

## **Myostatin is a negative regulator of adult neurogenesis in zebrafish**

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## ABSTRACT

1 Intrinsic and extrinsic inhibition of axonal and neuronal regeneration obstruct spinal cord (SC)  
2 repair in mammals. In contrast, adult zebrafish achieve functional recovery after SC damage.  
3 While studies of innate SC regeneration have focused on axon regrowth as a primary repair  
4 mechanism, how local neurogenesis impacts functional recovery is unknown. We uncovered  
5 dynamic expression of *myostatin b* (*mstnb*) in a niche of dorsal ependymal progenitors after  
6 complete SC transection in zebrafish. Genetic loss-of-function in *mstnb* impaired functional  
7 recovery, although glial and axonal bridging across the lesion were unaffected. Using a series of  
8 transgenic reporter lines, we quantified the numbers of stem, progenitor, and neuronal cells in the  
9 absence of *mstnb*. We found neural stem cell proliferation was reduced, while newborn neurons  
10 were increased in *mstnb* null tissues, suggesting *mstnb* is a negative regulator of neurogenesis.  
11 Molecularly, neuron differentiation genes were upregulated, while the neural stem cell  
12 maintenance gene *fgf1b* was downregulated in *mstnb* mutants. Finally, we show that human  
13 FGF1 treatment rescued neuronal gene expression in *mstnb* mutants. These studies uncover  
14 unanticipated neurogenic functions for *mstnb* in adult zebrafish, and establish the importance of  
15 local neurogenesis for functional SC repair.

## INTRODUCTION

16 Traumatic spinal cord injury (SCI) causes irreversible neuronal and systemic deficits (Hachem et  
17 al., 2017; Silva et al., 2014; Singh et al., 2014). In mammals, axon regrowth and neurogenesis  
18 are impeded by intrinsic and extrinsic inhibitory mechanisms that obstruct spinal cord (SC)  
19 regeneration (Alizadeh et al., 2019; Oyinbo, 2011; Sofroniew, 2018; Tran et al., 2021). Although  
20 multiple cell types including astrocytes and oligodendrocyte progenitor cells proliferate after SCI,  
21 the mammalian SC is incapable of generating mature neurons *in vivo* (Horky et al., 2006; Horner  
22 et al., 2000; Yamamoto et al., 2001). In contrast with mammals, highly regenerative vertebrates  
23 including teleost fish spontaneously recover after SCI. Following complete transection of SC  
24 tissues, adult zebrafish extend glial and axonal bridges across the lesion and achieve functional  
25 recovery within 6 to 8 weeks of injury. In addition to axon regrowth from hindbrain neurons,  
26 zebrafish regenerate motor neurons and interneurons around the lesion site (Becker et al., 1998;  
27 Becker et al., 1997; Kuscha et al., 2012a; Mokalled et al., 2016; Reimer et al., 2008). Yet, the  
28 contribution of local neurogenesis to functional recovery and the mechanisms that coordinate the  
29 regeneration of different neuronal subtypes remain to be determined.

30 Ependymal radial glial cells (ERGs) line the brain ventricles and SC central canal. ERGs co-  
31 express astroglial (*gfap* and *blbp*) and progenitor (*sox2* and *hey*) cell markers, and comprise  
32 populations of neurogenic stem cells in adult zebrafish (Kroehne et al., 2011; März et al., 2010;  
33 Ogai et al., 2014; Reimer et al., 2009; Than-Trong et al., 2020; Than-Trong et al., 2018). In  
34 uninjured neural tissues, the majority of ERGs are non-dividing, quiescent cells that possess  
35 epithelial-like features (Barbosa et al., 2015; Chapouton et al., 2010; März et al., 2010). Following  
36 brain and SC damage, ERGs undergo widespread proliferation and are thought to act as a major  
37 source of regenerated neurons (Adolf et al., 2006; Barbosa et al., 2015; Grandel et al., 2006;  
38 Kroehne et al., 2011). Activated ERGs undergo 3 modes of cell division: 1) symmetric division  
39 into ERGs, 2) asymmetric division into ERG and neural progenitors, or 3) symmetric division to  
40 generate differentiated neurons (Barbosa et al., 2015; Rothenaigner et al., 2011). Distinct lineage-  
41 restricted ERG domains emerge during SC regeneration in zebrafish. ERGs within the progenitor  
42 motor neuron (pMN) domain express *olig2* and generate *isl1/2<sup>+</sup>* and *hb9<sup>+</sup>* motor neurons after SCI  
43 (Reimer et al., 2008). On the dorsal and ventral sides of the pMN, ERGs give rise to *vsx1<sup>+</sup>* V2  
44 interneurons and serotonergic neurons, respectively (Barreiro-Iglesias et al., 2015; Kuscha et al.,  
45 2012b). We recently showed that ventral ERGs undergo epithelial-to-mesenchymal transition  
46 (EMT), and that EMT is required for glial bridging and functional regeneration (Klatt Shaw et al.,

47 2021). Thus, despite their morphological similarities, ERGs elicit compartmentalized injury  
48 responses and proliferate into lineage-restricted progenitors during SC regeneration.

49 Myostatin (Mstn), also known as Growth differentiation factor 8 (Gdf8), is a Tgf- $\beta$  superfamily  
50 member. Upon binding Activin type 2 and type 1 receptors, Mstn induces the phosphorylation and  
51 nuclear translocation of Smad 2/3 to regulate target gene expression (Massagué, 2012; Sartori  
52 et al., 2014; Sharma et al., 2015). Spontaneous and targeted Mstn loss-of-function mutations lead  
53 to double muscle phenotypes in zebrafish, mice, cattle and humans (Dogra et al., 2017;  
54 Kambadur et al., 1997; Schuelke et al., 2004; Whittemore et al., 2003). Mechanistically, Mstn  
55 controls lineage progression within myogenic stem cells (satellite cells) and progenitor cells  
56 (myoblasts). During muscle development, Mstn inhibits myoblast differentiation via negative  
57 regulation of the myogenic transcription factors MyoD and Myogenin. Mstn also suppresses  
58 satellite cell proliferation, differentiation, and muscle regeneration (Langley et al., 2002;  
59 McCroskery et al., 2003; McCroskery et al., 2005). However, it remains unclear whether  
60 recombinant MSTN proteins inhibit or stimulate myoblast proliferation (Rodgers et al., 2014;  
61 Taylor et al., 2001). Equally conflicting effects were reported for recombinant MSTN proteins on  
62 neuronal proliferation and neurite outgrowth *in vitro* (Kerrison et al., 2005; Wu et al., 2003),  
63 suggesting Mstn functions are dose- and context-dependent and that *in vivo* studies are required  
64 to decipher the role of Mstn in the nervous system.

65 Tgf- $\beta$  signaling directs immune, fibrotic, or regenerative injury responses across tissues and  
66 species. As zebrafish SCs regenerate without fibrotic scarring, we postulated that Tgf- $\beta$  signaling  
67 is pro-regenerative in adult zebrafish. By surveying the expression of Tgf- $\beta$  ligands after SCI, we  
68 found *mstnb* is induced in dorsal ERGs of lesioned SC tissues. *mstnb* mutants showed normal  
69 baseline swim capacity but failed to achieve functional recovery following SCI, despite having  
70 normal axonal and glial bridging across the lesion. Mstnb inhibition using genetic loss-of-function  
71 and pharmacological approaches enhanced neurogenesis and diminished ERG proliferation. A  
72 series of transgenic reporter lines was used to quantify the numbers of neural stem cells (NSCs),  
73 intermediate neural progenitors (iNPs), and interneurons. These studies revealed NSC  
74 proliferation was reduced, while regenerating neurons were increased in *mstnb* mutants. RNA  
75 sequencing showed neuron differentiation genes were upregulated in *mstnb* mutants. Finally, we  
76 show that the neural stem cell maintenance gene *fgf1b* was downregulated in *mstnb* mutants,  
77 and that human FGF1 treatment rescued neuronal gene expression. These studies indicate that  
78 *mstnb* acts as an essential negative regulator of adult neurogenesis in zebrafish, and that injury-

79 induced *mstnb* expression is required to maintain the potency and self-renewal of neurogenic  
80 ERGs during SC regeneration.

## RESULTS

### 81 **Tgf- $\beta$ signaling is activated in dorsal ependymal progenitors after SCI.**

82 Injuries to the central and peripheral nervous systems induce Tgf- $\beta$  signaling across vertebrates.  
83 In mammals, Tgf- $\beta$  activation directs a range of regenerative and anti-regenerative cell responses  
84 including immune cell activation, neurite outgrowth, and scar formation (Li et al., 2017). In  
85 zebrafish larvae, the anti-inflammatory effects of Tgfb1a are required for SC regeneration  
86 (Keatinge et al., 2021). We postulated that Tgf- $\beta$  signaling is pro-regenerative in adult zebrafish.  
87 To explore this hypothesis, we first surveyed Smad3 phosphorylation as a readout of Tgf- $\beta$  activity  
88 after zebrafish SCI. By immunohistochemistry, phosphorylated Smad3 (pSmad3) was strongly  
89 induced in dorsal SC tissues at 1 week post-injury (wpi) (Fig. 1A). pSmad3 expression gradually  
90 diminished between 2 and 3 wpi, and was minimally expressed in uninjured SCs (Fig. 1A). At 1  
91 wpi, pSmad3<sup>+</sup> cells accounted for 7% of dorsal SC cells, and were reduced by 3 wpi relative to 1  
92 wpi (Fig 1B, S1A). To determine the identity of Tgf- $\beta$  responsive cells after SCI, we co-labelled  
93 pSmad3 with either the neuronal markers HuC and HuD (HuC/D) or the ependymal progenitor  
94 marker Sox2. At 1 wpi, we rarely observed vesicular pSmad3 expression in some HuC/D<sup>+</sup>  
95 neurons. However, the majority of HuC/D<sup>+</sup> neurons were pSmad3<sup>-</sup> (Fig. S1B). pSmad3 was  
96 primarily expressed at high levels in Sox2<sup>+</sup> ERGs (Fig. 1C). Quantification revealed 66 to 76% of  
97 pSmad3<sup>+</sup> cells were Sox2<sup>+</sup> across timepoints (Fig. 1D, S1C). By EdU incorporation, ~10% of  
98 pSmad3<sup>+</sup> cells were proliferative at 1 wpi in dorsal SCs, and pSmad3<sup>+</sup> cell proliferation decreased  
99 to baseline levels by 3 wpi (Fig. S1D, E). These findings indicated Tgf- $\beta$  signaling is activated in  
100 dorsal ERGs after SCI.

### 101 ***mstnb* expression is upregulated in dorsal ependymal progenitors after SCI.**

102 To explore mechanisms of Tgf- $\beta$  activation during SC regeneration, we used a previously  
103 published RNA-seq dataset to survey the expression of Tgf- $\beta$  ligands at 2 wpi (Mokalled et al.,  
104 2016). We found *mstnb*, *bmp2a/b*, and *bmp5* are upregulated, while *gdf3*, *gdf6a*, *gdf9*, *bmp6*, and  
105 *ndr1* are downregulated after injury relative to uninjured SC tissues (Fig. S1F). By fluorescence  
106 *in situ* hybridization, *mstnb* expression was induced in dorsal SC tissues between 1 and 2 wpi  
107 and decreased at 3 wpi (Fig. 1E-G). *mstnb* transcripts were not detectable in uninjured SCs (Fig.  
108 1E-G). Co-labeling of *mstnb* transcripts with either ependymal Sox2 or neuronal HuC/D showed  
109 *mstnb* expression restricted to a subset of Sox2<sup>+</sup> ERGs in dorsal SCs (Fig. 1F). *mstnb* transcripts

110 were excluded from neuronal cell bodies (Fig. 1G). These studies revealed *mstnb* expression is  
111 induced in a subset of dorsal ERGs after SCI, and suggested *mstnb* expression correlates with  
112 Tgf- $\beta$  activation in dorsal SC tissues during SC regeneration.

### 113 ***mstnb* is required for functional SC repair.**

114 To examine the role of *mstnb* during SC regeneration, we analyzed the extent of functional and  
115 cellular recovery in genetic zebrafish mutants (*mstnb*<sup>bns5</sup>) (Dogra et al., 2017) (Fig. 2A). *mstnb*  
116 mutants are adult viable, and elicit skeletal and cardiac muscle hyperplasia. Interestingly, the  
117 growth phenotypes associated with *mstnb* mutants are thought to be muscle specific (Dogra et  
118 al., 2017). To establish baseline motor function, we first assessed the swim capacities of wild-  
119 type, *mstnb* heterozygous (*mstnb*<sup>+/-</sup>) and homozygous (*mstnb*<sup>-/-</sup>) siblings in an enclosed swim  
120 tunnel under increasing water current velocities (Klatt Shaw and Mokalled, 2021; Klatt Shaw et  
121 al., 2021; Mokalled et al., 2016) (Fig. 2B). In this swim endurance assay, wild-type animals swam  
122 for 41 min before reaching exhaustion. *mstnb*<sup>+/-</sup> and *mstnb*<sup>-/-</sup> fish showed comparable swim  
123 functions, averaging 43 and 39 min of swim time, respectively. These results indicated *mstnb*  
124 mutants show normal swim capacity and suggested the muscle phenotype of *mstnb* mutants does  
125 not impact swim endurance. Next, we performed SC transections on *mstnb*<sup>-/-</sup> and control siblings  
126 and evaluated their functional regeneration between 2 and 6 wpi. *mstnb*<sup>+/-</sup> fish displayed normal  
127 swim capacity at 2 and 4 wpi, but their functional recovery was slightly compromised at 6 wpi (Fig.  
128 2C). Relative to wild-type controls, functional recovery was 50% reduced in *mstnb*<sup>-/-</sup> fish at 2, 4,  
129 and 6 wpi (Fig. 2C).

130 To further rule out the contribution of skeletal muscle overgrowth to the functional regeneration  
131 output of *mstnb* mutants, we tracked the swim behavior of *mstnb* mutants in the absence of water  
132 current or under a constant, low current velocity of 10 cm/sec (Fig. 2D). We reasoned that, unlike  
133 the endurance test that required fish to swim against increasing current velocities, swim behavior  
134 under minimal current velocity is less likely to be dependent on muscle function. Fish position in  
135 the swim tunnel (Y position), percent activity, and burst frequency were quantified to assess  
136 overall swim competence. In this assay, *mstnb*<sup>-/-</sup> animals stalled in the back quadrant of the swim  
137 tunnel (Fig. 2E), were 65% less active than their wild-type siblings (Fig. 2F), and displayed less  
138 bursts under low current velocity (Fig. 2G). Consistent with a partial regeneration phenotype in  
139 heterozygous fish, *mstnb*<sup>+/-</sup> fish were 40% less active and their burst frequency was reduced by  
140 35% relative to wild types at 2 wpi. Swim parameters were comparable between *mstnb*<sup>+/-</sup> and  
141 wild-type siblings at 4 and 6 wpi, and were not statistically significant at 2 wpi (Fig. 2E-G).

142 To date, cellular growth across the lesion site has served as a primary readout of cellular  
143 regeneration in zebrafish (Goldshmit et al., 2012; Mokalled et al., 2016; Reimer et al., 2013). Glial  
144 bridging and axon tracing assays were performed to evaluate the extents of glial and axonal  
145 regeneration across the lesion site (Fig. 2A). By Gfap immunostaining, glial bridging was  
146 unaffected in *mstnb*<sup>-/-</sup> animals compared to wild-type siblings at 2 wpi (Fig. 2H). At 4 wpi,  
147 anterograde axon tracing using Biocytin showed comparable axon regrowth in proximal and distal  
148 SC sections between *mstnb*<sup>-/-</sup> and control animals (Fig. 2I). Together, these studies indicated  
149 *mstnb* is required for functional SC repair but is dispensable for glial bridging and axonal regrowth  
150 across the lesion. These findings prompted us to investigate mechanisms of SC regeneration that  
151 are independent of glial bridging and axon growth.

### 152 ***mstnb* is a negative regulator of adult neurogenesis after SCI**

153 In addition to glial bridging and axon regrowth, zebrafish regenerate lost motor neurons and  
154 interneurons around the lesion site (Barreiro-Iglesias et al., 2015; Kuscha et al., 2012b; Reimer  
155 et al., 2008). Dorsal ependymal progenitors are thought to give rise to regenerating interneurons  
156 in dorsal SC tissues after injury. Since *mstnb* is expressed in dorsal ERGs after SCI and *mstnb*  
157 mutants did not show glial bridging or axon growth defects, we postulated that *mstnb* plays a role  
158 in adult neurogenesis in zebrafish and that local neurogenesis around the lesion is required for  
159 functional SC repair. To test this hypothesis, we first examined the proliferation rates of Sox2<sup>+</sup>  
160 ERGs and of regenerating HuC/D<sup>+</sup> neurons in *mstnb* mutants. Uninjured and injured *mstnb*<sup>-/-</sup> and  
161 wild-type siblings were subjected to SCI and to a single EdU pulse 24 hours prior to SC collection  
162 and histological analysis (Fig. 3A and S2A). Cell counts revealed a significant increase in HuC/D<sup>+</sup>  
163 EdU<sup>+</sup> neurons in dorsal SCs with 1 wpi SCs showing the most pronounced differences (Fig. 3B,  
164 C and S2B). At 1 wpi, 7.7% of HuC/D<sup>+</sup> neurons were EdU<sup>+</sup> in wild-type SCs (Fig. S2B), and  
165 accounted for 0.9% of dorsal SC cells (Fig. 3C). Conversely, 15.2% of HuC/D<sup>+</sup> neurons were  
166 EdU<sup>+</sup> in *mstnb*<sup>-/-</sup> SCs (Fig. S2B), accounting for 1.5% of dorsal SC cells (Fig. 3C). The rates of  
167 neurogenesis were attenuated in wild-type SCs at 2 and 3 wpi relative to 1 wpi. However, *mstnb*  
168 mutants showed increased neurogenesis at 3 wpi (Fig. 3C and S2B). These differences were  
169 blunted in cell counts from total SCs (Fig. S2C), suggesting neuronal differentiation is specifically  
170 increased in dorsal SC tissues of *mstnb* mutants. On the other hand, we observed a minor, non-  
171 significant decrease in the number of Sox2<sup>+</sup> EdU<sup>+</sup> ERGs in *mstnb*<sup>-/-</sup> SCs at 1 and 3 wpi (Fig. 3D,  
172 E and S2D, E). These findings indicated the rates of neurogenesis are increased in *mstnb*  
173 mutants.

174 To evaluate how snapshots of increased neurogenesis at 1 wpi could impact the numbers of  
175 regenerating neurons in *mstnb* mutants, we performed SC transections on *mstnb*<sup>-/-</sup> and wild-type  
176 fish followed by daily EdU injections for 1 or 2 wpi (Fig. 4A and S3A). In this assay, daily EdU  
177 labeling allowed us to estimate the total numbers of regenerating neurons (HuC/D<sup>+</sup> EdU<sup>+</sup>  
178 neurons), and the extent of self-renewal in ependymal progenitors (Sox2<sup>+</sup> EdU<sup>+</sup> ERGs).  
179 Considering the dynamic rates of neurogenesis along the rostro-caudal axis, we quantified cell  
180 numbers at 150, 450, and 750 μm rostral to the lesion. At 1 wpi and in wild-type sections proximal  
181 to the lesion site (-150 μm), 10.9% of dorsal SC cells were HuC/D<sup>+</sup> neurons (Fig. 3C), and 2.7%  
182 of dorsal SC cells were regenerating neurons (HuC/D<sup>+</sup> EdU<sup>+</sup>) (Fig. 3D). On the other hand, 19%  
183 of dorsal SC cells were HuC/D<sup>+</sup> neurons (Fig. 3C), and 4.3% of dorsal SC cells were regenerating  
184 neurons (HuC/D<sup>+</sup> EdU<sup>+</sup>) in *mstnb*<sup>-/-</sup> SCs (Fig. 3D). Quantifications from total SC tissues confirmed  
185 that the increase in regenerating neurons in *mstnb* mutants is specific to dorsal SCs and blunted  
186 in total SCs (Fig. S3B,C). At 2 wpi, the numbers of HuC/D<sup>+</sup> EdU<sup>+</sup> neurons continued to be elevated  
187 in *mstnb*<sup>-/-</sup> SCs (Fig. 4D). Intriguingly, the overall increase in the total numbers of neurons was  
188 less pronounced at this time point (Fig. 4C), suggesting *mstnb* mutants elicit an early wave of  
189 increased neurogenesis at 1 wpi, but that compensatory mechanisms may be activated at later  
190 time points to counteract the loss of *mstnb*. Finally, despite increased neurogenesis rates in  
191 *mstnb*<sup>-/-</sup> fish, the numbers of Sox2<sup>+</sup> and Sox2<sup>+</sup> EdU<sup>+</sup> ERGs were comparable across genotypes  
192 (Fig. 4F,G), suggesting ERG self-renewal was maintained at normal levels in *mstnb* mutants.  
193 These studies revealed a 2-fold increase in regenerating neurons in *mstnb* mutants, and are  
194 consistent with *mstnb* acting as a negative regulator of adult neurogenesis in zebrafish.

195 Human MSTN proteins are translated as inactive full-length precursors that undergo  
196 proteolytic processing into mature MSTN peptide and MSTN proform (pro-MSTN) peptide. pro-  
197 MSTN exhibits high binding affinity for Myostatin and inhibits its function (Zhu et al., 2000). To  
198 examine whether the global effects of *mstnb* mutants could be reproduced by local Mstnb  
199 inhibition, we injured wild-type animals and performed daily injections of human recombinant  
200 MSTN Proform (pro-MSTN) peptide adjacent to the lesion site (Fig. S4A). We then assessed the  
201 numbers of HuC/D<sup>+</sup> neurons and Sox2<sup>+</sup> ERGs at 1 wpi, corresponding to 6 days after initial  
202 treatment. We found HuC/D<sup>+</sup> neurons were increased by 12% upon pro-MSTN treatment, though  
203 these differences were not significant (Fig. S4B). On the other hand, the numbers of Sox2<sup>+</sup>  
204 progenitors were decreased by 20% in pro-MSTN injected fish relative to vehicle controls (Fig.  
205 S4C). Consistent with genetic loss-of-function of *mstnb*, pharmacological Mstn inhibition at the



206 lesion site disrupted the relative ratios of HuC/D<sup>+</sup> neurons and Sox2<sup>+</sup> ERGs towards increased  
207 neurogenesis.

### 208 ***mstnb* mutants exhibit increased neuronal differentiation after SCI**

209 Our proliferation assays showed increased neurogenesis and suggested the rate of ERG self-  
210 renewal was slightly decreased in the absence of *mstnb*. To dissect the cellular basis for this  
211 phenotype, we evaluated the numbers of neural stem cells (NSCs) and intermediate neural  
212 progenitors (iNPs) in *mstnb* mutants at baseline, 1 and 2 wpi (Fig. 5A). We first combined a *nestin*  
213 reporter transgene (*nes:GFP*) with *mstnb*<sup>-/-</sup> background to quantify NSCs in *nes:GFP;mstnb*<sup>-/-</sup> fish  
214 (Lam et al., 2009) (Fig. 5B-D and S5A-C). *nes:GFP*<sup>+</sup> NSCs were rarely identified in uninjured SC  
215 sections from either mutant or wild-type animals, but were readily detectable after SCI (Fig. 5C  
216 and S5A). The proportions of *nes:GFP*<sup>+</sup> cells in dorsal or total SC tissues were comparable  
217 between *mstnb*<sup>-/-</sup> and wild-type siblings at either 1 or 2 wpi (Fig. 5C and S5A). At these time points,  
218 NSC proliferation showed decreasing trends in *mstnb* mutants (Fig. 5D), and was statistically  
219 significant when the numbers of *nes:GFP*<sup>+</sup> PCNA<sup>+</sup> cells were normalized to *nes:GFP*<sup>+</sup> NSCs (Fig.  
220 S5C). NSC proliferation was less pronounced in quantifications from total SC sections (Fig. S5B).  
221 These findings revealed NSC proliferation is reduced in *mstnb* mutants at 1 wpi.

222 The number of *nes:GFP*<sup>+</sup> NSCs were unaltered in *mstnb* mutants despite their decreased  
223 proliferation rate. We postulated that *mstnb* loss-of-function may bias NSC fate towards neuronal  
224 differentiation, and that compensatory mechanisms upstream of NSC activation maintain their  
225 total numbers across genotypes. To test this hypothesis, we examined the number of iNPs using  
226 *dbx1b:GFP* transgene bred into a *mstnb*<sup>-/-</sup> background (Pierani et al., 2001; Satou et al., 2012)  
227 (Fig. 5E-G and S5D,E). The proportion of *dbx1b:GFP*<sup>+</sup> iNPs averaged between 2.9 and 4.3 % of  
228 dorsal SC cells across time points, and showed an elevated trend in *mstnb*<sup>-/-</sup> relative to wild-type  
229 siblings at 2 wpi (Fig. 5F and S5D). These phenotypes were more pronounced in quantifications  
230 of *dbx1b:GFP*<sup>+</sup> PCNA<sup>+</sup> iNPs. Relative to wild-type siblings, *mstnb*<sup>-/-</sup> iNP proliferation was minimal  
231 prior to injury, increased by 2.3-fold at 1 wpi, and was normalized to wild-type levels by 2 wpi  
232 (Fig. 5G and S5E). These findings are consistent with accelerated neurogenesis in *mstnb*  
233 mutants.

234 To examine the differentiation and relative distribution of iNP-derived neurons after SCI, we  
235 labeled glycinergic neurons in *mstnb* mutants using *slc6a5:GFP* reporter line (Fig. 5H-J and S5F)  
236 (McLean et al., 2007). At 1 wpi, the proportions of glycinergic neurons comprised 3.9% and 3.4%

237 of dorsal SC cells in *mstnb*<sup>-/-</sup> and wild-type fish, respectively (Fig. 5I and S5F). By 2 wpi, glycinergic  
238 neurons accounted for 1.2% of dorsal SC cells in wild-type controls, but increased to 2.7% of  
239 dorsal cells in *mstnb*<sup>-/-</sup> SCs (Fig. 5I and S5F). By quantifying the proportions of glycinergic neurons  
240 within HuC/D<sup>+</sup> neurons, glycinergic neurons accounted for 6.1% and 3.7% of neurons in wild-type  
241 SC tissues at 1 and 2 wpi, respectively (Fig. 5J). These proportions were 2-fold elevated in *mstnb*  
242 mutants, accounting for 12% of neurons at 1 wpi and 8.5% of neurons at 2 wpi (Fig. 5J). Together,  
243 these results indicated *mstnb* mutants exhibit an expansion in glycinergic neurons, which are  
244 overrepresented relative to other neuronal cell populations.

### 245 **Neuronal genes are upregulated in *mstnb* mutants**

246 To determine the molecular mechanisms by which *mstnb* regulates the rates of neurogenesis, we  
247 deep sequenced SC tissues from *mstnb*<sup>-/-</sup> and wild-type siblings at 1 wpi, as well as uninjured  
248 controls (Fig. 6A-C). Principle component analysis confirmed clustering of biological replicates,  
249 and highlighted four distinct molecular signatures that are both injury- and genotype-induced (Fig.  
250 6A). At 1 wpi, 61 genes were downregulated and 359 genes were upregulated in *mstnb*<sup>-/-</sup> SCs,  
251 suggesting *mstnb* may be a negative regulator of gene expression after SCI (Fig. 6C). Genes  
252 upregulated in *mstnb*<sup>-/-</sup> SCs comprised several neuronal or neuron differentiation genes, including  
253 *birc5b*, *eloal*, *elavl2*, *htr2aa*, and *pou5f3*, which were either unchanged or downregulated in  
254 uninjured *mstnb*<sup>-/-</sup> SCs (Fig. 6B-D). These findings indicated neuronal gene expression changes  
255 in *mstnb* mutants are injury-dependent.

256 Fibroblast growth factor (Fgf) maintains the proliferation and self-renewal capacities of neural  
257 stem cells in mammals (Hsu et al., 2009). By RNA-seq and qRT-PCR, *fgf1b* was downregulated  
258 in *mstnb*<sup>-/-</sup> SCs at 1wpi, but was unchanged in uninjured SC tissues (Fig. 6E). The dysregulation  
259 of *fgf1b* in *mstnb* mutants suggested *mstnb*-mediated *fgf1b* expression inhibits neurogenesis by  
260 promoting progenitor cell proliferation and self-renewal. To test this hypothesis, we examined  
261 whether the neuronal gene expression changes observed in *mstnb* mutants could be rescued by  
262 localized delivery of human recombinant FGF1 into SC lesions. We injured *mstnb*<sup>-/-</sup> and wild-type  
263 siblings and applied FGF1 proteins adjacent to the lesion site at 5 dpi using a gelfoam sponge.  
264 Gene expression changes were assessed by qRT-PCR at 1 wpi, corresponding to 2 days after  
265 treatment (Fig. 6F). Consistent with increased neurogenesis in *mstnb* mutants, *birc5b*, *eloal*, and  
266 *pou5f3* transcript levels were increased in vehicle-treated *mstnb*<sup>-/-</sup> relative to vehicle-treated wild-  
267 types (Fig. 6G). Application of exogenous FGF1 proteins at the lesion rescued the upregulation

268 of neuronal genes in *mstnb*<sup>-/-</sup> SCs (Fig. 6G). These findings indicated Mstn-mediated Fgf signaling  
269 is a negative regulator of adult neurogenesis after zebrafish SCI.

## DISCUSSION

270 This study shows *mstnb* expression is induced in a subset of dorsal ERGs after SCI. Our results  
271 are consistent with a model in which *mstnb* regulates the rates of self-renewal and neuronal  
272 differentiation after SCI, and suggest *mstnb*-dependent Fgf signaling promotes self-renewal at the  
273 expense of neurogenesis (Fig. 6H).

274 Successful SC regeneration requires faithful recovery of the excitatory and inhibitory (E/I)  
275 balance in regenerating neural circuits. SCI alters the amount, strength, and relative locations of  
276 E/I inputs by disrupting descending hindbrain connections and promoting waves of axonal  
277 degeneration, neuronal death, and demyelination. Interneurons and motor neurons regenerate  
278 after zebrafish SCI. Notably, dopamine and serotonin signals from regenerating tracts control  
279 motor neuron regeneration by promoting the proliferation of pMN ERGs (Barreiro-Iglesias et al.,  
280 2015; Reimer et al., 2013). We found *mstnb* mutants display increased neuronal differentiation at  
281 1 wpi, with an overrepresentation in glycinergic interneurons among regenerating neurons.  
282 Glycinergic inhibition plays important roles in coordinating locomotor rhythms in different  
283 organisms (Hinckley et al., 2005; Jovanović et al., 1999; Sibilla and Ballerini, 2009). We propose  
284 that increased inhibitory neurotransmission disrupts E/I balance and may underlie the behavioral  
285 recovery defects observed in *mstnb* mutants. The mechanisms that underlie E/I balance  
286 disruption in *mstnb* mutants require further investigation into the time course of neuronal  
287 regeneration and the contribution of *mstnb*<sup>+</sup> ERGs to specific neuronal populations.

288 Our study highlights a niche of dorsal ependymal progenitors that express *mstnb* after SCI.  
289 Unlike tissues that undergo constant cell renewal such as skin or blood, the nervous system  
290 undergoes little turnover and does not harbor a constitutively active neurogenic niche. Instead,  
291 neural progenitors are quiescent and are only activated upon physiological or pathological  
292 stimulation. Lineage restricted ependymal progenitors emerge after zebrafish SCI. ERG niches  
293 include a ventro-lateral domain that gives rise to regenerating motor neurons, and a ventral  
294 domain that undergoes epithelial-to-mesenchymal transition and is required for glial bridging after  
295 SCI (Klatt Shaw et al., 2021; Reimer et al., 2008). Our findings support the emergence of a lineage  
296 restricted, neurogenic niche of dorsal ERGs during SC regeneration in zebrafish. Consistent with  
297 this model, the numbers of motor neurons and the extent of glial bridging across the lesion, which

298 have been respectively associated with ventro-lateral and ventral ERGs, are unaffected in the  
299 absence of *mstnb*. Instead, *mstnb* mutants showed specific neurogenesis defects in dorsal SCs,  
300 and a preferential increase in dorsal glycinergic neurons. The molecular identity and cellular  
301 contributions of *mstnb*<sup>+</sup> ERGs to neurogenesis and SC repair warrant further investigation.

302 Niches of progenitor cells require intricate regulatory mechanisms to balance the rates of self-  
303 renewal, differentiation, and quiescence (Li and Clevers, 2010). At the cellular level, the  
304 organization of progenitor cells into localized niches maintains quiescence under homeostatic  
305 conditions, and triggers progenitor cell activation following niche disruption (Bagheri-Mohammadi,  
306 2021). Molecularly, progenitor cell niches are hubs for Bmp, Wnt, and Notch signaling pathways,  
307 which control the rates of self-renewal, differentiation, and quiescence. Our study supports a  
308 model in which Mstn restricts neuronal differentiation and maintains neuronal progenitors in a  
309 proliferative, undifferentiated cell fate. Our findings are consistent with previously reported  
310 functions for Mstn in muscle, fat, and bone tissues (Dogra et al., 2017; Langley et al., 2002; Le  
311 and Yao, 2017; Lim et al., 2018; McCroskery et al., 2003; Wallner et al., 2017). Notably, previous  
312 findings have shown that Mstn is a regeneration limiting gene for zebrafish heart or fin  
313 regeneration, which are both dedifferentiation-based repair mechanisms (Dogra et al., 2017;  
314 Magga et al., 2019; Uribe et al., 2018). In contrast, in the context of SC regeneration, we find that  
315 Myostatin promotes regeneration by supporting regenerative FGF signaling, revealing a new role  
316 for Myostatin in this stem cell-based regeneration paradigm. Together these findings underline  
317 how tissue regeneration programs can coopt similar signaling pathways to achieve highly specific  
318 regenerative outcomes, and indicate a tissue-specific mechanism for Myostatin signaling.

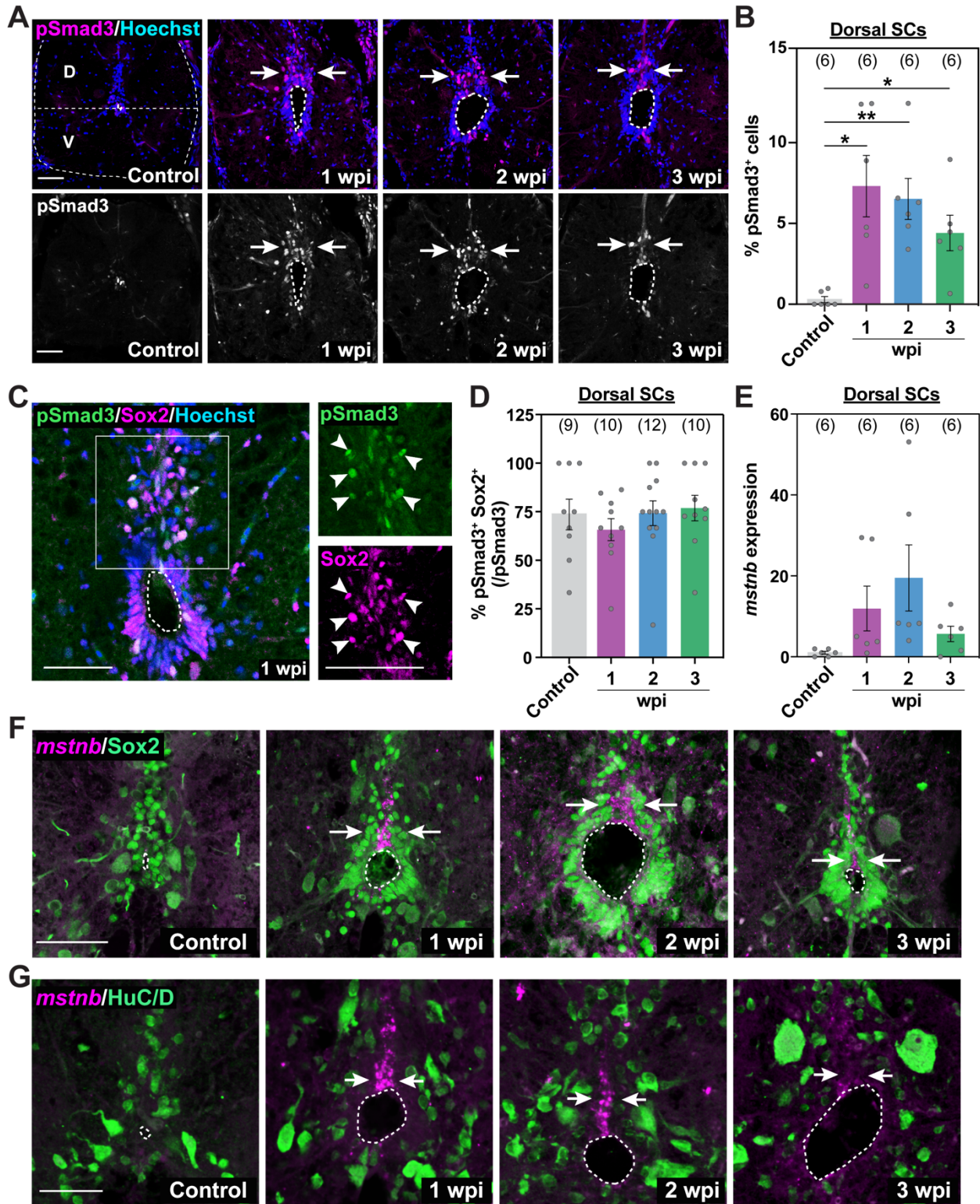
319 We propose that Fgf is a mediator of Mstn functions during SC regeneration, and that Mstn  
320 limits neuronal differentiation by promoting Fgf-dependent self-renewal in *mstnb*<sup>+</sup> ERGs. Similar  
321 regulatory mechanisms have been shown in muscle tissues, where Mstn inhibits the muscle  
322 differentiation transcription factors MyoD and Myogenin. Fgf signaling promotes NSC proliferation  
323 and self-renewal (Hsu et al., 2009). In mammals, isolated ependymal cells have the capacity to  
324 form neurospheres and produce neurons, astrocytes and oligodendrocytes *in vitro* (Meletis et al.,  
325 2008). However, although mammalian SCI induces the proliferation of ependymal cells lining the  
326 central canal (Horner et al., 2000), mammalian ependymal cells are incapable of forming neurons  
327 *in vivo* (Barnabe-Heider et al., 2010; Muthusamy et al., 2018; Ren et al., 2017; Shah et al., 2018).  
328 We propose that comparative studies between zebrafish ERGs and mammalian ependymal cells

329 could reveal new insights into their differential regenerative capacities and examine whether Mstn  
330 signaling is differentially regulated between zebrafish and mammals.

## **ACKNOWLEDGMENTS**

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334 from the NIH (R01 NS113915 to M.H.M.) and the McDonnell Center for Cellular Neuroscience (to  
335 M.H.M.).

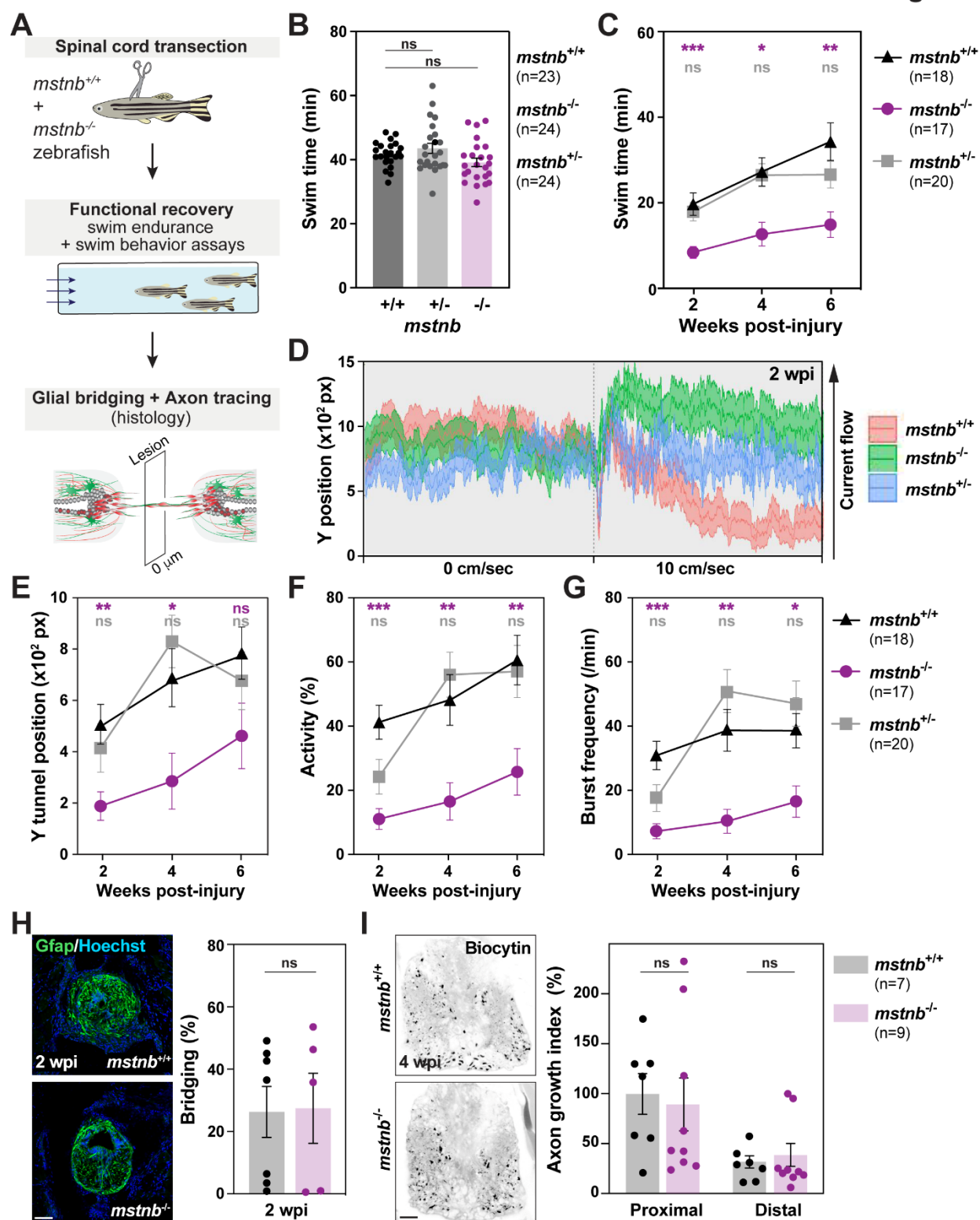
Figure 1



336 **Figure 1. *mstnb* is induced in dorsal ependymal progenitors during SC regeneration. (A)**  
337 Immunostaining for phosphorylated Smad3 (pSmad3) after SCI. Wild-type SC sections at 1, 2,  
338 and 3 wpi, and uninjured controls are shown. Cross sections 450 $\mu$ m from the lesion site are  
339 shown. Horizontal dotted line demarcates dorsal (D) and ventral (V) SC domains. Arrows point to  
340 pSmad3<sup>+</sup> nuclei in the dorsal domain. **(B)** pSmad3 quantification in dorsal sections of wild-type  
341 SCs. Percent pSmad3<sup>+</sup> cells was normalized to the number of nuclei in dorsal SCs. **(C)** pSmad3  
342 and Sox2 immunostaining in wild-type SCs at 1 wpi. High-magnification views of dorsal SCs are  
343 shown. Arrowheads point to pSmad3<sup>+</sup> Sox2<sup>+</sup> ERGs. **(D)** pSmad3 quantification in dorsal ERGs.  
344 Percent pSmad3<sup>+</sup> Sox2<sup>+</sup> cells was normalized to the number of pSmad3<sup>+</sup> cells. **(E)** Quantification  
345 of *mstnb* by *in situ* hybridization in dorsal SC tissues. **(F,G)** *mstnb* expression in wild-type SC  
346 sections after SCI. *mstnb* fluorescence *in situ* hybridization was followed by immunostaining for  
347 either Sox2 (F) or HuC/D (G) antibodies. Cross sections 450 $\mu$ m from the lesion site are shown at  
348 1, 2, and 3 wpi, and for uninjured controls. Arrows point to domains of *mstnb* expression in dorsal  
349 SCs. Dotted ovals delineate central canal edges. For all quantification, SC sections 450  $\mu$ m rostral  
350 to the lesion were analyzed and sample sizes are indicated in parentheses. \*P<0.05; \*\*P<0.01.  
351 Scale bars, 50  $\mu$ m.

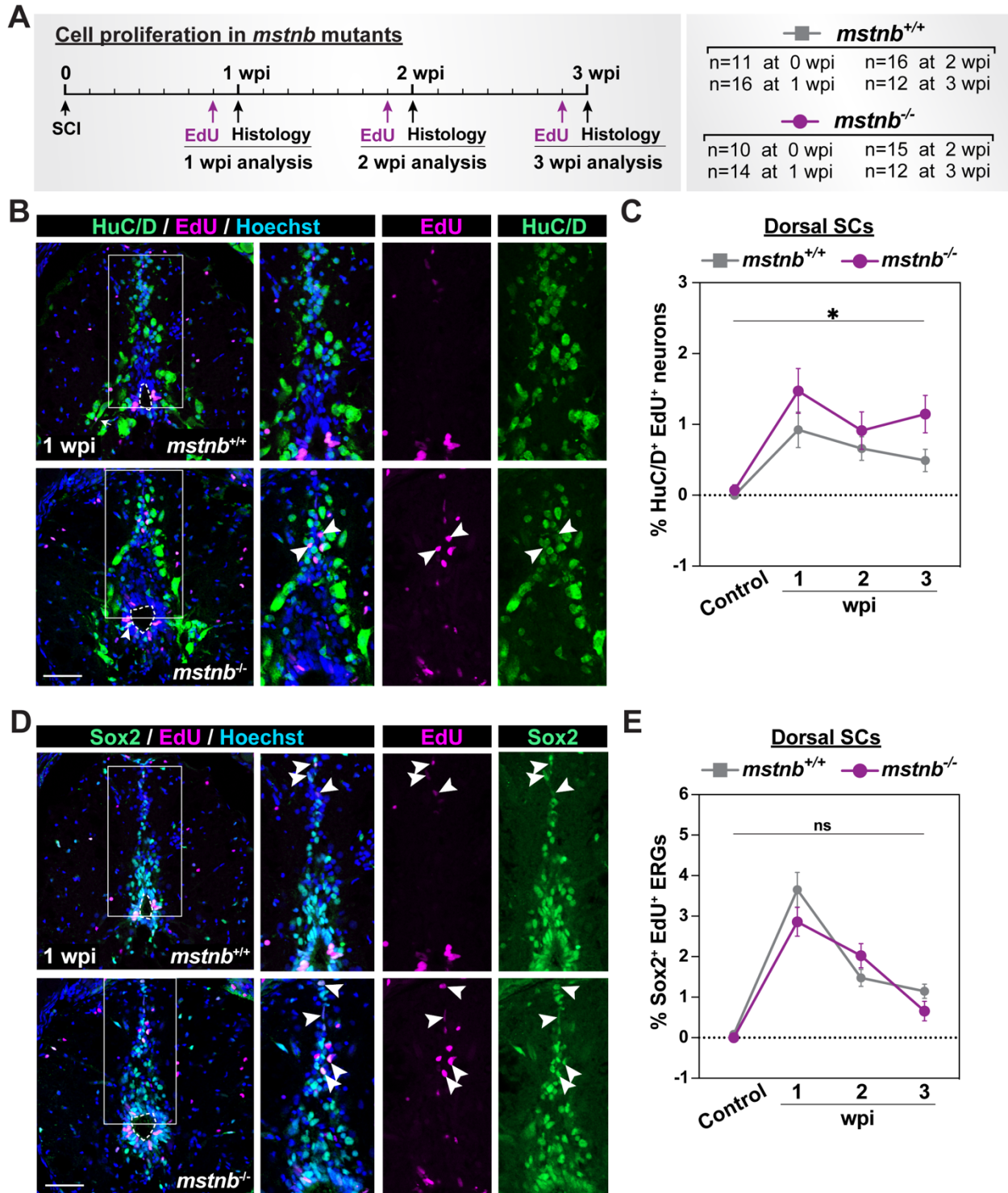


Figure 2



353 **Figure 2. *mstnb* is required for functional SC regeneration. (A)** Experimental pipeline to  
354 examine regeneration phenotypes after SCI. *mstnb*<sup>-/-</sup> fish and wild-type siblings were subjected  
355 to complete SC transection. Functional recovery was assessed between 2 and 6 wpi. Histology  
356 was used to assess glial and axonal bridging at 2 and 4 wpi, respectively. **(B)** Endurance swim  
357 assays determined baseline motor function for *mstnb*<sup>+/-</sup>, *mstnb*<sup>-/-</sup>, and wild-type fish. Dots  
358 represent individual animals from three independent clutches. **(C)** Endurance swim assays for  
359 *mstnb*<sup>+/-</sup>, *mstnb*<sup>-/-</sup>, and wild-type fish at 2, 4, and 6 wpi. Dots represent individual animals from two  
360 independent experiments. Statistical analyses of swim times are shown for *mstnb*<sup>+/-</sup> (grey) and  
361 *mstnb*<sup>-/-</sup> (magenta) relative to wild types. Recovery of *mstnb*<sup>-/-</sup> animals was not significant between  
362 2 and 6 wpi. **(D)** Tracking swim performance at minimal water current velocity for *mstnb*<sup>+/-</sup> (blue),  
363 *mstnb*<sup>-/-</sup> (green), and wild-type siblings (red). Average Y position is shown for each cohort at 2 wpi.  
364 Animals were tracked in the absence of current (0 cm/sec) for 5 min, and for a 10cm/sec current  
365 velocity for 5 min. The arrow shows the direction of the water current. **(E-G)** Average Y position  
366 in the tunnel (E), percent activity (F), and burst frequency (G) were quantified at 10 cm/sec water  
367 current velocity. *mstnb*<sup>+/-</sup>, *mstnb*<sup>-/-</sup>, and wild-type fish are shown at 2, 4, and 6 wpi. Statistical  
368 analyses of swim times are shown for *mstnb*<sup>+/-</sup> (grey) and *mstnb*<sup>-/-</sup> (magenta) relative to wild types.  
369 Two independent experiments are shown. **(H)** Glial bridging in *mstnb*<sup>-/-</sup> (magenta) and wild-type  
370 siblings (grey) at 2 wpi. Representative immunohistochemistry shows the Gfap<sup>+</sup> bridge at the  
371 lesion site. Percent bridging represents the cross-sectional area of the glial bridge at the lesion  
372 site relative to the the intact SC. Percent bridging was quantified for 7-9 animals per group. **(I)**  
373 Anterograde axon tracing in in *mstnb*<sup>-/-</sup> (magenta) and wild-type zebrafish (grey) at 4 wpi. Biocytin  
374 axon tracer was applied rostrally and analyzed at 100 μm (proximal) and 500 μm (distal) caudal  
375 to the lesion. Representative traces of biocytin are shown for each genotype animals at the  
376 proximal level. Quantification represents 7-9 animals per group. Axon growth was normalized to  
377 Biocytin labeling in wild-types at the proximal level. \*P<0.05; \*\*P<0.01; \*\*\*p<0.001; ns, not  
378 significant. Scale bars, 50 μm.

Figure 3

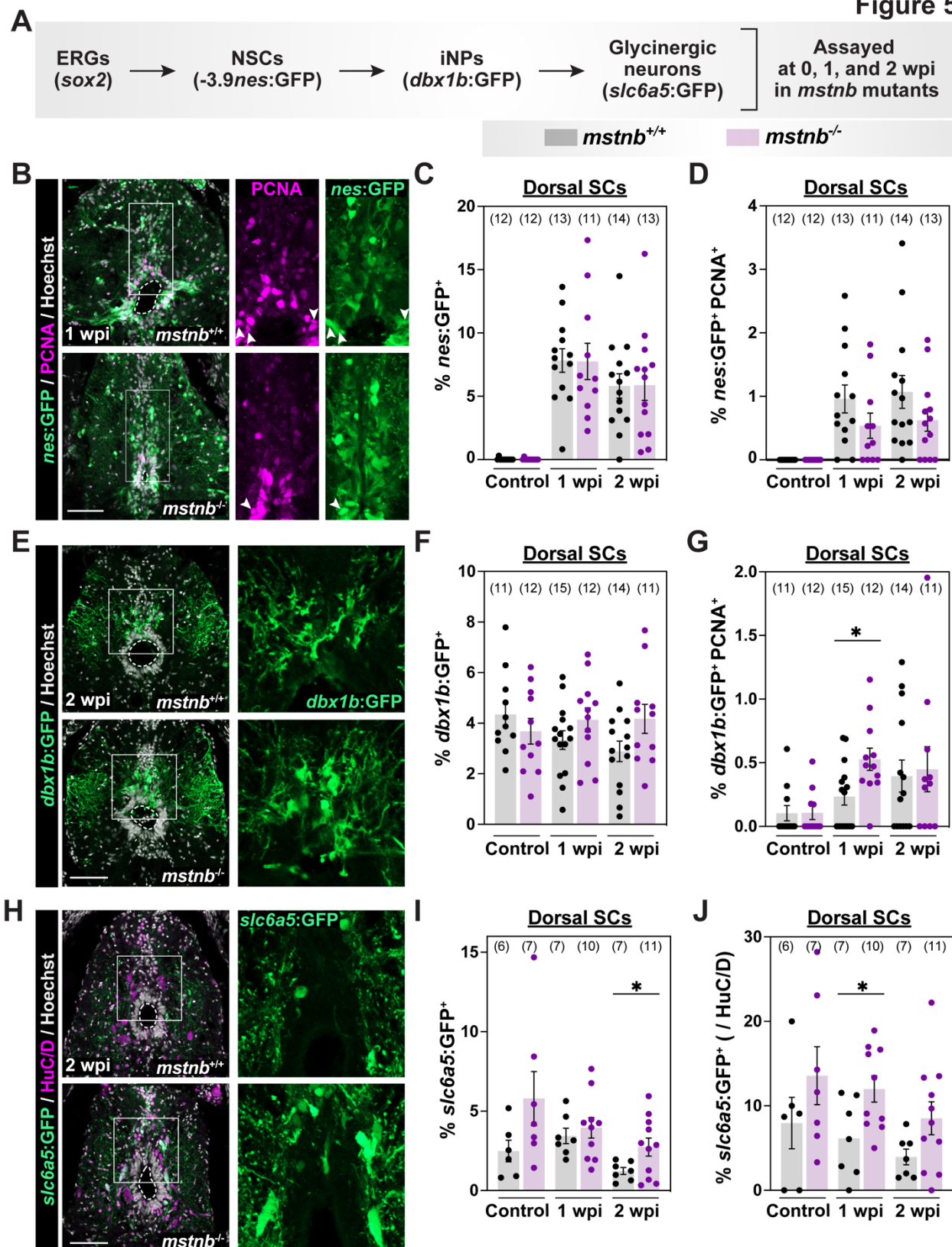


379 **Figure 3. Cell proliferation in *mstnb* mutant zebrafish. (A)** Experimental timeline to assess the  
380 rates of cell proliferation. *mstnb*<sup>-/-</sup> and wild-type siblings were subjected to SC transections. A  
381 single EdU injection was performed at either 6, 13, or 20 days post-injury. SC tissues were  
382 harvested for analysis at 1, 2, 3 wpi and at 24 hours after EdU injection. Animal numbers are  
383 indicated for each genotypes and two independent replicates are shown. **(B)**  
384 Immunohistochemistry for EdU and HuC/D in SC sections of *mstnb*<sup>+/+</sup> and *mstnb*<sup>-/-</sup> at 1 wpi. The  
385 region inside the rectangular box is shown in higher magnification. Arrowheads indicate HuC/D<sup>+</sup>  
386 EdU<sup>+</sup> neurons. **(C)** Regenerated HuC/D<sup>+</sup> EdU<sup>+</sup> neurons were quantified in dorsal SC sections at  
387 1, 2, 3 wpi and uninjured controls. Percent HuC/D<sup>+</sup> EdU<sup>+</sup> neurons was normalized to the total  
388 number of nuclei for each section. **(D)** Immunohistochemistry for EdU and Sox2 in SC sections of  
389 *mstnb*<sup>+/+</sup> and *mstnb*<sup>-/-</sup> at 1 wpi. The region inside the rectangular box is shown in higher  
390 magnification. Arrowheads indicate Sox2<sup>+</sup> EdU<sup>+</sup> ERGs. **(E)** Sox2<sup>+</sup> EdU<sup>+</sup> ERGs were quantified in  
391 dorsal SC sections at 1, 2, 3 wpi and uninjured controls. Percent Sox2<sup>+</sup> EdU<sup>+</sup> ERGs was  
392 normalized to the total number of nuclei for each section. For all quantifications, cross SC sections  
393 450 μm rostral to the lesion site were quantified. \*P<0.05; ns, not significant. Scale bars, 50 μm.  
394 Dotted ovals delineate central canal edges.



395 **Figure 4. Regenerative neurogenesis in *mstnb* mutant zebrafish. (A)** Experimental timeline  
396 to assess the rates of neurogenesis and ERG self-renewal. *mstnb*<sup>-/-</sup> and wild-type siblings were  
397 subjected to SC transections and daily EdU injections. SC tissues were harvested for analysis at  
398 1 or 2 wpi. Animal numbers are indicated for each genotypes and two independent replicates are  
399 shown. **(B)** Immunohistochemistry for EdU and HuC/D in SC sections of *mstnb*<sup>+/+</sup> and *mstnb*<sup>-/-</sup> at  
400 2 wpi. The region inside the rectangular box is shown in higher magnification. Arrowheads indicate  
401 HuC/D<sup>+</sup> EdU<sup>+</sup> neurons. **(C)** HuC/D<sup>+</sup> neurons were quantified in dorsal SC sections at 1 and 2 wpi.  
402 Percent HuC/D<sup>+</sup> neurons was normalized to the total number of nuclei for each section. **(D)**  
403 Regenerated HuC/D<sup>+</sup> EdU<sup>+</sup> neurons were quantified in dorsal SC sections at 1 and 2 wpi. Percent  
404 HuC/D<sup>+</sup> EdU<sup>+</sup> neurons was normalized to the total number of nuclei for each section. **(E)**  
405 Immunohistochemistry for EdU and Sox2 in SC sections of *mstnb*<sup>+/+</sup> and *mstnb*<sup>-/-</sup> at 2 wpi. The  
406 region inside the rectangular box is shown in higher magnification. Arrowheads indicate Sox2<sup>+</sup>  
407 EdU<sup>+</sup> ERGs. **(F)** Sox2<sup>+</sup> ERGs were quantified in dorsal SC sections at 1 and 2 wpi. Percent Sox2<sup>+</sup>  
408 ERGs was normalized to the total number of nuclei for each section. **(G)** Sox2<sup>+</sup> EdU<sup>+</sup> ERGs were  
409 quantified in dorsal SC sections at 1 and 2 w. Percent Sox2<sup>+</sup> EdU<sup>+</sup> ERGs was normalized to the  
410 total number of nuclei for each section. For all quantifications, cross SC sections at 150, 450, and  
411 750 μm rostral to the lesion site were quantified. \*\*P<0.01; ns, not significant. Scale bars, 50 μm.  
412 Dotted lines delineate central canal edges.

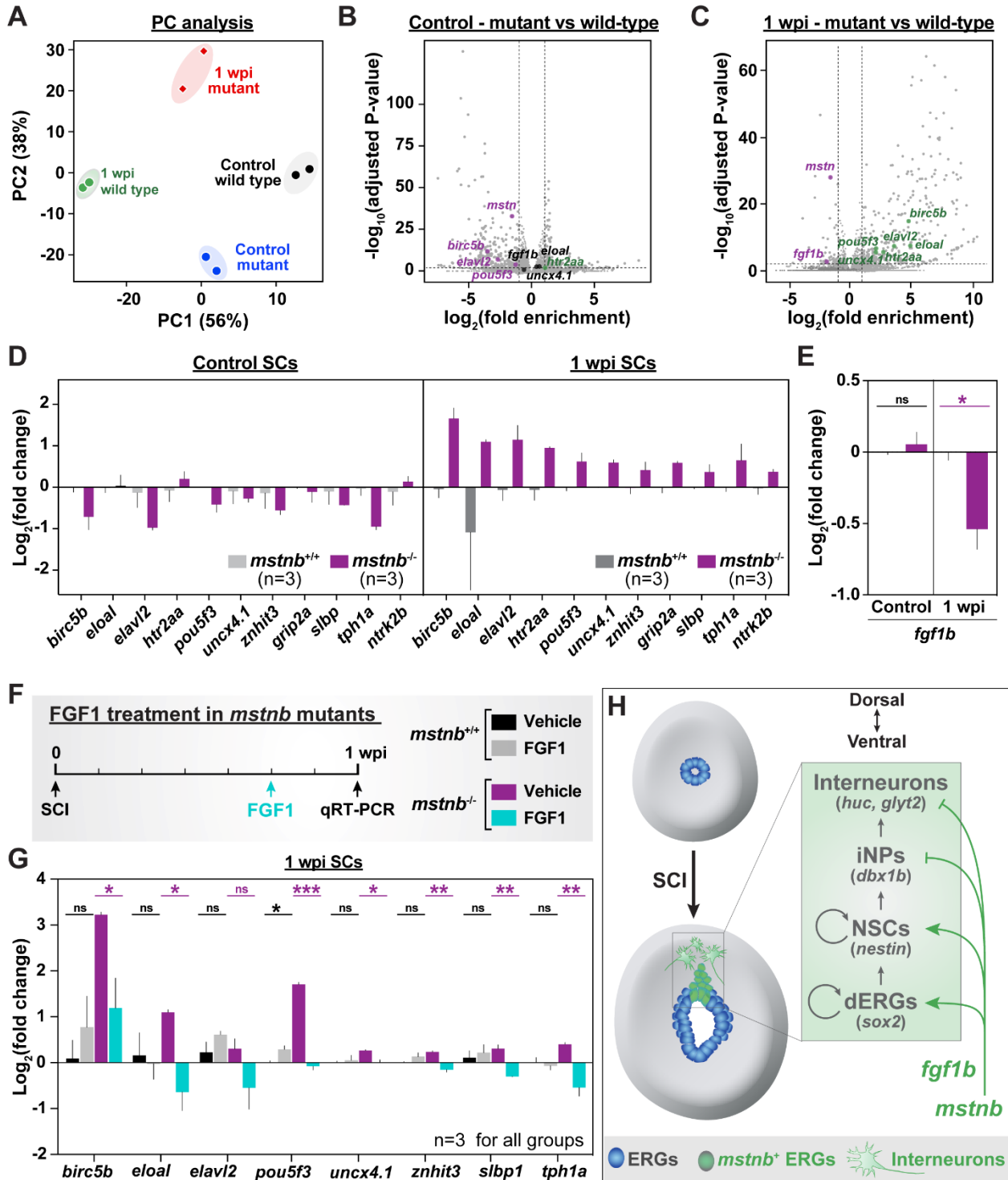
Figure 5



413 **Figure 5. Assessment of neuronal progenitors and neurons in *mstnb* mutant zebrafish. (A)**  
414 Experimental timeline to elucidate the dynamics of neurogenesis. *mstnb*<sup>-/-</sup> animals were crossed  
415 into either *-3.9nestin:GFP*, *dbx1b:GFP*, or *slc6a5:GFP* to evaluate the numbers of neural stem  
416 cells (NSCs), intermediate neural progenitors (iNPs), and glycinergic neurons, respectively.  
417 *mstnb*<sup>-/-</sup> and wild-type siblings were subjected to SC transections and collected at 1 or 2 wpi for  
418 analysis. Uninjured controls were used. Animal numbers are indicated for each genotypes and  
419 two independent replicates are shown. **(B)** GFP and PCNA staining in *-3.9nestin:GFP;mstnb*<sup>-/-</sup> SC  
420 sections at 1 wpi. *-3.9nestin:GFP;mstnb*<sup>+/+</sup> siblings are used as controls. The region inside the  
421 rectangular box is shown in higher magnification. **(C)** *nes*<sup>+</sup> NSCs were quantified in dorsal SC  
422 sections. Percent *nes*<sup>+</sup> NSCs was normalized to the total number of nuclei for each section. **(D)**  
423 *nes*<sup>+</sup> PCNA<sup>+</sup> NSCs were quantified in dorsal SC sections. Percent *nes*<sup>+</sup> PCNA<sup>+</sup> NSCs was  
424 normalized to the total number of nuclei for each section. **(E)** GFP staining in *dbx1b:GFP;mstnb*<sup>-/-</sup>  
425 SC sections at 2 wpi. *dbx1b:GFP;mstnb*<sup>+/+</sup> siblings are used as controls. The region inside the  
426 rectangular box is shown in higher magnification. **(F)** *dbx1b*<sup>+</sup> iNPs were quantified in dorsal SC  
427 sections. Percent *dbx1b*<sup>+</sup> iNPs was normalized to the total number of nuclei for each section. **(G)**  
428 *dbx1b*<sup>+</sup> PCNA<sup>+</sup> NSCs were quantified in dorsal SC sections. Percent *dbx1b*<sup>+</sup> PCNA<sup>+</sup> iNPs was  
429 normalized to the total number of nuclei for each section. **(H)** GFP staining in  
430 *slc6a5:GFP:GFP;mstnb*<sup>-/-</sup> SC sections at 2 wpi. *slc6a5:GFP:GFP;mstnb*<sup>+/+</sup> siblings are used as  
431 controls. The region inside the rectangular box is shown in higher magnification. **(I)** *slc6a5:GFP*<sup>+</sup>  
432 glycinergic neurons were quantified in dorsal SC sections. Percent *slc6a5:GFP*<sup>+</sup> neurons was  
433 normalized to the total number of nuclei for each section. **(J)** Percent *slc6a5:GFP*<sup>+</sup> neurons was  
434 normalized to the numbers of HuC/D<sup>+</sup> neurons for each section. Dotted ovals delineate central  
435 canal edges. For all quantifications, cross SC sections 450 μm rostral to the lesion site were  
436 quantified. \*P<0.05; ns, not significant. Scale bars, 50 μm.

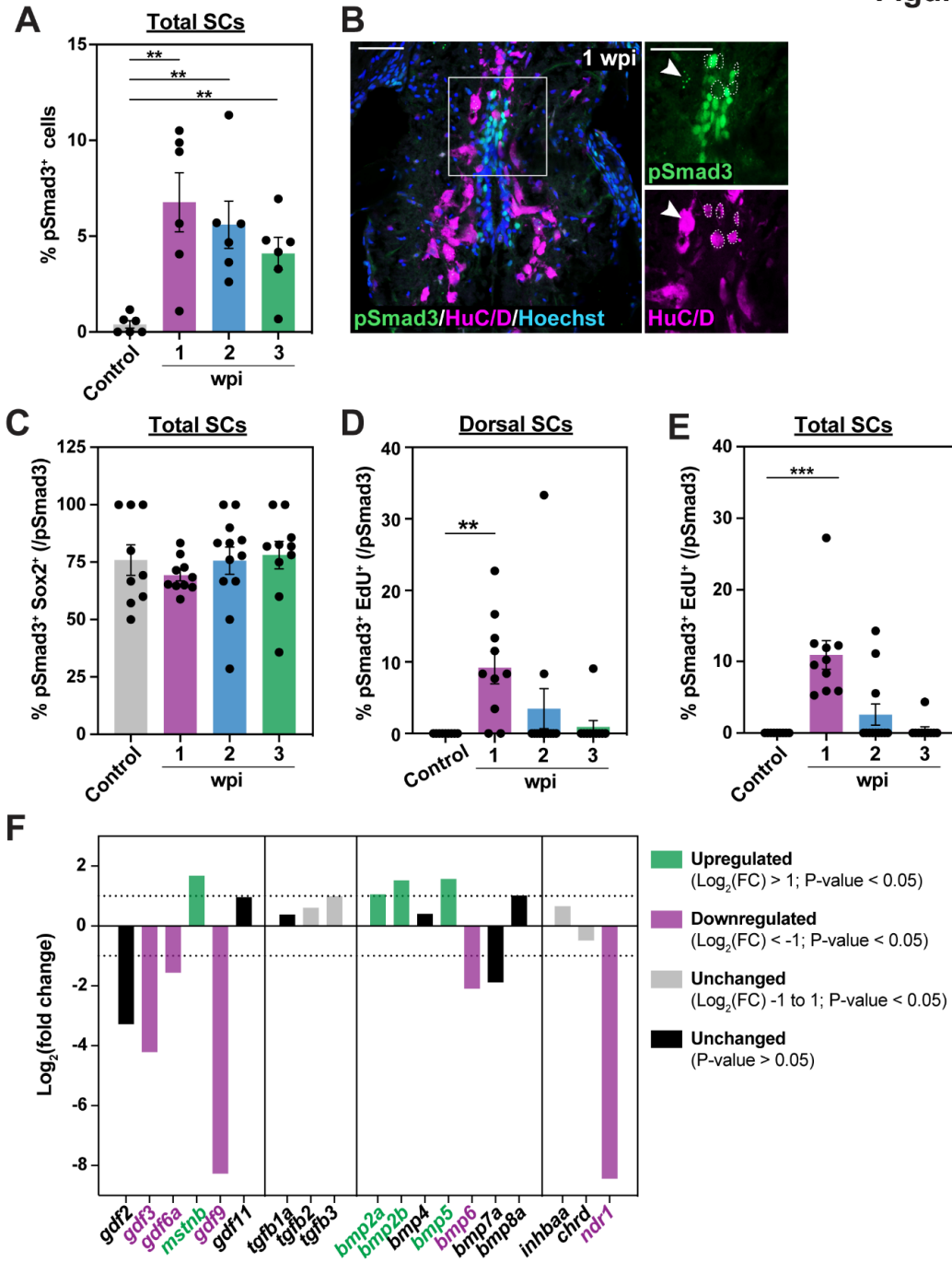


Figure 6



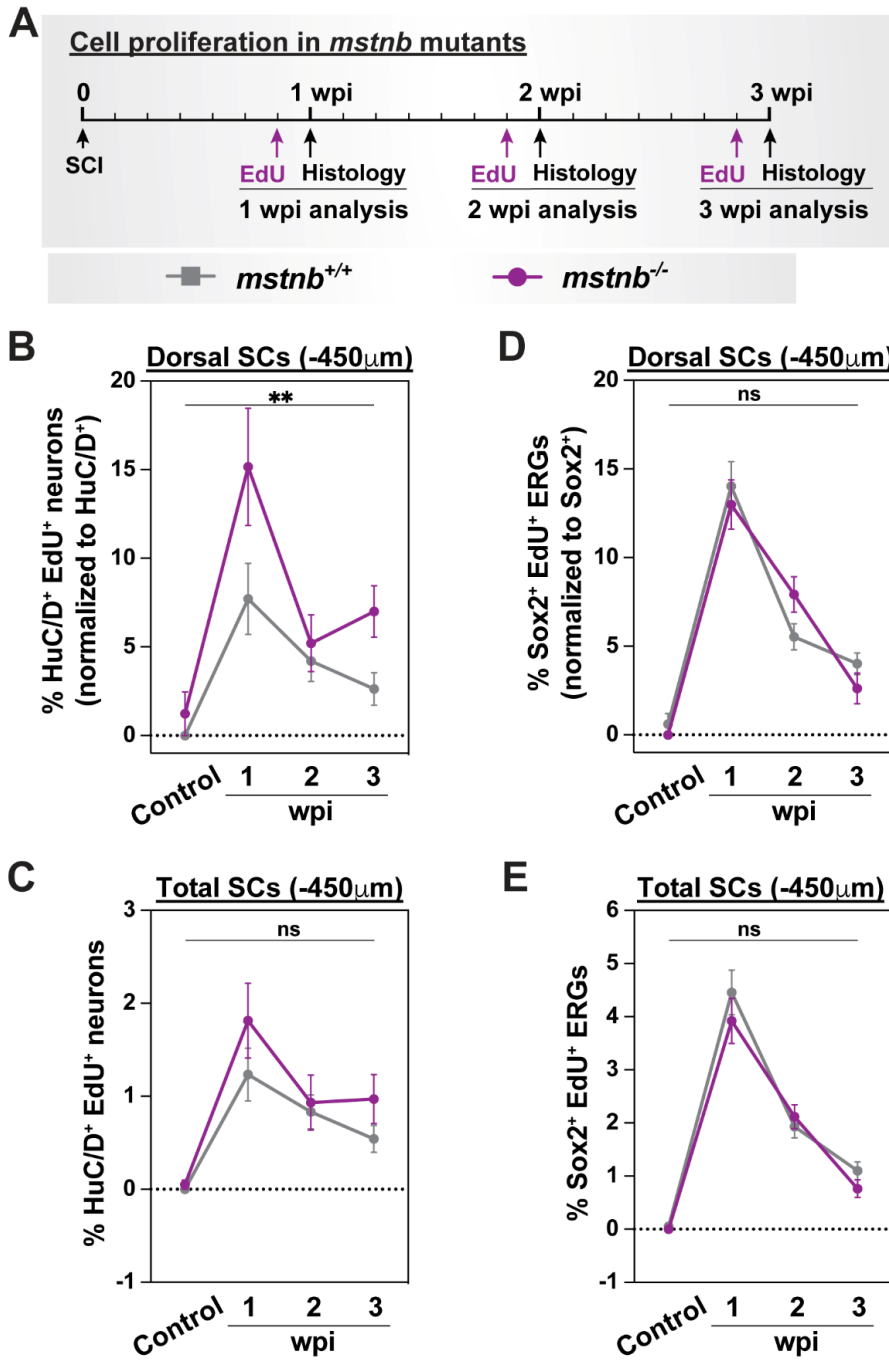
437 **Figure 6. *mstnb* regulates neuronal gene expression via *fgf1b*.** (A) *mstnb*<sup>-/-</sup> and wild-type  
438 siblings were subjected to complete SC transections and collected at 1 wpi for RNA sequencing.  
439 Control SC tissues were collected from uninjured fish. Sequencing was performed in independent  
440 duplicates. Principle component analysis shows clustering of biological replicates. Principle  
441 components 1 and 2 (PC1 and PC 2) show 56% and 38% variance, respectively. (B,C) Volcano  
442 plot representation of genes that are significantly upregulated or downregulated or depleted in  
443 *mstnb*<sup>-/-</sup> SCs relative to wild-type controls. Upregulated genes included genes with log<sub>2</sub> (fold  
444 enrichment) > 1 and adjusted P-value <0.01. Downregulated genes included genes with log<sub>2</sub> (fold  
445 enrichment) < -1 and adjusted P-value <0.01 are considered downregulated. Select upregulated  
446 (green) and downregulated (magenta) neuronal genes are indicated. Unchanged genes are  
447 labelled in black. (D) qRT-PCR for neuronal genes was performed on *mstnb*<sup>-/-</sup> and wild-type SCs  
448 at 1 wpi. Uninjured *mstnb*<sup>-/-</sup> and wild-type controls were used. For each time point, log<sub>2</sub> (fold  
449 change) was normalized to *eif1a* and to gene expression levels in *mstnb*<sup>+/+</sup> controls. (E) *fgf1b*  
450 qRT-PCR was performed on uninjured and injured *mstnb*<sup>-/-</sup> and wild-type animals. For each time  
451 point, *fgf1b* expression was normalized to *eif1a* as a loading control and to *fgf1b* levels in *mstnb*<sup>+/+</sup>  
452 controls. (F) *mstnb*<sup>-/-</sup> and wild-type siblings were subjected to SC transections and treated with  
453 gelfoam-soaked human recombinant FGF1 at 5 dpi. *mstnb*<sup>-/-</sup> and wild-type controls were treated  
454 with vehicle-soaked gelfoam. SCs were collected for gene expression analysis at 1 wpi, which  
455 corresponds to 2 days post-treatment. Animal numbers are indicated for each genotype. (G)  
456 Neuronal gene expression was analyzed by qRT-PCR from FGF1- and vehicle-treated SC  
457 tissues. For each gene, log<sub>2</sub> (fold change) was normalized to *eif1a* as a loading control and to  
458 gene expression levels in uninjured *mstnb*<sup>+/+</sup> SCs. (H) Schematic model shows *mstnb* is a  
459 negative regulator of neuronal differentiation in dorsal SC tissues after spinal cord injury. ERGs  
460 (blue) undergo extensive proliferation after SCI. A *mstnb* expressing niche (green) emerges in  
461 the dorsal ependyma.

Figure S1



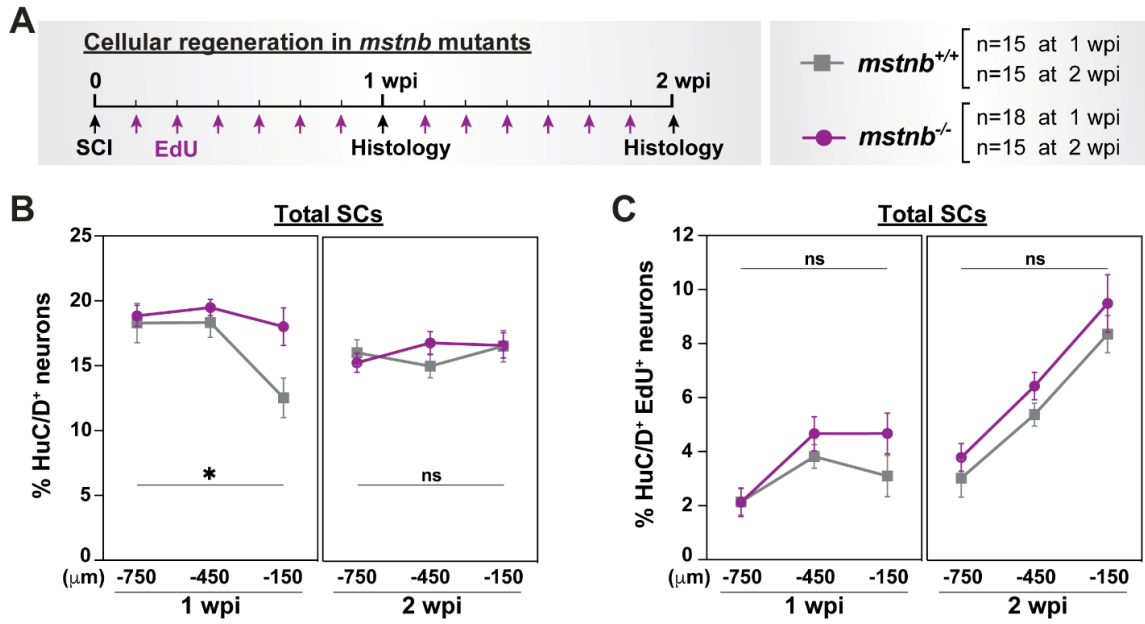
462 **Figure S1. TGF- $\beta$  signaling during SC regeneration. (A)** pSmad3 quantification in total SC  
463 sections. Wild-type SCs at 1, 2 and 3 wpi and uninjured SCS were analyzed. Percent pSmad3<sup>+</sup>  
464 cells was normalized to the number of nuclei in dorsal SCs. **(B)** pSmad3 and HuC/D  
465 immunostaining in wild-type SCs at 1 wpi. High-magnification views of dorsal SCs are shown.  
466 Dotted lines delineate pSmad3<sup>-</sup> HuC/D<sup>+</sup> neurons. Arrowheads point to dotted pSmad3 expression  
467 in a subset of HuC/D<sup>+</sup> neurons. **(C)** pSmad3 quantification in total ERGs. Wild-type SCs at 1, 2  
468 and 3 wpi and uninjured SCS were analyzed. Percent pSmad3<sup>+</sup> Sox2<sup>+</sup> cells was normalized to  
469 the number of pSmad3<sup>+</sup> cells. **(D, E)** pSmad3 and EdU quantification in dorsal (D) and total (E)  
470 SCs. EdU was administered for 24 hrs prior to SC collection. Wild-type SCs at 1, 2 and 3 wpi and  
471 uninjured SCS were analyzed. Percent pSmad3<sup>+</sup> EdU<sup>+</sup> cells was normalized to the number of  
472 pSmad3<sup>+</sup> cells. **(F)** Expression of Tgf- $\beta$  ligands in wild-type SCs at 2 wpi by bulk RNA sequencing.  
473 For each gene, log<sub>2</sub> (fold change) was normalized to gene expression levels in sham injured SCs.  
474 Upregulated (green) and downregulated (magenta) genes are shown. Unchanged genes are  
475 shown in grey and black. For all quantifications, cross SC sections 450  $\mu$ m rostral to the lesion  
476 site were quantified. \*\*\*P<0.001; \*\*P<0.01. Scale bars, 50  $\mu$ m.

Figure S2



477 **Figure S2. Cell proliferation in *mstnb* mutant zebrafish. (A)** Experimental timeline to assess  
478 the rates of cell proliferation. *mstnb*<sup>-/-</sup> and wild-type siblings were subjected to SC transections.  
479 A single EdU injection was performed at either 6, 13, or 20 days post-injury. SC tissues were  
480 harvested for analysis at 1, 2, 3 wpi and at 24 hours after EdU injection. Animal numbers are  
481 indicated for each genotypes and two independent replicates are shown. **(B)** Regenerated  
482 HuC/D<sup>+</sup> EdU<sup>+</sup> neurons were quantified in dorsal SC sections at 1, 2, 3 wpi and uninjured controls.  
483 Percent HuC/D<sup>+</sup> EdU<sup>+</sup> neurons was normalized to the total number of HuC/D<sup>+</sup> neurons for each  
484 section. **(C)** Regenerated HuC/D<sup>+</sup> EdU<sup>+</sup> neurons were quantified in total SC sections at 1, 2, 3 wpi  
485 and uninjured controls. Percent HuC/D<sup>+</sup> EdU<sup>+</sup> neurons was normalized to the total number of  
486 nuclei for each section. **(D)** Sox2<sup>+</sup> EdU<sup>+</sup> ERGs were quantified in dorsal SC sections at 1, 2, 3 wpi  
487 and uninjured controls. Percent Sox2<sup>+</sup> EdU<sup>+</sup> ERGs was normalized to the total number of Sox2<sup>+</sup>  
488 ERGs for each section. **(E)** Sox2<sup>+</sup> EdU<sup>+</sup> ERGs were quantified in total SC sections at 1, 2, 3 wpi  
489 and uninjured controls. Percent Sox2<sup>+</sup> EdU<sup>+</sup> ERGs was normalized to the total number of nuclei  
490 for each section. For all quantifications, cross SC sections 450 μm rostral to the lesion site were  
491 quantified. \*\*P<0.01; ns, not significant.

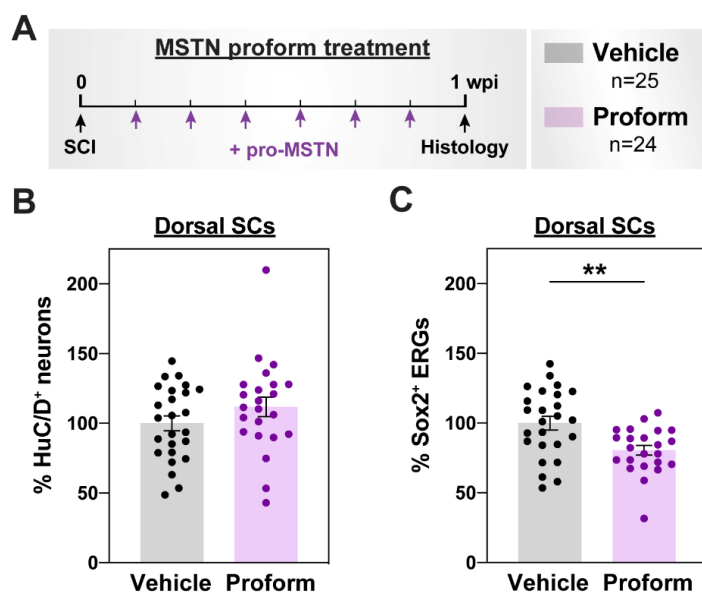
Figure S3



492 **Figure S3. Regenerative neurogenesis in *mstnb* mutant zebrafish.** (A) Experimental timeline  
493 to assess the rates of neurogenesis and ERG self-renewal. *mstnb*<sup>-/-</sup> and wild-type siblings were  
494 subjected to SC transections and daily EdU injections. SC tissues were harvested for analysis at  
495 1 or 2 wpi. Animal numbers are indicated for each genotypes and two independent replicates are  
496 shown. (C) HuC/D<sup>+</sup> neurons were quantified in total SC sections at 1 and 2 wpi. Percent HuC/D<sup>+</sup>  
497 neurons was normalized to the total number of nuclei for each section. (D) Regenerated HuC/D<sup>+</sup>  
498 EdU<sup>+</sup> neurons were quantified in total SC sections at 1 and 2 wpi. Percent HuC/D<sup>+</sup> EdU<sup>+</sup> neurons  
499 was normalized to the total number of nuclei for each section. For all quantifications, cross SC  
500 sections at 150, 450, and 750 μm rostral to the lesion site were quantified. \*\*P<0.01; ns, not  
501 significant.

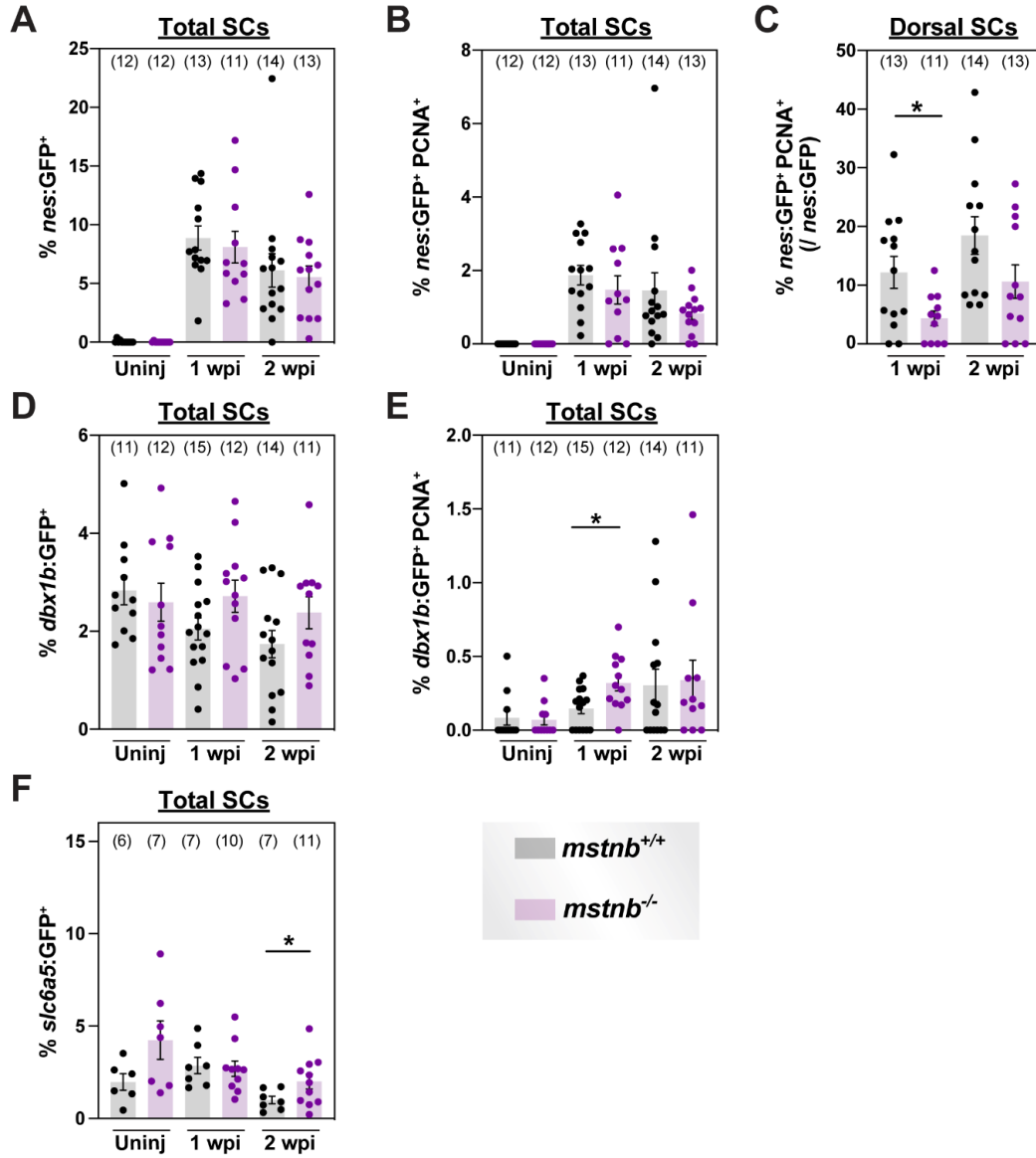


Figure S4



502 **Figure S4. Pharmacological Mstn inhibition during SC regeneration. (A)** For local Mstnb  
503 inhibition, wild-type SCs were subjected to SC transections and daily injections of human  
504 recombinant MSTN Proform (pro-MSTN) peptide adjacent to the lesion site. SC tissues were  
505 harvested for analysis at 1 wpi. Animal numbers are indicated for each genotypes and two  
506 independent replicates are shown. **(B)** HuC/D<sup>+</sup> neurons were quantified in pro-MSTN- and  
507 vehicle-treated SCs. Dorsal SC sections at 1 wpi were analyzed. Percent HuC/D<sup>+</sup> neurons was  
508 normalized to the total number of nuclei for each section. **(C)** Sox2<sup>+</sup> ERGs were quantified in pro-  
509 MSTN- and vehicle-treated SCs. Dorsal SC sections at 1 wpi were analyzed. Percent Sox2<sup>+</sup> ERGs  
510 was normalized to the total number of nuclei for each section. For all quantifications, cross SC  
511 sections 450 μm rostral to the lesion site were quantified. \*\*P<0.01.

Figure S5



512 **Figure S5. Assessment of neuronal progenitors and neurons in *mstnb* mutant zebrafish.**  
513 **(A)** *nes*<sup>+</sup> NSCs were quantified in total SC sections. Percent *nes*<sup>+</sup> NSCs was normalized to the  
514 total number of nuclei for each section. **(B)** *nes*<sup>+</sup> PCNA<sup>+</sup> NSCs were quantified in total SC sections.  
515 Percent *nes*<sup>+</sup> PCNA<sup>+</sup> NSCs was normalized to the total number of nuclei for each section. **(C)**  
516 *nes*<sup>+</sup> PCNA<sup>+</sup> NSCs were quantified in dorsal SC sections. Percent *nes*<sup>+</sup> PCNA<sup>+</sup> NSCs was  
517 normalized to the total number of *nes*<sup>+</sup> NSCs for each section. **(D)** *dbx1b*<sup>+</sup> iNPs were quantified  
518 in total SC sections. Percent *dbx1b*<sup>+</sup> iNPs was normalized to the total number of nuclei for each  
519 section. **(E)** *dbx1b*<sup>+</sup> PCNA<sup>+</sup> iNPs were quantified in total SC sections. Percent *dbx1b*<sup>+</sup> PCNA<sup>+</sup>  
520 NSCs was normalized to the total number of nuclei for each section. **(F)** *slc6a5*:GFP<sup>+</sup> glycinergic  
521 neurons were quantified in total SC sections. Percent *slc6a5*:GFP<sup>+</sup> neurons was normalized to  
522 the total number of nuclei for each section. For all quantifications, cross SC sections 450 μm  
523 rostral to the lesion site were quantified. \*P<0.05.

Gene	Primer Name	Sequence	Product size
<i>birc5b</i>	birc5b_exon1_fwd	CGAGAAGATTGCCAGTGACAC	70
	birc5b_exon2_rev	CATTCTCACTGGGACAGTGAAC	
<i>eloal</i>	eloal_exon1_fwd	GCTGAAGGAATGTAATGATG	74
	eloal_exon2_rev	CAAGTGTGATGTCCAATACTTC	
<i>elavl2</i>	elavl2_exon3_fwd	GAGATCGAGTCCTGCAAACCTCG	79
	elavl2_exon4_rev	GCTCCATATAGTTCACAAAGCCG	
<i>htr2aa</i>	htr2aa_exon1_fwd	GTCATGCCAGTCTCCATGGTG	74
	htr2aa_exon2_rev	CACATGGGACACAGTGATGC	
<i>pou5f3</i>	pou5f3_exon3_fwd	GAACGAGGCCGAAAACCTCCGAG	67
	pou5f3_exon4_rev	CGTGTGACAAACACCCGTTT	
<i>uncx4.1</i>	uncx4.1_exon2_fwd	GCTACGTCTAGACCTTGTTGAG	65
	uncx4.1_exon3_rev	GCCATTTGGCTCGCCGTTTTTG	
<i>znhit3</i>	znhit3_exon3_fwd	CAGATCCAGCCTCCAGCAAAC	75
	znhit3_exon4_rev	CATCCAGCAGATCCTCAACAGTC	
<i>grip2a</i>	grip2a_exon4_fwd	GCCATCCGCTTTATAGAGCCTG	72
	grip2a_exon5_rev	GAGAATTCTGTGCGCCAACTTG	
<i>slbp</i>	slbp_exon1_fwd	CGATTACAAATCTAGTGAAGACAG	84
	slbp_exon2_rev	CTCAGAATACCATCTGCTCCAC	
<i>tph1a</i>	tph1a_exon6_fwd2	GAAGACAACATCCCTCAGCTG	77
	tph1a_exon7_rev2	GCCACAGGCCTGATGGTGAAG	
<i>ntrk2b</i>	ntrk2b_exon6_fwd	CTTAAATTCCAGCGAACACCC	83
	ntrk2b_exon7_rev	CATTTAGAGTCAGCACCGACTGC	
<i>fgf1b</i>	fgf1b_exon1_fwd	GCAATGGACAAATGTGGAAG	76
	fgf1b_exon2_rev	CCTCCATCTTCTCAATGAAGAAAC	

524 **Table S1. Primer sequences for qRT-PCR.** Gene names, primer names, sequences, and  
525 product sizes are indicated.

## **METHODS**

526 **Zebrafish.** Adult zebrafish of the Ekkwill, Tubingen, and AB strains were maintained at the  
527 Washington University Zebrafish Core Facility. All animal experiments were performed in  
528 compliance with institutional animal protocols. Male and female animals between 3 and 9 months  
529 of ~2 cm in length were used. Experimental fish and control siblings of similar size and equal sex  
530 distribution were used for all experiments. SC transection surgeries and regeneration analyses  
531 were performed in a blinded manner, and 2 to 4 independent experiments were repeated using  
532 different clutches of animals. The following previously published zebrafish strains were used:  
533 *mstn*<sup>bn5</sup> (Dogra et al., 2017), Tg(*nes*:GFP) (Lam et al., 2009), Tg(*dbx1b*:GFP) (Satou et al., 2012),  
534 Tg(*slc6a5*:GFP) (McLean et al., 2007).

535 **SC transection and treatment.** Zebrafish were anaesthetized using MS-222. Fine scissors were  
536 used to make a small incision that transects the SC 4 mm caudal to the brainstem region.  
537 Complete transection was visually confirmed at the time of surgery. Injured animals were also  
538 assessed at 2 or 3 dpi to confirm loss of swim capacity post-surgery. For sham injuries, animals  
539 were anaesthetized, and fine scissors were used to transect skin and muscle tissues without  
540 inducing SCI.

541 For pro-MSTN treatment, lyophilized human MSTN proform peptide (BioVision, 4623P-10) was  
542 reconstituted in ddH<sub>2</sub>O to a concentration 100 ng/μl. Zebrafish were anaesthetized using MS-222.  
543 2 μl (200 ng) of reconstituted peptides were injected daily adjacent and lateral to the SC lesion  
544 site. 2 μl of ddH<sub>2</sub>O was injected for vehicle controls.

545 For FGF1 treatment, lyophilized human FGF1 protein (PeproTech, 100-17A) was reconstituted in  
546 heparin to a concentration 250 ng/μl. Sterile Gelfoam Absorbable Gelatin Sponge (Pfizer, 09-  
547 0315-08) was cut into 2 mm<sup>3</sup> pieces, soaked with 2 μl of recombinant FGF1, then cut into 10  
548 smaller pieces (50 ng per piece). Vehicle gelfoam pieces were soaked with 2 μl of heparin  
549 solution. At 5 dpi, zebrafish were anaesthetized using MS-222 and longitudinal incision lateral  
550 and parallel to the SC was made with fine scissors. Injured SC tissues were exposed without  
551 causing secondary injuries and gelfoam sponges were placed adjacent to the lesion site. The  
552 incision was closed and glued using Vetbond tissue adhesive material as previously described  
553 (Mokalled et al., 2016).

554 **Bulk RNA sequencing.** Two mm SC sections, including the lesion site plus additional rostral and  
555 caudal tissue proximal to the lesion, were collected from *mstnb* mutants and wild-type siblings at  
556 1 wpi. Uninjured *mstnb* mutants and wild-type SCs were also collected. Total RNA was prepared  
557 using NucleoSpin RNA Plus XS (Clontech, cat# 740990) and sent for bulk RNA sequencing.  
558 TruSeq libraries were prepared and sequenced on Illumina HiSeq 3000 using 50 bp single-end  
559 reading strategy. Quality QC and trimming of adapters and short sequences were performed  
560 using Fastx. Sequencing reads were mapped to the zebrafish genome (Zv11) using Bowtie2, then  
561 assembled and quantified using the Cufflinks and Cuffdiff algorithms. Genes with  $\log_2$ (fold  
562 enrichment) between -1 and 1 or adjusted p-value  $\geq 0.01$  were considered insignificant. RNA  
563 sequencing was performed at the Genome Technology Access Center at Washington University.  
564 Analysis was performed in the Bioinformatics Core at the Center for Regenerative Medicine at  
565 Washington University.

566 RNA-seq data (GEO accession number : GSE77025) was used to evaluate the expression of Tgf-  
567  $\beta$  ligands after complete SC transection.  $\log_2$ (fold change) is expressed for SCs at 1 wpi relative  
568 to the sham injured SCs (Mokalled et al., 2016).

569 **Histology.** 16  $\mu\text{m}$   $\mu\text{m}$  cross cryosections of paraformaldehyde-fixed SC tissue were used. Tissue  
570 sections were imaged using a Zeiss AxioVision compound microscope for *in situ* hybridization or  
571 a Zeiss LSM 800 confocal microscope for immunofluorescence. *In situ* hybridization for *mstnb*  
572 was performed as previously described (Mokalled et al., 2016).

573 For immunohistochemistry, tissue sections were rehydrated in PBT (0.1% Tween-20 in PBS),  
574 then treated with blocking agent (5% goat serum in PBT) for 1 hr at room temperature. For nuclear  
575 antigens, sections were treated with 0.2% TritonX-100 in PBT for 5 minutes and washed  
576 thoroughly in PBT prior to the blocking step. Sections were incubated overnight with the indicated  
577 primary antibodies, washed in PBT, and treated for 1 hr with secondary antibodies. Following  
578 washes, sections were incubated in 1 mg/mL of Hoechst and mounted in Fluoromount-G  
579 mounting media. Primary antibodies used in this study were: rabbit anti-Smad3(S423/425)  
580 (Abcam, ab52903, 1:50), rabbit anti-PCNA (GeneTex, GTX124496, 1:500), mouse anti-HuC/D  
581 (Invitrogen, A21271, 1:500), mouse anti-Gfap (ZIRC, Zrf1, 1:1000), mouse anti-acetylated  $\alpha$ -  
582 tubulin (Sigma, T6793, 1:1000), chicken anti-GFP (Aves Labs, 1020, 1:1000), rabbit anti-Sox2  
583 (GeneTex, 124477, 1:250). Secondary antibodies (Invitrogen, 1:200) used in this study were

584 Alexa Fluor 488- or Alexa Fluor 594- conjugated goat anti-rabbit, anti-mouse, or anti-chicken  
585 antibodies.

586 For simultaneous labeling with rabbit anti-Sox2 (GeneTex, 124477, 1:250) and rabbit anti-  
587 pSmad3(S423/425) (abcam, ab52903, 1:50) (Fig. 1B), unconjugated Fab Fragment Goat Anti-  
588 Rabbit IgG(H+L) (Jackson ImmunoResearch, 111-007-003) and donkey anti-goat 568 (Thermo  
589 fisher: A-11057) antibodies were used for pSmad3 labeling. Sox2 was labeled using donkey anti-  
590 rabbit-488 (Jackson ImmunoResearch, 711-547-003).

591 EdU Staining was adapted from a a previously described protocol (Salic and Mitchison, 2008).  
592 Briefly, zebrafish were anaesthetized using MS-222 and subjected to intraperitoneal EdU  
593 injections. 12.5 mM EdU (Sigma 900584) diluted in PBS was used. A single injection (Fig. 3 and  
594 S2) or multiple, daily injections (Fig. 4 and S3) were performed and paraformaldehyde-fixed  
595 cryosections were used. Sections were rehydrated in PBT for 10 min then incubated with freshly  
596 prepared staining solution for 30 min (100 mM Tris (Sigma, T6066) pH 8.5; 1 mM CuSO<sub>4</sub> (Sigma,  
597 C1297); 10 μM fluorescent azide; and 100 mM ascorbic acid (Sigma, A5960)).

598 **Cell counting.** Cell counting was performed using a customized Fiji script (adapting ITCN: Image  
599 based Tool for counting nuclei- <https://imagej.nih.gov/ij/plugins/itcn.html>). Orthogonal projections  
600 of individual image stacks were generated using Zen software. A Customized Fiji script  
601 incorporated user-defined inputs to define channels (including Hoechst), to determine the center  
602 of the central canal, and to outline SC perimeters. SC tissues dorsal to the central canal center  
603 was considered “dorsal SC”. SC tissues ventral to the central canal center was considered  
604 “ventral SC”. To quantify nuclei, the following parameters were set in ITCN counter: width, 15;  
605 minimal distance, 7.5; threshold, 0.4. For each staining, thresholds were user-defined. Raw  
606 counts from Fiji were processed using a customized R Studio script.

607 **Swim endurance assays.** Zebrafish were exercised in groups of 8-12 in a 5 L swim tunnel device  
608 (Loligo, cat# SW100605L, 120V/60Hz). After 10 minutes of acclimation inside the enclosed  
609 tunnel, water current velocity was increased every two minutes and fish swam against the current  
610 until they reached exhaustion. Exhausted animals were removed from the chamber without  
611 disturbing the remaining fish. Swim time and current velocity at exhaustion were recorded. Results  
612 were expressed as means ± SEM. An unpaired two-tailed Student's t-test with Welch correction



613 was performed using the Prism software to determine statistical significance of swim times  
614 between groups.

615 **Swim behavior assays.** Zebrafish were divided into groups of 5 in a 5 L swim tunnel device  
616 (Loligo, cat# SW100605L, 120V/60Hz). Each group was allowed to swim for a total of 15 min  
617 under zero to low current velocities (5 min at 0 cm/s, 5 min at 10 cm/s, and 5 min at 15 cm/s). The  
618 entire swim behavior was recorded using high-speed camera (iDS, USB 3.0 color video camera)  
619 with following settings: aspect ratio, 1:4; pixel clock, 344; frame rate, 70 frames/s; exposure time:  
620 0.29; aperture, 1.4 to 2; maximum frames; 63,000. Movies were converted to 20 frames/s and  
621 analyzed using a customized Fiji macro. For each frame, animals/objects > 1500 px<sup>2</sup> were  
622 identified, and the XY coordinates were derived for each animal/object. Frame were  
623 independently, and animal/object tracking was completed using a customized R Studio script. The  
624 script aligned coordinates, calculated swim metrics considering three separate frame windows  
625 (Frames 0-6000 at 0 cm/s; frames 6001-12000 at 10 cm/s, and frames 12001-18001 at 20 cm/s).

626 **Glial bridging.** GFAP immunohistochemistry was performed on serial transverse sections. The  
627 cross-sectional area of the glial bridge and the area of the intact SC rostral to the lesion were  
628 measured using ImageJ software. Bridging was calculated as a ratio of these measurements.  
629 Mann Whitney tests were performed using Prism software to determine statistical significance  
630 between groups.

631 **Axon tracing.** Anterograde axon tracing was performed on adult fish at 4 wpi. Fish were  
632 anaesthetized using MS-222 and fine scissors were used to transect the cord 4 mm rostral to the  
633 lesion site. Biocytin-soaked Gelfoam Gelatin Sponge was applied at the new injury site (Gelfoam,  
634 Pfizer, cat# 09-0315-08; Biocytin, saturated solution, Sigma, cat# B4261). Fish were euthanized  
635 6 hours post-treatment and Biocytin was histologically detected using Alexa Fluor 594-conjugated  
636 Streptavidin (Molecular Probes, cat# S-11227). Biocytin-labeled axons were quantified using the  
637 “threshold” and “particle analysis” tools in the Fiji software. Four sections per fish at 0.5 (proximal)  
638 and 2 (distal) mm caudal to the lesion core, and 2 sections 1 mm rostral to the lesion, were  
639 analyzed. Axon growth was normalized to the efficiency of Biocytin labeling rostral to the lesion  
640 for each fish. The axon growth index was then normalized to the control group for each  
641 experiment. Mann-Whitney tests were performed using Prism software to determine statistical  
642 significance between groups

643 **Quantitative real time PCR.** Two mm SC sections, including the lesion site plus additional rostral  
644 and caudal tissue proximal to the lesion, were collected for qRT-PCR. Total RNA was prepared  
645 using NucleoSpin RNA Plus XS (Clontech, cat# 740990) and cDNA was synthesized using the  
646 Maxima First Strand cDNA Synthesis Kit (ThermoFisher, cat# K1672) according to manufacturer's  
647 specifications. Quantitative PCR was completed using the Luna polymerase master mix (NEB,  
648 cat# M3003) using gene-specific primers (Table S1). Primers were designed to flank introns and  
649 were confirmed to not amplify product from genomic DNA. To determine primer efficiency, a  
650 standard curve was generated for each primer set using cDNA pooled from wild-type embryos at  
651 1, 3, and 5 days post-fertilization. qRT-PCR was performed on a Bio-Rad CFX Connect Real-  
652 Time System. For each gene,  $\log_2(\text{fold change})$  was calculated using the DCq method and  
653 normalized to *ef1a* as a loading control and to control gene expression for each experiment.

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