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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; Brockes 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 to cover 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 (Poleo 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 overlies 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^{y83} (Yeo et al., 2007), *hsp70l*:Gal41.5^{kca4} (Scheer et al., 2001), *UAS*:myc-Notch1a-intra^{kca3} (Scheer and Campos-Ortega, 1999), *OlSp7*:mCherry^{zf131} (Spoorendonk et al., 2008) and *Ola.Osteocalcin.1*:EGFP^{hu4008} (Knopf et al., 2011). About 50% of the caudal fin was amputated as previously described (Poss 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; Stoick-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^{hu4008}* expression

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

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 Bouin'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-desoxyuridine (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. 3I,J; supplementary material Fig. S4A) or 600 μm (Fig. 4H) of the mesenchyme of the regenerate.

Tissue dissociation and flow cytometry

Fin regenerates from 20 fish 4 dpa were dissociated in a collagenase/dispase solution (1 mg/ml, Roche) for 30 minutes, filtered using a 20 μm Filcon (Keul GmbH) filter, washed in HBSS (w/o $CaCl_2$, w/o $MgCl_2$, 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 Stratagene MX 3005 qPCR machine and expression values were normalized to those of β-actin1. Relative expression was calculated applying the $2^{-\Delta\Delta Ct}$ 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, *$P<0.05$, ***$P<0.001$.

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 response to amputation, making use of a transgenic line expressing

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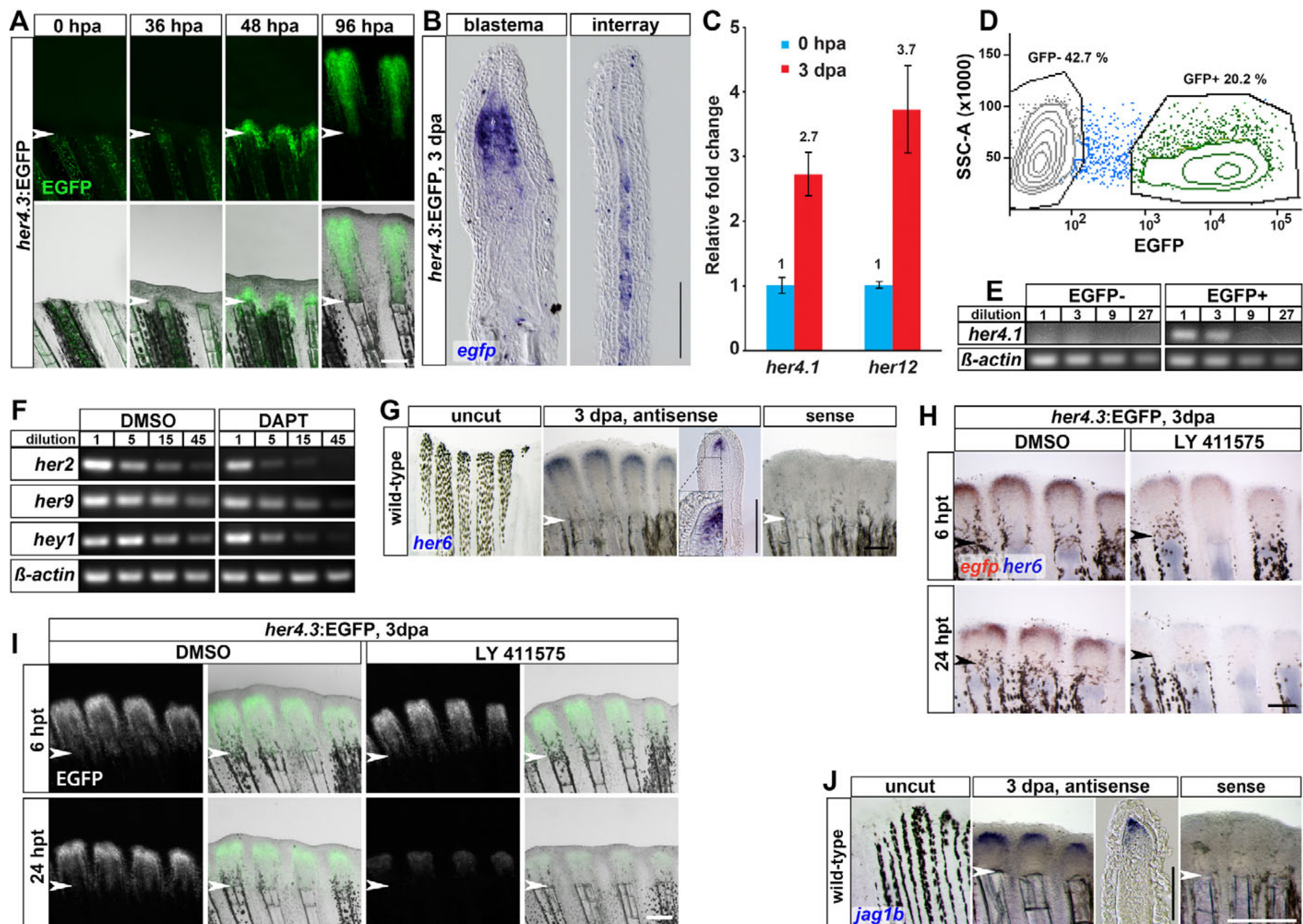

Fig. 1. Notch signaling is activated in the blastema during regenerative outgrowth. (**A**) EGFP fluorescence in *her4.3*:EGFP transgenics. By 96 hpa, EGFP signal is strongly intensified (exposure times: 0, 36 and 48 hpa for 7 seconds; 96 hpa for 0.5 seconds). (**B**) Longitudinal sections of *her4.3*:EGFP regenerates at 3 dpa stained for *egfp*. There is expression in the proximal but not the distal blastema, and weak expression in interray tissue. (**C**) *her4.1* and *her12* levels in 3 dpa regenerates measured by qRT-PCR, shown relative to the level in the distal-most stump segment at 50% fin length (0 hpa). (**D**) FACS scatterplot showing cell fractions of EGFP$^+$ and EGFP$^-$ cells from dissociated 4 dpa *her4.3*:EGFP regenerates. (**E**) Endogenous *her4.1* transcripts can be detected only 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.

EGFP under control of regulatory sequences of the Notch target gene locus *her4* (*her4.3*:EGFP^{y83}), 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 in the EGFP$^+$ cell fraction of FACS-sorted *her4.3*:EGFP transgenic regenerates, supporting the usefulness of the transgene as readout of Notch signaling activity (Fig. 1D,E). Although the expression levels of several of the endogenous *her* family members were too low to be

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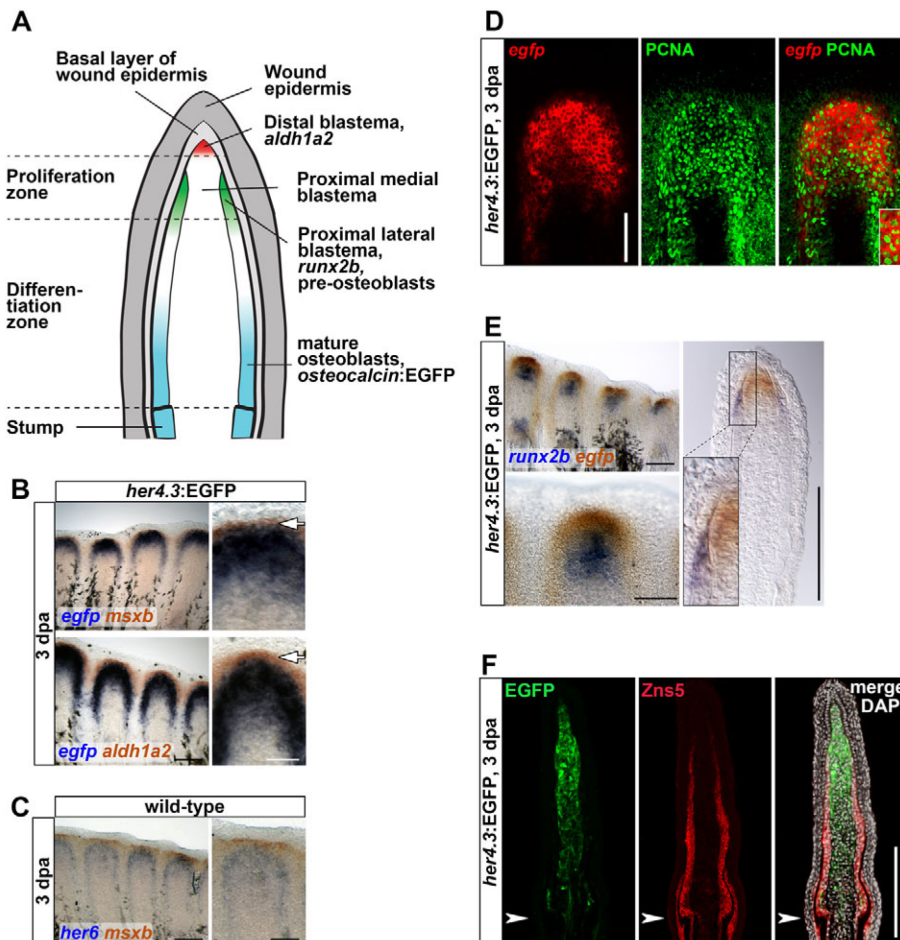

Fig. 2. Notch pathway activity is confined to the proliferative zone of the proximal medial blastema. (**A**) A longitudinal section of a regenerating fin during the outgrowth phase of regeneration (after 48 hpa) showing relevant anatomical structures and expression domains. (**B**) The *her4.3*:EGFP reporter (blue) is expressed proximally to *msxb* and *aldh1a2* (red) at 3 dpa. (**C**) *her6* (blue) is expressed proximal to *msxb* (red). (**D**) *her4.3*:EGFP is active in PCNA-positive cells. Confocal images of whole-mount regenerates stained for *egfp* transcripts and PCNA protein. (**E**) *her4.3*:EGFP (red) expression extends further distally than *runx2b* (blue), and is confined to the medial, *runx2b*-negative blastema. (**F**) Confocal images of longitudinal sections of *her4.3*:EGFP transgenic regenerates stained with Zns5 antibody (labeling all osteoblasts) and anti-GFP show no overlap. (B-E) Arrowheads indicate amputation plane. Scale bars: whole mounts, 200 μm; sections, 100 μm.

detected by *in situ* hybridization, we found *her6* to be robustly expressed specifically in the blastema at 3 dpa, but undetectable in noninjured fins (Fig. 1G). *her6* was co-expressed with *egfp* RNA in the *her4.3*:EGFP transgenics, and expression of both transcripts was downregulated within 6 hours, and extinguished within 24 hours of treatment with LY411575, another γ-secretase inhibitor (Fig. 1H). By contrast, EGFP protein expression persisted for a longer period of time in LY411575-treated fins (Fig. 1I). Together, these data indicate that Notch pathway activity is induced in regenerating fins predominately in the blastema at the onset of regenerative outgrowth at 48 hpa.

Of the eight zebrafish Delta, Delta-like and Jagged ligands, we could detect only *jagged1b* (*jag1b*) by in situ hybridization in regenerating fins. *jag1b* was not detected in uninjured fins, but was expressed in the blastema at 3 dpa (Fig. 1J). To characterize whether other ligands might be present, and to obtain some spatial information about their expression pattern, we assayed for ligand and Notch receptor expression by semi-quantitative PCR in FACS-sorted *her4.3*:EGFP-positive and -negative cell pools. *her4.1* was enriched in the EGFP$^+$ fraction, while *shh*, which is expressed in the epidermis (Laforest et al., 1998), was enriched in the EGFP$^-$ fraction, confirming the validity of the sorting (supplementary material Fig. S1C). PCR confirmed that *jag1b* was present at high levels and that it was enriched in the *her4.3*:EGFP-expressing cells. However, we also found that *jag1a* and, at lower levels, *jag2* were expressed in regenerating fins, but predominantly in EGFP-negative cells (supplementary material Fig. S1C). Delta transcripts could only be detected at low levels with no obvious enrichment in either pool, with the exception of *delta-like 4* (*dll4*), which was more strongly expressed in the EGFP$^+$ pool. Interestingly, all four Notch receptors were expressed in the regenerates, but only *notch2* could be detected in the EGFP-positive fraction. We also found that expression of *lunatic fringe*, a glycosyltransferase that promotes Notch activation (Appel et al., 2003; Nikolaou et al., 2009), was upregulated in regenerating fins (supplementary material Fig. S1D).

Together, these data suggest that *notch2* mediates Notch signaling in the *her4.3*:EGFP-positive cells, which largely represent blastemal mesenchyme. *jag1b* and *dll4* are candidates for ligands activating signaling within this population, whereas *jag1a* and *jag2* might be expressed in adjacent tissues such as the basal layer of the epidermis, and thus could mediate signaling across tissue 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 *aldh1a2* (*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

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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* domain, revealing that Notch activity is confined to the undifferentiated zone of the blastema (Fig. 2E). Transgene expression was confined to the medial mesenchyme and was excluded from 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 starting directly after fin amputation (Fig. 3A). Wound closure and formation of a wound epidermis were not affected by this treatment, but already by 2 dpa a reduction of regenerated mesenchymal tissue was evident in Notch-inhibited fins (Fig. 3B). Continued treatment completely repressed regenerative growth, resulting in fins whose stump appeared to be covered only by epidermis (Fig. 3B,C).

The complete blockade of fin regeneration suggests that Notch signaling might have a role in the earliest known cellular processes that result in blastema formation, namely dedifferentiation of mature stump osteoblasts and migration of these cells towards the amputation plane (Knopf et al., 2011). However, treatment of amputated fins with LY411575 for 3 days (Fig. 3A) did not affect downregulation of the osteoblast differentiation marker *osteocalcin* in *Ola.Osteocalcin.1*:EGFP^{hu4008} transgenic fins (see gaps of EGFP expression marked by arrowheads in Fig. 3D) nor accumulation of remaining EGFP signal at the amputation plane (arrows in Fig. 3E,F). Thus, Notch signaling is not required for osteoblast dedifferentiation and migration.

Next, we asked whether inhibition of Notch signaling after the blastema has formed is sufficient to interfere with regeneration. Indeed, when we treated fish with LY411575 for 4 days starting at 2 dpa (Fig. 3A), regenerative growth was severely impaired and LY411575-treated fins pretty much stalled at the length they had at the onset of treatment (Fig. 3G,H). Thus, the effect of continuous Notch signaling inhibition on regeneration can be largely explained by it being required for regenerative growth after the blastema has formed.

To test whether Notch signaling is required for blastema proliferation, 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+ blastemal 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 blastemal cells.

Notch overactivation impairs regenerative outgrowth

Our results so far are consistent with the hypothesis that Notch signaling maintains blastemal cells in an undifferentiated 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 (*hsp70l*:Gal4)1.5^{kca4}; *UAS*:myc-Notch1a-intra^{kca3} [which we will refer to as *hs*:Gal4; *UAS*:NICD (Scheer and Campos-Ortega, 1999)]. Application of two heatshocks at 66 and 72 hpa was sufficient to induce NICD expression in the regenerate, as detected by anti-Myc staining at 78 hpa, in a mosaic fashion in medial and lateral, Zns5-positive, mesenchymal cells, but less efficiently in the epidermis, which might be due to silencing of one or both of the transgenes in this tissue (supplementary material Fig. S3A). 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 regenerates of NICD overexpressing fins (arrow in Fig. 4C), confirming that

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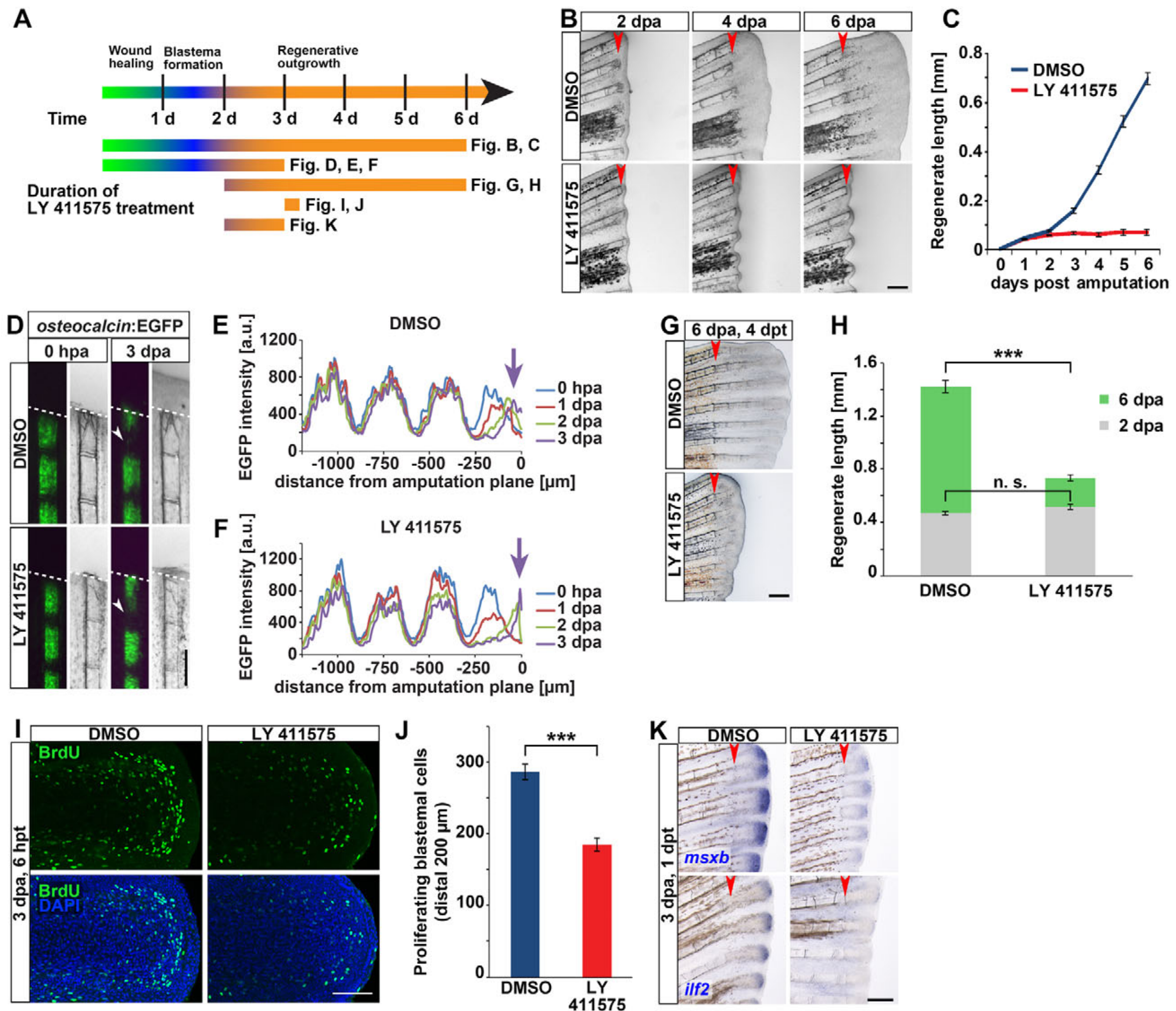

Fig. 3. Notch signaling is necessary for fin regeneration. (**A**) Schematic timeline of fin regeneration and experimental treatments. (**B**) Fins treated with LY411575 starting from the time of amputation fail to regenerate. (**C**) Regenerate length of fins treated with DMSO or LY411575 from the time of amputation, mean±s.e.m., n=6 fish, 18 fin rays each group (LY at day 6: n=5 fish, 15 fin rays). At all timepoints, except 1 and 2 dpa, regenerate lengths are significantly different (P<0.001) in control and LY411575-treated fins, Student's t-test. (**D**) LY411575 treatment from the time of amputation does not affect downregulation of EGFP expression (arrowhead) in the distalmost stump segment of *osteocalcin*:EGFP transgenic fins. Dashed line indicates amputation plane. (**E**,**F**) EGFP pixel intensity plots of representative *osteocalcin*:EGFP transgenic fin rays at the indicated time points after amputation treated with DMSO (E) or LY411575 (F), showing a decrease in intensity in a region 250 µm proximal to the amputation plane (0 on x-axis, right) and a shift of intensity towards the amputation plane (arrow). (**G**) Treatment with LY411575 for 4 days starting from 2 dpa is sufficient to interfere with regenerative outgrowth. (**H**) Regenerate length of fins treated as those in G at 2 dpa (prior to the start of treatment) and at 6 dpa, 4 days after the start of treatment (dpt). Mean±s.e.m. n=6 fins, 18 fin rays. ***P<0.001. (**I**) Confocal images of anti-BrdU immunofluorescence in the mesenchymal compartment of fin rays at 3 dpa treated with DMSO or LY411575 for 6 hours. (**J**) Number of BrdU-positive cells in the distal 200 µm of the mesenchyme of fins treated as in I. Mean±s.e.m. DMSO: n=7 fish, 27 blastemas; LY 411575: n=7 fish, 26 blastemas. ***P<0.001. (**K**) The blastema markers *msxb* (6/6 fins) and *ilf2* (5/6 fins) are downregulated in regenerates treated with LY411575 for 24 hours starting at 2 dpa. (B-K) Red arrowheads indicate amputation plane. Scale bars: 200 µm in B,D,G,K; 100 µm in I.

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

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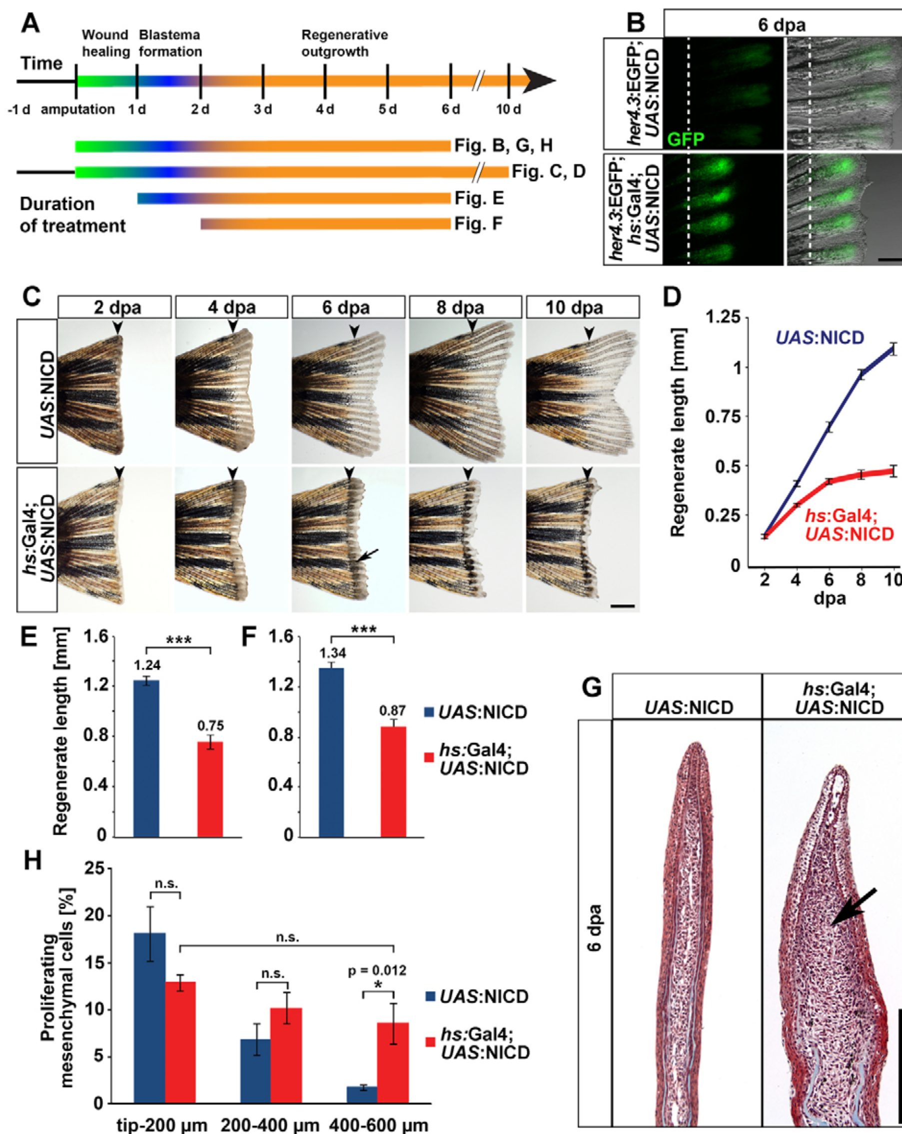

Fig. 4. NICD overexpression expands the proliferative blastema, yet stalls fin regeneration. (**A**) Schematic timeline of fin regeneration and experimental treatments. (**B**) Enhancement of EGFP expression in the blastema of *her4.3*:EGFP; *hs*:Gal4; *UAS*:NICD triple transgenic fins at 6 dpa after repeated heatshocks starting at the time of amputation. Dashed line indicates amputation plane. Scale bar: 200 µm. (**C**) Inhibition of regeneration in *hs*:Gal4; *UAS*:NICD fish heatshocked repeatedly starting 1 day prior to fin amputation. Pigment cells cluster in the stalled regenerate (arrow). Arrowheads indicate amputation plane. Scale bar: 500 µm. (**D**) Regenerate lengths of fins treated as in C; mean±s.e.m. Length differences at all timepoints, except 2 dpa, are highly significant ($P<0.001$). *UAS*:NICD: $n=8$ fins, 24 fin rays; *hs*:Gal4; *UAS*:NICD: $n=12$ fins, 36 fin rays. (**E**,**F**) NICD overexpression starting at 1 dpa (E) or 2 dpa (F) is able to delay fin regeneration, mean±s.e.m. $n=8$ fins, 24 fin rays each group. ***$P<0.001$. (**G**) Masson's trichrome staining reveals expansion of the mesenchymal compartment of regenerates (arrow) after sustained NICD overexpression for 6 days. Scale bar: 200 µm. (**H**) Percentage of BrdU-positive mesenchymal cells in three 200 µm regions along the distal-proximal axis of *UAS*:NICD and *hs*:Gal4; *UAS*:NICD fins heatshocked repeatedly for 6 days. NICD overexpression increases proliferation in the scarcely proliferating proximal 400-600 µm domain ('differentiation zone'). $n=5$ fish, six sections. *$P<0.05$.

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-expressing fins. By contrast, expression of *aldh1a2*, which is confined to the distal blastema, was not altered by NICD overexpression (supplementary material Fig. S4B). Together,

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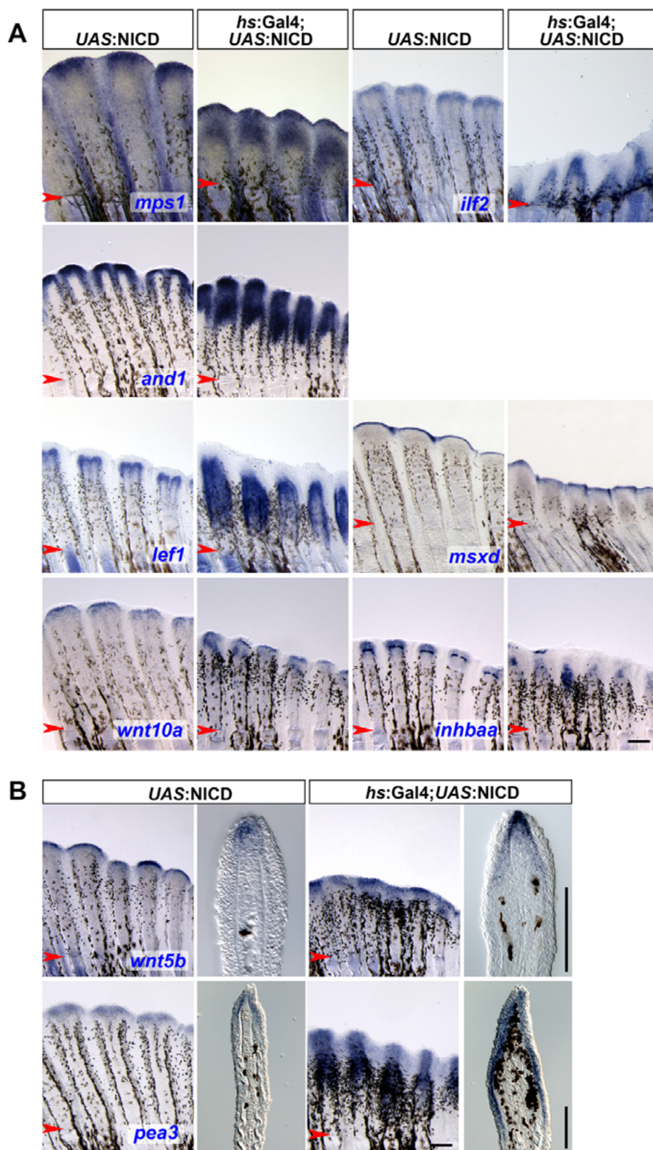

Fig. 5. NICD overexpression causes expansion of proximal mesenchymal and epidermal compartments. (**A**,**B**) Whole-mount *in situ* hybridization of *hs*:Gal4; *UAS*:NICD and *UAS*:NICD fins heatshocked repeatedly from 0 hpa to 6 dpa with the indicated markers reveals massive proximal expansion of proximal, but not distal, blastemal and epidermal domains upon NICD overexpression. *mps1*=6/6 fins; *ilf2*=6/6; *and1*=9/11; *lef1*=7/7; *wnt10a* and *inhbaa*=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 *lef1*, 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 *msxd* 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 *lef1* 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 *lef1* 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 *lef1* 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 (*osteocalcin*) 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*.1:EGFP^{hu4008}; *OlSp7*:mCherry^{zf131}). 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 *osterix*:mCherry expression was significantly expanded upon NICD overexpression (compare brackets in Fig. 6A) and the *osterix*:mCherry-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

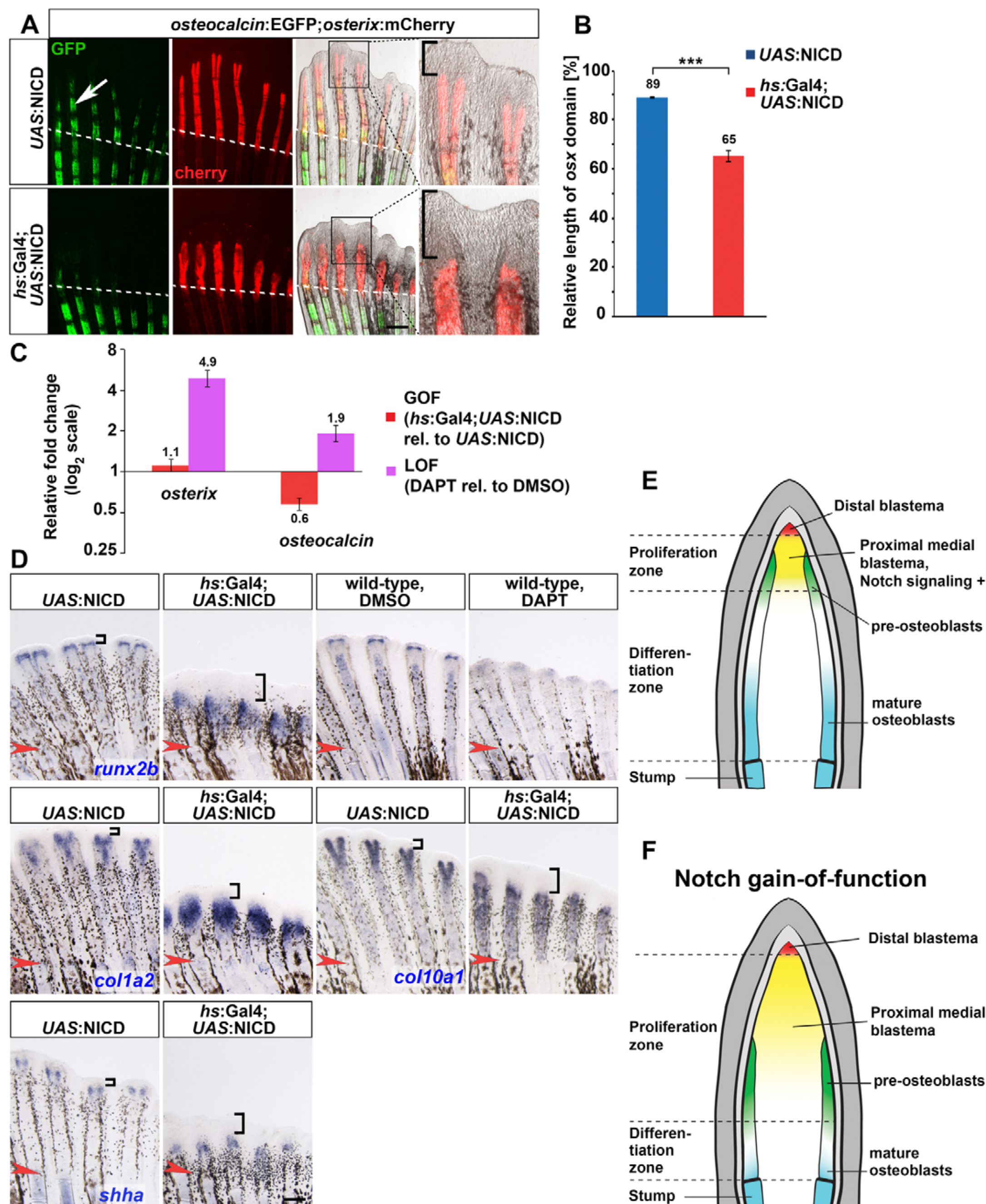

Fig. 6. Notch signaling negatively regulates osteoblast differentiation in the regenerating fin. (**A**) Altered bone marker expression in the regenerate of *osteocalcin*:EGFP; *osterix*:mCherry; *hs*:Gal4; *UAS*:NICD quadruple transgenic fish heatshocked repeatedly for 6 days. Dashed line indicates amputation plane. Scale bar: 200 μm. Arrows and brackets indicate *osteocalcin*: EGFP. (**B**) The fraction of the regenerate expressing *osterix*:mCherry in fish treated as in A. Mean±s.e.m. *n*=7 fins, 31 fin rays. ****P*<0.001. (**C**) qRT-PCR shows upregulation of *osteocalcin* and *osterix* at 6 dpa in regenerates treated with DAPT for 4 days from 2 dpa onwards (loss of function), whereas *osteocalcin* is downregulated upon sustained NICD overexpression (gain of function) for 6 days. Mean±s.d., *n*=15 regenerates transgenics, 10 drug treatments. (**D**) Whole-mount *in situ* hybridization of the indicated markers in 6 dpa regenerates overexpressing NICD or treated with DAPT for 6 days. *n*=5/6, *runx2b* (*hs*:Gal4; *UAS*:NICD); *n*=12/13, *runx2b* (DAPT); *n*=6/6, *col1a2*; *n*=4/5, *col10a1*; *n*=5/7, *shha*. Arrowheads indicate amputation plane. Brackets indicate distal region devoid of marker expression. (**E**,**F**) Model depicting the spatial distribution of Notch signaling-positive cells in a longitudinal section of a wild-type fin regenerate (E) and the patterning consequences of Notch gain of function (F).

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 *col1a2* and *col10a1*, which mark regions of skeletogenesis (compare brackets in Fig. 6D). *shha* expression in a subregion of the basal layer of the

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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 noninjured 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

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(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 Bejjani 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
The authors declare no competing financial interests.

Supplementary material
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