Cell Reports
Article

Cell PRESS
Open ACCESS

Wnt/β-Catenin Signaling Defines Organizing Centers that Orchestrate Growth and Differentiation of the Regenerating Zebrafish Caudal Fin

Daniel Wehner,[1] Wiebke Cizelsky,[1] Mohankrishna Dalvoy Vasudevaro,[1] Günes Özhan,[2] Christa Haase,[2] Birgit Kagermeier-Schenk,[2] Alexander Röder,[2] Richard I. Dorsky,[3] Enrico Moro,[4] Francesco Argenton,[5] Michael Kühl,[1] and Gilbert Weidinger[1,*]

[1]Institute for Biochemistry and Molecular Biology, Ulm University, Albert-Einstein-Allee 11, 89081 Ulm, Germany
[2]Biotechnology Center, Technische Universität Dresden, Tatzberg 47-49, 01307 Dresden, Germany
[3]Department of Neurobiology and Anatomy, University of Utah, Salt Lake City, UT 84112, USA
[4]Department of Molecular Medicine, University of Padova, 35121 Padova, Italy
[5]Department of Biology, University of Padova, 35121 Padova, Italy
*Correspondence: gilbert.weidinger@uni-ulm.de
http://dx.doi.org/10.1016/j.celrep.2013.12.036

SUMMARY

Zebrafish regenerate their fins via the formation of a population of progenitor cells, the blastema. Wnt/β-catenin signaling is essential for blastemal cell proliferation and patterning of the overlying epidermis. Yet, we find that β-catenin signaling is neither active in the epidermis nor the majority of the proliferative blastemal cells. Rather, tissue-specific pathway interference indicates that Wnt signaling in the non-proliferative distal blastema is required for cell proliferation in the proximal blastema, and signaling in cells lining the osteoblasts directs osteoblast differentiation. Thus, Wnt signaling regulates epidermal patterning, blastemal cell proliferation, and osteoblast maturation indirectly via secondary signals. Gene expression profiling, chromatin immunoprecipitation, and functional rescue experiments suggest that Wnt/β-catenin signaling acts through Fgf and Bmp signaling to control epidermal patterning, whereas retinoic acid and Hedgehog signals mediate its effects on blastemal cell proliferation. We propose that Wnt signaling orchestrates fin regeneration by defining organizing centers that instruct cellular behaviors of adjacent tissues.

INTRODUCTION

The capacity to regenerate organs or appendages after injury varies widely between species. Although adult mammals have limited regenerative potential, urodele amphibians and teleosts like the zebrafish (*Danio rerio*) can fully regenerate many internal organs and appendages (Azevedo et al., 2011; Stoick-Cooper et al., 2007a). The zebrafish caudal fin has emerged as a highly successful model for studying basic mechanisms of tissue regeneration. Following partial amputation, the fin, which consists of bony rays and soft interray tissue, regenerates very robustly through establishment of blastemas—populations of lineage-restricted mesenchymal progenitor cells that form via dedifferentiation of mature stump cells—distally to each fin ray (Knopf et al., 2011; Tu and Johnson, 2011) (Figure 1A). At 28°C, blastema formation is completed within 48 hr postamputation (hpa) succeeding an initial wound healing process in which a multilayered wound epidermis is formed. Following blastema formation the regenerative outgrowth phase is initiated leading to restoration of the fin within 3 weeks.

Although a number of signaling pathways including Activin (Jazwinska et al., 2007), Bmp (Smith et al., 2006), Fgf (Poss et al., 2000), Hh (Quint et al., 2002), Igf (Chablais and Jazwinska, 2010), Notch (Grotek et al., 2013; Münch et al., 2013), retinoic acid (RA) (Blum and Begemann, 2012), and Wnt/β-catenin (Kawakami et al., 2006; Stoick-Cooper et al., 2007b) have been shown to be required for fin regeneration, our understanding of their tissue-specific roles or how these pathways interact to regulate distally oriented growth during fin regeneration is limited.

Wnt ligands can trigger several signaling pathways, of which the β-catenin-dependent pathway is the best characterized. Ligand binding to Frizzled receptors and Lrp5/Lrp6 coreceptors results in stabilization and accumulation of cytoplasmic β-catenin, which translocates to the nucleus and modifies target gene transcription together with transcription factors of the Tcf/Lef family (MacDonald et al., 2009). It is well documented that the Wnt/β-catenin pathway directly regulates proliferation and maintenance of several stem or progenitor cell types during development and tissue homeostasis (Reya and Clevers, 2005). Importantly, Wnt/β-catenin signaling also plays critical roles in most naturally regenerating systems studied to date, ranging from hydra and planarian whole-body regeneration to organ

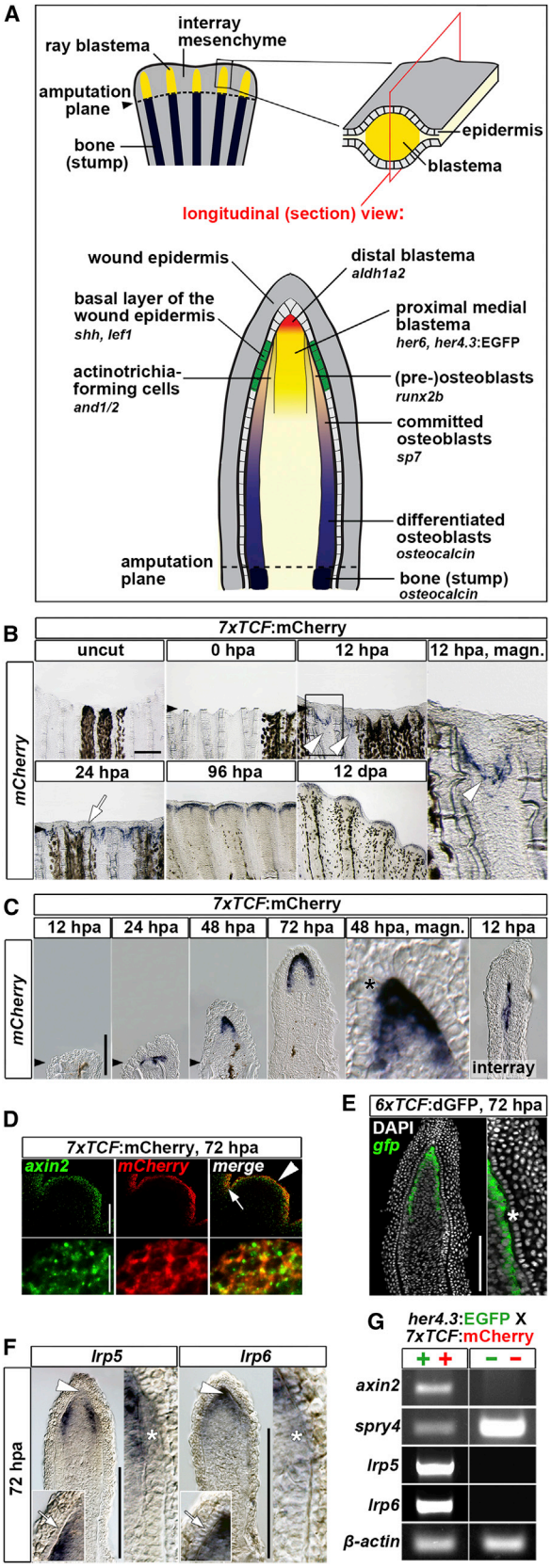


Figure 1. Wnt/β-Catenin Pathway Activation during Zebrafish Caudal Fin Regeneration

(A) Cartoons summarizing relevant anatomical structures and expression domains of a fin regenerate during the outgrowth phase. Whole-mount and longitudinal section views are shown.

(B) *mCherry* RNA expression in *7xTCF*:mCherry transgenic regenerates. Note transcript expression at 12 hpa in the interray tissue (arrowheads) and at 24 hpa distally to the bony ray (arrow).

(C) *mCherry* expression is confined to the mesenchyme at all stages analyzed. The basal epidermal layer is devoid of staining (asterisk).

(D) *axin2* (green) and *mCherry* RNA (red) are coexpressed in the distal blastema (arrowhead) and interray cells (arrow) in *7xTCF*:mCherry transgenic regenerates.

(E) *gfp* RNA expression in *6xTCF*:dGFP transgenic regenerates is confined to the blastema. The basal epidermal layer is devoid of staining (asterisk).

(F) *lrp5* transcripts are confined to proximal mediolateral domains of the blastema and are absent in the distal (arrowhead) blastema, the osteoblast progenitors (asterisk), and the basal epidermal layer (arrow). *lrp6* transcripts are predominantly detected in the distal blastema (arrowhead). Asterisk: (pre) osteoblasts; arrow: basal epidermal layer.

(G) RT-PCR of indicated genes on cDNA derived from fluorescence-activated-cell-sorted *7xTCF*:mCherry; *her4.3*:EGFP double transgenic regenerates at 72 hpa. Endogenous *lrp5/6* transcripts are not detected in the fluorescence-negative, blastema-free cell fraction that contains epidermal cells whereas *spry4* is.

In (B)–(G), confocal images of a whole-mount (D) or longitudinal section (E) are shown. Black arrowheads, amputation plane. Scale bars, 200 μm, 100 μm (D, whole mount),10 μm (D), and 100 μm (sections).

and tissue regeneration in lower vertebrates and mammals—all of which involve populations of proliferative precursor cells (Stoick-Cooper et al., 2007a; Whyte et al., 2012). However, a lack of sensitive reporters to monitor the spatiotemporal dynamics of pathway activation, the absence of genetic tools for temporally and spatially controlled manipulation of pathway activity, and the limited knowledge about downstream target genes have hindered further progress in understanding the precise functions of the β-catenin pathway during many of these regenerative processes.

Although the blastema is sometimes described as a "mass" of proliferative cells, the regenerate in fact becomes highly organized into several compartments during regenerative outgrowth (Figure 1A). The blastema comprises at least four domains: (1) the distal-most, scarcely proliferative blastema, characterized by expression of *aldh1a2* (*raldh2*), the rate-limiting enzyme in retinoic acid synthesis (Mathew et al., 2009; Nechiporuk and Keating, 2002), (2) the proximal medial blastema, which is rapidly proliferating and positive for readouts of Notch signaling (*her6* and the transgenic reporter *her4.3*:EGFP^{y83}; Grotek et al., 2013), (3) highly proliferative bilateral zones containing *runx2b*$^{+}$ osteoblast progenitors, and—further proximally—*sp7*$^{+}$ (*osterix/osx*) committed osteoblasts (Brown et al., 2009), and (4) domains directly medial to the osteoblast progenitors. These mediolaterally located cells constitute a subset of the Notch signaling-positive proximal medial blastema, are positive for *and1/2* transcripts, and are thought to be required for formation of unmineralized skeletal elements specific to fish fins, the actinotrichia (Zhang et al., 2010). The epidermis overlying the blastema likewise comprises different regions. In particular, a subdomain of the basal epidermal layer that abuts the (pre)osteoblasts expresses the transcription factor *lef1* and the Hedgehog (Hh) ligand *shh* and is thought to

regulate the maturation of underlying osteoblast progenitors (Laforest et al., 1998; Lee et al., 2009).

We have reinvestigated the role of the Wnt/β-catenin pathway during zebrafish caudal fin regeneration utilizing sensitive transgenic pathway reporters, tissue-specific pathway manipulation, and identification of the Wnt targetome. Surprisingly, our results suggest that main functions of β-catenin signaling during regenerative outgrowth, including regulation of blastemal cell proliferation and osteoblast maturation, are mediated by secondary signals. We suggest that a main function of Wnt/β-catenin signaling during fin regeneration is to define organizing centers within the blastema. There it regulates the activity of a number of signaling pathways that control cellular proliferation, differentiation, and tissue patterning in other compartments of the fin.

RESULTS

Transgenic Wnt Reporter Lines Reveal Spatiotemporal Dynamics of Pathway Activation

We and others have shown that fin regeneration requires β-catenin-dependent Wnt signaling for blastema formation and proliferation (Kawakami et al., 2006; Stoick-Cooper et al., 2007b). However, it has remained unexplained how Wnt signaling exerts these effects. Thus, we set out to characterize the spatiotemporal pattern of pathway activation utilizing transgenic reporters of β-catenin-dependent transcription. Transcripts of *7xTCF-Xla.Siam*:nlsmCherry^{ia5} (*7xTCF*:mCherry; Moro et al., 2012) could be detected in interray tissue starting at 6–12 hpa (white arrowheads in Figures 1B and S1A). These interray domains were lost upon overexpression of the Wnt inhibitor *dickkopf-1b* (*dkk1b*) in *7xTCF*:mCherry; *hsp70l*:dkk1b-GFP^{w32} (*hs*:dkk1; Stoick-Cooper et al., 2007b) double transgenic fish, indicating that they indeed reflect sites of active Wnt/β-catenin signaling (Figure S1B; see Table S4 for statistical data). At 24 hpa, *mCherry* transcripts were additionally detected distally to the bony rays where the blastemas form (white arrow in Figure 1B). Expression in rays and interrays persisted throughout the entire regenerative process (Figures 1B and S1A) and was restricted to the mesenchyme both in rays and interrays (Figures 1C and S1C). mCherry fluorescence was detectable from 16 hpa in a pattern resembling that of the *mCherry* transcripts (Figure S1D).

Expression of *axin2* is under feedback control by the Wnt/β-catenin pathway in many systems and thus commonly used as readout for pathway activity; its expression was dependent on β-catenin signaling in fin regenerates as well (Figure S1E). *axin2* transcripts colocalized with the *7xTCF*:mCherry reporter in the distal blastema (arrowhead in Figure 1D) and in the interray mesenchyme (arrow in Figure 1D), supporting that the transgene reports sites of endogenous β-catenin transcriptional activity. The expression pattern of a second transgenic reporter *6xTCF/Lef-miniP*:2dGFP (*6xTCF*:dGFP; Shimizu et al., 2012) was similar to that of the *7xTCF*:mCherry reporter being detectable in the distal blastema and extending proximally in lateral domains at 72 hpa (Figure 1E). Expression of a third Wnt reporter line, *Top*:dGFP^{w25} (Dorsky et al., 2002) could not be detected before 24 hpa, which is consistent with its lower sensitivity during zebrafish embryogenesis (Moro et al., 2012), but expression from 24 hpa onward resembled that of the other reporters (Figure S1F). Thus, Wnt/β-catenin signaling is activated soon after fin amputation and maintained in the ray and interray mesenchyme throughout the course of fin regeneration.

Expression of all five zebrafish Tcf/Lef family members was upregulated upon fin amputation (Figure S1G) and detected in partially overlapping patterns in different blastemal domains, and also in the basal epidermal layer (Figure S1H). *lef1* and *tcf1*(*tcf7*) are candidates for mediating β-catenin signaling in the distal blastema, where they are coexpressed with the *7xTCF*:mCherry reporter (arrowheads in Figures S1H and S1J), whereas *tcf1* and *tcf4* (*tcf7l2)* might mediate signaling in proximal regions of the blastema (Figures S1H and S1J). However, Tcf/Lef family members are also expressed in domains where we could not detect active β-catenin signaling, in particular the epidermis (*lef1* and *tcf1*) and the medial proximal blastema (*tcf3a* [*tcf7l1a*], *tcf3b* [*tcf7l1b*], *tcf4* [*tcf7l2*], Figures S1H and S1I).

The Wound Epidermis Is Devoid of Wnt/β-Catenin Signaling

lef1 is a Wnt target gene in many systems, and its expression in the regenerating fin was dependent on Wnt signaling as well (Figure S1K; Stoick-Cooper et al., 2007b). This was surprising, given that *lef1* is expressed in the epidermis, yet we have failed to detect Wnt signaling in this tissue. To gain further evidence that epidermal *lef1* is indirectly regulated by β-catenin signaling, we asked whether epidermal cells express the Wnt coreceptors *lrp5* and *lrp6,* which are considered essential for β-catenin pathway activation (He et al., 2004). We found that *lrp5* and *lrp6* transcripts were upregulated upon fin amputation (Figure S1L) and restricted to the blastema, whereas the epidermis was devoid of expression (arrows in Figure 1F). Likewise, when we sorted regenerates of *7xTCF*:mCherry; *her4.3:*EGFP^{y83} (Yeo et al., 2007) double transgenic fish (Figure S1M) by flow cytometry into a GFP$^+$ Cherry$^+$ fraction that contains blastemal cells, and a GFP$^-$ Cherry$^-$ population, which encompasses epidermis and other cell types, *lrp5* and *lrp6* transcripts could not be detected by RT-PCR in the GFP$^-$ Cherry$^-$ fraction (Figure 1G). Furthermore, overexpression of *wnt8* could not ectopically activate the β-catenin reporter in the epidermis in *hsp70l:*wnt8a-GFP^{w34} (*hs*:wnt8; Weidinger et al., 2005); *7xTCF*:mCherry double transgenic fish (Figure S1N). Finally, *dkk1* overexpression was sufficient to repress the *7xTCF*:mCherry reporter (arrow in Figure S1O), but not *lef1* expression within 3 hr (asterisk in Figure S1O), suggesting an indirect and thus delayed regulation of *lef1*. Together, these data show that the epidermis is devoid of Wnt/β-catenin signaling and that epidermal *lef1* expression must be indirectly regulated by the pathway.

Wnt/β-Catenin Signaling Is Highly Active in the Distal-Most Nonproliferative Blastema

During regenerative outgrowth, Wnt/β-catenin signaling is essential for blastemal cell proliferation (Stoick-Cooper et al., 2007b). We thus wondered whether Wnt signaling activity in the blastema correlates with sites of cellular proliferation. *6xTCF*:dGFP, *7xTCF*:mCherry and *axin2* expression were most strongly detected in the distal blastema and more weakly in lateral domains of the proximal blastema (Figures 1C, 2A, and 2C, arrowheads in Figures S2A and S2B). The strong expression

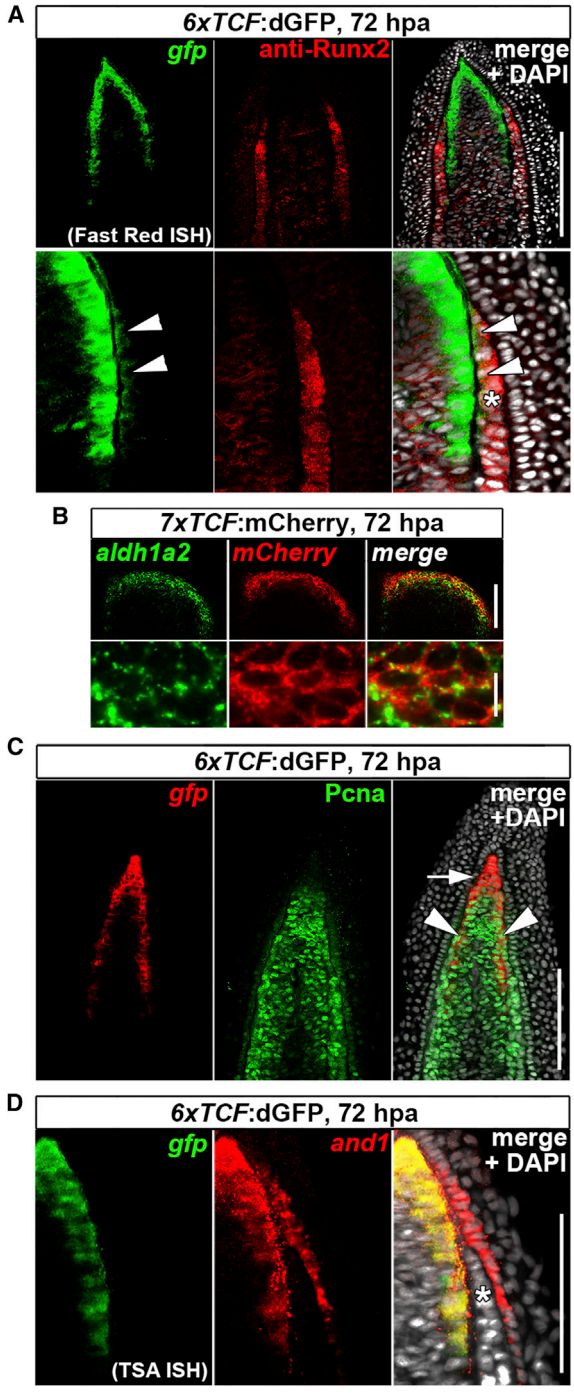


Figure 2. β-Catenin Pathway Activity Is Detected in Different Blastemal Compartments with Distinct Proliferative Properties

(A) *gfp* transcripts (green) in *6xTCF*:dGFP transgenic regenerates are strongly detected in cells of the distal blastema and in cells medially to the Runx2$^+$ osteoblast progenitors (red; asterisk). *gfp* transcripts are only weakly detected in Runx2$^+$ preosteoblasts (arrowheads).

(B) *aldh1a2* (green) and *mCherry* RNA (red) are coexpressed in the distal-most blastema in *7xTCF*:mCherry transgenic regenerates.

(C) *gfp* RNA in *6xTCF*:dGFP regenerates is detected in the distal-most, Pcna-negative blastema (arrow) and in a subpopulation of the Pcna$^+$, proximal blastema (arrowheads).

(D) *gfp* is coexpressed with *and1* in the actinotrichia forming cells of *6xTCF*:dGFP transgenics. Asterisk: (pre)osteoblasts. Note that the absence of Wnt reporter activity in the (pre)osteoblasts is due to a less sensitive staining procedure.

In (A)–(D), confocal images of longitudinal sections (A and C–D) or whole mounts (B). Scale bars, 100 μm and 10 μm (whole mount) and 100 μm (sections).

domain of the *7xTCF*:mCherry reporter colocalized with *aldh1a2*, which labels the distal-most blastema (Figures 2B and S2C). In contrast, in *7xTCF*:mCherry; *her4.3*:EGFP double transgenic fish, *mCherry* expression was detected distally to *egfp* expression that marks the proximal medial blastema (Figures S2D and S2E; Grotek et al., 2013). The distal-most blastemal domain is largely nonproliferative during regenerative outgrowth, whereas proximal domains proliferate at a high rate (Nechiporuk and Keating, 2002). Indeed, the distal expression domains of the *6xTCF*:dGFP and *7xTCF*:mCherry transgenics hardly overlapped with proliferative cell nuclear antigen (Pcna; arrow in Figure 2C; Figure S2F). Thus, during regenerative outgrowth Wnt/β-catenin signaling is highly active in the nonproliferative distal-most blastema.

Short-term fate mapping of the distal-most row of blastemal cells using a transgenic line expressing the photoconvertible fluorescent protein Kaede under control of the *7xTCF siamois* promoter (*7xTCF*:3xKaede; Figures S2G and S2H) showed that some of these cells maintained their distal positions for 48 hr after photoconversion (arrowhead in Figure S2I) despite the marked increase in regenerate length (~400 μm) that occurred within this time (Figure S2J). These data support a model in which the distal Wnt-receiving cells maintain their position during regenerative growth and hardly contribute cells to the process of tissue restoration.

Wnt/β-Catenin Signaling Activity in Actinotrichia-Forming Cells

In proximal regions of the blastema, the lateral *6xTCF*:dGFP Wnt reporter domains were positive for Pcna (arrowheads in Figure 2C). However, with the exception of these domains, the remaining proliferative proximal blastema was found to be largely negative for Wnt reporter activity (Figure 2C). Proximal reporter expression was mainly confined to domains of approximately two to three cells medial to the basal layer of the epidermis (Figure 1E) and, further proximally, medial to the Runx2$^+$ osteoblast progenitors (asterisk in Figure 2A). These cells expressed *and1*, a marker for actinotrichia-forming cells (Zhang et al., 2010) (Figure 2D). Although we could only detect little *6xTCF*:dGFP reporter mRNA in Runx2$^+$ osteoblast progenitors under specific staining conditions (arrowheads in Figure 2A), the preosteoblasts and adjacent actinotrichia-forming cells stained positive for *axin2* transcripts (asterisk and arrowhead in Figure S2B). Thus, in the proximal blastema Wnt/β-catenin signaling was active in actinotrichia-forming cells lining the osteoblasts and in Runx2$^+$ osteoblast progenitors, whereas further proximally located committed osteoblasts appeared to be devoid of signaling.

Interestingly, analysis of *lrp5* and *lrp6* Wnt coreceptor expression revealed similar distinct patterns. *lrp5* transcripts were confined to proximal mediolateral domains and absent from

the distal blastema (arrowhead in Figure 1F) and from the (pre) osteoblasts (asterisk in Figure 1F), whereas *lrp6* appeared to be predominantly expressed in the distal blastema and weakly in (pre)osteoblasts (arrowhead and asterisk in Figure 1F).

In summary, we found that the β-catenin pathway is only active in distinct subregions of the blastema during regenerative outgrowth and absent from most of the proliferative blastemal cells, where it is confined to actinotrichia-forming cells and pre-osteoblasts. Given that Wnt/β-catenin signaling in osteoblasts is well described to be essential for mammalian bone development and bone injury repair (Regard et al., 2012), we concentrated our further functional analyses on the distal blastema and the actinotrichia-forming cells, where Wnt signaling might play previously unrecognized functions in the regulation of fin regeneration. Indeed, a cell-autonomous role for Wnt signaling in preosteoblast maintenance is described in the accompanying article from Stewart et al. (2014) in this issue of *Cell Reports*.

Tissue-Specific Inhibition of Wnt/β-Catenin Signaling Confirms that the Pathway Has No Essential Role in the Wound Epidermis nor in Committed Osteoblasts

Systemic inhibition of Wnt/β-catenin signaling by heat shock promoter-driven overexpression of *dkk1* or *axin1* in *hs*:*dkk1* or *hsp70l*:Mmu.Axin1-YFP^{w35} (*hs*:Axin1; Kagermeier-Schenk et al., 2011) transgenics strongly reduced cell proliferation in the proximal blastema and blocked further regenerate growth upon prolonged inhibition (Figures S3A–S3C; Stoick-Cooper et al., 2007b). Because Wnt signaling does not seem to be active in many of the cells affected by Wnt loss-of-function (LOF), it appears that β-catenin signaling regulates blastemal cell proliferation largely indirectly. To test this hypothesis, we utilized the TetON system, which allows for doxycycline (DOX)-inducible, tissue-specific pathway manipulation (Figure 3A). To interfere with Wnt/β-catenin signaling, we used *TetRE*:Mmu.Axin1-YFP^{tud1} (*TetRE*:Axin1-YFP) transgenic fish, a TetResponder line that we have shown to cause severe Wnt LOF phenotypes when ubiquitously activated during gastrulation (Knopf et al., 2010). Treatment of *TetRE*:Axin1-YFP single transgenic fish with DOX or EtOH vehicle control for 12 days starting immediately after amputation did not affect regenerative growth, confirming low DOX toxicity and transgene leakiness (Figure S3D; Knopf et al., 2010). DOX-induced *axin1* overexpression directed by a *ubiquitin* promoter-driven TetActivator line (*ubiquitin*:irtTAM2(3F)-p2a-AmCyan, [*ubiquitin*:TetA AmCyan]; Figure S3E) in the distal and the majority of the proximal blastema and in the wound epidermis from 3 dpa until 12 dpa strongly interfered with regenerate growth, whereas EtOH had no effect (Figure 3B). Similarly, DOX treatment starting before amputation interfered with regeneration initiation (Figure S3F). Thus, expression levels achievable using the TetON system are suitable for studying the tissue-specific roles of Wnt/β-catenin signaling in adult fish.

Next, we used a *keratin4*:TetA AmCyan line (Figure S3G) to direct *axin1* overexpression to all layers of the wound epidermis except for its basal layer (Figure 3C), a *keratin18*:TetA AmCyan line (Figure S3H) for specific *axin1* overexpression in the basal epidermal layer (plus ectopically in the proximal medial blastema) (Figure 3D), and a *sp7*:TetA Cerulean line for inducible Wnt LOF specifically in committed osteoblasts (Figures 3E and S3I). Regenerate growth was not inhibited when fish were treated with DOX for 12 days starting immediately after amputation (Figures 3C–3E). Thus, Wnt LOF in tissues in which we have not detected signaling activity is of no gross phenotypic consequences, indicating that our efforts to map β-catenin signaling have not missed major sites of activity.

Wnt Signaling Must Be Inhibited in the Distal Nonproliferative Blastema and the Proliferative Proximal Blastema Together to Suppress Blastemal Cell Proliferation

We next used a *her4.3*:TetA AmCyan line to direct *axin1* overexpression specifically to the proximal medial blastema, including the *and1*$^{+}$ actinotrichia-forming cells but excluding the osteoblasts (Grotek et al., 2013; Figure 3F, asterisk in Figure S3J). Triple transgenic fish with the *7xTCF*:mCherry reporter confirmed that Wnt signaling in the distal blastema was not affected using this TetActivator (Figure S3K). Interestingly, *axin1* overexpression in the proximal proliferative blastema was able to slow, but not block regenerate growth (Figure 3F). Despite this modest effect on regenerate length, activation of *axin1* overexpression for 48 hr was not sufficient to significantly reduce blastemal cell proliferation as determined by antiphosphohistone 3 (H3P) staining (Figure 4A). In contrast, proliferation was severely reduced (to <50%) when *axin1* overexpression was driven by the *ubiquitin* promoter under the same conditions (Figure 4A). We conclude that β-catenin signaling must be inhibited in the proliferative proximal blastema and in the nonproliferative distal blastema at the same time, as achieved by the *ubiquitin*:TetA driver, for blastemal cell proliferation to be blocked. Furthermore, mild systemic inhibition of β-catenin signaling, as achieved by incubation of *6xTCF*:dGFP; *hs*:Axin1 double transgenic fish at 35°C, reduced reporter expression in the proximal, but not the distal blastema, confirming that Wnt signaling activity is stronger in the distal blastema (Figure S4A). When we used these conditions to interfere with Wnt signaling in the proximal domain, we found that regenerate growth was only mildly affected (Figure S4B).

Together, these data suggest that Wnt/β-catenin signaling in the distal-most, nonproliferative blastema indirectly regulates proliferation of proximal compartments of the blastema.

Wnt/β-Catenin Signaling in the Actinotrichia-Forming Cells Is Required for Osteoblast Differentiation

Although mild systemic inhibition of β-catenin signaling using incubation of *hs*:Axin1 fish at 35°C hardly effected regenerate growth, it resulted in malformed and shorter calcified bones, suggesting a role of proximal Wnt/β-catenin signaling in regulation of bone regeneration (Figure S4B). Because Wnt signaling activity in the proximal blastema is found in preosteoblasts and in adjacently located actinotrichia-forming cells, we asked whether β-catenin signaling in the latter might indirectly regulate regenerative osteogenesis—in addition to potential cell-autonomous roles of Wnt signaling in osteoblasts themselves. To test this, we directed *axin1* overexpression to the medial proximal blastema including the actinotrichia-forming cells, but excluding (pre)osteoblasts using the *her4.3*:TetA AmCyan driver and examined bone calcification as detected by Alizarin Red or

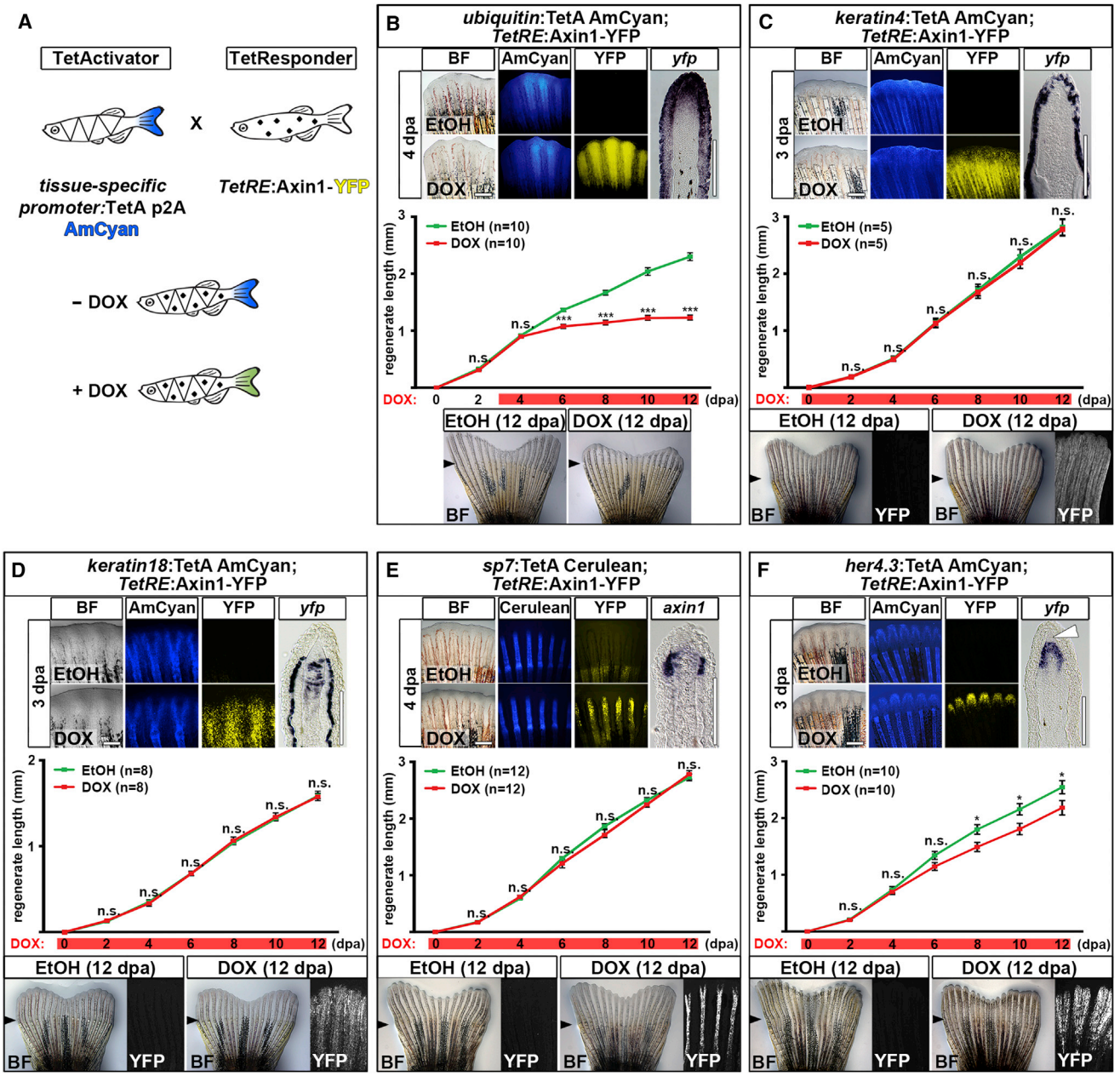


Figure 3. Wnt/β-Catenin Signaling Must Be Inhibited in the Distal and Proximal Blastema for Regenerative Growth to Be Stalled

(A) Cartoon showing strategy for tissue-specific inducible overexpression of the Wnt/β-catenin signaling inhibitor *axin1* using the TetON system.

(B) *ubiquitin* promoter-driven *axin1* overexpression in the epidermis and the blastema is sufficient to block regenerative growth.

(C) *keratin4* promoter-driven *axin1* overexpression in the epidermis excluding the basal layer does not interfere with regenerative growth.

(D) *keratin18* promoter-driven *axin1* overexpression in the basal epidermal layer (and ectopic expression in the proximal medial blastema) does not interfere with regenerative growth.

(E) *axin1* overexpression in the committed *sp7*$^{+}$ osteoblasts does not interfere with regenerative growth.

(F) *axin1* overexpression in the medial proximal blastema has only little impact on regenerative growth in *her4.3*:TetA AmCyan; *TetRE*:Axin1-YFP double transgenic fish. White arrowhead: distal-most blastema.

In (B)–(F), BF, bright field. Small arrowheads, amputation plane. Scale bars, 200 μm (whole mounts); 100 μm (sections). Error bars indicate error of the mean.

calcein staining (Figure S3J). DOX treatment for 8 days starting at 2 dpa reduced regenerate length by ~20% compared to EtOH controls (Figure S4C). In controls, ~85% of the regenerate was calcified as detected using either staining method, whereas upon Wnt inhibition this fraction was reduced to ~50% (Alizarin Red; Figure 4B) or ~60% (calcein; Figure S4D). Thus, Wnt signaling inhibition in the actinotrichia-forming cells interfered considerably more severely with bone calcification than with

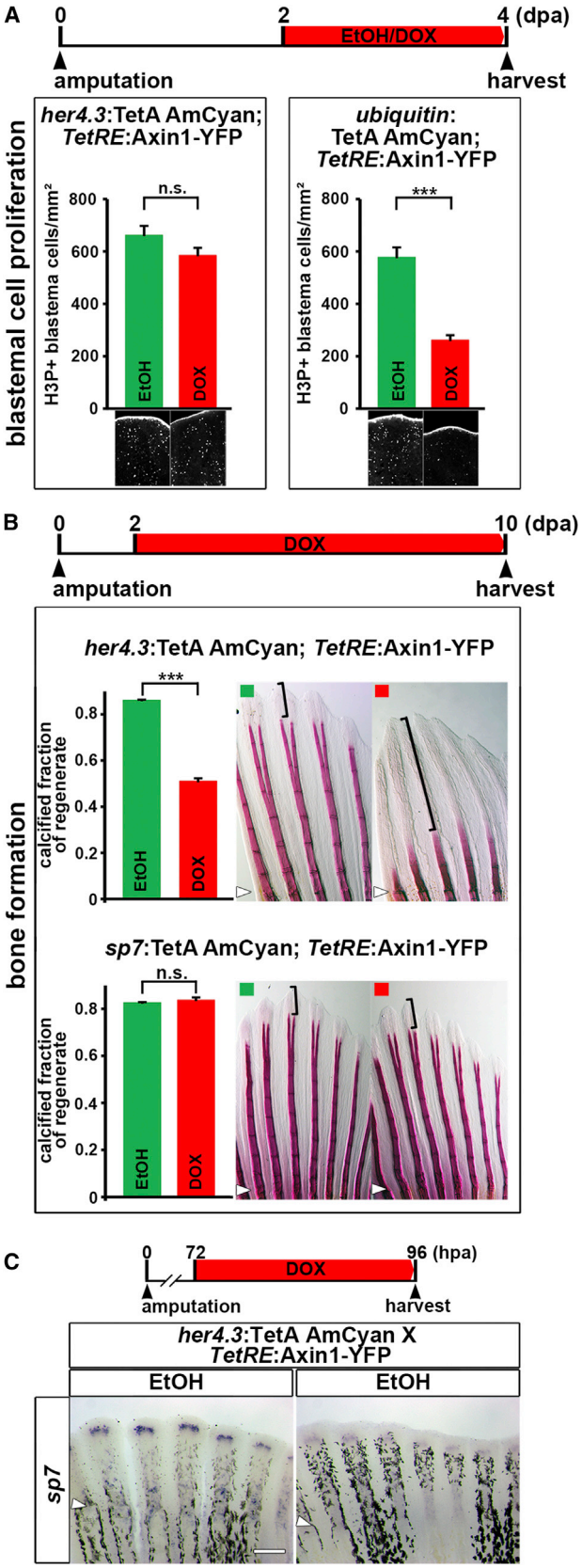


Figure 4. Wnt/β-Catenin Signaling in the Proximal Medial Blastema Is Required for Bone Formation

(A) *axin1* overexpression in the medial proximal blastema does not significantly reduce H3P$^+$ blastemal cells in *her4.3*:TetA AmCyan; *TetRE*:Axin1-YFP double transgenic fish. In contrast, proliferation is strongly reduced when *axin1* is directed to both the distal and medial proximal blastema using the *ubiquitin* promoter.

(B) *axin1* overexpression in the medial proximal blastema but not in committed osteoblasts reduces the fraction of the regenerate stained with the bone calcification marker Alizarin Red. Bracket indicates the noncalcified distal region of the regenerate.

(C) *axin1* overexpression in the proximal blastema reduces expression of the osteoblast commitment marker *sp7*.

In (A)-(C), arrowheads, amputation plane. Scale bar, 200 μm. Error bars indicate error of the mean.

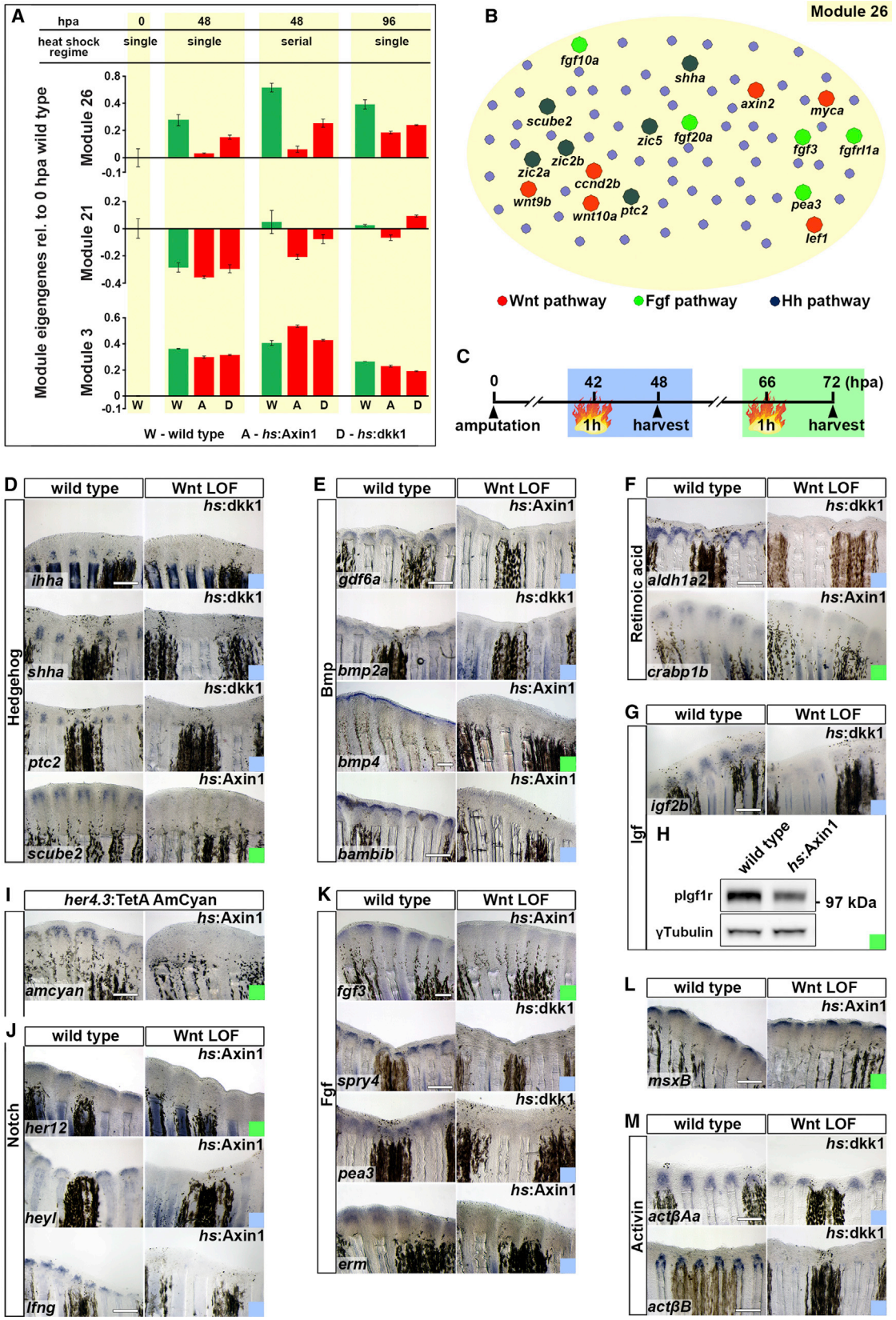
regenerate growth. Furthermore, *sp7* expression, a marker for committed osteoblasts, was strongly reduced within 24 hr of *axin1* induction (Figure 4C). Importantly, *sp7* downregulation was not due to enhanced apoptotic cell death as detected by TUNEL assay (Figures S4E and S4F). In contrast, *axin1* overexpression in committed osteoblasts in *sp7*:TetA Cerulean; TetRE:Axin1-YFP double transgenics had no effect on bone calcification (Figures 4B and S4D). These data suggest that Wnt/β-catenin signaling in the actinotrichia-forming cells indirectly regulates commitment and differentiation of adjacently located osteoblast progenitors.

Identification of the Wnt Targetome in the Regenerating Fin

Our data strongly suggest that Wnt/β-catenin signaling regulates fin regeneration largely indirectly. To identify secondary signals that could act downstream of β-catenin signaling to mediate its cell-nonautonomous effects, we characterized the Wnt/β-catenin targetome during wound healing (6 hpa), blastema formation (48 hpa), and regenerative outgrowth (96 hpa) by performing gene expression profiling after inducible inhibition of Wnt/β-catenin signaling using *hs*:Axin1 and *hs*:dkk1 transgenic fish (Table S1; Data S1–S3). Genes could be grouped into 33 modules based on their expression profile throughout regeneration and in response to Wnt inhibition, using weighted gene expression correlation network analysis (WGCNA; Data S4–S6; Langfelder and Horvath, 2008). Fifteen of these modules were significantly enriched for distinct biological processes (Table S2). Three modules (26, 21, and 3) showed reduction in gene expression upon Wnt inhibition with module 26 responding most consistently (Figure 5A). Module 26 was significantly enriched in genes involved in Wnt, Fgf, and Hh signaling, whereas modules 21 and 3 contained critical components of Bmp/Tgfβ, Hh, Notch, RA, and Mapk signaling (Figure 5B; Table S3). This suggests that Wnt/β-catenin signaling regulates key signaling pathways involved in regeneration.

Wnt/β-Catenin Signaling Is Required for Expression of Ligands, Pathway Components and Target Genes of Numerous Signaling Pathways Involved in Regeneration

In situ hybridization verified that expression of the Wnt pathway components *axin2*, *lef1*, *sost*, and *wnt10a* was indeed downregulated within 6 hr of *dkk1* or *axin1* induction (Figures



(legend on next page)

S1E, S1K, and S5A), as was the expression of ligands, target genes, and pathway components of many signaling pathways known to be required for fin regeneration (Figures 5C–5M). These included Hh signaling (ligands *ihha* and *shha*, target gene *ptc2,* pathway component *scube2*; Figure 5D), Bmp signaling (ligands *gdf6a/bmp13*, *bmp2a*, *bmp4*, feedback inhibitor *bambib*; Figure 5E), retinoic acid signaling (RA synthesizing enzyme *aldh1a2*, RA binding protein *crabp1b*; Figure 5F), Igf signaling (ligand *igf2b* and phosphorylation of the Igf1 receptor; Figure 5G), Notch signaling (activity of the Notch dependent *her4.3* promoter, target genes *her12* and *heyl*, pathway component *lunatic fringe* [*lfng*]; Figures 5I–5J), and Fgf signaling (ligands *fgf3* and *fgf20a* (Stoick-Cooper et al., 2007b), target genes *spry4*, *pea3* and *erm*; Figure 5K). *bmp4* transcripts colocalized with *7xTCF*:mCherry reporter activity in the distal blastema suggesting a direct regulation of *bmp4* transcription by β-catenin signaling (Figure S5B).

Importantly, some factors required for fin regeneration were insensitive to inhibition of Wnt/β-catenin signaling using the same heat shock protocol, namely, *msxB* (Figure 5L) and *actβAa* (Figure 5M), indicating that gene expression is not nonspecifically suppressed in *axin1* or *dkk1* overexpressing fins. In contrast to *actβAa, actβB* expression, which colocalized with *7xTCF*:mCherry in the distal-most blastema, was dependent on β-catenin signaling (Figure 5M, arrowhead in Figure S5C).

Together, these data strongly suggest that Wnt/β-catenin signaling regulates the activity of multiple signaling pathways required for fin regeneration including Hh, Bmp, RA, Igf, Notch, Fgf, and Activin. To determine whether these pathways also reciprocally control β-catenin signaling, we assayed *7xTCF:*mCherry reporter activity and expression of the endogenous Wnt target *axin2* after genetic or pharmacological inhibition of these pathways. Yet, Wnt reporter activity could still be detected after prolonged (for 48 or 72 hr) inhibition of Igf, Fgf, Notch, Activin, or RA signaling, even though these treatments were sufficient to block regenerative growth (Figures S5D–S5H). Similarly, whereas expression of *axin2* was abolished within 6 hr after Wnt/β-catenin signaling inhibition using *hs*:Axin1 fish or the IWR-1 drug (Figures S1E and S5P), inhibition of Activin, Igf, Hh, Notch, Fgf, RA, or Bmp signaling for 6 hr had no or very little effect on *axin2* expression (Figures S5I–S5O). These data suggest that Wnt/β-catenin signaling has a stronger effect on activity of other pathways than vice versa, indicating that Wnt signaling acts upstream of a network of signals during fin regeneration.

Wnt/β-Catenin Signaling Indirectly Regulates Epidermal Patterning through Fgf and Bmp Signaling

We have provided evidence that epidermal *lef1* expression cannot be directly controlled by β-catenin signaling. We thus looked for candidate secondary signals that could mediate its effects on the epidermis. In other systems, *lef1* expression has been shown to be regulated by Bmp signaling (Kratochwil et al., 1996). We found that epidermal *lef1* expression but not Wnt reporter activity was strongly reduced 6 hr after overexpression of the Bmp inhibitor *noggin3* in *hsp70l*:noggin3^{fr14} (*hs*:nog3; Chocron et al., 2007); *7xTCF*:mCherry double transgenic fish (Figures S6A and 6A). Thus, the Bmp pathway appears to regulate epidermal *lef1* expression independently of β-catenin signaling. Similarly, *lef1* expression was strongly diminished 6 hr after overexpression of a dominant-negative Fgf receptor 1 (Fgfr1) in *hsp70l*:dnfgfr1-EGFP^{pd1} (*hs*:dnfgfr1; Lee et al., 2005); *7xTCF*:mCherry double transgenics, whereas in the same fins the Wnt reporter was unaffected (Figure 6B). Together with our finding that Bmp and Fgf signaling are regulated by the Wnt/β-catenin pathway, these data suggest that these pathways control epidermal *lef1* expression downstream of β-catenin signaling.

Expression of the Fgf target genes *spry4*, *pea3*, and *erm*, which are all expressed in distal domains of the basal epidermal layer (Lee et al., 2005; Lee et al., 2009), likewise depended on Wnt/β-catenin signaling (Figure 5K). Although Fgf ligands regulate *erm* and *pea3* expression via the Ras/Mapk pathway, *lef1* expression is controlled via a Ras-independent route in the regenerating fin (Lee et al., 2009). To test whether Wnt signaling regulates epidermal *pea3* and *erm* expression through the Fgf/Ras pathway, we asked whether Ras activation could rescue *erm* and *pea3* expression in Wnt-inhibited fins using *hs*:Axin1; *hsp70l*:vHRAS,cryaa:DsREDEx^{pd8} (*hs*:v-ras; Lee et al., 2009) double transgenic fish. In agreement with published data (Lee et al., 2009) a single heat shock upregulated *pea3* and *erm* expression in *hs*:v-ras transgenic regenerates 6 hr after heat shock, whereas their expression was abolished in *hs*:Axin1 transgenic fish (Figure 6C). Intriguingly, upon simultaneous overexpression of *axin1* and *v-ras*, *erm* and *pea3* expression was restored (Figure 6C). Thus, we propose that Wnt/β-catenin signaling in the blastema regulates *pea3* and *erm* transcription in the epidermis via activation of Fgf/Ras signaling.

Blastemal Proliferation Is Regulated by the β-Catenin Pathway via Hedgehog and Retinoic Acid Signaling

Hh signaling is a candidate pathway that could act downstream of β-catenin to mediate its mitogenic effects in the blastema,

Figure 5. Wnt/β-Catenin Signaling Regulates Many Other Signaling Pathways

(A) Eigengene values of three correlated WGCNA modules, representing the average relative expression values of all genes in the module, in ten experimental conditions. Values shown are the average of triplicate samples relative to 0 hpa wild-type samples. Error bars indicate error of the mean.

(B) Cartoon of genes found in module 26 with genes annotated to be associated with Wnt, Fgf, and Hh signaling highlighted.

(C) Samples in (D)–(M) were either heat shocked at 42 hpa (blue) or 66 hpa (green) and harvested 6 hr later.

(D–M) Inhibition of Wnt/β-catenin signaling via *axin1* or *dkk1* overexpression interferes with pathway activity or expression of ligands, pathway components, and target genes of Hh (D), Bmp (E), RA (F), Igf (G), Notch (I and J), and Fgf (K) signaling. Expression of *msxB* and *actβAa* but not *actβB* remains unaffected using the same heat shock protocol (L and M).

(H) *axin1* overexpression for 6 hr reduces Igf1r phosphorylation as detected in western blots of whole regenerates at 72 hpa.

(I) *axin1* overexpression interferes with Notch signaling-dependent *her4.3* promoter activity in *her4.3*:TetA AmCyan; *hs*:Axin1 double transgenic fish.

In (D)–(M), scale bars, 200 μm.

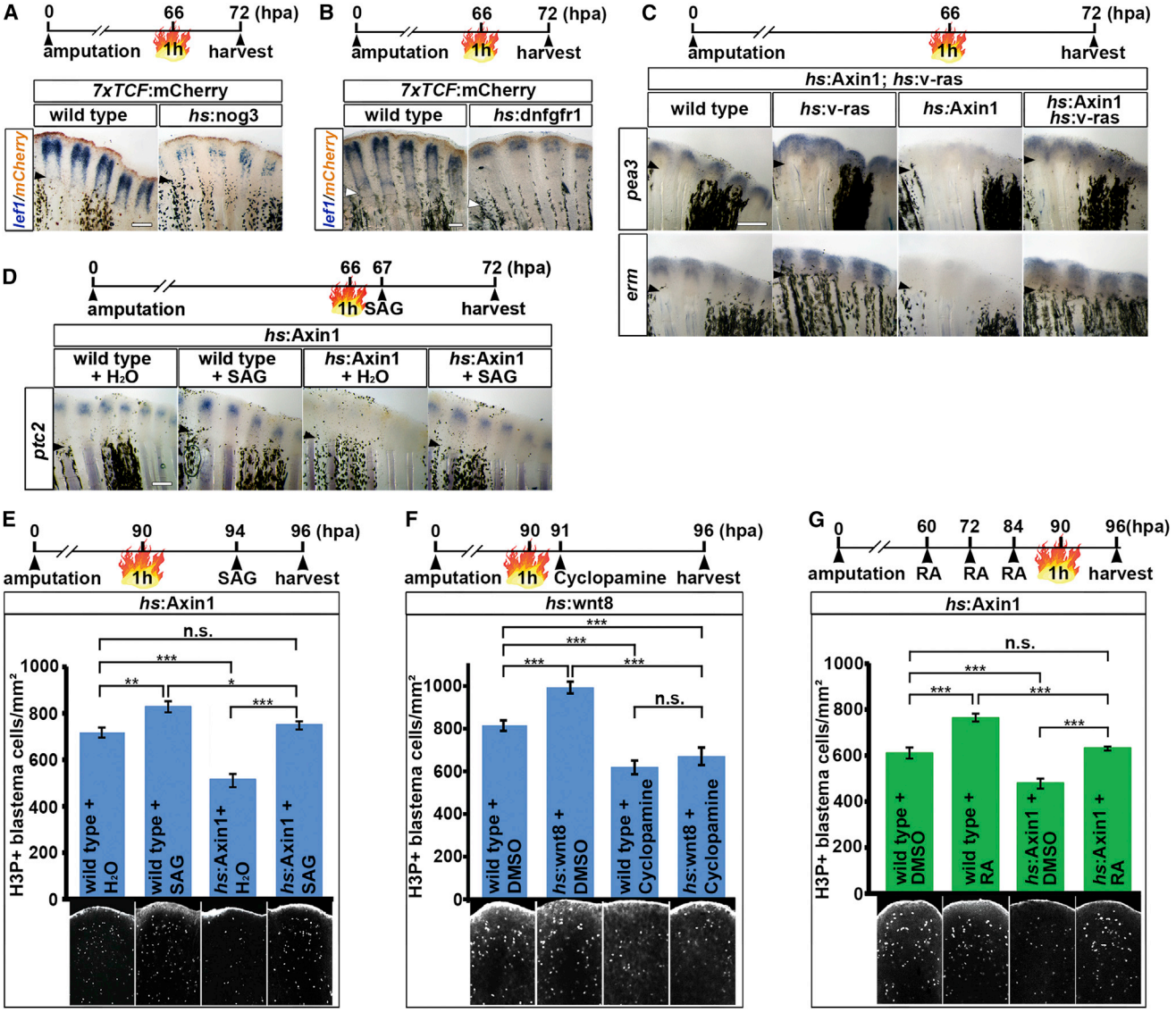


Figure 6. Wnt/β-Catenin Signaling Regulates Blastema Proliferation via Hedgehog and Retinoic Acid Signaling

(A) *noggin3* overexpression reduces *lef1* (blue) but not *mCherry* (brown) expression in *7xTCF*:mCherry; *hs*:nog3 double transgenic regenerates.

(B) Overexpression of a dominant negative *fgfr1* reduces *lef1* (blue) but not *mCherry* (brown) expression in *7xTCF*:mCherry; *hs*:dnfgfr1 double transgenic regenerates.

(C) *axin1* overexpression inhibits expression of the Fgf targets *pea3* and *erm*, which is rescued by concomitant activation of Fgf/Ras signaling via overexpression of *v-ras*.

(D) *axin1* overexpression inhibits expression of the Hh target *ptc2*, which is rescued by concomitant activation of Hh signaling using the pharmacological compound SAG.

(E) *axin1* overexpression reduces the number of $H3P^+$ cells in the blastema, which is rescued by concomitant activation of Hh signaling using SAG.

(F) Inhibition of Hh signaling through exposure to cyclopamine reduces the number of $H3P^+$ cells in the blastema, which is not rescued by concomitant activation of Wnt signaling following *wnt8* overexpression.

(G) Administration of RA via intraperitoneal injection rescues the reduction of $H3P^+$ blastemal cell numbers caused by *axin1* overexpression.

In (A)–(G), arrowheads indicate amputation plane. Scale bars, 200 μm. Error bars indicate SEM.

because it is required for blastema proliferation (Quint et al., 2002) and Hh ligands are transcriptionally regulated by Wnt signaling (see Figure 5D). Activation of Hh signaling using the Smoothened agonist SAG could rescue epidermal and blastemal (Figure S6B) expression of the direct Hh target gene *ptc2* in *hs*:Axin1 transgenic fins (Figure 6D), indicating that this treatment can restore Hh signaling lost after inhibition of β-catenin signaling. We therefore asked whether Hh signaling could rescue blastemal cell proliferation in Wnt inhibited fins. In agreement with recent studies, *axin1* overexpression decreased,

whereas SAG treatment increased the number of H3P$^+$ cells in the blastema (Lee et al., 2009; Stoick-Cooper et al., 2007b; Figure 6E). Importantly, SAG treatment rescued the number of H3P$^+$ cells in *hs*:Axin1 transgenic fish back to wild-type levels (Figure 6E). In contrast, activation of Wnt/β-catenin signaling through overexpression of *wnt8* in *hs:*wnt8 transgenic fish failed to rescue blastemal proliferation that was reduced upon inhibition of Hh signaling using Cyclopamine (Figure 6F). These results indicate that Wnt/β-catenin signaling acts upstream of Hh signaling and regulates blastema cell proliferation indirectly via activation of Hh ligand expression.

Another candidate diffusible signal that could mediate the mitogenic effect of the Wnt/β-catenin pathway on the proximal blastema is RA, which has been shown to be essential for blastema formation and proliferation (Blum and Begemann, 2012). Consistent with previous results, RA injection increased the number of H3P$^+$ blastemal cells, whereas overexpression of *axin1* in *hs*:Axin1 transgenic fish suppressed proliferation (Blum and Begemann, 2012; Stoick-Cooper et al., 2007b; Figure 6G). Intriguingly, proliferation was rescued back to wild-type levels in RA-treated *axin1* overexpressing fins, indicating that RA signaling regulates blastemal cell proliferation downstream of Wnt/β-catenin signaling during regenerative outgrowth (Figure 6G). Although repetitive administration of RA rescued blastemal cell proliferation when Wnt/β-catenin signaling was concomitantly inhibited, it was not sufficient to rescue morphological regenerative growth in *hs:*dkk1 transgenic fish (Figure S6C), suggesting that Wnt/β-catenin signaling orchestrates fin regeneration through regulation of multiple signaling pathways.

We next asked how Wnt/β-catenin activity in the distal-most blastema regulates other signaling pathways that mediate its effects on adjacent tissues. *fgf3* is a candidate Fgf ligand for mediating the interaction of the distal blastema with the epidermis, because its expression colocalized with β-catenin activity in the distal-most blastema (arrowhead in Figure S6D), and it was dependent on β-catenin signaling (Figure 5K). Likewise, expression of the RA synthesizing enzyme *aldh1a2* was also largely confined to the distal-most blastema, where its expression was dependent on the β-catenin pathway (Figures 2B, S2C, and 5F).

fgf3 and *aldh1a2* Are Direct Wnt Targets in the Distal Blastema

Thus, we wondered whether *fgf3* and *aldh1a2* expression is directly regulated by β-catenin signaling in the distal blastema. Using chromatin immunoprecipitation (ChIP) analysis, we found that immediate upstream sequences of the transcriptional start site (TSS) of *fgf3* could be precipitated from genomic DNA isolated from 3 dpa fin regenerates using antibodies against β-catenin or against Lef1 or Tcf1 (Figure 7A), both of which are expressed in the distal-most blastema (Figures S1H and S1J). This suggests that the *fgf3* promoter interacts with enhancers that are regulated by β-catenin/Lef1 and β-catenin/Tcf1 complexes. Importantly, negative control *fgf3* genomic sequences located 3.8 kb downstream of the TSS that contained no consensus Tcf/Lef binding sites did not precipitate with any of the antibodies, and the TSS of *msxB*, which is not Wnt responsive (Figure 5L), also did not (Figure 7A). The TSS of *aldh1a2* could also be precipitated with anti-β-catenin, anti-Lef1, and anti-Tcf1, whereas a negative control *aldh1a2* genomic fragment 4 kbp downstream could not (Figure 7A). We conclude that *fgf3* and *aldh1a2* are direct transcriptional targets of β-catenin in the distal-most blastema. Thus, we propose that Fgf3 and RA represent secondary signals that mediate the effect of Wnt/β-catenin signaling in the distal blastema on regenerative growth and patterning of surrounding tissues.

DISCUSSION

The work presented here advances our understanding of the mechanisms underlying fin regeneration. Our results suggest that the blastema, the population of undifferentiated mesenchymal cells characteristic of regenerating appendages, displays previously unrecognized functional diversity, given that it not only provides progenitor cells for tissue restoration, but also contains signaling centers that orchestrate growth and differentiation of the regenerate. We propose that Wnt/β-catenin signaling has essential functions in defining these organizing centers, and that it acts upstream of a large number of other developmental signaling pathways that mediate the effects of these organizers on surrounding tissues. Specifically, our data support the following model of Wnt/β-catenin pathway function during fin regeneration (Figures 7B–7E).

Wnt/β-Catenin Signaling Is Active in Specific Subregions of the Blastema

Wnt/β-catenin signaling is activated during wound re-epithelialization (at 6 hpa) in the interray mesenchyme, making β-catenin pathway activation one of the earliest known responses to fin amputation (Figure 7B). Pathway activation at 6 hpa is consistent with the induction of *wnt10a* expression at this time point, which we have reported previously (Stoick-Cooper et al., 2007b). Signaling activity is maintained in interray mesenchyme throughout regeneration but the functional significance of this site of β-catenin signaling remains to be determined. Here, we have concentrated on Wnt functions in the ray blastema, where pathway activation starts around 24 hpa. Subsequently, the blastema is compartmentalized into several subregions with distinct proliferative properties and cell fate (Figure 7C): Wnt-receiving cells localize to the distal-most compartment where they remain largely quiescent and retain their distal position until regeneration is completed. Additionally Wnt activity is detected in subregions of the proliferative proximal blastema, in actinotrichia-forming cells that lie medial to the osteoblasts, and—likely more weakly as judged by transgenic reporter expression—in Runx2$^+$ osteoblast progenitors. No β-catenin pathway activation appears to occur in the epidermis, in committed osteoblasts (*sp7*$^+$) or the majority of the proximal medial blastema. Thus, Wnt/β-catenin signaling is absent from a large part of the proliferating compartment of the fin during regenerative outgrowth.

Wnt Signaling Acts Indirectly via Regulation of Other Secreted Factors

We have not analyzed the function of β-catenin signaling in preosteoblasts, where it could play a cell-autonomous role in

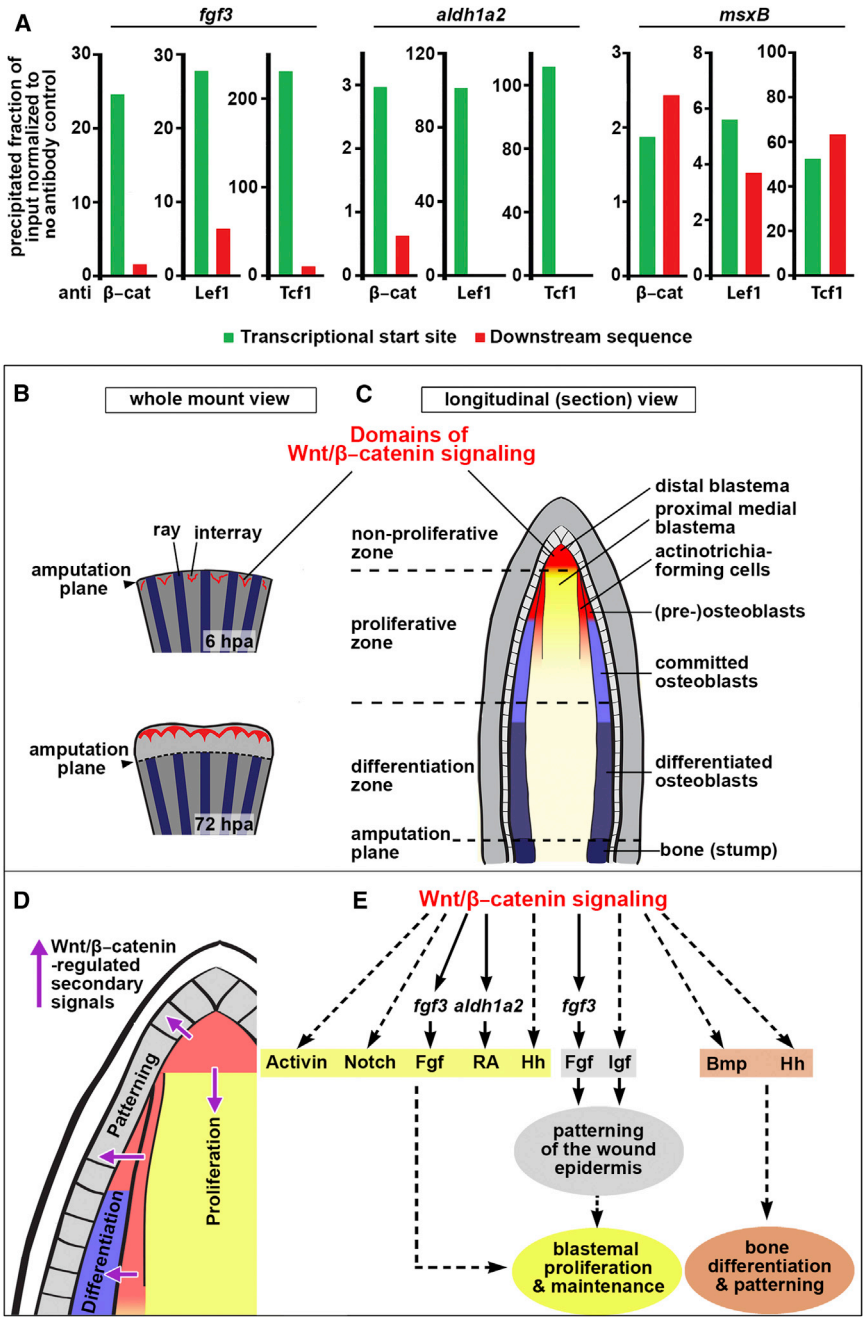


Figure 7. *fgf3* and *aldh1a2* Are Directly Regulated by Wnt/β-Catenin Signaling

(A) β-catenin, Lef1 and Tcf1 proteins are enriched at DNA sequences immediately 5′ of the transcriptional start site of the *fgf3* and *aldh1a2* gene compared to control sequences located 3.8 and 4 kbp further downstream. β-catenin, Lef1 and Tcf1 protein are not enriched at the transcriptional start site of the *msxB* gene. For each sequence, the amount of precipitated genomic DNA quantified by quantitative RT-PCR is shown as a fraction of the input and relative to the amount precipitated in a negative control reaction lacking the antibody.

(B and C) Schematic summary of the Wnt/β-catenin activity domains (in red color) during fin regeneration. Whole-mount (C) and longitudinal section views (D) are shown.

(D) Wnt pathway activity is required for production of (diffusible) signals that regulate proliferation of proximal blastemal regions, differentiation of osteoblasts, and patterning of the epidermis.

(E) Schematic summary of the hierarchical position of Wnt/β-catenin signaling within the molecular signaling network controlling fin regeneration. Arrows indicate direct regulation, dashed arrows indicate direct or indirect regulation.

regulation of osteoblast proliferation and/or differentiation as it does during mammalian bone development and repair (Regard et al., 2012). Intriguingly, however, our results suggest that β-catenin signaling has at least three additional indirect functions that are mediated by secondary signals (Figure 7D): (1) signaling in the nonproliferative distal blastema regulates proliferation in the proximal blastema, (2) signaling in the actinotrichia-forming cells regulates commitment and differentiation of adjacent osteoblasts, and (3) one or both of these domains regulate(s) epidermal patterning. We conclude that β-catenin signaling controls regenerate growth, differentiation, and patterning largely indirectly. Wnt/β-catenin signaling is considered to directly induce cell proliferation in many systems, and inappropriate activation of the pathway causes cancer (Reya and Clevers, 2005). Although organ regeneration requires a burst of cell proliferation and rapid tissue growth, Wnts surprisingly appear to have little role as mitogens in the regenerating fin; rather Wnt signaling regulates regenerative cell proliferation largely indirectly. This hypothesis is supported by the following findings: (1) the strongest β-catenin signaling activity is in nonproliferative cells of the distal blastema, (2) Wnt signaling inhibition in the proliferative domain is not sufficient to block cell proliferation, (3) RA acts functionally downstream of Wnt signaling in blastema proliferation, (4) RA is likely only produced in the distal blastema, because *aldh1a2*, the rate-limiting enzyme in RA production, is only expressed in this domain, and (5) *aldh1a2* expression is directly regulated by β-catenin signaling. Thus, we propose that Wnt signaling promotes RA production in the distal blastema, from where RA diffuses to regulate cell proliferation in proximal domains. However, definitive proof of this model will require the establishment of a distal-most blastema-specific TetActivator line. It will also be interesting to determine to what extent Wnt/β-catenin signaling directly regulates regenerative proliferation in other regenerating systems.

In addition, many other signaling pathways that have previously been shown to be involved in controlling fin regeneration

are regulated by Wnt/β-catenin signaling, with Hh and RA signals likely mediating its effects on blastemal cell proliferation and Fgf and possibly Igf and Bmp signaling on epidermal patterning (Figure 7E). Although Igf ligand expression in the blastema is regulated by Wnt/β-catenin signaling, Igf receptor activation occurs specifically in the epidermis (Chablais and Jazwinska, 2010). Thus, Wnt appears to regulate epidermal Igf signaling through transcriptional control of Igf ligands in the blastema. *fgf3* expression is exclusive to the distal-most blastema and is directly regulated by β-catenin, making it a prime candidate ligand that diffuses from this blastemal domain to activate epidermal Fgf signaling.

Our data indicate that Wnt/β-catenin signaling receives little reciprocal input from the signals it regulates, supporting a model in which Wnt signaling acts on top of a likely cooperative signaling network that instructs fin regeneration (Figure 7E). Interestingly, transgenic rescue experiments have shown that Fgf signaling acts downstream of Wnt/β-catenin signaling during *Xenopus* tadpole tail regeneration as well (Lin and Slack, 2008). Furthermore, in regenerating frog tadpole limbs and tails expression of Fgf ligands (*fgf8* in limbs, *fgf20a* in tails) depends on Wnt/β-catenin signaling (Lin and Slack, 2008; Yokoyama et al., 2007), suggesting that Wnt/β-catenin signaling regulates Fgf signaling through transcriptional control of Fgf ligands in both *Xenopus* and zebrafish. In contrast to the zebrafish fin however, Bmp signaling appears to act upstream of Wnt/β-catenin signaling in the regenerating *Xenopus* tadpole tail (Lin and Slack, 2008). During newt limb regeneration, activation of the Wnt or Hh pathway promotes blastemal cell proliferation (Singh et al., 2012), yet Wnt signaling appears to act downstream of Hh in this system. Thus, although a Wnt-Fgf axis appears to be conserved in appendage regeneration, differences in the hierarchical relationships between Wnt and Hh and Wnt and Bmp signaling pathway might exist between species.

In summary, we propose that Wnt signaling sets up two signaling centers, one in the distal-most blastema and one in the presumptive actinotrichia-forming cells in the proximal blastema, from which other diffusible signals emanate that direct growth, differentiation, and patterning of the regenerating zebrafish fin.

Wnt Signaling in Osteoblast Differentiation

During mammalian embryonic development, Wnt/β-catenin signaling is well accepted to have essential roles in promoting osteoblast differentiation (Long, 2012; Regard et al., 2012). Our data indicate that β-catenin signaling likewise is required for bone regeneration in the zebrafish fin. Although we have not addressed the role of Wnt/β-catenin signaling in the osteoblast progenitors, which is described in the accompanying paper by Stewart et al. (2014), we surprisingly discovered an additional indirect role of Wnt/β-catenin signaling in the regulation of bone maturation. We find that β-catenin pathway activity in the actinotrichia-forming cells, which are located adjacent to the osteoblasts, directs osteoblast commitment and differentiation. Thus, Wnt/β-catenin signaling likely promotes osteoblast maturation via stimulation of diffusible factors that are yet to be determined. The dermal bone found in fish fins, the lepidotrichia, has been suggested to be distinct from other skeletal tissues based on morphological and gene expression characteristics (Marí-Beffa et al., 2007). Furthermore, little is known about the role of Wnt signaling in skeletogenesis during fish embryonic development. Thus, it remains to be determined whether the indirect role of β-catenin signaling in osteoblasts observed here reflects a general difference in skeletal development between fish and mammals, is a special feature of the fin ray bones during development and regeneration, or occurs only during regeneration.

Conclusions

Wnt/β-catenin signaling is a major regulator of cellular proliferation and cell fate during fin regeneration, just as it is in numerous other systems, but stunningly our results suggest that it does so largely indirectly in the fin. We propose that blastemal cell proliferation is regulated by β-catenin signaling activity in nonproliferative distal blastemal cells, and osteoblast maturation and epidermal patterning are regulated via β-catenin activity in adjacent cells. Thus, our results highlight the emerging view that the regeneration blastema is not simply a homogenous mass of progenitor cells but is highly organized into distinct domains, some of which act as organizing centers that orchestrate regeneration via production of secreted factors. We conclude that Wnt/β-catenin signaling is essential in setting up and regulating the complex tissue interactions required for successful regeneration.

EXPERIMENTAL PROCEDURES

Experiments with adult animals have been approved by the state of Baden-Württemberg and the animal protection representative of Ulm University. Additional experimental procedures are described in the Supplemental Experimental Procedures.

Drug Treatments, Heat Shocks of Adult Zebrafish, and Regenerate Length Measurements

Drug treatments were performed by incubating fish in fish system water containing the drugs except for RA treatments. Fish were kept in the dark and water was exchanged daily. Doxycycline (Sigma) was dissolved in 50% EtOH and used at 25 μg/ml. Alk4/5/7 inhibitor SB431542 (Tocris) (10 μM), 5 μM Igf1r inhibitor NVP-AEW541 (Selleck), 10 μM γSecretase inhibitor LY411575 (Selleck), 5 μM Smoothened agonist (SAG; Calbiochem), 50 μM Smoothened inhibitor Cyclopamine (Sigma-Aldrich), or 12 μM Axin stabilizer IWR-1 (Sigma-Aldrich) were used as previously described (Chablais and Jazwinska, 2010; Grotek et al., 2013; Jazwinska et al., 2007; Lee et al., 2009; Lu et al., 2009). Ten microliters of 3 mM RA (Sigma) was administered by intraperitoneal injections as described (Blum and Begemann, 2012). If not indicated otherwise, heat shocks were performed for 1 hr at 37°C or for 1 hr at 38°C for *hsp70l*:cyp26a1^{kn1} transgenic fish. Heat shocks and drug treatments were performed according to the schematic timelines shown with each experiment. Regenerate length and calcified bone length measurements of the second, third, and fourth lateral ray of each lobe were performed using ImageJ software.

Statistics

Error bars indicate error of the mean. For nonquantitative data the number of specimen that showed the phenotype displayed in the figures and the total number of specimens is given in Table S4. Significance of differences in mean regenerate/calcified bone length and number of proliferative cells was tested using Student's t test. n.s., not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, six tables, and six data files and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.12.036.

ACKNOWLEDGMENTS

We thank Nicole Rund (Biotec FACS Facility), Ellen Geibelt and Markus Burkhardt (Biotec Light Microscopy Facility), Saintin Donakonda, Quang Huy Le for technical support, Marika Fischer, Katrin Sippel, Jitka Michling, Brigitte Korte, and Doris Weber for excellent fish care. Thanks to present and former members of the Weidinger lab for critical reading of the manuscript. This work was supported by a grant of the Deutsche Forschungsgemeinschaft (WE4223/3-1) to G.W., grant AIRC IG 10274 to F.A., Italian Ministry of Health grant GR2008-1139743 to E.M., and EU ZF-HEALTH grant 242048-2) to F.A.

Received: March 30, 2013
Revised: August 30, 2013
Accepted: December 24, 2013
Published: January 30, 2014

REFERENCES

Azevedo, A.S., Grotek, B., Jacinto, A., Weidinger, G., and Saúde, L. (2011). The regenerative capacity of the zebrafish caudal fin is not affected by repeated amputations. PLoS ONE *6*, e22820.

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

Brown, A.M., Fisher, S., and Iovine, M.K. (2009). Osteoblast maturation occurs in overlapping proximal-distal compartments during fin regeneration in zebrafish. Dev. Dyn. *238*, 2922–2928.

Chablais, F., and Jazwinska, A. (2010). IGF signaling between blastema and wound epidermis is required for fin regeneration. Development *137*, 871–879.

Chocron, S., Verhoeven, M.C., Rentzsch, F., Hammerschmidt, M., and Bakkers, J. (2007). Zebrafish Bmp4 regulates left-right asymmetry at two distinct developmental time points. Dev. Biol. *305*, 577–588.

Dorsky, R.I., Sheldahl, L.C., and Moon, R.T. (2002). A transgenic Lef1/beta-catenin-dependent reporter is expressed in spatially restricted domains throughout zebrafish development. Dev. Biol. *241*, 229–237.

Grotek, B., Wehner, D., and Weidinger, G. (2013). Notch signaling coordinates cellular proliferation with differentiation during zebrafish fin regeneration. Development *140*, 1412–1423.

He, X., Semenov, M., Tamai, K., and Zeng, X. (2004). LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. Development *131*, 1663–1677.

Jazwinska, A., Badakov, R., and Keating, M.T. (2007). Activin-betaA signaling is required for zebrafish fin regeneration. Curr. Biol. *17*, 1390–1395.

Kagermeier-Schenk, B., Wehner, D., Ozhan-Kizil, G., Yamamoto, H., Li, J., Kirchner, K., Hoffmann, C., Stern, P., Kikuchi, A., Schambony, A., and Weidinger, G. (2011). Waif1/5T4 inhibits Wnt/β-catenin signaling and activates noncanonical Wnt pathways by modifying LRP6 subcellular localization. Dev. Cell *21*, 1129–1143.

Kawakami, Y., Rodriguez Esteban, C., Raya, M., Kawakami, H., Martí, M., Dubova, I., and Izpisúa Belmonte, J.C. (2006). Wnt/beta-catenin signaling regulates vertebrate limb regeneration. Genes Dev. *20*, 3232–3237.

Knopf, F., Schnabel, K., Haase, C., Pfeifer, K., Anastassiadis, K., and Weidinger, G. (2010). Dually inducible TetON systems for tissue-specific conditional gene expression in zebrafish. Proc. Natl. Acad. Sci. USA *107*, 19933–19938.

Knopf, F., Hammond, C., Chekuru, A., Kurth, T., Hans, S., Weber, C.W., Mahatma, G., Fisher, S., Brand, M., Schulte-Merker, S., and Weidinger, G. (2011). Bone regenerates via dedifferentiation of osteoblasts in the zebrafish fin. Dev. Cell *20*, 713–724.

Kratochwil, K., Dull, M., Farinas, I., Galceran, J., and Grosschedl, R. (1996). Lef1 expression is activated by BMP-4 and regulates inductive tissue interactions in tooth and hair development. Genes Dev. *10*, 1382–1394.

Laforest, L., Brown, C.W., Poleo, G., Géraudie, J., Tada, M., Ekker, M., and Akimenko, M.A. (1998). Involvement of the sonic hedgehog, patched 1 and bmp2 genes in patterning of the zebrafish dermal fin rays. Development *125*, 4175–4184.

Langfelder, P., and Horvath, S. (2008). WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics *9*, 559.

Lee, Y., Grill, S., Sanchez, A., Murphy-Ryan, M., and Poss, K.D. (2005). Fgf signaling instructs position-dependent growth rate during zebrafish fin regeneration. Development *132*, 5173–5183.

Lee, Y., Hami, D., De Val, S., Kagermeier-Schenk, B., Wills, A.A., Black, B.L., Weidinger, G., and Poss, K.D. (2009). Maintenance of blastemal proliferation by functionally diverse epidermis in regenerating zebrafish fins. Dev. Biol. *331*, 270–280.

Lin, G., and Slack, J.M. (2008). Requirement for Wnt and FGF signaling in Xenopus tadpole tail regeneration. Dev. Biol. *316*, 323–335.

Long, F. (2012). Building strong bones: molecular regulation of the osteoblast lineage. Nat. Rev. Mol. Cell Biol. *13*, 27–38.

Lu, J., Ma, Z., Hsieh, J.C., Fan, C.W., Chen, B., Longgood, J.C., Williams, N.S., Amatruda, J.F., Lum, L., and Chen, C. (2009). Structure-activity relationship studies of small-molecule inhibitors of Wnt response. Bioorg. Med. Chem. Lett. *19*, 3825–3827.

MacDonald, B.T., Tamai, K., and He, X. (2009). Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev. Cell *17*, 9–26.

Marí-Beffa, M., Santamaría, J.A., Murciano, C., Santos-Ruiz, L., Andrades, J.A., Guerado, E., and Becerra, J. (2007). Zebrafish fins as a model system for skeletal human studies. ScientificWorldJournal *7*, 1114–1127.

Mathew, L.K., Sengupta, S., Franzosa, J.A., Perry, J., La Du, J., Andreasen, E.A., and Tanguay, R.L. (2009). Comparative expression profiling reveals an essential role for raldh2 in epimorphic regeneration. J. Biol. Chem. *284*, 33642–33653.

Moro, E., Ozhan-Kizil, G., Mongera, A., Beis, D., Wierzbicki, C., Young, R.M., Bournele, D., Domenichini, A., Valdivia, L.E., Lum, L., et al. (2012). In vivo Wnt signaling tracing through a transgenic biosensor fish reveals novel activity domains. Dev. Biol. *366*, 327–340.

Münch, J., González-Rajal, A., and de la Pompa, J.L. (2013). Notch regulates blastema proliferation and prevents differentiation during adult zebrafish fin regeneration. Development *140*, 1402–1411.

Nechiporuk, A., and Keating, M.T. (2002). A proliferation gradient between proximal and msxb-expressing distal blastema directs zebrafish fin regeneration. Development *129*, 2607–2617.

Poss, K.D., Shen, J., Nechiporuk, A., McMahon, G., Thisse, B., Thisse, C., and Keating, M.T. (2000). Roles for Fgf signaling during zebrafish fin regeneration. Dev. Biol. *222*, 347–358.

Quint, E., Smith, A., Avaron, F., Laforest, L., Miles, J., Gaffield, W., and Akimenko, M.A. (2002). Bone patterning is altered in the regenerating zebrafish caudal fin after ectopic expression of sonic hedgehog and bmp2b or exposure to cyclopamine. Proc. Natl. Acad. Sci. USA *99*, 8713–8718.

Regard, J.B., Zhong, Z., Williams, B.O., and Yang, Y. (2012). Wnt signaling in bone development and disease: making stronger bone with Wnts. Cold Spring Harb. Perspect. Biol. *4*, 4.

Reya, T., and Clevers, H. (2005). Wnt signalling in stem cells and cancer. Nature *434*, 843–850.

Shimizu, N., Kawakami, K., and Ishitani, T. (2012). Visualization and exploration of Tcf/Lef function using a highly responsive Wnt/β-catenin signaling-reporter transgenic zebrafish. Dev. Biol. *370*, 71–85.

Singh, B.N., Doyle, M.J., Weaver, C.V., Koyano-Nakagawa, N., and Garry, D.J. (2012). Hedgehog and Wnt coordinate signaling in myogenic progenitors and regulate limb regeneration. Dev. Biol. *371*, 23–34.

Smith, A., Avaron, F., Guay, D., Padhi, B.K., and Akimenko, M.A. (2006). Inhibition of BMP signaling during zebrafish fin regeneration disrupts fin growth and scleroblasts differentiation and function. Dev. Biol. *299*, 438–454.

Stewart, S., Gomez, A.W., Armstrong, B.E., Henner, A., and Stankunas, K. (2014). Sequential and opposing activities of Wnt and BMP coordinate

zebrafish bone regeneration. Cell Rep. *6*. Published online January 30, 2014. http://dx.doi.org/10.1016/j.celrep.2014.01.010.

Stoick-Cooper, C.L., Moon, R.T., and Weidinger, G. (2007a). Advances in signaling in vertebrate regeneration as a prelude to regenerative medicine. Genes Dev. *21*, 1292–1315.

Stoick-Cooper, C.L., Weidinger, G., Riehle, K.J., Hubbert, C., Major, M.B., Fausto, N., and Moon, R.T. (2007b). Distinct Wnt signaling pathways have opposing roles in appendage regeneration. Development *134*, 479–489.

Tu, S., and Johnson, S.L. (2011). Fate restriction in the growing and regenerating zebrafish fin. Dev. Cell *20*, 725–732.

Weidinger, G., Thorpe, C.J., Wuennenberg-Stapleton, K., Ngai, J., and Moon, R.T. (2005). The Sp1-related transcription factors sp5 and sp5-like act downstream of Wnt/beta-catenin signaling in mesoderm and neuroectoderm patterning. Curr. Biol. *15*, 489–500.

Whyte, J.L., Smith, A.A., and Helms, J.A. (2012). Wnt signaling and injury repair. Cold Spring Harb. Perspect. Biol. *4*, a008078.

Yeo, S.Y., Kim, M., Kim, H.S., Huh, T.L., and Chitnis, A.B. (2007). Fluorescent protein expression driven by her4 regulatory elements reveals the spatiotemporal pattern of Notch signaling in the nervous system of zebrafish embryos. Dev. Biol. *301*, 555–567.

Yokoyama, H., Ogino, H., Stoick-Cooper, C.L., Grainger, R.M., and Moon, R.T. (2007). Wnt/beta-catenin signaling has an essential role in the initiation of limb regeneration. Dev. Biol. *306*, 170–178.

Zhang, J., Wagh, P., Guay, D., Sanchez-Pulido, L., Padhi, B.K., Korzh, V., Andrade-Navarro, M.A., and Akimenko, M.A. (2010). Loss of fish actinotrichia proteins and the fin-to-limb transition. Nature *466*, 234–237.