Wnt/β–catenin Signaling Defines Organizing Centers That Orchestrate Growth and Differentiation of the Regenerating Zebrafish Caudal Fin

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Abbreviations

+ Positive
– Negative
AER Apical ectodermal ridge
Alk4 Activin receptor-like kinase receptor 4
AmpR Ampicillin resistance
Ap Alkaline phosphatase
Apc Adenomatous polyposis coli
Ck Casein kinase
BCIP 5-Brom-4-chlor-3-indolylphosphat
Bmp Bone morphogenetic protein
bp Base pairs
BrdU 5-bromo-2-deoxyuridine
BSA Bovine serum albumin
β–Trcp β–Transducin repeat containing protein
bzw beziehungsweise
°C Degrees celsius
CDS Coding sequence
Da Dalton
Dkk Dickkopf
Dapi 6-diamidino-2-phenylinodole
DIG Digoxigenin
DMSO Dimethylsulfoxid
DNA Deoxyribonucleic acid
DOX Doxycycline
dNTP Deoxyribonucleotide triphosphate
dpa Days post amputation
Dvl Dishevelled
ECM Extracellular matrix
EDTA Ethylenediaminetetraacetic acid
e.g. Exempli gratia (for example)
EGFP Enhanced green fluorescent protein
ER Endoplasmatic reticulum
EtOH Ethanol
Fgfr Fgf receptor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>Fgf</td>
<td>Fibroblast growth factor</td>
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<td>Figure</td>
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<tr>
<td>Fzd</td>
<td>Frizzled</td>
</tr>
<tr>
<td>g</td>
<td>Gram or gravity</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>Gsk3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>Hdac</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>Hh</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>H3P</td>
<td>Phospho-histone 3</td>
</tr>
<tr>
<td>hpa</td>
<td>Hours post amputation</td>
</tr>
<tr>
<td>Hrp</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Igf</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>Igf1r</td>
<td>Insulin-like growth factor 1 receptor</td>
</tr>
<tr>
<td>Igfbp4</td>
<td>Insulin-like growth factor binding protein 4</td>
</tr>
<tr>
<td>INT</td>
<td>Iodonitrotetrazolium chloride</td>
</tr>
<tr>
<td>ISH</td>
<td>In situ hybridization</td>
</tr>
<tr>
<td>Jnk/Sapk</td>
<td>c-Jun N-terminal kinase/Stress-activated protein kinase</td>
</tr>
<tr>
<td>l</td>
<td>Liter</td>
</tr>
<tr>
<td>Lef</td>
<td>Lymphoid enhancer factor</td>
</tr>
<tr>
<td>Lrp5/6</td>
<td>Low-density lipoprotein receptor-related protein 5/6</td>
</tr>
<tr>
<td>m</td>
<td>Meter</td>
</tr>
<tr>
<td>M</td>
<td>Molare</td>
</tr>
<tr>
<td>Mapk</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
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<tr>
<td>min</td>
<td>Minutes</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>N</td>
<td>Normal</td>
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<tr>
<td>n.a.</td>
<td>No information available</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitro Blue Tetrazolium</td>
</tr>
<tr>
<td>NCS</td>
<td>Newborn calf serum</td>
</tr>
<tr>
<td>Nicd</td>
<td>Notch intracellular domain</td>
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<tr>
<td>n.s.</td>
<td>Not significant</td>
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<tr>
<td>o/n</td>
<td>Over night</td>
</tr>
<tr>
<td>Osx</td>
<td>Osterix</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>Pena</td>
<td>Proliferative cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>Pdgf</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>Pkc</td>
<td>Protein kinase C</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Pod</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription PCR</td>
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<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SAG</td>
<td>Smoothened agonist</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>sFrp</td>
<td>Secreted frizzled-related protein</td>
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<tr>
<td>Sost</td>
<td>Sclerostin</td>
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<tr>
<td>Suppl.</td>
<td>Supplemental</td>
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<tr>
<td>Tcf</td>
<td>T cell factor</td>
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<tr>
<td>TetA</td>
<td>TetActivator</td>
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<tr>
<td>TetRE</td>
<td>TetResponder</td>
</tr>
<tr>
<td>Tgfβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TFM</td>
<td>Tissue freezing medium</td>
</tr>
<tr>
<td>Tle</td>
<td>Transducin-like enhancer of split</td>
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<td>U</td>
<td>Unit</td>
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<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>WGCNA</td>
<td>Weighted gene expression correlation network analysis</td>
</tr>
<tr>
<td>WPI</td>
<td>World precision instruments</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>wt</td>
<td>Wild type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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List of Publications Based on Doctoral Studies


Grotek, B.*, Wehner, D.*, and Weidinger, G. (2013). "Notch signaling coordinates cellular proliferation with differentiation during zebrafish fin regeneration". Development 140, 1412-1423. *these authors contributed equally to this work


Parts of this thesis have been published in Grotek et al., 2013.
Summary

Adult mammals, including humans, have limited regenerative potential. In contrast, many other animal groups possess remarkable capabilities to fully regenerate body appendages or organs that have been lost or damaged due to injury or disease. A key goal of regeneration research is to understand the cellular and molecular mechanisms underlying naturally occurring regeneration in these organisms, which will provide clues for the development of regenerative therapies in humans.

Adult zebrafish robustly regenerate the caudal fin following amputation which provides an excellent model for studying basic mechanisms of tissue regeneration. Fin regeneration occurs via dedifferentiation of mature stump cells, which form a proliferative mass of undifferentiated precursor cells, the blastema. During regenerative outgrowth, the blastema and the overlying epidermis become compartmentalized into several zones defined by molecular markers, distinct proliferation characteristics and cell fates. Little is known about the molecular mechanisms specifying these regions and the signaling pathways mediating their interaction. This is in particular due to 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.

Here, I provide evidence that Wnt/β-catenin signaling has an essential role in defining two signaling centers in the blastema that orchestrate regenerative growth and cellular differentiation. Using sensitive transgenic pathway reporters I found that Wnt/β-catenin signaling is activated soon after amputation and maintained in the very distal tip of the blastema, where cells are non-proliferative, plus in proliferative proximal domains which line osteoblast precursors and which are thought to form non-mineralized skeletal elements called actinotrichia. I utilized a panel of transgenic TetON driver lines to induce expression of the Wnt pathway inhibitor axin1 specifically in different regions of the fin to probe the tissue-specific roles of the Wnt pathway, and found that β-catenin signaling is not required in the epidermis or in committed osteoblasts for regenerative
growth or bone formation. Intriguingly, signaling in the proliferative actinotrichia-forming cells lining the osteoblasts appeared to be required for osteoblast differentiation but had little role in controlling blastemal cell proliferation, while pathway activity in the distal, non-proliferative domain was essential for regenerative growth. This strongly suggests that Wnt/β–catenin signaling controls regenerate growth and differentiation largely indirectly. To identify candidate signals that could act downstream of β–catenin signaling and thus mediate its effect on the surrounding tissue, a gene expression profiling was performed after inducible inhibition of the Wnt/β–catenin pathway. Analysis of the Wnt targetome revealed that Wnt/β–catenin signaling controls expression of ligands, pathway components and target genes of many signaling pathways required for caudal fin regeneration including Hh, Fgf, retinoic acid, Bmp, Notch, Activin and Igf. In contrast, Wnt/β–catenin signaling appears to receive little reciprocal input from the signaling pathways it regulates. Rescue experiments showed, that Wnt/β–catenin signaling acts through Fgf, Hh and retinoic acid signaling to indirectly regulate blastemal cell proliferation and gene expression in the epidermis. Thus, Wnt/β–catenin signaling orchestrates fin regeneration by forming signaling centers that instruct cellular behaviors of adjacent tissues.
Zusammenfassung

Adulte Säugetiere, so auch der Mensch, haben nur ein relativ eingeschränktes Regenerationspotenzial. Im Gegensatz dazu gibt es Tiere, die eine beachtliche Fähigkeit besitzen, Körperteile und Organe vollständig zu regenerieren, welche krankheits- bzw. verletzungsbedingt beschädigt oder verloren gegangen sind. Ein Schwerpunkt der Regenerationsforschung ist daher die Aufklärung zellulärer und molekularer Mechanismen, die diesen natürlich auftretenden Regenerationsphänomenen zugrunde liegen. Neue Erkenntnisse können einen wichtigen Beitrag zur Entwicklung regenerativer Therapien für den Menschen liefern.


Die in der vorliegenden Arbeit präsentierten Ergebnisse liefern Hinweise für eine essentielle Rolle des Wnt/β-catenin-Signalweges in der Etablierung zweier blastemaler Signalzentren, welche regeneratives Wachstum und zelluläre Differenzierung regulieren. Mittels sensitiver, transgener Wnt-Reportergene konnte gezeigt werden, dass der
Zusammenfassung

Zusammenfassung

Flossenregeneration durch die Bildung von Signalzentren organisiert, welche das zelluläre Verhalten und damit die Entwicklung benachbarter Gewebe instruiert.
1 Introduction

1.1 Motivation

Within the animal kingdom the ability to regenerate organs or appendages that have been lost or severely damaged due to injury or disease varies greatly. With a few exceptions, adult mammals including humans, have limited regenerative potential and most tissues respond to severe injury by formation of fibrotic scar tissue which is poorly organized and lacks functionality. In contrast, certain lower vertebrates, such as urodele amphibians (newts and salamanders) or the teleost Danio rerio (zebrafish), possess an elevated regenerative capacity and efficiently restore complex organs and body structures upon injury throughout lifetime. A thorough understanding of the cellular and molecular mechanisms underlying naturally occurring regeneration in these organisms will provide valuable clues for the development of regenerative therapies in humans.

Central goals of regeneration research are to identify the cellular source(s) of tissue restoration, and to characterize the molecular signals, which 1) initiate the regenerative program in response to injury, and 2) control the regenerative program until tissue integrity and functionality is restored. While studies on several regenerating systems, foremost the zebrafish caudal fin, have revealed a number of signals that are required for complete regeneration, our knowledge about their exact cellular functions during these processes remains incomplete. Hence, further efforts are needed to elucidate their tissue specific functions and how do they incorporate into complex signaling networks that guide regeneration. Therefore, this study was conducted to gain a more detailed understanding of the molecular mechanisms underlying regeneration in the vertebrate appendage.
1.2 Cellular Mechanisms of Tissue Regeneration

Successful tissue regeneration requires a series of instructive signals and a source of cells that restore the damaged tissue. While many signals are shared between naturally regenerating systems, it has become apparent that the cellular source of newly formed, regenerated tissue differs. Possible modes are (Fig. 1.1) (King and Newmark, 2012; Zhang et al., 2013): 1) the activation of resident stem cells, which self-renew and generate a differentiated daughter cell (e.g. skeletal muscle), 2) proliferation of lineage-restricted progenitor cells that are derived via dedifferentiation of mature cells (e.g. osteoblasts in the zebrafish caudal fin), 3) proliferation of differentiated, mature cells (e.g. hepatocytes after partial hepatectomy of the mammalian liver), or 4) transdifferentiation of mature cells into another cell type, either directly or via dedifferentiation to a progenitor cell (e.g. newt lens).

Fig. 1.1. Cellular mechanisms of tissue regeneration (adapted and modified from Günes Özhan and Gilbert Weidinger). Tissue regeneration can involve different cellular mechanisms: 1) activation of resident stem cells, which self-renew and generate one or more differentiating daughter cells, 2) dedifferentiation of mature, differentiated cells into cells with progenitor-like status, which however remain lineage restricted, 3) transdifferentiation of mature, differentiated cells into another cell type, which can occur either directly or involves dedifferentiation to a progenitor cell, and 4) proliferation of mature, differentiated cells.
The regenerative capacity among metazoan species can be categorized by their scale. Certain invertebrates such as planarian flatworms and the freshwater polyp *Hydra* are capable of regenerating large body parts from small pieces of tissue (Gierer *et al*., 1972; Salo, 2006). When a planarian’s head or tail is amputated, resident pluripotent stem cells (called neoblasts) form a mass of undifferentiated cells, the blastema, which provides a source for all cell types required to restore the missing body part (Reddien and Sanchez Alvarado, 2004). Head regeneration in *Hydra*, following midgastric bisection (at 50 % of the body length), likewise occurs through activation of stem cells (Tanaka and Reddien, 2011). Though less efficiently than planarians and the *Hydra*, lower vertebrates including frogs (*Xenopus laevis*) at tadpole stage, adult urodele amphibians (newts and salamanders) and adult teleosts (e.g. zebrafish) can robustly regenerate lost appendages and in the case of newts, salamanders and teleosts also many internal organs (Poss, 2010). During zebrafish heart regeneration, mature cardiomyocytes provide a source for precursor cells, which proliferate and restore the damaged heart muscle (Jopling *et al*., 2010). Appendage regeneration occurs via establishment of a blastema, a heterogeneous population of undifferentiated, proliferative cells, which are however unlike the planarian’s neoblasts lineage restricted (King and Newmark, 2012). Solid evidence has recently been provided that these progenitor cells are derived via dedifferentiation of mature, differentiated cells as it is the case during bone regeneration in the zebrafish caudal fin (Stoick-Cooper *et al*., 2007a, Knopf *et al*., 2011; Tu and Johnson, 2011). Some evidence exists, which suggests that many other lineages likewise regenerate via dedifferentiation of mature cells in fish and salamander appendages (Tanaka and Reddien, 2011). Although dedifferentiation of mature cells to a progenitor-like status appears to be an important mechanism for many cellular lineages during vertebrate appendage regeneration, activation of resident stem cells has also been identified as a possible mode for regeneration of some cellular lineages in the vertebrate appendage, in particular muscle (Morrison *et al*., 2006). Regeneration of complex tissues in mammals, including humans, is very limited. Examples of tissues with elevated regenerative potential are the liver and skeletal muscle. While regeneration of skeletal muscle is based on myogenic precursor cells, liver regeneration can occur via different cellular mechanisms that involve progenitor cells or proliferation of mature hepatocytes (Duncan *et al*., 2009; Poss, 2010). Mammals are also able to regenerate their digits after amputation, which involves the concerted action of nerve stem cells and
populations of fate-restricted mesenchymal progenitors (Lehoczky et al., 2011; Rinkevich et al., 2011; Takeo et al., 2013).

1.3 Appendage Regeneration in the Teleost Danio Rerio

The zebrafish Danio rerio is of central biomedical interest since it is amenable to large-scale drug screens and has the remarkable ability to fully regenerate its appendages and several internal organs, including the retina, brain, spinal cord, heart and kidney (Poss, 2010; Stoick-Cooper et al., 2007a). In the recent past the zebrafish caudal fin has emerged as a highly successful model for studying basic mechanisms of tissue regeneration due to its easy access to surgery, rapid and robust regeneration, apparently unlimited regenerative potential (Azevedo et al., 2011), relatively simple tissue architecture and largely transparent appearance (Fig. 1.2).

The caudal fin consists of 16-18 bifurcated, bony rays, the lepidotrichia, which extend along the whole length of the fin and are separated by soft interray tissue (Fig. 1.2A-B). Each fin ray consist of two concave, segmented hemirays, which enclose blood vessels, nerves, pigment cells and fibroblast-like cells (Fig. 1.2B-C) (Poss et al., 2003). A monolayer of bone-secreting cells, the osteoblasts, covers on both surfaces the calcified lepitodrichia bone which is formed via intramembranous ossification without a cartilage intermediate (Mari-Beffa et al., 2007; Poss et al., 2003). Additionally to the lepitodrichia bone, rigid, non-calcified, collagenous, skeletal elements are found at the distal tip of each ray, the actinotrichia (Fig. 1.2B) which provide further support to the fin edge and are thought to be specific to fish fins (Duran et al., 2011; Zhang et al., 2010).

The caudal fin completely regenerates following partial amputation (Fig. 1.2). The regenerative process can be grossly divided into three phases (Fig. 1.3; Stoick-Cooper et al., 2007a): 1) wound healing, 2) blastema formation, and 3) regenerative outgrowth and regeneration termination. During the early wound healing phase (1-3 hours post amputation; hpa) epidermal cells migrate over the wound edges and cover the wound (Stoick-Cooper et al., 2007a). When fish are kept at 28°C, wound healing continues until 24-36 hpa giving rise to a multi-layered wound epidermis, whose basal layer is composed of characteristic cuboidal-shaped cells (Stoick-Cooper et al., 2007a). Until 48 hpa
populations of lineage-restricted proliferative mesenchymal progenitor cells are established via dedifferentiation of mature stump cells distally to each ray, called blastema (Stoick-Cooper et al., 2007a, Knopf et al., 2011; Tu and Johnson, 2011). Once the blastema has been formed the regenerative outgrowth phase is initiated leading to restoration of the fin within two to three weeks.

Fig. 1.2. The zebrafish caudal fin. (A) A regenerating caudal fin at 2, 4, 6 and 20 days post amputation (dpa). The caudal fin is composed of bony rays and soft interray tissue, and regenerates completely within ~2-3 weeks following partial resection. Arrowheads: amputation plane. (B) Cartoon representing the (uninjured) fin ray skeleton comprising bifurcated, segmented lepidotrichia bone and distally-located actinotrichia (adapted and modified from Quint et al., 2002). (C) Cartoon of a fin ray segment which composed of a pair of mineralized hemirays.

Fig. 1.3. Stages of caudal fin regeneration (modified from Stoick-Cooper et al., 2007a). Within 24 following amputation (hpa) the wound is covered by epidermal cells and a multilayered wound epidermis is formed. Dedifferentiation of mature stump cells form populations of proliferative, lineage-restricted progenitor cells, the blastema, distally to each fin ray. Blastema formation is completed at 48 hours post amputation (hpa) and followed by the regenerative outgrowth phase leading to restoration of the fin within 2-3 weeks. The time frame applies when fish are kept at 28°C.
During regenerative outgrowth, the regenerate is organized into several tissue compartments as revealed on longitudinal sections (Fig. 1.4). The blastemal mesenchyme comprises at least four domains: 1) the distal-most, scarcely proliferative blastema, characterized by expression of aldha1a2 (raldh2), the synthesis rate-limiting enzyme in retinoic acid synthesis (Mathew et al., 2009; Nechiporuk and Keating, 2002), 2) the medial proximal blastema which is rapidly proliferating and positive for the mitotic checkpoint kinase ttk (mps1) (Poss et al., 2002), and for readouts of Notch signaling (her6 and the transgenic reporter her4.3:EGFP<sup>83</sup>; Grotek et al., 2013), 3) highly proliferative bilateral zones containing runx2b, sp7 (osterix, osx) and Zns5-positive osteoblast progenitors (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 medial proximal blastema, are positive for and1/2 transcripts, and are thought to be required for formation of actinotrichia (Zhang et al., 2010). The wound epidermis overlying the blastemal mesenchyme likewise comprises different regions. In particular, a subdomain of the basal epidermal layer that abuts the runx2b/sp7-positive mesenchyme expresses the transcription factor lef1 and the Hedgehog (Hh) ligand shh, and is thought to instruct the underlying osteoblast progenitors to differentiate (Laforest et al., 1998; Lee et al., 2009).

Recent studies have revealed the requirement of a number of signaling pathways for caudal fin regeneration, including Activin (Jazwinska et al., 2007), Bone morphogenetic protein (Bmp; Smith et al., 2006), Fibroblast growth factor (Fgf; Poss et al., 2000b; Whitehead et al., 2005), Hedgehog (Hh; Quint et al., 2002), Insulin-like growth factor (Igf; Chablais and Jazwinska, 2010), Notch (Grotek et al., 2013; Munch et al., 2013), retinoic acid (RA; Blum and Begemann, 2012) and Wnt/β–catenin (Kawakami et al., 2006; Stoick-Cooper et al., 2007b). All of these pathways have been shown to promote proliferation of the blastemal progenitor cells and/or are essential for formation of the blastema. However, our understanding of how these signals regulate regeneration is incomplete. Little is known about their tissue-specific roles or how different pathways interact to regulate distally-oriented growth during fin regeneration.
1.4 Wnt Signal Transduction Pathways

A fundamental property of all multicellular organisms is the ability of individual cells or cell populations to communicate and thus to coordinate their spatiotemporal directed response to environmental changes. Communication between cells is achieved by the activation of different signal transduction pathways, either by direct cell-cell contact in adjacent cells or through secreted signaling molecules, which allow information to be spread over short and long distance. Once activated, these pathways elicit a specific cellular response including cell fate decisions, cell differentiation, proliferation, apoptosis, or changes in cell polarity and morphology. Complex biological phenomena including embryogenesis and tissue regeneration are governed by the concerted action of multiple signaling pathways tightly coordinated in space and time.
Among the many pathways identified, those that are initiated by members of the highly conserved Wnt family of secreted proteins are of central importance. This is due to their ability to regulate a multitude of cellular mechanisms, including cell proliferation, migration, cell polarity, and cell fate determination during embryonic development and tissue homeostasis (Logan and Nusse, 2004; MacDonald et al., 2009). Importantly, Wnt signaling also appears to play critical roles in essentially all naturally regenerating systems studied to date, ranging from hydra and planarian whole body regeneration to organ and tissue regeneration in lower vertebrates and mammals (Poss, 2010; Stoick-Cooper et al., 2007; Whyte et al., 2012). Hence, the following chapters will provide a brief introduction and background on Wnt signaling.

1.4.1 Wnt Genes and Proteins

Wnt proteins are encoded by the large family of \textit{wnt} genes of which the first, \textit{wnt1}, was discovered more than three decades ago in mouse (Nusse and Varmus, 1982). Today up to 19 different \textit{wnt} genes are known to be present in mammalian genomes (Willert and Nusse, 2012). \textit{wnt} genes are expressed in all metazoan species ranging from simple cnidarians and sponges to humans (Holstein, 2012; Kusserow et al., 2005; Nichols et al., 2006; Willert and Nusse, 2012). In contrast, they are not found within genomes of single-cell organisms, which led to the hypothesis that the Wnt signaling pathway has been instrumental in the evolution of multicellularity (Clevers and Nusse, 2012). Genes of the \textit{wnt} family code for cysteine-rich secreted growth factor proteins of approximately 40 kDa in size, which act as signaling molecules over short and long distances (Clevers and Nusse, 2012; Holstein, 2012). The latter however likely requires association with additional factors such as the Swim protein or lipoprotein particles (Bartscherer and Boutros, 2008; Mulligan et al., 2012). Several posttranslational modifications have been reported for Wnt proteins of which acylation (palmitoylation) on a highly conserved cysteine residue and several glycosylation events are crucial for Wnt secretion and signaling activity (Fung et al., 1985; Komekado et al., 2007; Kurayoshi et al., 2007; Willert et al., 2003; Willert and Nusse, 2012). Following procession through endoplasmic reticulum (ER) and Golgi, Wnt proteins are secreted with the help of the Wntless protein into the extracellular space where they
bind to cell-surface receptors of target cells (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006).

Historically Wnt proteins have been categorized into two groups based on their ability to induce formation of a secondary body axis in *Xenopus laevis* embryos and to morphologically transform cells of the mouse mammary epithelial cell line C57MG (Du et al., 1995; McMahon and Moon, 1989; Wong et al., 1994). Wnt proteins with these capacities were classified as transforming or “canonical” Wnts, while Wnt proteins that lacked the former were categorized as non-transforming or “non-canonical” Wnts (Du et al., 1995; van Amerongen, 2012). The transforming ability of canonical Wnts was soon discovered to be attributable to an increase in the cellular levels of the transcriptional co-activator β-catenin (Shimizu et al., 1997). By contrast non-canonical Wnt ligands employ alternative, β-catenin-independent signaling mechanisms including intracellular calcium release and activation of protein kinase C (Pkc), small and heterotrimeric G proteins, or the c-Jun N-terminal kinase (Jnk) (van Amerongen, 2012). It has become apparent however, that whether the canonical, β–catenin-dependent signaling pathway is triggered or whether non-canonical, β–catenin-independent signaling pathways are employed in a particular system, is not determined by the Wnt protein, but rather by the cellular context and the presence of specific combinations of Wnt receptors (van Amerongen, 2012; Willert and Nusse, 2012).

### 1.4.2 The β–catenin-Dependent Wnt Signaling Pathway

Of the various signaling pathways triggered by Wnt ligands, the β–catenin dependent pathway is the best characterized. Wnt/β–catenin signaling has important functions during embryonic development and adult tissue homeostasis by controlling many biological processes including cell fate determination and cell proliferation. Since this pathway is highly regulated at many levels with still growing complexity, only the signaling steps and components that are most relevant for this study will be introduced (Fig. 1.5) (Clevers and Nusse, 2012; MacDonald et al., 2009): The central signaling molecule of the β–catenin-dependent Wnt pathway is the transcriptional co-activator β–catenin, whose stability is tightly controlled. In the absence of a Wnt ligand, cytoplasmic β–catenin is bound by a so
called “destruction complex”, which is a multi-protein complex consisting of the scaffolding protein Axin, the tumor suppressor Adenomatous polyposis coli (Apc), and two serine-threonine kinases (Glycogen synthase kinase 3, Gsk3α/β and Casein kinase 1 α/β, Ck1α/β). Both kinases sequentially phosphorylate β-catenin at N-terminal serine/threonine residues. Phosphorylated β-catenin is then ubiquitinated and thereby targeted for degradation by the proteasome pathway (Fig. 1.5A). Thus, in the absence of a Wnt signal very low levels of β-catenin are present in the cytoplasm, which are insufficient to trigger signaling. Additionally, in the nucleus proteins of the T cell factor/Lymphoid enhancer family (Tcf/Lef) bind to regulatory sequences of Wnt target genes and recruit co-repressors (e.g. Histone deacetylases, Hdacs and Transducin-like enhancer of split, Tle) to inhibit gene expression.

**Fig. 1.5. The β-catenin-dependent Wnt signaling pathway** (modified from MacDonald et al., 2009). Shown is a simplified overview of signaling events and protein interactions in the absence (A) or in the presence (B) of Wnt ligands activating β-catenin signaling. (A) In the absence of a Wnt ligand, β-catenin is bound by a multi-protein “destruction” complex composed of Axin, Apc, Gsk3 and Ck1 which phosphorylates β-catenin. Phosphorylated β-catenin is recognized by β-Trcp, a subunit of the E3 ubiquitin ligase complex, subsequently ubiquitinated and targeted for degradation by the proteasome. Transcriptional repressor complexes in the nucleus prevent activation of Wnt target genes. (B) Wnt ligand binding to Frizzled receptors and Lrp5/6 co-receptors results in Lrp5/6 phosphorylation and Axin recruitment to Lrp5/6, which inactivates the destruction complex. Subsequently, newly synthesized β-catenin accumulates, translocates to the nucleus and activates transcription of target genes together with transcription factors of the Tcf/Lef family. The scaffolding protein Dvl is thought to facilitate Lrp5/6 phosphorylation.
Wnt/β–catenin signaling is initiated by binding of a Wnt ligand to a seven-pass transmembrane receptor of the Frizzled (Fzd) family and its single-pass co-receptor low-density lipoprotein receptor-related protein 5 or 6 (Lrp5/6) (Fig. 1.5B). Subsequently, the scaffolding protein Dishevelled (Dvl) is recruited to the receptor complex and Lrp5/6 is phosphorylated on multiple conserved sites (PPPSPxS motifs) in its intracellular domain by the concerted action of Gsk3β and Ck1γ. Phosphorylated Lrp5/6 then serves as docking site for the recruitment of the cytoplasmic scaffolding protein Axin, which renders the destruction complex inactive. Consequently, newly synthesized β–catenin accumulates and translocates to the nucleus. Binding of nuclear β–catenin to Tcf/Lef proteins and recruitment of additional cofactors converts the Tcf/Lef repressor complex into a transcriptional activator which initiates transcription of target genes. While many target genes are cell-type specific due to the numerous biological processes regulated by the Wnt/β–catenin pathway, a number of genes such as the negative feedback inhibitor axin2 are considered to represent common targets and thus are frequently used as readout for active signaling (Jho et al., 2002; Lustig et al., 2002; Weidinger et al., 2005).

Due to its pivotal role in development and tissue homeostasis it is not surprising that the β–catenin pathway activity is tightly regulated by a wide range of modifiers whose number is still growing. These effectors act in a promoting or antagonizing manner at different levels in the signaling cascade including intracellularly to modulate components of the signal transduction machinery, or extracellularly to modulate ligand-receptor interactions (reviewed in Cruciat and Niehrs, 2013; MacDonald et al., 2009). Among the secreted antagonists which include Frizzled-related proteins (sFRPs), Dickkopf (Dkk) proteins, Wise/Sost proteins, Cerberus and insulin-like growth factor binding protein 4 (Igfbp4), proteins of the Dickkopf (Dkk) family are the best characterized (Cruciat and Niehrs, 2013). The founding member of the Dkk family, Dkk1, inhibits Wnt/β–catenin signaling by binding to Lrp5/6 co-receptors which disrupts the Wnt-induced Wnt/Lrp/Fzd complex and additionally induces cell surface removal of Lrp6 (Yamamoto et al., 2008). Crucial cytoplasmic regulators of Wnt/β–catenin signaling are Apc and Axin, which promote β–catenin phosphorylation and degradation. Ectopic expression of dkk1 or axin1 has become a frequently used strategy to efficiently interfere with Wnt/β–catenin signaling as knock-out approaches are often hindered through frequently occurring functional
Introduction

redundancy of Wnt/β–catenin pathway components, and β–catenin’s additionally role in regulating cell-cell adhesion.

1.5 Wnt/β–catenin Signaling in Tissue Regeneration

Among the many molecular signals that have been identified to be involved in tissue regeneration, Wnt signaling has emerged as an important player in essentially all naturally regenerating systems studied to date, ranging from Hydra and planarian whole body regeneration to organ and tissue regeneration in lower vertebrates and mammals (Poss, 2010; Stoick-Cooper et al., 2007a; Whyte et al., 2012). Particularly, activation of the Wnt/β–catenin pathway has been found to be a crucial signal in controlling many cellular processes during tissue regeneration. The functions of β–catenin pathway activation however, if known, are diverse and include promotion of progenitor cell proliferation and maintenance, regulation of progenitor cell differentiation into mature cells and patterning of the newly formed tissue (Whyte et al., 2012). During regeneration in planarians β–catenin signaling has an essential role in establishment of axial tissue patterning since pathway manipulation can result in worms with two heads or two tails (Gurley et al., 2008). Head regeneration in Hydra likewise relies on Wnt/β–catenin signaling. Following midgastric bisection, apoptotic cells provide a source for ligands activating the β–catenin pathway, which promotes proliferation of interstitial stem cells (Chera et al., 2009). Additionally, Wnt/β–catenin signaling also plays a critical role in axial patterning during Hydra head regeneration via establishment of the head organizer (Bode, 2009; Broun et al., 2005). During zebrafish caudal fin regeneration Wnt/β–catenin pathway activation is essential for formation and proliferation of the blastema (Kawakami et al., 2006; Stoick-Cooper et al., 2007b). Similarly, interference with β–catenin pathway activation prevents complete regeneration of adult Axolotl limbs and larval Xenopus limbs and tails (Kawakami et al., 2006; Yokoyama et al., 2007). Importantly, Wnt/β–catenin signaling also promotes regenerative processes in mammals, including liver and muscle regeneration. Here, β–catenin pathway activation appears to not only promote cellular proliferation of the involved progenitor cells to provide the cellular source for tissue
regeneration, but it is also thought to have additional roles in directing differentiation of muscle and liver progenitors (Boulter et al., 2012; Brack et al., 2008; Nejak-Bowen and Monga, 2011; Otto et al., 2008). During mammalian digit regeneration Wnt/β–catenin signaling directs differentiation of nail stem cells which prerequisites complete digit regeneration (Takeo et al., 2013). Thus, injury-induced β–catenin signaling appears to represent a pro-regenerative signal in most regenerative systems examined, though its exact role remains to be elucidated.
2 Aims of the Study

Two independent studies have shown that β–catenin pathway activation is essential for fin regeneration to occur (Kawakami et al., 2006; Stoick-Cooper et al., 2007b). Interference with Wnt/β–catenin signaling, before or immediately after amputation through systemic overexpression of a dominant negative form of a tcf transcription factor or the secreted Wnt antagonist dkk1, prevented specification of the wound epidermis and formation of the blastema. Inhibition of Wnt/β–catenin signaling during regenerative outgrowth resulted in incomplete regeneration due to reduced cellular proliferation. Thus, Wnt/β–catenin signaling appears to be required for fin regeneration, yet the precise function of pathway activation during this regenerative process remains largely unidentified. This is due to several reasons. First, the spatiotemporal dynamics of pathway activation have not been identified due to a lack of sensitive transgenic reporter. Transcripts of the direct Wnt/β–catenin target axin2 were detected in the blastema while lef1 expression was detected in both, the blastema and the basal epidermal layer (Poss et al., 2000a; Stoick-Cooper et al., 2007b). However, it remains controversial whether lef1 expression in the basal epidermal layer indeed reflects sites of active Wnt/β–catenin signaling (Poss et al., 2000a). Furthermore, whether β–catenin signaling is activated throughout the blastemal mesenchyme or confined to certain subregions has not been elucidated. Second, the cellular mechanisms regulated by the β–catenin pathway are essentially unknown. Third, while it has been suggested that Wnt/β–catenin signaling regulates Fgf signaling during fin regeneration (Stoick-Cooper et al., 2007b), its position within the molecular signaling network remains unknown. Little is known about the downstream targets that mediate the effects of pathway activation. Similarly, signals that act upstream of Wnt/β–catenin signaling have not been reported during caudal fin regeneration.

Therefore, the aim of this study was to investigate the role Wnt/β–catenin signaling plays during zebrafish caudal fin regeneration. Specifically I aimed:
i) to determine the spatiotemporal pattern of Wnt/β–catenin pathway activation

ii) to probe the tissue-specific roles of Wnt/β–catenin signaling

iii) to investigate the hierarchical relationships between Wnt/β–catenin signaling and other signaling pathways known to be required for fin regeneration.

Answering these questions will further our understanding of the molecular mechanisms underlying appendage regeneration and thus might have implications for regenerative medicine.
# Material and Methods

## 3.1 Materials

### 3.1.1 Technical Equipment

Additional technical equipment that was used for a particular experimental procedure is found within the chapter ‘3.2 Methods’ and is not listed here.

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3.1.2 Chemicals and Consumables

All generally used chemicals were, if not indicated otherwise, purchased from AppliChem, Merck, Roth, Sigma or VWR. General plastic ware was purchased from Sarstedt. Additional chemicals or consumables that were used for a particular experimental procedure are found within the chapter ‘3.2 Methods’ and are not listed here.

**Chemicals:**
Alizarin Red, Sigma  
3-Aminobenzoic acid ethylester (Tricaine), Sigma  
5-Brom-4-chlor-3-indolylphoshpat (BCIP), Roche  
5-Bromo-2-deoxyuridine (BrdU), Sigma  
Fast red TR/Naphthol AS-MX Tablets, Sigma  
Iodonitrotetrazolium chloride (INT), Sigma  
Nitro Blue Tetrazolium (NBT), Roche  
Tissue freezing medium (TFM), Triangel Biomedical Sciences

**Consumables:**
Chromatography paper (3 mm), Whatmann  
Scalpels, Techno Cut scalpel, HMD Healthcare, LTD  
Syringe needles (different size), BD Microlance

3.1.3 Reagents and Buffers

Additional reagents or buffers that were used for a particular experimental procedure are found within the chapter ‘3.2 Methods’ and are not listed here.

**Buffers:**
Alizarin Red solution: 0.5 % Alizarin Red, 1 % KOH  
Bleaching buffer: 0.8 % KOH, 0.6 % H_{2}O_{2}  
Blocking solution (ISH): 5 % heat-inactivated sheep serum, 10 mg/ml BSA in PBT  
Carnoy’s Fixative: 60 % EtOH, 30 % Chloroform, 10 % Acetic Acid  
E3 embryo medium: 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl_{2}· 2 H_{2}O, 0.33 mM MgSO_{4}· 7 H_{2}O, 0.2 ‰ (w/v) methylene blue, pH 6.5
### Hybridization buffer

- 5x SSC, 500 µg/ml type VI Torula yeast RNA (Sigma), 50 µg/ml Heparin, 0.1 % Tween 20, 9 mM Citric Acid (Monohydrate), 50 % deionized formamide, pH 6.0

### INT/BCIP staining buffer

- 175 µg INT, 175 µg BCIP per 1ml NTMT

### Mounting Medium

- 75 % Glycerol in PBS

### NBT/BCIP staining buffer

- 350 µg NBT, 175 µg BCIP per ml NTMT

### NCS-PBT

- 10 % Newborn calf serum, 1 % DMSO, 89 % PBT

### NTMT

- 50 mM MgCl₂, 100mM NaCl₂, 100 mM Tris-HCl, pH 9.5, 0.1 % Tween 20

### PBS

- 1.7 mM KH₂PO₄, 5.2 mM Na₂HPO₄, 150 mM NaCl

### PBT

- PBS with 0.1 % Tween 20

### PBTx

- PBS with 0.3 % Triton X-100

### PFA 4%:

- 4 % (w/v) paraformaldehyde in PBS

### RIPA buffer

- 1 mM EGTA, 50 mM HEPES, 10 % Glycerol, 1.5 mM MgCl₂, 150 mM NaCl, 100 mM NaF, 0.1 % SDS, 1 % Sodiumdeoxycholate, 1 mM Sodium orthovandate, 1 % Triton X-100, pH 7.4

### SDS PAGE Running buffer

- 1 M MOPS, 1 M Tris Base, 69.3 mM SDS, 20.5 mM EDTA

### SDS sample buffer (3x):

- 100 ml: 40 ml 0.5 M Tris, 228 mg EGTA, 6 g SDS, 28 ml Glycerol, 70 mg Brompehnol Blue, top up with H₂O, pH 6.8

### SSC (20x):

- 300 mM NaCl, 200 mM Na-Citrate, pH 7

### SSCT:

- 0.1 % Tween 20 in SSC (1x)

### STOP Solution:

- 1 mM EDTA, 0.1 % Tween 20, 0.05 M phosphate buffer, pH 5.8

### Stripping buffer (ISH):

- 0.1 % Tween 20 in 0.1 M Glycin-HCl, pH 2.2

### Transfer buffer (10x):

- 0.5 M Bicine, 0.5 M Bis-Tris, 20.5 mM EDTA, 10 % MeOH

### Tricaine solution (24x):

- 0.4 % Tricaine powder in 15 mM Tris, pH 7

#### Reagents:

- BSA, Roche
- NCS, Sigma
- Horse serum, Sigma

### 3.1.4 Molecular Biology / Biochemistry Kits

All restriction enzymes and DNA modifying enzymes (*e.g.* T4 Ligase) were purchased from New England Biolobas (NEB) or Thermo Scientific. Additional kits that were used
for a particular experimental procedure are found within the chapter ‘3.2 Methods’ and are not listed here.

Deoxynucleotide Solution MIX, NEB
Fast Plasmid Mini Kit, 5 Prime
Gene Ruler 1 kbp Plus DNA ladder, Fermentas
I-SceI, Roche
QIAquick gel extraction kit, Quiagen
Plasmid Plus Midi Kit, Quiagen
Proteinase K, Invitrogen

3.1.5 Antibodies


<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Host</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-BrdU</td>
<td>monoclonal</td>
<td>rat</td>
<td>Abd seroTec</td>
</tr>
<tr>
<td>Anti-DIG Ap conjugated</td>
<td>polyclonal</td>
<td>sheep</td>
<td>Roche</td>
</tr>
<tr>
<td>Anti-DIG Pod conjugated</td>
<td>polyclonal</td>
<td>sheep</td>
<td>Roche</td>
</tr>
<tr>
<td>Anti-Fluorescein Ap conjugated</td>
<td>polyclonal</td>
<td>sheep</td>
<td>Roche</td>
</tr>
<tr>
<td>Anti-Fluorescein Pod conjugated</td>
<td>polyclonal</td>
<td>sheep</td>
<td>Roche</td>
</tr>
<tr>
<td>Anti-GFP</td>
<td>polyclonal</td>
<td>chicken</td>
<td>Abcam</td>
</tr>
<tr>
<td>Anti-Pcnα</td>
<td>monoclonal</td>
<td>mouse</td>
<td>DakoCytomation</td>
</tr>
<tr>
<td>Anti-phospho Igf1</td>
<td>polyclonal</td>
<td>rabbit</td>
<td>SCBT</td>
</tr>
<tr>
<td>Anti-phospho-histone 3 (H3P)</td>
<td>polyclonal</td>
<td>rabbit</td>
<td>Millipore</td>
</tr>
<tr>
<td>Anti-γTubulin</td>
<td>monoclonal</td>
<td>mouse</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti-Zn5</td>
<td>monoclonal</td>
<td>mouse</td>
<td>ZIRC</td>
</tr>
</tbody>
</table>

Secondary Alexa fluor-conjugated antibodies were purchased from Invitrogen.
Secondary Hrp-conjugated antibodies were purchased from Roche.

3.1.6 Plasmids and Constructs

Amplification of DNA fragments used for generating of below listed constructs (Table 3.3 & 3.4) was performed using Advantage HF 2 PCR Kit (Clontech). Oligos that were used are listed in Table 3.7 & 3.8 (see chapter ‘3.1.7 Oligos’). All constructs were sequenced.

Table 3.2. Constructs used for generation of capped sense RNA.

<table>
<thead>
<tr>
<th>Insert</th>
<th>Vector</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>tol2 transposase (full CDS)</td>
<td>pCSP2⁺</td>
<td>(Suster et al., 2009)</td>
</tr>
</tbody>
</table>
### Table 3.3. Constructs used for synthesis of in situ hybridization probes

<table>
<thead>
<tr>
<th>Insert</th>
<th>Vector</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>actB4a/inhB4a (cDNA fragment)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>actB4b/inhB4b (CDS)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>aldH1n2 (cDNA)</td>
<td>pCR4</td>
<td>M. Brand, Technische Universität Dresden</td>
</tr>
<tr>
<td>ancevan (CDS)</td>
<td>pCSP2+</td>
<td>generated in this study</td>
</tr>
<tr>
<td>andl (CDS)</td>
<td>pBK-CMV</td>
<td>M.-A. Akimenko, University of Ottawa</td>
</tr>
<tr>
<td>axin1 (mouse, cDNA fragment)</td>
<td>pCRII</td>
<td>generated in this study</td>
</tr>
<tr>
<td>axin2 (n. a.)</td>
<td>pSPORT1</td>
<td>n. a.</td>
</tr>
<tr>
<td>bang1b (CDS)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>bmp2a (CDS)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>bmp4 (n. a.)</td>
<td>pBS</td>
<td>M. brand, Technische Universität Dresden</td>
</tr>
<tr>
<td>cebp1b (cDNA fragment including CDS)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>efgp, humanized (CDS)</td>
<td>pCSP2+</td>
<td>(Stoick-Cooper et al., 2007b)</td>
</tr>
<tr>
<td>erm (cDNA fragment)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>fgf3 (cDNA fragment including CDS)</td>
<td>pCSP2+</td>
<td>M. brand, Technische Universität Dresden</td>
</tr>
<tr>
<td>fd3b (cDNA fragment)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>gd40a (cDNA fragment)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>her12 (cDNA fragment including CDS)</td>
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<td>generated in this study</td>
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<tr>
<td>herv (CDS)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>igf2b (cDNA fragment including CDS)</td>
<td>pCRII</td>
<td>A. Wood, Center for Reproductive Biology, Boston</td>
</tr>
<tr>
<td>ihha (CDS)</td>
<td>pCRII</td>
<td>generated in this study</td>
</tr>
<tr>
<td>kaeae (CDS)</td>
<td>pCSP2+</td>
<td>generated in this study</td>
</tr>
<tr>
<td>lef1 (n. a.)</td>
<td>n. a.</td>
<td>(Stoick-Cooper et al., 2007b)</td>
</tr>
<tr>
<td>lfh (CDS)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>hrb5 (cDNA fragment)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>hrb6 (cDNA fragment)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>msx2 (n. a.)</td>
<td>pBluescript SK</td>
<td>(Akimenko et al., 1995)</td>
</tr>
<tr>
<td>peaz (cDNA fragment)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>ptc2 (cDNA fragment)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>rmnc2b (n. a.)</td>
<td>n. a.</td>
<td>H. Roesl, University of Sheffield</td>
</tr>
<tr>
<td>scube2 (cDNA fragment)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>ssh2a (CDS)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>sos3b (cDNA fragment)</td>
<td>pCRII</td>
<td>generated in this study</td>
</tr>
<tr>
<td>sost (cDNA fragment)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>sp3 (n. a.)</td>
<td>n. a.</td>
<td>H. Roesl, University of Sheffield</td>
</tr>
<tr>
<td>spry4 (cDNA fragment)</td>
<td>pSPORT1</td>
<td>(Stoick-Cooper et al., 2007b)</td>
</tr>
<tr>
<td>tcf3 (cDNA fragment)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>tcf5a (n. a.)</td>
<td>pCSP2+</td>
<td>R. Dorsky, University of Utah</td>
</tr>
<tr>
<td>tcf5b (full CDS)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>tcf4 (cDNA fragment)</td>
<td>pGEM-t</td>
<td>generated in this study</td>
</tr>
<tr>
<td>waf1a 5' UTR + mCherry (CDS)</td>
<td>pCSP2+</td>
<td>B. Kagermeier-Schenk, Technische Universität Dresden</td>
</tr>
<tr>
<td>wnt10a</td>
<td>pCRII</td>
<td>(Stoick-Cooper et al., 2007b)</td>
</tr>
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### Table 3.4. Constructs used for generation of transgenic zebrafish lines

<table>
<thead>
<tr>
<th>Construct</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>7xTCF-Xa:Stom:3xKaeae; Tol2, I-SceI, attp entry sites</td>
<td>generated in this study</td>
</tr>
<tr>
<td>her4: 3x rTAM2(3F)-p2A-AmCyan; Tol2, I-SceI sites</td>
<td>C. Haase, Technische Universität Dresden</td>
</tr>
<tr>
<td>keratin4: rTAM2(3F)-p2A-AmCyan; Tol2, I-SceI sites</td>
<td>C. Haase, Technische Universität Dresden</td>
</tr>
<tr>
<td>keratin18: rTAM2(3F)-p2A-AmCyan; Tol2, I-SceI sites</td>
<td>C. Haase, Technische Universität Dresden</td>
</tr>
<tr>
<td>sp7: rTAM2(3F)-p2A-Cerulean; Tol2, I-SceI sites</td>
<td>C. Haase, Technische Universität Dresden</td>
</tr>
<tr>
<td>ubiquitin: rTAM2(3F)-p2A-Cerulean; Tol2, I-SceI sites</td>
<td>generated in this study</td>
</tr>
</tbody>
</table>
The 7xTCF-Xla.Siam:3xKaede construct was created by cloning the 7xTCF-Xla.Siam regulatory sequence (Moro et al., 2012) upstream of 3 in-frame fused Kaede (Ando et al., 2002) CDS which were separated via a linker sequences containing either a SmaI (5’-CTGGAGGAAGCGGACCCGGGGGA-3’) or ApaI (5’-CCTGGAGGAAGCGGGCCGGCGGA-3’) restriction site (Fig. 3.1A).

The ubiquitin:irTAM2(3F)-p2A-AmCyan construct was generated by cloning the previously described regulatory sequence of the ubiquitin gene (Mosimann et al., 2011) upstream of the doxycycline (DOX)-inducible transcriptional activator [irtTAM2(3F)] tagged with p2a and AmCyan (Knopf et al., 2010) (Fig. 3.1B). The remaining TetActivator constructs were generated by Christa Haase (Technische Universität Dresden) by cloning the previously described regulatory sequences of the her4.3 (Yeo et al., 2007), sp7/osx (Spoorendonk et al., 2008), keratin4 (Gong et al., 2002) or keratin18 (Wang et al., 2006) genes upstream of the doxycycline (DOX)-inducible transcriptional activator [irtTAM2(3F)] tagged with p2a and AmCyan or Cerulean (Knopf et al., 2010).

Fig. 3.1. Plasmid maps of constructs used for generation of 7xTCF-Xla.Siam:3xKaede (A) and ubiquitin:irTAM2(3F)-p2A-AmCyan (B) transgenic lines.
### 3.1.7 Oligos

<table>
<thead>
<tr>
<th>Gene</th>
<th>RefSeq</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>axin2</td>
<td>NM_131561</td>
<td>GTGACCCCGGAAAATCCTAAT</td>
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<td>β-actin</td>
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<td>GAGGAGATCCACTCTCTGCTG</td>
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<tr>
<td>lrp5</td>
<td>NM_001177458</td>
<td>CACATGTTAAGGGATATCTG</td>
<td>CATGTGATACCTAAAAAGCT</td>
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<tr>
<td>lrp6</td>
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<td>TCGTCCACCTACAAGGAGCAT</td>
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<td>spry4</td>
<td>NM_131826</td>
<td>CAGCAGGAGTCTCTACGAGC</td>
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### Table 3.6. Primer used for genotyping.

<table>
<thead>
<tr>
<th>Transgenic line</th>
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<tbody>
<tr>
<td>hsp70::cyp26a1&lt;sup&gt;1&lt;sup&gt;1&lt;/sup&gt;</td>
<td>CAGCAGGAGGATTAGGAGC</td>
<td>GACCTGCTAGGGGAGGA</td>
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<tr>
<td>HG21C</td>
<td>GTTCTTCCACACATGATAGG</td>
<td>GACTGTCTTCTATCTAGG</td>
</tr>
<tr>
<td>hsp70::noggin&lt;sup&gt;3&lt;sup&gt;14&lt;/sup&gt;</td>
<td>GCAGGGAAAGAGCACTTGAG</td>
<td>CGGTTTGGACTGACAG</td>
</tr>
</tbody>
</table>

### Table 3.7. Primer used for generation of 7xTCF-Xla.Siam:3xKaede and ubiquitin:irTAM2(3F)-p2A-Am Cynan constructs.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>7xTCF-Xla.Siam: 3xKaede</td>
<td>TTAATTCTCGAGTACGATTAAAGGGACC</td>
<td>TTACCAGATGACAGAACCAGA</td>
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<tr>
<td></td>
<td>(A)TTATTTACGTCGACACCATGCTGA</td>
<td>(A)TTATTTACGTCGACACCATGCTGA</td>
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<tr>
<td></td>
<td>(B)CCCTGGAGAAGGCGCCCAGCGG</td>
<td>(B)CCCTGGAGAAGGCGCCCAGCGG</td>
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<tr>
<td></td>
<td>ATGGGTGACAGCATGATACAAACAGGA</td>
<td>ATGGGTGACAGCATGATACAAACAGGA</td>
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<tr>
<td></td>
<td>ATGGGTGACAGCATGATACAAACAGGA</td>
<td>ATGGGTGACAGCATGATACAAACAGGA</td>
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<tr>
<td></td>
<td>ATGGGTGACAGCATGATACAAACAGGA</td>
<td>ATGGGTGACAGCATGATACAAACAGGA</td>
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### Table 3.8. Primer used for generation of in situ hybridization probe templates.

<table>
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<th>Reverse primer</th>
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<tbody>
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<td>actβAa/ mblβAa</td>
<td>NM_130916</td>
<td>GGGATCCTGCTCGATATGCCTCGTAT</td>
<td>GTGGAGCTGGGACACGCA</td>
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<tr>
<td></td>
<td>actβB/ InhβB</td>
<td>NM_131068</td>
<td>ATGGGACATGATATTATGAAATGACGCT</td>
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<tr>
<td>amcyan</td>
<td>Clontech</td>
<td>TTAATTCCTCGAGGCTAAGGAGG</td>
<td>TTAATTCCTCGAGGCTAAGGAGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCAACAAAGGTTTCAT</td>
<td>ATGCAAGGCTAGGAGG</td>
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</table>

35
3.1.8 Transgenic Zebrafish Lines

Table 3.9. Transgenic zebrafish lines used.

<table>
<thead>
<tr>
<th>Line</th>
<th>Abbreviation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>6xTCF/lef-miniP:2dGFP</td>
<td>αTCF:2dGFP</td>
<td>(Shimizu et al., 2012)</td>
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</tbody>
</table>
### Table 3.9, continued

<table>
<thead>
<tr>
<th>Line</th>
<th>Abbreviation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>7xTCF-XlaSlam:3xKaede</td>
<td>7xTCF:3xKaede</td>
<td>generated in this study</td>
</tr>
<tr>
<td>7xTCF-XlaSlam:nlsCherry&lt;sup&gt;1s8&lt;/sup&gt;</td>
<td>7xTCF:nCherry</td>
<td>(Moro et al., 2012)</td>
</tr>
<tr>
<td>her&lt;sup&gt;4.3&lt;/sup&gt;:EGFP&lt;sup&gt;23&lt;/sup&gt;</td>
<td>her&lt;sup&gt;4.3&lt;/sup&gt;:EGFP</td>
<td>(Yeo et al., 2007)</td>
</tr>
<tr>
<td>her&lt;sup&gt;4.3&lt;/sup&gt;:iptTAM2(3F)-p2a-AmCy &lt;sup&gt;1s1&lt;/sup&gt;</td>
<td>her&lt;sup&gt;4.3&lt;/sup&gt;:TetA AmCy</td>
<td>C. Haase, Technische Universität Dresden, G. Weidinger (Ulm University)</td>
</tr>
<tr>
<td>HG21C</td>
<td>-</td>
<td>(Nagayoshi et al., 2008)</td>
</tr>
<tr>
<td>hsp70:MuMuAxin1-YFP&lt;sup&gt;2s6&lt;/sup&gt;</td>
<td>hsp:Axin1</td>
<td>(Kagermeier-Schenk et al., 2011)</td>
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<tr>
<td>hsp70:cy2961&lt;sup&gt;2s1&lt;/sup&gt;</td>
<td>hsp:cy2961</td>
<td>(Blum and Bégemann, 2012)</td>
</tr>
<tr>
<td>hsp70:dkk1-GFP&lt;sup&gt;2s2&lt;/sup&gt;</td>
<td>hsp:dkk1</td>
<td>(Stoick-Cooper et al., 2007b)</td>
</tr>
<tr>
<td>hsp70:dnfgr1-EGFP&lt;sup&gt;2s11&lt;/sup&gt;</td>
<td>hsp:dnfgr1</td>
<td>(Lee et al., 2005)</td>
</tr>
<tr>
<td>keratin4:iptTAM2(3F)-p2a-AmCy &lt;sup&gt;1s1&lt;/sup&gt;</td>
<td>keratin4:TetA AmCy</td>
<td>C. Haase, Technische Universität Dresden, G. Weidinger (Ulm University)</td>
</tr>
<tr>
<td>keratin18:iptTAM2(3F)-p2a-AmCy &lt;sup&gt;1s1&lt;/sup&gt;</td>
<td>Keratin18:TetA AmCy</td>
<td>generated in this study</td>
</tr>
<tr>
<td>hsp70:noggin&lt;sup&gt;2s4&lt;/sup&gt;</td>
<td>hsp:noggin</td>
<td>(Chocron et al., 2007)</td>
</tr>
<tr>
<td>hsp70:vIRAS,ery2a:dsREDEx&lt;sup&gt;2s8&lt;/sup&gt;</td>
<td>hsp:v-raf</td>
<td>(Lee et al., 2009)</td>
</tr>
<tr>
<td>hsp70:wnt8-GFP&lt;sup&gt;2s4&lt;/sup&gt;</td>
<td>hsp:wnt8</td>
<td>(Weidinger et al., 2005)</td>
</tr>
<tr>
<td>sp7:iptTAM2(3F)-p2a-Cerulean</td>
<td>sp7:TetA Cerulean</td>
<td>C. Haase Technische Universität Dresden, G. Weidinger (Ulm University)</td>
</tr>
<tr>
<td>Top:GFP&lt;sup&gt;2s5&lt;/sup&gt;</td>
<td>Top:GFP</td>
<td>(Dorsky et al., 2002)</td>
</tr>
<tr>
<td>TetRF:Axin1-YFP&lt;sup&gt;2s11&lt;/sup&gt;</td>
<td>TetRF:Axin1-YFP</td>
<td>(Knopf et al., 2010)</td>
</tr>
<tr>
<td>ubiquitin:iptTAM2(3F)-p2a-AmCy</td>
<td>Ubiquitin:TetA AmCy</td>
<td>generated in this study</td>
</tr>
</tbody>
</table>

### 3.2 Methods

#### 3.2.1 Fish Husbandry and Fish Transgenesis

Transgenic and wild type zebrafish (strains: AB, WIK, GOL, ALBINO, PANTHER) were raised and maintained under standard laboratory conditions in a 14-hour on/10-hour off light/dark cycle at 26-27°C and pH 7.5 (Westerfield, 2000). Embryos were kept for 6 days in E3 embryo medium at 28.5°C followed by maintenance under standard conditions. Transgenic fish lines created in this study were either established by Tol2-mediated transgenesis (keratin18:TetA AmCy, 7xTCF:3xKaede) or using the I-SceI meganuclease technique (ubiquitin:TetA AmCy) (Suster et al., 2009; Thermes et al., 2002). The her<sup>4.3</sup>:TetA AmCy, keratin4:TetA AmCy, and sp7:TetA Cerulean transgenic fish lines were generated by Christa Haase (Technische Universität Dresden) using the I-SceI meganuclease technique. 50 pg plasmid DNA of interest, containing either an I-SceI meganuclease site or Tol2 transposable elements, was injected into fertilized eggs together.
with I-SceI protein for I-SceI-mediated transgenesis or capped sense RNA coding for Tol2 transposase for transposon-mediated transgenesis, respectively. Germ-line integration was assessed by outcross and appearance of fluorescence.

3.2.2 Fin Amputations, Heat Shocks, Drug Treatments, Irradiations and Regenerate Length Measurements

For fin amputations, fish were anesthetized in 1x Tricaine solution and approximately 50 % of the caudal fin was removed with a scalpel as previously described (Poss et al., 2000a), after which fish were returned to 28.5°C water.

    Heat shocks were performed for 1 h at 37°C or for 1 h at 38°C for hsp70l:cyp26a1 transgenic fish, according to the schematic timelines shown with each experiment. Water temperature was raised to 37°C or 38°C within 10 min.

    Drug treatments were performed by incubating fish in fish system water containing the drugs except for retinoic acid treatments. Fish were kept in the dark and water was exchanged daily. Doxycycline (DOX; Sigma) was dissolved in 50 % EtOH and used at 25 µg/ml. 10 µM Alk4/5/7 inhibitor SB431542 (Tocris), 5 µM Igf1r inhibitor NVP-AEW541 (Selleck), 10 µM γSecretase inhibitor LY411575 (Selleck), 5 µM Smoothened agonist (SAG; Calbiochem), 5 µM Jnk inhibitor SP60125 (Tocris), 50 µM Smoothened inhibitor Cyclopamine (Sigma) or 12 µM Axin stabilizer IWR-1 (Sigma) was used as previously described (Chablais and Jazwinska, 2010; Grotek et al., 2013; Ishida et al., 2010; Jazwinska et al., 2007; Lee et al., 2009; Lu et al., 2009). NADPH-oxidase inhibitor VAS2780 (Enzo life science) was dissolved in DMSO at 20 µM and used at 0.5 µM. 10 µl of 3 mM retinoic acid (Sigma) were administered by repeated intraperitoneal injections as described (Blum and Begemann, 2012).

    Sublethal irradiation of zebrafish was performed in collaboration with Nona Shayegi (CRTD, Dresden). In brief, fish were transferred to plastic bags containing 1 l of fish system water, placed in a 137Cesium source irradiator (Gammacell 3000 Elan) and exposed until a total dosage of 40 Gy was achieved.

    Regenerate length and calcified bone length measurements were performed using ImageJ software. The regenerate length or calcified bone length of the 2nd, 3rd and 4th lateral ray of each lobe was determined.
3.2.3 **Synthesis of Capped Sense RNA and Labeled RNA Probes**

Capped sense RNA was synthesized *in vitro* from 0.5-1 µg of linearized plasmid DNA of interest using mMessage mMachine Kits (Ambion) according to manufacturer instruction. Subsequently, DNA template was removed using DNaseI (Promega), and RNA was purified using RNeasy Mini Kit (Quiagen) following the instruction manual. Correct integrity of purified RNA was confirmed by agarose gel electrophoresis and RNA was stored at -80°C.

DIG- or Fluorescein-labeled antisense RNA probes were *in vitro* synthesized from 1 µg linearized plasmid DNA of interest using SP6/T7/T3 RNA polymerases (Promega) and either DIG-labeled or Fluorescein-labeled Nucleotides (Roche). Reaction was performed according to SP6/T7/T3 RNA Polymerase instruction manuals. Following probe synthesis reaction mix was further processed as described for synthesis of capped sense RNA. Purified RNA probes were mixed with 100 µl Hybridization buffer and stored at -20°C.

3.2.4 **RNA Whole Mount in situ Hybridization (ISH) and Cryosectioning**

All ISHs were performed on whole mount regenerates and stained samples were subsequently cryosectioned. For chromogenic whole mount ISH, fins were fixed overnight (o/n) at 4°C in 4 % PFA. Next day fins were washed twice in PBT and stepwise dehydrated by successive incubation 5 min each in 25 % MeOH-PBT, 50 % MeOH-PBT, 75 % MeOH-PBT, 100 % MeOH, and stored until needed in 100 % MeOH at -20°C. When required, fins were rehydrated at room temperature (RT) by successive incubation 5 min each in 75 % MeOH-PBT, 50 % MeOH-PBT and 25 % MeOH-PBT followed by 4 washes 5 min each in PBT. Subsequently, fins were incubated at RT for 20 min in PBT containing 20 µg/ml Proteinase K (Invitrogen) followed by 2 brief washes in PBT to stop the digest. Fins were re-fixed at RT for 20 min in 4 % PFA followed by 5 washes 5 min each in PBT. Thereafter, fins were incubated at 65°C for >1 hour (h) in pre-warmed Hybridization buffer. Hybridization buffer was replaced with ISH probes diluted in Hybridization buffer (1:100-1:200; pre-warmed to 72°C for 10 min) and incubated o/n at 65°C. The next day
fins were washed at 65°C 20 min each once in Hybridization buffer, 3 times in 50 % 2xSSCT / 50 % deionized Formamide, and twice in 2xSSCT, followed by 4 washes at 65°C 30 min each in 0.2x SSCT. Thereafter, fins were washed twice at RT 5 min each in PBT and incubated for >1 h in Blocking solution under slow agitation. Subsequently, fins were incubated at 4°C o/n in Blocking solution containing anti-DIG-Ap antibody (1:4000). The next day fins were washed 6 times at RT 20 min each in PBT, followed by 3 washes at RT 5 min each in NTMT. Blue colour reaction was performed by incubating fins at RT in NBT/BCIP staining buffer in the dark until signal had reached the desired intensity. The staining reaction was terminated by washing fins several times in PBT and/or STOP solution. Background staining was cleared by incubating fins 2 times 15 min each in 100 % EtOH, once for 5 min in 50 % EtOH-PBT, followed by 5 washes 5 min each in PBT. Fins were stored in STOP solution or mounted in 75 % Glycerol-STOP solution. If required, fins were further processed for a 2nd color reaction as follows. Anti-DIG-Ap antibody was removed by washing fins 5 min each two times in Stripping buffer and five times in PBT. Afterwards fins were blocked in Blocking solution for >1 h at RT and incubated at 4°C o/n with anti-Fluorescein-Ap antibody diluted in Blocking solution (1:4000). Fins were further processed as described for 1st staining reaction using INT/BCIP (brown precipitate) staining solution or using FastRed tablets (fluorescent red precipitate).

TSA fluorescence ISH was performed using the TSA Plus System Kit (Perkin Elmer). Dual color TSA fluorescence ISH was performed as described for chromogenic dual color ISH with some modifications: 1.) Before pre-hybridization endogenous peroxidase activity was quenched by incubating fins at RT for 20 min in 1-3 % H2O2 followed by 5 washes 5 min each in PBS. 2.) Before incubation with anti-DIG or anti-Fluorescein antibody, fins were blocked at RT for >1 h in Blocking reagent (1 % in PBT) provided with the Kit. 3.) Antibodies used to detect labeled RNA hybrids were anti-DIG-Pod or anti-Fluorescein-Pod diluted 1:500 in Blocking reagent. 4.) Color reaction was performed at RT for 5-30 min in Amplification buffer containing Tyramid substrate (1:500) provided with the Kit, followed by 6-8 washes 20 min each in PBT.

For cryosectioning, fins were incubated at 4°C o/n in 0.5 mM EDTA in PBS. The next day, fins were incubated at RT in 10 % sucrose-PBS, 20 % sucrose-PBS, 30 % sucrose-PBS and 30 % sucrose-PBS:tissue freezing medium (TFM; Triangel Biomedical Sciences) at 1:1 for 30 min each. Subsequently, fins were incubated at 4°C for >2 h in
tissue freezing medium. Fins were frozen in cryomolds and 12-14 µm cryosections were obtained with a Cryostat HM560 (Microm) and stored at -20°C until needed.

3.2.5 Immunohistochemistry

For immunohistochemistry on fin sections, sections were treated with 100 % MeOH (chilled to -20°C) for >30 min to improve adherence to the microscope slides followed by two washes at RT 5 min each in PBT. Sections were blocked at 37°C for 1 h in NCS-PBT containing 2.5 % horse serum, and incubated o/n at 4 °C with primary antibody of interest (1:300-1:500) diluted in NCS-PBT. The next day, sections were washed at RT 4 times 10 min each with PBT and incubated for 2 h at RT with secondary antibody of interest diluted 1:400 in NCS-PBT. Thereafter, sections were washed 10 min each two times in with PBT and one times in PBS. Subsequently, sections were incubated for 10 min with 4’, 6-diamidino-2-phenylindole (Dapi; Sigma) to visualize nuclei followed by two washes 10 min each in PBS. Fin section were re-fixed for 7.5 min in 4 % PFA, washed two times 5 min each in PBS and mounted in Mounting medium.

Simultaneous detection of transcripts and proliferative nuclear antigen protein (Pcna) in whole mount regenerates was performed as described previously (Nechiporuk and Keating, 2002). During sample incubation with anti-DIG-Ap antibody (see chapter ‘3.2.4 RNA Whole Mount in Situ Hybridization (ISH) and Cryosectioning’) an anti-Pcna antibody (1:100) was added. The next day, fins were washed at RT 8 times 20 min each in PBT and incubated o/n at 4 °C with secondary Alexa Fluor-conjugated antibody of interest (1:200 in Blocking solution). The next day, fins were washed at RT 8 times 20 min each in PBT and processed for ISH staining reaction using FastRed tablets.

For simultaneous detection of transcripts and BrdU-labeled DNA, ISH-stained whole mount regenerates where subsequently washed twice in 2 N HCl-PBTx and incubated at RT for 30 min in 2 N HCl-PBTx to retrieve antigen. Fins were further processed as described below for whole mount H3P staining. Anti-BrdU antibody was used at 1:100.

Whole mount phospho-histone 3 (H3P) staining and imaging of H3P+ cells was performed as described previously (Lee et al., 2005; Poss et al., 2002). Fin regenerates were harvested and fixed o/n at 4 °C in Carnoy’s fixative. Next day fins were washed at
RT twice in 100 % MeOH and stepwise rehydrated by successive incubation 5 min each in 75 % MeOH-PBTx, 50 % MeOH-PBTx, 25 % MeOH-PBTx and PBTx. Subsequently fins were briefly washed at RT twice in 2 N HCl-PBTx and incubated for 30 min in 2 N HCl-PBTx to retrieve antigen. Fins were washed several times in PBTx, blocked at RT for 4 h in PBTx containing 0.25 % BSA and incubated o/n at 4 °C with anti-H3P antibody (1:200 in 0.25 % BSA-PBTx). Next day fins were washed at RT 8 times 20 min each in 0.25 % BSA-PBTx and incubated o/n at 4 °C with secondary Alexa Fluor-conjugated antibody of interest (1:200 in 0.25 % BSA-PBTx). Next day fins were washed at RT 8 times for 20 min each in PBS, mounted in Mounting medium and blastemal H3P+ cells were imaged using a Zeiss 510 Meta confocal microscope. Following H3P staining, the epidermis developed a non-specific fluorescence (unpublished observations; Lee et al., 2005; Poss et al., 2002). Thus, during the scans the confocal depth range was set between the highly stained epidermal layers. H3P+ cells in the 2nd and 3rd lateral ray of each lobe were then counted on maximum projections of confocal stacks (1 µm optical sections) covering the blastemal mesenchyme (~22-25 µm).

3.2.6 Detection of cell death

Apoptotic cells in regenerating fins were detected using the TUNEL assay. TUNEL staining on whole mount regenerates was performed using the ApopTag Red In Situ Apoptosis Detection Kit according to the manufacturer’s instructions with some modifications. Regenerates were fixed, de-hydrated, re-hydrated, treated with Proteinase K and re-fixed as described in chapter ‘3.2.4 RNA Whole Mount in situ Hybridization (ISH) and Cryosectioning’. Subsequently, fins were equilibrated and incubated with TdT enzyme according to the user’s manual, followed by several washes in Stop/Wash buffer. Thereafter, fins blocked for 30 min and incubated with ant-DIG-Ap diluted 1:2000 in Blocking solution provided with the Kit. Subsequently, fins were washed 20 min 6 times each in PBT and staining reaction was performed with NBT/BCIP as described in chapter ‘3.2.4 RNA Whole Mount in situ Hybridization (ISH) and Cryosectioning’. A positive control was generated by incubating the fins at RT for 10 min in 3000 U/ml DNase I in 50 mM Tris-HCl, pH 7.5.
3.2.7 BrdU Incorporation

Fish were incubated for 2 days in 1 l fish system water containing 50 µg/ml BrdU. Water was changed daily. At the end of the BrdU incubation fish were briefly rinsed in water before regenerates were harvested.

3.2.8 Tissue Histology

Alizarin Red staining of whole mount regenerates was performed as described previously (Munch et al., 2013). Fins were fixed o/n at 4°C in 4 % PFA. Next day fins were washed twice in PBT and rehydrated through a MeOH series as described for chromogenic ISH (see chapter ‘3.2.4 RNA Whole Mount in Situ Hybridization (ISH) and Cryosectioning’). Subsequently, fins were washed twice in distilled H₂O and bleached for 30 min in Bleaching buffer followed by two washes in distilled H₂O. Subsequently fins were incubated for 20 min in Alizarin Red solution and destained in H₂O until background staining disappeared.

Calcein staining of regenerates was performed on living fish. Fish were incubated for 30 min in fish system water containing 0.1 % Calcein (Sigma). Subsequently, fish were briefly rinsed once in fish system water and incubated for 20 min in fish system water to remove unbound excess Calcein. Calcein fluorescence was detected in the GFP channel using a Leica M205FA stereo microscope.

3.2.9 Preparation of Fin Regenerate Lysates and Immunoblotting

Fin regenerates were washed once in ice-cold PBS, transferred to RIPA buffer supplement with protease and phosphatase inhibitors (Calbiochem), snap frozen in liquid nitrogen and mechanically homogenized using a pestle. Samples were centrifuged at 4°C and 16,000 g for 5 min to pellet debris, and protein extracts were quantified by BCA protein assay (Pierce). Protein extracts were mixed with 3x SDS Page Sample buffer, separated using NuPage 10 % Bis/Tris gels (Invitrogen), transferred to Immobilon-FL PVDF membranes
(Millipore), and immunoblotted for protein of interest. Blots were developed using SuperSignal West Femto Maximum Sensitivity Substrate ECL detection (Pierce). Signals were visualized using a luminescent image analyzer LAS-3000 (Fujifilm).

3.2.10 Dissociation of Fin Regenerates and Flow Cytometry

Adult zebrafish caudal fin regenerates were harvested, briefly rinsed in PBS and incubated in 2 ml PBS containing 1 mg/ml Collagenase/Dispase mix (Roche). During incubation regenerates were mechanically dissociated by carefully pipetting them up and down using Pasteur glass pipettes with decreasing tip diameter. Cell suspension was filtered using a 20 µm Filcon (Keul GmbH) mesh. Flow cytometry was performed using a Becton Dickinson FACS ARIA II SORP (at the FACS Facility Biotec Dresden, Germany).

3.2.11 RNA Extraction and cDNA Synthesis

All centrifuge steps were performed at 4°C and 16,000 g. Zebrafish embryos, regenerates or FACS-sorted cells were incubated for 5 min in 500 µl Trizol (Gibco) and subsequently vigorously vortexed. Thereafter, samples were centrifuged for 10 min to pellet debris. Supernatant was mixed with 269 µl Chloroform, incubated for 10 min at RT and centrifuged for 15 min. The resulting upper (aqueous) phase was transferred to a new tube, mixed with 500 µl Isopropanol and centrifuged for 30 min. After decanting the Isopropanol, RNA pellet was washed in 500 µl 70 % EtOH and samples were centrifuged for 10 min. Ethanol was aspirated, RNA pellet air-dried followed by re-suspension in 20 µl RNase-free water. cDNA was synthesized from maximum 1µg RNA using ThermoScript RT-PCR system (Invitrogen) according to manufacturer’s instructions.
3.2.12 RT-PCR

PCR on cDNA derived from FACS-sorted cells or zebrafish embryos was performed as follows using 10 mM of each dNTP and 10 µM of each primer: 1x (120 s, 94°C); 25-35x (30 s, 94°C; 30 s, Tn – 2°C; 30 x, 68°C); 1x (120 s, 68°C).

3.2.13 Genotyping

For isolation of genomic DNA, caudal fins were amputated and incubated at 95°C for 20 min in 120 µl 50 mM NaOH. Subsequently samples were cooled to 8°C and 14 µl 1M Tris-HCl, pH 8 was added. Samples were vortexed and centrifuged at 4°C and top speed for 5 min to pellet debris. 2 µl of each sample were used for PCR reaction. The PCR conditions for genotyping of hs:nog3, HG21C and hs:cyp26a1 transgenic fish were as follows using 10 mM of each dNTP and 10 µM of each primer: 1x (120 s, 95 °C); 25-35x (30 s, 95°C; 30 s, Ths:nog3 = 55°C / ThG21C = 58°C / Ths:cyp26a1 = 58°C; 30 x, 68 °C); 1x (120 s, 68 °C).

3.2.14 Microarray Analysis

The microarray analysis was performed by Birgit Kagermeier-Schenk (Technische Universität Dresden) and Gilbert Weidinger (Ulm University). Probes for an Agilent 44K custom microarray were created using cDNA sequences derived from RefSeq, Unigene and ZFin databases (design LOLLSG01). Samples were prepared as detailed in Table 3.10. 10 fish were used for each sample and the regenerate plus 2 bony segments of the stump harvested for total RNA isolation using Trizol (Invitrogen), followed by a DNaseI digest and 21 subsequent purification with the NucleoSpin RNA extraction kit (Macherey & Nagel). RNA quality was analyzed using the Agilent Bioanalyzer. The Low RNA Input Fluorescent Linear Amplification Kit (Agilent) was used to obtain fluorescent cRNA. Two-color hybridizations were performed where arrays were simultaneously hybridized with 850 ng Cy3-labeled cRNA derived from one sample and a mix of Cy5-labeled cRNA.
from all samples. All microarray experiments were performed with three independent biological samples.

<table>
<thead>
<tr>
<th>Stage of regeneration</th>
<th>Fish line</th>
<th>Manipulation</th>
<th>Timepoint of harvest</th>
<th>Sample Identifier</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>wild type</td>
<td>heat shock 2 h before harvest</td>
<td>0 hpa</td>
<td>0hpa_1hs_wt1/2/3</td>
</tr>
<tr>
<td>control</td>
<td>wild type</td>
<td>none</td>
<td>0 hpa</td>
<td>0hpa_no hs_wt1/2/3</td>
</tr>
<tr>
<td>wound healing</td>
<td>wild type</td>
<td>heat shock 2 h before harvest</td>
<td>6 hpa</td>
<td>6hpa_no hs_wt1/2/3</td>
</tr>
<tr>
<td>wound healing</td>
<td>hs:dkk1</td>
<td>heat shock 2 h before harvest</td>
<td>6 hpa</td>
<td>6hpa_1hs_dkk1/2/3</td>
</tr>
<tr>
<td>wound healing</td>
<td>hs:Axin1</td>
<td>heat shock 2 h before harvest</td>
<td>6 hpa</td>
<td>6hpa_1hs_axin1/2/3</td>
</tr>
<tr>
<td>blastema formation</td>
<td>wild type</td>
<td>none</td>
<td>48 hpa</td>
<td>48hpa_no hs_wt1/2/3</td>
</tr>
<tr>
<td>blastema formation</td>
<td>wild type</td>
<td>heat shock 6 h before harvest</td>
<td>48 hpa</td>
<td>48hpa_1hs_dkk1/2/3</td>
</tr>
<tr>
<td>blastema formation</td>
<td>hs:dkk1</td>
<td>heat shock 6 h before harvest</td>
<td>48 hpa</td>
<td>48hpa_1hs_dkk1/2/3</td>
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<tr>
<td>blastema formation</td>
<td>hs:Axin1</td>
<td>heat shock 6 h before harvest</td>
<td>48 hpa</td>
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<tr>
<td>blastema formation</td>
<td>wild type</td>
<td>heat shock 2 h before amputation plus heat shocks at 10 hpa, 22 hpa, 34 hpa, 46 hpa</td>
<td>48 hpa</td>
<td>48hpa_serialhs_wt1/2/3</td>
</tr>
<tr>
<td>blastema formation</td>
<td>hs:dkk1</td>
<td>heat shock 2 h before amputation plus heat shocks at 10 hpa, 22 hpa, 34 hpa, 46 hpa</td>
<td>48 hpa</td>
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<tr>
<td>blastema formation</td>
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<td>heat shock 2 h before amputation plus heat shocks at 10 hpa, 22 hpa, 34 hpa, 46 hpa</td>
<td>48 hpa</td>
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</tr>
<tr>
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<td>96 hpa</td>
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<td>heat shock 6 h before harvest</td>
<td>96 hpa</td>
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</tr>
</tbody>
</table>

For annotation, probe sequences were mapped by BLASTn against Ensembl transcript sequences from version 9 of the zebrafish genome (derived via BioMart). Out of the 43219 probes that were used, 30081 probes had at least one hit using an E value cutoff < 10-9, while 13138 produced no hits. The latter probes were re-blasted against a transcript set including 1000 bp genomic sequence 3' of the predicted 3' end of the cDNA. 2334 probes produced a hit in the 3'flanking region of the cDNA set. These were assumed to represent 3'UTRs and the probes thus annotated to detect the corresponding transcript. Thus, in total, 32449 probes had at least one hit, with 1578 however being homologous to transcripts from more than 1 gene. These ambiguous probes were manually inspected and removed if they showed homology to transcripts of different genes. This resulted in a set of 31537 non-ambiguous annotated probes that have been used further (for differentially
expressed genes and co-expression analysis). Ortholog information for each transcript predicted by Ensembl was also extracted for mouse and humans.

The Agilent feature extraction software version 9.1 with all default parameters from Agilent was used to generate the feature extraction data from scans of hybridized arrays. Data analysis was performed using the Bioconductor package Limma (Smyth, 2005) for “two color” microarray with a “common reference”. Feature extraction data was corrected for background using the “backgroundCorrect” function and MA values were extracted after normalization using “normalizeWithinArrays” (by loess). To make the arrays comparable with each other “normalizeBetweenArrays” function was used and the “M values” extracted for further analysis (Suppl. file 1_M_values.xlsx). The function “lmfit” was used to get lists of fold changes and standard errors. “eBayes” was invoked for smoothing to the standard errors. A “design” was constructed based on the user guidelines in Limma Package for analysis of arrays with common reference. axin1 and dkk1 overexpressed samples were compared to the respective wild type controls. Since a Cy5 labeled common reference was used the design was multiplied by -1. The function “topTable” gave a list of differentially expressed genes. All probes were considered that showed absolute fold change of 1.2 and a Benjamini adjusted p-value <0.05. To remove multiple probes that were annotated to the same gene only the probe showing the biggest fold change was considered further (Suppl. 2_Differentially_expressed_genes.xlsx).

3.2.15 Weighted Gene Expression Correlation Network Analysis

The weighted gene expression correlation network analysis (WGCNA) was performed in collaboration with Mohankrishna Dalvoy Vasudevaro and Gilbert Weidinger (Ulm University). Expression data of probes that were significantly differentially expressed (up- or downregulated) between control and dkk1 or control and axin1 overexpressed samples in at least 1 of the following conditions were used to perform weighted gene co-expression network analysis (WGCNA) using the WGCNA package (Langfelder and Horvath, 2008): 48 hpa single heat shock group; 48 hpa serial heat shock 22 group; 96 hpa single heat shock group. A total of 8761 probes fulfilled these criteria and were used for the analysis. A signed (bidirectional) network was constructed where only the positively correlated
genes are clustered into modules. Default values were used (“power = 6” and “mergeCutHeight =0.25”). “Module membership” for each probe was retrieved by using the “datKME” function and tabulated for all probes (Suppl. 3_Module_membership.xlsx). Module eigene gene information was extracted using the “net$MEs” function of WGCNA (Suppl. file 4_Module eigene genes.xlsx). To visualize the expression patterns of modules using module eigene genes, the eigene gene information for the “wild type heat shock”, “Axin1” and “Dkk1” samples was used. Normalization was done by adjusting the values in a manner where eigene genes for the wild type samples at 0 hpa were set to zero. Averaging of the eigene genes was performed for the three biological replicates and the “Standard error of the means” calculated. The modules of our interest with respect to the pattern of expression (reduction in expression upon Wnt inhibition) were modules 26 (darkorange), 21 (darkred) and 3 (brown). To find the correlation between these modules, “eigene gene adjacency” was calculated using the “cor(MEs)” function. Module 26 was found to be positively correlated with module 21 and 3 by a positive correlation co-efficient of 0.37 and 0.42 respectively (Suppl. 5_Eigene gene adjacency.xlsx).

Gene Ontology (GO) and KEGG and PANTHER Pathway enrichment analysis was performed using “DAVID Bioinformatic Resource” (Huang da et al., 2009). Since functional annotation of zebrafish Ensembl gene IDs was poor in DAVID, the mouse ortholog Ensembl IDs for enrichment analysis was used. A specificity level of 5 was used for analysis of biological processes. Only those processes and pathways were considered that were composed of at least 4 genes with a Benjamini FDR corrected p value < 0.05. Out of the 33 modules clustered by WGCNA, 15 were significantly enriched for biological processes, and 14 showed enrichment for pathways (Table 3.11).

<table>
<thead>
<tr>
<th>Module number</th>
<th>Gene ontology</th>
<th>Benjamini corrected p-value</th>
<th>KEGG/Panther pathway</th>
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<td>Heart development</td>
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### Table 3.11, continued

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<th>KEGG/Panther pathway</th>
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<td></td>
<td>Hedgehog signaling</td>
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</tr>
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<td>Organ morphogenesis</td>
<td>9.23E-06</td>
<td>Wnt signaling pathway</td>
<td>9.87E-05</td>
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<td>Translation</td>
<td>2.85E-30</td>
<td>Ribosome</td>
<td>4.09E-44</td>
</tr>
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#### 3.2.16 Statistics

Error bars shown indicate error of the mean. Significance of differences in mean regenerate/calcified bone length and number of proliferative cells was tested using Student's t-test. n.s. indicates not significant, * = p<0.05, ** = p<0.01, *** = p<0.001.

#### 3.2.17 Nomenclature

The nomenclature of genes and proteins in this thesis has been simplified and is as follows: gene symbol: *dkk1* (small, italic), protein symbol: Dkk1 (*1*st letter is capitalized). Excluded from this rule are fluorescent proteins (*e.g.* EGFP) and transgene names of transgenic lines. Transgenic zebrafish lines were annotated according to the Zebrafish database ‘ZFIN’. Abbreviations for each line used are listed in Table 3.9 (chapter ‘3.1.8 Transgenic Zebrafish Lines’).
4 Results

4.1 Transgenic Wnt Reporter Lines Reveal Spatiotemporal Dynamics of Pathway Activation

It has recently been demonstrated that zebrafish caudal fin regeneration requires β-catenin-dependent Wnt signaling for both blastema formation and proliferation (Kawakami et al., 2006; Stoick-Cooper et al., 2007b). However, it has remained unexplained how Wnt signaling exerts these functions. To identify the mechanisms of Wnt action during fin regeneration, I first set out to characterize the spatiotemporal pattern of pathway activation taking advantage of sensitive transgenic reporters of β-catenin-dependent transcription. Transcripts of $7xTCF-Xla.Siam:nlsmCherry^{ia5}$ ($7xTCF:mCherry$; Moro et al., 2012) could be detected from 6 hours post amputation (hpa) in the interray tissue extending approximately 100-200 µm proximal to the amputation plane (big arrowhead in Fig. 4.1A). Intray expression persisted by 12 hpa throughout further regeneration (big arrowhead in Fig. 4.1A). These interray domains were lost upon overexpression of the Wnt inhibitor $dickkopf-1$ ($dkk1$) in $7xTCF:mCherry$; $hsp70l:dkk1$-GFP$^{w32}$ ($hs:dkk1$; Stoick-Cooper et al., 2007b) double transgenic fish, indicating that they indeed reflect sites of active Wnt/β-catenin signaling (Fig. 4.1B). At 24 hpa $mCherry$ transcripts were additionally detected distally to the fin ray stumps, where the blastema is forming (arrow in Fig. 4.1A). Following blastema formation, reporter activity was maintained in the distal part of the regenerate throughout the regenerative process (Fig. 4.1A and data not shown). Longitudinal sections revealed that within fin rays reporter activity was restricted to the mesenchyme of the blastema at all stages analyzed, while the epidermis was devoid of signal (asterisk in Fig. 4.1C). Similarly, the early interray expression domain was mesenchymal as well (Fig. 4.1C). mCherry fluorescence was detectable from 16 hpa and resembled that of the $mCherry$ transcript expression pattern throughout the entire course of regeneration (Fig. 4.1D and data not shown).
Fig. 4.1. The 7xTCF:mCherry transgenic reporter line reveals Wnt/β-catenin pathway activation during caudal fin regeneration.

(A) mCherry transcripts in 7xTCF:mCherry transgenic regenerates. Note transgene expression at 6 and 12 hpa in the interray tissue (big arrowheads).

(B) 7xTCF:mCherry reporter activity in the interray tissue (white arrowhead) is abolished upon dkk1 overexpression in 7xTCF:mCherry; hs:dkk1 double transgenic regenerates 6 hours post heat shock. \( n(7xTCF\; mCherry;\; hs:dkk1) = 11/11,\) \( n(7xTCF\; mCherry;\; hs:dkk1) = 11/11.\) Heat shocks were performed according to the schematic timeline.

(C) mCherry expression in 7xTCF:mCherry transgenic regenerates is confined to the mesenchyme at all stages analyzed. The basal layer of the epidermis is devoid of staining (asterisk).

(D) mCherry fluorescence in 7xTCF:mCherry transgenic caudal regenerates.

(A-D) Small arrowheads: amputation plans. Scale bars: whole mounts, 200 µm; sections, 100 µm.
Expression of the negative pathway regulator \( axin2 \) is directly regulated by the Wnt/\( \beta \)-catenin pathway in many systems and thus frequently used as readout for active signaling (Jho et al., 2002; Lustig et al., 2002 and others). \( axin2 \) expression was likewise found to be dependent on Wnt signaling in the regenerating fin as revealed by its suppression after overexpression of the Wnt inhibitor \( axin1 \) in \( hsp70l:Mu.Axin1-YFP^{35} \) (hs:Axin1; Kagermeier-Schenk et al., 2011) transgenic fish (Fig. 4.2A). 7xTCF:mCherry and \( axin2 \) expression largely co-localized in the distal blastema, supporting that the transgene reports sites of endogenous \( \beta \)-catenin transcriptional activity (arrowhead in Fig. 4.2B, Fig. 4.2C).

Fig. 4.2. \( axin2 \) is expressed in the 7xTCF:mCherry-positive blastema where it depends on Wnt/\( \beta \)-catenin signaling. (A) Downregulation of \( axin2 \) expression in \( hs:Axin1 \) transgenic fish 6 h post heat shock. n(wild type) = 5/5, n(hs:Axin1) = 4/6. Heat shocks were performed according to the schematic timeline. (B) \( axin2 \) (blue) and mCherry RNA (brown) are co-expressed in the distal blastema (arrowhead) in 7xTCF mCherry transgenic regenerates. (C) \( axin2 \) (green) and mCherry RNA (red) are co-expressed in distal blastema cells (arrowhead) and interray cells (arrow) in 7xTCF mCherry transgenic regenerates. Shown is an optical section through a whole mount blastema. (A-C) Small arrowheads: amputation plane. Scale bars: whole mounts, 200 \( \mu \)m (A), 100 \( \mu \)m (B, C) and 10 \( \mu \)m (C); sections, 100 \( \mu \)m.

The expression pattern of a second transgenic reporter line 6xTCF/Lef-miniP:2dGFP (6xTCF:dGFP; Shimizu et al., 2012) was similar to that of the 7xTCF:mCherry reporter at 72 hpa (Fig. 4.3A). Expression of a third Wnt/\( \beta \)-catenin reporter line, \( Top:dGFP^{25} \) (Dorsky et al., 2002) could not be detected before 24 hpa by \textit{in situ} hybridization (ISH), which is consistent with its lower sensitivity during zebrafish embryogenesis (Moro et al., 2012), but expression from 24 hpa onwards likewise was confined to distal domains of the
Results

regenerate and restricted to the blastemal mesenchyme (Fig. 4.3B). These results show that Wnt/β–catenin signaling is activated soon after fin amputation and that it is maintained in the tip of the blastemal mesenchyme throughout the course of tissue restoration.

Fig. 4.3. 6xTCF:dGFP and Top:dGFP transgenic reporter lines reveal Wnt/β–catenin pathway activation during fin regeneration. (A) gfp RNA expression in 6xTCF:dGFP transgenic regenerates is confined to the distal and lateral blastemal mesenchyme. The basal layer of the epidermis is devoid of staining (asterisk). Shown is a confocal image of a longitudinal section. (B) gfp RNA expression in Top:dGFP transgenic caudal regenerates. Note transgene expression is detected in the blastemal mesenchyme while the basal epidermal layer is devoid of signal (asterisk). (A-B) Small arrowheads: amputation plane. Scale bars: whole mounts, 200 µm; sections, 100 µm.

4.2 Tcf/Lef Transcription Factor Expression in the Regenerating Caudal Fin

Wnt target gene expression is regulated by binding of stabilized β–catenin to transcription factors of the Tcf/Lef family that includes Lef1, Tcf1 (Tcf7), Tcf3a (Tcf7l1a), Tcf3b (Tcf7l1b) and Tcf4 (Tcf7l2). In the absence of β–catenin, Tcf/Lefs can interact with transcriptional co-repressors to repress transcription, while β–catenin binding causes recruitment of co-activators and transcriptional activation of target genes (Arce et al., 2006; Hoppler and Kavanagh, 2007). Genetic studies in several systems have revealed that different Tcf/Lefs mainly act as repressors (Tcf3), in an activating role (Lef1) or both (Tcf1, Tcf4) (Arce et al., 2006; Hoppler and Kavanagh, 2007). To further identify the
tissues responding to Wnt signals during fin regeneration, I characterized the expression patterns of the zebrafish Tcf/Lef members. ISH on 72 hpa regenerates showed that expression of all 5 Tcfs was upregulated upon amputation (Fig. 4.4A). In agreement with previous findings, lef1 expression was detected weakly in the distal blastema (arrowhead in Fig. 4.4B) and stronger signal was observed in the proximal basal wound epidermis (Fig. 4.4B) (Poss et al., 2000a). tcf1 was not only expressed in the distal blastema (arrowhead in Fig. 4.4B) and the basal epidermal layer, but also in the lateral blastema where osteoblast progenitors are located (asterisk in Fig. 4.4B). tcf3a and tcf3b were largely confined to the medial blastema, and largely absent from the (pre-)osteoblast domains (Fig. 4.4B), while tcf4 appeared to be weakly expressed in the medial and lateral (asterisk in Fig. 4.4B) blastema. Epidermal transcripts of tcf1 co-localized with that of lef1 (Fig. 4.4C). lef1, tcf1 and tcf3a were co-expressed with the 7xTCF:mCherry reporter in the distal blastema mesenchyme (black arrowheads in Fig. 4.4D), while tcf3b and tcf4 expression hardly overlapped with distal reporter activity (white arrowheads in Fig. 4.4D). Thus, β–catenin signaling seems to be only activated in a subset of cells expressing Tcf/Lef family members. Since Tcf3a acts as a transcriptional repressor in many systems (Arce et al., 2006; Hoppler and Kavanagh, 2007), Lef1 and Tcf1 are primary candidates for mediating the response to Wnt ligands in the 7xTCF:mCherry reporter-positive distal blastema during fin regeneration. Interestingly, transposon-mediated loss of tcf1-function in the transgenic enhancer trap line HG21C did not impair regenerative growth suggesting redundant roles for Lef1 and Tcf1 during caudal fin regeneration (Fig. 4.4E), similar to pectoral fin development (Nagayoshi et al., 2008).
Fig. 4.4. Tcf/Lef transcription factor expression during caudal fin regeneration. (A) lef1, tcf1, tcf3a, tcf3b and tcf4 are expressed in the regenerating fin. Hybridization with sense control probes revealed no signal. (B) Sections of whole mount stained regenerates shown in (A) reveal lef1 expression in the distal blastema (arrowhead) and the basal layer of the epidermis, tcf1 expression in the basal epidermis, lateral (asterisk) and distal blastema (arrowhead), tcf3a, tcf3b and tcf4 expression in the medial and lateral (asterisk) blastema. (C) tcf1 and lef1 are co-expressed in the epidermis of the regenerating fin. (D) Dual color ISH of Tcf/Lef transcription factors and mCherry in 7xTCF:mCherry transgenic regenerates. tcf1, lef1 and tcf3a are co-expressed with mCherry in the distal blastema (black arrowheads), while tcf3b and tcf4 are not expressed in the distal mCherry-positive domain (white arrowheads). (E) Transposon-mediated loss of tcf1(tcf7)-function in HG21C transgenic fish does affect fin regeneration. n(tcf1+/+) = 10/10, n(tcf1−/−) = 10/10. (A-E) Small arrowheads: amputation plane. Scale bars: whole mounts, 200 µm and 100 µm (D); sections, 100 µm.
4.3 The Wound Epidermis is not Competent to Respond to Wnt Ligands Activating β–catenin Signaling

*lef1* does not only mediate signaling by Wnt proteins but its expression is directly activated by the pathway itself in several systems (Filali *et al*., 2002; Hovanes *et al*., 2001; Li *et al*., 2006). Furthermore, it was reported that *lef1* expression is suppressed by *dkk1* overexpression in the regenerating fin (Stoick-Cooper *et al*., 2007b) and I found it to be downregulated in all of its expression domains 6 hours after a single heat shock in *hs:Axin1* transgenic fish as well (Fig. 4.5).

*Fig. 4.5. lef1 expression depends on Wnt/β–catenin signaling during caudal fin regeneration.* Downregulation of *lef1* expression in *hs:Axin1* fish 6 hours post heat shock. n(wild type) = 8/8, n(*hs:Axin1*) = 7/8. Heat shocks were performed according to the schematic timeline. Arrowheads: amputation plane. Scale bar: 200 µm.

Thus, *lef1* expression in the wound epidermis is regulated by Wnt signaling, yet all three of the transgenic Wnt reporter lines that I have used failed to detect pathway activation in the epidermis. To further test whether β–catenin signaling is indeed restricted to the blastemal mesenchyme, I asked whether epidermal cells express the Wnt co-receptors *lrp5* and *lrp6*, which are considered to be essential for β–catenin pathway activation by Wnt ligands (He *et al*., 2004; Wehrli *et al*., 2000). I found that both, *lrp5* and *lrp6* transcripts were upregulated upon fin amputation (Fig. 4.6A) and restricted to the blastemal mesenchyme, while the epidermis was devoid of expression (asterisk in Fig. 4.6B). To exclude that I missed potential low expression levels of *lrp5* or *lrp6* in the epidermis due to insufficient sensitivity of the employed ISH protocol, I developed a flow-cytometry approach that allowed the separation of regenerated epidermal from mesenchymal tissue. I used double transgenic fins of *7xTCF*:mCherry, which is most strongly active in the distal blastema, and *her4.3:EGFP*83 (Yeo *et al*., 2007), which labels the proliferative proximal blastema but excludes the laterally located osteoblasts (Fig. 56)
4.7A-D, Fig. 4.8A). Thus, the GFP+ mCherry+ fraction of such fins should contain blastemal cells, while the GFP– mCherry– fraction should encompass epidermal cells and other cell types.

Fig. 4.6. *lrp5* and *lrp6* co-receptor expression during caudal fin regeneration. (A) *lrp5* and *lrp6* are expressed in the regenerating fin. Hybridization with sense control probes revealed no signal. (B) *lrp5* and *lrp6* transcripts are detected in the blastemal mesenchyme while the basal layer of the epidermis is devoid of signal (asterisk). Arrowheads: amputation plane. Scale bar: whole mounts, 200 µm; sections, 100 µm.

Fig. 4.7. *her4.3*:EGFP reporter activity is confined to the proliferative zone of the proximal medial blastema. (A) The *her4.3*:EGFP reporter (blue) is expressed proximally to the distal-most blastema marker *aldh1a2* (brown; big arrowhead). (B) *her4.3*:EGFP is active in the proliferative cell nuclear antigen (Pcna)-positive cells of the proximal blastema. Optical section through the center of a blastema stained for *egfp* transcripts and Pcna protein is shown. (C) *her4.3*:EGFP activity (brown) is detected medial to the *runx2b*-positive osteoblast progenitors. (D) Confocal image of a longitudinal section of a *her4.3*:EGFP transgenic regenerate stained with Zns5 antibody (labeling all osteoblasts) and GFP antibody show no overlap. Small arrowheads: amputation plane. Scale bar: whole mounts, 200 µm; sections, 100 µm.
By RT-PCR, transcripts of the distal blastema marker *axin2* were only detected in the GFP+ mCherry+ cell fraction while the Fgf target *spry4*, which is expressed in the blastema and the basal layer of the wound epidermis (Lee *et al.*, 2005), was found in both GFP+ mCherry+ and GFP– mCherry– fractions, confirming that the cell sorting was successful (Fig. 4.8B). Importantly, *lrp5* and *lrp6* transcripts were only detectable in the GFP+ Cherry+ fraction, confirming the absence of these co-receptors in the epidermis. Furthermore, overexpression of *wnt8* was sufficient to increase Wnt reporter expression in the mesenchyme, but could not ectopically activate the reporter in the wound epidermis in *hs*p70l:*wnt8-GFP<sup>W34</sup> (*hs*:wnt8; Weidinger *et al.*, 2005); 7xTCF:mCherry double transgenic fish (Fig. 4.8C). Together, these data strongly suggest that Wnt/β–catenin signaling is restricted to the blastemal mesenchyme and that epidermal *lef1* expression must be indirectly regulated by β–catenin signaling.

![Fig. 4.8. The wound epidermis is not competent to respond to Wnt ligands activating β–catenin signaling.](image)

(A) Confocal image showing mCherry+ and EGFP+ cells on a longitudinal section of a 7xTCF:mCherry; her4.3:EGFP double transgenic regenerate at 72 hpa. (B) RT-PCR of indicated genes on cDNA derived from FACS sorted 7xTCF mCherry; her4.3:EGFP double transgenic regenerates at 72 hpa shown in (A). Note that endogenous *lrp5* and *lrp6* transcripts are not detected in the fluorescence-negative, blastema-free cell fraction that contains epidermal cells while *spry4* is. (C) *wnt8* overexpression does not cause ectopic Wnt reporter activation in the wound epidermis in 7xTCF:mCherry; *hs*:wnt8 double transgenic fish. Heat shocks were performed according to the schematic timeline. n(7xTCF:mCherry) = 8/8, n(7xTCF+mCherry; *hs*:wnt8) = 8/8. (A-C) Arrowheads: amputation plane. Scale bars: whole mounts, 200 µm; sections, 100 µm.
4.4 Frizzled Receptor Expression in the Regenerating Caudal Fin

Pathway activation by Wnt ligands requires binding to receptors of the Frizzled (Fzd) family and to Lrp5/6 co-receptors (Angers and Moon, 2009). Both lrp5 and lrp6 co-receptors were found to be expressed in the blastemal mesenchyme during fin regeneration (Fig. 4.6, Fig. 4.8). To identify candidate Fzd receptors that potentially mediate Wnt/β-catenin signaling in the 7xTCF:mCherry-positive, distal blastema, deep sequencing-based gene expression profiling on mCherry+ and mCherry– cell fractions derived from 72 hpa 7xTCF:mCherry transgenic regenerates was performed in collaboration with the Dresden Genome Center (Technische Universität Dresden). Of the 14 fzd genes present in the zebrafish genome (Nikaido et al., 2013; Ensembl Genome Browser) fzd2, fzd7a, fzd7b and fzd9b showed considerable expression levels in the samples (Fig. 4.9A). Among these, fzd9b was found to be highly enriched (~8.5 fold) in the mCherry+ fraction relative to the mCherry– fraction (Fig. 4.9B). ISH against fzd9b on regenerates at 72 hpa confirmed robust fzd9b expression in the distal domains of the regenerate (Fig. 4.9C). Thus, Fzd9b represent a candidate for mediating Wnt/β-catenin signaling in the 7xTCF:mCherry-positive distal blastema during fin regeneration.

![Image of graphs and micrographs](image-url)

**Fig. 4.9. Frizzled receptor expression during caudal fin regeneration.** (A) fzd2, fzd7a, fzd7b and fzd9b are most abundantly expressed at 72 hpa as detected by deep sequencing. Shown is the number of reads in mCherry+ and mCherry– samples per 1 kbp transcript length of the indicated genes. (B) fzd9b is enriched in the 7xTCF:mCherry-reporter-positive cells. Shown is the expression of indicated genes in the mCherry+ fraction relative to the mCherry– fraction. (C) fzd9b is expressed in the distal regenerate at 72 hpa. Arrowhead: amputation plane. Scale bar: 200 µm.
4.5 Wnt/β–catenin Signaling is Active in Different Blastemal Compartments with Distinct Proliferative Properties and Wnt Co-Receptor Expression

The data presented so far strongly suggest that Wnt/β–catenin signaling is restricted to the blastemal mesenchyme. Since the blastema is organized into several domains during regenerative outgrowth (see Fig. 4.10), with distinct proliferative potential and cell fate, I next sought to map the relative spatial localization of the Wnt-receiving cells within the blastema.

![Fig. 4.10. Tissue compartments in a caudal fin regenerate at 72 hpa. Cartoon summarizing relevant anatomical structures and expression domains of a caudal fin regenerate during the outgrowth phase. Longitudinal section view is shown.](image)

In 6xTCF:dGFP regenerates reporter activity was robustly detected in the distal blastema plus in lateral domains of the proximal blastema (arrowheads in Fig. 4.11A-C), while these proximal domains were difficult to detect in 7xTCF:mCherry regenerates (arrowhead in Fig. 4.11B), indicating that the 6xTCF:dGFP reporter is more sensitive and that β–catenin signaling activity is strongest in the distal blastema. The proximal gfp expression was confined to thin lateral domains of approximately 2-3 cells medial to the basal layer of the epidermis and medial to the osteoblast progenitors further proximally (asterisk in Fig. 4.11C, Fig. 4.11E). The mesenchymal expression domains of and1, a marker for actinotrichia-forming cells (Zhang et al., 2010) appeared very similar to those of 6xTCF:dGFP (Fig. 4.11D), and double ISH indicated that 6xTCF:dGFP expressing cells
were also positive for \textit{andl} (asterisk in 4.11E, F). In contrast, no overlap of 6\textit{xTCF:dGFP} or 7\textit{xTCF:mCherry} reporter activity with the osteoblast marker Zns5, which labels all osteoblasts irrespectively of their differentiation state (Fig. 4.11G) (Johnson and Weston, 1995), or the (pre-)osteoblast markers \textit{runx2b} (Fig. 4.11H-I) or \textit{sp7} (Fig. 4.11J) was observed. Thus, Wnt/\(\beta\)-catenin signaling is active in the presumptive actinotrichia-forming cells lining the osteoblasts, but cannot be detected in the bone lineage itself.

\textbf{Fig. 4.11.} Wnt activity in the proximal blastema is confined to the actinotrichia-forming cells. (A) Whole mount view of GFP fluorescence in 6\textit{xTCF:dGFP} transgenic regenerates. Arrowhead points to expression extending further proximally in the center of each blastema. (B) Reporter activity in proximally located blastemal regions, as robustly detected in 6\textit{xTCF:dGFP} transgenic regenerates (big white arrowhead), can only be detected in 7\textit{xTCF:mCherry} transgenics in a minority of samples after prolonged staining (big white arrowhead, 1/8 regenerates). (C) \textit{gfp} RNA in 6\textit{xTCF:dGFP} transgenic regenerates is expressed in cells medial to the (pre-)osteoblasts (asterisk) in the proximal blastema (arrowhead). (D) \textit{andl} expression is detected in the basal layer of the wound epidermis and in the blastema, where it resembles 6\textit{xTCF:dGFP} reporter activity shown in (C). Arrowhead: actinotrichia-forming cells. Asterisk: (pre-)osteoblasts. (E-F) \textit{gfp} transcripts are co-expressed with \textit{andl} in the distal and proximal blastema of 6\textit{xTCF:dGFP} transgenics (arrowhead, asterisk). (G) Antibody staining shows that proximal GFP-positive cells are located adjacent to Zns5-positives osteoblasts in 6\textit{xTCF:dGFP} transgenic regenerates. (C-E, G) Confocal images of a longitudinal section are shown. \textbf{Continued on the next page.}
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Fig. 4.11. continued. (H) gfp expression (brown, asterisk) is detected medial to runx2b transcripts (blue) in 6xTCF:dGFP transgenic regenerates. (I-J) runx2b or sp7 (blue) and mCherry transcripts (brown) do not co-localize in 7xTCF mCherry transgenic regenerates at any stage analyzed. (A-J) Small arrowheads: amputation plane. Scale bars: whole mounts, 200 µm (A-B) and 100 µm (E-H); sections, 100 µm and 50 µm (E).

In addition to these mediolateral proximal domains reporter expression of both transgenic lines was strongly detected in the distal blastema (arrow in Fig. 4.13A). 7xTCF:mCherry reporter expression co-localized with aldha1a2 and mxB, which in whole mount stainings label the distal-most blastema (Fig. 4.12A-B). In contrast, in 7xTCF:mCherry; her4.3:EGFP double transgenic fish, mCherry expression was detected distally to egfp expression marking the proximal medial blastema (Fig. 4.12C-D).

Fig. 4.12. Wnt/β-catenin signaling is active in the distal-most blastema. (A) Double ISH shows co-expression of the 7xTCF:mCherry reporter and aldha1a2 in the same cells in the distal-most blastema. Confocal image through the center of a blastema. (B) aldha1a2 and mxB co-localize with mCherry transcripts in the distal-most blastema in 7xTCF:mCherry transgenic regenerates. Note that samples were stained for mCherry expression only shortly, so
that only the strongest expression domain in the distal blastema was detected. (C) mCherry transcripts are detected distally to egfp transcripts in 7xTCF mCherry; her4.3:EGFP double transgenic regenerates. (D) mCherry fluorescence is detected distally to the EGFP fluorescence-positive proximal blastema in 7xTCF:mCherry; her4.3:EGFP double transgenic regenerates. Cartoon showing the plane of imaging and an optical section through the center of a blastema is shown. (A-D) Scale bars: whole mounts, 100 µm and 10 µm (A); sections, 100 µm.

The distal-most blastemal domain has been shown to consist of largely non-proliferative cells during regenerative outgrowth, while cells of proximal domains proliferate at a high rate (Nechiporuk and Keating, 2002). Indeed, I found that the distal expression domains of the 6xTCF:dGFP and 7xTCF:mCherry transgenics did hardly overlap with proliferative cell nuclear antigen (Pcna) activity (arrow in Fig. 4.13A-B). Furthermore, a number of 7xTCF:mCherry reporter-positive cells located in the distal-most row of blastemal cells did not incorporate the S-phase marker bromodeoxyuridine (BrdU) even when treated for 48 h (arrowheads in Fig. 4.13C). Thus, Wnt/β–catenin signaling is active in the scarcely proliferative distal-most blastema.

Fig. 4.13. The Wnt-receiving cells in the distal-most blastema define a scarcely-proliferative cell population. (A) gfp RNA in 6xTCF:dGFP regenerates is detected in the distal-most, Pcna-negative blastema (arrow) and in a subpopulation of the Pcna-positive proximal blastema (arrowheads). Confocal image of a longitudinal section is shown. Asterisk: lack of Pcna staining due to a blood vessel. (B) 7xTCF mCherry reporter activity is detected in the Pcna-negative distal-most blastema (arrow). Confocal images of a longitudinal section and a whole mount regenerate stained for mCherry transcripts and Pcna protein are shown. (C) A number of 7xTCF mCherry reporter-positive cells remain BrdU-negative even when treated for 48 h (arrowheads). BrdU treatment was performed according to the schematic timeline. Confocal image of a longitudinal section is shown. (A-C) Scale bars: whole mounts, 100 µm and 20 µm (C); sections, 100 µm.
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; Fig. 4.14A-B) showed that some of these cells maintained their distal positions for 48 h after photoconversion (arrowhead in Fig. 4.14C) despite the marked increase in regenerate size (approximately 400 µm) that occurred within this time (Fig. 4.14D). These data support a model in which the distal Wnt-receiving cells maintain their position during regenerative growth and hardly contribute progenitors to the process of tissue restoration.

![Fig. 4.14. The Wnt-receiving cells in the distal-most blastema retain their relative localization during regenerative growth. (A-B) Kaede fluorescence (A) and transcripts (B) in 7xTCF:3xKaede transgenic regenerates resembles 7xTCF:mCherry reporter activity. (C-D) Kaede-positive Wnt-receiving cells located in the distal-most row of mesenchymal cells of a 72 hours-old 7xTCF:3xKaede transgenic blastema were photoconverted from green to red fluorescence and traced for two consecutive days using a confocal microscope. Cells containing the converted Kaede protein were still detected in the distal-most cell row 24 h and 48 h post conversion (white arrowheads in C) despite the substantial regenerative growth that occurred in these fins (D). Small arrowheads: amputation plane. Scale bars: whole mounts, 100 µm and 20 µm (C); sections, 100 µm.](image)

In contrast to the Wnt-receiving cells located in the distal-most blastema, the proximal Wnt reporter-positive domains were positive for PcnA (arrowheads in Fig. 4.13A). With the exception of these mediolateral domains, the remaining proliferative proximal blastema was found to be largely negative for Wnt reporter activity as revealed in optical stacks of 6xTCF:dGFP regenerates (asterisk in Fig. 4.15). Interestingly, analysis of
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lrp5 and lrp6 Wnt co-receptor expression revealed similar distinct patterns. lrp5 transcripts were confined to proximal mediolateral domains and absent from the distal-most blastema (arrowhead in Fig. 4.16) and the (pre-)osteoblast domains (asterisk in Fig. 4.16), while lrp6 appeared to be predominantly expressed in the distal-most blastema (arrowhead in Fig. 4.16). Thus, during regenerative outgrowth, Wnt/β–catenin signaling is confined to two blastemal domains with distinct proliferative properties and Wnt co-receptor expression: the distal-most, non-proliferative, lrp6+ blastema and proximal, mediolateral, proliferative, lrp5+ cells that are likely to form actinotrichia.

Fig. 4.15. 6xTCF:dGFP reporter activity in the regenerating caudal fin at 72 hpa. 10 confocal optical sections spanning the entire mesenchymal region of a whole mount 6xTCF:GFP transgenic regenerate are shown, the distal tip of the regenerate is up. Note that GFP fluorescence is absent from the medial proximal blastema (asterisk in section 6), while expression extends further proximally in the lateral regions of the blastema (e.g. in section 2 and 3). Scale bar: 200 µm.

Fig. 4.16. lrp5 and lrp6 co-receptors are distinctly expressed in the blastema during regenerative outgrowth of the caudal fin. lrp5 transcripts are confined to proximal mediolateral domains and absent in the distal-most blastema (arrowhead) and the osteoblast progenitors (asterisk). lrp6 transcripts are predominantly detected in the distal-most blastema (arrowhead). Asterisk: osteoblast progenitors. Scale bars: 100 µm.
4.6 Tissue-Specific Pathway Manipulation Indicates That Wnt/β-catenin Signaling in the Proximal Blastema, in Committed Osteoblasts, or the Wound Epidermis Has no Role in Regulating Regenerative Cell Proliferation

Loss of Wnt/β-catenin signaling as achieved by 

\[ \text{dkk1} \]

overexpression in the entire regenerate – including the distal blastema – interferes with blastemal cell proliferation and regenerative growth as reported previously (Stoick-Cooper et al., 2007b). Likewise, systemic overexpression of \( axin1 \) in \( hs:Axin1 \) fish at 72 hpa strongly reduced PcnA expression in the proximal mesenchyme and blocked further growth when expression was maintained by continuous heat shocks (Fig. 4.17A-C).

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Fig. 4.17. Systemic overexpression of \( axin1 \) strongly inhibits blastemal cell proliferation and regenerative growth. (A) Confocal images of YFP fluorescence in longitudinal cryosections of \( hs:Axin1 \) transgenic regenerates 6 hours post heat shock. Note that expression is ubiquitous, although levels differ greatly between cells. (B) Systemic overexpression of \( axin1 \) reduces the number of PcnA-positive cells in \( hs:Axin1 \) transgenic regenerates. A single optical section through the mesenchyme of a whole mount regenerate is shown. \( n = 5 \) fins, 2 blastema each. (C) Overexpression of \( axin1 \) starting at 3 dpa strongly interferes with regenerative outgrowth in \( hs:Axin1 \) transgenic fish. (A-C) Heat shocks were performed according to the schematic timeline. Arrowheads: amputation plane. Scale bars: whole mounts, 200 μm; sections, 100 μm. n.s., indicates not significant; \( * = p < 0.05; *** = p < 0.001 \), student’s t-test.
Yet, the data presented so far suggest that Wnt/β–catenin signaling is largely absent in proliferating regions of the fin during regenerative outgrowth, namely the osteoblasts, the epidermis, and the majority of the medial proximal blastema, raising the possibility that Wnt signaling regulates cell proliferation in these domains and therewith regenerative growth indirectly. To test this, I specifically interfered with Wnt signaling in these tissues utilizing the TetON system that has recently been adapted for use in zebrafish (Huang et al., 2005; Knopf et al., 2010). The TetON system comprises a driver line (TetActivator) in which a doxycycline (DOX)-inducible transcriptional activator (TetA; [irtTAM2(3F)]) is under control of a tissue-specific promoter, and a responder line (TetResponder) that harbors a transgene of interest under transcriptional control of a Tet response element (TetRE, Fig. 4.18A). To interfere with Wnt/β–catenin signaling in a cell autonomous manner, I used a TetRE:Axin1-YFP<sup>rad1</sup> TetResponder line that was shown to cause severe Wnt loss-of-function phenotypes when ubiquitously activated during zebrafish gastrulation (Knopf et al., 2010). Treatment of TetRE:Axin1-YFP single transgenic fish with DOX or EtOH for 12 days starting immediately after amputation did not affect regenerative growth, confirming low DOX toxicity and transgene leakiness (Fig. 4.18B, Knopf et al., 2010). I next asked whether overexpression of axin1 in the entire fin using this system is sufficient to interfere with regenerative growth and thus phenocopies hs:Axin1 fish. I created a TetActivator line expressing irtTAM2(3F) and the fluorophore AmCyan under control of the ubiquitin promoter (ubiquitin:irtTAM2(3F)-p2a-AmCyan [ubiquitin:TetA AmCyan]) (Mosimann et al., 2011). Although this driver line was not ubiquitously expressed in the regenerating adult fin (Fig. 4.18C), DOX treatment of ubiquitin:TetA AmCyan; TetRE:Axin1-YFP double transgenics robustly induced axin1-yfp transgene expression in the majority of the blastema and in the wound epidermis within 6 hours after start of the treatment (Fig. 4.18E). Administration of DOX for 5 days starting 1 day before amputation efficiently interfered with regeneration initiation (Fig. 4.18D). Similarly, DOX treatment from 3 dpa until 12 dpa strongly inhibited further regenerative growth, while EtOH had no effect (Fig. 4.18E). Furthermore, DOX treatment for 24 hours was sufficient to strongly reduce epidermal lefl expression (Fig. 4.18F). Thus, expression levels achievable using the TetON system are suitable for studying the tissue-specific roles of Wnt/β–catenin signaling in adult zebrafish.
Fig. 4.18. The TetON system is suitable for studying the tissue-specific role of Wnt/β-catenin signaling during caudal fin regeneration. (A) Cartoon showing the strategy for tissue-specific inducible expression of the Wnt inhibitor axin1 using the TetON system. (B) DOX treatment starting immediately after amputation does not affect regenerative growth in TetRE:Axin1-YFP transgenic fish compared to EtOH-treated controls. (C) AmCyan fluorescence and transcripts in ubiquitin:TetA AmCyan transgenic regenerates. Note that expression is quite ubiquitous in the epidermis and the distal blastema, and covers a substantial part of the proximal blastema. (D-F) ubiquitin promoter-driven axin1-yfp overexpression in the wound epidermis and the blastema is sufficient to block regeneration initiation (D) and regenerative growth (E) as well as inhibits lef1 transcription (F). lef1: n(EtOH) = 6/6, n(DOX) = 5/6. (B-F) DOX treatments were performed according to the schematic timeline. Arrowheads: amputation plane. Scale bars: whole mounts, 200 µm; sections, 100 µm. n.s. indicates not significant; *** = p<0.001, student’s t-test.
To interfere with β–catenin signaling in a tissue-specific manner, a panel of TetActivator lines was established in collaboration with Christa Haase and Gilbert Weidinger (Technische Universität Dresden; Ulm University). To specifically interfere with Wnt/β–catenin signaling in the epidermis, two TetActivator lines were created using regulatory elements of the keratin4 (Gong et al., 2002) and keratin18 (Wang et al., 2006) genes (keratin4:irtTAM2(3F)-p2a-AmCyan [keratin4:TetA AmCyan] and keratin18: irtTAM2(3F)-p2a-AmCyan [keratin18:TetA AmCyan]). While the keratin4 promoter drove TetActivator expression in all layers of the wound epidermis excluding the basal layer (asterisk in Fig. 4.19A), the keratin18 driven TetActivator was only expressed in the latter (Fig. 4.19B). Although these drivers robustly induced the TetRE:Axin1-YFP transgene in the respective domains of the wound epidermis after DOX treatment (plus ectopically in the proximal medial blastema in case of the keratin18:TetA AmCyan line), regenerative growth was not inhibited when fish were treated with DOX for 12 days starting immediately after amputation or 1 day before amputation (Fig. 419C-D and data not shown).

Fig. 4.19. Wnt/β–catenin signaling is not required in the wound epidermis during caudal fin regeneration. (A) amcyan RNA expression in keratin4:TetA AmCyan transgenic regenerates is detected in the epidermis excluding the basal epidermal layer (asterisk). (B) amcyan RNA expression in keratin18:TetA AmCyan transgenic regenerates is confined to the basal epidermal layer. (C) keratin4 promoter-driven axin1-yfp expression in the wound epidermis excluding the basal layer does not interfere with regenerative growth. (D) keratin18 promoter-driven axin1-yfp expression in the basal epidermal layer (plus ectopic expression in the proximal medial blastema) does not interfere with regenerative growth. (A-D) DOX-treatments were performed according to the schematic timelines. Arrowheads: amputation plane. Scale bars: whole mounts, 200 µm; sections, 100 µm. n.s. indicates not significant, student’s t-test.
Similarly, continuous overexpression of *axin1-yfp* specifically in committed osteoblasts mediated by a *sp7*:irtTAM2(3F)-p2a-Cerulean driver (*sp7*:TetA Cerulean), whose expression pattern in the fin at 72 hpa resembled that of endogenous *sp7*:osx did not reduce regenerate growth (Fig. 4.20A-B). In contrast, *axin1-yfp* overexpression in the proximal medial blastema (including the *and1*+ cells) driven by a *her4.3*:irtTAM2(3F)-p2a-AmCyan Activator line (*her4.3*:TetA AmCyan, Fig. 4.21A-C) was able to slow, but not block regenerative growth (Fig. 4.21C). Intriguingly, despite this effect on regenerate length, overexpression of *axin1-yfp* in the *her4.3* domain for 48 hours was not sufficient to significantly reduce mesenchymal cell proliferation as determined by confocal imaging of anti-phospho-histone 3 (H3P)-stained whole mount regenerates (Fig. 4.22). In contrast, proliferation was severely reduced (>50 %) in *ubiquitin*:TetA AmCyan; *TetRE*:Axin1-YFP double transgenic fish under the same conditions (Fig. 4.22). I conclude that β–catenin signaling must be inhibited in the mediolateral proximal blastema and in the distal-most blastema at the same time, as achieved by the *ubiquitin*:TetA driver, for blastemal proliferation to be blocked. Together, these data suggest that Wnt/β–catenin signaling is not directly required for cell proliferation in the wound epidermis, in committed osteoblasts or in the fibroblasts of the proximal medial blastema during the outgrowth phase of fin regeneration. Rather Wnt signaling in the distal, non-proliferative blastema appears to indirectly regulate cell proliferation in the other compartments of the regenerate.

Fig. 4.20. Wnt/β–catenin signaling is not required in committed osteoblasts during caudal fin regeneration. (A) cerulean expression recapitulates endogenous *sp7* expression in *sp7*:TetA Cerulean transgenic regenerates. (B) *axin1-yfp* overexpression in the committed *sp7*-positive osteoblasts does not interfere with regenerative growth. (A-B) DOX treatments were performed according to the schematic timeline. Arrowheads: amputation plane. Scale bars: whole mounts, 200 µm; sections, 100 µm. n.s. indicates not significant, student’s t-test.
Fig. 4.21. Wnt/β–catenin signaling in the proximal medial blastema is not essential for regenerative growth during caudal fin regeneration. (A) amcyan transcripts are detected from 48 hpa (big arrowhead) in her4.3:TetA AmCyan transgenic regenerates. (B) amcyan and yfp transcripts are detected proximally to mCherry in regenerates of her4.3:TetA AmCyan; TetRE:Axin1-YFP; 7xTCF:mCherry triple transgenic fish treated with DOX for 12 hours. (C) axin1-yfp overexpression in the medial proximal blastema has only little impact on regenerative growth in her4.3:TetA AmCyan; TetRE:Axin1-YFP double transgenic fish. The big arrowhead marks the distal-most blastema which is devoid of yfp transcripts. (A-C) Small arrowheads: amputation plane. Scale bars: whole mounts, 200 µm; sections, 100 µm. n.s. indicates not significant; * = p< 0.05, student’s t-test.

Fig. 4.22. Wnt/β–catenin signaling in the medial proximal blastema has little role in regulating blastemal cell proliferation. axin1-yfp overexpression in the medial proximal blastema does not significantly reduce blastemal cell proliferation in her4.3:TetA AmCyan; TetRE:Axin1-YFP double transgenic fish. The number of H3P+ cells in the blastemal mesenchyme is shown. In contrast, proliferation is strongly reduced when axin1-yfp is directed to the distal plus medial proximal blastema using the ubiquitin promoter. n = 4 fish, 16 blastemas. DOX treatments were performed according to the schematic timeline. n.s. indicates not significant; *** = p< 0.001, student’s t-test.
4.7 Wnt/β–catenin Signaling in the Actinotrichia-Forming Cells is Required for Osteoblast Differentiation

I have recently contributed to the discovery that regenerative outgrowth can be halted under conditions where overall levels of cell proliferation are not reduced but osteoblast differentiation fails, as is the case when Notch signaling is overactivated (Grotek et al., 2013). Since inhibition of β–catenin signaling in the proximal blastema also reduced regenerate length without strongly affecting cell proliferation, I asked whether Wnt signaling in this domain regulates osteoblast differentiation. To test this, I examined calcification of regenerating bone as detected by Alizarin Red or Calcein staining in her4.3:TetA AmCyan; TetRE:Axin1-YFP double transgenic fish following DOX treatment. In fish exposed to DOX for 8 days starting at 2 dpa (Fig. 4.23A) regenerate length was reduced by ~20 % at 10 dpa compared to EtOH controls (Fig. 4.23B). In controls, ~85 % of the regenerate was calcified as detected using either of the staining methods, while upon Wnt inhibition in the proximal medial blastema this fraction was reduced to ~50 % as detected by Alizarin Red staining (Fig. 23C) or ~60 % as detected by Calcein staining (Fig. 4.23D). Importantly, axin1-yfp overexpression in committed osteoblasts in sp7:TetA Cerulean; TetRE:Axin1-YFP double transgenics had no effect on bone calcification (Fig. 4.24C-D). Furthermore, DOX treatment of her4.3:TetA AmCyan; TetRE:Axin1-YFP double transgenic fish for 24 hours strongly reduced sp7 expression, a marker for osteoblast commitment (Fig. 23E). Importantly, the reduction in sp7 expression was not due to enhanced apoptotic cell death as detected by the TUNEL assay (Fig. 23F). I conclude that Wnt/β–catenin signaling in the actinotrichia-forming cells indirectly regulates differentiation of adjacently located osteoblast progenitors.
**Results**

Fig. 4.23. Wnt/β-catenin signaling in the medial proximal blastema is required for differentiation of adjacently located osteoblasts. (A) Scheme showing the experimental set-up used in (B-D). (B) **her4.3::TetA AmCyan; TetRE::Axin1-YFP** overexpression in the proximal medial blastema for 8 days reduces regenerate length in **her4.3::TetA AmCyan; TetRE::Axin1-YFP** double transgenic fish treated with DOX, while **axin1-yfp** overexpression in the committed osteoblasts does not affect regenerate length. n = 9 fish, 6 rays each. (C-D) **axin1-yfp** overexpression in the proximal blastema but not in committed osteoblasts reduces the fraction of the regenerate stained with the bone calcification marker Alizarin Red (C) or Calcein (D). n = 9 fish, 6 rays each. (E-F) **axin1-yfp** overexpression in the proximal medial blastema for 24 hours strongly reduces expression of the osteoblast commitment marker sp7 (E) but does not enhance apoptotic cell death as detected by TUNEL assay (F). sp7: n(EtOH) = 6/6, n(DOX) = 4/6. TUNEL: n(EtOH) = 5/6, n(DOX) = 5/6. DOX treatments were performed according to the schematic timelines. (B-F) Arrowheads: amputation plane. Scale bars: 200 µm. n.s. indicates not significant; *** = p < 0.001, student’s t-test.
Results

4.8 Identification of the Wnt Targetome in the Regenerating Caudal Fin

The data presented here strongly suggest that Wnt/β–catenin signaling indirectly regulates proliferation, differentiation and gene expression in several territories of the regenerate. To identify signaling pathways that could mediate these effects, I next aimed to characterize the Wnt targetome in the regenerating fin. This analysis was conducted in collaboration with Birgit Kagermeier-Schenk, Mohankrishna Dalvoy Vasudevaro, and Gilbert Weidinger (Technische Universität Dresden; Ulm University). Identification of the Wnt targetome in the fin was performed during wound healing (6 hpa), blastema formation (48 hpa) and regenerative outgrowth (96 hpa) by profiling gene expression after inducible inhibition of Wnt/β–catenin signaling using hs:Axin1 and hs:dkk1 transgenic lines (see Table 3.10 in chapter ‘3.2.14 Microarray Analysis’). Transgenic fish and their wild type siblings were heat shocked and regenerates were harvested 6 hours post heat shock to enrich for directly regulated targets. In addition, fins were profiled in which dkk1 or axin1 had been continuously overexpressed via serial heat shocks from amputation till 48 hpa. To identify genes which showed similar expression dynamics in response to the experimental conditions and thus might be functionally related, a supervised co-expression analysis of genes differentially expressed in 48 or 96 hpa samples was performed using weighted gene expression correlation network analysis (WGCNA; Langfelder and Horvath, 2008). Thereby, expression patterns could be grouped into 33 different modules. DAVID Bioinformatics resource-based gene ontology analysis (Huang da et al., 2009) revealed that 15 out of these 33 modules were enriched for distinct biological processes (Benjamini corrected p-value <0.05) which were considered for further analysis (see Table 3.11 in chapter ‘3.2.15 Weighted Gene Expression Correlation Network Analysis’). Plotting of the module eigengene value, which represents the weighted average expression of all genes present in a module, revealed that three modules (26, 21 and 3) showed reduced expression under conditions of Wnt inhibition (Fig. 4.24A). Module 26 contained genes whose expression in wild type fish is increased at 48 hpa and 96 hpa relative to 0 hpa fins and whose expression is reduced by inhibition of Wnt/β–catenin signaling (Fig. 4.24A). It was significantly enriched in genes involved in Wnt signaling, but also in genes associated with Fgf and Hh signaling pathways (Fig. 4.24B, Table 4.1). Modules 21 and 3 contained
critical components of not only Wnt signaling pathways but also Bmp/Tgβ3, Hh, Notch, retinoic acid (RA) and Mapk signaling (Table 4.1). This suggests that Wnt/β-catenin signaling regulates key signaling pathway involved in regeneration.

**Table 4.1. Gene composition of WGCNA modules 26, 21 and 3.** (Genes associated with signaling pathways are listed). Table was generated in collaboration with Mohankrishna Dalvoy Vasudevaro (Ulm University).

<table>
<thead>
<tr>
<th>Signaling pathway</th>
<th>Module 26</th>
<th>Module 21</th>
<th>Module 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wnt</td>
<td>wnt9b, wnt10a, axin2, lef1, myc, ccnd2</td>
<td>wnt6, nst, prickle1a, kremen1, notum, sfrp1</td>
<td>wnt4a, fkh2</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>shha, pch2, zic2a, zic3, scler2</td>
<td>hhlp, hhk</td>
<td>smo, sufu, gfl2a, fbxw11a</td>
</tr>
<tr>
<td>Fgf</td>
<td>fgf10a, fgf20a, fgf3, fgfr1a, per3</td>
<td>fgf18, fgfr1a, fgfr4</td>
<td>none</td>
</tr>
<tr>
<td>Notch</td>
<td>hey1</td>
<td>None</td>
<td>jag1a</td>
</tr>
<tr>
<td>Bmp</td>
<td>bmp7a, bambia</td>
<td>bmp2a, bmp4, bmp8a, inkbfb, bambib</td>
<td>bmp2b, mhh4a, smad5</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>None</td>
<td>retg, retbb</td>
<td>aldh1a2</td>
</tr>
<tr>
<td>Mapk</td>
<td>None</td>
<td>igf2a</td>
<td>igf2b, bfgf</td>
</tr>
</tbody>
</table>

*Fig. 4.24. Genes of WGCNA modules 26, 21 and 3 are co-expressed under conditions of Wnt inhibition.* (A) Eigengene values of three correlated WGCNA modules, representing the average relative expression values of all genes in the module, in 10 experimental conditions. Values shown are the average of triplicate samples relative to 0 hpa wild type samples. (B) Cartoon of genes found in module 26 with a selection of genes highlighted that are associated with Wnt, Fgf and Hh signaling. The above shown figure was generated in collaboration with Mohankrishna Dalvoy Vasudevaro (Ulm University).
4.9 Wnt/β–catenin Signaling is Required for Expression of Ligands, Pathway Components and Target Genes of numerous Signaling Pathways Involved in Regeneration

ISH revealed that expression of the Wnt pathway components axin2, lef1, sost and wnt10a was indeed strongly dependent on Wnt/β–catenin signaling supporting the usefulness of the WGCNA analysis and suggesting that these genes are under feedback control (Fig. 4.2A, 4.5, 4.25).

![Fig. 4.25. wnt10a and sost expression depends on Wnt/β–catenin signaling during caudal fin regeneration. sost and wnt10a expression is strongly reduced upon axin1 overexpression for 6 hours in hs:Axin1 transgenic regenerates. sost: n(wild type) = 7/8, n(hs:Axin) = 8/9; wnt10a: n(wild type) = 6/7, n(hs:Axin1) = 6/7. Heat shocks were performed according to the schematic timeline. Arrowheads: amputation plane. Scale bar: 200 µm.](image)

Consistent with the co-expression analysis I found that overexpression of dkk1 or axin1 suppressed expression of ligands, target genes and pathway components of a large number of signaling pathways that have been reported to be required for fin regeneration. Specifically, I found that Hh signaling was dependent on Wnt/β–catenin signaling, since expression of Hh ligands (ihha and shha), the target gene ptc2 as well as the pathway component scube2 were reduced upon dkk1 or axin1 overexpression (Fig. 4.26B). Hh signaling has been shown to be important for regenerative growth, differentiation of the regenerating osteoblasts and patterning of the bony fin rays (Laforest et al., 1998; Quint et al., 2002). Wnt/β–catenin signaling was also required for expression of the Bmp family members gdf6a/bmp13, bmp2a, bmp4, as well as the Bmp pathway feedback inhibitor bambib (Fig. 4.26C). Bmp signaling is thought to regulate osteoblast differentiation and patterning of the dermoskeleton (Laforest et al., 1998; Smith et al., 2006). Interestingly, bmp4 transcripts co-localized with 7xTCF:mCherry Wnt reporter activity in the distal-most
blastema suggesting that \(bmp4\) transcription could be directly controlled by Wnt/\(\beta\)-catenin signaling (arrowhead in Fig. 4.27A). Wnt signaling inhibition also abolished expression of the RA synthesizing enzyme \(aldh1a2/raldh2\) and of the RA binding protein \(crabp1b\) (Fig. 4.26D). RA signaling has recently been shown to be essential for blastemal cell proliferation and survival and it also appears to have roles in fin patterning (Blum and Begemann, 2012; Geraudie et al., 1995; White et al., 1994). Expression of the IGF signaling ligand \(igf2b\) was dependent on Wnt/\(\beta\)-catenin signaling as well (Fig. 4.26E). IGF ligands are expressed in the mesenchyme and have been shown to activate IGF receptors in the basal wound epidermis, which is thought to be required for maintenance of basal wound epidermis integrity as a prerequisite for formation of the blastema and its maintenance (Chablais and Jazwinska, 2010). I found that a single heat shock in \(hs:Axin1\) transgenic fish was sufficient to reduce IGF1 receptor (IGF1R) phosphorylation in the regenerate 6 hours post-heat shock, suggesting that Wnt/\(\beta\)-catenin signaling regulates epidermal IGF signaling through transcriptional control of IGF ligands (Fig. 4.26F).

I have recently contributed to the discovery that Notch signaling is active in the proliferative, proximal medial blastema where it coordinates osteoblast progenitor proliferation with differentiation during regenerative outgrowth (Grotek et al., 2013). Intriguingly, I found that inhibition of Wnt/\(\beta\)-catenin signaling strongly reduced activity of the Notch-dependent \(her4.3\) promoter in the \(her4.3:TetA\) AmCyan transgenic line (Fig. 4.26G) and suppressed expression of the Notch target genes \(her12\) and \(heyl\) and the Notch pathway component \(lunatic fringe (lfng)\) (Fig. 4.26H). Finally, a recent study has provided evidence that Wnt signaling regulates Fgf signaling during fin regeneration via transcriptional regulation of the Fgf ligand \(fgf20a\) (Stoick-Cooper et al., 2007b) and here I found that Wnt/\(\beta\)-catenin signaling was required for expression of the Fgf ligand \(fgf3\) and the Fgf-target genes \(spry4\), \(pea3\) and \(erm\) as well (Fig. 4.26I). Fgf signaling is essential for fin regeneration since it regulates blastema proliferation and epidermal patterning (Lee et al., 2005; Poss et al., 2000b; Whitehead et al., 2005). Interestingly, I also found expression of \(socs3b\), a negative regulator of cytokine signaling, to be dependent on Wnt/\(\beta\)-catenin signaling (Fig. 26J). A role for cytokine signaling in caudal fin regeneration is yet to be described.
Results

Fig. 4.26. Wnt/β-catenin signaling is required for expression of ligands, pathway components and target genes of many signaling pathways involved in regeneration of the zebrafish caudal fin. (A) Samples in (B-L) were either heat shocked at 42 hpa (blue) or 66 hpa (green) and harvested 6 hours later. (B-L) 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 (B), Bmp (C), RA (D), Igf (E-F), Notch (G-H), Fgf (I), and cytokine (J) signaling. Expression of msxB and actβAa but not actβB remains unaffected using the same heat shock protocol (K-L). (F) axin1 overexpression for 6 hours reduces Igf1r phosphorylation as detected in Western blots of whole regenerates at 72 hpa. (G) axin1 overexpression interferes with Notch signaling-dependent her4.3 promoter activity in her4.3:TetA AmCyan fish. ihha: n(wild type) = 5/5, n(hs:dkk1) = 5/5; shha: n(wild type) = 8/8, n(hs:dkk1) = 8/8; ptc2: n(wild type) = 5/5, n(hs:dkk1) = 5/5; scube2: n(wild type) = 8/8, n(hs:Axin1) = 6/8; gdf6a: n(wild type) = 7/7, n(hs:Axin1) = 7/7; bmp2a: n(wild type) = 7/9, n(hs:dkk1) = 7/9; bmp4: n(wild type) = 7/7, n(hs:dkk1) = 7/7; bambib: n(wild type) = 8/8, n(hs:Axin1) = 8/8; aldh1a2: n(wild type) = 7/7, n(hs:dkk1) = 7/7; crabp1b: n(wild type) = 7/8, n(hs:Axin1) = 6/8; igf2b: n(wild type) = 8/8, n(hs:dkk1) = 7/7; n(her4.3:TetA
Results

AmCyan(7/7; n(her4.3;TetA AmCyan; hs:Axin1) = 6/8; her12; n(wild type) = 6/7, n(hs:Axin1) = 6/7; hey1: n(wild type) = 6/7, n(hs:Axin1) = 6/7; ifng: n(wild type) = 9/10, n(hs:Axin1) = 9/10; fgf3: n(wild type) = 6/7, n(hs:dkk1) = 6/7; spry4: n(wild type) = 8/9, n(hs:dkk1) = 6/8; pea3: n(wild type) = 8/8, n(hs:dkk1) = 9/9; erm: n(wild type) = 8/8, (hs:Axin1) = 7/7; socs3b: n(wild type) = 7/8, n(hs:Axin1) = 5/8; mxb: n(wild type) = 7/7, n(hs:Axin1) = 7/7; actβAa: n(wild type) = 6/6, n(hs:dkk1) = 7/8; actβB: n(wild type) = 8/8, n(hs:Axin1) = 6/8. Scale bars: 200µm. Heat shocks were performed according to the schematic timeline.

Fig. 4.27. bmp4 and actβB are expressed in the Wnt-receiving cells. (A) bmp4 and mCherry transcripts co-localize in the distal-most blastema (arrowhead) in 7xTCF mCherry transgenic regenerates. (B) actβB and mCherry transcripts co-localize in the distal-most blastema in 7xTCF:mCherry transgenic regenerates (A-B) Note that samples were stained for mCherry expression only shortly, so that only the strongest expression domain in the distal blastema was detected. Scale bars: whole mounts, 100 µm; sections, 100 µm.

Importantly, some factors required for fin regeneration were insensitive to inhibition of Wnt/β–catenin signaling using the same heat shock protocol, namely mxb (Fig. 4.26K and data not shown) and actβAa/inhβAa (Fig. 4.26L and data not shown), indicating that gene expression is not non-specifically suppressed in axin1 or dkk1 expressing fins. Activin signaling has been shown to promote blastemal proliferation (Jazwinski et al., 2007). Interestingly, while actβAa/inhβAa was not dependent on Wnt/β–catenin signaling, actβB/inhβB expression, which co-localized with 7xTCF:mCherry in the distal-most blastema, was (Fig. 4.26L, arrowhead in Fig. 4.27B).

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.
4.10 Wnt/β-catenin Signaling Receives Little Reciprocal Input from the Signaling Pathways it Regulates

The data presented here indicate that Wnt/β-catenin signaling regulates many other signaling pathways required for fin regeneration. To determine whether these pathways also reciprocally control β-catenin signaling, I assayed 7xTCF:mCherry reporter activity or expression of the endogenous Wnt target axin2 after genetic or pharmacological inhibition of a number of these pathways. Yet, the Wnt reporter was hardly reduced after continuous inhibition of Igf signaling using the Igf1r inhibitor NVP-AEW541 or Fgf signaling by overexpression of a dominant negative zebrafish FGF receptor 1 (dnfgfr1) in hsp70l:dnfgfr1-EGFP<sup>hd</sup> (hs:dnfgfr1; Lee et al., 2005); 7xTCF:mCherry double transgenics for 3 days starting prior to amputation (Fig. 4.28A-B), although regenerative growth was severely reduced by both treatments. Reporter expression was reduced but still robustly detected in the severely diminished regenerates produced by continuous interference with Notch signaling using the γSecretase inhibitor LY411575 (Fig. 4.28C), with Activin signaling using the Activin receptor-like kinase receptor 4 (Alk4) inhibitor SB431542 (Fig. 4.28D) or with RA signaling using overexpression of zebrafish cyp26 in hsp70l:cyp26a1<sup>kn1</sup> (hs:cyp26a1; Blum and Begemann, 2012); 7xTCF:mCherry double transgenics (arrowhead in Fig. 4.28E). Furthermore, expression of the direct Wnt target axin2 was hardly reduced after interference for 6 hours with Activin signaling using the Alk4 receptor inhibitor SB431542 (Fig. 4.28F), with Igf signaling using the Igf1r inhibitor NVP-AEW541 (Fig. 4.28G), with Hh signaling using the Smoothened antagonist Cyclopamine (Fig. 4.28H), or with Notch signaling using the γSecretase inhibitor LY411575 (Fig. 4.28I). axin2 expression was likewise robustly detected 6 hours after heat shock-induced overexpression of the dnfgfr1 in hs:dnfgfr1 transgenics (Fig. 4.28J), of cyp26a1 in hs:cyp26a1 transgenics (Fig. 4.28K), or of the Bmp inhibitor noggin3 in hsp70l:noggin3<sup>fr14</sup> transgenics (hs:nog3; Chocron et al., 2007) (Fig. 4.28L). Importantly, axin2 expression was strongly reduced 6 hours after a single heat shock in hs:Axin1 transgenics (Fig. 4.2A) or when fish were treated for 6 hours with IWR-1, a pharmacological compound that inhibits Wnt/β-catenin signaling through stabilization of Axin (Fig. 4.28M). Thus, while interference with β-catenin signaling severely reduced or abolished expression of ligands or readouts of Activin, Bmp, Fgf, Hh, Igf, Notch and RA pathways within hours, inhibition of these
pathways to a level that severely affected regeneration was not sufficient to completely abolish Wnt signaling activation in the remaining blastemal tissue even after prolonged pathway interference. I conclude that Wnt/β-catenin signaling acts upstream of these pathways, but receives little reciprocal input.

Fig. 4.28. Wnt/β-catenin signaling receives little reciprocal input from other developmental signaling pathways during caudal fin regeneration. (A-E) 7xTCF:mCherry reporter expression is robustly detectable in severely reduced regenerates obtained after prolonged inhibition of Igf (A), Fgf (B), Notch (C), Activin (D) or RA (E) signaling for 48 or 72 hours using pharmacological compounds or heat shock-induced overexpression of pathway inhibitors. Continued on the next page.
Fig. 4.28. continued. (F-L) Expression of the endogenous Wnt target **axin2** is robustly detected after inhibition of Activin (F), Igf (G), Hh (H), Notch (I), Fgf (J), RA (K) or Bmp (L) signaling for 6 hours, while it is strongly reduced when Wnt/β-catenin signaling (M) is inhibited for 6 hours. Drug treatments or heat shocks were performed according to the schematic timeline. Small arrowheads: amputation plane. Scale bar: whole mounts, 200 µm. **7xTCF:mCherry:** NVP-AEW541: n(DMSO) = 6/6, n(NVP-AEW541) = 6/6; hs:dnfgfr1: n(7xTCF:mCherry) = 6/6, n(7xTCF:mCherry; hs:dnfgfr1) = 6/6; LY-411575: n(DMSO) = 5/6, n(LY-411575) = 4/6; SB431542: n(DMSO) = 6/6, n(SB431542) = 6/6; hs:cyp26a1: n(7xTCF:mCherry) = 4/4, n(7xTCF:mCherry; hs:cyp26a1) = 4/4. **axin2:** SB431542: n(DMSO) = 6/6, n(SB431542) = 7/7; NVP-AEW541: n(DMSO) = 6/6, n(NVP-AEW541) = 6/6; Cyclopamine: n(DMSO) = 6/6, n(Cyclopamine) = 6/6; LY-411575: n(DMSO) = 6/6, n(LY-411575) = 5/6; hs:dnfgfr1: n(wild type) = 8/8, n(hs:dnfgfr1) = 7/8; hs:cyp26a1: n(wild type) = 8/9, n(hs:cyp26a1) = 7/9; hs:nog3: n(wild type) = 5/5, n(hs:nog3) = 4/5, IWR-1: n(DMSO) = 7/7, n(IWR-1) = 6/7.

### 4.11 Wnt/β-catenin Signaling Indirectly Regulates Epidermal Patterning Through Fgf and Bmp Signaling

I have provided evidence that cell proliferation in several compartments of the regenerating fin appears to be indirectly regulated by Wnt/β–catenin signaling. Likewise, epidermal **lef1** expression cannot be directly controlled by the pathway. Consistent with this hypothesis we found that **mCherry** expression was already abolished 3 hours after a single heat shock in 7xTCF:mCherry; hs:dkk1 double transgenic regenerates (arrow in Fig. 4.29A) while in the same fins epidermal **lef1** expression was unaffected (asterisk in Fig. 4.29A). I thus
looked for candidate signals that could act downstream of β–catenin signaling to mediate its effects in the epidermis. In other systems, *lef1* expression has been shown to be regulated by Bmp signaling (Kratochwil *et al*., 1996). I found that epidermal *lef1* expression was strongly reduced 6 hours following heat shock-driven overexpression of the Bmp inhibitor *noggin* in the *hs:nog3* transgenic line (Fig. 4.29B-C), while Wnt reporter activity was hardly altered in *7xTCF:*mCherry; *hs:nog3* double transgenics (Fig. 4.29C). Thus, the Bmp pathway regulates epidermal *lef1* expression independently of β–catenin signaling. *lef1* has also been shown to be regulated by Fgf signaling in the regenerating fin (Lee *et al*., 2009). I found that *lef1* expression was strongly diminished 6 hours after activation of *dnfgf1* expression in *hs:dnfgf1; 7xTCF:*mCherry double transgenics, while in the same fin rays the Wnt reporter was unaffected (Fig. 4.29D). Together with the 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.

Interestingly, as shown in Fig. 4.26I, I have found that 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 depends on Wnt/β–catenin signaling. While 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). Thus, Wnt/β–catenin signaling might regulate *pea3* and *erm* expression through the Fgf/Ras pathway. To test this, we asked whether Ras activation could rescue *erm* and *pea3* expression in Wnt-inhibited fins using *hs:Axin1; hsp70l:vHRAS,cryaa:DsREDExpd8 (hs:v-ras; Lee *et al*., 2009) double transgenics. In agreement with published data (Lee *et al*., 2009) a single heat shock was sufficient to upregulate *pea3* and *erm* expression in *hs:v-ras* transgenic regenerates 6 hours post heat shock, while their expression was abolished in *hs:Axin1* transgenics (Fig. 4.30A). Intriguingly, upon simultaneous overexpression of *axin1* and *v-ras*, *erm* and *pea3* expression was restored (Fig. 4.30B). Thus, I conclude that Wnt/β–catenin signaling in the distal blastema regulates *pea3* and *erm* transcription in the adjacent wound epidermis via activation of Fgf/Ras signaling. *fgf3* is a candidate Fgf ligand mediating this interaction between tissues, since its expression co-localizes with β–catenin activity in the distal-most blastema (arrowhead in Fig. 4.30B), where it is dependent on β–catenin signaling (Fig. 4.26I).
Fig. 4.29. Epidermal *lef1* expression is regulated via Bmp and Fgf signaling independently of Wnt/β-catenin signaling. (A) *dkk1* overexpression interferes with *mCherry* (arrow) but not *lef1* (asterisk) expression 3 hours after a single heat shock in *7xTCF:mCherry; hs:dkk1* double transgenic regenerates. n(*7xTCF:mCherry*) = 7/8, n(*7xTCF:mCherry; hs:dkk1*) = 6/8. (B) Overexpression of the Bmp inhibitor *noggin3* for 6 hours interferes with *lef1* expression in *hs nog3* transgenic regenerates at 72 hpa. n(wild type) = 7/7, n(*hs nog3*) = 5/7. (C) *noggin3* overexpression reduces *lef1* (blue) but not *mCherry* (brown) expression in *7xTCF:mCherry; hs:nog3* double transgenic regenerates. n(*7xTCF:mCherry*) = 6/6, n(*7xTCF:mCherry; hs:nog3*) = 5/6. (D) Overexpression of a dominant negative *fgf receptor 1* reduces *lef1* (blue) but not *mCherry* (brown) expression in *7xTCF:mCherry; hs:dnfgfr1* double transgenic regenerates. n(*7xTCF:mCherry*) = 7/7, n(*7xTCF:mCherry; hs:dnfgfr1*) = 5/7. (A-D) Heat shocks were performed according to the schematic timeline. Small arrowheads: amputation plane. Scale bar: whole mounts, 200 µm.

Fig. 4.30. Wnt/β-catenin signaling regulates epidermal *pea3* and *erm* expression via Fgf/Ras signaling. (A) *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*. *pea3*: n(wild type) = 7/7, n(*hs:v-Ras*) = 7/7, n(*hs:Axin1*) = 7/7, n(*hs:Axin1; hs:v-ras*) = 6/7; *erm*: n(wild type) = 8/8, n(*hs:v-ras*) = 7/7, n(*hs:Axin1*) = 7/7, n(*hs:Axin1; hs:v-ras*) = 6/7. Heat shocks were performed according to the schematic timeline. (B) *fgf3* and *mCherry* transcripts co-localize in the distal-most blastema (arrowhead) in *7xTCF:mCherry* transgenic regenerates. Note that samples were stained for mCherry expression only shortly, so that only the strongest expression domain in the distal blastema was detected. (A-B) Small arrowheads: amputation plane. Scale bar: whole mounts, 200 µm (A) and 100 µm (B); section, 100 µm.
4.12 Wnt/β–catenin Signaling regulates Maintenance of the Wound Epidermis via a Retinoic Acid/Igf Signaling Axis

A recent study has proposed a model in which Igf signaling from the blastemal mesenchyme to the basal epidermal layer is essential for maintaining the integrity of the basal wound epidermis as a prerequisite for regeneration (Chablais and Jazwinska, 2010). Similarly, Wnt/β–catenin signaling is required for specification of the basal wound epidermis (Kawakami et al., 2006; Stoick-Cooper et al., 2007b). As shown in Fig. 4.26E-F one of the pathways regulated by Wnt signaling in the regenerating fin is Igf, suggesting that Wnt/β–catenin signaling regulates maintenance of the wound epidermis via activating epidermal Igf signaling. However, igf2b transcripts were found to be largely absent in regions of the regenerate with active Wnt/β–catenin signaling, namely the distal-most blastema and the actinotrichia-forming cells (Fig. 4.31A), indicating that it is not a direct target of the pathway. Interestingly, a recent study has provided evidence for RA signaling controlling igf2b transcription (Blum and Begemann, 2012) and I found that expression of the rate-limiting enzyme in RA synthesis aldhl2 depends on Wnt/β–catenin signaling (Fig. 4.26D). Thus I asked whether Wnt/β–catenin signaling regulates igf2b indirectly via RA pathway activation. I inhibited Wnt/β–catenin signaling using hs:dkk1 transgenic fish and concomitantly injected RA. Consistent with my previous results and those of others (Blum and Begemann, 2012), RA injections slightly enhanced, while dkk1 overexpression blocked igf2b expression. Intriguingly, upon RA administration I could again detect igf2b transcripts in hs:dkk1 transgenic regenerates (arrows in Fig. 4.31B). Thus Wnt/β–catenin signaling regulates at least in part igf2b expression indirectly via RA signaling. Together these data suggest that Wnt/β–catenin signaling possibly regulates maintenance of the wound epidermis via sequential activation of RA and Igf signaling.
Results

4.13 Blastemal Cell Proliferation is Regulated by the Wnt/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 blastemal mesenchyme, since Hh ligands are transcriptionally regulated by β–catenin signaling (see Fig. 4.26B) and the pathway has been shown to be required for blastema proliferation (Quint et al., 2002). Activation of Hh signaling using the Smoothened agonist SAG could rescue epidermal and blastemal expression of the direct Hh target gene \( ptc2 \) in \( hs:Axin1 \) transgenic fins (Fig. 4.32A-B), indicating that this treatment can restore Hh signaling lost after inhibition of β–catenin signaling. Thus, I asked whether Hh signaling could rescue blastemal proliferation upon Wnt pathway inhibition. \( hs:Axin1 \) transgenic fish and their wild type siblings were subjected to a single heat shock, treated with SAG or vehicle control and anti-phospho-histone 3 (H3P)-positive blastemal cells were quantified using confocal imaging. Consistent with previous reports \( axin1 \) overexpression significantly decreased, while SAG-treatment increased the number of H3P+ cells in the blastemal mesenchyme (Fig. 4.33A) (Lee et al., 2009; Stoick-Cooper et al., 2007b). Importantly, SAG treatment rescued the number of H3P+ cells in \( hs:Axin1 \) transgenic fish back to wild type levels (Fig. 4.33A). In contrast, activation of
Wnt/β–catenin signaling through overexpression of \textit{wnt8} in \textit{hs:wnt8} transgenic fish failed to rescue blastemal proliferation that was reduced upon inhibition of Hh signaling using Cyclopamine (Fig. 4.33B). Thus, Wnt/β–catenin signaling regulates blastemal proliferation through Hh signaling.

Fig. 4.32. Wnt/β–catenin signaling regulates Hedgehog signaling. (A) \textit{ptc2} expression is detected in the basal epidermal layer and in osteoblasts (arrowhead). (B) \textit{axin1} overexpression inhibits expression of the Hh target \textit{ptc2} which is rescued by concomitant activation of Hh signaling using the pharmacological compound SAG. n(wild type) = 5/6, n(wild type, SAG) = 5/6, n(hs:Axin1) = 6/6, n(hs:Axin1, SAG) = 4/6. Heat shocks and drug treatments were performed according to the schematic timeline. (A-B) Small arrowheads: amputation plane; scale bar: whole mount, 200 µm; section, 100 µm.

Fig. 4.33. Wnt/β–catenin signaling regulates blastemal cell proliferation via Hedgehog signaling. (A) \textit{axin1} overexpression reduces the number of H3P+ cells in the blastema which is rescued by concomitant activation of Hh signaling using the Smoothened Agonist SAG. n = 5 fish, 20 blastemas. (B) Inhibition of Hh signaling through exposure to the Smoothened antagonist Cyclopamine reduces the number of H3P+ cells in the blastema which is not rescued by concomitant activation of Wnt signaling following \textit{wnt8} overexpression. n = 5 fish, 20 blastemas. (A-B) Heat shocks and drug treatments were performed according to the schematic timeline.
Another candidate pathway that could mediate the mitotic effects of Wnt/β–catenin signaling in the proximal blastema is RA signaling, which has recently been shown to be essential for blastema formation and proliferation (Blum and Begemann, 2012). Expression of the RA synthesizing enzyme aldh1a2 is largely confined to the Wnt/β–catenin signaling-positive distal-most blastema, where its expression depends on the Wnt pathway (Fig. 4.12A-B, 4.26D). In contrast, the retinoic acid receptor γa (rarγa) was mainly expressed in the 7xTCF:mCherry negative (pre-)osteoblast domain in the lateral blastema, indicating that these cells are competent to respond to the distally synthesized RA (Fig. 4.34A, Blum and Begemann, 2012). To test whether RA acts downstream of Wnt/β–catenin signaling in regulation of blastemal cell proliferation, I assayed blastema mitosis in hs:Axin1 transgenic fish that were injected with RA. Consistent with previous results, RA increased the number of H3P+ mesenchymal cells, while overexpression of axin1 suppressed proliferation (Blum and Begemann, 2012; Stoick-Cooper et al., 2007b) (Fig. 4.34B). Intriguingly, I found that proliferation was rescued back to wild type levels in RA-treated, axin1 expressing fins, indicating that RA signaling regulates blastemal proliferation downstream of Wnt/β–catenin signaling during regenerative outgrowth (Fig. 4.34B). Although repetitive administration of RA rescued blastemal cell proliferation when Wnt/β–catenin signaling was concomitantly inhibited, it was not sufficient to rescue regenerative growth in hs:dkk1 transgenic fish (Fig. 4.34C), supporting the finding that Wnt/β–catenin signaling orchestrates fin regeneration through regulation of multiple signaling pathways. Together, these data indicate that Wnt/β–catenin signaling regulates blastemal cell proliferation indirectly through Hh and RA pathways.

Fig. 4.34. Wnt/β–catenin signaling regulates blastemal cell proliferation via retinoic acid signaling. (A) Transcripts of the RA receptor rarγa are detected proximally to mCherry expression in the distal-most blastema in 7xTCF:mCherry transgenic regenerates. Longitudinal sections reveal that rarγa expression is confined to the lateral blastema containing (pre-)osteoblasts. Continued on the next page.
Results

Fig. 4.34. continued. (B) axin1 overexpression reduces the number of H3P+ cells in the blastema which is rescued by concomitant activation of retinoic acid signaling through administration of RA. n = 5 fish, 20 blastemas. (C) dkk1 overexpression strongly inhibits regenerative growth which is not rescued by concomitant activation of retinoic acid signaling through administration of RA. n = 5 fish. (A-C) Heat shocks and drug treatments were performed according to the schematic timeline. Arrowheads: amputation plane. Scale bar: 100 µm.

4.14 Amputation-Induced Jnk Activity, ROS Production or Immune System Activation is Dispensable for Wnt/β–catenin Pathway Activation During Caudal Fin Regeneration

The data presented strongly suggest that the β–catenin pathway acts upstream of many other signaling pathways required for regeneration, indicating that Wnt signaling acts early in the hierarchy of signals. Thus, an important question arises: what signal acts upstream of Wnt/β–catenin signaling, that is, which wounding-induced signals activate Wnt ligand expression and thus β–catenin signaling? Potential candidate signals that have been shown to be activated very soon after wounding and are essential for initiation of adult fin or larval finfold regeneration are redox signaling via generation of reactive oxygen species (ROS) such as H₂O₂, activation of cytokine and chemokine-secreting immune cells, and c-Jun-N-terminal kinase/stress-activated proteinase kinase (Jnk/Sapk)-mediated activation of gene transcription (Ishida et al., 2010; Kyritsis et al., 2012; Niethammer et al., 2009; Yoo et al., 2012; Gauron et al., 2013). To test whether any of the aforementioned signals act upstream of β–catenin pathway activation I assayed 7xTCF:mCherry reporter expression
following immunosuppression or pharmacological inhibition of ROS production or Jnk signaling. 7xTCF:mCherry reporter expression was robustly detected after continuous inhibition of Jnk activity using the pharmacological compound SP60125 although regeneration was severely inhibited (Fig. 4.35A). Similarly, 7xTCF:mCherry reporter expression was detectable upon inhibition of NADPH oxidase-mediated H$_2$O$_2$ production using the pharmacological compound VAS-2780 (arrows in Fig. 4.35B) even though treated fish failed to form a wound epidermis. Finally, immunosuppression via sub-lethal irradiation (40 Gy; Traver et al., 2004), which was performed in collaboration with Nona Shayegi (CRTD, Dresden), completely blocked fin regeneration but did not inhibit 7xTCF:mCherry reporter activity (Fig. 4.35C). Together, these data suggest that redox signaling, Jnk/Sapk signaling and immune system-induced inflammation is dispensable for activation of Wnt/β–catenin signaling during caudal fin regeneration.
5 Discussion

5.1 A Model for Wnt/β–catenin Signaling Functions During Caudal Fin Regeneration

The work presented here significantly advances our understanding of the mechanisms underlying vertebrate appendage regeneration. The results presented here suggest that the blastema, the population of undifferentiated mesenchymal cells characteristic of regenerating appendages, displays previously unrecognized functional diversity, since it contains signaling centers that orchestrate growth and differentiation of the regenerate. I 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, my data support the following model of Wnt/β–catenin pathway action during fin regeneration (Fig. 5.1):

Wnt/β–catenin signaling is activated already 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 (Fig. 5.1A). Pathway activation at 6 hpa is consistent with the induction of wnt10a expression at this time point, which has been reported previously (Stoick-Cooper et al., 2007b). Pathway activity is maintained in interray mesenchyme throughout regeneration but the functional significance of this site of β–catenin signaling remains to be determined. Here, I 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 (Fig. 5.1B). 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 detectable in a subregion of the proliferative proximal blastema, namely the presumptive actinotrichia-forming cells that lie medial to the osteoblast progenitors. No β–catenin pathway activation appears to occur in the highly
proliferative wound epidermis, osteoblasts or the majority of the proximal medial blastema.

Surprisingly, the data obtained using tissue-specific Wnt signaling inhibition suggest that signaling in the non-proliferative distal-most blastema is required for proliferation of other blastemal domains, while signaling in the proliferative proximal blastema has little role in regulating cell division, but in directing differentiation of adjacent osteoblast progenitors (Fig. 5.1C). Furthermore, β–catenin signaling regulates the maintenance of gene expression domains in the wound epidermis and thus, likely its regionalization indirectly as well (Fig. 5.1C). Thus, I conclude that β–catenin signaling controls regenerate growth, differentiation and patterning largely indirectly. I found that almost all signaling pathways that have previously been shown to be involved in controlling fin regeneration are regulated by Wnt/β–catenin signaling and that Hh and RA signals act downstream of β–catenin in the promotion of blastemal cell proliferation, while Fgf and possibly Igf and Bmp signaling mediate its effects on the epidermis (Fig. 5.1C-D).

Interestingly, the data presented here indicate that Wnt/β–catenin signaling receives little reciprocal input from the signals it regulates. These findings support a model in which Wnt/β–catenin signaling acts on top of a likely cooperative signaling network that instructs fin regeneration (Fig. 5.1D). In contrast, a recent study has proposed that RA signaling promotes Fgf and Wnt/β–catenin signaling and that these pathways cooperatively regulate regeneration (Blum and Begemann, 2012). While the rescue experiments shown point towards a role of the Wnt pathway upstream of RA, Fgf and Hh signaling, epistatic relationships are difficult to assess in hypomorphic knock-down conditions. Thus, definitive conclusions about the hierarchical relationships between pathways regulating regeneration probably need to await the development of inducible genetic loss-of-function techniques in zebrafish.

In summary, I 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 mediolateral proximal blastema, from which other signals, either diffusible or via cell-cell contact, emanate that direct growth, differentiation and patterning of the regenerating zebrafish caudal fin.
Fig. 5.1. A model for Wnt/β-catenin function during caudal fin regeneration. (A-B) Schematic summary of the Wnt/β–catenin activity domains (in red color) during caudal fin regeneration. Whole mount (A) and longitudinal section views (B) are shown. (C) Wnt pathway activity is required for production of (diffusible) signals that regulate proliferation of proximal blastemal regions, differentiation of osteoblasts, and maintenance of gene expression domains in the epidermis. (D) Schematic summary of the hierarchical position of Wnt/β–catenin signaling within the molecular signaling network controlling caudal fin regeneration. Arrows indicate direct or indirect regulation.

An interesting aspect of this study is that these signaling centers not only show differences in proliferative properties and, according to the proposed model, exert distinct functions, but can also be defined by their Wnt co-receptor expression. The Wnt-receiving cells located in the distal-most blastema are rather quiescent and positive for lrp6 transcripts. In contrast, the Wnt-receiving cells that are found in mediolateral domains of the blastema, adjacent to the osteoblast progenitors, are highly proliferative and positive.
for lrp5 transcripts. A question thus arises: does the difference in lrp5 and lrp6 co-receptor expression account for the distinct proliferative potential and/or for the functional difference of these domains? A recent study provided evidence that Lrp6 exhibits stronger signaling activity as compared to Lrp5 and thus providing a possible mode of achieving differential signaling outputs (MacDonald et al., 2011). Consistent with this, I found that Wnt reporter activity is most strongly detected in the distal-most lrp6+ blastema. Thus, it would be interesting to test whether the difference in lrp5 and lrp6 co-receptor expression determines the functionality and proliferative properties of the blastemal signaling centers described here using genetic loss-of-function approaches.

5.2 Signaling Pathway Interactions During Vertebrate Appendage Regeneration

While many signaling pathways have been identified to be required for complete vertebrate appendage regeneration, their hierarchical relationships are poorly understood. This study provides for the first time a more detailed insight into the complex interaction network of signaling pathways that guide vertebrate appendage regeneration. Loss-of-function experiments revealed that expression of ligands for Fgf, Hh and RA signaling pathways depend on Wnt/β–catenin signaling and the rescue experiments performed indicate, that Wnt/β–catenin signaling acts upstream of Fgf, Hh and RA signaling in the regulation of blastemal cell proliferation and epidermal patterning. Interestingly, interaction of Wnt/β–catenin signaling with Hh or Fgf signaling pathways has also been reported for appendage regeneration in frogs and newts. In frog tadpole limbs, expression of the Fgf ligand family member fgf8 depends on Wnt/β–catenin signaling (Yokoyama et al., 2007). This suggests, that Wnt/β–catenin signaling regulates Fgf signaling, similar to fin regeneration, through transcriptional control of Fgf ligands. Furthermore, it has been shown that both, Wnt/β–catenin and Fgf signaling are required for Xenopus tadpole tail regeneration, and that Fgf acts downstream of Wnt/β–catenin signaling (Lin and Slack, 2008). Interestingly, in both regenerating systems, the fish fin and the frog tadpole tail, expression of the Fgf ligand fgf20a is regulated by Wnt/β–catenin signaling, suggesting conserved mechanisms of appendage regeneration (Stoick-Copper et al., 2007; Lin and
Slack, 2008). In contrast to the zebrafish fin however, Bmp signaling appears to act upstream of Wnt/β-cat signaling in the regenerating Xenopus tadpole tail (Lin and Slack, 2008). During newt limb regeneration, activation of the Wnt or Hh pathway promotes proliferation of blastemal cells (Singh et al., 2012). However, in contrast to the regenerating fin, Wnt signaling appears to acts downstream of Hh signaling during newt limb regeneration. Thus, Wnt and Fgf or Wnt and Hh signaling pathway interactions are conserved between different appendage regeneration systems, yet the hierarchy of events differs in some cases. Further studies are required to gain a more detailed insight into the hierarchical relationships of signaling pathways in these systems, which will allow for more substantial comparison.

5.3 Wound Signals Activating Wnt/β–cat Signaling During Caudal Fin Regeneration

In response to tissue injury signals are provided by the wound which, depending on the regenerative capacity of the organisms, lead to scarring of the wound or initiate a regenerative program. Activation of Wnt/β–cat signaling, possibly via Wnt10a, occurs within 6 hours after fin amputation and appears to regulate many other signaling pathways required for regeneration. Thus, it would be interesting to identify the wound signal acting upstream of Wnt/β–cat pathway activation. The data presented here suggest that key early wound signals of regenerative processes in fish and frogs, including redox signaling (Gauron et al., 2013; Love et al., 2013; Niethammer et al., 2009; Yoo et al., 2012), Jnk signaling (Ishida et al., 2010) and activation of immune cells (Kyritsis et al., 2012), are dispensable for amputation-induced Wnt/β–cat pathway activation, leaving the identity of the signal bridging injury and Wnt/β–cat pathway activation undiscovered. Further studies will be required to unravel this important mechanism, which will aid the understanding why mammals fail to initiate a regenerative response after severe injury while lower vertebrates do. An excellent candidate signal that remains to be tested is apoptosis. In many regenerating systems, including the Xenopus larval tail (Tseng et al., 2007), the zebrafish caudal fin (Gauron et al., 2013), planarians (Pellettiere et al., 2010), and the head of the freshwater water polyp Hydra (Chera et al., 2009), injury-induced
apoptosis has been identified to be a crucial event that precedes regeneration. Interestingly, Hydra head regeneration requires Wnt3 which is released by apoptotic cells (Chera et al., 2009). Thus it would be interesting to test whether apoptotic cells in the fin stump likewise provide a source of Wnt ligands and thereby activate the β–catenin pathway.

5.4 Tcf1/Lef1 Function in the Wound Epidermis

The data presented here indicate that the wound epidermis of the regenerating fin is not competent to respond to Wnt ligands activating β–catenin signaling. Yet, tcf1 and lef1 expression is robustly detected in the proximal basal epidermal layer. Thus, Tcf1 and Lef1 transcription factors in the wound epidermis must regulate gene transcription independently of Wnt/β–catenin signaling. Indeed, a number of examples are known where Lef/Tcf proteins together with co-activators can activate target gene transcription independently of β–catenin: 1) Lef1 (and also weakly Tcf1) can interact with Ets1, Runx1, Cbf and Aly at the T-cell receptor α enhancer to activate transcription (Bruhn et al., 1997; Hsu et al., 1998; Mayall et al., 1997), 2) during mouse palate development and Xenopus embryogenesis Lef1 and Smad factors cooperatively activate transcription of Tgfβ target genes (Labbe et al., 2000; Nawshad and Hay, 2003), and 3) in vitro evidence has also been provided for an interaction of the Notch intracellular domain (Nicd) or the Igf1r with Lef1 in regulation of target genes (Ross and Kadesch, 2001; Warsito et al., 2012). In addition to binding transcriptional co-activators, Lef/Tcf transcription factors are able to induce a strong bend in the DNA which facilitates gene expression (Koopman, 2010). Thus, beside for their function in Wnt/β–catenin signaling Lef/Tcf transcription factors have distinct roles in activation of target genes expression of other signaling pathways.

5.5 Parallels and Differences to Amniote Limb Development

A common feature of amniote limb development is the establishment of signaling centers, which provide instructive cues to pattern the forming appendage along three axes. Signals controlling elongation and patterning along the proximal-distal axis are mainly provided by
the apical ectodermal ridge (AER), a thickened epithelium covering the distal tip of the growing limb (Fernandez-Teran and Ros, 2008). Wnt/β–catenin pathway activation via ectodermally-derived Wnt3 is essential for induction and maintenance of the AER signaling center (Fernandez-Teran and Ros, 2008). Importantly, Wnt ligands activating the β–catenin pathway that emanate from surface ectoderm, including the AER, are essential for limb outgrowth by maintaining proliferation and concomitantly preventing differentiation of the underlying mesenchyme (ten Berge et al., 2008). Although development of pectoral fins, which are homologous to amniote limbs in zebrafish, is less well understood, many molecular signals and their interaction network are shared with limb organogenesis in mouse and chicken (Zeller et al., 2009). wnt3l is expressed in the AER of pectoral fin buds (Norton et al., 2005), and Lef1 and Tcf1-mediated Wnt/β–catenin signaling in the AER and the underlying mesenchyme is required for AER maintenance and pectoral fin growth (Nagayoshi et al., 2008; Shimizu et al., 2012; Veien et al., 2005).

During regenerative outgrowth of the zebrafish fin, I found Wnt/β–catenin signaling to be activated in the distal blastemal mesenchyme underneath the wound epidermis and that pathway activation is essential for maintaining proliferation of the mesenchymal progenitors in the proximal blastema and therewith distally-oriented regenerate growth. While the ligand responsible for β–catenin pathway activation remains to be elucidated, wnt3a has been reported to be expressed in the basal epidermis that abuts the distal blastemal mesenchyme, hinting at parallels between distally-oriented growth of developing limbs and regenerating fins (Poss et al., 2000a). However, more substantial comparisons between development and regeneration require a better characterization of the development of the adult caudal zebrafish fin – whose molecular regulation is essentially unknown.

5.6 Wnt Signaling in Osteoblast Differentiation

During mammalian development, Wnt/β–catenin signaling is well accepted to have essential roles in promoting osteoblast differentiation (Long, 2012; Regard et al., 2012). The data presented here indicate that β–catenin signaling likewise is required for bone maturation in the regenerating fin. Surprisingly however, my results suggest, that in
contrast to mammals, Wnt signaling does not directly regulate osteoblast differentiation during fin regeneration. Instead, I find β–catenin signaling not to be active or required in osteoblasts but rather in adjacent presumptive actinotrichia-forming cells. Thus, Wnt/β–catenin signaling likely promotes osteoblast maturation via stimulation of diffusible factors that are yet to be determined. An alternative explanation could be that Wnt/β–catenin signaling in the presumptive actinotrichia-forming cells directs osteoblast maturation by creating a local permissive environment based on the extracellular matrix (ECM) composition. It is well established that the ECM plays a central role in controlling cell behavior and that the degree of ECM stiffness based on its composition directs lineage specification and cellular differentiation (Engler et al., 2006; Izu et al., 2011; Mason, 2012). The actinotrichia-forming cells are known to express different collagens that form rigid hyperpolymerized collagen macrofibrils (Duran et al., 2011). Notably, it has been shown that rigid, collagenous matrices promote osteogenic lineage specification of mesenchymal stem cells and type XII Collagen has been shown to regulate osteoblast differentiation and maturation in mice (Engler et al., 2006; Izu et al., 2011). Indeed, genetic loss of collα1a function, a Collagen predominantly expressed in the actinotrichia-forming cells, results in aberrant lepidotrichia pattern and reduced ossification during caudal fin regeneration (Duran et al., 2011). Therefore, Wnt/β–catenin signaling in the actinotrichia-forming cells might indirectly direct osteoblasts maturation via regulation of the ECM composition.

While I cannot rule out a role of Wnt/β–catenin signaling in osteoblasts, the dermal bone found in fish fins has been suggested to be distinct from other skeletal tissues based on morphological and gene expression characteristics (Mari-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 lack of a direct 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. Yet, I suggest that Wnt/β–catenin signaling exerts a dual role during fin regeneration similar to limb development where the distal domain of Wnt signaling promotes proliferation of mesenchymal progenitors, while the proximal domains induce osteoblast maturation.
In summary, the study presented here greatly expands our knowledge about the molecular mechanisms regulating zebrafish appendage regeneration, since it

1) uncovers previously unknown organizing centers that direct cellular behavior of adjacent tissues in the regenerating caudal fin

2) identifies Wnt/β-catenin signaling as a crucial signal in specifying these signaling centers by regulating most other developmental signaling pathway known to be required for regeneration

3) hints at parallels, yet interesting differences in the role of Wnt signaling in bone formation between regeneration and development.

These findings may have implications in regenerative medicine.
6 References


References


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8 Curriculum Vitae

Der Lebenslauf wurde aus datenschutzrechtlichen Gründen aus diesem Dokument entfernt.
Erklärung (Statement of Original Contribution)

I herewith declare that I have produced this thesis without the prohibited assistance of third parties and without making use of aids other than those specified; notions taken over directly or indirectly from other sources have been identified as such. This paper has not previously been presented in identical or similar form to any other German or foreign examination board.

The thesis work was conducted from November 2009 to July 2013 under the supervision of Prof. Dr. Michael Kühl in the research group of Prof. Dr. Gilbert Weidinger, at the Biotechnology Center of the Technische Universität Dresden and at the Institute for Biochemistry and Molecular Biology of the Ulm University.

I declare that I have not undertaken any previous unsuccessful doctorate proceedings.

I declare that I recognize the doctorate regulations of the Fakultät für Naturwissenschaften of the Ulm University.

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Die Dissertation wurde von Prof. Dr. Michael Kühl, Institut für Biochemie und Molekularbiologie, Universität Ulm, in der Arbeitsgruppe von Prof. Dr. Gilbert Weidinger am Biotechnologischen Zentrum, Technische Universität Dresden und am Institut für Biochemie und Molekularbiologie, Universität Ulm, betreut und im Zeitraum vom 11/2009 bis 07/2013 verfasst.

Meine Person betreffend erkläre ich hiermit, dass keine früheren erfolglosen Promotionsverfahren stattgefunden haben.

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Supplemental Information

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