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

Juliane Münch, Alvaro González-Rajal and José Luis de la Pompa*

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 epitherms forms and seals the wound. Epithelial cells form a multilayered epidermis, which is required for blastema formation and proliferation (Chabrais 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, stumps 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...
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/CFB1/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(hsp70I:Gal4) expressing Tg(UAS:myc-Notch1a-intraγ-secretase inhibitor DAPT (565770, Calbiochem) or control DMSO.

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), Aldha1a2 (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 (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,J-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 TCF 2500 SP-E confocal microscope. For cell number analysis, we counted DAPIl and PCNAl, EGFPl or BrdUl cells within the regenerated tissue and calculated the total amount of labelled cells per DAPIl nuclei using Adobe Photoshop CS5.1. To analyse Osxl-stained fin sections, we used ImageJ64 to measure both the Osxl proximal fin region and the Osxl 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 ef1al 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), rbpjk (Sieger et al., 2003), Ifng (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 (dL2dlL1)/(vL2vL1), 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 dp, 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 dp, when the epidermis covers the wound but no blastema cells are visible distal to the amputation
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. 1J). 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...
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-pentfluoropropyl)-malonamide(1), 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 aldhl2 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), rhpgx (Sieger et al., 2003) and lfnl (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, lfnl or rhpgx 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
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-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
epidermis (Fig. 4E″) and have been identified as re-differentiating osteoblasts (Knopf et al., 2011). This cellular organization was altered in Tg(UAS:NICD) fins 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 lfn gene expression is regulated by Notch (Morales et al., 2002), we crossed the EGFP-lfn 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...
To investigate whether generation of this enlarged blastema was Notch dependent.

hybridization of fins compared with wild-type fins (Fig. 5J). Shock treatment was continued up to 12 dpa, the blastema retained (supplementary material Fig. S7A), whereas regeneration was fish showed no fin regeneration defect upon heat-shock treatment subsequently maintained at 28°C until 12 dpa. As before, wild-type fish, fins recovered the regeneration seen in wild-type (Fig. 5F). The overlap of msxb, msex and aldha2 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, msex and aldha2 transcripts in Tg(UAS:NICD) fins compared with wild-type fins (Fig. 5I). In situ hybridization of msxb and msex 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 msex 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.

**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 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 aldha2. 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 aldha2 (Fig. 5H). Moreover, the expansion of aldha2+ 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. S6I,J,K,K). The overlap of msxb, msex and aldha2 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, msex and aldha2 transcripts in Tg(UAS:NICD) fins compared with wild-type fins (Fig. 5I). In situ hybridization of msxb and msex 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 msex expression, and the activation of RA may be Notch dependent.

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**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
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) fins 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 otx 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 bc12 (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...
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.

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 aldl1a2 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.

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References

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