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Adult satellite cells and embryonic muscle progenitors have distinct genetic requirements

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Abstract

Myogenic potential, survival and expansion of mammalian muscle progenitors depend on the myogenic determinants *Pax3* and *Pax7* embryonically[1], and *Pax7* alone perinatally[2-5]. Several *in vitro* studies support *Pax7's* critical role in these functions of adult muscle stem cells[5-8], i.e. satellite cells, but a formal demonstration has been lacking *in vivo*. Applying inducible Cre/loxP lineage tracing[9] and conditional gene inactivation to the tibialis anterior muscle regeneration paradigm, we show unexpectedly that when *Pax7* is inactivated in adult mice, mutant satellite cells are not compromised in muscle regeneration, can proliferate and reoccupy the sublaminal satellite niche, and support further regenerative processes. Surprisingly, dual adult inactivation of *Pax3* and *Pax7* also results in normal muscle regeneration. Multiple time points of gene inactivation reveal *Pax7* is only required up to the juvenile period when progenitor cells transition into quiescence. We further demonstrate a cell intrinsic difference between neonatal progenitor and adult satellite cells in their *Pax7*-dependency. Our finding of an age-dependent change in the genetic requirement for muscle stem cells cautions against inferring adult stem cell biology from embryonic studies, and has direct implications for the use of stem cells from hosts of different ages in transplantation-based therapy.

Keywords

muscle regeneration; Pax3; Pax7; satellite cells

Skeletal muscle regeneration is of clinical importance to muscular dystrophies and sport injuries, and depends on a resident reservoir of muscle stem cells called satellite cells[10]. Satellite cells are set aside to become quiescent post-natally from a pool of highly proliferative muscle[1,11]. The survival and expansion of muscle progenitors rely on the transcription factor *Pax7*. As such, *Pax7* germline mutant mice which survive to adulthood have few or no myofiber-associated cells resembling satellite cells[2-5], and display severely compromised

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muscle regeneration[3,4]. Although it remains controversial whether *Pax7* mutant myofiber-associated cells are myogenic *in vivo*[3,4], *in vitro* studies corroborate the role of *Pax7* in myoblast survival, proliferation, and the establishment of myogenic potential[5,7-8]. Together with persistent *Pax7* expression in adult quiescent satellite cells[2,12], these data have raised the expectation that adult muscle stem cell self-renewal and injury-induced muscle regeneration depend on *Pax7*. Thus, we examined these highly anticipated roles of *Pax7in vivo*.

To determine *Pax7* function in adult satellite cells, we bypassed its earlier requirement via temporal gene inactivation in the adult. Previous analyses of *Pax7* mutants without lineage tracing have led to conflicting conclusions[3,4]. Therefore, we labelled conditionally inactivated *Pax7* cells and tracked their fate. Two new *Pax7* alleles were generated: $Pax7^{f}$, a conditional allele, that after Cre-mediated recombination becomes the null allele $Pax7^{\Delta}$ (Fig. S1), and $Pax7^{CE}$ which expresses a tamoxifen (tmx)-inducible Cre recombinase-Estrogen Receptor fusion protein, Cre-ER^{T2} (ref. [13]), in place of *Pax7*, and is therefore also null for *Pax7* (Fig. S2). We assayed for inducible Cre activity by crossing $Pax7^{CE}$ mice to *Rosa26*-Reporter *(R26R)* mice[14], which express *LacZ* following Cre-mediated recombination (Fig. S3). Tmx-dependent Cre-ER^{T2} activity was confirmed by β-gal activity (X-galactosidase reaction). Efficacy and specificity of tmx-induced satellite cell labeling was confirmed by 99.8% co-localization of β-gal and Pax7 in tibialis anterior (TA) muscle*s* (Fig. S3).

The $Pax7^{CE}$ and $Pax7^{f}$ alleles were combined ($Pax7^{CE/f};R26R^{+/-}$) for conditional inactivation of *Pax7* between postnatal days 60-90 (P60-90) by tmx. After five daily tmx injections (see Methods; Fig. 1a, b), no wild-type *Pax7* transcript, no Pax7 protein, and no $Pax7^{+}$ cells were detected in TA muscles (Fig. S4). Conditional *Pax7* inactivation in adult satellite cells did not lead to loss of β-gal^{+} cells (Fig. S5), indicating that *Pax7* is not required for their survival. Conditional mutant cells maintain satellite cell characteristics as evidenced by M-Cadherin (M-Cad), CD34, Integrin β1 and α7 expression[15,16] (Fig. S6).

To determine if *Pax7* conditional mutant cells can support injury-induced myogenesis, we used cardiotoxin (CTX) to induce injury. In *Pax7* heterozygotes ($Pax7^{+/CE};R26R^{+/-}$), all regenerating fibers at days 5 and 10 after injury are β-gal^{+} (Fig. 1c, d, S7). This result provides unequivocal evidence that *Pax7*-descendants are a major source of regenerating myofibers, extending data[15] that single $Pax7^{+}$ cells can form myofibers after transplantation into TA muscles. Contrary to expectation, regenerative myogenesis from *Pax7*-descendants was not impaired in conditional *Pax7* mutants ($Pax7^{CE/f};R26R^{+/-}$) as assessed by X-galactosidase reactions (Fig. 1e, f), regenerated fiber size (Fig. 1g, h), and number (Fig. S7). No reappearance of wild-type *Pax7* mRNA or protein was detected in injured muscles 10 days post injury (Fig. 1i, j). Thus, *Pax7* is not required for injury-induced myogenesis in adulthood.

These results contrast with *Pax7* germline mutants where only a minimal number of small regenerative myofibers are found after injury (refs. [3,4] and Fig. S1). These sparse regenerated fibers were proposed to be derived from alternate source(s), i.e. non-*Pax7* lineage[4]. To test this, we assayed for injury-induced myogenesis in surviving $Pax7^{CE/CE};R26R^{+/-}$ adults using the experimental paradigm described above. Surprisingly, the rare regenerative myofibers (Fig. 1k, l) were all of *Pax7*-descent. This result indicates that *Pax7* germline mutant cells surviving to adulthood do have myogenic potential and argues against alternate non-*Pax7* sources.

Isolated surviving cells associated with myofibers in adult *Pax7* germline nulls lack typical satellite cell characteristics[4]. Therefore, we examined whether conditional *Pax7* mutant cells also lose satellite cell characteristics after supporting one round of regeneration. For this, β-gal-marked cells were assessed for satellite niche occupancy using anti-M-Cad (for satellite/muscle junction) and anti-Laminin (for basal lamina). We found β-gal^{+}/M-Cad^{+}/$Pax7^{+}$ satellite cells in the sublaminal space in control regenerated tissue (Fig. 2a-d, S8). Surprisingly,

sublaminal β-gal$^+$/M-Cad$^+$/Pax7$^-$ cells were also found in *Pax7* conditional mutants (Fig. 2e-h). When we used EdU to monitor cells that had undergone proliferation, we found EdU$^+$ myonuclei and EdU-retaining sublaminal β-gal$^+$/M-Cad$^+$/Pax7$^-$ cells in conditional mutants (Fig. S8). We did not detect any Pax7$^+$ or β-gal$^-$/M-Cad$^+$ sublaminal cells in *Pax7*-inactivated regenerates, arguing against compensation by other lineages with or without *de novo Pax7* activation. These data indicate that adult-specific *Pax7* mutant descendants not only form muscle fibers but also proliferate and occupy the satellite niche.

To determine whether the β-gal$^+$/M-Cad$^+$/Pax7$^-$ sublaminal cells in conditional mutant regenerates still have myogenic potential, we induced a second round of injury (Fig. 2i, j). Twenty-eight days post first tmx injection, neither wild-type *Pax7* mRNA nor protein was detected in doubly injured conditional *Pax7* mutants (Fig. 2k, l). We found small and large β-gal$^+$ fibers comparable between controls (Fig. 2m) and conditional mutants (Fig. 2n) 6 days after the second injury. To identify second-round regenerated myofibers, we used the RNMy2/9D2 antibody, which labels new fibers only up to 6 days after injury[17]. Within the β-gal$^+$ regenerated area, small fibers were RNMy2/9D2$^+$ in control animals (Fig. 2o, q, r) and conditional mutants (Fig. 2s, u, v). To assess whether *Pax7*-descendants had proliferated prior to forming new fibers, EdU was administered after the second injury. Indeed, we found EdU$^+$ nuclei in RNMy2/9D2$^+$ fibers of control animals (Fig. 2p, r) and conditional mutants (Fig. 2t, v). Although we cannot formally exclude contribution from *Pax7*-independent lineages, our data suggest that adult-inactivated *Pax7* mutant cells can repeatedly contribute to muscle regeneration.

The normal regenerative capacity of conditional *Pax7* mutants sharply contrasts with the severe germline *Pax7* mutant defects (refs. [3,4] and here), and suggests the possibility of adult-specific compensation by another *Pax* family member. *Pax3* is a likely candidate as *Pax3* and *Pax7* compensate for each other in embryonic myogenesis[1]. We detected *Pax3* transcripts in adult TA muscles (Fig. 3a) but not Pax3 protein (not shown). To determine whether *Pax3* can compensate for *Pax7* in adult myogenesis, we conditionally inactivated both genes in adulthood. We used the *CreEsr* allele[18], which directs ubiquitous tmx-inducible Cre activity, to conditionally recombine a *Pax3* floxed allele *Pax3^f* (ref. [19]). Following the tmx regimen in Fig. 1a, both *Pax3^f* and *Pax7^f* were recombined (Fig. 3a, b). We were surprised again that muscle regeneration was not impaired in *Pax3;Pax7* doubly inactivated TA muscles as assessed by histology (Fig. 3d, compared to control in 3c) and regenerated fiber size (Fig. 3i, j). Furthermore, we found sublaminal M-Cad$^+$/Pax7$^-$ cells within the regenerate (Fig. 3g, h; compare to the control M-Cad$^+$/Pax7$^+$ cells, Fig. 3e, f). Thus, contrary to their essential roles for embryonic myogenesis, neither *Pax3* nor *Pax7* is required during adult TA muscle regeneration.

We next determined if and when myogenic progenitors become independent of *Pax7 in vivo*. To test this, we conditionally inactivated *Pax7* at different post-natal time points. Animals were then injured and regeneration assessed 10 days later. When *Pax7* was inactivated between P7-11, regeneration was severely compromised (Fig. 4b). However, when inactivation was carried out at P14-18 and P21-25 (Fig. 4c, d), regenerative capacity gradually increased to levels similar to control mice (Fig. 4a). Importantly, regenerated fibers were *Pax7*-descendants (β-gal$^+$). Thus, *Pax7* function is critical prior to P21. To elucidate muscle progenitor behavior at this post-natal period, we performed lineage tracing and found that myofiber fusion from *Pax7*-descendants sharply declines by P21 (Fig. S9), suggesting a transition to quiescent satellite cells. Intriguingly, myonuclei positioning in regenerated fibers also differs between P21 and adult animals, suggesting a coordinated transition of muscle biology around P21 (ref. [20]). By contrast, myofiber incorporation by *Pax7*-mutant descendants occurs for a prolonged period (beyond P31, Fig. S9), indicating that mutant cells have a greater propensity to differentiate. We therefore propose that in addition to survival and proliferation[3,5], *Pax7* also

directs myogenic progenitors to withdraw from myogenic differentiation and transition into quiescent satellite cells, thereby acquiring maximum regenerative capacity.

To determine whether this temporal difference is a cell-intrinsic or environmental effect, we cultured myoblasts isolated from *Pax7*$^{+/CE}$;*R26R*$^{+/-}$ and *Pax7*$^{CE/f}$;*R26R*$^{+/-}$ TA muscles at P0 (prior to quiescence) and P60 (after becoming quiescent), and assayed their properties after tmx treatment. Compared to controls, conditional *Pax7*-inactivated P0 myoblasts (β-gal^{+}) were defective in expansion and myogenic potential (Fig. 4e-g). At passage 1, residual conditional mutant cells still expressed Desmin (Fig. 4g, k-k″), displayed fibroblastic morphology at a high frequency (Fig. 4g), and some were positive for fibroblastic marker ER-TR7 (Fig. 4g, l-l″); however, many cells are of unknown fate(s) as they do not express either marker; which is in contrast to controls (Fig. 4g). This is reminiscent of aged *Pax7*-descendant cells after losing *Pax7* expression *in vitro*[21]. After passage 2, mutant cells were no longer myogenic (not shown) and most β-gal^{+} cells were fibroblastic. Thus, inactivation of *Pax7* in neonatal myoblasts *in vitro* decreases their expansive and myogenic capacity. By contrast, control and conditionally inactivated *Pax7*-descendant adult myoblasts had comparable expansive and myogenic potentials (Fig. 4h-j) as well as myogenic differentiation capacity (Fig. S10). No β-gal^{+}/ER-TR7^{+} cells were found despite the presence of rare fibroblastic cells (Fig. 4j). Tmx-treated *CreEsr*$^{+/-}$;*Pax7*$^{f/f}$;*Pax3*$^{f/f}$ adult myoblasts also had comparable proliferative and myogenic properties as mock-treated controls (Fig. S11). Together, these data support a cell autonomous change within satellite cells compared to their progenitor state.

We have defined a critical period of *Pax7*-dependency in the transition from muscle progenitor to adult stem cell state, which ensures muscles achieve regenerative capacity. Given the essential roles for *Pax3* and *Pax7* in embryonic and for *Pax7* alone in perinatal myogenic progenitors, it was entirely unexpected that adult satellite cells require neither *Pax7* nor *Pax3* for muscle regeneration. We imagine that post-natal changes of muscle organization, mechanics, and physiology demand stem cells to alter their transcriptional program as a means to adapt to these challenges. Changes in genetic requirement for muscle stem cells from embryonic to juvenile to adult stages elucidate the inadequacy of applying knowledge gained from developmental studies to adult stem cell biology. Our discovery should encourage future investigations into how widespread genetic transitions may occur in different adult stem cell types. Age-dependent differences in stem cell properties should also urge careful consideration of the age of stem cells used in transplantation-based regenerative medicine.

Methods Summary

Animals

Pax7^{f} and *Pax7*^{CE} alleles are in Fig. S1 and S2. The *Pax3*^{f} allele was described[19]. *R26R*[14] and *CreEsr*[18] mice were from the Jackson Laboratory. Tmx (Sigma) was administered intra-peritoneally (animals > 2 weeks) or subcutaneously (< 2 weeks) at 3 mg / 40 g body weight / injection. CTX (10 μM, Sigma) was injected into TA muscles (after anesthesia) at 100 μl (animals > 2 months) and 50 μl (< 2 months). EdU (Invitrogen) was injected at 0.1 mg / 30 g bodyweight per injection. All procedures were approved by IACUC.

PCR genotyping, RT-PCR, and Western Blot

Primers used for genotyping and RT-PCR are in Supplemental Tables 1 and 2. Antibodies used for Western Blots are in Supplemental Tables 3 and 4, and ECL (Amersham) was used for detection.

X-gal reactions, immunofluoresence, and histology

TA muscles were fresh frozen in isopentane/liquid nitrogen and cryo-sectioned at 10 μm. X-gal (Qiagen) reactions followed standard procedure[22]; counterstained by NFR (Lab Vision). Immunofluorescence was performed using antibodies in Supplemental Tables 3 and 4. Histology was performed according to manufacturer's instructions (Surgiopath). Edu was detected by Alexa649 (Invitrogen).

Cell culture

Myoblasts were isolated and cultured on collagen-coated dishes (VWR) as described[23]. 4-OH-Tmx (0.4 μM, Calbiochem) was added at the beginning of culture. For quantitative studies, cells were passed (1:3 dilution) every 4 days. For immunofluorescence, cells were plated onto 8-well chamber slides.

Muscle fiber size

Digital images of X-gal and histologically stained sections were processed by Metamorph software (Molecular Devices). Parameters were chosen to define muscle fiber boundaries, which were re-examined visually for accuracy prior to quantitation. Determination of fiber diameter and inner radius was assisted by Metamorph. Measurements for 300 fibers per animal were subjected to statistical analysis by Excel (Microsoft).

Methods

Animals

The experimental outlines for making the $Pax7^{f}$ and $Pax7^{CE}$ alleles are in Figs. S1 and S2, respectively. The precise constructions for these alleles will be described elsewhere. The genetic crosses to remove the neo selection marker with Actin-Flip mice are described (Fig. S1 and S2). Generation of the germ line $Pax7^{\Delta}$ allele with the Nestin-Cre mice is also outlined (Fig. S1). Methods for genotyping and characterization of these alleles are described in the figure legends therein.

To obtain $Pax7^{CE/f};R26R^{+/-}$ and $Pax7^{+/CE};R26R^{+/-}$ progenies for analysis, $Pax7^{+/CE}$ mice were mated to $Pax7^{+/f};R26R^{+/-}$ animals. To obtain $Pax7^{CE/CE};R26R^{+/-}$ animals, $Pax7^{+/CE};R26R^{+/-}$ mice were mated to $Pax7^{+/CE}$ animals. Similar strategies of genetic crosses were used (excluding $R26R^{+}$) to obtain $CreEsr^{+/-};Pax3^{f/f};Pax7^{f/f}$ and $CreEsr^{+/-};Pax7^{+/-}$ animals. Genotypes of these animals were determined by PCR using primers and conditions in Supplemental Table 1.

Tamoxifen (Tmx)

Tmx (Sigma) was prepared by dissolving a freshly opened bottle of 5 grams of tmx in corn oil (Sigma) at 20 mg / ml. The mixture was incubated at 37°C with periodic vortexing until tmx was completely dissolved (~2-4 hrs). It was then divided into 10 ml conical tubes (VWR) and frozen in a −80°C freezer. Each tube was thawed as needed and kept at 4°C to be used for no more than 1 week. A 1 ml syringe with a 26 gauge needle was used for injection. Either intraperitoneal (for animals more than 3 weeks old) or subcutaneous (for animals less than 3 weeks old) delivery routes were used. The regimens are outlined in Figs. 1a, 2i, and S8a.

Cardiotoxin (CTX)

CTX (Sigma) was prepared by dissolving a freshly opened tube in PBS at 10 μM. The solution was divided into Eppendorf tubes as 1 at −80°ml aliquot stocks, flash frozen, and stored C. Each tube was thawed fresh before injection and not re-used. Animals were anesthetized by intraperitoneal injection of Avertin (2,2,2-Tribromoethanol from TCI America at 15 μl / gram

body weight of 20 mg / ml solution). Right leg TA muscles were injected with 100 or 50 μl of CTX using an insulin needle (3/10cc Insulin Syringe from Becton-Dickinson). For two rounds of injury, right leg TA muscles were initially injected with 100 μl of CTX and allowed to regenerate for 2 weeks. Following, right leg TA muscles were re-injured via injection of 50 μl of CTX (see Fig. 2i). The volume and concentration of CTX are standard protocol used in published work (e.g. Hu et al., Dev. Cell, 2008; Kozlov et al., Hum Mol. Genet. 2006; Iessi et al., Dev. Cell, 2004; Meeson et al., Stem Cells, 2004). CTX Animals were kept under a warming lamp until recovery before being returned to a normal cage rack.

EdU

For cultured cells, EdU supplied in the Click-iT EdU Cell Proliferation Assay kit (Invitrogen) was used as instructed with minor modifications: EdU was used at 10 μM for 8 hrs in culture media; and for detection, we performed immunostaining for primary and secondary Abs (see below for detailed protocol) first, then the click chemical reaction using Alex647azide as a reactive fluorophore for detection, followed by DAPI staining (at 1 ug / ml).

For animals, EdU powder was dissolved in PBS at 0.5 mg / ml and frozen at −20°C for long-term storage. EdU was injected at 0.1 mg / 30 g bodyweight intraperitoneally. The regimen for in vivo EdU injection for short-term proliferation and long-term retention assays are outlined in corresponding figures (Figs. 2i and S8a, respectively). As for cultured cells, we performed the click chemical reaction using the same kit after immunostaining with primary and secondary Abs and prior to DAPI staining (when applicable).

PCR genotyping and RT-PCR

For adult animal, tail DNA was used for genotyping by PCR. For newborns and animals younger than 21 days, toe DNA was used. DNA was extracted using the ExtractN'Amp kit (Sigma) following instruction. 1 μl of neutralized DNA samples was used for PCR reaction using GoTaq polymerase (Promega) with buffers supplied by the manufacturer with 0.1 mM dNTPs and 2.5 mM $MgCl_2$. The PCR products were resolved in 2% agarose gel, stained with 0.5 μg /ml ethidium bromide (GIBCO), and digitally imaged with a Bio-Rad Gel Doc system for record keeping.

Total RNA from injured TA muscles was isolated using TRIzoL Reagent (Invitrogen) according to manufacturer's instructions. cDNA synthesis was performed using M-MLV Reverse Transcriptase (Invitrogen) also according to manufacturer's instructions. cDNA samples was then used for PCR as above.

Primer sequences, product sizes, and PCR conditions used for genotyping and RT-PCR are in Supplemental Tables 1 and 2.

P0 animals to be used for satellite cell isolation were genotyped in the morning and sacrificed in the afternoon according to their determined genotypes.

Western Blot

TA muscles were harvested (time as indicated in each figure) and snap frozen in 1.5 ml eppendorf tubes and stored at −80°C until use. For protein extraction, frozen samples were weighed and suspended in 1:9 (weight to volume) of ice cold RIPA buffer containing protease inhibitor cocktail (Roche) at 2X concentration and 1 mM PMSF, and ground with sterile plastic mini pestle (VWR) until no muscle bits were observable. The muscle homogenate was spun for 10 min in a microcentrifuge at maximum speed at 4°C. 4X SDS sample buffer was added to the supernatant to reach 1X concentration. Samples were boiled for 5 min before subjected to 7.5% SDS-PAGE. Kleidoscope wide-range molecular weight marker (Bio-Rad) was used

for size. Western transfer was performed with low molecular weight transfer buffer to an ECL membrane (Amersham) for overnight at 30 volts using a Bio-Rad miniprotein II transfer set up at 4°C. Membrane was blocked in 5% low fat Carnation milk powder in TBS for 30 min, incubated with primary Ab in blocking solution for overnight at 4°C (mouse IgG1 anti-Pax7 at 1:50 and mouse IgG1 anti-α-Tubulin (DM1α, from Sigma) at 1:10,000), washed in TBS, 3X, 10 min each, incubated with secondary Ab (HRP conjugated anti-mouse IgG1 from Zymed at 1:3,000) in blocking solution for 1 hour, washed in TBS, 3X, 10 min each, and then subjected to ECL reaction using the kit from Amersham (ECL Western Blotting Detection Reagents) and exposed to X-Ray films. Standard solutions prepared for SDS-PAGE, Western transfer, TBS, and 4X sample buffer were as described in "Antibodies, a laboratory manual" by Harlow and Lane (Cold Spring Harbor laboratory Press, 1988).

X-gal reaction and histology

10 μm frozen muscle sections (by Leica CM3000 Cryostat) were collected on Superfrost plus slides (VWR) and air dried for 30-90 min. Slides were either stored in −20°C freezer or used immediately.

For X-gal reactions, sections were fixed with 0.2% glutaraldehyde / 0.1 M phosphate buffer (pH 7.2) with 5 mM EGTA, 1 mM $MgCl_2$, for 5 min on ice, then washed and stained according to Ref. 22 for 8 to 24 hrs to obtain satisfactory staining intensity. Stained sections were then washed in H_2O and stained with NFR (Lab Vision, Corp.) for 5 min, rinsed with H_2O, dehydrated through graded EtOH series (25%, 50%, 75%, 95%, 100%), then in xylene and finally mounted in Permount mounting media (Fisher) with coverslip (VWR).

For histology, sections were fixed in 4% paraformaldehyde / 0.1 M phosphate (pH 7.2) for 30 min, rinsed with tap water, stained with Gill II Hematoxylin for 5 min, washed with tap water, treated with Scott's tap water substitute for 10-15 sec to blue, washed with tap water, stained with Eosin for 1 min, destained in 95% EtOH, followed by 100% EtOH, xylene, then mounted in Permount with coverslip. Staining reagents were purchased from Surgipath and used as instructed. Van Gieson stains were performed precisely as described in ref. S4.

Digital images of X-gal stained and histological sections were taken using a SPOT camera and a Nikon Eclipse E800 vertical microscope.

Immunostaining

Freshly prepared 4% paraformaldehyde / 0.1M phosphate buffer (pH 7.2) was filtered through 0.2 μm polyeurothane filter (VWR) and chilled on ice. Paraformaldehyde solution was used within 12 hrs. 10 μm frozen sections on Superfrost plus slides were fixed for exactly 12 min on ice, rinsed with 0.1 M phosphate buffer 3X, 5 min each, then permeabilized with 0.3% TritonX-100/PBS for 20 min. Sections were then incubated with mouse IgG blocking solution from the M.O.M. kit (Vector Lab) diluted in PBS / 0.01% TritonX-100 following the dilution instructed in the manual. They were then incubated in blocking solution provided in the M.O.M. kit with additional 15% goat serum in PBS / 0.01% TritonX-100 for 20 min. Slides were then incubated with primary Abs diluted (sources and dilution factors for each Ab are in Supplemental Table 3) in the blocking solution for overnight at 4°C. Next morning, the slides were washed 3X in PBS / 0.01% Triton X-100 (all washes used this solution), 5 min each. Fluorescently conjugated secondary Abs to each species-specific IgG in blocking solution was applied at dilutions in Supplemental Table 4 for 30 min. For Pax7 and M-Cad monoclonal IgG1 Abs, the anti-IgG1 isotype-specific secondary Ab gave us the best result. The slides were washed again as after the primary Ab. If EdU was to be detected, we performed the click chemical reaction after this step according to manufacturer's instructions using the Click-iT kit components (Invitrogen). The slides were then washed, incubated with DAPI when applicable

(at 1 ug / ml) for 10 min, washed two more times, and mounted in FluoromountG solution (Southern Biotechnology) with coverslip. All images were taken under an Axioscope equipped with AxioCam with filters of non-overlapping spectra (according to the fluorophores used) for multiple fluorescent signals. Images were pseudo-colored and superimposed (when necessary) using the MetaMorph program.

For cultured cells, 8-well chamber slides were fixed and processed the same way as sections except that the mouse IgG blocking (M.O.M. kit) step was omitted.

Myoblast cell culture

Genotyped animals were sacrificed by cervical dislocation, and hindlimb muscles were removed and minced by razor blades. Each sample was digested with collagenase and dispase as described in ref. [23]. Dissociated muscles were then transferred to 15 ml conical tubes with 10 ml myoblast media without FGF, spun down in the clinical centrifuge and resuspended in culture media with 2.5 ng / ml bFGF.

For cell counting and marker analysis, 0.4 μM 4-OH Tmx (Calbiochem) was applied at the same time of cell plating. Each sample was divided into 3 parts. One part was plated on a 3 cm collagen coated dish (prepared as described in ref. [23]) for cell counting four days later. One part was plated on another 3 cm collagen coated dish but used for sequential passaging at 1:3 dilution. The remaining part was plated into 8-well chamber slides (coated with collagen) for immunostaining. For passaging, cells were passaged at 1:3 dilution every four days. At each round, each sample was divided into 3 parts; the same routine as above for cell counting, continuous passaging, and immunostaining was repeated. For quantitation of β-gal$^+$ cell numbers, the 3-cm dish was fixed and subjected to X-gal staining (see above), and 9 random fields were imaged using the Canon EOS30D camera attached to the Zeiss SV11 APO microscope at the highest magnification (each frame is of 1.33 mm^2 area, 0.95 mm × 1.4 mm), and β-gal$^+$ cells (blue) counted and scored for their morphology. Cells that displayed large flattened multi-cellular processes were scored as “fibroblastic”. For immunostaining quantification, 9-16 random fields per well were chosen for imaging and quantified for EdU labeling or marker expression in conjunction with anti-β-gal Ab staining.

For myogenic marker analysis, myoblast cultures were first treated with or without 4-OH tmx for 2 days for gene inactivation. They were then assayed for myoblast markers (Pax3, Pax7, Desmin, or MyoD) by RT-PCR or immunostaining, or incubated with Edu (10 μM for 8 hrs) for proliferation assays. For differentiation, cells were switched to differentiation media (5% horse serum in DME; ref. [23]) for 3-4 days and assayed for Myogenin and MHC expression.

Muscle fiber quantitation

Cross-sections (by Cryostat sections at 10 μm) of TA muscles were stained by X-gal reactions or by Hematoxilin/Eosin. Digital images were acquired with a CCD camera by routine brightfield microscopy. Image processing and morphometry were carried out using MetaMorph software (Molecular Devices). Specifically, myofibers were automatically delineated. Myofiber outlines were visually examined to assure accuracy prior to further analysis. The centroid (geometric center) of each myofiber was then determined to calculate the myofiber inner radius, the distance from the centroid to the closest edge. A circle of this radius will be completely contained (inscribed) within the myofiber outline. Therefore, this is the most conservative measure of fiber size.

For myofiber diameter measurements, we used the ‘largest diameter of the lesser aspect of the myofiber’ criterion (according to Dubowitz, V: Muscle Biopsy: A Practical Approach (2nd ed), London, Bailliere, Tindall, 1985). This measurement is conventionally used to assess fiber

size. Automatic measurement of this dimension for outlined myofibers was also implemented in MetaMorph (termed "Breadth" within the software).

Statistical analysis

Quantitative data displayed as histograms are expressed as means ± the standard error of the mean (represented as error bars). Quantification of total percentages is shown in tables. Regenerated muscle fiber numbers are displayed as a distribution plot. Analysis of variance was conducted using Excel spreadsheet by using T-test for two-tailed paired comparison. Statistical significance was set at a p value <0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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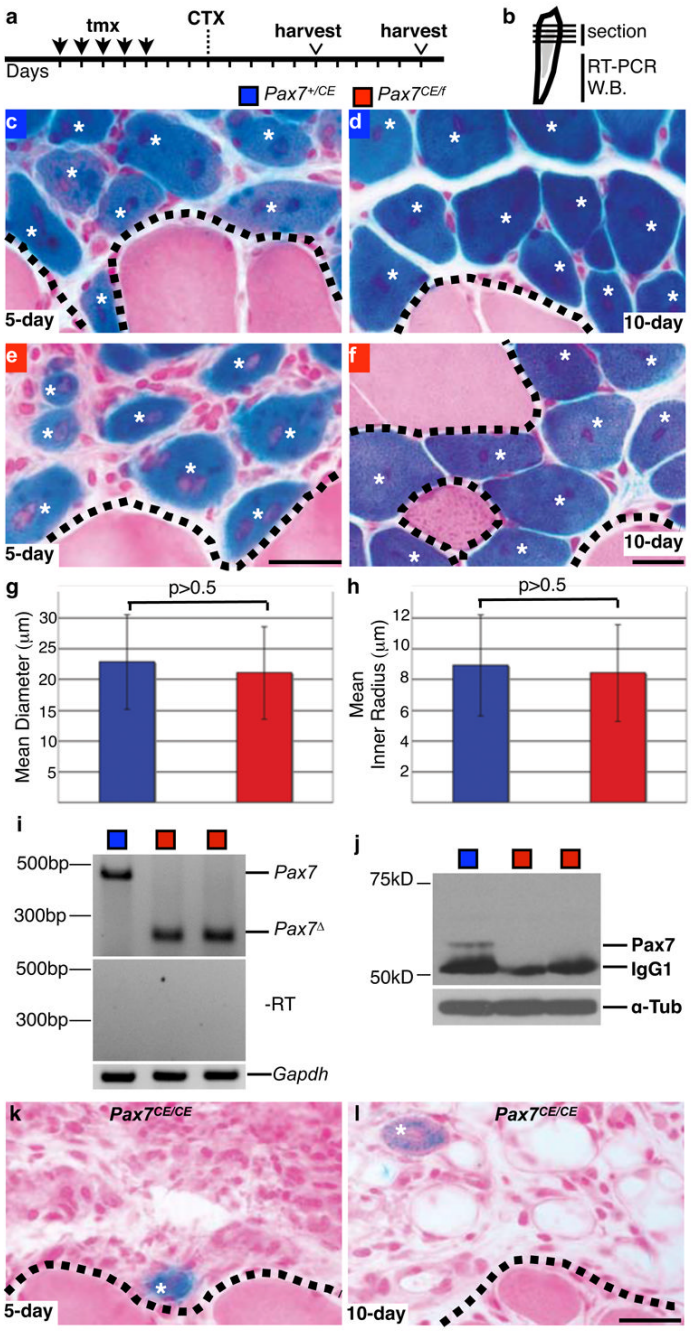

Figure 1. Adult-specific *Pax7* mutant cells regenerate muscle efficiently

a, Tmx and CTX regimen and regeneration assay scheme. Vertical lines, daily intervals. **b,** TA muscle diagram with injury in grey. Horizontal lines, cross sections; W.B., Western blot. **c-f,** β-gal (by X-galactosidase reaction) and Nuclear Fast Red (NFR) stained $Pax7^{+/CE}$ (**c**, **d**) and $Pax7^{CE/f}$ (**e**, **f**) muscles at 5 (**c**, **e**) and 10 (**d**, **f**) days post injury. Asterisks, regenerating fibers with central nuclei; dashed lines, boundary of injury. **g, h,** Mean diameter (**g**) and mean inner radius (**h**) of 10-day regenerated β-gal$^+$ fibers; 300 fibers / animal (n = 3 / genotype); error bars = s.d.; p, two-tailed student's t test. **i, j,** RT-PCR (**i**) and W.B. (**j**) of examples of $Pax7^{+/CE}$ and $Pax7^{CE/f}$ (2 shown) muscles; wild type *Pax7* and recombined ($Pax7^{\Delta}$) transcripts as indicated; -RT control, *Gapdh* control, endogenous IgG1, and α-tubulin (α-TUB, loading control) as

indicated. **k, l,** β-gal and NFR stained *Pax7*$^{CE/CE}$ muscles at 5 (**k**) and 10 (**l**) days post injury; regimen in **a**. All animals were *R26R*$^{+/-}$. Scale bars = 25 μm for **c**, **e** in **e**; for **d**, **f** in **f**; for **k**, **l** in **l**.

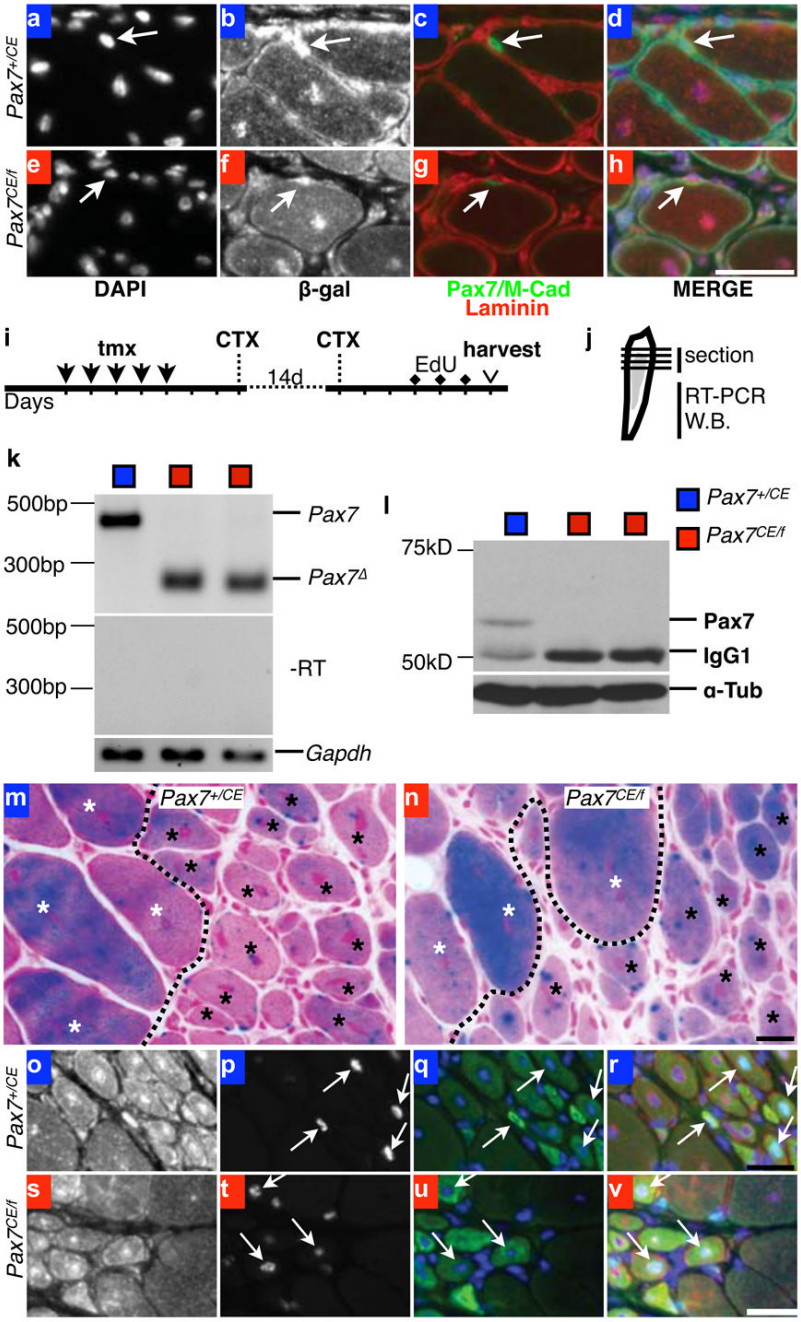

Figure 2. Adult-specific *Pax7* mutant cells are functional satellite cells

a-h, Fluorescent microscopy of $Pax7^{+/CE}$ (**a-d**) and $Pax7^{CE/f}$ (**e-h**) 10-day regenerates: DAPI (**a**, **e**); β-gal (**b**, **f**); Laminin (red), Pax7 and M-Cad (both in green: Pax7, nuclear, and M-Cad, satellite/muscle junction) (**c**, **g**). **d** and **h** are merged from **a-c** and **e-g**, respectively; β-gal pseudo-colored in red, DAPI in blue, Laminin in cyan, Pax7 and M-Cad in green; white arrows, satellite cells. **i**, Double injury regeneration assay scheme with EdU injections; labeling as in Fig. 1**a** with 14 days (14d) between two injuries. **j,** TA muscle diagram with 1st injury in grey, 2nd injury in black. **k, l,** RT-PCR (**k**) and W.B. (**l**) of TA muscles after second injury; labeling as in Fig. 1**i**, **j**. **m, n,** β-gal and NFR stained $Pax7^{+/CE}$ (**m**) and $Pax7^{CE/f}$ (**n**) regenerates at day-6 post 2nd injury; dashed lines, boundaries between 1st and 2nd regenerations based on

differences in fiber size; white and black asterisks, old and new regenerated fibers, respectively. All fibers are β-gal$^+$ compared to interstitial cells, but with varying staining signals. **o-v,** Fluorescent microscopy of *Pax7*$^{+/CE}$ (**o-r**) and *Pax7*$^{CE/f}$ (**s-v**) regenerates: β-gal (**o, s**); EdU (**p, t**); RNMy2/9D2 (green) and DAPI (blue) (**q, u**). **r** and **v** are merged from **o-q** and **s-u**, respectively; β-gal in red and other colors same as panels to the left; EdU$^+$ nuclei, white arrows. Scale bars = 25 μm for **a-h** in **h**; for **m**, **n** in **n**; for **o-r** in **r**; for **s-v** in **v**.

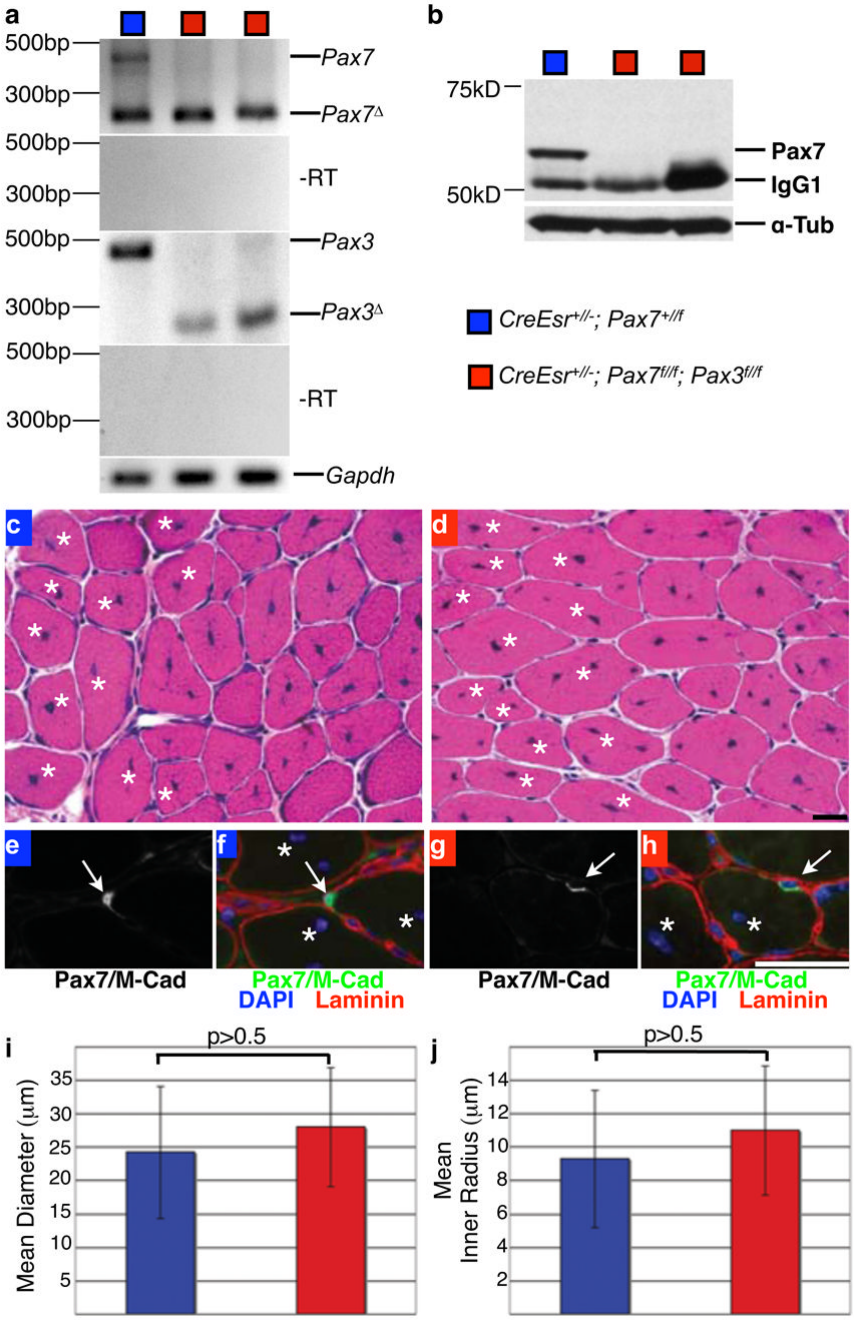

Figure 3. *Pax3* and *Pax7* are dispensable for adult muscle regeneration

a, b, RT-PCR (**a**) and W.B. (**b**) of indicated genotypes from regeneration assay scheme in Fig. 1**a**. Labeling is the same as Fig. 1**i**, **j**, with additions of *Pax3* wild type and recombined (*Pax3*$^{\Delta}$) transcripts. **c-h,** *CreEsr*$^{+/-}$;*Pax7*$^{+/f}$ (**c**, **e**, **f**) and *CreEsR*$^{+/-}$;*Pax3/7*$^{f/f}$ (**d**, **g**, **h**) day-10 regenerated muscles: hematoxylin and eosin stained (**c**, **d**); fluorescent microscopy of Pax7 and M-Cad together (arrowheads, M-Cad^{+} cells) (**e**, **g**); **f** and **h**, colored composites with co-stained Laminin (red), DAPI (blue), and Pax7/M-Cad (green) of **e** and **g**, respectively; asterisks, central nuclei; arrows, satellite cells. **i, j,** Mean diameter (**i**) and mean inner radius (**j**) of regenerated fibers; 300 fibers / animal (n = 3 / genotype); error bars = s.d.; p, two-tailed student's *t* test. Scale bars = 25 μm for **c**, **d** in **d**; **e-h**, in **h**.

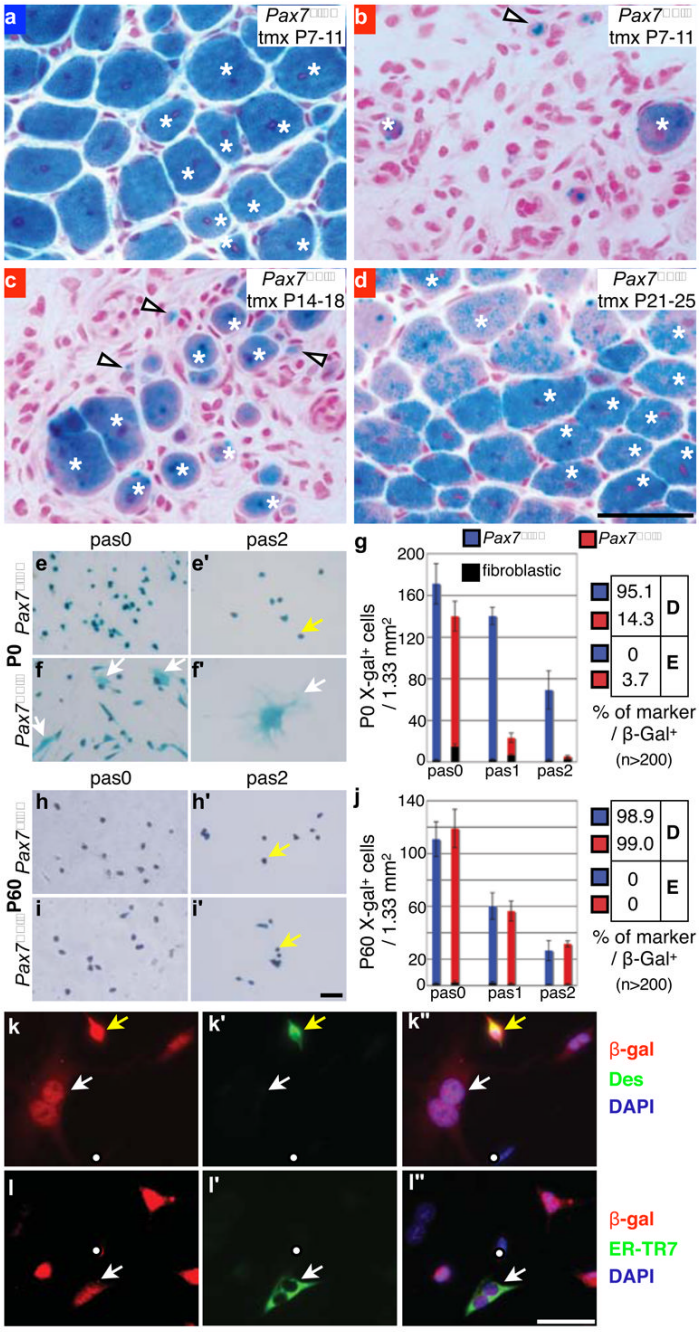

Figure 4. Age-dependent intrinsic change of *Pax7* requirement

a-d, Regeneration assessed at day-10 by β-gal and NFR staining: **a,** Control ($Pax7^{+/CE}$) regenerates of P7-P11 tmx-treated sample; essentially the same for other time points (not shown). **b-d**, Conditional inactivation of *Pax7* ($Pax7^{CE/f}$) by tmx at P7-11 (**b**), P14-18 (**c**), and P21-25 (**d**) followed by injury at P21 for (**b**, **c**), and P26 for (**d**); RT-PCR confirmed complete *Pax7* inactivation (not shown); new fibers, asterisks; triangles, β-gal$^+$ cells; n = 3 each; all are $R26R^{+/-}$. **e-j**, P0 (**e-g**) and adult (P60, **h-j**) tmx-treated (0.4 μM) and β-gal stained $Pax7^{+/CE};R26R^{+/-}$ (**e**, **e′, h, h′**) and $Pax7^{CE/f};R26R^{+/-}$ (**f**, **f′, i, i′**) myoblasts at passages 0 (pas0, **e**, **f, h, i**) and 2 (pas2, **e′**, **f′, h′, i′**); yellow arrows, round/short-spindled myoblasts; white arrows, fibroblastic cells. **g**, **j**, Left, average cell numbers (≥ 9 fields) at each passage (pas, x-

axes); n = 3 /genotype /stage; error bars = s. d.; right, percentages of Desmin^{+} (D) and ER-TR7^{+} (E) of β-gal^{+} cells at passage 1 (n ≥ 200 each). Fluorescent microscopy of tmx-treated P0 *Pax7*$^{CE/f}$;*R26R*$^{+/-}$ myoblasts at passage 1. **k, l**, β-gal (**k**, **l**), Desmin (**k′**), ER-TR7 (**l′**), and merged with DAPI (**k″** with **k**, **k′**; **l″** with **l**, **l′**); yellow arrows, Desmin^{+} cells; white arrows, ER-TR7^{+} or fibroblastic cells; white dots, negative cells for reference; note β-gal staining and immunofluorescence in nuclei. Scale bars = 50 μm for **a-d** in **d**, for **e-i′** in **i′**, and for **k-l″** in to **l″**.