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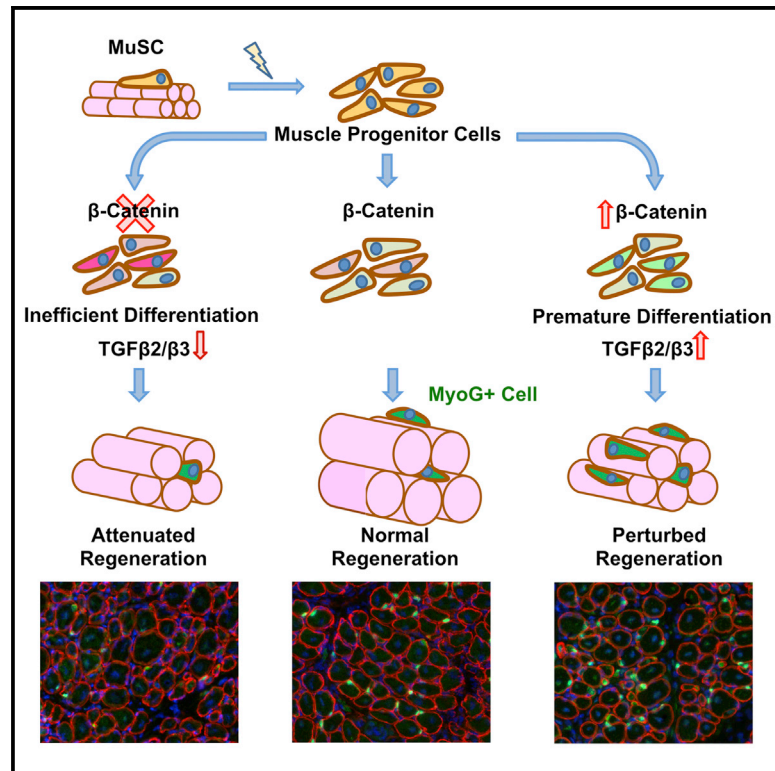
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## $\beta$ -Catenin Activation in Muscle Progenitor Cells Regulates Tissue Repair

### Graphical Abstract



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### In Brief

Using muscle stem cell-specific  $\beta$ -catenin gain-of-function and loss-of-function mutations, Rudolf et al. demonstrate that muscle progenitor cells (MPCs) require the canonical Wnt/ $\beta$ -catenin pathway during skeletal muscle regeneration. In vivo and in vitro, MPCs differentiate less efficiently when  $\beta$ -catenin is absent, whereas conditional activation of  $\beta$ -catenin pushes MPCs toward precocious differentiation.

### Highlights

- $\beta$ -catenin signaling in adult muscle progenitor cells (MPCs) controls muscle regeneration
- The absence of  $\beta$ -catenin signaling perturbs MPC differentiation
- Activation of  $\beta$ -catenin leads MPCs toward precocious differentiation
- TGF- $\beta$  injection restores MPC ability to differentiate in the absence of  $\beta$ -catenin

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# $\beta$ -Catenin Activation in Muscle Progenitor Cells Regulates Tissue Repair

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## SUMMARY

Skeletal muscle regeneration relies on a pool of resident muscle stem cells called satellite cells (MuSCs). Following injury-induced destruction of the myofibers, quiescent MuSCs are activated and generate transient amplifying progenitors (myoblasts) that will fuse to form new myofibers. Here, we focus on the canonical Wnt signaling pathway and find that either conditional  $\beta$ -catenin disruption or activation in adult MuSCs results in perturbation of muscle regeneration. Using both in vivo and in vitro approaches, we observed that myoblasts lacking  $\beta$ -catenin show delayed differentiation, whereas myoblasts with constitutively active  $\beta$ -catenin undergo precocious growth arrest and differentiation. Transcriptome analysis further demonstrated that Wnt/ $\beta$ -catenin signaling interacts with multiple pathways and, more specifically, TGF- $\beta$  signaling. Indeed, exogenous TGF- $\beta$ 2 stimulation restores the regenerative potential of muscles with targeted  $\beta$ -catenin disruption in MuSCs. We conclude that a precise level of  $\beta$ -catenin activity is essential for regulating the amplification and differentiation of MuSC descendants during adult myogenesis.

## INTRODUCTION

Regeneration of the adult skeletal muscle tissue relies on a pool of resident muscle stem cells located around myofibers called satellite cells (muscle stem cells [MuSCs]) that express the paired box transcription factor Pax7 (Seale et al., 2000). Upon damage to the myofibers, quiescent MuSCs are activated and give rise to a population of transient amplifying myoblasts expressing the basic helix-loop-helix (bHLH) transcription factors

MyoD and/or Myf5 (Megenev et al., 1996; Gayraud-Morel et al., 2007). Most of the myoblasts will then permanently exit the cell cycle, express the bHLH transcription factor Myogenin, and fuse to form new myofibers and regenerate the tissue, whereas a sub-population will re-populate the MuSC niche (Tedesco et al., 2010). In this process, signals from the local microenvironment orchestrate myoblast activation and amplification and control their myogenic fate choice (recently reviewed in Yin et al. (2013)). Our previous work highlighted that many Wnt proteins are secreted during muscle regeneration and also described an important role for non-canonical Wnt7a signals in regulating MuSC symmetric expansion during muscle tissue repair (Le Grand et al., 2009).

The canonical Wnt/ $\beta$ -catenin cascade is a critical regulator of stem cell biology in many adult tissues (Clevers and Nusse, 2012). Wnt proteins bind to receptors of the Frizzled and low-density lipoprotein receptor-relayed protein (LRP) families on the cell surface of receiving cells (MacDonald et al., 2009). When Frizzled/LRP receptors are not engaged,  $\beta$ -catenin is phosphorylated by a cytoplasmic destruction complex composed of Axin, adenomatous polyposis coli (APC), casein kinase I (CKI), and GSK3 $\beta$ . Phosphorylated  $\beta$ -catenin is ubiquitinated and targeted for rapid destruction by the proteasome. Upon receptor activation by Wnt ligands, Axin is recruited to the phosphorylated tail of LRP. Recent data show that, through this relocalization, the Wnt signal leads to inhibition of  $\beta$ -catenin ubiquitination, which normally occurs within the complex. Subsequently, the complex becomes saturated by the phosphorylated form of  $\beta$ -catenin, leading newly synthesized  $\beta$ -catenin to accumulate and translocate to the nucleus (Li et al., 2012). Nuclear  $\beta$ -catenin forms a complex with the TCF transcription factors to activate transcription of Wnt target genes. However, the mechanisms by which canonical Wnt proteins provoke specific cellular responses and regulate muscle progenitor cell biology remain poorly understood.

To date, conflicting reports proposed distinct roles for the canonical Wnt/ $\beta$ -catenin pathway during skeletal muscle regeneration. An earlier work implicated the Wnt1/ $\beta$ -catenin pathway in

muscle cell differentiation through activation and recruitment of MuSC-like reserve myoblasts for fusion with myotubes in vitro (Rochat et al., 2004). In contrast, a second set of in vitro experiments showed that Wnt/ $\beta$ -catenin signaling promotes MuSC proliferation in the first stages of their activation on isolated myofibers in vitro (Otto et al., 2008). Canonical Wnt signaling has also been implicated in promoting cultured myoblast myogenic potential through inhibition of Myostatin activity (Bernardi et al., 2011). When assessed in vivo, injection of recombinant Wnt proteins to a regenerating adult muscle appeared to control myogenic lineage progression by limiting Notch signaling and thus promoting differentiation in the later stages of muscle regeneration (Brack et al., 2008). Intriguingly, in vivo experiments suggested that canonical Wnt signaling is not required for muscle regeneration or satellite cell self-renewal (Murphy et al., 2014). In contrast, we recently showed that APC expression by MuSCs is required to dampen canonical Wnt signaling and allow cell-cycle progression (Parisi et al., 2015).

To address these issues and to comprehensively understand the roles of Wnt/ $\beta$ -catenin signals during muscle regeneration, we interrogated the regenerative capacity of skeletal muscle with either MuSC-specific  $\beta$ -catenin gain-of-function or loss-of-function mutations. Strikingly, we observed that, in both conditional mouse models, the fate of muscle progenitor cells was perturbed and that skeletal muscles were unable to regenerate properly following acute damage. We found that myoblasts fail to differentiate in the absence of  $\beta$ -catenin and that the genetic activation of the canonical Wnt pathway pushes myoblasts toward differentiation at the expense of amplification in vitro as well as in vivo. A Wnt/ $\beta$ -catenin-dependent transcriptome analysis identified the upregulation of, among others, the TGF- $\beta$  pathway. We further demonstrate that forced TGF- $\beta$  pathway activation, ex vivo and in vivo, can bypass the requirement of myoblasts for  $\beta$ -catenin to differentiate. Our results indicate that an adequate level of intrinsic Wnt/ $\beta$ -catenin signaling is essential within MuSC descendants for their function during tissue repair.

## RESULTS

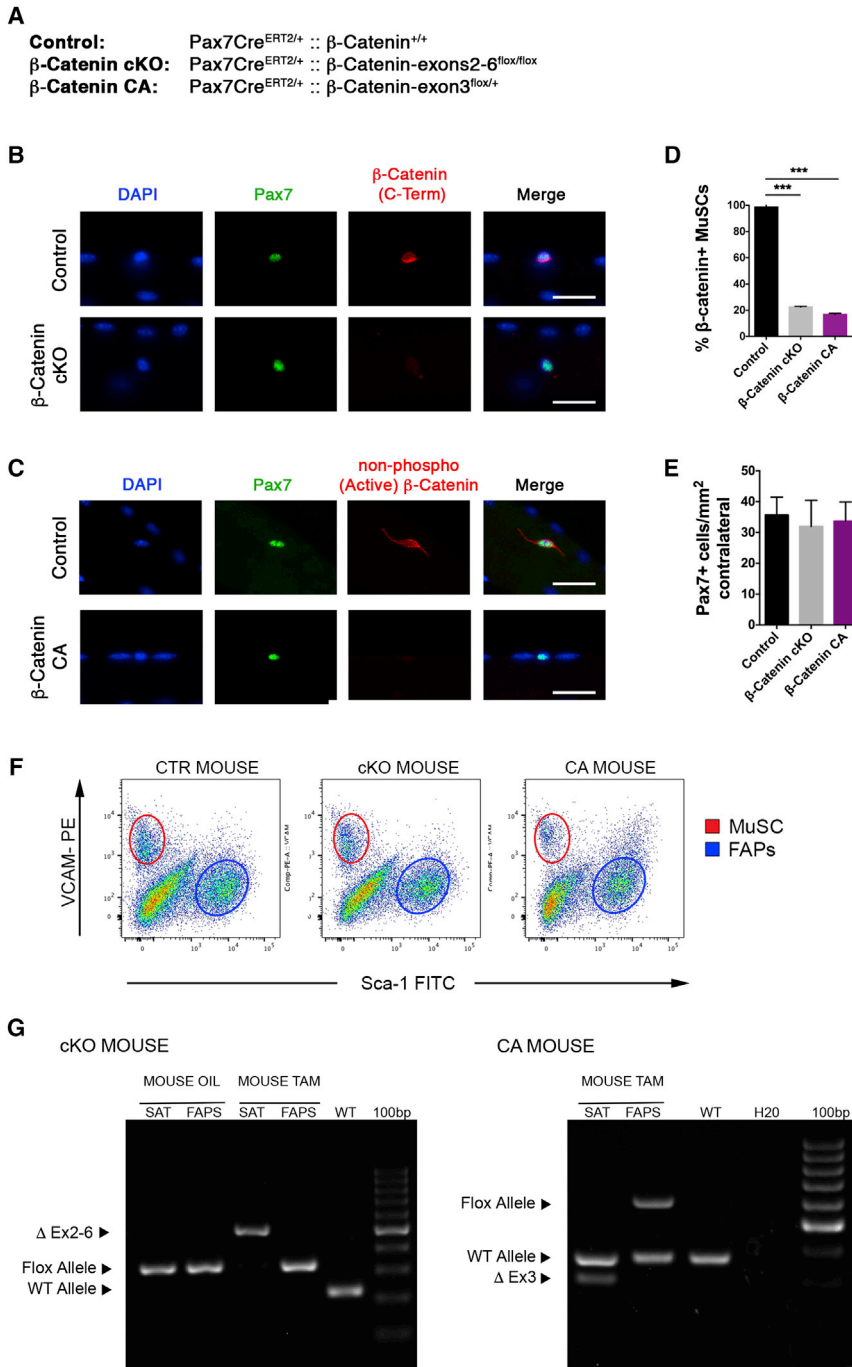
### Conditional Targeted $\beta$ -catenin Gene Disruption in MuSCs

Previous work has demonstrated that adult MuSCs and myoblasts express  $\beta$ -catenin. To reproduce these results, we performed immunohistochemical analysis of  $\beta$ -catenin expression on tissue sections of regenerating (7 days post-injury [d.p.i]) and regenerated (30 d.p.i) tibialis anterior (TA) muscle tissues using an antibody recognizing the C-terminal part of  $\beta$ -catenin. We observed that Myogenin+ and MyoD+ muscle progenitor cells express  $\beta$ -catenin in vivo (Figure S1A). To further evaluate  $\beta$ -catenin activation during adult myogenesis, we performed immunocytochemical analysis of single myofibers isolated from extensor digitorum longus (EDL) muscles using an antibody specifically recognizing the active forms of  $\beta$ -catenin (un-phosphorylated on Ser33/37 and Thr41). We observed that quiescent MuSCs stained immediately after isolation express only very low levels of active  $\beta$ -catenin proteins. Doublets of dividing MuSCs on cultured myofibers exhibited mainly cytoplasmic localization of

active  $\beta$ -catenin proteins when scored 42 hr after isolation (Figure S1B). Interestingly, foci of nuclear active  $\beta$ -catenin proteins were observed in MuSC descendants after 72 hr in culture ex vivo, at the time MuSCs undergo fate choice decision. Co-immunostaining of active  $\beta$ -catenin with Pax7 (to mark undifferentiated muscle progenitor cells) or Myogenin (to label differentiating muscle progenitor cells) indicated that  $\beta$ -catenin signaling is mainly active during differentiation (Figure S1C). These data are consistent with our previous analysis of the Wnt reporter allele *Axin2*<sup>LacZ</sup> that was not detected in uninjured muscles but strongly marked myogenin-expressing differentiating cells during muscle regeneration (Parisi et al., 2015).

To address the function of  $\beta$ -catenin in adult MuSCs and their progeny in vivo, we crossed mice with either the  *$\beta$ -catenin*<sup>Exons2-6-Lox</sup> (Brault et al., 2001) or  *$\beta$ -catenin*<sup>Exon3-Lox</sup> (Harada et al., 1999) conditional allele with mice expressing a tamoxifen (TAM)-inducible Cre recombinase estrogen receptor fusion protein, CreERT2, in cells that express Pax7 (Lepper et al., 2009). While the deletion of  $\beta$ -catenin exons 2–6 results in expression of an inactive protein (loss of function, conditional knockout [cKO]), the deleted protein fragment encoded by  $\beta$ -catenin exon 3 contains the GSK3 $\beta$  phosphorylation target serine/threonine residues necessary for ubiquitination. Thus, the resulting protein is constitutively active (CA, gain of function).

To induce recombination at the conditional  *$\beta$ -catenin* loci, we injected 8-week-old mice of the following genotypes with TAM diluted in corn oil: *Pax7*<sup>CreERT2/+</sup> (controls), *Pax7*<sup>CreERT2/+</sup>:: *$\beta$ -catenin*<sup>Ex3<sup>lox/+</sup></sup> ( $\beta$ -catenin CA), and *Pax7*<sup>CreERT2/+</sup>:: *$\beta$ -catenin*<sup>Ex2-6<sup>lox/lox</sup></sup> ( $\beta$ -catenin cKO) (Figure 1A). For controls, we also injected  $\beta$ -catenin cKO mice with corn oil. The efficacy and specificity of TAM-induced Cre activity was confirmed by 92% labeling of MuSCs one week following TAM administration in *Pax7*<sup>CreERT2</sup> mice crossed with Rosa26 reporter (R26R) mice (as described previously and repeated in our hands; data not shown). To evaluate the recombination efficiency at the protein level, we used the antibody recognizing the total  $\beta$ -catenin (C-terminal) for  $\beta$ -catenin cKO mice because the recombined allele cannot generate a functional protein (Figure 1B) and the antibody recognizing the non-phospho (active)  $\beta$ -catenin for  $\beta$ -catenin CA mice (Figure 1C) because the epitope is absent from the constitutively active protein when exon3 is deleted. Immunostaining on isolated myofibers further validated the recombination of targeted loci in MuSCs of  $\beta$ -catenin cKO and  $\beta$ -catenin CA mice 1 week after TAM administration (Figure 1D). Because  $\beta$ -catenin protein could be very stable in quiescent MuSCs, we sorted MuSCs from  $\beta$ -catenin cKO and CA mice by fluorescence-activated cell sorting (FACS) (Figure 1F), plated them in vitro for 48 hr to allow them to exit quiescence, and performed genotyping PCR for the conditional  $\beta$ -catenin alleles. As shown in Figure 1G, the deleted alleles can only be amplified in MuSCs from TAM-treated mice, and we could not detect the non-recombined alleles in  $\beta$ -catenin cKO and  $\beta$ -catenin CA MuSCs anymore. We used fibro-adipogenic progenitors (FAPs) sorted by FACS as an internal control to validate the tissue specificity of the *Pax7*<sup>CreERT2/+</sup> allele. Mice with conditional genetic disruption of either  $\beta$ -catenin allele did not show any overt phenotypes, and uninjured muscle appeared normal 1 month after TAM injection (data not shown). Quiescent MuSCs were not



**Figure 1. Conditional Targeted  $\beta$ -Catenin Gene Disruption in MuSCs**

(A) Genotypes of the mice used in the study. (B and C) Isolated single myofibers of control,  $\beta$ -catenin cKO (B), and  $\beta$ -catenin CA (C) animals 7 days after TAM-induced recombination of targeted loci in MuSCs. Recombination in Pax7+ cells of  $\beta$ -catenin cKO animals was confirmed by using an antibody that recognizes the C-terminal part of  $\beta$ -catenin. Recombination in Pax7+ cells of  $\beta$ -catenin CA animals was confirmed by using an antibody that recognizes an epitope encoded by exon 3 that is non-phosphorylated when  $\beta$ -catenin is active. (D) Quantification of  $\beta$ -catenin-positive MuSCs on isolated single myofibers. (E) Numbers of Pax7-positive cells in uninjured TA muscles 1 month after TAM-induced recombination. (F) FACS profiles of mononuclear cells derived from control (CTR),  $\beta$ -catenin cKO, and  $\beta$ -catenin CA muscles 1 week after TAM injection. The profiles were gated for CD31-neg, CD45-neg, and Ter119-neg fractions. FAPs are fibro-adipogenic progenitors cells. VCAM-PE, vascular cell adhesion molecule. (G) Genotyping PCRs of MuSCs sorted by FACS. For the  $\beta$ -catenin-exon2-6-lox locus, the PCR product of the deleted allele is longer than the flox allele, whereas the PCR product of the WT allele is shorter than the flox allele (left). For the  $\beta$ -catenin-exon3-Lox locus, the PCR product of the deleted allele is shorter than the WT allele, whereas the PCR product of the WT allele is shorter than the flox allele (right). FAP cells and H<sub>2</sub>O were used as negative controls. Scale bars, 20  $\mu$ m. n = 3 mice for each genotype. Values are mean  $\pm$  SEM. \*\*\*p < 0.001.

affected by  $\beta$ -catenin gain or loss of function because the number of sub-laminar Pax7-expressing cells was unchanged in  $\beta$ -catenin cKO and  $\beta$ -catenin-CA mice compared with control mice 1 month following induction of Cre activity (Figure 1E).

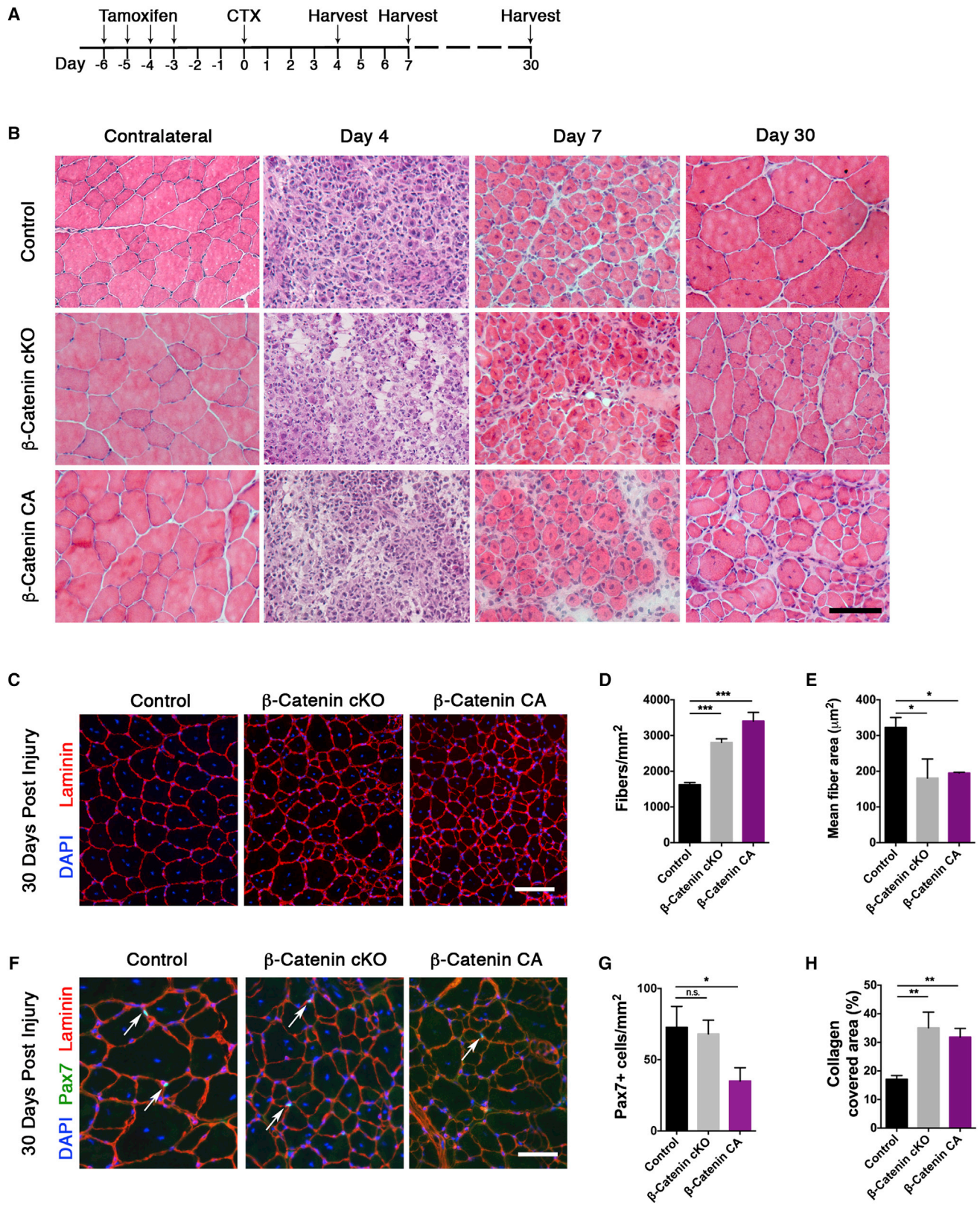
### Modulation of $\beta$ -Catenin Signaling in Adult MuSCs Impairs Muscle Regeneration

To determine whether  $\beta$ -catenin-conditional mutant MuSCs can support damage-induced myogenesis, we injected cardiotoxin

(CTX) into the TA muscle of TAM-treated control,  $\beta$ -catenin cKO, and  $\beta$ -catenin CA mice and sampled the injured tissue at different time points for histological analysis (Figure 2A). 4 d.p.i, regenerating tissues from all genotypes were composed of early regenerating myotubes and numerous mononucleated cells (likely proliferating myoblasts and infiltrating immune cells) and did not show overt phenotypes or abnormalities. 7 d.p.i, regenerating control muscles were already composed of newly formed myofibers with centrally located myonu-

clei, whereas  $\beta$ -catenin cKO and  $\beta$ -catenin CA muscles also contained a higher amount of fibrotic tissue and inflammatory cells. 30 d.p.i, control muscle had fully regenerated and muscle architecture was restored with normal myofiber hypertrophy, whereas  $\beta$ -catenin cKO and  $\beta$ -catenin CA muscles presented aberrant myofiber morphology and atrophy (Figure 2B).

To further visualize and quantify the differences in the regenerative process between control and  $\beta$ -catenin cKO/CA mice, we performed immunostaining for Laminin to visualize the myofiber



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basal lamina on 30-d.p.i muscle cryosections (Figure 2C). We first observed that  $\beta$ -catenin cKO and CA regenerated tissue sections contained more myofibers per field, suggesting smaller myofibers in mutant regenerated tissues, compared with controls (Figure 2D). We then determined the myofiber cross-sectional area (CSA). Representation of myofiber size distribution demonstrated that the proportion of small myofibers was significantly increased in  $\beta$ -catenin cKO and CA mice compared with control mice, whereas the proportion of myofibers with a higher CSA was decreased (Figure S2A). Calculation of the mean myofiber diameter further validated that regenerated myofibers were significantly smaller in both  $\beta$ -catenin cKO and CA tissues compared with control muscles (Figure 2E). Surprisingly, the size and weight of injured muscles were not altered in  $\beta$ -catenin cKO or CA mice compared with controls (Figure S2B; data not shown). This is explained by the increase in the total number of myofibers per TA muscle in both  $\beta$ -catenin cKO/CA mice compared with controls (Figure S2C). We next quantified the numbers of sub-laminar Pax7<sup>+</sup> cells (Figure 2F) and observed that, although  $\beta$ -catenin gene disruption in MuSCs does not perturb the renewal of the MuSC niche,  $\beta$ -catenin CA regenerated muscle contained a reduced MuSC pool (Figure 2G). Of note, no differences were observed in the numbers of MyoD<sup>+</sup> cells in regenerated muscles between controls and  $\beta$ -catenin cKO/CA mice (data not shown).

Defects or delays in the muscle-regenerative process are frequently associated with aberrant extracellular matrix (ECM) remodeling. To visualize the connective tissue, we performed immunostaining for collagen type I (Figure S2D). Quantification of the total collagen-positive areas revealed that both mutant mouse strains exhibited extensive deposition of connective tissue (Figure 2H). Interestingly, the number of ER-TR7<sup>+</sup> fibroblasts increased 3-fold in  $\beta$ -catenin cKO mice, whereas their proportion increased only slightly in  $\beta$ -catenin CA muscles (Figure S2E), suggesting that the cellular mechanisms responsible for aberrant ECM remodeling are different between the two mutant mice. These experiments demonstrate that correct levels of  $\beta$ -catenin expression and activation are critical for MuSC function in adult skeletal muscle.

### $\beta$ -Catenin Controls Muscle Progenitor Cell Dynamics during Muscle Regeneration

To further understand the alterations in the regenerative process in mice with targeted  $\beta$ -catenin mutations in MuSCs, we analyzed the early regeneration process at 7 d.p.i (Figure 3A).

At this point, both  $\beta$ -catenin cKO and  $\beta$ -catenin CA muscles were already composed of smaller new myofibers compared with control muscles (Figures 3B and 3C). In addition, the number of myonuclei per regenerated myofiber in  $\beta$ -catenin cKO muscles was reduced compared with control muscles when quantified at 7 and 30 d.p.i. (Figure 3D). Interestingly, the number of myonuclei per myofiber was comparable with controls in  $\beta$ -catenin CA mice at 7 d.p.i. but was significantly reduced compared with controls at the end of tissue regeneration. These results suggest an alteration in the differentiation/fusion process in  $\beta$ -catenin cKO muscles and an exhaustion of the myogenic progenitor pool at later time points in  $\beta$ -catenin CA muscles.

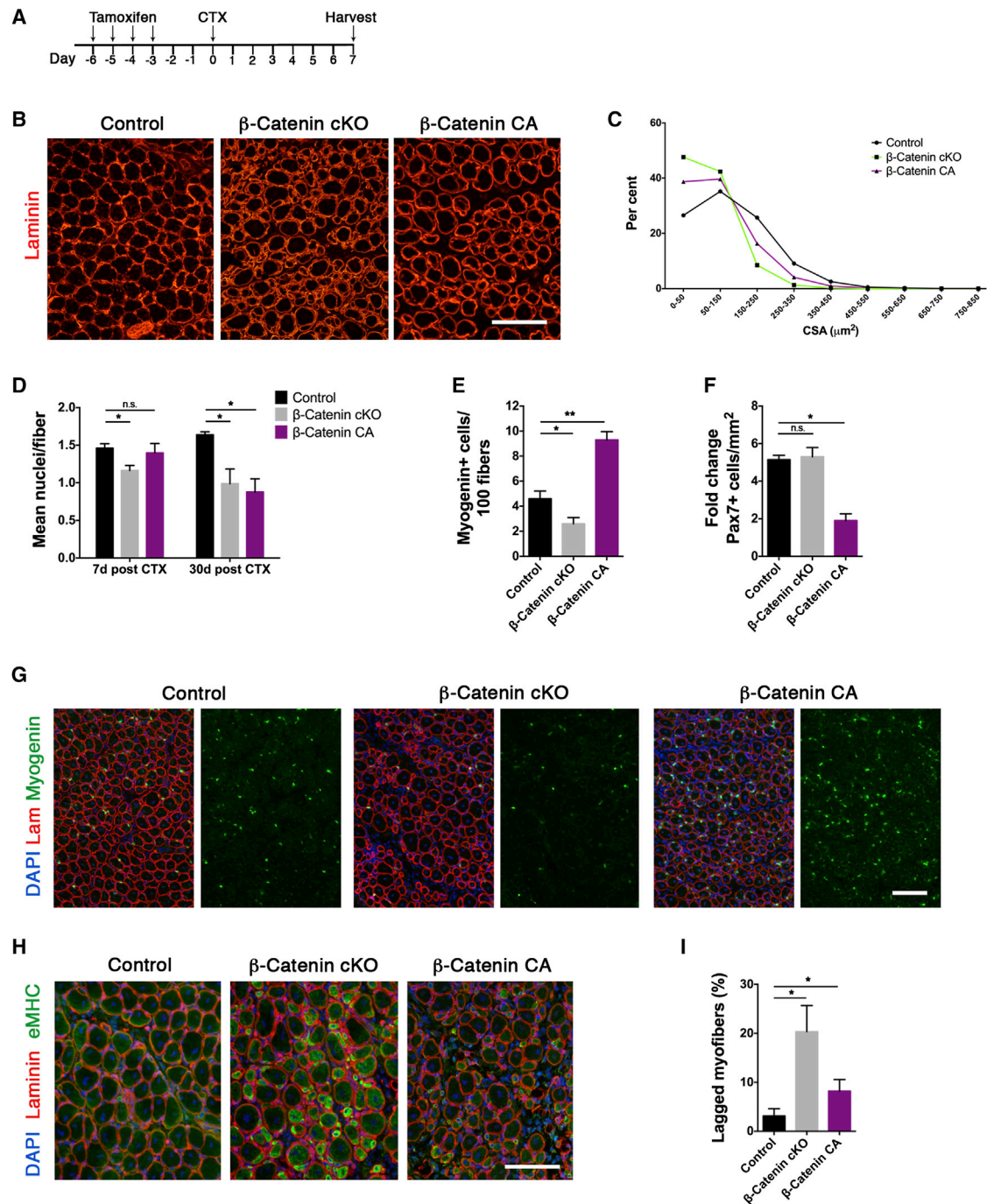
To further validate our hypothesis, we quantified the numbers of proliferating (Pax7-expressing) and differentiating (Myogenin-expressing) muscle progenitors in regenerating muscles at 7 d.p.i. (Figure 3G). Strikingly, we observed a reduction in  $\beta$ -catenin cKO muscles and a strong augmentation in  $\beta$ -catenin CA muscles of differentiating Myogenin<sup>+</sup> myocytes (Figure 3E). At the same time, the number of proliferating, Pax7<sup>+</sup> myoblasts in  $\beta$ -catenin CA muscles was reduced, whereas  $\beta$ -catenin cKO muscles did not show significant differences in the number of Pax7<sup>+</sup> cells compared with controls (Figure 3F). We then performed immunostaining for the embryonic forms of myosin heavy chain (eMHC), which marks all newly formed myofibers between 3 and 5 days post-injury and which is downregulated when the myofiber matures (Figure 3H). Control muscles only contained a very limited number of eMHC-positive myofibers, whereas  $\beta$ -catenin cKO muscles contained a high number of lagged myofibers expressing eMyHC (Figure 3I), suggesting a delay in the regeneration process in the absence of  $\beta$ -catenin expression in MuSCs. The proportion of lagged myofibers was, however, only slightly increased in  $\beta$ -catenin CA muscles compared with controls (Figure 3I). In summary, our results indicate that the state of  $\beta$ -catenin in MuSC descendants regulates the timing of myogenic progenitor cell differentiation during tissue repair in vivo.

### Delayed Differentiation of $\beta$ -Catenin cKO Myoblasts

Observation of muscle tissue regeneration in our mouse models suggested that  $\beta$ -catenin intrinsically regulates the balance between proliferation and differentiation in MuSC descendants. To further validate these results, we cultured MuSCs sorted by FACS from  $\beta$ -catenin cKO mice that had been subjected to TAM. We let the MuSCs proliferate to give rise to primary

## Figure 2. Conditional Modulation of $\beta$ -Catenin Function in MuSCs Impairs Proper Muscle Regeneration

- (A) Experimental setup. TAM was injected into mice on 4 consecutive days. TA muscles were injured by a single CTX injection and analyzed at different times following injury.
- (B) H&E staining on cryosections of uninjured and regenerating muscle tissue at 4, 7, and 30 d.p.i in control,  $\beta$ -catenin cKO, and  $\beta$ -catenin CA mice. Note the high number of inflammatory cells between the smaller myofibers in both conditional mutant muscles.
- (C) Anti-Laminin staining on cryosections of regenerated TA muscles in control,  $\beta$ -catenin cKO, and  $\beta$ -catenin CA mice at 30 d.p.i.
- (D) Quantification of the number of myofibers per field.
- (E) Regenerated muscles of conditional mutant mice are composed of smaller myofibers.
- (F) Anti-Pax7 and anti-Laminin staining on cryosections of regenerated TA muscles in control,  $\beta$ -catenin cKO, and  $\beta$ -catenin CA mice at 30 d.p.i.
- (G) Quantification of the number of sublaminar Pax7<sup>+</sup> cells per field.
- (H) Quantification of the collagen-covered area in regenerated TA muscles of control,  $\beta$ -catenin cKO, and  $\beta$ -catenin CA mice.
- Scale bars, 50  $\mu$ m (B and C) and 25  $\mu$ m (F). n = 4 mice/genotype. Values are mean  $\pm$  SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. n.s., not significant.



### Figure 3. $\beta$ -Catenin Controls the Proliferation/Differentiation Balance in Muscle Progenitor Cells In Vivo

For a Figure360 author presentation of Figure 3, see the figure online at <http://dx.doi.org/10.1016/j.celrep.2016.04.022#mmc3>.

(A) Experimental setup. TAM was injected into mice on 4 consecutive days. TA muscles were injured by a single CTX injection and analyzed at 7 d.p.i.

(B) Anti-Laminin staining of TA muscle cryosections. Note that early regenerating myofibers were smaller in diameter in  $\beta$ -catenin cKO mice than in controls, whereas both small and normally sized myofibers were present in  $\beta$ -catenin CA animals.

(C) Distribution of myofiber CSAs at 7 d.p.i.

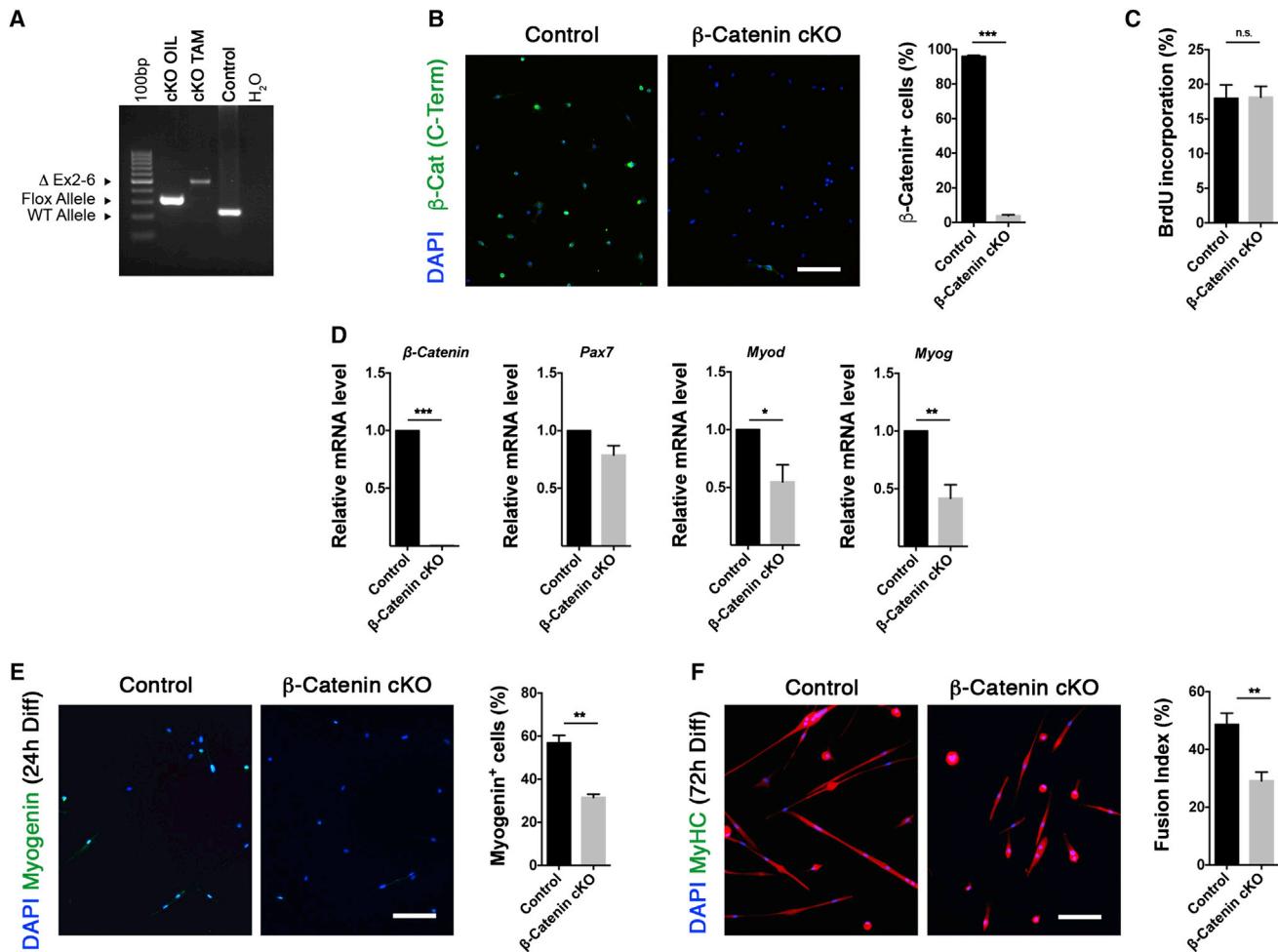
(D) Mean numbers of myonuclei per regenerating myofiber at 7 and 30 d.p.i. Conditional mutant mice show reduced myonuclei numbers compared with control mice.

(E) Numbers of Myogenin-positive (differentiating) cells in control and conditional mutant regenerating muscles at 7 d.p.i.

(F) Numbers of Pax7-positive (proliferating) cells in control and conditional mutant regenerating muscles at 7 d.p.i. (normalized by the number of Pax7-positive cells in the contralateral leg).

(legend continued on next page)





**Figure 4.  $\beta$ -Catenin Is Required for Primary Myoblast Differentiation**

Primary myoblasts were expanded from  $\beta$ -catenin cKO MuSCs sorted by FACS and cultured for two passages.

(A) Confirmation of  $\beta$ -catenin exon 2-6 deletion, which is generated in mutant cells at the DNA level. The floxed allele could be amplified by PCR on isolated DNA of myoblasts derived from TAM-treated  $\beta$ -catenin cKO mice, and the floxed allele could not be detected anymore. Myoblasts derived from TAM-treated control mice and corn oil-treated  $\beta$ -catenin cKO mice were used as negative controls.

(B) Immunocytochemistry and quantification of  $\beta$ -catenin expression in primary myoblasts using an antibody that recognizes the  $\beta$ -catenin C-terminal part.

(C)  $\beta$ -catenin cKO and control myoblasts have similar BrdU incorporation rates.

(D) qRT-PCR of myogenic genes of control and  $\beta$ -catenin cKO primary myoblasts under proliferating conditions.

(E) Myogenin immunocytochemistry and proportion of Myogenin-positive cells after 24 hr under differentiating conditions of control and  $\beta$ -catenin cKO primary myocytes.

(F) Myosin heavy chain (MyHC) immunocytochemistry and proportion of nuclei incorporated in myotubes (fusion index) after 72 hr under differentiating conditions of control and  $\beta$ -catenin cKO primary myocytes.

Scale bars, 50  $\mu$ m. n = 3 primary cell cultures. Values are mean  $\pm$  SEM. \*p < 0.05, \*\*p < 0.01.

myoblasts for two passages and validated  $\beta$ -catenin gene deletion by genotyping PCR. As shown in Figure 4A, the deleted allele was detected only in  $\beta$ -catenin cKO myoblasts and not in control myoblasts or in myoblasts derived from  $\beta$ -catenin cKO mice that

had not been treated with TAM. Immunostaining for  $\beta$ -catenin proteins showed that 97% of the  $\beta$ -catenin cKO myoblasts do not express  $\beta$ -catenin (Figure 4B). Similarly, qRT-PCR analysis demonstrated a complete absence of  $\beta$ -catenin expression at

(G) Anti-Myogenin staining of regenerating TA cryosections in control and conditional mutant mice at 7 d.p.i. revealed the number of differentiating myocytes outside the newly forming myofibers under control and mutant conditions.

(H) Lagging myofibers expressing eMHCs were present in regenerating muscles of  $\beta$ -catenin cKO mice and, to a lower extent, in  $\beta$ -catenin CA animals.

(I) Proportion of lagged, eMHC-expressing myofibers in controls and  $\beta$ -catenin cKO and  $\beta$ -catenin CA regenerating TA muscles at 7d.p.i.

Scale bars, 50  $\mu$ m. n = 4 mice/genotype. Values are mean  $\pm$  SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

the transcript level in  $\beta$ -catenin cKO myoblasts (Figure 4D). Interestingly,  $\beta$ -catenin cKO myoblasts did not exhibit any perturbation in their proliferation potential, as shown by BrdU incorporation assays (Figure 4C). In contrast, quantification of myogenic gene expression demonstrated that, although  $\beta$ -catenin cKO myoblasts express similar *Pax7* transcript levels compared with controls, they express a lower amount of *MyoD* and *Myogenin* transcripts, suggesting that they may be less efficient in their differentiation potential (Figure 4D). To test this hypothesis, we induced the myoblasts to differentiate and quantified a lower proportion of Myogenin<sup>+</sup> myocytes in  $\beta$ -catenin cKO cultures compared with controls (Figure 4E). After 3 days in differentiation medium,  $\beta$ -catenin cKO cells gave rise to smaller myotubes containing fewer nuclei compared with control cells (Figure 4F). Our data indicate that  $\beta$ -catenin controls myogenic progenitor cell differentiation.

### Impaired Proliferation and Precocious Differentiation of $\beta$ -Catenin CA Myoblasts

We next tried to grow  $\beta$ -catenin CA primary myoblasts from MuSCs sorted by FACS, but we observed that these cells immediately differentiated after plating in vitro (42% Myogenin<sup>+</sup> cells after 48 hr and 94% Myogenin<sup>+</sup> cells after 72 hr in vitro; data not shown). This phenotype led us to grow primary myoblasts from  $\beta$ -catenin CA mice that were not subjected to TAM and to perform exon 3 deletion in vitro. Recombination of  $\beta$ -catenin exon 3 was efficiently induced in vitro by treating the cultures with 1 mM 4-hydroxy-tamoxifen (Figures 5A and 5B). In this case, we observed that genetic activation of  $\beta$ -catenin signaling lead to a decrease in BrdU incorporation in primary myoblasts (Figure 5C). This was accompanied by a strong upregulation of canonical Wnt target *Axin2* and *Wisp1* transcription, a downregulation of *Pax7* and *MyoD* as well as of the cell-cycle regulator *Chek1*, and a strong induction of *Myogenin* expression in primary myoblasts (Figure 5D). Furthermore, we could observe that the number of Myogenin and embryonic-MHC positive cells 24 hr after shifting to differentiation medium increased 3-fold when 4-hydroxy-tamoxifen had been applied to myoblasts (Figure 5E). In summary, our findings indicate that activation of  $\beta$ -catenin signaling in myoblasts causes cells to switch from proliferation to growth arrest and differentiation independently of the muscle microenvironment.

### Identification of $\beta$ -Catenin-Dependent Genes in Muscle Progenitor Cells

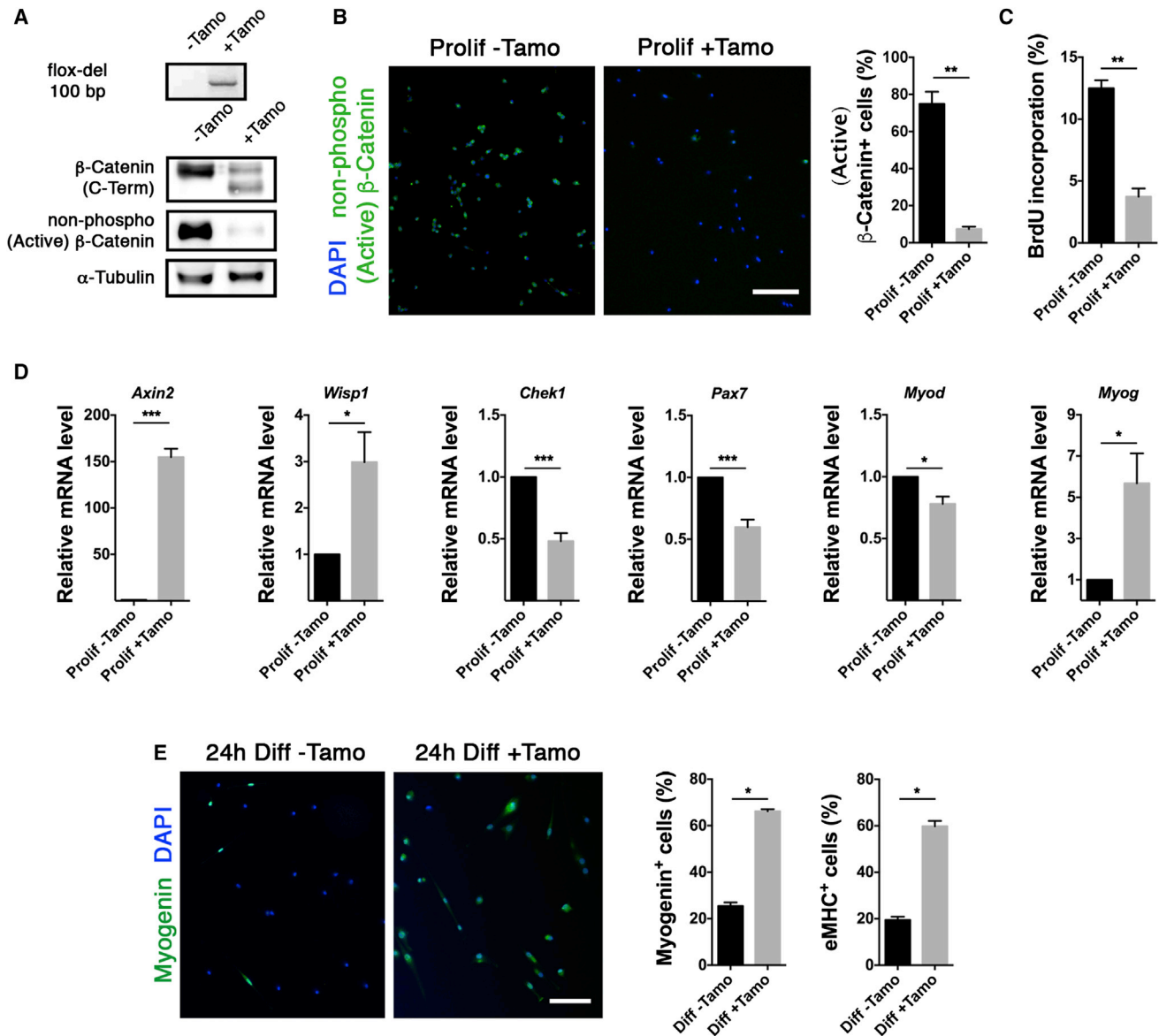
To identify genes that become transcribed following activation of the Wnt/ $\beta$ -catenin pathway in muscle cells, we performed microarray analysis of genetically disrupted  $\beta$ -catenin CA primary myoblasts compared with control cells (GEO: GSE72496). We observed that the transcription of many genes implicated in cell cycle and proliferation was significantly downregulated when the canonical Wnt pathway was constitutively active, whereas the transcription of many genes implicated in muscular development and function was upregulated (Figure 6A). Interestingly, genes encoding structural components of skeletal myofibers (identified under the category “muscular disorders and myopathies”) were also downregulated, suggesting that acceleration of the myogenic process in vitro perturbs normal muscle

cell maturation. We found that, when canonical Wnt signaling was activated, known Wnt target genes, along with components of the pathway itself, like *Porcupine*, *Frizzled1*, and *Nkd1*, become transcribed (Figure 6B). Furthermore, we observed that gene expression of other signaling pathways was also activated; i.e., components of the bone morphogenetic protein pathway, the Akt1/target of rapamycin (TOR) pathway, and the TGF- $\beta$  pathway. We validated the upregulation of gene expression of some genes found in the array by qRT-PCR (Figure 6C). Because a mutation in the TGF- $\beta$ 3 gene has been recently implicated in failed postnatal muscle growth (Rienhoff et al., 2013), we were interested in the signaling crosstalk of canonical Wnt and TGF- $\beta$  signaling pathways. We first validated that Wnt3a treatment resulted in elevated phosphorylation of the TGF- $\beta$  downstream effector proteins Smad2/3 in proliferating myoblasts (Figure 6D). Our microarray data indicated that TGF- $\beta$ 2 and TGF- $\beta$ 3 expression was elevated in primary myoblasts with the activated Wnt/ $\beta$ -catenin pathway, and we verified that both TGF- $\beta$ s' expression levels increased in  $\beta$ -catenin CA myoblasts as well as in wild-type proliferating and differentiating myoblasts when treated with Wnt3a by qRT-PCR (Figures 6E and 6F; data not shown). Accordingly, TGF- $\beta$ 2 and TGF- $\beta$ 3 expression was downregulated in  $\beta$ -catenin cKO myoblasts and in myoblasts treated with the *Porcupine* inhibitor LGK974 compared with control cells (Figures 6E and 6F). Taken together, these results indicate that activation of canonical Wnt/ $\beta$ -catenin signaling in myoblasts results in broad transcriptional changes associated with myogenic differentiation, including premature cell-cycle exit, precocious differentiation, and perturbed muscle cell differentiation. Furthermore, we identify the TGF- $\beta$  pathway as a potential effector of Wnt/ $\beta$ -catenin in adult muscle progenitor cells.

### Activation of the TGF- $\beta$ Pathway Restores the Ability of Myoblasts to Differentiate in $\beta$ -Catenin cKO Animals

A recent report demonstrated that TGF- $\beta$ 2 was induced in response to elevated canonical Wnt signaling in dystrophic muscles and that the resulting increase in TGF- $\beta$  activity lead to the conversion of dystrophic MuSCs to fibrogenic cells (Biressi et al., 2014). In contrast, we did not observe any changes in the expression of pro-fibrotic genes in our microarray data, and we further validated that activation of canonical Wnt signaling in healthy MuSCs descendants derived from non-dystrophic skeletal muscle tissue does not lead to increased expression of the *Col1a1* and *Col1a2* genes (Figure S3A). Additionally, treatment of control primary myoblasts with Wnt3a proteins during the course of myogenic differentiation did not lead to an increase in the proportion of non-myogenic cells in cell culture (Figure S3B). These results suggest that the Wnt/TGF $\beta$ 2 axis does not act similarly in healthy and dystrophic muscle progenitors.

To understand the role of the TGF- $\beta$  signaling pathway downstream of canonical Wnt signaling in MuSCs, we used the single myofiber assay. In this experimental setup, single myofibers from the EDL are carefully dissected out and cultured under “floating conditions.” Under these conditions, MuSCs become activated and proliferate on the surface of their host myofibers, generating clusters of amplifying progeny after 72 hr in culture (Figure 7A). At this time, MuSCs descendants either maintain



**Figure 5. Activation of  $\beta$ -Catenin Signaling in Proliferating Primary Myoblasts Promotes Growth Arrest and Myogenic Differentiation**

Primary myoblasts were prepared from untreated  $\beta$ -catenin CA mouse muscles.

(A) Confirmation of  $\beta$ -catenin exon 3 deletion, which generates a constitutively active  $\beta$ -catenin, in mutant cells at the DNA and protein levels. The flox-deleted allele could be amplified by PCR on isolated DNA of 4-hydroxy-tamoxifen (Tamo)-treated myoblasts, and a protein of smaller size could be detected by western blot. The amount of wild-type non-phosphorylated  $\beta$ -catenin was diminished in Tamo-treated cells compared with mock-treated cells.

(B) Immunocytochemistry and quantification of Tamo-treated and mock-treated  $\beta$ -catenin CA primary myoblasts using a  $\beta$ -catenin antibody that recognizes the protein fragment encoded by exon 3 (non-phospho active  $\beta$ -catenin).

(C) Myoblasts with constitutively active  $\beta$ -catenin do not incorporate BrdU.

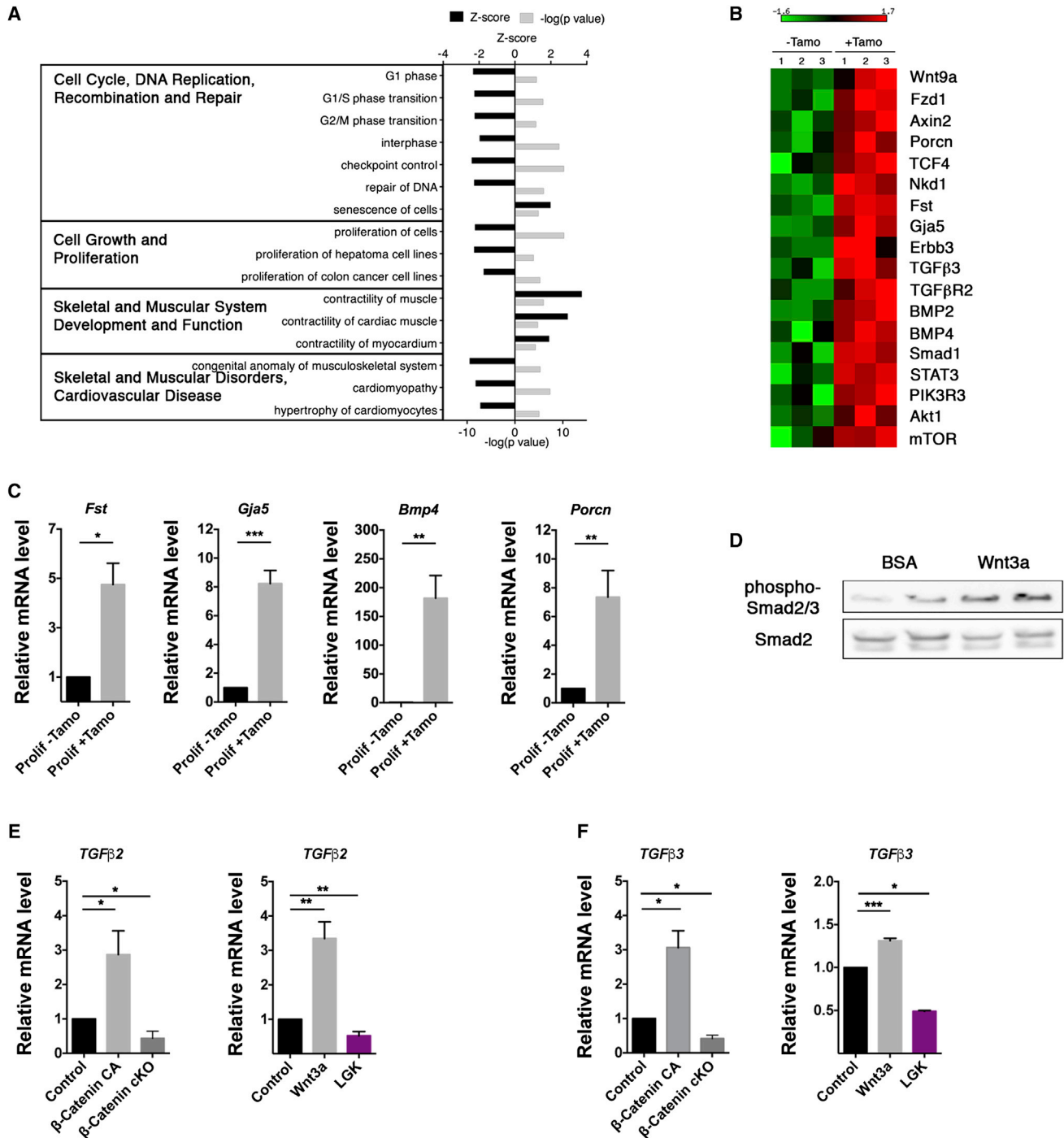
(D) qRT-PCR of Wnt targets, cell-cycle regulators, and myogenic genes of Tamo-treated and mock-treated  $\beta$ -catenin CA primary myoblasts under proliferating conditions.

(E) Myogenin immunocytochemistry and proportion of Myogenin and eMHC-positive cells after 24 hr under differentiating conditions of Tamo-treated or mock-treated  $\beta$ -catenin CA primary myocytes.

Scale bars, 50  $\mu$ m. n = 3 primary cell cultures. Values are mean  $\pm$  SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

the progenitor state (Pax7+) or start to differentiate (Myogenin+). As expected, LGK974 treatment lead to a diminution of the proportion of Myogenin+ cells compared with control conditions (Figure 7B). TGF- $\beta$ 2 addition alone to the culture medium did

not change the proportion of Myogenin-expressing cells (although cells appeared more dispersed around their host myofiber). Strikingly, co-treatment of single myofibers with both LGK974 and TGF- $\beta$ 2 completely restored the proportion



**Figure 6. TGF $\beta$  Signaling Is Induced in Primary Myoblasts with Conditionally Activated  $\beta$ -Catenin**

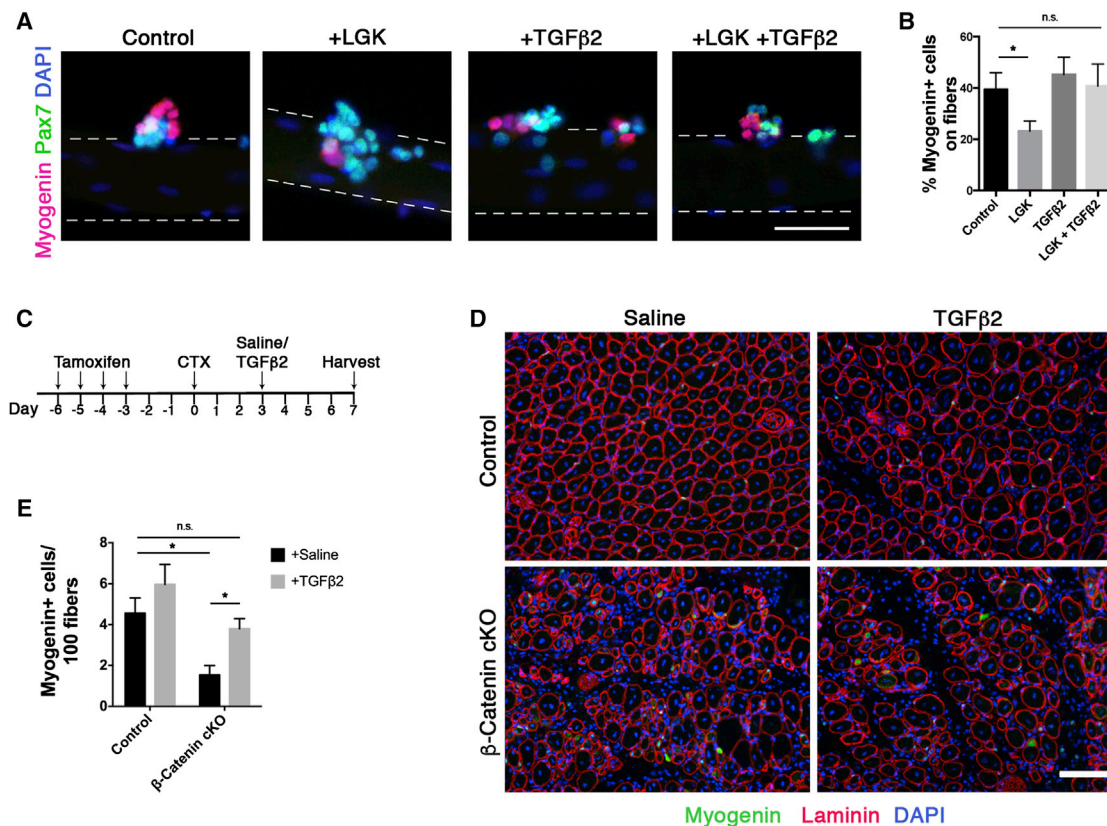
(A) Ingenuity pathway analysis of biological functions with their subcategories significantly ( $p < 0.05$ ) activated (Z score  $> 2$ ) or inhibited (Z score  $< -2$ ) in Tamoxifen-treated  $\beta$ -catenin CA myoblasts compared with mock-treated  $\beta$ -catenin CA myoblasts.

(B) Heatmap representation of the changes in selected gene expression levels in TAM-treated compared with mock-treated  $\beta$ -catenin CA primary myoblasts in three independent experiments (each row is normalized to have mean zero, variance one).

(C) qRT-PCR of Wnt target genes of Tamoxifen-treated and mock-treated  $\beta$ -catenin CA primary myoblasts under proliferating conditions.

(D) Activation of the canonical Wnt/ $\beta$ -catenin pathway in primary myoblasts by recombinant Wnt3a proteins results in phospho-Smad2/3 accumulation.

(legend continued on next page)



### Figure 7. TGF- $\beta$ 2 Addition Rescues Myogenic Differentiation in MuSCs Deficient for Wnt/ $\beta$ -catenin Signaling

(A) Single EDL myofibers were cultured ex vivo for 3 days under different conditions, fixed, and stained with Pax7 and Myogenin antibodies.  
 (B) Inhibition of Wnt secretion by LGK974 leads to a blockade in Myogenin expression compensated by TGF- $\beta$ 2 addition.  
 (C) Schematic of rescue experiments in TAM-treated control and  $\beta$ -catenin cKO mice. TA muscles were injured by a single CTX injection, recombinant TGF- $\beta$ 2 or saline solution was injected at 3 d.p.i., and muscles were analyzed at 7 d.p.i.  
 (D) Immunostaining for Laminin and Myogenin proteins on cryosections from 7 d.p.i. regenerating TA muscles.  
 (E) Quantification of the number of Myogenin-expressing cells per 100 myofibers. Exogenous TGF- $\beta$ 2 allows for MuSCs to differentiate in the absence of endogenous canonical Wnt signaling.  
 Scale bars, 25  $\mu$ m (A) and 50  $\mu$ m (E). n = 3 mice/condition. Values are mean  $\pm$  SEM. \*p < 0.05.

of Myogenin-expressing cells to control-treated ones. These results suggest that activation of TGF- $\beta$  signaling downstream of  $\beta$ -catenin controls the balance between proliferation and differentiation in muscle progenitor cells (Figure 7B).

We next tested whether activation of the TGF- $\beta$  pathway in vivo could compensate for the loss of  $\beta$ -catenin in MuSCs during regeneration and could bypass the failure of myoblasts to differentiate in  $\beta$ -catenin cKO animals. To this aim, we injected recombinant TGF- $\beta$ 2 protein into the TA of control and  $\beta$ -catenin cKO animals on day 3 of regeneration (Figure 7C). We observed that the number of Myogenin-positive myocytes outside the forming myofibers at 7 d.p.i. was significantly increased in  $\beta$ -catenin cKO animals when treated with TGF- $\beta$ 2 in comparison with  $\beta$ -cat-

enin cKO animals that were administered saline. Indeed, the number of Myogenin+ myocytes in TGF- $\beta$ 2-treated  $\beta$ -catenin cKO mice was not significantly different from the number of myocytes in control animals (Figures 7D and 7E). Moreover, myofibers were bigger and the tissue regenerated better in TGF- $\beta$ 2-injected  $\beta$ -catenin cKO animals than in saline-injected  $\beta$ -catenin cKO animals (Figure 7E; data not shown). These results suggest that exogenous TGF- $\beta$ 2 leads to a rescue of the proliferation/differentiation balance in myoblasts lacking  $\beta$ -catenin. From these data, we conclude that the Wnt/ $\beta$ -catenin signaling pathway in muscle progenitor cells activates the TGF- $\beta$  pathway, which in turn modulates their proliferation and differentiation to help orchestrate restoration of the injured skeletal muscle to its original size.

(E) Relative expression levels of *TGF- $\beta$ 2* in proliferating, control,  $\beta$ -catenin cKO, and TAM-treated  $\beta$ -catenin CA myoblasts and in wild-type proliferating myoblasts treated with recombinant Wnt3a proteins or the Porcupine inhibitor LGK974, respectively.  
 (F) Relative expression levels of *TGF $\beta$ 3* in proliferating, control,  $\beta$ -catenin cKO and TAM-treated  $\beta$ -catenin CA myoblasts and in wild-type proliferating myoblasts treated with recombinant Wnt3a proteins or the Porcupine inhibitor LGK974, respectively.  
 n = 3 primary cell cultures. Values are mean  $\pm$  SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

## DISCUSSION

Postnatal myogenesis is executed by a pool of adult stem cells, MuSCs. Upon injury to the tissue, MuSCs receive signals from their stem cell niche and microenvironment to be activated, proliferate, and give rise to muscle progenitor cells that will differentiate to fuse to their host myofibers or to each other to generate new muscle fibers de novo. Here we show that the canonical Wnt/ $\beta$ -catenin pathway is required by MuSC progeny during skeletal muscle regeneration. We found that, in mice with MuSC-specific  $\beta$ -catenin gain-of-function and loss-of-function mutations, muscle regeneration was disturbed following acute injury. Muscle progenitor cells with targeted  $\beta$ -catenin gene disruption differentiate less efficiently, whereas conditional activation of  $\beta$ -catenin pushed muscle progenitor cells prematurely toward differentiation.

During skeletal muscle development, Pax3/7+ myogenic progenitors arise from the mesoderm and are maintained as a proliferating population that sustains successive phases of myogenesis (Relaix et al., 2005). Canonical Wnt signaling has been shown to regulate both embryonic and fetal myogenesis.  $\beta$ -catenin activity is required for the maintenance of the epithelial structure of somites (Linker et al., 2005) and further for driving Myf5 expression in the somite expaxial domain in cells that will form the myotome (Borello et al., 2006). In the embryonic limb, different Wnt signals control myogenic differentiation (Anakwe et al., 2003), and, more specifically,  $\beta$ -catenin intrinsically controls fetal progenitor number as well as myofiber number and type (Hutcheson et al., 2009). The latter observations in the embryo agree well with our findings, suggesting that adult and fetal muscle progenitor cells share a requirement for  $\beta$ -catenin.

Previous work suggested that activation of canonical Wnt/ $\beta$ -catenin signaling in muscle cells is a necessary step for commitment to terminal differentiation (Zhuang et al., 2014), possibly by counteracting Notch (Brack et al., 2008) and Myostatin (Bernardi et al., 2011). Moreover, it has been shown that targeted recombination of the  $\beta$ -catenin transcriptional co-factors Bcl9 and Bcl9.2 in MuSCs inhibited the normal regenerative response of skeletal muscle (Brack et al., 2009). We demonstrate here that  $\beta$ -catenin function in adult myoblasts is crucial for their full regenerative potential to effectively repair skeletal muscle tissue after injury. Both our cell biology and transcriptome approaches validated that canonical Wnt signaling controls the proper timing of cell-cycle arrest and of the expression of differentiation markers by muscle progenitor cells both in vivo and in vitro.

Surprisingly, Murphy et al. (2014) recently used genetic approaches similar to ours but did not observe any obvious differences in the regenerative capacity of skeletal muscles with MuSCs homozygous for the deleted  $\beta$ -cateninEx2-6<sup>lox/lox</sup> allele in comparison to MuSCs heterozygous for  $\beta$ -catenin. In our hands, the regeneration deficits of both  $\beta$ -catenin cKO and  $\beta$ -catenin CA muscle were highly reproducible. There are multiple explanations for these discrepancies. First, it is possible that the use of  $\beta$ -catenin heterozygous mice as controls may already cause a regeneration phenotype and thus reduce the differences with conditional  $\beta$ -catenin-null mice. Second, targeted  $\beta$ -catenin recombination was not evaluated at the single

cell level but by FACS on MuSCs sorted from regenerating tissue by Murphy et al. (2014). It is possible that “escaper cells” already contributed to injury-induced myogenesis during the initial phases of regeneration. Third, the Pax7<sup>CreERT2</sup> mice used by Murphy et al. (2014) are not the same as we used, and it is well known that recombination efficiencies varies between different strains of mice. A high escaper ratio in conditional null MuSCs in the previous study could explain the absence of a regeneration phenotype. Importantly, our results are consistent with data obtained by injecting recombinant Wnt3a protein (Brack et al., 2008) or electroporating a Wnt3a expression plasmid (Le Grand et al., 2009) into regenerating muscles, which both perturbed muscle tissue repair.

Our transcriptome analysis allowed the identification of other signaling pathways regulated by  $\beta$ -catenin in primary myoblasts. We focused on TGF- $\beta$  signaling because this pathway is known to regulate muscle progenitor behavior and muscle regeneration. As such, mutation in the TGF- $\beta$ 3 gene has been shown to cause failed muscle growth after birth (Rienhoff et al., 2013). Further to that point, genetic disruption of Smad7 (Cohen et al., 2015) or Smad3 (Ge et al., 2012) in mice impairs muscle regeneration and leads to impaired myoblast differentiation in a similar fashion as the  $\beta$ -catenin cKO phenotype we report here. We observed that canonical Wnt signaling controlled the expression of both TGF- $\beta$ 2 and TGF- $\beta$ 3 in muscle progenitor cells and that injection of exogenous TGF- $\beta$ 2 during muscle regeneration was able to restore the ability of  $\beta$ -catenin cKO myoblasts to differentiate in vivo. We thus define TGF- $\beta$  pathway activation in MuSCs descendants, downstream of canonical Wnt signaling, as a necessary step for muscle tissue repair.

In contrast, the Wnt/TGF $\beta$ 2 axis was recently reported as being altered in dystrophic *mdx* mouse muscles (Biressi et al., 2014). In the diseased model of Duchenne muscular dystrophy, both canonical Wnt and TGF- $\beta$ 2 signaling are chronically elevated in the muscle tissue. In this pathological context, continuous Wnt/TGF $\beta$ 2 signals alter the muscle progenitor cell fate and lead to a significant proportion of the MuSCs losing their myogenic identity. However, we did not observe any changes in “fibrogenic” gene expression in myoblasts treated with Wnt3a proteins or with constitutively active  $\beta$ -catenin. In our hands, activation of canonical Wnt signaling in healthy muscle cells resulted in accelerated myogenic differentiation. Both results are not contradictory and can be explained in two ways. First, it is possible that MuSCs of a healthy or a dystrophic environment intrinsically respond differently to canonical Wnt and/or TGF- $\beta$  signals. It would be interesting to compare the transcriptomes as well as  $\beta$ -catenin and SMAD2/3 whole-genome occupancy in muscle progenitor cells from healthy and dystrophic muscle. Second, we think that the observed differences could be related to the duration of extrinsic signal reception by muscle cells in different environments. A short and high Wnt/TGF $\beta$  pulse during skeletal muscle repair will not have the same effect compared with continuous but low Wnt/TGF- $\beta$  systemic signaling.

We propose that a tight control of intrinsic  $\beta$ -catenin activity in MuSC progeny is required for their function within the skeletal muscle tissue. We delineated that either loss of  $\beta$ -catenin function or unscheduled  $\beta$ -catenin activity in muscle progenitor cells leads to defects in muscle regeneration that resemble a

pathological state (smaller myofibers, enhanced fibrosis, etc.). To that point, mis-regulation of canonical Wnt signaling has been reported in multiple muscle pathologies, such as fascio-scapulohumeral muscular dystrophy (Block et al., 2013), oculopharyngeal muscular dystrophy (Abu-Baker et al., 2013), and Duchenne muscular dystrophy (Trensz et al., 2010). Our findings represent a significant advance in our understanding of MuSC biology and muscle regeneration. Future experiments will investigate the utility of modulating the canonical Wnt/ $\beta$ -catenin pathway in vivo toward ameliorating the loss of muscle function in neuromuscular disease.

## EXPERIMENTAL PROCEDURES

### Mice and In Vivo Cre Activation

Animals were handled according to European Community guidelines, and experiments were performed in accordance with the guidelines of the French Veterinary Department and approved by the University Paris-Descartes Ethical Committee for Animal Experimentation.  $\beta$ -catenin-Exon2-6<sup>lox/lox</sup> (Brault et al., 2001) and  $\beta$ -catenin-Exon3<sup>lox/wt</sup> mice (Harada et al., 1999) (both provided by Sabine Colnot, Institut Cochin) were crossed to Pax7<sup>CreERT2</sup> (Lepper et al., 2009) mice (provided by Margaret Buckingham, Institut Pasteur). Progenitors were checked by genotyping as described before. Comparisons were made between age-matched littermates. To induce targeted gene disruption, 200  $\mu$ l of TAM (20 mg/ml in corn oil, Sigma) was applied intraperitoneally on four subsequent days to 8-week-old mice with an average weight of 20 g.

### Skeletal Muscle Injury

30  $\mu$ l of CTX (12  $\mu$ M in saline, Latoxan) was injected into hindlimb TA muscles to induce injury, and mice were euthanized 4, 7, and 30 days afterward. Recombinant mouse TGF- $\beta$ 2 (R&D Systems) was diluted in 4 mM HCl/saline at 10 ng/ml, and 25 ml was injected into the TA on day 3 of regeneration. Muscles were freshly frozen in Tissue-Tek CRYO-OCT compound (Fisher Scientific) and cut in cryostat sections of 10  $\mu$ m.

### Cell Culture

For single myofiber preparation, EDL muscles were dissected from hindlimbs, and myofibers were isolated and cultured like described before (Le Grand et al., 2012). Primary myoblasts were expanded from MuSCs sorted by FACS for  $\beta$ -catenin cKO mice (Pax7<sup>CreERT2</sup>;  $\beta$ -catenin-exon2-6<sup>lox/lox</sup>) or extracted from limb muscles using the pre-plate technique for  $\beta$ -catenin CA mice (Pax7<sup>CreERT2</sup>;  $\beta$ -catenin-exon3<sup>lox/+</sup>). Primary myoblasts were cultured on collagen-coated dishes as described previously (Le Grand et al., 2012). Proliferation medium consisted of Ham's F-10 with 20% FBS (Eurobio), 2 ng/ml basic fibroblast growth factor (FGF) (R&D Systems), and 1% penicillin/streptomycin (Pen/Strep) (Life Technologies). To induce ex vivo targeted gene disruption, medium containing 4-hydroxy-tamoxifen (Sigma) at 1  $\mu$ M was applied to low-passage  $\beta$ -catenin CA cells on 3 consecutive days, and cells were used for experiments after 3 more days. For differentiating conditions, cells were seeded at 20,000 cells/cm<sup>2</sup> on 1 mg/ml Matrigel matrix (Corning Life Sciences) in differentiation medium consisting of DMEM with 2% horse serum (Sigma) and 1% Pen/Strep (Life Technologies) and were analyzed after 24 hr. Recombinant mouse Wnt3a was applied to wild-type myoblasts at 50 ng/ml, and LGK974 Porcupine inhibitor (StemRD) was added at 2  $\mu$ M.

### Reverse Transcription and qPCR

RNA was isolated from TAM-treated and control cells with TRIzol reagent. After subsequent DNase treatment, cDNA was generated using a high-capacity reverse transcription kit (Ambion/Life Technologies). qPCR using LightCycler 480 SYBR Green Master Mix (Roche) was run in LC480 and performed in doublets (see Table S1 for the primer pairs sequences). Transcript levels were determined by absolute quantification using a four-point standard curve. Relative gene expression was calculated by normalization against the *TBP* and *Cyclophilin* reference genes.

### Immunostaining

H&E staining of muscle cryosections was performed according to standard procedures. For fluorescent antibody staining, cryosections were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 (Sigma) prior to unmasking with antigen unmasking solution (Vector Laboratories) at 95°C for 15 min and blocking with 5% BSA/2% goat serum for 1 hr. Cryosections were incubated overnight with primary antibodies at 4°C. Primary myoblasts were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked with 2% goat serum, and incubated with primary antibodies for 1 hr. For proliferation studies, cells were incubated with 30 mM bromodeoxyuridine (BrdU) (Sigma) for 40 min and fixed and treated with 2 N HCl for 20 min. DyLight-conjugated secondary antibodies (Abcam) were applied at 1:1,000, nuclei were counterstained with DAPI at 0.5  $\mu$ g/ml, and sections and cells, respectively, were mounted in Dako mounting medium. See Table S1 for the primary antibody list.

### Microscopy and Statistics

Labeling was visualized by epifluorescent illumination using a MacroScope AZ100 (Nikon). Quantification of muscle fiber sizes and the collagen-covered area were performed with ImageJ (<http://imagej.nih.gov/ij/>). Pictures were processed with Adobe Photoshop CS6. Histological analyses were conducted on at least three different animals per genotype. Experiments with cells were performed independently at least three times in duplicates. Statistics were performed with GraphPad Prism 6. All values are expressed as mean  $\pm$  SEM. Significance between two groups was determined by unpaired Student's t tests, and significance between multiple groups was determined using paired Student's t tests.  $p < 0.05$  was considered statistically significant.

### ACCESSION NUMBERS

The accession number for the expression data reported in this paper is GEO: GSE72496.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.04.022>.

### AUTHOR CONTRIBUTIONS

F.L.G. and A.R. conceived the experiments. A.R. performed most of the experiments. E.S., L.G., A.P., and F.L.G. also generated the experimental data. E.S. revised the manuscript. F.L.G., E.S., and A.R. analyzed the data. C.L. provided the Pax7Cre mice. M.M.T. provided the  $\beta$ -catenin-Exon3 mice. F.L.G. and A.R. generated the figures. F.L.G. wrote the manuscript. All authors corrected the manuscript.

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