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"DYNAMICS AND EPIGENETIC CONTROL OF PcG PROTEINS DURING SKELETAL MUSCLE CELL DIFFERENTIATION"

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La scienza puo' essere
solo cio' che e',
non cio' che potrebbe essere.
Albert Einstein

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1) "Differential Regulation of PRC2-EZH2 and PRC2-EZH1 by MSK1/H3Ser28ph controls skeletal muscle cell differentiation"

Submitted to Developmental Cell

ABSTRACT

During skeletal muscle differentiation, signal dependent switches in cell differentiation programs require global rearrangements in repression and activation of lineage specific genes, hence the importance of unravelling epigenetic mechanisms that control these dynamics and their integration with signaling pathways. Polycomb Group (PcG) proteins are transcriptional repressors that modify chromatin through epigenetic modifications that prevent changes in cell identity by maintaining transcription patterns, throughout development and in adulthood. PcG proteins form two major multiprotein complexes, Polycomb repressive complex 1 and 2 (PRC1 and PRC2, respectively), the latter containing the catalytic subunit, EZH2 that modifies histone H3 by tri-methylation of lysine 27 (H3K27me3). While EZH2 promotes transcriptional repression of muscle specific genes, transcriptional activation that accompanies skeletal muscle differentiation is characterized by loss of EZH2 and recruitment of transcriptional activators, such as MyoD and SRF, at muscle regulatory regions (Caretti et al. 2004). Although much is known about the processes regulated by PcG proteins, little is known about signaling dependent pathway mechanisms that regulate PcG dynamics onto the chromatin. We used C2C12 mouse cell line as a skeletal muscle differentiation model to gain insight into the role of different PRC2 components during this process and into the the signaling pathways that regulates PRC2 dynamics at muscle target loci. We report that two different PRC2 complexes are present during skeletal muscle differentiation: PRC2-EZH2, that is predominant in proliferating myoblasts and PRC2-EZH1 that is specific for post-mitotic myotubes. We show that these two complexes are differentially involved in the regulation of skeletal muscle differentiation. We demonstrate that the opposite dynamics of PRC2-EZH2 and PRC2-EZH1 at muscle regulatory regions is differentially regulated at the chromatin level by MAPK- (MSK1) dependent phospho/methyl switch mechanism involving phosphorylation of the lysine 28 of the histone H3 (H3Ser28ph), enabling to counteract the function of the PRC2 complex docking site H3K27me3. We report that, while MSK1/H3Ser28ph is crucial for the displacement of the PRC2-EZH2 from muscle regulatory regions, allowing gene activation and muscle differentiation, this pathway does not influence PRC2-EZH1 binding to chromatin, anticipating a novel PcG function in post-mitotic cells. Taken together, our results suggest that the phosphorylation of H3Ser28 by MSK1 plays a key role in epigenetic gene regulation and this modification is necessary for skeletal muscle differentiation.

1. INTRODUCTION

1.1 Epigenomes and Control of Cell Identity

"The difference between genetics and epigenetics can probably be compared to the difference between writing and reading a book. Once a book is written, the text (the genes or DNA: stored information) will be the same in all the copies distributed to the interested audience. However, each individual reader of a given book may interpret the story slightly differently, with varying emotions and projections as they continue to unfold the chapters. In a very similar manner, epigenetics would allow different interpretations of a fixed template (the book or genetic code) and result in different read-outs, dependent upon the variable conditions under which this template is interrogated."
Thomas Jenuwein

Even before the identification of DNA as genetic material, Conrad Waddington coined the term "*epigenetics*" to define the phenotypic execution of developmental programs. Epigenetics broadly describes the additional information superimposed on the genome that contributes to the heritable establishment and maintenance of transcriptional states and cellular identity (Grimaud et al. 2006b). As such, a single genome may be modified to produce multiple epigenomes allowing for cellular diversity, a necessary criterion for the development of metazoans (Richards 2006). The "Epigenome" contributes to the quality, stability and heritability of cell-specific transcription programs using different levels of control: DNA methylation, histone modifications, nucleosome mobility, domain organization, *in trans* interactions, nuclear organization that ensure the transmission of the epigenetic patterns from one cell to the daughter cells. During cell differentiation or metabolic switch, cells undergo profound changes in gene expression. These events are accompanied by complex modifications of chromosomal components and nuclear structures, including covalent modifications of DNA and chromatin up to topological reorganization of chromosomes and genes in the nucleus. To various extents, all these levels of organization contribute to the stability and heritability of transcription programmes and define what is meant as the *epigenomic* level of gene regulation (Lanzuolo and Orlando 2007). Epigenetic regulation consists of changes in chromatin structure mediated, primarily, by post-translational modification of histone tails, substitution of histone variants into nucleosomes, and DNA methylation (Jenuwein and Allis 2001; Turner 2005). Except in mammalian spermatozoa, the basic unit of chromatin is generally a nucleosome, which is comprised of a histone octamer (a histone H3-H4 tetramer and two H2A-H2B dimers) around which DNA, 147 base pairs in length, is wrapped in 1.75 superhelical turns (Barrera and Ren 2006). Histone H1 is located at the outer surface of the nucleosome and stabilizes its interaction with DNA. A multitude of post-translational modifications of the histone occur, which individually and collectively alter chromatin character and function, influencing gene expression and most DNA-dependent processes (Lee et al. 2010). What makes chromatin so unique is the extraordinary combination of mechanisms that

manage the huge amount of information stored in the genome. This regulation requires a strict and complex control that involves many different actors-DNA, histones and other proteins, ncRNAs–and a complex choreography where spatial and temporal aspects are crucial. It' s possible to discriminate between three hierarchically interconnected levels at which genome control takes place (van Driel et al. 2003). The first level of regulatory mechanism is found on the DNA molecule. Specific DNA sequences mediate the targeting of nuclear factors that regulate a multitude of processes in the genome. The regulatory elements found in the DNA nucleotide sequence cannot fully explain the different cellular identities in multicellular organism. Thus, this second regulatory level includes marks such as methylation of the DNA molecule, covalent post-translational modifications of the core histones, histone variants and other chromatin-associated proteins. Contrasting with DNA sequence level which, excluding mutation and recombination events, is stable throughout the life of the organism, the chromatin level may be regulated by enzymatic activities acting as a "switch" that can change its functional state (Jenuwein and Allis 2001). A third regulatory level that has been poorly studied, when compared to the other two, is the three-dimensional (3D) organization of chromatin in the nucleus. DNA molecules contained in the nucleus of, for example, human cells have a total length of 2 m if they are released from all other chromatin components. This has to be fitted into a nucleus of a diameter of ~ 10 μm. To achieve this high degree of compaction, the DNA molecule is organized together with the histones and other chromatin components into higher order 3D structures. 3D nuclear architecture reflects basic nuclear functions, such as transcription and replication. Transcription incorporates any available biological system to achieve the regulated expression of the transcriptome. Understanding the epigenetic modifications is required for comprehension of how gene expression is achieved and regulated. Chromatin provides a platform that becomes decorated with epigenetic marks that regulate transient interactions with transcriptional factors; this provides a fine-tuning level of regulation integrating primary sequence information with spatial and temporal control (Fig.1). As the language is defined " A systemic means of communicating ideas…using conventionalized signs…or marks having understood meanings", the complexity of the relationship between epigenetic marks and the biological processes could be explained in the same way. As scientists, it falls to us to learn and understand this "language", a task that we have only begun to undertake.

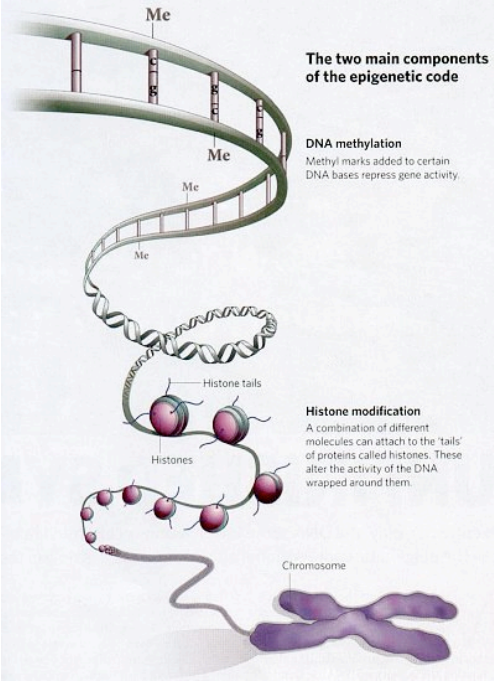

Fig.1: The two main components of epigenetic code. Epigenetic regulation consists of changes in chromatin structure mediated, primarily, by post-translational modification of histone tails, substitution of histone variants into nucleosomes, and DNA methylation. To various extent, all these levels of organization contribute to the stability and heritability of transcription programmes and define what is meant as the *epigenomic* level of gene regulation (www.epigenome-noe.net).

1.2 Polycomb Group Proteins as Epigenetic Regulators

Polycomb group genes exert essential memory function during all developmental processes. Together with a battery of components including sequence-specific DNA binding/accessory factors, signaling pathway intermediates, noncoding RNA, PcG proteins define cellular identity through tight regulation of specific expression patterns (Morey and Helin 2010). First characterized in *Drosophila*, PcGs were found to mediate silencing of the HOX subset of homeobox genes responsible for segment identity in metazoan (Lewis 1978; Schwartz and Pirrotta 2007). This group of gene clusters is essential for proper development across species. In PcG-mutant flies or mammals, Hox genes are expressed outside their normal spatial territories along the head-to tail axis. This causes characteristic defects in body patterning, which are the hallmark phenotypes that define fly PcG proteins (Fig. 2) (Simon and Kingston 2009).

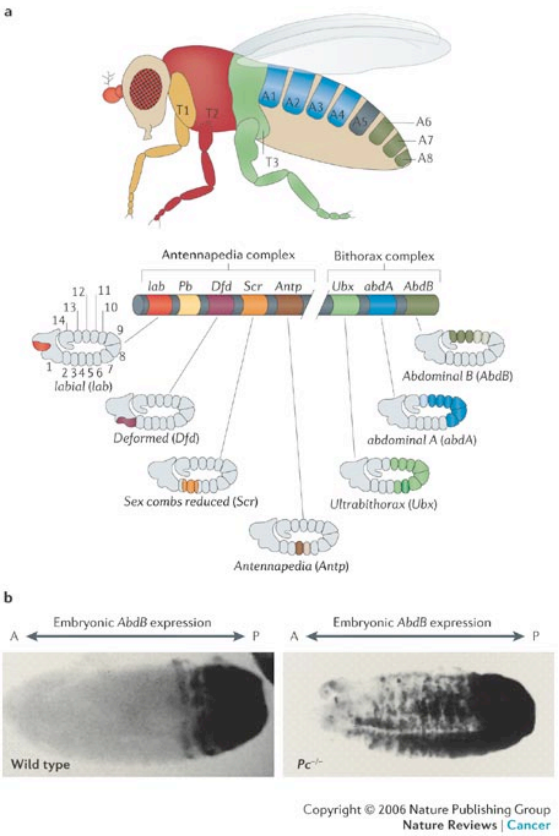

Fig.2: Polycomb proteins function as repressor of Hox genes. a) Hox gene expression in the Drosophila melanogaster embryo defines the positions of structures and appendages along the anterior-posterior axis of the adult body. **b)** PcG proteins mantein the correct spatial and temporal expression pattern of the Hox genes through transcriptional repression. Mutations in PcG genes therefore cause a de-repression of specific Hox members, which leads to homeotic transformation-transformations of one body segment into the identity of another. The term "Polycomb" was used to describe the first PcG mutations-extra sex combs (esc) and Polycomb (Pc)-because of additional sex combs on the second and third legs of male flies, a structure that is normally restricted to the first legs (Schwartz and Pirrotta 2007).

In addition to homeotic genes, PcG proteins control a sizable fraction of the genome. In metazoan animals, many PcG-regulated genes encode transcription factors and modifiers important in the control of cell fate decisions (Bracken et al. 2006). PcG proteins assemble as high molecular weight complexes. They were initially purified from *Drosophila*, but homologous complexes were later isolated in other organisms (Kohler and Villar 2008). *Drosophila* has four different PcG complexes - Polycomb repressive complex 1 (PRC1), Polycomb repressive complex 2 (PRC2), Pleiohomeotic repressive complex (PhoRC) and Polycomb

Repressive Deubiquitinase Complex (PR-DUB) that work together (Fig. 3). PRC2 is thought to be involved in the initiation of gene silencing, whereas PRC1

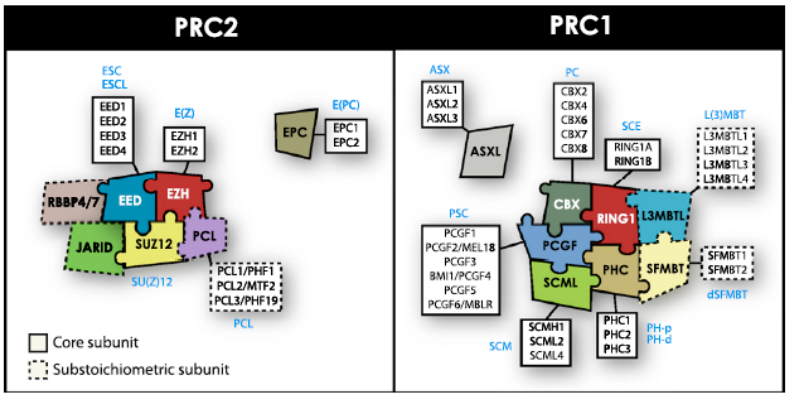

Fig.3: Diversity of PRC1 and PRC2 complexes formed by vertebrate PcG proteins. Subunits of the PRC1 (right panel) and PRC2 (left panel) complexes are indicated. The *Drosophila* homolog of each subunit is indicated in light blue. Multiple combinations of paralog subunits can generate a diversity of PRC1 and PRC2 complexes, which likely have specific and shared functions. Some subunits seem to be present in substoichiometric amounts and interact with the PcG complexes in a cell-context-dependent manner. The core and the substoichiometric subunits are identified. The contacts illustrated in the diagrams are not intended to represent the actual interactions. Involvement of EPC and ASXL subunits with PRC2 or PRC1 complexes is still unclear and requires further investigations (Sauvageau and Sauvageau 2010).

is implicated in the stable manteinance of gene repression (Lund and van Lohuizen 2004). PhoRC is required for the recruitment of both PRC2 and PRC1 complexes (Savla et al. 2008). PR-DUB complex is able to deubiquinate H2A (Scheuermann et al. 2010; Schuettengruber and Cavalli 2010) and this activity is required for a correct gene silencing *in vivo*. The PRC1 biochemically purified from flies contains a core quartet of PcG proteins: Polycomb (Pc), a chromodomain protein that can bind to trimethylated lysine 27 of histone H3 (H3K27me3); Posterior sex combs (PSC), a RING domain protein that, in a similar fashion to homologous proteins in mammals, probably enhances the catalytic activity of Ring; Ring, the catalytic subunit for histone H2A ubiquitination; Polyhomeotic (PH), a zinc finger protein as well as a lower amounts of Sex comb on midleg (SCM) (Franke et al. 1992; Rastelli et al. 1993; Shao et al. 1999; Francis et al. 2001). Many additional proteins were co-purified with these core components, including ZESTE, TBP (TATA-box binding protein)-associated factors TAFII250, TAFII110, TAFII85, TAFII62 and elements of other multiprotein complexes, such as MI2, SIN3A, SMRTER, indicating the possibility of a direct interaction between PcG complexes and

transcriptional machinery (Breiling et al. 2001; Francis et al. 2001; Saurin et al. 2001). The mammalian PRC1 (2-5 MDa) complex has been isolated from HeLa cells using exogenously expressed tagged protein components and affinity purification. The core components are similar to those of *D. melanogaster* PRC1 complex, although no TAFs have been detected in association with it (Levine et al. 2002; Lavigne et al. 2004; Wang et al. 2004a). Mouse and humans genomes carry various copies of some of PcG homologues that presumably are part of different PRC1 complexes acting at different targets or in different tissues. The purified complexes include HPC1, 2 and 3, HPH1, 2 and 3 RING1A and RING1B, BMI1 and potentially its homologue MEL18. These are, correspondingly, homologous to the fly PC, PH, RING and PSC (Gunster et al. 1997; Satijn et al. 1997; Bardos et al. 2000). Drosophila PRC2 is a 600 kDa complex composed of four core subunits: Enhancer of zeste [E(z)], a SET (Suvar 3-9, Enhancer of zeste, Tritorax) domain protein with histone methyltransferase activity specific for the lysine 27 of the histone H3 (H3K27me); Suppressor of zeste [Su(z)12]; and two WD 40 domain proteins, Extra sex comb (Esc) and Nurf55 (Nucleosome remodelling factor 55) (Cao et al. 2002; Czermin et al. 2002; Kuzmichev et al. 2002; Muller et al. 2002). Each of these components is required for binding to and efficient methylation of nucleosomes by the complex (Schwartz and Pirrotta 2007). The core components of human PRC2 are EZH2, EED, SUZ12, and the histone binding protein RbAp46/48 (Cao et al. 2002; Czermin et al. 2002; Kuzmichev et al. 2002; Muller et al. 2002). EZH2 protein is the human homologue of the *Drosophila* Enhancer of zeste protein. It contains histone methyltransferase (HMT) that is responsible for di- and trimethylation of histone H3 on lysine 27 (H3K27me2 and H3K27me3) the repression-associated epigenetic modifications (Cao and Zhang 2004a; Schuettengruber et al. 2007). SUZ12 is required for the methyltransferase activity of EZH2, along with EED (Cao and Zhang 2004b; Montgomery et al. 2005). In mammals, PRC2, PRC3 and PRC4 complexes have been biochemically characterized, and they differ by the presence of different isoforms of EED (Embrionic Ectoderm Development), the homologue of the fly ESC (Kuzmichev et al. 2004; Kuzmichev et al. 2005). In the presence of histone H1, PRC2, but not PRC3, preferentially methylates H1K26, which has an aminoacid context similar to that of H3K27, although it is present in only one of mammalian H1 variants and absent in *D. melanogaster* H1. PRC4 accumulates when EZH2 is overexpressed in cultered cells. It contains an EED isoform that is expressed only in undifferentiated ES cells, and SIRT1, a mammalian SIR2 homologue (Schwartz and Pirrotta 2007). Recently, a novel subunit of PRC2 called JARID2 was identified (Peng et al. 2009; Shen et al. 2009; Landeira et al. 2010; Li et al. 2010; Pasini et al. 2010). Interestingly JARID2 contains a Jumonji C domain, but is devoid of detectable histone demethylase activity. Indeed, the role of JARID2 appears to be quite complex. It colocalizes with PRC2 and H3K27me3 on the chromatin and modulates the function of this PcG complex in embryonic stem cells (ESCs). Genome-wide ChIP-seq analyses of JARID2, EZH2, and SUZ12 binding reveal that JARID2 and PRC2 occupy the same genomic regions (Peng et al. 2009; Pasini et al. 2010). Jarid2 is required for efficient binding of PRC2, indicating that the interplay of PRC2 and JARID2 fine-

tunes deposition of the H3K27me3 mark. However, evidence was reported for a different role of JARID2 in pluripotent ES cells in regulating RNA Polymerase II (RNA Pol II) recruitment at bivalent genes (Landeira et al. 2010). As described above, the PRC2 complex is responsible for setting the characteristic PcG chromatin mark, histone H3K27me3 that is recognized by PRC1 complex through the PC chromodomain. In *Drosophila*, loss of E(Z) or ESC/ESCL functions results in loss of monomethylation, dimethylation and trimethylation of H3K27, indicating that this is the only K27 methyltransferase activity. Diversely, in mammals, loss of EZH2 eliminates only dimethylation and trimethylation, impling that monomethylation of H3K27 is carried out by a different complex. The monomethylation complex may involve EZH1 instead of EZH2, though this has not been confirmed, and still requires EED since the loss of this component eliminates all three degrees of methylation (Montgomery et al. 2005). Mammals contain a second E(Z) homologue, called EZH1. Recently, it was demonstrated that the HMTase EZH1 could replace EZH2 in noncanonical PRC2 (PRC2-EZH1). While PRC2-EZH2 catalyzes H3K27me2/me3 and its knockdown affects global H3K27me2/me3 levels, PRC2-EZH1 performs this function weakly (Margueron et al. 2008; Shen et al. 2008). In accordance, EZH1 knockdown was ineffectual on global H3K27me2/me3 levels. Instead, PRC2-EZH1 directly and robustly represses transcription from chromatinized templates and compact chromatin. EZH1 targets a subset of EZH2 genes, yet EZH1 is more abundant in non proliferative adult organs while EZH2 expression is tightly associated with proliferation, suggesting that the two PRC complexes may have different functions in dividing versus post-mitotic cells (Ezhkova et al. 2009). The three levels of methylation have different distributions in the *Drosophila* as in mammalian genomes. In both organisms, H3K27me2 is virtually ubiquitous in euchromatin, found in more than 50% of nucleosomes. Instead, K27me3 is essentially confined to a PcG target sites and represents only 5-10% of the total histone H3 (Ebert et al. 2004). In this respect, the common identification of the 600 kDa PRC2 complex as the only one involved in the methylation is most probably incorrect. This relatively abundant complex carries out much more general function of dimethylating of H3K27. The function of K27 dimethylation is also unknown and it remains to be determined whether it is compatible with or permissive to transcriptional activity or whether it needs to be removed before gene activation (Schwartz and Pirrotta 2008). In contrast, H3K27 trimethylation is associated with stable binding of E(Z)-containing complexes to PcG target genes and with transcriptional repression. A possible structural difference in the form of the global PRC2 complex and the one associated with PcG repression may be the presence of the Polycomb-like protein (PCL in *Drosophila*, PHF in mammals). A larger (1 MDa) PRC2 complex containing PCL is found specifically at PcG target sites (Tie et al. 2003). Recent work suggests that in the absence of PCL function, H3K27 trimethylation but not dimethylation is specifically impaired (Sarma et al. 2008). The other PcG complex PhoRC, recently discovered in flies (Klymenko et al. 2006) has only two components: the specific DNA binding protein Pleiohomeotic (Pho), and dSfmbt, a homolog of mouse SFMBT [Scm (Sex comb on midleg)-related gene containing four MBT (malignant brain tumor) domains].

Drosophila dSfmbt is essential for Hox gene silencing and binds specifically to monomethylated and dimethylated H3K9 and H4K20 (Klymenko et al. 2006). Whether a similar complex is present in mammals is not known. In addition, however, other less central components may vary from one target site to another. Thus, different target genes, different tissues, or different developmental stages may involve PcG complexes with slightly different functionalities or sensitivity to external signaling.

1.2.1 The Gene Networks Controlled by PcG Proteins

Early studies in Drosophila revelead that aside Hox genes, the PC protein binds to multiple sites in the genome (Paro 1990; Franke et al. 1992). Recently, the gene networks controlled by PcG memory system have been identified. By taking a bioinformatic approach, Ringrose and coworkers predicted 167 PcG target sequences in the fly genome, some of which they confirmed experimentally. Among the targets they predict are mostly developmentally regulated genes and cell cycle regulators (Ringrose et al. 2003). At the same time Bracken and coworkers undertook a genome-wide mapping of PcG target genes in human embryonic fibroblasts, using a similar approach (Fig. 4) (Boyer et al. 2006; Bracken et al. 2006). Several targets of PRC1 and PRC2 proteins were found that were predicted for their *Drosophila* orthologs by Ringrose et al., as the *HOX* genes and orthologs of the segmentation genes. In addition, more than 1000 targets were identified, among these genes important for various differentiation processes, in particular neuronal differentiation, sex differentiation, bone differentiation, epidermal cell differentiation, hematopoiesis and muscle development (Bracken et al. 2006). Basically in parallel, PRC1 and PRC2 targets were mapped by ChiP on chip in murine and human embryonic stem cells, revealing the presence of PcG in these cells at many genes important for development, morphogenesis, organogenesis, neurogenesis, cell-cell signaling and transcriptional regulation. The vast majority of PcG binding sites in vertebrates are close to promoters (> 90% are whitin 1 kb of the transcription start site), whereas in flies only 30% of PcG binding sites are whitin 2 kb of a promoter, and many are several tens of kb away (Negre et al. 2006). The studies in mammals document two distinct classes of binding: ~70% of targets have a sharp peak of binding near the promoter. The remainder, which include the Hox and several other transcription factor foci, blanket the transcription unit (Bracken et al. 2006). Althought these studies still need to be extended to more tissues and developmental stages, the overall picture is that, in any given cell type, most alternative genetic programmes are shut down by PcG mechanisms, except for the subset that is required in that cell type.

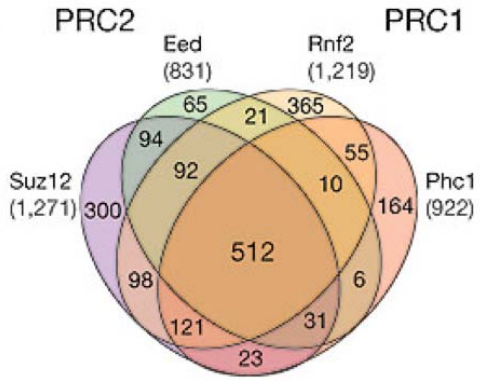

Fig.4: PRC1 and PRC2 colocalize at genes encoding developmental regulators. Genomic sites occupied by PcG proteins were identified as peaks of ChIP-enriched DNA using a validated algorithm. This analysis revealed that PRC1 and PRC2 components occupied an overlapping set of target genes and identified with high confidence 512 genes bound by all four PcG proteins in ES cells. Venn diagram showing the overlap among genes bound by PcG proteins within 1 kb of a transcription start site. Numbers in parentheses represent the total number of genes bound by the respective PcG protein (Boyer et al. 2006).

1.2.2 Recruitment of PcG Proteins to their Targets

A recurring question in the study of PcG mechanisms is how the complexes are recruited to their target genes. In *Drosophila*, specific PcG-dependent regulatory elements called Polycomb Response Elements (PREs) were identified (Chan et al. 1994). PREs are specialized epigenetic DNA modules that control the epigenetic state of homeotic genes as well as other developmentally regulated genes. Alike enhancers, they influence promoter activity and can act at a distance. The length of *Drosophila* PREs ranges from several hundred base pairs to some kilobases, although an ectopic "minimal" core PRE of a few hundred base pairs is sufficient to recapitulate temporal and spatial regulation of homeotic gene promoters (Breiling et al. 2007). PREs include many conserved short motifs, several of which are recognized by known DNA-binding proteins. So, the recruitment of PcG complexes to PREs is though to be mediated by DNA-binding proteins that directly interact with or facilitate the binding of PcG proteins. PHO has been shown to play such role (Mohd-Sarip et al. 2006) but it may not be true at all PREs. Other *Drosophila* DNA-binding proteins such as Zeste, GAGA factor (GAF), Pipsqueak, DSP-1, have been proposed to act as recruiters but, while these proteins often bind at or near known or presumptive PREs and PcG binding regions, the number and the distribution of their binding sites is quite

variable and none of them so far can be shown to be present at all PcG sites (Schwartz and Pirrotta 2008). Since no definitive signature of a PRE has been identified, the most likely scenario is that PcG proteins can be recruited in different ways by different proteins. A key aspect of PRE function is its ability to control promoters at a distance via chromatin structure and interference with RNA Polymerase II (Orlando 2003; Levine et al. 2004). PREs were shown to build up multiloop higher-order structures and that this topological organization is important for epigenetic inheritance (Fig. 5) (Lanzuolo et al. 2007). Similar results were reported also in mammalian cells (Tiwari et al. 2008). Notably, these long-distance effects seem to occur both *in cis* and *in trans* (Cleard et al. 2006).

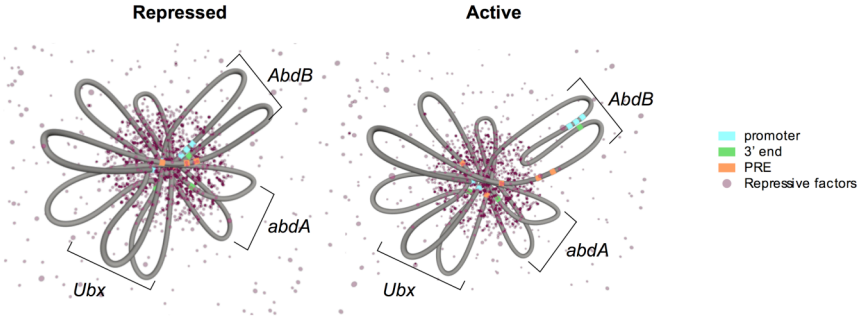

Fig. 5: PREs are thought to mediate the transcriptional memory function by building up higher-order structures in the nucleus. The Bithorax Complex (BX-C) adopts different spatial conformations relative to its transcriptional state. In the repressive state, the BX-C locus adopts a condensed structure in which all the PcG bound elements are interacting together. In this conformation, all PREs (orange in the figure), promoters (light blue) and 3' end of the genes (green) organize the BX-C in topologically discrete domains. In cells with a constitutively active *AbdB* gene, the PRE-promoter interaction, observed in the context of repression, is lost, whereas the other epigenetic elements that are still silenced retain their clusterd conformation (Lanzuolo et al. 2007).

Importantly, PcGs appear to be involved in other aspects of nuclear organization including insulator function (Gerasimova and Corces 1998) and pairing (Bantignies et al. 2003; Dejardin and Cavalli 2005). In mammals it remains unclear to what extent the DNA sequence plays a role in PcG targeting. Search of mammalian PREs is difficult and has only led to isolated successes (Sing et al. 2009; Woo et al. 2010). One impediment has been that PRC1 and PRC2 tend to broadly distributed across many kilobases of mammalian developmental genes (Ku et al. 2008) and nearly co-extensive with the domain of H3K27 trimethylation (Fig. 6). Novel insights can be gained by computational studies aimed at detection of discriminative DNA sequence features. Previous studies have identified a number of transcriptional factor (TF) motifs that are associated with PcG targets

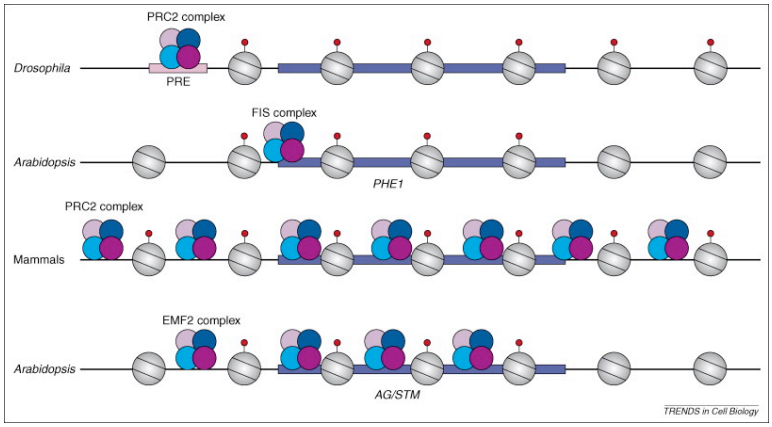

Fig.6: Different patterns of PRC2 and H3K27me3 localization in different organisms. In Drosophila, PRC2 is localized to defined PREs that are depleted of nucleosomes, whereas PRC2-dependent H3K27me3 (red lollipops) spreads over extended domains. Similarly, at the Arabidopsis PHE1 locus, the FIS PRC2-like complex binds close to the transcriptional start site, with H3K27me3 extending towards the 3′ end of the gene, but not into intergenic regions. By contrast, PRC2 complexes as well as H3K27me3 span extended domains into intergenic regions in mammals. Similarly, co-localization of the Arabidopsis EMF2 PRC2-like complex with H3K27me3 has been observed in the promoter and coding regions of the both AG and STM, but it does not spread into intergenic regions. Red lollipops, H3K27me3; grey circles, nucleosomes; blue rectangles, transcribed genic regions (Kohler and Villar 2008).

(Ku et al. 2008). As in *Drosophila*, a specific DNA binding component, the PHO homolog YY1 (Thomas and Seto 1999), is often associated with PcG complexes and it is reasonable to expect that multiple DNA-binding proteins are likely to act as recruiters and that the set of recruiters may vary in detail from one target gene to another. Furthermore, more general DNA sequence features have only been found to be associated with PcG targets, including high CpG density (Bernstein et al. 2006; Tanay et al. 2007), high sequence conservation score (Tanay et al. 2007), depletion of DNA transposons (Bernstein et al. 2006) and periodic patterns of dinucleotide frequencies (Yuan 2009). The completion of mammalian transcriptome allowed the identification of whole ncRNA output (Carninci et al. 2005). Specific classes of long ncRNA have been shown to play a major role in transcriptional regulation being part of major transcription complexes (Mercer et al. 2009). More recently, it has been recognized that PcG can physically interact with non-coding RNA (Fig. 7) (Khalil et al. 2009), providing another mechanism for sequence specific targeting. Because many issues remain unsettled about PREs and recruiters, more work is needed to define the rules for PcG targeting in mammalian cells. These rules seem likely to vary among different cell types and context (Rinn et al. 2007; Khalil et al. 2009; Simon and Kingston 2009; Gupta et al. 2010; Tian et al. 2010). This may include the potentially large complexity of ncRNA interacting with PcG complexes.

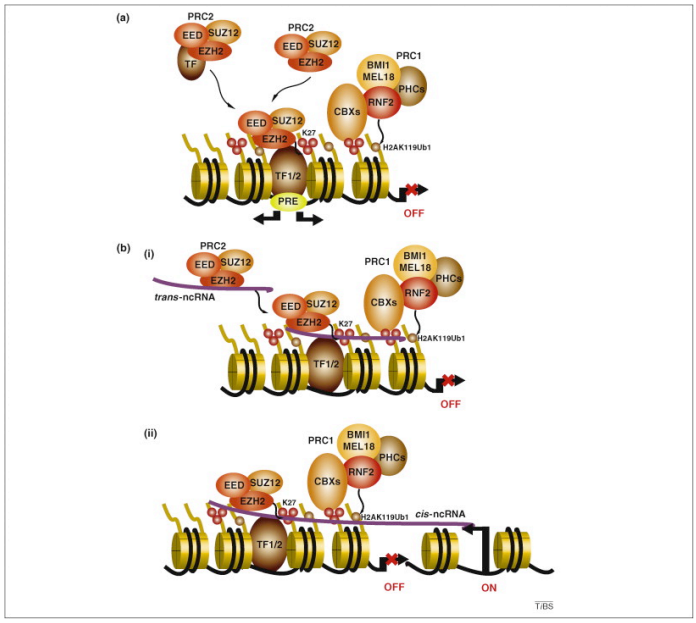

Fig.7: Possible mechanisms for PRC2 recruitment to chromatin. PRC2 is recruited to chromatin by different mechanisms involving transcription factors and ncRNAs. **a)** Cell-type specific transcription factors (TFs) and or gene-specific TFs (e.g. JARID2) associate with and recruit PRC2 to chromatin, peraphs through a PRE. The mammalian proteins that bridge the association between PRC2-PRE have not been identified. Arrows flanking the PRE indicate that PREs are longer than a nucleosome space. **b)** i) Trans-ncRNA binds and recruits PRC2 to chromatin. ii) PRC2 is recruited by a cis-ncRNA expressed upstream or downstream of the PRC2-target gene (Morey and Helin 2010).

1.2.3 PcG and Mechanisms of Transcriptional Repression

PcG proteins were thought to maintain gene silencing by locking inactive genes in heterochromatin-like environment that excludes transcriptional activators and that is incompatible with RNA synthesis. In this way, silent chromatin would irreversibly program differentiated cells to not leave their fate. Recent and less recent discoveries, however, depict a more dynamic situation in which PcG-mediated silencing is the result of an equilibrium between opposing transcriptional forces, which coexist and include activators and repressors, that maintain not only terminally determined states but also competence for switching (Orlando 2003). It is now clear that H3K27 methylation has a crucial role in the stable binding of PcG complexes. The discovery of the specific binding of chromodomains to methylated histones immediately indicated that methylation might recruit the chromodomain-containing complexes. The idea is that, if PRE-binding proteins can recruit the PRC2 complex, the ensuing methylation would then recruit the PRC1 complex. Wang *et al.* (Wang et al. 2004b) presented evidence suggesting this order of events. Binding of the PRC2 initiation complex to the Polycomb group target genes induces Ezh2-mediated methylation of

histone proteins, primarily at lysine 27 of histone H3 (H3K27). PRC1 is able to recognize the trimethylated H3K27 (H3K27me3) mark through the chromodomain of Polycomb (PC). This interaction might bring neighbouring nucleosomes into the proximity of the PRC2 complex to facilitate widespread methylation over extended chromosomal regions (Schwartz et al. 2006). In addition, PRC1 possesses ubiquitin E3 ligase activity that targets lysine 119 of the histone H2A (H2AK119) and this modification is associated with gene repression (Fig. 8) (Wang et al. 2004a; Cao et al. 2005).

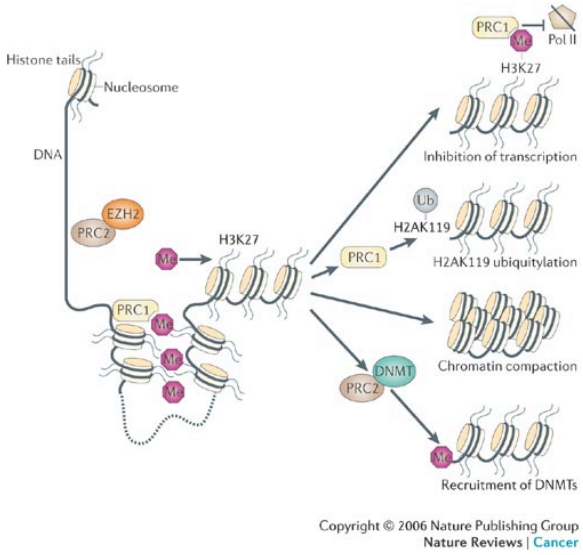

Fig.8: Epigenetic gene silencing by Polycomb protein complexes. Binding of the PRC2 initiation complex to the PcG target genes induces EZH2-mediated methylation of histone proteins, primarily H3K27. PRC1 is able to recognize H3K27me3 mark through the chromodomain of PC. This interaction might bring neighbouring nucleosomes into the proximity of the PRC2 complex to facilitate widespread methylation over extended chromosomal regions. Although the precise mechanisms for PRC-mediated stable gene silencing are still poorly understood, they are proposed to involve direct inhibition of the transcriptional machinery, PRC1-mediated ubiquitylation (Ub) of H2AK119, chromatin compaction and recruitment of DNA methyltransferase (DNMTs) to target gene loci by PRC2. Pol II, RNA Polymerase II (Sparmann and van Lohuizen 2006).

Furthermore, *in vitro* studies have demonstrated that PRC2 also exhibits methyltransferase activity towards lysine 26 of the linker histone H1 (H1K26) (Kuzmichev et al. 2004). Methylated H1K26 can tether heterochromatin binding protein 1 (HP1) to chromatin and could thereby influence higher order structure. In agreement with TrxG complexes that are involved in the formation of an "open" chromatin structure that is more accessible to the transcription machinery, it was suggested that PcG complexes may promote the formation of highly packed, transcriptionally repressed chromatin fiber. This view was supported by

the finding that histone deacetylases (HDACs) copurify with PcG proteins. Deacetylation of lysines in the N-terminal tails of histone H3 and H4 is an important mark of repressed chromatin and counteracts chromatin destabilization by histone acetyltransferases (HATs) activities (Jenuwein and Allis 2001). EED and EZH2 coimmunoprecipitate with HDAC1 and HDAC2 proteins from human cell line extracts (van der Vlag and Otte 1999). The same kind of interaction was described in *D. melanogaster*, where HDAC RPD3 is found together with PRC2 proteins (Tie et al. 2003). However, doubts concerning the idea that PcG repression creates compacted, inaccessible chromatin region were shed when Schlossherr and colleagues found that PcG repressed regions are as accessible for restriction nucleases as active regions (Schlossherr et al. 1994). More recently, general transcription factors (GTFs) and RNA Polymerase II have been mapped to PcG-repressed promoters, suggesting that PcG repression does not render the target regions inaccessible for the transcriptional machinery, but rather inhibits activated transcription or elongation (Breiling et al. 2001; Dellino et al. 2004). Nevertheless PcG-repressed regions seem to have a different structure, preventing active transcription events. In some way, PcG proteins change the state of the chromatin in their target region, although it does not become inaccesible. *In vitro*, PRC1 can bind and compact nucleosomal arrays, making them refractory to SWI/SNF-class ATP dependent chromatin remodelers (Francis et al. 2004; King et al. 2005). Thus, chromatin compaction could be an attractive mechanism for gene silencing, but where does it take place?

Drosophila PREs, bound by PRC1 and other PcG protein complexes, are nucleosome depleted region (Mohd-Sarip et al. 2006; Papp and Muller 2006), showing high histone replacement, suggesting a continuous disruption of nucleosomes at these sites (Mito et al. 2007). There is therefore little evidence that PRC1 would compact nucleosomes at PREs. It seems more plausible that PRE-tethered PRC1 performs this activity in the flanking chromatin to generate a less accessible chromatin structure, for example, at the promoter or in the coding region of target genes (Muller and Verrijzer 2009). However, any such models will have to accommodate the fact that, in *Drosophila*, PcG proteins have a residence time on chromosomes of only a few minutes (Ficz et al. 2005). Consistent with these observations, the antagonism between SWI/SNF recruitment and PcG repressor proteins seems to be dynamic rather than static (Kia et al. 2008). The results discussed above and other recent studies further corroborated the view that, although K27 methylation is important for the stable binding of PC, it is not sufficient. ChIP experiments detect PcG proteins of both PRC1 and PRC2 complexes, peaking sharply at known or presumptive PREs in *D. melanogaster* (Schwartz and Pirrotta 2007). By contrast, the distribution of trimethylated H3K27 at a silenced gene extends over the entire transcription unit and the upstream regulatory region, frequently involved many tens of kilobases (Kahn et al. 2006; Papp and Muller 2006). In similar experiments in mouse or human cells, PRC1, PRC2 proteins and H3K27 methylation have also been found together at target genes, but in most cases the proteins bind over broader regions than in *D. melanogaster* and their distributions are often co-extensive with the methylation domain (Fig. 6) (Boyer et al. 2006). If PcG proteins do not compact chromatin

and prevent access of the transcriptional machinery to the silenced genes, how do they silence? Experiments with reporter constructs indicate that the effects are likely to involve the higher order structures. The current evidence, much of which is derived from *D. melanogaster*, indicates that the PcG complex at the PRE must contact the promoter complexes to interfere with transcription initiation and perhaps other aspects of transcription. Histone methylation might stabilize the PRE complexes, but might also provide a means for the long-distance interaction between PRE and promoter. Indeed, PcG proteins can mediate specific silencing of genes that are away from PREs in the genome. This phenomena is called "long-range" gene silencing, and these regulating sequences are located many kilobases away from the star site (Fig. 5) (Mateos-Langerak and Cavalli 2008). Recent studies show a connection between the RNA interference (RNAi) pathway and long-distance PcG-mediated silencing (Grimaud et al. 2006a). The RNAi machinery does not affect recruitment of PcG proteins but rather the maintenance of long-distance contacts between PREs. A large pecentage of PRC2 target genes contain CpG-islands; therefore they might undergo DNA methylation as a mean to ensure a stable repressive state (Morey and Helin 2010). Some reports indicate that EZH2 directly interacts with DNA-methyltransferases (DNMTs) and that EZH2 is necessary for maintenance of DNA methylation for at least some promoters (Fig. 8) (Vire et al. 2006; Schlesinger et al. 2007). These results suggest a biochemical and functional link between these two repressive events. However, some genome-wide studies failed to show a significant overlap between DNA methylation and PRC2 binding (Fouse et al. 2008). Therefore, additional studies are needed to understand why some PRC2 target genes are selectively DNA-methylated. A flurry of papers in recent years reported the identification of long-awaited H3K27 demethylases: enzymes capable of specifically removing methyl groups from histone H3 trimethylated and dimethylated at lysine 27 (Agger et al. 2007; De Santa et al. 2007). These results show that even the histone modification associated with PcG silencing, long thought to be a relatively stable mark, may turnover rapidly under certain conditions. Together these results indicate that PcG complex binding is a dynamic process, sensitive to the antagonistic action of trxG complexes as well as to positive or negative input from other transcription factors. It is likely therefore that in each particular case, the functional state of the PcG target will be determined by the equilibrium between all these activities.

1.3 The Language of Histone Crosstalk

Inside an eukaryotic cell nucleus, chromatin is far more than a static carrier of the genetic information encoded in DNA, as it actively mediates dynamic changes in gene function and expression (Felsenfeld and Groudine 2003). The chromatin polymer integrates a variety of endogenous signals and functions as a biological relay station and signalling platform (Cheung et al. 2000). Emerging evidence suggests that covalent post-translational modifications of histones play key roles

in the controlling the capacity of the genome to store, release and inherit biological information (Fischle et al. 2003). Histone proteins, assembled with DNA to form nucleosomes, are the basic building block of chromatin. Not surprisingly, the four core histone molecules, H2A, H2B, H3 and H4, are among the most evolutionarily conserved proteins known (van Holde 1988; Luger et al. 1997) (Fig. 9). Structurally, histones consist of a highly compact globular core of alpha-helixs arranged in helix-turn-helix motifs that promote oligomerisation.

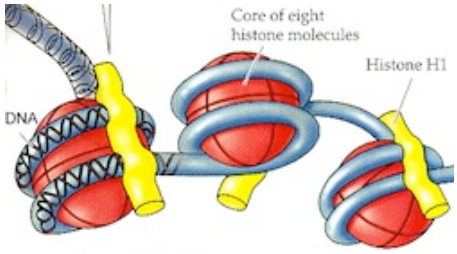

Fig. 9: Histone proteins assembled with DNA to form the nucleosomes. DNA wrapped around histone octamers to form nucleosomes (http://sciblogs.co.nz/code-for-life/2010/08/24/).

In contrast, the amino-terminal regions of histones are unstructured (Reid et al. 2009). These amino-terminal tails of histones are effectively a collection of linear peptide motifs that are subject to multiple post-translational modifications, including methylation of lysines (mono-, di- or tri-), methylation of arginines (mono-, symmetric or asymmetric di-methylation), acetylation of lysine, phosphorylation of serine and threonine residues (Turner 2005; Wang et al. 2007) ubiquitination (Schuettengruber and Cavalli 2010), sumoylation, deimination and proline isomerization (Fig. 10). How do these N-terminal tails participate in the modulation of chromatin architecture? One possible way is that they constitute targets for ATP-dependent chromatin remodelling factors, such as SWI/SNF and NURF (Cheung et al. 2000), that act to alter the position and/or the stability of nucleosomes. Another way is that diverse array of post-translational modifications on the N-terminal tails may modulate the contacts between histones and DNA. Because these modifications are reversible, they can act as chromatin-based "on/off" switches that regulate a multitude of DNA-related processes (Tab. 1). Moreover, since the histone tail domains are highly accessible to the nuclear environment, they constitute attractive targets for signaling-activated enzymes, and may function as important link between signal transduction and gene expression (Cheung et al. 2000). A well-characterized post-translational modification regulating chromatin structure is the acetylation of histone H3 N-terminal tails, which is thought to facilitate transcriptional activation either by charge neutralization of the tails-interaction with DNA or by forming a binding site for bromodomain-containing transcription factors, some of which can remodel nucleosomes (Lee et al. 2010).

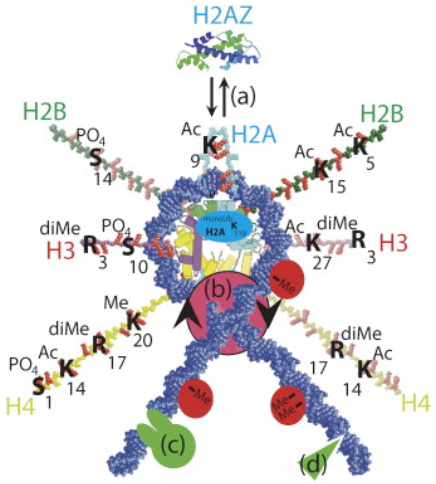

Fig 10: Covalent marks on chromatin. Chromatin consists of repeated units of 146bp of DNA wrapped 1,7 times around an octamer composed of two copies each of the four core histones. H2a, H2b, H3 and H4. Chromatin provides a structural platform that is subjected to extensive post-translational modifications. These include methylation, acetylation, phosphorylation and ubiquitination of specific histone residues, methylation of CpG dinucleotides, exange of histone **(a)**; changes in the position of the nucleosome mediated by ATP-dependent remodelling complexes **(b)**, induction of double-stranded DNA breaks by Topoisimerase II **(c)** and the generation of single-stranded DNA breaks by topoisomerase I **(d)** (Reid et al. 2009).

Chromatin Modifications	Residues Modified	Functions Regulated
Acetylation	K-ac	Transcription, Repair, Replication, Condensation
Methylation (lysines)	K-me1 K-me2 K-me3	Transcription, Repair
Methylation (arginines)	R-me1 R-me2a R-me2s	Transcription
Phosphorylation	S-ph T-ph	Transcription, Repair, Condensation
Ubiquitylation	K-ub	Transcription, Repair
Sumoylation	K-su	Transcription
ADP ribosylation	E-ar	Transcription
Deimination	R > Cit	Transcription
Proline Isomerization	P-cis > P-trans	Transcription

Tab.1: Overview of different classes of modification identified on histones. The functions that have been associated with each modification are shown. Some of these modifications are discussed in detail in the text (Kouzarides 2007).

Fuelled, in part, by discovery of enzymes responsible for bringing about the steady-state balance of this modification-histone acetyltransferases (HATs) and histone deacetyl-ase (HDACs)-compelling evidence has recently been provided that acetylation of specific lysine residues in the amino termini of the core histones plays a fundamental role in transcriptional regulation (Kornberg and

Lorch 1999). In H3 from most species, the main acetylation sites including lysine 9, 14, 18 and 23 (Thorne et al. 1990), and selected lysines become acetylated on H4 histone, during specific cellular processes (Grunstein 1997). Thus, in general, lysine acetylation is related to chromatin accessibility and transcription. Conversely, lysine methylation may have different impact on transcription, depending on the aminoacid position and degree of methylation. Three methylation sites on histones are implicated in activation of transcription: H3K4, H3K36 and H3K79. Two of these, H3K4me and H3K36me, have been implicated in transcriptional elongation. Other three lysine methylation sites are connected to transcriptional repression: H3K9, H3K27 and H4K20. Methylation at H3K9 is implicated in the gene silencing as well as forming silent heterochromatin. Repression involves the recruitment of methylating enzymes and the chromo-protein HP1 (Heterochromatin Protein 1) to the promoter of repressed genes (Kouzarides 2007). Intrestingly, the *dogma* that H3K9 methylation and HP1 recruitment are always repressive has recently been challanged by the finding that H3K9me3 and the isoform gamma of HP1 are enriched in the coding region of active genes (Vakoc et al. 2005). As described above, H3K27 methylation has been implicating in the silencing of HOX gene expression, in the silencing of the inactive X chromosome and during genomic imprinting. This tri-methyl H3 mark is recognized by PRC1 that in turn ubiquitinates histone H2A at K119 (Schuettengruber and Cavalli 2010). Very little is known regarding the repression function of H4K20 methylation. It has a role in the formation of heterochromatin and has a role in DNA repair (Kouzarides 2007). Phosphorylation appears to be a key regulatory modification linked to chromatin biology. The role of this modification will deeply discuss in the next paragraph. With the possibility of multiple histone modifications present at same time on the N-terminal histone tails, what kinds of interplay between them can occur? Could these modifications functionally regulate each other or could they be used in combination to affect the function of nucleosomes and chromatin? The identification of a multitude of histone modifications-some correlated with activation, some with repression- led to proposal that the modifications constitude a code (Strahl and Allis 2000) that could be recognized by transcription factors to determine the transcriptional state of a gene. However, additional research has since added layers of complexity, revealing a nuanced and intriguing language, not only a strict code, as the basis for transcriptional regulation through the chromatin signaling pathway (Fig 11) (Lee et al 2010). The abundance of modifications on the histone tail makes "crosstalk" between modifications very likely. Mechanistically such communications between modifications may occur at several different levels. Firstly, many different type of modification can occur on lysine residues. This undoubtedly results in some forms of antagonism since distinct types of modyfications on lysine are mutually exclusive. Secondly, the binding of a protein could be disrupted by an adjacent modification. An elegant way to circumvent H3K27me3-repressive marks is to mask them from their readers rather then erase them. This can be achieved by a signaling-induced phosphorylation of nearby residues for example (Gehani et al. 2010; Sawarkar and Paro 2010). Thirdly, the catalytic activity of an enzyme could be compromised by

modification of its substrate recognizion site. Fourthly, an enzyme could recognize its substrate more effectively in the context of a second modification (Kouzarides 2007). Thus, the study of regulation of gene expression has grown from identifying transcription factors to include a wide variety of other binding events associated with the modifications of histones that package the DNA.

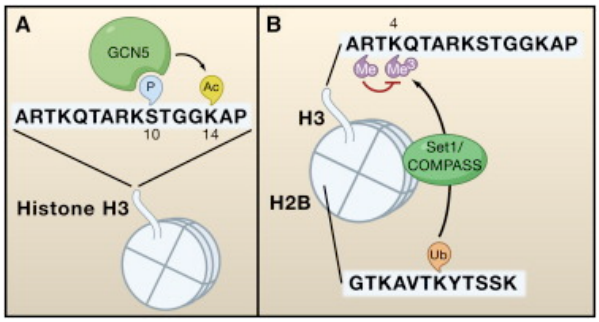

Fig.11: Examples of histone crosstalk. A) The first characterized example of histone crosstalk is the stimulation of acetyltransferase activity of GCN5 toward the histone H3 tail by prior phosphorylation (P) of serine 10. Acetylation, Ac. **B**) Crosstalk among histone modifications can span more than one histone. Monoubiquitination of histone H2B on lysine 120 of the C-terminal helix can lead to the trimethylation of lysine 4 in the histone 3 tail (H3K4) by SET1/COMPASS. However, H3K4 methylation by COMPASS and COMPASS-like complexes can be blocked if the nearby arginine of H3 is already methylated (Lee et al. 2010).

1.3.1 H3 Phosphorylation: Dual Role in Mitosis and Interphase

Phosphorylation of histone H3 was observed more than 30 years ago (Shoemaker and Chalkley 1978) and today there are several fairly well characterized and conserved phospho-H3 residues: Thr3, Ser10, Thr11 and Ser28. All of these phospho-H3 marks are present during mitosis, suggesting a possible role in chromatin condensation. Furthermore, phospho-H3 Ser10, Thr11 and Ser28 are known to be involved in transcriptional activation of specific genes. Therefore, histone H3 phosphorylation is associated with two opposed chromatin states: the highly condensed mitotic chromosomes and the more accessible chromatin structure observed at active genes during interphase (Fig. 12) (Prigent and Dimitrov 2003). This apparent paradox implies that the effect of H3 phosphorylation on chromatin structure might be context dependent and influenced by other histone PTMs. However, the precise role of H3 phosphorylation in epigenetic inheritance is unknown. During mitosis, histone H3 is phosphorylated at Ser10 in all eukariotes, but since this observation the function of this post-translational modification has continually been debated. In mammalian cells, phosphorylation of H3 at Ser10 is first evident in pericentromeric heterochromatin in late G2-interphase cells. Subsequently, the

H3-Ser10 phosphorylation spreads along the chromosomes and is completed at prophase and it is still evident at metaphase plate (Hendzel et al. 1997). On the other hand, dephosphorylation of H3 Ser 10 begins in late anaphase and is completed in early telophase before detectable post-mitotic decondensation of chromosomes (Hendzel et al. 1997). Observed later and less studied than Ser10 phosphorylation, phosphorylation of Ser 28 in the tail of H3 has kinetics very similar to those of Ser10. Both serines lie in the same consensus sequence -ARKS- and both phosphorylations occur early in mitosis when chromosomes begin to condense (Prigent and Dimitrov 2003). Aurora B, the major mitotic Ser10 kinase *in vivo*, was thus a good candidate for the mitotic Ser 28 kinase (Sugiyama et al. 2002). Furthermore, like Ser10 phosphorylation, Ser 28 is sensitive to treatment with PP1 (Goto et al. 2002), the phosphatase that counteracts Aurora B.

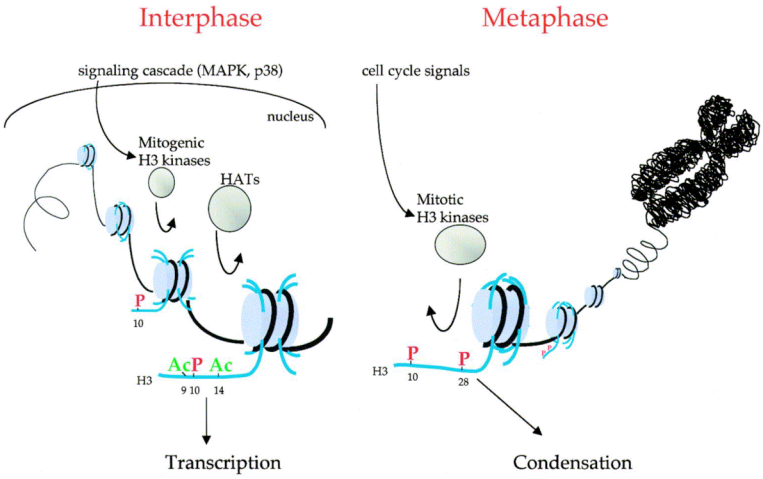

Fig.12: Duality of histone H3 phosphorylation in interphase and metaphase cells. (Left) Schematic representation of the sequential H3 phosphorylation and acetylation events seen upon mitogen (EGF) stimulation. Activation of MAP kinase or p38 pathways results in the activation of mitogenic H3 kinases (Rsk-2 or Msk1) and leads to rapid and transient phosphorylation (red P) of H3 at serine 10. This H3 phosphorylation is coupled to acetylation of nearby residues (green Ac's, acetylation of Lys9 and Lys14 are highlighted, but additional sites may also be involved). Together, these events can facilitate transcription of the immediate-early genes. The blue protrusions represent the N-terminal tails of the core histones. **(Right)** Schematic drawing of a mitotic chromosome with representative nucleosomes from the chromosome-arm region. During mitosis, not yet well-defined cell cycle signals activate mitotic H3 kinase which in turn phosphorylate the N-terminal tails of H3 (red P's on extended blue tails) at Ser10. Ser28 is also phosphorylated during mitosis. These phosphorylation events likely contribute to the chromosome condensation process (Cheung et al. 2000).

At interphase, in contrast to mitosis, the phosphorylation of histone H3 does not affect whole genome but only a subset of genes. Inducible phosphorylation of H3 at Ser10 and Ser28, and the time course of H3 phosphorylation closely followes

the transient expression of activated immediate-early genes, suggesting that this histone modification is linked to transcriptional activation. The best characterized link between signal transduction and histone modification was observed in mammalian cells upon exposure to mitogen or stress. By treating mouse fibroblasts with various growth factors or protein synthesis inhibitors, Mahadevan and colleagues found that H3 is rapidly and transiently phosphorylated, an effect subsequently termed "nucleosomal response" (Mahadevan et al. 1991). Further studies of this nucleosomal response showed that the RAS-mitogen-activated protein kinase (MAPK) signal transduction pathway can induce H3 phosphorylation (Davie et al. 2010). Growth factors (EGF) and phorbol esters (TPA) activate the RAS-MAPK pathway (RAS-RAF-MEK-ERK), while stressors such as UV irradiation stimulate the p38 MAPK pathway. Mitogen- and stress-activated protein kinases (MSK) 1 and MSK2 are activated by RAS-MAPK-ERK1/2 and p38 stress kinase pathways (Fig. 13) (Soloaga et al. 2003). The activity of MSK1 and 2 is regulated by multiple phosphorylation sites (McCoy et al. 2005). Using mouse knockout, MSK1 and MSK2 were shown to be required for the phosphorylation of CREB (cAMP-response-element-binding protein) and ATF1 (activating transcription factor 1) transcription factors (Arthur and Cohen 2000; Wiggin 2002) and the chromatin proteins histone H3 and HMG-14 (high mobility group protein 14) (Thomson et al. 1999), in response to mitogens and stress. By phosphorylation of H3Ser10 and H3Ser28, MSKs regulates the full induction of several immediate early genes, including *c-fos*, *junB*, *mkp-1* (MAPK phosphatase-1) and *nurr1* in response to various stimuli (Arthur et al. 2004), suggesting a direct link between phosphorylation and gene activation. The impact of H3 kinase activity and subsequent H3 Ser10 phosphorylation on gene expression could be explained by recruitment of factors necessary for transcription. MSK1 was shown to modulate phosphorylation of H3 at Ser10 as well as recruitment of transcription factors, chromatin-remodeling enzymes and RNA polymerase II to target genes (Vicent et al. 2006a). Inducible phosphorylation of H3 at Ser28 on the nucleosomes of specific genes with regard to transcriptional activation has not been as well studied as H3 Ser10 phosphorylation. Recently, in G0 phase cells like chicken eritrocytes, the ChIP assay provided direct evidence for H3 Ser28 phosphorylation at the promoter regions of transcriptionally active genes. This H3 phosphorylation event may act as an active mark of gene activation characterized by labile nucleosome structure (Sun et al. 2007). Furthermore, in RAS-transformed mouse fibroblasts, TPA-induced phosphorylation of H3 Ser10 and Ser28 colocalized with transcriptionally active RNA polymerase II isoform (Dunn et al. 2009). In the same cells, it was shown that TPA-induced H3 Ser28 phosphorylation was present at the immediate early *c-jun* promoter, providing further evidence that this H3 modification is associated with transcriptional gene activation (Dunn et al. 2009). Another possible role of phospho-H3 Ser10 and Ser28 in transcriptional activation could involve the 14-3-3 family of proteins, recently proposed as chromatin binding proteins. In mammalian cells, certain 14-3-3 isoforms have been characterized as phospho-H3 Ser10 or Ser28 binding proteins (Macdonald et al. 2005). The binding affinity for phospho-H3 Ser10 was more stable if the Lys14 residue was

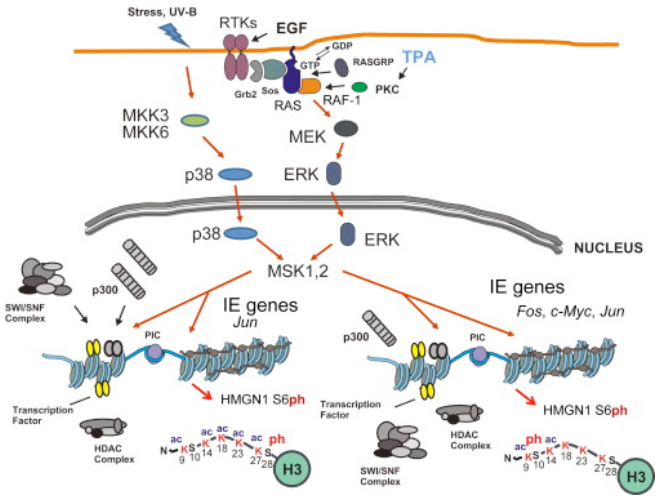

Fig.13: The nucleosomal response downstream from p38 and ERK1/2 MAPK pathways. Activated MSK1/2 phosphorylates HMGN1, which dissociates from nucleosomes and histone H3 on serine 10 or serine 28. Following H3 phosphorylation, chromatin remodelers (SWI/SNF) and KATs (p300) are recruited to the promoter and remodel chromatin in preparation for transcription. Both KATs and HDACs are present at the promoter, resulting in dynamic protein acetylation (Davie et al. 2010).

acetylated (Walter et al. 2008). Furthermore, a specif class of signaling-associated factors called "14-3-3 gamma proteins" are recruited to promoters of *c-jun* and *c-fos* along with ph-Ser10-acetyl-K14-H3 (Macdonald et al. 2005). These observations indicate that 14-3-3 proteins recognize phospho-acetyl H3 modifications and in turn they may mediate transcriptional activation of genes. However, the precise mechanism is unknown. It may be possible that 14-3-3 proteins are recruited within a multiprotein complex to genomic region where 14-3-3 could stabilize the complex at the nucleosomal level by directly binding the pre-existing H3 modifications. In addition, binding of 14-3-3 proteins to phospho-acetylated histone H3 tail could recruit other histone modifying and (or) chromatin-remodeling activities to aid in the initiation of gene activation (Perez-Cadahia et al. 2009). Strikingly, high resolution fluorescence microscopy analyses revealed that the majority of H3Ser10ph foci do not coincide with H3Ser28ph foci, indicating that Ser10ph and Ser28ph do not coexist in the same histone tail or even adjacent nucleosomes (Dunn and Davie 2005). H3 tails with Ser28ph have higher steady state levels of acetylation than that of H3 tails with Ser10ph (Dunn and Davie 2005). Thus, these two phosphorylation events at Ser10 and Ser28 are indipendent and act separately to promote gene expression. The mechanism by which MSKs phosphorylate H3 at either Ser10 or Ser28 is not known. This differential localization of H3 phosphorylation may be mediated by other proteins already bound on the nucleosomes, such as HMGN1 (Lim et al. 2004). Also, recruitment of the kinase by a specific protein or multiprotein

complex might dictate which of the two serine residues will be phosphorylated on H3 tail, which most likely contains preexisting modifications, such as acetylation and (or) methylation (Perez-Cadahia et al. 2009).

1.3.2 A role for H3 Phospho-Switch in Chromatin Regulation

What functional roles could these phospho-marks be involved in at these promoters? Experimental data suggest that phospho-H3 Ser10 and Ser28 might have a role as a part of the binary switch process, as has been proposed during mitosis (Fischle et al. 2005). Differential readout of distinct combinations of marks could, for example, be achieved by "local switching" mechanism such as the "methyl/phospho" switch. Phosphorylation of a site adjacent to (or nearby) a methyl mark that engages an effector module could lead to consecutive loss of binding to that factor. Whereas the binding of certain modules (e.g. bromodomains and chromodomains) to histone marks is well documented, little is known about the release of such factor, especially from sites of modification that are presumed to be more stable (e.g. lysine methylation). It has been shown that in response to cellular stimulation, the recruitment of MSK1 and the levels of H3Ser10ph and H3K14ac increased at a transcriptionally active gene promoter (Vicent et al. 2006b). Interestingly, this increase was paralleled by a reduction in HP1 gamma binding at the promoter, without changes in the levels of H3K9me3. Together, these series of events might suggest that the establishment of phospho-H3 Ser10 could influence gene induction by ejecting HP1 gamma and previously placed repressive complexes at gene promoter (Fig. 14) (Vicent et al. 2006b). Other methyl-mark specific modules have been identified whose binding might be sensitive to neighbouring phospho-marks. The best studied methyl marks in H3 tail (Lys4, Lys9 and Lys27), for example, are all adjacent to novel (Thr3) or previously described (Ser10 and Ser28) phospho-acceptors (Fischle et al. 2003). Recent report showed that H3Ser28ph affects PRC2 binding to H3K27me3 chromatin template (Gehani et al. 2010). This H3K27me3Ser28 phospho switch is functional in response to stress and mitogenic signaling and retinoic acid (RA)-induced neuronal differentiation (Gehani et al. 2010). These data suggest that PcG-repressed genes are dynamically regulated and that MSK recruitment and H3K27me3Ser28 phosphorylation are very important in this respect. Similarly, phosphorylation of histone H1 on Serine 27 (H1Ser27ph) was found to prevent binding of HP1 to the adjacent methylated Lysine 26 (H1K26me) (Daujat et al. 2005), an additional target of the PRC2 complex (Kuzmichev et al. 2004).

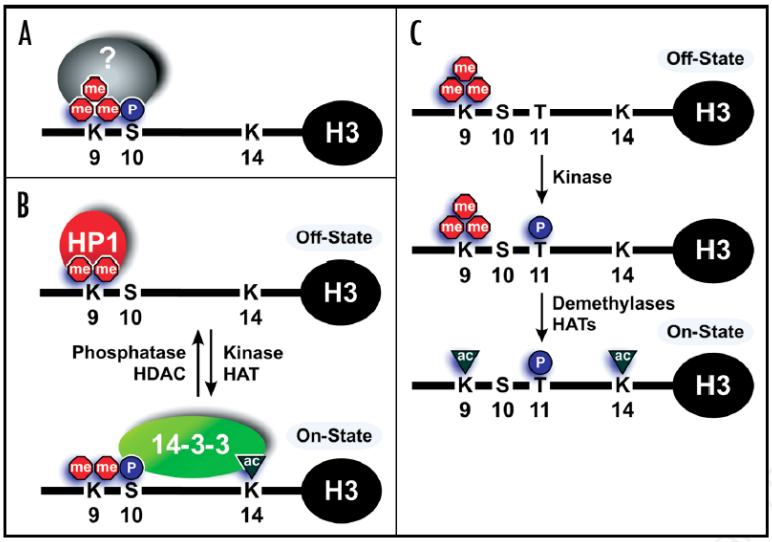

Fig.14: Models for the role of histone H3 phosphorylation in transcriptional regulation during interphase. A) The double H3K9me3/H3Ser10ph modification may generate a novel binding platform for a protein(s) involved in facultative heterochromatin organization and gene silencing. **B)** Phosphorylation of H3Ser10 and acetylation of H3K14 may trigger the displacement of repressive HP1, without removal of H3K9me3, the binding of 14-3-3 proteins and promoter activation. HAT, histone acetyltransferase; HDAC, histone deacetylase. **C)** Phosphorylation of H3T11 may enhance demethylation of H3K9 and histone H3 acetylation, resulting in chromatin changes conducive to transcriptional activation. HATs, histone acetyltransferases (Cerutti and Casas-Mollano 2009).

When considering all four core-histones, the number and placement of putative binary switches, inside and outside of histone tails, is provocative. Lys79 of H3, for example, represents the first known methylation site in the histone fold regions (Lachner et al. 2003). It also lies adjacent to a potential phosphorylation site, Thr80, and recent genetic screens have implied Lys79Thr80 in a genomic "silencing cluster" (Park et al. 2002; Thompson et al. 2003). A similar region is found in H4, H4Lys79/Thr80, suggesting that "methyl/phospho switches" might regulate the critical interface between H3 and H4. The idea that a "methyl/phospho switch" may operate on a critical interface of the H3:H4 dimer is attractive given the importance of this boundary for nucleosome structure and gene regulation (Park et al. 2002). The exercise of identifying all LysSer/Thr or Ser/ThrLys pairs in all major core histones reveals other surprises. All potential switches are located either in the exposed histone tails, at the edges of helical stretches, or in the connecting loops of the histone fold domains. These sites could therefore all be accessible to post-translational modification and function as potential switches (Fischle et al. 2003). Thus, mapping histone modification patterns may help to unravel specific gene networks being dependent on specific signalling pathways regulating gene expression.

1.4 The Genetic Networks Regulating Muscle Differentiation

Skeletal muscle formation in vertebrates constitutes an excellent system to study the signals and the molecular mechanism that govern cellular differentiation. Myogenesis is a multistep process, which begins with the commitment of multipotent mesodermal precursor cells (MPCs) to the muscle fate. These committed cells, the myoblasts, then differentiate and fuse into multi-nucleated myotubes. The final step of muscle differentiation is the maturation of differentiated myotubes into myofibers (Yahi et al. 2006). The specification, proliferation and terminal differentiation of skeletal muscle cells is controlled by the combinatorial activities of several transcription factors. The interactions of these transcription factors with enzymes that modify the structure of the nucleosomes enable temporally regulated formation and recruitment of specific protein complexes at the chromatin of discrete muscle gene loci. Classically, Pax3/7 transcription factors are considered the major regulators of muscle specification and tissue formation during development, playing a similar role during adult muscle regeneration (Fig. 15).

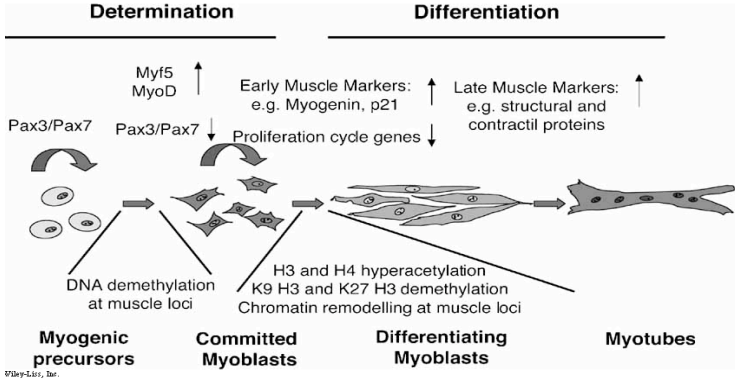

Fig.15: A schematic representation of the sequential stages that underlie skeletal myogenesis. Determination of the myogenic lineage is conferred by the expression of the early myogenic bHLH proteins, MyoD and Myf5, likely due to DNA de-methylation at their regulatory regions. These muscle regulatory factors (MRFs) initiate myogenesis by activating transcription of the muscle genes at previously silent loci, via the combined histone acetylation and demethylation of particular lysines, and the local chromatin remodelling. These events are promoted by the recruitment of chromatin-modyfing enzymatic complexes by sequence-specific myogenic activators (e.g. myogenic bHLH and MEF2 proteins) (Palacios and Puri 2006).

Pax3 gene acts upstream of the myogenic programme and is necessary for MyoD activation in the absence of Myf5 (see below) (Tajbakhsh et al. 1997; Kassar-Duchossoy et al. 2004). Most of the function of *Pax3* can be replaced by its paralogue *Pax-7* (Relaix et al. 2004). Many other regulatory factors involved in these steps are identified, including the early marker *Msx1*, that can inhibit the

myogenesis (Lee et al. 2004), the *Six* family transcription factors (Spitz et al. 1998), the homeobox *Lbx1* (Brohmann et al. 2000) and the proto-oncogene *c-met*, all regulating the migration of the MP cells from the lateral dermomyotome. During migration, the MPCs express all these specification factors, but do not yet express the determination factors, such as MyoD and Myf5. Interestingly, after birth, myogenic progenitors adopt a satellite position outside the myofiber (under the basal lamina), entering a quiescente state (Buckingham 2006). Upon environmental activating signals derived from injury or stress, satellite cells undergo apical-basal asymmetric cell divisions to both maintain their population through self-renewal and to give rise to committed myogenic cells; the latter cells, the myoblasts, proliferate, migrate, differentiate and fuse into new fibers (Buckingham 2006; Kuang et al. 2008), thereby sustaining post-natal muscle repair and growth. Muscle differentiation is controlled also by a family of muscle specific basic helix-loop-helix (bHLH) transcription factors called Muscle Regulatory Factors (MRFs) that cooperate with members of the MEF2 family of proteins (MEF2A-C) in the activation of muscle genes from previously silent loci (Fig. 15) (Molkentin et al. 1995; Puri and Sartorelli 2000). The myogenic bHLH family comprises MyoD, Myf5, myogenin (MyoG) and MRF4. Targeted gene disruption of any of these four bHLH family members in mice has shown that they play central roles in myogenesis (Yun and Wold 1996). Progenitor cells remain multi-potent in the absence of these factors, they do not locate correctly to sites of myogenesis and adopt other cell fates (Tajbakhsh et al. 1996). Moreover, in the absence of MyoD and Myf5, myogenin and MRF4 are not observed (Rudnicki et al. 1993; Kablar et al. 2003). On the contrary, it has been shown that mice lacking myogenin or MRF4 still develop skeletal muscle, although double knockout mice totally lack skeletal muscle fibers and myoblasts (Rudnicki et al. 1993; Kablar et al. 2003). MyoD is the pivotal player in myogenic differention. MyoD positively auto-regulates its own expression, and activates the "early" transcription factor myogenin. Myogenin is expressed in non- proliferating myoblasts as they enter the differentiation pathway. Myogenin controls later steps of muscle cell differentiation and triggers differentiation with activation of muscle specific genes, such as muscle creatine kinase (*mCK*) and myosin heavy chain (*MHC*). MyoD and Myf5 are expressed in undifferentiated myoblasts yet, in this cellular context, they do not activate transcription. Once cellular cues are interpreted by the undifferentiated myoblasts as pro-differentiation signals, MyoD and Myf5 become transcriptionally competent and activate the skeletal myogenic program. It appears that multiple and distinct mechanisms ensure that transcription is not prematurely activated in undifferentiated muscle cells (Sartorelli and Caretti 2005). The presence of bHLH proteins MyoD and/or Myf5 in the nucleus of myoblasts poses a fundamental question: how do MyoD and Myf5 confer and maintain the myogenic identity to proliferating muscle precursors, without activating the differentiation program? Each of the MRF members has been shown to heterodimerise *in vitro* and *in vivo* with *quasi*-ubiquitous E proteins that are the alternatively spliced products of the *E2A* gene: E12 and E47 (Murre et al. 1989; Chakraborty et al. 1991). Basically, dimers formed from bHLH proteins differ in their abilities to bind to DNA. For example, MyoD-E47 heterodimers forms

efficiently and bind strongly to DNA, whereas MyoD homodimers only poorly bind DNA. A simplistic, former model of inactivation of myogenic bHLH proteins in myoblasts relies on serum-induced expression of the anti-myogenic Id proteins, which sequestrate the heterodimeric partners of MyoD, E12 and E47 (Lassar et al. 1991). This model assumes that in myoblasts MyoD is unable to bind its DNA recognition sequences-the E box sites- on the regulatory regions of muscle genes. However, early studies could not address two critical issues relative to DNA binding activity of MyoD in myoblasts: whether MyoD-MyoD homodimers can transiently recruited to the DNA during myoblasts proliferation, and whether this transient interaction is restricted to the promoter/enhancer elements of muscle-specific loci, or might extend to other genes. Chromatin immunoprecipitation (ChIP) coupled with mouse promoter DNA microarray hybridization (ChIP-chip) has enabled the identification of approximately 200 genes bound by MyoD and/or myogenin and MEF2 (Blais et al. 2005; Cao et al. 2006). Of its approximately 100 target genes, MyoD is bound to half in undifferentiated myoblasts and the other half in differentiated myotubes. Several targets bound by MyoD were activated neither in myoblasts neither in myotubes. Others were activated only in either myoblasts or myotubes. This profiling of global MyoD-binding sites by ChIP-chip technology has been confirmed by recent studies, using ChIP-seq approach (Cao et al. 2010). The prior ChIP-chip studies only surveyed the promoter regions of the annotated genes, whereas the ChIP-seq technology allowed us to survey the entire genome, showing that the ~ 24% of the MyoD peaks are located in the promoter-proximal regions. This may be significant because a recent work has shown that the chromatin state at promoters is largely shared by different tissues, whereas tissue-specific gene expression is highly correlated with the chromatin state of the enhancers located outside of the promoter region (Heintzman et al. 2009). The major finding of this study was the genome-wide MyoD binding associated with histone acetylation in both myoblasts and myotubes (Cao et al. 2010). Dynamic changes in nuclear organization are necessary for genes to be repositioned during differentiation (Hu et al. 2008; Schoenfelder et al. 2010), and progenitor cell commitment requires a transition between two different ordered nuclear states (Rajapakse et al. 2009). Although speculative, the genome-wide binding of MyoD and associated histone acetylation may initiate new patterns of gene looping and chromosome architecture necessary for the transition between nuclear states (Cao et al. 2010).

1.4.1 The Epigenetic Networks Regulating Muscle Differentiation

Muscle cell differentiation provided a highly informative system to study the interaction between signalling pathways, transcription factors and chromatin regulation. In proliferating myoblasts, the ability of the MRFs to activate the differentiation is countered by their association at muscle regulatory regions with histone deacetylases (HDACs) and co-repressor complexes, including YY1 and

PcG proteins, which preclude premature muscle-gene expression by promoting histone modifications (Lu et al. 2000a; Lu et al. 2000b; Puri et al. 2001; Zhang et al. 2002; Caretti et al. 2004). MyoD and MEF2 interact with HDACs, resulting in inhibition of myogenesis (McKinsey et al. 2001). The completion of the human genome project has revealed at least 17 HDACs, which fall into three classes: I, II and III. MyoD associates with the class I HDAC, HDACI, which repress the transcriptional activity of this bHLH transcription factor. HDACI represses MyoD-dependent transcription in part by deacetylating this transcription factor (Mal et al. 2001). In contrast, MEF2 does not interact with HDACI, but it is directly coupled to class II HDACs (HDAC-4, -5, -7 and -9) (McKinsey et al. 2002). Class I and II HDACs are expressed in undifferentiated myoblasts, providing a potential explanation for the inability of MyoD and MEF2 to activate certain genes in these cells. As described below, muscle cells possess signaling mechanisms that function to inactivate HDACs associated with MyoD and MEF2, thereby freeing the transcription factors to interact with HATs and stimulate genes that promote myogenesis. The silent information regulator 2, Sir2, belongs to the HDAC III family of enzymes. Its activity is stimulated by the cofactor nicotinamide adenine dinucleotide (NAD^+) and repressed by nicotinamide (NAM) (Bitterman et al. 2002). In cell extracts derived from undifferentited myoblasts, Sir2 is found in a protein complex containing the acetyltransferase pCAF (p300/CBP-associated factor) and MyoD. The ability of Sir2 to deacetylate H3K9 and H3K14 correlates with repression of muscle gene expression and cell differentiation (Fulco et al. 2003). A key inhibitory complex found at muscle regulatory regions contains the PcG group protein EZH2 that promotes transcriptional repression. Overexpression of EZH2 in either estabilished or primary skeletal muscle cells contrasts muscle gene expression and cell differentiation, a phenomenon that is dependent on the HKMT activity of EZH2 (Caretti et al. 2004). A protein complex comprising the transcription regulator YY1, EZH2 and HDACI can be detected on the chromatin of selected muscle gene regulatory regions when their correspondent genes are inactive and lysine 27 of histone H3 is hypermethylated. Transcriptional activation that accompanies skeletal muscle differentiation is characterized by loss of YY1, EZH2 and HDACI, and the recruitment of the transcriptional activators MyoD and SRF (Serum Response Factor) (Fig. 16). Chromatin engagement of EZH2 relies on YY1, because reducing the levels of YY1 by RNA interference results in a lack of EZH2 recruitment. Intriguingly, PRC4 contains both EZH2 and SIRT1 (Kuzmichev et al. 2005), and Sir2 is required for PcG-mediated silencing (Furuyama et al. 2004). These observations suggest the possibility that SIRT1 might be recruited on the chromatin by both MyoD-dependent (mediated by MyoD-pCAF-SIRT1 complex) and MyoD-independent (YY1-EZH2-SIRT1) pathways, and that Sir2 and EZH2 might cooperate in repressing muscle gene expression. The Ezh2 complex might not regulate expression of every muscle-specific gene. By ChIP analysis, Caretti et al. (2004) showed that EZH2 is associated with the regulatory regions of *MHCIIb* and *mCK* derived from undifferentiated myoblasts but cannot be detected on the chromatin of differentiated myotubes.

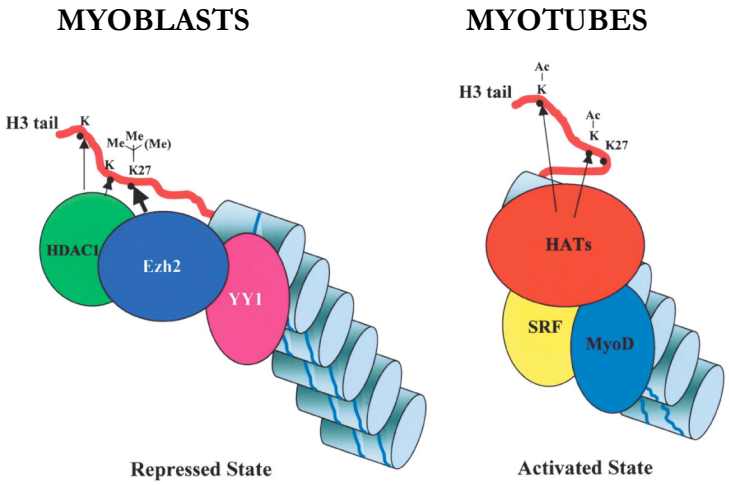

Fig.16: Two-step activation model of muscle gene expression. Regulatory regions of certain muscle-specific genes are occupied by a protein complex containing the DNA-binding protein YY1, the methyltransferase Ezh2, and the deacetylase HDAC1. Deacetylation of lysine residues by HDAC1 and di-/trimethylation of H3K27 by Ezh2 actively prevent transcription (repressed state). At the onset of transcriptional activation, YY1 is displaced from the chromatin—with consequent loss of Ezh2 and HDAC1—and replaced by SRF. H3K27 becomes hypomethylated, and loading of the MyoD family of transcription factors allows engagement of histone acetyltransferases (HATs) and permits initiation of transcription (activated state) (Caretti et al. 2004).

Recently, it was shown that also myogenin promoter is controlled by EZH2 in undifferentiated myoblasts (Juan et al. 2009). However, the role of all other PcG components and in particular PRC2 remains to be elucidated. It is interesting to note that in myoblasts lysine 9 methylation and class II HDACs were detected on myogenin promoter (Zhang et al. 2002), but not on *mCK* and *MHCIIb* promoter/enhancer sequences (Caretti et al. 2004). MyoD-dependent activation requires the SWI/SNF ATP-dependent chromatin remodeling complex through an interaction that can be regulated by the p38 MAPK pathway (de la Serna et al. 2001; Simone et al. 2004). At myogenic loci, the p38 kinase phosphorylates the SWI/SNF subunit BAF60 (Simone et al. 2004). Moreover, the forced activation of the p38 pathway promotes SWI/SNF recruitment, facilitates MyoD and MEF2 binding, leads to the recruitment of RNA polymerase II and anticipates expression of late muscle markers at early stages of differentiation (Penn et al. 2004). Inhibition of SWI/SNF activity, as well as inhibition of HAT activity, prevents the ability of MyoD to initiate transcription and chromatin remodeling at specific loci (de la Serna et al. 2001; Polesskaya et al. 2001). Indipendent studies have unambiguously demonstrated that p38 MAPK signaling pathway is a crucial regulator of skeletal muscle differentiation. Treatment with the p38 alpha and beta

inhibitor SB203580 prevented the fusion of myoblasts into myotubes, as well as the induction of muscle-specific genes (Cuenda and Cohen 1999; Li et al. 2000).
Thus, understanding the regulation of myogenesis and, in particular, how muscle stem cells change their behavior during this process represents a major aim in the skeletal muscle research area. Epigenetic studies that define the role of chromatin remodeling and modifying complexes in specifying developmental or differentiation programs, and in regulating the capacity of adult muscle to undergo efficient regeneration, will be of great interest. Recent studies performed in mouse models of muscular dystrophy have reported on the therapeutic potential of pharmacological interventions that target events downstream to genetic defect responsible for the disease. Deacetylase inhibitor treatment conferred on dystrophic muscle resistance to contraction-coupled degeneration and alleviated both morphological and functional consequences of the primary genetic defect (Minetti et al. 2006). These results provide a rationale for using deacetylase inhibitors in the pharmacological therapy of muscle dystrophies. With so many unresolved issues, the coming years of epigenetic research in myogenesis promise to be exciting.

2. AIM OF THE STUDY

It has been reported that EZH2 expression is developmentally regulated during myogenesis and its down-regulation coincides with activation of muscle gene expression and differentiation of myoblasts into myotubes (Caretti et al. 2004). However, the role of the other PRC2 core components in skeletal muscle differentiation has not been investigated. Since in mammals two E(z)-related genes have been isolated, EZH1 and EZH2 (Laible et al. 1997) and the existence of two partially redundant PRC2 complexes (PRC2-EZH2 and PRC2-EZH1) has previously been reported (Margueron et al. 2008; Shen et al. 2008; Ezhkova et al. 2009), we set out to analyze the role of these two complexes in controlling muscle gene program activation. The second step of our work was to identify the signaling pathway regulating PRC2 dynamics onto chromatin during the transition from myoblasts to myotubes. MSK1 and MSK2 are serine/threonine kinases known to phosphorylate histone H3 at serine 10 and serine 28 residues (Soloaga et al. 2003) that act downstream of p38 and ERK pathways (Deak et al. 1998). In particular, putative lysine "switch" sites followed by phospho-acceptors (-ARKS-), such as Lys9/Ser10 and Lys27/Ser28 in histone H3 were predicted to regulate the activity of silencing complexes (Fischle et al. 2003). Indeed, methylated lysines are bound by repressive proteins, which can be displaced by phosphorylation of the adjacent serine residues. In particular, phosphorylation of histone H3 at Ser10 was shown to inhibit methylation of the adjacent Lys9 by SUV39H1 (Rea et al. 2000) and subsequently to reduce HP1 binding to H3K9me3 (Fischle et al. 2003; Fischle et al. 2005). Since MSK1 is known to phosphorylate H3Ser28 *in vitro* and this histone mark is associated with gene activation (Dyson et al. 2005; Kim et al. 2008), we hypothesized that MSK1-mediated H3Ser28ph could regulate PRC2 chromatin dissociation at muscle specific genes, allowing gene activation and muscle differentiation.

On the basis of these observations, the work will be divided in two sections. In the first section, Section A, we'll analyze the role of the PRC2 components during skeletal muscle differentiation. In the second section, Section B, we'll investigate the signaling mechanisms that regulate the PRC2 chromatin association during the switch from myoblasts to myotubes.

3. MATERIALS AND METHODS

Cell Culture and Reagents

C2C12 mouse myoblasts cells (ATCC) were cultured in Dulbecco Modified Eagel's Medium (DMEM) supplemented with penicillin/streptomycin and 10% fetal bovine serum (Euroclone). Differentiation was induced when cells reached about ~80% confluency using DMEM containing ITS media supplement (Sigma). LY294002 was obtained from Sigma while H89 was from Alexis Corporation. The inhibitors were replaced freshly every 24h.

RNA Isolation and Quantitative Real-Time PCR

RNA was extracted from cells using TriReagent (Sigma) according to manufacturer's insctructions. cDNA synthesis was performed using the QuantiTec reverse transcription kit (Qiagen). Quantitative real-time PCR reactions were performed in triplicate using QuantiTect SYBR Green master mix (Qiagen) on a DNA Engine Opticon 2 machine (MJ Research) controlled by Opticon Monitor 2 software. C(T) values were calculating by Opticon Monitor 2 software, relative C(T) values were normalized to the housekeeping gene *GAPDH*. Fold enrichment refers to the enrichment relative to myoblasts values. *GAPDH* and *mCK* primers were already described (Caretti et al. 2004). Remaining primer sequences are the following:

MyoG-for: 5′-GCAGCGCCATCCAGTTTG-3′
MyoG-rev: 5′-GCAACAGACATATCACCG-3′
SUZ12-for: 5′-GTGCCTTTGAATCCTCTC-3′
SUZ12-rev: 5′-CTTCGCGATTTCGTTTTTC-3′
EZH1-for: 5′-AGACTCAGTGCAATACCAAGCA-3′
EZH1-rev: 5′-AGTTTCACCACCTT-3′
EZH2-for: 5′-TGATAAAGAAACTTGCCCACCT-3′
EZH2-rev: 5′-CTTTGCTCCCTCTGAACATT-3′
EED-for: 5′-ATAACCAGCCATTGTTTGGAGT-3′
EED-rev: 5′-TGTTGCTATCATAGGTCCATGC-3′
MSK1-for: 5′-GCCGATGAAACTGAAAGAGC-3′
MSK1-rev: 5′-TGCTCATTTCCTGGGGATAC-3′
MSK2-for: 5′-TCACACTGCACTACGCCTTC-3′
MSK2-rev: 5′-GATACCCAGCTTGTGCAGGT-3′

RNA Interference

siRNA EZH1#1 (SI00997766), siRNA EZH1#2 (SI00997773), siRNA SUZ12#1 (SI01438416), siRNA SUZ12#2 (SI01438402), siRNA MSK1 #1 (SI01407483), siRNA MSK1 #2 (SI01407504) as well as negative control siRNA (scrambled sequence not targeting mouse genome, 1027313) were purchased from Qiagen and used at the final concentration of 20 nM. Remaining siRNA sequences are the following:

siRNA Ezh2 #1: AAGGAAAGAACTGAAACTTA
siRNA Ezh2 #2: AAGCTGAAGCCTCCATGTTTA

Cells were transfected with HiPerfect (Qiagen) following the manufacturer's instructions. 48h after transfection the cells were induced to differentiate and collected at the indicated time-points.

Cell Lysis and Immunoblot

Cells were harvested and washed twice with PBS. Cell lysis of total cell extracts was performed on ice in 50 mM Tris–HCl pH 8, 125 mM NaCl, 1% NP-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitory cocktail (Roche) for 25 min. Insoluble material was pelleted by centrifugation at 16000 g for 3 min at 4°C. Histone extracts were prepared following Abcam protocol (www.abcam.com). Protein concentration was determined using the Bradford assay (Bio-Rad). The proteins were denatured, reduced, separated by SDS–PAGE and transferred to nitrocellulose transfer membrane (PROTRAN-Whatman, Schleicher & Schuell). The membranes were blocked with 5% non-fat dry milk in TBST for 60 min, incubated with primary antibodies overnight at 4°C, washed three times with TBST for 10 min, incubated with the peroxidase-conjugated secondary antibody in TBST with 2.5% non-fat dry milk for 60 min and washed three times with TBST for 10 min. Immunoreactive proteins were detected using Supersignal West Dura HRP Detection Kit (Pierce).
For cytoplasmatic and nuclear extracts preparation the cells were resuspended first in Buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA and 0.1 mM EGTA) supplemented with protease inhibitory cocktail (Roche), 1 mM dithiothreitol (DTT) and 1mM PMSF. After incubation on ice for 10 min, NP-40 was added to a final concentration of 0.5% and the samples were vortexed for 5 s. Nuclei were pelleted at 13200 rpm for 10 s and the cytoplasmatic proteins were collected. The pellet was then washed five times with Buffer A and resuspended in Buffer C (20 mM Hepes pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, protease inhibitory cocktail (Roche) and 1 mM PMSF). After 10 min on ice, the samples were sonicated, centrifuged at 13200 rpm for 10 min and the nuclear proteins were transferred to a fresh vial. For immunoprecipitation studies, nuclear extracts were first precleared using protein A/G beads (Santa Cruz) for 30

min at 4°C and then immunoprecipiated with EZH2 or SUZ12 antibody for 2h at 4°C. Normal rabbit IgGs (SantaCruz) were used as a negative control.

Chromatin Binding Assay

The method of Llano *et al.* (Llano et al. 2006) was used. At the indicated times, cells were divided equally in 2 tubes that were lysed for 15 min on ice in cold CSK I buffer (10 mM Pipes pH 6.8, 100 mM NaCl, 1 mM EDTA, 300 mM sucrose, 1 mM $MgCl_2$, 1 mM DTT) supplemented with 0.5% (v/v) Triton X-100, protease inhibitory cocktail (Roche) and 1 mM PMSF. After centrifugation at 500g at 4°C for 3 min, the first tube was divided in two fractions: the supernatant that contains Triton-soluble proteins and the pellet (Triton insoluble fraction) that contains chromatin-bound, nuclear matrix-bound and insoluble proteins. Triton insoluble fraction was resuspended in RIPA buffer (150 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5% DOC, 0.1% (w/v) SDS, 1% (v/v) NP-40). The second tube was resuspended in CSK II buffer (10 mM Pipes pH 6.8, 50 mM NaCl, 300 mM sucrose, 6 mM $MgCl_2$, 1 mM DTT), treated with DNase (Promega) for 30 min followed by extraction with 250 mM $(NH_4)_2SO_4$ for 10 min at 25 °C. The sample treated with DNase and salt was centrifuged at 1200g for 6 min at 4°C and the supernatant represents the DNase-released chromatin-associated proteins. All fractions were analyzed by immunoblotting where equal amount of proteins were loaded (15μg).

Antibodies

Histone H3 (1791), H3K4me3 (8580), RNA Pol II (4H8) and SUZ12 (12073) were from Abcam while EZH2 (3147), p38 (9228), phospho-p38$^{Thr180/Tyr182}$ (9211), phosho-Ser^{376} MSK1 (9591) were from Cell Signaling, H3K27me3 (07-449), H3S28ph (07-145), H3S10ph (05-817) and Acetyl H3 (06-599) were purchased from Upstate. MSK1 (9392, 25417), MyoG (12732), p21 (6346), MHCIIB (2064) and SUZ12 (46264) were from Santa Cruz. β–tubulin (T0198) was from Sigma. mCK antibody was kindly provided by Hidenori Ito. EZH1 antibody was kindly provided by R. Margueron.

Immunofluorescence Assay

Cells were grown on coverslips, washed in PBS, fixed in 3.7% formaldehyde/PBS (15 min, 4°C) and permeabilizaed in 0.2% Triton X-100/PBS (5 min, 4°C). The coverslips were then washed in PBS, and blocked with 3% low-fat milk/PBS for 1h at room temperature. Following overnight incubation with primary antibodies at 4°C, the coverslips were washed and incubated with secondary antibodies (Molecular Probes) for 60 minutes at 37°C, washed again

and counterstained with DAPI (1 g/l in Vectashield, Vector). Pictures were captured using epifluorescence microscope (Leica DM6000B) using Leica Application Suite software.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed as previously described (Breiling and Orlando doi:10.1101/pdb.prot4560, with adaptations) using a a crosslinking time of 10 min. Antibodies were incubated using Dynal magnetic beads overnight at 4°C. The day after, chromatin was added to Ab-beads complexes and incubated overnight at 4°C. The complexes were washed twice in Low Salt Solution, twice in High Salt Solution, once in LiCl, and once in TE buffer. DNA was extracted from beads by standard phenol/chloroform extraction, precipitated, and resuspended in 30 µl TE. To quantify the results, qPCR reactions were performed in triplicate (precipitated DNA samples as well as serially diluted input DNA) using QuantiTect SYBR Green master mix (Qiagen) on DNA Engine Opticon 2 machine (MJ Research) controlled by Opticon Monitor 2 software. C(T) values were calculating by Opticon Monitor 2 sotware. We calculated relative enrichment: the signal from the control immunoprecipiation experiment (Mock) was subtracted from that observed with the antibody of interest. Myoblasts values (GM) were set as 1 and values from differentiated cells in DM with or without inhibitor displays relative enrichment or reduction to those observed in GM. In case of ChIP with antibodies recognizing histone modifications, % of input was calculated first, and then a ratio of H3modified/H3total was calculated and set to 1 to myoblasts in GM. Values from DM show relative enrichment/reduction to those observed in GM. For CMA316 ChIP, data were presented as % of input.
Primers for ChIP Real Time-PCR:

MyoG promoter-for: 5′-CCGTCCGTCCAAGACAACCC-3′
MyoG promoter-rev: 5′-CCCCCCTCTAAGCTGTTGC-3′
MyoG A-for: 5′-CTGTCCACCTTCAGGGCTTC-3′
MyoG A-rev: 5′-CCTCGAAGGCCTCATTCACT-3′
MyoG B-for: 5′-GTGGCCTTCTGGGCTCATAC-3′
MyoG B-rev: 5′-TTTGTTACGGTGGCTCCTGA-3′
MyoG C-for: 5′-AAGTGGGGCTGTCCTGATGT-3′
MyoG C-rev: 5′-TAACAAGGGGGCTCTCTGGA -3′
mCK promoter-for: 5′-CGCCAGCTAGACTCAGCACT-3′
mCK promoter-rev: 5′-CCCTGCGAGCAGATGAGCTT-3′
mCK enhancer-for: 5′-GACACCCGAGATGCCTGGTT-3′
mCK enhancer-rev: 5′-GATCCACCAGGGACAGGGTT-3′

Size Exclusion Chromatography

Size exclusion chromatography was performed using C2C12 cell nuclear extracts on a Superose 6 PC 3.2/30 gel filtration column (GE Healthcare) on an AKTA purifier system (GE Healthcare) in IP (300) buffer (50 mM Tris-HCl at pH 7.5, 300 mM NaCl, 5% glycerol, 0.2% Igepal [Sigma], Aprotein, Leupeptin, 100 mM PMSF, 1 mM DTT). Immuno-depletion was performed as described (Villa et al. 2007). Briefly, protein extracts were subjected to five serial depletions within 24h at 4°C using the AC22 EZH2 monoclonal antibody (Bracken et al. 2003) pre-coupled to Protein-A beads.

Histone Tail Peptides

Histone H3 peptides were synthesized in unmodified and modified form using Fmoc (N-(9-fluorenyl)methoxycarbonyl)-based solid-phase synthesis. Peptides used for kinase assays corresponded to amino acids 21-33 of H3 containing an artificial Y at the C-terminus:

H3 unmodified: ATKAARKSAPATGY
H3K27me3: ATKAARK(me3)SAPATGY
H3S28ph: ATKAARKS(ph)APATGY

Peptides used for pulldown experiments corresponded to amino acids 1-40 of H3 and contained a C-terminal non-native YCK sequence with the lysine biotinylated at the ε-amino group:

H3unmodified: ARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHR-YCK(biotin)
H3K27me3S28ph: ARTKQTARKSTGGKAPRKQLATKAARK(me3)S(ph)APATGGVKKPHR-YCK (biotin)
H3K27me3: ARTKQTARKSTGGKAPRKQLATKAARK(me3)SAPATGGVKKPHR-YCK (biotin)

In Vitro Peptide Kinase Assay

Recombinant MSK1 (Millipore) was used to phosphorylate H3 histone tail peptides (21-33). Kinase assays were performed according to the manufacture's protocol incubating 10 ng MSK1 with 1 μg peptide for 30 min at 30 °C. The reaction was stopped by adding 0.5 % phosphoric acid, spotted on P81 paper, washed three times with 0.5 % phosphoric acid and once with acetone. Filter circles were air-dried and counted in a scintillation counter.

Peptide Pulldown

For preparation of nuclear extracts, cells were lysed in buffer A (10 mM Hepes-KOH pH 7.8, 60 mM KCl, 1 mM EDTA, 1 mM DTT, protease inhibitor cocktail (Roche), 0.075 % NP-40). After incubation on ice for 15 min nuclei were pelleted and washed once with buffer A without NP-40. The nuclear pellet was suspended in buffer B (20 mM Tris pH 8.0, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25 % glycerol, 1 mM DTT, protease inhibitor cocktail, PhosSTOP (Roche)) and sonicated on ice in a Branson Sonifier (duty cycle 20 %, output 7.5). Extract was left on ice for 30 min before centrifugation for 15 min at 16,000 g. The supernatant was supplemented with 0.1% Triton X-100 and used for pulldown experiments.
For H3 peptide pulldown experiments, 10 μg of biotinylated histone peptides (1-40) were coupled to 50 μl streptavidin-coated paramagnetic beads in PBS/BSA (1 mg/ml) for 4 h at 4 °C. Beads were washed three times with PD150 (10 mM Tris pH 7.5, 150 mM NaCl, 0.1 % Triton X-100, 20 % glycerol, 1 mM DTT, protease inhibitor cocktail, PhosSTOP) to remove unbound peptides. Peptide-bound beads were incubated with nuclear extract for 2 h and washed four times with PD300. Bound proteins were eluted with SDS sample buffer, separated by SDS-PAGE and analyzed by immunoblotting.

Generation of H3S28ph-Specific Antibody

Mice were immunized with synthetic peptides KQLATKAARK(phospho-S)APATGGVKC and KQLATKAAR(me3-K)(phospho-S)APATGGVKC for CMA316 antibody and hybridomas were screened by ELISA using peptides listed in Kimura et al. (Kimura et al. 2008). For ELISA, microtiter plates were coated with the individual peptides conjugated with bovine serum albumin and incubated with 3-fold dilutions of each antibody. After washing with PBS, plates were incubated with peroxidase-conjugated anti-mouse IgG and washed with PBS. The colorimetric signal of tetramethylbenzidine was detected by measuring the absorbance at 405 nm using a plate reader. CMA316 was isotyped as IgG2a-k using a kit (Serotec; MMT-1). IgG was purified using a Protein A column (GE Healthcare). For immunoblotting, HeLa cells were transfected with H3-GFP

(Kimura and Cook 2001) and the amino acid substituted mutants using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. After 2 days, 100ng/ml nocodazole was added and further incubated for 6 hr. After washing three times with PBS, cells were lysed using 2x SDS-gel loading buffer, and total protein was separated on 13% SDS-polyacryamide gels, transferred on to PVDF membranes, and blotted with CMA316 (1:10 dilution of hybridomas culture supernatant), as previously described (Kimura et al. 2008).

4. RESULTS AND DISCUSSION

4.1 Two PRC2 complexes, PRC2-EZH2 and PRC2-EZH1, are present during myogenic differentiation and are differentially associated with muscle gene regulatory regions

PcG proteins are transcriptional repressor that modify chromatin through epigenetic modifications that prevents changes in cell identity by maintaining transcription patterns, throughout development and in adulthood (Schuettengruber and Cavalli 2009; Simon and Kingston 2009). Skeletal muscle formation in vertebrates constitutes an excellent system to study the signals and the molecular mechanism that govern muscle differentiation. Since PcG proteins play a key role during muscle specific gene regulation (Caretti et al. 2004; Juan et al. 2009; Seenundun et al. 2010), we set out to investigate the dynamics of the PRC2 core components (SUZ12, EZH2, EED) and EZH1 during the transition from proliferative myoblasts to differentiated myotubes. C2C12 cells were used as *in vitro* model of skeletal muscle differentiation. In this cellular system exchange of growth medium (GM) with differentiation medium (DM) induces myoblasts to exit the cell cycle, to express muscle-specific genes and subsequently to fuse into multi-nucleated myotubes (Fig. 17 A) (Buckingham 1996).

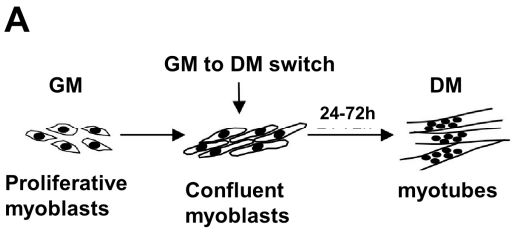

Fig.17: **PRC2 switches composition at muscle regulatory regions during differentiation. A)** Graphical representation of dynamics of C2C12 muscle cell line differentiation. Proliferative myoblasts at 80% confluency were induced to differentiate for 24-72h by replacing the growth (GM) with differentiation medium (DM).

Ezh2 protein levels are known to decline during skeletal muscle differentiation (Fig. 17 B) (Caretti et al. 2004). While EZH2, SUZ12 and EED protein levels declined during this process, EZH1 protein levels stayed steady throughout different phases of differentiation (Fig. 17 B). The same trend was observed analyzing PRC2 mRNA levels (Fig 17 C). Interestingly, also global levels of H3K27me3 followed EZH2, SUZ12 and EED dynamics (Fig. 17 B).

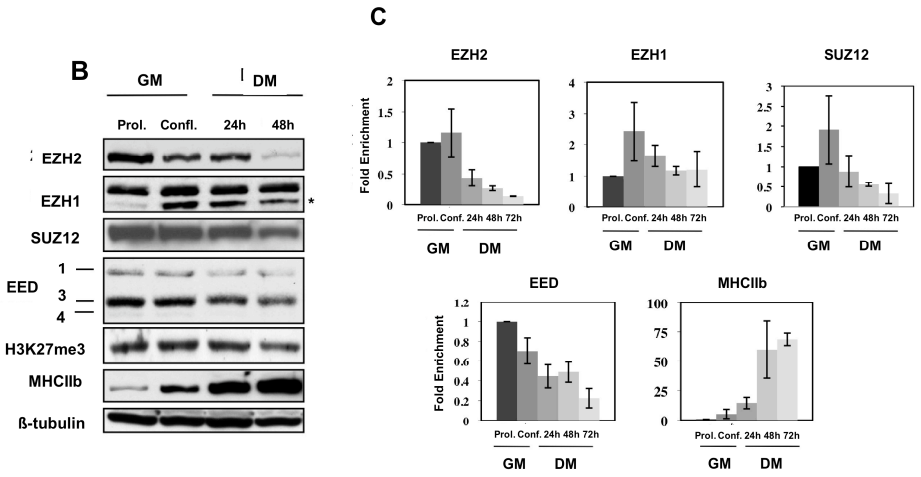

Fig.17: **B)** Immunoblot of EZH2, EZH1, SUZ12, EED, H3K27me3 and MHCIIb from whole cell extracts of C2C12 cells cultured as myoblasts in GM or as myotubes in DM. β-tubulin was used as a loading control. Asterisk indicates EZH1 unspecific band. Numbers represent EED isoforms. MHCIIb was used as a skeletal muscle differentiation control. **C)** Expression levels of EZH2, EZH1, SUZ12, EED and MHCIIb was measured by Real Time PCR in C2C12 myoblasts grown in GM or DM 24h, 48h and 72h after induction of differentiation. The transcription levels were normalized to GAPDH expression and represent the mean of three independent experiments +/- standard deviation (SD). Prol., proliferative myoblasts in growth medium (GM); conf., confluent myoblasts (80%) in growth medium (GM); DM, differentiation medium.

To investigate whether EZH1 can exist in a complex with other PRC2 components in our cellular system, we carried out size exclusion chromatography analyses from nuclear extract of undifferentiated and differentiated C2C12 cells, followed by immunoblot with EZH2, SUZ12, EED and EZH1 antibodies of the eluted fractions (Fig. 17 D). In myoblasts the majority of all four proteins were present in a 700 kDa complex that corresponds to the molecular weight of the PRC2 complex (Pasini et al. 2004). When myoblasts were induced to differentiate, EZH2 levels declined but SUZ12, EED and EZH1, still co-eluted in the same fractions (Fig. 17 D, middle panel). To exclude the possibility that the formation of an alternative complex between SUZ12, EED and EZH1 in differentiated myotubes is EZH2-dependent, we depleted EZH2 from C2C12 cultured in differentiation conditions, and re-analyzed the SUZ12, EED and EZH1 elution profile. As shown in Fig. 17 D (right panel), SUZ12, EED and EZH1 co-fractionated at the same 700 kDa molecular weight, suggesting that these proteins form a complex in myotubes also in the absence of EZH2. We refer to this complex as PRC2-EZH1. In support, co-immunoprecipitation experiments showed increased association of SUZ12 with EZH1 after C2C12 differentiation concomitant with decreased interaction of EZH2 (Fig. 17 E).

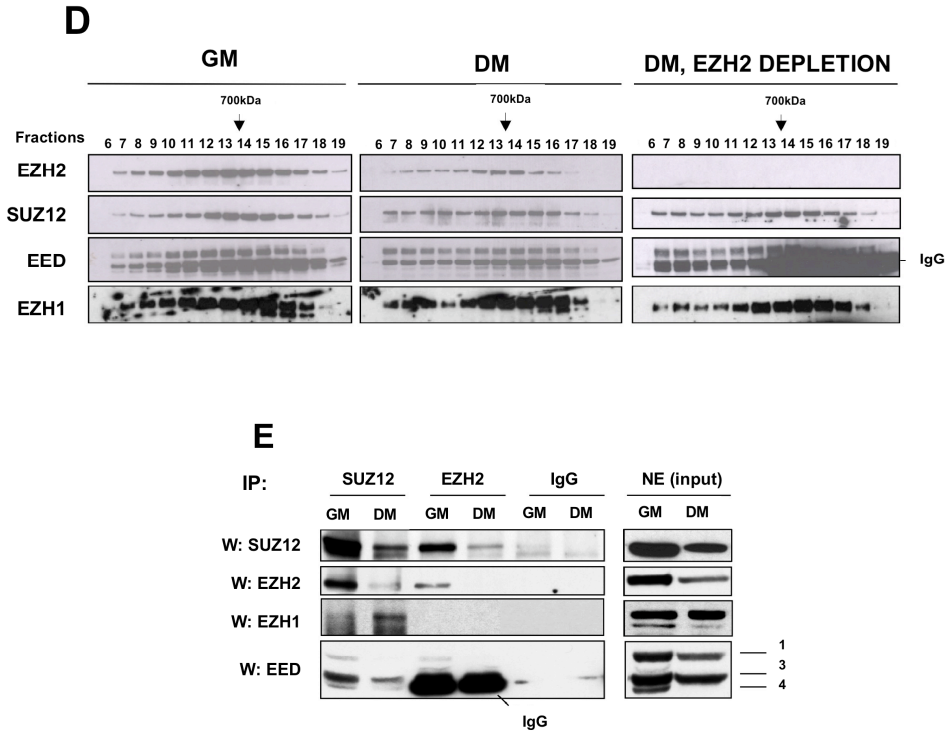

Fig.17: **D)** Size exclusion chromatography of nuclear extracts prepared from C2C12 myoblasts cultered in GM (left panel) or DM, 72h after induction of differentiation (middle panel) showing co-elution of EZH2, SUZ12, EED and EZH1 in high molecular weight fractions. The indicated fractions were analyzed by immunoblot. Right panel: EZH2 was depleted from C2C12 cells cultured in DM, 72h after induction of differentiation and nuclear extracts were analyzed as described previously. **E)** Immunoprecipitation (IP) of EZH2 and SUZ12 was performed from nuclear extracts (NE) of C2C12 myoblasts cultured in GM or DM (48h after differentiation induction). Co-IP of EZH2 with EED could not be properly assessed due to unspecific binding of IgG to EED antibody. Normal rabbit IgG antibody was used as a negative control.

To verify the presence of these two different PRC2 complexes in living cells during skeletal muscle differentiation, we performed Chromatin Immunoprecipitation (ChIP) experiments analyzing the dynamics of PRC2 components (SUZ12, EZH2, EZH1) and H3K27me3 repressive mark at regulatory regions of *mCK* and *MyoG*, previously reported to be PRC2 targets in myoblasts (Caretti et al. 2004; Juan et al. 2009; Seenundun et al. 2010). Two different *mCK* regulatory regions were analyzed corresponding to the promoter, located immediately upstream of the transcription start site (TSS) and to the enhancer, located -1,2 kb from the TSS (Nguyen et al. 2003). High ChIP levels of EZH2 and SUZ12 proteins were present on the *mCK* enhancer and promoter in undifferentiated myoblasts but they declined in differentiated myotubes (Fig. 17 F i) and ii). Interestingly and in agreement with previous reports, low ChIP levels of PRC2 components in myotubes was not followed by significant loss of H3K27me3 at the *mCK* promoter and only the *mCK* enhancer showed some

extent of H3K27me3 decrease (Fig. 17 F i) and ii) (Caretti et al. 2004; Juan et al. 2009). EZH1 protein was not detected on the *mCK* promoter or enhancer regions, neither in myoblasts nor in myotubes (ChIP-seq analysis, data not shown). PRC2 binding at *MyoG* promoter showed different dynamics. While EZH2 levels declined in the transition from myoblasts to myotubes, ChIP levels of SUZ12 and EZH1 as well as H3K27me3 increased (Fig. 17 F, iii). These data are in agreement with the previous observation that global levels of EZH1 were not downregulated during C2C12 differentiation (Fig. 17 B). We propose that, while EZH2 binding declines, a putative PRC2-EZH1 complex engages the *MyoG* promoter upon differentiation. Existence of two partially PRC2 complexes (PRC2-EZH2 and PRC2-EZH1) was previously reported (Margueron et al. 2008; Shen et al. 2008; Ezhkova et al. 2009). While EZH2 is mostly expressed in proliferative tissues and during embryogenesis, EZH1 appears to substitute for EZH2 in post-mitotic cells and in adult tissues (Ezhkova et al. 2009; Ho and Crabtree 2008). In our system, although PRC2-EZH2 and H3K27me3 are lost from the *mCK* enhancer, during differentiation we detected constant levels of H3K27me3 on both *MyoG* and *mCK* promoters (Fig. 17 F). Interstingly, we found that enrichment of PRC2-EZH1 (Fig. 17 F) was associated with *MyoG* expression in myotubes. This finding is not unprecedented, as presence of PcG has already been correlated with transcriptional activity (Ringrose et al. 2004; Papp and Muller 2006). Notably, recent studies show that 10-20% of PcG target genes in embryonic stem (ES) cells are transcriptionally active (Boyer et al. 2006; Lee et al. 2006). The presence of PRC2 components on active promoters that we proposed, may provide competence for subsequent re-silencing of genes whose expression needs to be only transiently activated. In keeping with this hypothesis, a recent reports showed that *Myog* is subsequently repressed after initial stage of differentiation (Berghella et al. 2008; Moresi et al. 2010).
Taken together, these results indicate that while *mCK* behaves as a classical PRC2 target where gene expression is associated with displacement of canonical PRC2-EZH2 complex, *MyoG* retains SUZ12 in complex with EZH1 following its activation during muscle differentiation. This shows that myotubes possess a PRC2-EZH1 complex that appears to replace PRC2-EZH2 in terminal differentiation.

From this moment on, the work will be divided in two sections. In the first section, the Section A, we'll analyze the role of the PRC2 components during skeletal muscle differentiation. In the second section, the Section B, we'll investigate the signaling mechanisms that regulate the PRC2 chromatin association during the transition from myoblasts to myotubes.

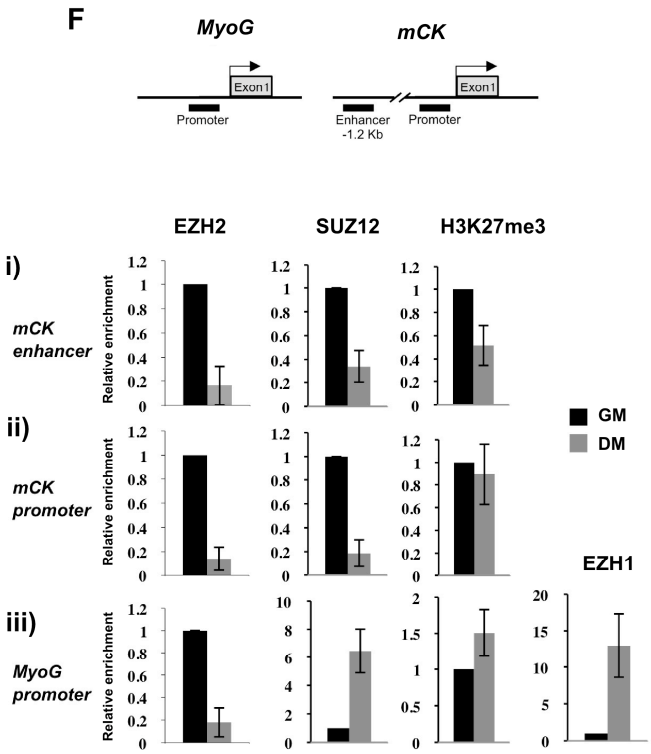

Fig.17: **F)** ChIP analysis of chromatin extracted from C2C12 cells cultured in GM or DM for 48h with SUZ12, EZH2, H3K27me3 and EZH1 antibodies. The precipitated DNA fragments were amplified using primers designed whitin *mCK* enhancer (i), *mCK* promoter (ii) and *MyoG* promoter (iii) as shown in the schematic representation in the top panel. ChIP enrichments are presented as relative enrichment to myoblasts. Data are shown as mean of three independent experiments +/- SD.

SECTION A

4.2-A PRC2 components are differentially involved in the myogenic differentiation

Since EZH1 was the unique PRC2 component that remained steady during differentiation (Fig. 17 B and C), we were interested in the possibility that EZH1 could regulate myogenic differentiation. In loss of function experiments, C2C12 myoblasts were transiently transfected with two different EZH1 siRNAs and induced to differentiate for 48h (Fig. 18 A).

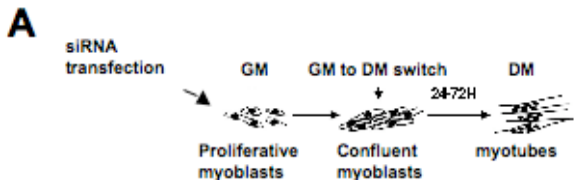

Fig.18: PRC2 components are differentially involved in the myogenic differentiation process. A) A scheme shows the design of siRNA knock down (k.d.) experiments used in this study.

In comparison with control samples, EZH1 depleted cells showed a significant impairment of their differentiation ability as revealed by phase contrast microscopy (Fig. 18 B). Conversely, and in agreement with published data (Caretti et al. 2004; Juan et al. 2009), muscle differentiation was not impaired after EZH2 depletion (Fig. 18 C). A delay in the activation of *MyoG* and *mCK* muscle markers was detected in EZH1 knock down cells, but not in EZH2 depleted cells. At this regards, lower protein levels of MyoG and mCK were found in EZH1 depleted cells (Fig. 18 D) together with only lower mRNA level of MyoG (Fig. 18 E, left panel). No decrease of mCK mRNA levels was detected after EZH1 knock down (Fig. 18 E, right panel), in keeping with our ChIP data (Fig. 17 F). The lower mCK protein levels in EZH1 depleted C2C12 coul be the consequence of the impairment of several connected pathways regulating muscle differentiation. The protein and mRNA levels of MyoG and mCK remained high after depletion of EZH2 (Fig. 18 F and G).

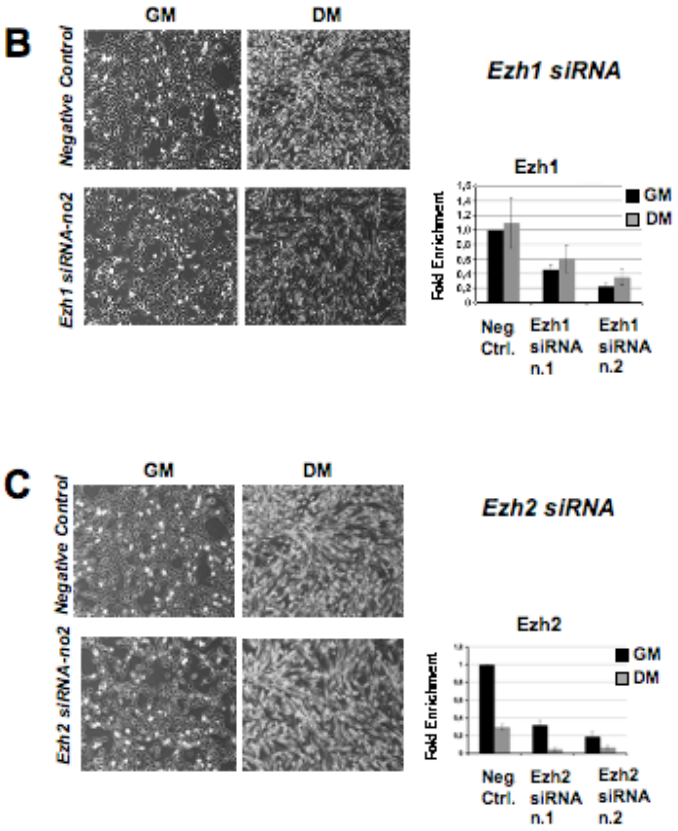

Fig.18: B) C2C12 myoblasts in GM were transfected with non-targeting siRNA (Neg. Ctrl.) or two different siRNAs against EZH1. 48h post transfection cells were placed in DM for additional 48h. Muscle differentiation phenotype was checked by phase contrast microscopy. Expression levels of EZH1 after siRNA k.d. were analyzed by Real Time-PCR. Data represent the mean from three different experiments +/- SD. Fold enrichment is calculated in comparison to negative control siRNA in GM. **C)** The same experiment described in **B)** was performed to analyze the phenotype obtained after EZH2 siRNA k.d.

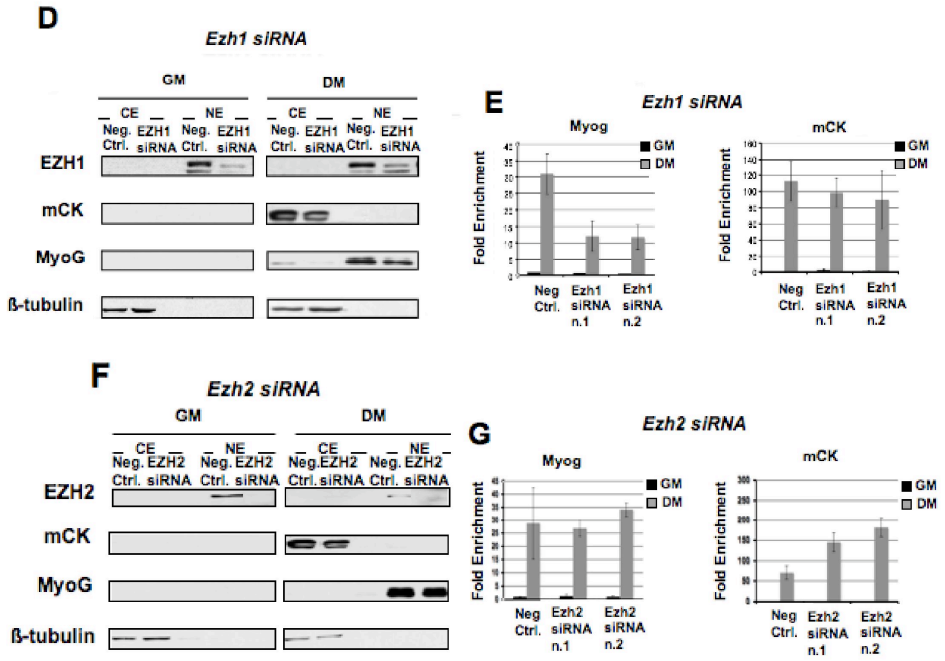

Fig.18: D) Immunoblot of EZH1, mCK and MyoG was performed after k.d. of EZH1 using the same siRNAs as in **B).** Nuclear (NE) and cytosolic (CE) cell extracts were used for analysis. β-tubulin was used as a control of cytosolic fractions. **E)** Expression levels of MyoG and mCK after siRNA EZH1 were analyzed using Real Time-PCR. Data represent the average from three independent experiments +/- SD. Fold enrichment is calculated in comparison to negative control siRNA in GM. **F)** Immunoblot of EZH2, mCK and MyoG was performed after k.d. of EZH2 using the same siRNAs as in **C)**. Nuclear (NE) and cytosolic (CE) cell extracts were used for analysis. β-tubulin was used as a control of cytosolic fractions. **G)** Expression levels of MyoG and mCK after siRNA EZH2 were analyzed using Real Time-PCR. Data represent the average from three independent experiments +/- SD. Fold enrichment is calculated in comparison to negative control siRNA in GM.

To verify that the delay of muscle differentiation in EZH1 depleted cells was not due to proliferation defect, we analyzed the proliferative capability of C2C12 cells after EZH1 knock down. As shown in Fig.19 A, EZH1 depleted myoblasts were able to correctly grow as the negative control. These data were confirmed by flow cytometry of cell cycle (Fig.19 B) and cell death analysis (Fig.19 C). At 24 and 48h after EZH1 depletion, the percentage of cells detected in G1/S phase (P1 fraction), S phase (P4 fraction) and G2/M phase (P5) was comparable between negative control and EZH1 knock down cells. The increase in cell number, both in the negative control and in EZH1 depleted samples, in G1/S phase at 72 and 96h cells is a physiological consequence of the growing cells that become confluent and start differentiating (Fig. 19 B). During the complete time course (from 24h to 96h), no significant cell death was detected both in negative control

and in EZH1 depleted cells (Fig. 19 C). Taken together, these data suggest that EZH1 is able to regulate muscle differentiation.

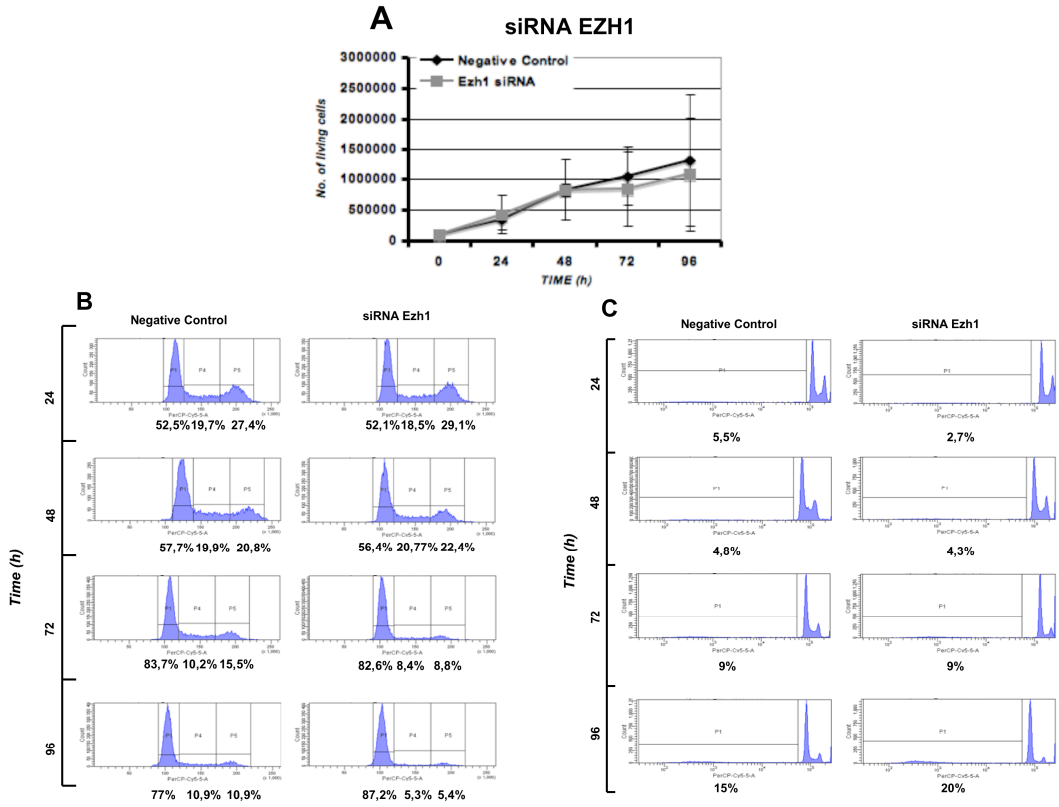

Fig.19: EZH1 depleted C2C12 are able to correctly grow. A) Effect of EZH1 siRNA on cell proliferation analyzing the growth capability of the Neg. Ctrl. and EZH1 depleted cells at 24h, 48h, 72h and 96h after transfection. Graph shows data from two independent experiments, error bars represent the standard deviation. **B)** Cell cycle analysis by FACS technique was performed after EZH1 siRNA analyzing the same time course of the growth curve as in **A). C)** Cell death analysis by FACS technique was performed after EZH1 siRNA analyzing the same time course of the growth curve as in **A).**

Since EZH1 was found in a complex with SUZ12 and EED in myotubes (Fig. 17 D), we performed the same knock down approach targeting SUZ12. As revealed by phase contrast microscopy (Fig. 20 A), a delay of muscle differentiation was detected after SUZ12 depletion, confirmed by lower protein and mRNA levels of Myog and mCK muscle markers (Fig. 20 B and C). In contrast to EZH1 knock down cells, the proliferation capability of SUZ12 depleted cells was also impaired (Fig. 20 D). Interestingly, flow cytometric analysis of the cell cycle of these cells revealed an accumulation in G1/S phase (P4) already at 48h after SUZ12 transfection (Fig. 20 E), whereas cell death profile was comparable to a negative control (Fig. 20 F). These results are in agreement with previously reported studies suggesting that SUZ12 is required for G1/S transition (Aoto et al. 2008).

Our data may be explained by an autonomous cell cycle defect induced by the specific derepression of PRC2 target genes such as cytokines (Bracken et al. 2006; Lee et al. 2006).

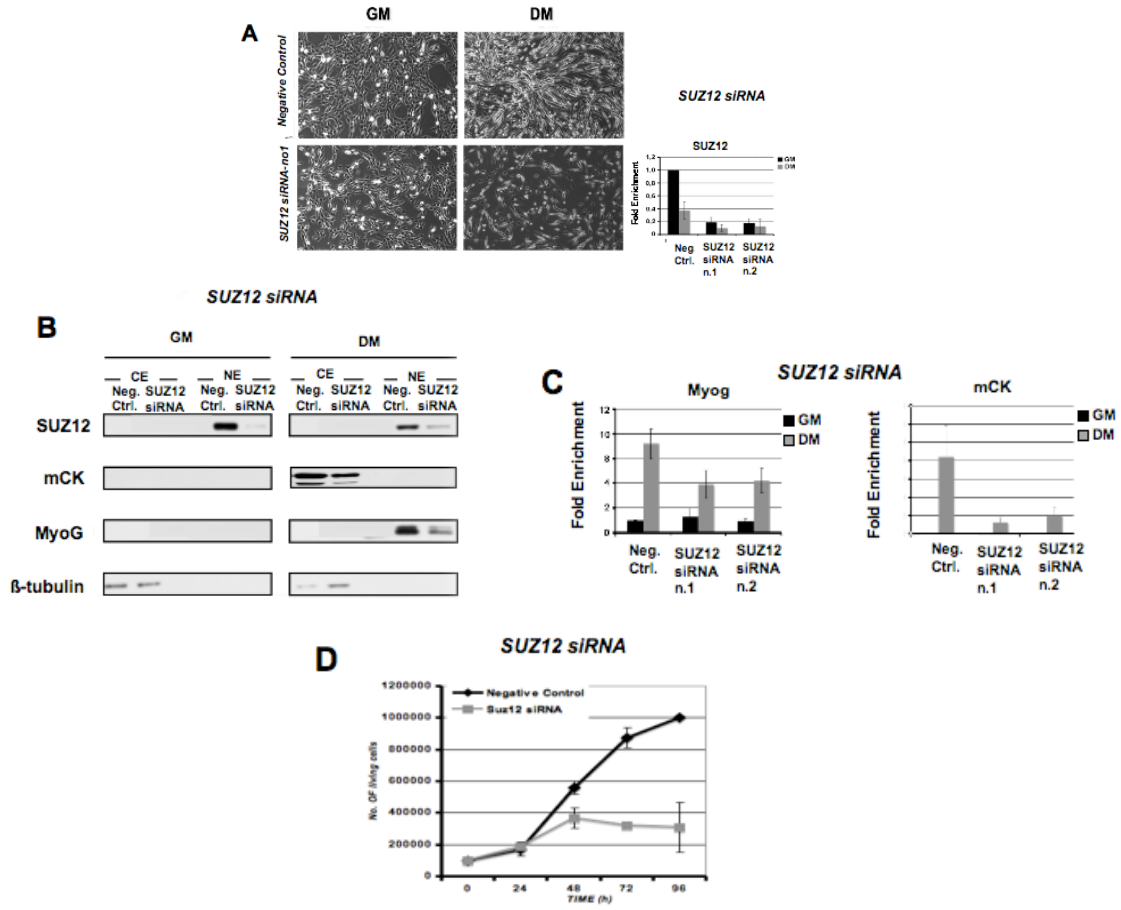

Fig.20: SUZ12 siRNA impairs proliferation and differentiation in C2C12 cell line. A) C2C12 myoblasts in GM were transfected with non-targeting siRNA (Neg. Ctrl.) or two different siRNAs against SUZ12. 48h post transfection cells were placed in DM for additional 48h. Muscle differentiation phenotype was checked by phase contrast microscopy. Expression levels of SUZ12 after siRNA k.d. were analyzed by Real Time-PCR. Data represent the mean from three different experiments +/- SD. Fold enrichment is calculated in comparison to negative control siRNA in GM. **B)** Immunoblot of SUZ12, mCK and MyoG was performed after k.d. of SUZ12 using the same siRNAs as in **A)**. Nuclear (NE) and cytosolic (CE) cell extracts were used for analysis. β-tubulin was used as a control of cytosolic fractions. **C)** Expression levels of MyoG and mCK after siRNA SUZ12 were analyzed using Real Time-PCR. Data represent the average from three independent experiments +/- SD. Fold enrichment is calculated in comparison to negative control siRNA in GM. **D)** Effect of SUZ12 siRNA on cell proliferation analyzing the growth capability of the Neg. Ctrl. and SUZ12 depleted cells at 24h, 48h, 72h and 96h after transfection. Graph shows data from two independent experiments, error bars represent the standard deviation.

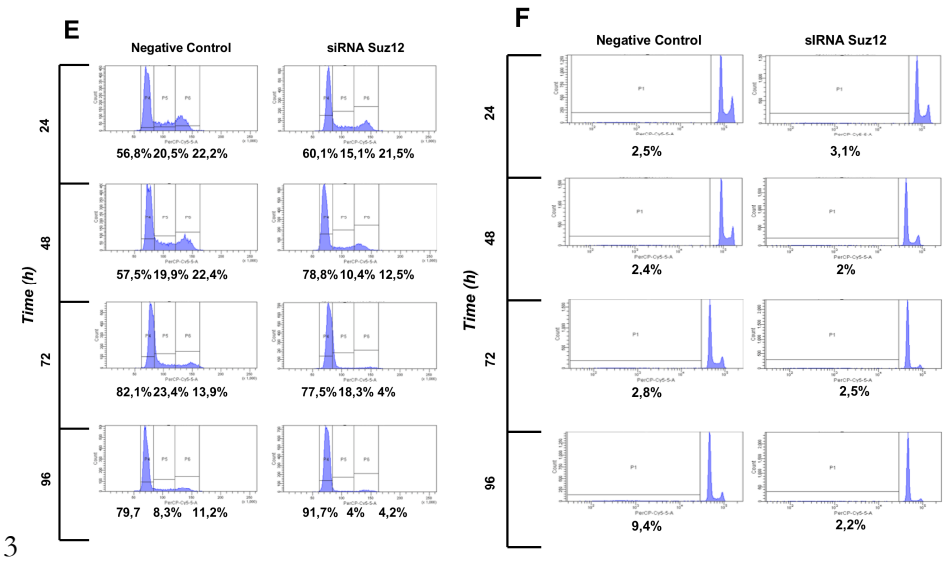

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Fig.20: E) Cell cycle analysis by FACS technique was performed after SUZ12 siRNA analyzing the same time course of the growth curve as in **D). F)** Cell death analysis by FACS technique was performed after SUZ12 siRNA analyzing the same time course of the growth curve as in **D).**

4.3-A PRC2-EZH1 complex is required for skeletal muscle differentiation

In order to analyze if there would be a common element in the different phenotypes after PRC2 component depletion to explain the delay of muscle differentiation, we examined the reciprocal protein levels of EZH1, SUZ12 and EZH2 in each silencing experiments. In SUZ12 depleted C2C12 cells (Fig. 20 A, B and C), a strong decrease of EZH1 protein levels was observed both in myoblasts and in myotubes (Fig. 21 A i). An opposite results was obtained after EZH2 silencing, in which the cells fused normally in myotubes (Fig. 18 C, F and G). Notably, in this case EZH1 protein levels increased both in proliferative and in differentiated cells (Fig. 21 A ii). Indeed, it was already reported that the depletion of SUZ12 induces the proteosomal degradation of other PRC2 components (Pasini et al. 2007; Tan et al. 2007; Pasini et al. 2010). On the basis of these results, we conclude that C2C12 differentiation occurred only in the presence of EZH1 protein.

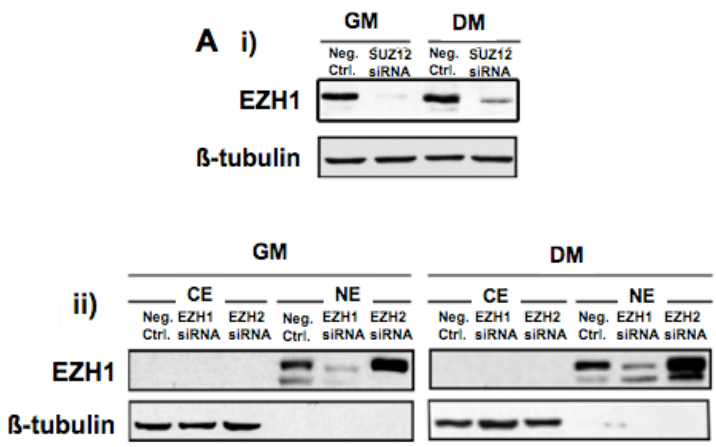
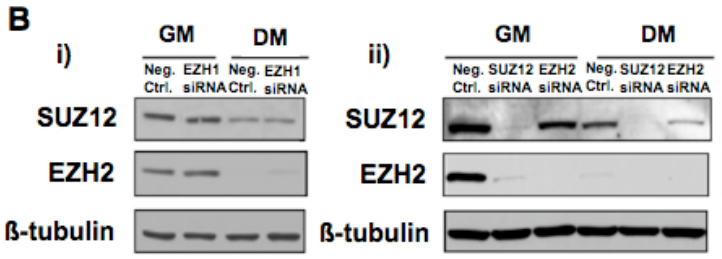


Fig.21: EZH1 is essential for skeletal muscle differentiation. A) Immunoblot analysis of EZH1 was performed in C2C12 myoblasts cultured in GM and DM, 48h after differentiation induction in SUZ12 (i), EZH1 and EZH2 (ii) depleted cells. Whole cell extracts were used in i) and nuclear (NE) and cytosolic (CE) cell extracts were used in ii). β-tubulin was used as a loading control in i) and as a control of cytosolic fractions in ii).

Moreover, we didn't detect any difference in SUZ12 protein levels in EZH1 depleted cells (Fig. 21 B i), whereas lower levels of SUZ12 protein were detected after EZH2 knock down (Fig. 21 B ii). The protein levels of EZH2 did not change neither in EZH1 nor in SUZ12 depleted cells (Fig. 21 B i) and ii), respectively). Thus, SUZ12 and EZH2 *per se* were not required for muscle differentiation program. The obtained data are summarized in Fig. 21 C. Taken together, these data suggest that EZH1 protein, that is part of a specific PRC2 complex, is required for skeletal muscle differentiation.



C

		PRC2 depletion		
		si Suz12	si Ezh1	si Ezh2
PRC2 reciprocal levels	*Suz12*	--	+	-
	Ezh1	-	--	++
	Ezh2	-	+	--
	Myogenic differentiation	-	-	++

Fig.21: B) Immunoblot analysis of SUZ12 and EZH2 was performed in C2C12 myoblasts cultured in GM and DM, 48h after differentiation induction in EZH1 (i), SUZ12 and EZH2 (ii) depleted cells. β-tubulin was used as a loading control. Whole cell extracts were used for this analysis. **C)** Schematic representation of the different muscle differentiation phenotypes obtained after siRNAs of each PRC2 components (SUZ12, EZH1 and EZH2) in comparison with PRC2 reciprocal protein levels previously analyzed. PRC2 reciprocal levels: - indicates that the protein declines; -- indicates that the protein strongly declines; + indicates that the protein increases; ++ indicates that the protein strongly increases. Myogenic differentiation: - indicates a delay of muscle differentiation; ++ indicates an acceleration of muscle differentiation. In grey, siRNA phenotypes showing a delay of muscle differentiation; in black, those showing an accelaration.

4.4-A Myogenin activation, via recruitment of MyoD and RNA Pol II, requires PRC2-EZH1

Differentiating C2C12 cells depleted of EZH1 showed strong defects in the correct timing of transcriptional activation of *Myog* (Fig. 18 E). Since MyoD transcription factor is considered one of the key elements involved in *MyoG* activation (Weintraub et al. 1989; Tapscott 2005) we asked if this muscle differentiation delay could be a consequence of the impairment of MyoD binding at *MyoG* promoter. To test this hypothesis, we performed ChIP experiments analyzing MyoD binding at *MyoG* promoter and *mCK* enhancer after EZH1 and EZH2 depletion (Fig. 22 A). As expected upon differentiation induction, MyoD binding increased in control myotubes, whereas EZH1 depleted cells showed lower levels of MyoD at *MyoG* promoter. Conversely, normal MyoD levels were present at *MyoG* promoter in EZH2 depleted myotubes (Fig. 22 A, left panel). Interestingly, MyoD binding at *mCK* enhancer was not impaired after EZH1 and EZH2 depletion (Fig. 22 A, right panel), suggesting that the delay of myogenic

differentiation in EZH1 depleted C2C12 could be considered a direct consequence of a deficient *MyoG* activation. In keeping with these data, we already observed that *MyoG* gene was a target of EZH1, whereas *mCK* gene was not (Fig. 17 F). To confirm these results, we analyzed the recruitment of RNA Polymerase II (RNA Pol II) and the active chromatin mark H3K4me3 (tri-methylation of lysine 4 of H3 histone) at the *MyoG* promoter and three different regions along the coding sequence of this gene (Reg. A, Reg. B and Reg. C) (Fig. 22 B) in EZH1 and EZH2 depleted cells. As shown in Fig. 22 B, the recruitment of RNA Pol II at the coding region of *MyoG* gene (Reg. A) was impaired in myotubes after EZH1 and not EZH2 knock down, further supporting the role of EZH1 in regulating transcriptional activation of *MyoG* gene. As expected, in EZH1 and not in EZH2 depleted C2C12, a strong reduction of H3K4me3 active mark was detected in myotubes along the promoter and the three coding regions of MyoG, in agreement with loss of transcriptional activation of this gene (Fig. 18 E). We can conclude that efficient recruitment of MyoD and RNA Pol II at *MyoG* gene requires EZH1 and these events are crucial for *MyoG* activation and for a correct timing of muscle differentiation.

The results described here show that PcG proteins shift the control of developmental regulatory genes from myogenic committed cells to terminal differentiated not proliferative cells, revealing a dual role for these proteins in developmental control. Thus, the epigenetic function of PcG proteins is apparently propagated in post-mitotic myotubes, with continued PcG (EZH1, SUZ12) occupancy. These results suggest a model in which myogenic signals induce a switch in the PcG distribution and function from multipotent myoblasts to differentiated myotubes, where they contribute to cell cycle exit and specification. Therefore, the PcG proteins play a dual role to facilitate the completion of myogenic differentiation, first acting in an epigenetic fashion to heritably silence developmental regulators and prevent premature differentiation in muscle precursors, then transitioning to act non-epigenetically to promote the completion of terminal differentiation in myotubes.

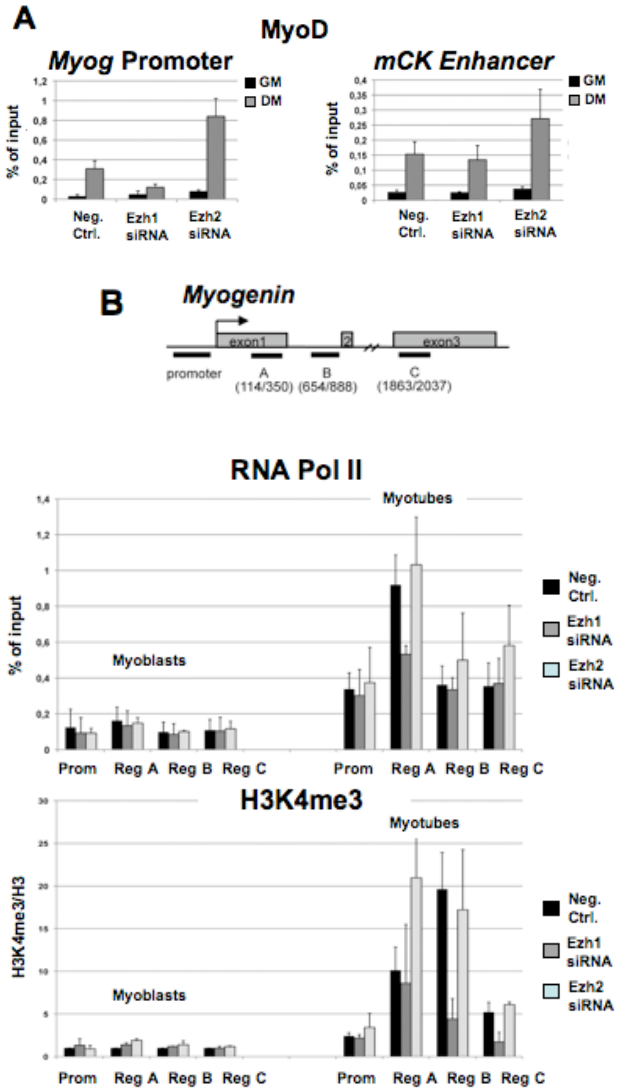


Fig.22: EZH1 depletion impairs MyoD and RNA Pol II recruitment at MyoG promoter. ChIP analysis was performed on chromatin extracted from C2C12 cultured in GM or DM 48h from the induction of differentiation, after EZH1 and EZH2 siRNA, using MyoD antibody at *MyoG* promoter and *mCK* enhancer in **(A)** and RNA Pol II and H3K4me3 antibodies at different regulatory regions of *MyoG* gene in **(B)**. The precipitated DNA fragments were subjected to Real Time-PCR. Levels of H3K4me3 were normalized to histone H3 density. The values represent the mean of three independent experiments. Error bars represent standard deviation.

SECTION B

4.2-B Msk1 kinase activity is required for muscle differentiation

In the present section we set out to investigate the role of MSK1/H3Ser28ph pathway and PRC2 association in the context of skeletal muscle cell differentiation (Fig. 23 A). We first investigated whether activation of MSK1/2 occurs during muscle differentiation. Since MSK2 RNA levels decreased in differentiated cells (Fig. 23 F, neg. ctr, right panel), we focused our attention on MSK1. MSK1 can autophosphorylate on Serine 376, a modification known as a hallmark of its active state (McCoy et al. 2005). When C2C12 myoblasts were induced to differentiate we detected increased level of phosphorylated MSK1 (Fig. 23 B).

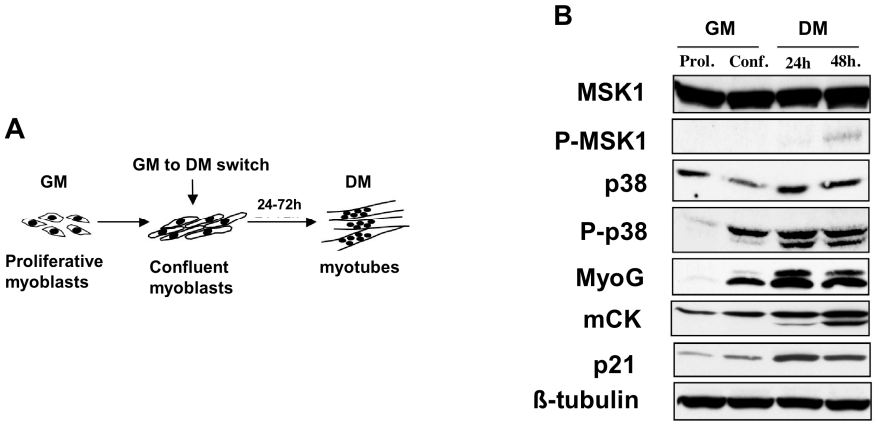


Fig. 23: MSK1 activity is required for muscle differentiation. A) Graphical representation of dynamics of C2C12 muscle cell line differentiation. Proliferative myoblasts at 80% confluency were induced to differentiate for 24-72h by replacing the growth (GM) with differentiation medium (DM). **B)** Immunoblot of MSK1 and its active form (phospho-MSK1, P-MSK1) was performed on whole-cell C2C12 extracts using specific antibodies. C2C12 differentiation was confirmed using antibodies against muscle cell markers (MyoG and mCK) and p38 (total and phosphorylatyed form, P-p38). p21 was used as a marker of cell cycle arrest. β-tubulin was used as a loading control. Prol., proliferative myoblasts (GM); conf., confluent myoblasts (80%) (GM); 24h and 48h indicate the time after GM to DM switch. Double bands of MyoG and p38 in DM represent their phosphorylated forms.

To assess the role of MSK1 kinase during muscle differentiation, we used H89, a compound which is known to inhibit MSK1 activity (Thomson et al. 1999; Davies et al. 2000). Although H89 works at concentration as high as 20 μM (Thomson et al. 1999; Vicent et al. 2006a), lower doses (i.e. 5 μM and 10 μM) inhibit MSK1 kinase more specifically (Brami-Cherrier et al. 2007). When C2C12 myoblasts were induced to differentiate in the presence of lower H89 concentration (5 μM and 10 μM) (Fig. 23 C), muscle differentiation was impaired as shown by phase contrast microscopy (Fig. 23 D) and immunofluorescence for the muscle specific differentiation marker mCK (Fig. 23 E).

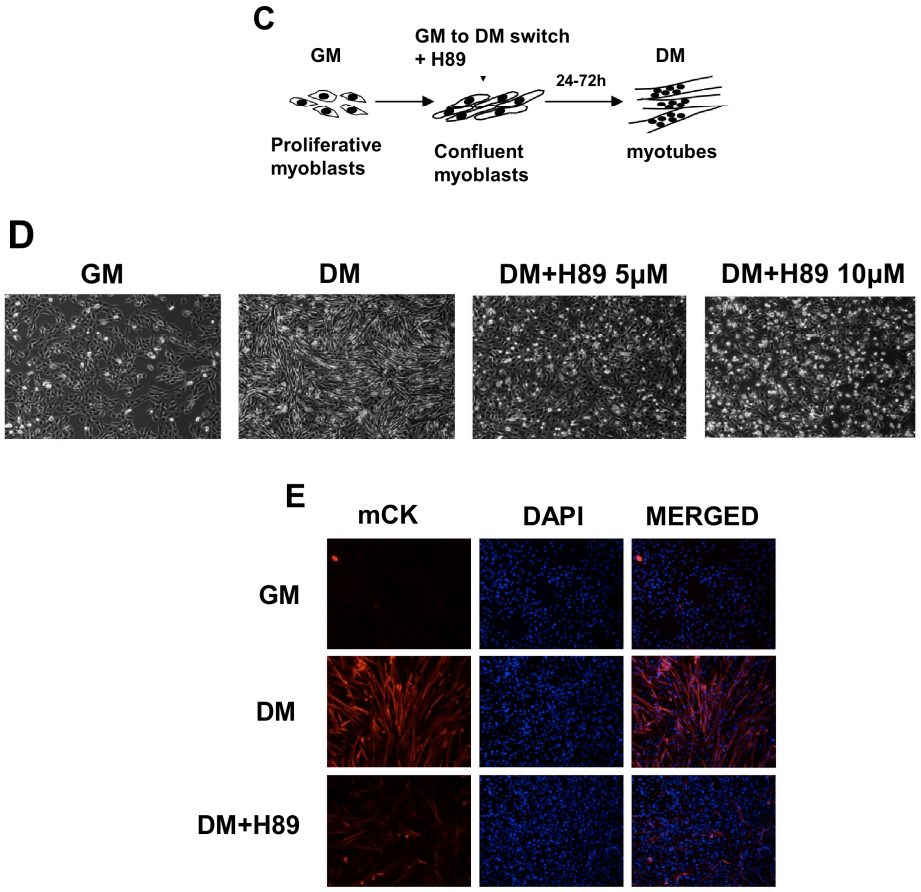


Fig. 23: C) and **D)** Proliferative myoblasts were induced to differentiate for 48h by replacing the GM with DM in the presence or not of MSK1 inhibitor (5μM and 10 μM H89). Effect of H89 treatment on muscle differentiation was evaluated by phase contrast microscopy. **E)** Expression of mCK was assayed by immunofluorescence in C2C12 cells cultured in GM and left to differentiate at 48h after the switch to DM with and without MSK1 inhibitor (5μM H89). DAPI staining visualizes nuclei.

Since H89 can also inhibit other signaling pathways such as PKA, p70S6K and RSK2 (Davies et al. 2000), we used siRNA knock down targeting MSK1 kinase to assess its specific involvement in muscle differentiation. Knock down of MSK1 by two different siRNA oligos caused a delay in muscle differentiation, as known by reduction in MyoG and mCK at mRNA and protein levels (Fig. 23 F and G). As a control, to exclude side effects on other closely related MAPK (e.g. MSK2) we checked for MSK2 stability in MSK1 depleted cells. We found that siRNA for MSK1 showed no effects on MSK2 mRNA levels (Fig 23 F, right panel). These results indicate that MSK1 kinase is specifically required for correct timing of C2C12 skeletal muscle differentiation.

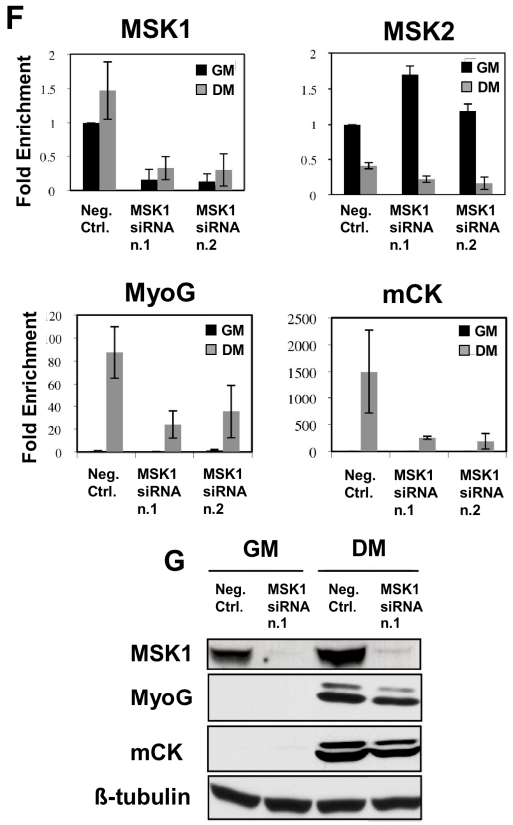


Fig. 23: F) C2C12 myoblasts in GM were transfected with non-targeting siRNA (Neg. Ctrl.) or two different siRNAs against MSK1. 48h post transfection cells were placed in DM for additional 48h. Expression levels of MSK1, MSK2, MyoG and mCK were analyzed using Real Time-PCR. Data represent the average from three independent experiments +/- SD. Fold enrichment is calculated in comparison to negative control siRNA in GM. **G)** Immunoblot of MSK1, MyoG and mCK was performed after knock down of MSK1 using the same siRNAs as in **F)**. β-tubulin was used as a loading control. Whole cell extracts were used for analysis.

4.3-B Inhibition of MSK1 kinase prevents down-regulation of PRC2 components during muscle differentiation

We analyzed the effect of MSK1 inhibition on the stability of PRC2 components, EZH2, EZH1, SUZ12 and EED by H89 treatment (Fig. 23 C). EZH2, SUZ12 and EED protein levels were significantly affected by MSK1 inhibition, in that their differentiation-dependent down-regulation was not observed upon H89 drug treatment, as shown by immunoblot in Fig. 24 A. Consistently, H89 treatment did not impair EZH1 protein levels (Fig. 24 A). Of note, stabilization of EZH2, SUZ12 and EED occurred also at the mRNA level (Fig. 24 B). Delay of differentiation by H89 treatment was confirmed by a decrease in MyoG and mCK levels (Fig. 24 A and B).

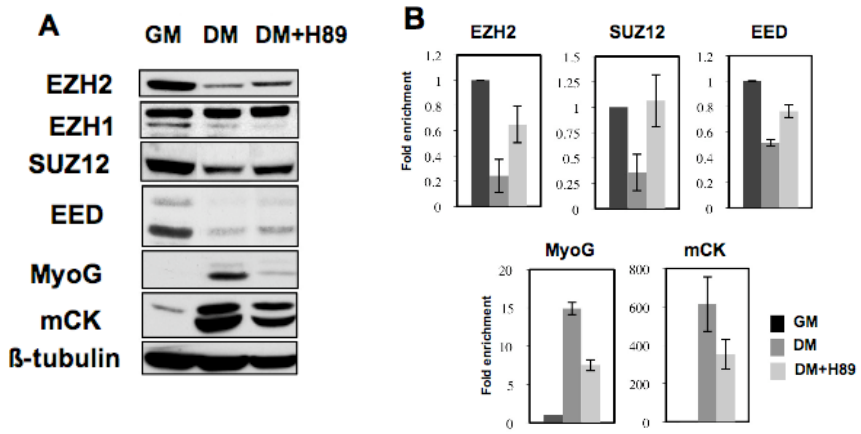


Fig.24: MSK1 inhibition by H89 affects PRC2 levels. A) Immunoblot of EZH2, EZH1, SUZ12, EED, MyoG and mCK from whole cell extracts of C2C12 myoblasts (GM) and myotubes (DM; 48h after differentiation induction) with and without MSK1 inhibitor (5μM H89). β-tubulin was used as a loading control. **B)** Expression levels of EZH2, SUZ12, EED, MyoG and mCK were measured by Real Time-PCR in C2C12 myoblasts cultured in GM and DM (48h after induction of differentiation) with or without MSK1 inhibitor (5μM H89). Transcription levels were normalized to GAPDH expression and represent the mean of three independent experiments +/- SD. Fold enrichment is calculated in comparison to myoblasts.

The sustained PRC2-EZH2 levels could be due to general interference with myoblasts differentiation by H89 rather than a direct consequence of MSK1 inhibition. To exclude this possibility C2C12 cells were treated with LY294002 (LY), which inhibits the PI3K/AKT pathway and has been shown to regulate muscle differentiation (Fig. 24 C) (Serra et al. 2007). Indeed, inhibition of this pathway led to failure of differentiation similar to H89 treatment as shown by phase contract microscopy (Fig. 24 D) and reduced levels of muscle markers (Fig. 24 E and F). However, LY treatment did not result in increased mRNA and

protein levels of core PRC2 components (Fig. 24 E and F). These results indicate that PRC2 stabilization upon H89 treatment is a specific effect of MSK1 inhibition and not an indirect consequence of impaired muscle differentiation. Thus, MSK1 inhibition is causally involved in stabilization of PRC2 components.

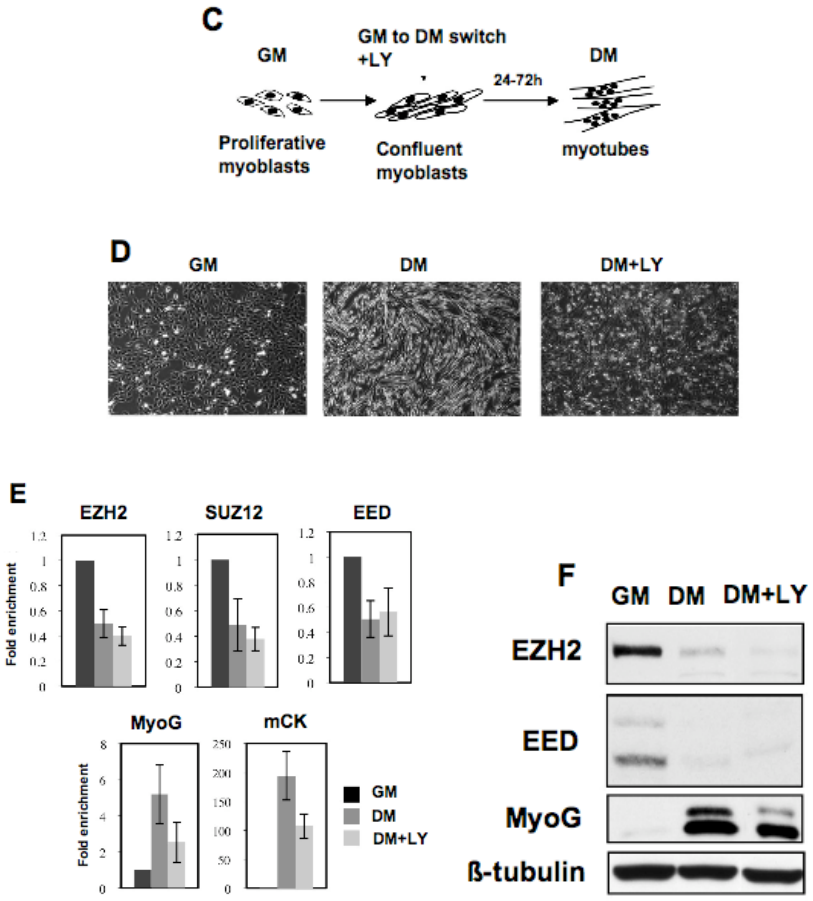


Fig.24: C) and **D)** Proliferative myoblasts were induced to differentiate for 48h by replacing the GM with DM in the presence or not of PI3K/AKT inhibitor (10μM of LY294002). Effect of LY treatment on muscle differentiation was evaluated by contrast microscopy. **E)** Expression levels of EZH2, SUZ12, EED and muscle markers (MyoG and mCK) cultured in GM or DM in the presence or not of PI3K/AKT inhibitor (10μM of LY294002) were measured by Real Time-PCR in the same conditions as described in **C). F)** Immunoblot of EZH2, EED and MyoG from whole cell extracts of C2C12 myoblasts cultured in GM or DM for 48h with and without PI3K/AKT inhibitor (10μM of LY294002). β-tubulin was used as a loading control.

4.4-B MSK1 regulates PRC2-EZH2 chromatin association during muscle differentiation

We then asked if the MSK1-dependent regulation of PRC2-EZH2 protein levels could reflect a modulation of their binding on chromatin during skeletal muscle differentiation. Therefore, we performed subcellular fractionation experiments (Fig. 25 A) in normal and MSK1 depleted C2C12 cells. This analysis permits to separate different subcellular protein fractions, using distinct lysis and extraction conditions. Cytoplasmic proteins were extracted in the form of a Triton-soluble fraction as shown by the presence of β-tubulin (Fig. 25 B, lanes 1, 4, 7 and 10), whereas chromatin bound proteins were isolated from the nuclear Triton-insoluble fraction (Fig. 25 B, lanes 2, 5, 8 and 11) after DNase and ammonium sulfate ($(NH_4)_2SO_4$) extraction (Fig. 25 B, lanes 3, 6, 9 and 12) (Llano et al. 2006). In myoblasts, MSK1 was present mainly in the soluble fraction (Fig. 25 B, lane 1) and was almost undetectable in insoluble (Fig. 25 B, lane 2) and chromatin bound (Fig. 25 B, lane 3) fractions. Consistently, we detected reduced levels of nuclear MyoG only in MSK1-depleted samples (Fig. 25 B, compare lane 8 with 11). Interestingly, when cells were induced to differentiate, MSK1 levels markedly increased with a significant fraction found in the cell nucleus and associated to chromatin (Fig. 25 B, lanes 8 and 9). As expected EZH2 and SUZ12 were present exclusively in the two nuclear fractions, with decreasing levels as cells differentiate (Fig. 25 B, lanes 2, 3, 8 and 9). Notably, depletion of MSK1 brought about a specific enrichment of EZH2 and SUZ12 in the chromatin-bound fraction, both in undifferentiated and differentiated cells, indicating a retention of these two components on chromatin (Fig. 25 B, compare lane 3 with lane 6 and lane 9 with lane 12). Taken together these data show that during muscle differentiation MSK1 acts in the nucleus modulating the interaction of PRC2-EZH2 components with chromatin.

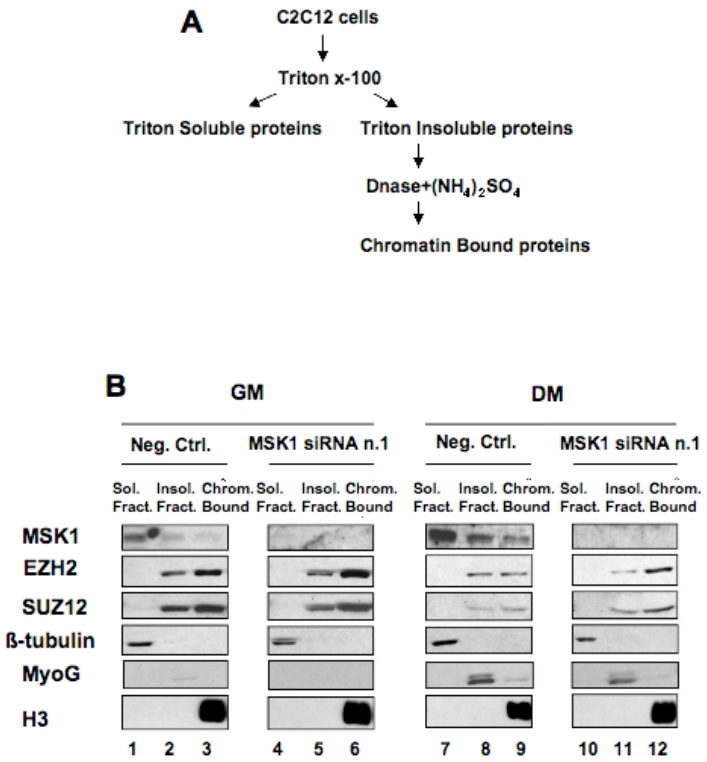


Fig. 25: Depletion of MSK1 leads to PRC2 chromatin retention. A) Schematic representation of different phases of the chromatin-binding assay. Three main fractions were recovered, Triton-soluble proteins (cytoplasmatic), Triton-insoluble proteins (nuclear and not chromatin bound) and chromatin bound. **B)** C2C12 myoblasts were transfected in GM with non-targeting siRNA (Neg. Ctrl.) or siRNA against MSK1. 48h after transfection, cells were cultured in DM for additional 48h. Enrichment of MSK1, EZH2 and SUZ12 in the different fractions was tested by immunoblot. Histone H3 was used as a control for chromatin bound proteins, β-tubulin as a marker for triton-soluble proteins and MyoG for triton-soluble fraction and differentiation induction.

4.5-B MSK1 controls an H3K27me3/Ser28ph switch and PRC2-EZH2 binding to muscle specific gene promoters

Putative lysine switch sites followed by phospho-acceptors (-ARKS-consensus sequence) such as Lys9/Ser10 and Lys27/Ser28 in histone H3 were predicted to regulate the activity of silencing complexes (Fischle et al. 2003). Indeed, methylated lysines are bound by repressive proteins, which can be then displaced by phosphorylation of the adjacent serine residues. In particular, phosphorylation of histone H3 at Ser10 was shown to inhibit methylation of the adjacent Lys9 by SUV39H1 (Rea et al. 2000) and subsequently to reduce HP1 binding to H3K9me3 (Fischle et al. 2003; Fischle et al. 2005). Since MSK1 is known to phosphorylate H3Ser28 *in vitro* and this histone mark is associated with gene activation (Dyson et al. 2005; Kim et al. 2008), we hypothesized that MSK1-mediated H3Ser28ph could regulate PRC2-EZH2 chromatin dissociation at muscle specific genes, neutralizing the PRC2 docking site H3K27me3. To test this hypothesis, we performed ChIP experiments analyzing MSK1, EZH2, SUZ12 and associated histone marks (H3Ser28ph and H3K27me3, respectively) at *mCK* and *MyoG* regulatory regions (Fig. 26 A). As shown in Fig. 26 B (left panels), simultaneously with activation of these muscle genes, H3Ser28ph and the other active mark acetylated histone H3 (AcH3) peaked at the *mCK* promoter, at the *mCK* enhancer and at the *MyoG* promoter in differentiated cells. Enrichment of H3Ser28ph at these three regions was associated with recruitment of MSK1 kinase (Fig. 26 B, left panels). Interestingly, in myotubes, the H3Ser28ph histone mark correlated with retention of H3K27me3 and displacement of the PRC2-EZH2 complex at the *mCK* and *MyoG* promoter regions (Fig. 26 B, right panels). At the *mCK* enhancer, instead, loss of PRC2-EZH2 occurred in parallel to H3Ser28ph enrichment and loss of H3K27me3 during muscle differentiation (Fig 26 B). Conversely to wild type conditions, treatment with H89 impaired the recruitment of MSK1 kinase at the *mCK* promoter, *mCK* enhancer and *MyoG* promoter, in association with loss of H3Ser28ph (Fig. 26 B, left panels) and failure of activation of these genes (Fig 24 A and B). These events were accompanied by retention of PRC2-EZH2 components at the *mCK* and *MyoG* promoter regions (Fig. 26 B, right panel). The differences in SUZ12 and EZH2 binding at two *mCK* regulatory regions and *MyoG* promoter could be explained by directly proportional modifications in H3K27me3, in that this repressive mark increased upon H89 treatment at *mCK* and *MyoG* promoters but not at *mCK* enhancer (Fig. 26 B, right panel).

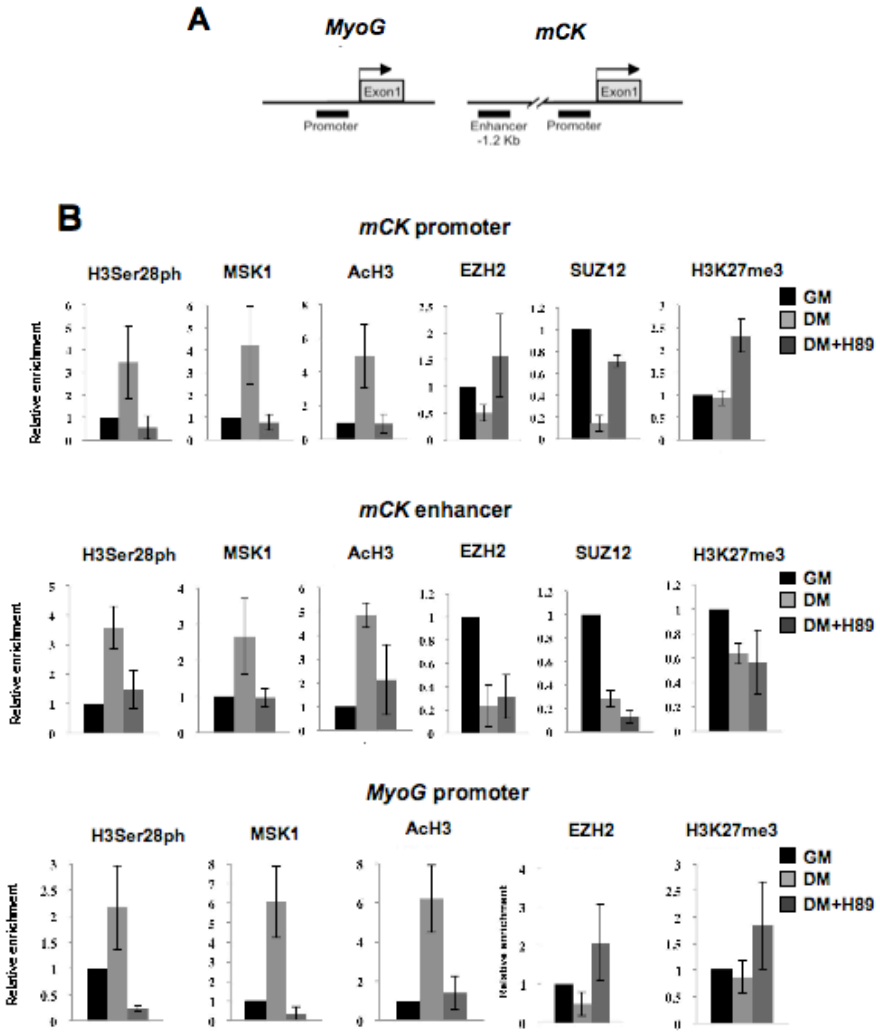


Fig. 26: MSK1-dependent H3Ser28 phosphorylation affects PRC2 dynamics at muscle regulatory regions. ChIP analysis was performed on chromatin extracted from C2C12 cultured in GM or DM 48h from the induction of differentiation with and without MSK1 inhibitor (5μM H89) using EZH2, SUZ12, H3K27me3, H3Ser28ph, MSK1 and acetyl-H3 (AcH3) antibodies **(B)**. The precipitated DNA fragments were subjected to Real Time-PCR analysis with primers amplifying *mCK* promoter, *mCK* enhancer and *MyoG* promoter **(A)**. Levels of H3K27me3, H3Ser28ph and AcH3 were normalized to histone H3 density. The values represent the mean of three independent experiments. Error bars represent standard deviations.

To validate the existence of the double mark H3K27me3/Ser28ph at the *mCK* and *MyoG* regulatory regions, ChIP experiments were performed using an antibody (CMA316) capable of recognizing H3Ser28ph regardless of the methylation status of H3K27 (Fig. 27 A). Specificity of this antibody was shown by immunoblot analysis of histone prepared from nocodazole treated cells (Fig. 27 B). Interestingly, we detected an increase of this double mark antibody from myoblasts to myotubes, both at *Myog* promoter and *mCK* enhancer (Fig. 27 C), suggesting that H3Ser28ph can occur even in the presence of H3K27me3. All together these data suggest that the displacememt of PRC2-EZH2 complex from the *mCK* and *MyoG* promoter, a functional event for muscle differentiation, is regulated by an H3K27me3/Ser28ph switch mechanism, via MSK1 recruitment to chromatin.

Previous work estabilished that the same muscle genes require p38 signal-dependent chromatin remodeling for their activation (Simone et al. 2004). Indeed MSK1 was shown to interact with BRG1 ATPase, a subunit of chromatin remodeling SWI/SNF complex involved in p38-dependent muscle gene activation (Simone et al. 2004; Drobic et al. 2010). Besides displacing repressive Polycomb proteins, how does H3Ser28ph regulate transcription? One possibility is that phospho-mark on histone H3 may favor the recruitment of SWI/SNF via 14-3-3 family proteins, that have high affinity for H3Ser10ph and are required for transcriptional activation (Winter et al. 2008; Niessen et al. 2009; Drobic et al. 2010). The conclusion of this work add a vast body of evidences in support of the importance of adjacent histone residues as potential targets for co-evolved epigenetic regulatory mechanism (Winter and Fischle 2010). Phosphorylation of Ser28 was shown to inhibit methylation at Lys27 (Manzur et al. 2003) and in *Tetrahymena* mutation of H3Ser28 (H3Ser28E) disrupted the proper methylation of both H3K9 and H3K27 during macronuclear development (Liu et al. 2007). Similarly, phosphorylation of histone H1 on Ser27 (H1Ser27ph) was found to prevent binding of HP1 and PRC2 to the adjacent methylated Lys26 (H1K26me3) (Kuzmichev et al. 2004; Daujat et al. 2005). Notably, PRC1/chromatin dissociation was correlated with H3Ser28ph further suggesting analogy with H3Ser10ph and HP1 displacement (Voncken et al. 2005). Recent report shows that phosphorylation of Ser28 inhibits tri-methylation at Lys27, leading to displacement of PcG proteins from target gene promoters, in response to stress and mitogenic signaling and retinoic acid (RA)-induced neuronal differentiation (Gehani et al., 2010). In conclusion, our data provide evidence that the MSK1/H3Ser28ph pathway is essential for skeletal muscle cell differentiation determining PRC2-EZH2 displacement from chromatin.

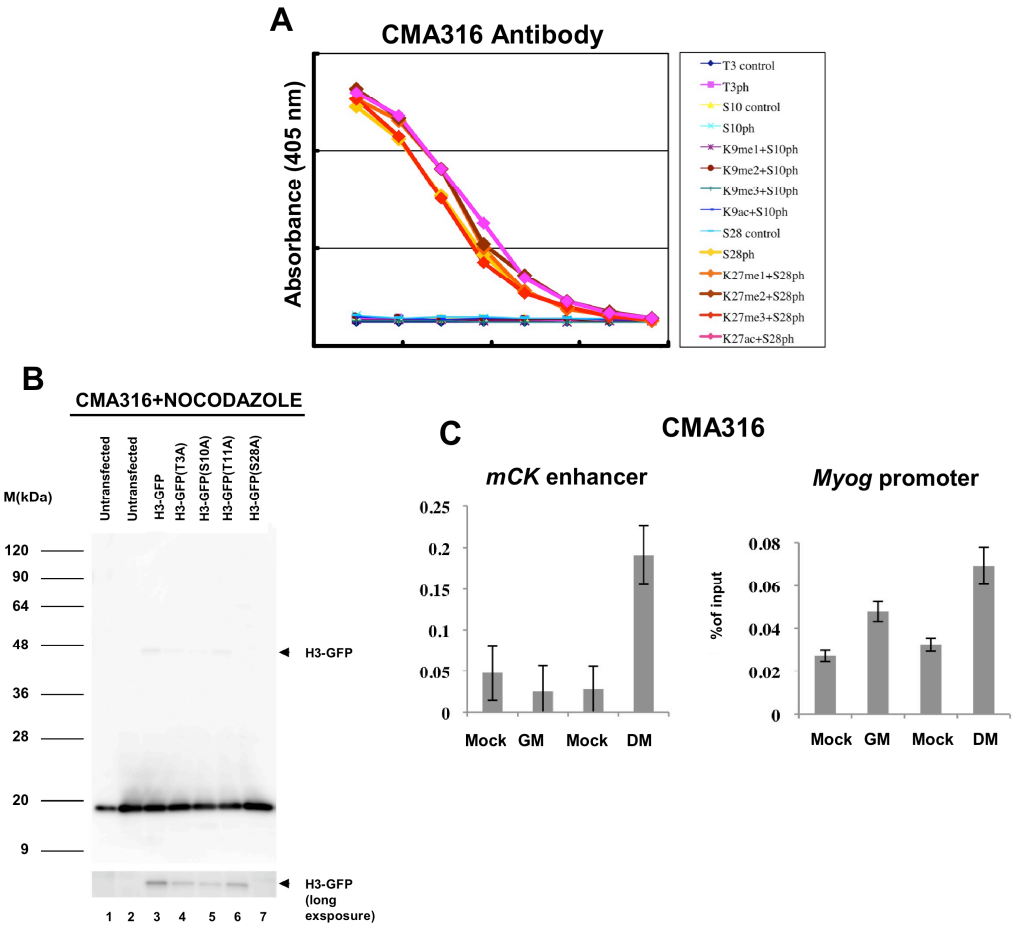


Fig. 27: Characterization of CMA316 mouse monoclonal antibody directed against phosphorylated histone H3 at Serine 28. A) Specificity of mAb was evaluated by ELISA. Microtiter plates were coated with the indicated peptides and incubated with 3-fold dilutions of each mAb (starting from 1:300 dilution of a hybridoma culture supernatant). **B)** HeLa cells were left untransfected (lanes 1, 2) or transfected with H3-GFP (lane3) and its phosphorylation-site mutants (lanes 4-7) and treated with nocodazole to enrich for mitotic cells (lanes 2-7). Whole cell proteins were separated on 13% SDS-polyacrylamide gels, transferred on to PVD membranes and blotted with CMA316 antibody that detects single bands at the size of histone H3 (lane1). Signal intensity is increased in nocodazole-treated, M-phase enriched, cell population (lane3). A mutant H3-GFP, which harbors Ser28A substitution, was not detected by these antibodies (lane7). **C)** ChIP analysis was performed on chromatin extracted from C2C12 cultured in GM or DM, 24h after induction of differentiation, using CMA316 antibody. The precipitated DNA fragments were subjected to Real Time-PCR analysis with primers amplifying *mCK* enhancer and *MyoG* promoter. The values represent the mean +/- SD of two indipendent experiments.

4.6-B Binding of EZH2, EED and SUZ12 but not EZH1 to the H3K27me3 mark is impaired by phosphorylation of H3Ser28

In order to provide direct mechanistic evidence for the involvement of H3Ser28ph in PRC2 displacement we first analyzed global levels of this mark in C2C12 myoblasts cultured in GM and DM with or without H89. As shown in Fig. 28 A, the levels of H3Ser28ph and H3Ser10ph declined in differentiated cells, which is in general agreement with their suggested roles as mitotic markers of proliferation (Goto et al. 1999; Prigent and Dimitrov 2003). Treatment of cells with the MSK1 inhibitor H89 caused a further decreased in global levels of H3Ser28ph and H3Ser10ph, whereas H3K27me3 and H3K4me3 remained unchanged. In light of the known role of MSK1 in H3Ser10 phosphorylation and H89 effects on global levels of H3Ser10ph (Fig. 28 A) (Thomson et al. 1999) we asked whether H3Ser10ph was also involved in muscle gene activation. Indeed, we did not observe any increase of this modification at *mCK* and *MyoG* regulatory regions during muscle differentiation, excluding a role for H3Ser10ph in muscle gene activation (Fig. 28 B). This observation is in agreement with earlier findings indicating that *in vivo* the two marks occur indipendently from each other and act separately to induce gene expression (Dunn and Davie 2005; Dyson et al. 2005). Also, H3Ser28ph has been associated with transcriptionally active promoter regions and with transcriptionally active chromatin, whereas H3Ser10ph did not differ between active and repressed chromatin regions (Sun et al. 2007).

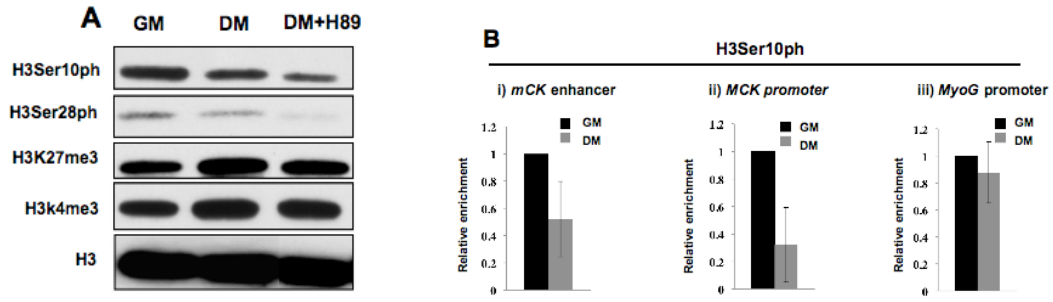


Fig.28: Binding of EZH2, SUZ12, and EED but not EZH1 is specifically affected by MSK1-mediated H3Ser28ph. A) Immunoblot of different histone H3 modifications in C2C12 cultured in GM or DM, 48h from induction of differentiation, with or without H89 (5μM). H3 was used as a loading control. **B)** ChIP analysis of *mCK* enhancer (i), *mCK* promoter (ii) and *MyoG* promoter (iii) was performed on chromatin extracted from C2C12 cultured in GM or DM for 48h after induction of differentiation, using H3Ser10ph antibody. Levels of H3Ser10ph were normalized to histone H3 density. The precipitated DNA fragments were subjected to Real Time-PCR analysis. The values represent the mean +/- SD of three independent experiments.

Our results shown until now indicate that MSK1 is required for the dissociation of the PRC2-EZH2 complex from the chromatin of muscle gene regulatory regions. To address whether MSK1 mediates this function by phosphorylating H3Ser28, we first analyzed if MSK1 can phosphorylate this site in the context of pre-existing H3K27me3. Recombinant MSK1 kinase was incubated with a histone H3 (21-33 residues) peptide either unmodified or carrying the K27me3 or Ser28ph modifications. As shown in Fig. 28 C, the H3K27me3 substrate was phosphorylated with similar kinetics as the unmodified peptide. Importantly, no phosphorylation of the H3Ser28 substrate was observed indicating that this is the only residue phosphorylated by MSK1 in this peptide. To investigate whether H3Ser28ph impairs binding of PRC2-EZH2 components to H3K27me3 docking site we performed affinity-purification experiments using long (1-40 residues) histone H3 tail peptides from nuclear extracts prepared from myoblasts and myotubes cells. In agreement with early findings (Hansen et al. 2008; Margueron et al. 2009) EZH2, SUZ12 and EED were found to be recruited to the H3K27me3 peptide but showed significantly lower binding to the unmodified control (Fig. 28 D). Interestingly, interaction of all PRC2 core components with H3K27me3 was significantly weakened in presence of neighboring H3Ser28ph. Similar observations were made for extracts prepared from undifferentiated myoblasts as well as from differentiated myotubes (Fig. 28 D). We therefore infer that the PRC2-EZH2 containing complex to bind H3K27me3 and to show sensitivity to H3Ser28ph is inherent to the complex and not depend on differentiation. Since we observed EZH1 binding (Fig. 17 F) and H3Ser28ph enrichment (Fig. 26 B) at *MyoG* gene regulatory regions upon differentiation, we next asked whether EZH1 is retained on H3K27me3 even in the presence of the adjacent phosphorylation. As Fig. 28 E shows, comparable amounts of EZH1 were recruited to H3K27me3 and H3K27me3Ser28ph peptides from extract of differentiated myotubes. Thus, MSK1 mediated phosphorylation of H3Ser28ph appears to impair PRC2-EZH2 binding onto H3K27m3 chromatin, but not PRC2-EZH1.

However, we cannot exclude that, besides H3Ser28ph, MSK1 (and/or MSK2) may target also PRC2 components, perhaps adding another layer of complexity in PcG regulation. PcG proteins are indeed broadly regulated by post-translational modifications (Niessen et al. 2009). For example, p38 kinase has been shown to phosphorylate the PRC1 component RING1B (Rao et al. 2009) and the PRC2 components EZH2 (Palacios et al. 2010) and AKT-dependent phosphorylation impairs EZH2 histone methyltransferase activity (Cha et al. 2005). In addition, the PRC1 component BMI1 is phosphorylated by MAPKAP kinase 3pK resulting in dissociation from chromatin (Voncken et al. 2005). More work will be needed to elucidate whether the MSK1/2 pathway directly regulates PRC2 activity.

In conclusion, our data provide strong evidence that MSK1/H3Ser28ph signaling pathway plays a key role in epigenetic gene regulation and it is essential for skeletal muscle differentiation.

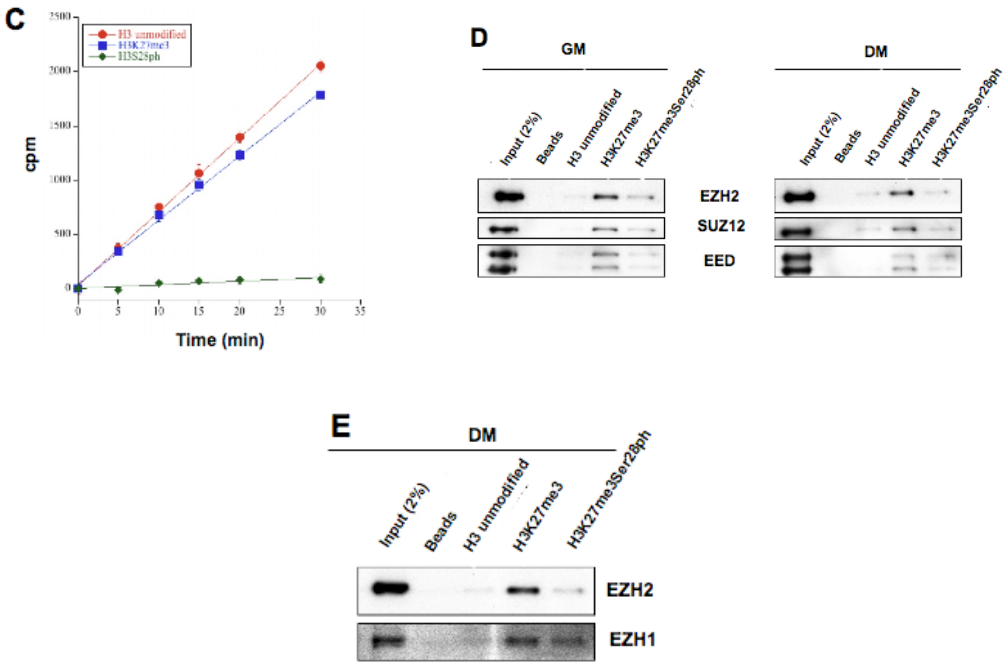


Fig.28: C) Recombinant MSK1 was incubated with unmodified histone H3, H3K27me3 and H3Ser28ph peptides and kinase assay was performed. **D)** Nuclear extracts from C2C12 cultured in GM or in DM, 48h after induction of differentiation, were incubated with peptides representing either unmodified H3 or H3 trimethylated at Lysine 27 (H3K27me3) or peptide that is both trimethylated at Lysine 27 and phosphorylated at Serine 28 (H3K27me3Ser28ph). Binding of EZH2, SUZ12 and EED was tested by immunoblot. **E)** The same experiment described in **D)** was performed in order to compare the binding of EZH2 and EZH1.

5. CONCLUSIONS

Our work addresses the dynamics of PRC2 complexes and the role of signaling pathways operating during skeletal muscle cell differentiation that by changing epigenomic codes control derepression of developmentally regulated genes, to allow their transcriptional switch. We show that two different PRC2 complexes are present during skeletal muscle differentiation, PRC2-EZH2, that is predominant in myoblasts and PRC2-EZH1 that is specific of post-mitotic myotubes. Since EZH1 is the unique PRC2 component that stays steady throughout different phases of differentiation, we hypothesize that EZH1 could regulate myogenic differentiation. Indeed, we show that depletion of EZH1 specifically impaires muscle differentiation due to the lack of transcriptional activation of the early muscle marker, MyoG. Surprisingly we find that EZH1 is required for the recruitment of the transcription factor, MyoD and RNA Pol II at MyoG promoter and these events are crucial to allow gene activation and muscle differentiation. Thus, more than one PRC2 entities has to be regulated to accomplish skeletal muscle cell differentiation. At this regard, we demonstrate that the opposite dynamics of PRC2-EZH2 and PRC2-EZH1 at muscle regulatory regions are differentially regulated at the chromatin level by signal-dependent phospho/methyl switch mechanism. Removal of Polycomb repressive complexes bound to their histone docking site H3K27me3 is a prerequisite to allow activation of muscle specific genes. In this work we explore the potential role of signal-dependent histone H3 phosphorylation as potential regulator of skeletal muscle differentiation. Because of its adjacent position to PcG H3K27me3 docking site, we hypothesize that H3S28ph could be a negative regulator of PcG silencing function necessary for regulating PRC2 binding to chromatin. Indeed, we show that depletion of MSK1 specifically stabilizes PRC2 complex on chromatin, hence inhibiting epigenome switch. By extensive ChIP experiments we show that upon differentiation MSK1 is directly recruited at *Myog* and *mCK* regulatory regions, H3Ser28 is phosphorylated and that these events perfectly follow loss of PRC2-EZH2 binding from the same regions and activation of muscle specific genes. Interestingly, in contrast to PRC2-EZH2, we find that during differentiation, PRC2-EZH1 is recruited to chromatin but this complex is insensitive to H3S28ph, indicating that MSK1/H3Ser28ph might be necessary but not sufficient to remove all PRC2 complexes from chromatin. Finally, by using an *in vitro* pull-down assay we provide mechanistic evidence in that H3Ser28ph modification neutralizes H3K27m3 mark as it greatly reduces binding of PRC2-EZH2 complex onto its docking site. Notably, we show that the same phospho-methyl switch does not influence EZH1 binding suggesting that MSK1/H3Ser28ph pathway is specifically required for de-repressing PRC2-EZH2 controlled genes at the time of differentiation. Our data provides strong evidence that MSK1/H3Ser28ph signalling pathway plays a key role in epigenetic gene regulation and it is essential for skeletal muscle differentiation. These findings may add important information to explore novel pharmacological strategies towards selective manipulation of gene expression via epigenome regulation to promote somatic cell reprogramming and tissue regeneration.

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Differential regulation of PRC2-EZH2 and PRC2-EZH1 by MSK1/H3S28ph controls skeletal muscle cell differentiation

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Running title: MSK1 regulates Polycomb during muscle differentiation

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SUMMARY

In differentiating cells epigenetic gene regulation allows the cell to respond dynamically to developmental cues. In particular, the Polycomb complex PRC2 has emerged as a key regulator of cell fate and differentiation. In this work we investigate how signalling pathways change PRC2 dependent epigenetic code of developmentally regulated genes during skeletal muscle cell differentiation. We show that MSK1 pathway is essential for muscle differentiation and regulates PRC2-EZH2 binding to muscle specific genes through phosphorylation of histone H3 at Ser 28 (H3S28ph). Furthermore, we report the existence of two PRC2 complexes, PRC2-EZH2 and PRC2-EZH1, the latter being the prominent PRC2 complex in myotubes. We show that while H3S28ph dependent displacement of PRC2-EZH2 is necessary for muscle gene derepression, H3S28ph does not influence EZH1 binding to chromatin. These results indicate that the epigenetic switch controlled by MSK1/H3S28ph pathway plays a key role in skeletal muscle cell differentiation in that it is specifically required to remove EZH2 from skeletal muscle gene regulatory regions.

Keywords: Epigenetics, MSK1 signaling, Polycomb, chromatin, muscle differentiation

INTRODUCTION

Polycomb group (PcG) proteins are transcriptional repressors that modify chromatin through epigenetic modifications that prevent changes in cell identity by maintaining transcription patterns, throughout development and in adulthood (Schuettengruber and Cavalli, 2009; Simon and Kingston, 2009). PcG proteins form two major multiprotein complexes, Polycomb repressive complex-1 and -2 (PRC1 and PRC2). PRC1 is the larger sized complex that contains several polypeptides whose functions, though not fully understood, include ubiquitination of histone H2A at Lysine 119 (H2AK119), chromatin compaction and regulation of the basal transcription machinery (Levine et al., 2004; Wang et al., 2004). The core of PRC2 is made up of three proteins, SUZ12, EED and EZH2, the latter being the catalytic subunit that modifies histone H3 by trimethylation of Lysine 27 (H3K27me3). Once H3K27me3 has been established, PRC2 is able to bind to this mark via the EED subunit, which in turn activates the histone methyltransferase activity (HMT) of the complex. This process allows maintenance of the repressive mark and its transmission to daughter cells (Hansen et al., 2008; Margueron et al., 2009). Recently, EZH1, which has a high degree of similarity to EZH2 but a weaker HMT activity, was found to interact with PRC2 to form a distinct complex (PRC2-EZH1; Margueron et al., 2008; Shen et al., 2008). A key aspect of PcG biological function is its signal dependent dynamics allowing developmental switches and possibly reprogramming (Maurange et al., 2006; Pereira et al., 2010). To date, little is known about how signaling pathways regulate PcG composition at target genes during cell differentiation.

During development, signal dependent switches in cell differentiation programs require global rearrangements in repression and activation of lineage specific genes. Hence it is important to unravel epigenetic mechanisms that control these dynamics and their integration with signaling pathways. In skeletal muscle cells, transcription factor driven processes cause proliferating

myoblasts to fuse into multinucleated myotubes. These require signal dependent activity of chromatin remodeling complexes (e.g. SWI/SNF) as well as removal of epigenetic repressors like PcG proteins and associated histone marks from specific regulatory regions (Caretti et al., 2004; Guasconi and Puri, 2009; Simone et al., 2004). The extracellular signals that regulate myogenic differentiation are transduced into the nucleus by mitogen-activated protein kinases (MAPKs), p38 and extracellular signal-regulated kinase (ERK; Simone et al., 2004; Wu et al., 2000). The two mitogen- and stress-activated protein kinases (MSK) -1 and -2 are known as downstream targets of the p38 or ERK pathways (Deak et al., 1998), however it is not known whether MSK1/2 have a role in muscle differentiation.

MSK1/2 function has been linked to histone H3 phosphorylation at Serine 28 (H3S28ph) or Serine10 (H3S10ph) (Thomson et al., 1999; Soloaga et al., 2003). Phosphorylation of histone H3 has different biological functions. It has been shown to occur either at gene regulatory regions where it activates transcription in response to cytokines and mitotic stimuli, or more globally at the level of histone H3, where it has been suggested to mediate chromosome condensation during mitosis (Cheung et al., 2000; Strahl and Allis, 2000). While mitotic phosphorylation by Aurora kinase is a long lasting event, which occurs on the majority of histone H3, interphase phosphorylation by MSK1/2 kinases is a transient phenomenon that affects only a small fraction of histone H3 at active gene sites (Clayton and Mahadevan, 2003). According to the histone code theory (Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 2002) and the binary switch model (Fischle et al., 2003) phosphorylation of residues next to methylated lysines can revert their repressive function and allow transcriptional activation. In fact, it has been shown that during mitosis H3S10ph can hamper the interaction between heterochromatin protein (HP1) and tri-methylated Lysine 9 of histone H3 (H3K9me3) and control heterochromatin formation (Chen et al., 2008; Fischle et al., 2005; Kloc et al., 2008). Displacement of HP1 from H3K9me3 by H3S10ph is not restricted to mitosis but can occur also during transcriptional activation of specific target genes

(Vicent et al., 2006; Winter et al., 2008). Since the characteristic Polycomb associated histone mark H3K27me3 is embedded in a sequence similar to Lysine 9 (-ARKS-), we were intrigued by the possibility that a signaling pathway controlling H3S28ph could regulate skeletal muscle differentiation via modulation of PRC2 chromatin binding at target genes. This has been very recently shown to occur during neuronal differentiation, stress response and mitogenic signaling (Gehani et al., 2010).

In the present work we show that MSK1 function is essential for skeletal muscle cell differentiation and that H3S28ph controls PRC2 dynamics on *Myogenin* (*MyoG*) and *muscle Creatine Kinase* (*mCK*). Transcriptional activation of *MyoG* and *mCK* in differentiated cells is associated with recruitment of MSK1 and increased levels of H3S28ph at their regulatory regions, while inhibition of MSK1 kinase correlates with loss of H3S28ph, retention of EZH2, SUZ12 and H3K27me3 and failure of *MyoG* and *mCK* activation. Further, we show that H3S28ph impairs PRC2-EZH2 binding to its H3K27me3 docking site. A novel PRC2-EZH1 complex appears to substitute for PRC2-EZH2 in myotubes. Interestingly, chromatin binding of EZH1, characteristic of non-proliferating cells, is not affected by MSK1/H3S28ph. Taken together, our studies provide the first evidence that a MSK1/H3S28ph signaling pathway controls skeletal muscle cell differentiation and allows a PRC2-EZH2 to PRC2-EZH1 switch at muscle specific genes.

RESULTS

MSK1 kinase activity is required for muscle differentiation

C2C12 cells were used as an *in vitro* model of skeletal muscle differentiation. In this cellular system exchange of growth medium (GM) to differentiation medium (DM) induces myoblasts to exit the cell cycle, to express muscle-specific genes and subsequently to fuse into multi-nucleated myotubes (Figure 1A) (Buckingham, 1996). We first investigated whether activation of MSK1/2 occurs during muscle differentiation. Since MSK2 RNA levels decreased in differentiated cells (Figure 1D, negative control, right panel) we focused our attention on MSK1. MSK1 can autophosphorylate on Serine376, a modification known as a hallmark of its active state (McCoy et al., 2005). When C2C12 myoblasts were induced to differentiate we detected increased level of phosphorylated MSK1 (Figure 1B). This post-translational modification occurred together with p38 phosphorylation and induction of muscle markers (*MyoG* and *mCK*) as well as the cell cycle inhibitor p21 (Figure 1B).

To assess the role of MSK1 kinase during muscle differentiation, we used H89, a compound which is known to inhibit MSK1 kinase activity (Davies et al., 2000; Thomson et al., 1999). Although H89 has been used at concentration as high as 20μM (Thomson et al., 1999; Vicent et al., 2006), lower doses (i.e. 5μM and 10μM) were shown to inhibit MSK1 kinase more specifically (Brami-Cherrier et al., 2007). When C2C12 myoblasts were induced to differentiate in the presence of low doses of H89 (5μM), muscle differentiation was impaired as shown by phase contrast microscopy (Figure S1) and immunofluorescence for the muscle specific differentiation marker *mCK* (Figure 1C). Since H89 has been shown to inhibit other signaling pathways such as PKA, p70S6K and RSK2 (Davies et al., 2000), we used siRNA knock down targeting MSK1 kinase to assess its specific involvement in muscle cell differentiation. Knock down of MSK1 by two different siRNA oligos caused a delay in muscle differentiation as shown by reduction in MyoG and mCK at mRNA

and protein levels (Figure 1D and 1E). Since MSK1 and MSK2 have a high degree of similarity and are functionally redundant (Deak et al., 1998), we confirmed that these siRNAs specifically depleted MSK1, but not MSK2 (Figure 1D). These results indicate that MSK1 kinase is necessary for correct timing of C2C12 skeletal muscle cell differentiation.

Two PRC2 complexes, PRC2-EZH2 and PRC2-EZH1, are differentially associated with muscle gene regulatory regions during myogenic differentiation

EZH2 protein levels are known to decline during skeletal muscle differentiation (Caretti et al., 2004). Thus, we set out to investigate PRC2 dynamics in this cellular system. In particular, we analyzed PRC2 core components (EZH2, SUZ12, EED) and EZH1 in the transition from myoblasts to myotubes. EZH2 was almost undetectable in differentiated cells, whereas SUZ12 and EED protein levels despite downregulation were still detectable in myotubes. Notably, EZH1 levels remained constant throughout different phases of differentiation (Figure 2A). A similar trend was observed analyzing PRC2 mRNA levels (Figure S2). Global levels of H3K27me3 followed EZH2, SUZ12 and EED dynamics (Figure 2A).

Next, we analyzed PRC2 components (SUZ12, EZH2, EZH1) and H3K27me3 dynamics by chromatin immunoprecipitation (ChIP) at the regulatory regions of *mCK a*nd *MyoG*, previously reported to be PRC2 targets in myoblasts (Caretti et al., 2004; Juan et al., 2009; Seenundun et al., 2010). Two different *mCK* regulatory regions were analyzed corresponding to the promoter located immediately upstream of the transcription start site (TSS) and to the enhancer located -1.2 kb from the TSS (Nguyen et al., 2003). EZH2 and SUZ12 were present on the *mCK* enhancer and promoter in undifferentiated myoblasts but could not be detected at these sites in differentiated myotubes (Figure 2B). In agreement with previous reports, displacement of PRC2 components was not followed by significant loss of H3K27me3 at the promoter and only the *mCK* enhancer showed some extent of H3K27me3 decrease (Caretti et al., 2004; Juan et al., 2009). EZH1 was not detected

on the *mCK* promoter or enhancer regions, neither in myoblasts nor in myotubes (including ChIP-seq analysis; data not shown). Interestingly, PRC2 binding at *MyoG* promoter showed different dynamics. While EZH2 was displaced, ChIP levels of SUZ12 and EZH1 as well as H3K27me3 increased (Figure 2B). These findings are in agreement with the fact that global levels of EZH1 were not downregulated during C2C12 differentiation (Figure 2A). We concluded that while EZH2 is lost, EZH1 and SUZ12 bound the *MyoG* promoter upon differentiation.

To investigate whether EZH1 can exist in complex with other PRC2 components in our cellular system, we carried out size exclusion chromatography analyses from nuclear extracts of undifferentiated and differentiated C2C12 cells followed by immunoblot with EZH2, EZH1, SUZ12 and EED antibodies of the eluted fractions (Figure 2C). In myoblasts the majority of all four proteins were present in a 700kDa complex that corresponds to the molecular weight of the PRC2 complex (Pasini et al., 2004). When myoblasts were induced to differentiate, EZH2 levels declined but EZH1, SUZ12 and EED still co-eluted in the same fractions. To exclude the possibility that the formation of an alternative complex between EZH1, SUZ12 and EED in differentiated myotubes is EZH2-dependent, we depleted EZH2 from C2C12 cells cultured in DM, and reanalyzed the SUZ12, EZH1 and EED elution profiles. As shown in Figure 2C (right panel) EZH1, SUZ12 and EED co-fractionated at the same 700kDa molecular weight, suggesting that these proteins form a complex in myotubes also in the absence of EZH2. We referred to this complex as to PRC2-EZH1. To support this conclusion, co-immunoprecipitation experiments were performed and showed increased association of SUZ12 with EZH1 after C2C12 differentiation, concomitantly with decreased binding to EZH2 (Figure 2D). Taken together, these results indicate that while *mCK* behaves as a classical PRC2 target where gene expression is associated with displacement of EZH2 and SUZ12, *MyoG* retains SUZ12 in complex with EZH1 following its activation during muscle differentiation. This shows that myotubes possess a PRC2-EZH1 complex that appears to replace PRC2-EZH2 in terminally differentiated cells.

Inhibition of MSK1 kinase prevents downregulation of PRC2 components during muscle differentiation

In order to analyze the effect of MSK1 on the PRC2 complexes, EZH2, EZH1, SUZ12 and EED levels were analyzed after pharmacological inhibition of this kinase by H89. EZH2, SUZ12 and EED protein levels were significantly affected by MSK1 inhibition as, their differentiation-dependent downregulation was not observed upon H89 drug treatment, as shown by immunoblot in Figure 3A and by immunofluorescence for nuclear EZH2 in Figure 3B. In contrast, H89 treatment did not impair protein levels of EZH1 (Figure 3A). Of note, stabilization of EZH2, SUZ12 and EED occurred at the mRNA level (Figure 3C) as well as at the protein level. The delay of differentiation by H89 treatment was confirmed by lower mRNA and protein levels of mCK and MyoG (Figure 3A and 3C). A possibility existed that the sustained PRC2-EZH2 levels could be due to general interference with myoblasts differentiation by H89 rather than be a direct consequence of MSK1 inhibition. To exclude this possibility C2C12 cells were treated with LY294002 (LY), which inhibits the PI3K/AKT, a pathway shown to regulate muscle differentiation (Serra et al., 2007). Indeed, inhibition of this pathway led to failure of differentiation similar to H89 treatment as shown by phase contrast microscopy (Figure S3) and resulted in reduced levels of muscle markers (Figure 3D and 3E). However, LY treatment did not result in increased mRNA and protein levels of core PRC2 components (Figure 3D and 3E). These results indicate that PRC2 stabilization by H89 treatment is a specific effect of MSK1 inhibition and not an indirect consequence of impaired muscle differentiation. All together, these experiments suggest that MSK1 inhibition is causally involved in stabilization of PRC2 components.

MSK1 regulates PRC2-EZH2 chromatin association during muscle differentiation

We then asked whether MSK1-dependent regulation of PRC2-EZH2 during skeletal muscle differentiation could reflect a modulation of its binding to chromatin. Therefore, we performed subcellular fractionation experiments (Figure 4A) in MSK1 depleted C2C12 cells. Cytoplasmic proteins were extracted in the form of a Triton-soluble fraction as shown by the presence of β-tubulin (Figure 4B, lanes 1, 4, 7, 10), whereas chromatin bound proteins were isolated from the nuclear Triton-insoluble fraction (Figure 4B, lanes 2, 5, 8, 11) after DNase treatment and ammonium sulfate ($(NH_4)_2SO_4$) extraction (Figure 4B, lanes 3, 6, 9, 12) (Llano et al., 2006). In myoblasts, MSK1 was present mainly in the soluble fraction (Figure 4B, lane 1) and was almost undetectable in insoluble (Figure 4B; lane 2) and chromatin bound fractions (Figure 4B, lane 3). As expected, muscle differentiation was impaired after MSK1 depletion as shown by reduced levels of nuclear MyoG (Figure 4B, compare lane 8 with 11). Interestingly, when cells were induced to differentiate, MSK1 levels markedly increased with a significant fraction found in the cell nucleus and associated to chromatin (Figure 4B, lanes 8 and 9). Fractionation experiments were also used to follow the subcellular distribution of PRC2-EZH2 components during muscle differentiation in MSK1 depleted cells. As expected EZH2 and SUZ12 were present exclusively in the two nuclear fractions, with decreasing levels as cells differentiate (Figure 4B, lanes 2, 3, 8, 9). Interestingly, depletion of MSK1 brought about an enrichment of EZH2 and SUZ12 specifically in the chromatin-bound fraction, both in undifferentiated and differentiated cells (Figure 4B, compare lane 3 with lane 6 and lane 9 with lane 12). Taken together these data show that during muscle differentiation MSK1 acts in the nucleus modulating the interaction of PRC2-EZH2 with chromatin.

MSK1 controls a H3K27me3/S28ph switch and PRC2-EZH2 binding to muscle specific gene promoters

Since MSK1 is known to phosphorylate H3S28 *in vitro* and this histone mark is associated with gene activation (Dyson et al., 2005, Kim et al., 2008), we hypothesized that MSK1-mediated H3S28ph could regulate PRC2-EZH2 chromatin dissociation at muscle specific genes. To test this hypothesis, we performed ChIP experiments analyzing MSK1, EZH2, SUZ12 and associated histone marks (H3S28ph, H3K27me3, respectively) at *mCK* and *MyoG* regulatory regions As shown in Figure 5A-C, activation of these muscle genes was accompanied by an increase at their regulatory regions of H3S28ph and the other active mark, acetylated histone H3 (AcH3). Enrichment of H3S28ph at these regions was associated with recruitment of MSK1 kinase (Figure 5A-C). Interestingly, in myotubes, the H3S28ph histone mark correlated with displacement of the PRC2-EZH2 complex but retention of H3K27me3 at the *mCK* and *MyoG* promoter regions (Figure 5A and 5C). However, at the *mCK* enhancer, loss of PRC2-EZH2 occurred in parallel with H3S28ph enrichment and a decrease of H3K27me3 during muscle differentiation (Figure 5B). To validate the existence of the double mark H3K27me3/S28ph at *mCK* and *MyoG* regulatory regions, ChIP experiments were performed using an antibody (CMA316) capable of recognizing H3S28ph regardless of the methylation status of H3K27 (Figure S4A). Specificity of this antibody was shown by immunoblot analysis of histones prepared from nocodazole treated cells (Figure S4B). As expected, an increase of this double mark was observed at the *mCK* enhancer and *MyoG* promoter during muscle differentiation (Figure S4C). As opposed to the wild type conditions, treatment with H89 impaired the recruitment of MSK1 kinase and the establishment of the H3S28ph mark at the *mCK* promoter, *mCK* enhancer and *MyoG* promoter (Figure 5A-C), causing failure of activation of these genes (Figure 3A and 3C). These events were accompanied by retention of PRC2-EZH2 at the *mCK* and *MyoG* promoter regions (Figure 5A and 5C). The differences in SUZ12 and EZH2

binding between *mCK* enhancer and the two other promoters are in keeping with the levels of H3K27me3, in that this repressive mark increased upon H89 treatment at the *mCK* and *MyoG* promoters (Figure 5A and 5C) but not at the *mCK* enhancer (Figure 5B). All together these data suggest that the displacement of PRC2-EZH2 complex from the *mCK* and *MyoG* promoter, a functional event for muscle differentiation, is regulated by a H3K27me3/S28ph switch mechanism, via MSK1 recruitment to chromatin.

Binding of EZH2, EED and SUZ12 but not EZH1 to the H3K27me3 mark is impaired by phosphorylation of H3S28

Our data so far strongly suggest that MSK1 is necessary for muscle differentiation where it regulates dissociation of PRC2-EZH2 components from chromatin possibly through H3S28ph. To confirm that this is due to H3S28ph we first analyzed global levels of this mark in C2C12 myoblasts cultured in GM and DM with or without H89. As shown in Figure 6A the levels of H3S10ph and H3S28ph declined in differentiated cells, which is in general agreement with their suggested roles as mitotic markers of proliferation (Goto et al., 1999; Prigent et al., 2003). Treatment of cells with the MSK1 inhibitor H89 caused a further decrease in global levels of H3S28ph and H3S10ph, whereas H3K27me3 and H3K4me3 levels remained unchanged. In light of the known role of MSK1 in phosphorylation of H3S10 and of the assays showing that H89 affects global levels of H3S10ph (Figure 6A) (Thomson et al., 1999), we asked whether H3S10ph was also involved in muscle gene activation. However, we did not observe any increase of this modification at the *mCK* and *MyoG* regulatory regions during muscle differentiation, excluding a role for H3S10ph in muscle gene activation (Figure S5).

Our results indicated that MSK1 kinase is required for the dissociation of the PRC2-EZH2 complex from the chromatin of muscle gene regulatory regions. To address whether MSK1 mediates this function by phosphorylating H3S28, we first analyzed if MSK1 can phosphorylate this site in the

context of preexisting H3K27me3. Recombinant MSK1 kinase was incubated with a histone H3 (residues 21-33) peptide either unmodified or carrying the K27me3 or S28ph modifications. As shown in Figure 6B, the H3K27me3 substrate was phosphorylated with similar kinetics as the unmodified peptide. Importantly, no phosphorylation of the H3S28ph substrate was observed indicating that this is the only residue phosphorylated by MSK1 in this peptide. To investigate whether H3S28ph impairs binding of PRC2-EZH2 complex to H3K27me3 we performed affinity-purification experiments using long (residues 1-40) histone H3 tail peptides incubated with nuclear extracts prepared from myoblasts and myotubes. In agreement with earlier findings (Hansen et al., 2008; Margueron et al., 2009), EZH2, SUZ12 and EED were found to be recruited to the H3K27me3 peptide but showed significantly lower binding to the unmodified control (Figure 6C). Interestingly, interaction of all three PRC2 core components with H3K27me3 was significantly weakened in the presence of neighboring H3S28ph. Similar observations were made for extracts prepared from undifferentiated myoblasts as well as from differentiated myotubes. We therefore infer that the ability of the PRC2-EZH2 containing complex to bind H3K27me3 and to show sensitivity to H3S28ph is inherent to the complex, and does not depend on differentiation.

Since we observed enrichment of EZH1 on *MyoG* regulatory regions upon differentiation occurring together with H3S28ph, we next asked whether EZH1 is retained on H3K27me3 even in the presence of the adjacent phosphorylation. As Figure 6D shows, comparable amounts of EZH1 were bound to H3K27me3 and H3K27me3S28ph peptides from extracts of differentiated myotubes. Overall, these results are in agreement with MSK1 mediated phosphorylation of H3S28 impairing binding of EZH2, but not EZH1 containing, PRC2 complexes to H3K27me3.

DISCUSSION

A signal dependent H3K27/S28 methyl-phospho switch controls skeletal muscle gene activation by displacing PRC2-EZH2 from chromatin target sites

During cell differentiation, the coordination of different signal-dependent pathways has to account for derepression and concomitant activation of lineage specific genes. In this view, the cross-talk between extracellular signaling and chromatin remodeling factors on gene promoters becomes essential for executing regulated developmental programs.

Skeletal muscle differentiation provides an excellent cellular system to study the interface between these signals and chromatin. Indeed, it is already established that removal of Polycomb repressive complex and concomitant recruitment of SWI/SNF chromatin remodeling complex controls muscle gene expression (Caretti et al., 2004; Guasconi and Puri, 2009; Simone et al., 2004). To accomplish these regulatory processes pro-differentiation signals have to be transduced into the nucleus. p38 MAPK, ERK and AKT signaling pathways have been showed to transmit cues that regulate myogenic differentiation (Serra et al., 2007; Simone et al., 2004; Wu et al., 2000). MSK1 and MSK2 are serine/threonine kinases known to phosphorylate histone H3 at Ser10 and Ser28 residues (Soloaga et al., 2003) and are known to act downstream of p38 and ERK (Deak et al., 1998). In the present work we set out to investigate the role of MSK1/H3S28ph pathway and PRC2 association to chromatin in the context of skeletal muscle cell differentiation.

Our data show that MSK1 is recruited to muscle gene regulatory regions to phosphorylate H3S28 in differentiating muscle cells. This event is concomitant with displacement of PRC2-EZH2 and activation of MyoG and mCK. Previous work established that the same genes require p38 signal-dependent chromatin remodeling for their activation (Simone et al., 2004). Thus, our data help understand another important layer of epigenome regulation, the control of the developmental switching of skeletal muscle genes. Our findings add to the vast body of evidence in support of the

importance of adjacent histone residues as potential targets for co-evolved epigenetic regulatory mechanisms (Winter and Fischle, 2010). In particular, putative lysine "switch" sites followed by phospho-acceptors (-KS/T- consensus sequence) such as Lys9/Ser10 and Lys27/Ser28 in histone H3 were predicted to regulate the activity of silencing complexes (Fischle et al., 2003). Methylated lysines are bound by repressive proteins, which can be then displaced by phosphorylation of the adjacent serine residues. Phosphorylation of histone H3 at Ser10 was shown to inhibit methylation of the adjacent Lys9 by SUV39H1 (Rea et al., 2000) and subsequently to reduce HP1 binding to H3K9me3 (Fischle et al., 2005). Phosphorylation of Ser28 was shown to inhibit methylation at Lys27 (Manzur et al., 2003) and in *Tetrahymena* mutation of H3S28 (H3S28E) disrupted the proper methylation of both H3K9 and H3K27 during macronuclear development (Liu et al., 2007). Similarly, phosphorylation of histone H1 on Ser27 was found to prevent binding of HP1 to the adjacent methylated Lys26, which is an additional product of PRC2 methyl-transferase activity (Daujat et al., 2005; Kuzmichev et al., 2004). Notably, PRC1/chromatin dissociation was correlated with H3S28ph further suggesting an analogy with H3S10ph and HP1 displacement (Voncken et al., 2005). Very recently MSK1/2 signaling has been linked to displacement of PRC2 from target genes via H3S28ph during neuronal differentiation and in response to stress and mitogenic signaling (Gehani et al., 2010).

Our data support a role for MSK1/H3S28ph pathway in counteracting Polycomb docking to H3K27me3 and therefore displacing PRC2-EZH2 from chromatin. Although MSK1 has been shown to phosphorylate both H3S28 and H3S10 *in vitro*, we show that MSK1 recruitment to muscle gene regulatory regions associate with an increase in H3S28ph but not H3S10ph. This observation is in agreement with earlier findings indicating that *in vivo* the two marks occur independently from each other and act separately to induce gene expression (Dunn et al., 2005; Dyson et al., 2005). In keeping with our data, H3S28ph has been associated with promoter regions

of transcriptionally active chromatin, whereas H3S10ph did not differ between active and repressed chromatin regions (Sun et al., 2007).

An interesting question concerns additional mechanism of transcriptional regulation by MSK1/H3S28ph pathway besides the displacement of repressive Polycomb proteins. One possibility is the recruitment of 14-3-3 proteins that have high affinity for this phospho mark on histone H3 (Macdonald et al., 2005; Winter et al., 2008). Indeed MSK1 was shown to interact with BRG1 ATPase, a subunit of chromatin remodeling SWI/SNF complex involved in p38-dependent muscle gene activation (Drobic et al., 2010; Simone et al., 2004). An additional layer of regulation could involve direct phosphorylation of PRC2 components by MSK1/2 kinases. PcG proteins are indeed broadly regulated by post-translational modifications (Niessen et al., 2010). For example, EZH2 has been shown to be phosphorylated by both p38 and AKT kinase, leading to enhancement of its repressive function and impairment of its HMT activity, respectively (Cha et al., 2005; Palacios et al., 2010). In addition, the PRC1 component BMI1 is phosphorylated by MAPKAP kinase 3pK resulting in dissociation from chromatin (Voncken et al., 2000). More work will be needed to elucidate whether the MSK1/2 pathway directly regulates PRC2 activity.

PRC2-EZH2 and PRC2-EZH1 complexes are differentially regulated by MSK1/H3S28ph signaling pathway

Unlike EZH2, EZH1 levels remain constant during C2C12 differentiation and it can be found in a PRC2 complex devoid of EZH2. This suggests that a specific PRC2-EZH1 complex may be required in non-cycling, differentiated muscle cells. Moreover, the MSK1/H3S28ph pathway specifically negatively regulates PRC2-EZH2 while chromatin binding of PRC2-EZH1 complex appears to be unaffected. Existence of two partially redundant PRC2 complexes (PRC2-EZH2 and PRC2-EZH1) was previously reported (Ezhkova et al., 2009; Margueron et al., 2008; Shen et al., 2008). While EZH2 is mostly expressed in proliferative tissues and during embryogenesis, EZH1

appears to substitute for EZH2 in post-mitotic cells and in adult tissues (Ezhkova et al., 2009; Ho and Crabtree, 2008). Although EZH1 has been reported to have weak HMT activity on H3K9 and H3K27 residues (Margueron et al., 2008; Shen et al., 2008), our data do not seem to favor the conclusion that its function relates to the maintenance of H3K27me3 on gene regulatory regions. In our system, although *mCK* enhancer behaved as a classical PRC2 target that loses PRC2-EZH2 and H3K27me3 upon activation, we detected constant levels of H3K27me3 during differentiation on both *MyoG* and *mCK* promoters. However, only *MyoG* promoter was targeted by PRC2-EZH1. This is the first evidence of EZH1 being recruited on active genes, even though a correlation between PcG binding to gene regulatory regions and transcriptional activity has already been described (Papp and Müller, 2006; Ringrose et al., 2004). Notably, recent studies have showed that 10-20% of PcG target genes in embryonic stem (ES) cells are transcriptionally active (Boyer et al., 2006; Lee et al., 2006).

Co-existence of active (AcH3 and H3S28ph) and repressive mark (H3K27me3) at *MyoG* and *mCK* promoters is reminiscent of bivalent domains that were shown not to be restricted exclusively to ES cells (Mohn et al., 2008). Although our study does not fully address the role of EZH1 on transcribed genes in post-mitotic differentiated cells, we hypothesize that presence of PRC2 components on active promoters may provide the possibility off subsequent re-silencing of genes whose expression needs only to be transiently activated. In keeping with this hypothesis, a recent report showed the *MyoG* up-regulation during initial stages of skeletal muscle differentiation is followed by subsequent repression (Berghella et al., 2008). Similarly *MyoG* was shown to be activated in the early stages of neurogenic muscle atrophy and downregulated at a later phase (Moresi et al., 2010).

The differential dynamics of EZH2 and SUZ12 at the two muscle genes are also in keeping with the formation of an alternative PRC2 complex which is insensitive to negative regulation by MSK1/H3S28ph signaling. In fact, we show that upon differentiation, while EZH2 is lost from both *MyoG* and *mCK* regulatory regions, SUZ12 is retained at the *MyoG* promoter in combination

with EZH1. Of note, both genes showed enrichment of MSK1 and H3S28ph. These results are in agreement with our *in vitro* data showing that H3S28ph does not affect EZH1 binding to a H3K27me3 peptide, whereas EZH2 binding is clearly reduced. This supports a scenario whereby the MSK1/H3S28ph signaling promotes displacement of PRC2-EZH2, but allows a switch with PRC2-EZH1 to differentially mark genes that, although synchronously activated at early stages of differentiation, may receive subsequently different types of regulation.

In conclusion, the present works identifies novel signal-dependent, chromatin-mediated mechanisms of regulation of skeletal muscle cell differentiation promoting removal of PRC2-EZH2 complex from target genes but allowing a switch with PRC2-EZH1. The present discovery of a role of MSK1/H3S28ph signaling in epigenetic regulation of skeletal muscle differentiation may be an important add to a field where novel targets are needed to increase the pool of molecular therapeutic targets to be used to treat muscular diseases.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents

C2C12 mouse myoblasts cells (ATCC) were cultured in Dulbecco Modified Eagel's Medium (DMEM) supplemented with penicillin/streptomycin and 10% fetal bovine serum (Euroclone). Differentiation was induced when cells reached about ~80% confluency using DMEM containing ITS media supplement (Sigma). LY294002 was obtained from Sigma while H89 was from Alexis Corporation. The inhibitors were replaced freshly every 24h.

RNA Isolation and Quantitative Real-Time PCR

RNA was extracted from cells using TriReagent (Sigma) according to the manufacturer's insctructions. cDNA synthesis was performed using the QuantiTect reverse transcription kit (Qiagen). Quantitative real-time PCR reactions were performed in triplicate using QuantiTect SYBR Green master mix (Qiagen) on a DNA Engine Opticon 2 machine (MJ Research) controlled by Opticon Monitor 2 software. C(T) values were calculating by Opticon Monitor 2 software, relative C(T) values were normalized to the housekeeping gene *GAPDH*. Fold enrichment refers to the enrichment relative to myoblasts values. *GAPDH, MHCIIB* and *mCK* primers were already described (Caretti et al., 2004). Remaining primer sequences are the following:

MyoG-for GCAGCGCCATCCAGTACATTG

MyoG-rev GCAACAGACATATCCTCCACCG

SUZ12-for GTGCCTTTGAATCCTGACCTC

SUZ12-rev CTTCGCGATTTCGTTTTCTTC

EZH1-for AGACTCAGTGCAATACCAAGCA

EZH1-rev AGTTTTTGCAAGACACCACCTT

EZH2-for TGATAAAGAAACTTGCCCACCT

EZH2-rev CTTTGCTCCCTCTGAACAGATT

EED-for ATAACCAGCCATTGTTTGGAGT

EED-rev TGTTGCTATCATAGGTCCATGC

MSK1-for GCCGATGAAACTGAAAGAGC

MSK1-rev TGCTCATTTCCTGGGGATAC

MSK2-for TCACACTGCACTACGCCTTC

MSK2-rev GATACCCAGCTTGTGCAGGT

RNA Interference

siRNA MSK1 #1 (SI01407483), siRNA MSK1 #2 (SI01407504) as well as negative control siRNA (scrambled sequence not targeting mouse genome, 1027313) were purchased from Qiagen and used at the final concentration of 20 nM. Cells were transfected with HiPerfect (Qiagen) following the manufacturer's instructions. 48h after transfection the cells were induced to differentiate and collected at the indicated time-points.

Cell Lysis and Immunoblot

Cells were harvested and washed twice with PBS. Cell lysis of total cell extracts was performed on ice in 50 mM Tris–HCl pH 8, 125 mM NaCl, 1% NP-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitory cocktail (Roche) for 25 min. Insoluble material was pelleted by centrifugation at 16000 g for 3 min at 4°C. Histone extracts were prepared following Abcam protocol (www.abcam.com). Protein concentration was determined using the Bradford assay (Bio-Rad). The proteins were denatured, reduced, separated by SDS–PAGE and transferred to nitrocellulose transfer membrane (PROTRAN-Whatman,

Schleicher&Schuell). The membranes were blocked with 5% non-fat dry milk in TBST for 60 min, incubated with primary antibodies overnight at 4°C, washed three times with TBST for 10 min, incubated with the peroxidase-conjugated secondary antibody in TBST with 2.5% non-fat dry milk for 60 min and washed three times with TBST for 10 min. Immunoreactive proteins were detected using Supersignal West Dura HRP Detection Kit (Pierce).

For cytoplasmic and nuclear extracts preparation the cells were resuspended first in Buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA and 0.1 mM EGTA) supplemented with protease inhibitory cocktail (Roche), 1 mM dithiothreitol (DTT) and 1mM PMSF. After incubation on ice for 10 min, NP-40 was added to a final concentration of 0.5% and the samples were vortexed for 5 s. Nuclei were pelleted at 13200 rpm for 10 s and the cytoplasmic proteins were collected. The pellet was then washed five times with Buffer A and resuspended in buffer C (20 mM Hepes pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, protease inhibitory cocktail (Roche) and 1 mM PMSF). After 10 min on ice, the samples were sonicated, centrifuged at 13200 rpm for 10 min and the nuclear proteins were transferred to a fresh vial. For immunoprecipitation studies, nuclear extracts were first precleared using protein A/G beads (Santa Cruz) for 30 min at 4°C and then immunoprecipiated with EZH2 or SUZ12 antibody for 2h at 4°C. Normal rabbit IgGs (Santa Cruz) were used as a negative control.

Chromatin Binding Assay

The method of Llano *et al.* was used (Llano et al., 2006). At the indicated times, cells were divided equally in 2 tubes that were lysed for 15 min on ice in cold CSK I buffer (10 mM Pipes pH 6.8, 100 mM NaCl, 1 mM EDTA, 300 mM sucrose, 1 mM $MgCl_2$, 1 mM DTT) supplemented with 0.5% (v/v) Triton X-100, protease inhibitory cocktail (Roche) and 1 mM PMSF. After centrifugation at 500g at 4°C for 3 min, the first tube was divided in two fractions: the supernatant that contains Triton-soluble proteins and the pellet (Triton insoluble fraction) that contains chromatin-bound,

nuclear matrix-bound and insoluble proteins. Triton insoluble fraction was resuspended in RIPA buffer (150 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5% DOC, 0.1% (w/v) SDS, 1% (v/v) NP-40). The second tube was resuspended in CSK II buffer (10 mM Pipes pH 6.8, 50 mM NaCl, 300 mM sucrose, 6 mM $MgCl_2$, 1 mM DTT), treated with DNase (Promega) for 30 min followed by extraction with 250 mM $(NH_4)_2SO_4$ for 10 min at 25 °C. The sample treated with DNase and salt was centrifuged at 1200g for 6 min at 4°C and the supernatant represents the DNase-released chromatin-associated proteins. All fractions were analyzed by immunoblotting where equal amount of proteins were loaded (15μg).

Antibodies

Histone H3 (1791), H3K4me3 (8580) and SUZ12 (12073) were from Abcam while EZH2 (3147), p38 (9228), phospho-p38$^{Thr180/Tyr182}$ (9211), phosho-Ser^{376} MSK1 (9591) were from Cell Signaling. H3K27me3 (07-449), H3S28ph (07-145), H3S10ph (05-817) and Acetyl H3 (06-599) were purchased from Upstate. MSK1 (9392, 25417), MyoG (12732), p21 (6346), MHCIIB (2064) and SUZ12 (46264) were from Santa Cruz. β–tubulin (T0198) was from Sigma. *mCK* antibody was kindly provided by Hidenori Ito.

Immunofluorescence

Cells were grown on coverslips, washed in PBS, fixed in 3.7% formaldehyde/PBS (15 min, 4°C) and permeabilizaed in 0.2% Triton X-100/PBS (5 min, 4°C). The coverslips were then washed in PBS, and blocked with 3% low-fat milk/PBS for 1h at room temperature. Following overnight incubation with primary antibodies at 4°C, the coverslips were washed and incubated with secondary antibodies (Molecular Probes) for 60 minutes at 37°C, washed again and counterstained with DAPI (1 μg/μl in Vectashield, Vector). Pictures were captured using epifluorescence microscope (Leica DM6000B) using Leica Application Suite software.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed as previously described (Breiling and Orlando, 2006; with adaptations) using a crosslinking time of 10 min. Antibodies were incubated using Dynal magnetic beads (Invitrogen) overnight at 4°C. The day after, chromatin was added to antibody-beads complexes and incubated overnight at 4°C. The complexes were washed twice in Low Salt Solution, twice in High Salt Solution, once in LiCl, and once in TE buffer. DNA was extracted from beads by standard phenol/chloroform extraction, precipitated, and resuspended in 30 μl TE. To quantify the results, qPCR reactions were performed in triplicate (precipitated DNA samples as well as serially diluted input DNA) using QuantiTect SYBR Green master mix (Qiagen) on DNA Engine Opticon 2 machine (MJ Research) controlled by Opticon Monitor 2 software. C(T) values were calculating by Opticon Monitor 2 software. To calculate relative enrichment the signal from the control immunoprecipiation experiment (Mock) was subtracted from that observed with the antibody of interest. Myoblasts values (GM) were set as 1 and values from differentiated cells in DM with or without inhibitor display relative enrichment or reduction to those observed in GM. In case of ChIP with antibodies recognizing histone modifications, % of input was calculated first, and then a ratio of H3modified/H3total was calculated and set to 1 to myoblasts in GM. Values from DM show relative enrichment/reduction to those observed in GM. For CMA316 ChIP, data were presented as % of input.

Primers for ChIP Real Time-PCR:

MyoG promoter-for CCGTCCGTCCAAGACAACCC

MyoG promoter-rev CCCCCCTCTAAGCTGTTGC

mCK promoter-for CGCCAGCTAGACTCAGCACT

mCK promoter-rev CCCTGCGAGCAGATGAGCTT

mCK enhancer-for GACACCCGAGATGCCTGGTT

mCK enhancer-rev GATCCACCAGGGACAGGGTT

Size Exclusion Chromatography

Size exclusion chromatography was performed using C2C12 cell nuclear extracts on a Superose 6 PC 3.2/30 gel filtration column (GE Healthcare) on an AEKTA purifier system (GE Healthcare) in IP (300) buffer (50 mM Tris-HCl at pH 7.5, 300 mM NaCl, 5% glycerol, 0.2% Igepal [Sigma], Aprotein, Leupeptin, 100 mM PMSF, 1 mM DTT). Immuno-depletion was performed as described (Villa et al., 2007). Briefly, protein extracts were subjected to five serial depletions within 24h at 4°C using the AC22 EZH2 monoclonal antibody (Bracken et al., 2003) pre-coupled to Protein-A beads.

Histone Tail Peptides

Histone H3 peptides were synthesized in unmodified and modified form using Fmoc (N-(9-fluorenyl)methoxycarbonyl)-based solid-phase synthesis. Peptides used for kinase assays corresponded to amino acids 21-33 of H3 containing an artificial Y at the C-terminus: H3 unmodified, ATKAARKSAPATGY; H3K27me3, ATKAARK(me3)SAPATGY, H3S28ph, ATKAARKS(ph)APATGY. Peptides used for pulldown experiments corresponded to amino acids 1-40 of H3 and contained a C-terminal non-native YCK sequence with the lysine biotinylated at the e-amino group:

H3 unmodified, ARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHR-YCK (biotin);

H3K27me3, ARTKQTARKSTGGKAPRKQLATKAARK(me3)SAPATGGVKKPHR-YCK (biotin);

H3K27me3S28ph, ARTKQTARKSTGGKAPRKQLATKAARK(me3)S(ph)APATGGVKKPHR-YCK (biotin).

In vitro Peptide Kinase Assay

Recombinant MSK1 (Millipore) was used to phosphorylate H3 histone tail peptides (21-33). Kinase assays were performed according to the manufacture's protocol incubating 15 ng of MSK1 with 1 μg of peptide for 30 min at 30˚C. The reaction was stopped by adding 0.5% phosphoric acid, spotted on P81 paper, washed three times with 0.5% phosphoric acid and once with acetone. Filter circles were air-dried and counted in a scintillation counter.

Peptide Affinity Purification

For preparation of nuclear extracts, cells were lysed in buffer A (10 mM Hepes-KOH pH 7.8, 60 mM KCl, 1 mM EDTA, 1 mM DTT, protease inhibitor cocktail (Roche), 0.075 % NP-40). After incubation on ice for 15 min nuclei were pelleted and washed once with Buffer A without NP-40. The nuclear pellet was suspended in Buffer B (20 mM Tris pH 8.0, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25 % glycerol, 1 mM DTT, protease inhibitor cocktail, PhosSTOP (Roche)) and sonicated on ice in a Branson Sonifier (duty cycle 20 %, output 7.5). Extract was left on ice for 30 min before centrifugation for 15 min at 16,000 g. The supernatant was supplemented with 0.1% Triton X-100 and used for pulldown experiments.

For H3 peptide pulldown experiments, 10 μg of biotinylated histone peptides (1-40) were coupled to 50 μl streptavidin-coated paramagnetic beads in PBS/BSA (1 mg/ml) for 4 h at 4 ˚C. Beads were washed three times with PD150 (10 mM Tris pH 7.5, 150 mM NaCl, 0.1 % Triton X-100, 20 % glycerol, 1 mM DTT, protease inhibitor cocktail, PhosSTOP) to remove unbound peptides. Peptide-bound beads were incubated with nuclear extract for 2 h and washed four times with PD300. Bound proteins were eluted with SDS sample buffer, separated by SDS-PAGE and analyzed by immunoblotting.

Generation of H3S28ph specific antibody

Mice were immunized with synthetic peptide KQLATKAAR(me3-K)(phospho-S)APATGGVKC for CMA316 antibody and hybridomas were screened by ELISA using peptides listed in Kimura et al., 2008. For ELISA, microtiter plates were coated with the individual peptides conjugated with bovine serum albumin and incubated with 3-fold dilutions of each antibody. After washing with PBS, plates were incubated with peroxidase-conjugated anti-mouse IgG and washed with PBS. The colorimetric signal of tetramethylbenzidine was detected by measuring the absorbance at 405 nm using a plate reader. CMA316 was isotyped as IgG2a-k using a kit (Serotec; MMT-1). IgG was purified using a Protein A column (GE Healthcare). For immunoblotting, HeLa cells were transfected with H3-GFP (Kimura and Cook, 2001) and the amino acid substituted mutants using Lipofectamine2000 (Invitrogen) according to the manufacturer's instruction. After 2 days, 100 ng/ml nocodazole was added and further incubated for 6 hr. After washing three times with PBS, cells were lysed using 2x SDS-gel loading buffer, and total protein was separated on 13% SDS-polyacryamide gels, transferred on to PVDF membranes, and blotted with CMA316 (1:10 dilution of hybridomas culture supernatant), as previously described (Kimura et al., 2008).

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FIGURE LEGENDS

Figure 1. MSK1 activity is required for muscle differentiation.

A) Graphical representation of dynamics of C2C12 muscle cell line differentiation. Proliferative myoblasts at 80% confluency were induced to differentiate for 24-48h by replacing the growth (GM) with differentiation medium (DM).

B) Immunoblot of MSK1 and its active form (phosphorylated at Serine 376, Phospho-MSK1) was performed on whole-cell C2C12 extracts using specific antibodies. C2C12 differentiation was confirmed using antibodies against muscle cell markers (MyoG and mCK) and p38 (total and phosphorylated and activated form). p21 was used as a marker of cell cycle arrest. β-tubulin was used as a loading control. prol, proliferative myoblasts; conf, confluent myoblasts (GM); 24h and 48h indicate the time after GM to DM switch. Double bands of MyoG and p38 in differentiated cells represents their phoshorylated forms.

C) Expression of *mCK* was assayed by immunofluorescence in C2C12 cells cultured in GM and left to differentiate at 48h after the switch to DM without and with MSK1 inhibitor (5μM H89). DAPI staining visualizes DNA.

D) C2C12 myoblasts growing in GM were transfected with non-targeting siRNA (Neg ctrl) or two different siRNAs against MSK1. 48h post-transfection cells were placed in differentiation medium for additional 48h before analysis. Expression levels of MSK1, MSK2, *MyoG* and *mCK* were analyzed using Real Time-PCR. Data represent the average from three independent experiments and error bars represent standard deviation. Fold enrichment is calculated in comparison to negative control siRNA in GM.

E) Immunoblot of MSK1, MyoG and mCK was performed after knock down of MSK1 using the same siRNAs as in **D**. β-tubulin was used as loading control. Whole cell extracts were used for analysis.

Figure 2. PRC2 switches composition at muscle regulatory regions during differentiation.

A) Immunoblot of EZH2, EZH1, SUZ12, EED, H3K27me3 and MHCIIB from whole cell extracts of C2C12 cells cultured as myoblasts in GM or as myotubes in DM. β-tubulin was used as a loading control. Asterisk indicates EZH1 unspecific band. Numbers represent EED isoforms.

B) ChIP analysis of chromatin extracted from C2C12 cells cultured in GM or in DM for 48h with SUZ12, EZH2, H3K27me3 and EZH1 antibodies. The precipitated DNA fragments were amplified using primers designed within *mCK* promoter (i), *mCK* enhancer (ii) and *MyoG* promoter (iii) as shown in the schematic representation in the top panel. ChIP enrichments are presented as relative enrichment to myoblasts (GM). Levels of H3K27me3 are normalized to histone H3 density. Data are shown as mean of three independent experiments +/–SD (standard deviation).

C) Size exclusion chromatography of nuclear extracts prepared from C2C12 myoblasts cultured in GM (*left panel*) or DM 72h after induction of differentiation (*middle panel*) showing co-elution of EZH2, SUZ12, EED and EZH1 in high molecular weight fractions. The indicated fractions were analyzed by immunoblot. *Right panel:* EZH2 was depleted from C2C12 cells cultured in DM (72h after induction of differentiation) and nuclear extracts were analyzed as described previously showing formation of SUZ12, EED and EZH1 complex in differentiated myotubes independently of EZH2.

D) Immunoprecipitation (IP) of EZH2 and SUZ12 was performed from nuclear extracts (NE) of C2C12 myoblasts cultured in GM or DM (48h after differentiation induction). Immunoblot analysis showed co-immunoprecipitation of SUZ12 with all other PRC components in GM, while interaction with EZH2 in DM is lost. EZH2 bound efficiently only to SUZ12 in myoblast, and this

interaction was lost in DM. Co-immunoprecipitation of EZH2 with EED could not be properly assessed due to unspecific binding of IgG to EED antibody. Normal rabbit IgG antibody was used as a negative control.

Figure 3. Inhibition of MSK1 by H89 affects PRC2 levels, which is not due to the block of muscle differentiation.

A) Immunoblot of EZH2, EZH1, SUZ12, EED, MyoG and mCK from whole cell extracts of C2C12 myoblasts cultured in GM or DM (48h from induction of differentiation) without and with the MSK1 inhibitor (5 μM H89). β-tubulin was used as a loading control.

B) Immunofluorescence for EZH2 was performed in C2C12 myoblasts cultured in GM or DM (48h from induction of differentiation) without and with the MSK1 inhibitor (5 μM H89). The inhibitor was replaced freshly every 24h. DAPI staining visualizes DNA.

C) Expression levels of EZH2, SUZ12, EED, *MyoG* and *mCK* were measured by Real Time-PCR in C2C12 myoblasts cultured in GM or DM (48h from induction of differentiation) in the presence or in the absence of MSK1 inhibitor (5 μM H89). Transcription levels were normalized to *GAPDH* expression and represent the mean of three independent experiments, error bars represent standard deviation. Fold enrichment is calculated in comparison to myoblasts in GM.

D) Expression level of EZH2, EED, SUZ12 and muscle marker genes (*MyoG* and *mCK*) cultured in GM or DM without and with the PI3K/AKT inhibitor (10μm LY294002) were measured by Real Time-PCR in the same conditions as described in **C**.

E) Immunoblot of EZH2, EED and MyoG from whole cell extracts of C2C12 myoblasts cultured in GM or DM for 48h in the presence or in the absence of PI3K/AKT inhibitor (10μM LY294002). β-tubulin was used as a loading control.

Figure 4. Depletion of MSK1 leads to PRC2 chromatin retention.

A) Schematic representation of different phases of the chromatin-binding assay. Three main fractions were recovered, Triton-soluble proteins (cytoplasmic), Triton-insoluble proteins (nuclear not chromatin bound) and chromatin bound.

B) C2C12 myoblasts were transfected in GM with non-targeting siRNA (Neg ctrl) or siRNA against MSK1. 48h post-transfection cells were cultured in GM (*left panel*) or DM (*right panel*) for additional 48h before harvesting. Enrichment of MSK1, EZH2 and SUZ12 in the different fractions was tested by immunoblot. Histone H3 served as control for chromatin bound proteins, β-tubulin served as marker for Triton-soluble proteins and MyoG for Triton-insoluble proteins and differentiation induction.

Figure 5. MSK1-dependent H3S28 phosphorylation affects PRC2 dynamics at muscle regulatory regions.

ChIP analysis was performed on chromatin extracted from C2C12 cultured in GM or DM 48h from the induction of differentiation without and with MSK1 inhibitor (5μM H89) using EZH2, SUZ12, H3K27me3, H3S28ph, MSK1 and acetyl H3 antibodies. The precipitated DNA fragments were subjected to Real Time-PCR analysis with the primers amplifying *mCK* promoter **(A)**, *mCK* enhancer **(B)** and *MyoG* promoter **(C)**. Levels of H3K27me3, H3S28ph and AcH3 are normalized to histone H3 density. The values represent the mean of three independent experiments. Error bars represent standard deviations.

Figure 6. Binding of EZH2, SUZ12 and EED, but not EZH1 is affected by MSK1-mediated H3S28ph.

A) Immunoblot of different histone H3 modifications in C2C12 cultured in GM or DM 48h from induction of differentiation without or with H89 (5μM).

B) Recombinant MSK1 was incubated with unmodified histone H3 peptide, H3K27me3 and H3S28ph and kinase assay was performed.

C) Nuclear extracts from C2C12 cultured in GM or in DM 48h from induction of differentiation were incubated with peptides representing either unmodified H3, or H3 trimethylated at Lysine 27 (H3K27me3) or peptide that is both trimethylated at Lysine 27 and phosphorylated at Serine 28 (H3K27me3S28p). Binding of EZH2, SUZ12, EZH1 and EED was tested by immunoblot.

D) Nuclear extracts from C2C12 cultured in DM 48h from the induction of differentiation were incubated with the same peptides as in C and the binding of EZH2 and EZH1 was tested by immunoblot.

Inventory of supplemental information

Figure S1. This figure supports the evidence that MSK1 regulates muscle differentiation (figure 1) by showing phase contrast microscopy of cells with impaired differentiation potential upon treatment with an MSK1 inhibitor (H89).

Figure S2. This figure provides RNA levels of Polycomb proteins during muscle differentiation in support of figure 2 where protein levels are shown.

Figure S3. This figure supports figure 3 by showing the phase contrast microscopy of cells treated with PI3K/AKT inhibitor (LY294002) whose Polycomb protein levels are shown in figure 3.

Figure S5. This figure provides evidence additional to figure 5 that H3S28ph coexists with H3K27me3 on muscle specific genes with the use of an antibody (CMA316) validated to bind the double mark.

Figure S6. This figures shows the levels on muscle genes upon H89 of H3S10ph whose global levels during muscle differentiation are shown in figure 6.

Experimental procedures

Real time qPCR and ChIP primers sequences are detailed in this section. We have been very detailed about experimental procedures in the main text. However we can transfer detailed material and methods into supplemental information at a further stage in order to shorten the main text.

Supplemental figure legends

Figure S1. Inhibition of MSK1 kinase by H89 blocks muscle differentiation. Proliferative myoblasts were induced to differentiate for 48h by replacing the GM with DM in the presence or in the absence of MSK1 inhibitor (5 and 10μM H89). Effect of H89 treatment on muscle differentiation was evaluated by phase contrast microscopy.

Figure S2. Differential expression of PcG proteins during muscle differentiation. Expression levels of EZH2, SUZ12, EZH1, EED and MHCIIB was measured by Real Time PCR in C2C12 myoblasts grown in GM or DM 24h, 48h and 72hr after induction of differentiation. The transcription levels were normalized to GAPDH expression and represent the mean of three independent experiments +/-SD (standard deviation). prol, proliferative myoblasts in growth medium (GM); conf, confluent myoblasts in growth medium (GM); DM, differentiation medium.

Figure S3. Inhibition of PI3K/AKT pathway by LY blocks muscle differentiation. Proliferative myoblasts were induced to differentiate for 48h by replacing the GM with DM in the presence or in the absence of PI3K/AKT inhibitor (10μM LY294002). Effect of LY treatment on muscle differentiation was evaluated by phase contrast microscopy.

Figure S5. Characterization of CMA316 mouse monoclonal antibody directed against phosphorylated histone H3 at Serine 28 (H3S28ph). A) Specificity of mAb was evaluated by ELISA. Microtiter plates were coated with the indicatedpeptides and incubated with 3-fold dilutions of each mAb (starting from 1:300 dilution of a hybridoma culture supernatant). CMA316 reacts with H3s28phh regardless of the modification status of H3K27. B) HeLa cells were left untransfected (lanes 1 and 2) or transfected with H3-GFP (lane 3) and its

phosphorylation-site mutants (lanes 4-7), and treated with nocodazole to enrich for mitotic cells(lanes 2-7). Whole cell proteins were separated on 13% SDS-polyacryamide gels, transferred on to PVDF membranes and blotted with CMA316 antibody that detect single bands at the size of histone H3 (lane 1). Signal intensity is increased in nocodazole-treated, M-phase-enriched, cell population (lane 2). A mutant H3-GFP, which harbors S28A substitution, was not detected by these antibodies (lane 7). C) ChIP analysis was performed on chromatin extracted from C2C12 cultured in GM or DM for 24h after induction of differentiation using CMA316 antibody. The precipitated DNA fragments were subjected to Real Time-PCR analysis with the primers amplifying mCK enhancer and MyoG promoter. ChIP enrichment is normalized to histone H3 density. The values represent the mean +/SD (standard deviation) of two independent experiments.

Figure S6. Levels of H3S10ph are not enriched on mCK and MyoG during muscle differentiation. ChIP analysis of mCK enhancer (i), mCK promoter (ii) and MyoG promoter (iii) was performed on chromatin extracted from C2C12 myoblasts cultured in GM and DM for 48hr after induction of differentiation using H3S10ph antibody. Levels of H3S10ph are normalized to histone H3 density. The precipitated DNA fragments were subjected to Real Time-PCR analysis. The values represent the mean +/-SD (standard deviation) of three independent experiments.

Experimental procedures

Primers for Real Time-PCR:

MyoG-for GCAGCGCCATCCAGTACATTG

MyoG-rev GCAACAGACATATCCTCCACCG

SUZ12-for GTGCCTTTGAATCCTGACCTC

SUZ12-revCTTCGCGATTTCGTTTTCTTC

EZH1-for...AGACTCAGTGCAATACCAAGCA

EZH1-rev AGTTTTTGCAAGACACCACCTT

EZH2-for TGATAAAGAAACTTGCCCACCT

EZH2-rev...CTTTGCTCCCTCTGAACAGATT

EED-for ATAACCAGCCATTGTTTGGAGT

EED-rev TGTTGCTATCATAGGTCCATGC

MSK1-for GCCGATGAAACTGAAAGAGC

MSK1-rev TGCTCATTTCCTGGGGATAC

MSK2-for TCACACTGCACTACGCCTTC

MSK2-rev GATACCCAGCTTGTGCAGGT

Primers for ChIP Real Time-PCR:

MyoG promoter-for CCGTCCGTCCAAGACAACCC

MyoG promoter-rev CCCCCCTCTAAGCTGTTGC

mCK promoter-for CGCCAGCTAGACTCAGCACT ù

mCK promoter-rev CCCTGCGAGCAGATGAGCTT

mCK enhancer-for GACACCCGAGATGCCTGGTT

mCK enhancer-rev GATCCACCAGGGACAGGGTT

Stojic et al.

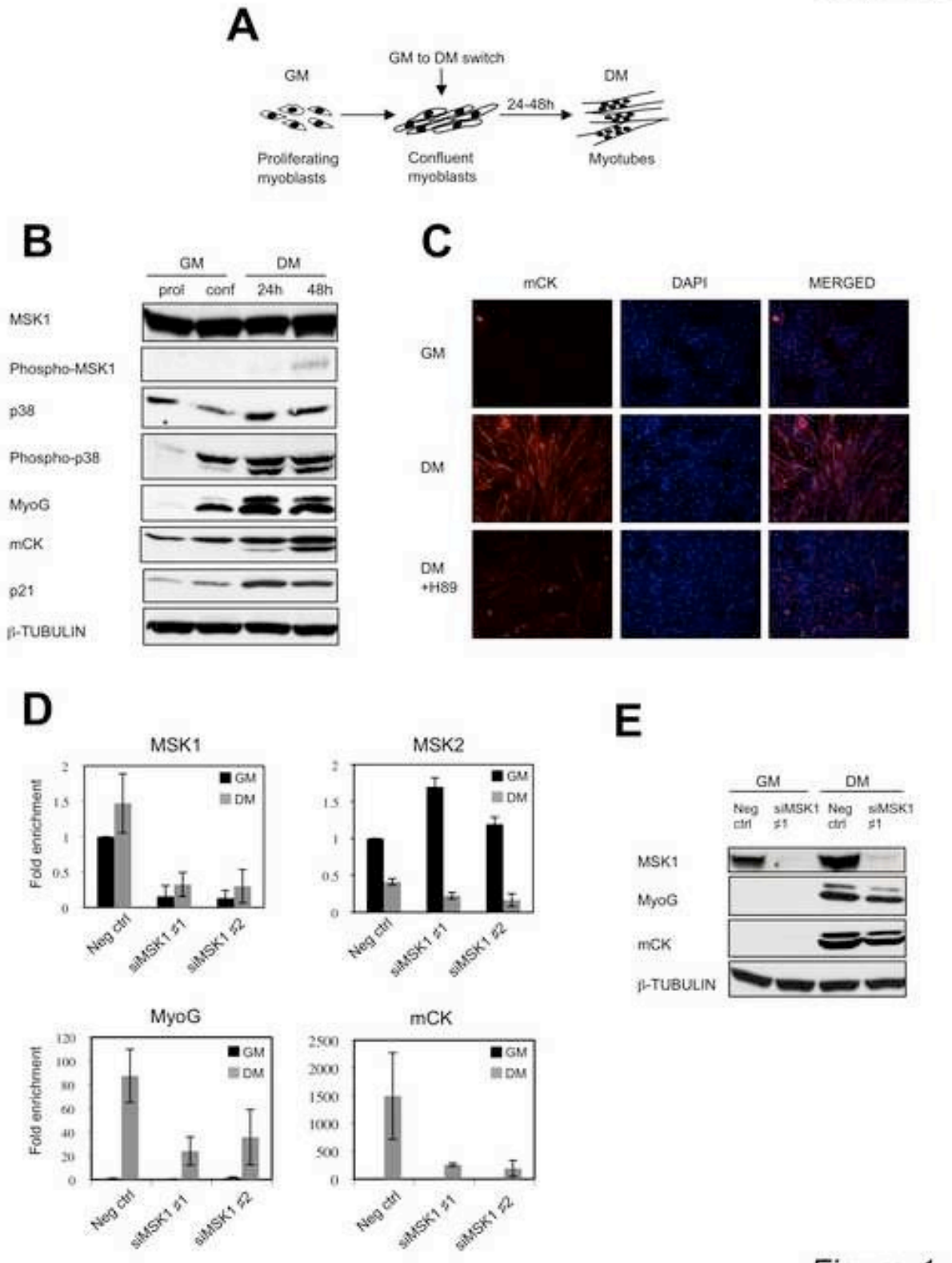

Figure 1.

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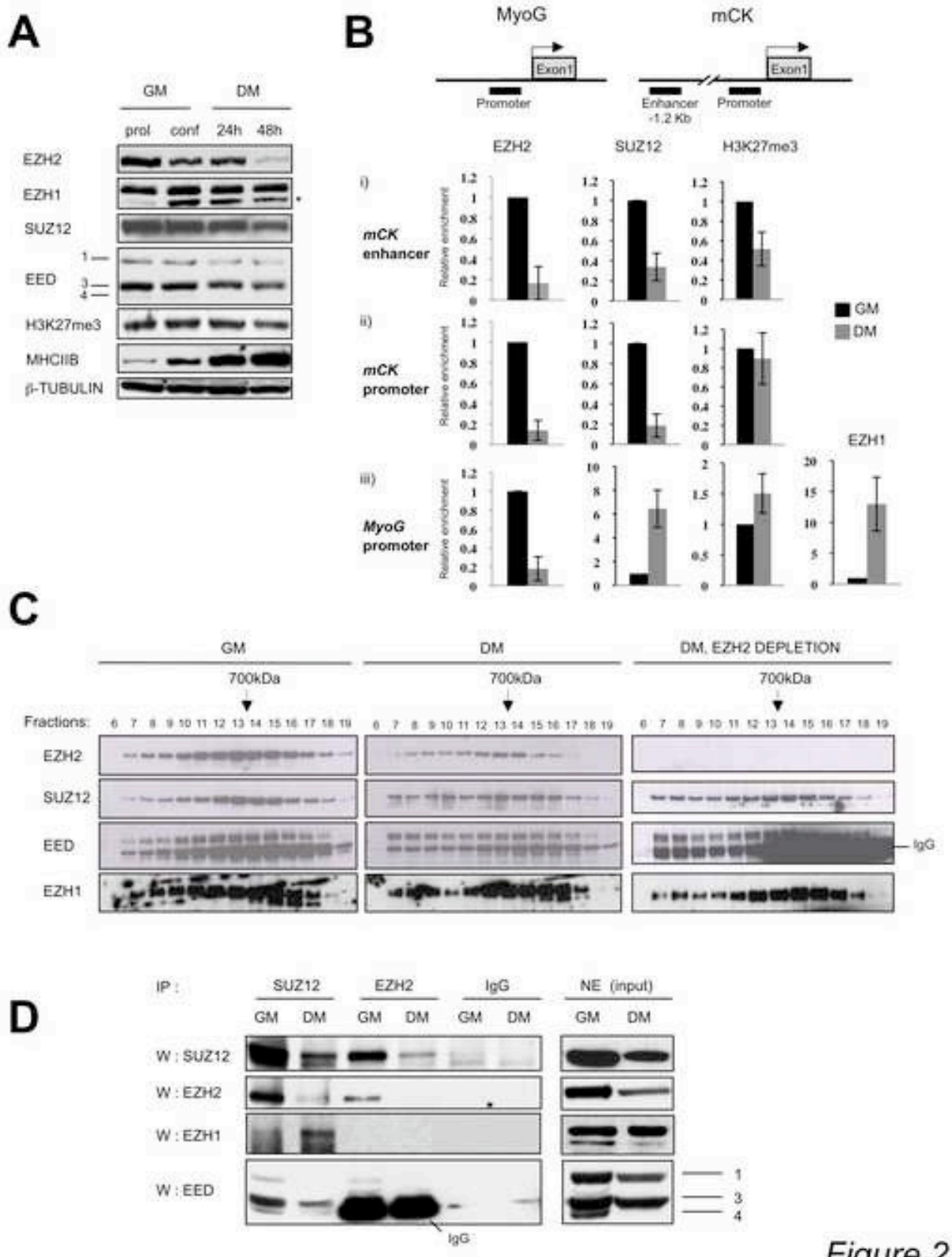

Figure 2.

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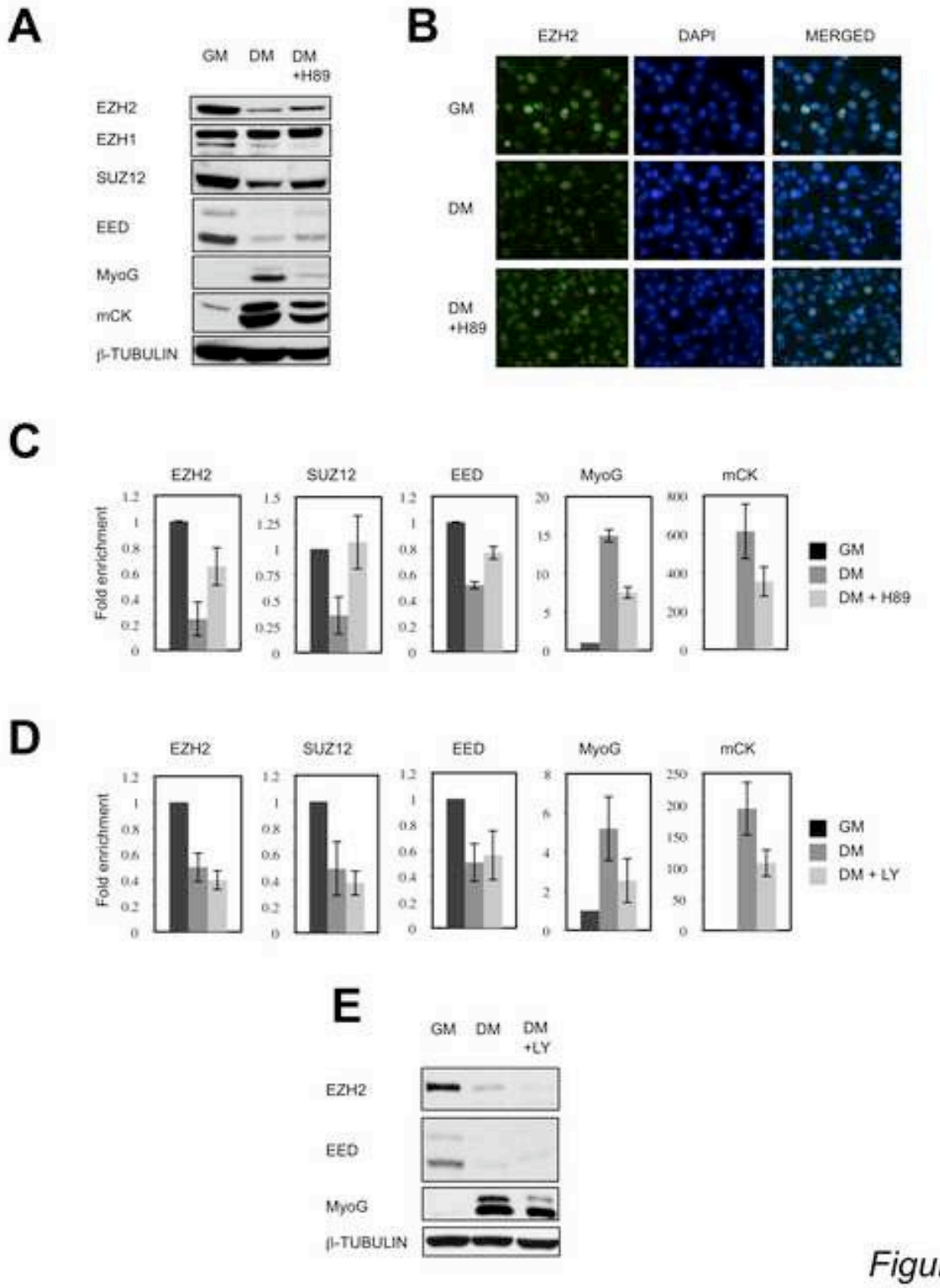

Figure 3.

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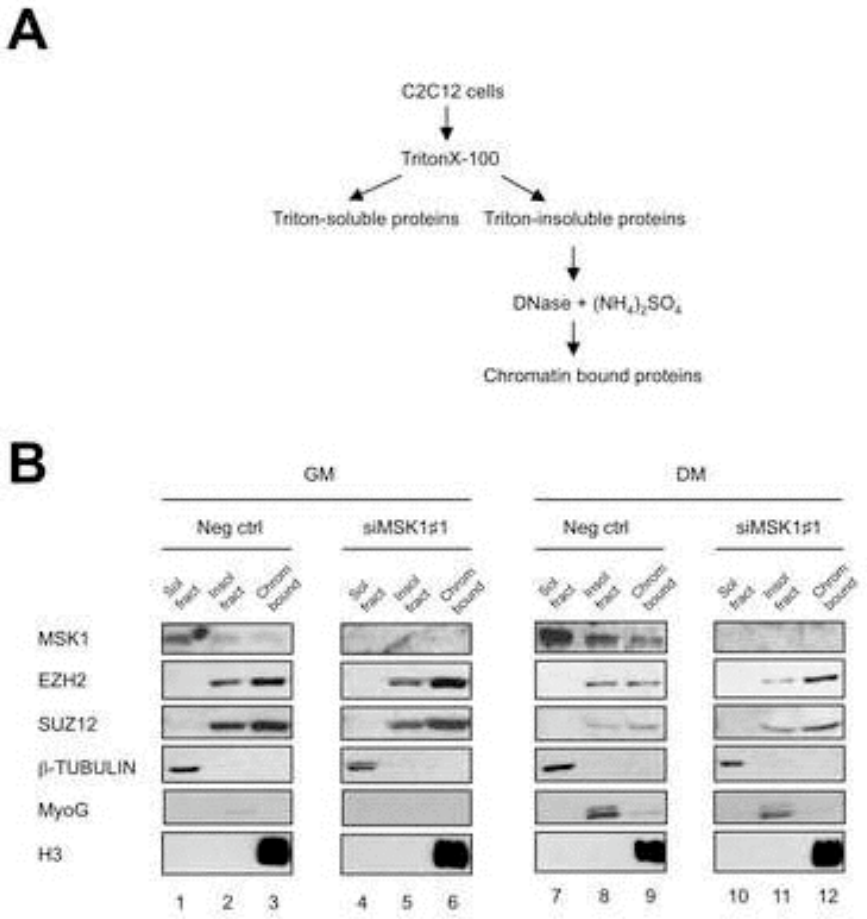

Figure 4.

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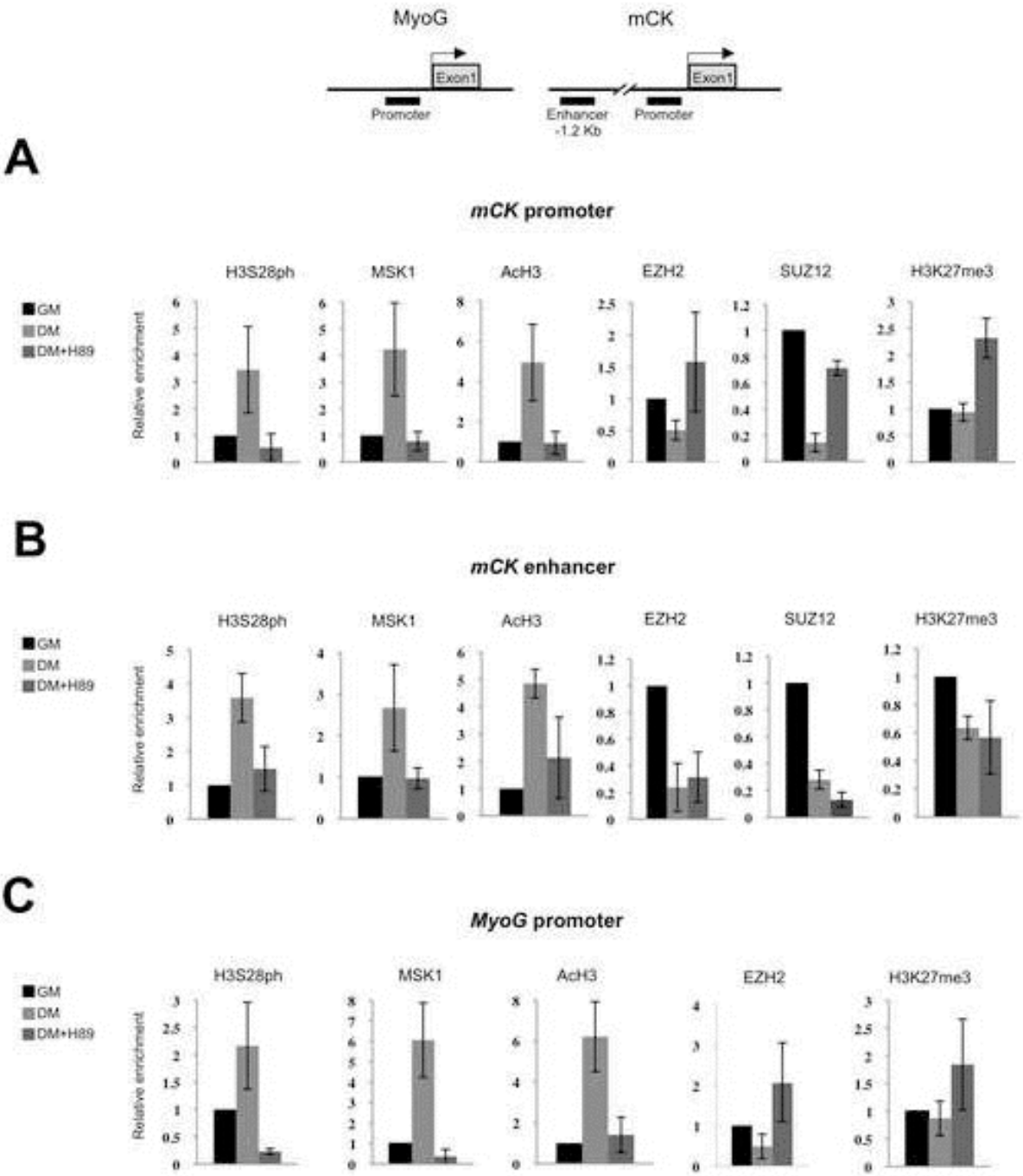

Figure 5.

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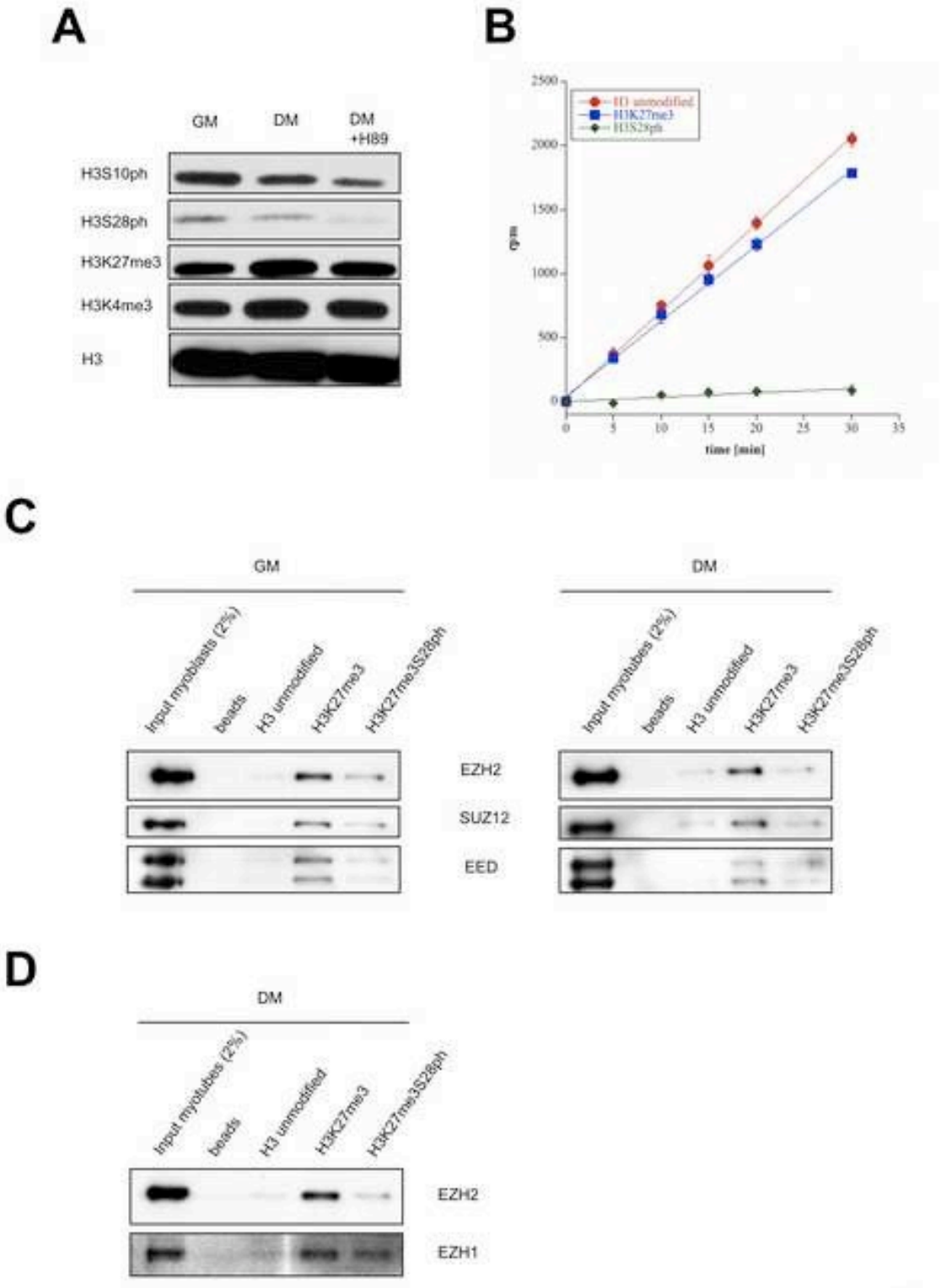


Figure 6.

SUPPLEMENTAL INFORMATION

Figure S1

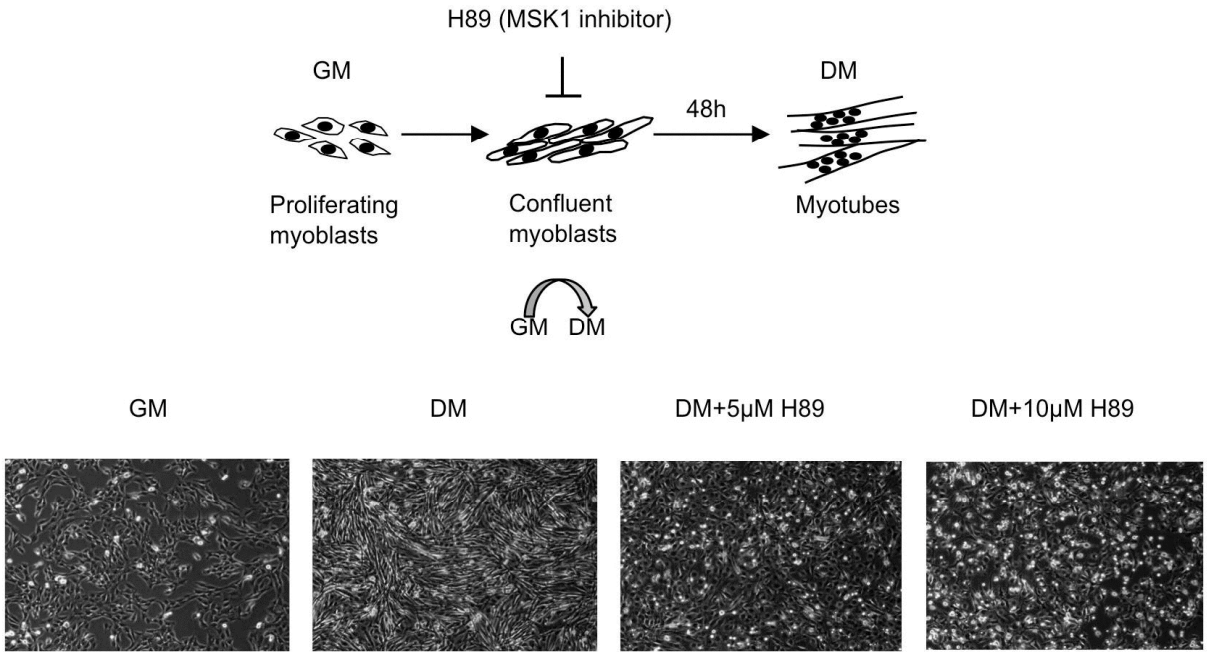


Figure S2

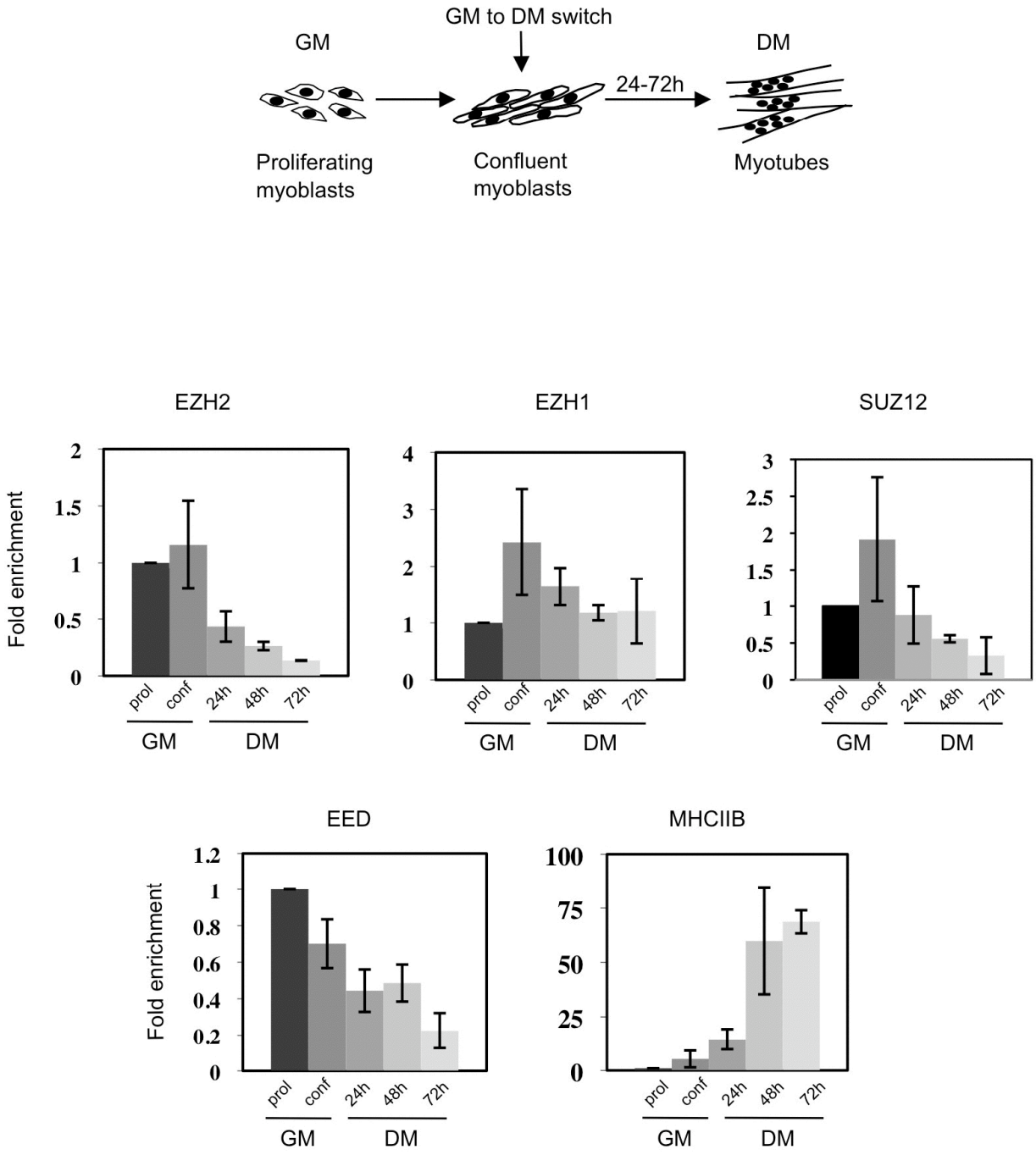

GM
GM to DM switch
DM
24-72h
Proliferating myoblasts
Confluent myoblasts
Myotubes
EZH2
EZH1
SUZ12
EED
MHCIIB
Fold enrichment
prol
conf
24h
48h
72h
GM
DM

Figure S3

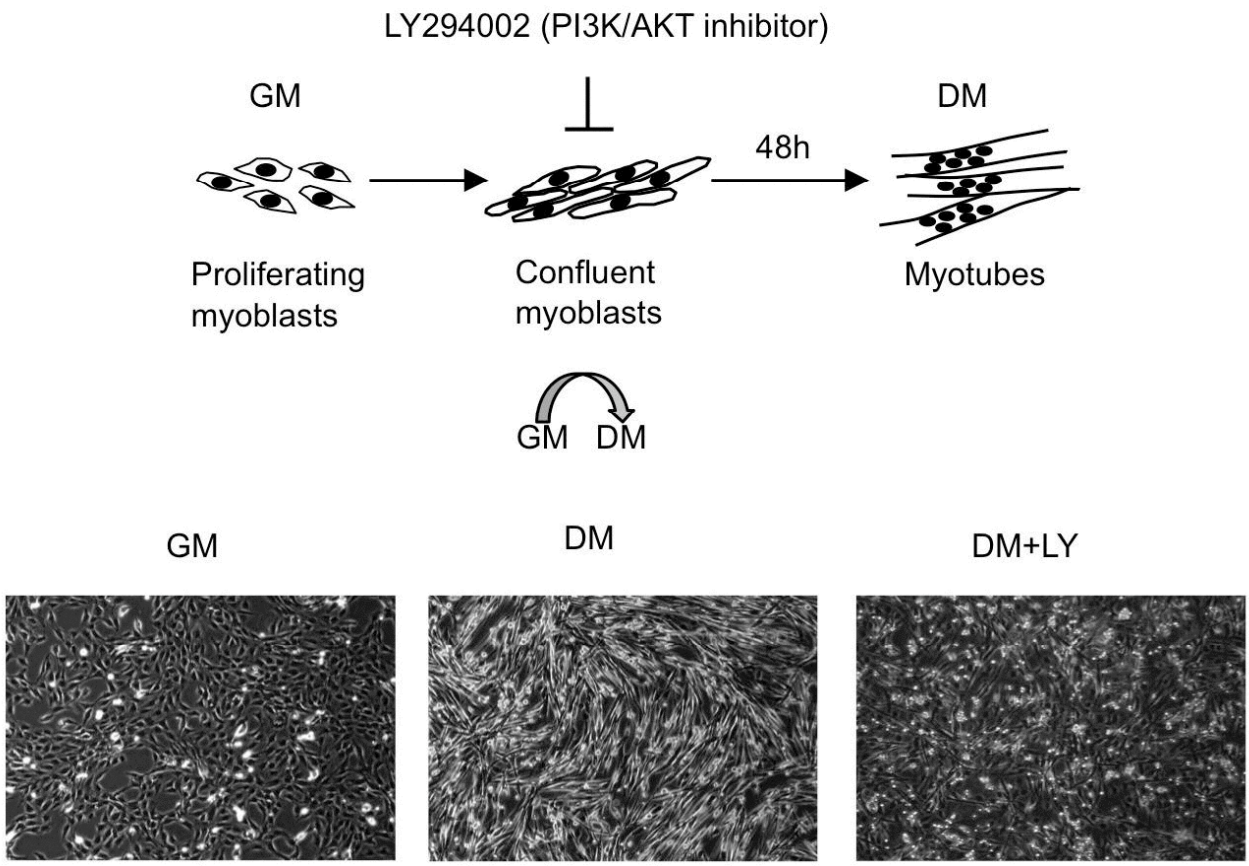

LY294002 (PI3K/AKT inhibitor)
GM
DM
48h
Proliferating myoblasts
Confluent myoblasts
Myotubes
GM DM
GM
DM
DM+LY

Figure S5

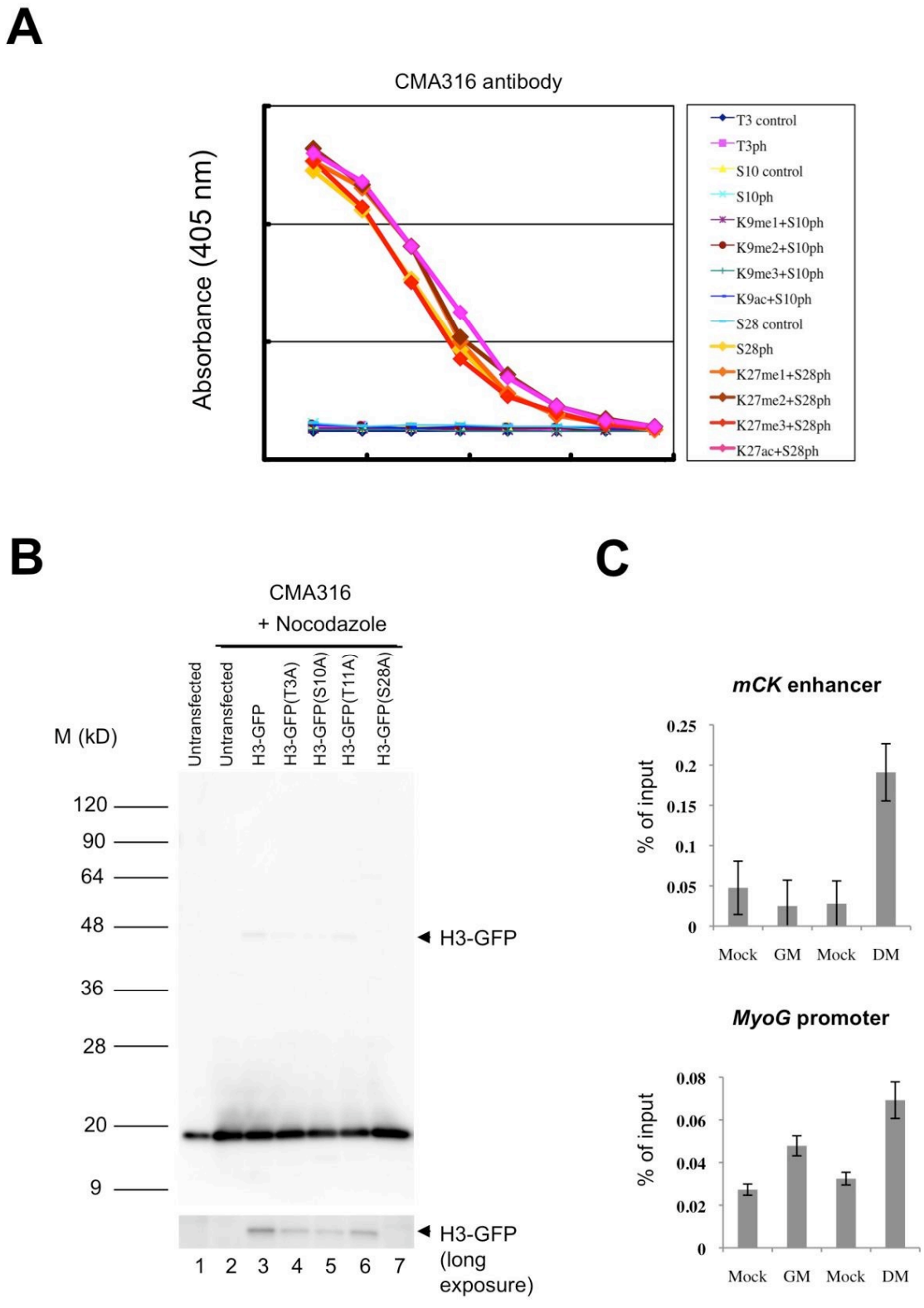
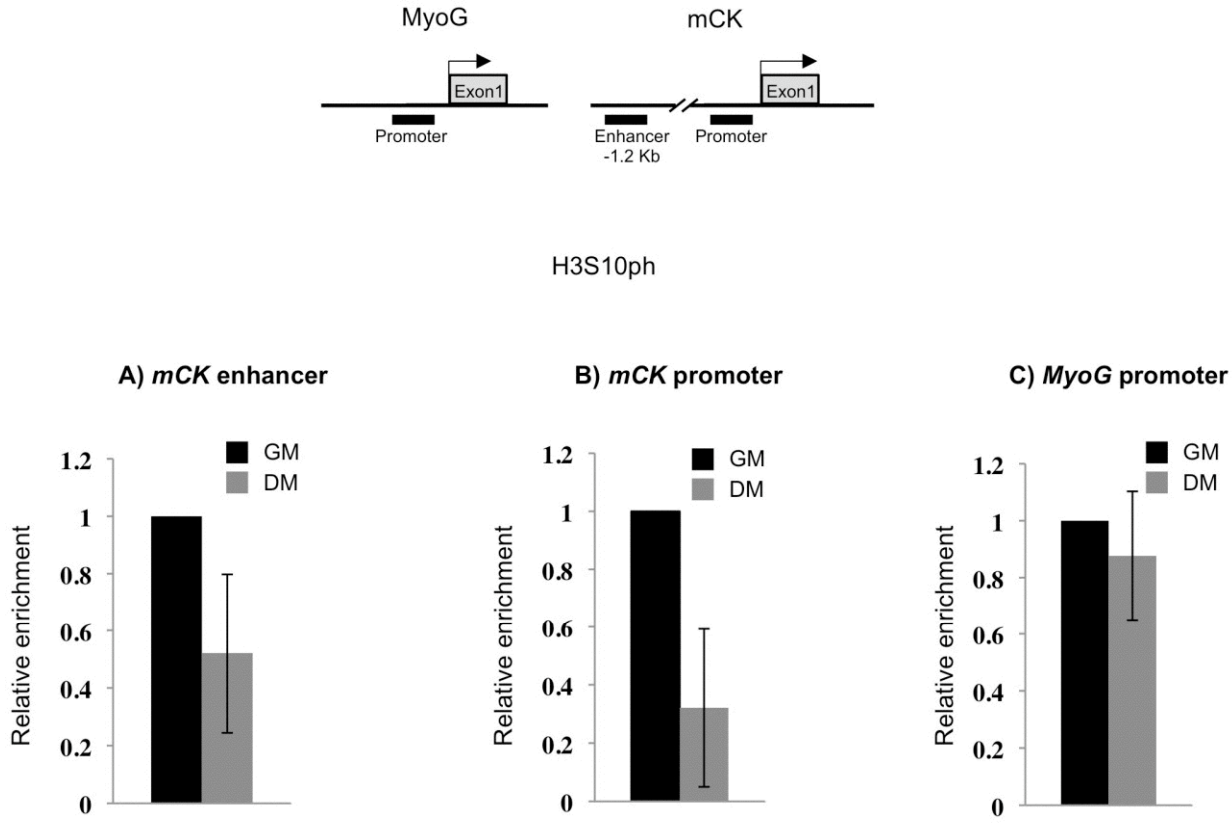

A
CMA316 antibody
Absorbance (405 nm)
T3 control
T3ph
S10 control
S10ph
K9me1+S10ph
K9me2+S10ph
K9me3+S10ph
K9ac+S10ph
S28 control
S28ph
K27me1+S28ph
K27me2+S28ph
K27me3+S28ph
K27ac+S28ph
B
CMA316
+ Nocodazole
Untransfected
Untransfected
H3-GFP
H3-GFP(T3A)
H3-GFP(S10A)
H3-GFP(T11A)
H3-GFP(S28A)
M (kD)
120
90
64
48
36
28
20
9
H3-GFP
H3-GFP
(long
exposure)
1 2 3 4 5 6 7
C
mCK enhancer
% of input
0.25
0.2
0.15
0.1
0.05
0
Mock GM Mock DM
MyoG promoter
% of input
0.08
0.06
0.04
0.02
0
Mock GM Mock DM

Figure S6


MyoG
mCK
Exon1
Exon1
Promoter
Enhancer
-1.2 Kb
Promoter
H3S10ph
A) *mCK* enhancer
B) *mCK* promoter
C) *MyoG* promoter
GM
DM
Relative enrichment
1.2
1
0.8
0.6
0.4
0.2
0