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A Critical Requirement for Notch Signaling in Maintenance of the Quiescent Skeletal Muscle Stem Cell State

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ABSTRACT

Notch signaling plays a key role in virtually all tissues and organs in metazoans; however, limited examples are available for the regulatory role of this pathway in adult quiescent stem cells. We performed a temporal and ontological assessment of effectors of the Notch pathway that indicated highest activity in freshly isolated satellite cells and, unexpectedly, a sharp decline before the first mitosis, and subsequently in proliferating, satellite cell-derived myoblasts. Using genetic tools to conditionally abrogate canonical Notch signaling during homeostasis, we demonstrate that satellite cells differentiate spontaneously and contribute to myofibers, thereby resulting in a severe depletion of the stem cell pool. Furthermore, whereas loss of *Rbpj* function provokes some satellite cells to proliferate before fusing, strikingly, the majority of mutant cells terminally differentiate unusually from the quiescent state, without passing through S-phase. This study establishes Notch signaling pathway as the first regulator of cellular quiescence in adult muscle stem cells. STEM CELLS *2012;30:243–252*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

During development, founder stem cells proliferate, and cell cycle exit is restricted to differentiating cells. In contrast, adult stem cells can assume different cellular states, either quiescent or proliferating [1]. Quiescent stem cells are arrested in a reversible G_0 phase and the mechanisms that maintain this state remain largely unknown. Elucidation of these mechanisms is critical for understanding how adult stem cells and tissue homeostasis are modulated.

Adult skeletal muscle stem (satellite) cells are quiescent or slow cycling and are associated with myofibers. External stimuli, like muscle injury or exercise, can activate satellite cells and trigger the expression of the muscle regulatory factor *Myod* and entry into the cell cycle [2, 3]. One candidate for controlling satellite cell quiescence is the Notch signaling pathway, whose activity has been shown to be instrumental for the regulation of cell fates and proliferation in a variety of tissues [4–6]. Notch was reported to have antimyogenic activity [7, 8], and disruption of this pathway during development leads to premature muscle differentiation at the expense of muscle stem/progenitors [9, 10]. In spite of its pleiotropic effects, a role for Notch in maintaining quiescent adult stem cells was reported only for neural stem cells in the adult brain [11–14]. In contrast, Notch was shown to be dispensable for quiescent hematopoietic stem cells [15]. Recently, Notch signaling was shown to be essential also for the maintenance of the proliferating stem cells located at the bottom of intestinal crypts [16].

Using a developmental transcriptome analysis of purified muscle stem/progenitor cells, we uncover a dynamic regulation of specific Notch signaling molecules in proliferating and quiescent muscle stem cells. We show that conditional loss of *Rbpj* in quiescent satellite cells also results in their differentiation and their subsequent depletion. Intriguingly, the majority of these aberrantly differentiating cells fail to undergo DNA replication.

MATERIALS AND METHODS

Animals

Rbpj-floxed mice were generously provided by Prof. T. Honjo [17]. For germline deletion of *Rbpj*, $Rbpj^{flox}$ mice were crossed to transgenic *Tg:PGK-Cre* mice [18]. *Tg:Pax7-nGFP* mice were used to isolate stem/progenitors [19]. Reporter line $Rosa26^{mTomato\text{-}STOP\text{-}mGFP}$ [20] was purchased from Jackson Laboratories (Stock 007576). Generation of $Tg{:}Pax7\text{-}CreER^{T2}$ and $Pax7^{STOP\text{-}nlacZ}$ mice are described in Supporting Information Figure S2A. Comparisons were done

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between age-matched littermates using 8–12-week-old mice. Animals were handled as per European Community guidelines.

Tamoxifen, Thymidine Analogs, and Notexin Administration

Tg:Pax7-CreER^{T2} mice were injected twice (13–16 hours apart) intraperitoneally with tamoxifen (250–300 μl, 20 mg/ml; Sigma #T5648, St. Louis, MO, www.sigma-aldrich.com) diluted in sunflower seed oil/5% ethanol. For muscle regeneration, mice were injected three times with tamoxifen. 5-Bromo-2′-deoxyuridine (BrdU, Sigma #B5002) was administered immediately after the last injection in drinking water (1 mg/ml, 2.5% sucrose) for 7–16 days. Drinking bottles were protected from light and changed every 3 days. Injuries were performed by intramuscular injections of 10 μl of snake venom notexin 10 μg/ml (Notexin, Lotaxan #37223-96-4, Valence, France, www.latoxan.com) on anesthetized mice (0.5% Imalgene/2% Rompun), and *Tibialis anterior* (TA) muscles were collected 12 days postinjection. For proliferation experiments in vivo, 5-ethyl-2′-deoxyuridine (EdU, Invitrogen #E10187, Carlsbad, CA, www.invitrogen.com) was injected 4 hours prior to sacrifice at a concentration of 50 μg/g of mouse. For primary myoblasts in culture, EdU was used at a concentration of 2×10^{-6} M and BrdU at 1×10^{-7} M.

Statistical Analysis

For comparison between two groups, two-tailed Student's t test was performed to calculate p values and to determine statistically significant differences (*, $p < .05$; **, $p < .01$; ***, $p < .001$). For analysis of three or more groups, one-way analysis of variance ANOVA was used. All statistical analyses were performed with PRISM software. A minimum of three and up to five independent mice were analyzed for all experiments. Data are presented as means ± SEM or SD, as indicated. Additional experimental methods and reagents can be found in Supporting Information.

RESULTS

Notch Signaling Declines During Myogenic Lineage Progression from Stem Cells

To identify the specific stages where Notch signaling and downstream targets exert their effects, we performed a developmental series of comparative microarrays. Transgenic *Tg:Pax7-nGFP* mice [19] were used to isolate stem/progenitor cells by fluorescence-activated cell sorting (FACS) from E12.5 to adult stages [Fig. 1A, Supporting Information Fig. S1A; R. Sambasivan and S. Tajbakhsh, unpublished data). Analysis showed a strict regulation of specific paralogs of Notch ligands (*Dll1* and *Jag1*) and receptors (*Notch1*, *2* and predominantly *3*) and of defined target genes (*Hes1*, *Hey1*, *HeyL*, and *Nrarp*), which are repeatedly induced throughout the developmental and postnatal periods in the muscle lineage (Fig. 1A). Notably, *HeyL* showed maximal expression in freshly isolated satellite cells (Fig. 1A-a, Supporting Information Fig. S1B, S1C), consistent with a previous report [21]. *Rbpj*, which is the main effector for canonical Notch signaling, was expressed at all developmental stages, including in satellite cells (Fig. 1A-c) and this was confirmed by antibody staining showing a characteristic localization in subnuclear bodies (Fig. 1B) [22]. In addition, endogenous Notch activity was validated by assessing the expression of *HeyL*/*Hey1*/*Hes1* and *Nrarp* after modulating Notch signaling in purified satellite cells (Fig. 1C; for assay validation see Supporting Information Fig. S1D). We note that *Hes1* is only slightly induced when Notch signaling is activated, whereas it is repressed when the pathway is inhibited, suggesting that in the myoblasts there are limiting factors regulating *Hes1* transcription. Therefore, Notch signaling is active in proliferating myoblasts with maximal activity in purified adult satellite cells.

We then fractionated Pax7-nGFP satellite cells into distinct subpopulations based on GFP intensity, representing the lineage progression from stem to committed cells. As *Pax7* expression decreases as cells differentiate, we analyzed Pax7^{Hi} (top 20%), Pax7^{Inter} (intermediate 40%), and Pax7^{Lo} (bottom 20%) (Fig. 1D). Accordingly, reverse transcription-quantitative polymerase chain reaction RT-qPCR analysis showed a downregulation of endogenous *Pax7* expression and an increase in the commitment (*Myod*) and differentiation (*Myogenin*) markers from Pax7^{Hi} to Pax7^{Lo} (Supporting Information Fig. S1E). Samples analyzed included perinatal (P8) and activated adult satellite cells, the latter obtained 4 days post-notexin injury of the TA muscle. Notch activity declined as cells progressed from stem to committed cells (Pax7^{Hi} to Pax7^{Lo}; Fig. 1D, upper graphs). This was linked with a concomitant decrease in *Notch* receptor expression (primarily *Notch3*) and an increase in *Dll1* (Fig. 1D, lower graphs). Therefore, highest Notch activity correlated with the Pax7^{Hi} fraction, which corresponds to a more stem cell state during growth and adult regeneration. This activity, apparently, is triggered and maintained by Dll1 expression from the more differentiated myoblasts (Pax7^{Lo} fraction).

A Critical Requirement for Notch for Maintaining Quiescence in Satellite Cells

The identification of active Notch signaling in satellite cells prompted us to investigate its potential functional importance in this cell population. To do so, we selectively abrogated *Rbpj* [17], using a transgenic mouse that we generated, which expresses a tamoxifen-inducible Cre recombinase/estrogen receptor fusion protein, CreER^{T2} [23], under the control of *Pax7* (*Tg:Pax7-CT2*; Fig. 2A, Supporting Information Fig. S2A-S2C). Mice heterozygous for *Rbpj* did not appear to have an obvious phenotype and they were used as control (*Tg:Pax7-CT2/+::Rbpj$^{flox/+}$*). A Cre-inducible green fluorescent protein (GFP) reporter (Rosa26$^{mTomato\text{-}STOP\text{-}mGFP}$, Fig. 2A-b–d; [20]) was also introduced to permanently mark the targeted cells (hereinafter, cKO mice are: *Tg:Pax7-CT2::Rbpj$^{flox/-}$::Rosa26$^{mTomato\text{-}STOP\text{-}mGFP/+}$*; control mice are: *Tg:Pax7-CT2::Rbpj$^{flox/+}$::Rosa26$^{mTomato\text{-}STOP\text{-}mGFP/+}$*). *Rbpj* depletion in satellite cells was highly efficient as at 7 days after tamoxifen injection (post-Tmx) Rbpj protein was undetectable in more than 93% of the GFP$^+$ cells (Fig. 2A-e). At day 4, however, the majority of the GFP$^+$ cells retained some immunoreactivity (Supporting Information Fig. S2D), suggesting that Rbpj protein is relatively stable in these noncycling satellite cells.

To investigate the consequence of the loss of canonical Notch signaling in quiescent satellite cells, GFP$^+$ satellite cells were isolated by FACS following tamoxifen injections (7, 16, and 40 days post-Tmx, Fig. 2B). Consistent with the absence of detectable Rbpj protein in the majority of targeted cells (Fig. 2A-e), *Rbpj* transcript levels were dramatically reduced (Fig. 2B). Interestingly, *HeyL*, *Hey1*, and *Hes1* (~80%–90%) as well as *Myod* (~50%) and *Pax7* (~80%) transcripts were significantly downregulated, whereas *Myogenin* transcript levels increased approximately 4.5–7.5-fold (Fig. 2B). The latter observation was confirmed by examining protein expression (Fig. 2B). Cell counts by immunostaining of muscle sections and isolated single myofibers showed more abundant expression of Myod and Myogenin at the expense of Pax7 (Fig. 2C); at 16 days post-Tmx Myod$^+$/GFP$^+$: 8.9 ± 2.6% in control and 24.9 ± 5.1% in cKO ($n = 3$ mice);

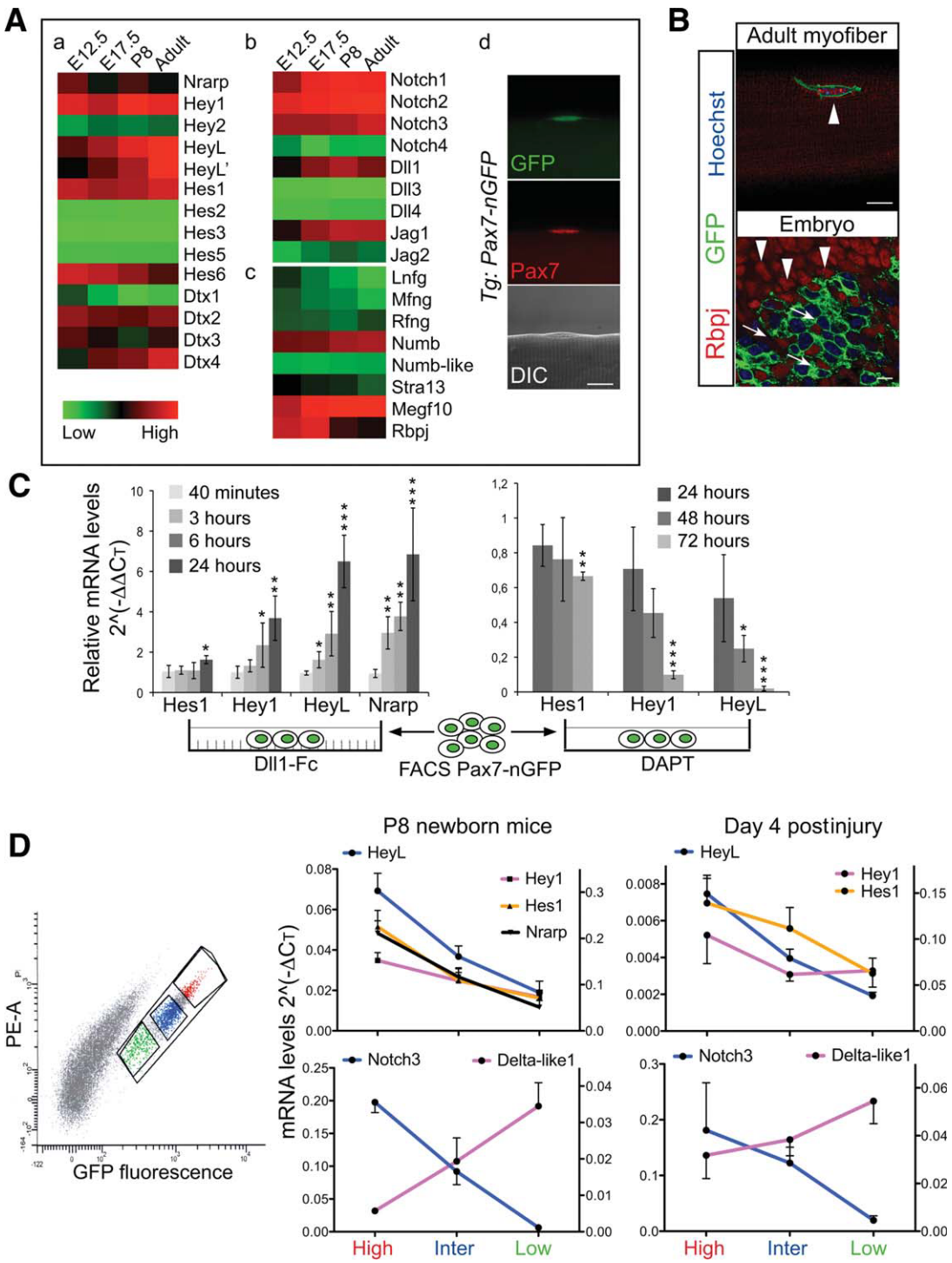

Figure 1. Notch signaling is active in muscle stem cells. **(A):** Heatmap of Notch-related genes from developmental microarrays of Pax7-nuclear GFP (nGFP) stem/progenitor cells (3 biological replicates/time point); **(a)** Notch targets, **(b)** ligands/receptors, **(c)** modifiers, and **(d)** isolated myofiber from *Tg:Pax7-nGFP* showing coexpression of nGFP (green) and Pax7 (red) proteins. **(B):** Rbpj protein in GFP^{+} satellite cells from isolated *Tg:Pax7-CT2::Rosa26*$^{mTomato\text{-}STOP\text{-}mGFP/+}$ myofibers induced by Tmx in the adult (upper) E11.5 mouse embryo cross-section; cells conditionally deleted for *Rbpj* using *Tg:Pax7-CT2* are marked by GFP (lower). Arrowheads, Rbpj expressing cells; arrows, *Rbpj* null cells. **(C):** Temporal response of Notch target genes in satellite cell-derived myoblasts in presence of Delta-like1 (Dll1-Fc, left) or a γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT, right). Graphs represent average $2^{-\Delta CT}$ ratios (treated/control) $\pm$ SEM from three independent experiments. **(D):** Subfractionation by FACS of stem/progenitors from *Tg:Pax7-nGFP* mice based on GFP intensity: Pax7^{Hi} (20%, red), Pax7^{Inter} (40%, blue), Pax7^{Lo} (20%, green); percentages of fluorescence correspond to number of cells. RT-qPCR analysis was performed on genes indicated. Graphs represent average $2^{-\Delta CT}$ values $\pm$ SEM (n = 3–4 mice/stage). Autofluorescence in red (PE-A) channel. Scale bars = 10 μm (A(d), B). Abbreviations: GFP, green fluorescent protein; PE-A: phycoerythrin.

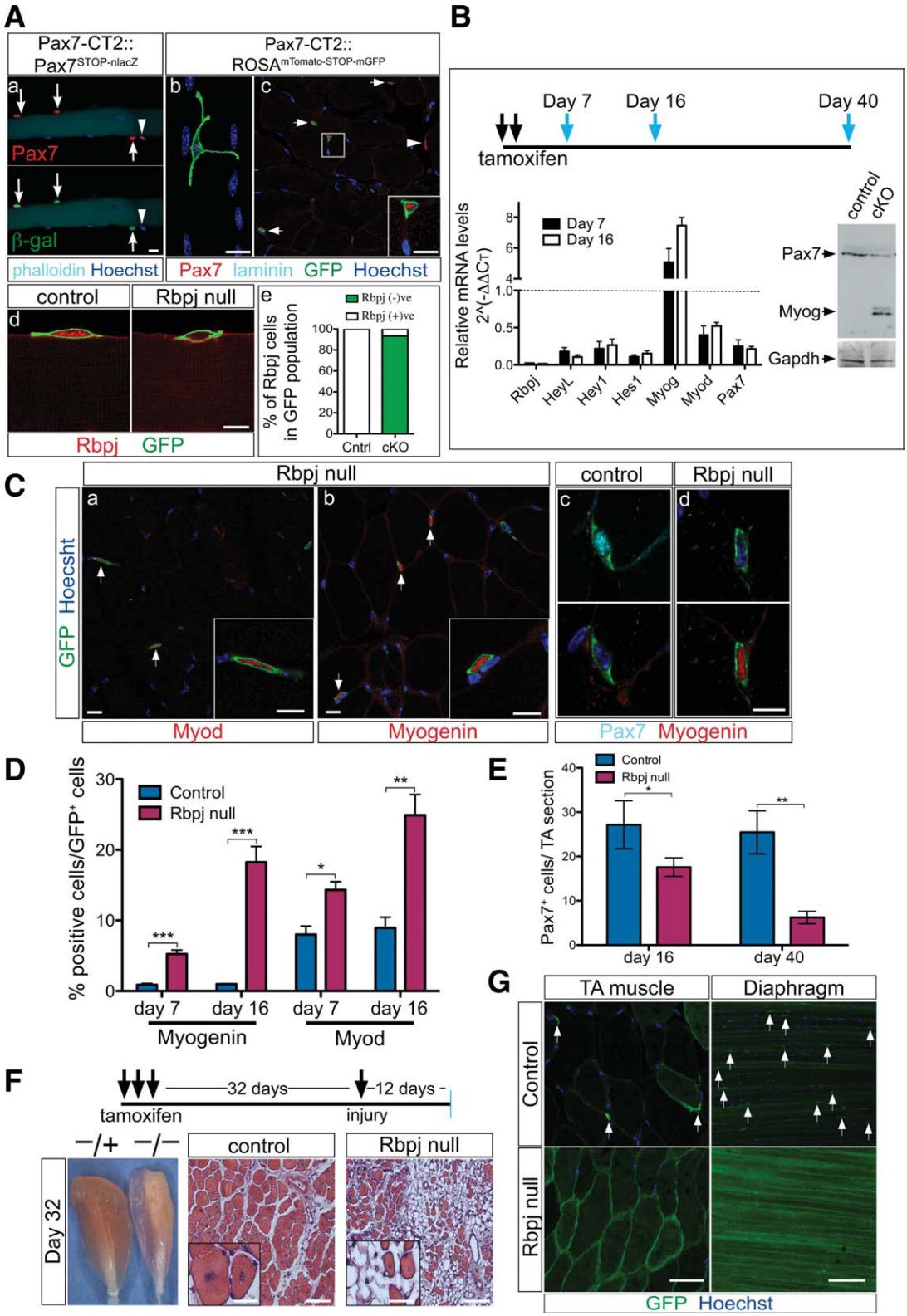

Figure 2. Precocious differentiation of satellite cells lacking Rbpj. **(A):** Characterization of *Tg:Pax7-CT2* mouse; **(a)** coexpression of β-galactosidase (green) and Pax7 (red) on isolated myofiber from a *Tg:Pax7-CT2::Pax7*$^{STOP\text{-}nlacZ}$ mouse, **(b)** inducibility scored with GFP reporter on EDL myofiber, **(c)** cross-section of TA muscle (a–c, 48 hours post-Tmx), **(d, e)** Immunostaining of isolated myofibers at day 7 post-Tmx (control, *Tg:Pax7-CT2::Rbpj*$^{flox/+}$*::Rosa26*$^{mTomato\text{-}STOP\text{-}mGFP/+}$; null, *Tg:Pax7-CT2::Rbpj*$^{flox/-}$*::Rosa26*$^{mTomato\text{-}STOP\text{-}mGFP/+}$); percentage of Rbpj-negative cells in GFP+ population (n = 4 mice). **(B):** RT-qPCR of genes from *Rbpj* null (control mice set to 1) (n = 3-4 mice/condition). Western blot (right) using purified adult satellite cells (16 days post-Tmx). **(C):** Immunostaining on sections from day 16 post-Tmx control and cKO resting TA muscles; **(a, b)** premature expression of Myod (a) and Myogenin (b) in *Rbpj* null GFP+ satellite cells (arrows), **(c)** control Pax7^{+} satellite cell, **(d)** note Myogenin^{+} satellite cell lacking Pax7. **(D):** Quantification of Myogenin and Myod-positive cells by immunostaining. Graphs represent average ± SEM (days post-Tmx indicated; $n \geq 3$ mice). **(E):** Average number of Pax7^{+} cells per cross-section in uninjured TA muscle at days 16 and 40 post-Tmx. Graphs represent average ± SD ($n = 3$ mice per condition). **(F):** TA muscles of control and cKO mice were injured 32 days post-Tmx and collected 12 days later; sections stained with hematoxylin and eosin. **(G):** Muscles isolated at day 40 post-Tmx stained with anti-GFP antibody. GFP-marked satellite cells are indicated (arrows). Scale bars = 15 μm (A), 15 μm (F), 40 μm (inset), 40 μm (G), 40 μm (TA), and 100 μm (diaphragm). Abbreviations: GFP, green fluorescent protein; TA, *Tibialis anterior*.

Myogenin$^+$/GFP$^+$: 0.96 ± 0.3% in control and 18.23% ± 4.5 in cKO ($n = 4$ mice), calculated as percentage of all *GFP*+ cells (Fig. 2D). Intriguingly, we noticed that the increase in the number of Myod-expressing cells (Fig. 2D) did not correlate with the level of its transcript, which was consistently found to be less abundant in the mutant cells (Fig. 2B). This observation suggests that *Myod* transcripts could be post-transcriptionally regulated in the Rbpj cKO satellite cells that exit quiescence.

The upregulation of differentiation proteins, like Myod and Myogenin, led to, or correlated with, a loss of satellite cells. Indeed, a continued decline in the number of Pax7$^+$ cells in resting muscle was observed over time after the depletion of Rbpj protein (35.2% reduction by 16 days, 75.5% reduction by 40 days post-Tmx; Fig. 2E, Supporting Information Fig. S2E). Interestingly, at day 40 the few remaining GFP$^+$ cells in the cKO were all positive for Rbpj protein (i.e., recombination escapers), suggesting that only satellite cells retaining Notch activity can persist. Importantly, when an injury was performed at 32 days post-Tmx, muscle regeneration failed in the cKO mice (Fig. 2F), with only a few residual myofibers present 12 days after the trauma. This compromised tissue regeneration provides functional evidence that prolonged blockage of Notch signaling results in the actual depletion of the satellite cells and not simply in the loss of its marker Pax7.

Notably, the sharp decline in satellite cell numbers in the cKO tissue (Fig. 2E, Supporting Information Fig. S2E, S2F) suggested that these were eliminated either by apoptosis, by fusion with myofibers, or by a change in cell fate. No evidence for the latter was noted as no GFP$^+$ cells were found in the interstitum or expressing nonmuscle markers (data not shown). Furthermore, staining for cleaved caspase-3 did not show an increase in cell death in the mutant tissue relative to the control, at any stage examined post-Tmx (Supporting Information Fig. S3A), suggesting that a progressive diminution of the satellite cell pool by differentiation was accompanied by the fusion of GFP-positive satellite-derived cells to myofibers. Accordingly, a significant increase in the number of GFP-marked myofibers was observed in the *Rbpj* cKO mice (Fig. 2G). These findings indicate that active Notch signaling in satellite cells is required to maintain their quiescent state and in its absence they spontaneously commit to differentiation in multinucleated myofibers. Intriguingly, although the majority of the GFP$^+$ cells lacked Rbpj protein by 7 days post-Tmx (Supporting Information Fig. S2D), loss of satellite cells was gradual over 4–5 weeks under homeostatic conditions (Fig. 2E), suggesting a combinatorial effect.

Notch Signaling Declines Sharply After Muscle Injury While Rbpj-Deficient Myoblasts Retain Proliferative Capacity

Notch activity was reported to be essential for muscle regeneration, as inhibition of the pathway immediately after injury by Notch ligand Jagged1-Fc administration resulted in poor muscle repair [24]. We used our mouse model to genetically address the role of Notch signals in the proliferating myoblast population. When injury was induced at 16 days post-Tmx, muscle regeneration was overtly normal, with occasional small zones containing poorly regenerated myofibers (data not shown). In spite of the fact that not all satellite cells were targeted in the cKO animals (efficiency ~80% of the total satellite cell population), our results suggested that *Rbpj*-deficient satellite cells can contribute to muscle regeneration. To investigate this phenotype in more detail, we measured the proliferation potential of activated satellite cells in vivo, as transient amplification of myogenic cells is a major event that follows injury and a requirement for generating myoblasts to effect regeneration. After 6 days of the last tamoxifen injection, control and cKO TA muscles were injured with notexin and GFP$^+$ cells were enumerated by FACS. Although the cKO cells on day 5 after activation were significantly less compared to the control (on average 51% less), they retained their potential to proliferate (on average 7.4-fold amplification in control and 5.4-fold in cKO; Fig. 3A).

This was examined further by scoring the fraction of the cells that were in S-phase of the cell cycle at a given time point, by injecting the thymidine analog EdU 4 hours prior to dissection of the regenerating muscle (diagram, Fig. 3A). Purified GFP$^+$ cells were plated, allowed to adhere for 5 hours, and stained for the presence of EdU. As shown in Figure 3A, a population of *Rbpj* null cells that cycled was reproducibly observed, albeit there were fewer than those in the control samples. Furthermore, the proliferation potential of *Rbpj* mutant myoblasts was monitored by videomicroscopy (Fig. 3B). In this case, the source of the purified satellite cells was noninjured TA muscle, as isolation of these cells invariably leads to their activation and entry into the cell cycle. As these cells were quiescent, there is a relatively long lag time of approximately 30 hours before their first cell division [25]. Cell divisions were then analyzed by videomicroscopy for the first 60 hours after cell isolation. We observed that, overall, *Rbpj* null myoblasts entered the cell cycle with the same kinetics as the control, exhibiting similar division times, although slightly more variability was observed in the null (Fig. 3B).

We then monitored the potential of the *Rbpj* mutant cells to grow in culture, where the presence of growth stimuli is abundant (20% calf serum, Matrigel substrate). Equal number of isolated satellite cells were plated, and the total number of nuclei was counted (mononucleated, and in multinucleated myotubes). After 4 days in culture, when cell fusion was initiating, the number of cells with or without Rbpj function was comparable. All cells were actively dividing as evidenced by BrdU incorporation (Fig. 3C). However, after 12 days *Rbpj* mutant cultures contained significantly less nuclei and almost no dividing *Rbpj* null cells (Fig. 3C). Taken together, our in vivo and ex vivo data demonstrate that satellite cells without canonical Notch signaling can exit quiescence and perform several rounds of divisions, yet they have a reduced growth potential possibly due to an impairment in self-renewal capacity.

Our data indicated that Notch activity is critical for the quiescent state but partially dispensable in the proliferating phase. Thus, we investigated whether this requirement correlated with the level of Notch activity. To do so, we measured endogenous Notch activity in wild-type myoblasts at different time points following trauma. GFP$^+$ cells were purified from 20 hours to 30 days postinjury from *Tg:Pax7-nGFP* mice; 20 hours being prior to the first division of the activated satellite cells [25] and 30 days the time by which self-renewal and homeostasis are being re-established. Surprisingly, Notch activity declined sharply immediately after injury, even before the first cell division, as evidenced by the dramatic downregulation of *Hey1*, *HeyL*, and *Hes1* already at 20 hours poststimulation (Fig. 3D). The expression of these genes was restored by days 20–30, corresponding to self-renewal. As expected, *Myod* transcript levels were upregulated directly postinjury. These observations suggest that a transient (several days) and dramatic reduction of Notch activity might be required for exit from quiescence and expression of *Myod*, which Notch activity normally inhibits [7].

Besides proliferation, the ability to self-renew is another hallmark of primary myogenic cells, ensuring that some cells

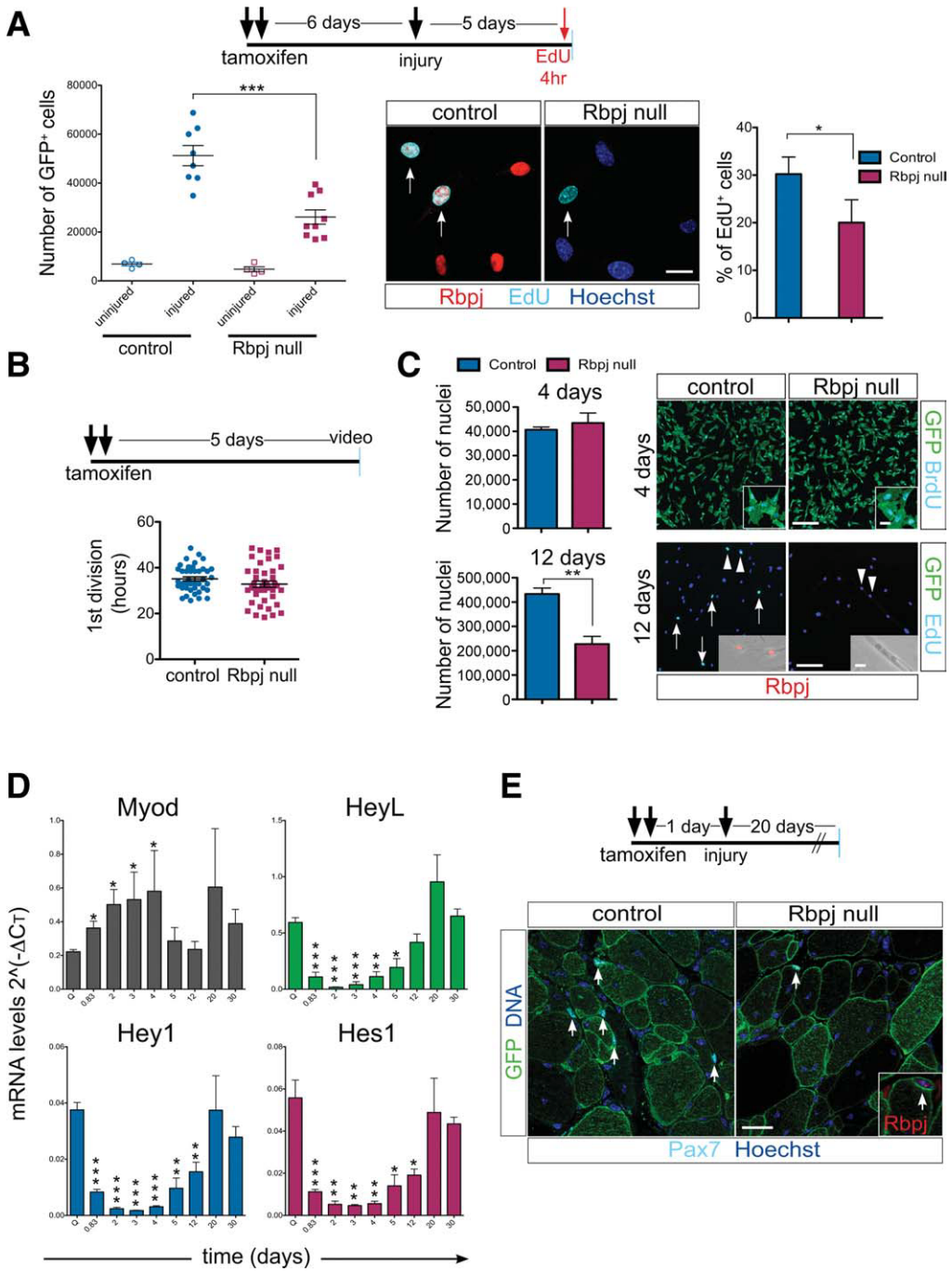

Figure 3. Proliferation and self-renewal potential of Rbpj null myoblasts. **(A):** Proliferation assay in vivo in control (*Rbpj*$^{+/-}$) and cKO (*Rbpj*$^{-/-}$) mice, measuring the number of GFP^{+} cells 5 days after notexin-induced trauma (left graph, 4-9 TAs examined/condition; n = 3-5 mice). Images show purified cells stained for EdU (arrows double positive, control). Note EdU^{+}/Rbpj^{-} cell in right panel (arrow); corresponding numbers as percentage of GFP+ cells isolated by FACS from the injured TA (right graph, n = 3 mice/condition). **(B):** Purified cells from control and cKO mice were filmed for 60 hours and the first divisions were scored (control: n = 44, cKO: n = 47). **(C):** Nuclei counts on days 4 and 12 after plating. Cells were incubated overnight with a thymidine analog to score dividing cells. Arrows, EdU^{+} cells; arrowheads, cells highlighted in insets stained for Rbpj (n = 3 mice/condition; hind and forelimb muscles). **(D):** Assay of endogenous Notch activity on purified myogenic cells from notexin injured *Tg:Pax7-nGFP Tibialis anterior* muscles. Graphs represent average $2^{-\Delta CT}$ values ± SEM (n = 3 mice per time point). **(E):** Pax7 staining 20 days after notexin injury in control and cKO mice. Note a rare Pax7 expressing cell in the cKO muscle that is Rbpj positive (inset, recombination "escaper"). Control, *Tg:Pax7-CT2::Rbpj*$^{flox/+}$*::Rosa26*$^{mTomato\text{-}STOP\text{-}mGFP/+}$ and *Rbpj* null, *Tg:Pax7-CT2::Rbpj*$^{flox/-}$*::Rosa26*$^{mTomato\text{-}STOP\text{-}mGFP/+}$ (control n = 2 mice, cKO: n = 4 mice). Arrows: Pax7^{+} cells. Scale bars = 10 μm (A), 100 μm (B), 10 μm (inset), 25 μm (D), and 10 μm (inset). Abbreviations: GFP, green fluorescent protein; EdU, 5-ethyl-2′-deoxyuridine; cKO, conditional Knock-Out.

will retain *Pax7* expression and replenish the quiescent satellite cell pool. We measured the capacity of activated cells to self-renew in vivo by scoring the number of Pax7^{+} cells 20 days postinjury. In control muscles, abundant Pax7^{+} cells were associated with newly formed myofibers. In marked contrast, cKO muscles were virtually devoid of Pax7^{+} cells in

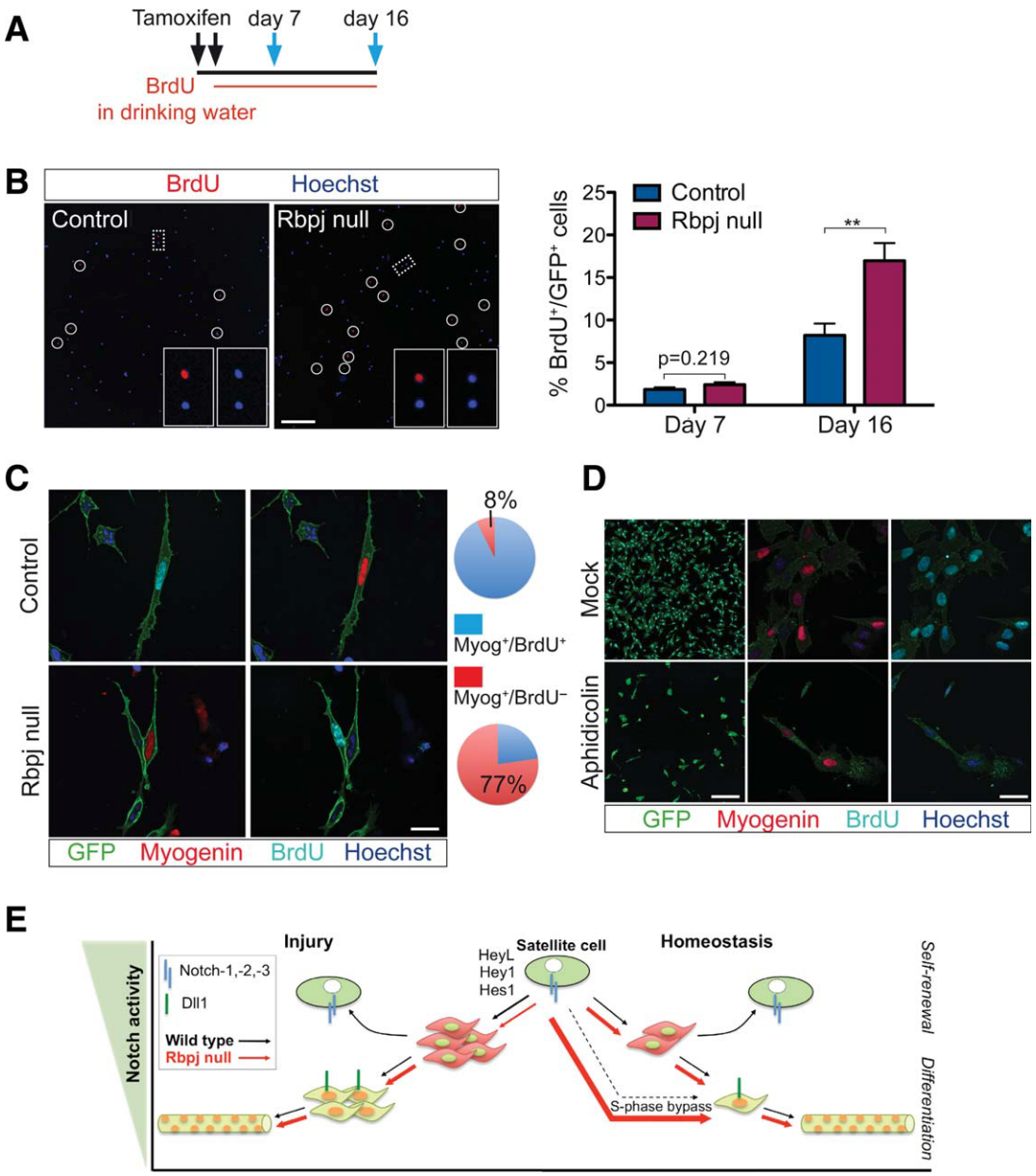

Figure 4. Inhibition of Notch signaling provokes differentiation and bypass of S-phase in the majority of satellite cells. **(A):** Scheme of continuous BrdU administration initiated immediately after second Tmx injection (see text, Fig. 3 for genotype). **(B):** GFP$^+$ satellite cells immunostained for BrdU immediately after isolation by FACS, 16 days post-Tmx, and quantification of BrdU cells. Graphs represent average ± SEM ($n = 3$ mice per condition). **(C):** GFP$^+$ cells double immunostained for BrdU and Myogenin (double-positive/Myogenin$^+$; control, 10/130, $n = 3$ mice; cKO 585/760, $n = 4$ mice). Mice were treated with Tmx/BrdU as shown in (A). Hind limb muscles were enzymatically dissociated and cells were plated for 5 hours prior to fixation to allow adhesion. Similar results were obtained with purified cells fixed immediately after isolation (Supporting Information Fig. S3D). Note that control is *Rbpj*$^{+/-}$. **(D):** Purified satellite cells from control mice (*Tg:Pax7-CT2::Rbpj*$^{flox/+}$*::Rosa26*$^{mTomato\text{-}STOP\text{-}mGFP/+}$, 5 days post-Tmx) treated with aphidicolin in the presence of BrdU, fixed after 4 days in culture (n = 3 mice). **(E):** Model for role of Notch signaling in adult muscle stem cells. Notch activity is highest in satellite cells and it declines on activation. A further decrease occurs during differentiation, whereas Notch activity is required for satellite cell self-renewal. The predominant signaling in early and late stages is indicated. During homeostasis, the majority of *Rbpj* null cells differentiate without executing S-phase. Scale bars = 100 μm (B, C); 100 μm (D, two left panels), and 50 μm (other four panels). Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; GFP, green fluorescent protein.

regions of regeneration (Fig. 3E). Interestingly, those Pax7-expressing cells found in the cKO muscle were also expressing Rbpj due to incomplete recombination (inset, Fig. 3E). Therefore, the Notch pathway is essential for replenishment of the satellite cell pool after injury. These results strongly suggest that Notch activity is essential for maintaining the balance between the differentiating and the proliferating cells during muscle regeneration. In the absence of Notch activity, proliferating myoblasts fail to self-renew and instead differentiate and fuse.

The Majority of Differentiating *Rbpj* Null Satellite Cells Bypass S-Phase

As loss of *Rbpj* function resulted in the loss of cellular quiescence and differentiation of muscle stem cells, we investigated if this cell state transition was accompanied by exit

from G_0 and entry into the cell cycle. To do this, mice were exposed to uninterrupted BrdU administration through the drinking water immediately following the tamoxifen injections. Mice were then analyzed at 7 days post-Tmx, when Rbpj protein was no longer observed in most satellite cells (Supporting Information Fig. S2D) and precocious differentiation was initiating (Fig. 2B–2D), and also at 16 post-Tmx (Scheme, Fig. 4A).

After 1 week of *Rbpj* gene inactivation, no significant difference was observed in BrdU incorporation or immunoreactivity with other proliferation markers (Ki67, phospho-Histone3) when compared to controls ($n = 3$ mice; Fig. 4B (see day 7), Supporting Information Fig. S3C; data not shown). In contrast, at 16 days post-Tmx a significant fraction of the *Rbpj* null satellite cells entered S-phase (17.0% $BrdU^+$ vs. control 8.3%; $n = 4$ mice, Fig. 4B, see day 16). Further examination of the *Rbpj* mutant cells revealed that at 16 days post-Tmx and continuous BrdU administration, the majority of the aberrantly differentiated cKO cells (~77% $Myog^+$) bypassed S-phase (BrdU-negative; Fig. 4C, Supporting Information Fig. S3D). In control *Rbpj* heterozygous mice, approximately 92% of the rare, spontaneously differentiating $Myog^+$ cells were BrdU positive (Fig. 4C, Supporting Information Fig. S3D). Interestingly, a small fraction of $Myog^+/BrdU^-$ cells was noted in the heterozyous control mice (~10-fold less than in the *Rbpj* null), suggesting that S-phase bypass can occur in cells competent to transduce Notch signal, albeit the possibility of a haploinsufficiency effect cannot be excluded.

Under the experimental conditions used, the BrdU was in excess, as in control regenerated muscle more than 98% of centrally located myonuclei were labeled (Supporting Information Fig. S3B). One possibility for the presence of $Myog^+/BrdU^-$ cells is that pre-existing $Myog^+$ cells were present in the cKO mice due to haploinsufficiency (*Tg:Pax7-CT2*/+ *::Rbpj*$^{flox/-}$). However, this is unlikely as they would have to remain as mononuleated, $Myog^+$ cells for 16 days, without fusing to the muscle fibers. In addition, these $Myog^+$ cells would need to express *Pax7* to be GFP^+ after Cre-mediated recombination, also an unlikely event. Nevertheless, to determine whether all $Myog^+/GFP^+$ cells were generated uniquely after Tmx injection, double-positive cells were scored in the cKO at 40 hours post-Tmx. No double-positive cells were detected over background (data not shown); therefore, the $Myog^+/GFP^+/BrdU^-$ cells observed were generated during BrdU administration in the period post-Tmx. To assess the capacity of quiescent cells to execute a G_0-to-differentiation transition, purified satellite cells were then cultured in the presence of BrdU and the S-phase inhibitor aphidicolin. After an overnight culture, all treated cells were activated ($Myod^+$) but had not transitioned yet through S-phase ($BrdU^-$). After 4 days in culture, $Myog^+/BrdU^-$ cells were observed (7% of total cells; Fig. 4D), indicating that some primary myogenic cells undergo a G_0-to-differentiation transition without undergoing DNA replication.

Discussion

Cellular quiescence is a property of most adult vertebrate stem cells. How this cell state is regulated as stem cells effect repair and self-renew to re-adopt the quiescent state is a central theme in stem cell biology; however, it remains poorly understood. We uncover a novel function for Notch signaling in adult muscle stem cells by showing that genetic abrogation of *Rbpj* function specifically in these cells, results in the loss of the quiescent state and spontaneous differentiation. The ultimate outcome of this event is an exhaustion of the stem cell pool and failure of muscle regeneration after induced muscle trauma. Strikingly, interruption of Notch activity in vivo favors an atypical G_0-to-differentiation transition and the bypass of S-phase in the majority of mutant cells (Fig. 4D). This is the first demonstration in vertebrates of direct adoption of the differentiated state from cellular quiescence without transiting through S-phase and mitosis. Taken together, our results establish Notch signaling as the first regulator of the quiescent state in muscle stem cells.

In the context of muscle repair, inhibition of Notch has been shown to result in failed regeneration [24]. In that report, block of Notch was achieved by injection of recombinant Jagged1-Fc 2 days after injury, when myoblasts are actively proliferating. Therefore, the current model suggested that a burst of Notch activity follows satellite cell activation, an event that is required for the expansion of the myoblast population. In the current report, by measuring the Notch target genes *Hey1*, *HeyL*, and *Hes1* in purified satellite cells in vivo (*Tg:Pax7-nGFP*), we report that Notch activity is dramatically reduced immediately after injury, relative to the nonactivated satellite cells (Fig. 3D). Notably, the reduction takes place even at 20 hours postinjury, that is, before the first cell division occurs in vivo [25]. Nevertheless, it is possible that the remaining activity is critical for full amplification of the myoblasts. Although *Rbpj* null myoblasts did continue to proliferate (EdU positive), the overall number of satellite cell progeny is significantly reduced in the absence of Rbpj function. Therefore, some Notch activity is critical for maintaining the proliferating cells, but it is not essential for cells to enter cycle after activation.

Muscle with approximately 80% of the satellite cells null for *Rbpj* can efficiently regenerate (*Tg:Pax7-CT2::Rbpj*$^{flox/-}$), whereas muscle in which Notch signaling is globally inhibited (Jagged1-Fc administration) fails to regenerate [24]. One interpretation for this difference is that the small fraction of cells that escape recombination can account for the repair; when all satellite cells are blocked for Notch signaling, regeneration collapses. Nevertheless, during regeneration, activated satellite cells interact with other cell types, like infiltrating inflammatory cells [26, 27] and fibro/adipogenic progenitors [28]. These interactions, which facilitate efficient regeneration, could be mediated, at least in part, by Notch signaling. Therefore, the block of regeneration on administration of Jagged1-Fc could be the outcome of a broader interference of cell–cell communications. Finally, Jagged-Fc acts antagonistically at the level of the membrane receptor, whereas in this study canonical Notch signaling is blocked by removal of DNA-binding protein Rbpj, the main downstream effector of canonical Notch signaling. It is then possible that the stronger effect seen with Jagged1-Fc is due to interference with a noncanonical Notch signaling pathway.

In this study, we also identify Notch signaling as a key regulator of the quiescent satellite cells, while this pathway has been shown to block differentiation during myogenesis [7, 8]. Therefore, the maintenance of quiescence and the inhibition of cellular differentiation involve similar molecular regulators and Rbpj activity appears to mediate both of these processes in quiescent stem cells. The Notch target Hes1 was reported to be essential for controlling the reversibility of quiescence in nondividing fibroblasts, by preventing permanent cell cycle exit (i.e., terminal differentiation or senescence) [29]. Our finding that Hes1, Hey1, and HeyL repressors are sharply downregulated in differentiating *Rbpj* null cells, is consistent with this notion. In myogenic cells, however, neither Hes1 nor HeyL, but only Hey1 overexpression can block

myogenesis by directly suppressing the *Myogenin* promoter [30]. Therefore, Hey1 would be a good candidate to mediate, at least in part, Notch regulation of satellite cell maintenance by preventing their differentiation.

Our observations that the majority of differentiating *Rbpj* null cells bypass S-phase demonstrates that DNA synthesis and mitosis are dispensable events in the transition from G_0 to permanent cell cycle exit. That this also occurs in a minor fraction of muscle stem cells in heterozygous mice warrants further investigation, also in other tissues. Some examples of direct transition from a cell cycle arrested to differentiation state have been described in lower eukaryotes [31, 32] and under certain conditions in cultured cell lines [33–35]. Our in vivo results, together with these reports, challenge the notion that at least one round of mitosis is required for the transition from an undifferentiated cell state to a fusion competent cell [36].

One question that arises from our observations is why the exit from quiescence is not accompanied by entry into the cell cycle. Is this due to Notch directly regulating the cell cycle machinery or is it a default fate acquired in the absence of proliferation signals in resting muscle? Notch signaling can act either to promote or block cell cycle progression depending on the cellular context [37]. Analysis of Notch null clones in the presence of strong mitogenic signals (the second mitotic wave of the *Drosophila* larva eye anlagen) clearly demonstrates that Notch is required to overcome the G_1–S checkpoint [38], consistent with our observations. In contrast, we show that higher Notch activity is critical for maintaining cells in a quiescent, noncycling state, suggesting that here Notch antagonizes cell cycle progression. These seemingly opposing actions of Notch can be reconciled if one presumes that in the context of resting satellite cells there are limited mitogenic cues. This notion is further supported by the fact that isolated *Rbpj* cKO satellite cells can actively proliferate after an induced trauma or when found in a stimulating milieu (i.e., cell culture conditions). Accordingly, a fraction of satellite cells that lose Notch activity in vivo do proliferate. We speculate that this divergent behavior is the outcome of a fine balance between proliferation and differentiation that is tuned by the relative environmental (e.g., vicinity to vessels or tendons) and cell intrinsic cues (e.g., levels of Hes/Hey), and possibly the levels of Notch activity.

Furthermore, distinct extrinsic and intrinsic properties could explain the diverse phenotypic consequences of loss of *Rbpj* function between muscle and neural stem cells, where the latter were reported to collectively enter the cell cycle and undergo transit amplification [12, 13]. Finally, stimulatory signals above those encountered during homeostasis, such as muscle trauma, override the maintenance of quiescence by Notch signaling. The delay in response to the loss of Rbpj may be due to the time required for the turnover of Hes/Hey effector proteins and/or the relative activity of parallel signaling pathways that maintain quiescence or stimulate proliferation. The intriguing observation that most *Rbpj* null satellite cells fail to undergo transit amplification in the absence of muscle injury indicates that this pathway plays a distinct role in regulating cell cycle kinetics during homeostasis.

Another issue which needs to be addressed in the future, is the nature of the quiescent state—in other words, is this an active cell state, or passive defined by the absence of proliferation or differentiation? The transition of satellite cells from G_0 to differentiation reported here, and their subsequent fusion to myofibers in the absence of Rbpj, argues in favor of a full cell transition taking place, and not simply a misfiring of some differentiation markers. Whether the role of Notch is to suppress differentiation or actively regulate cellular quiescence itself remains unclear. These questions await the identification of other regulators of cellular quiescence. Taken together, these findings point to a crucial role for Notch signaling in maintaining muscle stem cell quiescence and unveil an atypical and spontaneous G_0-to-differentiated cell transition in its absence.

Conclusion

We have developed a mouse model to study Notch signaling in quiescent adult muscle stem cells. Conditional deletion specifically in these cells of *Rbpj*, which is the major effector of Notch activity, results in the loss of cellular quiescence and spontaneous differentiation. This event leads to the depletion of the muscle stem cell pool and failure of tissue regeneration after muscle injury. Notably, abrogation of canonical Notch signaling favors an atypical G_0-to-differentiation transition and the bypass of S-phase. Taken together, this study establishes Notch signaling as the first factor regulating quiescence in muscle stem cells.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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