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Notch regulates blastema proliferation and prevents differentiation during adult zebrafish fin regeneration

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SUMMARY

Zebrafish have the capacity to regenerate several organs, including the heart and fins. Fin regeneration is epimorphic, involving the formation at the amputation plane of a mass of undifferentiated, proliferating mesenchymal progenitor-like cells, called blastema. This tissue provides all the cell types that form the fin, so that after damage or amputation the fin pattern and structure are fully restored. How blastema cells remain in this progenitor-like state is poorly understood. Here, we show that the Notch pathway plays an essential role during fin regeneration. Notch signalling is activated during blastema formation and remains active throughout the regeneration process. Chemical inhibition or morpholino-mediated knockdown of Notch signalling impairs fin regeneration via decreased proliferation accompanied by reduced expression of Notch target genes in the blastema. Conversely, overexpression of a constitutively active form of the Notch1 receptor (N1ICD) in the regenerating fin leads to increased proliferation and to the expansion of the blastema cell markers *msxe* and *msxb*, as well as increased expression of the proliferation regulator *aldh1a2*. This blastema expansion prevents regenerative fin outgrowth, as indicated by the reduction in differentiating osteoblasts and the inhibition of bone regeneration. We conclude that Notch signalling maintains blastema cells in a plastic, undifferentiated and proliferative state, an essential requirement for fin regeneration.

KEY WORDS: Caudal fin, Regeneration, Blastema, Proliferation, Differentiation, Notch

INTRODUCTION

Organ regeneration in mammals is limited. By contrast, other vertebrates including teleost fish have an impressive regeneration capacity and can easily replace lost tissues and organs (Poss et al., 2002a; Poss et al., 2003). The zebrafish caudal fin provides a useful model of limb regeneration and bone repair because it is easily accessible and not essential for survival (Akimenko et al., 2003; Poss et al., 2003). The fin is a complex structure, with 16-18 segmented bony fin rays (lepidotrichia) separated by soft interray tissue. Each fin ray is formed by two concave hemirays, which are lined with osteoblasts that secrete the bone matrix. The hemirays serve to protect an intraray core of mesenchymal cells, blood vessels, nerves, melanocytes and fibroblasts. The interray space is composed of mesenchymal cells, and an epithelial cell layer covers ray and interray tissue (Tal et al., 2010). Fin regeneration occurs through a mechanism called epimorphic regeneration, whereby a population of mesenchymal cells, the blastema, appears at the wound site (Akimenko et al., 2003; Schebesta et al., 2006). The blastema is a source of progenitor cells that divide, differentiate and organize to restore the lost tissue. Complete caudal fin regeneration takes around 14 days, and consists of three phases: (1) wound healing, (2) blastema formation and (3) regenerative outgrowth. Immediately after fin amputation, a wound epidermis forms and seals the wound. Epithelial cells form a multilayered epidermis, which is required for blastema formation and proliferation (Chablais and Jazwinska, 2010; Liu et al., 2010). By 2 days post amputation (2 dpa), a blastema consisting of undifferentiated, highly proliferative cells has formed beneath the wound epidermis distal to each amputated fin ray. These cells express the Msx homeobox family members *msxb* and *msxe*, which label undifferentiated/progenitor-like cells in a variety of regenerating tissues (Akimenko et al., 1995; Han et al., 2003; Barker and Beck, 2009; Yoshinari et al., 2009). In the third phase, differentiating blastema cells progressively reconstitute the lost tissue until the complete fin is regenerated. There is a gradient of differentiation within the regenerating fin: the blastema remains in the distal region underneath the epidermis, whereas the proximal region contains progressively more differentiated cells in the direction of the amputation plane. This can be observed for osteoblasts, which in the blastema align to the epidermis, differentiate and deposit bone matrix in more proximal locations (Smith et al., 2006; Brown et al., 2009).

Several groups have examined the nature of blastema cell identity and lineage relationships in the regenerating fin (Knopf et al., 2011; Sousa et al., 2011; Tu and Johnson, 2011). Genetic fate mapping and marker analysis have shown that, upon amputation, stump osteoblasts dedifferentiate, populate the blastema and redifferentiate, giving rise to new osteoblasts that will replace the lost bone (Knopf et al., 2011; Sousa et al., 2011). Further studies demonstrated that this lineage restriction occurs not only with osteoblasts but also with fibroblast-like cells and epidermis (Stewart and Stankunas, 2012) and that distinct fate-restricted progenitor cells exist for each lineage that makes up the adult fin (Tu and Johnson, 2011). By contrast, genetic ablation of the osteoblast lineage has revealed that osteoblasts can arise *de novo*, presumably from a lineage different from osteoblasts, suggesting that multiple cellular sources can contribute to bone regeneration in the fin (Singh et al., 2012).

The highly conserved Notch signalling pathway regulates embryonic cell fate determination, differentiation and patterning in a variety of tissues (reviewed by Artavanis-Tsakonas et al., 1999; de la Pompa and Epstein, 2012). Notch proteins constitute an

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evolutionarily conserved group of type I transmembrane receptors, with a large extracellular region that interacts with membrane-bound ligands of the Delta or Serrate/Jagged families on neighbouring cells. Ligand-receptor interaction leads to proteolytic cleavage of the receptor by γ-secretase activity to generate the Notch intracellular domain (NICD), which translocates to the nucleus (Kopan, 2002). In the nucleus, NICD heterodimerizes with the RBPJK/CBF1/Su(H) effector transcription factor (Fortini and Artavanis-Tsakonas, 1994; Jarriault et al., 1995), converting it from a repressor to an activator. Notch target genes include those encoding repressor transcription factors of the Hes and HRT/Hey/Herp families (Iso et al., 2003). Notch pathway genes are expressed during zebrafish fin regeneration (Raya et al., 2003; Schebesta et al., 2006). The established role of Notch in the regulation of stem/progenitor cell fate maintenance (Liu et al., 2010) prompted us to study Notch function during zebrafish fin regeneration. Here, we show that Notch signalling is essential for fin regeneration and provide mechanistic evidence for a function of Notch in the maintenance of blastema cells in an undifferentiated and proliferative state.

MATERIALS AND METHODS

Zebrafish husbandry and fin amputation

Zebrafish were raised under standard conditions at 28°C (Kimmel et al., 1995). Experiments were performed with 6 to 16-month-old adults, except for DAPT/DMSO treatment in which we used 1-month-old fish. The lines used were: wild-type AB strain, *Tg(hsp70l:Gal4)^{kca4};Tg(UAS:myc-Notch1a-intra)^{kca3}* (Scheer et al., 2001) and the *lfng* reporter line *ET(krt4:EGFP)$^{sqet33\text{-}mi60A}$* (*ET33-mi60A*) (Poon et al., 2010). For regeneration experiments, half the caudal fin was amputated and allowed to regenerate at 28°C. Heat shocks were applied automatically to wild-type and *Tg(hsp70l:Gal4)^{kca4};Tg(UAS:myc-Notch1a-intra)^{kca3}* fish using the immersion thermostat LAUDA E300. Two heat-shock protocols were used: for long-term treatment (days), 1 hour at 38°C followed by 3 hours at 28°C; for short-term treatment (hours), 1 hour at 38°C, 1.5 hours at 28°C. Fins were harvested up to 1 hour after heat shock treatment. Experimental protocols were approved by the local ethics committee, and conformed to EU Directive 2010/63EU regarding the protection of animals used for experimental purposes, enforced in Spanish law under Real Decreto 1201/2005.

BrdU, RO4929097 and DAPT treatments

Fish were transferred to water containing 10 mM bromodeoxyuridine (BrdU, Sigma) for 30 minutes immediately before tissue collection (Poss et al., 2002b). Fish were incubated in water containing 10 μM (for long-term, 72 hours) or 15 μM (for short-term, 10-12 hours) of the γ-secretase inhibitor RO4929097 (S1575, selleckchem.com) or 50 μM of the γ-secretase inhibitor DAPT (565770, Calbiochem) or control DMSO.

Histology

PFA (4%) overnight-fixed tissue (4°C) was washed for 24 hours in 0.5 M EDTA, dehydrated, paraffin wax-embedded and sectioned. Haematoxylin and Eosin staining followed standard methods. For whole-mount Alizarin Red staining, 4% PFA-fixed fins were rehydrated through a decreasing methanol series, bleached for 30 minutes in 0.8% KOH, 0.6% H_2O_2. Subsequently, fins were washed twice with water and washed for 20 minutes in a saturated Alizarin Red solution containing 1% KOH, followed by several washes with water and transferred to glycerol.

In situ hybridization

Whole-mount *in situ* hybridization and *in situ* hybridization were performed as described (de la Pompa et al., 1997; Kanzler et al., 1998). Probe details can be found in supplementary material Table S1.

Immunohistochemistry

Paraffin sections were stained according to standard protocols (Zhang et al., 2010). Primary antibodies used were against BrdU (1:30, BD), GFP (1:100, Living Colors), Osx/sp7 (1:100, Abcam), Aldh1a2 (1:400, GeneTex), PCNA (1:100, Santa Cruz) and Myc (1:100, Santa Cruz). Secondary antibodies used were biotin-conjugated antibody (Invitrogen), anti-rabbit Alexa-488, anti-mouse Cy3 (Jackson) and streptavidin-Cy5 (Vector).

Imaging and photography

Fins were photographed with an Olympus DP71 camera fitted in a Leica stereomicroscope. ImageJ64 was used to measure the size of regenerates from the amputation plane to the distal tip of each fin ray (Fig. 4A-C, Fig. 6A,B, supplementary material Fig. S4A-C) or just of the seven dorsal and ventral rays of both fin halves (Fig. 3A-C,I-N). Mean fin length was calculated for each animal. Fin width, excluding the epidermal layer, was measured on Haematoxylin and Eosin-stained sections with ImageJ64. Histological samples were photographed with a DP71 camera fitted in an Olympus BX51 microscope. Confocal images were obtained with a Leica TCE 2500 SP-E confocal microscope. For cell number analysis, we counted DAPI$^+$ and PCNA$^+$, EGFP$^+$ or BrdU$^+$ cells within the regenerated tissue and calculated the total amount of labelled cells per DAPI$^+$ nuclei using Adobe Photoshop CS5.1. To analyse Osx-stained fin sections, we used ImageJ64 to measure both the Osx$^+$ proximal fin region and the Osx$^-$ distal region. We estimated the proportion of both regions within the whole regenerated fin. Statistical significance was calculated using Student *t*-test.

Quantitative real-time PCR

Fish were treated following the short-term heat shock protocol described above. For RNA extraction, regenerated tissue from three to five fins, including one ray segment of the stump, was harvested per experiment. Each experiment was repeated at least three times, and data are presented as means±s.d. of several experiments. RNA was extracted with TRI reagent (Sigma). Equal amounts of RNA per sample were reverse transcribed with the SuperScript III first Strand Kit (Invitrogen). qPCR was performed with an ABI PRISM 7900HT FAST Real-Time PCR System using Power SYBRGreen PCR mastermix (Applied Biosystem). Expression was normalized to transcripts levels of *ef1a* and *gapdh* using Biogazelle qbasePLUS software. See supplementary material Table S2 for primer sequences.

Morpholino knockdown

We used the following fluorescein-coupled morpholino oligonucleotides (MO; GeneTools): *jag1b*, *notch1b* (Lorent et al., 2004), *rbpjκ* (Sieger et al., 2003), *lfng* (Nikolaou et al., 2009) and a standard negative control. MOs were injected and electroporated as described previously (Thummel et al., 2006) into the dorsal half of fins after regeneration for 2 days in water at 28°C. Fins were photographed immediately after injection and again on the following days. Regenerative outgrowth was determined by measuring the length of regenerated tissues from the amputation plane to the distal tip of each fin radial at each time point. The value for the ventral half of each fin served as an internal control. Outgrowth size was calculated from the formula $(dL_{t2}\text{-}dL_{t1})/(vL_{t2}\text{-}vL_{t1})$, where dL is the mean length of the dorsal regenerate, vL the mean length of the ventral regenerate, t1 is the day of MO-transfection and t2 is 2 days later.

RESULTS

Notch pathway genes are expressed in blastema cells

Expression profiling has shown that Notch signalling is upregulated during fin regeneration (Raya et al., 2003; Schebesta et al., 2006). qPCR analysis of regenerating fins at 3 dpa, when blastema formation has begun and regenerative outgrowth is initiated, revealed upregulated expression of the genes encoding Notch1b, lunatic fringe (Lfng), glycosyl transferase and the transcription factors Her6 and Her15 (Fig. 1A), whereas other pathway components were unchanged (supplementary material Fig. S1A). Analysis of the expression pattern of these genes during fin regeneration showed that at 1 dpa, when the epidermis covers the wound but no blastema cells are visible distal to the amputation

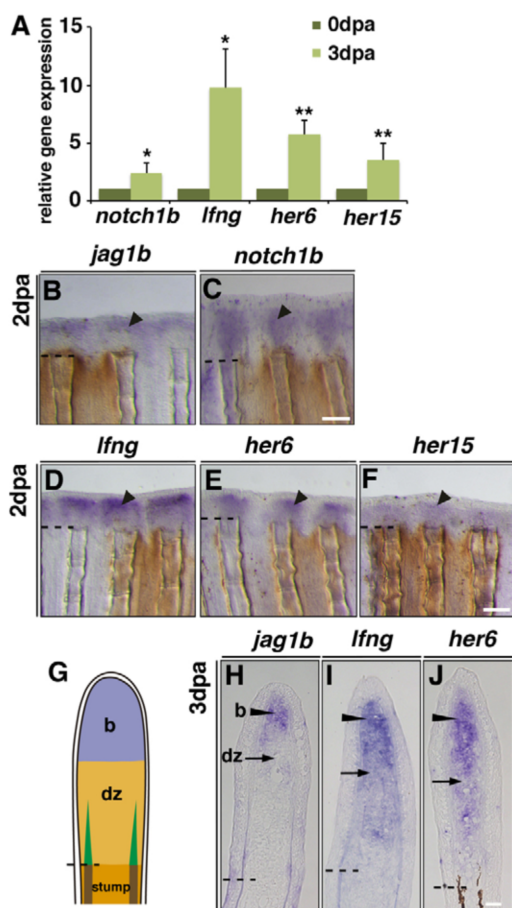

Fig. 1. Notch signalling is activated during fin regeneration. (**A**) qPCR analysis of *notch1b*, *lfng*, *her6* and *her15* mRNA in regenerating fins at 3 dpa relative to expression in non-amputated fins (0 dpa). **P*<0.05, ***P*<0.01. (**B-F**) Whole-mount *in situ* hybridization in 2 dpa fins for *jag1b* (B), *notch1b* (C), *lfng* (D), *her6* (E) and *her15* (F), showing Notch pathway activation within the blastema (arrowheads). (**G**) Scheme of a 3 dpa fin section, indicating the two regions within the regenerate: the blastema (b) and the differentiation zone (dz). Green indicates regenerating fin rays. (**H-J**) *In situ* hybridization on 3 dpa fin sections for *jag1b* (H), *lfng* (I) and *her6* (J) reveals Notch pathway activation in the blastema (b, arrowhead), but weak or no gene expression within the dz (arrow). Scale bars: 100 μm in C,F; 10 μm in J. Broken lines mark amputation plane.

plane, there is no Notch expression in the epidermis or within the fin stump (not shown). At 2 dpa, blastema formation has started and some cells are present beyond the epidermis and distal to the amputation plane. Whole-mount *in situ* hybridization indicated that *jag1b*, *notch1b*, *lfng*, *her6* and *her15* were expressed in the blastema (Fig. 1B-F), indicating that Notch signalling is activated during blastema formation. At 3 dpa, two regions can be distinguished within the fin regenerate, the distally located blastema and the proximal differentiation region (Fig. 1G). The blastema contains highly proliferative, de-differentiated cells, whereas in the proximal region close to the amputation plane, cells undergo differentiation (Knopf et al., 2011; Stewart and Stankunas, 2012). We detected *msxb*, a marker of de-differentiated cells and of the distal blastema (Nechiporuk and Keating, 2002), in the entire blastema and in the differentiation zone of the 3 dpa regenerating fin (supplementary material Fig. S1B), consistent with the reported expression of *msxb* in osteoblasts (Smith et al., 2008). A second Msx-family member, *msxe*, was restricted to blastema cells (supplementary material Fig. S1C) (Yoshinari et al., 2009). The blastema shows the highest proliferation rate (Poleo et al., 2001; Nechiporuk and Keating, 2002). At 3 dpa, almost all cells within the blastema were BrdU labelled, with the exception of a few positive proximal cells (supplementary material Fig. S1D). During fin regeneration, proliferation is controlled by retinoic acid (RA) (Blum and Begemann, 2012). We found that the expression of *aldh1a2*, which encodes the enzyme that catalyses RA synthesis from retinaldehyde, was restricted to distal blastema cells at 3 dpa (supplementary material Fig. S1E).

We next examined whether Notch signalling is activated in the entire regenerate or is restricted to a specific region. At 3 dpa, *jag1b* was expressed exclusively in blastema cells (Fig. 1H). Similarly, *lfng* and *her6* expression was especially strong in the blastema, but was low in proximal regions, where differentiation occurs (Fig. 1I,J). Indeed, the pattern of Notch activation coincided with *msxe* and *aldh1a2* expression domains. At later stages (5 dpa) Notch activity was similarly restricted to the blastema, consistent with *msxe* and *aldh1a2* expression (supplementary material Fig. S1F-K). These data indicate that Notch activity is predominant in the distal region of the regenerating fin, which is distinguishable from the more proximal differentiation zone by its high proliferation rate and expression of *aldh1a2* and *msxe*.

Lunatic fringe mediates Notch signalling in proliferating blastema cells

To examine whether Notch activation was related to proliferation, we used the *ET33-mi60A* enhancer trap line, which expresses EGFP upstream of *lfng* (Poon et al., 2010), allowing us to track Lfng-mediated Notch signalling during fin regeneration. Weak EGFP expression was observed in the caudal fin before amputation (Fig. 2A,A′), but expression increased sharply by 3 dpa (Fig. 2B,B′). EGFP expression was not detected at 1 dpa during wound closure and epidermis formation, confirming non-involvement of Notch in this early phase of regeneration (Fig. 2C,C′). Expression was first visible at 2 dpa, during blastema formation (Fig. 2D,D′) and was strongly upregulated at 3 dpa (Fig. 2E,E′) and at later stages (Fig. 2I, supplementary material Fig. S1G). Combined immunohistochemistry against EGFP and *her6* in situ hybridization confirmed that EGFP expression in *ET33-mi60A* fish coincided with Notch activation, and was stronger in distal blastema cells and weaker in proximal ones (Fig. 2F,F′).

To investigate whether blastema cells with Notch activity were proliferating, we examined the expression of proliferating cell nuclear antigen (PCNA). At 2 dpa, 51% (±7%) of cells expressed EGFP and were located beneath the epidermis (Fig. 2G, supplementary material Fig. S2A). Moreover 79% (±14%) of these cells expressed PCNA (supplementary material Fig. S2B). A similar correlation was evident at 3 dpa, with EGFP-expressing cells constituting 51% (±9%) of total blastema cells (supplementary material Fig. S2A); however, by this stage EGFP-expressing cells were concentrated in the distal region and more proximal EGFP-expressing cells were located close to the epidermis. These regions containing EGFP-expressing cells coincided with PCNA expression domains (Fig. 2H, supplementary material Fig. S2A), and 78% (±7%) of $EGFP^+$ cells co-expressed PCNA (Fig. 2H, supplementary material Fig. S2B). At 5 dpa, when regeneration had resulted in a progressive differentiation of proximal tissue (Fig. 2I), we observed a marked decrease in the number of EGFP- and PCNA-expressing

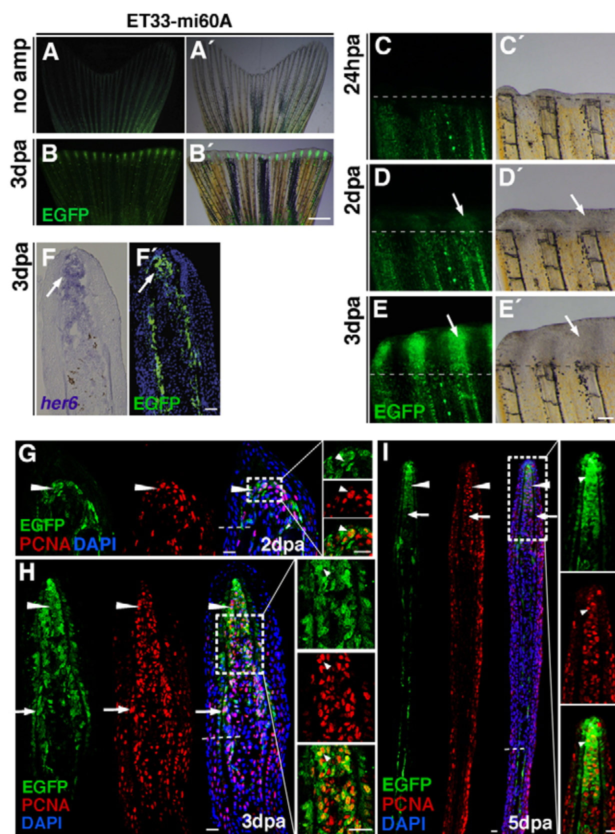

Fig. 2. Lunatic fringe-mediated Notch signalling in proliferating blastema cells. (**A-B′**) EGFP expression in the enhancer trap line *ET33mi60* is weak in non-amputated fins (A,A′), but strong in the fin regenerate at 3 dpa (B,B′). (**C-E′**) Time-course of *ET-33mi60A* regenerating fins. No EGFP expression is seen in the wound epidermis at 1 dpa. (C,C′). EGFP expression starts at 2 dpa within the blastema (D,D′, arrows). EGFP expression is strong within the blastema at 3 dpa (E,E′, arrows). (**F**,**F′**) *her6 in situ* hybridization and EGFP immunohistochemistry on a fin at 3 dpa reveals EGFP expression in cells in which Notch is activated (arrows). (**G-I**) EGFP and PCNA double immunohistochemistry. (G,H) Confocal microscopy images: EGFP is expressed during blastema formation (2 dpa) in cells co-expressing PCNA (G, arrowheads). At 3 dpa, EGFP expression is strong in all blastema cells (H, arrowheads), and is mosaic in proximal regions (H, arrows). Most cells are co-labelled with PCNA (H,I, arrowheads). At 5 dpa, EGFP expression is strong in the blastema (I, arrowheads) but weak in the proximal region (I, arrows). Most cells in the EGFP$^+$ area express PCNA (I, arrowheads). Scale bars: 1 mm in B′; 100 µm in E; 10 µm in E′,I. Broken lines mark the amputation plane.

cells within the whole fin regenerate compared with earlier stages (Fig. 2I, supplementary material Fig. S2A). However the proportion of proliferating EGFP-expressing cells remained high (66%±5) (Fig. 2I, supplementary material Fig. S2B). These double-labelled cells were mostly restricted to the distal region of the regenerate (Fig. 2I), consistent with the reported progression of regeneration through proliferation of cells in the distal blastema region (Blum and Begemann, 2012). Moreover, double immunohistochemistry in *ET33mi60A* fins revealed that EGFP$^+$ blastema cells also express Aldh1a2 (supplementary material Fig. S2C), which controls blastema cell proliferation (Blum and Begemann, 2012). Very few epidermal cells co-expressed EGFP and PCNA, suggesting that Notch is exclusively involved in the proliferation of blastema mesenchymal cells. Notch is thus active in the recently formed blastema (2 dpa) and is sustained at later regeneration stages. At all stages examined, Notch activation followed the pattern of proliferation within the mesenchyme, and was distally restricted, suggesting a role in proliferating blastema cells during regeneration.

Notch signalling inhibition reduces proliferation and impairs fin regeneration

To investigate the requirement of Notch during regeneration, we treated zebrafish embryos for 2 days with DMSO or 10 µM RO4929097 (2,2-dimethyl-N-(S)-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl)-N′-(2,2,3,3,3-pentafluoro-propyl)-malonamide]), which prevents γ-secretase-mediated Notch receptor cleavage and attenuates Notch signalling (Huynh et al., 2011). The treatment induced looped tail and impaired somitogenesis (supplementary material Fig. S3A,B), developmental defects that result from reduced Notch signalling (Zhang et al., 2007). Expression of the Notch target *her6* and *her15* was also reduced (supplementary material Fig. S3C,D), confirming effective downregulation of Notch signalling. Treatment of fins with 10 µM RO4929097 for 3 days starting just after fin amputation blocked Notch activation, indicated by reduced *her6* expression (supplementary material Fig. S3E,F) and blocked fin regeneration, whereas DMSO-treated fish showed no defects in fin regeneration (Fig. 3A-C). The blastema formed in RO4929097-treated fish, as indicated by *msxe, msxb* and *aldh1a2* expression (supplementary material Fig. S3G-L), but regeneration then stopped abruptly. To test whether this was due to a blockade of proliferation, we analysed BrdU-incorporation in fish treated with 15 µM RO4929097 between 62 and 72 or 98-110 hpa. RO4929097 strongly reduced cell proliferation in the regenerating fin blastema during regenerative outgrowth initiation (62-72 hpa; Fig. 3F-H) and further during outgrowth (98-110 hpa; supplementary material Fig. S3M-O), and this was accompanied by severely reduced *her6* expression (Fig. 3D,E). Notch signalling thus appears to regulate proliferation during regenerative outgrowth. We also observed a block in Notch activation (supplementary material Fig. S4D,E) and impaired regenerative outgrowth using another γ-secretase-inhibitor, DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) (supplementary material Fig. S4A-C).

We confirmed these findings using morpholinos (MO) against *notch1b*, *jag1b* (Lorent et al., 2004), *rbpjκ* (Sieger et al., 2003) and *lfng* (Nikolaou et al., 2009). MOs were injected into the dorsal halves of fins after regeneration for 2 days at 28°C and transfection was induced by electroporation. Transfection with control MO triggered only minor differences in regeneration (Fig. 3I,N). By contrast, MOs targeting *notch1b*, *jag1b*, *lfng* or *rbpjκ* decreased regeneration compared with the non-electroporated ventral region (Fig. 3J-N). BrdU analysis revealed a markedly reduced blastema cell proliferation in *notch1b* MO-transfected fin halves 24 hours post transfection, confirming association of the impaired regeneration with decreased proliferation (Fig. 3O-Q).

Notch gain of function leads to blastema expansion and inhibits regenerative outgrowth

To analyse the effect of Notch gain-of-function during regeneration, we used the double transgenic line *Tg(hsp70l:Gal4);Tg(UAS:myc-notch1a-intra)*, abbreviated as *Tg(UAS:NICD)*. Heat-shock promoter activation in this line triggers Gal4 expression, which activates NICD expression by

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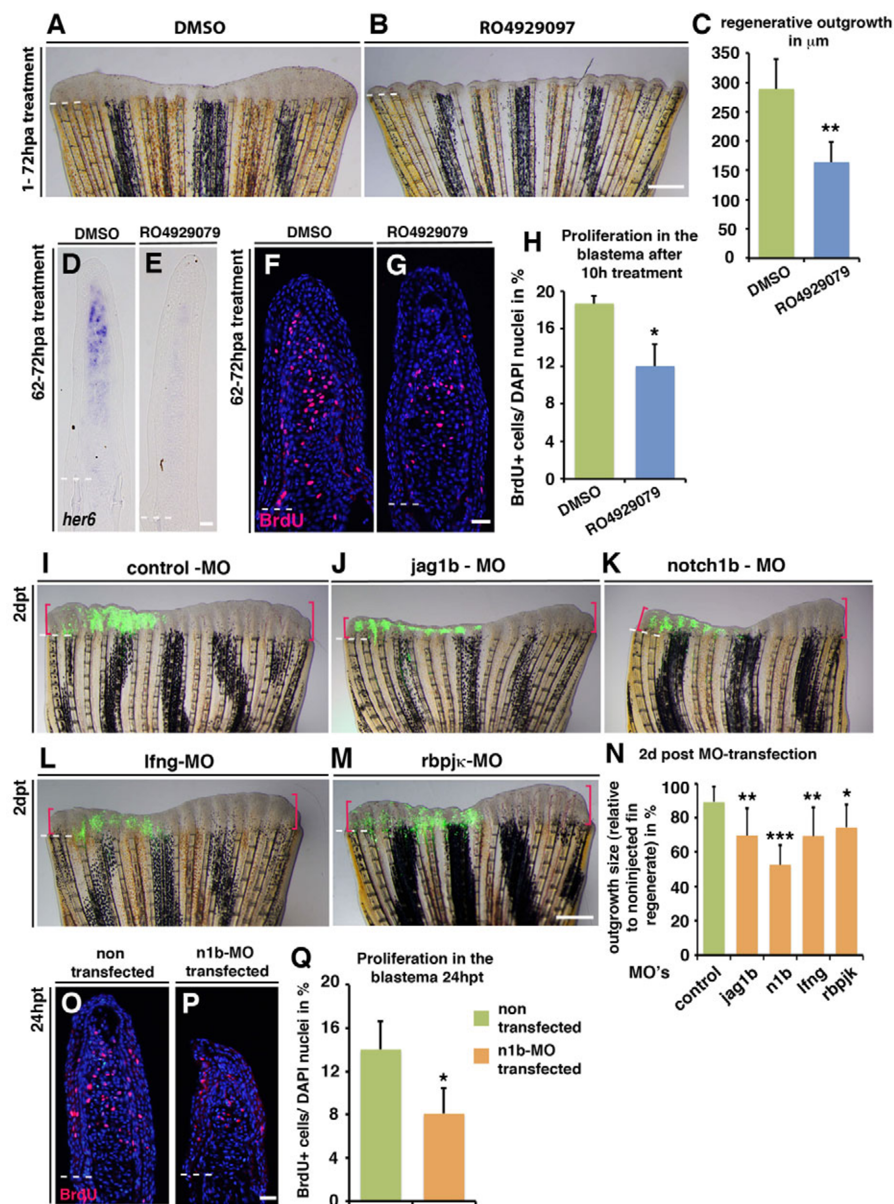

Fig. 3. Notch signalling inactivation impairs regeneration by decreasing proliferation. (**A**,**B**) Fish were treated with DMSO or 10 μM RO4929097 for 3 days starting at 1 hpa. Fin regeneration is blocked by RO4929097 treatment. (**C**) Mean length of fin regenerates (*n*=5); $^{**}P<0.01$. (**D**,**E**) *In situ* hybridization of *her6* showing Notch activity in regenerating fins treated with DMSO from 62 to 72 hpa (D) but not in RO4929097-treated fins (E). (**F-H**) BrdU-stained fin sections and quantification of $BrdU^+$ cells within the mesenchyme of RO4929097-treated fins (G; *n*=5) and DMSO-treated fins (F; *n*=6). $^*P<0.05$. (**I-M**) Fins microinjected in the dorsal half with fluorescein-labelled morpholinos (MO, green) and electroporated at 2 days post-transfection (dpt): control (I; *n*=9), *jag1b* (J; *n*=12), *notch1b* (K; *n*=9), *lfng* (L; *n*=12) or *rbpjκ* (M; *n*=6). The ventral half serves as an internal control. Pink brackets mark maximal regenerative outgrowth at 2 dpt. (**N**) Mean outgrowth size of the dorsal (MO-electroporated) half of the fin relative to the ventral half at 4 dpa/2 dpt. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.005$ versus control. (**O**,**P**) BrdU-stained fin sections and quantification of $BrdU^+$ cells within the mesenchyme. (**Q**) Mean percentage of $DAPI^+$ blastema cells in fin sections incorporating BrdU at 3 dpa/24 hpt (*n*=3). $^*P<0.05$. Proliferation is reduced in notch1b-MO-transfected fin halves. Scale bars: 1 mm in B; 10 μm in E,G; 1 mm in M; 10 μm in P. Broken lines indicate the amputation plane.

binding to the UAS sequence (Scheer et al., 2001), allowing us to control the timing of Notch activation. We applied a series of heat-shocks to wild-type and transgenic fish starting from amputation and continuing over 6 days of regeneration. Heat-shocks did not affect regeneration in wild-type fish, and there was no obvious morphological difference or defect in regenerative outgrowth between wild-type and *Tg(UAS:NICD)* fish at 1 dpa and 3 dpa (Fig. 4A-B′,C). However, from 5 dpa onwards, *Tg(UAS:NICD)* fish showed markedly weaker regenerative outgrowth (Fig. 4A″,B″,C). Notch gain of function also produced a swelling of the blastema distal to each fin ray (Fig. 4A″,B″). This relatively late phenotype is consistent with our finding that Notch is active throughout the blastema early during regeneration (Fig. 1B-F; Fig. 2G,H). As regeneration proceeds (5 dpa), endogenous Notch activation is restricted to blastema cells (supplementary material Fig. S1F-H), and this is when we observe the full impact of ectopic N1ICD expression on fin regeneration. NICD expression can be tracked in *Tg(UAS:NICD)* fish with an antibody against the Myc tag. Myc (Myc-NICD) was detected in 24% (±1%) of the blastema mesenchyme cells at 5 dpa, although there was little Myc expression in epidermal cells (Fig. 4D). Most Myc-expressing cells reside in the blastema periphery, close to the epidermis, although a few Myc-positive cells were present in the central mesenchyme (Fig. 4D). In the fin stump only a few cells, presumably fibroblasts and osteoblasts, expressed the transgene (supplementary material Fig. S5A,B). Moreover, Myc-NICD expression varied between *Tg(UAS:NICD)* fish, corresponding directly to the severity of the regeneration phenotype, with stronger expression of the transgene causing a more severe fin regeneration defect.

Haematoxylin and Eosin staining of regenerating fins at 5 dpa confirmed the swelling of the blastema, with the increased width in regenerating Notch gain-of-function fins, giving the sections a pear-shaped appearance (Fig. 4E,F, supplementary material Fig. S5C). The blastema cells in regenerating wild-type fins were densely packed, whereas more proximal central cells were loosely organized (Fig. 4E,E′). Lateral cells were aligned with the

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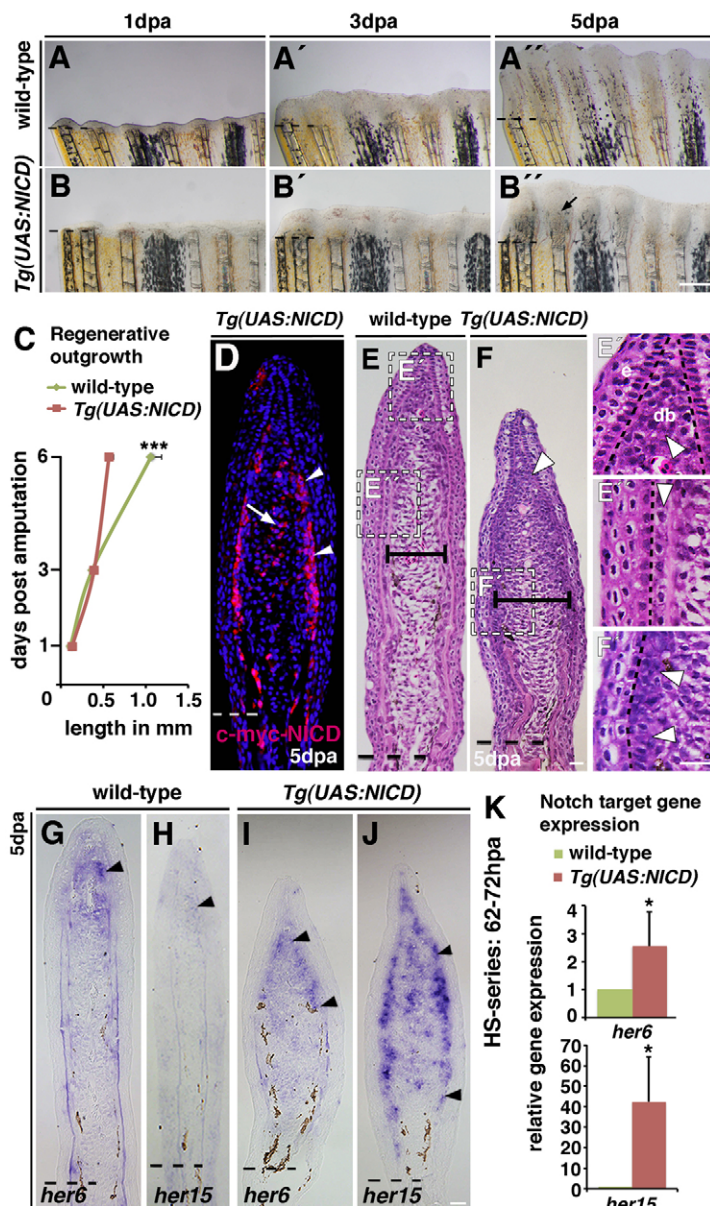

Fig. 4. Notch gain of function leads to blastema expansion and impaired fin regeneration. Wild-type and *Tg(hsp70l:Gal4);Tg(UAS:myc-notch1a-intra)* fish, referred to as *Tg(UAS:NICD)*, were heat-shocked throughout the regeneration period. (**A-B″**) Live images of wild-type and *Tg(UAS:NICD)* fins at 1, 3 and 5 dpa. No phenotypic differences were observed at 1 (A,B) and 3 (A′,B′) dpa between wild-type and *Tg(UAS:NICD)*. Regenerative outgrowth progresses in 5 dpa wild-type fish (A″) but is blocked in *Tg(UAS:NICD)* fish (B″). The blastema is swollen proximal to each fin radial (B″, arrow). (**C**) Mean length of the regenerate at 1, 3 and 6 dpa: regenerative outgrowth is significantly reduced in *Tg(UAS:NICD)* fish at 6 dpa; ****P*<0.001. (**D**) Immunohistochemistry against the Myc tag on the NICD transgene (Myc-NICD) in 5 dpa *Tg(UAS:NICD)* fin sections. Expression of Myc-NICD is mosaic in the blastema, in two or three cell layers beneath the epidermis (arrowheads) and in intraray blastema cells (arrow). (**E-F′**) Haematoxylin and Eosin stained fin sections (5 dpa): the blastema of regenerated *Tg(UAS:NICD)* fins is broader than wild-type blastema (E,F, horizontal bars; supplementary material Fig. S4C). Higher magnification views of wild-type fins reveal densely packed distal blastema cells (E″, arrowhead), the looser organization in the central proximal region and the strict alignment in the periphery (E″, arrowhead). In *Tg(UAS:NICD)* fins, cells are densely packed and disorganized in both blastema regions (arrowheads, F,F′). This structure is similar to the blastema (E′,F,F′). (**G-J**) *In situ* hybridization at 5 dpa. *her6* and *her15* transcription expands into the proximal blastema region in *Tg(UAS:NICD)* fins (arrowheads, G-J). (**K**) qPCR analysis after a 10-hour heat-shock series (62-72 hpa). **P*<0.05. Scale bars: 200 μm in B″;10 μm in F,F′,J.

epidermis (Fig. 4E″) and have been identified as re-differentiating osteoblasts (Knopf et al., 2011). This cellular organization was altered in *Tg(UAS:NICD)* fin sections (Fig. 4F). Densely packed and disorganized cells were observed in the entire regenerate (Fig. 4F,F′). Furthermore, instead of an alignment of prospective osteoblasts, we found a dense accumulation of disorganized cells in the equivalent region close to the epidermis (Fig. 4F,F′). Interestingly, the distinct blastema cell organization in *Tg(UAS:NICD)* fish (Fig. 4F,F′) coincided with the expression pattern of Myc-NICD (Fig. 4D).

Ectopic Notch activation in transgenic fish was confirmed by analysing *her6* and *her15* at 5 dpa. In wild-type fish, *her6* and *her15* expression was restricted to the distal region of regenerated fins (Fig. 4G,H) but expanded proximally in *Tg(UAS:NICD)* fish (Fig. 4I,J). To investigate whether expanded target gene expression is a direct response to Notch gain of function when regenerative outgrowth starts, we applied a series of short-term heat shocks restricted to the 10 hours (62-72 hpa) before tissue collection. qPCR detected significant upregulation of *her6* and *her15* in *Tg(UAS:NICD)* fins (Fig. 4K). Similar results were obtained when the heat-shocks were applied during outgrowth (98-110 hpa). *In situ* hybridization of *her6* and *her15* revealed a strong activation of Notch signalling (supplementary material Fig. S6A-D), suggesting that *her6* and *her15* are regulated by Notch throughout the fin regeneration process. As *lfng* expression is regulated by Notch (Morales et al., 2002), we crossed the *EGFP-lfng* reporter line (*ET33mi60A*) with *Tg(UAS:NICD)* fish and examined EGFP expression in response to heat-shock treatment. EGFP expression within the blastema was stronger in triple transgenic *Tg(hsp70l:Gal4); Tg(UAS:myc-notch1a-intra);ET33mi60A* fish than in *ET33mi60A* animals at 5 dpa (supplementary material Fig. S5D-E′).

Notch signalling regulates cellular proliferation and blastema marker gene expression

To investigate whether the impaired regenerative outgrowth and blastema swelling in the Notch gain-of-function fish is due to increased blastema cell proliferation, we examined BrdU

incorporation after the 10-hour heat-shock protocol. In wild-type regenerating fins, proliferation was detected in just 8% (±2%) of blastema cells (Fig. 5A,C). By contrast, in regenerating *Tg(UAS:NICD)* fins, 13%±1% of cells were proliferating (Fig. 5B,C). Many BrdU$^+$ cells were co-labelled with Myc-NICD, suggesting that Notch may directly promote cellular proliferation (supplementary material Fig. S6I).

Undifferentiated cells within the fin blastema express *msxb*, which is also required for proliferation in the regenerating fin (Akimenko et al., 1995; Nechiporuk and Keating, 2002; Thummel et al., 2006). At 5 dpa, wild-type fins expressed *msxb* weakly throughout the regenerated fin and more strongly distal in the blastema (Fig. 5D). *Tg(UAS:NICD)* fins showed a similar pattern throughout the blastema, but this was accompanied by strong *msxb* expression in peripheral cells (Fig. 5E), again coinciding with Myc-NICD expression (Fig. 4D).

We next investigated the effect of ectopic Notch signalling on the expression of *msxe* and *aldh1a2*. Compared with the distally restricted *msxe* expression in regenerating wild-type fins at 5 dpa (Fig. 5F), *Tg(UAS:NICD)* fins showed a marked expansion of *msxe* expression to the proximal blastema region, restricted to cells underlining the epidermis (Fig. 5G). Similarly expanded expression was detected for *aldh1a2* (Fig. 5H,I). Moreover, the expansion of aldh1a2$^+$ cells into proximal regions in *Tg(UAS:NICD)* fins predominantly occurs in PCNA$^+$ proliferating cells, whereas in wild-type fins both proteins are restricted to cells in the distal region at 5 dpa (supplementary material Fig. S6J,J′,K,K′). The overlap of *msxb*, *msxe* and *aldh1a2* expression in *Tg(UAS:NICD)* fins with regions expressing *Myc-NICD* suggests regulation of these blastema markers by Notch signalling. To confirm this, we applied the 10-hour (62-72 hpa) heat-shock series. qPCR revealed high levels of *msxb*, *msxe* and *aldh1a2* transcripts in *Tg(UAS:NICD)* fins compared with wild-type fins (Fig. 5J). *In situ* hybridization of *msxb* and *msxe* in fins after heat-shock during outgrowth (98-110 hpa) revealed that over-activation of Notch signalling also increased expression at later stages of regenerative outgrowth (supplementary material Fig. S6E-H). These results indicate that *msxb* and *msxe* expression, and the activation of RA may be Notch dependent.

Our data also suggest that Notch gain of function in the regenerating fin leads to the formation of an oversized blastema. To investigate whether generation of this enlarged blastema was reversible, we subjected *Tg(UAS:NICD)* fish to long-term heat-shock over 12 dpa, whereas wild-type and another group of *Tg(UAS:NICD)* fish were heat-shocked for the first 5 dpa and subsequently maintained at 28°C until 12 dpa. As before, wild-type fish showed no fin regeneration defect upon heat-shock treatment (supplementary material Fig. S7A), whereas regeneration was impaired in *Tg(UAS:NICD)* fish (supplementary material Fig. S7B,C). However, when heat-shock treatment was halted at 5 dpa in transgenic fish, fins recovered the regeneration seen in wild-type fish (supplementary material Fig. S7A′,B′). By contrast, when heat-shock treatment was continued up to 12 dpa, the blastema retained the altered form seen at 5 dpa (supplementary material Fig. S7C′). Notch signalling therefore may be transiently activated in blastema cells during fin regeneration.

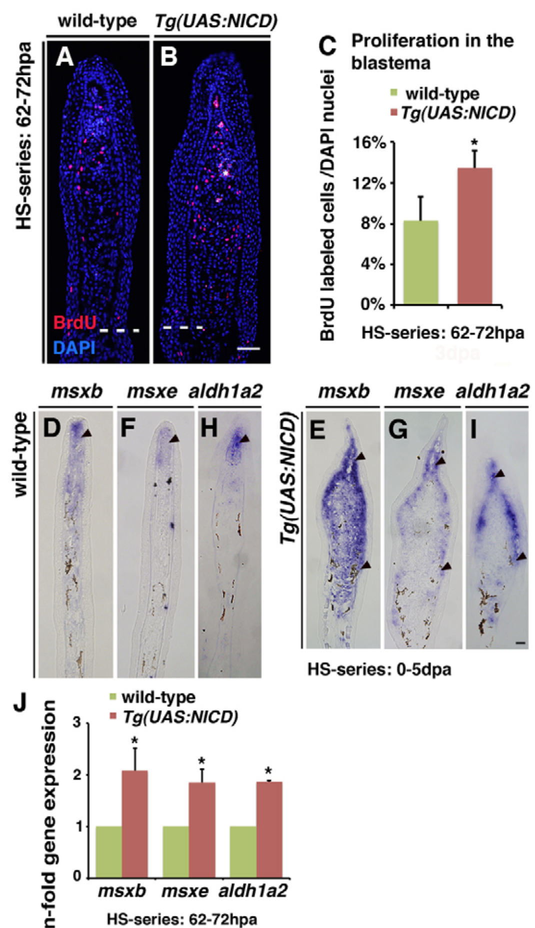

Fig. 5. Notch gain of function increases proliferation and is accompanied by an expansion of distal blastema marker expression into proximal regions. (**A-C**) BrdU-stained fin sections after a 10-hour heat-shock series (62-72 hpa) and quantification of the percentage of BrdU-labelled DAPI nuclei in the blastema: more cells incorporate BrdU in *Tg(UAS:NICD)* fins (B) than in wild-type fins (A). Asterisk in B indicates autofluorescent blood vessels. *$P<0.05$. (**D-I**) *msxb*, *msxe* and *aldh1a2 in situ* hybridization (5 dpa). Arrowheads indicate expression of *msxb*, *msxe* and *aldh1a2*. (**J**) qPCR analysis after a 10-hour heat-shock series (62-72 hpa): *msxb*, *msxe* and *aldh1a2* transcripts are increased in *Tg(UAS:NICD)* fins. *$P<0.05$. Scale bars: 10 μm in B,I.

Notch gain of function prevents fin bone regeneration

To investigate why Notch gain of function impaired regenerative outgrowth even though proliferation was increased, we assessed the progress of fin radial regeneration by examining calcification at 5dpa. Wild-type fins showed regular regenerated fin rays proximal to each stump (Fig. 6A). By contrast, *Tg(UAS:NICD)* fins showed much weaker bone calcification and malformations (Fig. 6B,C).

Bone regeneration in the fin proceeds via de-differentiation of stump cells, which proliferate and re-differentiate into osteoblasts. During wild-type regeneration, transcription factor 7 (tcf7), a marker of early osteoblasts during zebrafish head development (Li et al., 2009), was expressed in cells aligned with the epidermis in the distal fin region (Fig. 6D), suggesting that these cells are de-differentiated or early differentiating osteoblasts. The proximal regions of the fin did not express *tcf7*, suggesting its downregulation during osteogenesis in the regenerating fin. By contrast, *Tg(UAS:NICD)* fins showed expansion of *tcf7* expression up to the fin stump (Fig. 6E), indicating the presence of de-differentiated and early differentiating osteoblasts throughout the fin regenerate. To determine whether the progression of osteoblast differentiation was blocked in transgenic fins, we examined

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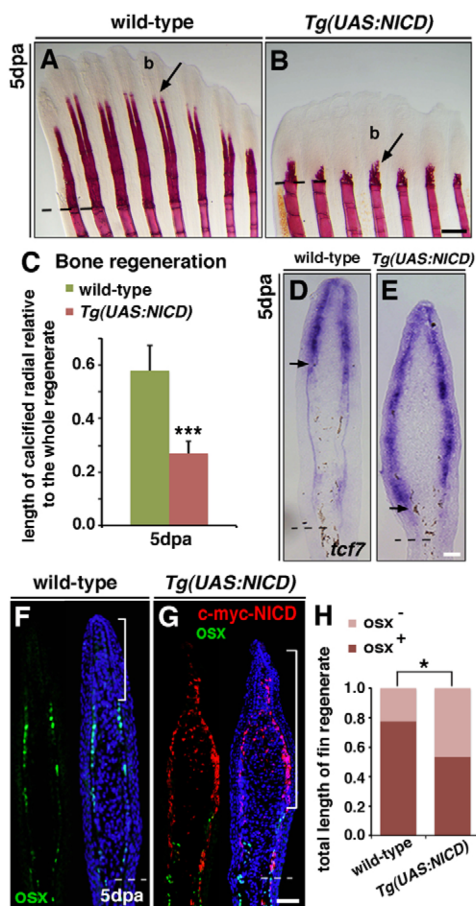

Fig. 6. Notch signalling inhibits osteoblast differentiation and prevents bone regeneration. (**A**,**B**) Alizarin Red staining of fins at 5 dpa reveals calcified bony rays in a wild-type fin regenerate (A, arrow). Fin rays are poorly formed in *Tg(UAS:NICD)* fins, whereas the blastema (b) is expanded (B, arrow). (**C**) Length of regenerated calcified radials of *Tg(UAS:NICD)* fins, relative to wild-type fins. ****P*<0.001. (**D**,**E**) *In situ* hybridization at 5 dpa. Wild-type fins express *tcf7* in peripheral cells in the distal region (D, arrow), whereas transcription is expanded proximally in *Tg(UAS:NICD)* fins (E, arrow). (**F**,**G**) Immunostaining for Osx and Myc-NICD. In wild-type fins, Osx^+ cells (green) are present in the proximal fin region but scarce in the distal region (F, bracket). Osx^+ cells are less numerous in regenerating *Tg(UAS:NICD)* fins and more proximally limited. The region of high Myc-NICD expression does not contain Osx^+ cells (G, bracket). (**H**) Relative length of the proximal region of the fin regenerate, which contains Osx^+ cells, and the distal region (brackets, F,G), which does not ($Osx^–$). The distal, $Osx^–$ region is larger in *Tg(UAS:NICD)* fins than in wild-type fins. **P*<0.05. Scale bars: 200 μm in B; 10 μm in E,G. Broken lines indicate the amputation plane.

expression of osterix (Osx), an intermediate-differentiation osteoblast marker (Knopf et al., 2011; Sousa et al., 2011; Singh et al., 2012). In the 5 dpa wild-type fin, Osx was expressed in cells aligned with the epidermis, within the stump and proximal to the amputation plane, but not in the distal region of the regenerate (Fig. 6F). By contrast, *Tg(UAS:NICD)* fins contained only a few Osx-positive cells close to the amputation plane (Fig. 6G).

To analyse the differentiation state of regenerating fins subjected to long-term heat-shock, we measured the Osx-positive and Osx-negative areas within the fin regenerate at 5 dpa. In wild-type fish, the Osx-negative undifferentiated distal blastema region constituted 23% (±7%) of the regenerating fin. By contrast, in *Tg(UAS:NICD)* fish up to 47% (±14%) of the fin regenerate lacked Osx-expressing cells (Fig. 6H), indicating blockade of differentiation in this region. This suggests that ectopic Notch activation in the regenerating fin does not only trigger expansion of dedifferentiated blastema cells but also prevents the progression of osteoblast differentiation. When regenerating fins were subjected to short-term heat-shock (98-110 hpa), we found no significant difference in the numbers of osteoblasts (supplementary material Fig. S6L,M), indicating that Notch does not directly regulate *osx* expression.

DISCUSSION

Our results show that Notch signalling is activated early during fin regeneration and plays fundamental roles in the regulation of blastema proliferation and differentiation. The first signs of Notch activity are observed at 2 dpa in blastema cells of the regenerating fin, suggesting a requirement for Notch from early stages of regeneration, when blastema cells appear distal to the amputation plane. As regeneration proceeds, Notch activity is restricted to the blastema, where the undifferentiated progenitor cell populations of the regenerating fin are located. This region also coincides with the area of highest proliferation throughout regeneration. Indeed, our signalling inhibition experiments indicate that Notch abrogation does not affect blastema formation but instead reduces blastema cell proliferation. However, constitutive Notch activation increases proliferation, while also expanding blastema markers and inhibiting differentiation. These observations are consistent with a role of Notch as a promoter of cell proliferation and an inhibitor of the terminal differentiation of progenitor cells in the blastema. The function of Notch in stem/progenitor cell biology is context dependent, and the consequences of its activation can vary from maintenance or expansion of stem cells to the promotion of stem cell differentiation (reviewed by Liu et al., 2010). In the embryonic and adult nervous system (Chitnis et al., 1995; Henrique et al., 1995; de la Pompa et al., 1997; Imayoshi et al., 2010), skeletal muscle (Mourikis et al., 2012; Wen et al., 2012) and intestine (Fre et al., 2005), Notch activation maintains progenitor cells in an undifferentiated state; in skeletal muscle it also sustains progenitor cell proliferation, similar to our findings in the zebrafish fin.

An important issue is what position Notch holds in the signalling network regulating fin regeneration. RA controls blastema formation, proliferation and survival (Blum and Begemann, 2012). Our findings suggest that Notch acts upstream of RA, as ectopic Notch activation induces *aldh1a2* expression. Unlike *aldh1a2*, Notch is not activated within the fin stump upon amputation, but at later stages appears distal to the amputation plane, where it remains active. This suggests that blastema cell proliferation may be regulated by more than one mechanism. One possibility is that amputation triggers an initial RA signalling response that regulates early proliferation of dedifferentiated osteoblasts and fibroblasts within the stump to form the blastema. Later, in the regenerative outgrowth phase, the proliferation of distal blastema cells is regulated by Notch, with RA signalling being one of its effectors. Our data show that as regeneration proceeds, a pool of fast-cycling cells persists at the distal tip of the fin, characterized by expression of *msxe* and *aldh1a2* and strong *msxb* expression. RA maintains this blastema region by enhancing expression of the pro-survival gene *bcl2* (Blum and Begemann, 2012), whereas Msx homologs are important regulators of de-differentiation in several regenerating structures, such as the mouse digit tip (Han et al., 2003) and *Xenopus* and *Amphioxus* tails (Barker and Beck, 2009; Somorjai et al., 2012). Our data demonstrate that constitutive Notch activation expands this $msxe^+$, $aldh1a2^+$ region, a phenotype that

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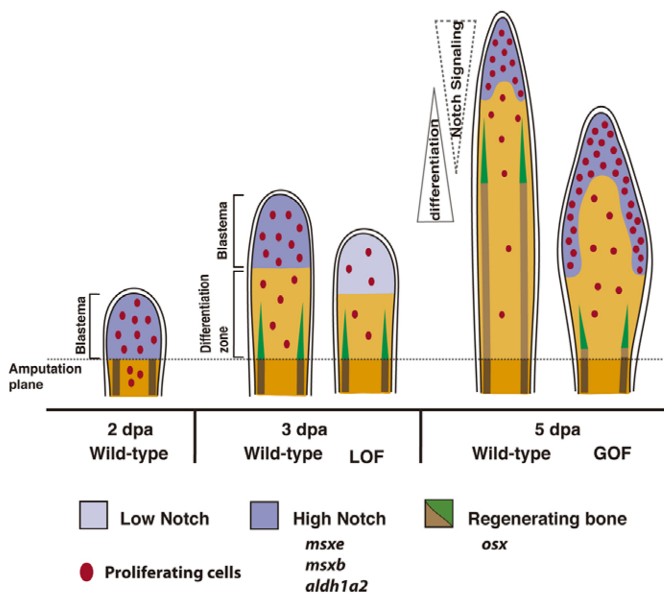

Fig. 7. Model of Notch function during fin regeneration. The role of Notch in fin regeneration is to maintain blastema cells in a proliferative and undifferentiated state so that the different cell lineages arise progressively. At 2 dpa, Notch is activated throughout the blastema and is restricted to the distal blastema at 3 dpa. In this region, *msxb*, *msxe* and *aldh1a2* are expressed and proliferation is higher. Notch loss-of-function decreases blastemal proliferation. At 5 dpa, Notch activation persists in the distal region of the blastema, whereas in the proximal region it is downregulated and cells progressively differentiate to originate the different fin lineages, including the osteoblasts. Notch gain of function leads to an accumulation of proliferating, *msxb*-, *msxe*- and *aldh1a2*-expressing progenitor-like cells, and bone regeneration is inhibited.

is reversed when Notch induction is stopped, indicating that Notch maintains an undifferentiated, proliferative state of blastema cells by promoting *msxb*, *msxe* and *aldh1a2* expression.

Various studies show that osteoblasts undergo de-differentiation and proliferation during zebrafish fin regeneration (Knopf et al., 2011; Sousa et al., 2011). Osteoblasts migrate distally to the amputation plane and re-differentiate to rebuild the lost bone. Fibroblasts may be an additional source of bone tissue in the regenerating fin (Singh et al., 2012). Lineage restriction has been proposed to occur in all fin cell lineages (Tu and Johnson, 2011); however, to date, the lack of proper lineage markers has precluded confirmation of this. Our data suggest that high Notch signalling prevents the progression of osteoblast differentiation, as indicated by the low number of osx-expressing cells in *Tg(UAS:NICD)* fins. By contrast, expression of *tcf7*, a marker of the early stages of osteoblast differentiation (Li et al., 2009), is expanded throughout the blastema in transgenic fins.

Fig. 7 shows a model of Notch activity during fin regeneration. At 2 dpa, Notch signalling is present throughout the blastema but becomes restricted to the distal fin region as regeneration proceeds. Proximal cells that differentiate then downregulate Notch signalling (3 dpa). However, maintenance of Notch activation throughout the fin regenerate in our gain-of-function model leads to the accumulation of progenitor-like cells, which are characterized by *msxe*, *msxb* and *ald1a2* expression (5 dpa). This has several consequences: (1) the marked thickening of the blastema; (2) the disorganized appearance of blastema cells in the proximal region; (3) the blockade of differentiation, which prevents bone regeneration; and (4) the inhibition of regenerative outgrowth. We propose that in the wild-type regenerating fin Notch promotes cell proliferation and maintains the undifferentiated state of blastema cells. Notch activity is complementary to a proximodistal gradient of cellular differentiation, so that the highest Notch activity occurs in the blastema, where de-differentiated osteoblasts and fibroblasts are located. We have shown that this model holds true for the osteoblast lineage, as early differentiation markers are expanded from the distal blastema to the differentiation zone in the Notch gain-of-function experiments. A role for Notch in the regulation of osteoblast differentiation in the regenerating zebrafish fin is consistent with results from mice supporting a role for Notch in bone homeostasis. Loss-of-function experiments in mice reveal that Notch maintains a pool of mesenchymal progenitor cells and suppresses osteoblast differentiation (Hilton et al., 2008), whereas gain-of-function experiments indicate that Notch stimulates the proliferation of immature osteoblasts (Engin et al., 2008). Further studies will reveal whether this also holds true for the other main lineage in the blastema, the fibroblasts (Tu and Johnson, 2011). Our findings contribute to a more detailed understanding of the molecular mechanisms underlying the development and maintenance of the regeneration blastema, a key process in epimorphic regeneration. It will be of interest in the future to extend the study of Notch function to examine its role in bone repair and regeneration, using mouse and zebrafish models.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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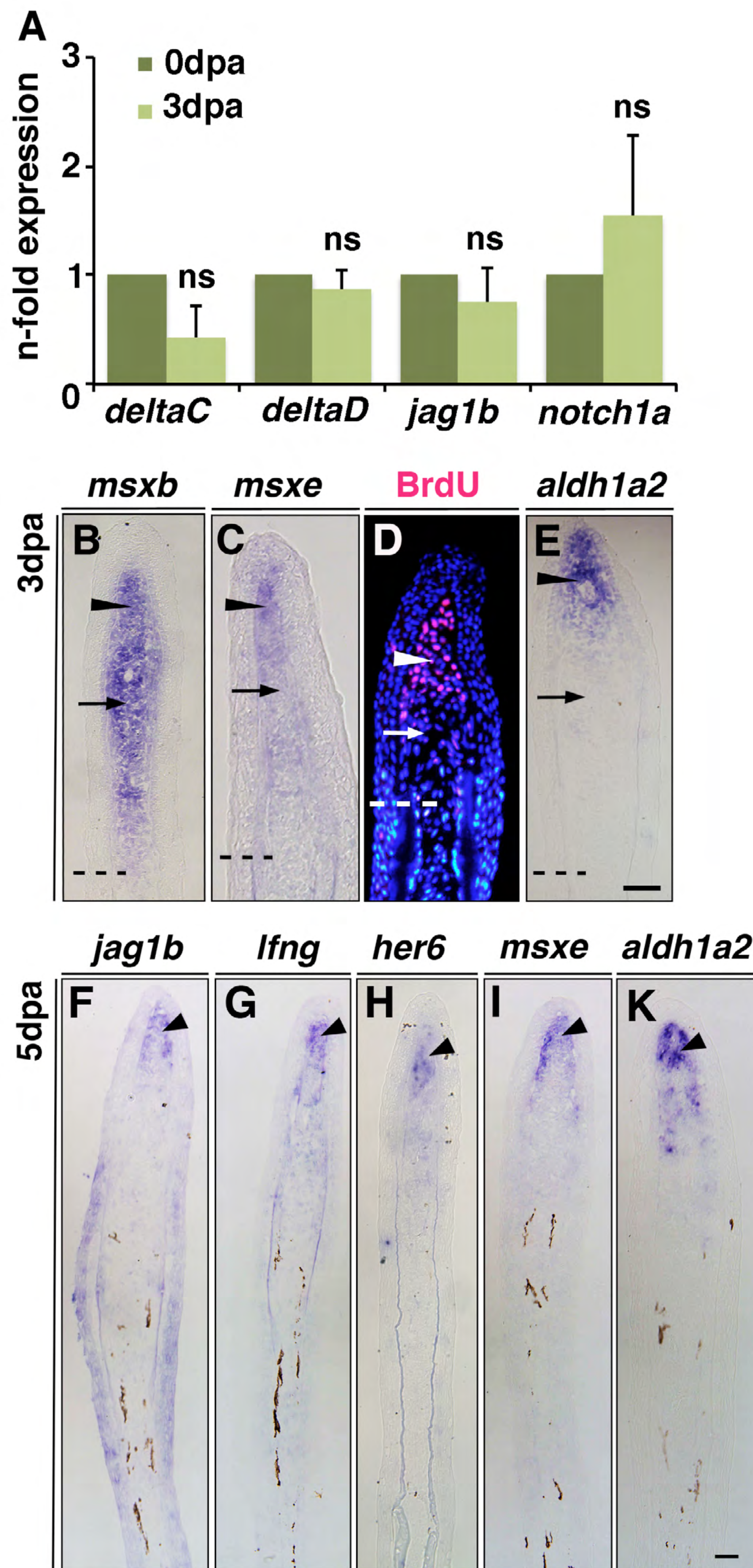
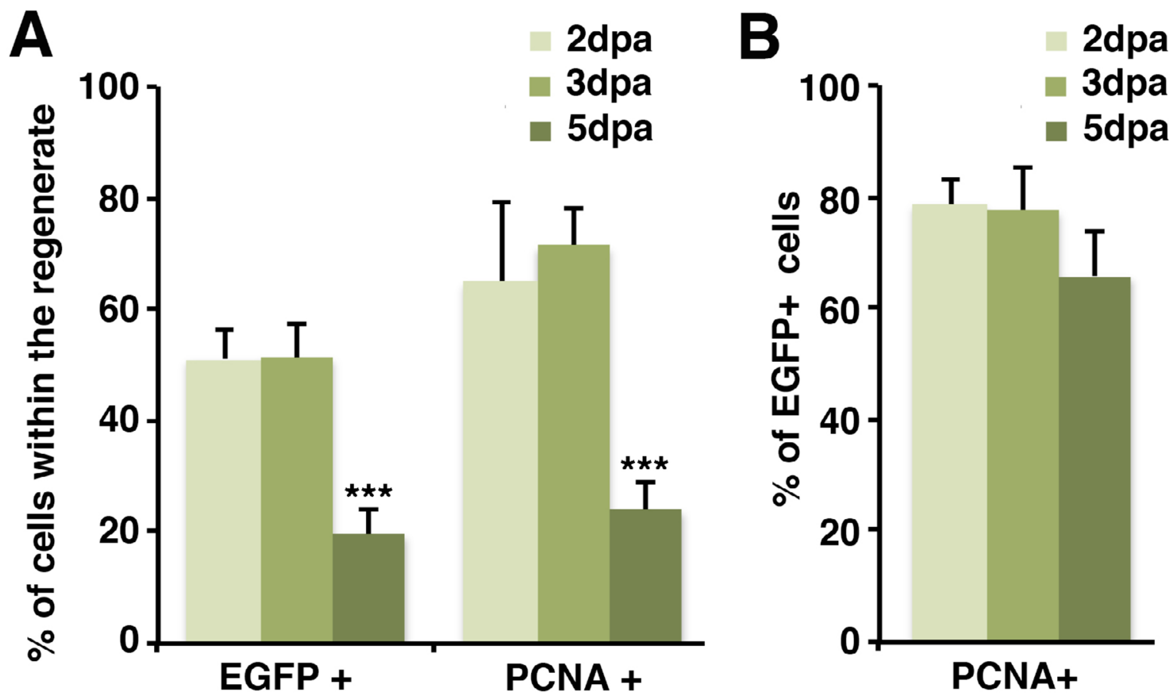


Fig. S1. Notch signalling is active in the blastema. (**A**) qPCR determination of *deltaC*, *deltaD*, *jag1b* and *notch1a* transcripts in regenerating fins at 3 dpa relative to non-amputated fins (0 dpa). (**B**,**C**,**E**) *In situ* hybridization on 3 dpa fin sections. *msxb* is expressed in the whole blastema (B) whereas *msxe* (C) and *aldh1a2* (E) expression is restricted to the distal region (arrowheads) but absent in the proximal region (arrows). (**D**) BrdU-labelled cells are densely packed in the distal region of the blastema (arrowhead), but dispersed proximally (arrow). (**F**-**K**) *In situ* hybridization on 5 dpa fin sections. *jag1b* (F), *lfng* (G) and *her6* (H) are expressed in the distal region of the blastema (arrowheads) but not in the more proximal differentiation zone, similar to *msxe* (I) and *aldh1a2* (K). Scale bar: 10 μm in E,K. Broken lines mark the amputation plane.



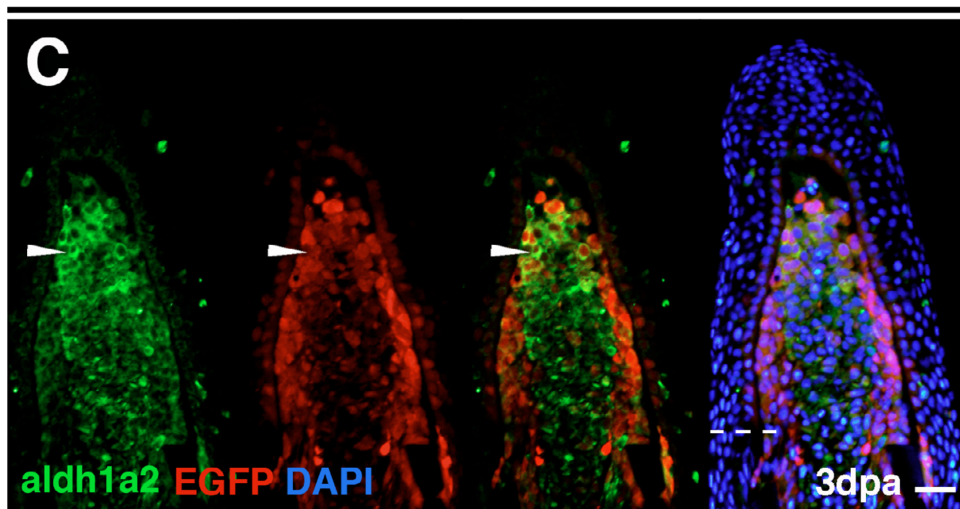


Fig. S2. Lunatic-fringe-mediated Notch signalling is activated in proliferating, *aldh1a2*-expressing cells. (**A**) Mean percentage of EGFP-expressing cells in the regenerate of *ET33-mi60A* fin sections at 2 dpa, 3 dpa and 5 dpa. ****P*<0.05. (**B**) Mean percentage of $EGFP^+$ blastema cells co-labelled for PCNA in fin sections at 2 dpa, 3 dpa and 5 dpa. (**C**) Representative immunhistochemistry for EGFP and *aldh1a2* in a 3 dpa fin section. Cells are double positive in the distal region of the blastema (arrowhead). Scale bars: 100 µm in C. Broken line marks the amputation plane.

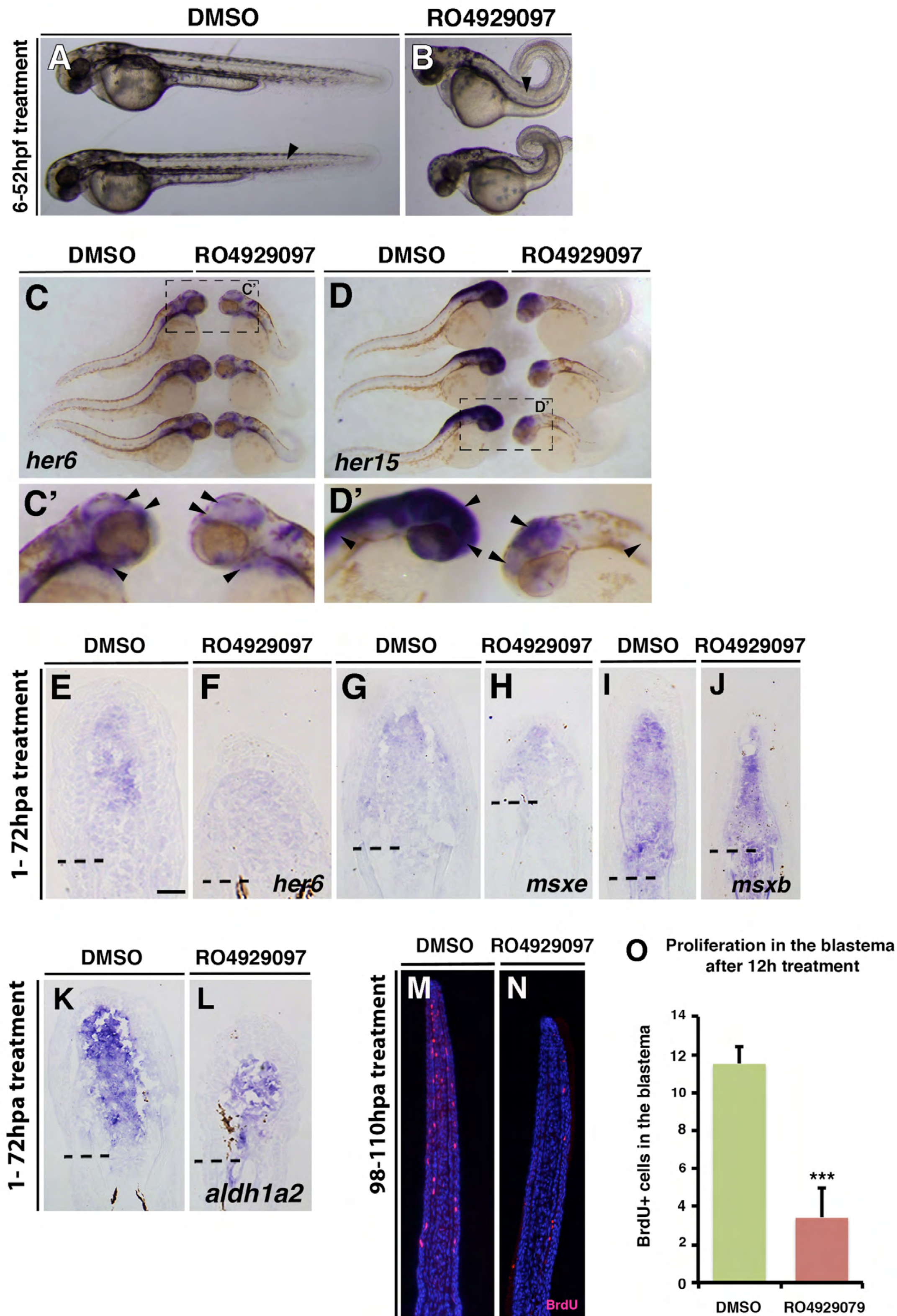


Fig. S3. RO929097 treatment leads to Notch signalling knockdown in embryos and regenerating fins and reduces proliferation. (**A-D**) Embryos treated with either DMSO or 10 μM RO929097 from 6 to 52 hpf. RO929097-treated embryos show defects in somitogenesis (arrow) and a looped tail (B). (C,D) Whole-mount *in situ* hybridization: *her6* gene expression is reduced in the brain and the gill mesenchyme of RO929097-treated embryos (arrowheads). *her15* gene expression is reduced in the brain and spinal cord of RO929097-treated embryos (arrowheads). (**E-L**) In situ on fin sections after 72 hours of DMSO or 10 μM RO929097 treatment. *her6* gene expression is reduced in RO929097-treated fins (E,F), but *msxe* (H), msxb (J) and *aldh1a2* (L) expression seems to be unchanged in RO929097-treated compared with DMSO-treated fins (G,I,K). (**M-O**) Anti-BrdU-stained fin sections and quantification of BrdU$^+$ cells within the distal most 300 μm of the mesenchyme. RO4929097-treated fins (*n*=4) (N) exhibit fewer BrdU-labelled cells than DMSO-treated fins (M) (*n*=4). ****P*<0.001.

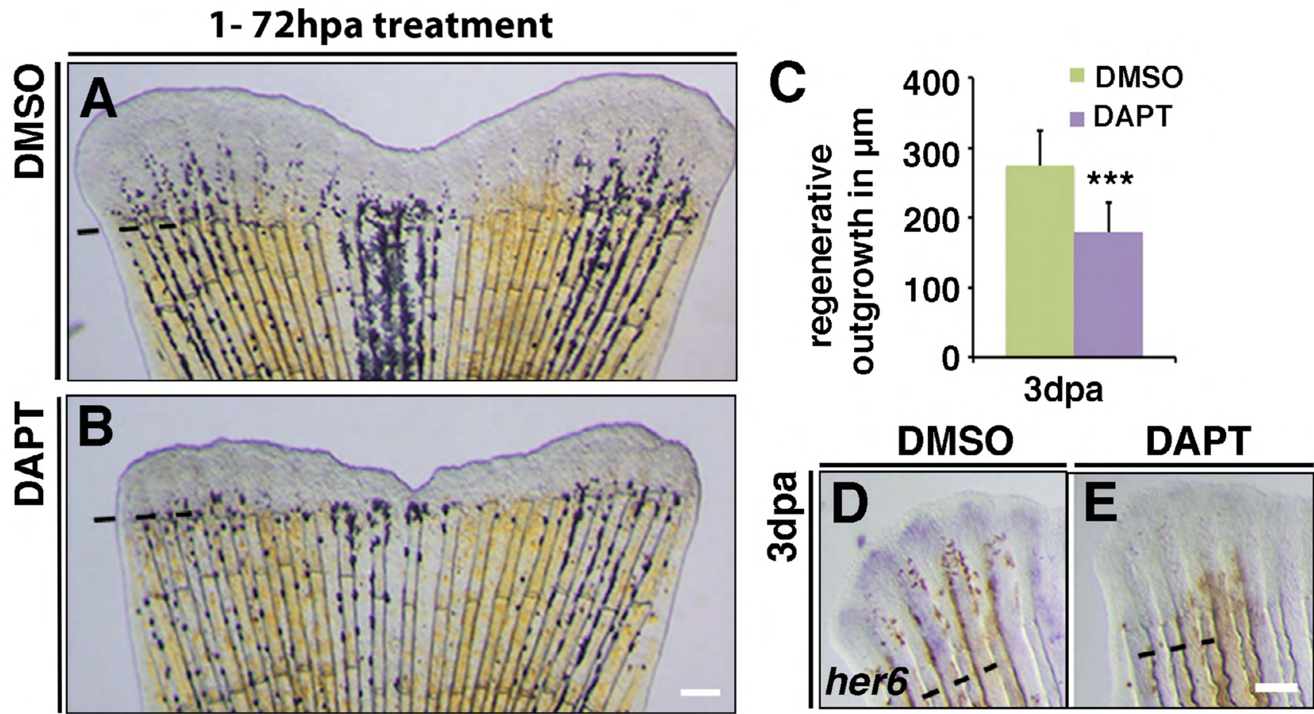


Fig. S4. DAPT treatment leads to Notch signalling downregulation in regenerating fins and reduces regenerative outgrowth. (**A**,**B**) Juvenile fish treated with 50 µM DMSO or DAPT for 3 days. Fin regeneration is impaired by DAPT treatment. (**C**) Mean length of fin regenerates; DAPT treatment (n=10) decreased regenerate length compared with DMSO-treated fins (n=6); ***P<0.005. (**D**,**E**) *her6* whole-mount *in situ* hybridization reveals Notch activity in DMSO-treated fins (D) but not in DAPT-treated fins (E).

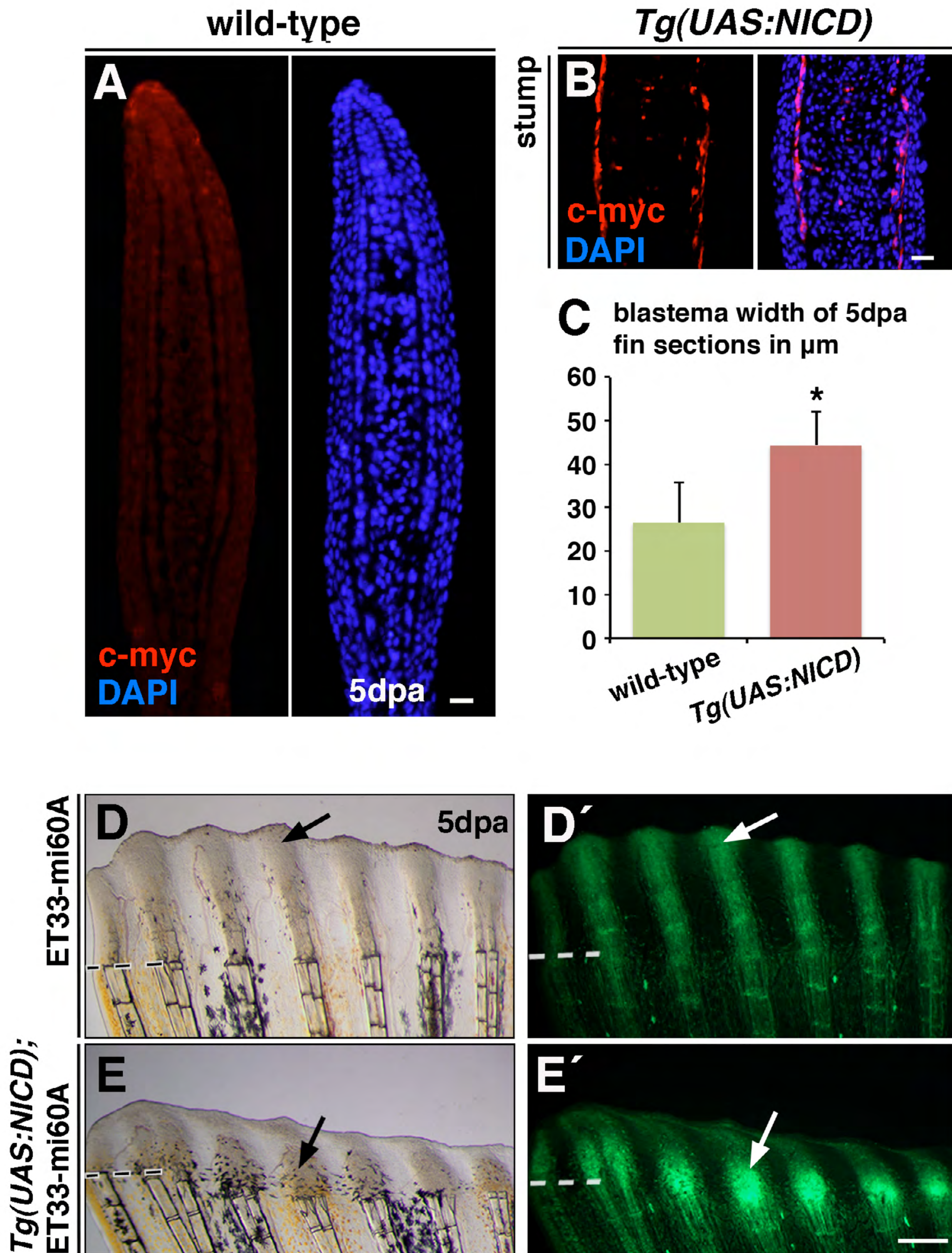


Fig. S5. NICD overexpression leads to increased blastema width and increases EGFP expression in *Tg(UAS:NICD);Et33-mi60A* fish. (**A**,**B**) Immunohistochemistry for Myc does not label wild-type fin sections (A), whereas Myc-NICD expression is detected in peripheral cells, most likely osteoblasts, and cells within the ray in the fin stump of *Tg(UAS:NICD)* fish (B). (**C**) Mean blastema width of wild-type and *Tg(UAS:NICD)* fin sections at 5 dpa. ***P<0.05. (**D-E′**) *Tg(UAS:NICD)* fish were crossed with *Et33-mi60A* fish and the *Tg(UAS:NICD);Tg Et33-mi60A* fish were exposed to a series of heat shocks during 5 days of regeneration. EGFP expression is increased in *Tg(UAS:NICD);ET33-mi60A* fish at 5 dpa (E′) compared with *ET33-mi60A* fish. Scale bars: 10 µm. Broken lines mark the amputation plane.

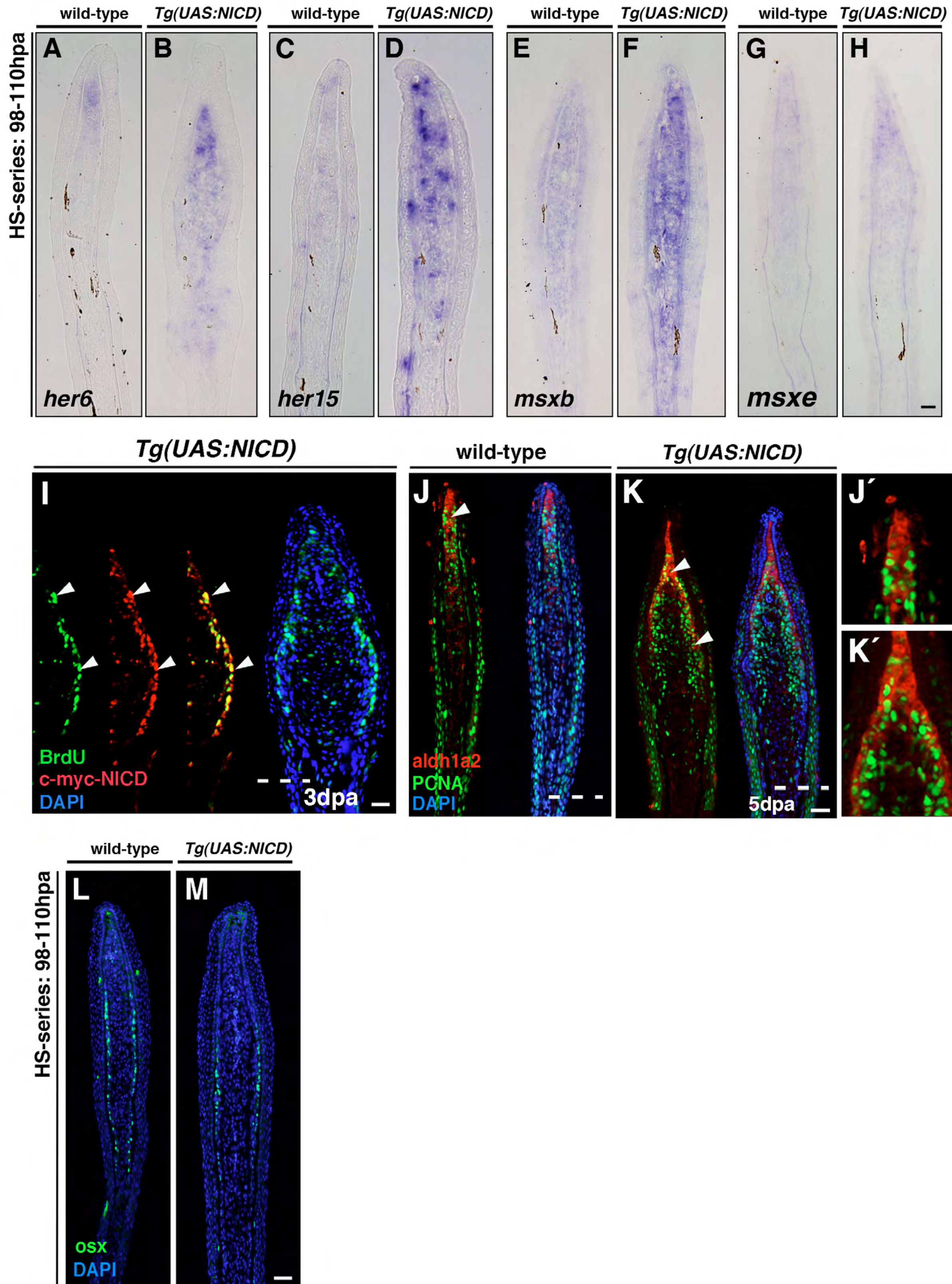


Fig. S6. Notch signalling pathway overactivation leads to increased blastema marker expression and higher proliferation. (**A-H**) *In situ* hybridization of fins after a 12-hour heat-shock period (98-110 hpa). *her6*, *her15*, *msxb* and *msxe* expression is present in the distal region of the blastema in wild-type fins (A,C,E,G). Gene expression is stronger and expanded proximally in *Tg(UAS:NICD)* fish (B,D,F,H). (**I**) Double immunohistochemistry reveals co-labeling of Myc-NICD and BrdU in many cells (arrowheads). (**J-K′**) Immunohistochemistry of PCNA and *aldh1a2* on 5 dpa fin sections. Double labelled cells (J′,K′) are restricted to the distal region of the wild-type fin (J, arrowhead) but is expanded proximally in *Tg(UAS:NICD)* fins at 5 dpa (K, arrowheads). (**L**,**M**) Immunohistochemistry against Osx of fins after a 12-hour heat-shock period (98-110 hpa). Scale bars: 10 μm in H,I,K,M. Dashed lines indicate the amputation plane.

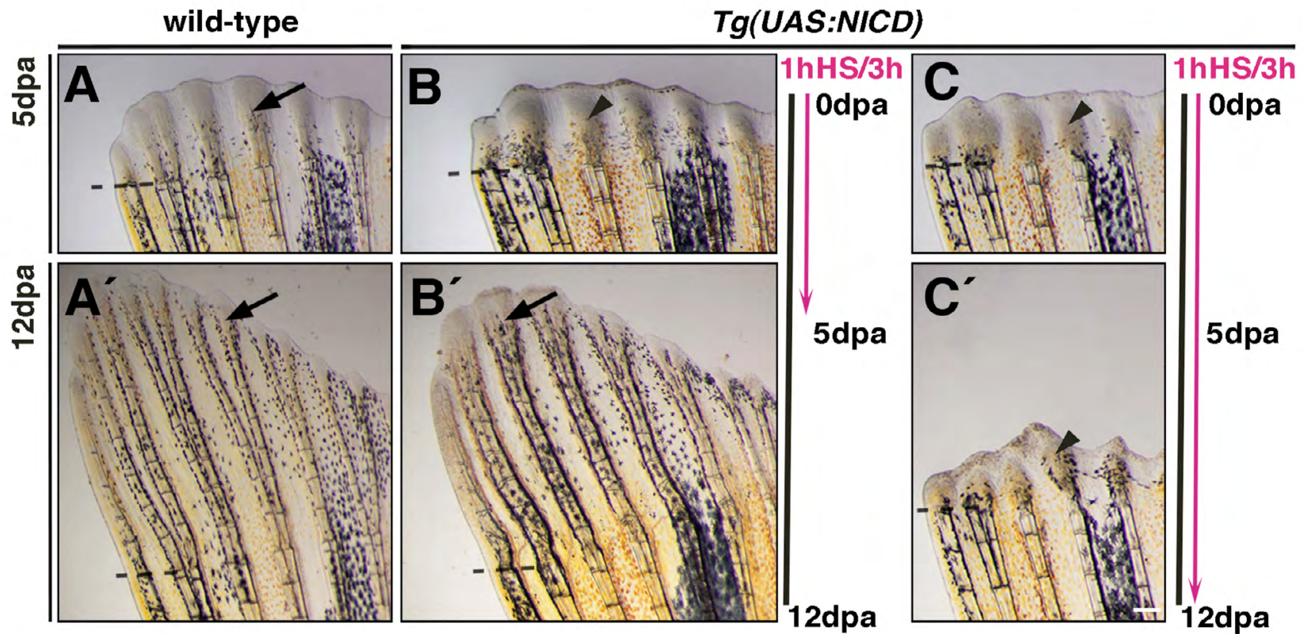


Fig. S7. NICD-induced blastema expansion is reversible. (A-C′) Heat-shock cycles were applied over 5 days of regeneration (A-B′) or 12 days (C,C′). Black line indicates time of regeneration. Pink line indicates time of heat-shock treatment (1 hour of heat-shock every 3 hours for 5 or 12 days). Arrows indicate regenerating radials. Arrowheads indicate expanded blastemas. Regeneration is inhibited in *Tg(UAS:NICD)* (B,C) fins but not in wild-type fins (A). Regeneration reverts to normal when heat-shock treatment is stopped (A′,B′, arrows) but the blastema remains close to the amputation plane (arrowhead) when heat-shocked continued up to 12 days (C′). Scale bars: 100 μm. Dashed lines mark the amputation plane.

Table S1. ISH probes

Probe	**Reference**
msxb	(Akimenko et al., 1995)
msxe	(Akimenko et al., 1995)
tcf7	(Li et al., 2009)
aldh1a2	(Grandel et al., 2002)
notch1b	(Westin and Lardelli, 1997)
jagged1b	(Zuniga et al., 2010)
her15	(Shankaran et al., 2007)
lunatic fringe	(Prince et al., 2001)
her6	This report: Forward, 5'-CATCATTGCCGCACCA-3'; Reverse, 5'-TGTGTTTAGGGCAGCGGTCAT-3'

Table S2. qPCR primers

Gene	Forward (5′-3′)	Reverse (5′-3′)
deltaC	CGCAGAAACCTCTGACCAGT	CAGTCCTCACTGATAGCGAGTC
deltaD	GTTCACCAACCCCATTCCTT	TGTGCAGCGCTTCAATAATC
jag1b	ACATGCGAGTGTCAAGAAGGT	CATGGGTTACTTTCACAATCGTT
notch1a	TGTGAATGCACCCCAGGT	GACGCACACTCGTTGATGTC
notch1b	GGGCACCTGCGTACAGAA	CAAATTCCTGCCGACCTG
lfng	TCTGTTGAGGAGGACCCATC	GCACCAAGGAGTGTCTGGAT
her6	GGCTTCGGAACACAGAAAGT	TGACCCAAGCTTTCGTTGa
her15	TCGCTCTGCTCAGAAACA	ACCACTGGCTTTCGAAT
msxb	GGTCAAACTCTTCATCTTTCACATC	TCTTGTGCTTGCGTAAGGTG
msxe	GAGCGGAGCACATGGGTA	CCGGTTGGTTTTGTGTTTTC
aldh1a2	GGGGGAAGCTACTGTTCAAAT	TCCAGAGACTCCAGGGTAGC