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Supplemental Information

CRISPR-Mediated Genomic Deletion of *Sox2* in the Axolotl Shows a Requirement in Spinal Cord Neural Stem Cell Amplification during Tail Regeneration

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Supplemental Figures and Figure Legends

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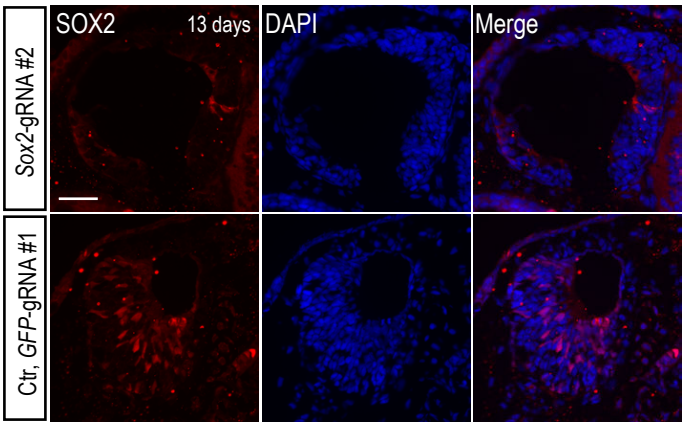
A



B

Sox2-gRNA#2 / Neural plate stage animals-**unselected**				
animals #	phenotype	total clones	modified clones	percentage(%)
1	no	12	3	25
2	no	12	12	100
3	no	12	8	67
4	no	12	11	92
5	no	12	10	83
6	no	12	12	100
7	no	10	3	30
8	no	12	12	100
9	no	12	8	67
10	no	12	12	100
11	no	12	7	58

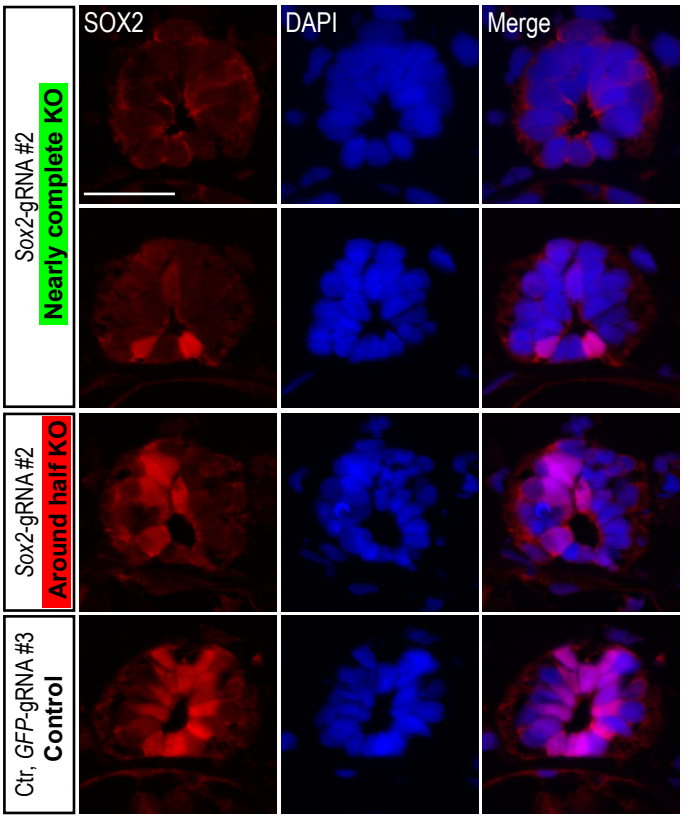
C

Sox2-gRNA#2 / 13 day animals-**curved body selected**				
animals #	phenotype	total clones	modified clones	percentage(%)
1	curved body	10	10	100
2	curved body	11	11	100
3	curved body	12	12	100
4	curved body	12	12	100
5	curved body	12	12	100
6	curved body	12	12	100
7	curved body	9	8	89
8	curved body	12	12	100
9	curved body	11	7	64
10	curved body	10	10	100
11	curved body	12	10	83

D

Sox2-gRNA#2 / animals-**no/mild phenotype selected**				
animals #	phenotype	total clones	modified clones	percentage(%)
1	no	10	1	10
2	no / mild	12	2	17
3	no / mild	12	1	8
4	no / mild	11	2	18

E



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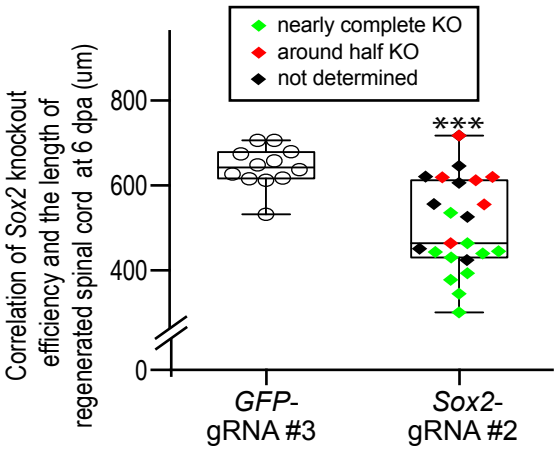


Fei et al. Supp Fig. 1

Suppl. Figure 1. Characterization and genotyping of *Sox2*-CRISPR animals shows a high penetrance of gene deletions even in some animals at embryonic stages.

A. Cross sections of the olfactory bulb from CRISPR *Sox2*-gRNA#2 versus *GFP*-gRNA#1 injected embryos were immunostained for SOX2 (red), combined with DAPI (blue). Only a few $SOX2^{+}$ cells are present in the *Sox2*-gRNA#2 injected animals, while controls have a number of positive cells. The pseudostratified architecture is also disrupted in the *Sox2*-gRNA#2 injected animals. Scale bar, 100μm.

B-D. Analysis of the genomic *Sox2* locus in early (B) and late (C and D) stage animals, injected with *Sox2*-gRNA#2 at single cell stage, by PCR amplification and sequencing of the clones. Note: the early stage15–neural plate stage embryos (B) used for genotyping showed normal development compared to controls and thus individuals were selected randomly for genotyping, since no phenotype could be detected yet. Late stage animals (day 13 larvae) showed either a curved body phenotype (C), or no / mild phenotype (D) and were segregated into these classes prior to genotyping. Individuals in which all clones contained deletions are highlighted in green and are present even in unselected, early stage embryos (B, 4/11). 13 day old larvae showing the curved body phenotype had a very high penetrance of deletions at the locus (C), while animals showing no / mild phenotype had a low penetrance of deletions at the locus (D) correlating the late phenotype and the genotype.

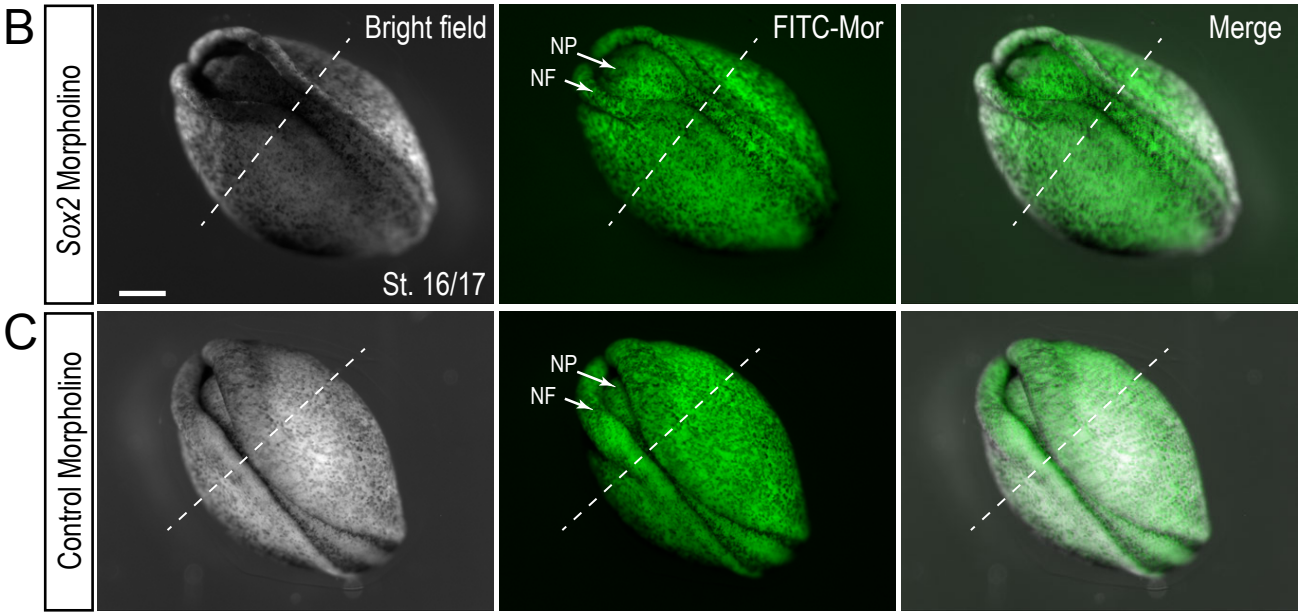
E. Categorization of SOX2 knockdown penetrance in *Sox2*-CRISPR animals used for correlating extent of *Sox2* deletion with the spinal cord regeneration phenotype in panel F. Cross sections of removed portion of the tail next to the amputation plane from *Sox2*-gRNA#2 and control *GFP*-gRNA#1 injected, 13 day old larvae (as illustrated in Fig. 4A “A“, and shown in Fig. 4B) were immunostained for SOX2 (red), combined with DAPI (blue). The same animals were used later for the quantification of spinal cord and tail length at 6 days of regeneration shown in Fig. 4C-E and S1F. Note: in all the *Sox2*-gRNA#2 injected animals (upper panels), SOX2 immunostaining shows cells lacking SOX2 signal in the spinal cord compared to the control (lower panel). Green highlights animals that showed complete or nearly complete loss of SOX2 signal. Red (middle panel)

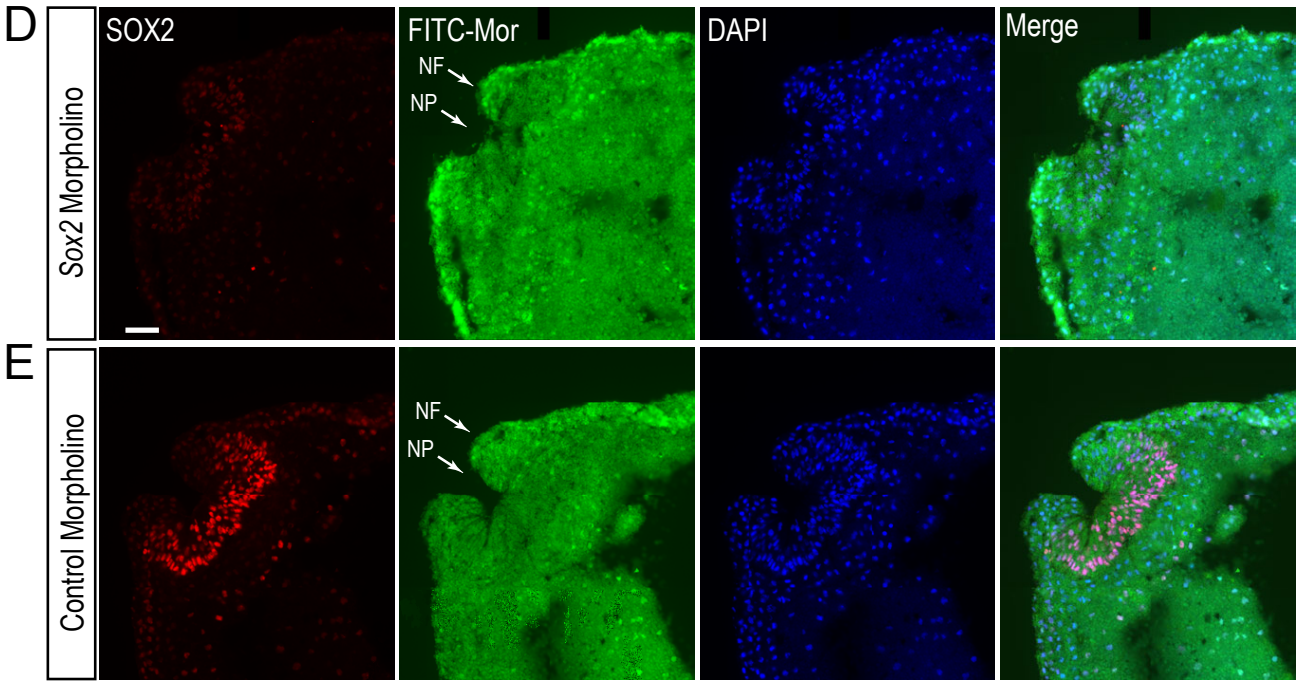
highlights animals that showed about half of the ependymal cells with loss of SOX2 prior to amputation. Scale bar, 50μm.

F. Highlighting of data in panel 4D to correlate the length of the regenerated spinal cord at 6 days with the SOX2 knockdown efficiency prior to amputation. The green and red highlighted bullets in *Sox2*-gRNA#2 samples correspond to the color categories illustrated in panel E. The green highlighted bullets that had complete or nearly complete loss of $SOX2^+$ cells in the spinal cord show a stronger spinal cord regeneration defect whereas the red highlighted bullets that had loss of around half of $SOX2^+$ cells in the spinal cord, showed a less severe spinal cord regeneration defect.

A

Amount of Morpholino injected per egg / Experiments	5 pmol	10 pmol	20 pmol
Sox2 Morpholino	17/20	18/20	17/21
Sox2 Morpholino + *Sox2* mRNA	15/21	18/21	18/21
Control Morpholino	34/40	34/40	32/39





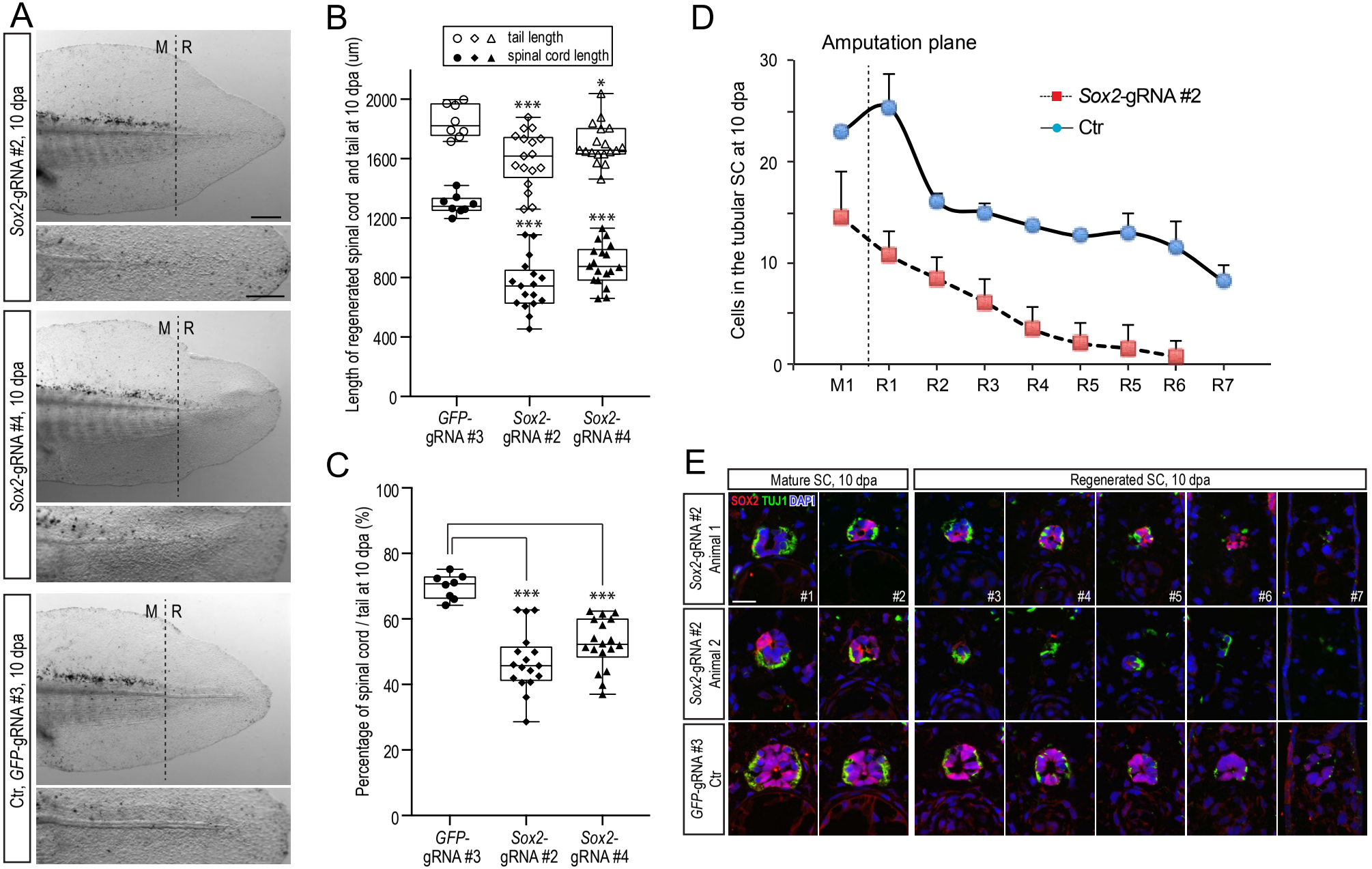
Fei et al. Supp Fig. 2

Suppl. Figure 2. Morpholino-mediated knockdown of SOX2 expression yields no detectable embryonic phenotype.

A. Embryo survival at stage 37 after injection of anti-*Sox2* Morpholinos.

B, C. Stage (St.) 16-17 axolotl embryos that had been injected at the one cell stage with 20 pmol of FITC-coupled (green) *Sox2* morpholino (B) or Control morpholino (C). NP, neural plate; NF, neural fold. Dashed lines indicate the rough positions of the cross sections in (D, E). Scale bar, 500μm.

D, E. Immunofluorescence for SOX2 (red) on cross sections of early embryos as shown in (B, C). SOX2 expression is inhibited, though not completely blocked, in *Sox2* Morpholino injected embryos. FITC-Morpholino (green), and DAPI (blue). Scale bar, 100μm.



Fei et al. Supp Fig. 3

Suppl. Figure 3. Analysis of the spinal cord in 10 day regenerates.

A. Images of live, 10 day regenerates. Upper: *Sox2*-gRNA#2; middle: *Sox2*-gRNA#4 and lower: control, *GFP*-gRNA#3. Lower panels show higher magnification image of regenerating spinal cord area. In *Sox2*-gRNA#2 and *Sox2*-gRNA#4 injected animals, a clear spinal cord tube that extends into the blastema is not visible compared to the control sample (*GFP*-gRNA#3). “M“, mature spinal cord area. “R“, regenerated area. Dashed lines, amputation planes. Scale bars, 500µm upper panel and 200µm lower panel.

B. Quantification of spinal cord and tail length in *Sox2*-gRNAs versus control *GFP*-gRNA#3 injected animals at 10 days of regeneration. By 10 days, the regenerated tail length in *Sox2*-gRNA#2 (n=17), *Sox2*-gRNA#4 (n=18) injected animals is slightly reduced compared to control *GFP*-gRNA#3 (n=8) injected animals. The spinal cord length shows a more severe reduction in length. Each bullet means one individual animal. Error bars, SD. *p<0.05, ***p<0.001.

C. Spinal cord length to tail length ratio in 10 day regenerates. The spinal cord represents a smaller fraction of the total tail length in *Sox2*-gRNAs injected animals compared to controls. The same data set from (B) was used for data plotting. Each bullet means one individual animal. Error bars, SD; ***p<0.001.

D. Quantification of the number of nuclei in the spinal cord at different points along the regenerating spinal cord shows that reduced cell number along the length of the regenerate in *Sox2*-gRNA#2 injected animals compared to control *GFP*-gRNA#3 injected animals. Data are the mean of 2 determinants from control (Ctr) and 6 from *Sox2*-gRNA#2 injected animals (each determinant is the average value derived from 3 adjacent sections, with 50µm distance), Each bar on X axis stands for 150µm. Error bar in control indicate the variation of the individual values from the mean; error bar in *Sox2*-gRNA #2, SD.

E. Immunostaining for SOX2 (red) and TUJ1 (green) with DAPI (blue) shows the reduced number of cells in the spinal cord, and the disorganization of $TUJ1^{+}$ cells in the animals injected with the *Sox2*-gRNA#2 compared to *GFP*-gRNA#3 injected animals. Sections from two different animals injected with *Sox2*-gRNA#2 are shown. From left to right, shown sections along A-P axis including mature and regenerated spinal cord (SC). Scale bar, 50µm.

Supplemental Tables

Table S1. Embryo survival at 10 days after injection of *GFP*-TALEN mRNA

[TALEN mRNA] / TALEN	500pg	250pg	125pg
GFP-TALEN #1	0/44	5/50 (10%)	20/55 (36%)
GFP-TALEN #3	2/52 (4%)	21/59 (36%)	34/61 (56%)

Table S2. Embryo survival at 10 days after injection of *GFP-CRISPR* RNAs

[Cas9 mRNA] [gRNA] / CRISPR gRNA	50 pg 450 pg	500 pg 500 pg	1000 pg 1000 pg
GFP-gRNA #1	92/123 (75%)	91/106 (86%)	-
GFP-gRNA #3	187/243 (77%)	58/92 (63%)	71/89 (80%)

Table S3. Phenotype penetrance of GFP TALEN and CRISPR injected animals.

"low conc." 500 pg each RNA. "high conc." 1000 pg each RNA

	Total injected eggs (50% GFP^+ genotype)	Survived GFP^+ genotype embryos	Phenotype: modification of GFP expression (# of animals)		
			Strong >50% loss of GFP^+ cells	Weak <50% loss GFP^+ cells	No visible phenotype (all GFP^+)
GFP-TALEN #1	639	78	28		50
GFP-TALEN #3	871	157	40		117
Control,1L+3R	169	38	0		38
CRISPR: *GFP*-gRNA #3 low conc.	229	153	51	34	68
CRISPR: *GFP*-gRNA #3 high conc.	181	62	40	16	6
Control CRISPR: *Tyr*-gRNA #1	155	52	0	0	52

Table S4. Phenotype penetrance of *Tyr* TALEN and CRISPR injected animals.

	Total injected eggs	Survived	Phenotype: loss of pigmentation (# of animals)			
			Complete loss	Strong >90% loss	Medium >50% loss	Weak <50%
Tyr-TALEN #4	104	92	2	5	15	15
Control: *Tyr*-TALEN 3L	124	103	0	0	0	no modification
CRISPR: *Tyr*-gRNA #1	205	144	124	19	1	0
Control CRISPR: *GFP*-gRNA #3	277	219	0	0	0	no modification

Supplemental Experimental Procedures

CRISPR design and RNA synthesis

For better comparison, relevant TALENs and CRISPRs were designed against the identical sequences of each targeted gene locus. The TALENs were designed using two different programs; the 'TALEN targeter' program from Voytas'lab (Cermak et al., 2011) and the 'TALEN hit' program from the Cellectis website, with the following criteria: 17-20 RVDs in each arm of TALEN (Left or right) and 15-20 base pairs of spacer for each pair of TALEN. According to the published protocols (Bedell et al., 2012; Cermak et al., 2011), the designed TALENs were assembled into RCIscript-GoldyTALEN vector (Addgene 1000000024 and 38142), and TALEN mRNAs were synthesized using mMESSAGE mMACHINE T3 Transcription Kit (Invitrogen). According to the previous established method (Hwang et al., 2013), CRISPR gRNAs were designed using the "ZiFiT" program, and assembled into DR274 vector (Addgene 38142), then Cas9 mRNA and gRNAs were synthesized using mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Invitrogen) and MAXIscript T7 Kit (Invitrogen), respectively.

List of other TALENs and CRISPR gRNA used in this study:

TALENs	Left arm sequence	Spacer	Right arm sequence
GFP-TALEN #1	CTTCAAGGACGACGG CAACT	ACAAGACCCGCG CCG	AGGTGAAGTTCGAG GGCG
GFP-TALEN #3*	TCGAGCTGGACGGCG AC	GTAAACGGCCACA AG	TTCAGCGTGTCCGG CGA
Tyr-TALEN #3	TATGCAATGCCACAGA C	GAAGGGCCCATAA TA	CGTAACCCCGGCAA TCA
Tyr-TALEN #4*	CACTATCCCCTACTGG G	ACTGGAGGGACG CCC	AGGGCTGCGCCGT CTGC

* corresponding sequences are also listed in the related figures.

gRNAs	gRNA sequence
GFP-gRNA #1	TGAACCGCATCGAGCTGA
GFP-gRNA #3*	CCACAAGTTCAGCGTGTC
Tyr-gRNA #1*	ACTTCACTATCCCCTACT
Tyr-gRNA #2	CGTCCCTCCAGTCCCAGT
Tyr-gRNA #3	TGCAGACGGCGCAGCCCT
Sox2-gRNA #1	TCTCCATCATGCTGTACA
Sox2-gRNA #2*	AGGTCTGCTGCGGGGCGG
Sox2-gRNA #3	CGTTGCTGCTGTTGTTGT
Sox2-gRNA #4*	ATTACAAGTACCGGCCCC
Sox2-gRNA #5	ACAAGTACACGCTGCCCG
Sox2-gRNA #6	GGTCTCCTCGGGCCACCT
Sox2-gRNA #7	GCACCGGCGGCGGCGTGG

* corresponding sequences are also listed in the related figures.

Axolotl care and egg injection

Animal experiments were carried out according to German animal welfare legislation.

Axolotl egg injection was performed according to previously published protocols (Khattak et al., 2014). Briefly, 125-500pg, typically 125pg of TALEN mRNAs (mixture of left and right arm mRNAs), CRISPR RNAs (equal mixture of 500-1000pg Cas9 mRNA and gRNA) or 5-20 pmol of FITC-coupled Morpholinos (*Sox2*: 5'-CGGTCTCCATCATGCTGTACATGGC-3'; Control: 5'-CCTCTTACCTCAGTTACAATTTATA-3', Gene Tools, LLC) was injected into freshly laid single-cell-stage embryos. For TALENs and CRISPR mediated GFP knockout experiments, eggs derived from the breeding of heterozygous *GFP* transgenic and *white* axolotl animals were used for injection. For other experiments, eggs derived from either wildtype or *white* animals were used for injection. Axolotl larvae were kept individually in plastic cups with a change of fresh tap water every

second day, and fed Artemia daily. Axolotl larvae were anaesthetized within 0.01% ethyl-p-aminobenzoate (benzocaine; Sigma) prior to imaging or amputation.

For the germ line transmission test, *GFP*-TALEN #1 and #3 injected embryos, which showed middle-high level loss of GFP expression, were raised to adulthood, and then were crossed with *white* animals. The ratio of GFP^+ : GFP^- embryos within F1 generation populations was calculated. Then genotyping and sequencing was carried out within GFP^- population as described in "DNA extraction, genotyping and sequencing".

DNA extraction, genotyping and sequencing

Genomic DNA extraction and PCR were carried out using REDExtract-N-Amp™ Tissue PCR Kit (Sigma) according to the manufactory instructions. *GFP* and *Tyr* locus were PCR amplified with primer pairs *GFP*-fw & *GFP*-re and *Tyr*-fw & *Tyr*-re, respectively; *Sox2* locus were PCR amplified with *Sox2*-#2fw & *Sox2*-#2re, when injected with *Sox2*-gRNA#2, and with *Sox2*-#4fw & *Sox2*-#4re, when injected with *Sox2*-gRNA#4. The resulting PCR products were cloned into pGEMT vector (Promega). Individual clones were sequenced with T7 primer.

Genotyping PCR primers:

Primer name	Primer sequences
GFP-fw	5'-GTTCGGCTTCTGGCGTGTGAC-3'
GFP-re	5'-GCTGTTGTAGTTGTACTCCAGCTTGTGC-3'
Tyr-fw	5'-GCTCTTCCTGCTGCTCTGGGAG-3'
Tyr-re	5'-CTGCCAAGAAGCGAAGAAGGAG-3'
Sox2-#2fw	5'-GTACCTCACAAAAGACTGAAGTGAC-3'
Sox2-#2re	5'-TGCTGATCTCCGAGTTGTGCATC-3'
Sox2-#4fw	5'-ATGTACAGCATGATGGAGACCGAC-3'
Sox2-#4re	5'-GGATTCATGGAGTTGTACTGCAGG-3'

In situ hybridization

To obtain the Digoxin-labeled antisense RNA probes, the corresponding EST clones harboring *Sox2* (Genebank accession number: KJ999995) and *Sox3* (Genebank accession number: KJ999996) coding sequences (in pCMVsports6 vector) were linearized with KpnI and EcoR1, respectively. The probes were then synthesized using T7 RNA polymerase (Roche). Whole-mount in situ hybridizations on albino axolotl embryos, and in situ hybridizations on 10μm longitudinal paraffin sections, were carried out as previously described (Epperlein et al., 2000; McHedlishvili et al., 2012).

Quantitative RT-PCR

500μm developing tail sections, measured from the amputation plane towards the tip, were collected at the day of amputation. 500μm response zones (see Figure 7C, upper panel) of the same animals were harvested at 1 day post amputation. Total RNA was extracted using TRIzol reagent. qRT-PCR was performed according to the published method (Kragl et al., 2009). *EF1* was used as internal control.

qRT-PCR primers:

Primer name	Primer sequences
EF1-RTfw	5'-CGCGAGTTCTAATAGGTTCTGATATT-3'
EF1-RTre	5'-ATGCAATTACTTTAGCGAGTACCAC-3'
Sox2-RTfw	5'-CTGCAGTACAACTCCATGAATCC-3'
Sox2-RTre	5'-ATGCTAATCATGTCCCTCAGGTC-3'
Sox3-RTfw	5'-AGGACACGCTTTGTTTATAGATGTAAT-3'
Sox3-RTre	5'-TCTGTTCTCAAGTCAAAAACAGTCTAA-3'

Immunohistochemistry

Axolotl tissue processing and immunohistochemistry were performed in according with previously published protocols (Kragl et al., 2009). Briefly, tails or embryos were fixed overnight in 4%

MEMFA buffer, infused overnight with 30% sucrose, and then embed into OCT. 10μm cryosections were collected and stained with respective antibodies.

Microscopy, quantifications and statistics

Fluorescence images were acquired with a Zeiss Observer or confocal microscope. Bright field or color images were acquired with Olympus dissecting microscope. The length of the regenerated spinal cords and tails were measured with "CellSens Standard" software. Spinal cord cells were counted using Photoshop software. $EdU^{+}TUJ1^{-}NeuN^{-}$ cells, within 300μm from amputation plane, in the regenerated spinal cord were used for quantification in Figure 6C. EdU fluorescence intensity (in Figure 6E) was measured with Software Image J, restricted to the spinal cells expressing overall EdU ($TUJ1^{-}$, $NeuN^{-}$), versus epidermal expressing overall EdU. The punctate EdU labeled cells were excluded, because likely, they were at either the beginning or the end of S phase during EdU pulse. After subtracting the background value, the relative EdU intensity was calculated by dividing the average EdU intensity value of each individual spinal cells with the average EdU intensity value, minimally derived from five epidermis of the same section. Data plotting was carried out using Microsoft Excel and Prism. Student's T test was used for the p value calculation.

Supplemental References

Bedell, V.M., Wang, Y., Campbell, J.M., Poshusta, T.L., Starker, C.G., Krug, R.G., 2nd, Tan, W., Penheiter, S.G., Ma, A.C., Leung, A.Y., et al. (2012). In vivo genome editing using a high-efficiency TALEN system. Nature *491*, 114-118.

Cermak, T., Doyle, E.L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, J.A., Somia, N.V., Bogdanove, A.J., and Voytas, D.F. (2011). Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res. *39*, e82.

Epperlein, H., Meulemans, D., Bronner-Fraser, M., Steinbeisser, H., and Selleck, M.A. (2000). Analysis of cranial neural crest migratory pathways in axolotl using cell markers and transplantation. Development *127*, 2751-2761.

Hwang, W.Y., Fu, Y., Reyon, D., Maeder, M.L., Tsai, S.Q., Sander, J.D., Peterson, R.T., Yeh, J.R., and Joung, J.K. (2013). Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat. Biotechnol. *31*, 227-229.

Khattak, S., Murawala, P., Andreas, H., Kappert, V., Schuez, M., Sandoval-Guzman, T., Crawford, K., and Tanaka, E.M. (2014). Optimized axolotl (Ambystoma mexicanum) husbandry, breeding, metamorphosis, transgenesis and tamoxifen-mediated recombination. Nat. Protoc. *9*, 529-540.

Kragl, M., Knapp, D., Nacu, E., Khattak, S., Maden, M., Epperlein, H.H., and Tanaka, E.M. (2009). Cells keep a memory of their tissue origin during axolotl limb regeneration. Nature *460*, 60-65.

McHedlishvili, L., Mazurov, V., Grassme, K.S., Goehler, K., Robl, B., Tazaki, A., Roensch, K., Duemmler, A., and Tanaka, E.M. (2012). Reconstitution of the central and peripheral nervous system during salamander tail regeneration. Proc Natl Acad Sci USA *109*, E2258-2266.