Wnt signaling controls pro-regenerative Collagen XII in functional spinal cord regeneration in zebrafish

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The inhibitory extracellular matrix in a spinal lesion site is a major impediment to axonal regeneration in mammals. In contrast, the extracellular matrix in zebrafish allows substantial axon re-growth, leading to recovery of movement. However, little is known about regulation and composition of the growth-promoting extracellular matrix. Here we demonstrate that activity of the Wnt/β-catenin pathway in fibroblast-like cells in the lesion site is pivotal for axon re-growth and functional recovery. Wnt/β-catenin signaling induces expression of col12a1a/b and deposition of Collagen XII, which is necessary for axons to actively navigate the non-neural lesion site environment. Overexpression of col12a1a rescues the effects of Wnt/β-catenin pathway inhibition and is sufficient to accelerate regeneration. We demonstrate that in a vertebrate of high regenerative capacity, Wnt/β-catenin signaling controls the composition of the lesion site extracellular matrix and we identify Collagen XII as a promoter of axonal regeneration. These findings imply that the Wnt/β-catenin pathway and Collagen XII may be targets for extracellular matrix manipulations in non-regenerating species.
Aft er a spinal injury, non-neural cells invade the lesion site and set up a complex extracellular matrix (ECM)1–2. The lesion site ECM consists of a plethora of molecules that may be inhibitory, permissive, or promoting for axon re-growth. In mammals, the balance of ECM molecules is shifted towards an inhibitory matrix that does not allow axons to cross the lesion site, in turn preventing recovery of function1–2. In vertebrates capable of functional spinal cord regeneration, such as fishes and salamanders, the lesion site ECM naturally allows crossing of regenerating axons, offering potential insight into how this ECM is controlled and which ECM molecules may promote regeneration3–6.

A candidate to control the composition of the lesion site ECM in zebrafish is the Wnt/β-catenin pathway, because Wnt/β-catenin signaling has been shown to control fibroblast biology and ECM deposition7–10. Moreover, axonal reconnection, which is necessary for functional recovery in adult11 and larval zebrafish12, 13, also depends on Wnt/β-catenin pathway activity in both systems8,9, 14, 15. Wnt/β-catenin signaling involves extracellular Wnt ligand-induced stabilization and translocation of β-catenin into the nucleus where it modifies target gene transcription together with Tcf/Lef family proteins.16 To understand how Wnt/β-catenin signaling promotes spinal cord regeneration, cell types of action and downstream mechanisms of pathway activation, such as deposition of ECM factors, need to be identified.

Different ECM molecules have specific functions for axon growth and regeneration. For example, among the collagens, col1a1, col1ba1, and col15a1b promote growth of developing motor axons, whereas only col4a5 is necessary for the correct growth of regenerating motor axons in zebrafish.17–20, Hence, it is important to identify specific axon re-growth-promoting ECM components in the lesion site.

Adult and larval zebrafish show robust axonal and functional regeneration after complete spinal cord transection11–13, 21. Importantly, axon regeneration in larvae is rapid and can be observed in real-time. Lesion-induced paralysis at 3 days-post fertilization (dpf) is followed by recovery of touch-evoked swimming within 2 days post-lesion (dpf).13 Swimming capacity is lost upon re-transection15, 16. This makes zebrafish a powerful tool to identify mechanisms responsible for establishing a lesion environment that is conducive to axone growth. Moreover, sensitive reporter lines and inducible cell-type specific pathway inhibition using the TetON system can be used to interrogate the Wnt/β-catenin signaling pathway in zebrafish.22–25

Here, we find that Wnt/β-catenin signaling in fibroblast-like cells accumulating in the lesion site is pivotal for axon re-growth and functional regeneration. A gene expression screen identifies col12a1a/b expression and deposition of Collagen XII downstream of Wnt/β-catenin signaling to be required for regeneration. Hence, Wnt/β-catenin signaling in non-neural cells controls the composition of an axon-growth promoting ECM environment in the spinal lesion site in zebrafish.

**Results**

**Axonal bridging correlates with functional recovery.** To identify molecular mechanisms regulating the spinal lesion ECM, we first analysed dynamics of axon regeneration in zebrafish larvae. Using anti-acetylated Tubulin immunohistochemistry we observed little to no axonal re-growth within the first 12 h after a spinal lesion. At 12 h post-lesion (hpl) mainly axonal debris, indicated by fragmented anti-acetylated Tubulin immunoreactivity, and only very few and thin re-growing axon fascicles were found in the lesion site (5/20 animals; Supplementary Fig. 1a). We found no animals with a completely bridged lesion site (0/20 animals). Analysis of stills from time-lapse video microscopy imaging at this time point confirmed little axonal re-growth (2/9 animals with axons in the lesion site, 0/9 animals with axonal bridge; Supplementary Movies 1 and 2). After this initial lag phase axons rapidly re-grew (Supplementary Fig. 1a). At 1 dpl, 57% of all larvae showed axonal bridging of the lesion site, as indicated by continuous axonal labelling between rostral and caudal spinal cord (n = 112). Bridging further increased to >80% of the animals at 2 dpl (n = 111; Fig. 1c). Remarkably, we found that in the 18% of lesioned larvae in which no axonal bridge was established by 2 dpl, functional recovery, as measured by touch-evoked swim distance, was also impaired (analysed in Xla.Tubb:DsRed26 transgenic fish; Supplementary Fig. 1b,c). This underscores the importance of axonal re-growth for functional recovery in larvae.

**Axons navigate a non-neural lesion site that is rich in ECM.** To determine how axons cross the lesion site, we analysed their relationship with astroglia-like processes, as these have been shown to be supportive for axon regeneration in zebrafish.27, 28

We imaged axonal and glial processes in time-lapse video microscopy using double transgenic fish for Xla.Tubb:DsRed, to visualize neuronal processes, and either gfp:GFP29 (n = 6 fish, 33 fascicles) or her4.3:EGFP30 (n = 3 fish, 30 fascicles) to label astroglia-like processes. This revealed that the majority of fascicles that entered the lesion site were composed of axons without detectable glial processes (49–53%). Some fascicles contained both glial and axonal processes (20–21%) and some pure glial fascicles were observed (27–30%; Fig. 1a and Supplementary Movies 1 and 2; see Methods section for scoring criteria). To confirm these findings we used a range of combinations of immunohistochemical and transgenic markers for neuronal and glial processes in static images of live animals and histological preparations at 1 dpl. This showed that 63–77% of axon-containing fascicles (64–62% of all fascicles analysed) were devoid of detectable glial processes (Fig. 1b). Nineteen to 26% contained both axonal and glial processes and 19–28% of all analysed fascicles were only composed of glial processes. Moreover, double-immunohistochemistry for anti-acetylated Tubulin and anti-GFAP showed that at 1 dpl, only 36% of animals had glial processes bridging the lesion site, in contrast to 57% of the fish with an axonal bridge. At 2 dpl, glial processes had caught up (Fig. 1c). This suggests that axons cross the lesion site more rapidly than glial processes. In summary, these observations support that many axons rapidly extend into the lesion site and cross it independently of detectable glial processes.

To experimentally test a role of astroglia-like processes in supporting axonal regeneration, we ablated the majority of glial cells using gfp:Gal4ff:UAS:NTT-mCherry transgenic fish that express the enzyme nitroreductase under regulatory sequences of the gfp gene.31 Adding the prodrug Metronidazole (MTZ) at 1 dpf, which is converted by nitroreductase into a cytotoxic compound, almost completely eliminated transgene-expressing glial cells by 3 dpf (Fig. 1d)31. A dramatic depletion of astroglia-like processes in the lesion site was confirmed by anti-GFAP immunohistochemistry (Fig. 1e). Remarkably, this treatment did not reduce the proportion of animals with axonal bridge (anti-acetylated Tubulin; Fig. 1e). This further supports that glial processes do not have a major role in facilitating axonal re-growth across a spinal lesion site. However, we cannot rule out that glial processes that were not detected with our markers and were spared by the ablation still supported axonal regeneration.

To explore possible substrates for the highly dynamic growth cones other than glia, we determined regulation and protein deposition for two major ECM components, collagen1a2 (coll1a2) and fibronectin1a (fn1a). Both genes showed robust upregulation
of expression in the lesion site (Fig. 1f). Using specific antibodies we indeed observed an accumulation of Collagen I (Col I) and Fibronectin proteins in the lesion matrix (Fig. 1g). The Col I and Fibronectin immunoreactivity appeared diffuse, but also formed fibrous structures, often with a longitudinal orientation.

Double immunohistochemistry at 1 dpl revealed close apposition of regenerating axonal fascicles (anti-acetylated Tubulin*) with anti-Col I (11 of 13 fascicles associated with Col I immunoreactivity; n = 7 fish) or anti-Fibronectin (15 of 22 fascicles associated with Fibronectin immunoreactivity; n = 6...
fish) immunoreactive fibres in the lesion site (arrows in Fig. 1g, Supplementary Movies 3 and 4). Hence, regenerating axons navigate the non-neural lesion environment in close association with ECM, suggesting that axon-ECM interactions might be important for axonal re-growth.

**Wnt signaling is activated after spinal cord lesion.** To determine a potential role of the Wnt/β-catenin pathway in controlling the composition of the spinal lesion ECM, we first characterized the cells in which the pathway was active after lesion. We used in situ hybridisation to detect transcripts of three different transgenic reporter lines of Wnt/β-catenin-dependent transcription, 6xTCF:dGFP, TopdGFP, 7xTCF:mCherry. We also determined expression levels of direct Wnt/β-catenin target genes axin2 and lef1, which are commonly used as read-outs of pathway activation and as feedback inhibitor (axin2) and transcriptional activator (lef1) in the pathway, respectively (see Fig. 2a). All probes showed increased labelling in the lesion site at 2 dpf (Fig. 2b; Supplementary Fig. 2a,b). Time course analysis in the highly sensitive 6xTCF:dGFP Wnt reporter line indicated incipient pathway activity in the lesion site at 0.5 dpf (12 hpl; arrows in Supplementary Fig. 2c), increased activity at 1 dpf, maximal activation at 2 dpf and return to undetectable control levels by 5 dpf after dorsal incision lesion or lateral stab lesion (Fig. 2b, Supplementary Fig. 2d,e). This correlates well with the time course of axonal regeneration. The 6xTCF:dGFP reporter also labelled a few additional cells within the spinal cord at 1 and 2 dpf (empty arrows in Fig. 2c). These cells have previously been described as glial cells14.

6xTCF:dGFP reporter labelling was specific for Wnt/β-catenin pathway activity in our experimental paradigm, as pharmacological inhibition (IWR-1) or heat shock-induced ubiquitous overexpression of the negative regulator axin1 in hs:Axin1 transgenic larvae strongly reduced the signal in the lesion site (Supplementary Fig. 2f,g). Importantly, using anti-β-catenin immunohistochemistry on whole mounts and sections confirmed the strong signal in the lesion site and only sparse labelling in the adjacent spinal cord in 6xTCF:dGFP transgenic animals (Supplementary Fig. 2h,i). Moreover, our in situ hybridization protocol allowed us to detect gfp-expressing glial cells throughout the spinal cord in whole mount preparations of 5 dpf hera.3:EGFP transgenic fish (Supplementary Fig. 2j). This shows that lack of labelling in neural tissue was not due to potential probe penetration issues in whole mount preparations. Hence, after spinal cord lesion, Wnt/β-catenin signaling is mainly increased in the non-neural lesion site, which is a major, previously undescribed, domain of Wnt/β-catenin pathway activity.

Wnt signaling is active mainly in fibroblast-like cells. To characterize the Wnt-responding cell types in the non-neural lesion site environment, we utilized 6xTCF:dGFP transgenic fish. GFP protein and mRNA was mainly detectable at the lesion edge at 1 and 2 dpf (Fig. 2c; Supplementary Fig. 2h). Wnt-responding cells did not co-label with markers of muscle fibres (MyHC/F5935, n > 10; Supplementary Fig. 3a,b) or immune cells (L-Plastin36, n > 10; Supplementary Fig. 3c). In contrast, 31% of GFP-labeled cells (cell counts in immunolabeled sections of individual animals, n = 10) were co-labeled with the basal keratinocyte marker p6337 (arrowheads in Fig. 2d; Supplementary Fig. 3d). The remaining two thirds of the Wnt-responsive cells (6xTCF:dGFP+/p63+) were located subepidermally in the lesion site and could thus be fibroblasts. There is no definitive fibroblast marker. However, fibroblasts are known to express the ECM core components col1a2 and fn1α during dermal stroma formation in developing zebrafish38,39. Indeed we found that at 1 dpf and 2 dpf, Wnt-responding subepidermal cells were positive for col1a2 and fn1α transcripts (arrows in Fig. 2e; Supplementary Fig. 3e-g), which were both upregulated in the lesion site (Fig. 1f). Interestingly, in the peripheral lesion area, where the myotome was still intact, a population of 6xTCF:dGFP+/p63+ cells, continuous with the subepidermal cells in the lesion site, were found sandwiched between basal keratinocytes (p63+ and slow twitch muscle fibres (MyHC/F59+) (insets in Supplementary Fig. 3a,b). Additional 6xTCF:dGFP+/p63+ cells were found covering the spinal cord (arrows in Supplementary Fig. 3h). These positions correspond to previously described dermal and meningeal fibroblast locations in the developing zebrafish and adult el39,40. Based on these observations, we tentatively identify the vast majority of Wnt-responding cells as fibroblast-like. Importantly, we found fibroblast-like Wnt-responding cells (6xTCF:dGFP+/p63+) in close proximity to regenerating axons (anti-acetylated Tubulin+) in the spinal lesion site at 1 dpf (arrows in Fig. 2f). Hence, the majority of Wnt-responding cells after a lesion are non-neural fibroblast-like cells that are part of the lesion environment through which axons navigate.

**Regeneration requires Wnt signaling in non-neural cells.** To functionally inhibit Wnt/β-catenin pathway activity selectively in lesion site cells, we took advantage of the TetON system that allows for Doxycycline (DOX)-inducible (Supplementary Fig. 4a), cell type-specific pathway manipulation41. To interfere with Wnt/β-catenin signaling, we used TetRE:Axin1-YFP transgenic fish, a TetResponder line that has been shown to efficiently inhibit the pathway when activated during zebrafish development or adult tail fin regeneration42,43. To specifically target cells that activate...
the Wnt/β-catenin pathway in the lesion site, we generated a TetActivator line using regulatory elements of the direct Wnt/β-catenin target **lef1** (**lef1**:TetA AmCyan; Supplementary Fig. 4b). Indeed, this strategy showed injury-induced TetActivator expression (**amcyan** mRNA) in Wnt-responding cells in the lesion site in **lef1**:TetA AmCyan;6xTCF:dGFP double-transgenic fish (Supplementary Fig. 4c). YFP fluorescence was likewise concentrated in the lesion site in **lef1**:TetA AmCyan;TetRE:Axin1-YFP double-transgenic fish after DOX treatment (Fig. 3a,b). This indicates successful selective targeting of Wnt-responding cells in the lesion site. The inhibition was effective, as lesion site expression of **axin2**, an established read-out for Wnt/β-catenin pathway activity33 (Fig. 2a), was strongly reduced (Supplementary Fig. 4d).

To determine whether functional spinal cord regeneration was impaired in **lef1**:TetA AmCyan; TetRE:Axin1-YFP...
double-transgenic fish after DOX treatment, we scored fish showing the presence of axonal labeling over the lesion site (anti-acetylated Tubulin) and quantified swim distance after touch at 2 dpl. The proportion of larvae with bridged lesion sites was reduced by 48% (Fig. 3c). Moreover, larvae recovered to swim only less than half the distance (42%) of unlesioned Wnt-inhibited animals, whereas lesioned control larvae recovered to swim the same distance as their unlesioned age-matched controls (Fig. 3d).

Importantly, neuronal (Xla.Tubb:TetA AmCyan) and glial (her4.3:TetA AmCyan) specific Axin1 induction did not induce a phenotype, whereas ubiquitous expression (ubi:TetA AmCyan) recapitulated the axonal and behavioural phenotypes of lesion site-specific manipulations (Fig. 3e–m; Supplementary Fig. 4e). Of note, Axin1 was correctly targeted in all of these lines, as indicated by YFP fluorescence of the Axin1-YFP fusion protein in neurons (Xla.Tubb:TetA AmCyan), glia (her4.3:TetA AmCyan) or throughout the larvae (ubi:TetA AmCyan). Moreover, axin1 induction in neural cells reached functional levels. This is indicated in the neuronal-driven TetActivator line (Xla.Tubb:TetA AmCyan), in which axin2 expression in the lesion site remained unaffected but was strongly reduced in a constitutively Wnt-responsive domain in the brain (Supplementary Fig. 4f,g). Hence, Wnt/β-catenin pathway activity in neurons or glia is not detectably involved in axonal regeneration. To exclude non-specific effects of the TetON system or Axin1-YFP induction, we also interfered systemically with Wnt/β-catenin signaling using a pharmacological approach (IWR-1) or via heat shock-induced ubiquitous overexpression of the pathway antagonists axin1 or dkk1 in hs:Axin1 or hs:dkk1 transgenic fish, respectively. Using dkk1 overexpression as an additional control was important, as it specifically antagonizes the pathway at the receptor level, whereas axin1 overexpression and IWR-1 promote degradation of the transcriptional co-activator β-catenin (Fig. 2a; Supplementary Note 1). All of these manipulations robustly reduced the proportion of larvae with an axonal bridge (reduction by 51–72%) and functional recovery (<48% of the swim distance covered by unlesioned Wnt-inhibited animals; Supplementary Fig. 5a–c). Inhibition of Wnt/β-catenin signaling (IWR-1 treatment) starting at different time points post-lesion showed that axon re-growth was still maximally inhibited when the treatment was started at 0.5 dpl (12 hpl), but the effect of IWR-1 was greatly diminished when the treatment was started at 1 dpl and had no effect when it was started at 1.5 dpl (Supplementary Fig. 5d). Hence, Wnt/β-catenin pathway activity is required mainly between 0.5 dpl and 1 dpl for axon regeneration, when axons actively grow across the lesion site.

Two lines of evidence support that effects of Wnt/β-catenin pathway inhibition on axon regeneration were not merely a secondary consequence of potential defects in wound healing. Firstly, a lateral stab lesion, which generates minimal tissue disruption (Supplementary Fig. 2e), showed a similar reduction in axonal regeneration and functional recovery after pharmacological inhibition, as was observed after dorsal incision lesion (Supplementary Fig. 5e). Second, wound re-epithelialization by basal keratinocytes, providing a protective layer over the lesion site, robustly occurred in controls (30/30) and in larvae in which Wnt/β-catenin signaling had been systemically inhibited using IWR-1 (30/30) (visualized in krt1l1c1e:EGFP transgenic fish; arrow in Supplementary Fig. 6; also see Fig. 4h). We conclude that Wnt/β-catenin signaling in fibroblast-like cells in the lesion site, but not in neural cells, is necessary for functional axon regeneration.

Interestingly, systemic but not glial cell-specific Wnt-inhibition also reduced the number of animals with glial bridges. This suggests that glial crossing of the lesion site also depends on Wnt/β-catenin signaling in a non-cell autonomous way (Supplementary Fig. 7a,b).

Wnt signaling promotes Col XII deposition. We hypothesized that Wnt/β-catenin signaling in lesion site fibroblast-like cells controls ECM deposition, as fibroblasts are a major source of ECM under physiological and pathological conditions. To identify relevant Wnt/β-catenin-controlled ECM components, we compared labelling intensities between control and IWR-1-treated stab-lesioned larvae in an in situ hybridisation screen for two fibronectins and 44 collagen chain-encoding genes at 1 dpl (Fig. 4a; Supplementary Fig. 8a). These genes were chosen, because of roles of these ECM protein classes in axon growth and regeneration17–20, 45. In DMSO-treated controls we found expression of both fibronectins and 10 collagens to be locally upregulated in the lesion site compared to adjacent unlesioned trunk tissue. Of these upregulated genes, only expression of colta2 and of the paralogs col12a1a and col12ab was noticeably reduced when Wnt/β-catenin signaling was inhibited (IWR-1 treatment). We did not find any increased expression of ECM molecules after IWR-1 treatment. We focused our further analysis on col12a1a and col12ab, because both paralogs code for the essential α-chain of Collagen XII (Col XII) and are regulated by Wnt/β-catenin signaling. Since functional Col XII molecules assemble as α-chain trimers, Col XII protein expression likely also depends on Wnt/β-catenin signaling, as no other paralogs were found in the genome that could substitute for col12a1a and col12ab.

First we determined the time course of col12a1a/b expression after a lesion. Parallel to the time course of Wnt/β-catenin pathway activation, col12a1a/b transcripts were detectable in the lesion site at 0.5 dpl (12 hpl). Further increased expression was detected at 1 dpl, when axons started to regenerate. Expression peaked at 2 dpl and had returned to baseline levels by 5 dpl (Fig. 4b; Supplementary Fig. 8b).

To verify control of col12a1a/b expression by Wnt/β-catenin signaling, we quantified fluorescence in situ hybridisation signals for col12a1a/b transcripts at 1 dpl after 24 h of IWR-1 treatment. This confirmed transcriptional downregulation of col12a1a and...
Fig. 3 Wnt/β-catenin signaling in non-neural cells is required for axon regeneration and functional recovery. a Schematic timeline of the experimental design using the TetON system. b–d axin1 overexpression selectively in cells in the lesion site (b, left *) is sufficient to interfere with axon regeneration (c, Fischer’s exact test: ***P < 0.001) and functional recovery (d, two-way ANOVA: F1,156 = 14.82, P < 0.0005; t-test: ***P < 0.001). e–g axin1 overexpression throughout the fish e interferes with axon regeneration (f, Fischer’s exact test: *P < 0.05) and functional recovery (g, two-way ANOVA: F1,129 = 4.716, P < 0.05; t-test: **P < 0.01). h–j Neuronal axin1 overexpression, indicated by YFP labelling of parenchymal cell bodies and neuropil in the spinal cord h, does not interfere with axon regeneration i or functional recovery j (two-way ANOVA shows no interaction between lesion and treatment). k–m axin1 overexpression in ependymoradial glial cells, indicated by YFP labeling in cell bodies lining the central canal, their radial processes and pial end feet k does not interfere with axon regeneration l or functional recovery m (two-way ANOVA shows no interaction between lesion and treatment). b–e Views are transversal (b.e.h.k; dorsal is up) or lateral (c.f.i.l; dorsal is up, rostral is left). BF: brightfield. n.s.: not significant. Scale bars: whole mounts, 200 and 100 μm; sections: 100 μm. Error bars indicate s.e.m.
coli2a1b by 88% and 98% respectively (Fig. 4c). RT-PCR of lesion site-enriched trunk tissue confirmed that IWR-1 treatment interfered with lesion-induced coli2a1a and coli2a1b upregulation at 1 dpl, while coli1a1a expression levels remained unaffected by Wnt/β-catenin pathway inhibition (Supplementary Fig. 8c). Consistent with our finding that Wnt/β-catenin pathway activity is mainly required within the first 24 h after a lesion for axonal re-growth (Supplementary Fig. 5d), IWR-1 treatment for 24 h between 1 dpl and 2 dpl had no effect on coli2a1a/b expression. This indicates that sustained Wnt/β-catenin pathway activity was not needed for coli2a1a/b transcription.

**Table 3.** Analysis of lesion site fluorescence (AU × 10^6)

| 3 dpf | 4 dpf | 5 dpf | 6 dpf | 8 dpf |
|-------|-------|-------|-------|-------|
| DMSO | IWR-1 | DMSO | IWR-1 |
| fn1a | 16/18 | 20/20 | 10/12 | 11/13 |
| fn1b | 11/10 | 12/13 | 9/11 |
| col1a1a | 10/12 | 18/14 | 9/11 |
| col1a1b | 12/13 | 27/27 | 18/27 |
| col5a1 | 10/12 | 12/12 | 8/9 |

**Graph 4.** Fluorescence intensity in lesion site (AU × 10^6)
Activation of Wnt/β-catenin signaling through overexpression of wnt8 for 24 h in hs:wnt8 transgenic fish enhanced expression of both col12a1 genes at 1 dpl specifically in the lesion site (Fig. 4d). Thus, gain- and loss-of-function experiments for Wnt/β-catenin pathway activity support that zebrafish orthologs of the col12a1 gene are transcriptionally controlled by Wnt/β-catenin signaling in a spinal lesion site in zebrafish larvae during the initial phase of axon regeneration.

To determine whether Wnt/β-catenin signaling regulates col12a1a/b transcription in the lesion site likely directly or indirectly, we compared the kinetics of down-regulation of col12a1a/b expression after pathway inhibition with that of the direct transcriptional target gene axin2 at 1 dpl. Short-term inhibition of Wnt/β-catenin pathway activity through axin1 overexpression in hs:Axin1 transgenic fish for 6 h was sufficient to abolish expression of axin2 while col12a1a and col12a1b expression was not affected at that early time point (Supplementary Fig. 8e).

This indicates delayed and thus indirect control of col12a1a/b transcription by Wnt/β-catenin signaling. Taken together, these data support a scenario in which Wnt/β-catenin signaling promotes axonal regeneration by initiating col12a1a and col12a1b expression after a lesion.

To characterize the col12a1a and col12a1b-expressing cells in the lesion site, we used multicolour fluorescence in situ hybridization. The majority of col12a1a and col12a1b-expressing cells co-labeled with pdgfrb-GFP reporter transcripts, a marker for pericytes and reactive fibroblasts, at 1 dpl (Supplementary Fig. 8f,g). Inhibition of Wnt/β-catenin signaling (IWR-1 treatment) did not overly inhibit appearance of pdgfrb-GFP+ cells, indicating that Wnt/β-catenin signaling does not act on potential migration of col12a1a/b-expressing cells into the lesion site (Supplementary Fig. 8h). Some of the col12a1a and col12a1b-expressing cells were also positive for 6xTCF4dGFP reporter transcripts at 1 dpl (arrows in Fig. 4e). However, a substantial proportion of col12a1a/b-expressing cells were not co-labelled with 6xTCF4dGFP reporter transcripts, indicating that expression of col12a1a and col12a1b was not exclusive to acutely Wnt-responding cells. Hence, Wnt/β-catenin pathway activity could have already ceased in the majority of col12a1a and col12a1b-expressing cells at the time point of analysis or Wnt-responding cells could induce additional non-neural cells in the lesion site to express these genes.

We next assessed whether col12a1a/b protein deposition was altered by inhibition of Wnt/β-catenin signaling. We found increased immunoreactivity of Col XII in the lesion site at 1 dpl and 2 dpl in control lesioned larvae compared to adjacent unlesioned trunk tissue or unlesioned control animals (arrows in Figs. 4fg and Supplementary Fig. 9a). Inhibition of Wnt/β-catenin signaling by IWR-1 treatment or after heat shock-induced overexpression of dkk1 in hs:dkk1 transgenic fish, led to lower Col XII immunoreactivity in lesioned larvae than in DMSO-treated controls (Fig. 4h, Supplementary Fig. 9b). Hence, strongly reduced levels of Col XII matrix are laid down in the lesion site under conditions of Wnt/β-catenin pathway inhibition.

Col XII promotes functional spinal cord regeneration. To determine whether Col XII deposition is required for functional axonal regeneration, we co-injected Vivo-Morpholinos (MO), which act as cell-permeable translation blocking anti-sense molecules for col12a1a and col12a1b, into the circulation for efficient distribution in 3 dpf larvae (Supplementary Fig. 11a). This approach effectively reduced Col XII immunoreactivity in the lesion site compared to control MO-injected larvae (Fig. 5a). In contrast, immunoreactivity for Fibronectin and Collagen I was unaffected (Supplementary Fig. 11b), indicating specificity of the manipulation for col12a1a/b expression.

The proportion of larvae with an axonal bridge (determined in Xla.Tubb:DsRED transgenic animals) was reduced by 43% in col12a1a/b morphants compared to control MO-injected larvae at 2 dpl (Fig. 5c). In lesioned col12a1a/b morphants swimming...
distance was reduced by 58% compared to unlesioned col12a1a/b MO-injected larvae (Fig. 5d). Importantly, Vivo-Morpholino treatment was not toxic, since lesioned control MO-injected larvae recovered to the same swimming capacity as unlesioned age-matched control MO-injected larvae at 2 dpf, and col12a1a/b MO treatment did not impair swimming in unlesioned animals. Impaired functional regeneration after MO knockdown supports that Col XII is part of the axon-growth promoting ECM in the lesion site.

To independently confirm the function of col12a1a/b, we used transient CRISPR/Cas9-mediated gene editing. Injection of small guide-RNA (gRNA) and cas9 mRNA into the zygote efficiently generates biallelic mutations of targeted genes in a very high proportion of cells, leading to loss-of-function phenotypes. Indeed, targeting of col12a1a and col12a1b efficiently disrupted both genes in CRISPR-injected embryos, as shown by loss of targeted endonuclease restriction sites (Fig. 6a, b). Quantitative RT-PCR indicated reduced abundance of transcripts at 1 dpf, likely caused by non-sense mediated RNA decay in CRISPR-manipulated larvae (hereafter referred to as Crispants; Fig. 6c). Similarly, after a spinal lesion in Crispants the abundance of both col12a1a and col12a1b mRNAs in the lesion site was also reduced, as determined by quantification of fluorescence in situ hybridisation signals (Fig. 6d). Targeting of only col12a1a or col12a1b did not affect axonal regeneration, possibly due to compensation of the non-targeted paralog (Supplementary Fig. 12a). In contrast, in Crispants in which both col12a1 genes were targeted, the proportion of larvae with axonal continuity (anti-acetylated Tubulin+) between spinal cord ends was reduced by 31% compared to controls (Fig. 6e).

Single larvae analysis showed that lack of axonal bridging in col12a1a and col12a1b-targeted animals (analysed in Xla.Tubb;DsRED transgenic fish) correlated with high gene editing efficiency of both col12a1 paralogs, supporting specificity of the CRISPR/Cas9 manipulation (Supplementary Fig. 12b). Hence, both MO knockdown and CRISPR manipulation indicate an important contribution of Col XII to functional spinal cord regeneration in zebrafish.

To determine the relevance of Col XII as a downstream effector of Wnt/β-catenin signaling, we asked whether experimentally increasing expression of col12a1a was sufficient to rescue the regeneration phenotype induced by inhibition of the Wnt/β-catenin pathway. To this end, we created zebrafish that are transgenic for heat shock-inducible expression of full-length col12a1a (hs:col12a1a-p2a-Cerulean, short hscol12a1a; Supplementary Fig. 13a). Activation of the transgene in unlesioned animals increased anti-Col XII immunoreactivity in developmental Col XII domains but also in ectopic sites, including the spinal cord (arrow in Supplementary Fig. 13b). To increase Col XII deposition in the lesion site, we applied repetitive heat shocks to hscol12a1a transgenic animals until immediately before the lesion (3 dpf). This treatment increased detectability of transgene mRNA for at least 24 h after the last heat shock (Fig. 7a) and was sufficient to increase Col XII immunoreactivity in the lesion site of larvae in which the Wnt/β-catenin pathway was inhibited (IWR-1 treatment; analyzed at 2 dpf; arrow in Fig. 7b). We found that under these experimental conditions, col12a1a overexpression more than doubled the proportion of IWR-1-treated larvae with axonal bridge to 77% of all lesioned larvae, compared to 32% in only IWR-1-treated lesioned larvae (determined in Xla.Tubb;DsRED transgenic animals; Fig. 7c). This represents a rescue of the axonal bridging phenotype to near wild type levels (compare Supplementary Fig. 1b). col12a1a overexpression also improved functional recovery in IWR-1-treated larvae (+55% of the swim distance covered by Wnt/β-catenin-pathway-inhibited lesioned animals; Fig. 7d). This identifies Col XII as a major downstream effector of Wnt/β-catenin signaling in the regenerating zebrafish spinal cord.

We next asked whether col12a1a overexpression is sufficient to augment axon regeneration. To this end, we assessed
axonal bridging in hscol12a1a transgenic animals and their wild type control siblings at 18 hpf (Fig. 7e). At this early time point, 41% of lesioned control larvae showed axonal continuity (anti-acetylated Tubulin+) between spinal cord ends. Remarkably, col12a1a overexpression increased the proportion of larvae with axonal bridge to 63%. Thus, Col XII is sufficient to enhance axon regeneration after a spinal lesion in zebrafish. Taken together, these findings determine Col XII as major pro-regenerative, Wnt/\(\beta\)-catenin-controlled ECM factor in the spinal lesion site.

**Discussion**

Here, we demonstrate an important role for Wnt/\(\beta\)-catenin signaling in controlling the composition of the spinal lesion ECM, which is essential for functional axon regeneration in zebrafish. Our data support a model (Fig. 8) in which spinal cord transection triggers upregulation of Wnt/\(\beta\)-catenin signaling in non-neural cells. The vast majority of these are fibroblast-like cells accumulating in the lesion site. Wnt/\(\beta\)-catenin signaling regulates col12a1a transcription and deposition of Col XII by fibroblast-like cells in the spinal lesion ECM. The presence of Col XII in the ECM promotes axon growth across the lesion site and functional recovery.

How does Col XII support axonal regeneration? Col XII is abundant in the spinal lesion site in species of high regenerative capacity, such as in larval and adult zebrafish (this report) and salamanders. Col XII is a member of the FACITs family of collagens, which do not form supramolecular aggregates but are associated with Collagen fibrils. Previously, Col XII was found to interact with Col I fibrils. However, we find that Col I immunoreactivity remained grossly unchanged after interfering with col12a1a/b translation. Col XII also interacts with Col VI in forming matrix bridges between developing osteoblasts. Interestingly, we find in our expression screen that col6a2, coding for a Col VI \(\alpha\)-chain, is co-regulated with col12a1a/b by Wnt/\(\beta\)-catenin signaling, which may be related to functional interactions of the proteins. In addition, Col XII binds a variety of other matrix proteins, such as Tenascin-X, Decorin, and Fibromodulin. Hence, Col XII could promote growth of axons indirectly by organising the lesion site ECM. Another possibility is that Col XII could directly interact with axons via specific receptors. This is supported by our observation that overexpression of col12a1a alone is sufficient to enhance axonal regeneration. While specific axonal receptors for Col XII have not been identified, potential direct Col XII-axon interactions could be mediated by its integrin-binding site, the NC1 domain, as reported for Col IV.

While our results identify Col XII as a critical factor for axon regeneration, several additional ECM molecules likely contribute to establishing the growth promoting lesion site environment found in zebrafish. For example, Tenascin-C has been shown to be important for spinal cord regeneration in zebrafish, but possible interactions with Col XII are not known. Furthermore, Col I and Fibronectin, which we find to be abundantly present in the lesion site, are also known ECM components that promote axons growth.
How does Wnt/β-catenin signaling act on col12a1a/b expression? Wnt-responding cells co-express col12a1a/b. Hence, Wnt/β-catenin pathway activity likely acts in the same cells to increase expression of col12a1a/b after a lesion. Regulation of Collagens in fibroblasts by Wnt/β-catenin signaling has also been observed in dermal fibroblasts or myofibroblasts in the context of fibrosis. However, a sizable population of fibroblast-like cells showed Wnt-dependent col12a1a/b upregulation without having detectable Wnt/β-catenin pathway activity. Our results show that the effect of Wnt/β-catenin pathway inhibition on col12a1a/b expression is delayed and therefore likely indirect and that Wnt/β-catenin signaling does not need to be sustained for col12a1a/b expression and successful regeneration. These observations are consistent with the possibility that Wnt/β-catenin signaling is only transiently active in col12a1a/b-expressing cells. Alternatively, secondary signals from Wnt-responding cells could instruct transcriptional regulation of col12a1a/b in non-Wnt-responsive cells.

Are astroglia-like cell bridges a necessary substrate for axonal regeneration across a spinal lesion site? Regenerating axons could not be sustained for col12a1a/b expression and successful regeneration. These observations are consistent with the possibility that Wnt/β-catenin signaling is only transiently active in col12a1a/b-expressing cells. Alternatively, secondary signals from Wnt-responding cells could instruct transcriptional regulation of col12a1a/b in non-Wnt-responsive cells.
Is larval regeneration comparable to adult spinal cord regeneration? In both larvae and adult zebrafish, functional regeneration after complete spinal cord transaction depends on axons successfully crossing a non-neural lesion site (this report and refs. 11, 13, 28) and forming connections1, 12. Both show lesion-specific upregulation of col12a1a and axons regenerate in close proximity to Col XII in larval and adult zebrafish (this report). At least some axons grow independently of glia at both developmental stages (this report and ref. 27) and, crucially both larval and adult regeneration depend on Wnt/β-catenin signaling (this report and refs. 14, 15). This suggests that key aspects of regeneration are conserved across developmental stages. However, regeneration times in adults are much longer than in larva28, such that timing of signaling processes may differ in adults.

In conclusion, Col XII depletion, controlled by Wnt-responsive fibroblasts, promotes spinal cord regeneration in zebrafish. Interestingly, transplanting Wnt3a-secreting fibroblasts into a spinal lesion site in rats enhances functional recovery63. Thus, elucidating the molecular cues that promote axon regeneration in zebrafish could provide strategies in mammals to modulate the composition of the lesion site to tip the balance towards a growth-conducive ECM environment.

**Methods**

**Animals.** All fish were kept and bred in our laboratory fish facility according to standard procedures64, and all experiments have been approved by the British Home Office (project license no.: 70/8805). Details of the transgenic zebrafish lines used can be found in Supplementary Note 1. We used the WIK wild type strain of zebrafish and the following transgenic zebrafish lines: 6xTCF/Lef-mintm12D2;GFP (6xTCF4;GFP25); 7xTCF-Xa.Siam:nla:AmCyan (7xTCF-mCherry); gfp.Ga41829;UAS:Elk.Eco:NeGB-mCherry23; gfp.Ga41829;UAS:NT-kr::mCherry23; her4.3:EGFP104 (formerly known as her4.1: EGFPh29); her4.3;Ir:iRTAM23F(S)-p2a-AmCyanLum26; her4.1: Tet-GRD-p2a-MCherry23 (her4.3:mCherry41); hsp70dkd1::GFP25 (herd1k29); hsp70DMumu.Axin1-YFP23; hsp70,:Mmu.Axin1::Axin134; hsp70hf:Gal4FFh28. Primer were designed to span an exon of interest.

**DNA constructs.** The left1:iRTAM23F(S)-p2a-AmCyan construct was generated by replacing the translation start codon of left1 in the BAC clone CT73-1646H6 with the previously described iRTAM23F(S)-p2a-AmCyan TetActivator cassette65, 66. Similarly, a standard BAC recombination technique67. Additionally, Tol2 inverted repeats were introduced into the BAC backbone. The final BAC was purified using the HiPure Midiprep kit (Invitrogen). The Xla.Tubb1:iRTAM23F(S)-p2a-AmCyan construct was generated by cloning the previously described sequence of the Xenopus laevis neural-specific beta tubulin regulatory element (Xla.Tubb26) upstream of the published doxycycline (DOX)-inducible transcriptional activator [iRTAM23F(S)] tagged with p2a and AmCyan26, 41, 46. For generation of the hsp70Col212a1a::p2a-Cerulean construct, 3 overlapping fragments spanning the full-length col212a1a CDS were amplified from 1 dpl CDNA (primer sequences are given in Supplementary Table1) and fused via internal restriction sites (BsiWI, BsrGI). The complete col212a1a CDS was then subcloned into the hsp70Birtam23F(S)-p2a-AmCyan construct68, thereby replacing the iRTAM TetActivator CDS.

**Reverse transcriptase PCR and quantitative RT-PCR.** To assess the effect of IWR-1 treatment on lesion-induced gene expression total RNA from lesion site-enriched trunk tissue was extracted using TRIzol (Invitrogen). Twenty larvae were pooled for each experimental condition. To assess the effect of COL12A1A/b gRNA/cas9 mRNA microinjection on developmental col212a1a/b mRNA levels total RNA from ten 24 hpf embryos was extracted using TRIzol. Reverse transcription of 500 ng RNA was performed with the SuperScript III kit (Invitrogen) using a combination of oligo(DT) and random hexamer primer. Standard RT-PCR (38°C, 30 cycles) was performed using 10 µM of each dNTP and 10 µM of each primer. qRT-PCR was performed at 58°C using Roche Light Cycler 96 and relative mRNA levels determined using Roche Light Cycler 96 SW1.1 software. Samples were run in triplicate and expression levels were normalized to β-actin control. Primer were designed to span an exon-exon
Spinal cord lesions and behavioral recovery. Zebrafish larvae (3 dpf) were anesthetized in PBS containing 0.02% aminobenzoic acid ethyl methyl ester (MS222; Sigma-Aldrich). For dorsal incision lesions, larvae were transferred to a lid of a plastic Petri dish. Following aspiration of excess water, which placed the larvae in a lateral position, the tip of a sharp 30 G syringe needle was used to inflict a lesion in the dorsal trunk at the level of the anal pore. Survival of larvae was typically 70-80%. Larvae that had undergone extensive damage to the notochord showed the formation of a notochord-derived bulgy tissue structure (~10%). Those larvae were excluded from further analysis. For less invasive stab lesions, larvae were transferred to an agarose-coated Petri dish, and were placed in a lateral position through removal of excess water. The tip of a sharp 30 G syringe needle was pushed into the larval spinal cord at the level of the anal pore immersed in the agarose and passed through the notochord. Kinetics of anatomical/functional spinal cord regeneration were comparable in larvae that underwent dorsal incision lesion or stab lesion. After surgery, larvae were returned to E3 medium for recovery and kept at 28.5 °C. Wherever possible, Xlal.Tubb:DsRED transgenic larvae were used for experiments to visually verify completeness of spinal cord transection after lesion. Analysis of behavioral recovery was performed as described previously[15]. Behavioral data are shown as distances travelled within 15 s after touch, averaged for triplicate measures per larvae. Adult spinal cord lesions have been described previously[16]. Briefly, fish (≤ 3 month post-fertilization) were anesthetized by immersion in 0.02% MS222 in PBS for 10 min. Larvae were exposed to E3 embryo medium, containing 50 μM DMSO (6 mM stock) and used at a concentration of 50 μM. The Axin1 stabilizer IWR-1 (Sigma-Aldrich) was dissolved in DMSO (6 mM stock) and used at a final concentration of 5 μM. Doxycycline (DOX; Sigma-Aldrich) was dissolved in 50% EtOH (50 mg/ml stock) and used at a final concentration of 40 μg/ml. For targeted cell ablation, the pro-drug Metranidazole (MTzI; Sigma-Aldrich) was dissolved in DMSO (200 mM) and used at a final concentration of 2 mM.

Morpholino injection. Knockdown of col2a1a/b was performed by injecting Vivo-Morpholinos (MO) to col2a1a/b, which act as cell-permeable translation blocking anti-sense molecules, into the circulation for efficient distribution in 3 dpf larvae (Supplementary Fig. 11a). Two translation blocking Morpholinos were designed (Gene Tools, Philomath, OR, USA): col2a1a/b MO 5′-CGGCCAAAGA-TGGAGTGTGGGAAGAGAAAACTT-3′ and col2a1a/b MO 5′-TGCAAAATGCTGACGAGACCATC TTCC-3′. 100% homology of MO sequences to target sites were confirmed by sequence analysis. For all quantifications, the observer was blinded to the experimental treatments. Scores of key experiments were validated by a second independent observer.

Quantiﬁcations and statistics. Re-established axonal or glial connections (bridges) were scored in static preparations (fixed immunolabelled samples, live transgenic animals) or in still images of time-lapse movies at time points of interest. Larvae were directly visualized using a compound fluorescence microscope (Zeiss Imager.Z1), or after confocal imaging (Zeiss LSM 710, 880). A larva was scored as having a bridged lesion site when continuity of the neuronal or glial labeling between the rostral and caudal part of the spinal cord was observed. Continuity of labeling was defined as at least one fascicle being continuous between rostral and caudal spinal cord ends irrespective of the fascicle thickness. Larvae in which the lesion site was obscured by melanocytes were excluded from analysis. For all quantifications, the observer was blinded to the experimental treatments.

Live imaging of zebrafish larvae and time-lapse imaging. For live confocal imaging, zebrafish larvae were anesthetized in PBS containing 0.02% MS222 and mounted in the appropriate orientation in 1% low melting point agarose (Ultra Pure, Sigma-Aldrich). Larvae were left to settle down and were then imaged in time-lapse imaging containing fish water to keep preparations from drying out. For time-lapse imaging, agarose covering the lesion site was gently removed after gelation. Time-lapse imaging was performed for 19 h starting at 6 hpl. After initial visual inspection, acquired time-lapse images were denoised to improve image quality (enhance detail of background noise, cause the low signal to noise ratio, was removed completely). This was done by inspecting single optical sections and 3D renderings of the lesion site. If more than half of the length of a fascicle contained fluorescence for both glial and neuronal markers it was scored as “mixed”, otherwise it was scored as purely “axonal” or “glial” depending on the markers used. The threshold of half the length was used to exclude situations in which one type of process followed the other long after that one was established.

For quantification of axonal and glial fascicle composition in time-lapse movies we included only fascicles that grew during the observation period.

Fascicles were considered as purely “axonal” or “glial” when the distal part of a glial or axonal fascicle was labelled by the respective marker throughout the entire observation period. In “mixed” fascicles, both markers were present in the distal portion of the fascicle.

Fascicles were considered to be in close apposition with ECM in histological preparations when > 50% of an axonal fascicle that had entered or crossed the lesion site was aligned with ECM immunoreactivity. This was assessed in single optical sections and 3D renderings of the lesion site.

Quantification of immunohistochemistry or fluorescent in situ hybridization signal in the lesion site was performed on captured images of whole mount samples using ImageJ software as described in subsection “Image analysis”. Samples were imaged using a compound fluorescence microscope (Zeiss Imager.Z1) or confocal microscope (Zeiss LSM 710, 880). Measurements were performed in a blinded fashion.

Quantification of GFP+/?+3 cells and GFP+/?+6TCF+ delaminated transgenic larvae was performed on immunolabeled sections of individual animals (n ≥ 10).

For non-quantitative data, the number of specimens that showed a given phenotype and the total number of specimens analyzed is given as a ratio in each figure. Figures were scored in a blinded fashion.

All experiments were performed at least three times. Animals were randomly assigned to different experimental groups but no formal method of randomisation was used. Power analysis of pilot experiments informed minimum samples size. All quantitative data were tested for normality and analyzed with parametric and non-parametric tests as appropriate. An F-test was used to check for equal variances. We used one-way ANOVA followed by Dunn’s multiple comparison test if the data showed good normality and homoscedasticity. For non-normal data, a Kruskal-Wallis test was used as the non-parametric analog and post hoc Dunn’s test was used. All p values are two-tailed, and p < 0.05 was considered statistically significant. Error bars always indicate the standard error of the mean (SEM).

Image analysis. Quantitative analysis of immunohistochemistry or fluorescent ISH signals in the lesion site was performed on captured images. Samples were
formamide, and twice in 2xSSCT for 20 min each, followed by four washes at 65 °C for 1 h incuba-
tion at 65 °C overnight. On the following day, larvae were washed at 65 °C six times in PBTx for 20 min each, followed by incubation in 100% Acetone (pre-chilled to –20 °C) for 10 min. Thereafter, larvae were washed twice in distilled H2O for 5 min each, followed by incubation with secondary antibody of interest diluted in blocking buffer (1:300) at 4 °C overnight. On the following day, larvae were incubated in blocking buffer containing primary antibody (1:300–1:500) diluted in blocking buffer at 4 °C overnight. For all measurements except in Fig. 7b, ImageJ software was used to determine the number of pixel above an intensity threshold (thresholded pixel area) in the region of interest (ROI) (an example of such a measurement is shown in Supplementary Fig. 14). In brief, two thresholds was applied to raw images from the same dataset, limiting pixels to only those of equal or higher intensity. The intensity threshold was determined for each experimental data set individually, using untreated lesioned control samples as reference. The threshold was set such that the area included the fluorescent domain and lesion in the lesion site while low background signal in the vicinity of the lesion site was largely excluded (efficient image segmentation). After thresholding, a ROI was additionally defined manually to exclude pixels from analysis that fell within the threshold intensity range but were located outside the lesion site. This was necessary in some samples to exclude autofluorescence (e.g., from blood vessels), or constitutively labelled domains (e.g., myosepta for Col XII). Measurements of immunoreactivity in the lesion site, shown in Fig. 7b, was performed by determining the pixel area in a predefined ROI of constant size without prior thresholding. This was necessary due to low signal to noise ratio. In brief, the image was converted to a binary image using the automated binary thresholding function of ImageJ. The ROI was placed in the center of the lesion site. All measurements were performed in a blinded fashion.

In situ hybridization. All incubations were performed at room temperature unless stated otherwise.

For whole mount acetylated Tubulin/GFAP immunolabeling, larvae were fixed in 2% TCA-PBTx (1% Triton X-100 in PBS) for 3 h, followed by brief washes in PBTx and PBS. Thereafter, larvae were incubated for 30 min in 0.25% Trypsin (Gibco) diluted in PBTx, followed by five washes in PBTx for 5 min each. Larvae were blocked in 4% BSA-PBTx for 1 h, followed by incubation in blocking buffer containing primary antibody (1:300) diluted in blocking buffer at 4 °C overnight. On the following day, larvae were washed six times in PBTx for 20 min each, followed by incubation with secondary antibody of interest diluted in blocking buffer (1:300) at 4 °C overnight. On the following day, larvae were washed six times each in PBTx for 20 min each, followed by brief washes in PBS or 75% Glycerol-PBS.

For whole mount GFP/acytlated Tubulin/p63 immunolabelling in 6xTGF-dGFP transgenic larvae, larvae were fixed in 4% PFA-PBS containing 1% DMSO at 4 °C overnight. On the following day, larvae were washed four times in PBTx, followed by two washes in PBTx (0.2% Triton X-100 in PBS) for 5 min each. Thereafter, larvae were permeabilized by incubation in PBS containing 2 µg/ml Collagenase (Sigma-Aldrich) for 25 min. To terminate Collagenase digest, larvae were briefly rinsed in PBTx. Subsequently, larvae were incubated in 50 mM glycine in PBTx for 10 min, followed by a brief rinse in PBTx. Larvae were blocked in blocking buffer (0.7% PBTx, 1% DMSO, 1% normal donkey serum, 1% BSA) for 1 h. Thereafter, larvae were incubated in blocking buffer containing primary antibody (1:300–1:500) diluted in blocking buffer at 4 °C overnight. On the following day, larvae were washed three times in PBTx for 15 min each, followed by incubation with secondary antibody of interest diluted in blocking buffer (1:300) at 4 °C overnight. On the following day, larvae were washed three times in PBTx and once in PBS for 15 min each, before mounting in 75% Glycerol-PBS.

For whole mount acetylated Tubulin/GFP immunolabelling, as well as all other combinations of immunolabelling on whole mounts (e.g., acetylated Tubulin/Col XII), larvae were fixed in 4% PFA-PBS for 1 h, followed by brief washes in 0.1% PBT (0.1% Tween-20 in PBS). Thereafter, larvae were stepwise dehydrated by incubation in 70% (v/v) ethanol for 15 min, 90% (v/v) ethanol for 15 min, 95% (v/v) ethanol for 15 min, 100% MeOH for 5 min each, and stored at –20 °C until need. When required, head and tail of larva were removed and lesion site-enriched trunk tissue was incubated in 100% MeOH at –20 °C overnight. On the following day, samples were rehydrated by successive incubations in 75% MeOH-PBT, 50% MeOH-PBT and 25% MeOH-PBT for 5 min each, followed by four washes each in PBS for 5 min each. Larvae were incubated in pre-tissue in distilled H2O for 5 min each, followed by incubation in 100% Acetone (pre-chilled to –20 °C) for 10 min. Thereafter, larvae were washed twice in distilled H2O for 5 min each, followed by brief washes in 75% Glycerol-PBS. For immunohistochemistry on floating agarose sections of larval zebrafish, larvae were fixed in 4% PFA-PBS at 4 °C overnight. After two brief washes in PBTx (0.1% Tween-20) for 5 min each, larvae were washed in 4% PFA-PBS for 15 min each. Subsequently, larvae were permeabilized in 1% Triton X-100 in PBS for 15 min, followed by incubation in blocking buffer containing primary antibody (1:300) diluted in blocking buffer at 4 °C for 2–3 days. Thereafter, larvae were washed eight times in PBTx for 20 min each, followed by incubation with secondary antibody of interest diluted in blocking buffer (1:300) at 4 °C for 1–2 days. Thereafter, larvae were washed six times in PBTx for 20 min each and once in PBS for 15 min before mounting in 75% Glycerol-PBS.

For immunofluorescence. All incubations were performed at room temperature unless stated otherwise.

For whole mount acetylated Tubulin/GFP immunolabelling, larvae were fixed in 2% TCA-PBTx (1% Triton X-100 in PBS) for 3 h, followed by brief washes in PBTx and PBS. Thereafter, larvae were incubated for 30 min in 0.25% Trypsin (Gibco) diluted in PBTx, followed by five washes in PBTx for 5 min each. Larvae were blocked in 4% BSA-PBTx for 1 h, followed by incubation in blocking buffer containing primary antibody (1:300) diluted in blocking buffer at 4 °C overnight. On the following day, larvae were washed six times in PBTx for 20 min each, followed by incubation with secondary antibody of interest diluted in blocking buffer (1:300) at 4 °C overnight. On the following day, larvae were washed six times each in PBTx for 20 min each, followed by brief washes in PBS or 75% Glycerol-PBS.

For whole mount GFP/acytlated Tubulin/p63 immunolabelling in 6xTGF-dGFP transgenic larvae, larvae were fixed in 4% PFA-PBS containing 1% DMSO at 4 °C overnight. On the following day, larvae were washed four times in PBTx, followed by two washes in PBTx (0.2% Triton X-100 in PBS) for 5 min each. Thereafter, larvae were permeabilized by incubation in PBS containing 2 µg/ml Collagenase (Sigma-Aldrich) for 25 min. To terminate Collagenase digest, larvae were briefly rinsed in PBTx. Subsequently, larvae were incubated in 50 mM glycine in PBTx for 10 min, followed by a brief rinse in PBTx. Larvae were blocked in blocking buffer (0.7% PBTx, 1% DMSO, 1% normal donkey serum, 1% BSA) for 1 h. Thereafter, larvae were incubated in blocking buffer containing primary antibody (1:300–1:500) diluted in blocking buffer at 4 °C overnight. On the following day, larvae were washed three times in PBTx for 15 min each, followed by incubation with secondary antibody of interest diluted in blocking buffer (1:300) at 4 °C overnight. On the following day, larvae were washed three times in PBTx and once in PBS for 15 min each, before mounting in 75% Glycerol-PBS.
Thereafter, sections were washed eight times in PBTx for 20 min each, followed by incubation with secondary antibody of interest diluted in blocking buffer (1:250) at 4 °C overnight. On the following day, larvae were washed six times in PBTx and twice in PBS for 20 min each. Sections were stained for DAPI (Sigma-Aldrich) to visualize nuclei, followed by two washes in PBS and mounted in 75% Glycerol-PBS.

For immunohistochemistry on floating agarose sections of adult zebrafish tissue, zebrafish were sacrificed and perfused with 4% PFA-PBS (0.1M). The lesion site with surrounding non-neural tissue and spinal cord was dissected in a semi-intact preparation and the tissue was post-fixed in 4% PFA for 1 h. Tissue was embedded in 4% Agarose-PBS and sectioned at 100–200 μm using a vibratome. Sections were briefly rinsed in PBS and washed several times in PBTx (1% Triton X-100 in PBS) for a total of 1 h followed by incubation in blocking buffer (4% BSA in PBTx). Thereafter, sections were incubated with primary antibody (1:250) diluted in blocking buffer at 4 °C for 3 days. Thereafter, sections were washed eighteen times in PBTx for 10 min each, followed by incubation with secondary antibody of interest diluted in blocking buffer (1:250) at 4 °C for 2 days. On the following day, sections were washed twelve times in PBTx and twice in PBS for 20 min each. Sections were stained for DAPI (Thermo Fisher) to visualize nuclei, followed by two washes in PBS and mounted in 75% Glycerol-PBS.

All primary antibodies used are given in Supplementary Table 3. Primary antibodies used are specific for zebrafish antigens as shown in the indicated publications. To detect basal keratinocytes, we used anti-p63 antibodies (Cell Signaling Technology) and anti-p63 antibodies (Sigma-Aldrich), replicates. The antibody used in this study, anti-p63 antibody (Sigma-Aldrich), replicates. Secondary fluorophore-conjugated antibodies were from Jackson Immuno Research.

Data availability. The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files, or from the corresponding authors on reasonable request.

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