Supporting Information (SI Appendix)

Efficient gene knock-in in axolotl and its use to test the role of satellite cells in limb regeneration

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SI Results

Generation and characterization of double-transgenic axolotls

To generate a double transgenic animal harboring the ***Pax7:*** *ER^{T2}-Cre-ER^{T2}* knock-in and the *LoxP* reporter alleles, we injected the targeting plasmid, pGEMT-*Pax7*-ORF^{b}-*P2A-memCherry-T2A-ER^{T2}-Cre-ER^{T2}*-PA into eggs produced from a parental *LoxP* reporter transgenic strain ***CAGGS*****:** *LoxP-GFP-STOP-LoxP-Cherry* (Fig. S7*A*, top). The ***CAGGS*****:** *LoxP-GFP-STOP-LoxP-Cherry* allele, ubiquitously expresses GFP until exposed to CRE-activity, upon which the GFP sequences are excised and CHERRY expression is initiated (Fig. S7*A-C*) (1). It should be noted that although the *CAGGS* promoter drives expression in all cells, the expression level found in muscle is more intense compared to surrounding satellite and connective tissue cells.

We first checked whether undesired *Cre*-mediated recombination occurs prior to Tamoxifen treatment in the double transgenic axolotls. Immunohistochemistry on limb cross sections confirmed that the membrane-tagged CHERRY is specifically expressed in the PAX7-positive, MHC-negative cells in 3-month old double transgenic animals (Fig. S8*A* and *C*) but not in the control ***CAGGS*****:** *LoxP-GFP-STOP-LoxP-Cherry* single transgenic axolotls (Fig. S8*B* and *D*). Further immunohistochemical analysis on tail cross sections and limb longitudinal sections of the sexually mature double transgenic axolotl also showed that the CHERRY signal is found surrounding PAX7-positive nuclei and also fills the long satellite cell processes that stretch along the cell surface of the muscle fiber-- consistent with membrane-localization of the CHERRY (Fig. S8*E, F* and Fig. S9).

We did not detect any cytosolic CHERRY expression in muscles (a sign of undesired recombination) (Fig. S8*C*, *F* and Fig. S9*C-E*). Overall, we demonstrated here that there was no sign of conversion of the *LoxP* reporter cassette prior to Tamoxifen administration in ***Pax7:*** *Pax7*-ORF^{b}Δ-*P2A-memCherry-T2A-ER^{T2}-Cre-ER^{T2}*; ***CAGGS:*** *LoxP-GFP-STOP-LoxP-Cherry* double transgenic axolotls.

SI Materials and Methods

Molecular Cloning

1) pGEMT-*Sox2*-ORF-*T2A*-*Cherry*-PA: Targeting construct pGEMT-*Sox2*-ORF-*T2A*-*Cherry*-PA was cloned using Gibson assembly method from three individual PCR products, *Sox2*-ORFa, T2A-*Cherry*-PA and pGEMT-a. Axolotl *Sox2* open reading frame (*Sox2*-ORFa, without stop codon) was PCR-amplified with the primer pair Sox2-forward & Sox2-reverse1. Anchored-Oligo(dT)–primed cDNA prepared from axolotl spinal cord total RNA was used as template. *T2A*-*Cherry*-PA was PCR-amplified with the primer pair Cherry-forward & pA-reverse using a plasmid harboring *Cherry* and rabbit β-globin polyadenylation sequence (e.g. pCAGGS-*Cherry* plasmid) as template. The vector backbone pGEMT-a was PCR-amplified with primer pair pGEMT-forward & pGEMT-reverse using pGEMT vector (Promega, A3600) as template.

2) pGEMT-*Sox2*-ORF-*P2A-memCherry-T2A-ER^{T2}-Cre-ER^{T2}*-PA: Targeting construct pGEMT-*Sox2*-ORF-*P2A-memCherry-T2A-ER^{T2}-Cre-ER^{T2}*-PA was cloned using Gibson assembly method from four individual PCR products, *Sox2*-ORFb, *P2A*-mem*Cherry*, *T2A-ER^{T2}-Cre-ER^{T2}*-PA and pGEMT-a. Axolotl *Sox2* open reading frame (*Sox2*-ORFb, without stop codon) was PCR-amplified with the primer pair Sox2-forward & Sox2-reverse2, using cDNA prepared from axolotl spinal cord as template. *P2A*-mem*Cherry* was PCR-amplified with the primer pair memCherry-forward & Cherry-reverse using a plasmid harboring a *GAP43*-membrane-localization-signal tagged *Cherry* coding sequence (e.g. pCAGGS-*GAP43-Cherry* plasmid, the sequence is listed in the Dataset2) as template. *T2A-ER^{T2}-Cre-ER^{T2}*-PA was PCR-amplified with primer pair ER^{T2}-forward

& pA-reverse, using a plasmid harboring *ER^{T2}-Cre-ER^{T2}*-PA as template (1). The PCR product pGEMT-a vector backbone is identical as the one for cloning of pGEMT-*Sox2*-ORF-*T2A*-*Cherry*-PA plasmid.

3) Other targeting constructs: To obtain the targeting plasmids pGEMT-*Pax7*-ORF^{a}-*T2A*-*Cherry*-PA and pGEMT-*Pax7*-ORF^{b}-*P2A-memCherry-T2A-ER^{T2}-Cre-ER^{T2}*-PA, *Pax7* ORF were PCR-amplified with primer pairs Pax7-forward & Pax7-reverse using axolotl cDNA as template, enzymatically digested with MluI and SphI, and ligated into the pGEMT-*Sox2*-ORF-*T2A*-*Cherry*-PA and pGEMT-*Sox2*-ORF-*P2A-memCherry-T2A-ER^{T2}-Cre-ER^{T2}*-PA plasmids opened with the same enzymes (to remove *Sox2*-ORF).

List of primers for cloning:

Cherry-forward: 5' GAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGG

AGAATCCCGGCCCTATGGTGAGCAAGGGCGAGGAG

Cherry-reverse: 5' AGGGCCGGGATTCTCCTCCACGTCACCGCATGTTAGAAGAC

TTCCTCTGCCCTCCTTGTACAGCTCGTCCATGCC

memCherry-forward: 5' GGAAGCGGAGCTACTAACTTCAGCCTGCTGAAGCAGGC

TGGAGACGTGGAGGAGAACCCTGGACCTATGCTGTGCTGTATGAGAAGAAC

ER^{T2}-forward: 5' TGGAGGAGAATCCCGGCCCTATGGCCGGCGATATGAGAGCC

pA-reverse: 5' GATCTTCATAAGAGAAGAGGGACAG

Pax7-forward: CTATAGacgcgtATGGCTTGTCTCCCCGGAGCTG

Pax7-reverse: GCTTCCgcatgcGTATGCTTGTCCCGTCTCCAC

pGEMT-forward: 5' GTCCCTCTTCTCTTATGAAGATCTATTCTATAGTGTCACCT

AAATAGC

pGEMT-reverse: 5' GTCGGTCTCCATCATGCTGTACATacgcgtCTATAGTGAGTC

GTATTACAATTC

Sox2-forward: 5' ATGTACAGCATGATGGAGACCGAC

Sox2-reverse1: 5' TAGAAGACTTCCTCTGCCCTCgcatgcCATGTGCGAGAGGGGCA

GCGT

Sox2-reverse2: 5' GAAGTTAGTAGCTCCGCTTCCgcatgcCATGTGCGAGAGGGGCA

GCGT

gRNA targeting sites:

Pax7-gRNA#1: 5' GGTCCTGGCCGCATCATTCT

Pax7-gRNA#2: 5' GGGTAGTTCTGCCCTGGTCC

Pax7-gRNA#3: 5' GGGCAGAACTACCCACGGAC

Sox2-gRNA#3: 5' TACCAGAGCGCGCCCGTGCC

Sox2-gRNA#4: 5' GAGGGGCAGCGTGCCGTTGA

Sox2-gRNA#5: 5' GTGCCGGGCTCGTCCATCAA

Animal experiments. All axolotl experiments were performed in accordance with German animal laws. For the generation of gene knock-in axolotls, 5nl of a mixture of CAS9 protein (0.5 μg/μl), gRNA (0.2-0.4 μg/μl) and circular targeting plasmid (50 ng/μl) was injected into single cell stage fertilized eggs. Surviving axolotl embryos were raised in fresh tap water and fed daily. Prior to sample collection, amputation, transplantation or imaging, axolotls were anaesthetized in a solution of 0.01% ethyl-p-aminobenzoate

(Benzocaine, Sigma) in water. Normally, we examined reporter gene expression in atleast three individual F0 founders. If applicable, we also checked the F1 generation. We collected the limbs or tails from the strong transgenics for characterization. The ages of axolotls used in each experiment are listed in the respective figure legends. We bred our transgenic founders with white axolotls (d/d) for germ-line transmission assessment. Genotyping PCRs were carried out as described previously (2).

List of primers for genotyping:

P1: 5' CCAACTCCTCCCAAGAACTCTG

P2: 5' CAGCTTCACCTTGTAGATGAACTC

P3: 5' GATCTTCATAAGAGAAGAGGGACAG

P4: 5' GTACCTCACAAAAGACTGAAGTGAC

In-situ-hybridization. In-situ-hybridizations were carried out on 10-µm cryosections from axolotl tails as previously described (3). We used PCR products harbouring the T7 promoter as template for Dig-labelled antisense RNA probe synthesis by in vitro transcription.

List of primers for RNA probe synthesis:

Cherry-forward: 5' ATGGTGAGCAAGGGCGAGGAG

T7-Cherry-reverse: 5'

TTGAAATTAATACGACTCACTATAGGGCTTGTACAGCTCGTCCATGCC

Pax7-forward: 5' ATGGCTTGTCTCCCCGGAGCTG

T7-Pax7-reverse: 5'

TTGAAATTAATACGACTCACTATAGGGGTATGCTTGTCCCGTCTCCACAG

Sox2-forward: 5' ATGTACAGCATGATGGAGACCG

T7-Sox2-reverse: 5'

TTGAAATTAATACGACTCACTATAGGGCATGTGCGAGAGGGGCAGCGTGC

Immunohistochemistry. Amputated tail or limb tissue, or axolotl larva were immediately fixed in 3.7% formaldehyde prepared in MEM buffer overnight at 4ºC. For cryosections, tissues were cryopreserved in 30% sucrose and embedded in Tissue-Tek (O.C.T Compound, Sakura Finetek). 10-20 μm cryosections were collected for immunohistochemical analysis. All washing steps were carried out in PBST (0.3% Triton X-100 in PBS). The sections were blocked in blocking buffer (0.3% Triton X-100, 5% fetal calf serum in PBS) for 1 hour at room temperature, then incubated with primary antibodies overnight at 4ºC. After several intensive washes in PBST, secondary antibodies (Invitrogen, 1:500) and DAPI diluted in blocking buffer were applied to sections and incubated for 2 hours at room temperature. The slides were then washed several times in PBST and mounted with Mowiol medium. For PAX7 immunohistochemistry, we performed antigen retrieval by treating the samples in sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0) at 85 ºC for 10 minutes before the blocking step. If GFP fluorescence is present in the samples for antigen retrieval, we either applied GFP antibody to regain GFP signal, or MHC antibody to label muscle. For CHERRY fluorescence from the transgenic loci, we always use RFP antibody to enhance the signal. Whole-mount immunohistochemical staining on the fixed

axolotl larvae was carried out similarly as section staining. We kept the stained larvae in PBS for imaging.

List of primary antibodies:

Primary antibodies against the following antigens were used in this study, GFAP (goat polyclonal antibody, Abcam, ab53554, 1:1000), GFP (goat polyclonal antibody, MPI-CBG antibody facility, 1:1000), MHC (mouse monoclonal antibody, DSHB, 1:500), FITC-coupled MHC (self-made, 1:500), NEUN (mouse monoclonal antibody, Millipore, MAB377, 1:200), PAX7 (mouse monoclonal antibody, DSHB, 1:200), RFP (rabbit polyclonal antibody, Rockland, 600-401-379, 1:1000), RFP (Rat monoclonal antibody, Chromotek, 5f8-100, 1:500) and SOX2 (rabbit polyclonal antibody, 1:500) (4), TUJ-1 (mouse monoclonal antibody, R&D, MAB1195, 1:500).

SI References

1. Khattak S, et al. (2013) Germline Transgenic Methods for Tracking Cells and Testing Gene Function during Regeneration in the Axolotl. *Stem Cell Rep* 1(1):90-103.
2. Fei JF, et al. (2014) CRISPR-mediated genomic deletion of Sox2 in the axolotl shows a requirement in spinal cord neural stem cell amplification during tail regeneration. *Stem Cell Rep* 3(3):444-459.
3. Knapp D, et al. (2013) Comparative transcriptional profiling of the axolotl limb identifies a tripartite regeneration-specific gene program. *PloS one* 8(5):e61352.
4. Fei JF, et al. (2016) Tissue- and time-directed electroporation of CAS9 protein–gRNA complexes in vivo yields efficient multigene knockout for studying gene function in regeneration. *npj Regen Med* 1(1):16002.

SI Figures and Figure Legends

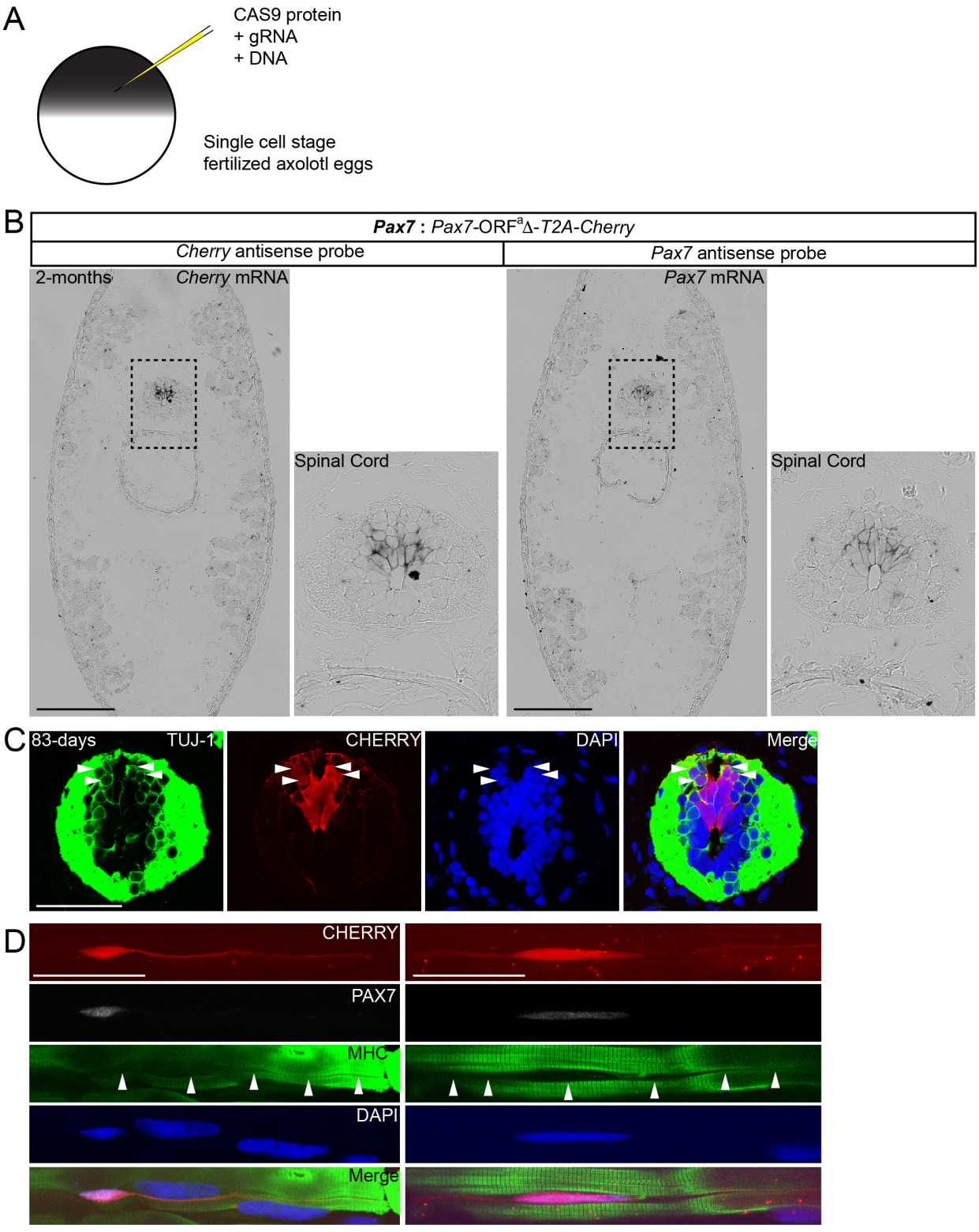

Fig. S1 Characterization of *Pax7*: *Pax7*-ORF^{a}Δ-T2A-*Cherry* knock-in axolotls.

(A) Scheme of axolotl egg injection.

(B) Correspondence between *Pax7* and *Cherry* mRNA expression in transgenic animals. *Cherry* (left panels) and *Pax7* (right panels) mRNA in situ hybridization on adjacent 10 μm tail cross sections of 2-month old F0 ***Pax7*:** *Pax7*-ORF^{a}Δ-T2A-*Cherry* axolotls using DIG-labeled antisense *Cherry* (left) and *Pax7* (right) probes shows close correspondence between signals. Boxed area shows higher zoom of the spinal cord. Scale bars, 200 μm.

(C) Protein localization shows dim CHERRY expression in a few newborn daughters reflecting protein perdurance. Immunofluorescence for TUJ-1 (green, to mark neurons), CHERRY (red) combined with DAPI (blue) on 10 μm tail cross-cryosections of 83-day old ***Pax7*:** *Pax7*-ORF^{a}Δ-*T2A-Cherry* knock-in F0 axolotls. Arrowheads indicate the few TUJ-1 and CHERRY double positive dorsal spinal cord cells. Scale bar, 100 μm.

(D) Longitudinal sections of muscle show elongated morphology of CHERRY-expressing, PAX7^{+} satellite cells. Immunofluorescence for CHERRY (red), PAX7 (white), MHC (green, to mark muscle fibers) combined with DAPI (blue) on 10 μm limb longitudinal-cryosections of around 6-month old ***Pax7*:** *Pax7*-ORF^{a}Δ-*T2A-Cherry* knock-in F0 axolotls. Note the axolotl PAX7^{+} satellite cells extend their elongated processes along MHC-positive muscle fibers. Arrowheads indicate the interspace of muscle fibers. Scale bars, 50 μm.

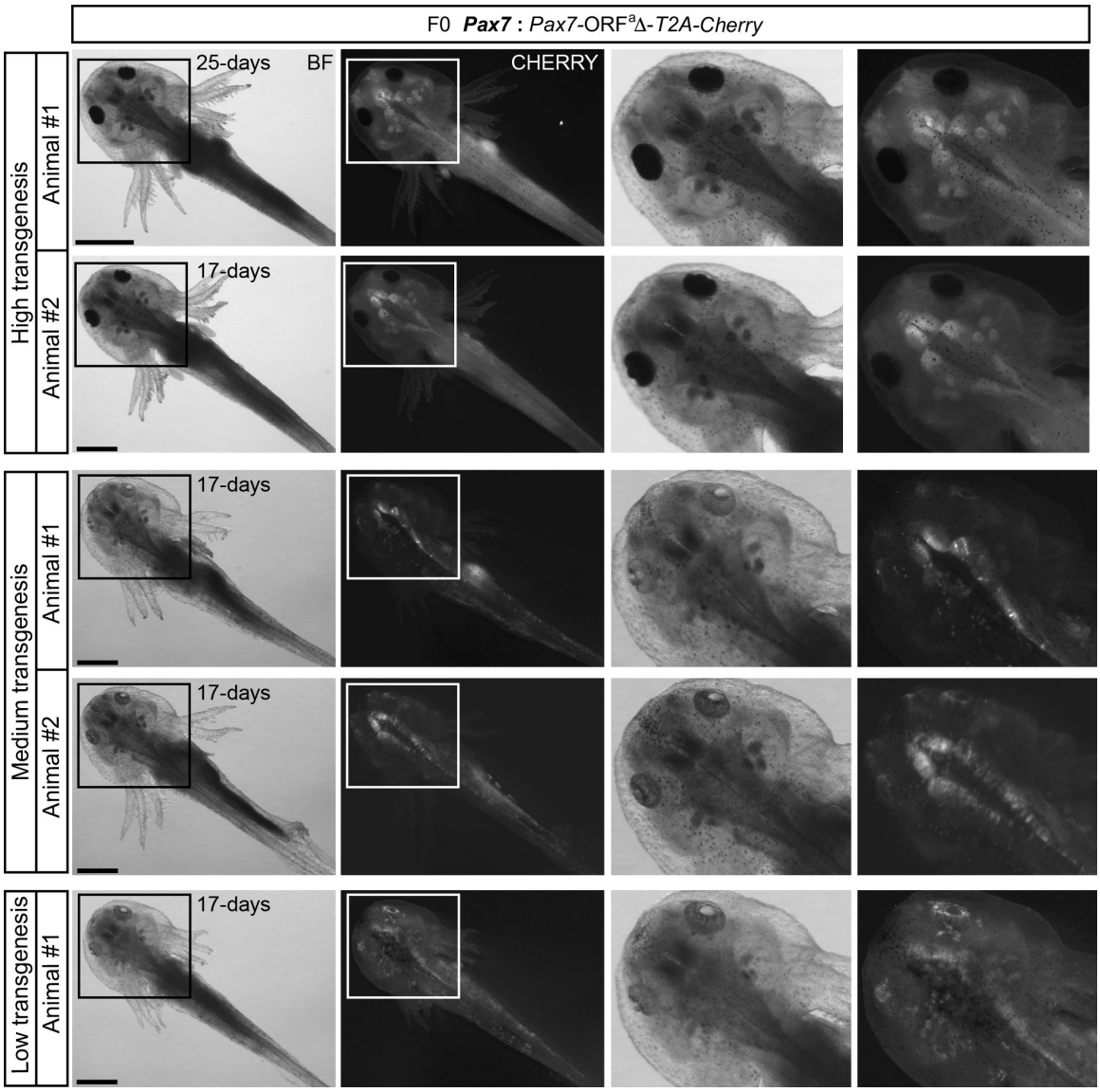

Fig. S2 Phenotypic evaluation of the transgene insertion efficiency in F0 *Pax7*: *Pax7*-ORF^{a}Δ-T2A-*Cherry* knock-in axolotls.

Bright field (BF) and CHERRY fluorescence images of 17- or 25-day old ***Pax7*:** *Pax7*-ORF^{a}Δ-*T2A-Cherry* knock-in F0 axolotls. The rectangular areas are shown at higher magnification. The efficiency of the transgene integration is evaluated by the uniformity and level of the CHERRY expression in the expected PAX7-expressing domains. Scale bars, 200 μm.

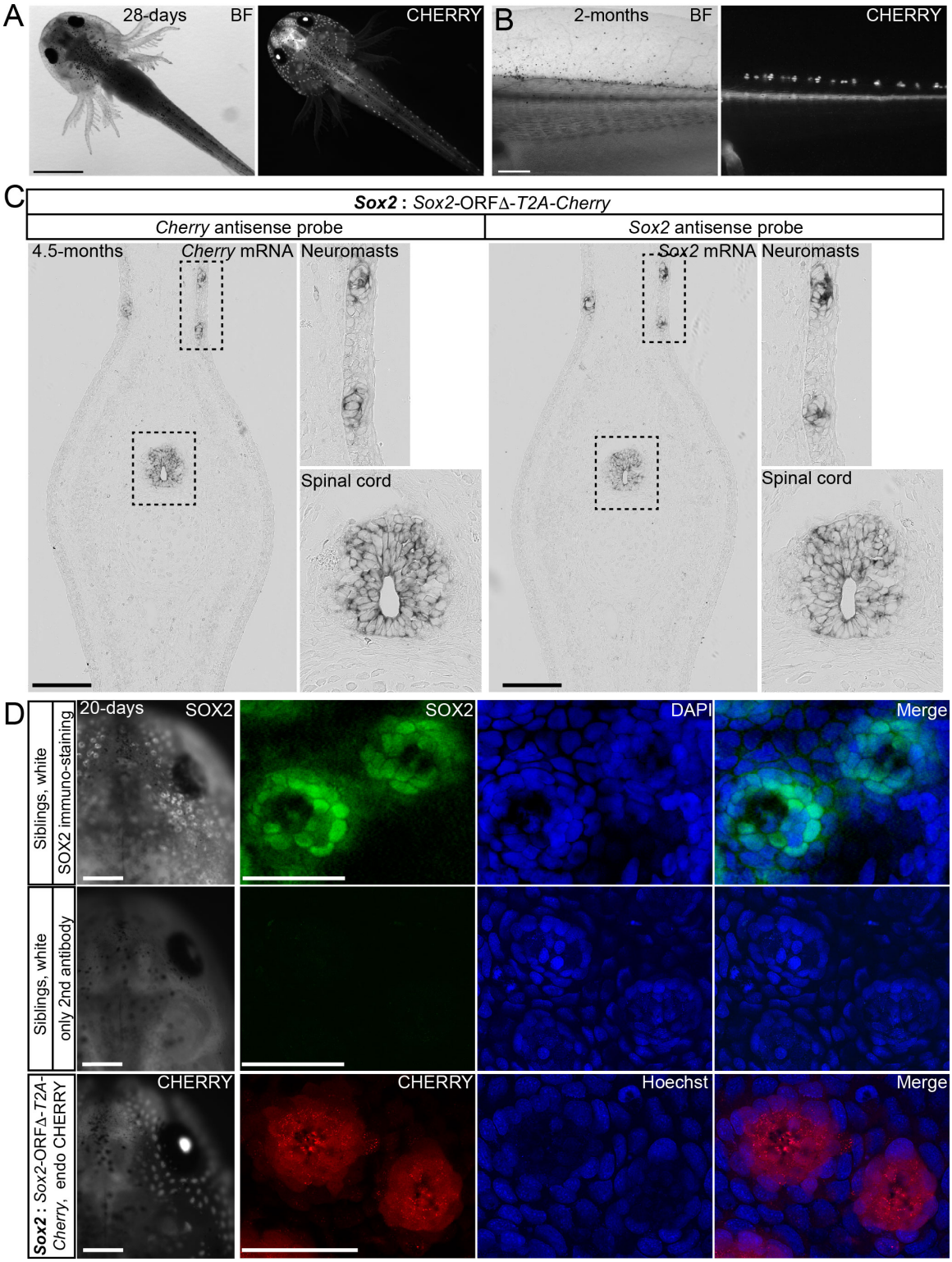
A
28-days
BF
CHERRY
B
2-months
BF
CHERRY
C
Sox2 : Sox2-ORFΔ-T2A-Cherry
Cherry antisense probe
Sox2 antisense probe
4.5-months
Cherry mRNA
Neuromasts
Spinal cord
Sox2 mRNA
Neuromasts
Spinal cord
D
Siblings, white
SOX2 immuno-staining
20-days
SOX2
SOX2
DAPI
Merge
Siblings, white
only 2nd antibody
Sox2 : Sox2-ORFΔ-T2A-Cherry, endo CHERRY
CHERRY
CHERRY
Hoechst
Merge

Fig. S3 Characterization of *Sox2*: *Sox2*-ORFΔ-T2A-*Cherry* knock-in axolotls.

(A and B) Wholemount, bright field (BF) and CHERRY fluorescence images of 28-day old (A) and 2-month old (B) live ***Sox2*:** *Sox2*-ORFΔ-*T2A*-*Cherry* knock-in F1 axolotls. Note the CHERRY expression pattern in ***Sox2*:** *Sox2*-ORFΔ-*T2A*-*Cherry* F1 and F0 (Fig. 1I-K') axolotls is indistinguishable. Scale bar, 2 mm in (A), 1 mm in (B).

(C) Correspondence between *Sox2* and *Cherry* mRNA expression in transgenic animals. *Cherry* (left panels) and *Sox2* (right panels) mRNA in situ hybridization on adjacent 10 μm tail cross sections of 4.5-month old F1 ***Sox2*:** *Sox2*-ORFΔ-T2A-*Cherry* axolotls using DIG-labeled antisense *Cherry* and *Sox2* probes. Note the correspondence of expression pattern of *Cherry* and *Sox2* mRNA in the spinal cord and lateral line neuromasts in ***Sox2*:** *Sox2*-ORFΔ-T2A-*Cherry* axolotls. Scale bars, 200 μm.

(D) SOX2 and CHERRY expression in the head lateral line neuromasts. Top: Whole mount immunostaining for SOX2 (white and green in top panels), and the control stained with only secondary antibody (middle panels) in 20-day old non-transgenic axolotls. Bottom panels: CHERRY expression in the head lateral line neuromasts from the 20-day old ***Sox2*:** *Sox2*-ORFΔ-*T2A*-*Cherry* knock-in F1 axolotl resembles the pattern of SOX2-immunostaining seen in the top panels. Scale bars, 500 μm in (left panels), 100 μm in (right panels).

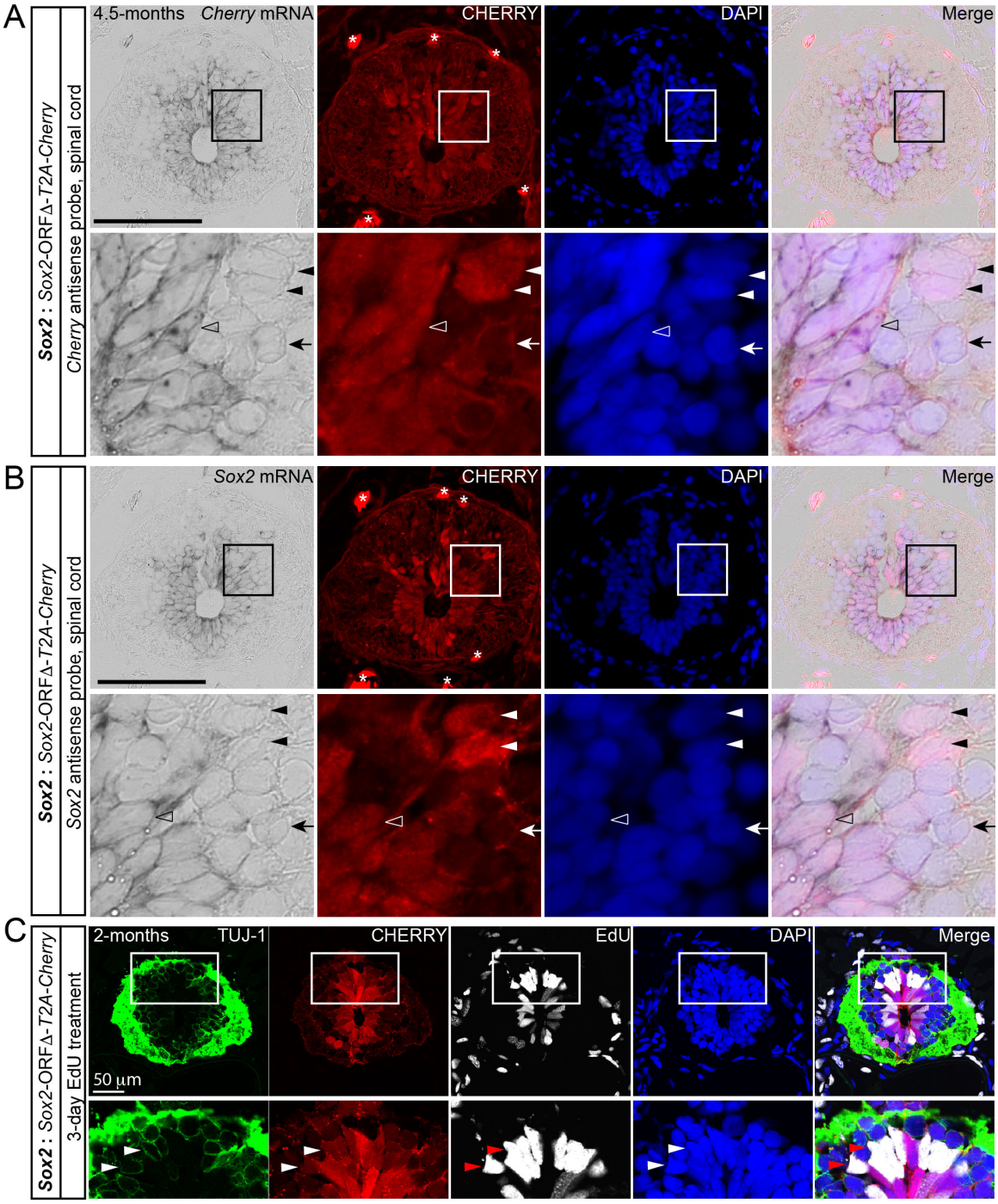
A
Sox2 : Sox2-ORFΔ-T2A-Cherry
Cherry antisense probe, spinal cord
4.5-months Cherry mRNA
CHERRY
DAPI
Merge
B
Sox2 : Sox2-ORFΔ-T2A-Cherry
Sox2 antisense probe, spinal cord
Sox2 mRNA
CHERRY
DAPI
Merge
C
Sox2 : Sox2-ORFΔ-T2A-Cherry
3-day EdU treatment
2-months
TUJ-1
CHERRY
EdU
DAPI
Merge
50 μm

Fig. S4 Analysis of CHERRY protein inheritance into the differentiated progeny of CHERRY-labeled stem cells in *Sox2*: *Sox2*-ORFΔ-T2A-*Cherry* knock-in axolotls.

(A and B) *Cherry* (A) and *Sox2* (B) mRNA in situ hybridization, using DIG-labeled antisense *Cherry* (A) or *Sox2* (B) probes, followed by immunostaining for CHERRY (red) and DAPI staining (blue) on adjacent 10 μm tail cross sections (showing spinal cord) of 4.5-month old F1 ***Sox2*:** *Sox2*-ORFΔ-T2A-*Cherry* axolotls. Rectangular regions are shown at higher magnification. Empty arrowhead, *Cherry* (A) or *Sox2* (B) mRNA positive cells that express CHERRY protein. Arrows point to cells that are negative for *Cherry* (A) or *Sox2* (B) transcripts and also negative for CHERRY protein. Solid arrowheads, *Cherry* (A) or *Sox2* (B) mRNA negative cells that express CHERRY protein. Asterisks indicate the unspecific red fluorescent signals, perhaps the blood vessels. Scale bars, 200 μm.

(C) Birthdating of new neurons shows CHERRY protein is inherited in newborn neurons. Immunofluorescence for TUJ-1 (green) to mark neurons, CHERRY (red) combined with EdU (white) and DAPI (blue) staining on 10 μm tail cross-cryosections (showing spinal cord) of 2-month old F1 ***Sox2*:** *Sox2*-ORFΔ-T2A-*Cherry* axolotls treated with EdU for 3-days. Rectangular regions are shown at higher magnification. Arrowheads indicate TUJ-1, CHERRY and EdU triple-positive newborn neurons. Scale bar, 50 μm.

Fig. S5 Genomic PCR and sequence analysis of *Sox2*: *Sox2*-ORFΔ-T2A-*Cherry* knock-in axolotls.

(A) Genomic PCR. Around 2.8-kb PCR product from ***Pax7*:** *Pax7*-ORF[a]Δ-T2A-*Cherry* knock-in axolotls (#1-4) and control (Ctr) using the primers P1 and P3 (Fig. 1A iii).

(B) The 5' integration junctions in ***Pax7*:** *Pax7*-ORF[a]Δ-T2A-*Cherry* knock-in axolotls. Note: the in-frame integration junctions in the limb (limb junct) and tail (tail junct) from the same individuals are identical in three different knock-in axolotls, animal #1, #2 and #4.

(C) The 5' integration junction in a ***Sox2*:** *Sox2*-ORFΔ-T2A-*Cherry* knock-in axolotl. A six-nucleotide deletion has occurred at the integration junction from the analysis of tail tissue.

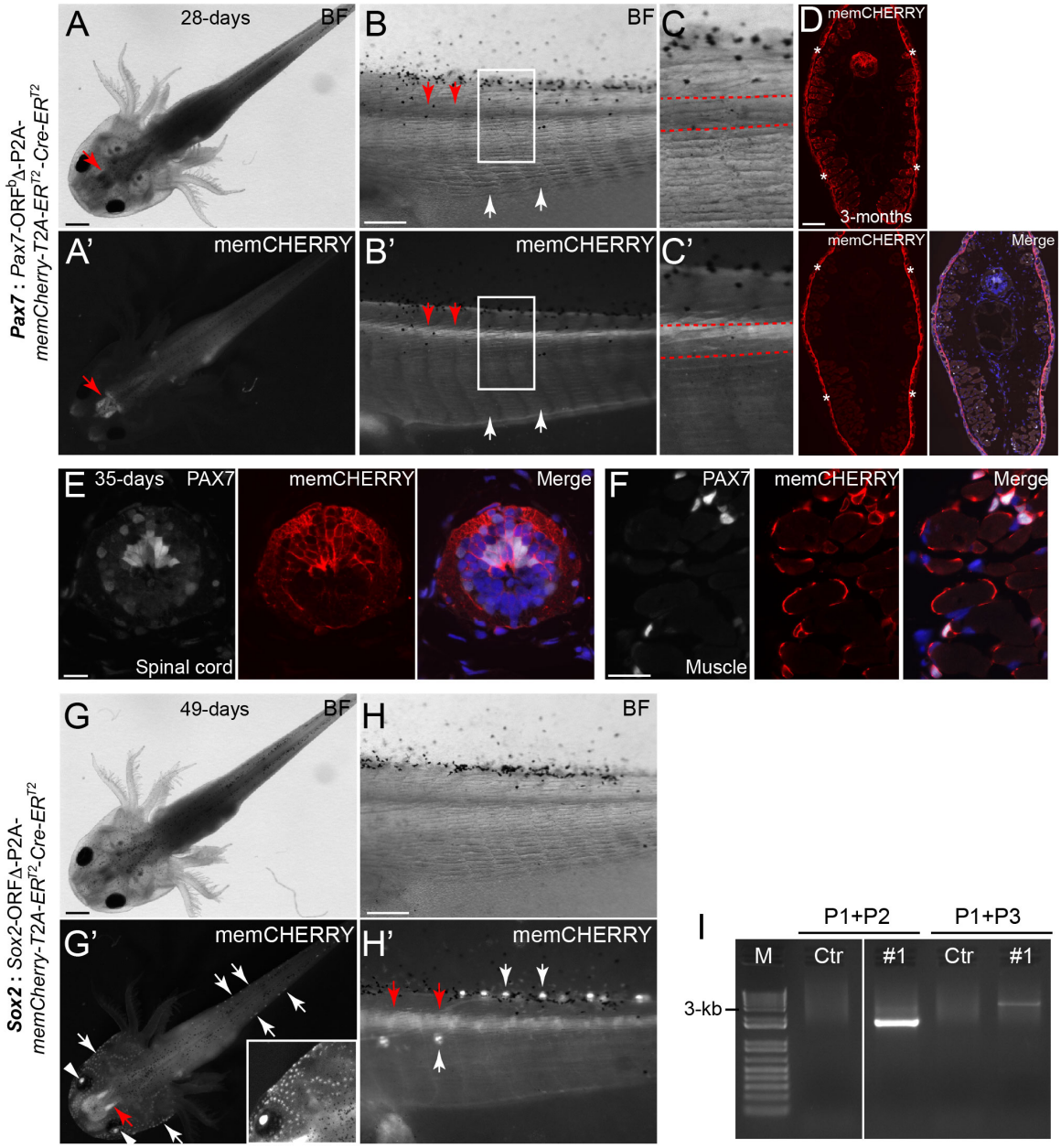
Pax7 : Pax7-ORFΔ-P2A-memCherry-T2A-ERT2-Cre-ERT2
A
28-days
BF
B
BF
C
D
memCHERRY
3-months
A'
memCHERRY
B'
memCHERRY
C'
memCHERRY
Merge
E
35-days
PAX7
memCHERRY
Merge
Spinal cord
F
PAX7
memCHERRY
Merge
Muscle
Sox2 : Sox2-ORFΔ-P2A-memCherry-T2A-ERT2-Cre-ERT2
G
49-days
BF
H
BF
G'
memCHERRY
H'
memCHERRY
I
P1+P2
P1+P3
M
Ctr
#1
Ctr
#1
3-kb
J
Pax7-gRNA#3 target PAM
Pax7 bait 5' CCCCAGAATGATGCGGCCAGGACCA GGGCAGAACTACCCACGGAC CGGTTTCCCACTAGAAGTGTCCACTCCTCT 3'
#1 tail junct 5' CCCCAGAATGATGCGGCCAGGACCA GGGCAGAACTACCCACGT-- -GGTTTCCCACTAGAAGTGTCCACTCCTCT 3'
K
Sox2-gRNA#5 target PAM
Sox2 bait 5' GTCGCAGCACTACCAGAGCGCGCCC GTGCCGGGCTCGTCCATCAA CGGCACGCTGCCCCTCTCGCACATG 3'
Tail junct 5' GTCGCAGCACTACCAGAGCGCGCCC GTGCCGGGCTCGTC------ ---CACGCTGCCCCTCTCGCACATG 3'

Fig. S6 Knock-in of a large gene cassette into axolotl genomic loci through CRISPR/Cas9 mediated homology-independent integration.

(A-C') Bright field (BF, upper panels) and memCHERRY fluorescence (lower panels) images of 28-day old ***Pax7*****:** *Pax7*-ORF^{b}Δ-*P2A-memCherry-T2A-ER^{T2}-Cre-ER^{T2}* knock-in F1 axolotls. Dorsal (A, A') and lateral (B-C') view images highlight the memCHERRY expression in the brain (A, A', arrows), the spinal cord (B, B', red arrows) and the trunk muscle (B, B', white arrows) compartments. Rectangular regions are shown at higher-magnification in (C, C') showing that the memCHERRY expression is restricted to the dorsal domain of the spinal cord (red dashed lines). Scale bars, 1 mm in (A), 500 μm in (B).

(D) Expression of the memCHERRY in dorsal spinal cord and satellite cells in 3-month old ***Pax7*****:** *Pax7*-ORF^{b}Δ-*P2A-memCherry-T2A-ER^{T2}-Cre-ER^{T2}* F0 transgenic animals. Immunofluorescence for memCHERRY (red) on 10 μm tail cross-cryosections of 3-month old transgenic (top) and control non-transgenic axolotls showing specific memCHERRY immunostaining in the dorsal spinal cord and in muscle satellite cells of the transgenic, and non-specific staining of the dermal matrix in both types of animals (asterisks). PAX7-immunostaining of the control sample (white) shows the native PAX7 expression domain in the dorsal spinal cord and muscle satellite cells. Scale bars, 200 μm.

(E and F) High magnification views of memCHERRY immunostaining in the spinal cord (E) and in muscle satellite cells (E). memCHERRY signal encases PAX7^{+} nuclei. Immunofluorescence for PAX7 (white), memCHERRY (red) combined with DAPI (blue)

staining on 10 μm tail cross-cryosections of 35-day old ***Pax7*****:** *Pax7*-ORF^{b}Δ-*P2A-memCherry-T2A-ER^{T2}-Cre-ER^{T2}* knock-in F1 axolotls. Scale bars, 50 μm.

(G-H') Expression of memCHERRY in ***Sox2*****:** *Sox2*-ORFΔ-P2A-*memCherry*-T2A-*ER^{T2}-Cre-ER^{T2}* transgenic animals. Bright field (BF, upper panels) and memCHERRY fluorescence (lower panels) images of 49-day old ***Sox2*****:** *Sox2*-ORFΔ-P2A-*memCherry-T2A-ER^{T2}-Cre-ER^{T2}* knock-in F0 axolotls. The dorsal (G, G') and lateral (H, H') view images highlight the memCHERRY expression in the brain (G', red arrow), the lens (G', arrowheads), the spinal cord (H', red arrows) and the tail lateral line neuromasts (white arrows). The head region is shown at higher magnification in the inset. Scale bars, 1 mm in (G), 500 μm in (H).

(I) Genomic PCR analysis of ***Pax7*****:** *Pax7*-ORF^{b}Δ-*P2A-memCherry-T2A-ER^{T2}-Cre-ER^{T2}* knock-in axolotl tissue shows bands at 2.0-kb and 5.3-kb from knock-in axolotls (#1) but not control (Ctr) animals, when using the primer pairs P1 and P2 (P1+P2), or P1 and P3 (P1+P3) for PCR (Fig. 2A iii).

(J and K) Representative 5' junction sequences upon the integration of the *ER^{T2}-Cre-ER^{T2}* targeting constructs into the axolotl *Pax7* (J) or *Sox2* (K) genomic loci. Red characters, gRNA binding sites; Underlined characters, protospacer adjacent motif (PAM).

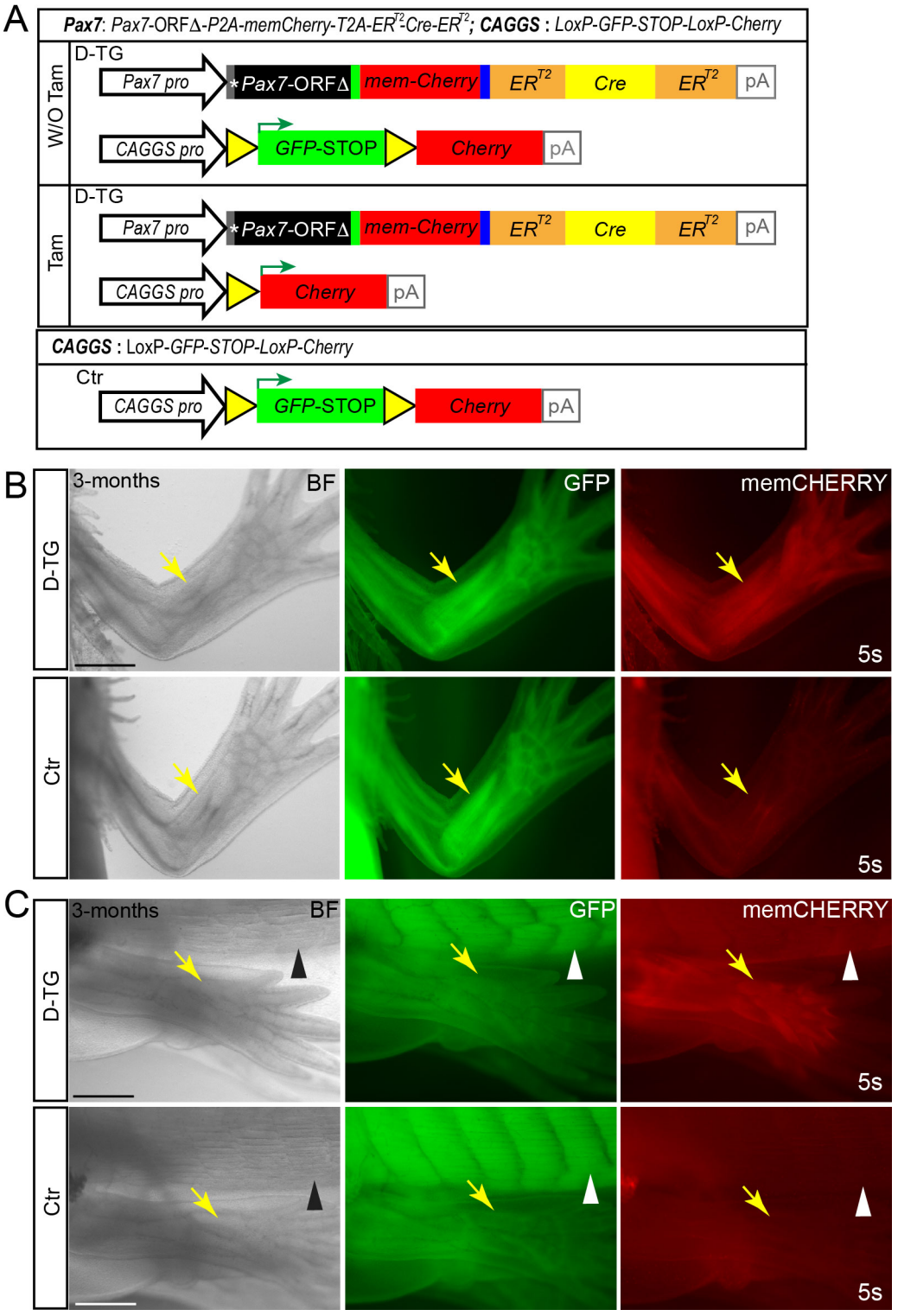
A
Pax7: Pax7-ORFΔ-P2A-memCherry-T2A-ER^T2-Cre-ER^T2; CAGGS : LoxP-GFP-STOP-LoxP-Cherry
W/O Tam
D-TG
Pax7 pro
*Pax7-ORFΔ
mem-Cherry
ER^T2
Cre
ER^T2
pA
CAGGS pro
GFP-STOP
Cherry
pA
Tam
D-TG
Pax7 pro
*Pax7-ORFΔ
mem-Cherry
ER^T2
Cre
ER^T2
pA
CAGGS pro
Cherry
pA
CAGGS : LoxP-GFP-STOP-LoxP-Cherry
Ctr
CAGGS pro
GFP-STOP
Cherry
pA
B
D-TG
3-months
BF
GFP
memCHERRY
5s
Ctr
5s
C
D-TG
3-months
BF
GFP
memCHERRY
5s
Ctr
5s

Fig. S7 Generation and characterization of *Pax7*: *Pax7*-ORF^{b}Δ-*P2A-memCherry-T2A-ER^{T2}-Cre-ER^{T2}*; *CAGGS: LoxP-GFP-STOP-LoxP-Cherry* double transgenic axolotls.

(A) Scheme of double transgenic animals generated for labeling and lineage tracing of satellite cells. Prior to tamoxifen treatment the *Cherry* gene driven by *CAGGS* promoter is silent. After tamoxifen (Tam) treatment, *Cherry* gene is switched on specifically in PAX7-positive satellite cells in double transgenic (D-TG) axolotls. Single transgenic ***CAGGS*:** *LoxP-GFP-STOP-LoxP-Cherry* axolotls are used as control (Ctr).

(B and C) Dim memCHERRY signal in wholemount images of double transgenic limbs. Bright field (BF), GFP and memCHERRY fluorescence limb images of the forelimb (B) and hind limb (C) region of the 3-month old double transgenic (D-TG, upper panels) and control (Ctr, lower panels) axolotls. Arrows indicate the limb muscle compartments; Arrow heads indicate the tail muscles. Exposure time for CHERRY, 5 s. Scale bars, 1 mm.

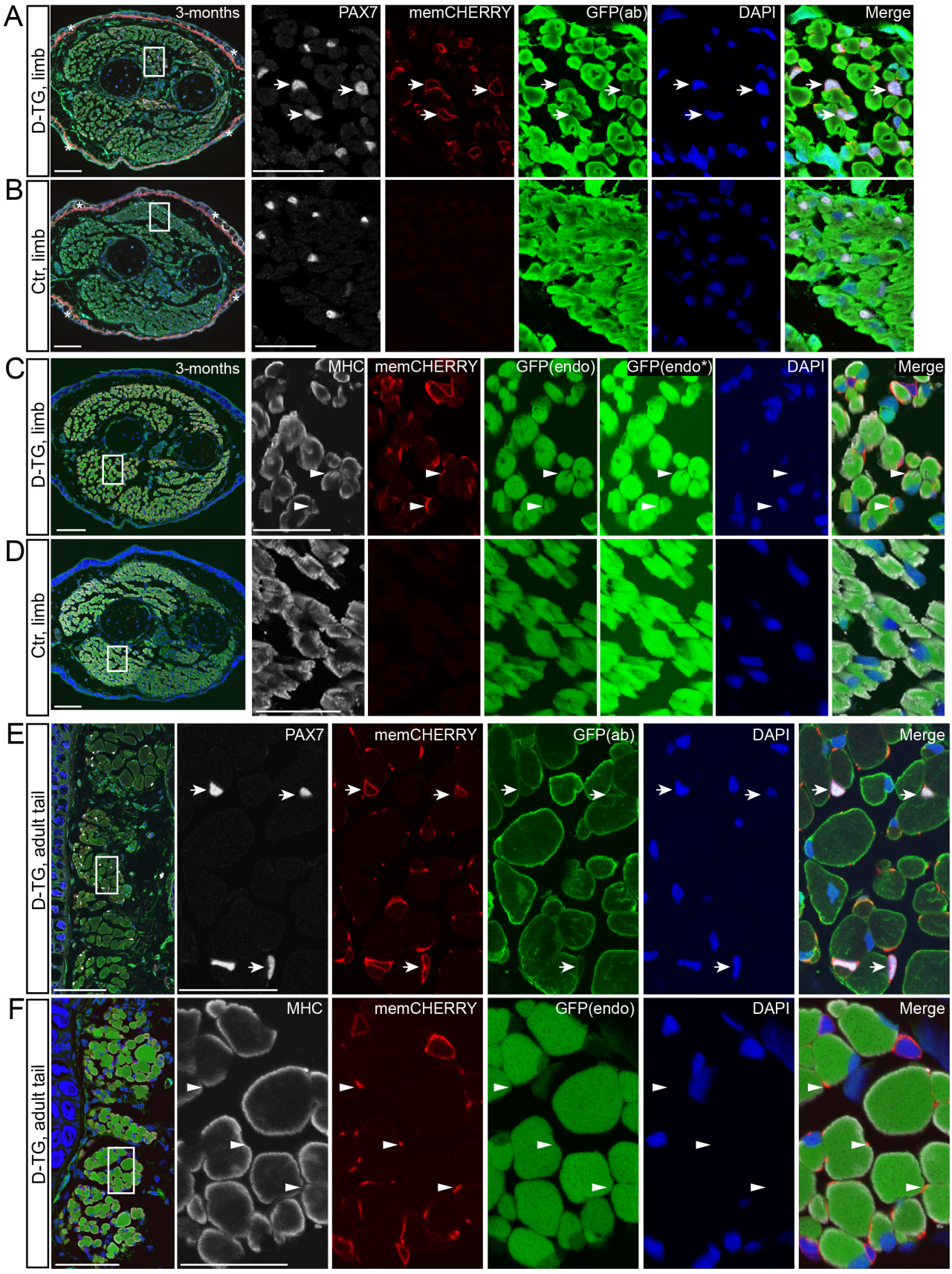
A
D-TG, limb
3-months
PAX7
memCHERRY
GFP(ab)
DAPI
Merge
B
Ctr, limb
C
D-TG, limb
3-months
MHC
memCHERRY
GFP(endo)
GFP(endo*)
DAPI
Merge
D
Ctr, limb
E
D-TG, adult tail
PAX7
memCHERRY
GFP(ab)
DAPI
Merge
F
D-TG, adult tail
MHC
memCHERRY
GFP(endo)
DAPI
Merge

Fig. S8 Absence of recombination prior to Tamoxifen treatment in double transgenic *Pax7*: *Pax7*-ORF^{b}Δ-*P2A-memCherry-T2A-ER^{T2}-Cre-ER^{T2}*; *CAGGS: Loxp-GFP-STOP-LoxP-Cherry* axolotls.

(A-D) Comparison of membrane-tagged CHERRY (memCHERRY) expression in limbs of 3-month old double transgenic (D-TG) axolotls (A and C) versus control (Ctr) single transgenic *LoxP* reporters (B and D).

(E and F) show memCHERRY expression in tail tissue from sexually mature, double transgenic animals.

Immunofluorescence for CHERRY shows that the membrane tagged CHERRY (memCHERRY from *Cre*-driver cassette, red) signal surrounds PAX7+ nuclei (white, in A and E), consistent with membrane localization to PAX7+ cells. The ring-shaped or dotted memCHERRY signal does not overlap with Myosin Heavy Chain (MHC)-labeled muscle fibers (white, in C and F). Note that the cytoplasmic CHERRY expression is not detected in MHC-positive muscle fibers (C and F). memCHERRY expression is not detectable in controls (B and D). This confirms the specific memCHERRY expression in satellite cells and lack of ectopic *Cherry* conversion in double transgenic axolotls prior to tamoxifen administration.

Arrows indicate memCHERRY^{+}/DAPI^{+}/PAX7^{+} satellite cells; Arrowheads point to memCHERRY signal associated with satellite cell processes (see Fig. S1D and S9). GFP panels (green) show the expression from the unconverted *LoxP* reporter. The ubiquitous *CAGGS* promoter drives strong GFP expression in muscle and weaker expression in satellite cells (arrows). (ab): GFP signal was amplified by antibody staining; (endo): unamplified endogenous GFP signal. Asterisk (in C) indicates the GFP images with

higher (saturated) exposure, in order to show the dim-GFP labeled satellite cells. Asterisks (in A and B) indicate red signal observed in dermis, representing non-specific antibody staining with a different lot of anti-CHERRY antibody than in Figure S8C-F. The areas depicted by rectangles in the left panels are shown as separate or merged images at higher-magnification in right panels. Scale bars, 200 μm in (left panels), 50 μm in (right panels).

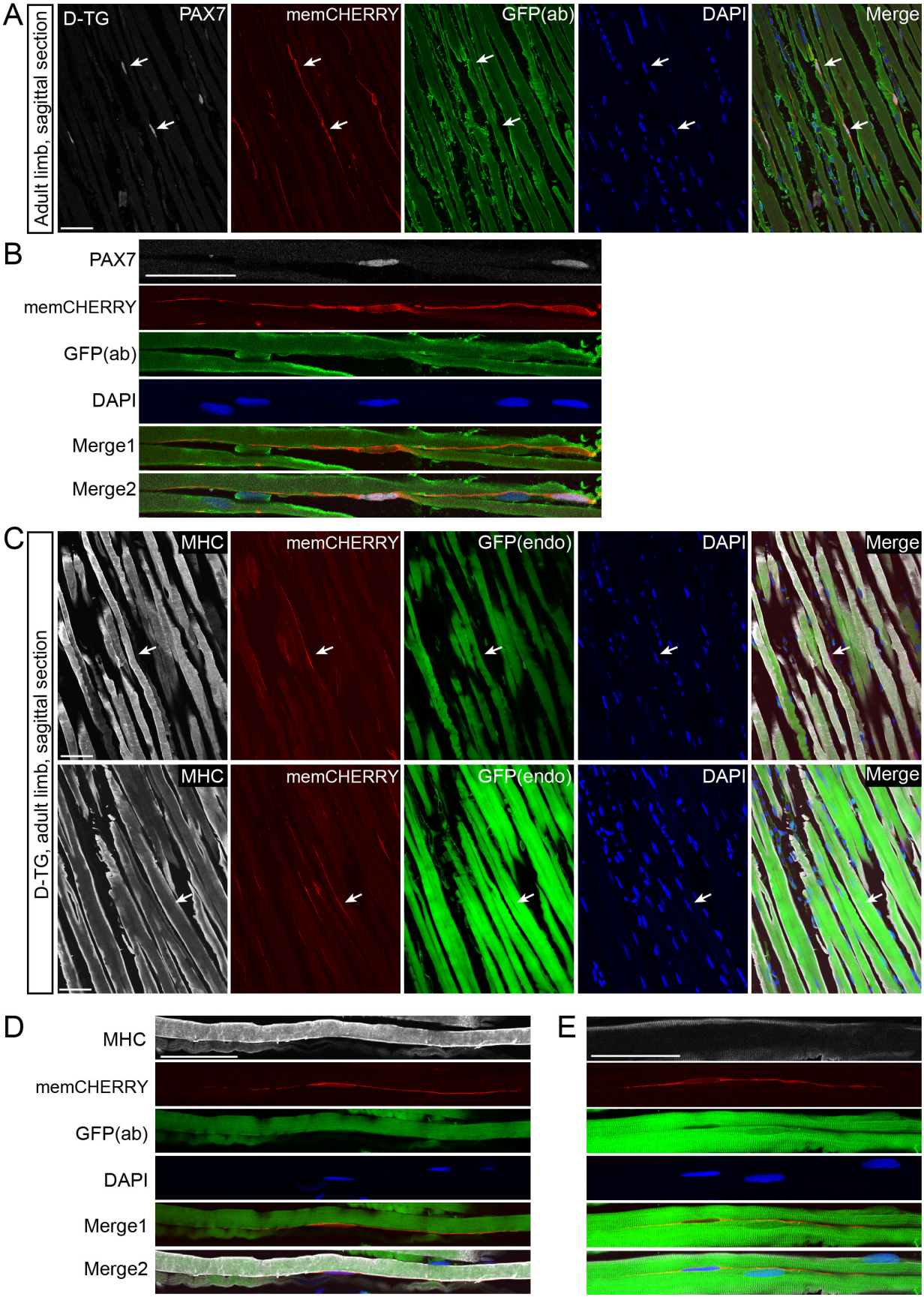
A
Adult limb, sagittal section
D-TG
PAX7
memCHERRY
GFP(ab)
DAPI
Merge
B
PAX7
memCHERRY
GFP(ab)
DAPI
Merge1
Merge2
C
D-TG, adult limb, sagittal section
MHC
memCHERRY
GFP(endo)
DAPI
Merge
D
MHC
memCHERRY
GFP(ab)
DAPI
Merge1
Merge2
E

Fig. S9 memCHERRY expression is restricted to elongated satellite cells prior to Tamoxifen treatment in double transgenic *Pax7*: *Pax7*-ORF^{b}Δ-*P2A-memCherry-T2A-ER^{T2}-Cre-ER^{T2}*; *CAGGS: Loxp-GFP-STOP-LoxP-Cherry* axolotls.

(A and B) Longitudinal (Sagittal) sections of limb muscle immunofluorescence for PAX7 (white), CHERRY (red), GFP (green, antibody staining (ab)) combined with DAPI (blue) of adult ***Pax7*:** *Pax7*-ORF^{b}Δ-*P2A-memCherry-T2A-ER^{T2}-Cre-ER^{T2}*; ***CAGGS:*** *Loxp-GFP-STOP-LoxP-Cherry* double transgenic (D-TG) axolotl without tamoxifen-treatment. The area depicted by arrows (in A) is shown as separated or merged images at higher-magnification (in B). Note that satellite cells have elongate processes that extend between muscle fibers. Scale bars, 100 μm.

(C-E) Immunofluorescence for MHC (white), CHERRY (red), endogenous (endo) GFP fluorescence combined with DAPI (blue) on 10 μm tail longitudinal (sagittal)-cryosections (showing limb muscle) of adult ***Pax7*:** *Pax7*-ORF^{b}Δ-*P2A-memCherry-T2A-ER^{T2}-Cre-ER^{T2}*; ***CAGGS:*** *Loxp-GFP-STOP-LoxP-Cherry* double transgenic (D-TG) axolotl without tamoxifen-treatment. The areas depicted by arrows (in C, upper panels and lower panels) are shown as separated or merged images at higher-magnification in (D and E), respectively. Note that the elongated processes of memCHERRY-expressing PAX7-positive, MHC-negative satellite cells lie between the muscle fibers. No CHERRY expression is detected in the muscle fibers. Scale bars, 100 μm.

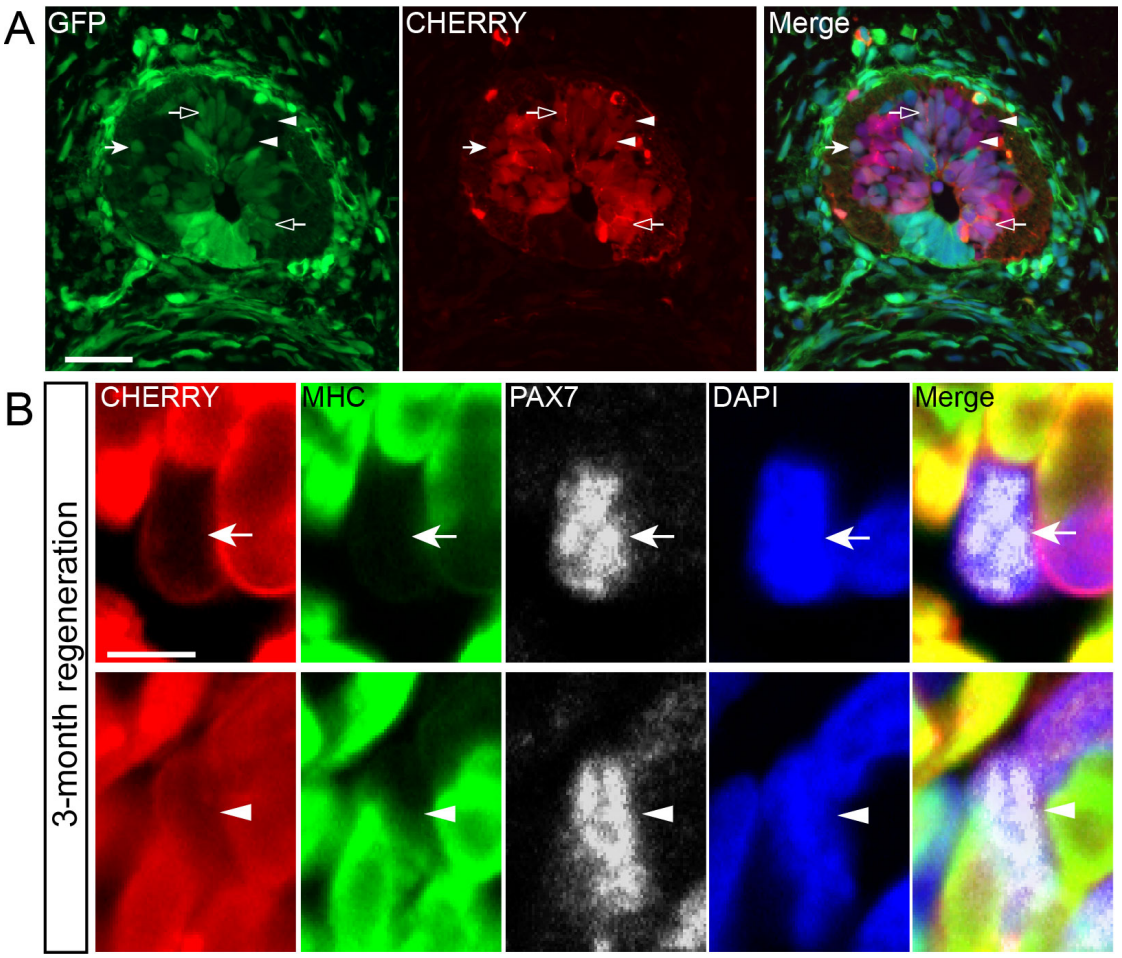

Fig. S10 Some cells display partial-conversion or no conversion of tandemly arrayed *LoxP* reporter cassettes after *Cre* induction

(A) The presence of GFP in subsets of CHERRY-converted spinal cord cells in ***CAGGS:*** *Loxp-GFP-STOP-LoxP-Cherry* reporter after *Cre*-mediated conversion (1.5-months). Solid arrow indicates CHERRY and GFP double positive neuron; Empty arrows indicate CHERRY and GFP double positive spinal cord ependymal cells; Arrowheads indicate CHERRY-positive GFP-negative cells. Scale bar, 100 μm.

(B) Unconverted (memCHERRY only, arrow in top panels) and converted (cytoplasmic CHERRY, arrowhead in lower panels) PAX7-positive (white), MHC-negative (green)

satellite cells on cross sections of 3-month regenerated double transgenic limb post Tamoxifen treatment. Scale bar, 20 μm.

Table S1. Evaluation of gRNA mediated genomic DNA cleavage activity

gRNAs efficiency (%)	Modification at the gRNA targets
Pax7-gRNA#1 30.8% (12/39)	wt 5' GGGTAGTTCTGCCCT GGTCCTGGCCGCATCATTCT GGGGACAGCTCCGGG 27x 5' GGGTAGTTCTGCCCT GGTCCTGGCCGCATCATTCT GGGGACAGCTCCGGG 2x 5' GGGTAGTTCTGCCCT GGTCCTGGCCGCATCATTC- -GGGACAGCTCCGGG 1x 5' GGGTAGTTCTGCCCT GGTCCTGGCCGCATCAT--- GGGGACAGCTCCGGG 1x 5' GGGTAGTTCTGCCCT GGTCCTGGCCGCATCA---- -GGGACAGCTCCGGG 1x 5' GGGTAGTTCTGCCCT GGTCCTGGCCGCATCAT--- --GGACAGCTCCGGG 1x 5' GGGTAGTTCTGCCCT GGTCCTGGCCGCATC----- -GGGACAGCTCCGGG 1x 5' GGGTAGTTCTGCCCT GGTCCTGGCCGCATC----t --GGACAGCTCCGGG 1x 5' GGGTAGTTCTGCCCT GGTCCTGGCCGCATC----- GGGGACAGCTCCGGG 1x 5' GGGTAGTTCTGCCCT GGTCCTGGCCGCAT------ -GGGACAGCTCCGGG 1x 5' GGGTAGTTCTGCCCT GGTCCTGGCC---------- --GGACAGCTCCGGG 1x 5' GGGTAGTTCTGCCCT GGTCCTGGCCGCAT-ac--- GGGGACAGCTCCGGG 1x 5' GGGTAGTTCTGCCCT GGTCCTGGCCGCATC----- ---GACAGCTCCGGG
Pax7-gRNA#2 76.3% (29/38)	wt 5' GGGAAACCGGTCCGT GGGTAGTTCTGCCCTGGTCC TGGCCGCATCATTCT 9X 5' GGGAAACCGGTCCGT GGGTAGTTCTGCCCTGGT-- TGGCCGCATCATTCT 1X 5' GGGAAACCGGTCCGT GGGTAGTTCTGCCCTGGTCC -GGCCGCATCATTCT 1X 5' GGGAAACCGGTCCGT GGGTAGTTCTGCCCTGGTC- --GCCGCATCATTCT 2X 5' GGGAAACCGGTCCGT GGGTAGTTCTGCCCTGG--- TGGCCGCATCATTCT 4X 5' GGGAAACCGGTCCGT GGGTAGTTCTGCCCTG---- --GCCGCATCATTCT 1X 5' GGGAAACCGGTCCGT GGGTAGTTCTGCCCT----- TGGCCGCATCATTCT 1X 5' GGGAAACCGGTCCGT GGGTAGTTCTGCCCT----- -GGCCGCATCATTCT 1X 5' GGGAAACCGGTCCGT GGGTAGTTCTGCCC------ -GGCCGCATCATTCT 2X 5' GGGAAACCGGTCCGT GGGTAGTTCTGCC------- TGGCCGCATCATTCT 3X 5' GGGAAACCGGTCCGT GGGTAGTTCTGCC------- --GCCGCATCATTCT 1X 5' GGGAAACCGGTCCGT GGGTAGTTCTGCCCTGG--- -----GCATCATTCT 1X 5' GGGAAACCGGTCCGT GGGTAGTTCTGCCCT----- ----CGCATCATTCT 1X 5' GGGAAACCGGTCCGT GGGTAGTTCT---------- --GCCGCATCATTCT 3X 5' GGGAAACCGGTCCGT GGGTA--------------- -GGCCGCATCATTCT 1X 5' GGGAAAC-------- --------------------------CATCATTCT 1X 5' GGGAAACCGGTCCGT GGGTAGTTCTG--------- -GGCCGCATCATTCT 2X 5' GGGAAACCGGTCCGT GGGTAGTTCTGCC-gct--- TGGCCGCATCATTCT 1X 5' GGGAAACCGGTCCGT GGGTAGTTCTGCCCTGG-aa ----CGCATCATTCT 1X 5' GGGAAACCGGTCCGT GGGTAGTTCT---ttg---- -GGCCGCATCATTCT 1X 5' GGGAAACCGGTCC-- --------tcgat------- --GCCGCATCATTCT
Pax7-gRNA#3 94.9% (37/39)	wt 5' ATGCGGCCAGGACCA GGGCAGAACTACCCACGG----AC CGGTTTCCCACTAGA 2x 5' ATGCGGCCAGGACCA GGGCAGAACTACCCACGG----AC CGGTTTCCCACTAGA 4x 5' ATGCGGCCAGGACCA GGGCAGAACTACCCACGG----AC -GGTTTCCCACTAGA 2x 5' ATGCGGCCAGGACCA GGGCAGAACTACCCACGG----A- CGGTTTCCCACTAGA 3x 5' ATGCGGCCAGGACCA GGGCAGAACTACCCACGG------ CGGTTTCCCACTAGA 2x 5' ATGCGGCCAGGACCA GGGCAGAACTACCCACGG------ -GGTTTCCCACTAGA 2x 5' ATGCGGCCAGGACCA GGGCAGAACTACCCACG------- --GTTTCCCACTAGA 1x 5' ATGCGGCCAGGACCA GGGCAGAACTACCCAC-------- CGGTTTCCCACTAGA 3x 5' ATGCGGCCAGGACCA GGGCAGAACTACCC---------- ---TTTCCCACTAGA 2x 5' ATGCGGCCAGGACCA GGGCAGAACTACCCACG------- CGGTTTCCCACTAGA

	4x 5' ATGCGGCCAGGACCA GGGCAGAACTACCC---------- --GTTTCCCACTAGA 1x 5' ATGCGGCCAGGACCA GGGCAGAACTACCCACGG------ -----TCCCACTAGA 3x 5' ATGCGGCCAGGACCA GGGCAGAACTACCCA--------C CGGTTTCCCACTAGA 1X 5' ATGCGGCCAGGACCA GGGCAGAACTACCCACG-ct---- CGGTTTCCCACTAGA 1x 5' ATGCGGCCAGGACCA GGGCAGAACTAC-----atca--- -------CCACTAGA 2x 5' ATGCGGCCAGGACCA GGGCAGAACTACCCAC-------- ----TTCCCACTAGA 1x 5' ATGCGGCCAGGACCA GGG-------------------- -------CCACTAGA 1x 5' --------------- ----------------------- --------CACTAGA 1x 5' ATGCGGCCAGGACCA G---------------------- --GTTTCCCACTAGA 1x 5' ATGCGGCCAGGACCA GGGCAGAACTACCCACGG---gct gcgTTTCCCACTAGA 2x 5' ATGCGGCCAGGACCA GGGCAGAACTACCCACGtacgtag aGGTTTCCCACTAGA
Sox2-gRNA#5 89.7% (26/29)	wt 5' TACCAGAGCGCGCCC GTGCCGGGCTCGTCCATCAA------ CGGCACGCTGCCCCT 3X 5' TACCAGAGCGCGCCC GTGCCGGGCTCGTCCATCAA------ CGGCACGCTGCCCCT 1X 5' TACCAGAGCGCGCCC GTGCCGGGCTCGTCCATCA------- -GGCACGCTGCCCCT 1X 5' TACCAGAGCGCGCCC GTGCCGGGCTCGTCCATC-------- -GGCACGCTGCCCCT 1X 5' TACCAGAGCGCGCCC GTGCCGGGCTCGTCCAT--------- CGGCACGCTGCCCCT 2X 5' TACCAGAGCGCGCCC GTGCCGGGCTCGTCCAT--------- -GGCACGCTGCCCCT 1X 5' TACCAGAGCGCGCCC GTGCCGGGCTCGTCC----------- -GGCACGCTGCCCCT 3X 5' TACCAGAGCGCGCCC GTGCCGGGCTCGT------------ CGGCACGCTGCCCCT 1X 5' TACCAGAGCGCGCCC GTGCCGGGCTCG------------- -GGCACGCTGCCCCT 2X 5' TACCAGAGCGCGCCC GTGCCGGGCTC-------------- CGGCACGCTGCCCCT 1X 5' TACCAGAGCGCGCCC GTGCCGGGCTCGT------------ -GGCACGCTGCCCCT 2X 5' TACCAGAGCGCGCCC GTGCCGGGCTCG------------- --GCACGCTGCCCCT 1X 5' TACCAGAGCGCGCCC GTGCCGGGCTCGTC------------ ---CACGCTGCCCCT 2X 5' TACCAGAGCGCGCCC GTGCCGGGC----------------- --GCACGCTGCCCCT 1X 5' TACCAGAGCGCGCCC GTGCCGGG----------------- -GGCACGCTGCCCCT 1X 5' TACCAGAGCGCGCCC GTGCCGGGC-----gat--------- --GCACGCTGCCCCT 1X 5' TACCAGAGCGCGCCC GTGCCGG------------------ ---CACGCTGCCCCT 1X 5' TACCAGAGCGCGCCC GTGCCGGGCTCGTCCA---ccag--- --GCACGCTGCCCCT 1X 5' TACCAGAGCGCGCCC GTGCC--------------------- ----ACGCTGCCCCT 1X 5' TACCAGAGCGCGCCC GTG---------------------- ---CACGCTGCCCCT 1X 5' --------------- ------------------------- -----------CCCT 1X 5' TACCAGAGCGCGCCC GTGCCGGGCTCGTCCATgatgataac taGCACGCTGCCCCT

1. The PAM sequences are highlighted in green.

2. The number of clones harboring either non-modified (wild type) or modified targeted loci are indicated as "nX"

Table S2. Summary of CRISPR/Cas9 mediated homologous independent knock-in (KI) in axolotl

Genomic loci *[1]	gRNAs	KI sequences	KI efficiency (%) (Positive larva / total injected eggs)*[2]	Avg./Max. KI efficiency in individuals *[3]	Germ-line transmission
Pax7 (N-ter)	*Pax7*-gRNA#1	*Pax7*-ORF[a]-*T2A*-*Cherry*-PA	0% (0/146)[Phe]	N. D.	N. D.
Pax7 (N-ter)	*Pax7*-gRNA#2	*Pax7*-ORF[a]-*T2A*-*Cherry*-PA	0.8% (1/122)[Phe]	low-medium	N. D.
Pax7 (N-ter)	*Pax7*-gRNA#3	*Pax7*-ORF[a]-*T2A*-*Cherry*-PA	12.7% (20/157)[Phe]	medium-strong / strong	Yes
Pax7 (N-ter) *[4]	*Pax7*-gRNA#3	*Pax7*-ORF[a]-*T2A*-*Cherry*-PA	7.6% (9/118)[Phe]	low-medium / medium	N. D.
Pax7 (N-ter)	*Pax7*-gRNA#1	*Pax7*-ORF[b]-*P2A*-*memCherry*-*T2A*-*ER^{T2}*-*Cre*-*ER^{T2}*-PA	4.3% (9/211)[Phe]	low-medium / strong	Yes
Sox2 (C-ter)	*Sox2*-gRNA#5	*Sox2*-ORF-*T2A*-*Cherry*-PA	14.5% (45/310)[Phe]	medium-strong / strong	Yes
Sox2 (C-ter)	*Sox2*-gRNA#5	*Sox2*-ORF-*P2A*-*memCherry*-*T2A*-*ER^{T2}*-*Cre*-*ER^{T2}*-PA	5.8% (12/207)[Phe]	medium / strong	N.D.

*1: Targeting the N-terminal (N-ter) or C-terminal (C-ter) of the gene locus.
*2: Efficiency is evaluated based on the phenotype (Phe) or genotype (Gen) in F0 embryos at around 20-days post injection.
*3: The estimated average (Avg.) knock-in efficiency of individuals in all positive embryos, and the maximum (Max.) knock-in efficiency in individual embryos. For images of animals, see Fig. S2.
*4: This single knock-in experiment was carried out using Cas9 mRNA, all rest with CAS9 protein.