Distinct Wnt signaling pathways have opposing roles in appendage regeneration

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INTRODUCTION

All organisms mount a biological response to damage, but they vary widely in their ability to recover. Humans constantly renew components of blood, skeletal muscle and epithelia. Such homeostatic renewal is thought to be mediated by resident stem cells of a specific lineage. Although humans can regenerate an injured liver and repair limited insults to bone, muscle, digit tips and cornea, they do not regenerate the heart, spinal cord, retina or limbs. Thus, humans and other mammals are somewhat disadvantaged when compared with amphibians and teleost fish, which have a remarkable capacity to regenerate damaged organs including heart, spinal cord, retina and limbs/fins (Akimenko et al., 2003; Brockes and Kumar, 2002; Poss et al., 2003; Poss et al., 2002). A dramatic example of organ regeneration is that of amphibian limbs and fish fins, where intrinsic structures consisting of multiple cell types that are patterned into complex tissues are faithfully restored after amputation. The mechanisms that enable lower vertebrates to re-establish such structures and the reasons why mammals are not able to do so, are incompletely understood. Elucidation of these mechanisms and an understanding of why regenerative capacity has diminished in vertebrate evolution hold the potential to revolutionize clinical medicine, with practical applications ranging from organ disease and wound treatment to possible alternatives to prosthetics for amputees.

Whereas repair of many organs, such as the chicken retina or mouse liver, is thought to be mediated through activation of resident stem cells or proliferation of normally quiescent differentiated cells, respectively (Fausto et al., 2006; Fischer and Reh, 2001), amphibian and fish appendages regenerate through a process termed ‘epimorphic regeneration’, sometimes called ‘true’ regeneration. This occurs in three steps: (1) wound healing and formation of the wound epidermis; (2) formation of a regeneration blastema, a population of mesenchymal progenitor cells that is necessary for proliferation and patterning of the regenerating limb/fins; and (3) regenerative outgrowth and pattern reformation (Akimenko et al., 2003; Poss et al., 2003). Progenitor cells of the blastema in the regenerating axolotl tail can be formed by reprogramming and de-differentiation of differentiated cells (Casimir et al., 1988; Echeverri et al., 2001; Echeverri and Tanaka, 2002; Kintner and Brockes, 1984; Lentz, 1969; Lo et al., 1993). These cells express transcriptional repressors of the msx gene family that may help maintain a pluripotent state (Akimenko et al., 1995; Yokoyama et al., 2001). Recently, activation of resident muscle stem cells has been reported in regenerating salamander limbs (Morrison et al., 2006). Thus, it is likely that de-differentiation and stem cell activation both contribute to formation of the blastema. Although de-differentiation of cells has not yet been shown to occur in regenerating structures other than amphibian limbs and tails, the morphology, ontology and gene expression profile of the zebrafish blastema in the regenerating tail fin suggest that zebrafish tail regeneration occurs by similar mechanisms.

In contrast to mammals, lower vertebrates have a remarkable capacity to regenerate complex structures damaged by injury or disease. This process, termed epimorphic regeneration, involves progenitor cells created through the reprogramming of differentiated cells or through the activation of resident stem cells. Wnt/β-catenin signaling regulates progenitor cell fate and proliferation during embryonic development and stem cell function in adults, but its functional involvement in epimorphic regeneration has not been addressed. Using transgenic fish lines, we show that Wnt/β-catenin signaling is activated in the regenerating zebrafish tail fin and is required for formation and subsequent proliferation of the progenitor cells of the blastema. Wnt/β-catenin signaling appears to act upstream of FGF signaling, which has recently been found to be essential for fin regeneration. Intriguingly, increased Wnt/β-catenin signaling is sufficient to augment regeneration, as tail fins regenerate faster in fish heterozygous for a loss-of-function mutation in axin1, a negative regulator of the pathway. Likewise, activation of Wnt/β-catenin signaling by overexpression of wnt8 increases proliferation of progenitor cells in the regenerating fin. By contrast, overexpression of wnt5b (pipetail) reduces expression of Wnt/β-catenin target genes, impairs proliferation of progenitors and inhibits fin regeneration. Importantly, fin regeneration is accelerated in wnt5b mutant fish. These data suggest that Wnt/β-catenin signaling promotes regeneration, whereas a distinct pathway activated by wnt5b acts in a negative-feedback loop to limit regeneration.

KEY WORDS: Wnt, zebrafish, regeneration, β-catenin, dickkopf, wnt8, wnt5, pipetail, axin1, masterblind

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A major question that remains incompletely answered involves the identification of the extracellular signals that regulate the formation or activation of stem cells during regeneration. Although hedgehog signaling has been implicated in newt tail and chick retina regeneration (Schnapp et al., 2005; Spence et al., 2004), and BMP signaling in newt lens and Xenopus tail regeneration (Beck et al., 2003; Grogg et al., 2005), the strongest evidence to date points to FGF signaling as an essential regulator of progenitor cell formation in limb and fin regeneration. FGF-10 is sufficient to reactivate regeneration in Xenopus limbs at later stages of development where limbs have lost their regenerative capacity (Yokoyama et al., 2001), and FGF-2-soaked beads can stimulate chick limbs, which normally do not regenerate, to do so (Taylor et al., 1994). Inhibition of FGF signaling by pharmacological inhibitors or expression of a dominant-negative FGF receptor blocks blastema formation in zebrafish caudal fin regeneration (Lee et al., 2005; Poss et al., 2000b), and a mutation in zebrafish fgf20a causes an early block in blastema formation (Whitehead et al., 2005).

Wnt/β-cat signaling regulates progenitor cell fate and proliferation during embryonic development and in adult tissue homeostasis (Logan and Nusse, 2004; Reya and Clevers, 2005), raising the possibility that it is also involved in progenitor cell function during regeneration. Several studies have documented expression of Wnt ligands and components of the β-catenin signaling pathway in regenerating amphibian and fish appendages (Caubet et al., 1997a; Caubet et al., 1997b; Poss et al., 2000a), and other studies have suggested that Wnt/β-catenin signaling is functionally involved in the proliferation of cells during regeneration of mammalian muscle, liver and bone (Poleskaya et al., 2003; Sodhi et al., 2005; Zhong et al., 2006). However, whether Wnt/β-catenin signaling plays an essential role in the epimorphic, ‘true’ regeneration of complex structures has not been tested.

Many Wnt ligands can activate β-catenin-independent ('noncanonical') signaling pathways (Slusarski et al., 1997; Veeman et al., 2003) that are well documented to regulate cell polarity and cell migration during embryonic development (Veeman et al., 2003). However, other than reports which indicate that β-catenin-independent Wnt signaling might act to suppress tumor formation (Dejmek et al., 2005; Jonsson et al., 2002; Kremenevskaja et al., 2005), nothing is known about its role in adults. Here, we provide evidence that both β-catenin-dependent and -independent Wnt signaling pathways regulate zebrafish fin regeneration.

MATERIALS AND METHODS

Zebrafish surgeries

Zebrafish of 6-12 months of age were used for all studies. Zebrafish heart and fin amputations were performed as previously described (Poss et al., 2000a; Raya et al., 2004), after which fish were returned to 28-30°C water.

Partial hepatectomy in TOPGAL mice

TOPGAL mice (a gift from E. Fuchs, Rockefeller University, NY) have been described previously (DasGupta and Fuchs, 1999). We performed 2/3 partial hepatectomy (Campbell et al., 2006) and sham laparotomy on 8- to 11-week-old male TOPGAL mice in the morning after a night of fasting. Resected lobes were collected and served as control tissue for subsequent experiments; remnant livers were harvested 48 hours later. β-galactosidase activity was determined in whole liver lysates as per manufacturer's instructions (Promega, Madison, WI), and normalized to total protein concentration as determined by the Bradford assay (Bio-Rad, Hercules, CA). X-Gal staining was performed on glutaraldehyde-fixed 5 μm frozen liver sections as per manufacturer’s instructions (Gold Biotechnology, St Louis, MO).

Cloning of zebrafish wnt5a

Zebrafish genomic sequence was searched for sequences homologous to the previously known zebrafish Wnt5 ortholog, pipetalll (ppt). A sequence distinct from ppt was identified and a partial cDNA coding for this wnt5 paralog cloned by RT-PCR from a mixture of RNA isolated at different stages of embryonic development. The 5′ end of the cDNA was defined by RACE and by homology to EST 052-H12-2. The very 3′ end of the open reading frame and a putative 3′ UTR were predicted from genomic sequences, but have not been experimentally verified. BLAST searches, multiple sequence alignments of the predicted protein sequence with Wnt5 paralogs from other species and phylogenetic analysis using the PAUP program support the conclusion that the previously described zebrafish wnt5 paralog ppt is the zebrafish ortholog of wnt5b, whereas the newly cloned paralog described here is most likely to be the ortholog of wnt5a (see Fig. S2 in the supplementary material). We thus deposited the new sequence as wnt5a in GenBank (accession number DQ465921) and suggested that wnt5 (pipetalll; ppt) should be renamed wnt5b, which has now been done.

In situ hybridization

Whole-mount in situ hybridization was performed on amputated fins and hearts as described previously (Poss et al., 2000a). For Digoxigenin-labeled probe synthesis, published templates were used, except for wnt5a cDNA, which was cloned by RT-PCR from RNA isolated from embryos at different stages of development. When assaying for differences in expression, the development of the staining reaction was monitored carefully and fins or hearts of the same comparative groups were stopped at exactly the same time. Cryosectioning of the fins was performed as described previously (Poss et al., 2000b).

Heat-shock inducible transgenic zebrafish lines

The hsDkk1GFP and hsWnt5bGFP lines were established as follows (see Fig. S3 in the supplementary material). mmGFP5 (Siemerling et al., 1996) was fused to the C-terminus of zebrafish dkk1 (Genbank accession # AB023488). Upon injection into zebrafish embryos, RNA encoding this fusion protein was found to cause posterior truncations, and increased the size of eyes and forebrain at similar doses as the wild-type dkk1 RNA (data not shown). Likewise, mmGFP5 was fused to the C-terminus of zebrafish wnt5b/pipetalll (see Fig. S2 in the supplementary material for nomenclature; Genbank accession # DRU51268). Injection of RNA coding for this fusion protein into early zebrafish embryos caused similar gastrulation defects as RNA coding for the wild-type Wnt5b protein, but the fusion protein appeared to be significantly less active (data not shown). Both fusion proteins were cloned downstream of a 1.5 kb fragment of the zebrafish hsp70-4 promoter (Halloran et al., 2000) and upstream of the SV40 polyadenylation signal of the vector pCS2+. An I-SceI meganuclease restriction site was inserted 5′ of the transgene. Supercoiled plasmid DNA containing the transgenes was injected together with I-SceI meganuclease (Thermes et al., 2002) into one-cell-stage embryos to create mosaic G0 founder fish. Founders that transmitted a functional transgene through their germline were identified by crossing them to wild-type fish, heat shocking the resulting F1 embryos and screening them for GFP expression. Transgenic F1 embryos were found to be viable when heat shocked at 24 hpf or later and therefore could be raised to adulthood. To establish transgenic lines, identified heterozygous F1 fish were crossed to wild-type fish and the F2 generation raised. For most experiments on adult fish, wild-type siblings from such crosses served as controls. When siblings could not be used, age-matched wild types served as controls.

Heat shocks for these lines and the hsTUCGF/GFP and hsWnt5GFP lines were performed twice daily by transferring fish from 28-30°C water to water preheated to 38°C with subsequent incubation in an air incubator at 39°C for 1 hour.

Tissue sectioning and histology

Hematoxylin staining and histology were performed as previously described (Poss et al., 2002) on 20 μM cryostat sections.

BrdU incorporation and mitosis analysis

BrdU incorporation and mitosis analysis were performed as previously described (Nechiporuk and Keating, 2002). All BrdU incorporations were performed for the final 1-2 hours of the experiment. Sections were rinsed.
three times in PBS, then incubated in 2N HCl for 30 minutes at 37°C. Sections were then briefly rinsed in PBS three times and incubated in blocking solution (1% Triton X-100 and 0.25% BSA in PBS) for at least 1 hour. Slides were incubated in mouse anti-BrdU (1:200; Sigma, St Louis, MO) and rabbit anti-phosphorylated histone H3 (PH3; 1:200, Upstate Biotechnology, Charlotteville, VA) antibodies overnight at room temperature. Slides were then washed all day with multiple changes of PBS and then incubated in secondary antibodies (goat anti-mouse Alexa-fluor-546; goat anti-rabbit Alexa-fluor-488, Molecular Probes) for 1-2 hours at room temperature. Slides were rinsed three times in PBS (20 minutes each) and mounted with DAPI mounting media and coverslipped. DAPI-stained nuclei, BrdU-positive cells and PH3-positive cells were counted from 3-6 sections per fin from three fins per wild-type or transgenic sample. n-number of blastemas counted per experiment.

**Fin length measurements in axin1 and wnt5b mutant fish**

Heterozygous carriers of the axin1 mutation masterblind (mb

**Semi-quantitative and quantitative RT-PCR**

Total RNA was extracted from zebrafish fin regenerates using TRIZOL according to the manufacturer’s protocol (Invitrogen). RNA was digested with DNase and purified using the Qiagen RNeasy Kit. Equal amounts of total RNA from each sample were reverse transcribed with Thermoscript reverse transcriptase (Invitrogen) using oligo(dT) and random hexamer primers. For semi-quantitative PCR, amplification of ornithine decarboxylase (odc1) was used as the loading control, fgf20a (primers 5'-GCAGATTGTTGATATGGGACACAT-3' and 5'-CTGACAACATCTTTGAAGTCAGG-3') and odc1 (primers 5'-ACTTTGAGGTCTGGGAGGAT-3' and 5'-CAAGGACCATACGGCG-3') PCR products were detected on Ethidium Bromide-stained agarose gels. Quantitative PCR was performed using a Roche Lightcycler and the SYBR Green Labeling System. wnt10a was amplified using primers 5'-ATTCACTCCCCAGGAGTTCACTG-3' and 5'-GGTTGAGATGTTGGGCTTTGTG-3' wnt10a expression levels were normalized to β-actin (primers 5'-GGATCCAGAAAAAGACAG-3' and 5'-AGAGTCATCAGACGATCAG-3') or 18S rRNA (primers 5'-CGCTATTGAGCTGGAATTACC-3' and 5'-GAAACCAGCTCACATCCAA-3') levels. Primers for quantitative PCR of fgf20a were 5'-CAGCTTCTCTTCACGCTTG-3' and 5'-AAAGCTCAGGAACTGCTCTG-3' (Whitehead et al., 2005).

**RESULTS**

**Wnt/β-catenin signaling is activated during regeneration**

Since the zebrafish tail fin is a good model for epimorphic regeneration (Poss et al., 2003) and is highly amenable to experimental manipulation, we studied the role of Wnt signaling in regeneration using the zebrafish fin model. Expression of Wnt ligands and of components of the β-catenin signaling pathway has been reported in regenerating amphibian and fish appendages (Caubit et al., 1997a; Caubit et al., 1997b; Poss et al., 2000a), suggesting that Wnt/β-catenin signaling is upregulated during regeneration. The endpoint of Wnt/β-catenin signaling is transcriptional regulation of target genes; however, because Wnt signaling is tightly regulated by extracellular, cytoplasmic and nuclear inhibitors, expression of Wnt ligands does not necessarily result in activation of transcription. Thus, to test whether the Wnt/β-catenin pathway is functional during zebrafish fin regeneration, we asked whether a transcriptional reporter of Wnt/β-catenin signaling, ToppGFP (Dorsky et al., 2002), is activated in response to fin amputation in ToppGFP transgenic zebrafish. We found that ToppGFP is detectable in the blastema of the regenerating fin at 2 days post-amputation (dpa) (Fig. 1A). We also found that the expression of axin2, which has been shown to be a direct Wnt target gene in several systems (Jho et al., 2002; Leung et al., 2002; Lustig et al., 2002; Weidinger et al., 2005) and of sp8, which is regulated by Wnt/β-catenin signaling in fin and limb development (Kawakami et al., 2004), were upregulated in regenerating zebrafish tail fins (Fig. 1B). We then investigated which Wnt ligands might be responsible for activation of Wnt/β-catenin signaling during regeneration of the tail fin. We found that wnt10a, which has been shown to activate Wnt/β-catenin signaling during limb development (Narita et al., 2005), is expressed early during regeneration. Expression of wnt10a was detected in the distal tip of the blastema (Fig. 1B). Using quantitative PCR, we found that expression of wnt10a was upregulated very early during regeneration, expression being 2.3-fold higher than in uncut fins 3 hours post-amputation (hpa) and 5.3-fold higher at 6 hpa (Fig. 1C). Thus, wnt10a is an excellent candidate for a Wnt ligand responsible for early activation of the β-catenin signaling pathway during fin regeneration. Interestingly, we found that Wnt signaling activity, as detected by transgenic reporters, is also upregulated during zebrafish heart and mouse liver regeneration (see Fig. S1 in the supplementary material), suggesting that activation of Wnt/β-catenin signaling may be a conserved feature of regeneration.

Wnt/β-catenin signaling is required for fin regeneration

To test the requirement of Wnt/β-catenin signaling for fin regeneration we created a line of zebrafish that are transgenic for heat-shock inducible Dickkopf1 (hsDkk1GFP, see Fig. S3 in the supplementary material), a secreted inhibitor of Wnt/β-catenin signaling (Glinka et al., 1998). Activation of the transgene during embryogenesis phenocopies the effects of wnt8 loss-of-function (see Fig. S3G-I in the supplementary material) and is sufficient to suppress expression of the ToppGFP Wnt/β-catenin reporter in doubly transgenic embryos 3 hours after induction (see Fig. S3J-K in the supplementary material). Heat shock induces ubiquitous
expression of the transgene (as monitored by GFP expression) in embryos and regenerating adult tail fins (see Fig. S3C-F in the supplementary material). Thus, this transgenic line represents an excellent tool to study the functions of Wnt/β-catenin target genes (Lewis et al., 2004). When we shocked the fish 2 hours before fin amputation and continued to heat shock twice daily for 7 days, we found that regeneration was completely blocked in both hsDkk1GFP and hsΔTcfGFP transgenic fish, whereas regeneration in heat-shocked wild-type fish was unperturbed (Fig. 2A, B).

Fin regeneration can be divided into three phases: wound healing, which happens within 24 hpa at 29°C; blastema formation (approximately 24–48 hpa); and regenerative outgrowth (starting around 48 hpa). Our inducible transgenic system allowed us to test when Wnt/β-catenin signaling is required during regeneration. Interestingly, when we started to inhibit Wnt/β-catenin signaling by heat shock after wound healing had taken place, but before the regeneration blastema had formed (24 hpa, Fig. 2A), regeneration was again completely blocked (Fig. 2C, left panel). Thus, impaired regeneration in Dkk1-overexpressing fish is not a consequence of failed wound healing, but instead is due to a specific requirement for Wnt/β-catenin signaling during blastema formation. We also asked whether Wnt/β-catenin signaling is important for the outgrowth phase of fin regeneration. To do this, we began heat shocking hsDkk1GFP transgenic fish at 72 hpa (Fig. 2A). These fish displayed incomplete regeneration (Fig. 2C, right panel), indicating that Wnt/β-catenin signaling is not only required for formation of the blastema, but subsequently for blastema maintenance and/or proliferation.

**Wnt/β-catenin signaling regulates blastema formation and subsequent proliferation**

To characterize the cell biological functions of Wnt/β-catenin during fin regeneration, we carried out assays to test for specific effects of Dkk1 overexpression on cell specification and proliferation. Heat shock of hsDkk1GFP fish starting shortly before amputation resulted in a loss of expression of left1, a marker for the basal epidermis (Poss et al., 2000a), by 24 hpa, indicating that the basal layer of the wound epidermis was not being specified correctly (Fig. 3A). We also found that expression of msxb, a marker for the mesenchymal progenitor cells of the regeneration blastema (Poss et al., 2000b), and of shh, which is normally expressed within basal epidermal cells (Poss et al., 2000b), was lost by 72 hpa in Dkk1-expressing fins (Fig. 3A). Histological examination confirmed that formation of the regeneration blastema was severely impaired in hsDkk1GFP fish, although the wound healed properly (Fig. 3B). These data show that neither the blastema mesenchyme nor the overlying epithelium are specified correctly following loss of Wnt/β-catenin signaling.
To test whether Wnt/β-catenin signaling is required for proliferation of the blastema, we inhibited Wnt signaling by a single pulse of Dkk1 expression in regenerating fins during the outgrowth phase of regeneration at 3 dpa. We assayed for cell proliferation 6 hours after heat shock using BrdU incorporation and staining for phosphorylated histones. We observed that loss of Wnt/β-catenin signaling lead to a reduction in proliferation of both the blastema mesenchyme and the overlying epithelium (Fig. 3C,D). Thus, Wnt/β-catenin signaling is required for the formation and subsequent proliferation of the blastema.

**Wnt/β-catenin signaling is sufficient to enhance regeneration**

We next investigated whether enhanced Wnt/β-catenin signaling is sufficient to augment regeneration. To activate Wnt/β-catenin signaling we used transgenic fish that overexpress Wnt8 after heat shock (hsWnt8GFP) (Weidinger et al., 2005). During embryogenesis, heat shock of these fish causes characteristic Wnt/β-catenin gain-of-function phenotypes (Weidinger et al., 2005).

Induction of Wnt8 during fin regeneration increased expression of the Wnt/β-catenin target gene *axin2* (Fig. 4A), showing that overexpression of Wnt8 in the fin is sufficient to augment β-catenin signaling. Importantly, overexpression of Wnt8 at 72 hpa significantly increased proliferation of the blastema mesenchyme and overlying epithelium 6 hours after induction of the transgene, as...
detected by BrdU incorporation and anti-phosphorylated histone H3 antibody staining (Fig. 4B). Despite its ability to increase proliferation, overexpression of Wnt8 had no consistent effect on fin length by 10 dpa (Fig. 4C). However, the short half-life of Wnt proteins and the pulsed activation of the transgene raise the question of whether a more prolonged and consistent activation of the pathway might be sufficient to augment overall fin regeneration.

To test this, we made use of fish in which one copy of axin1, an inhibitor of the Wnt/β-catenin signaling pathway, is mutated (Heisenberg et al., 2001), and asked whether axin1+/– fins regenerate more rapidly. To minimize effects of the genetic background, we used wild-type and axin1+/– fish that were siblings derived from a cross of a wild-type fish with an axin1 heterozygous carrier. We genotyped the fish, amputated fins of 12 wild-type and 9 axin1 heterozygous mutant fish, allowed them to regenerate for 7 days, photographed the fins, blinded the photographs, measured the length of the regenerate (from the amputation plane to the distal tip of the fin) in the third, fourth and fifth dorsal fin ray in each fish and calculated the average length of the regenerate of each fish (experiment 1). The same fish were

Fig. 4. β-catenin-dependent and β-catenin-independent Wnt signaling pathways have opposing roles in zebrafish fin regeneration. (A, D) Overexpression of Wnt8 in hsWnt8GFP transgenic fish induces the Wnt/β-catenin target gene axin2 in regenerating fins 6 hours after heat shock at 3 dpa (A; 3 of 4 fins), whereas overexpression of Wnt5b in hsWnt5bGFP transgenic fish represses axin2 expression (D; n=4). Note that staining reactions were stopped as soon as a robust signal could be detected in most samples of one experimental group. Robust signal was first detected in hsWnt8GFP fins (versus wild-type controls) after a short amount of time and reactions were stopped (A), whereas robust signal was first detected in wild-type controls (versus hsWnt5GFP fins) after a longer staining reaction (D), thus accounting for the difference in wild-type signal between groups. (B, E) Cell proliferation in regenerating fins, as detected by BrdU incorporation and staining with an anti-PH3 antibody, is increased by overexpression of Wnt8 (B; n=14), and repressed by overexpression of Wnt5b (E; n=10). Fish were heat shocked once at 66 hpa and fixed at 72 hpa. The percentage of BrdU-positive or PH3-positive cells relative to the total number of cells in sections of regenerating fins is shown. Error bars represent the s.e.m. (C, F) Whereas overexpression of Wnt8 for 10 days starting shortly before amputation has no obvious effect on overall length of the regenerate (C; n=16), overexpression of Wnt5b completely inhibits regeneration (F; n=16). (B) *P=0.0579; **P=0.0082; ***P=0.0002 (two-tailed). (E) *P=0.0377; **P=0.0123; ***P=0.0006 (two-tailed).

Fig. 5. Fins regenerate faster in fish heterozygous for a loss-of-function mutation in axin1. (A) Average length of regenerating tail fins at 7 dpa is increased in fish heterozygous for an axin1 loss-of-function mutation (mblm013) compared with wild-type siblings. Results of one representative experiment of three are shown. To determine the length of the regenerate for individual fish, the average length of the third, fourth and fifth dorsal regenerating fin ray was calculated. n=12 wild-type fish and 9 mbl heterozygous fish; error bars represent the s.e.m. of the average regenerate lengths; ***P=0.0009 (one-tailed). (B) The number of fin rays (in percent of the total number counted) is plotted against the length of the regenerate (in 0.1 mm intervals) for wild-type (upper bar graph) and axin1 heterozygous fish (lower bar graph). The curves represent a fifth-order polynomial trendline. The average regenerate length is marked by black bars at the x-axis. 148 fin rays were counted (combined results from three experiments) in 19 wild-type fish and 94 rays were counted in 10 axin1+/– fish.
Wnt signaling regulates regeneration

As Wnts that can act through the Wnt/β-catenin pathway (wnt10a) and through β-catenin-independent pathways (wnt5a, wnt5b) are expressed during fin regeneration (Fig. 1), we next tested whether these distinct Wnt pathways might have different roles in fin regeneration. We compared the effects of activation of Wnt/β-catenin signaling with those produced by activation of β-catenin-independent Wnt signaling. To this end, we generated a transgenic zebrafish line carrying a heat-shock inducible Wnt5bGFP transgene (hsWnt5bGFP; see Fig. S3 in the supplementary material). Wnt5b has been shown to activate β-catenin-independent signaling pathways in zebrafish embryos (Westfall et al., 2003). Accordingly, heat-shocked hsWnt5bGFP embryos display the characteristic phenotypes associated with gain-of-function of β-catenin-independent Wnt pathways, namely defects in convergence-extension cell movements during gastrulation and somitogenesis (see Fig. S3L,M in the supplementary material).

Interestingly, whereas overactivation of Wnt/β-catenin signaling enhances regeneration, overexpression of Wnt5b represses regeneration. Heat shock of hsWnt5bGFP transgenic fish for 10 days starting shortly before fin amputation completely inhibited fin regeneration (Fig. 4F). This is in marked contrast to the effects of overexpressing Wnt8, which had no obvious effect on overall fin morphology (Fig. 4C), but closely resembled the defects caused by inhibition of Wnt/β-catenin signaling via Dkk1 overexpression (Fig. 2B). As with overexpression of Dkk1, but in contrast to Wnt8, overexpression of Wnt5b significantly reduced proliferation of the blastema mesenchyme and overlying epithelium 6 hours after induction of the transgene, as detected by BrdU incorporation and anti-phosphorylated histone H3 antibody staining (Fig. 4E). Thus, activation of Wnt5b inhibits fin regeneration.

Although it is difficult to test which signaling pathways Wnt5b activates in the regenerating fin, the fact that it causes dramatically different effects than Wnt8, which signals via β-catenin, suggests that it is likely to act through β-catenin-independent pathways. Since Wnt5b overexpression causes the same phenotypes as Wnt/β-catenin loss-of-function, and because β-catenin-independent Wnt signaling has been reported to be able to inhibit Wnt/β-catenin signaling in other systems (Weidinger and Moon, 2003), we hypothesize that Wnt5b overexpression inhibits fin regeneration by repressing Wnt/β-catenin signaling. In support of this model, we found that Wnt5b overexpression abolished expression of the direct Wnt/β-catenin target gene axin2 6 hours after heat shock at 3 dpa (Fig. 4D).

wnt5b loss-of-function augments fin regeneration

We next tested whether endogenous wnt5b acts as an essential modulator of fin regeneration. If non-canonical Wnt signaling activated by wnt5b inhibits regeneration in vivo, loss of wnt5b function in the regenerating fin might result in enhanced or faster regeneration. To test this prediction, we made use of homozygous adult wnt5b (pipetail) mutant fish. We amputated tail fins of wnt5b mutant and age- and size-matched wild-type fish of the same genetic background, measured the length of the third, fourth and fifth dorsal fin ray at 4 and 7 dpa, and calculated the average length of the regenerate for each fish (Fig. 6A). In two independent sets of experiments using different fish (experiments 1 and 2), we found that wnt5b mutants had significantly longer regenerates than wild types at both 4 and 7 dpa (Fig. 6A,B and see Table S2 in the supplementary material). The difference in length between wild-type and wnt5b mutant regenerates increased between 4 and 7 dpa.

Fig. 6. Fins regenerate faster in wnt5b mutant fish. (A) Dorsal half of regenerating tail fins of wild-type and wnt5b (ppt) homozygous mutant fish at 7 dpa. The amputation plane is indicated by a dashed red line, the length of the third fin ray by red or green bars and arrows. Note that the regenerate is longer in ppt than in wild-type fish. (B) The average length of the regenerate of wild-type and ppt mutant fish at 4 and 7 dpa in two independent experiments. To determine the length of the regenerate for individual fish, the average length of the third, fourth and fifth dorsal regenerating fin ray was calculated. Experiment 1: n=14 wild-type, 12 ppt fish. Experiment 2: n=12 wild-type, 11 ppt fish. Error bars represent the s.e.m. of the average regenerate lengths. For P values, see Table S2 in the supplementary material. Note that absolute fin lengths cannot be compared between experiments because water temperatures and thus regenerative speed and exact times of photography varied between experiments.
showing that wnt5b mutant fins regenerate faster (Fig. 6B). These data provide genetic evidence that wnt5b acts as a negative modulator of fin regeneration. Wnt5b mutant regenerating fins did not show any obvious patterning defects or indications of tumor formation or other signs of inappropriate growth (Fig. 6A), suggesting that wnt5b is only required to modulate the overall rate of regeneration.

It is tempting to speculate that this antagonistic role of β-catenin-independent signaling regulated by wnt5b (and possibly wnt5a) during fin regeneration represents a negative feedback mechanism that regulates the levels and/or duration of Wnt/β-catenin signaling. If so, we would expect the expression of wnt5 paralogs to be regulated by Wnt/β-catenin signaling in the regenerating fin. Indeed, we found that wnt5b expression is downregulated in Dkk1-overexpressing fins 6 hours after heat shock at 3 dpa (see Fig. S4 in the supplementary material). Taken together, these data strongly suggest that β-catenin-independent Wnt signaling activated by wnt5b and possibly wnt5a acts in a negative feedback loop to inhibit Wnt/β-catenin signaling during fin regeneration (Fig. 8).

**Fig. 7. Wnt/β-catenin signaling regulates FGF signaling during fin regeneration.** (A) fgf20a expression as detected by semi-quantitative RT-PCR is greatly reduced in Dkk1-overexpressing fins at 3 hpa. Wild-type and hsDkk1GFP transgenic fish were treated according to the schematic (hs, heat shock; amp, amputation; the green line indicates inhibition of Wnt/β-catenin signaling), and RNA was harvested from the tissue adjacent to the amputation plane of nine wild-type and two groups of nine hsDkk1GFP transgenic fins. odc1 amplification serves as a loading control. The experiment was repeated four times using two sets of biological samples and representative results are shown. (B) fgf20a expression is greatly reduced by in situ hybridization in hsDkk1GFP transgenic fins (5 of 6 fins) at 24 hpa as compared with wild-type fins. (C) sprouty4 expression is greatly reduced 6 hours after heat shock in hsDkk1GFP fins (n=3) at 72 hpa.

**Fig. 8. A model of signaling events regulating zebrafish fin regeneration.** We propose that injury of the tail fin activates a β-catenin-dependent signaling pathway that positively regulates fgf20a expression, which has been shown to be required for blastema formation and subsequent regeneration (Whitehead et al., 2005). In addition to its role in regulating fgf20a expression, Wnt/β-catenin signaling might also regulate other genes that are required for blastema formation and proliferation (gray arrow). We propose that wnt5b employs a β-catenin-independent signaling pathway that antagonizes Wnt/β-catenin signaling. However, we cannot exclude the possibility that such β-catenin-independent pathways also inhibit regeneration without impairing Wnt/β-catenin signaling (gray arrow). Arrows do not imply direct events.

Wnt/β-catenin signaling regulates FGF signaling during fin regeneration

FGF signaling has been shown to be required for regeneration of amphibian and fish appendages (Lee et al., 2005; Poss et al., 2000b; Yokoyama et al., 2001) and recently fgf20a was found to be induced early during zebrafish fin regeneration and to be required for blastema formation (Whitehead et al., 2005). Similarly, we observe that wnt10a is induced very early in regenerating fins and that Wnt/β-catenin signaling is essential for formation of the blastema. Therefore, to gain more mechanistic insight into the role of Wnt/β-catenin signaling in fin regeneration, we investigated whether Wnt/β-catenin signaling regulates FGF signaling during regeneration. Strikingly, we found that levels of fgf20a transcripts are suppressed 3 hours after amputation in Dkk1-overexpressing fins (Fig. 7A), and that fgf20a expression is still not detectable in hsDkk1GFP fins at 24 hpa (Fig. 7B). Quantitative PCR revealed that induction of Dkk1 2 hours prior to amputation resulted in severe downregulation of the baseline of fgf20a expression at the time of amputation and in the suppression of fgf20a upregulation during the first 48 hours of regeneration (see Fig. S5 in the supplementary material). These findings show that Wnt/β-catenin signaling is required for initiation of fgf20a expression during regeneration. The fast response and the repression of basic fgf20a levels in hsDkk1 fins indicate that fgf20a downregulation is not an indirect consequence of a failure of these fins to regenerate, but is likely to reflect a more direct regulation of fgf20a expression by Wnt/β-catenin signaling.

In addition, we observed that in fins that have been allowed to regenerate normally for 72 hours, a single pulse of Dkk1 expression quickly results in the repression of sprouty4, an FGF target gene (Lee et al., 2005) (Fig. 7C). We conclude that Wnt/β-catenin signaling is also required for the maintenance of FGF signaling. These findings indicate that Wnt/β-catenin signaling acts upstream
of FGF signaling during regeneration, placing Wnt/β-catenin signaling at the top of the hierarchy of signaling pathways known to be required for epimorphic regeneration (Fig. 8).

**DISCUSSION**

Our findings further an understanding of the molecular events that initiate regenerative processes by demonstrating a crucial role for Wnt/β-catenin signaling in epimorphic regeneration of the zebrafish tail fin, and an antagonistic role for β-catenin-independent Wnt signaling. Based on our results and those of others, we propose the following model for signaling pathways regulating zebrafish fin regeneration (Fig. 8). Injury of the fin activates signal(s) that rapidly induce expression of wnt10a and possibly other Wnt ligands that activate the β-catenin signaling pathway. The nature of these signals and whether they directly or indirectly regulate expression of Wnt ligands is unknown. One potential candidate that might indirectly activate Wnt expression is thrombin, which is activated by the wound-healing response and which has been shown to be involved in regeneration of newt lens and limb myotubes (Imokawa and Brookes, 2003; Imokawa et al., 2004; Tanaka et al., 1999). Wnt10a activates the β-catenin signaling pathway, which directly or indirectly activates expression of fgf20a, which in turn activates (directly or indirectly) the events resulting in blastema formation and thus regeneration (Whitehead et al., 2005). Although we have not tested whether wnt10a activates fgf20a expression directly in the regenerating fin, it is intriguing that fgf20a has been found to be a direct target of Wnt/β-catenin signaling in cultured human cells (Chamorro et al., 2005). Since we have not tested whether Wnt/β-catenin signaling acts solely through fgf20a to regulate blastema formation, we cannot exclude the possibility that β-catenin signaling also controls regeneration in parallel to FGF signaling (gray arrow in Fig. 8).

The same injury-activated signal(s) that regulate wnt10a expression might also activate expression of wnt5a and wnt5b and potentially other Wnt ligands that activate β-catenin-independent signaling. We postulate that these signaling pathways modulate regeneration by negatively regulating Wnt/β-catenin signaling. However, we cannot exclude the possibility that β-catenin-independent Wnt signaling also represses regeneration independently of its antagonistic effect on β-catenin signaling (gray arrow in Fig. 8). Because we find that expression of wnt5b is regulated by Wnt/β-catenin signaling, we hypothesize that these separate Wnt pathways establish a negative feedback loop whose function might be to ensure proper levels, duration or location of β-catenin signaling in the regenerating fin.

In addition to its role in blastema formation, FGF signaling appears to be absolutely required for the regenerative outgrowth of the fin, as drugs that block FGF signaling can inhibit fin regeneration during this phase (Poss et al., 2000b). Our experiments indicate that Wnt/β-catenin signaling is also required for regenerative outgrowth. However, overexpression of Dickkopf1 does not cause a complete inhibition of outgrowth. It is possible that the expression levels of Dkk1 are not sufficient to completely block β-catenin signaling during this regenerative phase. Alternatively, other signals that are partially redundant with Wnt/β-catenin signaling might compensate for the loss of Wnt signaling. We have found that Wnt/β-catenin signaling regulates FGF signaling during regenerative outgrowth, and thus it appears likely that β-catenin signaling acts through FGF signaling in this phase of regeneration as well.

Elucidation of the exact cell biological role of Wnt/β-catenin and FGF signaling in blastema formation awaits further experiments. Whereas regeneration of the zebrafish tail fin occurs in similar steps to salamander limb regeneration, blastema formation by de-differentiation of differentiated cells has so far only been reported in salamanders. Interestingly, a recent report has shown that resident muscle stem cells are activated during salamander limb regeneration and that progeny of these cells take part in the formation of the blastema (Morrison et al., 2006). It is likely that the relative contribution of de-differentiation and resident stem cell activation to the formation of progenitor cells during regeneration varies between organs and organisms, with amphibian limbs likely to represent one end of the spectrum where de-differentiation is prominent and, at the other end, processes like mammalian muscle or bone regeneration being driven only by activation of resident stem cells. Whether Wnt/β-catenin and FGF signaling regulate de-differentiation or stem cell activation or both in blastema formation is at present unclear. Interestingly, Wnt/β-catenin signaling has been shown to be important for regeneration or repair of systems that are thought to rely largely or solely on activation of resident stem cells. Inhibition of Wnt/β-catenin signaling reduces proliferation of CD45+ resident stem cells in mammalian muscle regeneration (Poleskaya et al., 2003) and inhibits proliferation of osteoblasts, which drive bone repair, in culture (Zhong et al., 2006). Wnt/β-catenin signaling has also been reported to be active during regeneration of deer antlers and to be required for survival of antler bone progenitor cells in culture (Mount et al., 2006). Very recently, Hayashi et al. have shown that Wnt/β-catenin signaling is necessary and sufficient for regeneration of newt lenses in culture (Hayashi et al., 2006). More specifically, Wnt signaling appears to regulate the second step of regeneration in which, subsequent to proliferation of the iris pigmented epithelium and activation of early lens genes in the whole iris, only the dorsal iris continues to develop (Hayashi et al., 2006). Thus, together with our results showing that β-catenin signaling is required for fin regeneration and our data showing that β-catenin signaling is activated during mouse liver and zebrafish heart regeneration, evidence is beginning to emerge that Wnt/β-catenin signaling might play central roles in many regenerative processes. However, the specific function of Wnt signaling in the regeneration of different organs is most likely to differ. For example, in the newt lens, Wnt signaling is only activated after the initial phase of proliferation and gene expression and is required for the second step of regeneration. By contrast, we have shown that Wnt signaling regulates gene expression very early in fin regeneration and that it is required for the early events of blastema formation.

A better understanding of the role of Wnt/β-catenin and FGF signaling in de-differentiation and/or stem cell activation during epimorphic regeneration is hampered by the fact that our insights into signaling events that regulate epimorphic regeneration come mainly from systems such as zebrafish, where de-differentiation has not been reported. Thus, further insights into the role of these pathways awaits better characterization of the cell biological events of blastema formation in zebrafish or the development of tools that facilitate genetic and other in vivo functional studies in salamanders.

Our study not only demonstrates an important role for Wnt/β-catenin signaling during regeneration, but also adds to our knowledge about the functions of β-catenin-independent Wnt signaling in adults. In vertebrates, it is well established that β-catenin-independent Wnt signaling is required for cell polarity and cell movements during gastrulation, and has also been implicated in endoderm cell migration, pancreas cell migration, the migration of neurons and organization of hair cell polarity in the inner ear (Bingham et al., 2002; Carreira-Barbosa et al., 2003; Curtin et al., 2003; Jessen et al., 2002; Kim et al., 2005; Matsui et al., 2005; Wada et al., 2005). It is less clear whether β-catenin-independent Wnt
signaling plays roles in cell fate determination. Interestingly, however, it has been shown that β-catenin-independent Wnt signaling can inhibit Wnt/β-catenin signaling and thus can, indirectly at least, regulate cell fate. For example, overexpression of β-catenin-independent Wnt ligands in Xenopus blocks the ability of “canonical” Wnt ligands to activate β-catenin signaling and to induce a secondary body axis. Genetic evidence for the existence of such opposing roles of β-catenin-independent Wnt signaling on Wnt/β-catenin signaling comes from zebrafish, where maternal loss of wnt5b has been reported to result in ectopic β-catenin signaling and a consequent increase in dorsal cell fates (Withinger and Moon, 2003; Westfall et al., 2003). Furthermore, loss of wnt5a in mouse limb buds likewise results in ectopic β-catenin signalling, causing defective chondrocyte differentiation (Topol et al., 2003). We propose that β-catenin-independent Wnt signaling, activated by wnt5a and wnt5b, plays a similar antagonistic role in fin regeneration. Our finding that wnt5b expression appears to be regulated by β-catenin signaling suggests the existence of a negative feedback loop. Such a loop represents a mechanism for regulation of β-catenin signaling that, to our knowledge, has not been described before. It will be interesting to see whether the transcriptional activation of Wnt ligands that activate antagonistic β-catenin-independent pathways is a more widespread regulatory mechanism employed by organisms to keep β-catenin signaling in check.

Taken together, our findings add to our mechanistic insight into the regulation of regeneration by demonstrating separate and opposing roles for β-catenin-dependent and β-catenin-independent signaling pathways during fin regeneration. Furthermore, although regeneration of the mammalian liver and the zebrafish heart employ different cellular mechanisms than regeneration of the zebrafish fin or amphibian limbs (with only the latter two involving formation of a blastema), it is intriguing that Wnt/β-catenin signaling is upregulated during regeneration of all three organs. Although beyond the scope of the present study, it will be very interesting to test what role Wnt signaling plays in regeneration of these organs. It is conceivable that our findings will prove to be important for the goals of regenerative medicine, as the modulation of Wnt signaling is conceivable that our findings will prove to be important for the goals of regenerative medicine, as the modulation of Wnt signaling is.
Wnt signaling regulates regeneration


Table S1. *mbl* (*axin1*) heterozygous mutant fish regenerate their tail fins faster than wild-type fish

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Wild-type (mm)</th>
<th>mbl&lt;sup&gt;+&lt;/sup&gt; (mm)</th>
<th>Different?</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp 1</td>
<td>1.88±0.047 n=12</td>
<td>2.08±0.058 n=9</td>
<td>Yes</td>
<td>P=0.009</td>
</tr>
<tr>
<td>Exp 2</td>
<td>2.17±0.071 n=12</td>
<td>2.56±0.077 n=9</td>
<td>Yes</td>
<td>P=0.00083</td>
</tr>
<tr>
<td>Exp 3</td>
<td>2.53±0.058 n=12</td>
<td>2.73±0.085 n=9</td>
<td>Yes</td>
<td>P=0.039</td>
</tr>
</tbody>
</table>

The error represents the standard error of the average lengths of the individual fish. Student’s *t*-test was used to determine whether the average lengths of the wild-type and *axin1* mutant fish were significantly different; *P* value is one-tailed; note that in the first two experiments, the average lengths of the regenerates were also found to be significantly different using two-tailed *t*-tests.
Table S2. *ppt* (*wnt5b*) homozygous mutant fish regenerate their tail fins faster than wild-type fish

<table>
<thead>
<tr>
<th>Exp</th>
<th>Wild-type Average (mm) at 4 dpa</th>
<th>ppt⁻ Average (mm) at 4 dpa</th>
<th>Different?</th>
<th>Wild-type Average (mm) at 7 dpa</th>
<th>ppt⁻ Average (mm) at 7 dpa</th>
<th>Different?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.23±0.05 <em>n=14</em></td>
<td>1.35±0.03 <em>n=12</em></td>
<td>Yes (Mann Whitney)</td>
<td>P=0.022</td>
<td>1.76±0.05 <em>n=14</em></td>
<td>2.48±0.12 <em>n=12</em></td>
</tr>
<tr>
<td>Exp</td>
<td>1.06±0.02 <em>n=12</em></td>
<td>1.16±0.02 <em>n=11</em></td>
<td>Yes (t-test) <em>P=0.0018</em></td>
<td></td>
<td>1.78±0.03 <em>n=12</em></td>
<td>2.07±0.03 <em>n=11</em></td>
</tr>
</tbody>
</table>

The error represents the standard error of the average lengths of the individual fish. Normal distribution of the data was tested using a Shapiro-Wilk W Test. If data were normally distributed, a Student’s t-test was used to determine whether the average lengths of wild-type and ppt mutant fish were significantly different, otherwise a Mann Whitney tested was used. The *P* value is one-tailed for t-tests.