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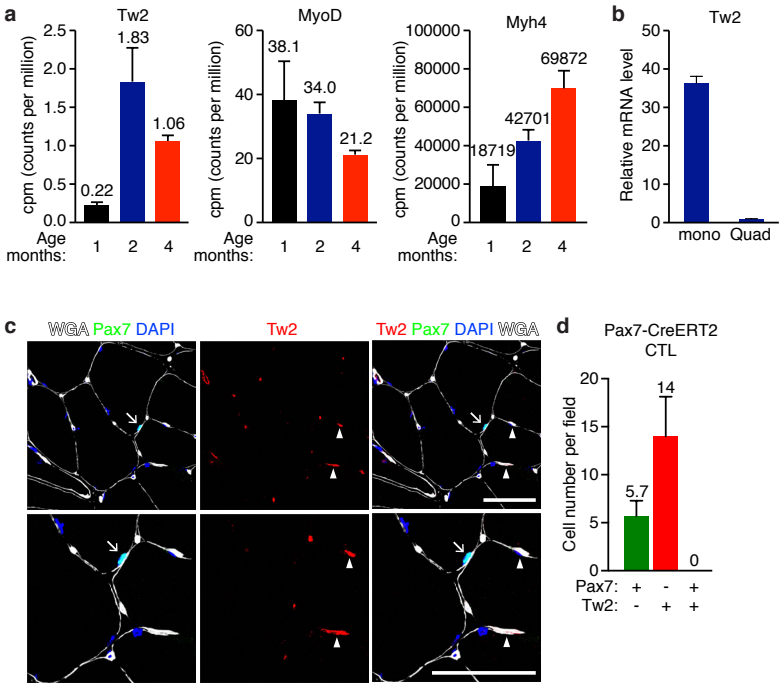
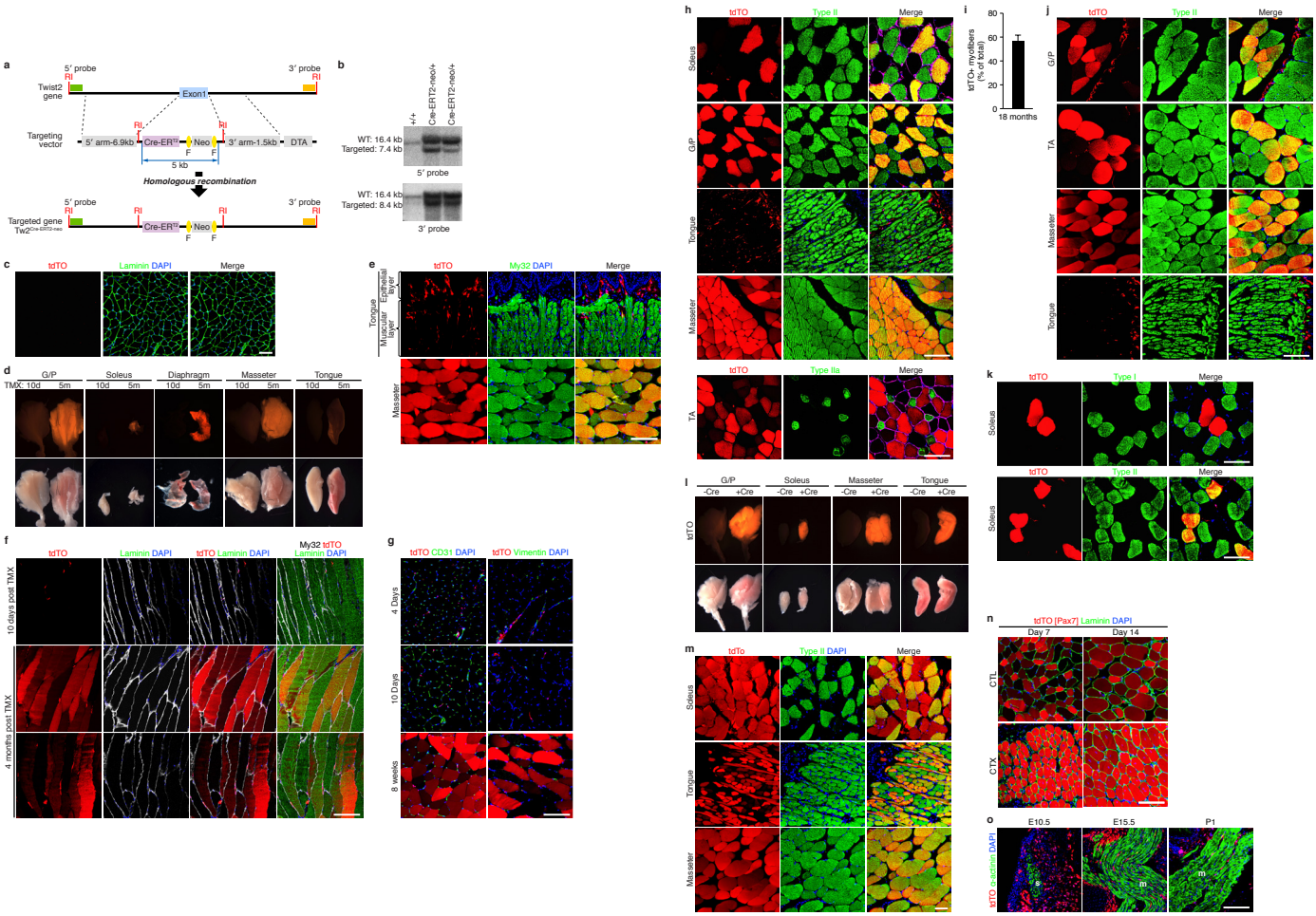
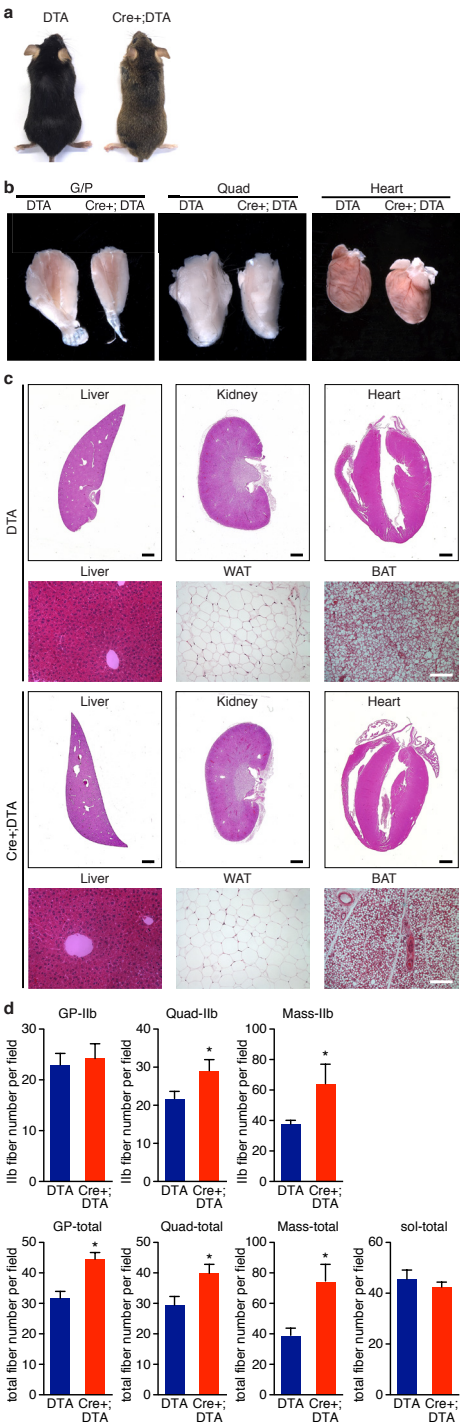


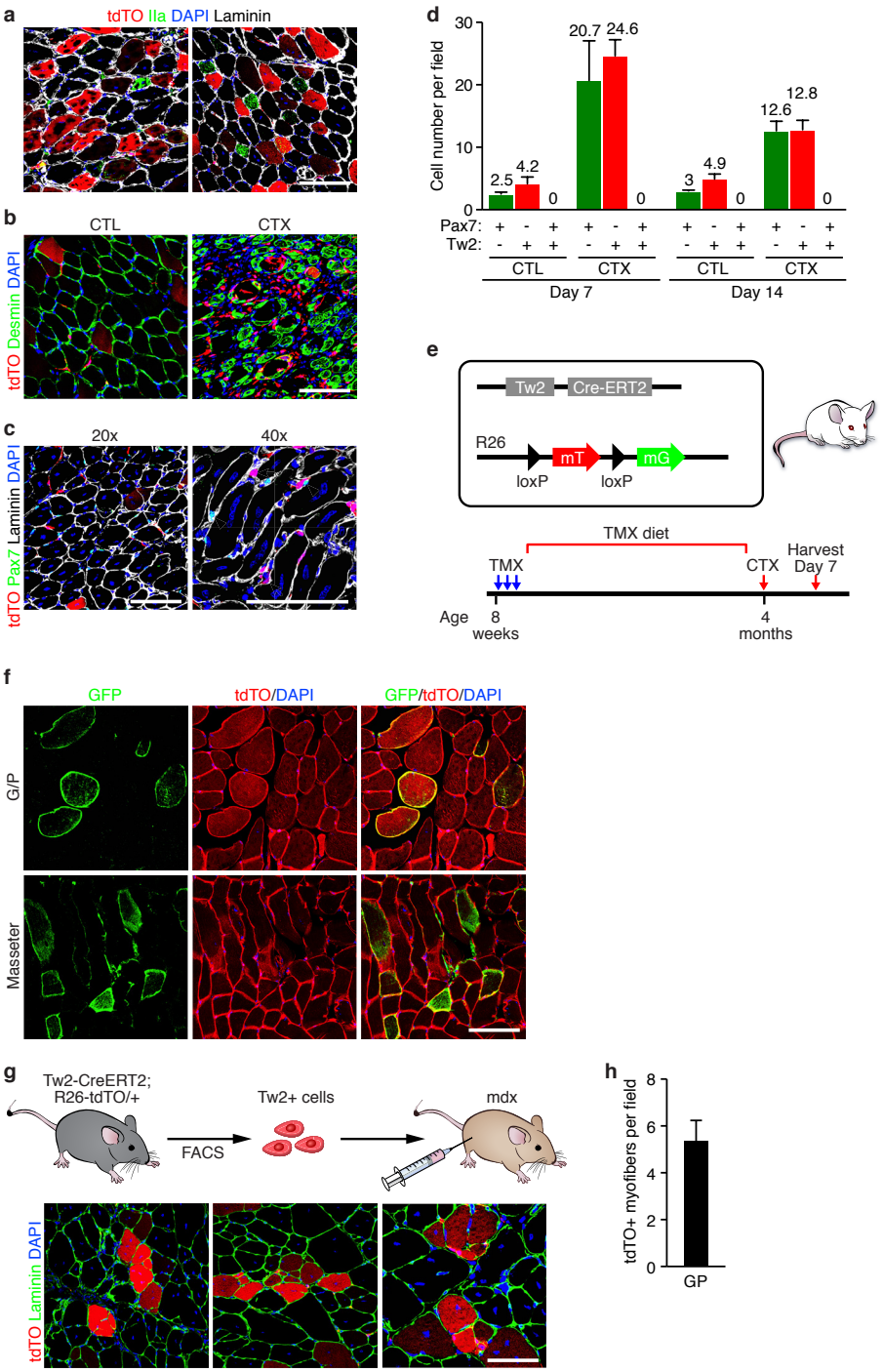
Supplementary Figure 1 Analysis of Tw2 expression in skeletal muscle. (**a**) CPM (counts per million) of Tw2, MyoD and Myh4 expression by RNA-seq in G/P muscles of WT mice at 1, 2 and 4 months of age. Data are mean ± S.E.M. N=3 mice for each time point. (**b**) Real-time RT-PCR demonstrates Tw2 mRNA is enriched in mononuclear cells of the adult muscle (mono) compared to whole quadriceps muscle (quad). Data are mean ± S.E.M; N=3 independent experiments. (**c**) Immunostaining of Tw2 (red) and Pax7 (green) on transverse sections of G/P muscle of 12-month old WT mice. Myofibers were co-stained with wheat germ agglutinin (white) and DAPI (blue). Arrows indicate Pax7+ cells and arrowheads indicate Tw2+ cells. Scale bar: 50 um. (**d**) Quantification of the number of Pax7+ (tdTO+), Tw2+, and Pax7+/Tw2+ double positive cells per field in CTL TA muscles of Pax7-CreERT2; R26-tdTO mice. For each muscle section, at least 6 different fields were quantified and averaged. Data are mean ± S.E.M; N=3 mice. Statistic source data for **a,b,d** are provided in Supplementary Table 3.



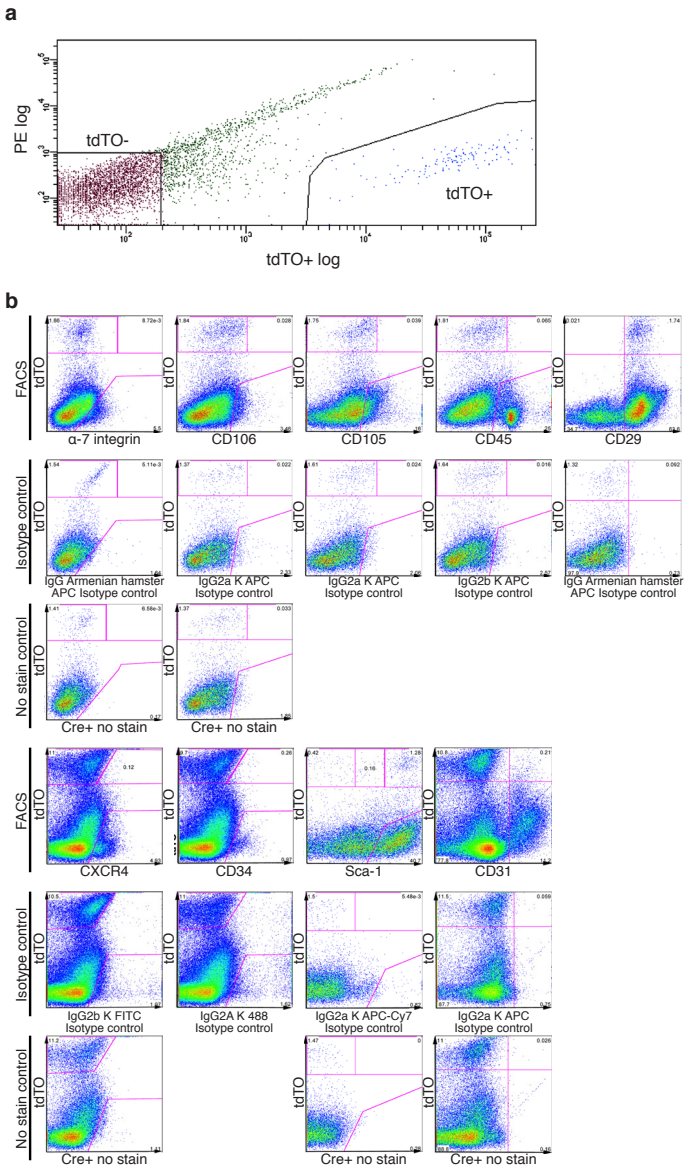
Supplementary Figure 2 Generation of Tw2-CreERT2 mice and analysis of the Tw2 lineage tracing. (**a**) Targeting strategy of the Tw2-CreERT2 allele. Coding region of the Tw2 gene was replaced by a CreERT2-Frt-Neo-Frt cassette by homologous recombination in ES cells. Out of 500 ES cell clones 10% were correctly targeted. (**b**) Southern blot analysis of ES cells using 5' probe and 3' probes to demonstrate correct targeting. Unprocessed original scans of blots are shown in Supplementary Fig. 9. (**c**) In the absence of TMX treatment, tdTO is not detected in muscles of Tw2-CreERT2; R26-tdTO mice at 9 months of age. Scale bar: 100 um. (**d**) Whole mount images showing tdTO+ muscles at 10 days (left) and 5 months (right) post-TMX. At 5 months, all muscles examined showed strong tdTO signals except for the tongue muscle. (**e**) Transverse-sections of tongue and masseter muscles from Tw2-CreERT2; R26- tdTO mice at 4 months post-TMX were co-stained with My32 (green) and DAPI (blue). Scale bar: 100 um. (**f**) Longitudinal-sections of TA muscle from Tw2-CreERT2; R26-tdTO mice at 10 days and 4 months post-TMX were co-stained with My32 (green), Laminin (white) and DAPI (blue). tdTO signal expands through the entire myofibers. Scale bar: 100 um. (**g**) Transverse sections of Tw2-CreERT2;R26-tdTO mice at indicated days post TMX were co-stained with wCD31 (green; left panel), an endothelial cell marker and vimentin (green; right panel), a fibroblast marker. Tw2+ cells do not give rise to endothelial cells or fibroblasts in adult skeletal muscle. Scale bar: 100 um. (**h**) Type II myofiber specificity after prolonged labeling of Tw2-CreERT2; R26-tdTO mice. Transverse sections of indicated muscles of Tw2-CreERT2; R26-tdTO mice were obtained at 18 months post-TMX. Myosin staining using a type II specific antibody (My32, green) showed only a subset of Type II fibers were labeled by tdTO. Myofibers in tongue muscle were excluded from tdTO expression. Bottom panels show co-staining for type IIa myofibers. Scale bar: 100 um. (**i**) Quantification of the percentage of tdTO+ myofibers among all myofibers in each field in G/P muscle at 18 months post-TMX. Data are mean ± S.E.M; N=3 mice. (**j**) Seven-month old Tw2-CreERT2;R26-tdTO/+ mice were injected with 3 doses of TMX as described in Fig. 2a. Three months later, muscles were harvested and stained for type II myofibers. Tw2+ cells strongly labeled a subset of type II fibers in G/P, TA, masseter muscles, but not in tongue. Scale bar: 100 um. (**k**) Soleus muscle of the same mice described in panel (b) were co-stained with antibodies against type I and type II myofibers. A subset of type II myofibers but not type I myofibers are labeled by tdTO. Scale bar: 100 um. (**l**) Whole mount images showed intense tdTO signals in G/P, soleus, masseter and tongue of Pax7-CreERT2; R26-tdTO mice (right) compared to muscles of R26-tdTO mice (left) 8 weeks post-TMX. Mice were treated with the same regimen as shown in Fig. 2a. (**m**) Myosin staining using a type II specific antibody (My32, green) showed tdTO signals in the majority of myofibers of soleus, tongue and masseter muscle from Pax7-CreERT2; R26-tdTO mice at 8 weeks post-TMX. Scale bar: 100 um. (**n**) Pax7-CreERT2; R26-tdTO mice were subjected to CTX injury and transverse sections of TA muscles were analyzed 7 and 14 days later. Contralateral uninjured muscle served as control (CTL). The results showed that Pax7+ cells contribute to all regenerating myofibers (indicated by centralized nuclei) on days 7 and 14 after CTX injury. Sections were co-stained with Laminin (green) and DAPI (blue). Scale bar: 100 um. **(o)** Tw2+ cells do not contribute to embryonic myogenesis during development. Sections of Tw2-Cre; R26-tdTO embryos at E10.5, E15.5 and P1 were stained with α-actinin to detect somites (s) and muscle cells (m). Body wall muscles were shown for E15.5 and P1. Scale bar: 100 um. Statistic source data for **i** are provided in Supplementary Table 3.



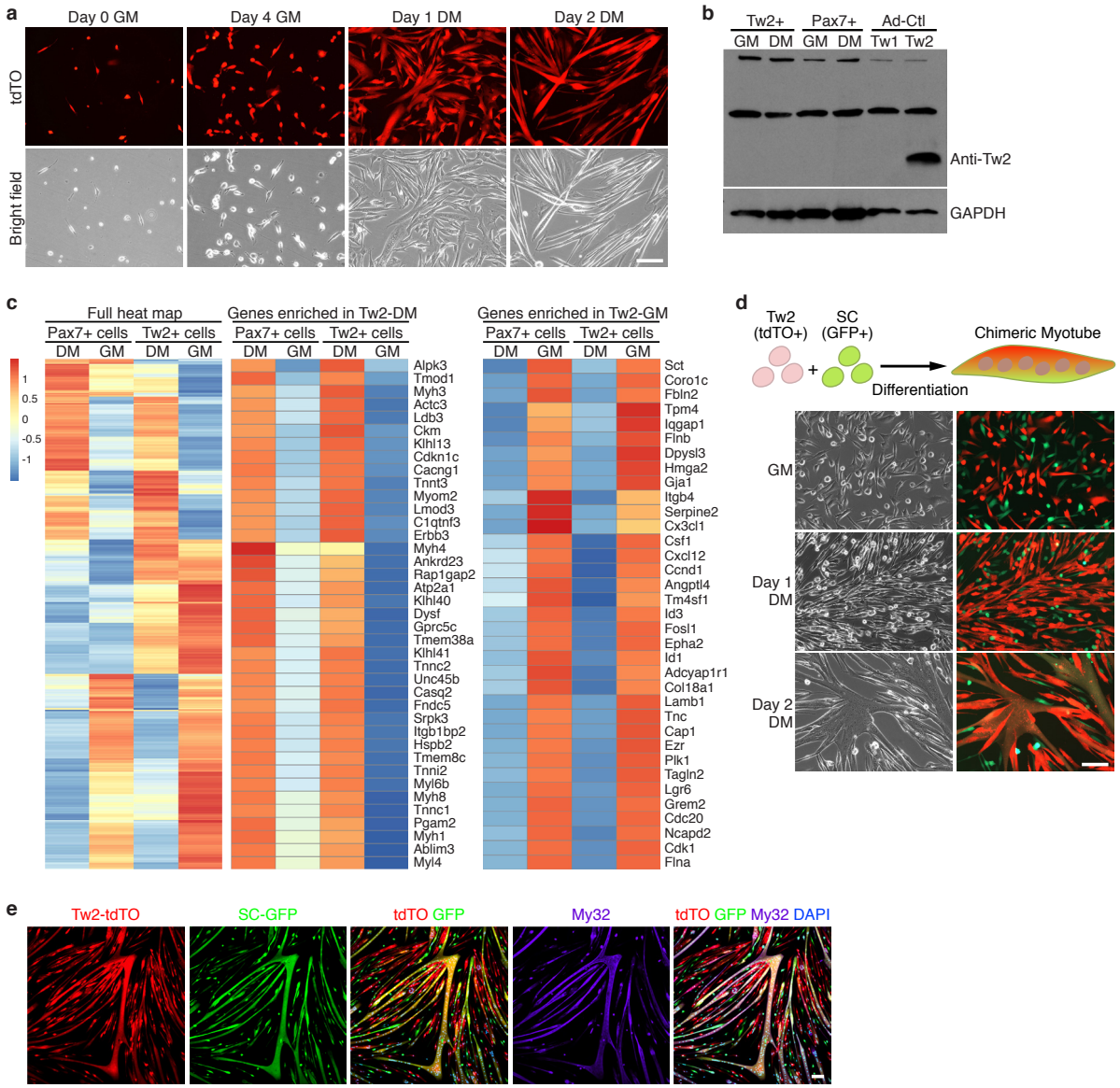
Supplementary Figure 3 Effect of Tw2+ cell ablation on non-muscle tissues. (**a**) Cre+;DTA mice were smaller than control DTA mice at 9 months post-TMX. The difference in coat color is due to the mixed genetic background. (**b**) Whole mount images of G/P, Quad and heart of Cre+;DTA and control DTA mice at 9 months post-TMX. (**c**) Hematoxylin and eosin staining of liver, kidney, heart, white adipose tissue (WAT) and brown adipose tissue (BAT) from DTA and Cre+; DTA mice at 9 months post TMX. Black scale bar: 1 mm; White scale bar: 100 um. (**d**) Quantification of type IIb myofibers per field and total myofibers per field in G/P, quad, mass and soleus muscles of DTA and Cre+; DTA mice. Data are mean ± S.E.M; N =5 mice for each genotype. Two sample t-test; *: $P < 0.05$. Statistic source data for **d** are provided in Supplementary Table 3.



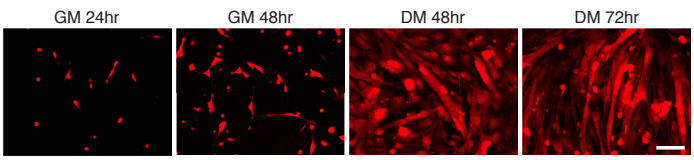
Supplementary Figure 4 Analysis of Tw2+ cells during regeneration following CTX injury and their engraftment capacity. (**a**) Co-staining for type IIa myosin revealed regenerated tdTO+ myofibers are not type IIa myofibers. Scale bar: 100 um. (**b**) Sections of TA muscle from Tw2-CreERT2;R26-tdTO mice were co-stained with desmin (green) on day 3 after CTX. The majority of tdTO+ cells are negative for desmin. Scale bar: 100 um. (**c**) Sections of TA muscle from Tw2-CreERT2;R26-tdTO mice were co-stained with Pax7 (green) on day 7 after CTX. tdTO+ cells are negative for Pax7. Scale bar: 100 um. (**d**) Quantification of the number of Pax7+, Tw2+ (tdTO+) and Pax7+/Tw2+ double positive cells per field in Tw2-CreERT2; R26-tdTO on days 7 and 14 post-CTX injury. CTL: contralateral TA muscle. For each muscle section, at least 6 different fields were quantified and averaged. Data are mean ± S.E.M; N=3 mice. (**e**) Schematic of lineage tracing with Tw2-CreERT2; R26-mT/mG/+ mice. (**f**) Transverse-sections of G/P and masseter muscles from Tw2-CreERT2; R26-mT/mG mice at 4 months post-TMX. Tw2+ cells are labeled by GFP expression. All GFP+ myofibers remained TdTO+ in G/P and master, indicating Tw2+ cells fuse with existing myofibers. Scale bar: 100 um. (**g**) Freshly isolated Tw2+ cells can engraft and form myofibers when transplanted into the TA muscle of mdx mice. 60,000 freshly isolated Tw2+ cells from Tw2-CreERT2; R26-tdTO mice at 10 days post-TMX were injected into TA muscle of 4 month-old mdx mice, which were injected with CTX 1 day prior to engraftment. TA muscles were harvested at 4 weeks post-injection and stained with laminin (green) and DAPI (blue) to visualize engrafted tdTO+ myofibers. Scale bar: 100 um. (**h**) Quantification of the number of tdTO+ myofibers in the engraftment experiemnts. For each mouse, at least 6 fields of G/P muscle sections were quantified and averaged. Data are mean ± S.E.M; N=3 mice. Statistic source data for **d,h** are provided in Supplementary Table 3.



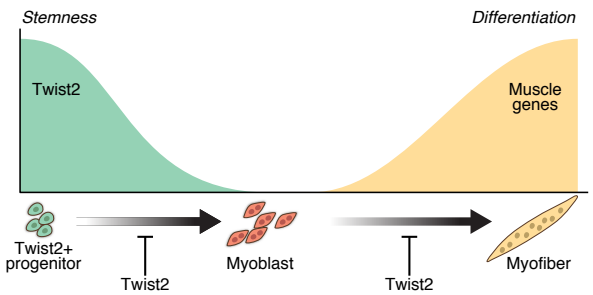
Supplementary Figure 5 FACS analysis of Tw2+ cells. (**a**) Representative FACS plots of Tw2+ (tdTO+) cells. Mononuclear cells from Tw2-CreERT2;R26-tdTO mice at 10 days post-TMX were sorted based on expression of tdTO. Approximately 3.3% of all mononuclear cells were positive for tdTO+. Sorting gates were drawn as indicated for both tdTO+ and tdTO- cell populations. (**b**) FACS plots of cell surface marker expression of tdTO+ cells from the Tw2-CreERT2; R26-tdTO mice at 10 days post-TMX.



Supplementary Figure 6 Properties of Tw2+ cells in culture. (**a**) Tw2+ cells isolated by FACS sorting proliferated efficiently in growth medium (GM) and differentiated into multinucleated myotubes in differentiation medium (DM). These cells remain tdTO+ in GM and DM. Bright field images are presented for comparison. Scale bar: 20 um. (**b**) Western blotting analysis for Tw2 protein of Tw2+ cells and Pax7+ cells in GM and DM. Ad-Ctl represents protein samples from neonatal rat cardiomyocytes infected with adenoviruses expressing either Twist1 (Tw1) or Twist2 (Tw2). The upper band present in all samples represents a non-specific band. GAPDH protein is detected as loading control. . Unprocessed original scans of blots are shown in Supplementary Fig. 9. (**c**) Heat map of genes expressed in Pax7+ cells and Tw2+ cells in GM and DM identified by RNA-seq analysis (left panel). Heat map of the top 39 genes enriched in Pax7-DM vs. Pax7-GM are shown in the middle panel, and the heat map of top 35 genes enriched in Pax7-GM vs. Pax7-DM are list on the right panel. Importantly, these genes showed the same trend of enrichment and repression in Tw2-DM vs. T w2-GM samples. (**d**) Tw2+ cells and SCs can fuse with each other to form multinucleated myotubes. Tw2+ cells were isolated by FACS sorting from adult Tw2-CreERT2; R26-tdTO mice 10 days post-TMX, which are labeled by tdTO expression. SCs, which are labeled by GFP expression, were isolated by FACS sorting from CAG-eGFP mice. Equal numbers of Tw2+ cells and SCs were mixed and grown in GM, followed by differentiation in DM. Cells were visualized by direct fluorescence. (**e**) Myosin immunostaining (My32) revealed formation of multi-nucleated myotubes that express both GFP and tdTO. Scale bar: 100 um.

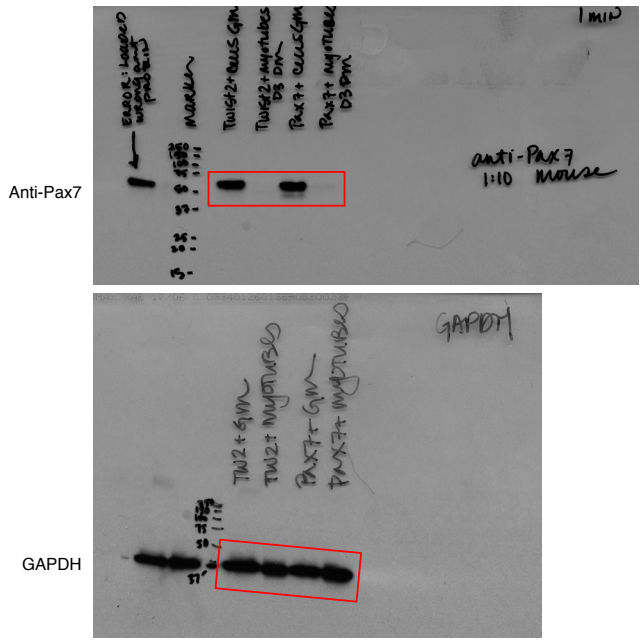


Supplementary Figure 7 Growth and myogenesis of tdTO+/CD34- cells in culture. Freshly sorted tdTO+/CD34- cells were grown in GM for 48 hours before being switched to DM to induce myogenesis. Scale bar: 20 um.



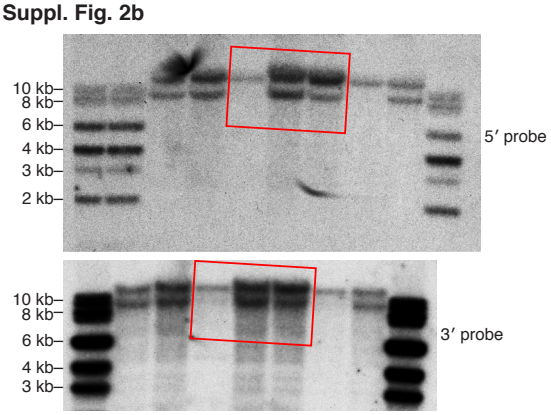
Supplementary Figure 8 Model of Tw2 maintains stemness and blocks myogenesis. Pax7 expression is not detectable in Tw2+ cells in vivo. However, when removed from their native milieu, Tw2+ cells rapidly down-regulate Twist expression and enter a Pax7+ state en route to a myogenic pathway.

Fig. 6e



Suppl. Fig. 6b

Suppl. Fig. 2b



Supplementary Figure 9 Unprocessed original scans of for Fig. 6e, Supplementary Fig. 2b, and Supplementary Fig. 6b.

Supplementary Table Legends

Supplementary Table 1 Summary of FACS analysis of tdTO+ cells in uninjured muscle at 10 days post-TMX.

Supplementary Table 2 Summary of FACS analysis of tdTO+ cells in CTX-injured muscle. TA muscle was injected with CTX at 1 week post the 1st dose of TMX, and analyzed at day 5 after CTX.

Supplementary Table 3 Statistic source data are provided.