Supplementary information

Tissue and time-directed electroporation of Cas9 protein-gRNA complexes in vivo yields efficient multigene knockout for studying gene function in regeneration

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Supplementary Materials and Methods: list of primers and gene sequences.

Supplementary Figure S1: Knockout of *GFP* in the axolotl spinal cord.

Supplementary Figure S2: Knockout of *Sox2* in the axolotl spinal cord.

Supplementary Figure S3: Knockout of *GFP* in the tail skin cells through CAS9-gRNA complex electroporation.

Supplementary Materials and Methods

List of primers:

For pCAGGS-*Cas9* cloning:
Cas9-for: 5'
CATTTTGGCAAAGAATTATTCCGCTAGCCGCCACCATGGATAAGAAATAC
TCAATAG
Cas9-rev: 5'
GCAGCCTGCACCTGAGGAGTGGATCCTTACTTGTACACTCATCCTGCAGCT
CCACCG

For pOCC97-*Cas9-NLS* cloning:
Cas9-for2: 5' GGGCCGGCGGCCGCAATGGATAAGAAATACTCAATAG
Cas9-rev2: 5' 5' CTATTACGGCGCGCCTCATCCTGCAGCTCCACCG.

List of gene sequences:

RGR-*GFP*-gRNA (IDT):
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RGR-*Sox2*-gRNA (IDT):
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RGR-*Tyr*-gRNA (IDT):
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Cas9-NLS expressing cassette in pOCC97 vector:
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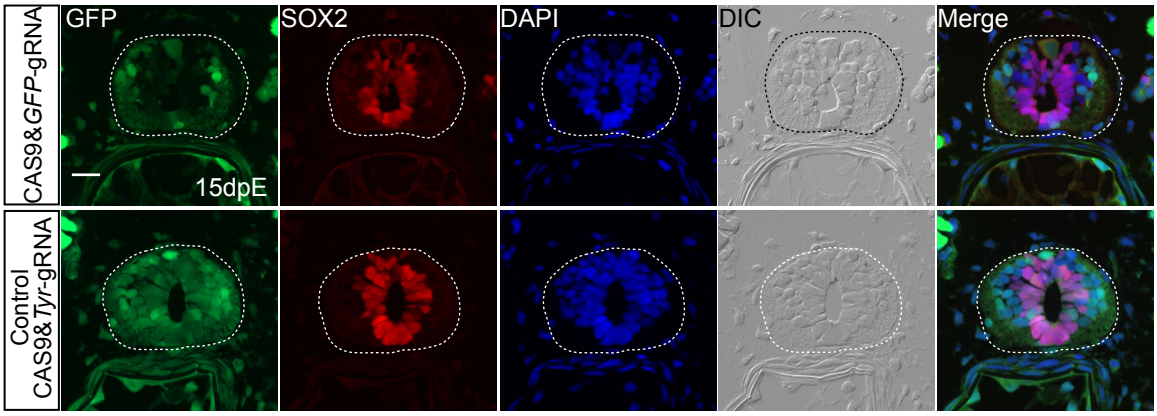

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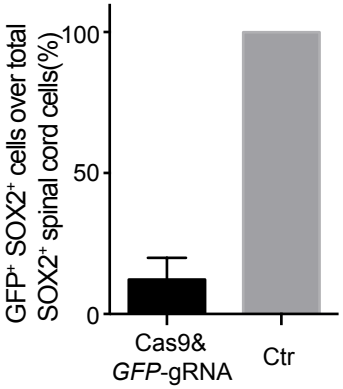

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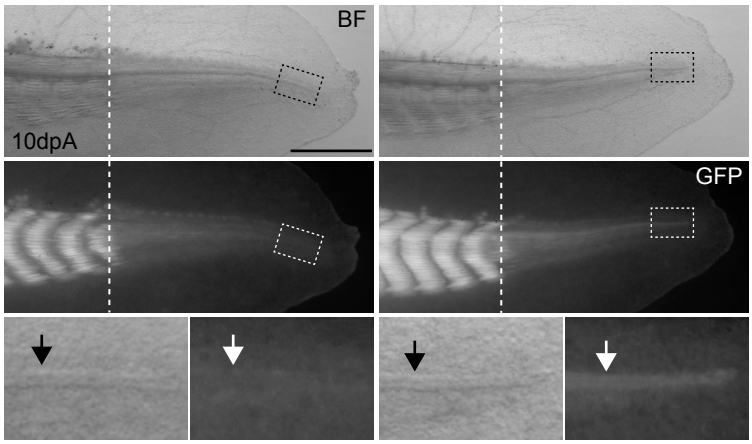

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        GFP-gRNA targeting sequence
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1/20 5' ACGTAAACG--------------------------------------GCCACCT 3' 38nt del
1/20 5' ACGTAAACGGC----------------------GAGGGCGAGGGCGATGCCACCT 3' 21nt del
1/20 5' ACGTAAACGGCCACAA----------------GAGGGCGAGGGCGATGCCACCT 3' 16nt del
1/20 5' ACGTAAACGGCCACAAGT---------CCGGCGAGGGCGAGGGCGATGCCACCT 3' 9nt del
1/20 5' ACGTAAACGGCCACAAGTT----------GGCGAGGGCGAGGGCGATGCCACCT 3' 10nt del
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2/20 5' ACGTAAACGGCCACAAGTTCAG-------GGCGAGGGCGAGGGCGATGCCACCT 3'  7nt del
1/20 5' ACGTAAACGGCCACAAGTTCAGCG----------GGGCGAGGGCGATGCCACCT 3' 10nt del
2/20 5' ACGTAAACGGCCACAAGTTCAGCGT--CCGGCGAGGGCGAGGGCGATGCCACCT 3'  2nt del


        GFP-gRNA targeting sequence
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2/20 5' ACGTAAACGGCCACAAGTTCAGCGTt-------GTCCGGCGAGGG 3' 1nt ins
1/20 5' ACGTAAACGGCCACAAGTTCAGCGTtcagttcaGTCCGGCGAGGG 3' 8nt ins
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Fei et al. Supp Fig. 1

Supplementary Figure S1. Knockout of *GFP* in the axolotl spinal cord NSCs.

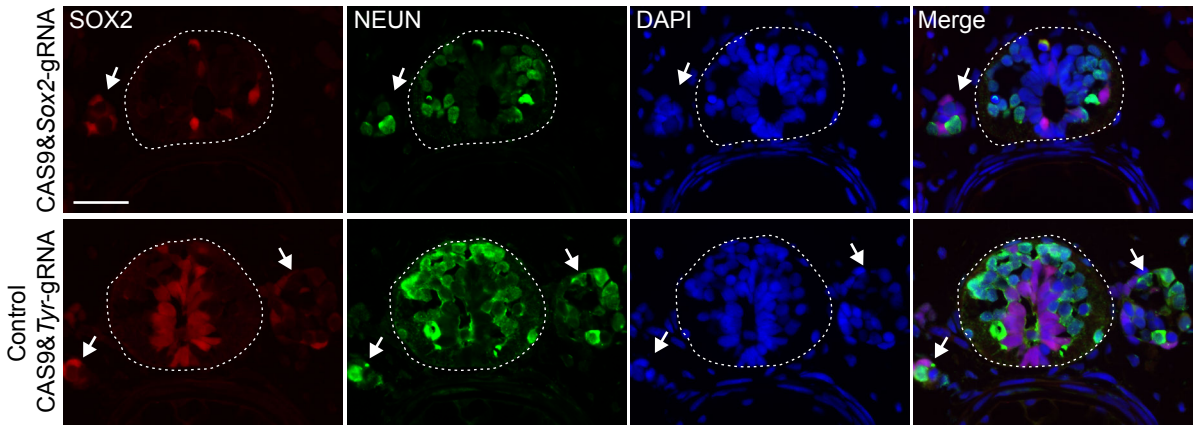
(A) GFP fluorescence (green), SOX2 (red) immunofluorescence combined with DAPI (blue) and DIC images of 10 μm cross-cryosections show the massive loss of GFP expression in NSCs ($SOX2^+$) electroporated with CAS9&*GFP*-gRNA (upper panel) compared to the control (CAS9&*GFP*-gRNA, lower panel). Dotted lines define the spinal cord area. dpE, day post electroporation; Scale bar, 50 μm.

(B) Quantification the percentage of GFP^+ cells over total spinal cord $SOX2^+$ NSCs. Loss of GFP expression is observed in approximately 90% spinal cord $SOX2^+$ NSCs. Data are collected from four CAS9&*GFP*-gRNA electroporated animals and four controls (4-5 cross cryosections per animals). Error bars, SD.

(E) Bright field (BF, upper panels) and GFP fluorescence (middle panels) images of 10-day regenerates from CAGGS-*GFP* transgenic axolotls electroporated with CAS9&*GFP*-gRNA (left panel) or CAS9&*Tyr*-gRNA (control, right panel) into the spinal cord lumen. Lower panel shows higher-magnification images of regenerating spinal cord area of corresponding rectangles. Note: there is a clear loss of GFP expression in the regenerating spinal cord tube treated with CAS9&*GFP*-gRNA compared to the control. Dotted lines indicate the amputation plans; arrows indicate the spinal cord; dpA, day post amputation. Scale bar, 2 mm.

(F) Sequence analysis of the *GFP* genomic locus in a single 10-day regenerated spinal cord electroporated with CAS9&*GFP*-gRNA. Nucleotides in red are the gRNA targeting sequence; nucleotides in green are the PAM sequence. Note: majority of the cloned PCR products include modifications at the target site, most of them with deletions (del), few with insertions (ins).

A



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Sox2-gRNA targeting sequence

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1/20  5' ATGATGGAGA-------------------------CAGACCTCCACCAACC 3' 26nt del
1/20  5' ATGATGGAGACCGA--------------CCCCGCAGCAGACCTCCACCAACC 3' 14nt del
1/20  5' ATGATGGAGACCGACCTGAAGC---------GCAGCAGACCTCCACCAACC 3'  9nt del
2/20  5' ATGATGGAGACCGACCTGAAGCCC-----CCCGCAGCAGACCTCCACCAACC 3'  5nt del
1/20  5' ATGATGGAGACCGACCTGAAGCCCC------CGCAGCAGACCTCCACCAACC 3'  6nt del
3/20  5' ATGATGGAGACCGACCTGAAGCCCC----CCCGCAGCAGACCTCCACCAACC 3'  4nt del
1/20  5' ATGATGGAGACCGACCTGAAGCCCCC-------CAGCAGACCTCCACCAACC 3'  7nt del
1/20  5' ATGATGGAGACCGACCTGAAGCCCCCC----CGCAGCAGACCTCCACCAACC 3'  4nt del
1/20  5' ATGATGGAGACCGACCTGAAGCCCCCCG-----CAGCAGACCTCCACCAACC 3'  5nt del
2/20  5' ATGATGGAGACCGACCTGAAGCCCCCCGC-----AGCAGACCTCCACCAACC 3'  5nt del
1/20  5' ATGATGGAGACCGACCTGAAGCCCCCCGC-------CA-ACCTCCACCAACC 3'  8nt del/ins
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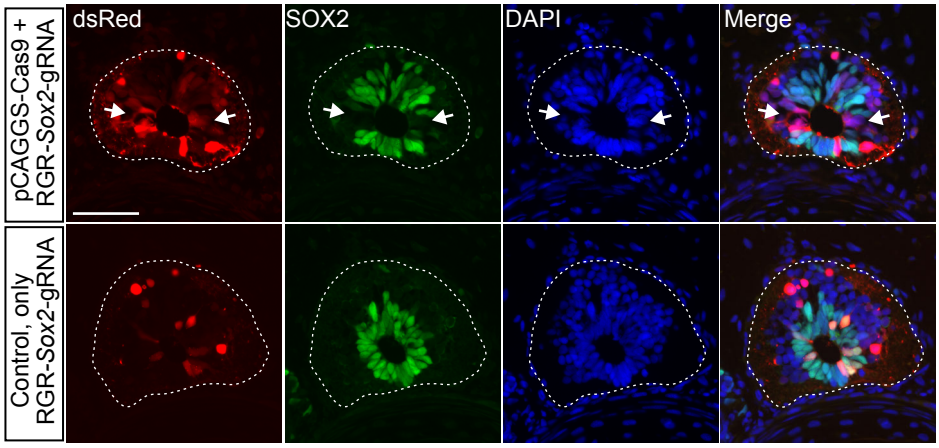

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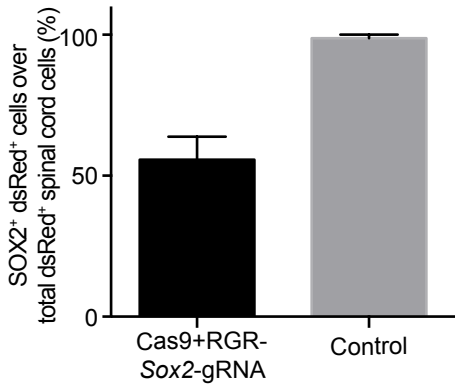

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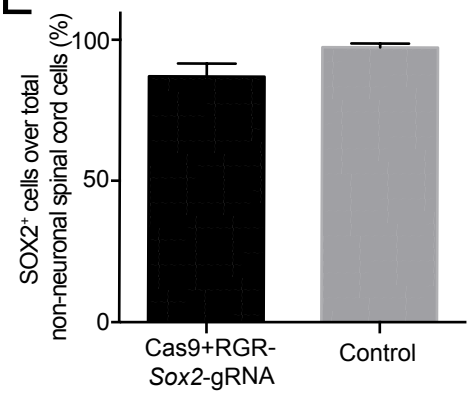


Fei et al. Supp Fig. 2

Supplementary Figure S2. Knockout of *Sox2* in the axolotl spinal cord NSCs.

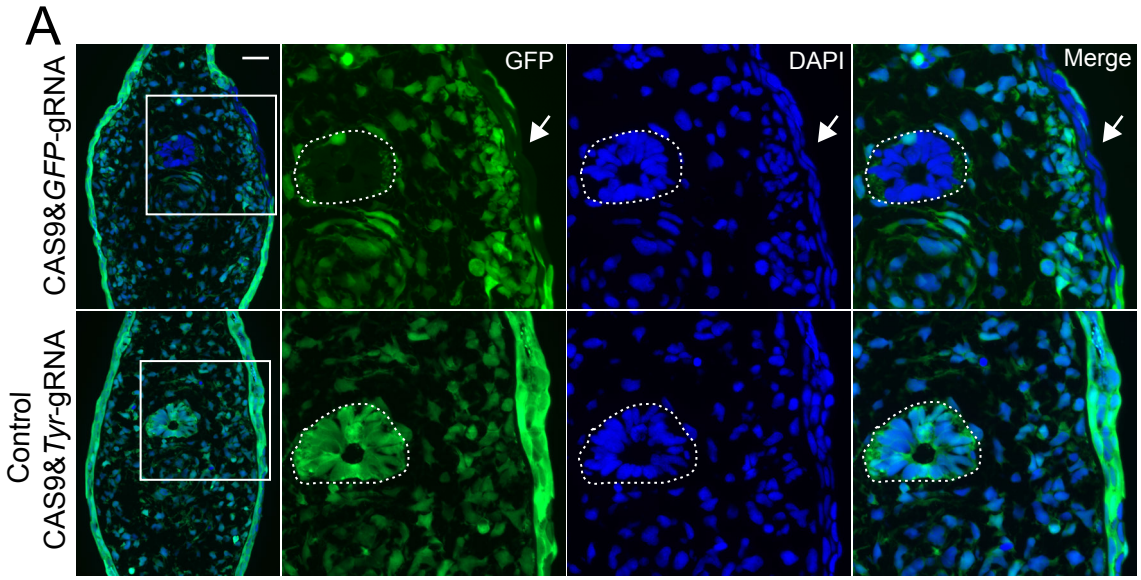
(A) Immunofluorescence for SOX2 (red), NEUN (green) combined with DAPI (blue) on 10 μm cross-cryosections shows the massive loss of SOX2 immunoactivity in NSCs at 15 days post electroporation of CAS9&*GFP*-gRNA (upper panel) compared to the CAS9&*Tyr*-gRNA control (lower panel). Note: SOX2 (labeling NSCs) and NEUN (labeling neurons) expression is mutually exclusive in the spinal cord in the control (lower panel). Dotted lines define the spinal cord areas; Arrows indicate DRG. Scale bar, 100 μm.

(B) Sequence analysis of the *Sox2* genomic locus in a single 6-day regenerating spinal cord electroporated with CAS9&*Sox2*-gRNA. Nucleotides in red are the complementary sequence of gRNA binding site; nucleotides in green are the complementary of PAM sequence. Note: majority of the cloned PCR products include modifications at the target site.

(C) Fluorescence images of dsRed (red), immunofluorescence for SOX2 (green) combined with DAPI (blue) on 10 μm cryosections showing the loss of SOX2 expression in a subpopulation of dsRed-labeled spinal cord cells (arrows) at 20-days post electroporation of CAGGS-*Cas9* and CMV-dsRed-RGR-*Sox2*-gRNA plasmids (pCAGGS-Cas9+RGR-*Sox2*-gRNA, upper panel) compared to control (only RGR-*Sox2*-gRNA plasmid, lower panel). Dotted lines define the spinal cord area. Scale bar, 100 μm.

(D and E) Quantification of the percentage of (D) $SOX2^+$ cells over electroporated $dsRed^+$ spinal cord cells and (E) $SOX2^+$ cells over total non-neuronal spinal cord cells. Neurons are determined by their location and the shape of DAPI-stained nuclei (see Material and Methods). Data are collected from three CRISPR plasmids (Cas9+RGR-

Sox2-gRNA) electroporated animals and three controls (only CMV-dsRed-RGR-*Sox2*-gRNA plasmid) (4-5 cross cryosections per animals).



Fei et al. Supp_Fig. 3

Supplementary Figure S3. Knockout of *GFP* in the tail skin cells through CAS9-gRNA complex electroporation.

(A) GFP fluorescence (green) combined with DAPI (blue) on 10 μm cross-cryosections from 6-day tail regenerates shows the loss of GFP expression in the spinal cord, and a subpopulation of skin cells, when treated with CAS9&*GFP*-gRNA compared to the control. The boxed areas are shown at higher-magnification as single channel or merged images. Dotted lines define the spinal cord; arrows indicate the skin cells. Scale bar, 100 μm.