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Compensatory growth and regeneration of skeletal muscle is dependent on the resident stem cell population, satellite cells (SCs). Self-renewal and maintenance of the SC niche is coordinated by the paired-box transcription factor Pax7, and yet continued expression of this protein inhibits the myoblast differentiation program. As such, the reduction or removal of Pax7 may denote a key prerequisite for SCs to abandon self-renewal and acquire differentiation competence. Here, we identify caspase 3 cleavage inactivation of Pax7 as a crucial step for terminating the self-renewal process. Inhibition of caspase 3 results in elevated Pax7 protein and SC self-renewal, whereas caspase activation leads to Pax7 cleavage and initiation of the myogenic differentiation program. Moreover, in vivo inhibition of caspase 3 activity leads to a profound disruption in skeletal muscle regeneration with an accumulation of SCs within the niche. We have also noted that casein kinase 2 (CK2)-directed phosphorylation of Pax7 attenuates caspase-directed cleavage. Together, these results demonstrate that SC fate is dependent on opposing posttranslational modifications of the Pax7 protein.

caspase | satellite cells | Pax7 | casein kinase 2 | self-renewal

Postnatal skeletal muscle retains a robust capacity for compensatory growth and regeneration in response to injury and disease. This physiologic adaptation of skeletal muscle is largely dependent on a resident stem cell population, termed satellite cells (SCs), which exist in a well-defined position juxtaposed to the myofiber (1, 2). In resting skeletal muscle, SCs have low homeostatic turnover and are maintained in a nonproliferative, quiescent state (3). Exogenous activation of SCs may lead to a process of self-renewal, which maintains the stem cell niche, or these cells may commit to the myogenic differentiation program, fusing with existing myofibers to accomplish muscle growth and repair (4, 5). The factors that direct an activated SC to self-renew versus initiate myogenesis are not fully defined, and yet a reasonable hypothesis would envision that acquisition of these divergent cell fates requires a mutually exclusive molecular milieu.

The establishment of the SC lineage is controlled by expression of Pax7, a paired-box transcription factor (6). Targeted deletion of Pax7 results in an almost complete loss of SCs, with a striking deficit in muscle regenerative capacity (6, 7). Although Pax7 is required to instruct the myogenic fate of SCs, continued expression of this protein inhibits the myoblast differentiation program (8, 9). Pax7 has also been reported to regulate distinct panels of genes that promote proliferation and antagonize myogenic differentiation, a function that is consistent with a SC-enriched function for this transcription factor (10). As such, the reduction or removal of Pax7 may denote a key prerequisite for SCs to abandon self-renewal and acquire differentiation competence. How the activated SC retains or eliminates Pax7 protein activity remains unknown.

A probable mechanism that may influence Pax7 protein activity (and by extension SC fate choice) is the restricted deployment of directed proteolysis. The caspase 3 protease, originally identified as the central effector of multiple cell death pathways, has been demonstrated to control cell fate determination independent of inducing cell death (11). Transient activation of caspase 3 is essential for inducing the differentiation program across a broad range of lineage restricted progenitor cells (12). In addition, caspase 3 has been observed to limit self-renewal of embryonic stem cells through direct proteolysis and inactivation of the key pluripotency factor Nanog (13). In skeletal muscle myoblasts, caspase 3 directs a differentiation specific gene expression program by activating the caspase-responsive DNase CAD. Once active, CAD reprograms the genome through targeted DNA damage/strand break events (14).

Previous observations have suggested Pax7 is subjected to caspase/proteasome-dependent regulation (15). Here, we provide evidence that Pax7 is a direct target of caspase 3 at aspartic acid residues D187 and D208. Furthermore, we examined whether limitation of SC self-renewal was dependent on caspase 3-targeted cleavage of Pax7. We noted that loss or inhibition of caspase 3 activity leads to the accumulation of Pax7 protein, expansion of the SC self-renewing compartment, and impeded muscle regeneration. Conversely, small molecule stimulation of caspase 3 depletes Pax7 protein and results in a down-regulation of Pax7 target genes. Moreover, the ability of caspase 3 to target Pax7 is subject to an additional regulatory control via CK2. Here, quiescent and self-renewing SCs maintain elevated CK2 activity, which phosphorylates Pax7 and prevents caspase-mediated degradation of Pax7. Together, these results demonstrate that caspase cleavage of Pax7 is an early and essential step to limit SC self-renewal, thereby establishing a cellular environment that is permissive for muscle differentiation.

Results and Discussion

Inhibition of Caspase 3 Promotes Satellite Cell Self-Renewal. To address the role of caspase 3 activity in SC function, we isolated single muscle fibers from juvenile mice and performed immunofluorescence

Significance

Satellite cells form the resident stem cell population in adult skeletal muscle, providing the foundation for postnatal growth and repair of this tissue. Satellite cell self-renewal is maintained by the paired-box transcription factor Pax7, suggesting that this protein is a key determinant in managing cell fate decisions for this niche. Here, we show activation of caspase 3 protease limits satellite cell self-renewal, through targeted cleavage and inactivation of Pax7 in a casein kinase (CK2)-dependent manner. Temporal regulation of caspase 3 activity may offer a robust mechanism to control the satellite cell compartment and enhance skeletal muscle regeneration.

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for active-caspase 3. Isolated and cultured myofibers provide an amenable model that accurately reconstructs the transition of SCs from quiescence to activation/early cell divisions, with well-defined temporal kinetics (T = 0 postculture, quiescence; T = 48 h, early activation; and T = 72 h, full activation) (9). No caspase 3 activity was observed immediately following fiber isolation; however, we observed focal caspase 3 activity in both early and late activated SCs (Fig. 1*A*). This temporal activity pattern is reminiscent of caspase 3 activation in other nondeath settings, which include differentiation of committed skeletal myoblasts and stem cells from a variety of lineages, including neuronal and hematopoietic progenitors (12, 13, 16, 17).

To examine the function of caspase 3 in SC activation and self-renewal, isolated myofibers were incubated with a cell permeable caspase 3 specific peptide inhibitor (20 μM z.DEVD.fmk) and assessed for markers of self-renewal (Pax7) versus commitment to differentiation (MyoD). Sustained Pax7 expression in the absence of myogenic markers is indicative of the self-renewing population (8). Alternatively, SCs with down-regulation of Pax7 and up-regulation of the transcription factors MyoD and myogenin are considered to be a cell population committed to differentiation. SCs expressing both Pax7 and MyoD are understood to be committed cells that remain in a proliferative state. Inactivation of caspase 3 resulted in a significant increase in the number of $Pax7^+/MyoD^-$ SCs on fibers at 3 d postisolation (34.85 ± 3.13% DEVD vs. 15.74 ± 4.61% DMSO; $P < 0.05$) with a corresponding decrease in the number of differentiating cells ($Pax7^-/MyoD^+$; 36.71 ± 3.79% DEVD vs. 54.67 ± 3.85% DMSO; Fig. 1 *B* and *C*). The number of SCs undergoing self-renewal was only found to be significantly increased at 3 and 4 d (38.69 ± 3.77% DEVD vs. 21.93 ± 2.69% DMSO) postisolation (Fig. 1*D*). We saw no significant difference in the number of SCs undergoing self-renewal at 2 d postisolation (Fig. 1*D*), suggesting caspases affect SC fate after the initial division occurs. To confirm this, we used the Myf5-Cre/Rosa-YFP lineage-tracing mouse, which irreversibly labels SCs that have at one time expressed the myogenic transcription factor Myf5. This strategy has been used to identify a population of SCs that are YFP-negative and can undergo a symmetric (producing two YFP- cells) or asymmetric division (producing one YFP^- and one YFP^+ cell) (4). We observed no significant change in these two populations on fibers treated with the caspase 3 inhibitor; however, we did see a decrease in the number of differentiating SCs ($YFP^+/MyoD^-$; 5.30 ± 1.55% in DEVD vs. 12.78 ± 3.44% DMSO) (Fig. S1 *A* and *B*). Importantly, the total number of SCs per fiber did not change with or without caspase 3 inhibition at any of the time points examined (Fig. 1*D* and Fig. S1*B*), confirming that caspase activation in the activated SC population impacts the self-renewal process post Myf5 induction, rather than influencing cell death/cell survival per se.

Caspase 3 Activity Is Required for Skeletal Muscle Regeneration. Muscle regeneration requires proper activation, proliferation, and differentiation of SCs to restore muscle function (18). To determine whether caspase 3 is important to this process, we injected the tibialis anterior (TA) muscle with an adenovirus containing a p35-IRES-GFP vector (or GFP alone as a control). The baculovirus p35 protein is a potent biologic inhibitor of caspase 3/7 activity, which we have previously used for in vivo characterization of caspase activity in the heart (19). Infection with the p35-containing virus (Adp35) at 2 d post-cardiotoxin (CTX) injury resulted in a significant reduction in the minimal Feret's diameter (with the majority of fibers measuring between 10–20 μm; 30.03 ± 2.27%) compared with control (AdGFP)-treated muscle (17.33 ± 4.52% of fibers between 10–20 μm) at 14 d postinjury (Fig. 1*E* and Fig. S1*C*). This was accompanied by a dramatic increase in the number of SCs in the niche (4.49 ± 0.37 GFP^+ cells/field Adp35 vs. 1.7 ± 0.26 GFP^+ cells/field AdGFP; Fig. 1 *F* and *G*), disorganized muscle ultrastructure, and ineffective repair (Fig. S1*D*). In addition, we also examined protein content in TA muscles at 3 and 7 d postinjury to assess the Pax7 protein levels as a proxy for monitoring the SC self-renewal response. We noted a robust increase in Pax7 protein in Adp35-infected muscle at day 7 compared with uninjured control, with a consistent increase in Pax7 protein in the Adp35-treated muscle compared with AdGFP control muscles at day 7 (Fig. 1 *H* and *I*).

Taken together with the isolated myofiber experiments, these observations are consistent with the hypothesis that caspase 3 activity may act as a critical step in the regulation of SC-mediated skeletal muscle repair. At this juncture, it is salient to consider that the in vivo experimental approach (Adp35 infection) may also result in the loss of caspase 3 activity in transduced non-muscle cell types, a condition that could separately influence the outcome of the regenerative process. For example, caspase 3 activity is an essential requirement for both macrophage and T-cell maturation/differentiation (12), and these immune cell types have been demonstrated to infiltrate damaged muscle tissue and enhance regeneration (20). Therefore, the impaired regeneration that follows Adp35 infection may derive from a loss of caspase function in both SCs and critical immune cell subtypes. As such, it will be of considerable interest to parse caspase 3 function within each tissue compartment and determine the relative contribution of each protease signal to the overall repair process.

Pax7 Protein Is Degraded via Caspase 3-Directed Cleavage. The increased numbers of self-renewing SCs following caspase 3 inhibition is consistent with the premise that this protease may act to cleave and inactivate the factor(s) that govern the self-renewal process. Pax7 is a logical target in this regard, because (*i*) the protein content has been shown to decline faster than message content in differentiating myoblasts (21); and (*ii*) simple inspection of the Pax7 protein reveals conserved caspase 3 cleavage sites (Fig. S2*A*). To address a prospective posttranslational regulation of Pax7, we used primary derived myoblasts as a reliable proxy for SCs (which can be harvested in sufficient quantity to conduct accurate biochemical analysis) (22). Pax7 protein is rapidly lost following low serum induction of differentiation, and yet peptide inhibition of caspase 3 maintains elevated Pax7 protein for a 24 h period (Fig. 2*A*). To determine whether caspase 3 directly targets the Pax7 protein, we conducted an in vitro cleavage assay using recombinant active-caspase 3 and recombinant Pax7 proteins. Addition of active-caspase 3 was sufficient to induce an immune-reactive Pax7 cleavage product of ∼40 kDa (ΔPax7; Fig. 2*B*). This cleavage event was blocked by addition of the caspase 3 peptide inhibitor, confirming the specificity of Pax7 as a caspase 3 substrate.

Next, we sought to identify the caspase 3 cleavage site(s) within Pax7 and whether the cleavage event is consistent with producing a loss of function for Pax7. Importantly, we observed both a 40-kDa band on a silver-stained gel, which corresponded to the immune-reactive fragment identified via Western blot analysis, as well as a 20-kDa fragment, as predicted, upon addition of active-caspase 3 (Fig. 2*C*, arrows). These two bands were excised and processed for liquid chromatography-tandem mass spectrometry (LC-MS/MS), identifying peptides that clustered to either the N terminus (and within the paired domain) in the case of the 20-kDa fragment or the C terminus (including the homeodomain) for the 40-kDa fragment (Fig. S2*B*). This analysis suggested a physiologic cleavage event that parsed these two regions. To identify the precise caspase 3 cleavage site (an exposed C-terminal aspartic acid), we subjected aliquots of the whole cleavage reaction to enzymatic digestion using three different endoproteases (Chymotrypsin, Trypsin, GluC). Analysis of the Pax7 cleavage reaction products revealed a number of peptides produced following cleavage at D187 that could not be attributed to cleavage via the selected diagnostic endoproteases (Table S1).

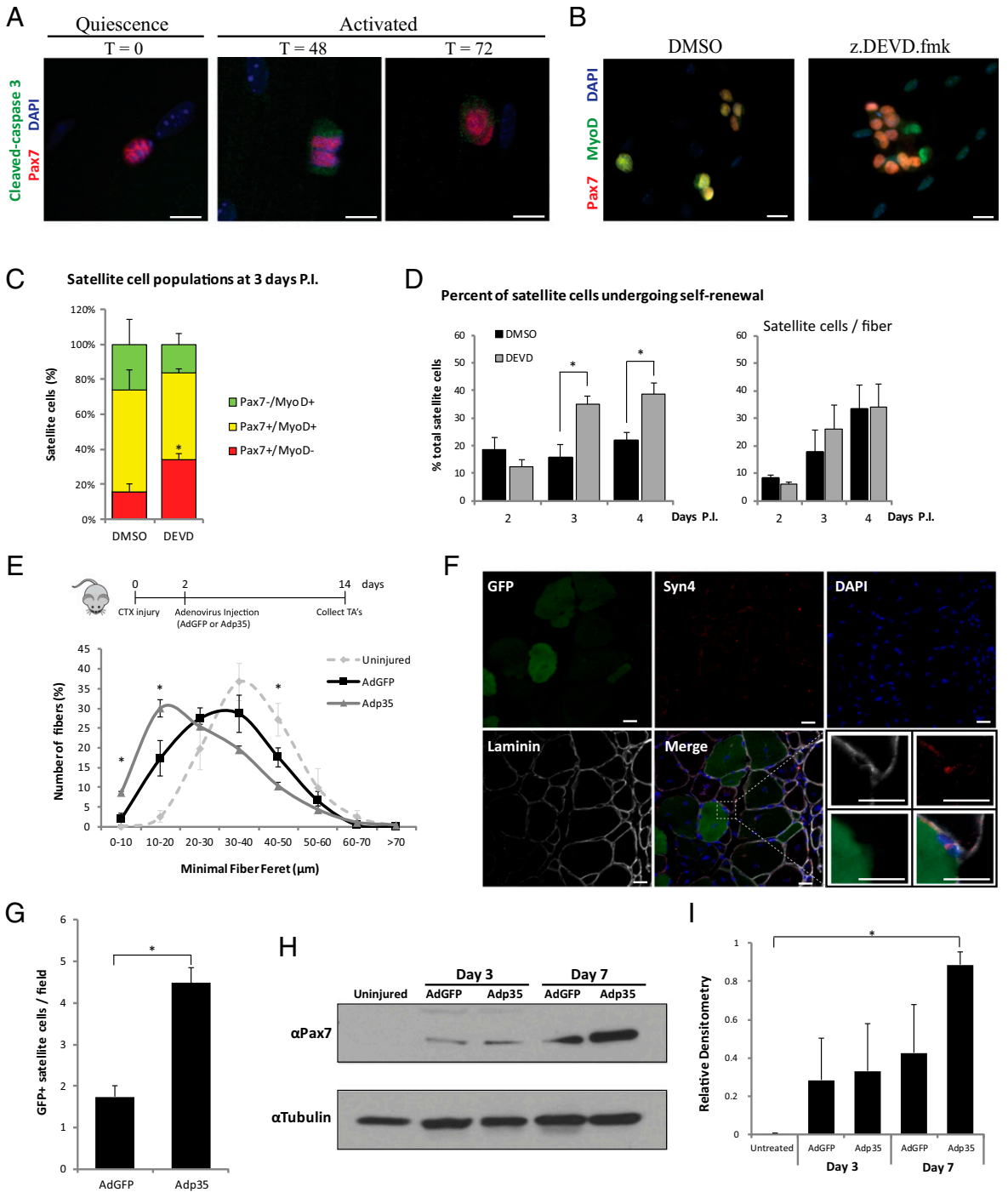

Fig. 1. Caspase 3 activity is required during early satellite cell fate decisions. (*A*) Fibers were stained for cleaved-caspase 3 (green), Pax7 (red), and DAPI (blue) at 48 and 72 h following isolation. Active caspase 3 was observed at a low frequency in fiber-associated SCs at 48 and 72 h but not at time 0 in quiescent SCs. (*B*) Fibers were cultured in z.DEVD.fmk (DEVD; 20 μM) (*Right*) or DMSO (control) (*Left*) for 3 d and stained for Pax7 (red), MyoD (green), and DAPI (blue). (Scale bars: 10 μm.) (*C*) Quantification of the number of SCs expressing each marker at 3 d postinjury (P.I.) expressed as a percentage of the total number of SCs (>30 fibers per treatment per mouse; $n = 4$ mice). (*D*) Quantification of the number of self-renewing SCs ($Pax7^+/MyoD^-$) at 2, 3, and 4 d postinjury (*Left*) and the total number of SCs per fiber (>30 fibers per treatment per time point per mouse; $n = 4$ mice) (*Right*). The percentage of SCs undergoing self-renewal was significantly increase in DEVD-treated fibers compared with control at 3 and 4 d postinjury (*$P < 0.05$). (*E*) Mouse TA muscle was injured with CTX and injected with AdGFP or Adp35 at 2 d postinjury. TAs were embedded and 10-μm-thick frozen sections were stained for immunofluorescence (IF) analysis (Fig. S1C), the minimal fiber Feret's diameter was calculated for GFP^+ fibers, and the frequency of fibers in each bin size was expressed as a percentage of total number of fibers counted (>100 fibers/mouse; $n = 3$ mice/condition; *$P < 0.05$ AdGFP vs. Adp35). (*F*) Representative IF of muscle sections stained for GFP (green), Syn4 (red), laminin (white), and DAPI (blue). (Scale bars: 100 μm.) (*G*) The number of $GFP^+/Syn4^+$ SCs were counted per field indicating an increase in the number of infected SCs in Adp35 infected muscle compared with AdGFP control (*$P < 0.05$; $n = 3$). (*H*) Western blot of whole TA lysate isolated from 3 and 7 d regenerating muscle injected with AdGFP or Adp35 at 1 d postinjury. (*I*) Densitometry results were quantitated from the Western blot (*G*) and averaged from three separate experiments. Pax7 levels were only found significantly increased in day 7 Adp35 mice compared with uninjured control (ANOVA, $P < 0.05$). Error bars ± SEM.

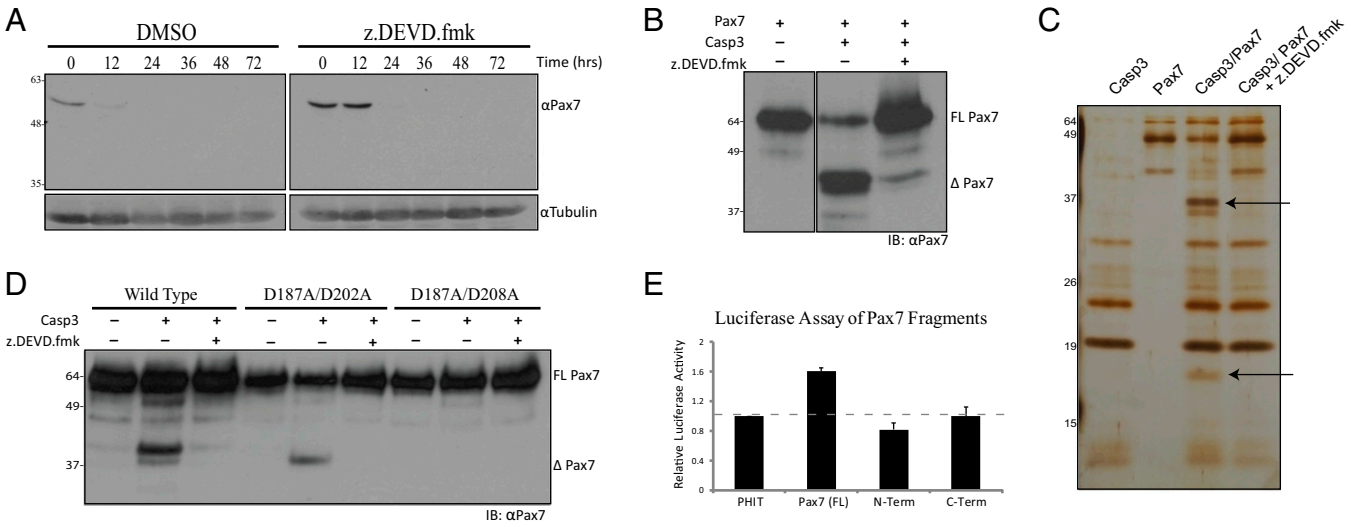

Fig. 2. Pax7 protein is cleaved by caspase 3 at a cryptic cleavage site. (*A*) Differentiation time course of primary myoblasts treated with the caspase 3 peptide inhibitor z.DEVD.fmk (20 μM) or DMSO control. Lysates were probed for αPax7 (upper blots) and α-alpha-tubulin (lower blots; loading control). (*B*) Recombinant Pax7 protein and recombinant active caspase 3 were incubated for 3 h in standard cleavage assay conditions containing either DMSO or z.DEVD.fmk (20 μM) as indicated. Reactions were then subjected to SDS/PAGE and Western blot analysis using αPax7 indicating a caspase 3-specific cleavage event (ΔPax7). (*C*) Pax7 was subjected to caspase 3 cleavage as in *B*, followed by SDS/PAGE and silver stained using silver nitrate. Arrows indicate the Pax7 cleavage fragments (corresponding to ∼40 and ∼20 kDa). (*D*) Recombinant Pax7 protein containing aspartic acid to alanine point mutations at site D187 and D202 or D187 and D208 were subjected to caspase 3 cleavage, followed by Western blot analysis. Pax7 containing D187A/D202A was cleaved; however, D187A/D208A was completely blocked compared with wild type. (*E*) Luciferase Assay performed in COS cells cotransfected with a Pax7 containing plasmid and a luciferase reporter plasmid containing the *Myf5* promoter. Error bars ± SEM; $n = 3$.

D187 mapped to a region that was not detected in either the 40-kDa or the 20-kDa fragments and was localized between the paired and the homeodomain in which the respective peptides clustered (Fig. S2*B*, underlined).

Interestingly, a recombinant Pax7 protein with an alanine substitution at D187 (D187A) displayed partial cleavage via caspase 3, suggesting that Pax7 contained additional cleavage site(s) (Fig. S2*C*). Examination of the Pax7 amino acid sequence indicated two additional aspartic acid residues (D202 and D208) that retain prototypical caspase 3 recognition sites (23). To test the caspase targeting of these residues, we generated the single aspartic acid to alanine Pax7 mutations (D202A and D208A), as well as the relevant Pax7 double mutants (D187A/D202A and D187A/D208A) for use in the caspase cleavage assay. Recombinant Pax7 containing the D187A/D202A double mutant, or any of the single mutants, did not provide effective blockade of caspase directed cleavage (Fig. 2*D* and Fig. S2*C*). However, Pax7 protein containing the dual D187A/D208A aspartic acid to alanine mutations was completely protected from caspase 3 cleavage (Fig. 2*D*), establishing these aspartic acids as de facto caspase target sites. Finally, we examined whether caspase cleavage of Pax7 resulted in loss of function for the resulting Pax7 fragments. COS cells were cotransfected with either full-length [Pax7 (FL)] or truncated Pax7 constructs and a Pax7-responsive promoter linked to a luciferase reporter (*Myf5*-57.5). COS cells transfected with Pax7 (FL) induced expression of the *Myf5*-linked reporter, whereas COS cells expressing either caspase-generated Pax7 fragment [amino acids 1–208 (N terminus) or 209–503 (C terminus)] did not activate the reporter (Fig. 2*E*). These results suggest that caspase cleavage of Pax7 results in the generation of nonfunctional Pax7 fragments.

Exogenous Activation of Caspase 3 Results in Loss of Pax7 and SC Differentiation. The combination of caspase inhibitor experiments and in vitro cleavage assays indicate that caspase activity is necessary to inactivate Pax7 and limit SC self-renewal. To determine whether caspase 3 activation is a dominant (sufficient) signal in determining SC fate, we used a specific small molecule activator of caspase 3, termed procaspase 3-activating compound 1 (PAC-1), to engage protease activity. PAC-1 is a potent and specific procaspase 3-targeted molecule that chelates the inhibitory zinc ions from the catalytic site of the enzyme, leading to autoactivation (24). We have successfully used PAC-1 to produce nonlethal levels of caspase 3 activation, confirming a role for this protease in the induction of cardiac hypertrophy (19). Isolated myofibers treated for 3 h with PAC-1 (50 μM) displayed a significant increase in the number of differentiating SCs (56.41 ± 1.00% $Pax7^{-}$/$Myogenin^{+}$) compared with control (40.81 ± 1.39%), suggesting caspase activation is sufficient to induce differentiation of SCs (Fig. 3 *A* and *B*). We also noted PAC-1 treatment caused a reduction in the number of Pax7 positive cells compared with control treated myofibers ($Pax7^{+}$/$syndecan4^{+}$ cells; Fig. S3 *A* and *B*). To independently confirm these observations, we also tested the capacity of PAC-1 to alter Pax7 levels and the commitment of FACS isolated SCs. Western blot analysis revealed that SCs treated with 25 or 50 μM PAC-1 (a nonlethal concentration in myoblast cultures; Fig. S3*C*) displayed a dramatic reduction in Pax7 protein in growth media conditions, a loss that was comparable to cells exposed to low serum induction of differentiation (Fig. 3 *C* and *D*). Moreover, the PAC-1–induced loss of Pax7 was concurrent to a reduction in the expression of the Pax7 target gene, *Myf5* (Fig. 3*E*). Collectively, these results support the hypothesis that caspase 3 is necessary and sufficient to limit SC self-renewal and establish the molecular conditions that are conducive for differentiation.

Phosphorylation of Pax7 via CK2 Prevents Caspase 3 Cleavage and Promotes SC Self-Renewal. The standalone capacity of caspase 3 to alter self-renewal implies that the SC may have evolved or coopted a mechanism(s) to restrain the protease targeting of Pax7. Interestingly, casein kinase 2 (CK2) has been shown to produce a steric inhibition on caspase 3 cleavage events via phosphorylation of serine residues that reside in close proximity to the caspase 3 cleavage site (25). Indeed, comprehensive proteomic analysis has

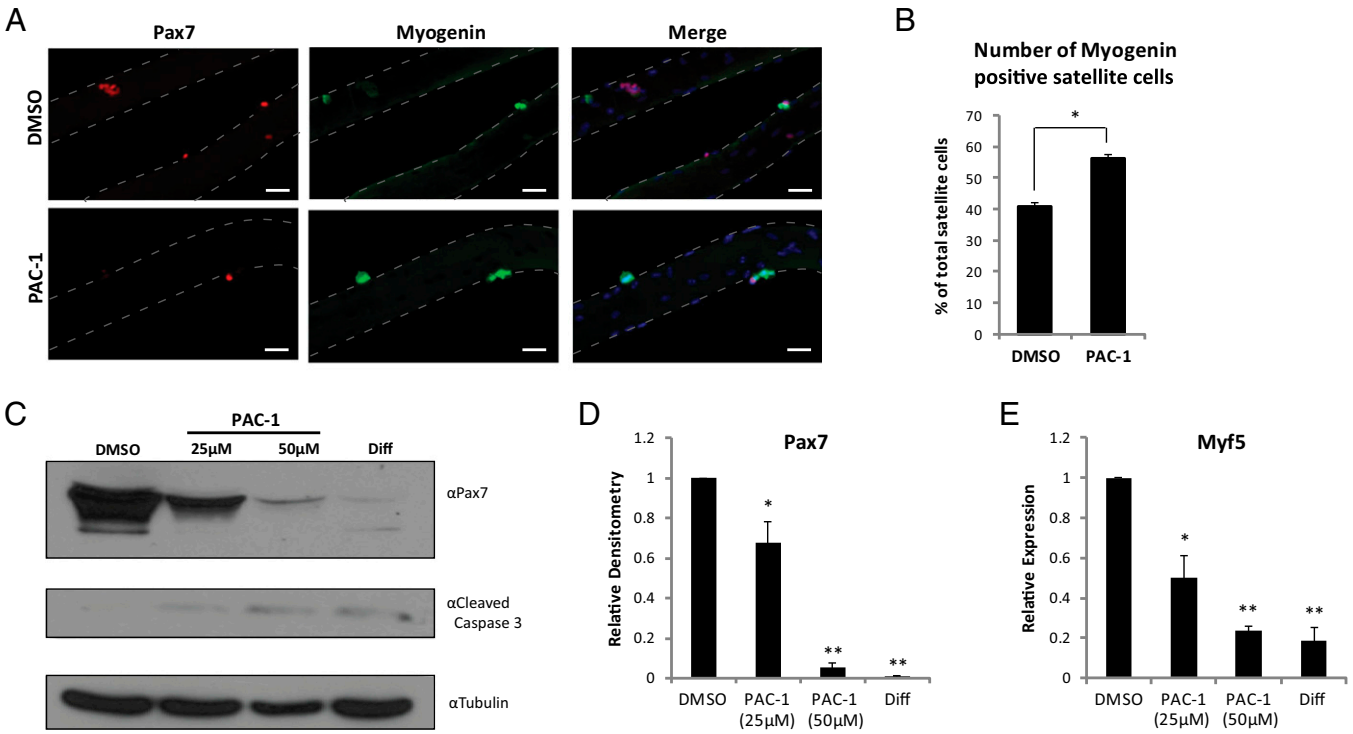

Fig. 3. Small molecule (PAC-1) activation of caspase 3 induces cleavage of Pax7 and loss of SC self-renewal. (*A*) Single fibers were treated with either 50 μM PAC-1 or DMSO 60 h postisolation for 3 h, washed with fresh fiber media, and left for an additional 9 h. At 72 h postisolation, fibers were fixed and stained for Pax7 (red), Myogenin (green), and DAPI (blue). (Scale bars: 20 μm.) (*B*) The number of SCs expressing each marker was counted and the number of myogenin positive SCs (Pax7^{-}/Myogenin^{+}) was expressed as a percentage of total number of SCs (>30 fibers per treatment per mouse; $n = 3$ mice; $^{*}P < 0.05$). (*C*) Western blot analysis of primary myoblasts treated with PAC-1 at the indicated concentrations for 24 h or differentiation media (Diff). Lysates were probed with αPax7, αCleaved-Caspase3, and αTubulin. (*D*) Densitometry analysis of Pax7 protein levels normalized to tubulin (loading control) shows a decrease similar to that seen in differentiation conditions ($n = 3$). (*E*) Primary myoblasts treated with 25 μM or 50 μM PAC-1, DMSO (control) or differentiation media (diff) for 24 h. RT-quantitative PCR (RT-qPCR) analysis using primers to *Myf5* show a decrease in *Myf5* expression in all treatment groups compared with DMSO control ($n = 3$; $^{*}P < 0.05$; $^{**}P < 0.005$). Error bars ± SEM.

established that caspase 3 cleavage sites and CK2 phosphorylation sites strongly overlap (25, 26). Here, we show that the constitutively active CK2 is present in all activated SCs and the majority (73.0 ± 3.5%) of quiescent SCs (Fig. 4*A*). To address the physiological relevance of CK2 activity and whether this kinase modulates SC self-renewal, we tested the effect of the CK2 specific inhibitor tetrabromobenzotriazole (TBBt) on single fiber cultures. We observed an increase in the differentiation potential of SCs when treated with TBBt (33.55 ± 4.89%) compared with vehicle control (DMSO; 23.68 ± 5.44%), as indicated by the increase in Pax7^{-}/MyoD^{+} SCs at 3 d postisolation (Fig. 4 *B* and *C*). There was no significant difference in the number of SCs per fiber, indicating that inhibition of CK2 activity does not impair cell survival.

The Pax7 amino acid sequence contains two serine residues (S201 and S205), which are consistent with a CK2 consensus sequence and in close proximity to the caspase-targeted aspartic acid residue at position D208 (Fig. 4*D*). To confirm Pax7 is a direct target of CK2, we performed an in vitro kinase assay (Fig. 4*E*), observing phosphorylation of Pax7 protein that was completely blocked by addition of the CK2 inhibitor TBBt (Fig. S4*A*). Point mutations of Pax7 at S201 or S205 revealed that only S201 in recombinant Pax7 protein was directly phosphorylated by CK2 in vitro (Fig. 4*F*). The specificity of this phosphorylation event was confirmed via MS peptide mapping of Pax7 protein following incubation with CK2 (Fig. S4*B*).

Next, we tested whether prior CK2 phosphorylation of recombinant Pax7 was sufficient to inhibit cleavage via active caspase 3. Interestingly, CK2 phosphorylation of Pax7 resulted in the generation of a single cleavage fragment of wild-type Pax7 (Fig. 4*G*, †), compared with the two cleavage products generated from unphosphorylated Pax7 protein (Fig. 4*G*, † and ‡). This result suggested that CK2 mediated phosphorylation of Pax7 S201 may shield only one of the caspase-directed cleavage sites. To identify the CK2 protected caspase cleavage site, we tested the D187A and D208A single Pax7 mutants in the same serial kinase/cleavage assay used above. CK2 phosphorylation followed by caspase 3 incubation of the D187A mutant displayed a similar cleavage pattern to uncleaved Pax7 (Fig. 4*G*), whereas the caspase cleavage products generated from the D208A mutant were unaffected by a prior CK2-mediated phosphorylation event (Fig. 4*G*, *Center*). This experiment revealed that CK2 phosphorylation at S201 was sufficient to block Pax7 cleavage at a single cleavage site, D208. Together these results demonstrate a physiologic interaction between CK2 and caspase 3 in the control of Pax7 protein stability.

The persistence of CK2 in cells throughout differentiation is related to the cell survival functions attributed to this kinase (27). Moreover, the CK2 holoenzyme is constitutively active (28), suggesting that the subcellular localization and binding partners of CK2 are the primary determining factor in its substrate-targeting capacity. To investigate the functional interplay between Pax7 and CK2, we performed proximity ligation assay (PLA) on primary myoblasts during a differentiation time course. We observed strong CK2:Pax7 interactions in a perinuclear position during growth (1.85 ± 0.13 puncta/cell) and at 6 h postdifferentiation (1.51 ± 0.14 puncta/cell), which was rapidly dissipated as the differentiation program proceeded (0.68 ± 0.10 puncta/cell by 24 h postdifferentiation) (Fig. 4*H*). The temporal loss of the CK2:Pax7 interaction coincides precisely with the

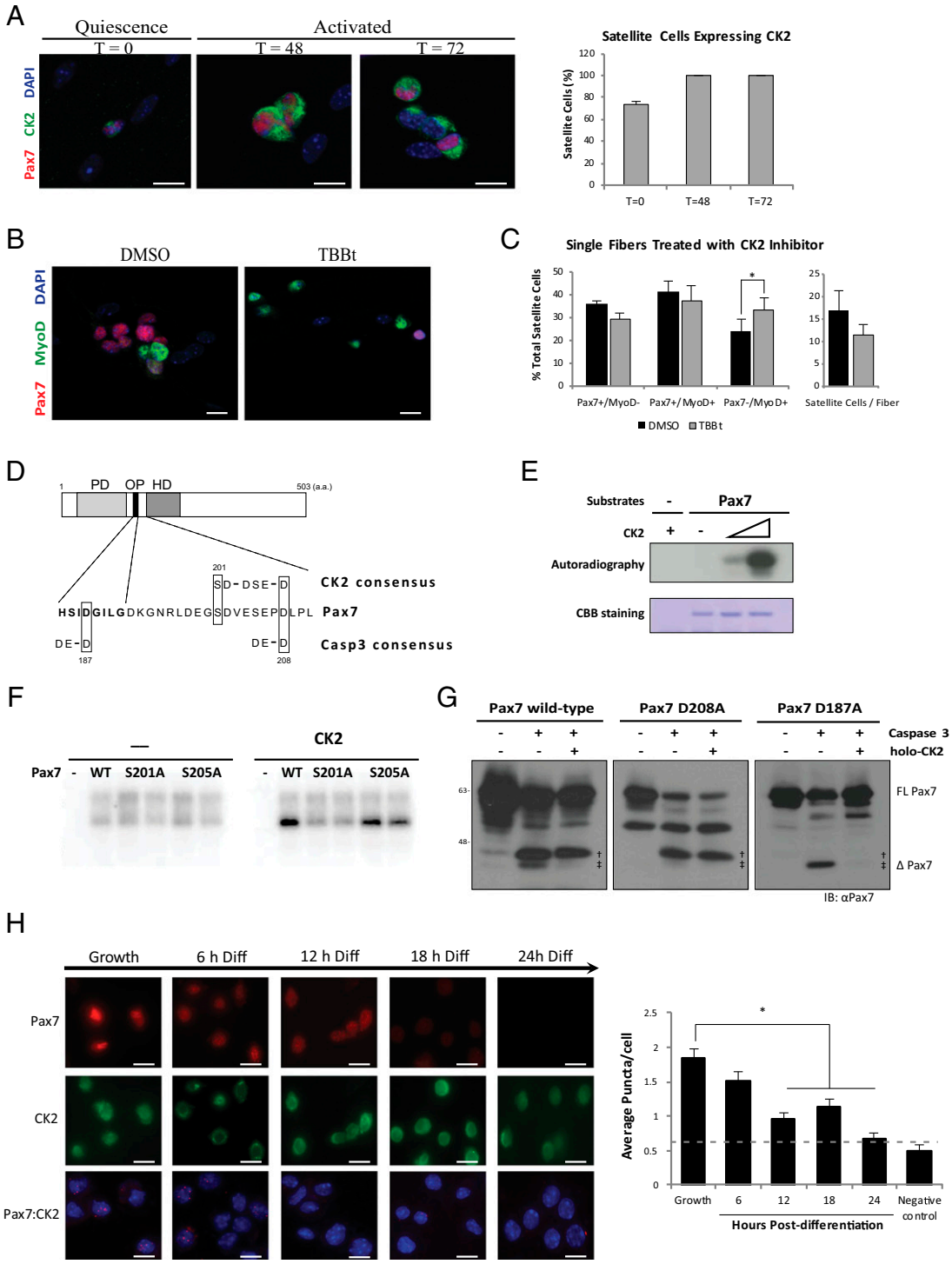

Fig. 4. CK2 phosphorylates Pax7 and restricts SC differentiation. (*A*) Fibers were stained for CK2α (green), Pax7 (red), and DAPI (blue) (*Left*). CK2 is expressed by fiber associated SCs at all time points tested ($n = 3$) (*Right*). (*B*) Fibers were cultured in the presence of the CK2 inhibitor TBBt (50 μM) or DMSO for 3 d and stained for Pax7 (red), MyoD (green), and DAPI (blue). (*C*) Quantification of the number of SCs expressing each marker expressed as a percentage of total SCs. Fibers treated with TBBt contained more differentiating $Pax7^{-}/MyoD^{+}$ SCs compared with DMSO control ($n = 4$; $^{*}P < 0.05$). (*D*) Schematic of Pax7 protein indicating the caspase 3 cleavage sites, as well as the CK2 phosphorylation site located between the paired domain (PD) and the homeodomain (HD) of Pax7. The octapeptide (OP) sequence is bolded. (*E*) In vitro kinase assay of CK2 and Pax7. Autoradiography indicates Pax7 is phosphorylated by CK2 in a dose-dependent manner. (*F*) In vitro kinase assay of wild-type Pax7 or Pax7 with site mutations at S201A or S205A. Autoradiography indicating loss of phosphorylation in the S201A mutant only. (*G*) Recombinant Pax7 was subjected to an in vitro kinase assay as in *E*. Aliquots from each reaction were then subjected to caspase 3 cleavage, SDS/PAGE, and Western blot analysis using αPax7. Production of the Pax7 cleavage fragments (ΔPax7, † and ‡) was impaired in the D187A mutant following preincubation with CK2. (*H*, *Left*) Primary myoblasts were fixed at the indicated times and stained for Pax7 (red) (*Top*), CK2 (green) (*Middle*), or PLA [red dots indicate PLA reaction, costained with DAPI (blue)] (*Bottom*). (*H*, *Right*) PLA showed a positive interaction between CK2 and Pax7 at growth and 6 h following differentiation (6 h Diff). The number of PLA-positive puncta per cell was quantified (80–150 cells/treatment; $n = 3$). Error bars represent ± SEM. $^{*}P < 0.05$. (Scale bars: 10 μm.)

endogenous caspase 3 activation profile (29) and the consequent loss of Pax7 protein. Additionally, we performed PLA on isolated single fibers to verify the CK2 and Pax7 interaction in SCs in situ. We detected a clear PLA signal in SCs at 48 h following myofiber isolation, a time point that coincides with SC activation/proliferation and before the initiation of the differentiation program (Fig. S4*C*). These results in combination with the results obtained using the CK2 inhibitor TBBt (Fig. 4 *B* and *C*) suggest that CK2 ensures protein stability of Pax7 in proliferating/self-renewing SCs and loss of this kinase function is a key step in initiating myogenic differentiation.

Caspase 3 activation is an essential cue for promoting differentiation of committed progenitor cells (12). Our study demonstrates that caspase 3 also acts at a much earlier step in the life cycle of muscle stem cells, by limiting the self-renewal process. Caspase 3 blocks SC self-renewal by cleavage inactivation of Pax7 protein, a mechanism that is counter balanced by CK2-mediated phosphorylation of Pax7. Blockade of caspase activity extends self-renewal in the SC pool, whereas inhibition of CK2 leads to increased numbers of differentiation committed cells, revealing that SC fate is strongly influenced by competing post-translational modifications of Pax7.

The mechanisms involved in the posttranslational regulation of Pax7 protein stability during myoblast differentiation has only been briefly analyzed (15). However, its close paralogue, Pax3, has been demonstrated to be regulated by ubiquitin-mediated degradation (30). Although these studies ruled out a role for the ubiquitin/proteasome pathway in the regulation of Pax7, a role for CK2 has never previously been examined. Here, we provide, to our knowledge, the first evidence that CK2 mediates Pax7 stability during muscle differentiation and is important for SC self-renewal. This mechanism may also have implications in the regulation of the Pax7-FOXO1 fusion protein, the overexpression of which plays a causative role in the tumorigenicity of the skeletal muscle cancer, alveolar rhabdomyosarcoma (31). Indeed, CK2 phosphorylation of Pax3-FOXO1 enhances its protein stability in transformed cells (32). This study did not address the mechanism of Pax3 degradation; however, we predict a similar mechanism involving caspase 3, as we have demonstrated here for Pax7.

In summary, our study is, to our knowledge, the first to demonstrate that caspase 3 limits self-renewal of a lineage restricted stem/progenitor cell. Caspase 3 has been previously reported to inhibit ES cell self-renewal through targeted cleavage of the pluripotency factor Nanog (13). Therefore, it is reasonable to conclude that a similar dichotomous phosphorylation/cleavage modification of Nanog controls ES cell self-renewal, with CK2 and caspase 3 acting as the respective competing enzymes. We speculate that the caspase 3/CK2 targeting of self-renewal factors is a broadly conserved phenomenon, affecting cell fate determination across all lineages.

Materials and Methods

For a more detailed discussion of the materials and methods, see *SI Materials and Methods*. All animal studies were approved by the University of Ottawa Animal Care Committee.

Fibers were isolated from 6- to 8-wk-old mice and cultured in DMEM Fiber Media with z.DEVD.fmk (20 μM; BioVision), TBBt (20 μM; Chemicon), PAC-1 (25–50 μM; Abcam), or DMSO (control). Inhibition of caspase 3 in vivo was accomplished by infecting TA muscles with adenovirus containing a p35-IRES-GFP cassette (or IRES-GFP control) at 1 or 2 d post-CTX injury, and tissue was collected at 3 and 7 d for Western blot analysis or fixed at 14 d for sectioning and immunostaining. For in vitro cleavage assays, recombinant Pax7 protein (100–300 ng) and recombinant active-caspase 3 (0.5 μg; Chemicon) were incubated at 37 °C for 3 h in cleavage assay buffer containing either DMSO or z.DEVD.fmk (20 μM; BioVision) as indicated. Kinase assays were performed by combining recombinant Pax7 (100 ng) with increasing concentrations of purified GST-CK2 in standard kinase assay conditions containing γ[^{32}P]ATP (0.04 μCi/μL) for 1 h at 30 °C. Reactions were stopped by addition of Laemmli sample buffer, subjected to SDS/PAGE, and either Western blot analysis using αPax7 primary antibody or autoradiography. All data are expressed as means ± SEM. The Student *t* test was used for comparisons between treatments unless specified, with $P < 0.05$ considered significant.

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