

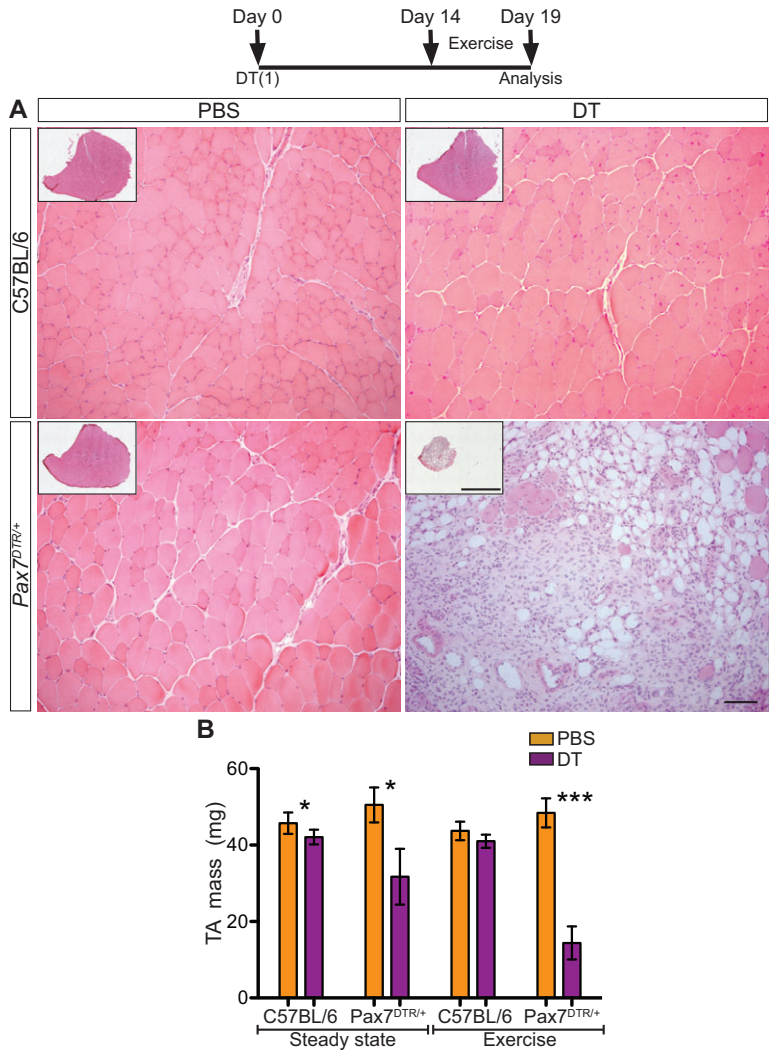
**Pax7-expressing satellite cells are indispensable for adult skeletal muscle regeneration**

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There was an error published in *Development* **138**, 3647-3656.

The panel labels on the left indicating genotypes were misaligned in Fig. 5A. The corrected Fig. 5 appears in full below.

The authors apologise to readers for this mistake.



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# Pax7-expressing satellite cells are indispensable for adult skeletal muscle regeneration

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## SUMMARY

Distinct cell populations with regenerative capacity have been reported to contribute to myofibres after skeletal muscle injury, including non-satellite cells as well as myogenic satellite cells. However, the relative contribution of these distinct cell types to skeletal muscle repair and homeostasis and the identity of adult muscle stem cells remain unknown. We generated a model for the conditional depletion of satellite cells by expressing a human diphtheria toxin receptor under control of the murine *Pax7* locus. Intramuscular injection of diphtheria toxin during muscle homeostasis, or combined with muscle injury caused by myotoxins or exercise, led to a marked loss of muscle tissue and failure to regenerate skeletal muscle. Moreover, the muscle tissue became infiltrated by inflammatory cells and adipocytes. This localised loss of satellite cells was not compensated for endogenously by other cell types, but muscle regeneration was rescued after transplantation of adult *Pax7*<sup>+</sup> satellite cells alone. These findings indicate that other cell types with regenerative potential depend on the presence of the satellite cell population, and these observations have important implications for myopathic conditions and stem cell-based therapeutic approaches.

**KEY WORDS:** Skeletal muscle stem cell, Diphtheria toxin, Regeneration, Mouse

## INTRODUCTION

Identifying tissue-specific regenerative cell types is a major challenge for regenerative biology and medicine. In skeletal muscle, satellite cells specifically express the *Pax7* gene, which is essential for their maintenance in the perinatal (Kuang et al., 2006; Oustanina et al., 2004; Relaix et al., 2006; Seale et al., 2000), but not adult (Lepper et al., 2009), period. Satellite cells can also be identified by cell surface markers, thereby permitting their isolation from the tissue by fluorescence-activated cell sorting (FACS) (Bosnakovski et al., 2008; Cerletti et al., 2008; Fukada et al., 2004; Kuang and Rudnicki, 2008; Montarras et al., 2005; Sacco et al., 2008; Sambasivan et al., 2009; Sherwood et al., 2004). After skeletal muscle injury, satellite cells are rapidly activated then enter the cell cycle and proliferate, generating myoblasts that will fuse to form new skeletal muscle fibres within a few days. Following this repair response, new quiescent satellite cells are generated during muscle homeostasis (Sambasivan and Tajbakhsh, 2007; Tedesco et al., 2010). *Pax7* is expressed in quiescent as well as activated satellite cells, and it is downregulated when satellite cells commit to muscle differentiation (Kuang and Rudnicki, 2008; Sambasivan and

Tajbakhsh, 2007; Tedesco et al., 2010). The finding that quiescent adult satellite cells do not require *Pax7* (or the paralogue *Pax3*) for self-renewal and regeneration (Lepper et al., 2009) highlights the differences between the adult satellite cell population and their embryonic or perinatal counterparts. Significantly, several studies have reported that a variety of non-satellite cell types can also participate in skeletal muscle regeneration in the adult. These include mesoangioblasts, which are associated with blood vessels in mice, dogs and humans (Dellavalle et al., 2007; Sampaolesi et al., 2006; Sampaolesi et al., 2003), and interstitial cells (PICs) which express *Pw1* (*Peg3* – Mouse Genome Informatics), an imprinted gene involved in regulating cell stress (Mitchell et al., 2010), as well as other cell types that are not fully characterised (Asakura et al., 2002; Gussoni et al., 1999; LaBarge and Blau, 2002; Poleskaya et al., 2003) (for a review, see Tedesco et al., 2010). Furthermore, cell-cell interactions play an important role in forming this tissue as multipotent fibroblastic/adipogenic mesenchymal progenitors (FAPs) interact with satellite cells to regulate muscle homeostasis (Joe et al., 2010; Uezumi et al., 2010), and endothelial cells were reported to affect satellite cell behaviour through secretion of signalling molecules (Christov et al., 2007). Therefore, the possibility that satellite cells can arise from another cell type and are endowed with potent short-term self-renewal capacity has not been formally excluded. Another possibility is that satellite cells are ‘paused’ precursor cells, having induced the downstream determination genes *Myf5* and *Myod* during development and subsequently downregulated them (Kanisicak et al., 2009; Kuang et al., 2007). Alternatively, satellite cells might constitute autonomous resident muscle stem cells with the capacity to generate differentiated myofibres and to self-renew. In any case, it is not known to what extent the different regenerative cell types in muscle are solicited in time and space either physiologically or in response to lesion, because models that can eliminate specific cell populations have not been available for investigation. Here, we describe a model

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in which satellite cells are ablated and, in combination with muscle injury, this model shows a sustained failure to regenerate the tissue despite the presence of other regenerative cells.

## MATERIALS AND METHODS

### Generation of the *Pax7<sup>tm1Mal</sup>* allele and *Pax7<sup>DTR/+</sup>* mutant mice

hDTR-IRES-EYFP-Cre-*neo<sup>r</sup>* containing a self-excising *neo* cassette [modified from Kissenpennig et al. (Kissenpennig et al., 2005)], was inserted in-frame with the ATG start codon in the first exon of the *Pax7* gene resulting in ablation of Pax7 protein expression (see Fig. S1A in the supplementary material for more details). Here, the *Pax7* endogenous promoter and regulatory elements drive expression of hDTR-IRES-EYFP, although YFP expression was not detected. PCR primers used for genotyping are listed in Table S1 in the supplementary material. The transgenic cassette was expressed specifically in *Pax7<sup>DTR/+</sup>* mice and specific sub-populations of muscle progenitor cells were enriched with the cassette, as shown by *DTR* mRNA levels (see Tables S2 and S3 in the supplementary material).

Experiments on mice were in accordance with French national regulations and local and international guidelines. For all experiments, a mixture of females and males were included in each group.

### Toxin injections

A total volume of 15–30  $\mu$ l was used for intramuscular injections of toxins (injected either alone or combined) or PBS into the TA muscle using 30G Hamilton syringes under anaesthesia induced by intraperitoneal injection of mice with ketamine (100 mg/kg) and xylazine (10 mg/kg) in sterile saline solution. Diphtheria toxin (DT) from *Corynebacterium diphtheriae* (Sigma Aldrich) was used at various concentrations ranging from 1 ng/g to 12.5 ng/g of mouse total body mass (weight). Cardiotoxin (Ctx) from *Naja mossaibica* (Sigma) was prepared at a concentration of 5–10  $\mu$ M.

### Histology

Dissected muscle was snap frozen in liquid nitrogen, sectioned (8  $\mu$ m thick) and stained with Haematoxylin and Eosin (HE) stain or Oil Red O (Sigma) using standard protocols. Microscopic examination was performed with a Nikon Eclipse E800 microscope. Photographs were taken with a Nikon DXM1200 digital camera.

### Measure of muscle mass and exercise

Dissected TA muscles were weighed with a precision balance (Sartorius, sensitivity 0.1 mg). As experiments combine male and female mice and to avoid intersubject variability, the muscle mass data were normalised relative to the PBS-injected contralateral muscle in the same mouse. When subjected to exercise, mice were placed on a forward-moving belt set at 12 m/min with a declining slope (30° for the first day and 15° for days 2 to 5) for 30 minutes per day for 5 days.

### Gene expression levels by RT-qPCR analysis

Total RNA was prepared from cells from total muscle by FACS using Ter119<sup>-</sup> cell subsets or from isolated fibres prepared from tibialis anterior (TA) or from the extensor digitorum longus (EDL) following 0.3% collagenase digestion at 37°C (80 isolated fibres per sample, two fibre preparations for each condition). Total RNA isolation was performed using RNeasy Micro and Mini Kits (Qiagen) and was reverse transcribed using the superscript II reverse transcriptase (Invitrogen). Gene expression was measured on an ABI PRISM 7700 sequence Detector (Applied Biosystems). Primers are listed in Table S3 in the supplementary material. Primers were used at a final concentration of 0.1  $\mu$ M, cycling conditions: 95°C for 10 minutes followed by 40 cycles of amplification (95°C for 15 seconds and 60°C for 1 minute). Each PCR was performed one to three times and in duplicate. CT values greater than 36 were considered to be a negative signal. Data were analysed using SDS 1.2 software (Applied Biosystems). Relative levels of expression of *Pax7*, *Myf5* or *Mrf4* to *Gapdh* mRNAs were normalised to a cDNA from an E10.5 C57Bl/6 mouse embryo (total extract) arbitrarily assigned a value of 1.0 for expression of each gene. Expression of *DTR* relative to *PO* [murine large ribosomal protein (NM\_007475.5)] was normalised with a cDNA obtained from the bone marrow (BM) of a CD11b-DTR transgenic mouse (Jackson Laboratory), which was assigned an arbitrary value of 55.87. This value was chosen because it provides

equivalent levels of *DTR/PO* and *Pax7/Gapdh* in total muscle from *Pax7<sup>DTR/+</sup>* mice (value of 2.6 for each). The primer pairs and Taqman probe used for amplification are listed in Table S3 in the supplementary material.

### Muscle mononuclear cell fractions and FACS

Skeletal muscles were digested with collagenase A 0.2% (w/v) and dispase II (2.4 U/ml (Roche), filtered successively through 100-, 70- and 40- $\mu$ m cell strainers (BD Biosciences). The resulting mononuclear cells (MNC) were suspended in PBS with 0.2% bovine serum albumin (BSA; Sigma) for staining with fluorescent monoclonal antibodies [APC-conjugated anti-CD45 (clone 30F11.1) or isotype control (Miltenyi); PE-anti-CD45 (Biolegend); PE-conjugated anti-Sca1 (clone D7) or isotype control IgG2a (Becton Dickinson); PE-Cy7 anti-Sca1 (clone D7, eBioscience); APC-conjugated anti-CD34 (clone RAM-34, eBioscience) or isotype control (clone eBR2a); PE-CY7-conjugated anti-TER119 or isotype control IgG<sub>2b</sub> (Becton Dickinson) and 7AAD (final concentration 10  $\mu$ g/ml) (Sigma)], washed and sorted by FACS (MoFlo, Cytomation or CyAn, Beckman Coulter) and was analysed by flow cytometry (MoFlo, Cytomation or CyAn, Beckman Coulter). Gates included cells with a low side scatter, cells negative for 7AAD and cells negative for the erythroid marker Ter119. Quadrants were established using a negative threshold based on isotype controls. Data were analysed post-acquisition by FlowJo (Tree Star).

### Cell sorting and transplantations

Muscles were dissected from *Tg:Pax7nGFP* mice and digested in 0.1% collagenase D (Roche 11088882) and 0.25% trypsin (Invitrogen 15090), then sorted based on GFP fluorescence using a FACSaria, BD and FACSDiva. For grafting, sorted cells were centrifuged and resuspended in 10  $\mu$ l of sterile Dulbecco's modified Eagle's medium. Mice were anaesthetised with 0.5% Imalgene/2% Rompun. Satellite cells isolated by FACS were injected into the TA muscle (30,000–50,000 cells) that had been subjected to toxin injections 48 hours before transplantation. Grafted muscles were collected 7 or 14 days post-transplantation for analysis.

### Immunohistochemistry

Muscles were fixed in 2% paraformaldehyde with 0.5% Triton X-100 for 2 hours at 4°C, washed several times in PBS for 2 hours, equilibrated in 15% sucrose overnight, cryofrozen in a 2-methylbutane bath on liquid nitrogen and cryosectioned transversally at a thickness of 12–14  $\mu$ m. Tissue was not fixed for immunostaining with neonatal MyHC antibody. Antibodies used in this study include rabbit anti-neonatal myosin heavy chain (kindly provided by V. Mouly, Institute of Myology, Paris, France; 1/500), anti-myogenin (SantaCruz Biotechnology; M-225; 1/200), anti-desmin (Dako, M0760; 1/200), anti-laminin (Sigma, 9393; 1/1000 or Abcam, ab14055; 1/100), anti-GFP (Abcam; 13970; dilution 1/750), anti-Pax7 (Aviva biosystems, ARP32742; 1/750), anti-CD31 (Abcam; ab7388; 1/100) and anti-Pw1 (kindly provided by D. Sassoon, Institute of Myology, Paris, France; 1/4000). Images were acquired using a LEICA SPE confocal and LAS software or Zeiss Observer and Axiovision software. Optical sections (1–1.5  $\mu$ m thick) were z-projected using ImageJ (NIH). Images were assembled using Adobe Photoshop and Adobe In-design.

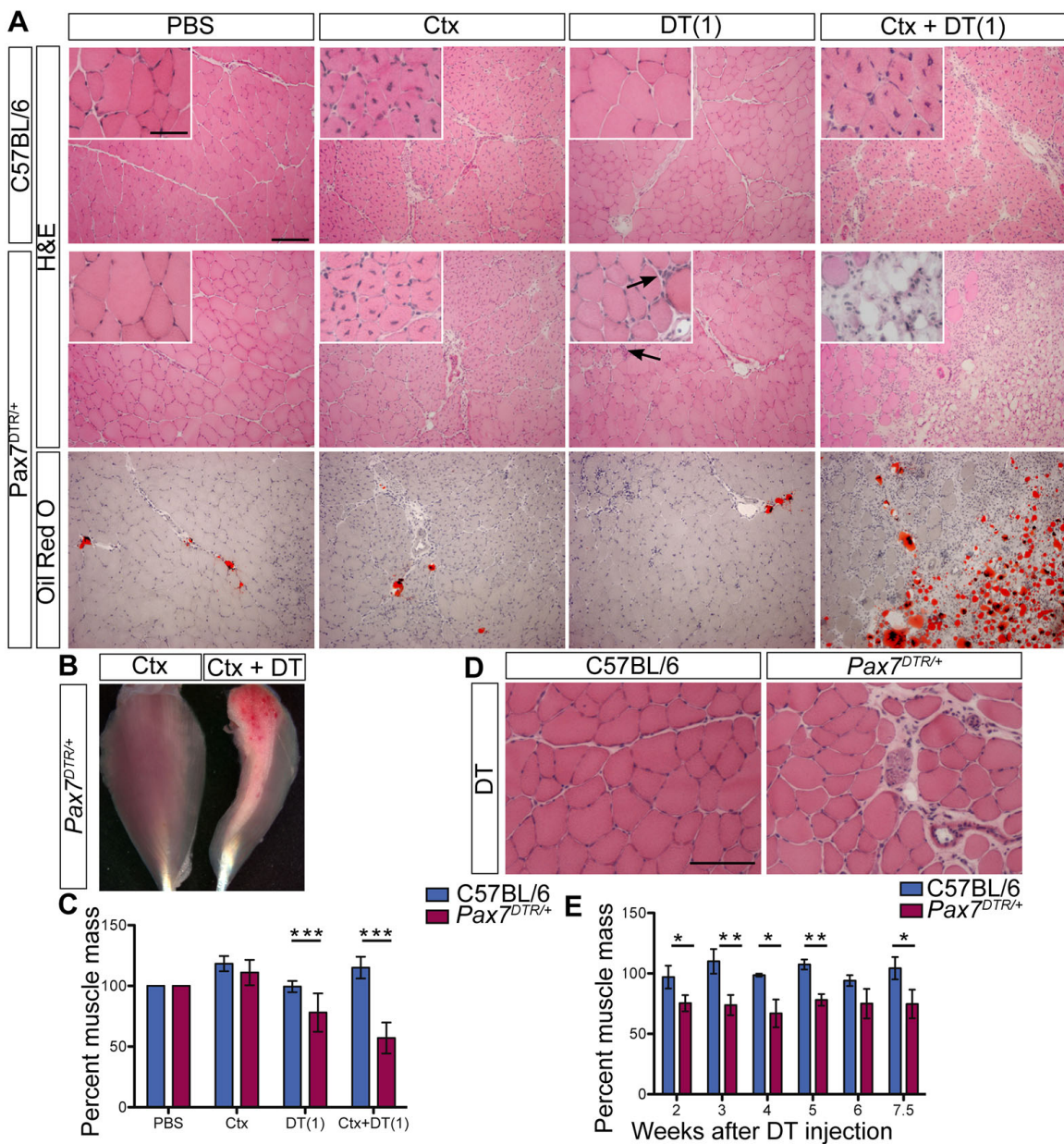
### Statistics

Statistical analysis was performed with GraphPad Prism software using appropriate tests and minimum of 95% confidence interval for significance. Specific details of the statistical tests are given in the figure legends.

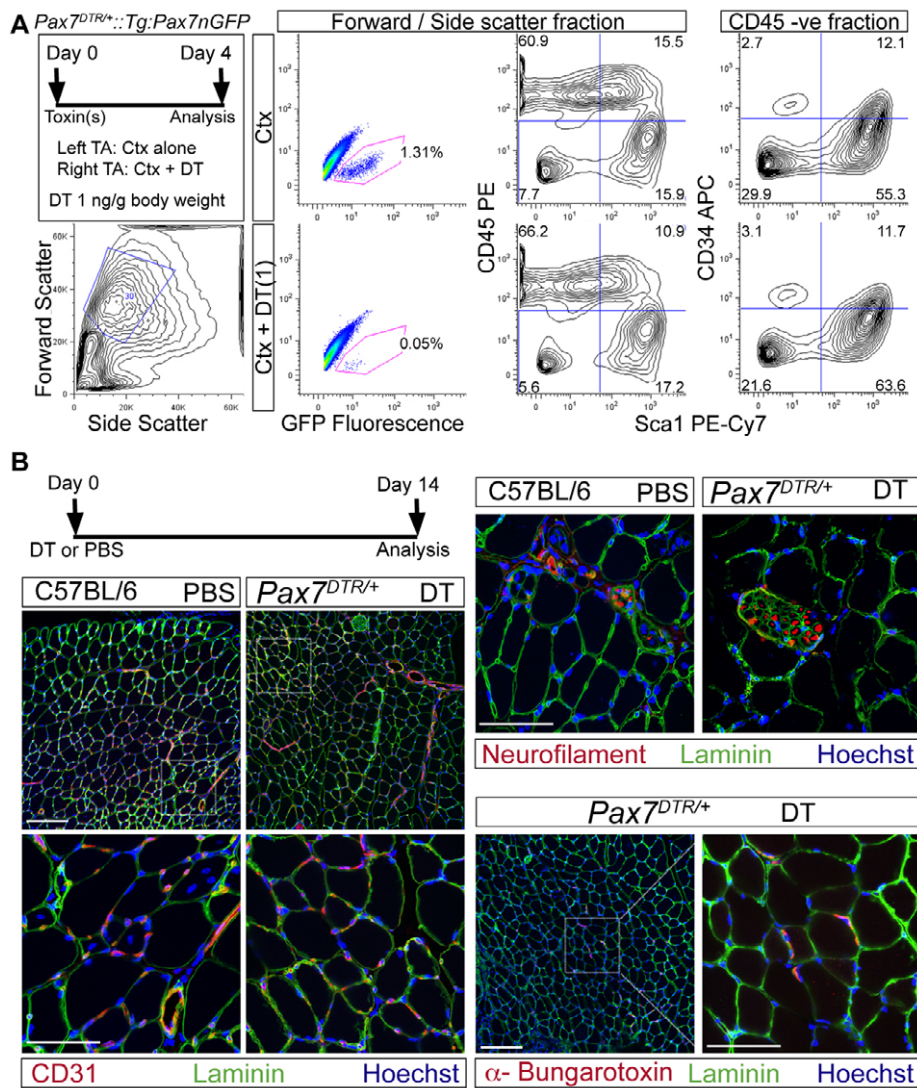
## RESULTS

### Muscle regeneration fails upon Pax7<sup>+</sup> cell depletion

To investigate the extent to which adult satellite cells contribute to regenerating skeletal muscles, a knock-in *Pax7<sup>DTR</sup>* mouse model was made by expressing a diphtheria toxin (DT) receptor (DTR) under control of the *Pax7* gene promoter (see Fig. S1A,B in the supplementary material). The DTR knock-in strategy was used successfully to ablate DTR<sup>+</sup> cells in different lineages at chosen times by inducing their death after administration of DT, a potent inhibitor of protein translation (Duffield et al., 2005;



**Fig. 1. Diphtheria toxin (DT) combined with myotoxic injury causes muscle damage, failure to regenerate and loss of differentiated muscle tissue in *Pax7<sup>DTR/+</sup>* mice.** (A) Histological analysis (H&E or Oil Red O stainings) of tibialis anterior (TA) muscle of adult C57BL/6 or *Pax7<sup>DTR/+</sup>* mice 14 days following injection of either cardiotoxin (Ctx) or DT (1 ng/g body weight) or both, compared with injection of PBS. Note *Pax7<sup>DTR/+</sup>* mice display normal muscle histology and regeneration (centronucleated fibres, a hallmark of muscle regeneration) after Ctx injection. *Pax7<sup>DTR/+</sup>* mice selectively responded to DT, as shown by discrete cellular infiltration (arrows), and display severe muscle destruction after combined administration of DT and Ctx; note loss of myofibres, marked cellular inflammation, and fat infiltration (bottom panels). Results are representative of at least three independent experiments using 6- to 14-week-old mice with at least nine mice/condition. Scale bar: 50  $\mu$ m for insets; 150  $\mu$ m for main panels. (B) Whole mount of *Pax7<sup>DTR/+</sup>* TA muscles 14 days post-toxin injections. (C) Selective loss of muscle mass following injection of DT in *Pax7<sup>DTR/+</sup>* mice. Intramuscular injections of either DT (1 ng/g body weight) or Ctx or both in one TA; the contralateral TA received PBS and muscle mass was measured 14 days after injection. Mass of toxin-injected TA are expressed as percentage relative to PBS-injected contralateral TA. Graph represents average percentages  $\pm$  s.e.m. from three to six independent experiments comprising a total of 10-18 mice/condition. The effect of DT or Ctx+DT on *Pax7<sup>DTR/+</sup>* mice is highly significant compared with PBS as determined using paired Student's *t*-test giving *P*-values of  $5 \times 10^{-4}$  and  $3 \times 10^{-9}$ , respectively (not represented). For comparison of C57BL/6 and *Pax7<sup>DTR/+</sup>* in each condition, *P*-values are obtained using Mann and Whitney non-parametric test. (D,E) Loss of muscle mass induced by DT injection in *Pax7<sup>DTR/+</sup>* mice is not restored over time. *Pax7<sup>DTR/+</sup>* or *Pax7<sup>+/+</sup>* littermate controls (6-9 weeks old) received a DT injection (1 ng/g body weight) in the TA and muscle mass was measured relative to PBS-injected contralateral TA. Histology (H&E) of TA, 6 weeks following DT injection. Scale bar: 100  $\mu$ m. (E) Histogram represents average percentages  $\pm$  s.e.m. from four mice per condition and in two independent experiments. DT caused a small but significant reduction in muscle mass in *Pax7<sup>DTR/+</sup>* mice compared with control mice (unpaired Student's *t*-test) but the analysis of variance (Kruskal-Wallis test, two-tailed) across all time points showed no significant difference, thus mass of DT-injected muscles from *Pax7<sup>DTR/+</sup>* mice does not recover or aggravate over time in steady-state conditions. \**P*<0.05; \*\**P*<0.005; \*\*\**P*<0.0005.



**Fig. 2. Specific elimination of adult muscle satellite cells by diphtheria toxin (DT) administration in *Pax7<sup>DTR/+</sup>* mice.**

(A) Representative flow cytometry profiles of mononuclear cells obtained from tibialis anterior (TA) muscles of *Pax7<sup>DTR/+</sup>::Tg:Pax7nGFP* mice ( $n=3$ ) injected with DT (1 ng/g body weight) and control mice ( $n=3$ ). A dramatic loss of satellite cells, varying from 20- to 100-fold is shown by the loss of GFP<sup>+</sup> cells. Non-satellite cell populations identified by combinations of surface markers (CD45, CD34 and Sca1) and fluorescence-activated cell sorting (FACS) are not overtly affected. Similar results were obtained with 12.5 ng DT/g body weight (data not shown). Ctx, cardiotoxin. (B) Staining with antibodies against CD31, neurofilament and  $\alpha$ -bungarotoxin (which binds acetylcholine receptors) shows that vasculature, innervation and neuromuscular junctions, respectively, are unaffected by DT in the TA muscle of *Pax7<sup>DTR/+</sup>* mice. Scale bars: 50  $\mu$ m for high magnifications; 100  $\mu$ m for low magnifications.

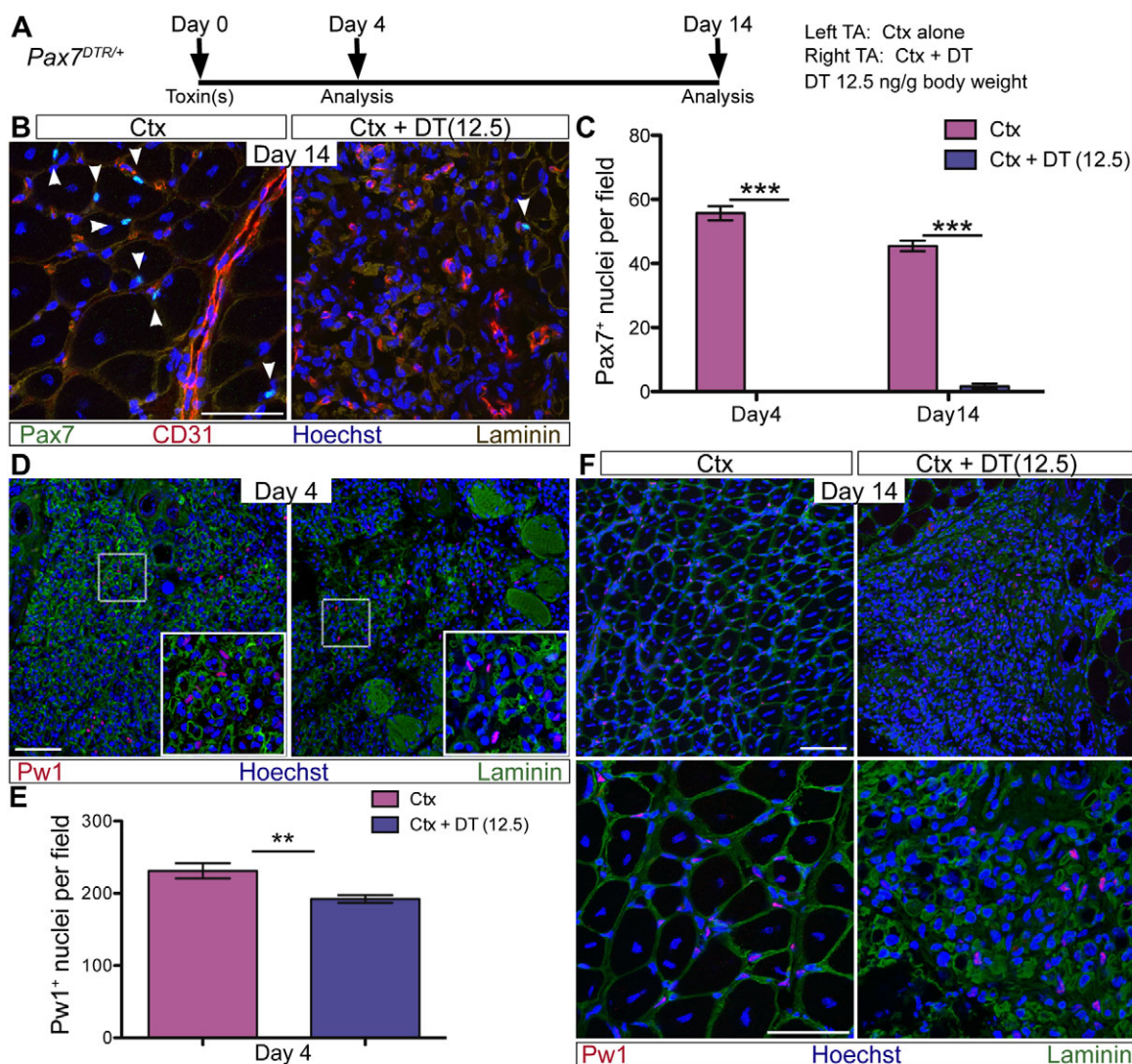
Kissenpennig et al., 2005; Probst et al., 2005; Saito et al., 2001). As expected (Kuang et al., 2006; Lepper et al., 2009; Oustanina et al., 2004; Relaix et al., 2006; Seale et al., 2000), *Pax7<sup>DTR/DTR</sup>*-null mice were born smaller in size and they died during young adulthood. Heterozygous adult *Pax7<sup>DTR/+</sup>* mice (6-14 weeks old) exhibited normal musculature and regenerative capacity, as shown by histology and gross morphology (Fig. 1A,B). In these mice, mRNA expression of *Pax7* was correlated with that of *DTR* and enriched in the CD45<sup>-</sup>/Sca1<sup>-</sup> cell fraction of skeletal muscle, which is known to be enriched in myogenic activity and to contain satellite cells (Cerletti et al., 2008; Fukada et al., 2004; Montarras et al., 2005; Sherwood et al., 2004), but not in the non-satellite containing fractions, CD45<sup>+</sup> blood-derived cells and CD45<sup>-</sup>/Sca1<sup>+</sup> cells, nor in differentiated muscle fibres, as expected from the known pattern of *Pax7*, *Myf5* and *Mrf4* (*Myf6* – Mouse Genome Informatics) gene expression (see Table S2 in the supplementary material).

Like wild-type mice (Gayraud-Morel et al., 2009), *Pax7<sup>DTR/+</sup>* mice regenerate skeletal muscle in response to snake venom-derived myotoxins such as cardiotoxin (Ctx; Fig. 1A) or notexin. Damage of the tibialis anterior (TA) muscle caused by intramuscular injection of Ctx led to regeneration of the tissue within 2 weeks, both in terms of

histological features (Fig. 1A) and the full recovery of muscle mass compared to the contralateral muscle, which was injected with phosphate buffered saline (PBS; Fig. 1C).

Compared with myotoxins, the intramuscular injection of a range of doses of DT (1-12.5 ng/g of body weight) caused little tissue remodelling in wild-type mice as determined 2 weeks later at the histological level (Fig. 1A; data not shown). In *Pax7<sup>DTR/+</sup>* mice, the injection of DT resulted in a modest cellular interstitial infiltration (Fig. 1A, arrows) that was composed primarily of macrophagic CD45<sup>+</sup>/CD11b<sup>+</sup> cells. This mild phenotype might be provoked by the removal of satellite cells and it was more pronounced at higher doses of DT (data not shown). We note that equivalent doses, or doses twice those used in the current study, were employed to target specific cell populations in other models (Arnold et al., 2007). It is possible that DT might bind to mouse receptors, albeit with a significantly lower affinity than to the human DTR (Saito et al., 2001); therefore, at high doses, an additional non-specific effect of DT cannot be excluded.

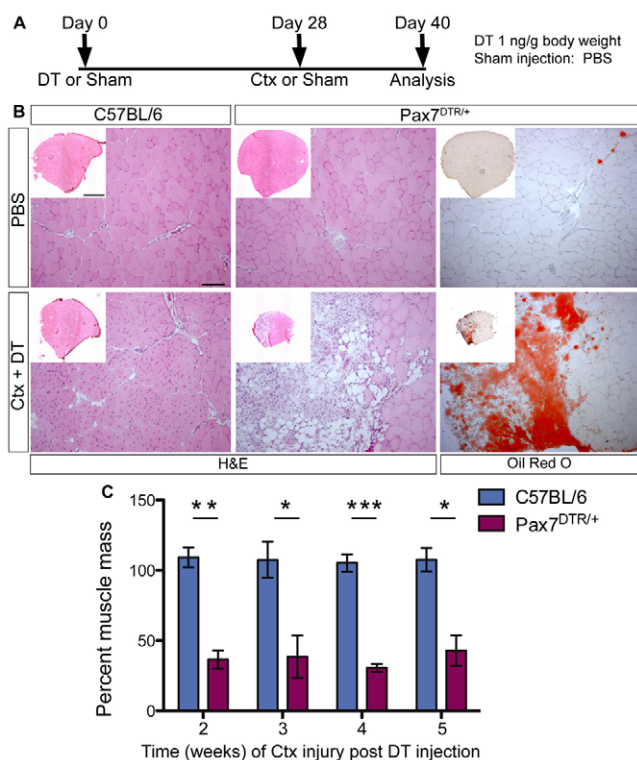
In spite of a modest effect on the anatomical structure of the muscle, injection of DT in *Pax7<sup>DTR/+</sup>* mice had a profound impact on the homeostasis and regenerative capacity of the muscle. A



**Fig. 3. Satellite cell-depleted skeletal muscle retains Pw1<sup>+</sup> cells.** (A) Scheme of experiments using *Pax7<sup>DTR/+</sup>* mice. Ctx, cardiotoxin; DT, diphtheria toxin; TA, tibialis anterior. (B,C) Anti-Pax7 and anti-CD31 antibody staining, and quantification of Pax7<sup>+</sup> cells at day 4 and 14 ( $n=3$  animals, six fields per TA). Arrowheads indicate positive cells in panel B. (D-F) Anti-Pw1 antibody staining and quantification ( $n=3$  animals, four to ten fields per TA, 400–1000 nuclei per TA, unit area=0.604 mm<sup>2</sup>, two to three TA cross-sections per animal;  $P$ -values calculated using unpaired Student's  $t$ -test). The moderate but statistically significant loss of Pw1<sup>+</sup> cells in panel E at day 4 might correspond to loss of a subset of Pw1<sup>+</sup> satellite cells (Mitchell et al., 2010). \*\* $P<0.005$ ; \*\*\* $P<0.0005$ . Scale bars: 50 μm in B and F (bottom); 100 μm in D and F (top).

single intramuscular injection of a low dose of DT in the TA of *Pax7<sup>DTR/+</sup>* adult mice led to a 20–40% loss of muscle mass (Fig. 1C) and this effect was sustained for at least 7 weeks without recovery or aggravation over time (Fig. 1D,E). Injection of DT induced, within 4 to 8 days and for at least 14 days, the complete loss of *DTR* mRNA as determined by RT-qPCR of CD45<sup>-</sup>/Sca1<sup>-</sup> cells isolated by FACS from injected muscles (data not shown). Accordingly, DT demonstrated specific toxic effects on this population of myogenic cells in vitro as addition of DT to cultures of CD45<sup>-</sup>/Sca1<sup>-</sup> cells isolated from *Pax7<sup>DTR/+</sup>* mice, but not from C57BL/6 control mice, abrogated myogenic growth and induced cell death (see Fig. S2A,B in the supplementary material). By contrast, addition of DT did not affect the growth and survival of cultured CD45<sup>-</sup>/Sca1<sup>+</sup> cells, which do not contain Pax7- or DTR-expressing cells as indicated by RT-qPCR (see Table S2 in the supplementary material).

Because skeletal muscle and satellite cells are thought to have a very low turnover in steady state conditions (Lepper and Fan, 2010; Spalding et al., 2005), we explored further how muscle regeneration occurs when *Pax7<sup>+</sup>* satellite cells are depleted. Combining DT injection with an acute myotoxic injury provoked by Ctx induces muscle regeneration in normal mice as shown by clear histological signs such as large areas of centrally nucleated myofibres (Fig. 1A) and by the recovery of complete muscle mass within about 2 weeks (Fig. 1C). By contrast, *Pax7<sup>DTR/+</sup>* mice responded to this combined toxin treatment with massive tissue infiltration, loss of myofibres and failure to reconstitute skeletal muscle tissue structure and mass (Fig. 1A-C). Interestingly, the loss of myofibres was accompanied by the presence of adipocytes as observed by Oil Red O staining as well as macroscopic observations (Fig. 1A, bottom panels; 1B), indicating that these cells expanded at the expense of skeletal muscle tissue as reported previously (Uezumi et al., 2010).



**Fig. 4. Sustained inability to regenerate muscle tissue following depletion of Pax7<sup>+</sup> satellite cells.** (A) Scheme showing muscle damage induced by a single injection of diphtheria toxin (DT; 1 ng/g body) in the tibialis anterior (TA) of 6- to 9-week-old mice or littermate controls, followed by cardiotoxin (Ctx) injection and analysis. (B) Histology with H&E and Oil Red O staining. Scale bars: 1 mm in insets; 150  $\mu$ m in panels. (C) Average muscle mass of TA relative to PBS-injected contralateral muscle  $\pm$  s.e.m. from three to five mice per group; *P*-value calculations using unpaired Student's *t*-test. Analysis of variance using Kruskal-Wallis showed that muscle mass was not recovered in Pax7<sup>DTR/+</sup> mice. \**P*<0.05; \*\**P*<0.005; \*\*\**P*<0.0005.

### Non-satellite regenerative cell types are inadequate for muscle regeneration

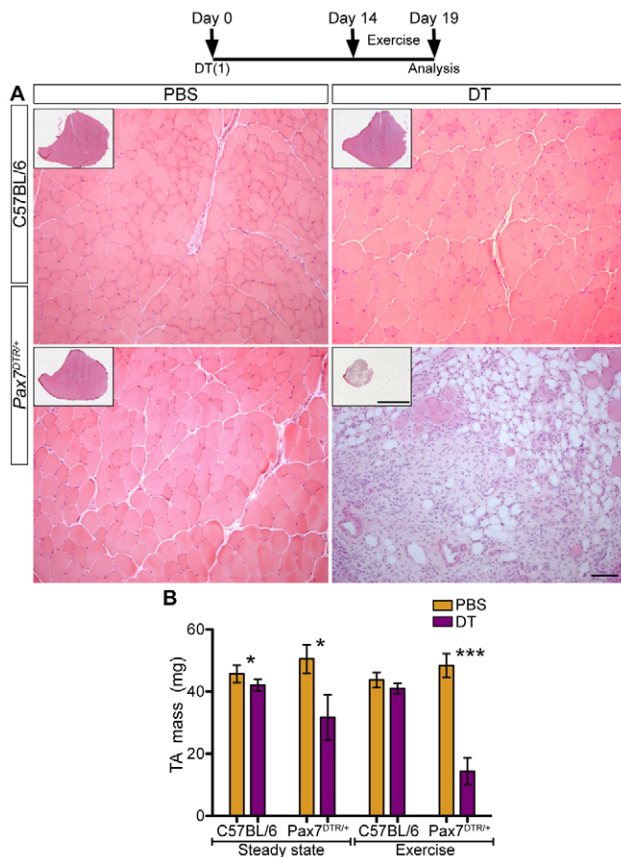
Injection of DT depleted Pax7<sup>+</sup> satellite cells as directly demonstrated by FACS using Pax7<sup>DTR/+</sup>::Tg:Pax7nGFP mice (Fig. 2A), where transgenic Tg:Pax7nGFP mice permit the isolation of all satellite cells based on GFP expression (Sambasivan et al., 2009) (see Fig. S3 in the supplementary material; B.G.-M. and S.T., unpublished). In these mice, injection of DT in addition to Ctx induced an almost total loss of Pax7nGFP<sup>+</sup> cells. FACS analysis of CD34 or Sca1 subsets of the CD45<sup>-</sup> cell population also showed little change in these populations after DT injection (Fig. 2A) suggesting that FAPs are not eliminated. This is also consistent with the adipogenesis shown in this model (Fig. 1). The loss of Pax7<sup>+</sup> cells following injection of DT alone was also confirmed by immunohistochemistry (data not shown); we also show that neither vasculature, as revealed by staining for the endothelial marker CD31, nor innervation, as shown by staining for neurofilaments and neuromuscular junctions ( $\alpha$ -bungarotoxin), is affected by DT injection in Pax7<sup>DTR/+</sup> muscle (Fig. 2B).

At least two populations of myogenic progenitor cells were described in skeletal muscle: Pw1<sup>+</sup>/Pax7<sup>+</sup> cells, representing the majority of the satellite cell population located under the basement membrane; and Pw1<sup>+</sup>/Pax7<sup>-</sup> cells, located in the interstitium with

the capacity to contribute to regeneration and to give rise to satellite cells after transplantation into previously injured muscle (Mitchell et al., 2010). Our satellite cell ablation model would eliminate the former, but not the latter population. To examine in more detail the cellular composition of the TA muscle following satellite cell ablation and muscle injury, the TA muscles of Pax7<sup>DTR/+</sup> mice were injected with either Ctx, or DT and Ctx (DT/Ctx), and the muscles were analysed by immunohistochemistry after 4 and 14 days (Fig. 3A-F). As expected, Ctx-injected muscles displayed regenerating features with centrally nucleated regenerating myofibres and numerous CD31<sup>+</sup> vascular endothelial cells (Fig. 3B). In addition, Pax7<sup>+</sup> cells associated with myofibres (55.67 $\pm$ 2.21 s.e.m. per unit area, day 4; 45.44 $\pm$ 1.63 s.e.m., day 14; Fig. 3B,C), as well as Pw1<sup>+</sup> cells, including PICs (231.1 $\pm$ 10.6 s.e.m. per unit area, day 4; Fig. 3D,E), were readily observed in muscles regenerating after Ctx injection as expected (see also the recovery of normal histology in Fig. 1A). Furthermore, at early and late stages after Ctx administration, muscle differentiation assayed by the presence of the differentiation specific transcription factor myogenin, as well as the structural protein desmin could be observed (see Fig. S4C,D in the supplementary material).

Significantly, DT/Ctx-injected muscle sections contained virtually no regenerating centrally nucleated myofibres, and only rare Pax7<sup>+</sup> cells (0.11 $\pm$ 0.11 s.e.m. per unit area, day 4; 1.67 $\pm$ 0.75 s.e.m., day 14) with both doses of DT (Fig. 3B,C; see also Fig. S4C,D in the supplementary material). By contrast, Pw1<sup>+</sup> (192.1 $\pm$ 5.29 s.e.m., day 4) and CD31<sup>+</sup> cells were present in these conditions (Fig. 3B,D,E,F). The severe loss of Pax7<sup>+</sup> cells induced by the injection of DT in combination with Ctx is confirmed at lower doses of DT (see Fig. S4A-D in the supplementary material) and by an almost total absence of Pax7 mRNA expression in the CD45<sup>-</sup> cell population 2 weeks after injection of toxins (data not shown). Accordingly, muscle differentiation was virtually undetectable using anti-myogenin or anti-desmin antibodies (see Fig. S4B-D in the supplementary material). Taken together, we show that the co-injection of DT/Ctx in Pax7<sup>DTR/+</sup> mice provoked a loss of tissue mass (Fig. 1C), resulting from a lack of myofibres, as well as a disappearance of Pax7<sup>+</sup>, myogenin<sup>+</sup> and desmin<sup>+</sup> cells by day 4, both of which persisted until later stages (Fig. 1A, Fig. 2A, Fig. 3C see Fig. S4A-D in the supplementary material). Therefore, we conclude that the loss of satellite cells results in failure to regenerate skeletal muscle after injury in spite of the presence of PICs or other non-satellite cells.

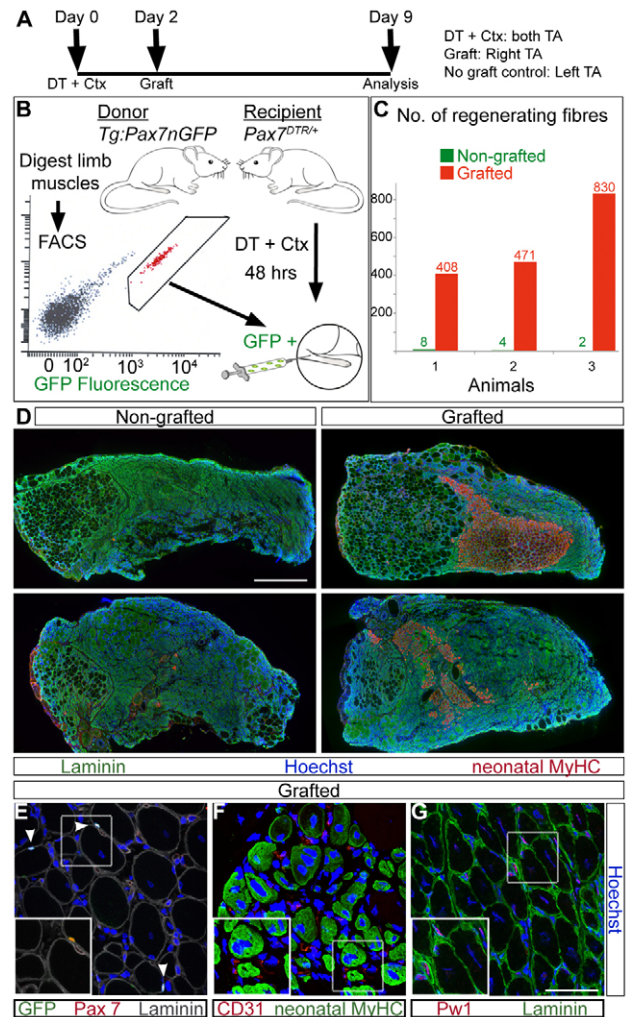
One concern in our experimental paradigm was that cells such as PICs, mesoangioblasts or other regenerative cell types that are not of satellite cell origin might be eliminated by the simultaneous injection of DT/Ctx, owing to a possible non-specific effect of DT and/or activation of Pax7 expression in those cells in response to Ctx. Indeed, PICs and mesoangioblasts were reported to express Pax7 and, at least in some cases, they might require this gene for participating in muscle regeneration (Mitchell et al., 2010). To address this issue, DT was injected into the TA muscle of Pax7<sup>DTR/+</sup> mice, followed by at least 14 days of latency, to allow DT clearance and tissue reconstitution, and muscle degeneration was provoked with one injection of Ctx at different time points (Fig. 4A). This protocol revealed that satellite cell-depleted Pax7<sup>DTR/+</sup> muscles persistently lost the capacity to regenerate for as long as 35 days post-DT injection (harvest at day 52). The response to Ctx challenge remained abnormal at the histological level, showing fatty infiltration, and tissue mass was not recovered, in contrast to what was observed in wild-type mice (Fig. 4B,C). Thus, even after 5 weeks of DT injection, the satellite cell compartment is not replenished by other regenerative cell types.



**Fig. 5. Failure to regenerate skeletal muscle following satellite cell depletion.** (A,B) Strenuous physical exercise after diphtheria toxin (DT) injection causes collapse of tibialis anterior (TA) muscle depleted in satellite cells. Mice (six to nine mice per condition) were injected with PBS or DT (1 ng/g) and were either forced to run for 30 minutes/day for five days or not (steady state). Note dramatically affected histology (A) of muscle (H&E stain) in DT-injected *Pax7<sup>DTR/+</sup>* mice; insets display whole muscle area. Scale bars: 150  $\mu$ m in main panels; 1 mm in insets. Histogram (B) represents average mass  $\pm$  s.e.m. of DT-injected TA relative to PBS-injected contralateral muscle in the different conditions. The effects of DT on *Pax7<sup>DTR/+</sup>* mice compared with PBS were statistically significant in steady-state conditions (paired Student's *t*-test). \**P*<0.05; \*\*\**P*<0.0005.

### Distinct injury models and satellite cell ablation

It is known that satellite cells are activated and that they re-enter the cell cycle in response to strenuous resistance exercise, thereby effecting muscle repair, and that regeneration and physical exercise is proposed to patients with sarcopenia (degenerative loss of muscle mass) to improve muscle mass and strength (Snijders et al., 2009; Shefer et al., 2010). Physical exercise was therefore tested as an alternative to chemical myotoxic injury, to induce endogenous muscle repair in DT-treated *Pax7<sup>DTR/+</sup>* mice under more physiological conditions. A strenuous exercise regimen was imposed 2 weeks after the depletion of satellite cells with a low dose of DT in one TA muscle. This regimen affected *Pax7<sup>DTR/+</sup>* mice specifically, but not controls, leading to a dramatic loss of myofibres, inflammation, fat infiltration and loss of muscle mass only in the DT-injected TA (Fig. 5A,B). The resulting effect of exercise was consistent with solicitation of satellite cells for maintenance of



**Fig. 6. Rescue of satellite cell depletion by transplantation of satellite cells alone.** (A) Scheme of experimental design for satellite cell transplantation in *Pax7<sup>DTR/+</sup>* mice. Ctx, cardiotoxin; DT, diphtheria toxin; TA, tibialis anterior. (B) Transplantation protocol and fluorescence-activated cells sorting (FACS) profile for satellite cells isolated from *Tg:Pax7nGFP<sup>+</sup>* mice (*n*=3 mice, 12.5 ng/g DT; *n*=3 mice, 1 ng/g DT; data not shown). The contralateral TA was injected with PBS. (C) Quantification of regenerating myofibres expressing neonatal MyHC. (D) Two separate examples (different animals) of transverse sections of whole TA muscle illustrate failure to regenerate non-grafted TA (top left and bottom left) and rescue of muscle tissue in grafted TA (top right and bottom right). (E-G) Presence of donor-derived GFP<sup>+</sup>, Pax7<sup>+</sup> cells in engrafted muscle; endothelial cells (CD31<sup>+</sup>) as well as Pw1<sup>+</sup> cells are shown associated with the graft-rescued muscle. Arrowheads indicate donor-derived satellite cells. Scale bars: 500  $\mu$ m in D; 100  $\mu$ m in G.

muscle regeneration and repair, and the outcome of this severe injury paradigm was consistent with the observations made above following myotoxin injury.

### Satellite cell transplantation rescues failed regeneration of muscle in *Pax7<sup>DTR</sup>* mouse

Taken together, our experiments provide compelling evidence that one cell type, the satellite cell, was eliminated in our genetic DTR ablation model following intramuscular DT injection in adult mice



and that this causes failure to regenerate skeletal muscle in two distinct injury models. To demonstrate unequivocally that the loss of satellite cells alone causes failure of muscle regeneration in this ablation model, we performed a rescue experiment. The experiment involved DT/Ctx injection of TA muscles of *Pax7<sup>DTR/+</sup>* mice followed 2 days later by transplantation of satellite cells isolated from *Tg:Pax7nGFP* donor mice (Fig. 6A,B). Muscle regeneration was evident by the presence of new myofibres expressing neonatal myosin heavy chain (MyHC) 7 days after transplantation, and this was observed only in skeletal muscles transplanted with satellite cells (Fig. 6C,D;  $n=6$  mice). Notably, *GFP<sup>+</sup>/Pax7<sup>+</sup>* cells (donor-derived satellite cells) were observed in the engraftment zone containing regenerating myofibres, in addition to *Pw1<sup>+</sup>* and *CD31<sup>+</sup>* cells (Fig. 6E-G).

## DISCUSSION

We describe, for the first time, a model for the conditional and localised depletion of *Pax7*-expressing muscle cells in mice. Given the recently revised role of *Pax7* in adult myogenesis, as well as reports that non-satellite cells can contribute to muscle growth and regeneration, this model provides an important paradigm to resolve the role of *Pax7*-expressing cells in adult skeletal muscle. Our findings provide unequivocal evidence for an essential role of *Pax7<sup>+</sup>* satellite cells in adult regenerative myogenesis, which contrasts with the dispensability of *Pax7* gene function for adult myogenesis (Lepper et al., 2009). Notably, in support of our findings, two distinct genetic models based on the conditional ablation of satellite cells using tamoxifen-induced expression of diphtheria from the *Rosa* locus resulted in a failure to regenerate skeletal muscle up to 2 months after ablation and muscle injury (Lepper et al., 2011; Murphy et al., 2011). In addition, and complementary to our study involving the transplantation of satellite cells to effect a rescue of muscle regeneration, skeletal muscle lacking satellite cells failed to regenerate even after transplantation of the affected muscle into a normal recipient muscle bed (Lepper et al., 2011). Together, the complementary experimental strategies of these two studies provide compelling evidence that other regenerative cell types in skeletal muscle cannot effect skeletal muscle regeneration after acute muscle injury or exercise.

*Pax7* is widely expressed in the central nervous system in addition to its expression in muscle satellite cells (see Buckingham and Relaix, 2007). It is noteworthy that in our model the DT is injected intramuscularly, thereby limiting the elimination of *Pax7<sup>+</sup>* cells to the target muscle and minimising the secondary effects on the other *Pax7*-expressing tissues. Although our experimental model indicates that *Pax7<sup>+</sup>* satellite cells play a crucial role in adult myogenesis, it does not exclude the possibility that other cell types that do not express *Pax7* during muscle homeostasis can participate in muscle regeneration as well. However, our findings show that those cells are not autonomously myogenic to an appreciable extent in the absence of satellite cells. This raises the intriguing possibility that satellite cells might orchestrate the intervention of non-satellite cells for muscle regeneration. This influence could be passive, i.e. secondary recruitment of non-satellite cells to fuse with satellite cell derived myofibres, and/or active by conferring myogenic fate on these cell types through instructive signalling. In this context, the adipogenic infiltration in satellite cell-depleted muscle in the absence of myotoxin-induced injury is interesting. This could either be the result of mild damage by the DT injection (needle injury and low affinity mouse DT receptor-mediated) or due to deregulation of FAPs (Joe et al., 2010; Uezumi et al., 2010) in homeostatic

muscle lacking satellite cells. Further experiments with this experimental model in this context would be interesting. Clearly, other cell types, including endothelial cells (Christov et al., 2007), fibroblasts (Murphy et al., 2011) and FAPs (Joe et al., 2010; Uezumi et al., 2010), could modulate the behaviour and survival of satellite cells. Alternatively, non-satellite cells might intervene during development, either prenatally or during perinatal growth, or acquire that capacity in a transplantation context. These possibilities merit further investigations, particularly for optimising stem cell-based therapeutic approaches in the context of acute myopathic diseases. We note also that spindle myofibres have higher numbers of satellite cells and they maintain embryonic gene expression (Kirkpatrick et al., 2008; Zammit et al., 2004). It would be of interest to examine satellite cell ablation in these myofibres in the future.

Our previous studies showed that the elimination of 80-90% of the satellite cell population still results in robust myofibre regeneration after 3-4 weeks (Gayraud-Morel et al., 2007), suggesting that in the present study at least this level of depletion was reached after DT injection. However, we noted that in some cases, satellite cells were not totally eliminated (Fig. 2); therefore, this observation raises the possibility that a threshold number of cells, or a quorum, is required for full muscle reconstitution to take place. This contrasts with the demonstration that single fibres containing up to ~20 satellite cells, or even single cell transplantations, contribute considerably to muscle regeneration (Collins et al., 2005; Sacco et al., 2008). These data might also reflect a heterogeneity in the regenerative potential of subpopulations of satellite cells. Reports that satellite cells constitute a heterogeneous population consisting of more stem-like and committed cells should also be taken into account in this context (Biressi and Rando, 2010; Tajbakhsh, 2009). Finally, residual satellite cells might also reflect a more committed subpopulation expressing lower levels of *Pax7*, and consequently *DTR* (resulting in their survival), but with a lower potential to self-renew if they are more primed to differentiate. These cells would be isolated using the *Tg:Pax7-nGFP* mouse owing to the perdurance of the *GFP* reporter gene. Such discrepancies might have important implications in understanding the physiology of the muscle stem cell niche, notably by physical constraints to the maintenance of stem cell properties (Gilbert et al., 2010; Lutolf et al., 2009).

The long-lasting depletion of myogenic activity following injection of DT shows that the loss of myogenic cells was sustained for at least several weeks whereas during satellite cell mediated regeneration, differentiated nascent myofibres are observed within a few days, suggesting that in this model the failure to regenerate could be permanent. These kinetics suggest that the depleted satellite cells are indeed self-renewing stem cells rather than an intermediate progenitor cell population that would be expected to be more rapidly reconstituted from cells upstream in the hierarchy. Heterogeneities reported among satellite cells (Biressi and Rando, 2010; Conboy et al., 2007; Day et al., 2010; Shinin et al., 2006; Tajbakhsh, 2009) also point to the existence of stem and progenitor cells within this population. Our present study favours the notion that satellite cells are muscle stem cells and that the predominant mode of satellite cell replenishment is direct self-renewal (for reviews, see Dhawan and Rando, 2005; Tajbakhsh, 2009). Although our study unambiguously favours self-renewal, we cannot formally exclude the possibility that non-satellite cell types could effect muscle regeneration over an extended time frame.

A thick fascia separates muscle groups, and it is believed that there is little movement of satellite cells from one muscle to another. Our findings are in accordance with this view as satellite cells from surrounding muscles apparently contribute neither to the reconstitution of the satellite cell compartment nor to regeneration of the DT-injected muscle. However, certain myogenic progenitor cells, such as the pericyte-derived mesoangioblasts, have the capacity to traverse tissues and they have been described as a potential source of stem cells capable of regenerating skeletal muscle (for a review, see Tedesco et al., 2010). Whereas these cells can be injected into the circulation in myopathic mouse models to reconstitute skeletal muscle (Sampaolesi et al., 2003), it is likely from the results obtained in our model that endogenous mesoangioblasts are not spontaneously mobilised for regenerative myogenesis in the absence of satellite cells. The transplantation of these and other cell types into the satellite cell-depleted model would be a future goal.

Apparently, a critical loss of satellite cells does not provoke overtly severe consequences for prolonged periods of time in homeostatic conditions. However, based on our findings in mice, we surmise that pathological conditions leading to a decrease in satellite cell numbers could abruptly evolve into irreparable muscle atrophy once a critical threshold is reached. Certain inherited myopathies are associated with reduced numbers of satellite cells. This is the case for SEPNI-related myopathies caused by selenoprotein N deficiency and characterised by early-onset muscle atrophy. Similarly to humans with this condition, *Sepn1*<sup>-/-</sup> mice are also defective in satellite cells and undergo a marked muscle atrophy with the presence of fat following repeated regenerative injury (Castets et al., 2010) in a manner that is reminiscent of DT-depleted *Pax7*<sup>DTR/+</sup> mice. The notion that a critical threshold for satellite cell numbers can be linked directly to the loss of muscle tissue may help to disentangle the metabolic and cellular intricacies involved in complex conditions, such as ageing or sarcopenic obesity. Satellite cells, as well as mesoangioblasts, PICs and other multipotent populations, have been considered as obvious candidates for a cell-therapy approach to treat various muscular dystrophies. Only recently have methods become available that permit the expansion of muscle stem cells in culture under appropriate mechanotransduction signals (Gilbert et al., 2010). However, to date, the recipient models are not optimal. The possibility of creating a model with a niche for candidate muscle stem cell populations by inducing the depletion of *Pax7*-expressing cells in adult mice with a single injection of DT creates a unique opportunity to design rational cell therapeutic protocols.

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#### Competing interests statement

The authors declare no competing financial interests.

#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.067587/-/DC1>

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