A Twist2-dependent progenitor cell contributes to adult skeletal muscle

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Skeletal muscle possesses remarkable regenerative potential due to satellite cells, an injury-responsive stem cell population located beneath the muscle basal lamina that expresses Pax7. By lineage tracing of progenitor cells expressing the Twist2 (Tw2) transcription factor in mice, we discovered a myogenic lineage that resides outside the basal lamina of adult skeletal muscle. Tw2+ progenitors are molecularly and anatomically distinct from satellite cells, are highly myogenic in vitro, and can fuse with themselves and with satellite cells. Tw2+ progenitors contribute specifically to type IIb/x myofibres during adulthood and muscle regeneration, and their genetic ablation causes wasting of type IIb myofibres. We show that Tw2 expression maintains progenitor cells in an undifferentiated state that is poised to initiate myogenesis in response to appropriate cues that extinguish Tw2 expression. Tw2-expressing myogenic progenitors represent a previously unrecognized, fibre-type-specific stem cell involved in postnatal muscle growth and regeneration.

Skeletal muscle is among the most regenerative adult tissues. Its remarkable regenerative capacity originates from a population of resident stem cells, termed satellite cells (SCs), located beneath the muscle basal lamina1. SCs are marked by expression of Pax7, a transcription factor critical for muscle regeneration1. In response to injury and disease, SCs become activated and undergo self-renewal and differentiation to form new myofibres1–3. While SCs are essential for muscle regeneration, their genetic ablation in adult mice does not accelerate sarcopenia4–6. Thus, additional mechanisms or cell types might contribute to maintenance of muscle mass during ageing.

Skeletal muscle is composed of heterogeneous myofibre types that differ in contractile and metabolic properties and expression of distinctive myosin isoforms. Four major fibre types are present in rodent muscles: one type of slow-twitch fibre (type I) and three types of fast-twitch fibre (type IIA, IIX/d and IIB). While type I and type IIA fibres exhibit oxidative metabolism and high endurance; type IIX and IIB fibres are glycolytic and display low endurance7. Slow- and fast-twitch fibres also differ in their responses to hypertrophic or atrophic stimuli. For example, type IIB and IIX myofibres are more susceptible than slow-twitch fibres to a variety of atrophic signals such as denervation, nutrient deprivation, cancer cachexia and chronic heart failure8–10. While SCs can fuse into all myofibre types in injured muscle11, it remains unknown whether fibre-type-specific myogenic progenitors might also exist.

The Drosophila basic helix–loop–helix transcription factor Twist is expressed in muscle progenitors during embryogenesis and is essential for the formation of mesoderm and muscle12–14. Within the adult musculature of Drosophila, Twist expression is restricted to muscle precursors that are normally quiescent but are activated by extracellular cues to regenerate the adult musculature during metamorphosis15–17. Two mammalian Twist genes, Twist1 (Tw1) and Twist2 (Tw2), are expressed in various mesenchymal cell types, but not in differentiated myofibres18,19. Tw1 and Tw2 have been shown to block myogenesis in vitro19–21, but their potential roles in muscle formation or regeneration in mammals have not been explored.

Here, we traced the fate of Tw2-dependent cell lineages in mice and discovered that Tw2 expression marks a previously unrecognized interstitial myogenic progenitor cell that forms type IIb/x myofibres in adult muscle. Tw2-expressing progenitors represent a population of myogenic progenitor cells that contributes to specific fibre types during muscle homeostasis and regeneration, highlighting the ancestral functions of Twist as a regulator of muscle formation.
RESULTS

Twist expression in interstitial cells within adult skeletal muscle

In adult muscle, Tw2 transcript is barely detectable in whole gastrocnemius and plantaris (G/P) muscle at 1, 2 and 4 months of age by RNA-seq analysis, in contrast to MyoD and Myh4, which are readily detected (Supplementary Fig. 1a). Real-time PCR with reverse transcription (RT–PCR) revealed that Tw2 was highly enriched in mononuclear non-myofibre cells compared with whole quadriceps muscle (Supplementary Fig. 1b). Immunostaining of transverse sections of gastrocnemius muscle from 3-month-old wild-type (WT) mice revealed Tw2 protein in interstitial cells outside the myofibres, but not within myofibres (Fig. 1a). Furthermore, Tw2 protein was not co-localized with Pax7, which was restricted to SCs beneath the basal lamina (Fig. 1a,b). Similar mutual exclusivity of expression of Tw2 and Pax7 was observed in muscles of 12-month-old mice (Supplementary Fig. 1c). We conclude that Tw2 is expressed in the myofibre interstitium and not in mature myofibres or SCs in adult muscle.

To analyse Tw2 expression during muscle regeneration, we performed cardiotoxin (CTX) injury on tibialis anterior (TA) muscle of WT mice, and harvested muscles on 4, 7 and 14 days post-CTX injury. Tw2+ and Pax7+ cells were detected by immunostaining. We did not observe cells that expressed both Pax7 and Tw2 proteins at any time point examined (Fig. 1c,d). Tw2+ cells declined immediately following CTX injury but the number of Tw2+ cells was rapidly restored by day 7 post-CTX.

By 3 weeks following TMX treatment, strong tdTO staining was observed in the G/P muscles, and the number of tdTO+ myofibres continued to increase up to 8 weeks post-TMX treatment, at which time 58% of myofibres were labelled by tdTO (Fig. 2c,d). No tdTO+ myofibres were observed in the absence of TMX (Supplementary Fig. 2e), validating the specificity of the lineage tracing method. Strong tdTO labelling was observed in all hindlimb muscles, diaphragm and masseter muscles by 5 months post-TMX with the exception of the tongue (Supplementary Fig. 2d,e). Instead, interstitial cells within the tongue and adjacent epithelial cells along the surface of the tongue were labelled with tdTO (Supplementary Fig. 2e).

Longitudinal sections of G/P muscle at 4 months post-TMX also revealed tdTO expression throughout the entire length of myofibres (Supplementary Fig. 2f). Tw2+ cells did not give rise to other cell types such as endothelial cells (marked by CD31) or fibroblasts (marked by vimentin) in adult muscle (Supplementary Fig. 2g).

To determine whether the Tw2+ lineage contributed to specific myofibre types, we analysed tdTO labelling in G/P and soleus muscles, which are comprised of different proportions of type I and type II myofibres. There was no overlap between type I myofibres and tdTO+ myofibres in the soleus, but a subset of type II fibres within G/P also expressed tdTO (Fig. 2e). Further staining revealed tdTO labelling of type Iib but not type Iia myofibres in G/P muscle (Fig. 2e). Some tdTO+ fibres were negative for Iib staining (Fig. 2e). By exclusion, we interpret these to be type IIX fibres. Therefore, we conclude that Tw2 cells specifically label type Iib/X fibres. The fibre-type-specific tdTO labelling remained even 18 months after the TMX pulse (Supplementary Fig. 2h,i). To determine whether the fibre-type specificity also remained in aged mice, we performed lineage tracing on 7-month-old Tw2-CreERT2; R26-tdTO mice, and observed robust contribution of tdTO+ cells to myofibres and the same type-II-fibre-specific labelling at 3 months post-TMX (Supplementary Fig. 2j,k).

To further assess the type-Iib/X-myofibre specificity of the Tw2 lineage, we compared the contribution of SCs to adult myofibres by treating Pax7-CreERT2; R26-tdTO mice with TMX at 8 weeks of age. Pax7+ SCs contributed to myofibres in all muscles including tongue at 8 weeks post-TMX (Supplementary Fig. 2l), consistent with prior studies.11 SCs also labelled all types of myofibre (Supplementary Fig. 2m). This finding is consistent with a recent study showing that Pax7+ SCs fuse into myofibres in uninjured skeletal muscle and that a fibre-type preference does not exist for Pax7+ SCs during fusion.11 Thus, in contrast to the broad muscular contributions of SCs, the Tw2-expressing lineage contributes specifically to adult type Iib/X myofibres.

To determine whether Tw2+ cells contribute to embryonic myogenesis during development, we performed lineage tracing of Tw2+ cells by breeding mice harbouring a constitutively active Cre expression cassette inserted into the Tw2 locus25,26 with R26-tdTO mice. At embryonic day (E) 10.5, Tw2+/tdTO+ cells were present in regions surrounding the somites, but not within somitic muscle (Supplementary Fig. 2o). At E15.5 and P1, tdTO was expressed in interstitial cells within various muscle groups but not within myofibres (Supplementary Fig. 2o). Together, these findings demonstrate that Tw2+ cells do not contribute to primary or secondary myogenesis in embryos, but are dedicated to the formation of muscle postnatally.
Figure 1 Tw2-expressing and Pax7-expressing cells are distinct cell types in skeletal muscle. (a) Immunostaining of Tw2 (red) and Pax7 (green) on transverse sections of gastrocnemius muscle of 3-month-old C57Bl6 WT mice. Myofibres were co-stained with wheat germ agglutinin (WGA; white) and DAPI (blue). The arrows indicate Pax7+ cells and the arrowheads indicate Tw2+ cells. Scale bars, 50 µm. (b) Quantification of the number of Pax7+, Tw2+, and Pax7+/Tw2+ double-positive cells per field in 3-month-old C57Bl6 WT mice. Quantification was performed on the top panel images in a. For each muscle section, at least 6 different fields were quantified and averaged. Data are mean ± s.e.m. N = 6 mice. (c) TA muscle of C57Bl6 WT mice was injured by CTX injection and harvested on days 4, 7 and 14 post injury. Muscle sections were stained for Pax7 (green), Tw2 (red), DAPI (blue) and WGA (white). CTL, contralateral TA muscle. Scale bar, 100 µm. (d) Quantification of the number of Pax7+, Tw2+, and Pax7+/Tw2+ double-positive cells per field in C57Bl6 WT mice on days 4, 7 and 14 post-CTX injury. For each muscle section, at least 6 different fields were quantified and averaged. Data are mean ± s.e.m. N = 3 mice. (e) Tw2 expression in TA muscle of Pax7-CreERT2; R26-tdTO mice after CTX injury. Adult Pax7-CreERT2; R26-tdTO mice were treated with TMX for 3 alternating days. One week after the first dose of TMX, CTX injury was performed on TA muscle. Muscles were harvested at days 4 and 7 post injury and immunostained for Tw2 (green) and DAPI (blue). Scale bar, 100 µm. (f) Quantification of the number of Pax7+/tdTO+ , Tw2+, and Pax7+/Tw2+ double-positive cells per field in Pax7-CreERT2; R26-tdTO on days 4 and 7 post-CTX injury. Pax7+ cells were detected by tdTO+ signal. 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 b,d,f are provided in Supplementary Table 3.

Ablation of the Tw2+ lineage causes atrophy of type IIB myofibres

We genetically ablated Tw2+ cells in adult mice by breeding the Tw2-CreERT2 mice to mice harbouring a diphtheria toxin (DTA) expression cassette in the Rosa26 locus (R26-DTA mice)27. Tw2-CreERT2; R26-DTA/+ (named Cre+; DTA) mice and control R26-DTA/+ (named DTA) mice were injected with TMX and maintained on a TMX-containing diet beginning at 4 weeks of age (Fig. 3a) and analysed at 13 months of age. G/P and quadriceps muscles were significantly smaller in Cre+; DTA mice compared with DTA control mice, whereas other tissues such as heart, kidney and liver were approximately comparable in size (Fig. 3b and Supplementary Fig. 3). Histological analysis of liver, WAT and BAT also revealed no significant difference between DTA and Cre+; DTA mice (Supplementary Fig. 3c). Intriguingly, in Cre+; DTA mice, we observed a significant decrease in the mean cross-sectional area of type IIB fibres in G/P, quadriceps and masseter muscles (Fig. 3c,d). The mean cross-sectional area of other fibre types remained unchanged...
Figure 2 Progressive contribution of Tw2<sup>+</sup> cells to adult skeletal muscle. (a) Schematic of TMX treatment. Tw2-CreERT2; R26-tdTO mice on a mixed genetic background were injected with TMX at 8 weeks of age on 3 alternating days. Mice were analysed at various time points following initial TMX injection. (b) At 10 days post-TMX, Tw2<sup>+</sup> cells marked by tdTO<sup>+</sup> (red) were located outside the basal lamina (green) (left panel). The middle panel is an enlarged image of the left panel showing the morphology of Tw2<sup>+</sup> cells. In contrast, Pax7<sup>+</sup> cells (stained with Pax7 antibody, green) were beneath the basal lamina (right panel). The arrow indicates a Pax7<sup>+</sup> cell. Scale bar, 100 μm. (c) Progressive tdTO labelling of myofibres of G/P muscle at the indicated times following TMX treatment. Transverse sections of G/P muscle were co-stained with laminin (green) and DAPI (blue). Scale bar, 100 μm. (d) Quantification of the percentage of tdTO<sup>+</sup> myofibres among all myofibres in each field in G/P muscle. Data are mean ± s.e.m. For each time point, N=3 mice were analysed. For each mouse, between 1,000 and 2,000 myofibres of G/P muscle were quantified. (e) Transverse sections of different muscle groups obtained from Tw2-CreERT2; R26-tdTO mice at 4 months post-TMX were stained with various fibre-type-specific antibodies (green as indicated). Scale bar, 100 μm. Statistic source data for d are provided in Supplementary Table 3.

(Fig. 3d). The number of type IIb fibres per field as well as the total fibre numbers were also increased in quadriceps and masseter but not G/P muscles (Supplementary Fig. 3d). No centralized nuclei were observed in Cre<sup>+</sup> DTA mice. These results indicate that ablation of Tw2<sup>+</sup> cells caused type-IIb-fibre-specific atrophy, supporting the notion that Tw2<sup>+</sup> cells are important for maintenance of type-IIb-myofibre size during adulthood.

The Tw2 lineage contributes to muscle regeneration

To determine whether the Tw2 lineage forms new myofibres in response to injury, we performed CTX injury on TA muscle (Fig. 4a). Seven days after CTX injection, we observed tdTO labelling of newly regenerated myofibres, marked by centralized nuclei, and the labelling became more robust by day 14 post-CTX when tdTO<sup>+</sup> myofibres represented ~27.4% of newly regenerated myofibres (Fig. 4b). Myosin staining revealed that 55% of the regenerated tdTO<sup>+</sup> myofibres were type IIb myofibres (Fig. 4c,d and Supplementary Fig. 4a).

To determine whether activated Tw2<sup>+</sup> cells differentiate autonomously or fuse with new myofibres, we immunostained muscle sections for desmin, which is highly expressed in immature muscle fibres during fetal life and regeneration<sup>28,29</sup> (Fig. 4e). Although most of the activated Tw2<sup>+</sup> cells were negative for desmin expression (Supplementary Fig. 4b), we occasionally observed small desmin<sup>+</sup>/tdTO<sup>+</sup> myofibres, indicating that Tw2<sup>+</sup> cells differentiated into desmin-expressing myofibres (Fig. 4e). Activated Tw2<sup>+</sup> cells did not express Pax7, as demonstrated by immunostaining (Supplementary Fig. 4c,d).

To further determine whether Tw2 cells contribute to regeneration by initiating myogenesis autonomously or by fusing with existing myofibres, we bred Tw2-CreERT2 mice to R26-mT/mG mice, which constitutively express a membrane-targeted tdTO protein (mT) from the Rosa26 locus<sup>30</sup> (Supplementary Fig. 4e). On Cre-activation, tdTO fluorescence is lost and membrane-targeted eGFP (mG) becomes expressed. If Tw2<sup>+</sup> cells fuse with existing myofibres, both signals will be present, but a de novo myofibre formed from Tw2<sup>+</sup> cells will only...
For comparison, we performed CTX injury on Pax7-Cre-ERT2; R26-DTA/DTA mice following the same treatment regimen as in Fig. 4a. Ablation of the Tw2\(^+\) lineage causes type-IIb-myofibre atrophy. (a) Schematic of TMX treatment. Tw2-CreERT2; R26-DTA/+ (Cre\(^+\); DTA) and R26-DTA/+ (DTA) mice on a mixed genetic background were injected with TMX at 4 weeks of age on 3 alternating days. Mice were kept on a TMX-containing diet until the time of analysis. (b) Measurement of body weight (BW), heart weight (HW) and muscle mass normalized to tibia length (TL) of mice. Data are mean ± s.e.m.; two-sample t-test; \(*< 0.05\). \(N = 5\) male mice for each genotype. (c) Type IIb (green) and laminin (red) immunostaining of transverse sections of quad, G/P and masseter muscles of Cre\(^+\); DTA and DTA mice. Scale bar, 100 \(\mu\)m. (d) Measurement of mean myofibre cross-sectional area (CSA) of type IIb fibres (top) and other fibres (bottom). Data are mean ± s.e.m.; two-sample t-test; \(*< 0.05\), \('*' < 0.01. NS, not significant. \(N = 5\) mice for each genotype. For each muscle, more than 300 fibres were measured and averaged. Statistic source data for b,d are provided in Supplementary Table 3.

Figure 3 Ablation of the Tw2\(^+\) lineage causes type-IIb-myofibre atrophy. 

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be green. We analysed muscles from Tw2-CreERT2; R26-mTimG/+ mice at 4 months post-TMX. In the absence of injury, almost all GFP\(^+\) myofibres retained tdTO expression (Supplementary Fig. 4f), indicating that Tw2\(^+\) cells contribute to myofibres via fusion with existing fibres during homeostasis, as reported for satellite cells\(^5\). On CTX injury, although the majority (93.8%) of mG\(^+\) new myofibres also expressed mT\(^+\); 6.2% of new myofibres expressed mG and lost mT expression (Fig. 4g). These findings demonstrate that Tw2\(^+\) cells can initiate myogenesis autonomously during regeneration, although the majority of these cells fuse with newly regenerated myofibres.

For comparison, we performed CTX injury on Pax7-Cre-ERT2; R26-tdT0 mice following the same treatment regimen as in Fig. 4a. The Pax7\(^+\) lineage labelled all regenerating myofibres (Supplementary Fig. 3n). We conclude that following injury Tw2\(^+\) cells contribute to a subset of new type IIb myofibres that are also derived from the Pax7 lineage.

To test whether Tw2\(^+\) cells also possess the capacity to engraft into injured muscle, we isolated Tw2\(^+\) (tdTO\(^+\)) cells by fluorescence-activated cell sorting (FACS) at 10 days post-TMX, and injected them into the TA muscles of mdx mice, which display extensive muscle degeneration and regeneration. Four weeks post-transplantation, tdTO\(^+\) myofibres were observed in the TA muscle of mdx mice, revealing the ability of exogenous Tw2\(^+\) cells to engraft and form new muscle (Supplementary Fig. 4g). However, the engraftment efficiency was limited with an average of only 5.5 myofibres per field (Supplementary Fig. 4h), which is lower than the engraftment capacity of SCs.

**Freshly isolated Tw2\(^+\) cells are distinct from Pax7\(^+\) SCs**

To further characterize Tw2\(^+\) cells, we examined expression of cell-surface markers on freshly isolated Tw2\(^+\) cells by FACS analysis from 8-week-old Tw2-CreERT2; R26-tdT0/+ mice 10 days post-TMX (Supplementary Table 1 and Supplementary Fig. 5a,b). Only 0.46% of Tw2\(^+\) cells expressed the SC-specific marker α7-integrin. Similarly, other SC markers, including CD34, Vcam1 (CD106) and CXCR4 (CD184), were expressed in only a small fraction of Tw2\(^+\) cells. The majority of tdTO\(^+\) cells also did not express markers for endothelial cells (CD31) or haematopoietic cells (CD45) (Supplementary Table 1 and Supplementary Fig. 5a,b).
Figure 4 Tw2+ cells contribute to skeletal muscle regeneration. (a) Schematic of TMX and CTX treatment on adult Tw2-CreERT2; R26-tdTO mice on a mixed genetic background. CTX was injected into the TA muscle at 1 week after the first dose of TMX. Both contralateral (CTL) and CTX-injured TA muscles were harvested at 7 and 14 days post-CTX. (b) Immunostaining of transverse sections of TA muscles revealed that Tw2+ cells are activated and contribute to regenerating myofibres (indicated by centralized nuclei) on days 7 and 14 after CTX injury. Sections were co-stained with laminin (white) and DAPI (blue). Scale bar, 100 µm. (c) Co-staining of IIb myosin (green) with newly regenerated tdTO+ myofibres on day 14 post-CTX. Scale bar, 100 µm. (d) Quantification of the percentage of tdTO+ myofibres that are IIb-positive among all tdTO+ myofibres on day 14 post-CTX. Data are mean ± s.e.m.; N = 5 mice. (e) Three days post-CTX, occasional small tdTO+ cells expressed desmin (green), indicating that these cells differentiated into desmin-positive myoblasts. Scale bar, 100 µm. (f) Transverse sections of TA muscle on day 7 post-CTX from Tw2-CreERT2; R26-mT/mG mice on a mixed genetic background at 4 months post-TMX. The asterisk represents a GFP+ regenerated new myofibre that lost mT (tdTO) expression. Scale bar, 100 µm. (g) Percentage of de novo versus fusion events in Tw2-CreERT2; R26-mT/mG mice on day 7 post-CTX. Data are mean ± s.e.m.; N = 3 mice were analysed. Statistic source data for d,g are provided in Supplementary Table 3.

and Supplementary Fig. 5a,b). Approximately 98.8% of tdTO+ cells were positive for CD29 (β1-integrin), a widely expressed marker of mesenchymal stem cells (MSCs), lymphocytes, haematopoietic stem cells and other cell types. In addition, tdTO+ cells positive for Sca1 were further separated as Sca1-high (68.8%) and Sca1-medium (8.6%) populations. Other MSC markers, including CD73 and CD105, were expressed in only a small fraction of tdTO+ cells (Supplementary Table 1). Together, these findings suggest that the Tw2 lineage represents a population of muscle precursors clearly distinct from SCs and most closely resembling cells of a mesenchymal lineage.

Expression of cell-surface markers remained largely unchanged in Tw2+ cells following CTX injury (Supplementary Table 2).

We performed RNA-seq analysis on freshly sorted Tw2+ and Tw2− cells from Tw2-CreERT2; R26-tdTO mice 10 days post-TMX treatment at 8 weeks of age. For comparison, freshly sorted Pax7+ and Pax7− cells from Pax7-CreERT2; R26-tdTO mice were also analysed. Freshly isolated Tw2+ and Pax7+ cells showed distinct transcriptome signatures, whereas Tw2− and Pax7− cells displayed similar expression profiles (Fig. 5a). SC-enriched transcripts such as Pax7, Cadh15 (M-cad), Fgfr4, Sdc4, Met, Itga7 and Vcam1 were all strongly enriched in
Figure 5 Molecular profiling of the Tw2+ lineage in skeletal muscle. (a) Heat map of 4,980 genes expressed in freshly sorted Tw2+, Tw2−, Pax7+ and Pax7− cells identified by RNA-seq. Cells were isolated by FACS sorting from Tw2-CreERT2; R26-tdTO mice and Pax7-CreERT2; R26-tdTO mice at 10 days post-TMX, respectively. (b) Heat map of the top 23 genes enriched in Pax7+ cells compared with Pax7− cells. (c) Counts per million (cpm) of Pax7, MyoD, Tw2 and Peg3 expression by RNA-seq in Tw2+, Tw2−, Pax7+ and Pax7− cells. (d) Heat map of the top 60 genes enriched in Tw2+ cells relative to Tw2− cells. (e) Counts per million of PDGFRα and PDGFRβ expression by RNA-seq in Tw2+, Tw2−, Pax7+ and Pax7− cells. (f) Pathways enriched in freshly sorted Tw2+ cells relative to Tw2− cells identified by Ingenuity Pathway Analysis.

Pax7+ cells. Strikingly, none of these messenger RNAs was enriched in Tw2+ cells (Fig. 5b,c). MyoD transcript was barely detectable in freshly isolated Tw2+ cells in contrast to Pax7+ cells (Fig. 5b,c). Similarly, Peg3/PW1, a marker for PW1+ interstitial cells, was not enriched in the Tw2+ cells relative to either Tw2− cells or Pax7+ cells (Fig. 5b,c). On the contrary, genes highly enriched in Tw2+ cells (relative to Tw2− cells) were all repressed in Pax7+ cells, again confirming that Tw2+ and Pax7+ cells have distinct transcriptome signatures (Fig. 5d). Furthermore, platelet-derived growth factor receptor alpha (PDGFRα), a marker for human and mouse MSCs,
Tw2+ cells differ-
were upregulated in Tw2+ cells but not Pax7+
cells. Pax7 protein was detected as loading control. Unprocessed original scans of blots are shown in Supplementary Fig. 9. (f) MyoD staining of Tw2+ (tdTO+) cells in GM revealed MyoD expression. Scale bar, 100 μm. Statistic source data for b,c,d are provided in Supplementary Table 3.

Tw2+ cells transition through a pre-myogenic Pax7+ state

Freshly isolated Tw2+ cells proliferated rapidly in growth medium (GM) and remained tdTO-positive (Supplementary Fig. 6a). When switched to differentiation medium (DM), 81% of Tw2+ cells differentiated into multinucleated myosin-positive myotubes (Fig. 6a,b and Supplementary Fig. 6a). Muscle genes such as Myogenin, Ckm and Myh4 were upregulated in Tw2+ cells after 2 days in DM (Fig. 6c).

Although Tw1 and Tw2 transcripts were highly expressed in freshly isolated Tw2+ cells, expression was extinguished after maintaining the cells in culture (Fig. 6d and Supplementary Fig. 6b). In contrast, cultured Tw2+ cells quickly became Pax7-positive and MyoD-positive in GM (Fig. 6e,f). Thus, whereas Tw2+ cells do not express Pax7 in their native interstitial location in vivo, they transition through a Pax7+ state en route to differentiation. In addition, the full transcriptome profiles between Tw2+ cells and Pax7+ cells in GM

Figure 6 Myogenic potential of Tw2+ cells in culture. (a) Myosin staining (My32, green) revealed that the majority of Tw2+ (tdTO+) cells expressed myosin and formed multinucleated myotubes starting at 1 and 2 days in DM. Scale bar, 20 μm. (b) Fusion index was calculated as the percentage of tdTO+ nuclei within multinucleated myotubes compared with the total number of tdTO+ nuclei in the culture. Data are mean ± s.e.m.; N = 4 independent experiments. (c) Real-time RT-PCR revealed that muscle genes such as Myog, Ckm and Myh4 were strongly activated when Tw2+ cells were cultured in DM. Values were normalized to those of the GM sample, which is set as 1. Data are mean ± s.e.m. N = 4 independent experiments. (d) Real-time RT-PCR revealed that Tw1 and Tw2 mRNAs were enriched in freshly sorted Tw2+ cells (Sort). Expression was extinguished once cells were plated in culture. Values were normalized to those of the GM sample, which is set as 1. Data are mean ± s.e.m. N = 4 independent experiments. (e) Western blot analysis for Pax7 protein in Tw2+ and Pax7+ cells. Pax7 protein is expressed in both Tw2+ and Pax7+ cells in GM, but not in cells cultured for 2 days in DM. GAPDH protein was detected as loading control. Unprocessed original scans of blots are shown in Supplementary Fig. 9. (f) MyoD staining of Tw2+ (tdTO+) cells in GM revealed MyoD expression. Scale bar, 100 μm. Statistic source data for b,c,d are provided in Supplementary Table 3.
Figure 7 Myogenic and osteogenic potential of tdTO+/CD34− cells in vitro. (a) Myosin staining (My32, green) revealed that tdTO+/CD34− cells expressed myosin and formed multinucleated myotubes after 4 and 10 days in DM. Freshly sorted tdTO+/CD34− cells were cultured in GM for 48 h before induction of myogenesis. Scale bar, 20 µm. (b) Fusion index was calculated as the percentage of tdTO+ nuclei within multinucleated myotubes compared with the total number of tdTO+ nuclei in the culture. Data are mean ± s.e.m. N = 3 independent experiments. (c) Oil Red O staining revealed that only very few tdTO− cells differentiated into adipocytes. Freshly sorted tdTO+/CD34− cells were cultured in GM for 48 h before induction of adipogenesis for 10 days. Cells were stained with My32 and Hoechst before Oil Red O staining. Scale bars, 20 µm. (d) Percentage of Oil Red O-positive cells per field. Data are mean ± s.e.m. N = 3 independent experiments. For each experiment, more than 500 nuclei were counted and averaged. (e) Alkaline phosphatase staining revealed that tdTO+/CD34− cells can form osteoblasts when exposed to osteogenic medium. These cells did not express myosin. Scale bar, 20 µm. (f) Clonal analysis of tdTO+/CD34− cells for myogenic potential. Single tdTO+/CD34− clones were grown on inactivated MEF feeder layers and induced for myogenesis. Myogenic clones were identified by My32 staining. DAPI staining identified both Tw2+ cells (tdTO+/CD34−) and MEFs. Scale bar, 100 µm. (g) Percentage of myogenic clones (My32+) of the total surviving clones in the clonal analysis. Data are mean ± s.e.m. N = 5 independent experiments. Statistical source data for b,d,g are provided in Supplementary Table 3.

and DM were largely similar (Supplementary Fig. 6c). Furthermore, when co-cultured at equal numbers in GM and exposed to DM, Tw2+ cells (tdTO+) and Pax7+ SCs (GFP+) fused with each other to form chimera myotubes that were positive for both GFP and tdTO (Supplementary Fig. 6d,e). We conclude that although Tw2+ cells are distinct from Pax7+ SCs, when removed from their native environment and cultured in vitro, Tw2+ cells acquire a Pax7+ cell fate and display similar myogenic potential to Pax7+ cells.

FACS analysis revealed that a small fraction (2.6%) of Tw2+ cells (tdTO+) expressed CD34, a marker commonly used to isolate SCs (Supplementary Table 1). To exclude the possibility that this small fraction of CD34+ cells might be responsible for the myogenic capacity of Tw2+ cells in vitro, we isolated the tdTO+/CD34− population by FACS. Freshly sorted tdTO+/CD34− cells proliferated rapidly within 48 h post isolation (Supplementary Fig. 7a). After 4 days in DM, 50% of tdTO+/CD34− cells formed multinucleated myotubes, and after 10 days in DM, the fusion index increased to 66%, with many myotubes contracting spontaneously (Fig. 7a,b). These results exclude the possibility that the myogenic potential of Tw2+ cells comes from a rare population of tdTO+/CD34− cells.

To examine whether the tdTO+/CD34− cells can form other cell lineages in addition to muscle cells, we exposed them to different conditions that support the formation of adipocytes and osteoblasts, respectively. After 10 days in adipogenic medium, the majority of tdTO+/CD34− cells formed multinucleated myotubes and only 1.5% of the cells differentiated into adipocytes (positive for Oil Red O staining), among more than 5,000 nuclei analysed (Fig. 7c,d). Thus, tdTO+/CD34− cells are not adipogenic in culture. Under osteogenic conditions, the majority of tdTO+/CD34− cells died within the first 24 h and after 10 days in culture, the few surviving cells...
Overexpression of Tw2 blocks myogenesis in vitro. (a) Tw2+ cells were infected with retroviruses expressing either GFP (Retro-GFP) or Tw2 (Retro-Tw2-IRES-GFP) for 24 h before switching to DM. After 5 days in DM, My32 staining (purple) was performed to detect myotubes. Scale bar, 100 µm. (b) Heat map of genes expressed in GFP- or Tw2-infected Tw2+ cells in GM and DM identified by RNA-seq. (c) Heat maps of the top genes enriched and repressed in GFP-DM versus Tw2-DM. (d) Pathways of genes enriched in Tw2-DM relative to GFP-DM identified by Ingenuity Pathway Analysis. (e) Overexpression of Tw2 inhibits myotube formation of Pax7+ cells. Pax7+ cells were infected with Retro-GFP or Retro-Tw2-IRES-GFP for 24 h before switching to DM. After 5 days in DM, My32 staining (purple) was performed to detect myotubes. Scale bar, 100 µm. (f) EdU labelling revealed increased DNA synthesis in Tw2-infected Pax7+ cells in DM. Infected Pax7+ cells were treated with EdU (10 µM) for 24 h in DM before staining. Scale bar, 100 µm.

proliferated and differentiated into osteoblasts, which stained for alkaline phosphatase (Fig. 7e). No My32-positive myotubes were observed under osteoblastogenic conditions. Together, these results indicate that the Tw2 cells that did not express CD34 are myogenic and osteogenic in vitro.

To further confirm the myogenic capacity of Tw2+ cells, we performed clonal analysis on tdTO+/CD34− cells. Briefly, single tdTO+/CD34− clones were seeded by FACS sorting onto each well of 96-well plates that were pre-coated with inactivated mouse embryonic fibroblasts (MEFs) as feeder cells. Single clones were grown in GM with bFGF for 1 week before being switched to DM. After 1 week in DM, 29.8% of surviving clones formed My32+ myotubes, while the others did not express My32 and had different morphologies (Fig. 7f,g). These results confirmed the intrinsic myogenic capacity of Tw2+ cells.

Tw2 blocks myogenesis in vitro

The finding that Tw2+ cells do not express Pax7 or MyoD in vivo, but quickly lose Tw2 gene expression and upregulate Pax7 and MyoD expression in vitro suggested that Tw2 maintains cells in a progenitor cell state, preventing them from entering the muscle differentiation pathway. To test this hypothesis, we overexpressed Tw2 by retrovirus infection in Tw2+ cells and analysed myotube formation. While control GFP retrovirus-infected cells formed multinucleated...
myotubes, Tw2-infected cells in DM underwent a dramatic change in morphology from spindle-shaped myoblasts to more flattened fibroblast-like shapes and did not form myotubes or express myosin (Fig. 8a). The few myosin-positive cells observed in Tw2-infected cultures were not infected by Tw2 retrovirus. The gene expression profiles in GM were very similar between GFP-infected and Tw2-infected cells (Fig. 8b). However, after 5 days in DM, muscle-specific genes that were upregulated in GFP-DM cells were not upregulated in Tw2-DM cells, and conversely, genes strongly upregulated in Tw2-DM cells were all downregulated in GFP-DM (Fig. 8c). Ingenuity Pathway Analysis revealed that genes involved in cell-cycle regulation, cancer metastasis and EMT were specifically upregulated in Tw2-DM cells (Fig. 8d). These findings confirm that Tw2-overexpression in Tw2+ cells inhibits myogenesis and induces EMT-like programs. Similarly, overexpression of Tw2 in Pax7+ cells inhibited myobute formation and caused morphological changes in DM (Fig. 8e). EdU labelling showed that Tw2-infected Pax7+ cells continued to proliferate in DM compared with GFP-infected cells, further demonstrating the ability of Tw2 to maintain cells in an undifferentiated state (Fig. 8f). Tw2 thus functions as a repressor of the myogenic program and its downregulation triggers the onset of myogenesis in Tw2 progenitors (Supplementary Fig. 8).

**DISCUSSION**

Our results reveal a previously unrecognized population of skeletal muscle progenitor cells marked by expression of Tw2. Tw2+ cells display a unique cell-surface marker expression profile and contribute specifically to type IIb/x fibres. These cells are distinct from Pax7+ SCs and appear to represent a subset of MSCs. In recent years, various non-SC muscle progenitors have been identified, including bone marrow-derived circulating stem cells, pericytes, PW1+ SC muscle progenitors have been identified, including bone marrow- and appear to represent a subset of MSCs. In recent years, various non-

specifically to type IIb/x fibres. These cells are distinct from Pax7+ muscle progenitor cells marked by expression of Tw2. Tw2+ muscle progenitor cells are specifically excluded from the tongue musculature. It will be interesting to investigate the potential contributions of Tw2+ cells in different muscle lineages during development.

Tw2+ progenitors are, to our knowledge, the first example of a fibre-type-specific myogenic progenitor population. In this regard, type IIb fibres are the most abundant fibres and are most susceptible to injury and disease in mice. Therefore, it is essential to maintain the size and integrity of type IIb fibres during ageing, and the contribution of Tw2+ cells to type IIb fibres may represent such a mechanism. Humans do not have type IIb fibres in skeletal muscle; instead the predominant fibre type is equivalent to mouse IId fibres. It will be interesting to study whether Tw2+ cells exist in human skeletal muscle and whether they contribute to specific fibre types.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

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AUTHOR CONTRIBUTIONS
Experiments were designed by N.L., G.A.G. and S.L. Experiments were performed by N.L., G.A.G., S.L., B.C., R.B.-D. and E.N.O. The paper was written by N.L. and E.N.O.

COMPETING FINANCIAL INTERESTS
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METHODS Generation of Tw2-CreERT2 mice. The targeting strategy for generation of Tw2-CreERT2 mice is shown in Supplementary Fig. 2a. A 7.0 kb EcoRI-NolI genomic fragment upstream of the mouse Twist2 coding region, and a BspEI-HindIII 1.5 kb fragment downstream of the Twist2 coding region were used as a long and short arm, respectively, in the construction of the targeting vector. A CreERT2-Frt-Neo-Frt expression cassette was inserted between the two regions, resulting in a vector designed to delete the entire coding region of Twist2. The targeting vector was linearized and electroporated into 129Esv-derived embryonic stem cells. Out of 500 embryonic stem cell clones, 10% were correctly targeted. Three clones with a properly targeted allele were injected into 3.5-d C57BL/6 blastocysts, no. 009669), CAG-eGFP (Twist2 resulting in a vector designed to delete the entire coding region of CreERT2-Frt-Neo-Frt expression cassette was inserted between the two regions, 1.5 kb genomic fragment downstream of the Twist2 genomic fragment upstream of the mouse Twist2-CreERT2 mice is shown in Supplementary Fig. 2a. A 7.0 kb EcoRI-NotI Fluor 647-CD106, FITC-Integrin-α100 × Fluorescence-activated cell sorting analysis. Following isolation, mononuclear cells area and fibre numbers were quantified by ImageJ. Skeletal muscles were harvested at desired ages, and & mg of TXM was administered by intraperitoneal injection to 8-week-old mice as schematized in the figures. Tw2-CreERT2; R26-DTA/+ mice and R26-DTA/+ in a TMX-containing diet (250 mg kg⁻¹) (Harlan Laboratories) immediately following TMX injection. Cardiotoxin (CTX, from Naja mossambica mossambica, Sigma Aldrich) was dissolved in sterile saline at 10 μM concentration. For CTX injury, 50 μL CTX (10 μM) was delivered by intramuscular injection to TA muscle, as described previously. TA muscles were harvested at 3, 7, and 14 days post-CTX injury. Immunohistochemistry. Skeletal muscles were harvested at desired ages, and fixed in 4% paraformaldehyde at 4°C for 1 h. Tissues were then washed to 10% sucrose/PBS overnight and in 18% sucrose/PBS at 4°C overnight, before they were frozen embedded and sectioned as previously described. For immunohistochemistry, the following antibodies were used: Twist2 (Abcam, no. ab66031, 1:200), Pax7, myosin Ila (SC-71), slow myosin (Abcam) (all from Developmental Studies Hybridoma Bank, 1:10), sarcomeric α-actinin (Sigma Aldrich, no. A8711, 1:100), laminin (Sigma Aldrich, no. L9393, 1:500), desmin (Dako, clone D353, 1:100), slow myosin (Sigma Aldrich, clone NOQ7, 1:250), Fast myosin (Sigma Aldrich, clone My32, 1:250), vimentin (Sigma Aldrich, no. V2258 1:100), MyHC (BD Pharlmingen, 593379, 1:200), Alexa Fluor secondary antibodies were used according to the manufacturer’s instructions. TdT signals were detected by direct fluorescent imaging. Immunohistochemistry of frozen sections was performed as described. Wheat germ agglutinin staining was performed on both frozen and paraffin-embedded sections, using WGA-Alexa Fluor 555 (W32464) or WGA-Alexa 647 conjugate (W34666) (Life Technologies, 50 μg ml⁻¹). Images were taken on a Zeiss LSM700 confocal microscope. Muscle cross-sectional area and fibre numbers were quantified by ImageJ.

Fluorescence-activated cell sorting analysis. Following isolation, mononuclear cells were resuspended in PBS/2% BSA at 1 × 10⁶ cells per 50 μL and aliquoted into 100 μL per tube, and incubated on ice for 1 h with Fc blocking agent and one of the following fluorescein-conjugated antibodies: APC-CD45 (1:50), APC-CD31 (1:50), APC-CD90.2 (1:50), APC-Cy7 Sc1 (1:50), Alexa Fluor 647-CD106 (1:50) (all from BD Biosciences-Pharmlingen), APC-CD29 (1:50) (eBioscience), Alexa Fluor 488-CD34 (1:50) (Abd Serotec), FITC-Cd184 (1:50), FITC-Integrin-α7 (1:50), Isotype-specific controls were also performed on these cells. Cells isolated from wild-type mice were used as stained negative controls using Alexa Fluor 488-CD34, Alexa Fluor 647-CD106, FITC-Integrin-α7, APC-Cy7 Sc1 and APC-CD29. Unstained cells isolated from Cre-positive and Cre-negative mice were used as unstained controls. Cells were analysed on FACScalibur (BD) flow cytometer and FACS data were analysed using Flowjo Software (TreeStar). Values of FACS analysis were averaged from three independent experiments.

Engrafment of Tw2+ cells into mdx mice. TA muscles of adult mdx mice were injected with 25 μL of CTX (10 μM) 1 day before transplantation. Tw2+ cells were isolated from cultures of Tw2-CreERT2; R26-tDTA mice 10 days post-CTX injury. freshly isolated cells were resuspended in sterile saline at a concentration of 2,400 cells μL⁻¹. For transplantation, 60,000 cells (25 μL) were injected into TA muscle of mdx mice with a Hamilton gastight syringe and 30G standard hypodermic needle (COVIDIEN). Four weeks post-transplantation, TA muscles were harvested, fixed, sucrose protected, frozen embedded and sectioned. Engrafment experiments were performed three times, each time with three mice.

Cell culture and myogenesis, adipsogenesis and osteogenesis in vitro. Tw2+ cells and Pax7+ cells were cultured as previously described. Satellite cells from CAG-eGFP mice were isolated by FACS sorting as the integrin-α7-positive and CD45/CD31/Scal-negative population. For mixing experiments, satellite cells from CAG-eGFP mice were co-cultured with Tw2+ cells at a 1:1 ratio in DM and then switched to DM to induce myoblast formation. Freshly sorted tdTom/CD34+ cells were grown in GM for 2 days and subsequently differentiated towards myoblasts, adipocytes or osteoblasts. For myoblast differentiation, the growth medium was replaced with DM for 4–10 days. Cells were then immunostained for MyHC to visualize differentiated myoblasts. For adipocyte differentiation, the growth medium was replaced with DMEM high glucose, 10% fetal bovine serum, Pen/Strep, 1.72 μg/ml insulin, 625 nM dexamethasone, 2 μM rosiglitazone and 0.5 μM isobutyl-methyl-xanthine for 10 days. Cells were then fixed for 15 min, stained with Oil Red O for 30 min, and then washed three times with PBS. Cells were then visualized with light microscopy. Cells were also stained with My32 and Hoechst to visualize myoblasts and nuclei. For osteoblast differentiation, the growth medium was replaced with DMEM high glucose, 10% fetal bovine serum, Pen/Strep, 50 μg ml⁻¹ ascorbic acid, 10 mM β-glycerol phosphate, and 100 nM dexamethasone. Cells were fixed and then stained for alkaline phosphatase using SigmaFAST BCIP/NBT. Cells were then washed and subjected to light microscopy. For immunocytochemistry, the detection of Oil Red O-positive cells, more than 500 nuclei per field and a total of 10 fields were counted and averaged.

Clonal analysis. Mouse embryonic fibroblasts (MEFs) were used as feeder cells for clonal analysis. One day before FACS sorting, confluent MEFs were treated with 8 μg ml⁻¹ of mitomycin C for 2.5 h at 37°C to inhibit cell cycling. Cells were then washed twice with PBS, followed by trypsinization. MEFs were then seeded into pre-Matrigel-coated 96-well plates at a density of 2 × 10⁶ cells well in DMEM/10%FBS/Pen/Strep and allowed to attach overnight at 37°C. Two hours prior to sorting, the medium was replaced with 100 μl of satellite cell growth cell medium (SCGM) comprising Ham’s F10, 20% FBS, 0.2% Primocin and 2.5 ng ml⁻¹ bFGF. Tw2+ cells isolated from hind limb muscle were isolated by sorting for the tdTom/CD34+ population. Single Tw2+ cells were sorted directly into each well of 96-well plates and were allowed to attach overnight. To prevent cell detachment, 50 μl of fresh SCGM containing the appropriate concentration of bFGF was added on top of existing media on a daily basis. When the maximum volume capacity of each individual well was reached (300 μl), 200 μl of SCGM was removed followed by replacement of 100 μl of fresh GM containing bFGF. Cells were cultured for 1 week in GM, followed by four days of differentiation in differentiation medium (DMEM, 2% horse serum and 0.2% Primocin). Differentiated cells were fixed and immunostained with My32 antibody.

Immunostaining of cultured cells. No commercial cell lines were used in this study. For immunostaining, cells grown on plates or chamber slides were fixed with 4% paraformaldehyde at room temperature for 15 min and subsequently permeabilized with 0.3% Triton X-100/PBS. Cells were blocked in 10% goat serum diluted in 0.3% Triton X-100/PBS at room temperature for 1 h. Cells were then blocked in 1% goat serum/0.1% Triton X-100 and incubated at room temperature for 2 h. Cells were washed and secondary antibodies were diluted in 1% goat serum/0.1% Triton X-100 and incubated at room temperature for 1 h. Cells were stained with Hoechst dye (1.2000 in PBS) at room temperature for 10 min. Primary antibodies include: fast myosin (Sigma-Aldrich, My32, 1:250), MyoD (Santa Cruz Biotechnology, sc-377400, 1:100). Fusion index was calculated as a ratio of the number of tdTom+ nuclei within a multinucleated myotube to the total number of tdTom+ nuclei. A minimum of 10 independent microscopic fields was used for each group over three independent differentiation experiments at 1 and 2 days in DM. For EdU labelling, EdU was used at 10 μM on Pax7+ cells in 24 h in DM. EdU detection was performed according to the Click-iT EdU imaging kit (Invitrogen). Images were taken using the Nikon Eclipse Ti microscope.

Retroviral infection of Tw2+ and Pax7+ cells. The following retroviral plasmid constructs expressing either eGFP or myc-Tw2-RES-eGFP were cloned into the retroviral vector pMX. Ten micrograms of retroviral plasmid DNA was transfected using FuGENE 6 (Roche) into Platinum E cells (Cell Biolabs), which were plated on a 10-cm tissue culture dish at a density of 3 x 10⁵ cells per dish, 24 h before transfection. After 48 h of transfection, viral medium was harvested and filtered through a 0.45 μm cellulose filter. The viral supernatant was mixed with Polybrene (Sigma) to a final concentration of 6 μg ml⁻¹. Tw2+ and Pax7+ cells were plated at a density of 200,000 cells per 35-mm plate in growth medium. After 24 h, the growth medium was replaced with freshly made.
viral mixture containing Polybrene and bFGF (5 ng ml\(^{-1}\)). Twenty-four hours later, viral medium was replaced with growth medium with bFGF. Twenty-four hours later, cells were switched to differentiation medium. Cells were either fixed and stained or harvested for NRA isolation at certain time points.

**Real-time RT–PCR analysis.** Total RNA was extracted from sorted cells with Trizol (Invitrogen) following the manufacturer's instructions. From this RNA, cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen). Selected gene expression was analysed by real-time RT–PCR using Taqman probes. Probes are twist2 (Mm00492147_m1), twist1 (Mm04208233_m1), myog (Mm00446195_g1), ckm (Mm00432536_m1), myh4 (Mm01332518_m1).

**RNA isolation and RNA-seq analysis.** Cells were resuspended in 1 ml of Trizol and homogenized using 20G needles. Following chloroform extraction, the supernatant was mixed with an equal volume of 75% ethanol and loaded on RNeasy mini-columns (Qiagen). Total RNA was isolated according to the manufacturer’s instructions (Qiagen). RNA quality was verified by the Agilent 2100 Bioanalyzer and RNA-seq was performed using Illumina HiSeq 2500 by UTSW Genomics and Microarray Core Facility. Quality assessment of the RNA-seq data was done using NGS-QC-Toolkit\(^{15}\). Reads with more than 30% nucleotide with phred quality scores less than 20 were removed from further analysis. Quality-filtered reads were then aligned to the mouse reference genome GRCm38 (mm10) using the TopHat2 (v 2.0.0) aligner\(^{15}\) using default settings except for –library-type = fr-firststrand. Aligned reads were counted using featurecount\(^{15}\) (v1.4.6) per gene ID. Differential gene expression analysis was done using the R package edgeR\(^{54}\) (v 3.8.6). For each comparison, genes were required to have 10 cpm (counts per million) in at least half of the total number of samples to be considered as expressed. They were used for normalization factor calculation. Gene differential expression analysis was done using the GLM approach following edgeR official documentation. (https://www.bioconductor.org/packages/3.3/bioc/vignettes/edgeR/inst/doc/edgeRUsersGuide.pdf). Cutoff values of fold change greater than 2 were then used to select for differentially expressed genes between sample group comparisons. Normalized gene cpm values were averaged within groups for heat map generation.

**Pathway enrichment analysis.** Significant pathway enrichment analysis was performed using Ingenuity Pathways Analysis (Ingenuity Systems). Differentially expressed genes from the RNA expression data are associated with a biological function supported by at least one publication in the Ingenuity Pathways Knowledge Base. Fisher's exact test was used to calculate the P value and determine the probability that each biological function was enriched in the data set due to chance alone. Statistically significant biological pathways were then identified by selection for pathways with P values less than 0.05.

**Statistics and reproducibility.** All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software). Data are presented as mean ± s.e.m. Differences between groups were tested for statistical significance by using the two-sample t-test. P < 0.05 was considered significant. The number of biological (non-technical) replicates for each experiment is indicated in the figure legends. Two independent sets of RNA samples from Tw2\(^{−}\) cells and Tw2\(^{+}\) cells, and one set of RNA samples from Pax7\(^{−}\) and Pax7\(^{+}\) cells were analysed by RNA-seq. For comparison between DTA and Cre\(^{−}\)-; DTA mice, n = 5 age- and gender-matched littermates were analysed to provide statistical significance of the study. Randomization was not used in most of the animal studies. However, images of type Iib/laminin staining (Fig. 3c) were taken by an investigator who was blinded to the group allocation. All immunofluorescence images are representative of at least three independent experiments or mice of the same genotypes. Western blots were representative of three independent experiments. No statistical method was used to predetermine sample size. Experiments described here were not randomized.

**Data availability.** RNA-seq data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) under accession codes GSE84377, GSE84378, GSE84379, GSE84380. Source data for Figs 1a,b,d, 2d, 3d, 4d, 6c,g and 7b,d and Supplementary Figs 1a,b,d, 2i, 3d and 4d,b have been provided as Supplementary Table 3. All other data supporting the findings of this study are available from the corresponding authors on request.

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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 Information**

**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. 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 Ila 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. Statistical 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 experiment. 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 39 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. Tw2-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 myogenisis 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 myogenisis. 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.
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.