

## RESEARCH COMMUNICATION

# Polycomb EZH2 controls self-renewal and safeguards the transcriptional identity of skeletal muscle stem cells

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**Satellite cells (SCs) sustain muscle growth and empower adult skeletal muscle with vigorous regenerative abilities. Here, we report that EZH2, the enzymatic subunit of the Polycomb-repressive complex 2 (PRC2), is expressed in both Pax7<sup>+</sup>/Myf5<sup>-</sup> stem cells and Pax7<sup>+</sup>/Myf5<sup>+</sup> committed myogenic precursors and is required for homeostasis of the adult SC pool. Mice with conditional ablation of *Ezh2* in SCs have fewer muscle postnatal Pax7<sup>+</sup> cells and reduced muscle mass and fail to appropriately regenerate. These defects are associated with impaired SC proliferation and derepression of genes expressed in nonmuscle cell lineages. Thus, EZH2 controls self-renewal and proliferation, and maintains an appropriate transcriptional program in SCs.**

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Adult skeletal muscle regenerates in response to traumatic injuries or degenerative conditions. This property is afforded mainly by satellite cells (SCs), a heterogeneous population of resident committed myogenic progenitors and noncommitted stem cells (Sherwood et al. 2004; Collins et al. 2005; Montarras et al. 2005; Kuang et al. 2007). In the mouse, postnatal SCs are mitotically active for the initial 2 wk after birth. After this period, they enter quiescence and their number declines. However, following muscle injury or degeneration, adult SCs undergo intense proliferation and efficiently differentiate. To replenish the reservoir, a subset of dividing SCs returns to the niche following a process of asymmetric and

symmetric cell division (Shinin et al. 2006; Conboy et al. 2007; Kuang et al. 2007; Shea et al. 2010). Approximately 10% of noncommitted Pax7<sup>+</sup>/Myf5<sup>-</sup> SCs can asymmetrically generate a self-renewing, noncommitted Pax7<sup>+</sup>/Myf5<sup>-</sup> cell and a committed Pax7<sup>+</sup>/Myf5<sup>+</sup> daughter cell in vivo. The noncommitted Pax7<sup>+</sup>/Myf5<sup>-</sup> cell returns to the niche to maintain the SC reservoir, while the committed Pax7<sup>+</sup>/Myf5<sup>+</sup> SC undergoes several rounds of cell division and the ensuing cells eventually differentiate into pre-existing or newly formed myofibers (Kuang et al. 2008).

Polycomb group (PcG) proteins regulate differentiation of totipotent embryonic stem (ES) cells and maintenance of multipotent and progenitor stem cell populations (Sauvageau and Sauvageau 2010). The Polycomb-repressive complex 2 (PRC2) subunit EZH2 methylates histone H3 Lys 27 (H3K27me3), establishing an epigenetic mark that identifies repressed chromatin regions. Ablation of PRC2 members in ES cells impairs their differentiation (Pasini et al. 2007; Chamberlain et al. 2008; Shen et al. 2008) and results in unscheduled expression of mixed cell lineage genes (Boyer et al. 2006; Lee et al. 2006). While PcG establishes and maintains positional patterning of the body axis through regulation of Hox genes in both *Drosophila* and mammals, its role in conferring cell identity by repressing inappropriate cell lineage-specific transcription in animal development has not been demonstrated. Indeed, derepression of mixed cell lineage genes does not occur in epidermal, neuronal, or pancreatic cells of *Ezh2* conditional null mice (Chen et al. 2009; Ezhkova et al. 2009; Hirabayashi et al. 2009).

We generated mice in which *Ezh2* was conditionally ablated in SCs (*Ezh2* muscle knockout, *Ezh2*<sup>mkO</sup>). While EZH2 was dispensable for fetal muscle development, it was required for postnatal muscle growth and adult muscle regeneration, ensuring appropriate homeostasis of the SC pool. Unlike other progenitor cells, reduced H3K27me3 in *Ezh2*<sup>mkO</sup> SCs was accompanied by RNA polymerase II (PolII) recruitment and transcriptional activation of genes normally repressed in SCs and expressed in other cell lineages, including cardiac progenitors, retinal cones, neurons, and chondrocytes. Thus, our findings indicate that EZH2, which regulates proliferation and maintains transcriptional identity of adult muscle stem cells, is an important molecular component of adult skeletal myogenesis.

## Results and Discussion

### *Pax7* and *Ezh2* are coexpressed in postnatal SCs

Pax7 is essential for SC specification and survival (Buckingham and Relaix 2007). While Pax7<sup>+</sup> cells were present at embryonic day 15.5 (E15.5) and E17.5, EZH2 could not be detected in skeletal muscle but was expressed in the epidermis (Supplemental Fig. S1A, Ezhkova et al. 2009). At postnatal day 1 (P1), EZH2 was expressed in limb muscles, where it remained detectable, along with Pax7, for ~14 d (P14) (Fig. 1A). Indeed, the vast majority of Pax7<sup>+</sup> cells coexpressed EZH2 in muscle cross-sections of P8 animals (Fig. 1B). Both EZH2 and Pax7 decreased and became undetectable by immunoblot after P21 (Fig. 1A), consistent with cessation of myonuclei accretion.

[*Keywords:* Polycomb; *Ezh2*; satellite cells; muscle growth; muscle regeneration]

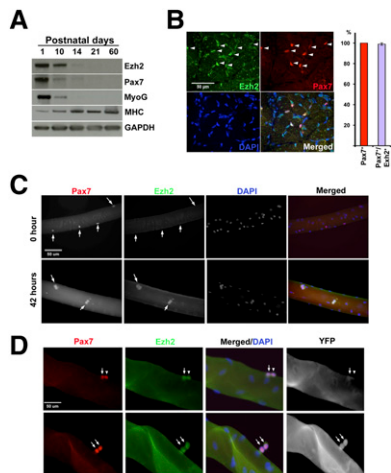
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**Figure 1.** Ezh2 expression in limb muscles and SCs. (A) Limb muscle extracts from mice at P1–P60 probed with the indicated antibodies. (B) In P8 hindlimb muscles, EZH2 is expressed in Pax7<sup>+</sup> cells. Arrowheads indicate cells positive for Pax7 and EZH2. Quantification of Pax7<sup>+</sup>/EZH2<sup>+</sup> cells in P8 EDL muscles. (C, top panel) Myofiber-associated quiescent (0 h) Pax7<sup>+</sup> SCs do not express a detectable level of EZH2. (Bottom panel) After 42 h, EZH2 is expressed in couplets of dividing Pax7<sup>+</sup> SCs. (D) In single myofibers of *Myf5-Cre/ROSA-YFP* mice cultured for 42 h, EZH2 is expressed in both Pax7<sup>+</sup>/YFP<sup>-</sup> (top panel) and Pax7<sup>+</sup>/YFP<sup>+</sup> (bottom panel) cells. Arrows indicate YFP<sup>+</sup> cells, and arrowheads indicate YFP<sup>-</sup> cells. Bars, 50  $\mu$ m.

### *Ezh2 is expressed in both noncommitted and myogenically committed proliferating SCs*

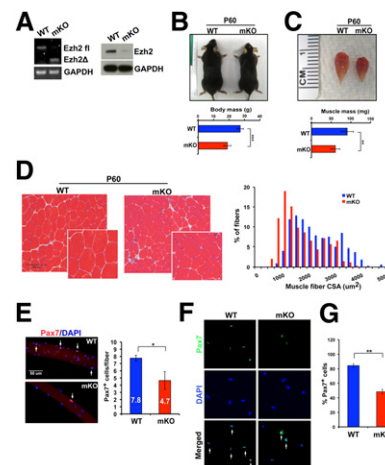
To evaluate EZH2 expression in quiescent and activated (i.e., cells that have entered the cell cycle) SCs, individual myofibers and their associated SCs were isolated from the extensor digitorus longus (EDL) muscle of 2-mo-old mice (P60) and either fixed immediately or cultured for 42 h to stimulate SC proliferation. While Pax7 was expressed in both conditions, EZH2 was detected only in Pax7<sup>+</sup> dividing SCs at 42 h (Fig. 1C). Neither Pax7 nor EZH2 was detected in myofiber nuclei (Fig. 1C). The low abundance of SCs at P21 prevented Pax7 detection (Fig. 1A).

The SC pool is composed of at least two hierarchical subpopulations (Kuang et al. 2007). Pax7<sup>+</sup>/Myf5<sup>-</sup> cells are noncommitted self-renewing stem cells maintaining the SC compartment and giving rise to Pax7<sup>+</sup>/Myf5<sup>+</sup> myogenic committed precursors fated to differentiate into myofibers. To investigate EZH2 expression in these two subpopulations, we used a lineage-tracing approach based on the *Myf5-Cre/ROSA-YFP* mouse, where cells that express or have ever expressed Myf5-Cre are YFP<sup>+</sup>, while cells that have never expressed Myf5-Cre are YFP<sup>-</sup> (Kuang et al. 2007). Immunostaining with Pax7 and EZH2 antibodies of *Myf5-Cre/ROSA-YFP* myofibers cultured for 42 h in growth medium revealed that Pax7 and EZH2 were coexpressed in both YFP<sup>-</sup> (Fig. 1D, top panel) and YFP<sup>+</sup> (Fig. 1D, bottom panel) cells (Fig. 1D). Thus, EZH2 is expressed in both dividing Pax7<sup>+</sup>/Myf5<sup>-</sup> noncommitted stem cells and Pax7<sup>+</sup>/Myf5<sup>+</sup> muscle progenitors.

### *Mice with conditional ablation of Ezh2 in Pax7-derived muscle precursor cells have reduced muscle mass with smaller myofibers*

*Ezh2* was selectively ablated in Pax7-derived cells by crossing knock-in mice expressing Cre recombinase from

the Pax7 locus (*Pax7-Cre*) (Keller et al. 2004) with mice bearing floxed *Ezh2* alleles (Su et al. 2003). In *Pax7-Cre<sup>+/+</sup>; Ezh2<sup>fl/fl</sup>* mice (*Ezh2<sup>mKO</sup>*), both EZH2 RNA and protein were largely and specifically reduced in muscle tissue (Fig. 2A). *Ezh2* deletion did not affect the expression of other members of the PRC2 complex, Suz12 and Eed, or the PRC1 protein Bmi1 (Supplemental Fig. S2A). *Ezh2* floxed alleles were not deleted in the kidney, heart, and white fat (data not shown). *Ezh2<sup>mKO</sup>* mice had no obvious feeding difficulties and were apparently healthy. At E17.5, when EZH2 is not expressed in muscle tissues (Supplemental Fig. S1A), we did not observe gross morphological changes or appreciable abnormalities in *Ezh2<sup>mKO</sup>* fetal myofibers (Supplemental Fig. S1B). In addition, a comparable number of Pax7<sup>+</sup> cells in the EDL of *Ezh2<sup>mKO</sup>* and littermate controls further suggested that EZH2 is not required for fetal muscle development (Supplemental Fig. S1B). However, at P8, the muscle size of *Ezh2<sup>mKO</sup>* animals was obviously smaller than that of littermate controls, and myofibers with a reduced cross-sectional area (CSA) were prevalent (Supplemental Fig. S2B,C). The myofiber number was comparable with that of littermate controls. These postnatal muscle defects persisted through adulthood, and both the body mass and the total muscle mass of P60 *Ezh2<sup>mKO</sup>* animals were significantly reduced (Fig. 2B,C), with a prevalence of smaller myofibers (Fig. 2D). Thus, *Ezh2* ablation in Pax7-derived skeletal muscle cells results in defects of postnatal muscle growth characterized by reduced muscle mass with smaller muscle fibers.



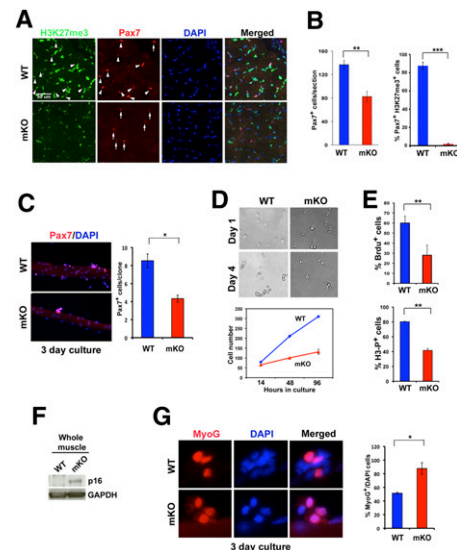
**Figure 2.** Conditional *Ezh2* ablation results in postnatal skeletal muscle defects and an impoverished SC pool. (A) RNA (left) and protein (right) analysis of *Ezh2* in wild-type (WT) (*Pax7-Cre<sup>-/-</sup>; Ezh2<sup>fl/fl</sup>*) and mKO (*Pax7-Cre<sup>+/+</sup>; Ezh2<sup>fl/fl</sup>*) littermates. EZH2 $\Delta$  and EZH2 $\Delta$  correspond to floxed and SET-deleted *Ezh2* RNAs, respectively. (B,C) Both body and muscle mass are significantly reduced in P60 mKO compared with control (wild-type) littermates. Data are presented as mean  $\pm$  SD ( $n = 6$ ); (\*\*\*)  $P < 0.0005$ ; (\*)  $P < 0.005$ . (D) H&E staining of transverse sections of P60 TA reveals a reduced myofiber CSA in *Ezh2<sup>mKO</sup>* animals ( $n = 3$ ). Ninety-five percent confidence intervals do not overlap. (E) EDL single myofibers from adult (P60) *Ezh2<sup>mKO</sup>* animals have a reduced number of SCs. Arrows indicate Pax7<sup>+</sup> cells. Data are presented as mean  $\pm$  SD ( $n = 6$ ); (\*)  $P < 0.05$ . (F,G) Pax7 immunostaining of FACS-isolated wild-type and *Ezh2<sup>mKO</sup>* SCs. Arrows indicate cells stained positive for Pax7 and DAPI. Data are presented as mean  $\pm$  SD ( $n = 3$ ); (\*\*\*)  $P < 0.005$ .

### *Ezh2 is required for maintenance of the adult muscle SC pool*

We examined the quiescent SC population of P60 mice. Pax7<sup>+</sup> cells on freshly isolated individual myofibers were reduced by ~40% in *Ezh2*<sup>mKO</sup> animals (Fig. 2E). To further substantiate these findings, we isolated the quiescent SC population from both wild-type and *Ezh2*<sup>mKO</sup> animals by FACS by gating on integrin  $\alpha$ -7<sup>+</sup> (positive selection) and Lin<sup>-</sup> (CD31<sup>-</sup>, CD45<sup>-</sup>, CD11<sup>-</sup>, Sca1<sup>-</sup>) (negative selection) cells. Although the percentage of the integrin- $\alpha$ 7<sup>+</sup>Lin<sup>-</sup> population was similar in wild type and *Ezh2*<sup>mKO</sup> (Supplemental Fig. 3A), only ~50% of the purified cells were Pax7<sup>+</sup> in *Ezh2*<sup>mKO</sup> compared with ~85% in wild type (Fig. 2F,G). In agreement with the results reported above, muscle cross-sections of *Ezh2*<sup>mKO</sup> animals contained only ~50% of the Pax7<sup>+</sup> cells of littermate controls (Supplemental Fig. S3B). Reduced Pax7 expression in *Ezh2*<sup>mKO</sup> is not the consequence of direct regulation by EZH2, since adenoviral Cre-mediated *Ezh2* excision did not affect the Pax7 level (Supplemental Fig. S3C). Together, these data suggest that EZH2 regulates establishment and/or maintenance of the adult SC pool.

### *Ezh2 affects SC proliferation and differentiation*

We evaluated the SC population by quantifying Pax7<sup>+</sup> cells in P8 mice, when SCs are highly proliferative. Pax7<sup>+</sup> cells, located under the basal lamina, were decreased by ~40% in *Ezh2*<sup>mKO</sup> animals and, consistent with *Ezh2* ablation, H3K27me3<sup>+</sup> cells were hardly detected (Fig. 3A,B; Supplemental Fig. S3D). The reduced number of Pax7<sup>+</sup> cells in *Ezh2*<sup>mKO</sup> animals suggested that SC proliferation may be impaired. To test this hypothesis, individual myofibers derived from either wild-type or *Ezh2*<sup>mKO</sup> EDL muscle were isolated and cultured for 3 d to allow for SC delamination and proliferation. The number of Pax7<sup>+</sup> cells derived from 3-d cultured *Ezh2*<sup>mKO</sup> myofibers was significantly curtailed, when compared with that of wild-type myofibers (Fig. 3C). An equivalent number of FACS-isolated cells obtained from either wild-type or *Ezh2*<sup>mKO</sup> animals was plated and cultured for 14, 48, and 96 h, respectively. Fewer *Ezh2*<sup>mKO</sup> cells were present after 48 and 96 h in culture (Fig. 3D). 5-bromo-2'-deoxyuridine (BrdU) cell incorporation and immunostaining with an antibody detecting histone H3Ser10 phosphorylation—a marker of the G2/M mitotic transition—indicated that *Ezh2*<sup>mKO</sup>-derived cells had reduced proliferative capacity (Fig. 3E). Apoptosis was not increased in *Ezh2*<sup>mKO</sup> SCs (Supplemental Fig. S4A). The proliferative defects of *Ezh2*<sup>mKO</sup> SCs are likely the consequence of derepression of the cell cycle inhibitor p16 (Cdkn2a) (Fig. 3F; Supplemental Table S1; Bracken et al. 2007; Kotake et al. 2007; Chen et al. 2009; Ezhkova et al. 2009; Pereira et al. 2010). To directly test this hypothesis, we reduced p16 in FACS-isolated and cultured SCs by siRNA (Supplemental Fig. S4B). A higher percentage of *Ezh2*<sup>mKO</sup> SCs with reduced p16 scored positive for H3Ser10 phosphorylation compared with control siRNA transfected *Ezh2*<sup>mKO</sup> SCs. Reducing p16 did not affect H3Ser10 phosphorylation of wild-type SCs. (Supplemental Fig. S4B). Thus, p16 up-regulation is partly responsible for the proliferative defects of *Ezh2*<sup>mKO</sup> SCs. Despite the reduced number of Pax7<sup>+</sup> cells per clone (Fig. 3C), the majority of *Ezh2*<sup>mKO</sup> SCs cultured for 72 h expressed myogenin (Fig. 3G), suggesting earlier differen-



**Figure 3.** Skeletal muscle progenitors derived from *Ezh2*<sup>mKO</sup> animals have proliferative defects and precociously activate myogenin. (A,B) Immunostaining of H3K27me3 and Pax7 of P8 EDL. Arrowheads indicate cells stained positive for both Pax7 and H3K27me3. Arrows indicate Pax7<sup>+</sup>/H3K27me3<sup>-</sup> cells. Data are presented as mean  $\pm$  SD ( $n = 3$ ); (\*\*\*)  $P < 0.0005$ ; (\*\*)  $P < 0.005$ . (C) Pax7 immunostaining of EDL single myofibers isolated from wild-type (WT) and *Ezh2*<sup>mKO</sup> mice cultured for 3 d. (\*)  $P < 0.05$ . (D) FACS-isolated wild-type and *Ezh2*<sup>mKO</sup> SCs after 1 or 4 d in culture. Cells ( $1.5 \times 10^4$ ) of wild-type and *Ezh2*<sup>mKO</sup> SCs were seeded, and the total number obtained from counting cells present in 10 independent microscopic fields after 14, 48, and 96 h in culture was plotted in the curve graph ( $n = 4$ ;  $P < 0.001$  and  $P < 0.003$  for 42-h and 96-h counts, respectively). (E) Percentage of FACS-isolated wild-type and *Ezh2*<sup>mKO</sup> SCs that have incorporated BrdU or that stained positive for histone-H3 (H3-P). Data are presented as mean  $\pm$  SD for BrdU ( $n = 3$ ; [\*]  $P < 0.005$ ) and H3-P ( $n = 4$ ; [\*]  $P < 0.005$ ). (F) Increased p16 protein in *Ezh2*<sup>mKO</sup> muscles. GAPDH serves as a loading control. (G) Myogenin immunostaining of SC clones from wild-type and *Ezh2*<sup>mKO</sup> myofibers. Data are presented as mean  $\pm$  SD; (\*)  $P < 0.05$ .

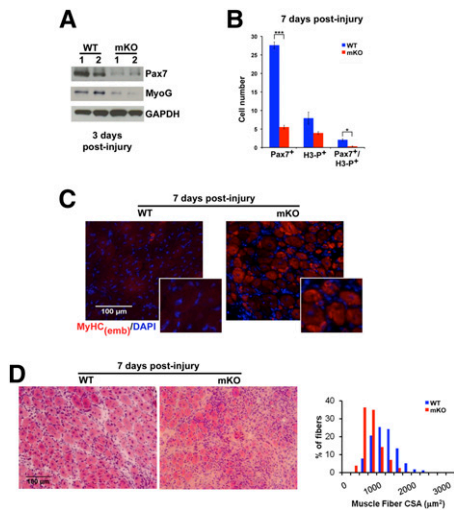
tiation potential (Juan et al. 2009). Overall, these results indicate that EZH2 plays an important role in the SC's proliferation and differentiation.

### *Muscle regeneration is compromised in Ezh2mKO animals*

The proliferative defects of SCs and the depletion of the quiescent SC pool observed in *Ezh2*<sup>mKO</sup> mice suggested that muscle regeneration may be compromised in these animals. To test this hypothesis, muscle damage was induced by injecting the tibialis anterior (TA) muscle of adult (P60) wild-type and *Ezh2*<sup>mKO</sup> animals with cardiotoxin (CTX). Three days after CTX injection, Pax7 and myogenin, which are normally undetectable by immunoblot in uninjured adult muscles (Fig. 1A), were readily seen in wild type but were reduced in *Ezh2*<sup>mKO</sup> animals (Fig. 4A). Reduced myogenin expression in *Ezh2*<sup>mKO</sup> animals is likely the consequence of defective SC proliferation rather than impaired differentiation, as *Ezh2*<sup>mKO</sup> SCs display anticipated myogenin expression (Fig. 3G). Seven days after CTX injection, proliferation of Pax7<sup>+</sup> cells in the *Ezh2*<sup>mKO</sup> regenerating muscle was severely impaired, as indicated by an almost 10-fold reduction of double-positive Pax7<sup>+</sup>/phospho-H3<sup>+</sup> cells (Fig. 4B). At this stage,



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**Figure 4.** Impaired muscle regeneration in *Ezh2<sup>mKO</sup>* animals. (A) Immunoblots of limb muscle extracts from wild-type (WT) or *Ezh2<sup>mKO</sup>* animals ( $n = 2$ ) 3 d after CTX, probed with myogenin, Pax7, and GAPDH antibodies. (B) Pax7 and H3-P immunostaining of TA muscles from wild-type and *Ezh2<sup>mKO</sup>* animals 7 d after CTX injection. Data are presented as mean  $\pm$  SD ( $n = 3$ ); (\*\*\*)  $P < 0.0005$ ; (\*)  $P < 0.05$ . (C) Embryonic MyHC [MyHC<sub>(emb)</sub>] immunostaining in wild-type and *Ezh2<sup>mKO</sup>* mice in 7-d regenerating myofibers. (D) H&E staining of transverse sections of wild-type or *Ezh2<sup>mKO</sup>* regenerating TA muscles 7 d after CTX injection indicates that the CSA of centrally nucleated myofibers is reduced in *Ezh2<sup>mKO</sup>* animals ( $n = 3$ ). (Right panel) Ninety-five percent confidence intervals do not overlap.

while regenerating myofibers of littermate controls had almost extinguished expression of embryonic myosin heavy chain (eMyHC), a marker of muscle regeneration, *Ezh2<sup>mKO</sup>* myofibers exhibited intense eMyHC staining and were smaller, indicating that muscle repair in *Ezh2<sup>mKO</sup>* lagged behind that of control mice (Fig. 4C). Indeed, the majority of newly formed myofibers, identified by the presence of nuclei located in a central position, had a smaller CSA in *Ezh2<sup>mKO</sup>* animals (Fig. 4D). Overall, these findings indicate that EZH2 is required for appropriate SC proliferation during muscle regeneration.

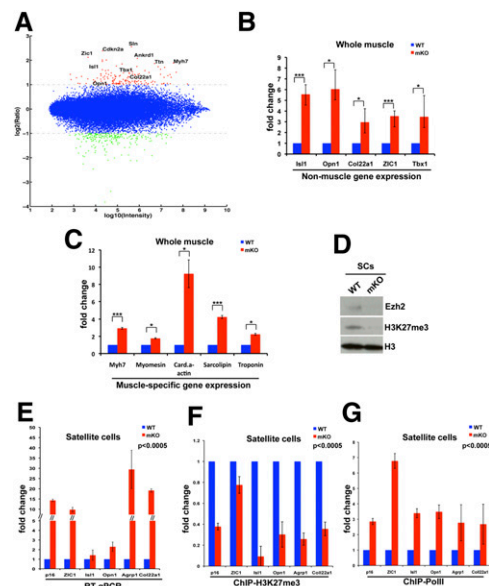
#### *Ezh2* maintains the transcriptional identity of SCs

We compared transcriptional profiles of wild-type and *Ezh2<sup>mKO</sup>* muscles by microarray analysis (Fig. 5A; Supplemental Table S1). Among the down-regulated transcripts in *Ezh2<sup>mKO</sup>* muscle were several corresponding to developmental regulators and structural proteins (Supplemental Table S1). It is likely that reduced transcription is an indirect effect of *Ezh2* ablation mediated by intermediate regulators, as H3K27me3 is not known to be directly associated with transcriptional activation. Gene ontology (GO) analysis for up-regulated genes found significant enrichment for functional classes, including those corresponding to muscle functions (Supplemental Table S2). Indeed, transcripts for muscle-expressed titin, sarcolipin, myosin Myh7, myomesin3, cardiac actin, Ankrd1, and troponin Tnni1 were increased in *Ezh2<sup>mKO</sup>* (Fig. 5A; Supplemental Table S1). In addition, several developmental regulators expressed in multiple lineages that are PRC targets in ES cells were also up-regulated (Fig. 5A; Supplemental Table S1). Among those were ZIC-1 (expressed in early somites and the cerebel-

lum), *Isl1* (expressed in cardiac progenitors and the pancreas), and *Tbx1* (expressed in cardiac progenitors). Transcripts of structural genes expressed in other cell lineages were also up-regulated in *Ezh2<sup>mKO</sup>*. For instance, expression of the agouti-related protein homolog 1 (*Agrp1*), expressed in neurons of the arcuate nucleus of the hypothalamus (Hahn et al. 1998); the collagen *Col22a1*, specifically expressed in chondrocytes (Koch et al. 2004); and *opsin1* (*Opn1*), encoding a retinal photopigment expressed in retinal cones (Shichida and Matsuyama 2009) was increased (Fig. 5A; Supplemental Table S1). Expression of some of the transcripts identified as up-regulated by microarray analysis was evaluated independently by quantitative PCR (qPCR) and confirmed to be increased in the limb muscles of *Ezh2<sup>mKO</sup>* animals. (Fig. 5B,C). Importantly, expression of mixed cell lineage genes and p16 was increased in FACS-isolated quiescent *Ezh2<sup>mKO</sup>* SCs (Fig. 5E). However, muscle-specific transcripts increased in *Ezh2<sup>mKO</sup>* muscles were not augmented in *Ezh2<sup>mKO</sup>* SCs (data not shown), suggesting that their misregulation occurs in myofibers.

#### *PolII* recruitment to mixed cell lineage genes and the *Ink4a/Arf* locus in *Ezh2<sup>mKO</sup>* SCs

To mechanistically investigate how *Ezh2* ablation causes gene derepression, we conducted chromatin immunoprecipitation (ChIP) assays with antibodies against



**Figure 5.** *Ezh2* maintains transcriptional identity of SCs. (A) Log intensity versus ratio scatter plot of gene expression obtained from microarray data derived from muscles of wild-type ( $n = 2$ ) and *Ezh2<sup>mKO</sup>* ( $n = 2$ ) animals. The X-axis represents the  $\log_{10}$  of the average intensity of the data, and the Y-axis represents the  $\log_2$  of their ratio. Each dot represents one gene. Up-regulated and down-regulated genes are colored red and green, respectively (fold change  $>2$ ). (B, C) Expression of selected up-regulated genes identified in A by qPCR in muscles of wild-type or *Ezh2<sup>mKO</sup>* animals. Data are presented as mean  $\pm$  SD ( $n = 3$ ); (\*\*\*)  $P < 0.0005$ ; (\*)  $P < 0.05$ . (D) EZH2 and H3K27me3 immunoblots of FACS-isolated *Ezh2<sup>mKO</sup>* SCs. Total histone H3 serves as loading control. (E) qPCR on selected up-regulated genes from FACS-isolated wild-type or *Ezh2<sup>mKO</sup>* SCs. Data are presented as mean  $\pm$  SD ( $n = 3$ );  $P < 0.0005$  for all the values. (F, G) ChIP-qPCR for H3K27me3 and PolII at selected genes up-regulated in *Ezh2<sup>mKO</sup>* SCs. Data are presented as mean  $\pm$  SD ( $n = 3$ );  $P < 0.0005$  for all the values.

H3K27me3 and PolII. ZIC-1, Ink4a/Arf (p16), Isl1, Opn1, and Coll22a1 genes were marked by H3K27me3, and PolII was undetectable in wild-type SCs. In contrast, H3K27me3 was significantly reduced and PolII was recruited at these genes in *Ezh2<sup>mkKO</sup>* SCs (Fig. 5D,F,G; Supplemental Fig. S4C). Overall, these results indicate that SCs are the source of mixed cell lineage and p16 gene misexpression, and that reduced H3K27me3 in *Ezh2<sup>mkKO</sup>* SCs allows PolII chromatin access, resulting in unwanted gene transcription.

*Conditional ablation of Ezh2 in MyoD-expressing cells recapitulates the muscle phenotypes observed in Ezh2<sup>mkKO</sup> mice*

Pax7 expression is not limited to SCs but extends to cells located in the CNS and craniofacial tissues (Jostes et al. 1990). Indeed, some *Pax7-Cre-Ezh2<sup>mkKO</sup>* mice with CNS defects were retrieved at E15.5 and E17.5 (data not shown). To ablate *Ezh2* in committed skeletal myogenic cells (*MyoD<sup>+</sup>* cells), we bred *MyoD-Cre* (Chen et al. 2005) and floxed *Ezh2* mice (Supplemental Fig. S5A). Body and muscle mass were greatly reduced in *MyoD-Cre-Ezh2<sup>mkKO</sup>* mice (Supplemental Fig. S5B–E). Moreover, the number of Pax7<sup>+</sup> cells was also reduced in muscle cross-sections (Supplemental Fig. S5F). Recent studies have documented an activation of the *MyoD* locus in prenatal SCs (Kanisicak et al. 2009). Thus, *MyoD-Cre-Ezh2<sup>mkKO</sup>* mice may have deleted the *Ezh2<sup>fl/fl</sup>* alleles in SCs capable of self-renewal. While p16 derepression in *MyoD-Cre-Ezh2<sup>mkKO</sup>* was comparable with that observed in *Pax7-Cre-Ezh2<sup>mkKO</sup>* mice, expression of mixed-lineage genes ZIC-1, *Agrp1*, and *Coll22a1* was much less pronounced (Supplemental Fig. S5G). Overall, the phenotypic similarities of *Pax7-Cre-Ezh2<sup>mkKO</sup>* and *MyoD-Cre-Ezh2<sup>mkKO</sup>* mice suggest that the observed muscle defects can be imputed to *Ezh2* ablation in skeletal myogenic cells.

*Conclusions*

The present study revealed that EZH2 influences several aspects of SC biology, including self-renewal, proliferation, and cell identity.

Unlike other studies in which *Ezh2* deletion was conditionally obtained in committed progenitors or differentiated cells (Chen et al. 2009; Ezhkova et al. 2009; Hirabayashi et al. 2009), we observed that Pax7-induced *Ezh2* deletion resulted in derepression of developmental regulators and structural genes physiologically not expressed in SCs. We speculate that, in committed or differentiated cells, the chromatin structure at selected genomic regions may be insensitive to epigenetic modifications caused by *Ezh2* ablation. Indeed, when *Ezh2* was deleted in committed myogenic precursors (*MyoD<sup>+</sup>*), gene misexpression was barely observed (Supplemental Fig. S5G). A more naive and plastic chromatin environment, such as that of ES cells or noncommitted Pax7<sup>+</sup>/*Myf5<sup>-</sup>* stem cells, may react to PcG gene ablation by dysregulating gene expression (Boyer et al. 2006; Lee et al. 2006). In contrast to mixed-lineage genes, the *Ink4a/Arf* locus is derepressed in committed and differentiated *Ezh2*-ablated cells (*MyoD-Cre-Ezh2<sup>mkKO</sup>*) (Chen et al. 2009; Ezhkova et al. 2009; Hirabayashi et al. 2009; this study). Thus, different genomic chromatin structures may be susceptible to PcG regulation only at defined developmental windows. Interestingly, derepression of some of

the mixed-lineage genes reported here has been observed in the skin of *Ezh1/Ezh2* double-knockout mice (Ezhkova et al. 2011).

EZH2 has been reported to repress Pax7 expression (Palacios et al. 2010). Specifically, EZH2 knockdown in cultured SCs results in Pax7 activation only if induced when the Pax7 level starts declining (Palacios et al. 2010). Since Cre expression in *Pax7-Cre* mice is coincident with Pax7 transcription (Keller et al. 2004), it is likely that *Ezh2* ablation in *Ezh2<sup>mkKO</sup>* mice occurred at the onset of Pax7 expression. In addition to the different temporal *Ezh2* inactivation strategies, cultured SCs may not experience the same physiological regulation attained in the animal, and may thus explain the different experimental outcomes.

In conclusion, the processes regulated by EZH2 are pivotal for SC homeostasis. As SCs hold therapeutic potential to treat muscle-wasting diseases (Kuang et al. 2008), PRC2 may be exploited as a target to promote ex vivo expansion and in vivo SC self-renewal.

**Materials and methods**

*Mice and animal care*

Mice were housed in a pathogen-free facility, and all experiments were performed according to the National Institutes of Health's (NIH) Animal Care and Use regulations. For a detailed animal description, see the Supplemental Material.

*Myotoxic injury*

The right TA muscle of 8-wk-old wild-type and *Ezh2<sup>mkKO</sup>* mice was surgically exposed and injected to holding capacity (~50  $\mu$ L) with 10  $\mu$ M CTX, as described previously (Schertzer et al. 2007). The left TA muscle was not injected and served as an uninjured control.

*Histology*

For a detailed histology protocol, see the Supplemental Material.

*FACS sorting, myofiber isolation, BrdU labeling, immunofluorescence, and image acquisition*

SC FACS sorting was performed as described previously (Joe et al. 2010). BrdU staining and immunofluorescence protocols are described in the Supplemental Material.

*Transferase dUTP nick end-labeling (TUNEL) assay*

FACS-sorted SCs were examined by terminal deoxynucleotidyl TUNEL assay (Roche Applied Science) according to the manufacturer's instructions.

*Gene expression analyses*

Total RNA from either whole-muscle or FACS-isolated SCs was reverse-transcribed using a cDNA synthesis kit (Applied Biosystems) and subjected to qPCR analysis. Gene expression analyses are detailed in the Supplemental Material.

*Immunoblots*

Muscles or FACS-isolated SCs were lysed, and their extracts were resolved by SDS-PAGE and transferred onto nitrocellulose filters. Specific antibodies used to perform immunoblots are described in Supplemental Table 4.

*ChIP assay*

Cross-linking and ChIP were performed as described (Caretti et al. 2004) with minor modifications (see the Supplemental Material).

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## Polycomb EZH2 controls self-renewal and safeguards the transcriptional identity of skeletal muscle stem cells

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