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TNF/p38 alpha/Polycomb signalling to Pax7 locus in satellite cells links inflammation to the epigenetic control of muscle regeneration

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Abstract

How regeneration cues are converted into the epigenetic information that controls gene expression in adult stem cells is currently unknown. We identified a novel inflammation-activated signalling in muscle stem (satellite) cells, by which the Polycomb Repressive Complex 2 (PRC2) represses Pax7 expression during muscle regeneration. TNF-activated p38alpha kinase promotes the interaction between YY1 and PRC2, via threonine 372 phosphorylation of EzH2, the enzymatic sub-unit of the complex, leading to the formation of repressive chromatin on Pax7 promoter. Anti-TNF 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 signalling – p38alpha and EzH2 - invariably promote Pax7 expression and expansion of satellite cells that retain their differentiation potential upon signalling resumption. Genetic knockdown of Pax7 impaired satellite cell proliferation in response to p38 inhibition, thereby establishing the biological link between p38/PRC2 signalling to Pax7 and satellite cell decision to proliferate or differentiate.

Keywords

Pax7; p38; muscle stem (satellite) cells; regeneration; chromatin; Polycomb complex

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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 on 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 TNFalpha, 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, as 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 signalling by which inflammatory cytokines deliver the information to individual genes in SCs.

p38 mitogen-activated protein kinases alpha, beta, gamma and delta respond to cellular stressors, such as inflammatory cytokines. In SCs, this group of kinases convert inflammatory cues into epigenetic information that controls gene expression (Lluis et al. 2006; Lassar 2009). The p38 alpha and beta kinases contribute to the assembly of the myogenic transcriptosome on the chromatin of muscle loci, by promoting MyoD-E47 heterodimerization (Lluis et al. 2005), the recruitment of SWI/SNF chromatin remodelling complex (Simone et al. 2004; Serra et al. 2007) and of Ash2L-containing mixed-lineage leukaemia (MLL) methyltransferase complex (Rampalli et al. 2007). By contrast, p38 gamma 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. 2006). De-repression 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. 2010; Panning 2010). The

repressive activity of PRC2 is counterbalanced by trithorax group (trxG)-associated H3-K4 methyltyransferases (Schuettengruber et al. 2007). The coordinated activity of PRC2 and trxG generates the simultaneous tri-methylation 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 de-repression of these genes coincides with the down-regulation of the catalytic subunit of PRC2 – the enhancer of Zeste (EzH2) (Caretti et al. 2004; Ezhkova et al. 2009; Juan et al. 2009).

While 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 re-distributed to repress genes characteristic of the undifferentiated state. Even more puzzling, the identity of the signalling that directs the PRC2 chromatin re-distribution 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 signalling, in SCs. The inflammation-activated p38/PRC2 signalling 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 signalling regulates Pax7 expression and satellite cell proliferation

To investigate the impact of inflammation-activated signalling 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 following muscle contraction. 5 week-old mdx mice were exposed to neutralizing antibodies against TNF alpha (Infliximab). TNF alpha is a cytokine that is secreted within the regenerative environment by the inflammatory infiltrate and regulates muscle regeneration by activating the pro-myogenic p38 signaling (Chen et al. 2007). Previous work showed that long-term treatment with anti-TNF 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 sub-laminar position within muscles from mdx mice that were exposed to anti-TNF antibodies, as compared to muscles from control-treated mdx mice (Fig. 1A and B). 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 anti-TNF 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 treated

mdx mice (Fig. 1C). Consistently, pharmacological inhibition of p38 alpha and beta kinases with SB 203580 (SB) replicated the effect observed with anti-TNF antibodies on Pax7 expression; whereas the inhibition of two other TNF-activated cascades, such as JNK and NFkB pathways, did not alter Pax7 expression (Suppl. Fig. 1A, upper panel). An increased expression of Pax7 was detected in myofibre-derived SCs that were isolated from mdx mice exposed to anti-TNF antibodies (Fig. 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 anti-TNF antibodies (Fig. 1D) or in SCs exposed to SB (Suppl. Fig. 1A, lower panel). Consistently, muscles from mdx mice treated with anti-TNF antibodies showed an increased number of smaller myofibers (Suppl. Fig. 1B) 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 signalling. The reversible nature of p38 inhibition by anti TNF antibodies suggests that restoration of the TNF-p38 signalling 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 anti-TNF 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 anti-TNF antibodies (Suppl. Fig. 2 A and B). Fluorescence-activated cell sorting (FACS) was used to determine the precise identity of these cells. Suppl. Fig. 2 C shows an increase in Pax7 expression levels only in CD34/alpha7integrin double-positive cells (a combination that defines the satellite cell identity) isolated from injured muscles of mice treated with anti-TNF 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 anti-TNF antibody-treated and control mice (Suppl. Fig. 2C).

Collectively, these results indicate that TNF-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. Fig. 1E and F 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 SC (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 (Fig. 1E and F). This indicates an effect of p38-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 (Fig. 2A – arrow indicates a multinucleated myotube) 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 (Fig. 2A and B). In these cells the expression of Pax7, but not Pax3 - the functional orthologue of Pax7 (Buckingham 2007) - was elevated (Fig. 2C and D), further indicating that Pax7 expression is specifically controlled by the p38 signaling in SCs. Notably, upon p38 alpha/beta blockade virtually all myofiber-derived SCs expressed both MyoD and Pax7 when placed in culture, and proliferate in the presence of the p38

inhibitor SB (Fig. 2A and B). 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 p38 alpha/beta in SCs cultured in DM prevented the formation of MyHC-positive myotubes and increased the number of proliferating (BrdU-positive) cells (Fig. 2E, compare top and middle panels) that continued to express Pax7 and cyclinA (Fig. 2F and G), but did not express muscle differentiation markers, such as muscle creatine kinase (MCK) (Fig. 2F) and myogenin (Fig. 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 (Fig. 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 (Fig. 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 p38 alpha, YY1 and Polycomb Repressive Complex 2 (PRC2) 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 (Suppl. Fig. 3A and B), and resulted in down-regulation of Pax7 (Suppl. Fig. 3 B and C). The levels of Pax7 were restored to those detected in control SCs by the p38 alpha/beta inhibitor SB (Suppl. Fig. 3C), further demonstrating that p38 alpha and/or beta control Pax7 expression in SCs.

Previous studies established that p38 alpha 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 p38 alpha deficient mice (Perdiguero et al. 2007), but not in mice deficient for the expression of p38 beta, p38 gamma and p38 delta kinases (Ruiz-Bonilla et al. 2008). The increased number of Pax7-positive cells observed in p38 alpha deficient mice is reminiscent of the phenotype observed in mice treated with anti-TNF alpha antibodies - Fig. 1 shows the magnitude of Pax7 upregulation in mice treated with anti-TNF alpha that is identical to that observed in p38 alpha-deficient mice by Perdiguero et al. (2007). This strong analogy indicates that p38 alpha 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 – p38 alpha and beta (Serra et al. 2007). Only knockdown of p38 alpha in SCs replicated the effect of SB on Pax7 expression (Suppl. Fig. 3D). The Pax7 repression by p38 alpha kinase likely occurs at the transcriptional level, as 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 Lluis et al. 2006; Karen 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 (Fig. 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; Muller 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 p38 alpha by signals that promote myoblast differentiation. We activated the p38 signalling in myoblasts by the ectopic expression of MKK6EE, which promotes differentiation and represses Pax7 (see Suppl. Fig. 3), and monitored the presence of endogenous p38 alpha, YY1 and the enzymatic sub-unit EzH2, on the chromatin at the Pax7 gene. To this purpose, we used C2C12 cells, as they provide abundant material for Chromatin Immuno-Precipitation (ChIP) and co-immunoprecipitation analyses that cannot otherwise be obtained from SCs. The simultaneous chromatin occupancy by p38 alpha, YY1 and EzH2 was detected on both PRE and on promoter region of the Pax7 gene only following MKK6EE-mediated activation of p38 signalling (Fig. 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 Fig. 5). By contrast, p38 alpha was not detected on the chromatin of Pax7 promoter in undifferentiated myoblasts. Chromatin occupancy by p38 alpha following MKK6EE-mediated activation of the p38 signalling was indicated by a two-fold enrichment, which was inhibited by SB, together with the inhibition of YY1 and EzH2 chromatin binding (Fig. 3A). Reciprocal co-immunoprecipitation experiments from nuclear extracts showed the formation of a complex containing endogenous p38 alpha, YY1 and EzH2 upon MKK6EE-mediated activation of the p38 pathway (Fig. 3B). Blockade of p38 alpha by SB attenuated these interactions (Fig. 3B). This is, to our knowledge, the first evidence of a signal-inducible formation of a nuclear complex containing components of the p38 signalling (MKK6 and p38 alpha) and of the PRC2-mediated repressive machinery (YY1 and EzH2).

Chromatin-bound p38 alpha 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 de-repression coincides with the EzH2 disengagement during late differentiation stages (Caretti et al. 2004; Juan et al. 2009). We therefore tested whether the p38 alpha signalling to PRC2 could also mediate EzH2 chromatin disengagement on these genes. MKK6EE-mediated chromatin recruitment of p38 alpha on MHCIIb promoter was detected in myoblasts in the same experimental conditions in which p38 alpha was detected on the chromatin of Pax7 genes (Fig. 3A). However, on MHCIIb promoter p38 alpha 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 hours). As a control, p38 alpha, EzH2 and YY1 were not detected on the chromatin of IgH enhancer (Fig. 3A), which is constitutively repressed in myoblasts, likely by a PRC2-independent mechanism.

Collectively, the results presented above demonstrate a signal-dependent interaction between p38 alpha, 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 de-repression of muscle genes at the same stage. This evidence indicates that the p38 signalling to PRC2 specifically directs the repression of genes that are typically down-regulated during muscle differentiation (e.g. Pax7), but has not direct impact on PRC2-mediated repression of muscle genes, which are induced in myotubes. This conclusion is also supported by our previous evidence that p38 alpha/beta 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).

p38 alpha-mediated phosphorylation of EzH2 enhances the interaction with YY1 to repress Pax7 expression

We next investigated the biochemical and molecular impact of p38 alpha kinase interaction with EzH2 and YY1. A reciprocal interaction between exogenous Flag-tagged EzH2 and myc-tagged p38 alpha was detected upon overexpression in heterologous cell lines, such as 293 cells, even in the absence of p38 activation (Suppl. Fig. 4 A and B), indicating that EzH2 is a potential substrate for p38 alpha kinase. Indeed, an *in vitro* kinase assay with immunoprecipitated Flag-EzH2 incubated with recombinant active p38 alpha showed an incorporation of radiolabelled phosphate in EzH2 only in the presence of ATP; and this effect was specifically inhibited by the inclusion of SB, but not the Pi3K inhibitor LY (Fig. 4A). By using Flag-EzH2 deletion mutants, we could map the p38 alpha-phosphorylated region within a C-terminal fragment encompassing aminoacids 320 and 752 – see phosphorylation of EzH2 1–417 and 321–752 aa fragments, but not EzH2 1–320 aa fragment (Fig. 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 non-phosphorylatable alanine generated a p38 alpha phosphorylation-resistant EzH2 point mutant – EzH2 T372A (Fig. 4C). This mutation does not impair the Ezh2 ability to interact with the other components of PRC2 - Suz12 and EED (Fig. 4D). Of note, MKK6EE-activated p38 alpha (Fig. 4E, right panel, see band indicated by arrow) enhanced the interaction of endogenous YY1 with exogenous EzH2 wild type, but not with the phosphorylation-resistant EzH2 T372A mutant, in C2C12 myoblasts. (Fig. 4E, left panel). By contrast, the kinase deficient MKK6AA mutant, which does not activate the p38 signalling, could not induce EzH2/YY1 interaction (Suppl. Fig. 4C). The failure of EzH2 T372A phospho-mutant to interact with YY1 in response to MKK6EE-activated p38alpha resulted in an impaired ability to repress Pax7 expression, as compared to the EzH2 wild type (Fig. 4E, right panel).

These results link p38alpha-mediated association of EzH2 with YY1 to Pax7 repression in SCs.

PRC2 converts the p38 signalling 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 (Fig. 5, middle vertical panels) and the promoter region (Fig. 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 elements of Pax7 during SC differentiation. We note that myofibre-derived satellite myoblasts (MB) 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 vs 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 (MT) (Fig. 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 (Fig. 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 p38 alpha 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 (Fig. 5). An analysis of Pax7 locus 12 kb upstream 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 (Fig. 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 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 SC

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 RNA expression in myofibre-derived SCs, during their 4 days of culture in GM and further incubation in DM (Fig. 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 down-regulation 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 Fig. 5). We therefore surmised that EzH2 knockdown at this stage could prevent Pax7 down-regulation. Indeed, when the EzH2 knockdown by siRNA was restricted to days 3 and 4 of SC culture in GM (see transparent box in Fig. 6A), we observed an induction of Pax7 transcripts that was proportional to the reduction of EzH2 levels (Fig. 6B). By contrast, MyoD levels remained unchanged, and myogenin and MCK levels were down-regulated, possibly because of the anti-differentiative action of Pax7 (Olguin et al 2007). Since the p38 signalling 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), it is formally possible that p38-directed SWI/SNF participates to Pax7 repression. However, knock-down of individual BAF60 sub-units 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) (Suppl. Fig. 5). This evidence further demonstrates that p38-SWI/SNF and p38-PRC2 are two distinct signalling 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 de-repressed 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.

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) (Fig. 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 (Fig. 7A), which is equivalent to the time of treatment with anti-TNF antibodies (see Fig. 1). After the exposure to dZNep, we isolated the myofibers for *ex vivo* evaluation of PRC2 blockade in primary SC. In these cells, the levels of EzH2 were drastically reduced by the exposure to dZNep (Fig. 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 (Fig. 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 Table in Materials and methods) 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 (Fig. 7D, E and F). Furthermore, SCs retained the ability to differentiate into myotubes surrounded by undifferentiated SCs, upon drug withdrawal - a feature also observed with SB (Fig. 2). Despite of these overlapping effects, it was evident that p38 inhibition by SB showed a more pronounced effect on SC number (see Fig. 7D and Suppl. Fig. 6A). By contrast, the EzH2 inhibitors MC1947 and MC1948 only moderately expanded the number of SC (Fig. 7D and Suppl. Fig. 6A, upper panel). The common ability of p38 and EzH2 inhibitors to decrease H3-K27^{3me} levels at the Pax7 regulatory elements (Suppl. Fig. 6B) reflects their impact on the p38-PRC2 signalling 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 (Suppl. Fig. 6C), 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 signalling.

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 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 p38alpha 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 if the control of H3-K4 methylation is directly regulated by a parallel p38 signalling 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 (Suppl. Fig. 6D). This evidence identifies two distinct levels of epigenetic regulation at the Pax7 locus by the p38-PRC2 signalling. One level reflects an "extrinsic" regulation of Pax7 by the regeneration-activated p38 signalling 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 p38alpha in SCs did not result in a synergistic effect on Pax7 expression (Suppl. Fig. 6E), supporting the concept that p38 and Ezh2 act on the same pathway.

Pax7 mediates the effect of p38 signalling on satellite cell proliferation

We next investigated the impact of Pax7 expression on p38-mediated control of SC proliferation, as p38-mediated phosphorylation of EzH2 reduces its ability to repress Pax7 in SCs (Fig. 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 (Suppl. Fig. 7A, left panel). Pax7 knockdown resulted in a reduced number of SCs (Suppl. Fig. 7B and C), which dowregulated proliferation markers, such as cyclin E1, as compared to control-transfected (siC) cells (Suppl. Fig. 7A, 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 (Suppl. Fig. 7B and C). This evidence demonstrates that Pax7 expression mediates the ability of p38 blockade to expand the number of SCs, thereby supporting the biological link between p38 alpha/PRC2 signalling to Pax7 and muscle-stem cell decision to proliferate or differentiate (see also Fig. 2).

Discussion

Although the instructive role of the inflammatory component of adult stem cell niche is well documented, the intracellular signalling 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 novel signalling 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 SC undergoing terminal differentiation. Key components of this signalling are the p38 alpha kinase and the enzymatic sub-unit 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 re-locates to loci that are typically repressed in differentiated myotubes – e.g. Pax7. The chromatin re-distribution of PRC2 in differentiating SCs is regulated by the p38alpha kinase, which promotes the formation of a complex containing p38 alpha, 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 p38alpha 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 p38alpha and/or beta are recruited via interactions with components of the muscle transcriptosome, including BAF60 (Simone et al. 2004), E47 (Lluis et al. 2005), 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; Yuan 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 detectable 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). De-repression 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 of SCs (Oustanina et al. 2004), 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 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), as its expression would otherwise antagonize the activation of the differentiation program (Olguin et al. 2007).

While recent studies have begun to identify downstreatm targets and effectors of Pax7 in SCs (McKinnell et al. 2008; Hu et al. 2008; Kumar et al. 2009), the upstream signalling that controls Pax7 expression in SCs in response to extrinsic signals is poorly characterized (Kuang and Rudnicki, 2008). Previous works indicated a role of beta-catenin in self-renewal of SCs, implicating beta-catenin/GSK3beta signalling as a potential upstream regulator of Pax7 expression (Perez-Ruiz et al. 2008). Myostatin- and Megf10-activated signalling 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 p38 alpha-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 anti-proliferative function of PRC2 that is in apparent conflict with the widely observed tumor-promoting activity of Polycomb proteins (Bracken et al. 2009; Sparmann and van Lohuizen 2006). However, recent works have also indicated an anti-proliferative 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 anti-proliferative 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 signalling 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 signalling 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 two 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 cells isolation and culture

Single muscle fibers were isolated by standard procedures. Briefly, 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 72h and then fixed for immunofluorescence or plated on matrigel (Sigma, 1mg/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 5uM), SP600 (10 uM), PDTC (100 nM), MC1947 (10 uM) and MC1948 (10 uM) were added when indicated and replaced every 24 hours. BrdU (Amersham) was added to the medium (diluted 1:800) 4 hours prior 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 using the oligonucleotide: siPax7-GGUAACAUCCCAGCUUUACTT (Silencer Pre-designed siRNA from Ambion), and following the Dharmafect3 (Dharmacon) transfection protocol. Unrelated oligonucleotide siC-AAGTAAGCTGATGAAAGACTG, was used as a control. Knockdown of p38 alpha and beta, 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 hour in serum free medium before being placed in growth medium.

In vitro kinase assay

HEK 293T cells were transfected with pCMV FlagMycEzh2 WT, deletion mutants or Ezh2T372A mutant plasmids by calcium phosphate method. 1.5mg of total extracts were incubated with Flag M2 agarose beads (Sigma) for 2 hours at 4C, extensively washed in lysis buffer and the immunoprecipitated material was eluted in lysis buffer containing 150ng/ul 3XFlag peptide(Sigma).

200ng of immunopurified proteins were incubated with 100ng of recombinant active p38 alpha (Cell Signaling) in the presence of 60 mM MgCl2, 60 μM ATP, 50 mM Tris-HCl pH7.5, 12 mM DTT and phosphatase inhibitors (Calbiochem), supplemented with 0.7μCi of γ-(32P)ATP at room temperature for 15 minutes. 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 anti-Flag (Sigma, M2) and anti-p38 alpha (Cell Signaling) antibodies.

Histology and inmunofluorescence

Quadriceps muscles were cut transversally, fixed in 4% PFA for 20 minutes and permeabilised with 100% methanol for 6 minutes 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 using the 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 using the Image J software downloaded from http://rsb.info.nih.gov/ij.

Single myofibers were fixed with 2% PFA for 20 minutes, permeabilized with 0,5% Triton/PBS and blocked with 20% goat serum 1h at room temperature. Satellite cells were fixed with 4% PFA for 20 minutes, permeabilized with 0,25% triton and blocked with 4% BSA in PBS 1h 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 Signalling) and tubulin (Thermo Fisher, MS581P1) were detected by western blot analysis on total cell or nuclear extracts after lysis in 50mM Tris-HCl (pH 8.0), 125 mM NaCl, 1mM DTT, 5mM MgCl2, 1mM EDTA, 10% glycerol, and 0.1% NP-40 supplemented with 1mM 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 Over-expressed 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 ug were retro-transcribed using the Taqman reverse transcription kit (Applied Biosystems). Real time quantitative PCR was performed to analyse relative gene expression levels using SYBR Green Master mix (Applied Biosystems) and following manufacturer indications. Primers sequences are described in the supplemental data

Coimmunoprecipitation studies

Endogenous CoIPs on nuclear cell extracts of C2C12 cells were performed by standard procedures. Briefly, nuclear extracts were pre-cleared with protein G agarose for 1 hour at 4 C, immunoprecipitated with anti-p38 alpha (kindly provided by Dr T Sudo), anti-YY1 (Santa Cruz, SC-281) or anti-EzH2 (Diagenode) antibodies for 3 hours at 4C 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 anti-p38 (Cell-Signaling), anti-Ezh2 (AC22, Cell Signaling), anti-YY1 (Santa Cruz, SC-7341) and anti-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-p38 alpha, 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 using the SYBR Green Master Mix (Applied Biosystems). Relative recruitment is calculated as the amount of amplified DNA normalised 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 data

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 data.

Statistical methods

Where indicated statistical significance was determined by the student t-test (* P<0,5; ** p<0,01; *** p<0,001)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL, Lander ES. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell. 2006; 125(2):315–26. [PubMed: 16630819]

Blais A, van Oevelen CJ, Margueron R, Acosta-Alvear D, Dynlacht BD. Retinoblastoma tumor suppressor protein-dependent methylation of histone H3 lysine 27 is associated with irreversible cell cycle exit. J Cell Biol. 2007; 179(7):1399–412. [PubMed: 18166651]

Boyer LA, Mathur D, Jaenisch R. Molecular control of pluripotency. Curr Opin Genet Dev. 2006a; 16:455–462. [PubMed: 16920351]

Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, Levine SS, Wernig M, Tajonar A, Ray MK, Bell GW, Otte AP, Vidal M, Gifford DK, Young RA, Jaenisch R. Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature. 2006b; 7091:349–53. 2006.

Bracken AP, Dietrich N, Pasini D, Hansen KH, Helin K. Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. Genes Dev. 2006; 20(9):1123–36. [PubMed: 16618801]

Bracken AP, Helin K. Polycomb group proteins: navigators of lineage pathways led astray in cancer. Nat Rev Cancer. 2009; 11:773–84. [PubMed: 19851313]

Buckingham M. Skeletal muscle progenitor cells and the role of Pax genes. C R Biol. 2007; 330:530–533. [PubMed: 17631448]

Buckingham M, Relaix F. The role of Pax genes in the development of tissues and organs: Pax3 and Pax7 regulate muscle progenitor cell functions. Annu Rev Cell Dev Biol. 2007; 23:645–673. [PubMed: 17506689]

Caretti G, Di Padova M, Micales B, Lyons GE, Sartorelli V. The Polycomb Ezh2 methyltransferase regulates muscle gene expression and skeletal muscle differentiation. Genes Dev. 2004; 18:2627–2638. [PubMed: 15520282]

Chen SE, Jin B, Li YP. TNF-alpha regulates myogenesis and muscle regeneration by activating p38 MAPK. Am J Physiol Cell Physiol. 2007; 292:1660–1671.

Chow CW, Davis RJ. Proteins kinases: chromatin-associated enzymes? Cell. 2006; 127:887–890. [PubMed: 17129776]

Classen AK, Bunker BD, Harvey KF, Vaccari T, Bilder D. A tumor suppressor activity of Drosophila Polycomb genes mediated by JAK-STAT signaling. Nat Genet. 2009; 41(10):1150–5. [PubMed: 19749759]

Collins CA, Gnocchi VF, White RB, Boldrin L, Perez-Ruiz A, Relaix F, Morgan JE, Zammit PS. Integrated functions of Pax3 and Pax7 in the regulation of proliferation, cell size and myogenic differentiation. PLoS ONE. 2009; 4:e4475. [PubMed: 19221588]

Conboy IM, Rando TA. The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. Dev Cell. 2002; 3:397–409. [PubMed: 12361602]

de Nadal E, Posas F. Multilayered control of gene expression by stress-activated protein kinases. EMBO J. 2010; 29(1):4–13. [PubMed: 19942851]

de la Serna IL, Ohkawa Y, Imbalzano AN. Chromatin remodelling in mammalian differentiation: lessons from ATP-dependent remodellers. Nat Rev Genet. 2006; 7(6):461–473. [PubMed: 16708073]

Dhawan J, Rando TA. Stem cells in postnatal myogenesis: molecular mechanisms of satellite cell quiescence, activation and replenishment. Trends Cell Biol. 2005; 15:666–673. [PubMed: 16243526]

Ezhkova E, Pasolli HA, Parker JS, Stokes N, Su IH, Hannon G, Tarakhovsky A, Fuchs E. Ezh2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells. Cell. 2009; 136:1122–1135. [PubMed: 19303854]

Gillespie MA, Le Grand F, Scimè A, Kuang S, von Maltzahn J, Seale V, Cuenda A, Ranish JA, Rudnicki MA. p38-{gamma}-dependent gene silencing restricts entry into the myogenic differentiation program. J Cell Biol. 2009; 187(7):991–1005. [PubMed: 20026657]

Gopinath SD, Rando TA. Stem cell review series: aging of the skeletal muscle stem cell niche. Aging Cell. 2008; 7:590–598. [PubMed: 18462272]

Guasconi V, Puri PL. Chromatin: the interface between extrinsec cues and the epigenetic regulation of muscle regeneration. Trends Cell Biol. 2009; 19(6):286–94. [PubMed: 19394225]

Hirabayashi Y, Suzki N, Tsuboi M, Endo TA, Toyoda T, Shinga J, Koseki H, Vidal M, Gotoh Y. Polycomb limits the neurogenic competence of neural precursor cells to promote astrogenic fate transition. Neuron. 2009; 63(5):600–13. [PubMed: 19755104]

Holterman CE, Le Grand F, Kuang S, Seale P, Rudnicki MA. Megf10 regulates the progression of the satellite cell myogenic program. J Cell Biol. 2007; 179:911–922. [PubMed: 18056409]

Hu P, Geles KG, Paik JH, DePinho RA, Tjian R. Codependent activators direct myoblast-specific MyoD transcription. Dev Cell. 2008; 4:534–546. [PubMed: 18854138]

Ito T, Yamauchi M, Nishina M, Yamamichi N, Mizutani T, Ui M, Murakami M, Iba H. Identification of SWI.SNF complex subunit BAF60a as a determinant of the transactivation potential of Fos/Jun dimers. J Biol Chem. 2001; 276(4):2852–7. [PubMed: 11053448]

Huang P, Zhao XS, Fields M, Ransohoff RM, Zhou L. Imatinib attenuates skeletal muscle dystrophy in mdx mice. Faseb J. 2009

Jones NC, Tyner KJ, Nibarger L, Stanley HM, Cornelison DD, Fedorov YV, Olwin BB. The p38alpha/beta MAPK functions as a molecular switch to activate the quiescent satellite cell. J Cell Biol. 2005; 169:105–116. [PubMed: 15824134]

Juan AH, Kumar RM, Marx JG, Young RA, Sartorelli V. Mir-214-dependent regulation of the polycomb protein Ezh2 in skeletal muscle and embryonic stem cells. Mol Cell. 2009; 36(1):61–74. [PubMed: 19818710]

Keren A, Tamir Y, Bengal E. The p38 MAPK signaling pathway: a major regulator of skeletal muscle development. Mol Cell Endocrinol. 2006; 252(1–2):224–30. [PubMed: 16644098]

Kuang S, Rudnicki MA. The emerging biology of SC and their therapeutic potential. Trends Mol Med. 2008; 14:82–91. [PubMed: 18218339]

Kuang S, Gillespie MA, Rudnicki MA. Niche regulation of muscle satellite cell self-renewal and differentiation. Cell Stem Cell. 2008; 2:22–31. [PubMed: 18371418]

Kumar D, Shadrach JL, Wagers AJ, Lassar AB. Id3 Is a Direct Transcriptional Target of Pax7 in Quiescent SC. Mol Biol Cell. 2009; 20(14):3170–7. [PubMed: 19458195]

Lassar AB. The p38 MAPK family, a pushmi-pullyu of skeletal muscle differentiation. J Cell Biol. 2009; 187(7):941–3. [PubMed: 20026653]

Lee TI, Jenner RG, Boyer LA, Guenther MG, Levine SS, Kumar RM, Chevalier B, Johnstone SE, Cole MF, Isono K, Koseki H, Fuchikami T, Abe K, Murray HL, Zucker JP, Yuan B, Bell GW, Herbolsheimer E, Hannett NM, Sun K, Odom DT, Otte AP, Volkert TL, Bartel DP, Melton DA, Gifford DK, Jaenisch R, Young RA. Control of developmental regulators by Polycomb in human embryonic stem cells. Cell. 2006; 125:301–313. [PubMed: 16630818]

Lepper C, Conway SJ, Fan CM. Adult SC and embryonic muscle progenitors have distinct genetic requirements. Nature. 2009; 460 (7255):627–31. [PubMed: 19554048]

Lickert H, Takeuchi JK, Von Both I, Walls JR, McAuliffe F, Adamson SL, Henkelman RM, Wrana JL, Rossant J, Bruneau BG. Baf60c is essential for function of BAF chromatin remodelling complexes in heart development. Nature. 2004; 432(7013):107–12. [PubMed: 15525990]

Lluis F, Perdiguero E, Nebreda AR, Munoz-Canoves P. Regulation of skeletal muscle gene expression by p38 MAP kinases. Trends Cell Biol. 2006; 16:36–44. [PubMed: 16325404]

LLuis F, Ballestar E, Suelves M, Esteller M, Munoz-Canoves P. E47 phosphorylation by p38 MAPK promotes MyoD/E47 association and muscle-specific gene transcription. EMBO J. 2005; 24:974–984. [PubMed: 15719023]

McFarlane C, Hennebry A, Thomas M, Plummer E, Ling N, Sharma M, Kambadur R. Myostatin signals through Pax7 to regulate satellite cell self-renewal. Exp Cell Res. 2008; 314:317–329. [PubMed: 17949710]

McKinnell IW, Ishibashi J, Le Grand F, Punch VG, Addicks GC, Greenblatt JF, Dilworth FJ, Rudnicki MA. Pax7 activates myogenic genes by recruitment of a histone methyltransferase complex. Nat Cell Biol. 2008; 10:77–84. [PubMed: 18066051]

Mai A, Valente S, Cheng D, Perrone A, Ragno R, Simeoni S, Sbardella G, Brosch G, Nebbioso A, Conte M, Altucci L, Bedford MT. Synthesis and biological validation of novel synthetic histone/protein methyltransferase inhibitors. ChemMedChem. 2007; 2:987–991. [PubMed: 17458842]

Merdes G, Paro R. About combs, notches, and tumors: epigenetics meets signaling. Dev Cell. 2009; 4:440–2. [PubMed: 19853558]

Martinez AM, Schuettengruber B, Sakr S, Janic A, Gonzalez C, Cavalli G. Polyhomeotic has a tumor suppressor activity mediated by repression of Notch signaling. Nat Genet. 2009; 41(10):1076–82. [PubMed: 19749760]

Montarras D, Morgan J, Collins C, Relaix F, Zaffran S, Cumano A, Partridge T, Buckingham M. Direct isolation of SC for skeletal muscle regeneration. Science. 2005; 309:2064–2067. [PubMed: 16141372]

Mozzetta C, Minetti G, Puri PL. Regenerative pharmacology in the treatment of genetic diseases: the paradigm of muscular dystrophy. Int J Biochem Cell Biol. 2009; 41:701–710. [PubMed: 18804548]

Muller J, Kassis JA. Polycomb response elements and targeting of Polycomb group proteins in Drosophila. Curr Opin Genet Dev. 2006; 16:476–484. [PubMed: 16914306]

Olguin HC, Yang Z, Tapscott SJ, Olwin BB. Reciprocal inhibition between Pax7 and muscle regulatory factors modulates myogenic cell fate determination. J Cell Biol. 2007; 177:769–779. [PubMed: 17548510]

Oustanina S, Hause G, Braun T. Pax7 directs postnatal renewal and propagation of myogenic SC but not their specification. EMBO J. 2004; 23:3430–3439. [PubMed: 15282552]

Panning B. Fine-tuning silencing. Cell Stem Cell. 2010; 1:3–4. 2. [PubMed: 20085734]

Pasini D, Cloos PA, Walfridsson J, Olsson L, Bukowski JP, Johansen JV, Bak M, Tommerup N, Rappsilber J, Helin K. JARID2 regulates binding of the Polycomb repressive complex 2 to target genes in ES cells. Nature. 2010 Jan 14. [Epub ahead of print] 3.

Pasini D, Hansen KH, Christensen J, Agger K, Cloos PA, Helin K. Coordinated regulation of transcriptional repression by the RBP2 H3K4 demethylase and Polycomb-Repressive Complex 2. Genes Dev. 2008; 22:1345–1355. [PubMed: 18483221]

Peng JC, Valouev A, Swigut T, Zhang J, Zhao Y, Sidow A, Wysocka J. Jarid2/Jumonji coordinates control of PRC2 enzymatic activity and target gene occupancy in pluripotent cells. Cell. 2009; 139(7):1290–302. [PubMed: 20064375]

Perdiguero E, Ruiz-Bonilla V, Gresh L, Hui L, Ballestar E, Sousa-Victor P, Baeza-Raja B, Jardi M, Bosch-Comas A, Esteller M, Caelles C, Serrano AL, Wagner EF, Munoz-Canoves P. Genetic analysis of p38 MAP kinases in myogenesis: fundamental role of p38alpha in abrogating myoblast proliferation. Embo J. 2007; 26:1245–1256. [PubMed: 17304211]

Perez-Ruiz A, Ono Y, Gnocchi VF, Zammit PS. beta-Catenin promotes self-renewal of skeletal-muscle SC. J Cell Sci. 2008; 121:1373–1382. [PubMed: 18397993]

Peterson JM, Guttridge DC. Skeletal muscle diseases, inflammation, and NF-kappaB signaling: insights and opportunities for therapeutic intervention. Int Rev Immunol. 2008; 27(5):375–87. [PubMed: 18853344]

Pokholok DK, Zeitlinger J, Hannett NM, Reynolds DB, Young RA. Activated signal transduction kinases frequently occupy target genes. Science. 2006; 313:533–536. [PubMed: 16873666]

Radley HG, Davies MJ, Grounds MD. Reduced muscle necrosis and long-term benefits in dystrophic mdx mice after cV1q (blockade of TNF) treatment. Neuromuscul Disord. 2008; 18:227–238. [PubMed: 18207402]

Rampalli S, Li L, Mak E, Ge K, Brand M, Tapscott SJ, Dilworth FJ. p38 MAPK signaling regulates recruitment of Ash2L-containing methyltransferase complexes to specific genes during differentiation. Nat Struct Mol Biol. 2007; 14:1150–1156. [PubMed: 18026121]

Relaix F, Montarras D, Zaffran S, Gayraud-Morel B, Rocancourt D, Tajbakhsh S, Mansouri A, Cumano A, Buckingham M. Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. J Cell Biol. 2006; 172:91–102. [PubMed: 16380438]

Relaix F, Rocancourt D, Mansouri A, Buckingham M. A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. Nature. 2005; 435:948–953. [PubMed: 15843801]

Ringrose L, Paro R. Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. Annu Rev Genet. 2004; 38:413–443. [PubMed: 15568982]

Ruiz-Bonilla V, Perdiguero E, Gresh L, Serrano AL, Zamora M, Sousa-Victor P, Jardí M, Wagner EF, Muñoz-Cánoves P. Efficient adult skeletal muscle regeneration in mice deficient in p38beta, p38gamma and p38delta MAP kinases. Cell Cycle. 2008; 7(14):2208–14. [PubMed: 18641461]

Sacco A, Doyonnas R, Kraft P, Vitorovic S, Blau HM. Self-renewal and expansion of single transplanted muscle stem cells. Nature. 2008; 456:502–506. [PubMed: 18806774]

Schwartz YB, Pirrotta V. Polycomb silencing mechanisms and the management of genomic programmes. Nat Rev Genet. 2007; 8:9–22. [PubMed: 17173055]

Schuettengruber B, Chourrout D, Vervoort M, Leblanc B, Cavalli G. Genome regulation by polycomb and trithorax proteins. Cell. 2007; 128(4):735–45. [PubMed: 17320510]

Seale P, Ishibashi J, Scimè A, Rudnicki MA. Pax7 is necessary and sufficient for the myogenic specification of CD45+:Sca1+ stem cells from injured muscle. PLoS Biol. 2004; 2:664–672.

Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA. Pax7 is required for the specification of myogenic SC. Cell. 2000; 102:777–786. [PubMed: 11030621]

Serra C, Palacios D, Mozzetta C, Forcales S, Ripani M, Morantte I, Jones D, Du K, Jahla U, Simone C, Puri PL. Functional interdependence at the chromatin level between the MKK6/p38 and IGF1/Pi3K/AKT pathways during muscle differentiation. Mol Cell. 2007; 28:200–213. [PubMed: 17964260]

Shen X, Kim W, Fujiwara Y, Simon MD, Liu Y, Mysliwiec MR, Yuan GC, Lee Y, Orkin SH. Jumonji modulates polycomb activity and self-renewal versus differentiation of stem cells. Cell. 2009; 139(7):1303–14. 4. [PubMed: 20064376]

Simon JA, Kingston RE. Mechanisms of polycomb gene silencing: knowns and unknowns. Nat Rev Mol Cell Biol. 2009; 10:697–708. [PubMed: 19738629]

Simone C, Forcales SV, Hill DA, Imbalzano AN, Latella L, Puri PL. p38 pathway targets SWI-SNF chromatin-remodeling complex to muscle-specific loci. Nat Genet. 2004; 36:738–743. [PubMed: 15208625]

Sing A, Pannell D, Karaiskakis A, Sturgeon K, Djabali M, Ellis J, Lipshitz HD, Cordes SP. A vertebrate Polycomb response element governs segmentation of the posterior hindbrain. Cell. 2009; 138(5):885–97. [PubMed: 19737517]

Sparmann A, van Lohuizen M. Polycomb silencers control cell fate, development and cancer. Nat Rev Cancer. 2006; 11:846–56. [PubMed: 17060944]

Squazzo SL, O'Geen H, Komashko VM, Krig SR, Jin VX, Jang SW, Margueron R, Reinberg D, Green R, Farnham PJ. Suz12 binds to silenced regions of the genome in a cell-type-specific manner. Genome Res. 2006; 16(7):890–900. [PubMed: 16751344]

Stoick-Cooper CL, Moon RT, Weidinger G. Advances in signaling in vertebrate regeneration as a prelude to regenerative medicine. Genes Dev. 2007; 21:1292–1315. [PubMed: 17545465]

Tan J, Yang X, Zhuang L, Jiang X, Chen W, Lee PL, Karuturi RK, Tan PB, Liu ET, Yu Q. Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. Genes Dev. 2007; 21:1050–1063. [PubMed: 17437993]

Wong ES, Le Guezennec X, Demidov ON, Marshall NT, Wang ST, Krishnamurthy J, Sharpless NE, Dunn NR, Bulavin DV. p38MAPK controls expression of multiple cell cycle inhibitors and islet proliferation with advancing age. Dev Cell. 2009; 17(1):142–9. [PubMed: 19619499]

Woo CJ, Kharchenko PV, Daheron L, Park PJ, Kingston RE. A region of the human HOXD cluster that confers polycomb-group responsiveness. Cell. 2010; 140(1):99–110. [PubMed: 20085705]

Zammit PS, Partridge TA, Yablonka-Reuveni Z. The skeletal muscle satellite cell: the stem cell that came in from the cold. J Histochem Cytochem. 2006a; 54:1177–1191. [PubMed: 16899758]

Zammit PS, Relaix F, Nagata Y, Ruiz AP, Collins CA, Partridge TA, Beauchamp JR. Pax7 and myogenic progression in skeletal muscle SC. J Cell Sci. 2006b; 119:1824–1832. [PubMed: 16608873]

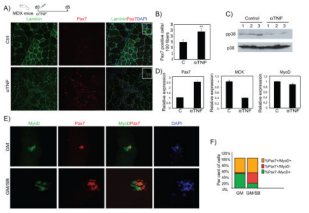

Fig. 1. *In vivo* treatment of mdx mice with anti TNF and *ex-vivo* blockade of the p38 pathway expand a population of activated muscle satellite cells and increase Pax7 expression

Top: Schematic representation of the experimental design. **A)** Immunofluorescence staining using antibodies against laminin (green), Pax 7 (red) and counterstained for DAPI (blue), on transversal quadriceps sections from six weeks old mdx mice treated for five days with 20mg/kg of control (upper panels) or anti TNF antibodies (lower panels), **B)** The reported data represent the number of Pax7 positive cells per cent fibres in the same conditions as above. For each quadriceps over four hundred fibres were counted and the graph represents the average of three mice per experimental group. Error bars indicate the standard deviation (** Student test: p<0,01). **C)** Levels of activated p38 in muscles from control and anti TNF treated mice were measured by western blot using an antibody that recognises 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 test: p<0,01). **E)** Co-immunostaining of myofibers isolated from the gastrocnemius of C57/Bl6 wild type mice using antibodies against MyoD (green), Pax 7 (red) and counterstained for DAPI (blue). The single myofibers were maintained in culture for 72 h 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 vs GM/SB. (See also Suppl. Fig. 1 and 2)

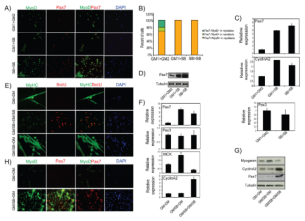

Fig. 2. p38 signalling represses Pax7 expression and proliferation of satellite cells

A) Immunofluorescence staining against Pax7 (red) and MyoD (green) on myofiber-derived satellite cells that were allowed to delaminate in culture for three 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 counter stain 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. **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)** Satellite cells cultured in the same conditions as above were tested for their differentiation and proliferation potential. After a BrdU pulse (4 h), cells were harvested and stained 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. **G)** Protein levels of Pax7, Cyclin A2 and Myogenin were quantified by Western Blot. Tubulin was used as a loading control. **H)** Co-immunostaining using 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 Suppl. Fig. 3 and 7)

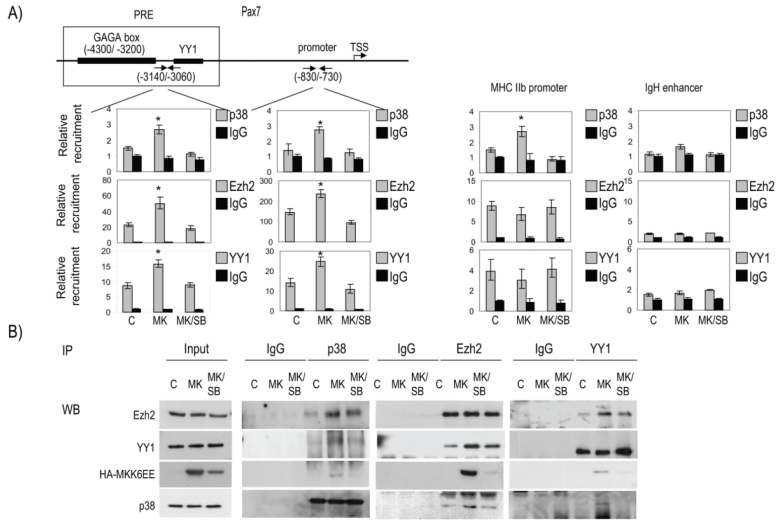

Fig. 3. MKK6EE-dependent interactions between Ezh2, YY1 and p38 alpha on the chromatin of Pax7 promoter

Top: Schematic representation of the PRE containing region and the proximal promoter of Pax7 gene. **A)** 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 normalised against the input DNA. Error bars show standard deviation from three independent experiments. p values showing statistical significance by the student t-test between control and MKK6EE are indicated (* indicates p<0,05). **B)** Co-immunoprecipitation 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 an anti-p38, anti-Ezh2 and anti-YY1 antibodies or control IgG and bound proteins were revealed by western blot using antibodies against Ezh2, p38, YY1 and HA. The images are representative of 3 independent experiments reproducing the same result.

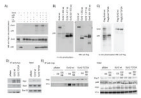

Fig. 4. p38 phosphorylation of Ezh2 on Threonine 372 is necessary for Ezh2 and YY1 interaction and for Pax7 repression in response to MKK6EE

A) In vitro kinase assay using exogenously expressed flag-tagged Ezh2 that was immunoprecipitated from HEK 293 cells and then incubated with recombinant active p38 alpha (upper panel), in the absence or presence of the p38 alpha/beta inhibitor SB or the Pi3K inhibitor LY. Middle and lower panel show control western blots for the expression of Flag-Ezh2 and p38. **B)** In vitro kinase assay using deletion constructs of Ezh2 were performed as in A (left panel). Western blot using antibodies against the flag tag is shown as a loading control (right panel). **C)** In vitro kinase assay with a point mutant of Ezh2 in which threonine 372 was replaced with an alanine was performed as in A and B (left panel). Western blot using antibodies against the flag tag is shown as a loading control (right panel). **D)** Co-immunoprecipitation from nuclear extracts of C2C12 cells stably expressing control (pBabe) or myc-tagged Ezh2 wt and Ezh2T372A mutant. Nuclear extracts were immunoprecipitaed with anti-myc and bound proteins were revealed by western blot using antibodies against myc tag, Suz12 and EED (left panel). Input nuclear extracts before immunoprecipitation are shown in the left panel (**E)** Co-immunoprecipitation from 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 the p38 inhibitor SB. Nuclear extracts were immunoprecipitated with an anti-myc and bound proteins were revealed by western blot using antibodies against myc tag and YY1. (Left panel) Western blot of input nuclear extracts before immunoprecipitation showing Pax7, p38, YY1 and myc-ezh2 expression levels (right panel). Arrow indicates super-shifted (active, phosphorylated) endogenous p38 alpha. (See also Suppl. Fig. 4)

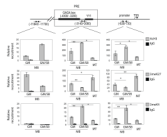

Fig. 5. p38-dependent modulation of PRC2-mediated histone modifications at the Pax7 promoter in satellite cells

Top: Schematic representation of the PRE containing region (−3140/−3060), the proximal promoter (−830−730) and distal elements (−11840/−11700) of the Pax7 gene. **A)** ChIP analysis of the PRE containing region, the proximal promoter and −12 kb region of Pax7 gene was performed using antibodies against AcH3, H3K27me3, H3K4me3 and control IgG in satellite myoblasts (MB) cultured for 5 days in growth medium in the presence (GM/SB) or absence (GM) of the p38 alpha/beta inhibitor SB. Note that SB was replaced every 24 hours in the GM for the 5 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 normalised against the input DNA. Error bars indicate the standard deviation (Student test: * indicates p<0,05; ** p<0,01).

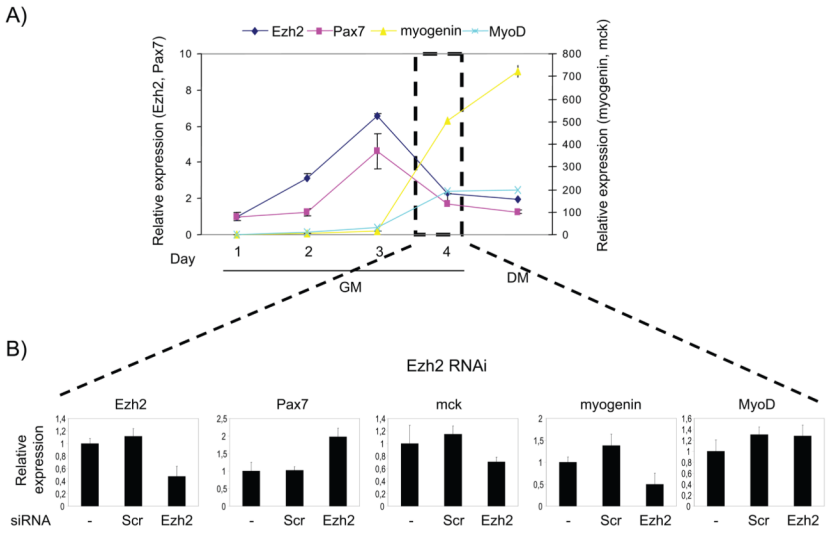

Fig. 6. Stage-specific upregulation of Pax7 by EzH2 knockdown in satellite cells

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 myofibre-derived satellite cells, during 4 days of culture in GM and further incubation in DM (Fig. 5A). **B)** satellite cells derived from wild type C57/BL6 mice were transfected with control scramble (scr) or EzH2 siRNA at day three 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 Suppl. Fig. 5)

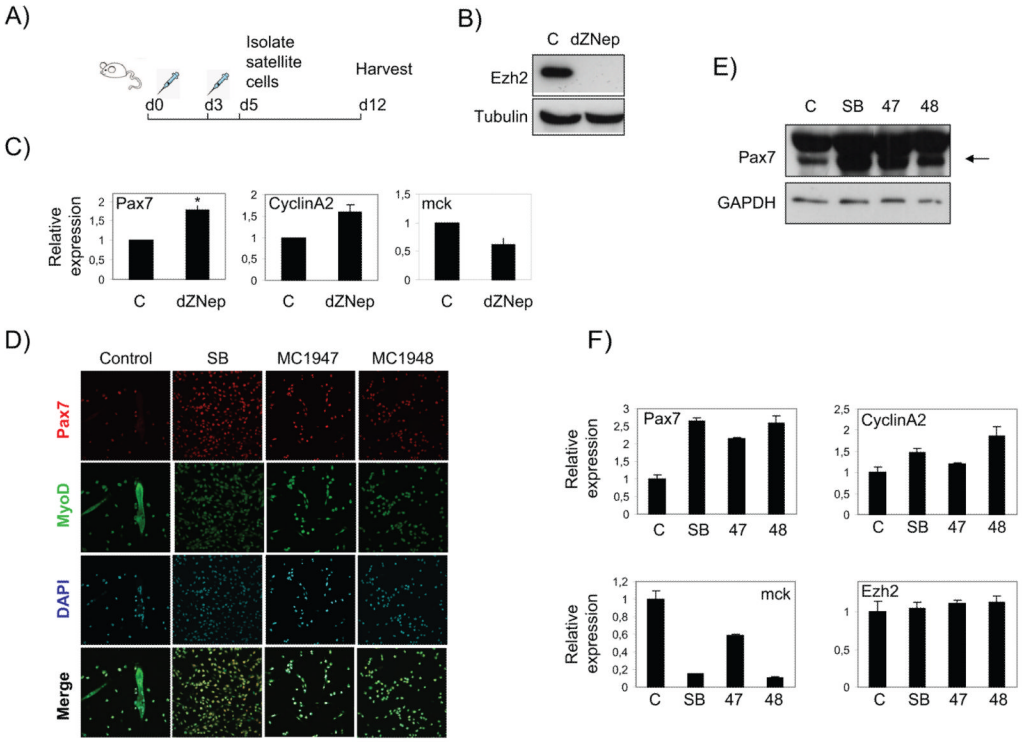

Fig. 7. Pharmacological blockade of PRC2 increases Pax7 expression in satellite cells both *in vivo* and *ex vivo*

A) Schematic representation of the experimental design. Briefly, five weeks old mdx mice were treated intra-peritoneally with dZNep (1 mg/kg). After five days mice were sacrificed and satellite cells were isolated and cultured in growing conditions (GM) for seven 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 studend t-test are indicated (* indicates 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 co-immunostaining. 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. (See also Suppl. Fig. 6).