

Transiently Active Wnt/ β -Catenin Signaling Is Not Required but Must Be Silenced for Stem Cell Function during Muscle Regeneration

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SUMMARY

Adult muscle's exceptional capacity for regeneration is mediated by muscle stem cells, termed satellite cells. As with many stem cells, Wnt/ β -catenin signaling has been proposed to be critical in satellite cells during regeneration. Using new genetic reagents, we explicitly test *in vivo* whether Wnt/ β -catenin signaling is necessary and sufficient within satellite cells and their derivatives for regeneration. We find that signaling is transiently active in transit-amplifying myoblasts, but is not required for regeneration or satellite cell self-renewal. Instead, downregulation of transiently activated β -catenin is important to limit the regenerative response, as continuous regeneration is deleterious. Wnt/ β -catenin activation in adult satellite cells may simply be a vestige of their developmental lineage, in which β -catenin signaling is critical for fetal myogenesis. In the adult, surprisingly, we show that it is not activation but rather silencing of Wnt/ β -catenin signaling that is important for muscle regeneration.

INTRODUCTION

Adult vertebrate muscle has an exceptional capacity for regeneration, mediated by a dedicated population of muscle stem cells. These muscle stem cells, termed satellite cells, were first identified by their unique anatomical position between the sarcolemma and basement membrane of myofibers (Mauro, 1961). Subsequently, satellite cells were found to express the transcription factor Pax7 (Seale et al., 2000), and Pax7 is required for their maintenance in adult mice (Günther et al., 2013; Kuang et al., 2006; Oustanina et al., 2004; Relaix et al., 2006; von Maltzahn et al., 2013). Recent genetic labeling and ablation studies in mouse, using *Pax7^{CreER}* mice, have definitively established that satellite cells are the endogenous stem cells necessary and sufficient for muscle regeneration (Lepper et al., 2009, 2011; Murphy et al., 2011; Sambasivan et al., 2011). During regeneration, satellite cells activate, proliferate, and give rise to transit-amplifying myoblasts, which differentiate into myocytes that fuse with one another to form multinucleate myofibers. In addition, like other stem cells, satellite cells self-renew.

Canonical Wnt/ β -catenin signaling is an important regulator of many adult stem cells (Holland et al., 2013) and has been proposed to be critical for satellite cells and muscle regeneration. Wnts are secreted glycoproteins that function as ligands, and β -catenin is the central mediator of canonical Wnt signaling (Niehrs, 2012). In the absence of Wnts, β -catenin is phosphorylated and targeted for degradation. The binding of Wnts to their receptors leads to the formation of stabilized, unphosphorylated β -catenin

that translocates to the nucleus, where it binds to TCF/LEF proteins and activates transcription of Wnt-responsive genes. Many studies have identified Wnt pathway components as being active during muscle regeneration (Brack et al., 2008, 2009; Le Grand et al., 2009; Poleskaya et al., 2003; Zhao and Hoffman, 2004). Based largely on gain-of-function, primarily *in vitro* experiments, multiple labs have proposed that Wnt/ β -catenin signaling is essential for muscle regeneration, although the conclusions of these papers are often contradictory (reviewed in von Maltzahn et al., 2012). However, no studies have explicitly examined *in vivo* whether Wnt/ β -catenin signaling is necessary and sufficient specifically within satellite cells and their derivatives for muscle regeneration.

In this study, we use a highly sensitive reporter of Wnt/ β -catenin signaling (*TCF/Lef:H2B-GFP^{Tg}*; Ferrer-Vaquer et al., 2010), as well as a reagent (*Pax7^{CreERT2}*) that our lab has generated to genetically manipulate satellite cells with high specificity and efficiency (Murphy et al., 2011), to test the role of this signaling pathway specifically within satellite cells and their derivatives during muscle regeneration. We find that Wnt/ β -catenin signaling is transiently active in myoblasts during regeneration. However, β -catenin is not required cell autonomously for muscle regeneration. Instead, downregulation of transiently activated β -catenin is critical for limiting the regenerative response, as continuous regeneration deleteriously leads to increased fibrosis and an increased number of small myofibers. Thus, surprisingly, we show that it is not activation of Wnt/ β -catenin signaling but rather silencing of this activation that is important for muscle regeneration.

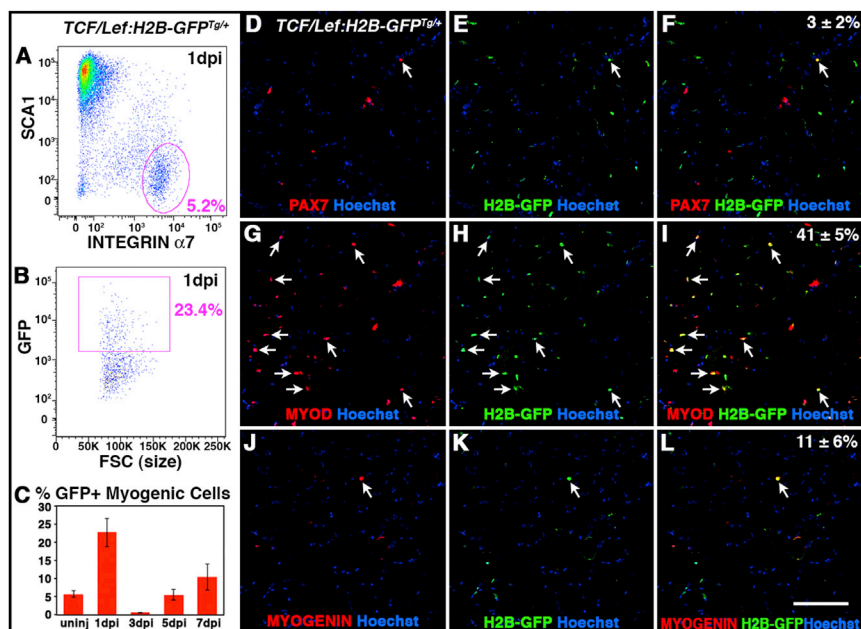


Figure 1. Wnt/ β -Catenin Signaling Is Transiently Active in Myoblasts after Injury

(A–C) Mononuclear myogenic cells (A) transiently express *TCF/Lef:H2B-GFP* reporter at 1 dpi ($n = 3$ mice for each time point; B and C).

(D–L) At 1 dpi, only 3% of satellite cells (D–F) but 41% of myoblasts (G–I) and 11% of myocytes (J–L) are GFP+ ($n = 3$ mice). Arrows show GFP+ cells. The scale bar represents 100 μ m.

Error bars in (C) represent one SEM.

RESULTS

Wnt/ β -Catenin Signaling Is Transiently Active in Myoblasts during Regeneration

Multiple studies have established that Wnts, Frizzled receptors, nuclear β -catenin, coactivator BCL9, TCF/LEF reporters, and also Wnt antagonists secreted Frizzled-related proteins (sFRPs) are expressed during muscle regeneration (Brack et al., 2007, 2008; Le Grand et al., 2009; Otto et al., 2008; Poleskaya et al., 2003; Zhao and Hoffman, 2004). However, activation of Wnt/ β -catenin signaling has not been explicitly tested and quantified within the myogenic lineage in vivo during the time course of regeneration. To test whether and when Wnt/ β -catenin signaling is active in myogenic cells, we used the sensitive *TCF/Lef:H2B-GFP^{Tg}* reporter (Ferrer-Vaquero et al., 2010), in which cells with active Wnt/ β -catenin signaling express nuclear localized GFP. To determine the percentage of myogenic cells with active Wnt/ β -catenin signaling during regeneration, the right tibialis anterior (TA) muscles of *TCF/Lef:H2B-GFP^{Tg/+}* mice were injured via BaCl₂ injection (Caldwell et al., 1990), injured TAs (and uninjured control TAs) collected at different days postinjury (dpi), and mononuclear myogenic cells analyzed via fluorescence-activated cell sorting (FACS). CD31-CD45-SCA1-INTEGRIN α 7+ cells were identified as myogenic (Yi and Rossi, 2011) and include satellite cells, myoblasts, and potentially myocytes (Figure 1A). In uninjured muscle, an average of 6% of myogenic cells was GFP+, indicating that Wnt/ β -catenin is active in few myogenic cells (Figures 1B and 1C). However, at 1 dpi, 23% of myogenic cells were GFP+, although

this declines to 0.6% by 3 dpi. To determine in which myogenic cells Wnt/ β -catenin signaling is transiently active, we analyzed sections of TAs from *TCF/Lef:H2B-GFP^{Tg/+}* mice at 1 dpi via immunofluorescence (Figures 1D–1L). Whereas only 3% of PAX7+ satellite cells and 11% of MYOGENIN+ myocytes were GFP+, 41% of MYOD+ cells were GFP+. MYOD+ cells may be either activated PAX7+MYOD+ satellite cells or PAX7–MYOD+ myoblasts. Because few PAX7+ cells were GFP+, we interpret the GFP+MYOD+ cells to be myoblasts. Thus we find that Wnt/ β -catenin signaling is transiently active during muscle regeneration at 1 dpi, particularly in myoblasts.

Canonical Wnt/ β -Catenin Signaling Is Effectively Abrogated in Satellite Cells and Their Progeny in *Pax7^{CreERT2/+}; β -Catenin ^{Δ /fl2-6}* Mice

Our analysis of *TCF/Lef:H2B-GFP^{Tg/+}* mice demonstrates that Wnt/ β -catenin signaling is transiently active in myoblasts during muscle regeneration. To test whether Wnt/ β -catenin signaling is necessary specifically within myogenic cells for regeneration, we conditionally deleted β -catenin in satellite cells using *Pax7^{CreERT2/+}; β -catenin ^{Δ /fl2-6}; Rosa^{mTmG/+}* mice. In *Pax7^{CreERT2}* mice, Cre-mediated recombination occurs specifically and efficiently (>94% recombination) in *Pax7*+ satellite cells after delivery of tamoxifen (TAM) (Murphy et al., 2011). The β -catenin loss-of-function allele creates a functional null following Cre-mediated deletion of exons 2–6, thus inactivating signaling (Brault et al., 2001). The fate of recombined cells was tracked via the *Rosa^{mTmG}* reporter, which ubiquitously expresses membrane-bound *Tomato* until Cre-mediated recombination excises *Tomato*,



resulting in membrane-bound GFP expression (Muzumdar et al., 2007). We analyzed $Pax7^{CreERT2/+};\beta\text{-catenin}^{\Delta/f12-6};Rosa^{mTmG/+}$ mice and compared them to $Pax7^{CreERT2/+};\beta\text{-catenin}^{\Delta/+};Rosa^{mTmG/+}$ mice to control for any possible $\beta\text{-catenin}$ heterozygous phenotype. Satellite cells are the only cells that express $Pax7$ in uninjured muscle (Murphy et al., 2011). Therefore, by delivering TAM before injury, in control $Pax7^{CreERT2/+};\beta\text{-catenin}^{\Delta/+};Rosa^{mTmG/+}$ mice, nearly all satellite cells and their progeny express GFP and all are heterozygous for $\beta\text{-catenin}$, whereas in mutant $Pax7^{CreERT2/+};\beta\text{-catenin}^{\Delta/f12-6};Rosa^{mTmG/+}$ mice, nearly all satellite cells and their progeny express GFP and are null for $\beta\text{-catenin}$.

We tested whether $\beta\text{-catenin}$ was efficiently and completely deleted in satellite cells and their progeny in $Pax7^{CreERT2/+};\beta\text{-catenin}^{\Delta/f12-6};Rosa^{mTmG/+}$ mice. To test that exons 2–6 of $\beta\text{-catenin}$ were genetically deleted, we isolated by FACS nonmyogenic TOMATO+ and myogenic GFP+ cells from TAs of control and mutant mice given five 10 mg doses of TAM, injured via $BaCl_2$, and harvested at 5 dpi (when there are maximal number of satellite cells; Murphy et al., 2011). We isolated genomic DNA and used PCR to identify $\beta\text{-catenin}$ wild-type (WT), $f12-6$, and $\Delta 2-6$ alleles (Brault et al., 2001). In control mice, TOMATO+ and GFP+ cells were positive for both WT and $\Delta 2-6$ alleles (Figure S1A available online). In contrast, in mutant mice, TOMATO+ cells contained both $f12-6$ and $\Delta 2-6$ alleles, whereas myogenic GFP+ cells contained only the $\Delta 2-6$ allele (Figure S1A). To test that genetic loss led to loss of $\beta\text{-catenin}$ protein, we analyzed $\beta\text{-catenin}$ protein expression in nonmyogenic TOMATO+ and myogenic GFP+ cells FACS isolated from TAs 5 dpi of control and mutant mice. Whereas $\beta\text{-catenin}$ was detectable in 23% and 17% of nonmyogenic TOMATO+ cells in control and mutant mice, respectively, its detection in myogenic GFP+ cells dropped from 76% in control to 1% in mutant mice (Figures S1C and S1D). Together, these experiments indicate that $\beta\text{-catenin}$ is effectively deleted in satellite cell-derived myogenic cells in $Pax7^{CreERT2/+};\beta\text{-catenin}^{\Delta/f12-6};Rosa^{mTmG/+}$ mice in response to TAM.

We next tested whether Wnt/ β -catenin signaling was effectively abrogated in $Pax7^{CreERT2/+};\beta\text{-catenin}^{\Delta/f12-6}$ mice. To do this, we generated control $Pax7^{CreERT2/+};\beta\text{-catenin}^{+/+};TCF/Lef:H2B-GFP^{Tg/+}$ and mutant $Pax7^{CreERT2/+};\beta\text{-catenin}^{\Delta/f12-6};TCF/Lef:H2B-GFP^{Tg/+}$ mice, gave them five TAM doses, injured TAs, and harvested muscle 1 dpi (when $TCF/Lef:H2B-GFP$ reporter levels are highest in myogenic cells). CD31-CD45-SCA1-INTEGRIN $\alpha 7$ + myogenic cells were isolated by FACS from control and mutant mice and analyzed for GFP. Whereas 25% of control myogenic cells were GFP+, only 2.5% of mutant myogenic cells were GFP+, indicating that, by 1 dpi, canonical Wnt/ β -catenin signaling is nearly completely abolished (Figures S1E and S1F). To further determine whether $Pax7^{CreERT2/+};\beta\text{-catenin}^{\Delta/f12-6}$ mice effec-

tively abolished Wnt/ β -catenin signaling in muscle, we analyzed these mice during development. We have previously shown, using $Pax7^{Cre/+};\beta\text{-catenin}^{\Delta/f12-6}$ mice, that $\beta\text{-catenin}$ regulates the number and slow fiber type of fetal myofibers (Hutcheson et al., 2009). If $Pax7^{CreERT2/+};\beta\text{-catenin}^{\Delta/f12-6}$ mice work as effectively, fetal mice given TAM during development should demonstrate a similar phenotype. To test this, timed pregnant dams were given TAM (E11.5, E13.5, and E15.5), pups harvested at E18.5, and hind limbs sectioned and analyzed as before (Hutcheson et al., 2009). We found that, similar to $Pax7^{Cre/+};\beta\text{-catenin}^{\Delta/f12-6}$, in $Pax7^{CreERT2/+};\beta\text{-catenin}^{\Delta/f12-6}$ mice, there were fewer myofibers and a loss of slow myofibers in many muscles, particularly in the soleus (Figures S2A and S2B). Thus, $Pax7^{CreERT2/+};\beta\text{-catenin}^{\Delta/f12-6}$ mice effectively abrogate Wnt/ β -catenin signaling in myogenic cells and recapitulate phenotypes previously reported for fetal myogenesis.

$\beta\text{-catenin}$ Is Not Required for Satellite Cells to Regenerate Muscle or to Self-Renew

Having established that $Pax7^{CreERT2/+};\beta\text{-catenin}^{\Delta/f12-6};Rosa^{mTmG/+}$ mice, upon TAM delivery, effectively abrogate $\beta\text{-catenin}$ signaling in myogenic cells, we tested whether satellite cells and their progeny require $\beta\text{-catenin}$ to regenerate muscle. $Pax7^{CreERT2/+};\beta\text{-catenin}^{\Delta/f12-6};Rosa^{mTmG/+}$ and littermate control $Pax7^{CreERT2/+};\beta\text{-catenin}^{\Delta/+};Rosa^{mTmG/+}$ mice were given five doses of TAM and then the right TA injured via $BaCl_2$. $BaCl_2$ injury induces a stereotyped pattern of muscle regeneration, with the peak of number of satellite cells and regenerating myofibers 5 dpi and regeneration complete by 28 dpi (Murphy et al., 2011). We found that, at 5 dpi, there was no difference between mutant and control muscle in either the number or proliferation of PAX7+ satellite cells (Figure 2A). Satellite cells give rise to MYOD+ cells, and the peak number of MYOD+ cells after $BaCl_2$ injury occurs at 3 dpi (Murphy et al., 2011; M.M.M. and G.K., unpublished data). Surprisingly, despite activation of the $TCF/Lef:H2B-GFP^{Tg/+}$ reporter in a large number of MYOD+ cells (see above), $\beta\text{-catenin}$ deletion did not alter the number or proliferation of MYOD+ cells (Figure 2B). Myoblasts differentiate into myocytes, and these myocytes fuse into regenerating myofibers, characterized by their expression of embryonic myosin heavy chain (MyHCemb), an immature form of MyHC replaced by slow (MyHCI) and fast isoforms (MyHCII) as nascent myofibers mature. However, we found no difference in the amount of MyHCemb between mutant and control muscle at 5 dpi (Figure 2C), indicating loss of $\beta\text{-catenin}$ does not affect regeneration of new myofibers. At 28 dpi, there continued to be no deleterious effect on muscle stem cells or regeneration. To determine whether $\beta\text{-catenin}$ is required for satellite cells to self-renew and

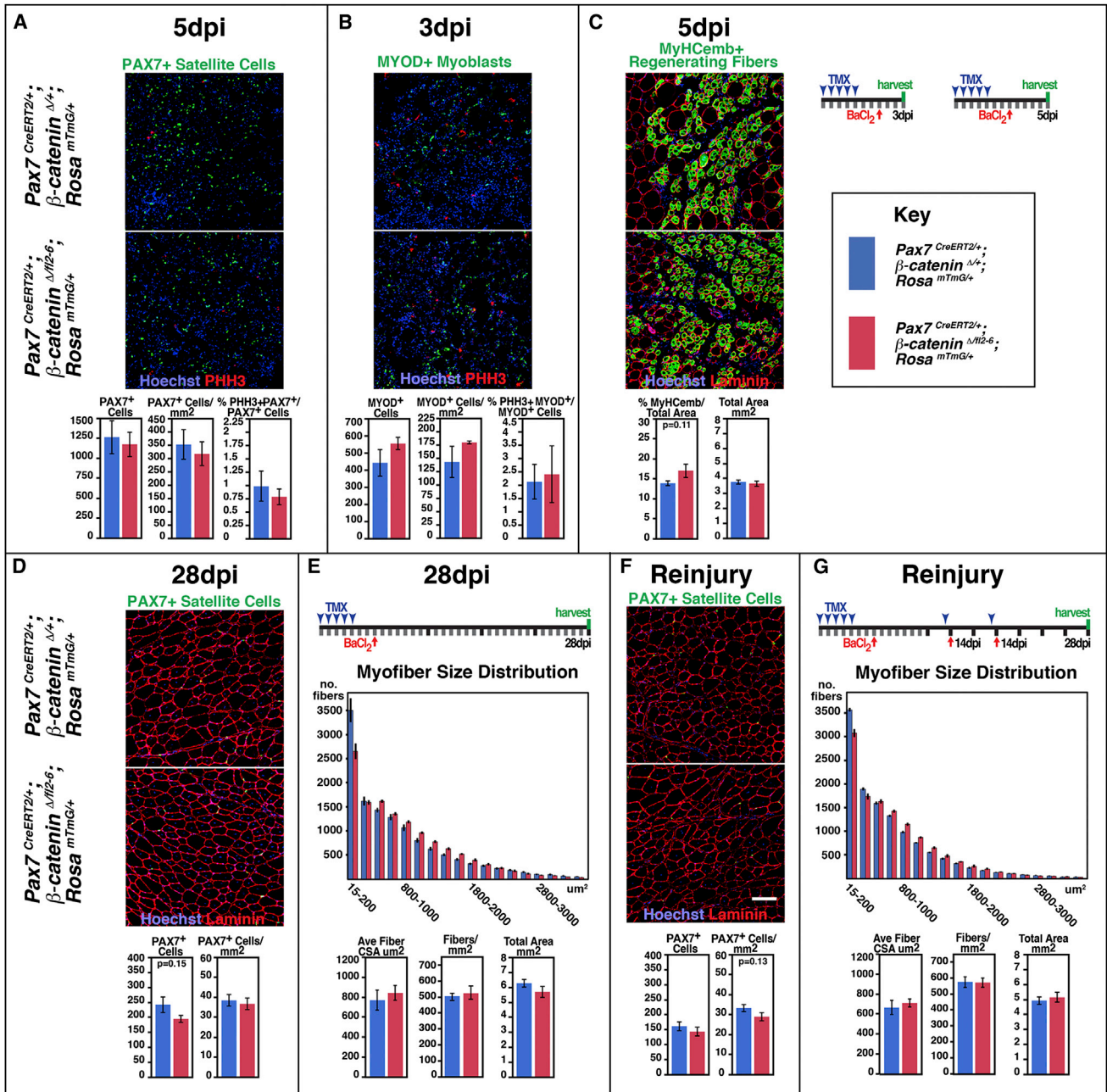


Figure 2. β -Catenin Is Not Required for Satellite Cells to Regenerate Muscle or Self-Renew

At 3 dpi (B; n = 3 control; n = 3 mutant), 5 dpi (A and C; n = 5 control; n = 5 mutant), and 28 dpi (D–G; n = 6 control; n = 5 mutant mice), β -catenin deletion does not affect number or proliferation of satellite cells (A and D), number or proliferation of myoblasts (B), amount of regenerating myofibers (C), or total muscle CSA (C and E). (F and G) After three rounds of injury (n = 5 control; n = 5 mutant mice), β -catenin deletion does not alter satellite cell self-renewal (F) or total muscle CSA (G). (E and G) At 28 dpi and after reinjury, myofibers are shifted to larger CSA when β -catenin is deleted. The scale bar (F) for all panels represents 100 μ m. See also Figures S1–S3. Error bars in all histograms represent one SEM.

return to their niche, we compared the number of PAX7+ satellite cells between mutant and control mice at 28 dpi. However, neither the number nor location of satellite cells within their niche beneath the myofibers' LAMININ+ basal

lamina differed (Figure 2D). In addition, regeneration was also unaffected, as neither the average myofiber cross-sectional area (CSA) nor the number of myofibers was affected by β -catenin deletion (Figure 2E). Interestingly,

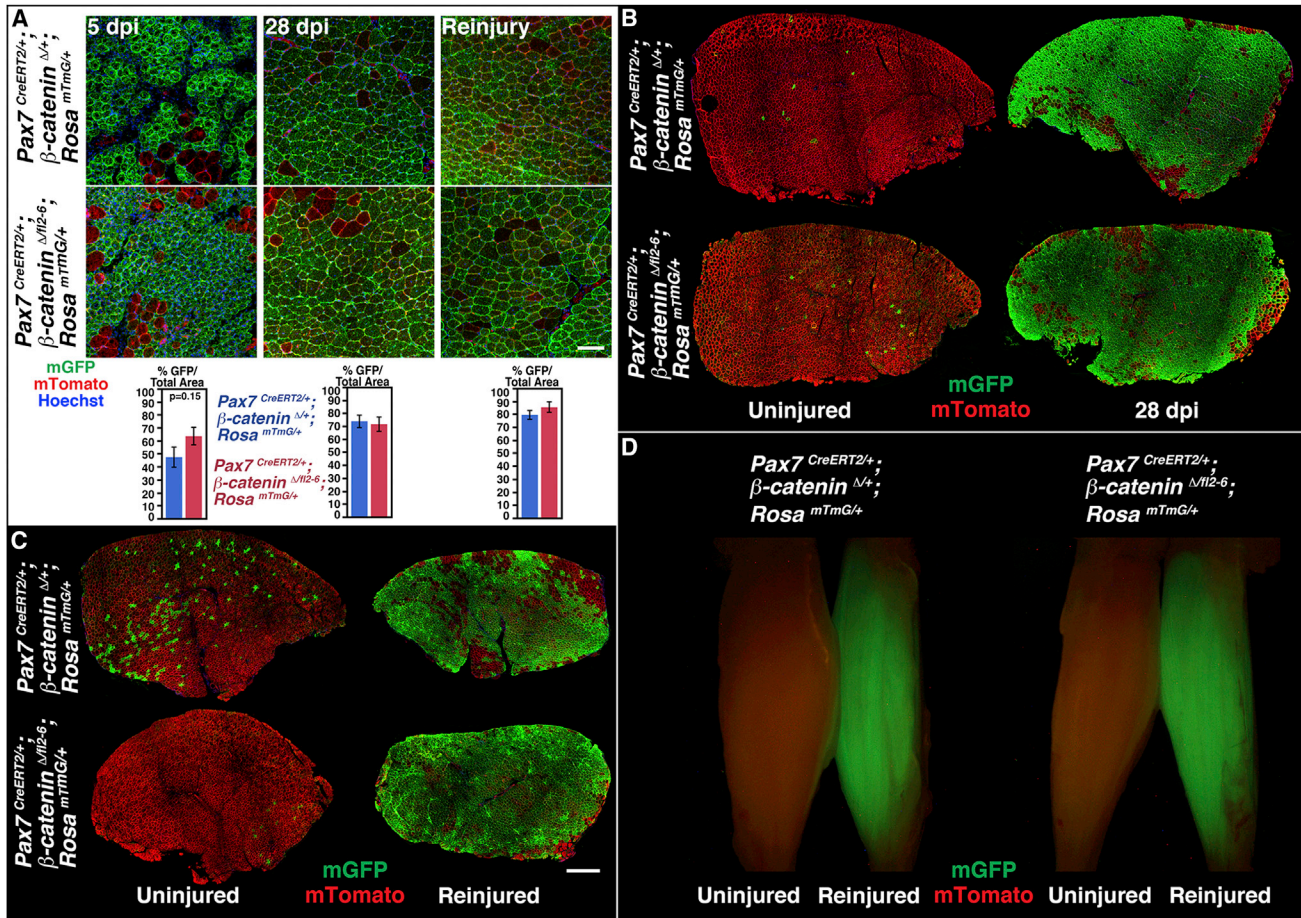


Figure 3. Loss of β -Catenin Does Not Affect Satellite Cell Contribution to Regenerated Myofibers

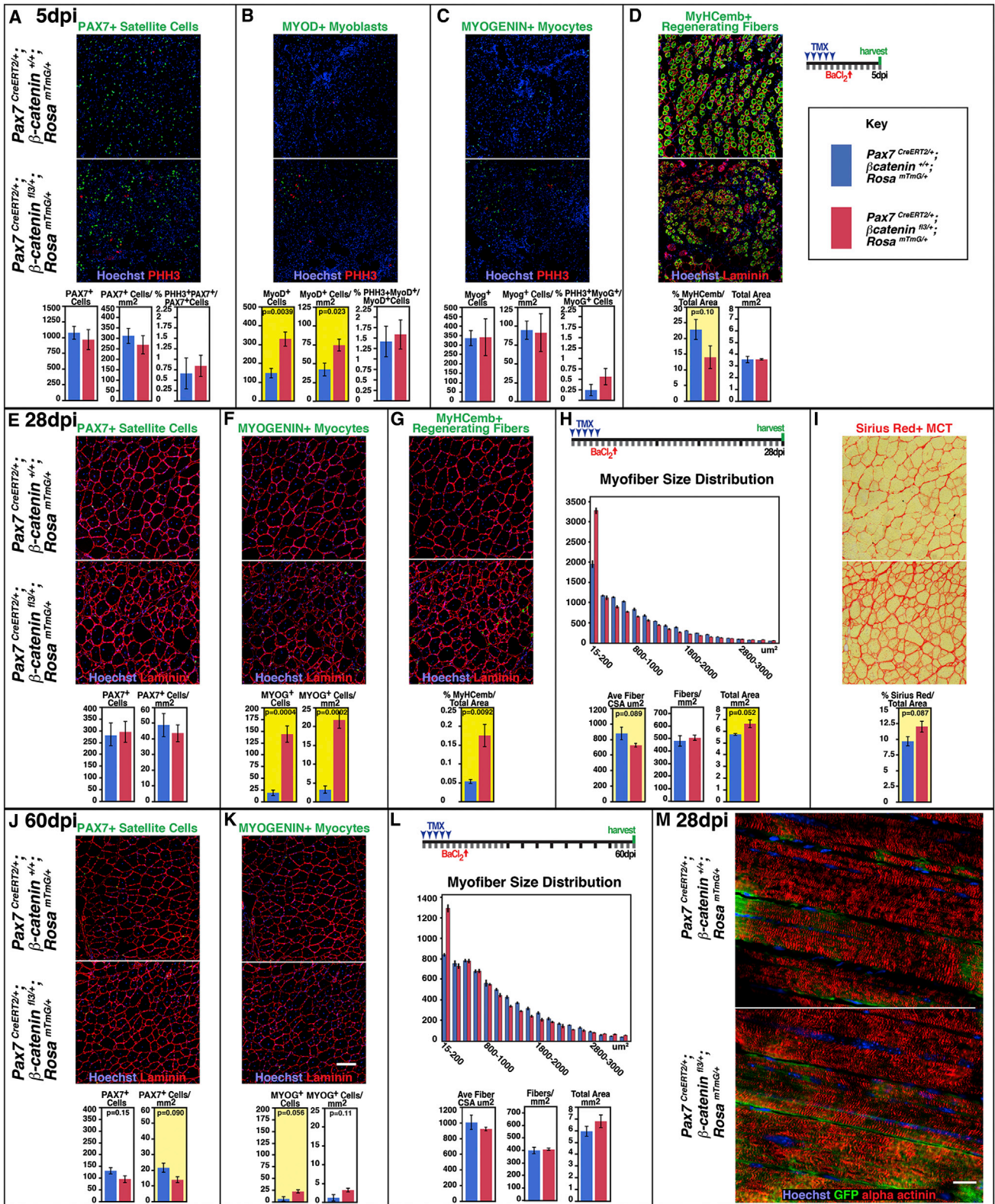
At 5 dpi (A; n = 5 control; n = 5 mutant), 28 dpi (A and B; n = 6 control; n = 5 mutant mice), or after reinjury (A, C, and D; n = 5 control; n = 5 mutant mice), β -catenin-null satellite cells regenerate GFP+ myofibers. Sections through entire contralateral and injured TA and extensor digitorum longus (EDL) muscles (B and C) and whole-mount images of contralateral and reinjured TAs (D). The scale bars represent 100 μ m (A) or 0.5 mm (B and C). See also Figures S1–S3. Error bars in (A) represent one SEM.

the distribution of the CSA of individual myofibers shifted to larger sizes with β -catenin deletion, but the overall area of the TA muscle was unaffected (Figure 2E). In summary, loss of β -catenin has no deleterious effect on the ability of satellite cells to self-renew, activate, proliferate, differentiate into myoblasts, or regenerate myofibers.

Although our data indicate that β -catenin is not required within myogenic cells for muscle regeneration, potentially the function of β -catenin may only be uncovered after multiple rounds of regeneration. To test this, we successively injured the TA and allowed it to regenerate three times (strategy in Figure 2G). Even after repeated rounds of regeneration, we detected no difference between mutant and control mice in satellite cell self-renewal, average myofiber CSA, or number of regenerated myofibers (Figures 2F and 2G). Similar to our findings at 28 dpi, we observed that

the CSA of individual myofibers was shifted to larger sizes with loss of β -catenin, although the overall area of the TA was not changed (Figure 2G). Thus, β -catenin is not required in myogenic cells to regenerate muscle even after multiple rounds of regeneration.

A possible explanation for the lack of a defect in muscle regeneration with deletion of β -catenin may be technical issues with the *Pax7*^{CreERT2/+};*β-catenin*^{Δ/12-6};*Rosa*^{mTmG/+} mice. As satellite cells are highly proliferative, a few nonrecombined “escaper” satellite cells, retaining one wild-type allele of *β-catenin*, could potentially outcompete *β-catenin*-null cells and regenerate muscle. To test this, we compared the amount of GFP, representative of the contribution of satellite cells, in muscle from mutant and control mice and found no difference in GFP at 5 or 28 dpi or after multiple rounds of reinjury (Figure 3). Recently, it has been



(legend on next page)



shown that continuous administration of TAM during muscle regeneration may be required to completely delete a gene of interest in satellite cells (Günther et al., 2013). We repeated our experiments injuring TA muscles of mutant and control mice but with continuous TAM administration (strategy in Figure S3C) and analyzed muscles at 28 dpi. Similar to our previous results, we found no difference in satellite cell self-renewal or their contribution to regeneration (Figures S3A, S3B, and S3D). As we saw previously, the distribution of the CSA of individual myofibers was shifted to larger sizes with loss of β -catenin but now resulted in a slight increase (but not significant; $p = 0.07$) in the average CSA of myofibers and a slight decrease (but not significant; $p = 0.08$) in the number of myofibers, although the overall CSA of the muscle was unaffected (Figure S3C). Thus, we show in $Pax7^{CreERT2/+};\beta\text{-catenin}^{fl3/+};Rosa^{mTmG/+}$ mice that satellite cells are able, despite loss of β -catenin, to effectively regenerate muscle. Also, the finding that two different stringent TAM strategies give similar results argues that the lack of a deleterious phenotype is unlikely to be a false-negative result.

In summary, we show by in vivo conditional deletion of β -catenin that, despite activation of Wnt/ β -catenin signaling within myogenic cells, β -catenin is not required within satellite cells or their derivatives for muscle regeneration or satellite cell self-renewal.

Constitutive Activation of β -Catenin in Satellite Cells Alters Myoblast Kinetics, Resulting in a Prolonged Regenerative Response

Our experiments demonstrate that, although β -catenin is not required, Wnt/ β -catenin signaling is transiently active in myogenic cells during muscle regeneration. This presents an alternative hypothesis: whereas Wnt/ β -catenin signaling is not required, once activated, prompt downregulation of signaling may be important for proper muscle regeneration. To test this, we constitutively activated β -catenin in satellite cells and their derivatives and assayed for effects on muscle regeneration.

To constitutively activate β -catenin, we used $Pax7^{CreERT2/+};\beta\text{-catenin}^{fl3/+};Rosa^{mTmG/+}$ mice. In the $\beta\text{-catenin}^{fl3}$ allele, Cre mediates deletion of exon 3 and the formation of a stabi-

lized, constitutively active form of β -catenin (Harada et al., 1999). We confirmed that GFP expression reflects recombination in the $\beta\text{-catenin}$ locus by isolating by FACS GFP+ myogenic and TOMATO+ nonmyogenic cells from TA muscles of control $Pax7^{CreERT2/+};\beta\text{-catenin}^{+/+};Rosa^{mTmG/+}$ and mutant $Pax7^{CreERT2/+};\beta\text{-catenin}^{fl3/+};Rosa^{mTmG/+}$ mice given five TAM doses, injured, and harvested 5 dpi. Using genomic DNA and PCR, we found in control mice both GFP+ and TOMATO+ cells contained only the WT allele (Figure S1B). In mutant mice, TOMATO+ cells contained both the $fl3$ and WT alleles, whereas GFP+ cells had only the WT allele because the primer-binding sites for the $fl3$ allele were deleted by recombination (Figure S1B). Therefore, after TAM delivery to $Pax7^{CreERT2/+};\beta\text{-catenin}^{fl3/+};Rosa^{mTmG/+}$ mice, GFP+ myogenic cells constitutively activate β -catenin.

We examined whether constitutive activation of β -catenin affected the expansion or self-renewal of satellite cells during muscle regeneration. Comparison of mutant $Pax7^{CreERT2/+};\beta\text{-catenin}^{fl3/+};Rosa^{mTmG/+}$ with littermate control $Pax7^{CreERT2/+};\beta\text{-catenin}^{+/+};Rosa^{mTmG/+}$ mice revealed that, at 5 dpi, when the number of satellite cells peaks, there was no difference in the number or proliferation of PAX7+ satellite cells with constitutive β -catenin activation (Figure 4A). At 28 dpi, when muscle regeneration is complete, there was no difference in the number of satellite cells that had self-renewed (Figure 4E), although at 60 dpi, there was a slight (but not significant; $p = 0.09$) decrease in satellite cells with constitutive β -catenin activation (Figure 4J). Thus, constitutive β -catenin activation did not alter the expansion of satellite cells or significantly impair their return to the niche during regeneration.

The transient activation of Wnt/ β -catenin signaling in myoblasts suggests that β -catenin may regulate myoblast expansion or differentiation during regeneration. Normally, the number of MYOD+ cells peaks at 3 dpi and declines by 5 dpi, and MYOD+ cells are absent at 28 dpi (Figures 2B and 4B; Murphy and Kardon, 2011; data not shown). At 5 dpi, there was a significant 1.79-fold increase in the number of MYOD+ cells ($p = 0.02$) with constitutive β -catenin activation, although there was no difference in proliferation of these cells (Figure 4B). There was no difference in either the number or proliferation of MYOGENIN+

Figure 4. Constitutive Activation of β -Catenin Alters the Kinetics of Myoblast Differentiation, Resulting in a Prolonged Regenerative Response

(A–D) At 5 dpi ($n = 6$ control; $n = 6$ mutant), constitutive β -catenin activation does not alter expansion or proliferation of satellite cells (A) but increases the number of myoblasts (B) at the expense of regenerating myofibers (D).

(E–I) At 28 dpi ($n = 4$ control; $n = 5$ mutant mice), β -catenin activation causes continued presence of myocytes (F) and regenerating myofibers (G), smaller regenerating myofibers (H), unresolved fibrosis (I), and results in TAs with larger CSA (H).

(J–L) At 60 dpi ($n = 3$ control; $n = 3$ mutant mice), with β -catenin activation, myocytes (K) and smaller myofibers (L) are still present. (M) β -catenin activation does not alter sarcomere structure of regenerated myofibers ($n = 3$ control; $n = 3$ mutant mice).

The scale bar in (K) for sections (A–C), (E–G), and (I–K) represents 100 μm . The scale bar in (M) represents 10 μm . See also Figure S1. Error bars in all histograms represent one SEM.

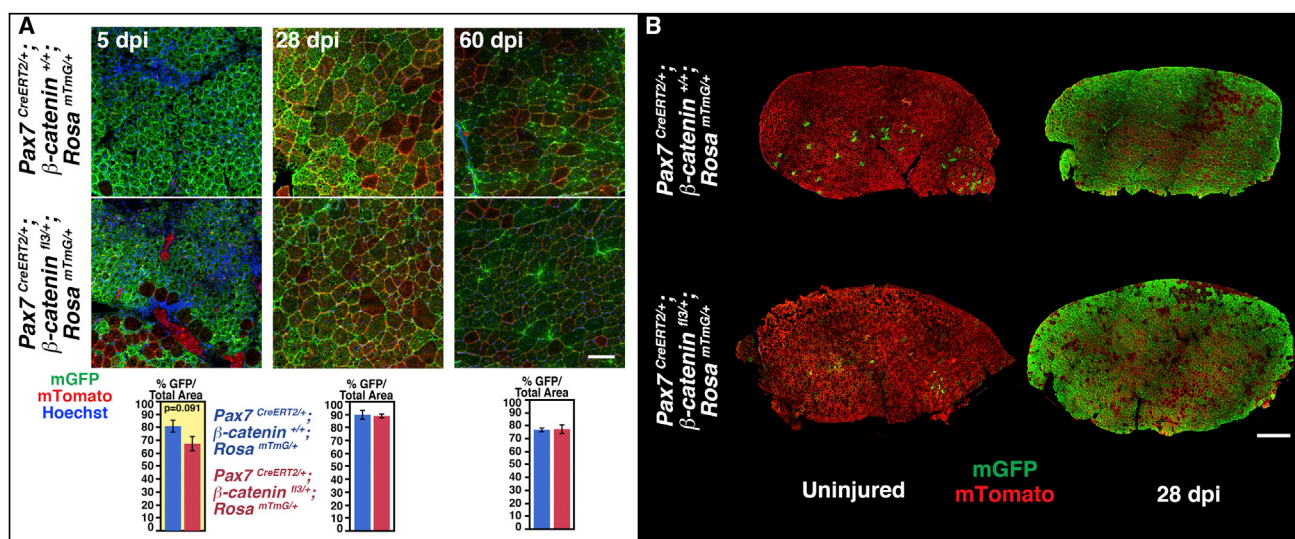


Figure 5. Constitutive Activation of β -Catenin Does Not Affect Satellite Cell Contribution to Regenerated Myofibers

At 5 dpi (A; n = 6 control; n = 6 mutant) or 28 dpi (A and B; n = 4 control; n = 5 mutant mice), satellite cells with constitutive β -catenin regenerate GFP+ myofibers. Sections through entire contralateral and injured TA and EDL muscles (B). The scale bars represent 100 μ m (A) or 0.5 mm (B). See also Figure S1. Error bars in (A) represent one SEM.

myocytes (Figure 4C), but there was a slight decrease (high variance precluded significance; $p = 0.10$) in the amount of MyHCemb+-regenerating myofibers (Figure 4D). The lack of change in PAX7+ cells, increased number of MYOD+ cells, and decreased area of MyHCemb+-regenerating myofibers suggest that constitutive activation of β -catenin prolongs the time that myogenic cells remain as MYOD+ myoblasts.

To test if constitutive activation of β -catenin blocks myofiber differentiation, we examined TAs at 28 dpi, when regeneration is normally complete. No MYOD was expressed in either *Pax7^{CreERT2/+}; β -catenin^{fl3/+};*Rosa^{mTmG/+}* or *Pax7^{CreERT2/+}; β -catenin^{+/+};*Rosa^{mTmG/+}* mice (data not shown), and so the MYOD+ myoblasts present at 5 dpi do not remain in an undifferentiated state. In control mice, few MYOGENIN+ myocytes or actively regenerating MyHCemb+ myofibers remained at this time point, but in *Pax7^{CreERT2/+}; β -catenin^{fl3/+};*Rosa^{mTmG/+}* mice, there was a 6.8-fold increase in myocytes ($p = 0.008$) and a 3.6-fold increase in regenerating myofibers ($p = 0.009$; Figures 4F and 4G). This increase in regenerating myofibers was reflected in a shift to smaller myofibers (Figure 4H). Interestingly, the total CSA of the TAs was significantly increased with constitutive β -catenin activation ($p = 0.05$; Figure 4H). This increased muscle size may partially result from a slight increase in Sirius Red+ connective tissue (but not significant; $p = 0.09$; Figure 4I), and this increase in fibrosis may reflect that the regenerative response is ongoing. In total, these data show that, at 28 dpi, when muscle regeneration is normally complete, constitutive activation of β -catenin leads to a prolonged***

regenerative response, which is reflected in the continued presence of myocytes, actively regenerating myofibers and unresolved fibrosis.

We examined regenerated TAs at 60 dpi to test whether constitutive β -catenin activation had long-term effects on muscle regeneration. We found there was a 2.7-fold increase in MYOGENIN+ myocytes in *Pax7^{CreERT2/+}; β -catenin^{fl3/+};*Rosa^{mTmG/+}* compared with control mice ($p = 0.05$; Figure 4K). Although there was no expression of MyHCemb in either genotype (data not shown), there was an increased number of smaller (likely newly regenerated) myofibers (Figure 4L). Thus, even at 60 dpi, the regenerative response is ongoing.*

Potentially, constitutive β -catenin activation could prevent myogenic cells from regenerating muscle, and all regenerated muscle could result from a small population of nonrecombined escaper cells. To test this, we compared GFP expression (which reflects constitutive β -catenin activation) in *Pax7^{CreERT2/+}; β -catenin^{fl3/+};*Rosa^{mTmG/+}* and control *Pax7^{CreERT2/+}; β -catenin^{+/+};*Rosa^{mTmG/+}* mice. At 5 dpi, there was slightly less GFP expression in mutant mice (but not significant; $p = 0.09$; Figure 5A), but this likely reflects the decrease in regenerated myofibers (Figure 4D). However, at 28 and 60 dpi, there was no difference in GFP expression between mutant and control mice, as nearly all regenerated myofibers with centralized myonuclei were GFP+ (Figures 5A and 5B). This demonstrates that, whereas constitutive activation of β -catenin prolongs the regenerative response, ultimately it does not prevent myogenic cells from regenerating muscle.**



β -catenin is also a member of the adherens junction complex and localizes to the membrane of muscle fibers, and its overexpression can cause muscle structural defects (Kramerova et al., 2006; Nastasi et al., 2004). Thus, constitutive β -catenin activation in myogenic cells might cause muscle structural defects. To test this, we analyzed myofibers from *Pax7^{CreERT2/+}; β -catenin^{fl3/+};Rosa^{mTmG/+}* mice 4 weeks after injury. However, analysis of regenerated GFP+ myofibers for sarcomere structure did not reveal any obvious defects (Figure 4M).

In summary, constitutive activation of β -catenin in satellite cells and their derivatives prolongs the regenerative response to muscle injury. Whereas constitutive β -catenin activation does not prevent muscle regeneration or affect sarcomere structure of regenerated myofibers, it significantly affects the kinetics of muscle regeneration. Activation and proliferation of satellite cells is unaltered, but myoblasts and subsequently myocytes and smaller regenerating myofibers are present for an extended period, long after regeneration is normally complete.

β -Catenin Is Not Necessary but Is Sufficient to Regulate Slow Myofiber Type during Muscle Regeneration

β -catenin has been implicated in the determination of muscle fiber type. Previously, we showed that β -catenin is a necessary and sufficient positive regulator of the differentiation of slow MyHCI+ myofibers during fetal myogenesis (Hutcheson et al., 2009; Figure S2). Therefore, we tested in vivo whether β -catenin regulates the differentiation of slow myofibers during muscle regeneration. However, examination of mutant *Pax7^{CreERT2/+}; β -catenin ^{Δ /fl2-6};Rosa^{mTmG/+}* and control *Pax7^{CreERT2/+}; β -catenin ^{Δ /+};Rosa^{mTmG/+}* mice revealed no difference in MyHCI expression at either 5 or 28 dpi (Figures 6A and 6B). In satellite cell-derived C2C12 cells, expression of fast MyHCIb is directly regulated by β -catenin binding, via TCF/LEF, to the MyHCIb promoter (Shanely et al., 2009). Again, examination of mutant and control mice revealed no difference in MyHCIb expression at 28 dpi (Figure 6C). Because constitutive β -catenin is sufficient to drive all fetal myogenic progenitors to differentiate into slow MyHCI+ myofibers (Hutcheson et al., 2009), we tested whether constitutive β -catenin activation in satellite cells would have a similar effect on regenerated myofibers. We found a 4.5-fold increase in MyHCI expression ($p = 0.0004$) in *Pax7^{CreERT2/+}; β -catenin^{fl3/+};Rosa^{mTmG/+}* versus *Pax7^{CreERT2/+}; β -catenin^{+/+};Rosa^{mTmG/+}* mice at 5 dpi (Figure 4D). At 28 dpi, there continued to be a 4-fold increase in MyHCI expression ($p = 0.009$; Figure 4E) with constitutive β -catenin activation, and this effect was somewhat maintained at 60 dpi (but not significant; $p = 0.09$; Figure 6F). However, whereas only a small percentage of the myofibers are MyHCI+, 80%–90% of the myofibers are GFP+ and satellite cell derived (Figure 5A). This small

number of MyHCI+ myofibers may reflect an incomplete conversion to a slow fiber type or that nerve-derived signals significantly modulate fiber type. Altogether, our data demonstrate that, during adult muscle regeneration, β -catenin is not necessary for differentiation of slow MyHCI+ or fast MyHCIb+ myofibers. However, β -catenin is sufficient to cell autonomously positively regulate MyHCI expression but, unlike during fetal myogenesis, cannot convert all myofibers to a slow MyHCI+ fiber type during regeneration.

DISCUSSION

Wnt/ β -catenin signaling has been proposed to be critical for adult muscle regeneration (reviewed in von Maltzahn et al., 2012). Here, we explicitly test the role of this signaling pathway specifically within satellite cells and their derivatives during muscle regeneration in vivo. We find that Wnt/ β -catenin signaling is transiently active in myoblasts during muscle regeneration. However, unlike previous studies, we find that β -catenin is not required in myogenic cells for regeneration, but instead downregulation of transiently activated β -catenin is critical for limiting the regenerative response. Thus, we show that it is not activation but rather silencing of Wnt/ β -catenin signaling that is important for muscle regeneration (summarized in Figure 7).

Using the highly sensitive *TCF/Lef:H2B-GFP^{T8}* reporter, we demonstrate that Wnt/ β -catenin signaling is transiently active during muscle regeneration, specifically in myoblasts 1 dpi. Our finding that myoblasts transiently transduce Wnt/ β -catenin signals agrees with previous analyses of nuclear β -catenin and *TOPGAL* reporter expression (Brack et al., 2007, 2008). Results from others (Abiola et al., 2009; Le Grand et al., 2009; Polesskaya et al., 2003; Zhao and Hoffman, 2004) show that Wnts are upregulated during regeneration, although the cellular origin of these Wnts is unresolved. The Wnt antagonists, sFRPs 1, 2, and 4 are also strongly upregulated during regeneration (Le Grand et al., 2009; Zhao and Hoffman, 2004), and this likely is the endogenous molecular mechanism by which Wnt/ β -catenin signaling, activated at 1 dpi, is subsequently silenced.

We explicitly tested the requirement for β -catenin in satellite cells and their derivatives for muscle regeneration. Despite efficient deletion of β -catenin, satellite cells were able to self-renew and regenerate muscle (although a subtle phenotype, undetectable in our assays, is possible). Interestingly, we did see that with β -catenin deletion myofibers shifted to larger cross-sectional areas at 28 dpi or with reinjury. Given that constitutive β -catenin activation prolonged regeneration and resulted in a shift to smaller, regenerating myofibers, the shift to larger myofibers with

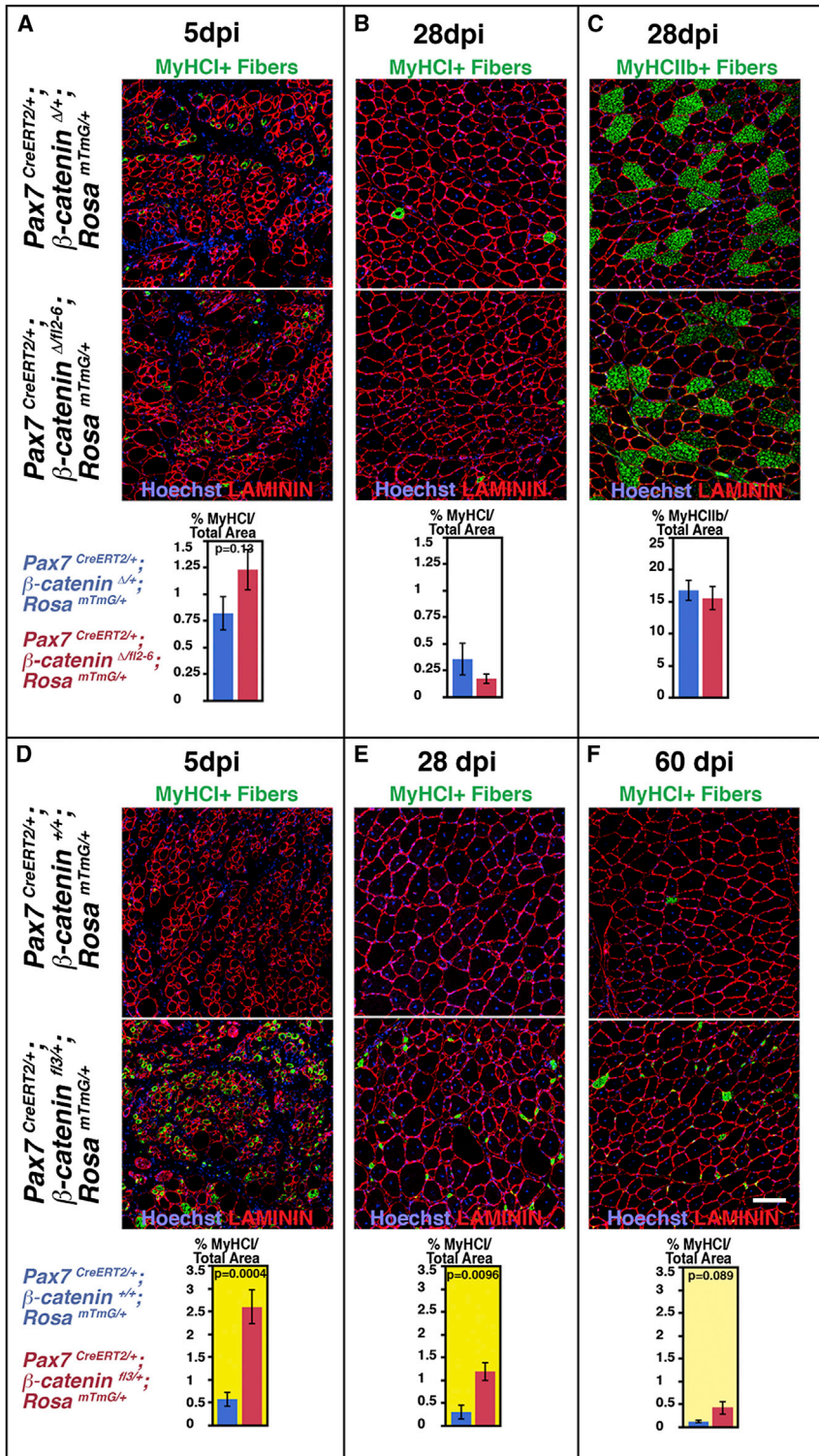


Figure 6. β -Catenin Is Not Necessary but Is Sufficient to Regulate Slow Myofibers during Regeneration

(A–C) β -catenin deletion does not alter the amount of slow MyHCI+ (A and B) or fast MyHCIIb (C) myofibers (at 5 dpi: n = 5 control, n = 5 mutant; at 28 dpi: n = 6 control, n = 5 mutant mice).

(D–F) β -catenin activation increases the amount of slow MyHCI+ myofibers (at 5 dpi: n = 6 control, n = 6 mutant; at 28 dpi: n = 4 control, n = 5 mutant).

Error bars in all histograms represent one SEM.

loss of β -catenin may indicate premature differentiation of myofibers. However, the finding that TAs regenerated from β -catenin satellite cells are GFP+ and do not differ in overall

size from control TAs suggests that a potential requirement of β -catenin to inhibit premature differentiation is modest, at best. Consistent with the lack of a significant phenotype

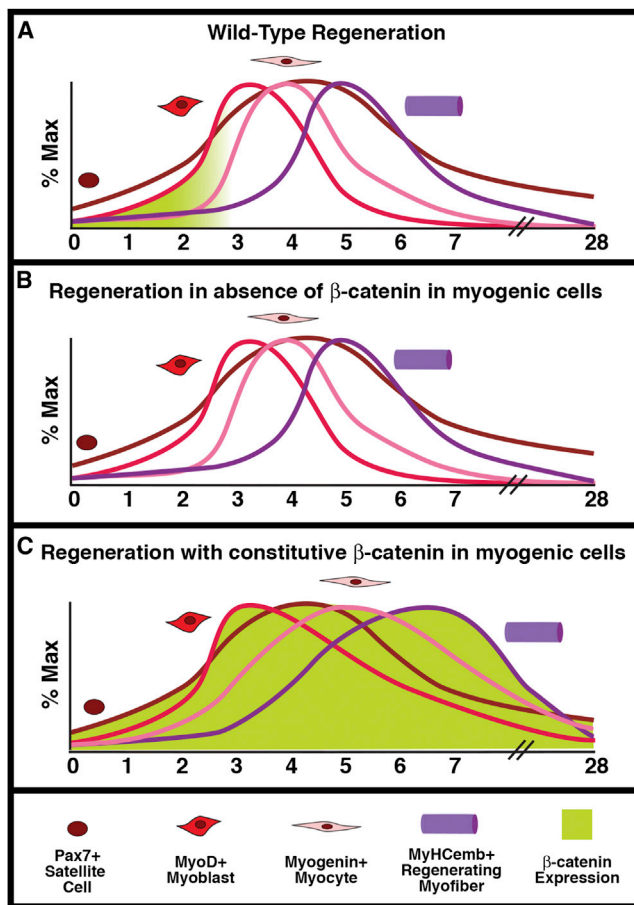


Figure 7. Model of Role of Wnt/ β -Catenin Signaling in Adult Muscle Regeneration

(A) During wild-type regeneration, Wnt/ β -catenin signaling is transiently active in myoblasts.

(B and C) Deletion of β -catenin in satellite cells and their derivatives does not alter muscle regeneration (B), but constitutive β -catenin activation alters the kinetics of myoblast differentiation (C), leading to a prolonged regenerative response.

with β -catenin loss, previous studies have produced contradictory findings. Most experiments have been conducted in vitro and, using a variety of techniques to inhibit β -catenin signaling, have found decreased satellite cell proliferation (Otto et al., 2008), less differentiation (Brack et al., 2008; Descamps et al., 2008; Kim et al., 2008), or more differentiation (Gavard et al., 2004; Tanaka et al., 2011). Only Brack et al. (2008, 2009) inhibited Wnt/ β -catenin signaling in vivo, via injection of sFRPs into regenerating TAs or genetic deletion of β -catenin coactivators BCL9 and BCL9-2 (via *Myf5^{Cre/+};Bcl9^{loxP/loxP};Bcl9-2^{loxP/loxP}* mice) after BaCl₂ or freeze injury. They concluded that Wnt/ β -catenin is necessary to promote muscle differentiation, but addition of sFRPs blocks both canonical and noncanonical Wnt signaling (Li et al., 2008) and does not specifically target

myogenic cells, and the genetic BCL9/BCL9-2 deletion potentially affects satellite cell development. Thus, previous phenotypes attributed to β -catenin necessity in satellite cells for regeneration may reflect in vitro conditions or in vivo reveal the role of canonical signaling in muscle progenitors during development or in other cell types involved in muscle regeneration or the function of noncanonical signaling in regeneration.

The transient activation of Wnt/ β -catenin signaling in myoblasts suggested the alternative hypothesis that not activation but silencing of signaling is critical for proper muscle regeneration. To test this, we examined the effects of constitutive β -catenin activation. Previous studies testing this have primarily been conducted in vitro, via Wnt3a or LiCl delivery to cultured satellite cells, and found constitutive Wnt/ β -catenin signaling either prevents differentiation (Gavard et al., 2004; Kuroda et al., 2013; Tanaka et al., 2011) or promotes differentiation and fusion (Bernardi et al., 2011; Brack et al., 2008; Han et al., 2011; Pansters et al., 2011). Two papers (Brack et al., 2008; Le Grand et al., 2009) tested in vivo (using either BaCl₂ or freeze injury) the effects of increased signaling and concluded that Wnt/ β -catenin signaling promoted premature differentiation. However, these in vivo experiments activated, via ectopic Wnt3a, signaling in all cell types (including muscle connective tissue fibroblasts and endothelial cells) during regeneration. Our experiments constitutively activating β -catenin specifically in satellite cells revealed that satellite cells are largely insensitive to increased β -catenin, as we saw no effects on satellite cell expansion or proliferation after injury. However, constitutive β -catenin activation did alter the kinetics of the regenerative process, as myoblasts (which normally transiently express β -catenin) and subsequently myocytes and regenerating myofibers are present for an extended period. Thus, continued activation of β -catenin signaling prolongs the myoblast phase of regeneration, although it does not ultimately block differentiation. This prolonged regeneration negatively impacts muscle structure as it results in smaller myofibers and increased fibrosis.

Our study is a cautionary warning about the conclusions of Wnt/ β -catenin signaling functional significance that can be drawn from reporter and gain-of-function experiments. Multiple reporters have been developed for Wnt/ β -catenin signaling (reviewed in Barolo, 2006). Although they differ in their sensitivities, a finding of reporter activity is considered a good indicator that endogenous Wnt/ β -catenin signaling is active. However, our data show that presence of activity does not necessarily imply a biological requirement for that activity. In addition, gain-of-function experiments reveal whether cells are sensitive to Wnt/ β -catenin signaling but again do not demonstrate signaling necessity. Loss-of-function studies, particularly when



conducted conditionally (to limit the spatial and temporal scope of deletion) and *in vivo*, are essential for determining the necessity of signaling. In fact, in our study, the combination of reporter and gain- and loss-of-functions experiments show that it is not activation but rather silencing of signaling that is important. In wild-type mice, this silencing is likely accomplished by the early and strong up-regulation of sFRPs during muscle regeneration (Le Grand et al., 2009; Polesskaya et al., 2003; Zhao and Hoffman, 2004).

The finding that Wnt/ β -catenin signaling must be silenced during adult regeneration naturally raises the question of why signaling is activated. Our previous analysis of Wnt/ β -catenin signaling during fetal myogenesis demonstrated that β -catenin is required for regulation of fetal myofiber differentiation (Hutcheson et al., 2009). Similar to the classic argument about the origin and function of the Spandrels of San Marcos (Gould and Lewontin, 1979), activation of Wnt/ β -catenin signaling in adult myoblasts might simply be a vestige of their developmental lineage, in which β -catenin signaling is required for fetal myogenesis (Hutcheson et al., 2009; Murphy and Kardon, 2011).

Comparison of the role of Wnt/ β -catenin signaling during fetal myogenesis and adult regeneration reveals intriguing similarities and differences between fetal and adult stem cells and myoblasts. Loss-of-function experiments demonstrate that, during fetal myogenesis, β -catenin is critical for regulating the number and fiber type of myofibers that differentiate from PAX7⁺ stem cells and yet, in the adult β -catenin, is dispensable for the differentiation and fiber type of myofibers regenerated from PAX7⁺ satellite cells. This difference between fetal and adult stem cells is reminiscent of the difference between the development and maintenance of hematopoietic stem cells (HSCs); β -catenin is essential for HSC generation (Ruiz-Herguido et al., 2012) but later appears to be dispensable for maintenance of embryonic HSCs (Ruiz-Herguido et al., 2012) and adult HSC function (Cobas et al., 2004). Gain-of-function experiments demonstrate that differentiating fetal and adult myofibers are similarly sensitive to β -catenin, as constitutive β -catenin activation is sufficient to convert both types to slow MyHC1⁺ myofibers, although this conversion is more complete during fetal myogenesis. However, most striking is the difference in β -catenin sensitivity between fetal and adult PAX7⁺ stem cells. In the fetus, constitutive activation of β -catenin causes a dramatic expansion of PAX7⁺ stem cells (Hutcheson et al., 2009). In contrast, in the adult β -catenin, activation does not expand the number of PAX7⁺ satellite cells but rather the number of transit-amplifying MYOD⁺ myoblasts. Altogether, our experiments indicate that, despite their close lineage relationship (Hutcheson et al., 2009; Murphy and Kardon,

2011), fetal and adult PAX7⁺ stem cells differ in their requirement of and sensitivity to β -catenin. Limiting the sensitivity of highly proliferative satellite cells to β -catenin may be important for decreasing the adult risk of cancer from oncogenic β -catenin signaling.

EXPERIMENTAL PROCEDURES

Mice

All mouse lines were previously reported: *Pax7^{CreERT2}* (Murphy et al., 2011); *β -catenin^{fl2-6}* (Brault et al., 2001); *β -catenin^{fl3}* (Harada et al., 1999); *Rosa^{mTmG}* (Muzumdar et al., 2007); and *TCF/Lef:H2B-GFP^{T3}* (Ferrer-Vaquer et al., 2010). Mice were bred onto C57/BL6J background and used at 6–8 weeks of age.

FACS Cell Isolation and Analysis

Mononuclear myogenic cells and fibroblasts were isolated from injured right and uninjured left TAs, incubated with antibodies if needed (Table S1), and analyzed via FACS (details in Supplemental Experimental Procedures). Myogenic cells and fibroblasts were isolated from *TCF/Lef:H2B-GFP^{T3}* mice using strategy of Yi and Rossi (2011). For *Pax7^{CreERT2/+}; β -catenin^{fl2-6};*Rosa^{mTmG/+}* and *Pax7^{CreERT2/+}; β -catenin^{fl3/+};*Rosa^{mTmG/+}* mice and their controls, myogenic cells were isolated via GFP and nonmyogenic cells via TOMATO. Genomic DNA of cells was isolated and alleles of *β -catenin* determined via PCR using primers of Brault et al. (2001) and Harada et al. (1999).**

Muscle Injury and Tamoxifen Delivery

Injury was induced by injecting 25 μ l of 1.2% BaCl₂ in normal saline into right TA. Left TA served as uninjured control. For administration prior to injury, TAM was delivered via gavage in 10 mg doses. For continuous delivery before and after injury, TAM was delivered intraperitoneally at 3 mg/40 g body weight per injection. All mouse experiments were conducted under the oversight of University of Utah Institutional Animal Care and Use Committee.

Immunofluorescence, Histology, and Microscopy

For section immunofluorescence, flash-frozen muscles were sectioned, fixed in paraformaldehyde, and labeled via immunofluorescence or stained with Sirius Red (details in Supplemental Experimental Procedures). Sirius Red sections were imaged on a Zeiss Axioplan2 microscope. Immunofluorescent sections were imaged on a Nikon AR1 confocal or widefield microscope. Each confocal image is a composite of maximum projections, derived from stacks of optical sections.

Quantification and Statistics

Quantification of PAX7⁺, MYOD⁺, or MYOGENIN⁺ nuclei and amount of GFP, MyHCemb, MyHCI, MyHCIIb, or ECM was quantified using Image J (details in Supplemental Experimental Procedures). For each variable, counts of two sections across the entire TA were averaged for three to six individuals of each genotype per time point and analyzed using a Student's two-tailed t test. On all bar charts, mean \pm 1 SEM shown. Fiber distribution was determined using MuscleQNT (developed by S.D.F. and M.Y. and available at <https://github.com/stevendflygare/muscleQNT>). In



brief, MuscleQNT is an image analysis pipeline implemented in Python designed to identify borders (through adaptive thresholding and a series of erosion and dilation steps) of LAMININ+ myofibers and quantify the number and CSA of all myofibers in a muscle cross-section. Histograms and summary statistics of myofiber sizes are generated, and histogram error bars are the result of permutation tests. All displayed histograms were statistically significant via the Kolmogorov-Smirnov test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2014.06.019>.

AUTHOR CONTRIBUTIONS

M.M.M., A.C.K., J.A.L., and G.K. designed experiments; M.M.M., A.C.K., and J.A.L. performed experiments; S.D.F. and M.Y. designed and implemented MuscleQNT; M.M.M., A.C.K., J.A.L., and G.K. analyzed data; and M.M.M. and G.K. wrote the manuscript.

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