalcin.1:EGFP<sup>0088</sup> expression

Fluorescence intensity was measured as previously described (Knopf et al., 2011) using ImageJ software version 1.47.

Heatshocks and fin length measurements

Fish were heatshocked four times daily, except for supplementary material Figs S1-4 where heatshocks were applied as indicated, by increasing water temperature from 27°C to 37°C within 10 minutes and incubating fish at 37°C for 1 hour with subsequent active cooling. Fish were fed and water was exchanged every second day. The lengths of fin rays 2, 3 and 4 of the dorsal fin lobe were measured on images using ImageJ software and compared with their respective control siblings.

Tissue sectioning and histology

Cryosections were prepared as described previously (Knopf et al., 2011). Masson’s trichrome stainings were performed on paraffin wax-embedded sections as described previously (Azevedo et al., 2011) with the following modifications: 3.5 µm sections were washed with xylene, with decreasing concentrations of ethanol and with distilled water before incubation in Boun’s fixative (1 hour, 56°C). Collagen was stained using Aniline Blue solution with subsequent rinsing in distilled water and washing in 1% acetic acid solution. Sections were mounted using Permount (Fisher Scientific).

BrdU incorporation and quantification of proliferating cells

Fish were kept in 5 mM 5-bromo-2-deoxyuridine (BrdU, Sigma) dissolved in fish system water for 1 hour prior to fixation and immunohistochemistry on cryosections or whole-mount fins. BrdU-positive cells were counted in 10 µm projections of confocal optical sections in the distal-most 200 µm (Fig. 3J; supplementary material Fig. S4A) or 600 µm (Fig. 4H) of the mesenchyme of the regenerate.

Tissue dissociation and flow cytometry

Fin fragments from 20 fish 4 dpa were dissociated in a collagenase/dispose solution (1 mg/ml, Roche) for 30 minutes, filtered using a 20 µm Filcon (Keul GmbH) filter, washed in HBSS (w/o CaCl<sub>2</sub>, w/o MgCl<sub>2</sub>, Gibco), spun down (10 minutes, 300 g) and resuspended in HBSS. Propidium iodide was added to a concentration of 1 µg/ml. Cells were sorted for EGFP fluorescence using a Becton Dickinson FACS Aria II SORP.

qRT-PCR and semi-quantitative PCR

Total RNA of 15 fin regenerates was extracted and cDNA prepared as previously described (Knopf et al., 2011). PCRs were performed in triplicates (for β-actin1 cDNA diluted 1:20) using a Strategene MX 3000 qPCR machine and expression values were normalized to those of β-actin1. Relative expression was calculated applying the 2<sup>−ΔΔCt</sup> method (Livak and Schmittgen, 2001). For semi-quantitative PCR, cDNA was prepared as for qRT-PCR, and PCR was performed on serial dilutions of cDNA. Oligo sequences are in supplementary material Table S1.

Statistics

Significance of differences in mean fin lengths and number of proliferative cells was tested using Student’s t-test. n.s. indicates not significant, *<i>P</i>&lt;0.05, **<i>P</i>&lt;0.001.

RESULTS

Notch signaling is activated in the blastema during regenerative outgrowth

To address a potential role of Notch signaling in zebrafish fin regeneration, we first asked whether the pathway is activated in the blastema, and forced activation of the Notch pathway was found during regenerative outgrowth (Spoorendonk et al., 2008) and :EGFPhu4008 (Knopf et al., 2011). Thus, the Notch pathway represents a good candidate for a signal regulating blastema progenitor cell maintenance versus differentiation. Notch signaling has been shown to be required for hematopoietic stem cells (Suzuki and Chiba, 2006). Despite these advances, much needs to be learned about proliferation and differentiation during the distal growth of the regenerating fin.
EGFP under control of regulatory sequences of the Notch target gene locus her4 (her4.3:EGFP)3), which has been shown to faithfully report Notch pathway activity during zebrafish development (Yeo et al., 2007). Although non-amputated adult fins and fins imaged immediately after amputation expressed EGFP only in few scattered cells, robust induction of EGFP fluorescence could be detected at 48 hpa in groups of cells distal to the amputation plane in each fin ray and spreading proximally into intraray regions (Fig. 1A). EGFP fluorescence was reduced in fin regenerates treated with the γ-secretase inhibitor DAPT, which interferes with Notch signaling, confirming that expression of the her4.3:EGFP transgene in the regenerating fin is regulated by Notch signaling (supplementary material Fig. S1A). EGFP expression persisted during further regenerative outgrowth in a distal high to proximal low gradient (Fig. 1A). In situ hybridization showed that the transgene transcript was largely confined to the blastema within fin rays at 3 dpa, while being weakly expressed also in interray mesenchyme (Fig. 1B). Additionally, a few scattered cells located in the epidermis appeared to express the transgene, which could be confirmed by imaging of EGFP fluorescence in whole-mount and cryosectioned fins (supplementary material Fig. S1B). The identity of these cells remains to be determined.

Semi-quantitative and quantitative RT-PCR (qRT-PCR) showed that several members of the Her and Hey families of putative Notch targets, her2, her4.1 (one of several genes of the repetitive zebrafish her4 locus), her9, her12 and hey1, were robustly expressed in regenerating fins at 3 dpa (Fig. 1C,F). Levels of her2, her9 and hey1 were reduced in fins treated with DAPT, confirming that these genes are Notch responsive in the regenerating caudal fin (Fig. 1F). Endogenous her4.1 expression could only be detected by semi-quantitative PCR in the EGFP+ fraction of her4.3:EGFP regenerates. (F) her2, her9 and hey1 expression is downregulated in 3 dpa regenerates treated with DAPT for 6 hours. (G) her6 is expressed in the blastema at 3 dpa. (H) her4.3-driven egfp transcripts (red) and endogenous her6 (blue) are downregulated in regenerates treated with LY411575 6 hours after the start of treatment (hpt) (n=6/6 fins) and not detectable at 24 hpt (n=5/6). (I) her4.3-driven EGFP fluorescence is downregulated in 3 dpa regenerates treated with LY411575 for 24 hours, but not for 6 hours. (J) jag1b is expressed in the blastema at 3 dpa. (A–J) Arrowheads indicate amputation plane. Scale bars: whole mounts, 200 μm; sections, 100 μm.
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During regenerative outgrowth, msxb (as detected in whole-mount in situ hybridization) and aldha1a2 (raldh2), mark the distal nonproliferative blastema (Fig. 2A) (Nechiporuk and Keating, 2002; Mathew et al., 2009). In her4.3:EGFP transgenics, both markers were expressed in cells distal to the egfp expression domain (arrows in Fig. 2B). Likewise, her6 expression was detected proximal to the boundaries. The expression of Notch receptors, in particular notch2, in her4.3-negative cells hints at the existence of additional sites of active Notch signaling in the regenerating fin that are not reported by the her4.3:EGFP transgenic.

**Notch pathway activity is confined to the proliferative zone of the blastema**

During regenerative outgrowth, msxb (as detected in whole-mount in situ hybridization) and aldha1a2 (raldh2), mark the distal nonproliferative blastema (Fig. 2A) (Nechiporuk and Keating, 2002; Mathew et al., 2009). In her4.3:EGFP transgenics, both markers were expressed in cells distal to the egfp expression domain (arrows in Fig. 2B). Likewise, her6 expression was detected proximal to the...
msxb domain (Fig. 2C). Furthermore, egfp transcripts in her4.3:EGFP transgenics localized to the PCNA-positive proliferative blastemal region (Fig. 2D). Thus, Notch pathway activity is confined to the proximal proliferative compartment of the blastema during regenerative outgrowth.

The fin regenerate grows distally by proliferation of cells in the proximal proliferative region of the blastema, followed by displacement of the newborn cells into further proximal regions, where they start to differentiate (Nechiporuk and Keating, 2002). Differentiation of the osteoblast lineage occurs in lateral regions of the regenerate; one of the earliest markers activated by cells differentiating along the skeletogenic fate is runx2b, which is expressed proximally to the distal blastema markers in the lateral intraray mesenchyme (Fig. 2A) (Brown et al., 2009). At 3 dpa, her4.3:EGFP was expressed mainly distally to the runx2b-positive pre-osteoblasts (Fig. 2E). We thus wondered whether her4.3:EGFP-positive cells do not give rise to osteoblasts. EGFP protein expression could be detected substantially further proximally than egfp RNA in her4.3:EGFP regenerates, as determined by comparing the relative domain lengths of EGFP fluorescence and RNA in situ hybridization staining (supplementary material Fig. S2A) and by combining egfp in situ hybridization with anti-EGFP immunofluorescence (supplementary material Fig. S2B). Thus, after cessation of transgene transcription EGFP protein appears to persist in cells that are born in the proximal blastema and subsequently become displaced further into the proximal differentiation zone, allowing for short-term cell fate tracing of cells transcribing the transgene. EGFP protein was not co-expressed with Zns5, an antigen specifically present on all osteoblasts irrespective of their differentiation status (Johnson and Weston, 1995; Knopf et al., 2011) (Fig. 2F). Thus, Notch signaling appears to be mainly active in the medial blastema that probably gives rise to fibroblasts, but is absent from lateral blastema cells that contain osteoblast precursors. However, her4.3:EGFP expression could also be detected in the forming joint regions between bony segments starting at 3 dpa, where a few EGFP-positive cells also expressed Zns5 (arrowheads in supplementary material Fig. S2C). Thus, joint osteoblasts appear to represent a population with characteristics distinct from segmental osteoblasts. We have concentrated our further analysis on Notch function in the medial blastema.

Notch signaling is required for fin regeneration

To test the role of the Notch pathway in fin regeneration, we treated fish with LY411575 for 6 hours starting at 3 dpa and labeled proliferative cells using BrdU incorporation, which resulted in a reduction in the number of BrdU+ blastema cells by 35% as detected on confocal optical sections of fin rays (Fig. 3I,J). Thus, Notch signaling regulates blastema proliferation. Furthermore, we found that LY411575 treatment for 24 hours starting at 3 dpa resulted in a severe downregulation of the blastema markers msxb and ilf2 (Fig. 3K), suggesting that Notch signaling is required for maintenance of blastema cells.

Notch overactivation impairs regenerative outgrowth

Our results so far are consistent with the hypothesis that Notch signaling maintains blastema cells in an undifferentiation state. To test this, we ectopically activated Notch signaling via misexpression of the Notch1a intracellular domain (NICD) using a heatshock inducible Gal4-UAS:NICD double transgenic system (hs:Gal4; UAS: NICD). Treatment of heatshocked UAS:NICD fish lacking the Gal4 driver were used as controls. In fish carrying these hs:Gal4; UAS: NICD transgenes plus the her4.3:EGFP reporter, repeated application of heatshocks for 6 days (Fig. 4A) resulted in strong upregulation of her4.3:EGFP activity in the fin ray mesenchyme, but was not able to induce ectopic her4.3:EGFP expression in the epidermis, which is consistent with the poor induction of NICD expression in this tissue (Fig. 4B). Thus, this system is well suited to assess the effects of sustained activation of Notch signaling in the blastema and of ectopic activation in the mesenchymal zone of differentiation.

We then tested the consequences of NICD overexpression throughout the entire regenerative process, starting 1 day prior to amputation (Fig. 4A). Intriguingly, this resulted in a severe reduction of regenerative growth (Fig. 4C), whereas overexpression of only Gal4 in hs:Gal4 single transgenics had no influence on regeneration (supplementary material Fig. S3B). In contrast to Notch loss of function, which completely blocked growth, NICD overexpression did allow for some increase in fin length; however, this progressively slowed over the course of the experiment (Fig. 4D). Pigment cells accumulated in the reduced regenerate of NICD overexpressing fins (arrow in Fig. 4C), confirming that...
pigment cell migration and expansion can be uncoupled from regenerative outgrowth (Rawls and Johnson, 2000).

NICD misexpression initiated at 24 hpa, after the wound epidermis had formed (Fig. 4A), was likewise sufficient to severely inhibit regenerative growth (Fig. 4E) as was overexpression initiated at 2 dpa, after the blastema had formed (Fig. 4A,F). In fact, NICD overexpression initiated at 2 dpa was sufficient to cause equally severe defects in fin growth as overexpression started immediately after amputation or at 1 dpa (supplementary material Fig. S3C), suggesting that perturbation of Notch signaling does not affect wound healing or blastema formation, but interferes with regenerative outgrowth.

**NICD misexpression causes ectopic cell proliferation**

NICD misexpression for 6 days resulted in a shortened regenerate displaying a lateral expansion of blastemal tissue compared with controls at 6 dpa (arrow in Fig. 4G). Thus, in contrast to Notch loss of function, which suppresses blastema proliferation, NICD
overexpression might interfere with distally oriented regenerative outgrowth by causing ectopic proliferation and by uncoupling proliferation and differentiation. To test this, we quantified the fraction of BrdU-incorporating mesenchymal cells at the tip of fin regenerates in three zones along the distal-proximal axis. The distal-most zone of 200 µm contained the distally located non-proliferating and the further proximally located highly proliferative regions of the blastema. The third zone (400-600 µm) encompasses the differentiation zone of the regenerate (see Fig. 2A). In control fins, cell proliferation was mainly localized to the distal 200 µm zone, and the fraction of proliferating cells progressively declined further proximally to less than 1.7% in the third 400 to 600 µm zone (Fig. 4H). By contrast, in fins overexpressing NICD for 6 days, no such gradient of proliferation was observed, as proliferation was significantly elevated in the proximal (400-600 µm) zone and the rate of proliferation in this zone was not significantly different from that in the distal (tip to 200 µm) zone (Fig. 4H). Of note, NICD overexpression did not increase cell division in the already proliferative distal zone. Thus, prolonged Notch overexpression caused ectopic blastema proliferation in the differentiation zone of the regenerate, suggesting that sustained Notch signaling activation locks blastema cells in a proliferative state. By contrast, NICD overexpression for only 24 hours, starting at 3 dpa, had no effect on blastema proliferation (supplementary material Fig. S4A). This is consistent with a model in which Notch signaling does not induce cellular proliferation, but rather keeps cells from exiting the cell cycle, an effect that will become obvious only at later stages of regeneration.

The blastema is proximally expanded in NICD-overexpressing fins

After 6 days of sustained NICD activation, the expression of the mitotic checkpoint kinase ttk (mps1), which marks the proliferative proximal blastema (Poss et al., 2002), was expanded into proximal regions of the regenerate (Fig. 5A). Likewise, the blastema marker ilf2 (Yoshinari et al., 2009) was ectopically expressed in proximal regions (Fig. 5A). Expression of and1, which is found in the blastema and in cells lining the bony rays (Zhang et al., 2010), was also massively expanded in NICD-overexpressing fins. By contrast, expression of aldha2, which is confined to the distal blastema, was not altered by NICD overexpression (supplementary material Fig. S4B). Together,
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Fig. 5. NICD overexpression causes expansion of proximal mesenchymal and epidermal compartments. (A,B) Whole-mount in situ hybridization of hs:Gal4; UAS:NICD and UAS:NICD fins heatshocked repeatedly from 0 hpa to 6 dpa with the indicated markers reveals massive proximal expansion of proximal, but not distal, blastemal and epidermal domains upon NICD overexpression. mps1=6/6 fins; ilf2=6/6; and1=9/11; left=7/7; wnt10a and inhban=4/5; wnt5b and pea3=5/6. Arrowheads indicate the amputation plane. Scale bars: whole mounts, 200 μm; sections, 100 μm.

these data indicate that sustained Notch pathway activation causes an expansion of the proximal, proliferative blastema, but not of the distal, nonproliferative blastemal territory. Interestingly, this proximal shift in expression domains was not restricted to the mesenchyme, as expression of left, which labels a proximally located subdomain of the basal layer of the wound epidermis (Poss et al., 2000), also extended proximally in NICD-expressing fins. By contrast, the distal epidermal domain marked by mssxd expression (Akimenko et al., 1995) did not change (Fig. 5A). Thus, NICD overexpression expands both epidermal and mesenchymal proximal, but not distal, territories. Overexpression of NICD for just 12 hours starting at 3 dpa did not alter left expression (supplementary material Fig. S4C), indicating that the patterning defects caused by sustained NICD overexpression are not due to a direct effect on cell fate but due to the accumulated failure of cells to exit the proliferation zone.

As Notch signaling activity is normally confined to the mesenchyme of the proliferative blastema and the hs:Gal4; UAS:NICD transgenic system did not seem to be able to cause efficient pathway misactivation in the fin epidermis, we asked whether NICD overexpression caused misexpression of secreted factors that could mediate the effects of NICD in the epidermis. Indeed, we observed a proximal expansion of expression of wnt10a, a ligand that activates β-catenin signaling and of inhibin beta Aa (activin beta Aa), a ligand that activates Alk4 receptor signaling (Fig. 5A), which are both essential for regeneration (Jaźwińska et al., 2007; Stoick-Cooper et al., 2007b).

Fin outgrowth and patterning is also dependent on the establishment of distinct functional domains within the fin epidermis (Lee et al., 2009). The proximal epidermal subregion characterized by left expression is positioned by a repressive function of FGF signaling in distal regions, acting via pea3 and wnt5b (Lee et al., 2009). Sustained NICD activation resulted in a proximal expansion of the expression domains of wnt5b and of pea3 (Fig. 5B), which might explain the observed proximal shift in the left epidermal domain upon NICD overexpression.

Notch signaling suppresses osteoblast differentiation

If Notch indeed acts to maintain blastema cells in an undifferentiated, proliferative state, expansion of the proliferative blastema in NICD-overexpressing fins should be accompanied by a reduction in cellular differentiation in proximal regions of the regenerate. Within the skeletogenic lineage, cells activate markers for pre-osteoblasts (runx2b), committed osteoblasts (sp7/osterix) and differentiated osteoblasts (ostecalcin) sequentially, both temporally during the course of regenerate growth and also spatially along the distal–proximal axis, with distal regions being devoid of commitment and differentiation markers (Brown et al., 2009; Knopf et al., 2011). We thus asked whether NICD overexpression effects transgenic readouts of osteoblast commitment and differentiation using quadruple transgenic fish (hs:Gal4; UAS:NICD; Ola.Osteocalcin::EGFPfluoro; OSp7::mCherry+yT31). Control triple transgenic fins lacking the Gal4 driver subjected to repeated heatshocks for 6 days robustly expressed osteocalcin:EGFP in the regenerate at 6 dpa, indicating that osteoblast differentiation had been correctly initiated (arrow in Fig. 6A). By contrast, sustained NICD expression resulted in a severe reduction of osteocalcin:EGFP expression in the regenerate (Fig. 6A). Furthermore, the distal region of the regenerate devoid of osteocalcin:EGFP expression was significantly expanded upon NICD overexpression (compare brackets in Fig. 6A) and the osteocalcin:EGFP-positive domain was reduced from 89% to 65% of the regenerate length (Fig. 6B). Expression of the endogenous osteocalcin gene was also reduced in NICD-overexpressing fins (Fig. 6C). Importantly, inhibition of Notch signaling with DAPT resulted in an upregulation of both endogenous osterix and osteocalcin expression (Fig. 6C). Together, these data suggest that Notch signaling acts to interfere with osteoblast commitment and differentiation in the regenerating fin.

To further test this hypothesis, we assessed the expression pattern of genes associated with osteoblast specification and differentiation. The domain positive for the pre-osteoblast marker runx2b was...
shifted proximally and expanded along the distal-proximal axis in NICD overexpressing fins, but strongly reduced upon DAPT treatment (Fig. 6D). Short-term activation of NICD for 12 hours did not effect *runx2b* expression (supplementary material Fig. S4C).

Sustained NICD overexpression also resulted in an expansion of the distal domains devoid of the collagens *coll1a2* and *coll10a1*, which mark regions of skeletogenesis (compare brackets in Fig. 6D). *shha* expression in a subregion of the basal layer of the
wound epidermis is thought to be important for commitment and differentiation of underlying osteoblasts (Quint et al., 2002). NICD overexpression resulted in a proximal shift and proximal expansion of the shha expression domain (Fig. 6D). Together, these data indicate that Notch signaling delays differentiation of cells along a skeletogenic fate in the regenerating fin.

**DISCUSSION**

**A model for Notch signaling function during fin regeneration**

Our work sheds light on the molecular mechanisms controlling the balance between cellular proliferation and differentiation during regenerative growth. We propose that Notch signaling is required for maintenance of blastemal progenitor cells in an undifferentiated proliferative state. Specifically, our results support the following model for Notch signaling function during zebrafish fin regeneration (Fig. 6E,F).

Notch signaling activity is not detected in mesenchymal cells of the uninjured adult caudal fin, but is upregulated following fin amputation in the blastema. During the regenerative outgrowth phase of fin regeneration (after 48 hpa at 28°C), the blastema organizes into three distinct compartments: a hardly proliferating distal compartment; a highly proliferative proximal medial zone; and bilateral regions that contain osteoblast progenitors, which also are highly proliferative. The Notch pathway – as detected by the her4.3:EGFP reporter – appears to be active only in the medial, proliferative compartment (yellow region in Fig. 6E). When Notch signaling is inhibited, blastemal proliferation is suppressed and blastemal cells are subsequently lost. Conversely, when Notch pathway activity is experimentally enhanced in the mesenchyme, cells that are derived from the proximal proliferative blastemal compartment fail to exit the cell cycle and stay proliferative, resulting in a proximal expansion of the proliferative blastema (Fig. 6F). Concomitantly, cellular differentiation of osteoblasts is suppressed in regenerating fins upon Notch overactivation, resulting in an expansion of the pre-osteoblast zone at the expense of the region containing mature osteoblasts. Despite the massive expansion of the blastema, the overall result of Notch signaling overactivation is a failure to regenerate, probably owing to reduced differentiation and perturbed coordination between proliferation and differentiation, resulting in a lack of distally oriented growth. Notch signaling thus seems to promote the undifferentiated proliferative state of regenerative cells.

**A cell non-autonomous role for Notch in osteoblast differentiation?**

The current cellular model of zebrafish fin regeneration indicates that the blastema is a heterogenous mixture of lineage-restricted cells, where laterally located cells give rise to osteoblasts, while the medial blastema forms fibroblasts (Tu and Johnson, 2011). Our short-term fate mapping based on EGFP protein persistence indeed indicates that the Notch-positive medial blastema cells do not give rise to pre-osteoblasts. Yet, we have shown that osteoblast differentiation is affected by Notch gain- and loss-of-function. Thus, it appears likely that Notch signaling does not directly interfere with cellular differentiation along the osteoblast lineage. Rather, we assume that secreted factors derived from the proliferative medial blastema, the size of which is regulated by Notch signaling, regulate osteoblast differentiation in the lateral blastema, possibly via setting up distinct expression domains in the wound epidermis (Lee et al., 2009). It will be interesting to identify the nature of such factors. Although many signaling pathways have been identified to be essential for fin regeneration (Stoick-Cooper et al., 2007a; Poss, 2010; Tal et al., 2010), only sonic hedgehog and BMP signaling have been implicated in regenerative bone patterning (Laforest et al., 1998; Quint et al., 2002; Smith et al., 2006). However, based on their expression patterns, none of the hedgehog or BMP ligands characterized in the fin so far are good candidates for mediators of an effect of the medial blastema on osteoblast differentiation (Smith et al., 2006).

Alternatively, it is possible that the her4.3:EGFP reporter does not reflect all domains of active Notch signaling. Our analysis of Notch receptor and ligand expression indicates that cells outside the her4.3:EGFP-positive domains are competent to activate the Notch pathway. Thus, Notch signaling might have additional roles in fin regeneration to the one described here. Therefore, although our data support an indirect effect of Notch signaling on the osteoblast lineage, we cannot fully exclude the possibility that it directly acts on these cells.

**Parallels and differences to mammalian skeletal development**

One intriguing aspect of our work is that it hints at interesting parallels, yet important differences between the role of Notch signaling in mammalian embryonic and zebrafish regenerative bone formation. During mouse development, Notch signaling was proposed to maintain mesenchymal osteoblast progenitor cells in an undifferentiated state (Engin et al., 2008; Hilton et al., 2008). A functional role for Notch signaling in mammalian bone repair has not yet been tested, but upregulation of Notch pathway components in mesenchymal cells during endochondral and intramembranous bone repair has been reported (Dishowitz et al., 2012).

Thus, both during mammalian bone development and zebrafish fin regeneration, Notch signaling appears to be required to maintain mesenchymal cells in an undifferentiated state and to suppress osteoblast differentiation. Although, in mice, Notch signaling is thought to interfere cell-autonomously with differentiation of mesenchymal progenitors into osteoblasts, during fin regeneration its effect on osteoblast differentiation appears to be indirect, raising intriguing questions about the conservation of the role of Notch signaling in bone formation between fish and mammals. It will be interesting to test what role Notch signaling plays in fish embryonic skeletogenesis. Alternatively, it is possible that osteoblast differentiation in the formation of lepidotrichia, the dermal bones of the fish exoskeleton, which are not homologous to mammalian bones, is regulated differently from that in other dermal or perichondral bones in fish (Apschner et al., 2011). Finally, although mature differentiated osteoblasts do give rise to blastemal osteoblast progenitors in a process of dedifferentiation during normal fin regeneration (Knopf et al., 2011; Sousa et al., 2011), bone regeneration can occur rather normally in fins depleted of committed osteoblasts, because another, yet unidentified, cell population appears to be able to substitute and to form osteoblasts (Singh et al., 2012). Thus, fish fins might contain a mesenchymal population of cells with osteogenic potential similar to that of MSCs in the mammalian bone marrow. It will be interesting to test a role for Notch signaling in this alternative mode of bone formation during fin regeneration.

**Functions for Notch signaling in regeneration of other systems**

Roles for Notch signaling in regeneration or repair of various organs have recently emerged. It positively regulates regeneration of *Xenopus* larval tails (Beck et al., 2003), mammalian skeletal muscle...
(Conboy and Rando, 2002), mouse tracheobronchial epithelium (Rock et al., 2011) and chick retina (Hayes et al., 2007). Interestingly, in the zebrafish lateral line sensory organ and in injured adult mouse inner ears, Notch signaling appears to act as inhibitor of hair cell regeneration (Lin et al., 2011) and it blocks axon regeneration in C. elegans (El Bejiani and Hammarlund, 2012).

Thus, both systems that regenerate via activation of stem cells and organs where mature cells are thought to dedifferentiate [our results and those of Hayes et al. (Hayes et al., 2007) in the retina] involve Notch signaling. Although the exact cellular functions of Notch vary widely between these systems, similar to its role during development, Notch signaling is often involved in controlling the balance between proliferation and differentiation of precursor cells during regeneration.

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Competing interests statement
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