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Article

TNF/p38α/Polycomb Signaling to *Pax7* Locus in Satellite Cells Links Inflammation to the Epigenetic Control of Muscle Regeneration

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SUMMARY

How regeneration cues are converted into the epigenetic information that controls gene expression in adult stem cells is currently unknown. We identified an inflammation-activated signaling in muscle stem (satellite) cells, by which the polycomb repressive complex 2 (PRC2) represses *Pax7* expression during muscle regeneration. TNF-activated p38α kinase promotes the interaction between YY1 and PRC2, via threonine 372 phosphorylation of EZH2, the enzymatic subunit of the complex, leading to the formation of repressive chromatin on *Pax7* promoter. TNF-α antibodies stimulate satellite cell proliferation in regenerating muscles of dystrophic or normal mice. Genetic knockdown or pharmacological inhibition of the enzymatic components of the p38/PRC2 signaling—p38α and EZH2—invariably promote *Pax7* expression and expansion of satellite cells that retain their differentiation potential upon signaling resumption. Genetic knockdown of *Pax7* impaired satellite cell proliferation in response to p38 inhibition, thereby establishing the biological link between p38/PRC2 signaling to *Pax7* and satellite cell decision to proliferate or differentiate.

INTRODUCTION

Upon tissue injury, the cues released by the inflammatory component of the regenerative environment instruct somatic stem cells toward repairing the damaged area (Stoick-Cooper et al., 2007). The elucidation of the molecular events underpinning the interplay between the inflammatory infiltrate and tissue progenitors is crucial to devise new strategies toward implementing regeneration of diseased or injured tissues.

Regeneration of diseased muscles relies on muscle stem cells (satellite cells [SCs]), which are activated in response to cytokines and growth factors (Dhawan and Rando, 2005; Kuang and Rudnicki, 2008). The current lack of knowledge of how external cues coordinate gene expression in these cells precludes their selective manipulation through pharmacological interventions.

The inflammatory infiltrate is a transient, yet essential, component of the SC niche and provides the source of locally released cytokines, such as interleukin 1, interleukin 6, and TNF-α, which regulate muscle regeneration (Gopinath and Rando, 2008; Kuang et al., 2008). As an inducible element of the SC niche, the inflammatory infiltrate provides an ideal target for selective interventions aimed at manipulating muscle regeneration (Peterson and Guttridge, 2008). However, because local inflammation regulates multiple events within the regeneration process, global anti-inflammatory interventions have both positive and negative effects on SCs (Mozzetta et al., 2009). Thus, it is important to elucidate the intracellular signaling by which inflammatory cytokines deliver the information to individual genes in SCs.

p38 mitogen-activated protein kinases α, β, γ, and δ respond to cellular stressors, such as inflammatory cytokines. In SCs, this group of kinases converts inflammatory cues into epigenetic information that controls gene expression (Lluís et al., 2006; Lassar, 2009). The p38α and β kinases contribute to the assembly of the myogenic transcriptosome on the chromatin of muscle loci, by promoting MYOD-E47 heterodimerization (Lluís et al., 2005) and the recruitment of SWI/SNF chromatin remodeling complex (Simone et al., 2004; Serra et al., 2007) and of ASH2L-containing mixed-lineage leukemia (MLL) methyltransferase complex (Rampalli et al., 2007). By contrast, p38γ represses MYOD transcriptional activity by direct phosphorylation, via association with the H3-K9 methyltransferase KMT1A (Gillespie et al., 2009). Thus, the p38 kinases can either activate or repress gene expression in SCs, depending on the engagement of specific p38 isoforms. Furthermore, chromatin-associated p38 kinases can control gene transcription by directly targeting components of the transcription machinery (Pokholok et al., 2006; Chow and Davis, 2006; de Nadal and Posas,

2010), suggesting that signaling via p38 kinases plays a general role in regulating how chromatin-modifying complexes redistribute across the genome in response to extrinsic signals.

During SC differentiation, a large subset of genes is repressed in concomitance with the activation of muscle gene expression (Guasconi and Puri, 2009). Gene repression in flies and mammals is typically associated with methylation of specific lysine residues within histone tails (H3-K27) by the methyltransferase-containing Polycomb repressive complexes 1 and 2 (PRC1 and PRC2) (Simon and Kingston, 2009). PRC-mediated repression of developmental genes is a general mechanism that ensures the maintenance of the undifferentiated phenotype in embryonic stem cells (ESCs) (Boyer et al., 2006a). Derepression of developmental genes in differentiating ESCs correlates with functional inactivation of the enzymatic activity of PRC2, through a physical and functional interplay with a recently described component of PRC2—the Jumonji- and ARID-domain-containing protein, JARID2 (Pasini et al., 2010; Shen et al., 2009; Peng et al., 2009; Panning, 2010). The repressive activity of PRC2 is counter-balanced by trithorax group (trxG)-associated H3-K4 methyltyransferases (Schuettengruber et al., 2007). The coordinated activity of PRC2 and trxG generates the simultaneous trimethylation of H3-K27 ($H3K27^{3me}$) and H3-K4 ($H3\text{-}K4^{3me}$), which typically defines the "bivalent" profile of developmental genes in ESCs; resolution of such bivalency can result either in productive transcription or permanent repression, depending on the relative levels of $H3K27^{3me}$ and $H3\text{-}K4^{3me}$ (Bernstein et al., 2006). H3-K4- and H3-K27-specific demethylases contribute to the resolution of bivalency in developmental genes during ESC differentiation (Pasini et al., 2008).

Likewise, in adult somatic stem cells (i.e., SCs, keratinocytes, and neural precursors), PRC2-mediated gene repression coordinates the temporal expression of differentiation genes (Caretti et al., 2004; Ezhkova et al., 2009; Hirabayashi et al., 2009). PRC2 represses the expression of differentiation genes in undifferentiated adult stem cells, and derepression of these genes coincides with the downregulation of the catalytic subunit of PRC2—the enhancer of Zeste (EZH2) (Caretti et al., 2004; Ezhkova et al., 2009; Juan et al., 2009).

Although PRC-mediated repression of developmental and differentiation genes in ESCs and adult stem cells, respectively, has been extensively investigated, the contribution of PRC2 in gene repression during terminal differentiation of adult stem cells, such as myogenic progenitors, has been suggested (Blais et al., 2007) but remains relatively unexplored. In particular, it is currently unknown whether in adult muscle stem cells exposed to regeneration signals, PRC2 is redistributed to repress genes characteristic of the undifferentiated state. Even more puzzling, the identity of the signaling that directs the PRC2 chromatin redistribution in response to these signals is still obscure.

In the present work, we have identified and characterized a signal-inducible repression of *Pax7* expression by PRC2, via inflammation-activated p38 signaling, in SCs. The inflammation-activated p38/PRC2 signaling to *Pax7* controls the size and the regeneration activity of SCs and might be exploited for pharmacological manipulation of muscle regeneration.

RESULTS

TNF-Activated p38 Signaling Regulates *Pax7* Expression and Satellite Cell Proliferation

To investigate the impact of inflammation-activated signaling on muscle regeneration, we have used a mouse model of muscular dystrophy—mdx mice—in which compensatory regeneration of dystrophin-deficient myofibers is elicited by repeated cycles of degeneration/regeneration after muscle contraction. 5-week-old mdx mice were exposed to neutralizing antibodies against TNF-α (Infliximab). TNF-α is a cytokine that is secreted within the regenerative environment by the inflammatory infiltrate and regulates muscle regeneration by activating the promyogenic p38 signaling (Chen et al., 2007). Previous work showed that long-term treatment with TNF-α antibodies produces a beneficial effect in mdx mice (Radley et al., 2008; Huang et al., 2009); however, the molecular mechanism underlying this effect remains unknown.

We observed an increased number of PAX7-positive cells predominantly located in sublaminar position within muscles from mdx mice that were exposed to TNF-α antibodies, as compared to muscles from control-treated mdx mice (Figures 1A and 1B). Because Pax7 is a typical marker of SCs (Seale et al., 2000; Montarras et al., 2005), the increased number of PAX7-positive cells in mdx muscles exposed to TNF-α antibodies indicates that TNF-activated pathway negatively regulates SC number and *Pax7* expression in regenerating muscles. Among the TNF downstream cascades, the p38 pathway, which is typically activated in SCs (Jones et al., 2005), was inhibited in muscles isolated from anti-TNF-α-treated mdx mice (Figure 1C). Consistently, pharmacological inhibition of p38α and β kinases with SB 203580 (SB) replicated the effect observed with TNF-α antibodies on *Pax7* expression, whereas the inhibition of two other TNF-α-activated cascades, such as JNK and NF-κB pathways, did not alter *Pax7* expression (Figure S1A, upper panel, available online). An increased expression of *Pax7* was detected in myofiber-derived SCs that were isolated from mdx mice exposed to TNF-α antibodies (Figure 1D). By contrast, the expression of *MyoD* was unchanged and the differentiation marker muscle creatine kinase (*MCK*) was downregulated in SCs from mdx mice exposed to TNF-α antibodies (Figure 1D) or in SCs exposed to SB (Figure S1A, lower panel). Consistently, muscles from mdx mice treated with TNF antibodies showed an increased number of smaller myofibers (Figure S1B) that reflected a delay in the regeneration process. This effect is presumably due to an expansion of SCs whose ability to differentiate into myofibers has been transiently impaired by the inhibition of TNF-p38 signaling. The reversible nature of p38 inhibition by TNF-α antibodies suggests that restoration of the TNF-p38 signaling in the expanded population of SCs might yield to a more robust muscle regeneration. And this can contribute to beneficial effect observed by long-term treatment with TNF-α antibodies in mdx mice (Radley et al., 2008; Huang et al., 2009). An increased number of PAX7-positive cells was also observed in muscles of normal mice that were induced to regenerate by acute injury (cardiotoxin injection), upon treatment with TNF-α antibodies (Figures S2A and S2B). Fluorescence-activated cell sorting (FACS) was used to determine the precise identity of these cells. Figure S2C shows an increase in *Pax7*

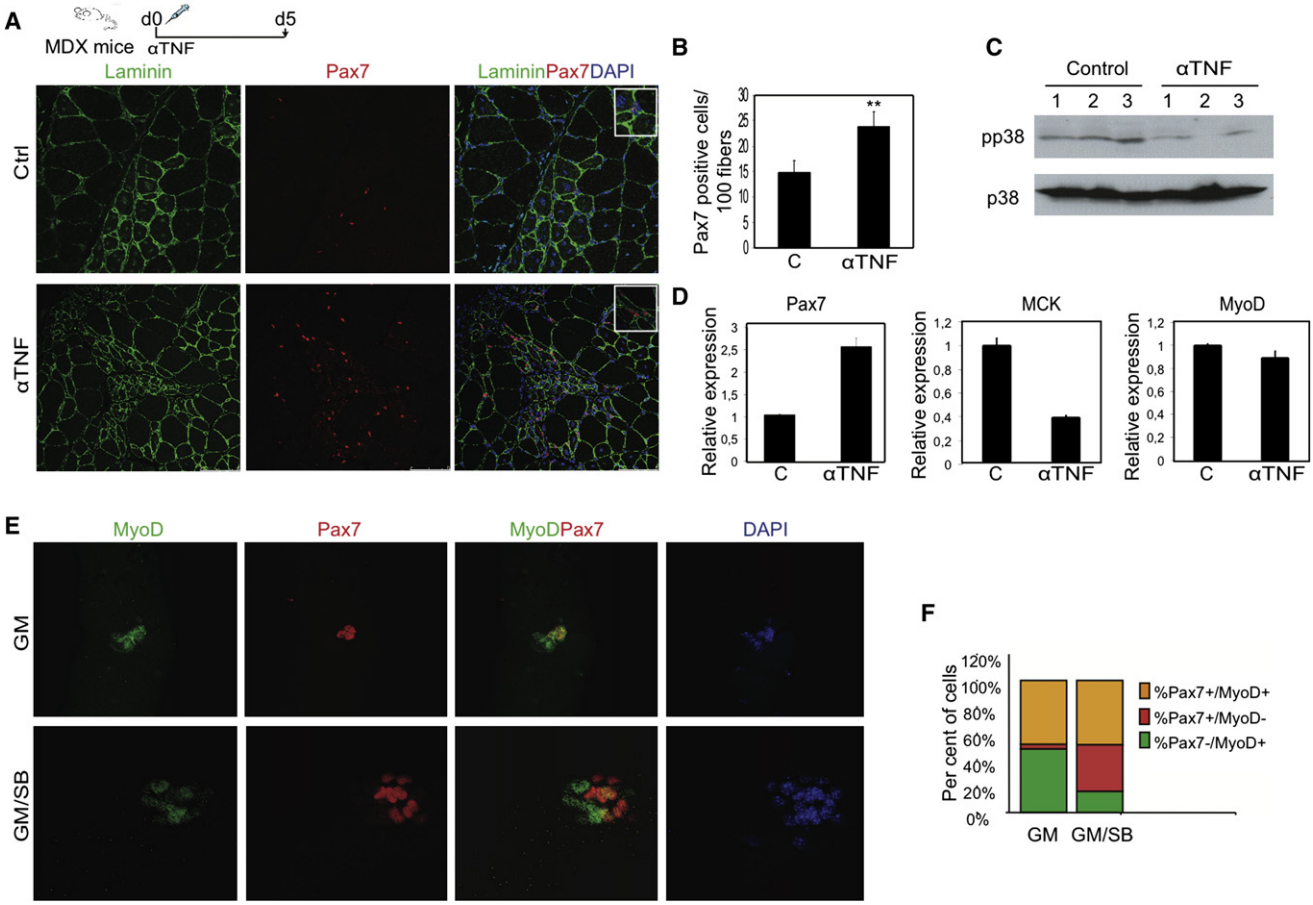

Figure 1. In Vivo Treatment of mdx Mice with Anti-TNF and Ex Vivo Blockade of the p38 Pathway Expand a Population of Activated Muscle Satellite Cells and Increase *Pax7* Expression

(A) Schematic representation of the experimental design (top). Immunofluorescence staining via antibodies against LAMININ (green) and PAX 7 (red) and counterstained for DAPI (blue), on transversal quadriceps sections from 5-week-old mdx mice treated for 5 days with 20 mg/kg of control (upper panels) or TNF antibodies (lower panels).

(B) The reported data represent the number of Pax7-positive cells percent fibers in the same conditions as above. For each quadriceps more than 400 fibers were counted and the graph represents the average of three mice per experimental group. Error bars indicate the standard deviation (**Student's t test: $p < 0.01$).

(C) Levels of activated p38 in muscles from control and anti-TNF-treated mice were measured by western blot via an antibody that recognizes the phosphorylated form of p38. Total p38 was used as a loading control.

(D) Real-time RT-PCR analysis of the expression of *Pax7*, muscle creatine kinase (*MCK*), and *MyoD* in satellite cells derived from control and treated mice. Error bars indicate the standard deviation (**Student's t test: $p < 0.01$).

(E) Coimmunostaining of myofibers isolated from the gastrocnemius of C57/Bl6 wild-type mice via antibodies against MyoD (green) and Pax7 (red) and counterstained for DAPI (blue). The single myofibers were maintained in culture for 72 hr either in growth medium alone (GM) or in the presence of the p38-specific inhibitor (SB).

(F) The graph represents the quantification of the $Pax7^+/MyoD^-$ and $Pax7^+/MyoD^+$ and $Pax7^-/MyoD^+$ cells per cluster, in the experimental conditions shown in (E). Data are represented as the mean percentage of positive cells per clone. 60 clones from 2 different experiments were analyzed for each experimental point for a total 200 cell counted for each experimental point. p value < 0.01 for differences between $Pax7^+/MyoD^-$ and $Pax7^-/MyoD^+$ cells in GM versus GM/SB.

See also Figures S1 and S2.

expression levels only in CD34/α-7integrin double-positive cells (a combination that defines the satellite cell identity) isolated from injured muscles of mice treated with TNF-α antibodies, as compared to injured muscles from control mice. By contrast, *Pax3* and *MyoD* levels were unchanged in FACS-sorted populations from injured muscles of TNF-α antibody-treated and control mice (Figure S2C).

Collectively, these results indicate that TNF-α-activated p38 pathway negatively controls the expansion of PAX7-positive SCs during the regeneration stages of dystrophic muscles.

The effect of p38 blockade was further explored in SCs within the myofibers isolated from normal mice. Figures 1E and 1F show that the exposure to the p38 inhibitor SB increased the number of PAX7-positive cells located within single myofibers. These cells clustered beneath the basal lamina—the typical anatomical position of SCs (Zammit et al., 2006a). The number of PAX7/MYOD double-positive SCs did not significantly change in these experimental conditions, but a significant increase in PAX7-positive/MYOD-negative cells was observed upon p38 blockade (Figures 1E and 1F). This indicates an effect of p38

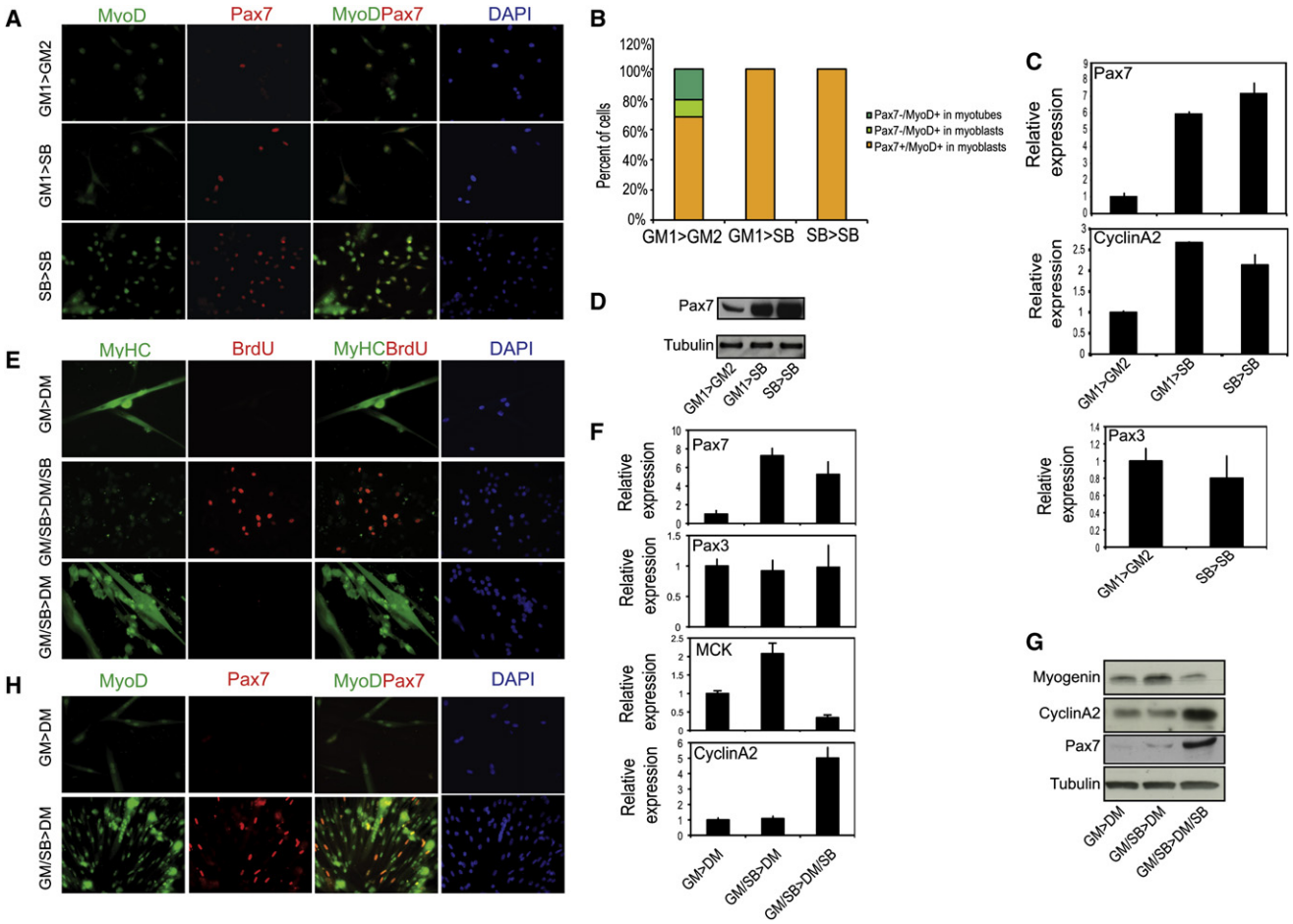

Figure 2. p38 Signaling Represses *Pax7* Expression and Proliferation of Satellite Cells

(A) Immunofluorescence staining against Pax7 (red) and MyoD (green) on myofiber-derived satellite cells that were allowed to delaminate in culture for 3 days in GM1 (10% HS+0.5% CEE) and then induced to proliferate in GM2 (20% FBS+10% HS+1% CEE) in the presence or absence of SB. DAPI (blue) was used to counterstain nuclei.

(B) Quantification of the mean percentage of $Pax7^{+}/MyoD^{+}$ and $Pax7^{-}/MyoD^{+}$ nuclei in myoblasts and myotubes in the experiments reported in (A).

(C) The expression of *Pax7*, *Pax3*, and *Cyclin A2* in satellite cells cultured in the presence or absence of the p38 inhibitor SB were analyzed by real-time RT-PCR. Error bar indicates standard deviation from three independent experiments.

(D) Pax7 protein levels (upper panel) on satellite cells treated as in (C) were measured by western blot. Tubulin (lower panel) was used as loading control.

(E) Immunofluorescence after a BrdU pulse (4 hours) on satellite cells grown in the presence (lower and middle panels) or absence (upper panel) of SB and induced to differentiate with (middle panel) or without (lower panel) the drug using antibodies against MyHC (green) and BrdU (red). Nuclei were counterstained with DAPI (blue).

(F) *Pax7*, *Pax3*, *Cyclin A2*, and *MCK* RNA levels were measured by real-time RT-PCR in satellite cells cultured as described in (E). Error bar indicates standard deviation from three independent experiments.

(G) Protein levels of Pax7, Cyclin A2, and Myogenin were quantified by western blot. Tubulin was used as a loading control.

(H) Coimmunostaining via antibodies against Pax7 (red) and MyoD (green) on satellite cells incubated in the presence (lower panel) or absence (upper panel) of SB and induced to differentiate after drug withdraw. Nuclei were counterstained with DAPI (blue).

See also Figures S3 and S7.

blockade on the segregation of SCs into PAX7-positive and -negative populations. Alternatively, an expansion of PAX7-positive/MYOD-negative SCs might occur at expenses of the MYOD-positive/PAX7-negative population.

Upon myofiber culture in growth medium (GM1), SCs are released and undergo rounds of proliferation. A unique feature of myofiber-derived SCs consists of their ability to initiate the differentiation program (Figure 2A) even when incubated with defined growth factors (GM2). This property coincides with the downregulation of *Pax7* (Zammit et al., 2006b) and other genes (e.g., proliferation-associated cyclins) and is reminiscent of the differentiation of SCs in vivo, in response to regeneration cues. SCs incubated with the p38 inhibitor SB continued to express *Pax7* and did not differentiate into myotubes (Figures 2A and 2B). In these cells the expression of *Pax7*, but not *Pax3*—the functional ortholog of *Pax7* (Buckingham, 2007)—was elevated (Figures 2C and 2D), further indicating that *Pax7* expression is specifically controlled by the p38 signaling in SCs. Notably,

upon p38α/β blockade, virtually all myofiber-derived SCs expressed both *MyoD* and *Pax7* when placed in culture and proliferated in the presence of the p38 inhibitor SB (Figures 2A and 2B). The simultaneous expression of *MyoD* and *Pax7* typically defines a dynamic population of activated SCs that undergoes terminal differentiation upon *Pax7* downregulation (Zammit et al., 2006b). We reasoned that if p38-mediated repression of *Pax7* was reversible, then the release of the p38 inhibition could convert an expanded population of SCs into a higher number of myotubes. To this purpose we have induced the simultaneous differentiation of SCs by incubation in differentiation medium (DM), which implements the formation of myotubes. Continuous inhibition of p38α/β in SCs cultured in DM prevented the formation of MyHC-positive myotubes and increased the number of proliferating (BrdU-positive) cells (Figure 2E, compare top and middle panels) that continued to express *Pax7* and *cyclinA* (Figures 2F and 2G) but did not express muscle differentiation markers, such as muscle creatine kinase (*MCK*) (Figure 2F) and *myogenin* (Figure 2G). However, upon the release of p38 inhibition by SB withdrawal, the expanded population of SCs differentiated massively and formed myotubes with an increased efficiency, as compared to control cells (Figure 2H, bottom panel). Interestingly, in these conditions, we still observed a fraction of PAX7-positive undifferentiated cells within the population of myotubes derived from SB-treated SCs (Figure 2H, bottom panel). Thus, reversible inhibition of p38 pathway can be used to implement the efficiency of satellite cell-mediated muscle regeneration ex vivo.

Physical and Functional Interactions between p38α, YY1, and Polycomb Repressive Complex 2 on *Pax7* Promoter

We began investigating the effect of p38 pathway on *Pax7* expression in SCs by using a tetracycline-regulated (Tet-off) expression of the p38 upstream activator MKK6EE. The activation of endogenous p38 in SCs was achieved by retroviral delivery of MKK6EE in the absence of doxycicline (Figures S3A and S3B) and resulted in downregulation of *Pax7* (Figures S3B and S3C). The levels of *Pax7* were restored to those detected in control SCs by the p38α/β inhibitor SB (Figure S3C), further demonstrating that p38α and/or β control *Pax7* expression in SCs.

Previous studies established that p38α controls the proliferation of adult stem cells, such as SCs and other tissue progenitors (Perdiguero et al., 2007; Wong et al., 2009). An impaired regeneration, accompanied by an increased number of PAX7-positive cells, was observed in p38α-deficient mice (Perdiguero et al., 2007), but not in mice deficient for the expression of p38β, p38γ, and p38δ kinases (Ruiz-Bonilla et al., 2008). The increased number of PAX7-positive cells observed in p38α-deficient mice is reminiscent of the phenotype observed in mice treated with TNF-α antibodies—Figure 1 shows the magnitude of *Pax7* upregulation in mice treated with anti-TNF-α that is identical to that observed in p38α-deficient mice by Perdiguero et al. (2007). This strong analogy indicates that p38α kinase selectively controls the expression of *Pax7* in SCs. To definitely address this issue, we compared the effect of RNAi-mediated knockdown of the p38 isoforms inhibited by SB—p38α and β (Serra et al., 2007). Only knockdown of p38α in SCs replicated the effect of SB on *Pax7* expression (Figure S3D). The *Pax7* repression by p38α kinase probably occurs at the transcriptional level, because p38 kinases regulate gene transcription by direct targeting of chromatin-associated proteins (Simone et al., 2004; Chow and Davis, 2006; Pokholok et al., 2006; Rampalli et al., 2007). Notably, most of the previous studies reported on p38-mediated activation of gene expression (reviewed in Lluís et al., 2006; Keren et al., 2006), but no evidence exists that p38 kinases repress gene transcription at the chromatin level. We have performed an analysis of the putative regulatory elements of *Pax7* along the evolution and found that the only conserved region is composed of a GAGA-rich sequence and a motif containing a consensus YY1 binding site located upstream of the *Pax7* promoter (Figure 3A). The combination of this two elements form a bona fide Polycomb response element (PRE) that mediates, both in *Drosophila* and mammals, the recruitment of YY1-associated PRCs, which repress transcription epigenetically (Ringrose and Paro, 2004; Müller and Kassis, 2006; Sing et al., 2009; Woo et al., 2010). The negative control of *Pax7* by PRC2 has already been suggested by the chromatin occupancy of the PRC2 component Suppressor of Zeste 12 (SUZ12) on *Pax7* promoter to repress *Pax7* expression in embryonic stem cells (Lee et al., 2006). Thus, we explored the possibility that PRC2 could mediate *Pax7* repression in response to the activation of p38α by signals that promote myoblast differentiation. We activated the p38 signaling in myoblasts by the ectopic expression of MKK6EE, which promotes differentiation and represses *Pax7* (see Figure S3), and we monitored the presence of endogenous p38α, YY1, and the enzymatic subunit EZH2 on the chromatin at the *Pax7* gene. To this purpose, we used C2C12 cells, because they provide abundant material for chromatin immunoprecipitation (ChIP) and coimmunoprecipitation analyses that cannot otherwise be obtained from SCs. The simultaneous chromatin occupancy by p38α, YY1, and EZH2 was detected on both PRE and on promoter region of the *Pax7* gene only after MKK6EE-mediated activation of p38 signaling (Figure 3A). Of note, pre-existing levels of chromatin-bound YY1 and EZH2 were detected on *Pax7* promoter in undifferentiated myoblasts, possibly reflecting the regulation of *Pax7* expression by a balanced activity of PRC2 and H3K4 methyltransferases (see also Figure 5). By contrast, p38α was not detected on the chromatin of *Pax7* promoter in undifferentiated myoblasts. Chromatin occupancy by p38α after MKK6EE-mediated activation of the p38 signaling was indicated by a 2-fold enrichment, which was inhibited by SB, together with the inhibition of YY1 and EZH2 chromatin binding (Figure 3A). Reciprocal coimmunoprecipitation experiments from nuclear extracts showed the formation of a complex containing endogenous p38α, YY1, and EZH2 upon MKK6EE-mediated activation of the p38 pathway (Figure 3B). Blockade of p38α by SB attenuated these interactions (Figure 3B). In this study, we present evidence of a signal-inducible formation of a nuclear complex containing components of the p38 signaling (MKK6 and p38α) and of the PRC2-mediated repressive machinery (YY1 and EZH2).

Chromatin-bound p38α was previously shown to direct SWI/SNF-mediated activation of muscle gene expression in differentiating myoblasts (Simone et al., 2004). Contractile muscle genes such as *MHCIIb* are repressed by PRC2 in myoblasts, and derepression coincides with the EZH2 disengagement during late

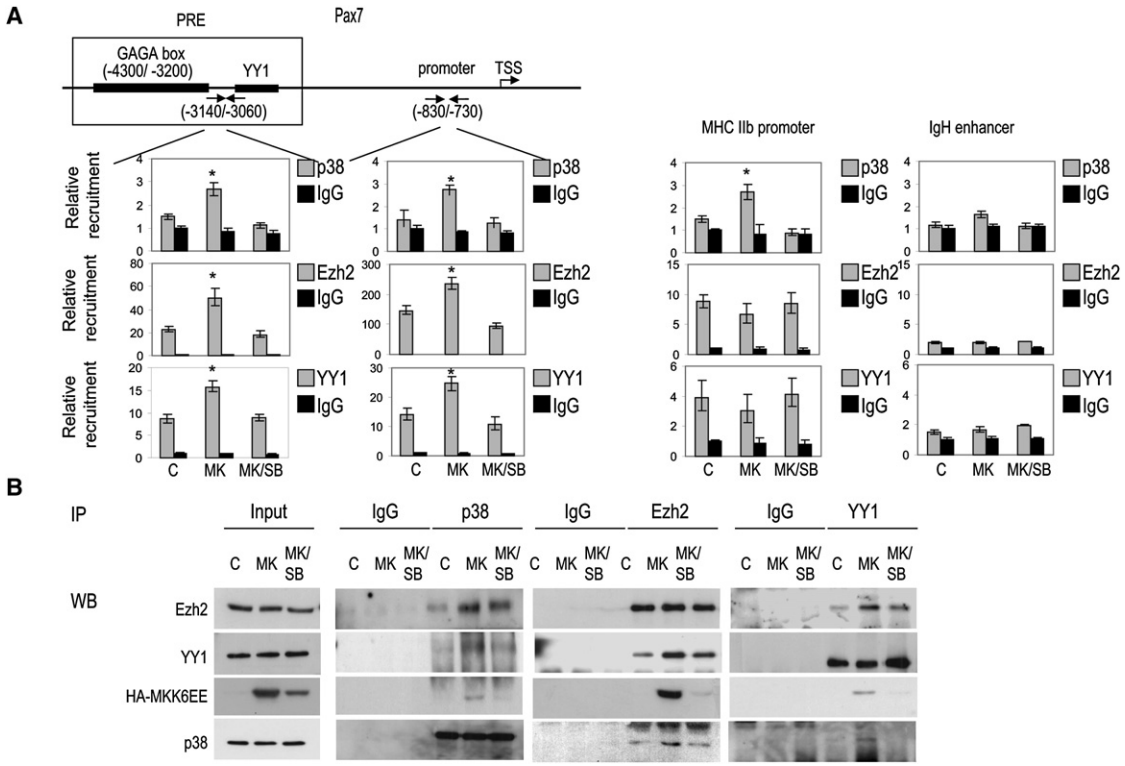

Figure 3. MKK6EE-Dependent Interactions between EZH2, YY1, and p38α on the Chromatin of *Pax7* Promoter

(A) Top: Schematic representation of the PRE-containing region and the proximal promoter of *Pax7* gene. Chromatin immunoprecipitation analysis (ChIP) of the *Pax7* PRE and promoter, *MHCIIb* promoter, and *IgH* enhancer were performed in C2C12 myoblasts cultured in growth medium and infected with control (C) or adeno-MKK6EE in the absence (MK) or presence (MK/SB) of the p38 inhibitor SB. ChIP was performed with antibodies against p38, EZH2, YY1, and control IgG. Graph shows real-time PCR values normalized against the input DNA. Error bars show standard deviation from three independent experiments. p values showing statistical significance by the Student's t test between control and MKK6EE are indicated (*$p < 0.05$).

(B) Coimmunoprecipitation from nuclear extracts of C2C12 cells infected with control (C) or adeno-MKK6 and cultured in the absence (MK) or presence (MK/SB) of the p38 inhibitor SB. Nuclear extracts were immunoprecipitated with p38, EZH2, and YY1 antibodies or control IgG and bound proteins were revealed by western blot via antibodies against EZH2, p38, YY1, and HA. The images are representative of three independent experiments reproducing the same result.

differentiation stages (Caretti et al., 2004; Juan et al., 2009). We therefore tested whether the p38α signaling to PRC2 could also mediate EZH2 chromatin disengagement on these genes. MKK6EE-mediated chromatin recruitment of p38α on *MHCIIb* promoter was detected in myoblasts in the same experimental conditions in which p38α was detected on the chromatin of *Pax7* genes (Figure 3A). However, on *MHCIIb* promoter p38α recruitment did not coincided with the changes in EZH2 and YY1 chromatin recruitment, consistent with the lack of expression of *MHCIIb* expression in these conditions (C2C12 myoblasts infected by MKK6EE and cultured in GM for 18 hr). As a control, p38α, EZH2, and YY1 were not detected on the chromatin of *IgH* enhancer (Figure 3A), which is constitutively repressed in myoblasts, probably by a PRC2-independent mechanism.

Collectively, the results presented above demonstrate a signal-dependent interaction between p38α, YY1, and EZH2 on the chromatin of the *Pax7* regulatory elements that coincides with p38-mediated repression of *Pax7* at early stages of myoblast differentiation. By contrast, the p38/YY1/EZH2 interactions do not appear to regulate PRC2-mediated derepression of muscle genes at the same stage. This evidence indicates that the p38 signaling to PRC2 specifically directs the repression of genes that are typically downregulated during muscle differentiation (e.g., *Pax7*), but has no direct impact on PRC2-mediated repression of muscle genes, which are induced in myotubes. This conclusion is also supported by our previous evidence that p38α/β blockade by SB does not restore EZH2 depletion and the relative decrease in H3-K27^{3me} on the chromatin of muscles genes in differentiating myoblasts (Serra et al., 2007).

p38α-Mediated Phosphorylation of EZH2 Enhances the Interaction with YY1 to Repress *Pax7* Expression

We next investigated the biochemical and molecular impact of p38α kinase interaction with EZH2 and YY1. A reciprocal interaction between exogenous Flag-tagged EZH2 and myc-tagged p38α was detected upon overexpression in heterologous cell lines, such as 293 cells, even in the absence of p38 activation (Figures S4A and S4B), indicating that EZH2 is a potential substrate for p38α kinase. Indeed, an in vitro kinase assay with immunoprecipitated Flag-EZH2 incubated with recombinant active p38α showed an incorporation of radiolabelled phosphate in EZH2 only in the presence of ATP, and this effect was

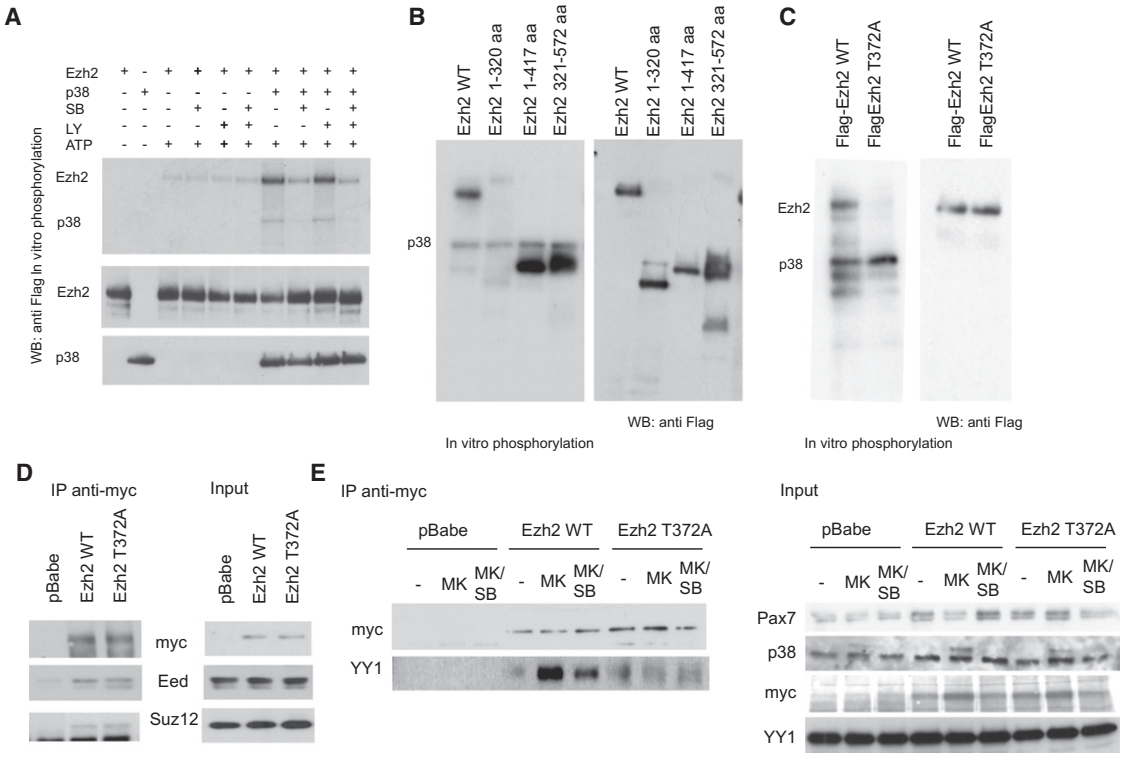

Figure 4. p38 Phosphorylation of EZH2 on Threonine 372 Is Necessary for EZH2 and YY1 Interaction and for *Pax7* Repression in Response to MKK6EE

(A) In vitro kinase assay with Flag-EZH2 immunoprecipitated from HEK293 cells and then incubated with recombinant active p38α (upper panel), in the absence or presence of SB or LY. Middle and lower panels show control western blots for the expression of Flag-EZH2 and p38.

(B) In vitro kinase assay via deletion constructs of EZH2 (left panel). Western blot with antibodies against Flag, as a loading control (right panel).

(C) In vitro kinase assay, comparing EZH2 WT and T372A mutant, performed as in (A) and (B) (left panel). Western blot with anti-Flag, as a loading control (right panel).

(D) Coimmunoprecipitation from nuclear extracts of C2C12 cells stably expressing control (pBabe) or myc-tagged EZH2 WT and EZH2T372A mutant. Anti-myc immunoprecipitates were analyzed by western blot via indicated antibodies (left panel). Input nuclear extracts before immunoprecipitation (right panel).

(E) Expression levels of the indicated proteins in nuclear extracts of the same cells as in (D), infected with control (C) or adeno-MKK6 and cultured in the absence (MK) or presence (MK/SB) of SB. The doublet in the p38 western blot indicates super-shifted (active, phosphorylated) endogenous p38α.

See also Figure S4.

specifically inhibited by the inclusion of SB, but not the PI3K inhibitor LY (Figure 4A). By using Flag-EZH2 deletion mutants, we could map the p38α-phosphorylated region within a C-terminal fragment encompassing amino acids 320 and 752—see phosphorylation of EZH2 1–417 and 321–752 aa fragments, but not EZH2 1–320 aa fragment (Figure 4B). Further analysis identified a conserved proline-directed threonine in position 372 as the only p38 kinase-target residue within the phosphorylated fragment of EZH2, and replacement of this threonine with nonphosphorylatable alanine generated a p38α phosphorylation-resistant EZH2 point mutant—EZH2 T372A (Figure 4C). This mutation does not impair the EZH2 ability to interact with the other components of PRC2—SUZ12 and EED (Figure 4D). Of note, MKK6EE-activated p38α (Figure 4E, right panel) enhanced the interaction of endogenous YY1 with exogenous EZH2 wild-type, but not with the phosphorylation-resistant EZH2 T372A mutant, in C2C12 myoblasts (Figure 4E, left panel). By contrast, the kinase-deficient MKK6AA mutant, which does not activate the p38 signaling, could not induce EZH2/YY1 interaction (Figure S4C). The failure of EZH2 T372A phospho mutant to interact with YY1 in response to MKK6EE-activated p38α resulted in an impaired ability to repress *Pax7* expression, as compared to the EZH2 wild-type (Figure 4E, right panel).

These results link p38α-mediated association of EZH2 with YY1 to *Pax7* repression in SCs.

PRC2 Converts the p38 Signaling to *Pax7* Promoter into Repressive Epigenetic Marks

We elucidated the functional relationship between p38 signaling to PRC2 in SCs by monitoring the levels of H3-K27^{3me}—the typical epigenetic mark of PRC2-mediated repression that reflects the enzymatic activity of EZH2. ChIP analysis of histone modifications requires much less amounts of chromatin, as compared to the analysis of chromatin occupancy of transcription factors or other proteins. Thus, we monitored the relative changes in H3-K27^{3me} at the PRE (Figure 5, middle vertical panels) and the promoter region (Figure 5, right vertical panels) of *Pax7* in undifferentiated SCs (myoblasts [MB]) and their differentiated progeny (myotubes [MT]). This analysis showed increasing levels of H3-K27^{3me} that spread over the regulatory

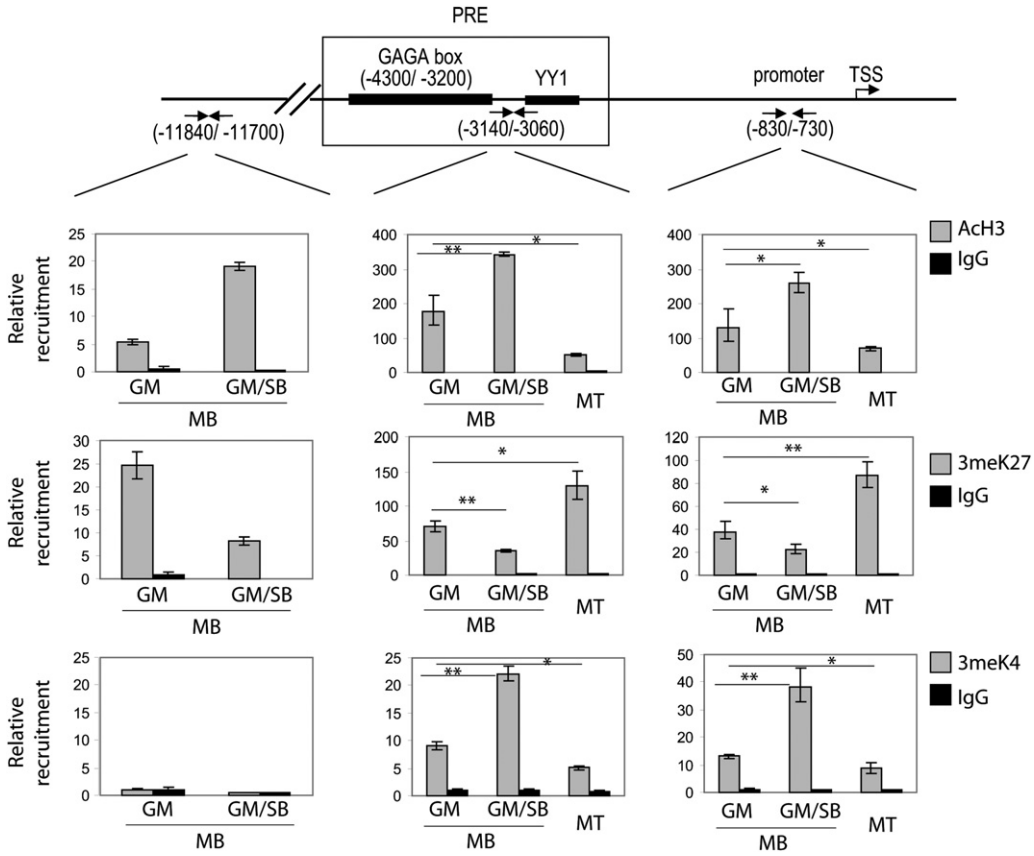

Figure 5. p38-Dependent Modulation of PRC2-Mediated Histone Modifications at the *Pax7* Promoter in Satellite Cells

Top: Schematic representation of the PRE-containing region (−3140/−3060), the proximal promoter (−830/−730), and distal elements (−11840/−11700) of the *Pax7* gene. ChIP analysis of the PRE-containing region, the proximal promoter, and −12 kb region of *Pax7* gene was performed with antibodies against AcH3, H3K27me3, and H3K4me3 and control IgG in satellite myoblasts (MB) cultured for 4 days in growth medium in the presence (GM/SB) or absence (GM) of the p38α/β inhibitor SB. Note that SB was replaced every 24 hr in the GM for the 4 days of culture. The same analysis was performed in satellite cell-derived myotubes (MT) after incubation in differentiation medium (DM). Graph shows real-time PCR values normalized against the input DNA. Error bars indicate the standard deviation (Student's t test: *$p < 0.05$; **$p < 0.01$).

elements of *Pax7* during SC differentiation. We note that myofiber-derived satellite myoblasts cultured in growth medium (GM) for 4 days (the time necessary to obtain an amount of cells sufficient to perform ChIP analysis) are composed of an asynchronous population of cells at different stages of the differentiation process. This mixed population tends to flatten the differences in H3-K27^{3me} levels detected on *Pax7* gene in MB versus MT. Still, the dynamic enrichment in this repressive mark at the *Pax7* locus was significantly detected and culminates with an increase in H3-K27^{3me} in myotubes (Figure 5A, middle transversal panels), which correlates with the downregulation of *Pax7*, as typically observed in SCs undergoing terminal differentiation. A simultaneous decrease in H3-K9-14 acetylation and H3-K4^{3me} was detected on the chromatin at same regions of the *Pax7* locus in satellite during MB to MT transition (Figure 5A, upper and lower panels, respectively). These data indicate that the relative enrichment in the epigenetic marks of PRC2- and TrxG-associated enzymatic activity—H3-K27^{3me} and H3-K4^{3me}, respectively—regulate *Pax7* expression during SC differentiation. Persistent inhibition of p38α by SC exposure to SB during MB to MT transition reversed the epigenetic pattern underlying *Pax7* repression, with reduced levels of H3-K27^{3me} and the consensual increase in levels of H3-K9/14 acetylation and H3-K4^{3me} observed in SB-treated SCs (Figure 5). An analysis of *Pax7* locus 12 kb upstream of the TTS showed reduced H3-K27^{3me} and increased H3-K9-14 acetylation in response to p38 blockade, while H3-K4^{3me} levels were undetectable in either condition (Figure 5, left vertical panels). This result is consistent with the notion that changes in H3-K27^{3me} and histone acetylation are spread over most of the gene locus and that changes in H3-K4^{3me} are restricted to the proximity of the TSS.

Genetic Knockdown or Pharmacological Inhibition of EZH2 Abolishes p38-Mediated Repression of *Pax7* in SCs

To definitely establish a causal relationship between PRC2 activity and Pax7 repression in SCs, we investigated the effect on *Pax7* expression of siRNA-mediated downregulation of *Ezh2* or pharmacological blockade of EZH2 methyltransferase activity in SCs. Previous work showed that *Ezh2* expression is high in undifferentiated myoblasts and drastically declines in myotubes (Caretti et al., 2004). However, when we monitored *Ezh2*

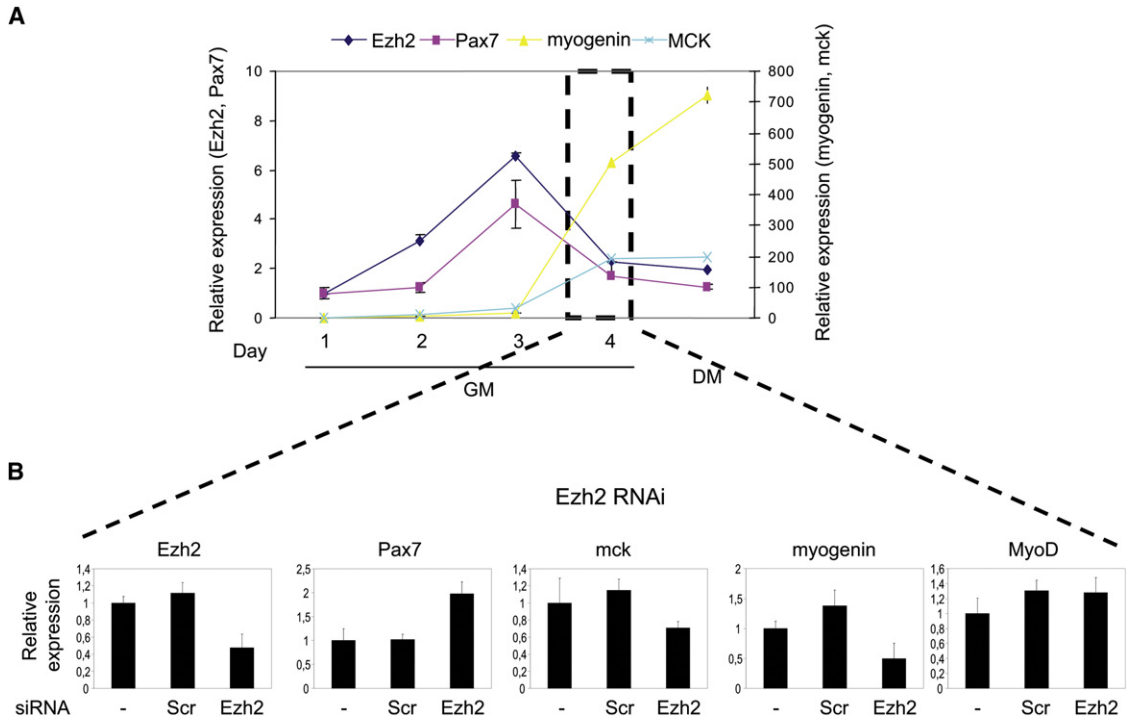

Figure 6. Stage-Specific Upregulation of *Pax7* by EZH2 Knockdown in Satellite Cells

(A) Real-time RT-PCR analysis of the expression of *Ezh2* (blue line), *Pax7* (pink line), *myogenin* (yellow line), and *MCK* (turquoise line) transcripts in myofiber-derived satellite cells, during 4 days of culture in GM and further incubation in DM (Figure 5A).

(B) Satellite cells derived from wild-type C57/BL6 mice were transfected with control scramble (scr) or *Ezh2* siRNA at day 3 and harvested at day 4, and the expression levels of *Ezh2*, *Pax7*, *MCK*, *myogenin*, and *MyoD* RNA were detected by RT-PCR. Error bars show standard deviation from two independent experiments.

See also Figure S5.

RNA expression in myofiber-derived SCs during their 4 days of culture in GM and further incubation in DM (Figure 6A), we observed that *Ezh2* transcripts (blue line) increase during the first 3 days of culture in GM, in coincidence with the higher proliferative activity of SCs, which express high levels of *Pax7* (pink line) and do not show detectable levels of differentiation markers, such as *myogenin* (yellow line) and *MCK* (turquoise line). This expression pattern indicates that in proliferating SCs, *Pax7* is not repressed by PRC2 and is consistent with the PRC2-mediated repression of *myogenin* and *MCK* in undifferentiated myoblasts and the induction of *myogenin* and *MCK* by siRNA- or miRNA-mediated depletion of EZH2 (Caretti et al., 2004; Juan et al., 2009). At this stage, siRNA-mediated knockdown of *Ezh2* does not change the expression levels of *Pax7* (not shown). During days 3 and 4 of culture in GM, a significant proportion of SCs initiate to differentiate spontaneously, as shown by the increased levels of *myogenin* and *MCK* and by the sharp decline of *Pax7* expression. *Pax7* downregulation during this transition is accompanied by PRC2-mediated deposition of epigenetic marks of transcriptional repression on the chromatin surrounding the regulatory elements of *Pax7* gene (see Figure 5). We therefore surmised that *Ezh2* knockdown at this stage could prevent *Pax7* downregulation. Indeed, when the *Ezh2* knockdown by siRNA was restricted to days 3 and 4 of SC culture in GM (see transparent box in Figure 6A), we observed an induction of *Pax7* transcripts that was proportional to the reduction of *Ezh2* levels (Figure 6B). By contrast, *MyoD* levels remained unchanged and *myogenin* and *MCK* levels were downregulated, possibly because of the antidifferentiative action of *Pax7* (Olguin et al., 2007). The p38 signaling also directs the SWI/SNF chromatin recruitment, via BAF60 phosphorylation (Simone et al., 2004), and SWI/SNF is involved in both gene activation and repression (de La Serna et al., 2006), so it is formally possible that p38-directed SWI/SNF participates to *Pax7* repression. However, knockdown of individual BAF60 subunits a, b, and c by siRNA did not affect *Pax7* expression in myoblasts but led to a decreased expression of genes previously shown to be direct target of BAF60a (the proliferation gene, *cFos*) (Ito et al., 2001), BAF60c (the muscle contraction gene, *Tnnt3*) (Lickert et al., 2004), and BAF60b (*Igf2*) (our unpublished data) (Figure S5). This evidence further demonstrates that p38-SWI/SNF and p38-PRC2 are two distinct signaling that control activation and repression, respectively, of different subsets of genes.

Collectively, these data show a stage-dependent effect of *Ezh2* depletion on muscle genes and *Pax7* expression. At earlier times, when EZH2 occupies the chromatin of muscle genes, *Ezh2* knockdown derepressed these genes (as shown in Caretti et al., 2004; Juan et al., 2009). A the onset of differentiation, when EZH2 occupies *Pax7* regulatory sequences, but not muscle genes, *Ezh2* knockdown induces *Pax7* expression, which in turn antagonizes muscle differentiation.

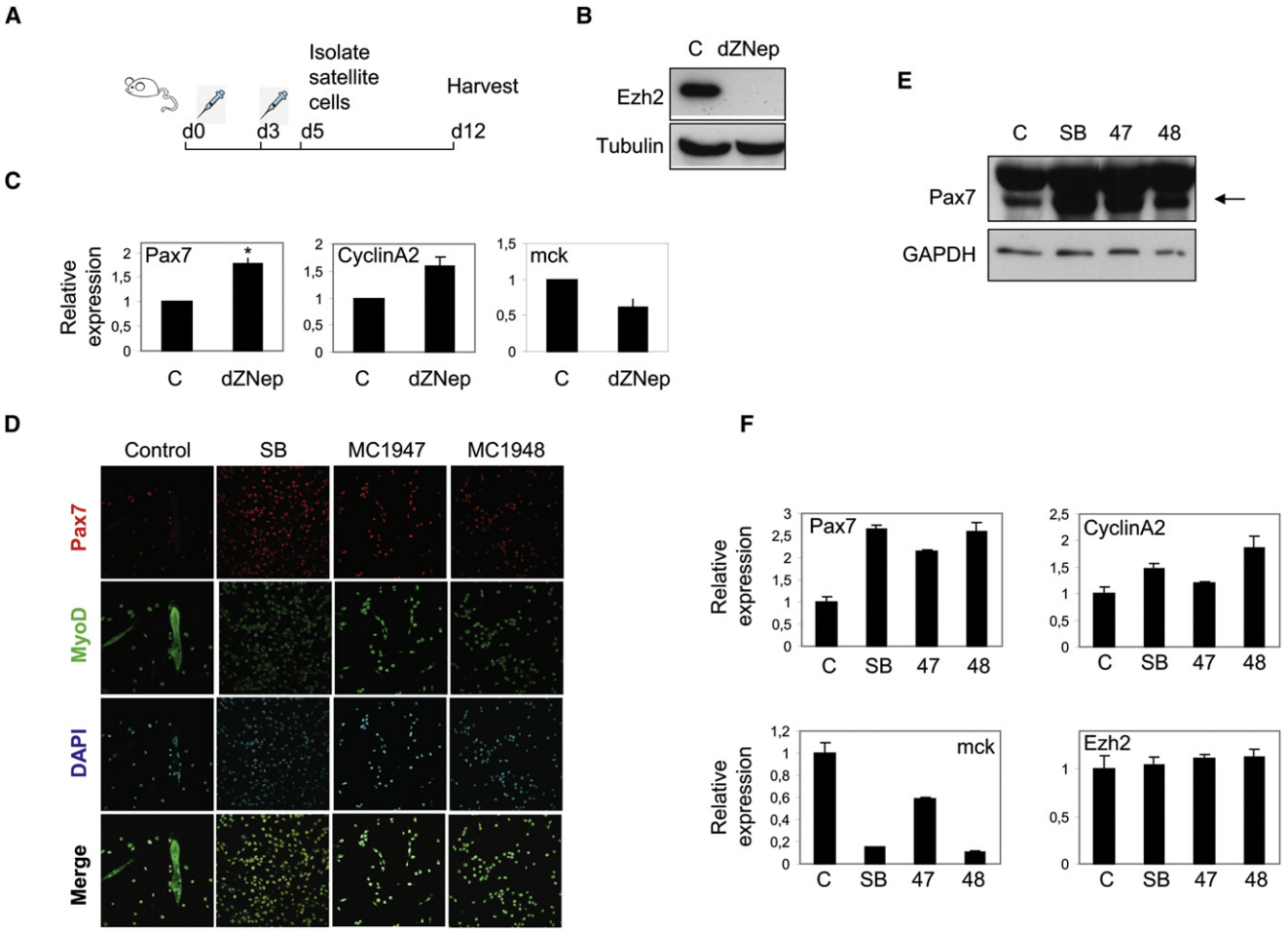

Figure 7. Pharmacological Blockade of PRC2 Increases *Pax7* Expression in Satellite Cells Both In Vivo and Ex Vivo

(A) Schematic representation of the experimental design. In brief, 5-week-old mdx mice were treated intraperitoneally with dZNep (1 mg/kg). After 5 days mice were sacrificed and satellite cells were isolated and cultured in growing conditions (GM) for 7 days.

(B) Protein levels of Ezh2 in satellite cells derived from control or dZNep-treated mdx mice were evaluated by western blot. Tubulin is shown as a loading control.

(C) Real-time RT-PCR analysis of *Pax7*, *MCK*, and *cyclinA2* on satellite cells obtained as in (A). Error bars show standard deviation from four independent experiments. p values showing statistical significance by the Student's t test are indicated (*$p < 0.05$).

(D) Satellite cells derived from wild-type C57/BL6 mice were isolated and incubated in growth medium in the absence (C) or presence of SB and the Ezh2 inhibitors MC1947 (47) and MC1948 (48) and the expression of *Pax7* and *MyoD* was detected by coimmunostaining. Nuclei were counterstained with DAPI.

(E) Western blot showing the levels of Pax7 in the same conditions as in (D). Gapdh was used as a loading control.

(F) Real-time RT-PCR analysis of *Pax7*, *cyclinA2*, *MCK*, and *Ezh2* on satellite cells treated as in (D). Error bar indicates standard deviation from three independent experiments.

See also Figure S6.

We further addressed the role of PRC2 in the regulation of *Pax7* expression during muscle regeneration in vivo by injecting young mdx mice with the S-adenosylhomocysteine hydrolase inhibitor 3-Deazaneplanocin A (DZNep) (Figure 7A). Pharmacological inhibition of PRC2 by this compound results in the elimination of EZH2 and other PRC2 components (Tan et al., 2007; and our data nor shown). We treated mdx mice with dZNep for 5 days (Figure 7A), which is equivalent to the time of treatment with TNF antibodies (see Figure 1). After the exposure to dZNep, we isolated the myofibers for ex vivo evaluation of PRC2 blockade in primary SCs. In these cells, the levels of EZH2 were drastically reduced by the exposure to dZNep (Figure 7B) as previously reported (Tan et al., 2007). SCs derived from mice exposed to dZNep expressed higher levels of *Pax7* and *cyclin A* and lower levels of the differentiation marker *MCK* (Figure 7C).

A number of new soluble compounds that are structurally unrelated to dZNep and share the ability to inhibit the methyltransferase activity of EZH2 (see Tables S1 and S2 in Supplemental Experimental Procedures) were screened for their ability to replicate the biological effect of p38 inhibition on SCs. Among them, MC1946, MC1947, and MC1948 were first selected by virtue of their effect on SC number. We further focused on MC1947 and MC1948 because of their ability to expand a population of PAX7-positive SCs and to induce *Pax7* and *cyclin A* expression, while inhibiting *MCK* expression, that was

reminiscent of the effect of SB (Figures 7D–7F). Furthermore, SCs retained the ability to differentiate into myotubes surrounded by undifferentiated SCs upon drug withdrawal—a feature also observed with SB (Figure 2). Despite these overlapping effects, it was evident that p38 inhibition by SB showed a more pronounced effect on SC number (see Figure 7D; Figure S6A). By contrast, the EZH2 inhibitors MC1947 and MC1948 only moderately expanded the number of SCs (Figure 7D; Figure S6A, upper panel). The common ability of p38 and EZH2 inhibitors to decrease H3-K27^{3me} levels at the *Pax7* regulatory elements (Figure S6B) reflects their impact on the p38-PRC2 signaling to *Pax7* at different levels and accounts for their shared ability to increase *Pax7* expression. However, MC1947 and MC1948, but not SB, caused a reduction of the global H3-K27^{3me} levels (Figure S6C), indicating that the effect of p38 blockade on H3-K27^{3me} is restricted only to those loci, such as *Pax7*, which are targeted by the p38-PRC2 signaling.

The shared ability of p38 and PRC2 inhibitors to increase *Pax7* expression in SCs relates to their effect on the epigenetic profile that governs the transcription of bivalent genes, as a result of the concerted action of H3-K4 and H3-K27 methyltransferases and demethylases (Pasini et al., 2008). According to this model, p38-dependent repression of *Pax7* entails the simultaneous engagement of PRC2 and H3-K4 demethylases to achieve selective enrichment of H3-K27^{3me} at the *Pax7* locus. Conversely, the induction of *Pax7* expression observed upon blockade of either p38α or EZH2 enzymatic activity results from the simultaneous enrichment in H3-K4^{3me} and decrease in H3-K27^{3me} levels at the *Pax7* regulatory elements. However, it is unclear whether the control of H3-K4 methylation is directly regulated by a parallel p38 signaling to H3-K4 methyltransferases/demethylases or is autonomously regulated by the enzymatic activity of p38-directed PRC2. To address this issue, we evaluated the effect of the inhibition of EZH2 enzymatic activity on H3-K4^{3me} levels at the *Pax7* locus in conditions permissive for p38-mediated recruitment of PRC2—that is, in differentiating SCs. Under these experimental conditions, EZH2 inhibition by MC1947 or MC1948 led to increased H3-K4^{3me} levels on *Pax7* regulatory regions (Figure S6D). This evidence identifies two distinct levels of epigenetic regulation at the *Pax7* locus by the p38-PRC2 signaling. One level reflects an ''extrinsic'' regulation of *Pax7* by the regeneration-activated p38 signaling to PRC2; therefore, p38 blockade prevents PRC2 chromatin binding and EZH2-mediated H3-K27^{3me}. A second level of control relates to the ''intrinsic'' ability of chromatin-bound PRC2 to regulate H3-K4 methylation and reveals an ''autonomous'' control of the epigenetic profile of *Pax7* by the enzymatic activity of chromatin-associated methyltransferases and demethylases. This model is consistent with the ability of p38 and EZH2 inhibitors to generate a common epigenetic profile at the *Pax7* locus. However, simultaneous inhibition of EZH2 and p38α in SCs did not result in a synergistic effect on *Pax7* expression (Figure S6E), supporting the concept that p38 and EZH2 act on the same pathway.

Pax7 Mediates the Effect of p38 Signaling on Satellite Cell Proliferation

We next investigated the impact of *Pax7* expression on p38-mediated control of SC proliferation, because p38-mediated phosphorylation of EZH2 reduces its ability to repress *Pax7* in SCs (Figure 4E). Previous works demonstrated that PAX7 contributes to SC proliferation and survival (Relaix et al., 2006; Collins et al., 2009). Efficient knockdown of *Pax7* was obtained by delivery of siRNA (siPax7) in SCs derived from single myofibers (Figure S7A, left panel). *Pax7* knockdown resulted in a reduced number of SCs (Figures S7B and S7C), which dowregulated proliferation markers, such as *cyclin E1*, as compared to control-transfected (siC) cells (Figure S7A, right panel). Moreover, SCs in which *Pax7* levels were reduced by siRNA did not expand in response to p38 blockade, as control cells did, but maintained the ability to differentiate in response to the activation of p38 by the upstream activator MKK6 (Figures S7B and S7C). This evidence demonstrates that *Pax7* expression mediates the ability of p38 blockade to expand the number of SCs, thereby supporting the biological link between p38α/PRC2 signaling to *Pax7* and muscle-stem cell decision to proliferate or differentiate (see also Figure 2).

DISCUSSION

Although the instructive role of the inflammatory component of adult stem cell niche is well documented, the intracellular signaling that converts the inflammatory cues released in the regenerative environment into the epigenetic information that coordinates gene expression in adult stem cells is unknown.

Our data uncover the existence of a signaling mechanism linking the regeneration cues released by the inflammatory infiltrate within the satellite cell niche to the epigenetic modifications that repress the expression of *Pax7* in SCs undergoing terminal differentiation. Key components of this signaling are the p38α kinase and the enzymatic subunit of PRC2, EZH2. This evidence illustrates a model of signal-directed chromatin recruitment of PRC2 to promote muscle stem cell differentiation that is different from the most commonly studied model of PRC-mediated gene repression to maintain pluripotency in embryonic stem cells.

The signal-inducible repression of a lineage-specific gene (*Pax7*) by PRC2 during muscle stem cell differentiation extends the role of PRC2 in coordinating gene expression during skeletal myogenesis. Previous work demonstrated that in undifferentiated myoblasts, PRC2-mediated repression precludes the unscheduled expression of muscle genes (Caretti et al., 2004). Our data show that when PRC2 is released from muscle genes, it relocates to loci that are typically repressed in differentiated myotubes—e.g., *Pax7*. The chromatin redistribution of PRC2 in differentiating SCs is regulated by the p38α kinase, which promotes the formation of a complex containing p38α, EZH2, and YY1, via direct phosphorylation of EZH2.

The different effect of chromatin-associated p38 kinases observed at regulatory sequences of muscle genes versus *Pax7* probably reflects the different composition of the chromatin-associated complexes. Differentiation-activated p38α is recruited to the *Pax7* promoter via interaction with EZH2—a direct substrate for phosphorylation-mediated association with YY1—to repress gene expression. On muscle-specific genes, p38α and/or β are recruited via interactions with components of the muscle transcriptosome, including BAF60 (Simone et al., 2004), E47 (Lluís et al., 2005), and MEF2 (Rampalli et al., 2007), which promote transcription. On these genes, the EzH2/YY1 complex dissociates from the chromatin, via

a differentiation-dependent (Caretti et al., 2004; Juan et al., 2009) but p38-independent mechanism.

Pax7 transcription is tightly regulated through development and adult life, to restrict its expression mainly to muscle progenitors, with detectable levels of *Pax7* also in the neural tube and adult brain. *Pax7* expression is silenced by PRC2 in embryonic stem cells and in most of the somatic cells (Lee et al., 2006; Boyer et al., 2006b; Bracken et al., 2006) and in human embryonal carcinoma F9 cells (Squazzo et al., 2006). Derepression of *Pax7* coincides with the specification of embryonic muscle progenitors and adult SCs (Relaix et al., 2005; Buckingham and Relaix, 2007). Genetic evidence shows that *Pax7* is implicated in the specification (Seale et al., 2000) and in the renewal and maintenance (Oustanina et al., 2004) of SCs and is necessary and sufficient to induce the myogenic phenotype in resident stem cells within adult skeletal muscles (Seale et al., 2004). However, the contribution of *Pax7* to the regeneration of adult muscles has been questioned by recent studies (Lepper et al., 2009). *Pax7* controls a number of satellite cell activities prior to differentiation, including renewal, maintenance of lineage identity, and survival (Relaix et al., 2006; Oustanina et al., 2004). At the onset of muscle differentiation, *Pax7* transcription is again repressed (Zammit et al., 2006b), because its expression would otherwise antagonize the activation of the differentiation program (Olguin et al., 2007).

Although recent studies have begun to identify downstream targets and effectors of PAX7 in SCs (McKinnell et al., 2008; Hu et al., 2008; Kumar et al., 2009), the upstream signaling that controls *Pax7* expression in SCs in response to extrinsic signals is poorly characterized (Kuang and Rudnicki, 2008). Previous works indicated a role of β-catenin in self-renewal of SCs, implicating β-catenin/GSK3β signaling as a potential upstream regulator of *Pax7* expression (Perez-Ruiz et al., 2008). Myostatin- and Megf10-activated signaling and the Notch pathways have also been implicated in the control of *Pax7* expression in SCs (McFarlane et al., 2008; Holterman et al., 2007; Conboy and Rando, 2002). The p38α-directed repression of *Pax7* by PRC2, reported here, illustrates a novel signal-inducible mechanism of PRC2-mediated gene repression during cellular differentiation that differs from the constitutive repression of developmental genes described in ESCs.

The link between p38-mediated arrest of cell proliferation in differentiating SCs and PRC2-dependent silencing of *Pax7* suggests an antiproliferative function of PRC2 that is in apparent conflict with the widely observed tumor-promoting activity of Polycomb proteins (Bracken and Helin, 2009; Sparmann and van Lohuizen, 2006). However, recent works have also indicated an antiproliferative function of PRC2, via silencing of mitogenic signaling, such as JAK/STAT and Notch pathways in *Drosophila* (Classen et al., 2009; Martinez et al., 2009; Merdes and Paro, 2009). Thus, it is possible that in certain cell types and in response to specific cues, PRC2 exerts an antiproliferative function, by silencing the expression of genes directly involved in cell cycle regulation. Future studies should identify *Pax7* downstream genes that promote SC proliferation, such as *Id3*—a recently identified direct transcriptional target of *Pax7* (Kumar et al., 2009).

In sum, the p38-PRC2 signaling to the *Pax7* locus uncovered by this study provides an unanticipated framework that links regeneration cues to the epigenetic modifications leading to repressive chromatin during satellite cell differentiation. Given the importance of pharmacological tools that can expand muscle stem cells in vitro before transplantation (Sacco et al., 2008), we anticipate that interventions targeting the p38-PRC2 signaling to *Pax7* will be the focus of future efforts toward enhancing the ability of muscle stem cells to regenerate diseased muscles, such as in muscular dystrophies.

EXPERIMENTAL PROCEDURES

Mice and In Vivo Treatments

All the experiments were performed in C57/Bl6 or mdx mice. For the experiments with anti-TNF, intraperitoneal injection of Infliximab (Centocor) (20 mg/kg) or control antibody was performed and mice were sacrificed 5 days later. For the experiments with dZNep, animals were injected intraperitoneally with 1 mg/kg of 3-Deazaneplanocin A or the same volume of vehicle (PBS). Three days after the first injection, a second dose was inoculated and mice were sacrificed 2 days later.

All experimental procedures were approved by the internal Animal Research Ethical Committee according to the Italian Ministry of Health and complied with the NIH Guide for the Care and Use of Laboratory Animals.

Satellite Cell Isolation and Culture

Single muscle fibers were isolated by standard procedures. In brief, the hind limb muscles were digested with collagenase and single myofibers were either cultured in GM1 (DMEM supplemented with 10% horse serum [GIBCO], 0.5% chick embryo extract [MP biomedicals], and penicillin-streptomycin [GIBCO]) at 37°C in suspension for 72 hr and then fixed for immunofluorescence or plated on matrigel (Sigma, 1 mg/ml ECM gel) -coated dishes for satellite cell culture. Three days later, the fibers were removed and the medium replaced with proliferation medium (GM2: 20% FBS, 10% horse serum, 1% chick embryo extract in DMEM). After 4–5 days, the medium was replaced with differentiation medium (DM: 2% HS and 0.5% chick embryo extract in DMEM). SB203580 (Calbiochem, final concentration 5 μM), SP600 (10 μM), PDTC (100 nM), MC1947 (10 μM), and MC1948 (10 μM) were added when indicated and replaced every 24 hr. BrdU (Amersham) was added to the medium (diluted 1:800) 4 hr prior to harvesting the cells.

Cell Lines and Plasmids

Cell lines and plasmids are described in the Supplemental Information.

RNA Interference

Downregulation of *Pax7* expression in myofiber-derived satellite cells was achieved by RNAi with the oligonucleotide siPax7-GGUAACAUCCCAGCUUUACTT (Silencer Pre-designed siRNA from Ambion) and according to the Dharmafect3 (Dharmacon) transfection protocol. Unrelated oligonucleotide siC-AAGTAAGCTGATGAAAGACTG was used as a control.

Knockdown of *p38α* and *β* and of *EzH2* in satellite cells was performed as previously described (Serra et al., 2007; Caretti et al., 2004).

Adenoviral Infections and Retroviral Infections

Adenoviral constructs have been described elsewhere (Simone et al., 2004). The viruses were amplified by transfection of 293 packaging cells and satellite cells or C2C12 myoblasts were infected with control or AdMKK6EE for 1 hr in serum-free medium before being placed in growth medium.

In Vitro Kinase Assay

HEK293T cells were transfected with pCMV FlagMycEZH2 WT, deletion mutants, or EZH2T372A mutant plasmids by calcium phosphate method. 1.5 mg of total extracts were incubated with Flag M2 agarose beads (Sigma) for 2 hr at 4°C and extensively washed in lysis buffer, and the immunoprecipitated material was eluted in lysis buffer containing 150 ng/μl 3XFlag peptide (Sigma).

200 ng of immunopurified proteins were incubated with 100 ng of recombinant active p38α (Cell Signaling) in the presence of 60 mM $MgCl_2$, 60 μM ATP,

50 mM Tris-HCl (pH 7.5), 12 mM DTT, and phosphatase inhibitors (Calbiochem), supplemented with 0.7 μCi of [γ-^{32}P]ATP at room temperature for 15 min. Reactions were stopped with Laemmli Buffer and resolved by SDS-PAGE; phosphorylated proteins were visualized by autoradiography. Protein loading was checked by western blot, with Flag (Sigma, M2) and p38α (Cell Signaling) antibodies.

Histology and Immunofluorescence

Quadriceps muscles were cut transversally, fixed in 4% PFA for 20 min, and permeabilized with 100% methanol for 6 min at −20°C. Inmunostaining with anti-PAX7 (Developmental Studies Hybridoma Bank, DSHB) and anti-LAMININ (Sigma) was performed overnight at 4°C after antigen retrieval with 100 mM sodium citrate and blocking first with a solution containing 4% BSA in PBS and then with anti-mouse AffiniPure Fab fragment (Jackson, 1:100) to avoid unspecific binding. Cy2-conjugated anti-rabbit (Jackson) and Biotin-conjugated anti-mouse (Jackson) secondary antibody followed by another incubation with Cy3-conjugated streptavidin (Jackson) were used to reveal LAMININ and PAX7 signal. Nuclei were visualized by counterstaining with DAPI.

Images were acquired with a Leica confocal microscope and edited with Photoshop software. Fields reported in the figures are representative of all examined fields. Average number of PAX7-positive cells per hundred fibers was obtained by counting multiple areas in several sections. Mean of the average number of cells in three mice per experimental group are shown. The cross-sectional area (CSA) was calculated with the Image J software downloaded from http://rsb.info.nih.gov/ij.

Single myofibers were fixed with 2% PFA for 20 min, permeabilized with 0.5% Triton/PBS, and blocked with 20% goat serum 1 hr at room temperature. Satellite cells were fixed with 4% PFA for 20 min, permeabilized with 0.25% triton, and blocked with 4% BSA in PBS 1 hr at room temperature. Immunostaining with anti-PAX7, anti-MYOD (Santa Cruz, SC-760), anti-BrdU (BD, 347580), and MyHC was performed O/N at 4°C and cy2 or cy3 conjugated secondary antibodies were then used.

Western Blot and RT-PCR

The levels of endogenous PAX7, MyHC (MF20, DSHB), MYOGENIN (DSHB), CYCLINA2 (Santa Cruz, SC-596), EZH2 (AC22, Cell Signaling), LAMIN A/C (Cell Signaling), and TUBULIN (Thermo Fisher, MS581P1) were detected by western blot analysis on total cell or nuclear extracts after lysis in 50 mM Tris-HCl (pH 8.0), 125 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, and 0.1% NP-40 supplemented with 1 mM PMSF and protease inhibitor mix. Anti-phospho p38 (Promega, V1211) and anti-p38 (Santa Cruz, SC-535) were used to detect phosphorylated and total p38 overexpressed proteins in C2C12 were detected by anti-MYC (Santa Cruz, SC-789) and anti-HA (Santa Cruz, SC-805) immunoblotting.

Total RNA was extracted with Trizol, and 0.5–1 μg were retro-transcribed with the Taqman reverse transcription kit (Applied Biosystems). Real-time quantitative PCR was performed to analyze relative gene expression levels with SYBR Green Master mix (Applied Biosystems) and according to manufacturer indications. Primers sequences are described in the Supplemental Information.

Coimmunoprecipitation Studies

Endogenous CoIPs on nuclear cell extracts of C2C12 cells were performed by standard procedures. In brief, nuclear extracts were precleared with protein G agarose for 1 hr at 4°C, immunoprecipitated with p38α (kindly provided by T. Sudo), YY1 (Santa Cruz, SC-281), or EZH2 (Diagenode) antibodies for 3 hr at 4°C, and incubated with protein G agarose. Inmunoprecipitates were extensively washed with the lysis buffer, resuspended in Laemmli buffer, separated on polyacrylamide gels, and transferred to nitrocellulose membranes. Precipitated proteins were revealed by western blot with p38 (Cell-Signaling), EZH2 (AC22, Cell Signaling), YY1 (Santa Cruz, SC-7341), and HA (Santa Cruz) antibodies.

Chromatin Immunoprecipitation

ChIP assay was performed as previously described (Simone et al., 2004). The following antibodies were used: anti-acetylated histone 3 (Upstate), anti-trimethyl lysine 27 histone 3 (Upstate), anti-trimethyl lysine 4 histone 3 (Upstate), anti-EZH2 (Diagenode), anti-p38α, anti-YY1 (Santa Cruz, SC-281), and anti-MYC (Santa Cruz, SC-789). Normal rabbit IgG was used as a "no antibody" control. Real-time PCR was performed on input samples and equivalent amounts of immunoprecipitated material with the SYBR Green Master Mix (Applied Biosystems). Relative recruitment is calculated as the amount of amplified DNA normalized to input and relative to values obtained after normal rabbit IgG immunoprecipitation, which were set as the background (one unit). Primers used are indicated in Supplemental Information.

Ezh2 Inhibitors

The small molecules MC1946, MC1947, and MC1948 were prepared according to the reported procedure (Mai et al., 2007). Detailed information about the chemistry is indicated in the Supplemental Information.

Statistical Methods

Where indicated statistical significance was determined by the Student's t test (*$p < 0.5$; **$p < 0.01$; ***$p < 0.001$).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at doi:10.1016/j.stem.2010.08.013.

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