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Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells

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The growth and repair of skeletal muscle after birth depends on satellite cells that are characterized by the expression of Pax7. We show that Pax3, the paralogue of Pax7, is also present in both quiescent and activated satellite cells in many skeletal muscles. Dominant-negative forms of both Pax3 and -7 repress *MyoD*, but do not interfere with the expression of the other myogenic determination factor, Myf5, which, together with Pax3/7, regulates the myogenic differentiation of these cells. In *Pax7* mutants, satellite cells are progressively lost in both Pax3-expressing and -nonexpressing muscles. We show that this is caused by satellite cell death, with effects on the cell cycle. Manipulation of the dominant-negative forms of these factors in satellite cell cultures demonstrates that Pax3 cannot replace the antiapoptotic function of Pax7. These findings underline the importance of cell survival in controlling the stem cell populations of adult tissues and demonstrate a role for upstream factors in this context.

Introduction

Pax genes play key roles during development. Members of this family of paired box/homeodomain transcription factors regulate the contribution of progenitor cells to different tissue types (Tremblay and Gruss, 1994). *Pax3* and its paralogue *Pax7* have been implicated in the specification of cells that will enter the myogenic program. In the absence of both Pax3 and -7, there is a major deficit in skeletal muscle, with arrest of myogenesis occurring during later embryonic and fetal development (Relaix et al., 2005). Cells in which the genes are activated become incorporated into other tissues or die in the double mutants. Normally, Pax3/7-positive skeletal muscle progenitor cells, which are derived from the central dermomyotome region of the somites (Ben-Yair and Kalcheim, 2005; Gros et al., 2005), activate the myogenic regulatory genes and differentiate into skeletal muscle fibers or remain as a proliferating reserve cell population within the muscle mass (Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005). In late-stage fetal muscle, these cells begin to adopt a satellite cell position (Kassar-Duchossoy et al., 2005; Relaix et al., 2005), suggesting that this somite-derived population also provides the progenitor cells of postnatal skeletal muscle (Gros et al., 2005). In these cells, the expression of *Myf5* and *MyoD* results in muscle cell determination. During the formation of early embryonic skeletal muscle in the somite, Myf5 and Mrf4 play a critical role in myogenic progenitors, which at this stage are derived from the edges of the dermomyotome (Braun et al., 1992; Tajbakhsh et al., 1996; Kassar-Duchossoy et al., 2004). Pax7 is not expressed in these cells in the mouse, where Pax3 is present. Early myogenesis occurs in the *Pax3* mutant; however, in a triple *Pax3/Myf5(Mrf4)* mutant no skeletal muscle forms and MyoD, which is required for skeletal muscle determination in the absence of Myf5 and Mrf4, is not expressed (Tajbakhsh et al., 1997). Therefore, Pax3, together with Myf5/Mrf4, regulates the activation of *MyoD*. Consistent with this conclusion, *MyoD* is up-regulated in embryos in which PAX3-FKHR, which acts as a strong transcriptional activator, has been targeted to an allele of *Pax3* (Relaix et al., 2003). Pax3 is essential for the survival of cells at the edges of the dermomyotome, particularly to those located hypaxially, where it is also required for the delamination and migration of muscle progenitor cells to other sites where skeletal muscle will form, such as the limbs (Franz et al., 1993; Bober et al., 1994; Goulding et al., 1994). When the coding sequence is targeted to the *Pax3* gene, Pax7 can substitute for Pax3 function in the trunk, but not in the

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Abbreviations used in this paper: β-galactosidase, β-gal; PI, propidium iodide.
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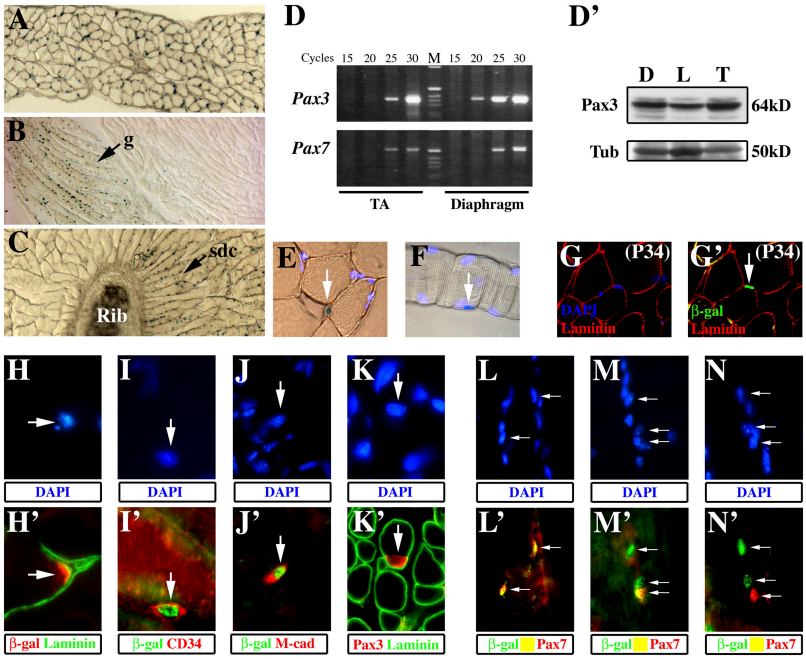

Figure 1. **Pax3 expression in muscle satellite cells.** (A–C) Expression of Pax3 in different muscles from 3-wk-old *Pax3*$^{IRESnLacZ/+}$ mice, revealed by X-Gal staining. (A) Diaphragm muscle; (B) hindlimb muscles, (g) gracilis muscle; (C) trunk muscles, (sdc) serratus dorsalis caudalis. (D) Semiquantitative RT-PCR of Pax3 and -7 transcripts in adult tibialis anterior (TA) and diaphragm muscle. The number of cycles is indicated on the top of each lane. M, molecular weight markers (see Materials and methods). (D') Pax3 protein is detected by Western blot in protein extracts from different muscles (D, diaphragm; L, hindlimb; T, ventral trunk muscles) in 3-wk-old mice. Tubulin (Tub) expression is shown as a loading control. (E and F) *Pax3*$^{IRESnLacZ/+}$ is expressed in a subset of diaphragm muscle nuclei (arrows) from 3-wk-old mice, as revealed by X-Gal and DAPI staining of a transverse section (E) or isolated fiber (F). (G and G') Detection of Pax3/7-dependent β-gal expression in transverse sections of adult diaphragm muscle from the transgenic line *P34*, which reports Pax3/7 transcriptional activity. (G) Immunodetection of laminin (red) and DAPI (blue) staining. (G') Coimmunodetection of laminin (red) and β-gal (green). (H–J') Coimmunodetection of β-gal–positive cells in the diaphragm muscle of 3-wk-old *Pax3*$^{IRESnLacZ/+}$ mice with laminin (H', green), CD34 (I', red), or M-cadherin (J', red). As indicated in the figure, β-gal is shown in red in H' and in green in I' and J'. Corresponding DAPI staining is shown (H, I, and J) for each panel. Arrows indicate the labeled satellite cell nuclei. (K) Coimmunodetection of Pax3 (red) and laminin (green, K'). Corresponding DAPI staining is shown, with the labeled nucleus indicated by an arrow (K). (L–N') Coimmunohistochemistry on diaphragm muscle from 3-wk-old *Pax3*$^{IRESnLacZ/+}$ mice for Pax7 (red) or β-gal (green). (L–N) Corresponding DAPI staining is presented with labeled nuclei indicated by arrows for each panel.

limbs, suggesting that after the duplication of a common *Pax3/7* gene, which is present before vertebrate radiation, the functions of Pax3 and -7 diverge in response to the requirements of appendicular muscle formation (Relaix et al., 2004).

Satellite cells, the myogenic progenitor cells of postnatal muscle, lie under the basal lamina of muscle fibers in a quiescent state until they become activated, proliferate, and form new skeletal muscle, which occurs during postnatal growth and in response to damage (Bischoff and Heintz, 1994). Myogenic regulatory genes are expressed during this process; *Myf5* is already expressed in quiescent satellite cells (Beauchamp et al., 2000), and *MyoD* is expressed as the cells become activated and subsequently differentiate with the expression of *myogenin* (Yablonka-Reuveni and Rivera, 1994). *Myf5:MyoD* double mutants have not yet been examined in this adult context because of the perinatal lethality of the original *Myf5* mutant. However, in the absence of MyoD, muscle regeneration is less efficient and the balance between proliferation and differentiation of myosatellite cells appears to be affected (Megeney et al., 1996). The most striking result, however, came from the examination of *Pax7* mutant mice (Seale et al., 2000). Pax7 is present in satellite cells, and in its absence muscle regeneration is severely affected. Satellite cells were not observed in the mutant, leading to the proposal that Pax7 is essential for the specification of adult muscle progenitor cells (Seale et al., 2000). However, it has recently been shown that satellite cells are present in the *Pax7* mutant, although in decreasing numbers as the mice mature, and it has been suggested that their proliferation is compromised in the absence of Pax7 (Oustanina et al., 2004). Pax7 is present in quiescent satellite cells and during their activation, but is down-regulated when they differentiate. A proportion of activated satellite cells remain undifferentiated, retain Pax7 expression, and are thought to reconstitute the satellite cell pool (Olguin and Olwin, 2004; Zammit et al., 2004). Therefore, Pax7 appears to play a predominant role in adult muscle progenitor cells. The presence of Pax3, however, has been noted after satellite cell activation, leading to the proposal that it is implicated in their proliferation (Conboy and Rando, 2002). It was also noted that the expression of a *Pax3*^{nLacZ} allele can be detected in quiescent satellite cells (Buckingham et al., 2003).

We now investigate the role of Pax7 in relation to Pax3, which we show is expressed in the quiescent satellite cells of a major subset of skeletal muscles. We show that both Pax3 and -7 control *MyoD* activation, and therefore regulate myogenesis in the adult. However, we demonstrate that in the absence of Pax7 satellite cells are progressively lost postnatally because of apoptosis accompanied by cell cycle defects. Pax7 has a critical antiapoptotic function in activated satellite cells for which Pax3 does not compensate. These results underline the critical role of upstream regulators of tissue formation and regeneration in assuring progenitor cell survival.

Results

Pax3 expression in the satellite cells of adult skeletal muscle

Because Pax3 plays a key role during the onset of skeletal myogenesis in the embryo, we investigated its status in adult muscle in relation to Pax7. Analysis of adult mice in which the *Pax3* gene is targeted with *nLacZ* reporters (Relaix et al., 2003) revealed the presence of β-galactosidase (β-gal)–positive cells in adult skeletal muscle. The number of such cells varies between muscles. They are particularly abundant in the diaphragm

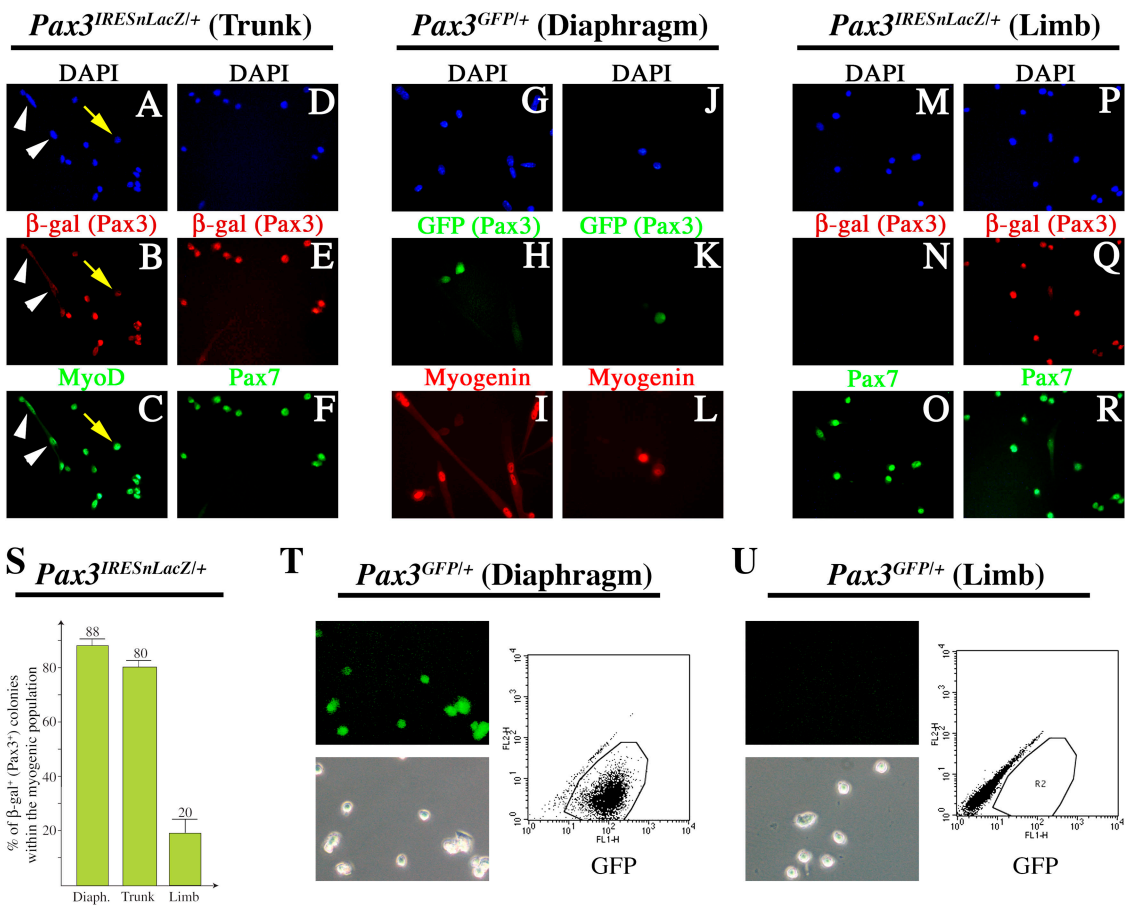

Figure 2. **Pax3 and -7 expression in activated satellite cells.** (A–F) Coimmunocytochemistry on primary cultures derived from the trunk muscles of 3-wk-old *Pax3*$^{nLacZ/+}$ mice after 4 d in culture, using DAPI staining (A and D), or an antibody recognizing β-gal (Pax3; B and E, red), MyoD (C, green) or Pax7 (F, green). Whereas β-gal (Pax3) and MyoD are coexpressed in proliferating myoblasts, upon terminal differentiation Pax3 (β-gal) is down-regulated (A–C, arrowheads), and is already lower in some mononucleated MyoD-positive cells (A–C, arrow). (E and F) Most colonies coexpress β-gal (Pax3) and Pax7. (G–L) Detection of GFP and myogenin in primary cultures of satellite cells from the diaphragm of 3-wk-old Pax3$^{GFP/+}$ mice after 5 d in culture; (G and J) DAPI staining; (H and K) direct detection of GFP (Pax3) fluorescence (green); and (I and L) immunodetection of myogenin (red). (M–R) Coimmunocytochemistry on primary cultures derived from the hindlimb muscles of 3-wk-old *Pax3*$^{nLacZ/+}$ mice after 4 d in culture, showing DAPI staining (M and P) or reaction with an antibody recognizing β-gal (N and Q, red) or Pax7 (O and R, green). Colonies expressing either Pax7 alone (N and O) or Pax3 and -7 (Q and R; ≤20%, see S) were identified. (S) Histograms showing the percentage (%) of β-gal (Pax3)–positive colonies of myogenic cells obtained from the diaphragm (Diaph.), ventral trunk (Trunk), and hindlimb (Limb) muscles of 3-wk-old *Pax3*$^{nLacZ/+}$ mice. Cells were plated at low density to permit the formation of colonies and stained with X-Gal 3–4 d after plating. The results are from three independent experiments after counting ≥100 colonies from triplicate culture plates. (T and U) Muscle satellite cells were isolated by flow cytometry from the lower hindlimb muscles and from the diaphragm of Pax3$^{GFP/+}$ adult mice as (Pax3) GFP-negative and (Pax3) -positive cells and maintained in culture as proliferating cells for 6 d before analysis of GFP expression by flow cytometry. Direct detection of GFP was performed on living cells under an inverted fluorescence microscope (T and U, top left) with corresponding phase-contrast detection of the cells (T and U, bottom left) and flow cytometry detection of GFP-positive cells (T and U, right, boxed R2 region).

(Fig. 1 A), but are much less frequent in hindlimb muscles, with the exception of the gracilis muscle (Fig. 1 B). In contrast, ∼50% of forelimb muscles express *Pax3*. As in the embryo (Relaix et al., 2004), expression of *Pax3* is not detectable in head muscles. Most ventral trunk muscles are positive, with a striking juxtaposition in the rib area, where intercostal muscles are mainly negative, whereas body wall muscles such as the serratus caudalis dorsalis are positive (Fig. 1 C). This difference is confirmed by semiquantitative RT-PCR analysis of Pax3 versus -7 transcripts in the tibialis anterior hindlimb muscle compared with the diaphragm (Fig. 1 D). The Pax3 protein is also present, as shown by Western blot analysis of different muscles (Fig. 1 D'). The *Pax3*$^{nlacZ/+}$-expressing cells are found to be associated with muscle fibers (Fig. 1, E and F). The Pax3/7 protein is transcriptionally active in adult muscle, as indicated by activation of the P34 reporter transgene in which Pax3/7 binding sites regulate *nLacZ* expression (Fig.1, G and G'; Relaix et al., 2004). These Pax3-expressing nuclei are present in satellite cells, as shown by coimmunolocalization of β-gal with the satellite cell markers CD34 and M-cadherin and by the inclusion of β-gal–positive cells within the basal lamina of the muscle fiber (Fig. 1, H–K'). Because Pax7 is present in satellite cells (Seale et al., 2000), the question of Pax3 expression in relation to Pax7 was addressed. In the diaphragm, the majority of Pax7-positive satellite cells also coexpress Pax3. About 15% of the cells only label with Pax7, and, occasionally, cells that express only Pax3 are also detected (<3%; Fig. 1, L–N'). We therefore conclude that Pax3, like Pax7, is expressed in quiescent satellite cells and that the frequency of this event varies between muscles. There is no direct relation to fiber type (Kelly and

Rubenstein, 1994) because the diaphragm muscle (type IIX, IIA, and I fibers) is positive, whereas in the hindlimb the soleus (type I and IIA) is negative. Similarly, in the hindlimb the gastrocnemius (mostly type IIB) is negative, whereas other fast muscles (type IIA and IIB) in the trunk and forelimb have Pax3-expressing satellite cells.

We next examined the expression of Pax3 and -7 in primary cultures of satellite cells prepared from different muscles (Fig. 2). Activated satellite cells, from the trunk muscle of *Pax3*$^{IRESnLacZ/+}$ mice, which are Pax3 (β-gal)-positive, also express MyoD and down-regulate Pax3 (β-gal) in differentiated muscle fibers (Fig. 2, A–C). This is also seen in cultures from *Pax3*$^{GFP/+}$ mice (Relaix et al., 2005), where expression of myogenin, which marks the onset of differentiation, is associated with rapid reduction in Pax3 (GFP) expression (Fig. 2, G–L). MyoD-positive cells in which Pax3 (β-gal) is low (Fig. 2, B and C) have probably activated *myogenin*. MyoD (Fig. 2 C), or later myogenin (Fig. 2, I and L), is expressed in most cells marked by DAPI staining. In cultures from trunk (Fig. 2, D–F) and diaphragm muscle (not depicted), most nondifferentiating satellite cells coexpress Pax3 (β-gal) and -7. However, in cultures from the hindlimb of *Pax3*$^{IRESnLacZ/+}$ mice this is not the case and many colonies only express Pax7 (Fig. 2, N and O). Some colonies (≤20%) express both *Pax* genes (Fig. 2, P–R). The distribution of these two types of colonies from different muscle sources is quantified in Fig. 2 S. To confirm that *Pax3* is not activated in satellite cells that only express *Pax7*, we isolated these cells using flow cytometry. Based on the isolation of GFP-positive satellite cells from the diaphragm muscle, we had previously established the gating window that contains these cells (Montarras et al., 2005), which is shown as a boxed area (R2) in Fig. 2 (T and U). When satellite cells isolated on this basis from the diaphragm muscle are cultured and reanalyzed by flow cytometry, they remain Pax3 (GFP)-positive (Fig. 2 T). However, when Pax3-negative satellite cells are sorted from the muscle of the lower hindlimb and cultured, no GFP-positive cells were found in the R2 window after re-sorting by flow cytometry (Fig. 2 U). These results demonstrate that activated satellite cells from Pax3-negative muscles, do not activate this *Pax* gene in cell culture.

The myogenic function of Pax3 and -7 in satellite cells

In the genetic hierarchy that regulates the onset of myogenesis in the embryo, Pax3 activates *MyoD* (Tajbakhsh et al., 1997; Relaix et al., 2003), and Pax7 can replace Pax3 in this function (Relaix et al., 2004). Therefore, we investigated the myogenic activity of the two Pax proteins in adult muscle by infecting cultured satellite cells with adenoviral vectors expressing wild-type or dominant-negative forms of Pax3 and -7, together with a GFP reporter. The dominant-negative proteins contain the repression domain of the *Drosophila melanogaster* engrailed transcription factor (Han and Manley, 1993) fused to the NH_2-terminal region of the Pax sequence, which retains its DNA-binding domain. An initial series of experiments was performed with satellite cells isolated from a Pax reporter line, *P34*, in which the transgenic mice express β-gal

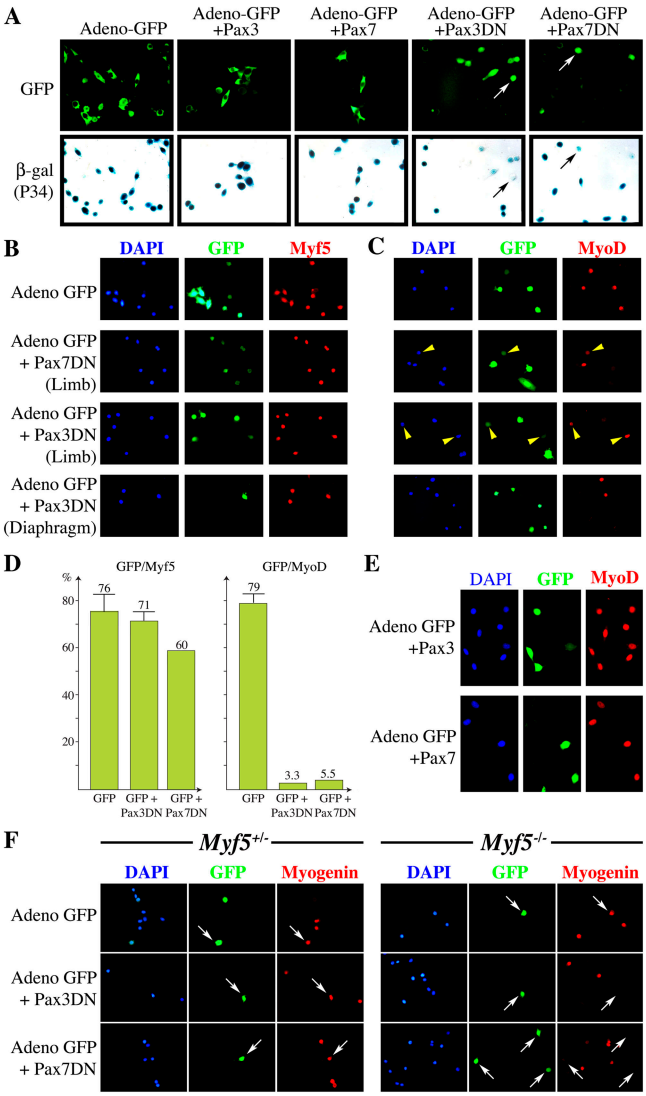

Figure 3. **The effect of dominant-negative forms of Pax3 and -7 on MyoD and Myf5 expression in satellite cells.** (A) Primary cultures prepared from adult diaphragm muscle from the Pax3/7 transcriptional reporter line, *P34*, were infected with adenoviruses encoding either GFP alone (Adeno-GFP), GFP and Pax3, GFP and Pax7, or GFP with dominant-negative versions of either Pax3 (Pax3DN) or Pax7 (Pax7DN). (top) Infected cells are identified as GFP positive. (bottom) The capacity of Pax3DN or -7DN to abrogate Pax3/7 transcriptional activity is revealed by weaker (arrows) or undetectable X-Gal staining seen in ≥80% of GFP-positive cells with both dominant-negative constructs. (B and C) Coimmunohistochemistry on primary cultures from hindlimb or diaphragm muscles of 3-wk-old wild-type mice infected with adenoviral vectors as in A. DAPI staining and antibodies recognizing either Myf5 (B) or MyoD (C) were used, whereas GFP fluorescence was detected directly. Cells expressing lower levels of PaxDN are indicated with an arrowhead. (D) Quantitation of results for satellite cell cultures infected with an adenovirus-expressing GFP and Pax3DN or GFP and Pax7DN, presented in B and C. Results presented as histograms of the percentage (%) of GFP-positive cells expressing Myf5 or MyoD are taken from two to five independent experiments. (E) Primary cultures of satellite cells isolated from the diaphragm muscle of adult wild-type mice were infected with adenoviruses expressing GFP and Pax3 or -7. Infected cells were identified by direct detection of GFP fluorescence, and MyoD expression was followed by immunodetection. (F) Primary cultures of muscle satellite cells from hindlimb muscles of 3-wk-old *Myf5*$^{GFP/+}$ and *Myf5*$^{GFP/GFP}$ mice were infected with adenoviral vectors encoding either GFP alone or GFP and Pax3DN or -7DN. Infected cells are identified by GFP fluorescence, and myogenesis is monitored by immunodetection of myogenin. The threshold of fluorescent GFP detection was set to capture only the strong adenovirus GFP reporter, excluding the weaker expression from the *Myf5*^{GFP} allele.

from an *nLacZ* reporter that is regulated by multimerized Pax3/7 binding sites (Relaix et al., 2004). Overexpression of Pax3 or -7 resulted in no obvious increase in reporter activity, which was already expressed at a high level in the Pax3/7-positive satellite cells. However, the dominant-negative versions of both of these factors (Pax3DN and -7DN) resulted in down-regulation of the reporter (Fig. 3 A). With decreasing levels of Pax3DN or -7DN, no significant difference in the repression exerted by either dominant-negative Pax was detectable (not depicted). We conclude that Pax3 and -7 bind to the consensus site with similar affinities and that their dominant-negative forms compete effectively with both endogenous proteins, which normally function as transcriptional activators in these adult muscle cells, as in the embryo (Relaix et al., 2003). When dominant-negative forms of Pax3 or -7 were expressed in satellite cell cultures, the level of the myogenic factor Myf5 was not markedly affected (Fig. 3 B), whereas MyoD was down-regulated in infected cells (Fig. 3 C). As for the Pax3/7 reporter transgene, similar dosage effects were seen for both Pax3DN and -7DN, with reduction, but not elimination, of MyoD in cells in which the adenoviral expression vector was expressed at a lower level (Fig. 3 C, arrowheads). These results are quantified in Fig. 3 D. In contrast to the striking down-regulation of MyoD seen with the dominant-negative constructs, overexpression of wild-type Pax3 or -7 is compatible with MyoD expression (Fig. 3 E). Because myogenesis still occurs in the absence of MyoD (Fig. 3 F and not depicted), we investigated whether this is regulated by Myf5, which is expressed independently of Pax3 or -7 in the satellite cell cultures. These cells were prepared from hindlimb muscles of $Myf5^{GFP/+}$ and $Myf5^{GFP/GFP}$ mice and infected with Pax3DN and -7DN adenoviral vectors (Fig. 3 F). Unlike infected cells from heterozygous mice, *Myf5* mutant cells do not express myogenin or differentiate in the presence of the dominant-negative Pax vectors. This demonstrates that either Pax3/7, acting via MyoD, or Myf5 are required for the myogenic differentiation of adult satellite cells.

The presence of satellite cells and muscle differentiation in Pax3-expressing muscles of *Pax7* mutant mice

Because Pax3-expressing satellite cells are found in adult muscles, their potential contribution to muscle growth and regeneration was investigated in the *Pax7* mutant mouse. We first evaluated whether Pax3 continues to be expressed in diaphragm and trunk muscles. This is the case as shown in Fig. 4 at postnatal day 3 (P3). Western blots show that Pax3 is present, although at a reduced level (Fig. 4 A), and immunohistochemistry confirms that Pax3 is expressed in satellite cells (Fig. 4 B). Satellite cells are also revealed by β-gal labeling of $Pax7^{LacZ/LacZ}$ muscle at P2 (Fig. 4 C) and, indeed, even at P10 cultures from the diaphragm contain MyoD-positive cells that differentiate into myofibers expressing troponin T (Fig. 4 D), although such cells are much rarer (≤15% of wild type). Similarly, single fiber experiments with the extensor digitorum longus hindlimb muscle (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200508044/DC1) show that satellite cells are still present in the mutant, but that their number is reduced

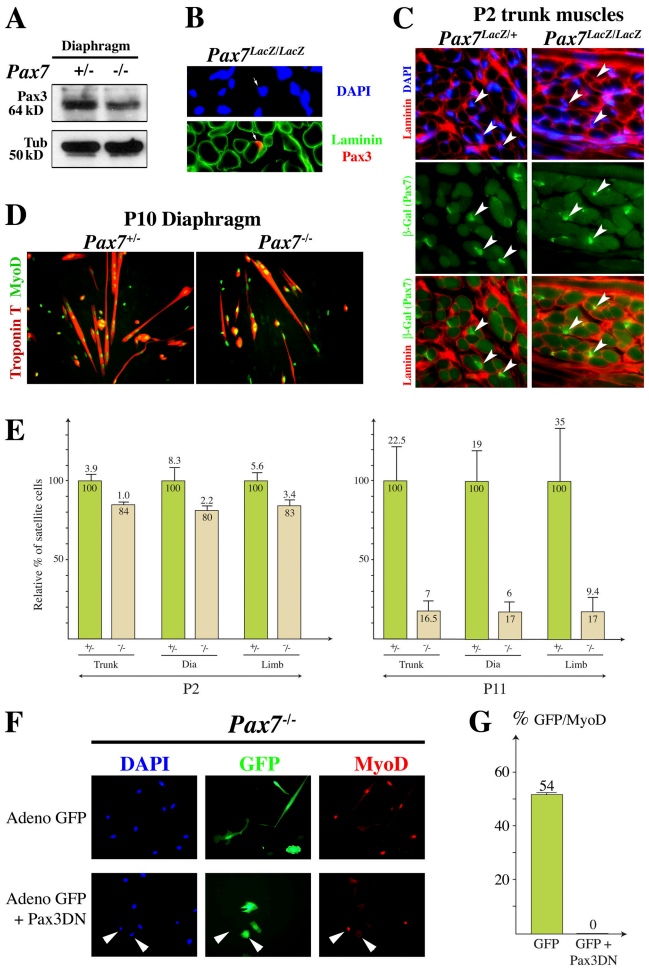

Figure 4. **Pax3 expression is maintained in Pax7-deficient satellite cells.** (A) Western blot analysis of Pax3 expression in the diaphragm of $Pax7^{LacZ/+}$ (+/−) or $Pax7^{LacZ/LacZ}$ (−/−) mice at P3. Tubulin (Tub) expression is shown as a loading control. (B) Coimmunohistochemistry on transverse sections of ventral trunk muscle of $Pax7^{LacZ/LacZ}$ mice at P2 using DAPI staining and an antibody that recognizes Pax3. Laminin staining shows a Pax3-positive cell (red) present in a satellite cell position. (C) Immunohistochemistry on transverse sections of ventral trunk muscle from $Pax7^{LacZ/+}$ or $Pax7^{LacZ/LacZ}$ mice at P2 using antibodies recognizing β-gal (green) and laminin (red). Corresponding DAPI staining is indicated. Arrowheads indicate *Pax7* (β-gal)–expressing cells, located under the basal lamina. (D) Coimmunocytochemistry on 7-d primary cultures derived from the diaphragm of $Pax7^{LacZ/+}$ or $Pax7^{LacZ/LacZ}$ mice at P10 using antibodies recognizing MyoD and troponin T. (E) Quantification of the number of β-gal–positive satellite cells in trunk, diaphragm (dia), and hindlimb (limb) muscles of $Pax7^{LacZ/+}$ (+/−) or $Pax7^{LacZ/LacZ}$ (−/−) mice, detected per fiber on 10-μm sections from ventral trunk muscle at P2 or P11. (F) Infection of primary cultures from $Pax7^{LacZ/LacZ}$ mutant mice ($Pax7^{-/-}$) at P4 with an adenovirus expressing a dominant-negative form of Pax3 (Adeno GFP+Pax3DN) shows elimination of MyoD expression in infected cells (right arrowhead, infected cell; left arrowhead, noninfected cell), whereas virus expressing GFP alone had no effect. Quantification is shown in G. No MyoD-positive myogenic cells are present after Pax3DN infection.

to ∼10% of normal levels at P10. The number of myonuclei is also reduced by ∼50% at this stage (Fig. S1 F) and muscle fibers are smaller (not depicted), consistent with the reduced size of mutant mice (Mansouri et al., 1996; Seale et al., 2000). The number of β-gal–positive satellite cells were counted on sections of $Pax7^{LacZ/LacZ}$ muscles and compared with heterozygotes at P2 and P11 (Fig. 4 E). At P2 there is only a small reduction

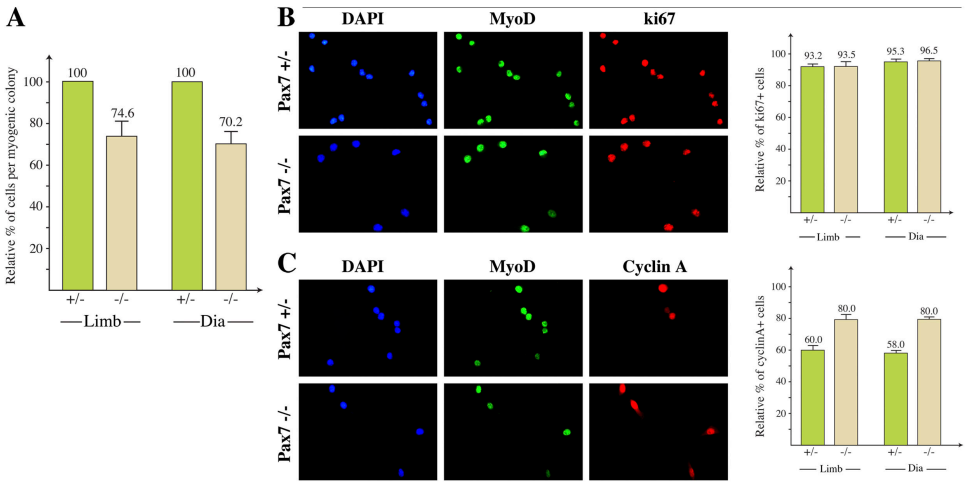

Figure 5. **Satellite cell proliferation in the *Pax7* mutant.** (A) Satellite cells from the diaphragm (dia) or hindlimb muscles (limb) of $Pax7^{lacz/+}$ (+/−) and $Pax7^{lacz/lacz}$ (−/−) mice at P4 were plated at low density. After 3 d, when colonies had formed, cells were processed for immunocytochemistry using a MyoD antibody. The number of cells per myogenic colony was determined in three independent experiments for each type of muscle and after counting at least 10 colonies per experiment. Results are expressed as the percentage of cells per myogenic colony from the Pax7 mutant (−/−) with respect to Pax7 heterozygotes (+/−), taken as 100%. (B) Determination of the percentage of cycling cells in normal and mutant myogenic colonies cultured as in A. Cells were costained with antibodies against MyoD and Ki67, which marks all phases of the cell cycle. Examples shown are for myogenic colonies from hindlimb muscles. Quantitation is from two independent experiments for each type of muscle. Results expressed as percentage of Ki67-positive cells per myogenic colony indicate that both mutant and wild type cells proliferate equally well. (C) Determination of the percentage of cyclin A–positive cells per myogenic colony cultured as in A. Cells were costained with antibodies against MyoD and cyclin A, which marks the S and G2 phases of the cell cycle. Examples shown are from hindlimb muscles treated for immunocytochemistry as in B. Results are expressed as percentage of cyclin A–positive cells per myogenic colony.

(∼18%) in the numbers of satellite cells in trunk, diaphragm, or hindlimb muscles. By P11, this reduction is striking (∼83%) and is similar for all three muscle sources, whether Pax3 is expressed in most satellite cells (e.g., diaphragm) or not (hindlimb). This demonstrates that Pax3 cannot compensate for Pax7 function during the postnatal development of skeletal muscle. This is not because of a failure in myogenesis in the mutant satellite cells. *MyoD* is still expressed and, as in wild-type cultures (Fig. 3), its expression is inhibited by a dominant-negative form of Pax3 (Fig. 4, F and G).

Satellite cell proliferation in the *Pax7* mutant

Because the numbers of satellite cells fall during postnatal development in *Pax7* mutant mice, and muscle fiber size is reduced, it is possible that activated satellite cells, which contribute to muscle growth, do not proliferate normally. This was examined in primary cultures from the diaphragm and hindlimb muscles of $Pax7^{LacZ/LacZ}$ and $Pax7^{LacZ/+}$ mice, plated at low density so that the number of cells per colony could be monitored. A reduction of 25–30% in the number of cells per myogenic colony was observed (Fig. 5 A), indicating that, in the absence of Pax7, proliferation is affected and that this is also the case for colonies from a muscle, such as the diaphragm, which expresses Pax3. To determine whether some cells have withdrawn from the cell cycle, a Ki67 antibody was used, which marks proliferating cells in all phases of the cell cycle (Scholzen and Gerdes, 2000). There was no difference between colonies from mutant or heterozygous mice, where the proportion of proliferating cells is concerned (Fig. 5 B). We next examined progression of cells through the cell cycle, using a cyclin A antibody that marks cells in the S and G_2 phases (Girard et al., 1991). There were 33% more cyclin A–positive cells in the mutant myogenic colonies (Fig. 5 C).

One possible explanation for the increase in the proportion of proliferating cells in the S and G_2 phases of the cycle in the *Pax7* mutant is that some cells exit the cycle during G1 and immediately undergo apoptosis, resulting in a reduction in the number of cells per colony observed in the absence of Pax7 (Fig. 5 A). In the colony assay, we did not detect a significant difference in the numbers of dying cells using standard markers of apoptosis (not depicted). This may be because such cells detach immediately. Alternatively, there may be a cell cycle defect independent of apoptosis, such as a cell cycle arrest in G_2, resulting in a slower progression through the cell cycle.

Apoptosis of satellite cells in the absence of *Pax7*

To investigate the survival of satellite cells in postnatal muscle in vivo, in the absence of Pax7, we used an antibody to the activated form of caspase-3 to label cells undergoing apoptosis (Patel et al., 1996). Coimmunohistochemistry was performed with an antibody to desmin, which marks activated satellite cells as they assume a myoblast phenotype (Creuzet et al., 1998), as well as muscle fibers. In the postnatal trunk muscle of *Pax7* mutant mice, activated caspase-3–labeled cells are observed, whereas in control mice labeled cells are not detected (Fig. 6, A–D). These results are quantitated in Fig. 6 E. The decrease observed from P0 to P6 reflects the decreasing numbers of satellite cells in the mutant. These cells also express desmin, suggesting that they correspond to activated satellite cells, probably contributing to the postnatal growth of muscle (Fig. 6, A and B, arrowheads). The identification of these cells was confirmed by labeling with a laminin (Fig. 6 C) or β-gal anti-

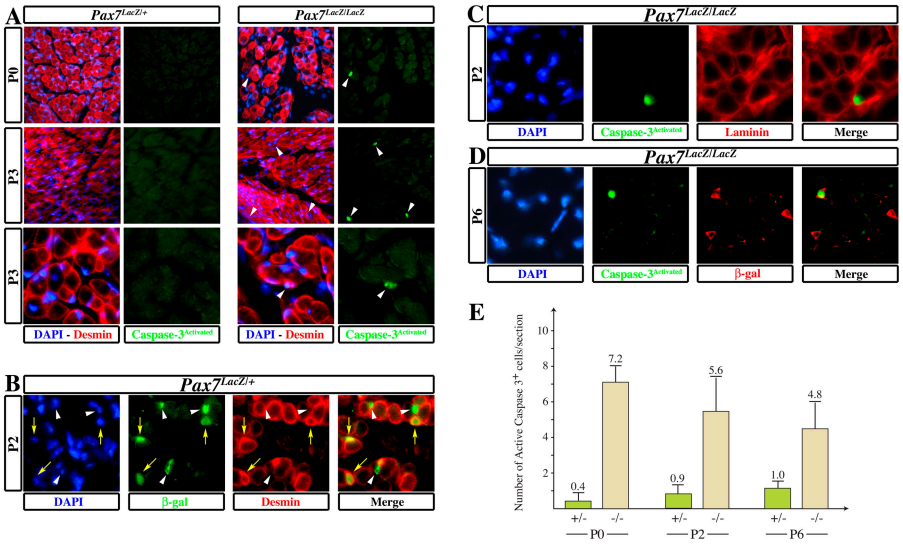

Figure 6. **Satellite cell survival in *Pax7* mutant mice.** (A) Coimmunohistochemistry on transverse sections of ventral trunk muscle of $Pax7^{LacZ/+}$ or $Pax7^{LacZ/LacZ}$ newborn mice at P0 or P3 using DAPI staining or antibodies recognizing desmin (red) or the activated form of caspase-3 (green). Apoptotic cells that are desmin positive are present in muscles from *Pax7* mutant mice (arrowheads). (B) Coimmunohistochemistry on transverse sections of ventral trunk muscle of $Pax7^{LacZ/+}$ mice at P2 using DAPI staining or antibodies recognizing desmin (red) or β-gal (green). Pax7 (β-gal)-expressing satellite cells are either desmin positive (arrows), which marks activated satellite cells, or desmin negative (arrowheads), indicating quiescent satellite cells. (C and D) Coimmunohistochemistry on transverse sections of ventral trunk muscle of $Pax7^{LacZ/LacZ}$ mice at P2 (C) or P6 (D) using DAPI staining or antibodies recognizing the activated form of caspase-3 (green), laminin (C, red), or β-gal (D, red) shows that the *Pax7* mutant cells located in a satellite cell position are subject to apoptosis. (E) Quantification of apoptotic cells at P0, P2, and P6, based on analysis of sections after coimmunohistochemistry with an antibody to activated caspase-3, and to desmin as a marker of muscle cells. Cells labeled with both antibodies were scored per standard transverse section of trunk and forelimb muscle.

body (Fig. 6 D). The latter detects *Pax7* transcription, which marks satellite cells. During postnatal development, apoptotic cells were detected in all trunk and limb muscles examined in the *Pax7* mutant, whereas they were very rare in the muscles of normal mice (Fig. 6 E). Therefore, we conclude that Pax7 has an antiapoptotic function and that in its absence satellite cells die, despite the presence of Pax3.

To compare the roles of Pax3 and -7 in protecting against apoptosis, wild-type satellite cells were transfected with GFP-marked adenoviral vectors coexpressing a dominant-negative form of Pax3 or -7. These cells were analyzed by flow cytometry on the basis of GFP expression, and their viability was measured by propidium iodide (PI) staining, which detects dying cells (Matteucci et al., 1999). Such an experiment is shown in Fig. 7 A for cells from hindlimb muscle infected with a dominant-negative form of Pax3 or -7, which led to 71% of dying cells in the GFP-positive population when Pax7DN was expressed. The results of these experiments are summarized in Fig. 7 B. Whereas Pax7DN led to substantial cell death, the Pax3DN-expressing virus at similar or sixfold higher multiplicities of infection did not show any effect on these cells, relative to control values. Similar results were seen in primary cultures from young mice (P7; not depicted) to those shown here for 3–4-wk-old animals (Fig. 7). No such apoptotic effect was observed when muscle cultures were infected with adenoviral vectors expressing Pax7. The induction of apoptosis is not related to the presence of MyoD (Peschiaroli et al., 2002) because in satellite cell cultures from $MyoD^{-/-}$ mice infected by

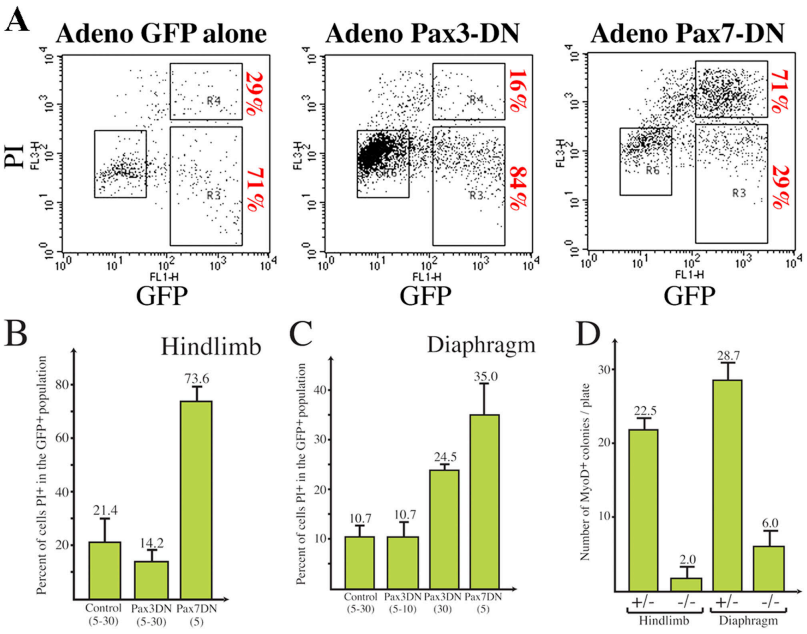

Figure 7. **The role of *Pax7* and *-3* in the survival of satellite cells.** (A) An example of the data obtained by flow cytometry after infection of cells cultured from hindlimb muscles with a GFP-labeled adenovirus expressing GFP alone or with a dominant-negative form of Pax3 (Pax3DN) or Pax7 (Pax7DN). The percentages indicate the number of GFP-positive cells that are undergoing cell death on the basis of PI uptake (R4) compared with those that survive (R3). (B) A summary of results obtained from the hindlimb on infection with both Pax7DN and Pax3DN, based on four independent experiments with triplicate cultures in each experiment. The numbers shown in the histograms are the percentage of dying cells (R4). Control experiments were performed with adenoviruses expressing only GFP. Numbers in brackets indicate the multiplicity of infection. (C) A summary of results obtained for cells isolated from the diaphragm. The histograms correspond to five independent experiments for the control (5–30) and Pax3DN (5–10), two for Pax3DN (30), and three for Pax7DN (5). Numbers in brackets indicate the multiplicity of infection. (D) The number of MyoD-positive colonies per culture dish obtained after plating the same number of cells from hindlimb or diaphragm muscle of $Pax7^{LacZ/+}$ or $Pax7^{LacZ/LacZ}$ mice at P4.

Pax7DN a similar extent of cell death was observed (not depicted). Furthermore, Pax7DN (or Pax3DN) did not provoke cell death in nonmyogenic cells, such as the OP9 bone marrow stromal cell line (not depicted). When cells isolated from the diaphragm (Fig. 7 C) were similarly infected, cell death caused by Pax7DN was about half of that witnessed in cells from hindlimb muscle. Equivalent levels of Pax3DN showed no effect, but in contrast with the observations on the hindlimb (Fig. 7 B), when the concentration of Pax3DN was increased, some cell death was observed. These results suggest that Pax3 can have a limited antiapoptotic effect in the muscles in which it is expressed. In keeping with this conclusion, more myogenic colonies are present in cells isolated from the diaphragm of *Pax7* mutant mice at P4, although in both diaphragm and hindlimb preparations, this number was strikingly lower than with the wild type (Fig. 7 D). At later stages no difference was detectable.

Discussion

Pax7 is a satellite cell marker; however, we now show that its paralogue, Pax3, is coexpressed in these cells in many skeletal muscles. This raises the question of their respective roles and whether Pax3 can compensate for the *Pax7* mutant phenotype. Both function similarly in regulating *MyoD* during the onset of myogenesis in satellite cell cultures, and our analysis of *Myf5* mutant cells demonstrates that Myf5 and Pax3 or Pax7 control the entry of these adult muscle progenitor cells into the myogenic program. The progressive postnatal loss of satellite cells that we document for *Pax7* mutant mice is seen in the presence of Pax3, and it is caused by a requirement for Pax7 in muscle satellite cell survival and cell cycle progression for which Pax3 cannot compensate.

Muscle progenitor cell specification and Pax3 expression in a subset of skeletal muscles

At birth the great majority of satellite cells are still present in the trunk and limb muscles of *Pax7* mutant mice (Oustanina et al., 2004), showing that Pax7 is not required for satellite cell specification as previously suggested (Seale et al., 2000). During prenatal development, a Pax3/7 population of myogenic progenitor cells is present and these somite-derived cells take up a satellite cell position in late fetal muscle (Gros et al., 2005). It is only when both Pax3 and -7 are absent that these cells die or fail to enter the myogenic program, with a major deficit in skeletal muscle in the double mutant (Relaix et al., 2005). We would therefore propose that satellite cell progenitors are specified prenatally by the action of Pax3 in the *Pax7* mutant.

It is not clear why Pax3 should be present in the quiescent, as well as in the activated satellite cells of some muscles and not others. Most hindlimb muscles, usually used as a source of satellite cells (Seale et al., 2000; Conboy and Rando, 2002), and some forelimb and trunk muscles are negative for Pax3. Even within a muscle that is positive, like the diaphragm, some satellite cells express only Pax7. These differences do not correlate with fiber type and, thus, they do not correlate with the type of innervation. A correlation with the embryological origin of the muscle is also not evident (Tajbakhsh and Buckingham, 2000).

Heterogeneity between muscles is a well known feature of myopathies in which the mutation of a gene expressed in all muscles may have a pathological effect on particular muscle groups (Hadchouel et al., 2003). It is also evident from the study of regulatory genes in the embryo that different sites of myogenesis are coordinated by different regulatory strategies. This is illustrated by the number of distinct sequences that control the spatiotemporal activation of the *Myf5* gene (Buchberger et al., 2003; Hadchouel et al., 2003) or by the effects of mutations in genes encoding homeobox proteins, such as Lbx1 (Schafer and Braun, 1999; Brohmann et al., 2000; Gross et al., 2000) or Meox2 (Mankoo et al., 1999), which lead to the loss of certain limb muscles and not others. Understanding the basis of such myogenic heterogeneity represents a challenge for the muscle field, which has tended not to think in these terms because of the apparently generalized effects of the MyoD family of myogenic regulatory factors in the embryo.

The role of Pax3 and -7 in myogenesis

Myogenesis in the embryo is initially orchestrated by the myogenic regulatory factors Myf5 and Mrf4 that, together with Pax3, lead to the subsequent activation of *MyoD*. Mrf4 is not detectable in adult satellite cells (unpublished data), although it is expressed when they differentiate (Cooper et al., 1999), and this gene does not play a role as a determination factor during late embryonic and fetal development (Kassar-Duchossoy et al., 2004, 2005). However, we show that the genetic hierarchy that controls the onset of myogenesis in the embryo is conserved in the adult and that *Pax3/7* and *Myf5* control the entry of cells into the myogenic program, acting in parallel genetic pathways. In the absence of all three factors, skeletal muscle differentiation does not occur, whereas in satellite cells from the *Myf5* mutant or from Pax3-negative hindlimb muscles of the *Pax7* mutant, myogenin is activated and the cells differentiate. In keeping with previous observations on *MyoD* mutant embryos (Rudnicki et al., 1992), Myf5 can activate *myogenin* directly, whereas activation of myogenesis by Pax3/7 depends on MyoD, as shown in the experiments reported here (Tajbakhsh et al., 1997). Expression of *Myf5* in satellite cells, which we show is not affected by dominant-negative forms of Pax3 or -7, suggests that these cells have progressed beyond the progenitor cell state, characterized by the presence of Pax3/7 and the absence of any myogenic regulatory factor (Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005). In prenatal progenitor cells, expression of both *Myf5* and *MyoD* depends on the presence of Pax3/7 factors (Relaix et al., 2005).

Dominant-negative forms of Pax3 or -7 down-regulate *MyoD* expression in the presence of Myf5. Presumably, the engrailed repression domain present in these constructs overrides transcriptional activation by Myf5. The direct repression exerted by these constructs was demonstrated by their effect on the *P34* reporter transgene, which is dependent on Pax3/7 binding sites. In these experiments, and in situations where

Pax3DN or -7DN are expressed at a lower level, as indicated by the GFP reporter, with only partial repression of *MyoD*, we have never detected any difference between the two Pax dominant-negative forms. Furthermore, satellite cells isolated from hindlimb muscles expressing only Pax7 down-regulate *MyoD* in the presence of Pax3DN. We therefore conclude that Pax3 and -7 play a similar role in the activation of *MyoD* and subsequent skeletal muscle differentiation. In experiments with adenoviral vectors in which Pax3 or -7 were overexpressed in satellite cells, we saw no down-regulation of *MyoD*. This is in contrast to the results of the study conducted by Olguin and Olwin (2004), although in the C_2 myogenic cell line, we did see some effect on MyoD (unpublished data). This may be a question of the cell system and the extent of overexpression, but we conclude that Pax3 and -7 normally act as activators of the myogenic program. The overexpression of *MyoD* that we observe in embryos that express the constitutively active PAX3-FKHR protein is consistent with this role. We show that Pax3, like Pax7 (Olguin and Olwin, 2004; Zammit et al., 2004), is rapidly down-regulated as satellite cells begin to differentiate. We observed the presence of Pax3/7-positive, MyoD-negative cells in older cultures in which differentiated myotubes were present. These cells probably correspond to reserve cells that will reconstitute the satellite cell pool, taking up a satellite cell position on newly formed fibers (Zammit et al., 2004).

Satellite cell proliferation

In satellite cultures from hindlimb muscles, we frequently saw colonies that expressed only *Pax7*, with no detectable expression of the *Pax3* reporter. In contrast, Pax7-positive satellite cells from the tibialis anterior muscle, also used in our experiments, have been reported to systematically activate *Pax3* in culture and it has been proposed that Pax3 is necessary for their proliferation (Conboy and Rando, 2002). This discrepancy may reflect problems with the Pax3 antibody used, which, in our experience, works poorly on cultured cells and may show cross-reactivity with Pax7. This is why we rely on different *Pax3* reporter lines, which consistently give reproducible results (Relaix et al., 2003, 2004, 2005). When we isolate satellite cells from $Pax3^{GFP/+}$ mice by flow cytometry after injury of the tibialis anterior, these cells remain Pax3 (GFP)-negative (Montarras et al., 2005), consistent with our ex vivo observations. Grafting experiments (Montarras et al., 2005), in addition to cell culture observations, show that satellite cells retain their Pax3-positive or -negative status independent of their environment.

In the *Pax7* mutant, satellite cells are present and can differentiate into skeletal muscle (Oustanina et al., 2004), but, as we show, there is a progressive loss of these cells during postnatal development. Their loss is equally evident in muscles where *Pax3* is also expressed in satellite cells. This indicates that there must be a function of Pax7 for which Pax3 cannot compensate. It had been suggested that there may be a proliferative defect in satellite cells in the absence of Pax7 (Oustanina et al., 2004). We examined the proliferation of satellite cell cultures from *Pax7* mutant mice and observed a 25–30% reduction compared with wild type. This was seen with Pax3-positive myogenic colonies from the diaphragm, as well as from Pax3-negative hindlimb muscles. All of the cells were still cycling, but the cell cycle was perturbed, with relatively more cells in S and G2 phases. We suggest that this effect may be attributable to the loss of cells in G1 caused by apoptosis (Abrams and White, 2004).

Pax7 and satellite cell survival

We show that apoptosis, marked by activated caspase-3, occurs in the skeletal muscle of the *Pax7* mutant. This is detectable immediately after birth and appears to occur in activated satellite cells, marked by desmin expression that would normally contribute to muscle growth. This suggests that self-renewal of satellite cells takes place via an activated cell state, as previously proposed (Zammit et al., 2004). We do not detect cell death in cultured satellite cells from *Pax7* mutant mice. This may be because the presence of serum in the medium provides some protection or because dying cells detach rapidly precluding detection with markers of apoptosis. Muscles, such as the diaphragm, in which *Pax3* is expressed, have more satellite cells, giving rise to myogenic colonies when satellite cell loss in vivo is still relatively minor (15–20%). However, by 15 d after birth only 5% of satellite cells remain in *Pax3*-expressing as well as *Pax3*-negative muscles. The difference between Pax3 and -7 is demonstrated by the flow cytometry experiments on satellite cells expressing dominant-negative constructs. Pax3DN at high levels does have some effect on the viability of the satellite cells in which it is expressed, but Pax7DN is much more potent, suggesting that the antiapoptotic targets of these two Pax factors are different in postnatal muscle. In the embryo, muscle progenitor cells in the somite are lost in the absence of Pax3, which is necessary for the survival of the hypaxial dermomyotome. Pax7 is not normally expressed in these cells, but can rescue this function (Relaix et al., 2004). The expression of a single *Pax3/7* gene in the muscle progenitor cells present in the somites of the cephalochordate Amphioxus (Holland et al., 1999) also suggests that Pax3 and -7 have similar functions in this embryonic context and that the distinct antiapoptotic activity of Pax7 evolved during vertebrate radiation, perhaps in response to the requirements of postnatal muscle growth and regeneration.

Members of the *Pax* gene family, which play key roles in the formation of other tissues (Tremblay and Gruss, 1994; Chi and Epstein, 2002), have been implicated in several functions discussed here for Pax3 and -7 in the skeletal muscle context. In addition to affecting cell fate choices, roles in proliferation and cell survival have been described. Notably, in the latter context, cells that do not express *Pax2* in the developing inner ear undergo apoptosis (Li et al., 2004) and apoptosis is seen in mesodermal cells, which would normally form the pronephros during kidney development, in the absence of Pax2 and -8, which are required for the emergence of the nephric cell lineage (Bouchard et al., 2002). Furthermore, Pax2 has been shown to prevent apoptosis in renal cell cultures (Torban et al., 2000; Cai et al., 2005) related to its function during later nephrogenesis (Porteous et al., 2000). In a screen of human cancer cell lines from a range of tissues, *PAX* gene expression was frequently observed, and repression of its expression led to ap-

optosis (Muratovska et al., 2003). The antiapoptotic function of Pax7, which we describe here in postnatal skeletal muscle, is therefore not unique to this Pax subgroup. A role for Pax proteins in assuring the survival of the progenitor cells in which they regulate cell fate determinants may be widespread. The fine-tuning of such an antiapoptotic function in progenitor cells in the adult may also be critical in regulating the maintenance of stem cell populations during tissue growth and regeneration.

Materials and methods

Mice

Pax3$^{nLacZ/+}$ (Relaix et al., 2004) and *Pax3*$^{IRESnLacZ/+}$ (Relaix et al., 2003) mice were genotyped on the basis of the "splotch" phenotype (Auerbach, 1954). *Pax7*$^{nLacZ/+}$ mice (Mansouri et al., 1996) were genotyped by PCR, using the following primers: DPax7Ex2A: CTTggCCAAggCCgggTCAATCAgCTTggTggg; RLacZ3: AAATTCAgACggCAAACgACTgTCCTggCC; and RPax7Ex3C: gATggACCCAgTCTCCTgATATCggCACAg. The wild-type band was amplified using DPax7Ex2A/RPax7Ex3C (800 bp), whereas the mutated allele was amplified using DPax7Ex2A/RLacZ3 (500 bp).

Cell culture

Cells were prepared from muscle tissue of mice at different time points after birth by enzymatic dissociation, as previously described (Pinset and Montarras, 1998; Montarras et al., 2000). Cells were plated on gelatin-coated dishes in a 1:1 mixture (vol/vol) of Ham's F12 and DME (GIBCO BRL) containing 20% (vol/vol) fetal calf serum (AbCys) and 2% (vol/vol) ultroser (Biosepra). This medium, which supports both the proliferation and differentiation of muscle cells (Montarras et al., 2000), was used in all experiments. To allow the formation of colonies of muscle cells, primary cultures were plated at a density of 100 and 200 cells/cm^{-2}. When plated under these conditions cultures were highly enriched in myogenic colonies. The number of mononucleated cells to be plated was determined by counting after labeling an aliquot with 5 μg/ml of the DNA dye bis-benzimide (Hoeschst).

Single fiber preparation and culture were performed according to Beauchamp et al. (2000).

Immunocytochemical analysis

Cells were treated as previously described (Montarras et al., 2000). In brief, after fixation with 4% (wt/vol) paraformaldehyde and permeabilization with 0.2% (wt/vol) Triton X-100, cells were incubated with antibodies diluted in PBS containing 0.2% (wt/vol) gelatin. All incubations were at room temperature. For immunofluorescence, cells were mounted in mowiol (Calbiochem) after the staining of DNA with 5 μg/ml bis-benzimide in the penultimate PBS wash.

Antibodies used were as follows: Myf5, rabbit polyclonal (Lindon et al., 2000), at a 1:1,000 dilution; MyoD, either a rabbit polyclonal (Santa Cruz Biotechnology, Inc.), at a 1:200 dilution, or a mouse monoclonal (clone 5.8A; DAKO), at a 1:200 dilution; troponin T, mouse monoclonal (clone JLT12, Sigma-Aldrich), at a 1:200 dilution; desmin, mouse monoclonal (clone D33; DakoCytomation), at a 1:200 dilution; laminin, rabbit polyclonal (Sigma-Aldrich), at a 1:200 dilution; M-cadherin, mouse monoclonal (clone 12G4; Nanotools GmBh), at a 1:200 dilution; antiactive caspase-3, rabbit polyclonal (BD Biosciences), at a 1:250 dilution; cyclin A, a rabbit polyclonal (gift from A. Fernandez and N. Lamb, Institut de Génétique Humaine, Montpellier, France), at a 1:200 dilution; Ki67, a mouse monoclonal (BD Biosciences), at a 1:100 dilution; Pax7, mouse monoclonal (Developmental Studies Hybridoma Bank), at a 1:100 dilution; Pax3, mouse monoclonal (provided by M. Bronner-Fraser, California Institute of Technology, Pasadena, CA), at a 1:100 dilution; β-gal, either rabbit polyclonal (Invitrogen), at a 1:4,000 dilution in cell culture experiments, or another rabbit polyclonal used on sections (provided by J.-F. Nicolas, Institut Pasteur, Paris, France), at a 1:500 dilution, or mouse monoclonal (clone Gal13; DakoCytomation), at a 1:100 dilution. Secondary antibodies were coupled to a fluorochrome, either Alexa 488 or 594 (Invitrogen), at a 1:250 dilution.

For X-Gal (Roche) staining, single fibers were fixed for 30 min with 4% paraformaldehyde in PBS, on ice. Fibers were rinsed twice with PBS, and then stained with X-Gal, using 0.4 mg/ml X-Gal in 2 mM $MgCl_2$, 0.02% NP-40, 0.1 M PBS, pH 7.5, 20 mM $K_4Fe(CN)_6$, and 20 mM $K_3Fe(CN)_6$ for 4–16 h at 37°C, with shaking. Fibers were rinsed in PBS, postfixed overnight in 4% paraformaldehyde, and mounted after washing in PBS-buffered mowiol with DAPI. Similar conditions were used for X-Gal staining of sections. X-Gal staining of cells was performed after a 5-min fixation in 4% paraformaldehyde in the same solution used for fibers, but without NP-40.

Images of cultured cells and sections were acquired using an Axiophot or an Apotome equipped with an Axiocam camera and Axiovision software (Carl Zeiss MicroImaging, Inc.). Neofluar lenses, 40×, NA 0.75, and 20×, NA 0.50, were used. Images were optimized globally for contrast and brightness and assembled using Photoshop CS software (Adobe).

Analysis of extracts by semiquantitative RT-PCR

RNA extracts were prepared from tibialis anterior and diaphragm muscles of 8-wk-old mice, using TRIzol (Invitrogen). Reverse transcripts were generated using Power Script reverse transcriptase (BD Biosciences). The primers for Pax3 were DPax3-740 (TGCCCTCAGTGAGTTCTATCAGC) and RPax3-1100 (GCTAAACCAGACCTGCACTCGGGC), which generate a 360-bp PCR fragment. The primers for Pax7 were Dpax7-140 (TGGAAGTGTCCACCCCTCTTGGC) and RPax7-650 (ATCCAGACGGTTCCCTTTGTCGCC), which generate a 510-bp PCR fragment. Three other primer pairs were used and gave similar results. PCR products were separated on 1.5% agarose gels, using standard techniques, and revealed by UV light (Image Master CVS; GE Healthcare).

Analysis of extracts by Western blotting

Protein extracts were prepared and analyzed by Western blotting, as previously described (Lindon et al., 2000). The antitubulin antibody (clone 5HI; BD Biosciences) was used at a 1:2,000 dilution and the Pax3 antibody at a 1:400 dilution.

Preparation of adenoviral vectors and cell infection

Adenoviral vectors were generated using standard molecular biology techniques. In brief, dominant-negative Pax3 and -7 constructs were made by fusing in-frame sequences encoding the *D. melanogaster* engrailed repression domain (298 amino acids; Han and Manley, 1993) to the first 340 amino acids of murine Pax7 (to generate the Pax7DN construct) or the first 374 amino acids of Pax3 (to generate the Pax3DN construct). Dominant-negative activity of Pax7DN and -3DN was verified by cotransfection in 293 cells with a plasmid containing polymerized Pax3/7 binding sites (Epstein et al., 1996) in front of a thymidine kinase (Herpes virus) minimal promoter followed by a *LacZ* reporter gene. Before their introduction into the adenovector, Pax7DN and -3DN were cloned into the Adtrack-CMV shuttle vector and recombinant adenoviruses with a GFP reporter sequence were generated as described previously (He et al., 1996). High-titer viral stocks were prepared by repeated infection into the packaging cell line 293T. Viruses were purified by CsCl banding followed by passage through a 2.5-ml Sephadex G25 column (GE Healthcare) for desalting and stored in aliquots at −80°C. The titer of each preparation was determined after infection of 293 cells by limiting dilution of virus and detection of GFP expression.

Primary cultures of muscle cells were infected 3 d after plating on 35-mm dishes. The medium was removed, but a film of medium (0.3 ml) was left to prevent drying and virus was added to each dish for 30 min at 37°C at a multiplicity of infection of 5–30 (Results). 1.5 ml of medium was added to the cells that were analyzed 3 d later by immunofluorescence and flow cytometry.

Flow cytometry

Flow cytometric analysis and characterization of (Pax3) GFP-positive cells present in skeletal muscles of adult *Pax3*$^{GFP/+}$ mice have permitted us to define parameters for isolating adult muscle progenitor cells both from Pax3-expressing muscles (e.g., diaphragm) and non-Pax3–expressing muscles (e.g., lower hindlimb muscles; Montarras et al., 2005). In the case of the latter, this was on the basis of the size and granularity of CD34$^+$ cells. GFP-positive muscle progenitor cells from diaphragm muscle and GFP-negative muscle progenitor cells from lower hindlimb muscles, isolated by flow cytometry, were maintained in culture for 6 d as proliferating cells before a second flow cytometric analysis for detecting the presence of GFP-positive cells.

Flow cytometry analysis was performed with an LSR analyzer (Becton Dickinson) and cell sorting was performed with a Moflo (Cytomation, Inc.). Antibody against CD34 was a mouse monoclonal clone (clone Ram [34]; Becton Dickinson), coupled to biotin, and detected with streptavidin coupled to phycoerythrin. Cell death was measured by PI staining of

adenovirus-infected cells, which were identified by GFP fluorescence. Cells were trypsinized and pooled with the culture supernatants before addition of 1 μg/ml PI and flow cytometry. Each determination was from triplicate plates.

Online supplemental material

Fig. S1 presents an analysis of satellite cells present on single fibers isolated from the extensor digitorum longus hindlimb muscle of Pax7+/− and Pax7−/− mice at P10. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200508044/DC1.

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In the original paper, an incorrect kiloDalton measurement appeared in the "Pax3" lane of Fig. 1 D'. The corrected image is shown below.

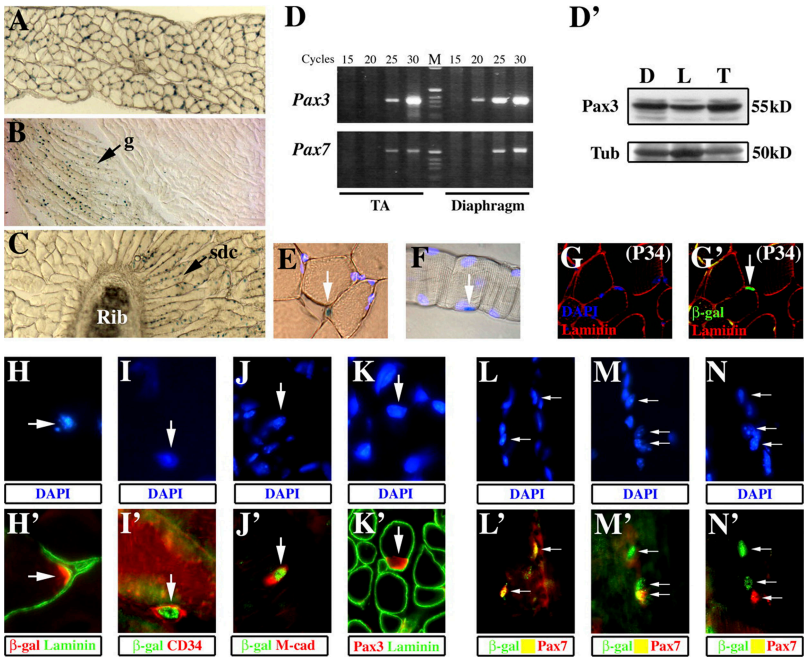

Figure 1. **Pax3 expression in muscle satellite cells.** (A–C) Expression of Pax3 in different muscles from 3-wk-old *Pax3*$^{IRESnLacZ/+}$ mice, revealed by X-Gal staining. (A) Diaphragm muscle; (B) hindlimb muscles, (g) gracilis muscle; (C) trunk muscles, (sdc) serratus dorsalis caudalis. (D) Semiquantitative RT-PCR of Pax3 and -7 transcripts in adult tibialis anterior (TA) and diaphragm muscle. The number of cycles is indicated on the top of each lane. M, molecular weight markers (see Materials and methods). (D') Pax3 protein is detected by Western blot in protein extracts from different muscles (D, diaphragm; L, hindlimb; T, ventral trunk muscles) in 3-wk-old mice. Tubulin (Tub) expression is shown as a loading control. (E and F) *Pax3*$^{IRESnLacZ/+}$ is expressed in a subset of diaphragm muscle nuclei (arrows) from 3-wk-old mice, as revealed by X-Gal and DAPI staining of a transverse section (E) or isolated fiber (F). (G and G') Detection of Pax3/7-dependent β-gal expression in transverse sections of adult diaphragm muscle from the transgenic line *P34*, which reports Pax3/7 transcriptional activity. (G) Immunodetection of laminin (red) and DAPI (blue) staining. (G') Coimmunodetection of laminin (red) and β-gal (green). (H–J') Coimmunodetection of β-gal–positive cells in the diaphragm muscle of 3-wk-old *Pax3*$^{IRESnLacZ/+}$ mice with laminin (H', green), CD34 (I', red), or M-cadherin (J', red). As indicated in the figure, β-gal is shown in red in H' and in green in I' and J'. Corresponding DAPI staining is shown (H, I, and J) for each panel. Arrows indicate the labeled satellite cell nuclei. (K) Coimmunodetection of Pax3 (red) and laminin (green, K'). Corresponding DAPI staining is shown, with the labeled nucleus indicated by an arrow (K). (L–N') Coimmunohistochemistry on diaphragm muscle from 3-wk-old *Pax3*$^{IRESnLacZ/+}$ mice for Pax7 (red) or β-gal (green). (L–N) Corresponding DAPI staining is presented with labeled nuclei indicated by arrows for each panel.