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Polycomb protein Ezh2 controls rhabdomyosarcoma formation

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Summary

ABSTRACT

Abstract

Rhabdomyosarcoma (RMS) is a sarcoma more frequent in children, which arises from the disruption of regulatory mechanisms that leads to the myogenic phenotype. RMS cells have low levels of muscle differentiation markers. Ezh2 is a histone methyltransferase part of the PRC2 complex and at the early stage of myogenesis it silences muscle specific genes (even MyoD target genes) consenting the proper timing of muscle specific gene expression. During skeletal differentiation in myoblasts, Ezh2 displaces from myogenic regulatory regions and MyoD dependent transcription is co-activated by the complex Cdk9/CycT2. The cyclin dependent kinase 9 and one of its cyclin partners, cyclin T2, are MyoD interacting proteins that together form a complex whose main activity is phosphorylating the carboxyl terminal domain of RNApolII on muscle specific promoters allowing stabilization of the nascent transcripts. Surprisingly, its kinase activity is abrogated in RMS. Our results show that Ezh2 depletion in a human rhabdomyosarcoma cell line causes a partial reactivation of the myogenic program, more in particular a reactivation of MyoD target genes such as myosin heavy chain (MyH) and myogenin (Myog) and, in addition these data correlate with an increase in the elongating RnapolII at Myog and Myh promoters. Further analyses demonstrated the restoration of Cdk9 occupancy at Myog and Myh promoters in RD-Ezh2-KD cells providing evidence for an inhibitory effect of EZh2 on Cdk9/CycT2 kinase activity in RMS.

Chapter 1

INTRODUCTION

1.1. Rhabdomyosarcoma: pathology and molecular pathogenesis

Rhabdomyosarcoma (RMS) is a rare soft tissue sarcoma, more frequent in children, which accounts for 3-4% of childhood cancers. It is caused by the disruption of regulatory mechanisms that lead to the myogenic phenotype in primitive mesenchymal stem cells. The annual incidence of this kind of tumor in children, adolescents, and young adults under the age of 20 is 4.3 cases per one million population and two-thirds of the cases are diagnosed in children younger than 6 years of age. 5-year overall survival rate is 68,9% (Okcu *et al*., 2011). The etiology of the disease is still unknown, but some familial syndromes have been associated with it such as neurofibromatosis, the Li-Fraumeni, Beckwith-Wiedemann, and Costello syndromes (Okcu *et al*., 2011; Xia *et al*., 2002). RMS comprises two histological subtypes, alveolar (aRMS) and embryonal (eRMS), each of them with different prognosis and various genetic and molecular alterations. As an example, 80% of aRMS harbor a translocation with fuses the 5' end of PAX3 or PAX7 (transcription factors that initiate myogenesis in muscle stem cells) with the 3' end of the FOXO1A gene. The resulting chimeric protein is a potent transcription factor that stimulates myogenesis and resists apoptosis (Jain *et al*., 2010; Linardic, 2008). The eRMS, on which we focused our attention, typically occurs in the head, neck and genitourinary sites and is associated with loss of heterozigosity on the short arm of chromosome 11 (the 11p15.5 region) which codify for various tumor suppressing genes (Loh *et al*., 1992; Koi *et al*., 1993) imprinted in physiological but not in pathologic conditions. One of them is the CDKN1C (p57/KIP2) gene, expressed during development in several tissues, including skeletal muscle, which encodes a cyclin-dependent kinase inhibitor.

Comparative genomic hybridization (CGH) and fluorescent in situ hybridization studies (FISH) have also identified in eRMS gains and losses in other chromosomes. Moreover, many studies support alterations of different pathways such as the pRb, the Ras, and the p53 pathways (Xia *et al*., 2002). On the basis of its genetic alterations, in rhabdomyosarcoma there is evidence of deregulation in the mechanisms controlling the cell cycle.

Rhabdomyoblasts appear like partially differentiated cells showing nuclear expression, to varying degrees, of myogenic nuclear regulatory proteins like myogenin and MyoD (Sebire and Malone, 2003), whose immunohistochemical analyses are necessary for the diagnosis of the tumor (Dias *et al*., 1990, 2000; Morgenstern *et al*., 2008). RMS cells have low levels of other late differentiation markers. Cluster analysis and comparison of the expression profiles of normal muscle, RMS, and fetal muscle confirmed the high degree of similarity between the fetal muscle and RMS transcriptomes. Nevertheless, gene and epigenetic alterations could further contribute to maintaining the proliferative state of RMS and consequently prevent myogenic differentiation (Schaaf *et al.,* 2005).

1.2. An overview of skeletal muscle differentiation and correlated critical points in rhabdomyosarcoma

Myoblasts are committed-muscle cell precursors that produce myogenic transcription factors. Until growth factors are present in the environment myoblasts proliferate without differentiating. When these factors are depleted, the myoblasts stop dividing and leave the cell cycle. The consequent step is the alignment of myoblasts together into chains that ends with cells fusion and finally multinucleated myotubes formation (Gilbert, 2000).

Several myogenic transcription factors such as MyoD, Myf5, myogenin (Myog), MRFs, coordinate together skeletal muscle differentiation. They belong to the family of bHLH myogenic transcription factors as they share the basic-helix-loop-helix regulatory motif. The basic domain is required for DNA binding of promoter binding sites termed E-boxes whereas the HLH domain is required for dimerization.

Among muscle specific transcription factors, MyoD is a master regulator of the myogenic program and upon condition permissive to myogenesis it initiates skeletal muscle differentiation. MyoD heterodimerizes with other bHLH proteins, such as the E2A gene products E12 and E47, also called E-proteins, to recognize its numerous target genes (Lassar *et al.,* 1991; Puri and Sartorelli, 2000). High-throughput ChIP-Seq analysis performed in differentiating myoblasts indicate that MyoD binds to several sites throughout the genome, although MyoD activity is regulated and MyoD occupancy at promoters doesn't always correlate with transcription (Aziz *et al.,* 2010). MyoD also recruits HATs on chromatin such as P300 and PCAF, and after the acetylation of MyoD itself and of lysine residues on histones, mediates the recruitment of the complex SWI/SNF and other myogenic transcription factors on muscle specific promoters, thereby inducing transcription of regulatory and structural muscle specific genes (Eckner *et al.,* 1994; Eckner *et al.,* 1996; Puri and Sartorelli, 2000; Iezzi *et al.,* 2002).

MRFs instead collaborate with MEF2 transcription factors family to activate muscle gene expression (Sartorelli and Caretti, 2005).

RMS is a suitable model to study pathological myogenesis because cells fail to undergo terminal differentiation. The transition from the G1 to the S phase of the cell cycle is regulated by cyclin dependent kinases (Cdks) coupled with their cyclin partners. The

major substrates of Cdks are the retinoblastoma tumor suppressor protein pRb and its related proteins, p107 and p130 (also named RB family proteins). Ipophosphorylated pRb binds the E2F transcription factor on the regulatory regions of its target genes in myoblasts repressing transcription from genes involved in DNA synthesis. When Cdks phosphorylate pRb, it detaches from its binding sites allowing transition to the S phase of the cell cycle (Sidle *et al.,* 1996).

During terminal myogenesis Cdks activities are downregulated and terminal differentiation correlates with cell cycle arrest (Franklin and Xiong, 1996). Cyclin-dependent kinase inhibitors (CdkIs) induce dephosphorylation of pRb by inhibiting the binding of cyclin-dependent kinases CDK4 and CDK6 to cyclin D1, resulting in G1 growth arrest (Serrano *et al.*, 1993).

The RD cell line is a human cell line of embryonal rhabdomyosarcoma whose model is currently used to study pathological myogenesis. RD cells maintain elevated levels of Cdk4, Cdk6 and their cyclin partners in conditions favoring differentiation compared to myoblasts. In fact, in RD cells the concomitant alterations of CdkIs activities preclude to attenuate them and fail to arrest the cell cycle (Knudsen *et al.,* 1998).

1.3. Role of the Cyclin dependent kinase 9 in muscle development

The Cyclin-Dependent Kinase 9 (Cdk9) is a member of the Cdks family widely expressed at high levels in terminally differentiated cells in all human and murine tissues (Bagella *et al*., 1998, 2000; Simone *et al.,* 2002). Cdks form heterodimers with their cyclin partners, regulating cell cycle progression and transcription. The heterodimer deriving from the association of Cdk9 and the cyclin of the T (T1 and T2a or T2b) and K families is the main component of the positive transcription elongating factor (P-TEFb) which stabilizes the

transcription machinery and RNA polymerase II (polII) on genes regulatory regions, displacing negative effectors like the negative transcription elongating factor (N-TEF) and allowing the elongation of the nascent RNA. The main role of the Cdk9/CycT complex is to phosphorylate the carboxyl terminal domain (CTD) of the RNA PolII (Fig.1) (reviewed from Romano and Giordano 2008).

Cdk9 has two isoforms named $Cdk9_{42}$ and $Cdk9_{55}$ on the basis of their molecular weight. $Cdk9_{55}$ differs from $Cdk9_{42}$ in 117 additional amino acid residues at the amino terminus region, in frame with the $Cdk9_{42}$ coding sequence (Shore *et al.,* 2003). Although the two isoforms are differentially expressed in tissues and particular stimuli can affect the ratio $Cdk9_{55}$ to $Cdk9_{42}$ (like in activated macrophages or in hepatocytes in culturing conditions (Shore *et al.,* 2005), $Cdk9_{55}$ is very similar to $Cdk9_{42}$ in many respects: ability to associate with cyclins, association with 7SK and HEXIM1 (that regulate kinase activity), and kinase

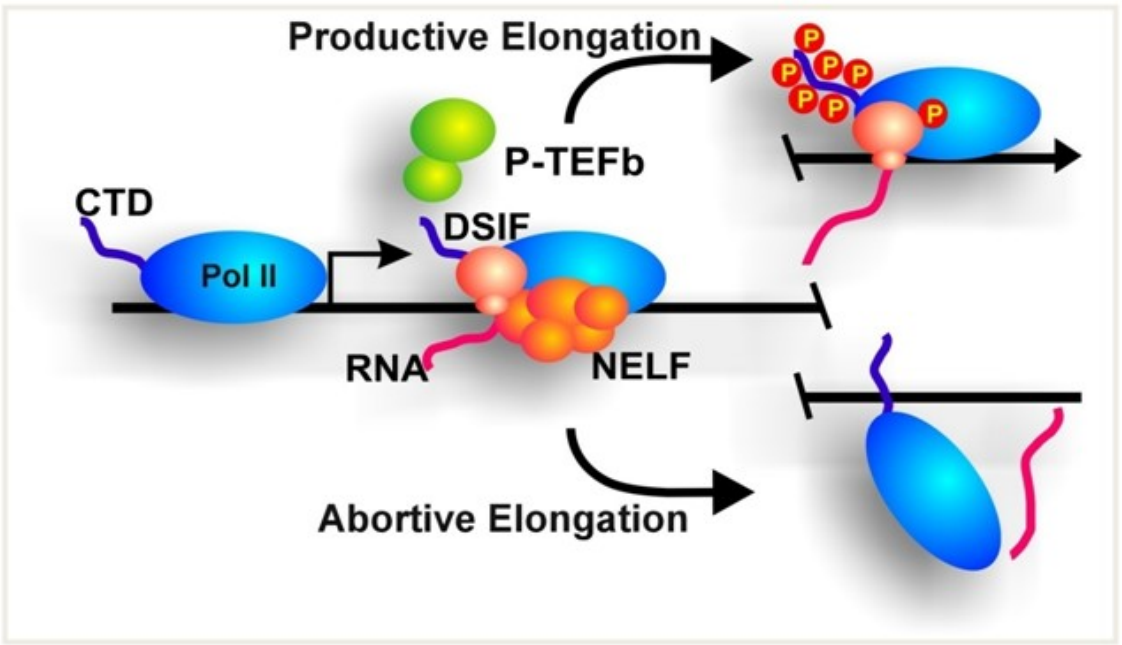

Fig.1. RNA polymerase II comes under the control of negative elongation factors (DSIF and NELF) shortly after initiation. PTEFb (also named CDK9/CyCT) mediates a transition into productive elongation by phosphorylating the CTD of the large subunit of RNA polymerase II.

The CTD of RNApolII is evolutionarily conserved, and in fungi, plants and animals it comprises from 25 to 52 tandem copies of the consensus repeat heptad $Y_1S_2P_3T_4S_5P_6S_7$ (Fig.2) (Corden, 1990). Phosphorylation occurs on Ser2 and Ser5 of the repeats (Dahmus, 1994; Marshall *et al*., 1996; Zhou et al., 2001; Sano *et al.,* 2002), and phosphorylation state changes as RNAPolII progresses through the transcription cycle (Weeks *et al.,* 1993; Dahmus, 1994). This way modification of the CTD by the Cdk9/CycT complex contributes to the activation of transcriptional events, and different states of the CTD are characteristic of different transcriptional stages. Thus, RNApolII phosphorylated at Ser2 (Ser2P CTD) and Ser5 (Ser5P CTD) is in the active state and its presence on promoter regions correlates with active transcription.

Fig.2. Heptapeptide repeat of RNApolII CTD. Yellow circles indicate Cdk9/CycT phosphorylation sites on Ser2 and Ser5. Tyr=tyrosine, Ser= serine, Pro=proline, Thr=threonine.

The Cdk9/CycT complex is recruited on its target genes by two different mechanisms: gene-specific recruitment through interactions with transactivators and indirect recruitment via the interaction between P-TEFb and Brd4. Several transcription factors have been reported to bind directly to P-TEFb and recruit the complex on specific promoters. P-TEFb was initially identified as the cellular cofactor for transactivator (Tat) protein of the human immunodeficiency virus type 1 in human cells (Lin *et al.,* 2002).

Several reports have demonstrated the interaction of Cdk9 and Cyclin T1 with factors such as NF-kB, androgen receptor, HIC, aryl hydrocarbon receptor, c-myc, and pRb which activate transcription by specific regulatory regions (Grana, X., *et al.,* 1994; Barboric *et al*., 2001; Lee *et al.,* 2001; Eberhardy and Farnham 2002; Simone *et al.,* 2002; Kanazawa *et al*., 2003; Young *et al*., 2003; Tian *et al*., 2003).

Further studies also suggest an important role of Cdk9 coupled with its cyclin partners in skeletal muscle development where Cdk9 exerts its activity in synergy with myogenic transcription factors. For example, Cdk9/CycT1 selectively enhances myocyte enhancer factor 2 (Mef2c) dependent transcription in murine myoblasts induced to differentiate where Cdk9/CycT1 and Mef2c co-occupy the promoters of MEF2c target genes (Nojima M *et al.,* 2008) 24h after induction of myogenesis. Similarly, Cdk9 together with cyclin T2a or T2b activates MyoD dependent transcription in myoblasts triggered to differentiate and the complex Cdk9/CycT2 binds MyoD through its bHLH region (Simone *et al.,* 2002a). Overexpression experiments of CDK9 and CycT2 increase the rate of the myogenic program while ectopic expression of dominant negative mutants of Cdk9 impairs muscle specific expression, such as myosin heavy chain (MyH) and muscle creatine kinase (MKC) levels (Simone *et al.,* 2002b). In addition, Cdk9/CycT2 occupies the Myog promoter and the enhancer of muscle creatine kinase during muscle differentiation and this event correlates with phosphorylation of the CTD of RNapolII (Giacinti *et al.,* 2006). Besides its kinase activity, it is important to stress that Cdk9 participates to the formation of a multimeric complex on myogenic gene regulatory regions containing MyoD, Mef2, histone acetyl transferases (HATs), like p300, PCAF and SWI/SNF besides several enzymes. This complex induces chromatin remodeling and promotes gene expression through the

acetylation of specific lysine residues on histones H3 and H4 and phosphorylation of the RNA polymerase II CTD (Fig.3) (Puri and Sartorelli 2000; Giacinti *et al.,* 2006). Significantly, cyclin T1 was not detected on the same regions, suggesting a Cyclin T2-depedent Cdk9 activation (Giacinti *et al.,* 2006).

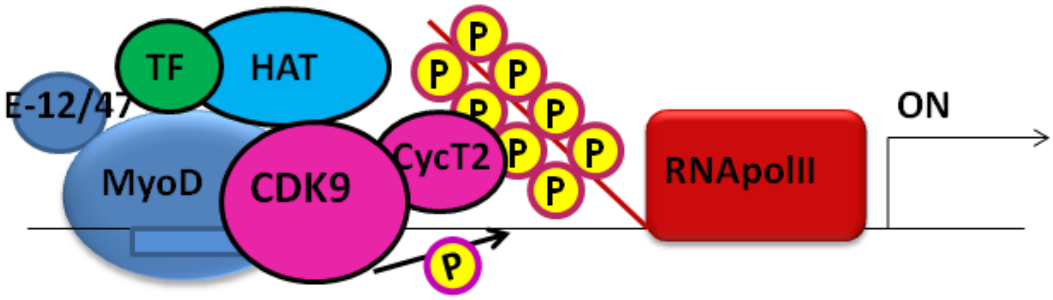

Fig.3. Activation of MyoD dependent-transcription by Cdk9/CycT2. A MyoD-dependent histone acetylation is the initial event at the promoter region of genes and it is mediated by HATs. MyoD recognizes an E-box site through an interaction with the E-protein. When MyoD recognizes its target it recruits and binds a muscle specific transcription factor (TF) and the complex Cdk9/CycT2, which phosphorylates the CTD of RnapolII (tail of the red rectangle) activating transcription. The blue rectangle represents the E-box sequence on the promoter region of MyoD target genes. Yellow circles represent phosphate groups.

Interestingly, RD cells fail to complete the myogenic program and Cdk9/CycT2 fails to phosphorylate MyoD in rhabdomyoblasts triggered to differentiate, despite Cdk9 and CycT2 protein levels are comparable with myoblasts and any mutation was detected in Cdk9 and CycT2 coding sequences. Enforced expression of MyoD in RD cell increases the production of late muscle specific markers but does not reverse the tumorigenic phenotype nor completely recapitulates the myogenic program. Moreover, overexpression of Cdk9/CycT2 or MyoD cannot rescue the function of the complex, suggesting the possible presence of a mechanism inhibiting the complex in rhabdomyosarcoma (Simone and Giordano 2007).

1.4. Role of Ezh2 in muscle development and RMS progression

During development differentiation programs global rearrangements are required in repression and activation of lineage-specific genes. Chromatin based epigenetic mechanisms ensure the correct integration of developmental signals at gene regulatory regions, allowing correct temporal expression of genes.

The Polycomb group, (PcG) proteins regulate developmental processes in embryonic stem cells (ESCs) leading to a particular cell fate. In human, two PcG multimeric complexes have been identified and named Polycomb repressive complex 1 (PRC1) and 2 (PRC2). These proteins repress the early differentiation genes to maintain the pluripotency of ESCs at the embryonic stage. At the initiation of cell fate commitment, those genes are activated while PcG proteins suppress the late differentiation genes for specific cell lineages (Chou *et al*., 2001).

The enhancer of Zeste homologue 2 (Ezh2) is part of the PRC2 complex. Human Ezh2 took its name from the fly homologue E(Z) in *Drosophila melanogaster,* where the protein was first discovered and characterized (Ketel *et al*., 2005; Cao and Zhang, 2004). Three PcG proteins, enhancer of zeste2 (Ezh2), embryonic ectoderm development (Eed), and suppressor of zeste 12 homolog (Suz12), comprise the core of PRC2, which mediates trimethylation of histone H3 lysine 27 (H3K27me3) (Cao *et al.,* 2002; Czermin *et al.,* 2002; Kuzmichev *et al.,* 2002; Muller *et al.,* 2002). Ezh2, a SET domain-containing histone methyltransferase (HMTase), is the catalytic subunit of PRC2. Its activity, in concert with histone deacetylases (DHACs) and DMTs, stabilizes chromatin and represses transcription of many key developmental regulators in embryonic stem cells through H3K27me3 and mediates X-chromosome inactivation (Lee *et al.,* 2006; Boyer *et al.,* 2006; Plath *et al.,* 2003).

The precise mechanisms of PRC-mediated gene silencing have not been completely understood yet. H3K27me3 PRC2-dependent is the fundamental feature of PcG-mediated repression whereas PRC1 is viewed as the direct executor of silencing (Simon and Kingston, 2009).

It has been suggested that the PRC1 can prevent the ATP-dependent nucleosome remodeling by the SWI/SNF and RNA polymerase II recruitment (Francis and Kingston, 2001; Dellino *et al.,* 2004; Wang *et al.,* 2004). Moreover the complex can induce gene silencing by chromatin compaction (Francis et al., 2004) and the ubiquitylation of H2A, modification associated with gene repression (Wang *et al.,* 2004; Cao *et al.,* 2005). Polycomb silencing and DNA methylation have often been considered biochemically independent gene silencing systems. However, recent studies show that EZH2 and DNMTs are physically and functionally linked and that EZH2 acts upstream of DNMTs to methylate and silence target chromatin (Virė *et al.,* 2006). The mechanism is not yet clear, but a hypothesis is that target genes are initially silenced through histone H3-K27 methylation by PRC2. PRC2 recruits DNA methyltransferases which methylate CpG DNA of target genes, leading to a more permanently or deeply silenced chromatin state (Ohm *et al.,* 2007; Schlesinger *et al.,* 2007; Widschwendter *et al.,* 2007).

As well as DNMTs, the PRC2 complex collaborates with other epigenetic silencing enzymes. In human cells, PRC2 can associate with histone deacetylases (HDACs) 1 and 2 providing functional synergy to silencing of target genes. HDACs may deacetylate H3-K27 or other lysine residues in order to adjust the local histone code for silencing (van Der Vlag and Otte, 1999; Muller *et al.,* 2002; Kuzmichev et al., 2002; Cao *et al.,* 2002; Czermin *et al.,* 2002; Varambally *et al.,* 2002; Cao and Zhang, 2004; Simon and Lange, 2008). The

precise mechanisms of this synergy at target genes are not yet clear. HDACs may deacetylate H3-K27 to make the ε-amino group available for methylation by PRC2. Alternatively, HDACs may deacetylate other histone lysines, such as H3-K9, H3-K14 or H4-K8, in order to adjust the local histone code for silencing (van Der Vlag and Otte, 1999; Muller *et al.,* 2002; Kuzmichev *et al.,* 2002; Cao *et al.,* 2002; Czermin *et al.,* 2002; Varambally *et al.,* 2002; Cao and Zhang, 2004). A hypothetic model for PcG-mediated silencing is shown in Fig.4.

Typically, in adult tissues, EZH2 is concentrated in undifferentiated progenitor cell populations, such as hematopoietic cells of the pro-B lymphocyte lineage (Su et al., 2003) and it is down-regulated in adult differentiated tissues (Varambally *et al.,* 2002; Bracken *et al.,* 2003; Kleer *et al.,* 2003). Moreover, in contrast to widespread EZH2 role in early mouse development (O'Carroll *et al.,* 2001; Erhardt *et al.,* 2003), post-embryonic EZH2 expression is limited (Hobert *et al.,* 1996; Laible *et al.,* 1997).

Interestingly, EZH2 is overexpressed in a variety of different tumors. EZH2 levels are abnormally elevated in cancer tissues versus corresponding normal tissues, with higher expression correlates with advanced stages of disease and poor prognosis (for review Simon and Lange 2008).

Indeed, Ezh2 has been linked to aggressive cancers of the prostate (Varambally *et al.,* 2002), breast (Raaphorst *et al.,* 2003), melanoma and endometrial cancer (Bachmann *et al.,* 2006) and its expression is higher in tumor tissues than in normal tissues (Bracken *et al.,* 2003).

It is thereby demonstrated that disruption of EZH2 expression may lead the cell cycle, in particular S-phase entry and G2/M transition. Furthermore, EZH2 is controlled by E2F

transcription factors, and its expression is higher in pRb-deficient tumor (Bracken *et al.,* 2003).

The predominant current view is that EZH2 functions in cancer as a transcriptional repressor that silences an array of target genes, including tumor suppressor and tissue specific differentiation genes.

Ezh2 implication in tumorigenesis is then strictly correlated with other associated chromatin modifying enzymes. For example, ectopic expression of Ezh2 in prostate cells induces gene silencing of a specific cohort of genes and this is strictly dependent on histone deacetylase activity (Varambally *et al.,* 2002).

For what regards muscle differentiation, it has been shown that PRC2 binds and represses numerous MyoD target genes in embryonic staminal cells by establishing H3K27 tri-methylation (Lee *et al.,* 2006). Moreover, although MyoD is expressed in undifferentiated skeletal myoblasts, Ezh2 forms a complex with YY1 on promoters and enhancers of the muscle-specific genes, thus silencing several MyoD target genes. After the commitment of myogenesis, PRC2-binding at MyoD loci is lost, inducing muscle gene expression and skeletal muscle differentiation (Caretti *et al.,* 2004). Indeed, accordingly to its role in repressing transcription Ezh2 is not detected on myogenic regulatory regions in terminally differentiated myotubes (Caretti *et al.,* 2004).

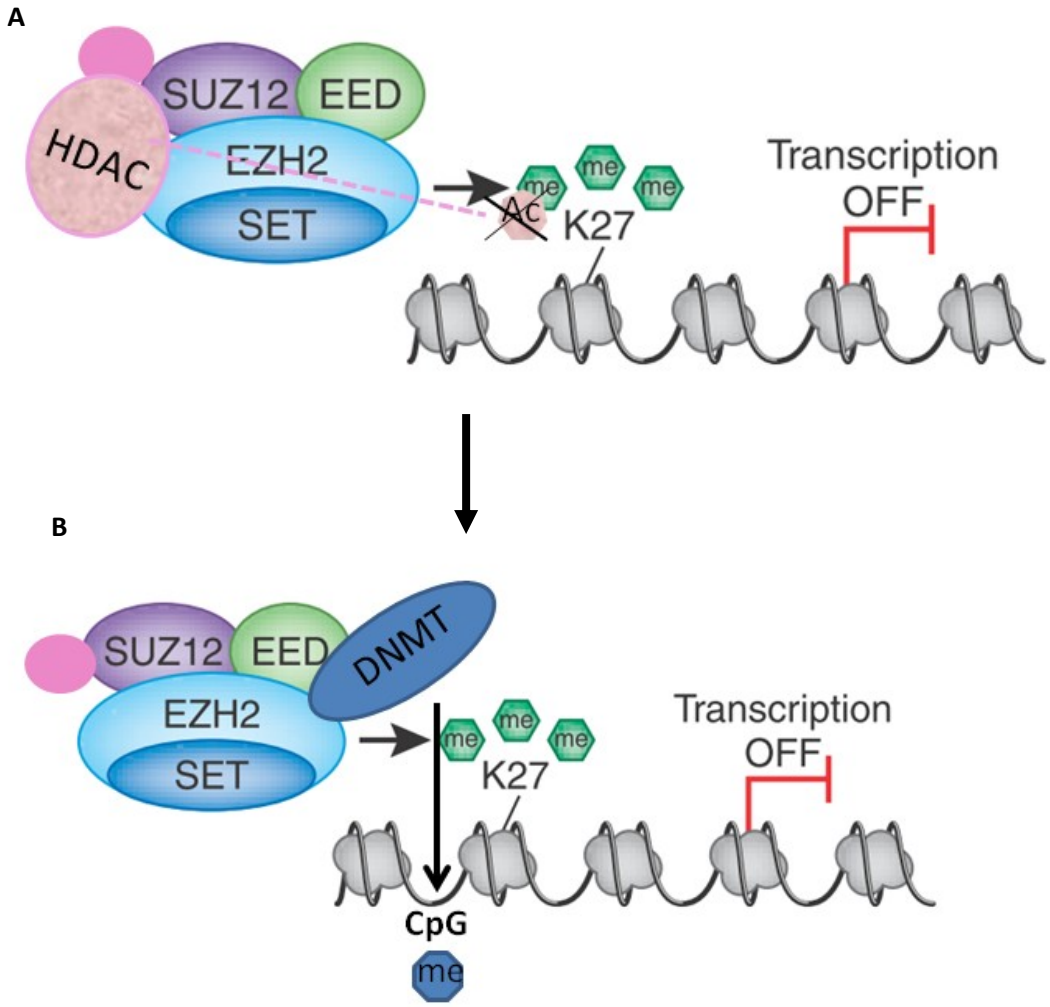

Fig.4. Hypothetic model for PcG-mediated gene silencing. A. First Ezh2 silences gene through methylation of H3K27. If an acetyl group occupies K27, it is removed by DHACs (that are known to interact with Ezh2). HDAC is shown in pink and the dotted line indicates its deacetylase activity at K27 before the methyltransferase activity of Ezh2. Ac indicates the acetyl group. **B.** Ezh2 recruits DNMTs, which methylate CpG islands on promoter regions, leading to a more permanent silencing of genes. DNMTs are shown in blue and me represents methyl groups.

To ensure proper spatial and temporal expression of muscle-specific genes during skeletal differentiation, PcG proteins are overcome by Trithorax group proteins (TrxG), through demethylation of H3K27me3 within the promoters of genes and methylation on H3K4,

which instead is a transcriptionally permissive mark. Removal of H3K27me3 requires RNA Pol II elongation (Seenundun *et al.,* 2010), a process mediated by CTD phosphorylation pTEFb-dependent (Ni *et al.,* 2004).

These data demonstrated that EZH2 plays a key role in skeletal-muscle differentiation, specifically in the maintenance of the undifferentiated state of muscle cell precursors and suggests that its deregulation may participate at RMS formation and progression by muscle specific genes silencing. Moreover these studies could suggest a role of EZH2 in the inhibition, Cdk9-dependent, of CTD phosphorylation in rhabdomyosarcoma.

Chapter 2

OBJECTIVES

Recent findings confirm the relevance of epigenetic and genetic alterations in the progression of cancer. Particular epigenetic patterns are associated with multiple cancer types as they frequently occur in tumor suppressor genes promoters. Our study focuses on Ezh2, a histone methyltransferase whose disruption has been linked to cancerogenesis in a wide variety of tumors. Previous data showed that Ezh2 regulates muscle gene expression during myogenesis, ensuring the proper timing of transcription from muscle specific promoters. RMS cells show lower levels of skeletal differentiation markers compared to myoblasts and have significantly elevated levels of Ezh2, whose overexpression has been linked to a large variety of cancers. Interestingly, Cdk9/CycT2 activity, previously shown to be a crucial coactivator of myogenesis, is abrogated in RMS cells although the expression levels of the two subunits of the complex are similar to normal myoblasts. The aim of the project is to understand if and how Ezh2 participates to rhabdomyosarcoma formation and verify the assumption that it could alter transcriptional regulation. To achieve this goal we choose to study in detail whether Ezh2 depletion changes the activation state of muscle specific promoters. In addition, our goal is to clarify if Ezh2 interferes with Cdk9/CycT2 activity, preventing terminal differentiation in RMS, in order to try to reverse the tumorigenic phenotype and highlight new therapeutic targets for cancer treatment.

Chapter 3

MATERIALS AND METHODS

3.1. Cell culture and differentiation

The RD cell line was obtained from the American Type Culture Collection (ATCC, Rochville, MD, USA). The stable RD-Ezh2-KD cell line had been previously created in our laboratory, introducing in the cells an shRNA against Ezh2 by lentiviral vectors. Both cell lines were grown at 37C, in a humidified incubator containing 5% CO_2, in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and antibiotics (Growth medium, GM) until reaching confluence. After, that skeletal differentiation was induced changing the medium with Differentiation Medium (DM), containing Dulbecco's modified Eagle's medium and 2% HS for 96h. Pellets were collected each 24h.

3.2. RT-PCR

Total RNA was extracted with HIGH PURE RNA ISOLATION KIT (Roche Applied Science, Indianapolis, USA) according to the manufacturer's protocol. 1ug of total RNA was retro-transcribed in 20ul of final volume, containing 10mM dNTPs, DTT 0,1M, random primers 2ug/ul and MML-V Reverse Transcriptase (Invitrogen) following the manufacturer's protocol. After reaction double distilled, water was added until reaching a final volume of 200ul.

Real time PCR was performed using primers specifically designed for human MyoD, MyH, Myog and Gapdh, using 4,5ul of cDNA and 5,5ul of Fast Start Universal SYBR Green Master (ROX) (Roche Applied Science, Indianapolis, USA), and 250nM of each primer. The primer efficiency was calculated for every target using 10 fold serial dilutions of PCR products.

Amplification conditions for all the amplicons were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15s, 60°C for 30s, 72°C for 30s. To ensure specific PCR amplification, every real time PCR run was followed by a dissociation phase analysis (denaturation curve).

Accumulation of fluorescent products was monitored using the BIORAD IQ5 instrument.

Each data point was obtained from three independent experiments. The relative expressions were calculated with the ΔΔCT method. Transcripts levels for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as a reference and RD and RD-EZH2-KD at 0h of differentiation was used as the calibrator point.

Primer sequences:

hMyoD forward: GACGGCATGATGGACTACAGC

hMyoD reverse: GGAGATGCGCTCCACGATGC

hMyH forward: GGAGGAGGCTGATGAACAAGC

hMyH reverse: GGAGGAGGCTGATGAACAAGC

hMyog forward: CCTGCTCAGCTCCCTCAACC

hMyog reverse: AGGGTCAGCCGTGAGCAGATG

hGapdh forward: GAAGGTGAAGGTCGGAGT

hGapdh reverse: CATGGGTGGAATCATATTGGA

3.3. Immunoblotting

Protein extracts were collected from whole cell lysates after 0h, 24h, 48h, 72h, 96h in DM in a non denaturing lysis buffer containing 20 mM Tris HCl pH 8; 137 mM NaCl; 10% glycerol, 1% Nonidet P-40; 2 mM EDTA, 20mM NaF and 0,1mM Na_3VO_4. Protein quantification was performed with a Bradford assay, using BSA as a standard. 60ug of

total proteins extracts were resolved in 8% and 10% PAA-GELS and transferred to a nitrocellulose membrane (Amersham, IL) at 4˚C and at 100V for 1 hour. The blots were blocked for 40' at room temperature with TBS-T containing 5% non-fat dry milk. Antibodies specific for MyoD, CyCT2, myogenin, MyH (Santa Cruz, CA), Cdk9 (Rockland), Ezh2 (Invitrogen), RNA Polymerase II (Santa CRUZ, CA), P-Serine2-CTD, P-Serine5-CTD (Bethyl Laboratories Inc., Montgomery, TX) were used to detect protein levels. Anti-70 kDa heat shock protein (Hsp70) and anti-Gapdh were used to control equal loading of the proteins. Anti-rabbit (1:10000), anti-mouse (1:10000) and anti-goat (1:2500) peroxidase conjugated antibodies (Pierce) and ECL detection system (PerkinElmer) were used for detection.

3.4. Chromatin Immunoprecipitation

ChIP was performed with ChIP-IT EXPRESS ENZYMATIC KIT (Active motif, CA, USA) according to the manufacturer's protocol. Cells were grown in 150 x 25mm tissue culture dishes in GM to 70-80% confluence and triggered to differentiate for 72h in DM. Cells were fixed for 5 minutes with 1% formaldehyde, pelleted and lysated in non denaturing lysis buffer, nuclei were isolated and chromatin was prepared according to the instructions of the protocol. It was then shared in a digestion buffer containing an enzymatic shearing cocktail for 15 minutes and the reaction was stopped with the addition of cold 0,5M EDTA. 200ul of chromatin was used to analyze shearing efficiency and chromatin was quantified after phenol/chloroform extraction and precipitation.

ChIP was performed with 50ug of chromatin and anti-Ezh2 (Millipore), anti-RNAPolII, anti-Ser2CTD, anti-Ser5CTD (Bethyl Laboratories Inc., Montgomery, TX) and anti-CDK9 (Rockland) antibodies O/N at 4˚C. Rabbit and mouse IgG were used as negative controls.

The sheared chromatin samples were subjected to cross-link reversal and treated with proteinase K.

PCR amplifications were performed with primers specifically designed within human Myog and MyH promoters.

Fold enrichments of ChIP were calculated as a ratio of the amplification efficiency of the ChIP sample over the IgG's (rabbit, mouse and goat IgG depending on the antibody used, all purchased from Santa Cruz) and each data point represents the medium of three independent experiments.

Real time PCR was performed with primers designed within the promoter regions of Myog and MyH promoters, and amplification efficiency was calculated using 10 fold dilutions of input DNA qPCR amplified along with the ChIP and IgG samples. Amplification conditions were 95°C for 10 minutes, followed by 45 cycles at 95°C for 15s, 58°C for 30s, 72°C for 30s.

hMyogenin promoter forward: GAGAGGGGAATGTGTCCTCC

hMyogenin promoter reverse: CTTCTTTCTCCCGCATGGCC

hMyh promoter forward: CTCGTCAGAAGCCGATTCTAC

hMyH promoter reverse: CTCGTCAGAAGCCGATTCTAC

Chapter 4

RESULTS

4.1. Analyses of transcriptional regulation in rhabdomyosarcoma and recovering of muscle gene expression in RD-EZH2-KD differentiating cells

Previous works revealed a crucial role of Ezh2 in skeletal muscle differentiation as its transcriptional and post-transcriptional downregulation is required to allow activation of muscle-specific genes (Caretti *et al* 2004, Juan *et al.,* 2009). The polycomb repressive complex 2 (PRC2) represses transcription on myogenic genes regulatory regions through the histone methyltransferase activity of Ezh2. Overexpression of this protein in C2C12 myoblasts impairs differentiation and inhibits skeletal muscle genes expression (Caretti *et al*., 2004). Thus, we supposed that such protein in rhabdomyosarcoma could interfere with the normal myogenic transcriptional program and cause defective enrollment of transcriptional factors on myogenic regulatory regions.

We previously generated an Ezh2-KD-RD cell line with a lentiviral vector containing an shRNA against Ezh2 (unpublished results). At first we validated the Knockdown by performing a Real time PCR for Ezh2 for both RD-WT and RD-EZH2-KD cells. Ezh2 mRNA expression levels in the RD-EZH2-KD cell line resulted reduced to 70% compared to the RD-WT (Fig.5). We supposed that Ezh2 overexpression in rhabdomyosarcoma could blockade the proper activation of genes during myogenesis, preventing cells to complete terminal differentiation. Thus, we chose early and late differentiation markers, such as Myog and MyH, whose expression has been already related to Ezh2 (Caretti *et al.,* 2004), to detect changes in the activation of the transcriptional myogenic program.

To evaluate the effect of Ezh2 depletion on muscle-specific expression we monitored the temporal expression of the myogenic markers in RD-WT and RD-EZH2-KD cells cultured in conditions favoring either growth or differentiation. Skeletal muscle differentiation was induced replacing growth medium (GM) with differentiation medium (DM) in confluent

cells. As Ezh2 controls timing of muscle specific gene expression and its expression is differentially regulated during myogenesis the transcripts and protein levels of MyoD, Myogenin (early markers of muscle differentiation) and Myh (late marker of muscle differentiation) were analyzed at 0, 24, 48, 72, 96h after induction of differentiation with Real Time PCR (Fig.6). As previously reported Ezh2 mRNA was robustly represented in RD-WT differentiating cells while MyoD, Myogenin and Myh transcripts levels were only detectable in all the stages analyzed. Conversely, the myogenic markers transcripts levels clearly increased in RD-EZH2-KD differentiating cells at every time point analyzed. A similar profile was obtained by Western blot analyses in both RD-WT and RD-EZH2-KD although to a lesser extent for MyoD (Fig.7). Those data together indicate that Ezh2 inhibits muscle gene expression in RD-WT differentiating cells. Although a partial reactivation of the myogenic program was obtained, we could not observe a complete reversion of the tumorigenic phenotype but a significant change was detected in cells morphology. In fact RD-EZH2-KD cultured for 72h in DM showed a more lengthened phenotype (Fig.8).

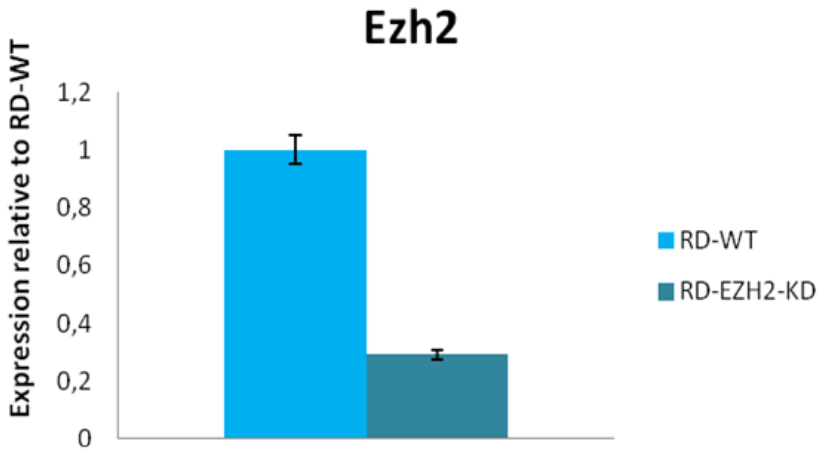

Fig.5. Expression levels of Ezh2 measured by Real Time PCR in human RD-WT and RD-EZH2-KD at 48h in DM. Transcription levels were normalized to GAPDH expression.

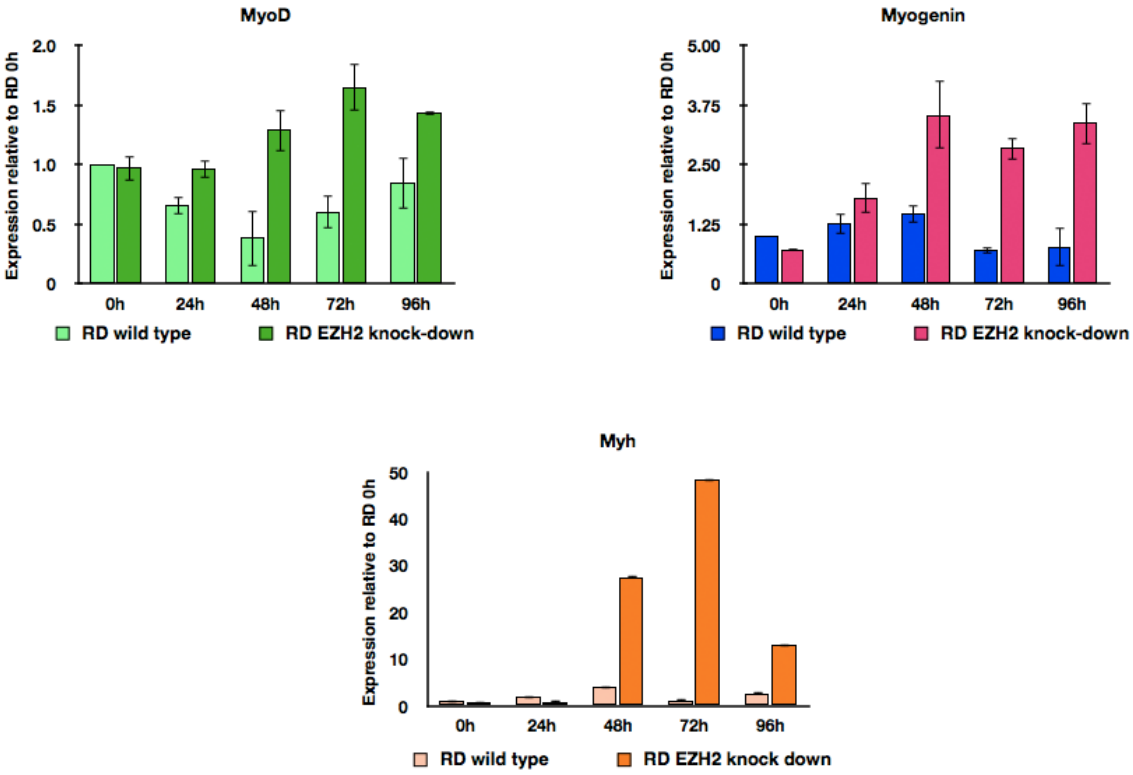

Fig.6. Expression levels of MyoD, MyoG, MyhC measured by real-time PCR in human RD-WT and RD-EZH2-KD at 0h in GM and at 24, 48, 72, 96h in DM. Transcription levels were normalized to GAPDH expression and represent the mean of three independent experiments ± SD. Fold enrichment was calculated in comparison to RD in GM.

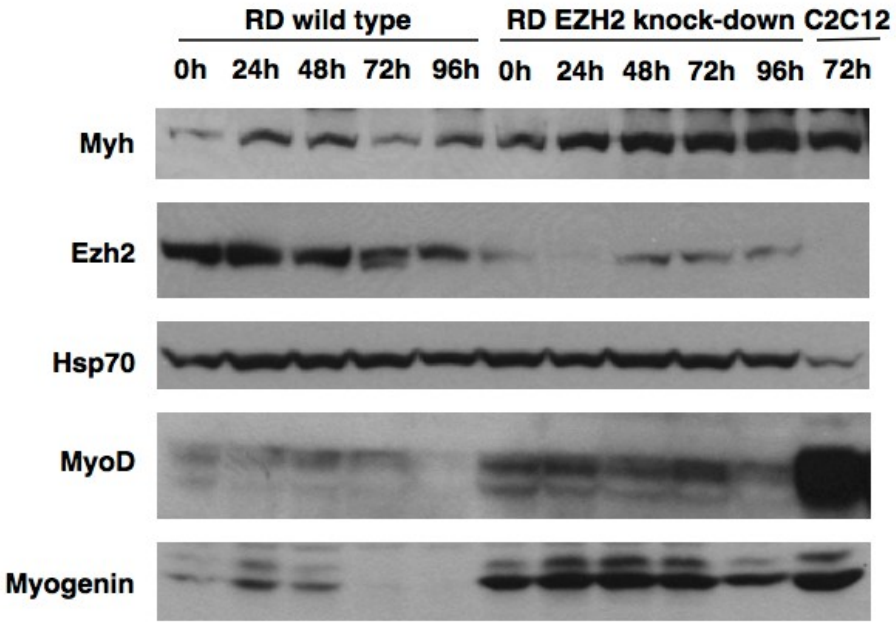

Fig.7. Immunoblot of MyoG, MyoD, MyhC, Ezh2 from whole cell extracts of human RD-WT and RD-EZH2-KD at 0h in GM and at 0, 24, 48, 72, 96h in DM. C2C12 murine myoblasts at 72h in DM were used as a positive control and equal loading was performed with an anti Hsp70 antibody.

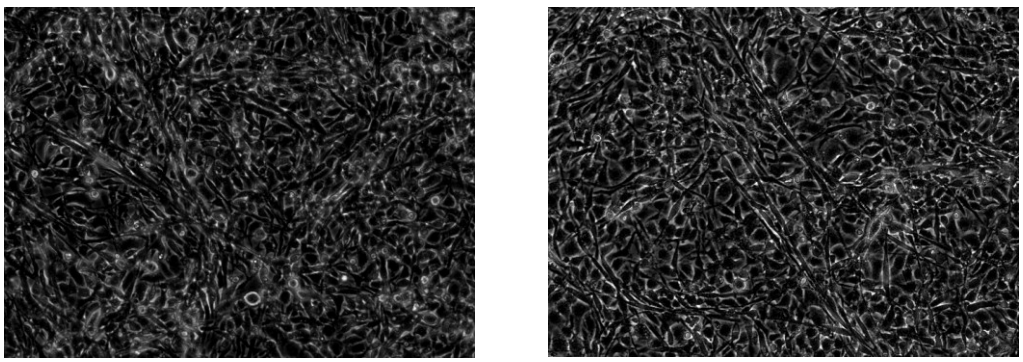

Fig.8. RD-WT (on the left side) and RD-EZH2-KD (on the right side) cells cultured for 72h in DM. RD-EZH2-KD have more cells with a longer and more differentiated phenotype.

4.2. Ezh2 occupies Myog and MyH promoters in human RD rhabdomyosarcoma cells inhibiting gene expression

Once established that Ezh2 overexpression in RD-WT impedes transcription, a chromatin immmunoprecipitation (ChIP) was performed with an antibody against Ezh2 to evaluate if this protein directly mediated the inhibition of muscle gene expression. RD-WT were induced to differentiate and chromatin was precipitated from whole cell protein extracts through an antibody against Ezh2 and analyzed for the occupancy of Myog and

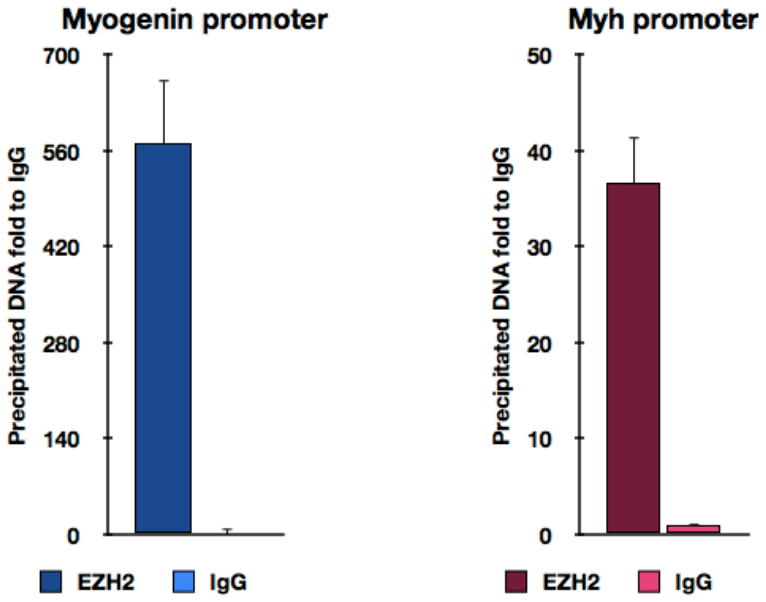

Fig.9. ChIP analysis of chromatin prepared from RD-WT cells cultured in GM with an Ezh2 antibody. The precipitated DNA fragments were subjected to real-time PCR analysis using primers designed within MyoG and MyH promoters. Fold enrichments are presented as a ratio of the amplification efficiency over that of the IgG. Data are shown as an average of three independent experiments ± SD.

Myh promoters (Fig.9). The ChIP analysis showed that Ezh2 strongly occupies Myog and Myh promoters in RD-WT, at a much greater extent compared with the negative control immunoprecipitated with IgG. Recent findings recognize the displacement of Ezh2 from myogenic regulatory regions as a necessary step for the activation of muscle gene

transcription (Aziz *et al* 2010, Stojic *et al.,* 2011). This data provide further evidence for a possible role of Ezh2 in preventing activation of transcription in rhabdomyosarcoma.

4.3. Ezh2 depletion in human RD cells results in a general increase in elongating RnaPolII

The Cdk9/CycT2 complex is a crucial coactivator of myogenesis. The complex coactivates MyoD-dependent transcription and its recruitment on myogenic regulatory regions correlates with phosphorylation of the CTD of RNApolII (Simone *et al.,* 2002a; Giacinti et al., 2006). Our previous results showed that knockdown of Ezh2 determine an increase in early and late muscle differentiation markers (Fig.6 and Fig.7). In order to evaluate alterations in Cdk9 kinase activity and in RNA processing machinery on gene regulatory regions after depletion of Ezh2 we analyzed the CTD phosphorylation state of RNApolII. RD-WT and RD-EZH2-KD were cultured in growth medium until reaching confluence. Growth medium was then replaced with differentiation medium and total proteins were extracted at 0h, 24h, 48h, 72h, 96h after induction of myogenesis. Immunoblot analyses were performed against total RNApolII, Ser2P-RnapolII, Ser5P-RNApolII, Ezh2, Cdk9, CycT2a-b, and hsp70 and Gapdh as loading controls (Fig. 10).

Ser2P-RnapolII and Ser5P-RnapolII in RD-EZH2-KD are significantly higher than in RD-WT at every time point analyzed. This consent to sustain that there is a general increase in the activation of transcription after Ezh2 depletion. The amount of total RNApolII in RD-WT is similar to the amount in RD-EZH2-KD and these data exclude the possibility that a general increase in transcription is due to an augment in RNApolII expression levels. Interestingly, Cdk9 and CycT2 levels are equivalent in both cell lines at every point investigated.

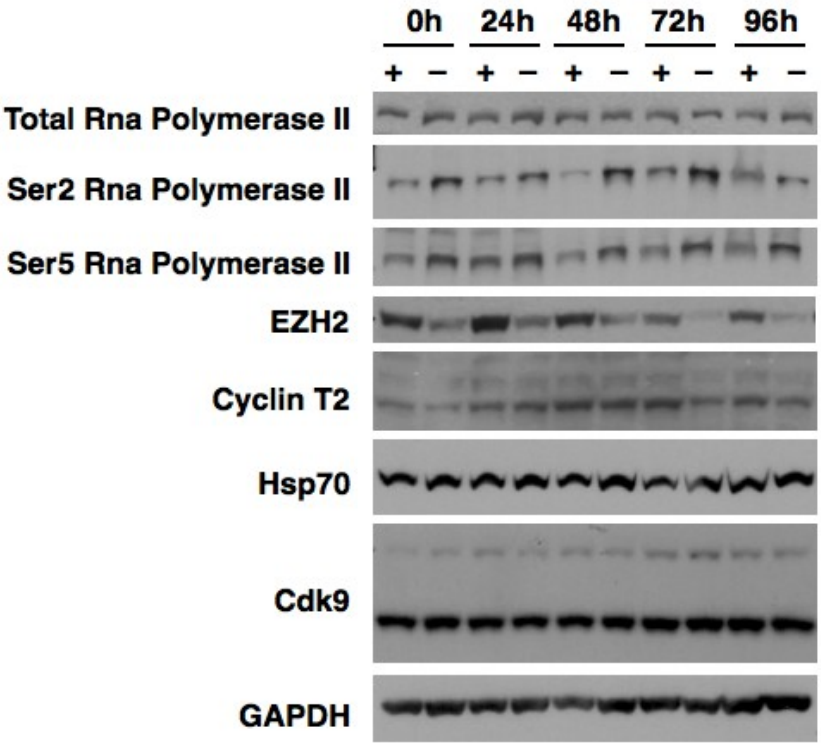

Fig.10 Immunoblot analyses of MyoG, MyoD, MyhC, Ezh2 from whole cell extracts of human RD-WT (+) and RD-EZH2-KD (-) at 0h in GM and at 0, 24, 48, 72, 96h in DM. C2C12 murine myoblasts at 72h in DM were used as a positive control and equal loading was performed with an anti-Hsp70 antibody.

It was previously shown by Simone and Giordano (2007) that the signal-dependent activation of the Cdk9/CycT2 complex is abrogated in human RD cells. The observed increase in the elongating RNApolII confirms that Ezh2 inhibits the transcriptional program although it does not impede the normal expression of Cdk9 and CycT2. This puts forward the presence of a different mechanism inhibiting the complex.

4.4. Analysis of CTD phosphorylation of RNApolII on Myog and Myh promoters 72h after induction of myogenesis

Upon induction of muscle differentiation, Cdk9 and CycT2 were recruited on regulatory regions of myogenin and MyH in myoblasts and this event correlates with phosphorylation of the CTD of RnapolII (Simone *et al.,* 2002b; Giacinti *et al.,* 2006). The aim of our study was to examine MyoD dependent transcription in Rhabdomyosarcoma because it is coactivated by the complex Cdk9/CyCT2 whose activity is abrogated in this kind of tumor. To assess how Ezh2 influenced Cdk9 kinase activity we examined the occupancy of Ser2P-RNApolII, Ser5P-RNApolII and total RNapolII at Myog and Myh promoters. We chose 72h of differentiation as a point to analyze since it was already observed that after 72 hours both Myog and MyH proteins were expressed in the knockdown RD cell line (Fig.6 and Fig.7). The results demonstrated that fold enrichments in Ser2P-RNApolII and Ser5P-RNApolII were higher in RD-EZH2-KD than in RD-WT, with a greater occupancy of Ser5P RNApolII (Fig.11 and Fig.11) at both the promoters. These data imply a greater activity of the Cdk9/CycT2 complex on the regulatory regions and support western blot analyses, which revealed increased Myog and MyH expression in RD-EZH2-KD (Fig.7).

4.5 Cdk9 occupies Myog and Myh promoters in 72h RD-EZH2-KD differentiating cells

To test whether deregulation of muscle-gene expression in RD cells was the result of defective enrollment of transcriptional factors, and more in particular to test whether Cdk9 recruitment was affected by Ezh2 and to further support the previous experiments the same analysis on 72 hours differentiating cells was made with an antibody against Cdk9. Interestingly, the ChIP analyses proved that Cdk9 binds Myog and MyH promoters

in RD-EZH2-KD but not in RD WT, (Fig.12). This result confirms the hypothesis that the activity of Cdk9/CycT2 complex is influenced by the presence of EZH2.

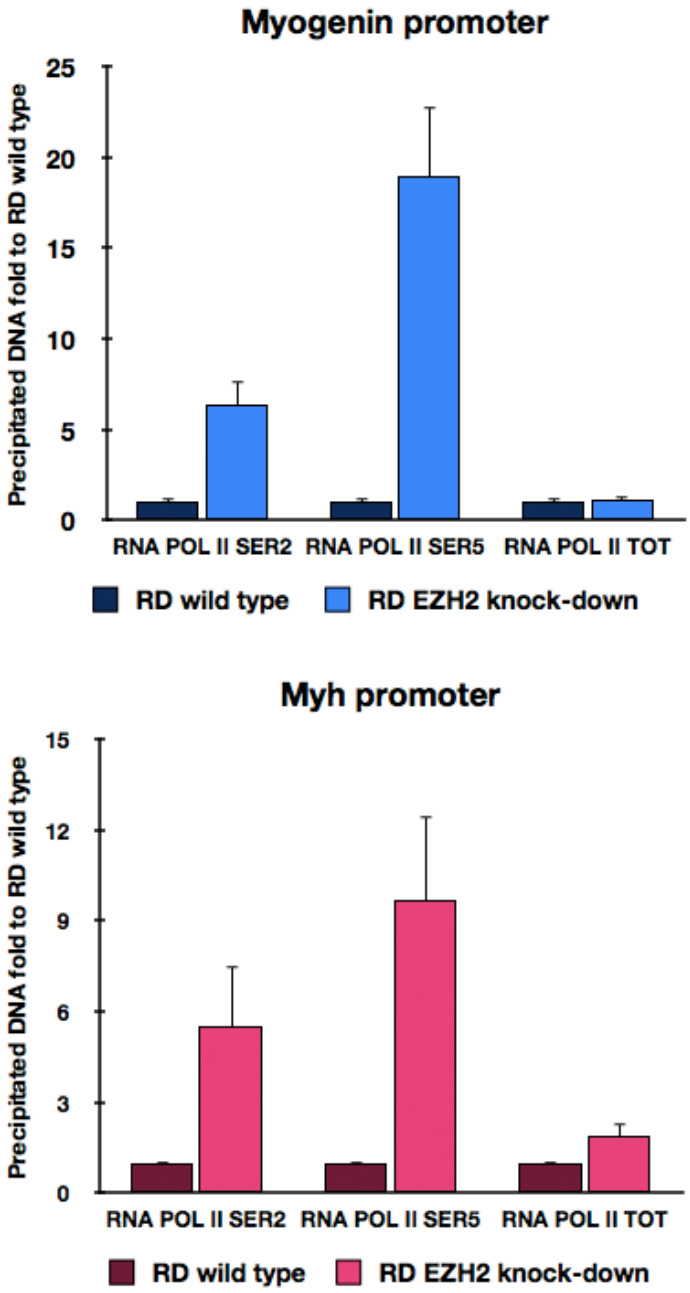

Fig.11. ChIP analysis of chromatin prepared from RD-WT cells and RD-EZH2-KD cells 72h after induction of myogenesis with anti-total RNApolII, anti-Ser2P RNApolII, and anti-Ser5P RNapolII. The precipitated DNA fragments were subjected to real-time PCR analysis using primers designed within MyoG and MyH promoters. Fold enrichments are calculated as a ratio of the amplification efficiency over that of the IgG. Data are shown as an average of three independent experiments ± SD.

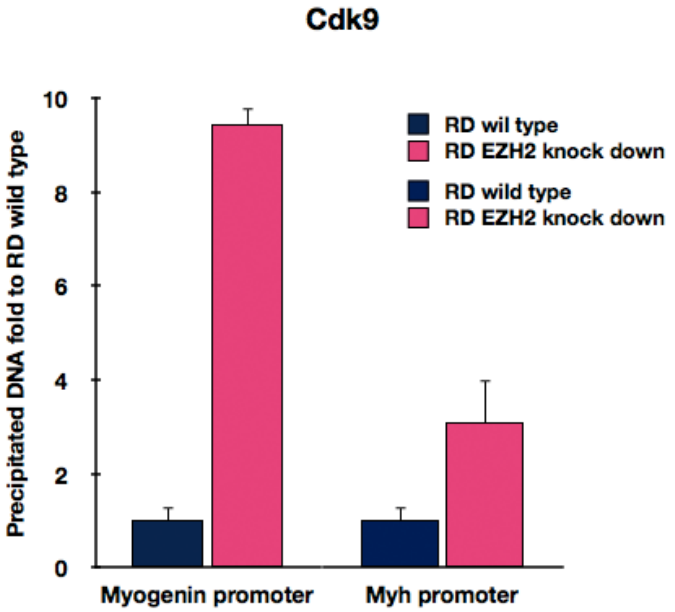

Fig.12. ChIP of chromatin derived from RD WT and RD-EZH2-KD after 72h in DM with an anti-Cdk9 antibody. The precipitated DNA fragments were subjected to real-time PCR analysis using primers designed within MyoG and MyH promoters. Fold enrichments are normalized to the precipitated DNA folds in RD wild type. Data are shown as an average of three independent experiments ± SD.

Chapter 5

DISCUSSION

Epigenetic alterations in cancer cells are recognized as an important aspect of tumor biology. Unlike genetic alterations, epigenetic modifications can be reversed with specific inhibitors or enzymes. Indeed, epigenetically silenced tumor suppressor genes can be reactivated to induce apoptosis or senescence. This peculiarity makes epigenetic changes an interesting target for therapeutic intervention in cancer. There are at least eight distinct types of modifications found on histones: acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination, proline isomerization (Kouzarides, 2007). In particular specific pattern of acetylation and methylation of histones H3 and H4 are associated with several cancer types and for some tumors, epigenetic changes enable to distinguish diseases subtype (Fraga *et al.,* 2005; Seligson *et al.,* 2005).

Polycomb group proteins are regulators of embryonic development and stem cell maintenance (Pietersen and van Lohuizen; 2008; Kerppola, 2009; Simon and Kingstone, 2009) and their deregulation contributes to cancer (Sparmann and van Lohuizen 2006; Bracken and Helin, 2009).

Altered expression of Ezh2 has been related to cancer pathogenesis while high Ezh2 transcript and protein levels have been associated with poor clinical outcome in breast, prostate and bladder cancer patients (Varambally *et al.,* 2002; Kleer *et al.,* 2003; Tonini *et al.,* 2004). The same protein has been promoted as a diagnostic marker of invasion and aggressiveness (Varambally *et al.,* 2002; Raaphorst *et al*., 2003).

RMS cells show elevated levels of Ezh2 compared to normal myoblasts. In the early phase of myogenesis, this protein maintains repression of muscle specific transcription ensuring proper timing in tissue-specific gene expression (Caretti *et al.,* 2004; Stojic *et al.,* 2010).

The myogenic transcriptional program is assisted by the complex Cdk9/CycT2, which activates MyoD dependent transcription in myoblasts, triggered to differentiate and stabilizes the elongating RNApolII on muscle-specific promoters even in synergy with other myogenic transcription factors (Simone *et al.,* 2002; Giacinti *et al.,* 2006; Nojima *et al.,* 2008). Skeletal differentiation markers are lower in RMS compared to normal myoblasts, although the time course of RD-WT induced to differentiate shows that Cdk9 and CycT2 in RMS are normally expressed (Fig.10). Since Cdk9/CycT2 is a crucial coactivator of myogenesis and its activity is abrogated in RD cells (Simone and Giordano, 2007), these data led us to suppose that Ezh2 could interfere with the kinase activity of the complex and to test this hypothesis we compared an RD-WT cell line with an RD-EZH2-KD cell line. Our results demonstrated that MyoD, myogenin and MyH expressions are partially restored after Ezh2 depletion in the RD-Ezh2-KD cell line (Fig.6 and Fig.7). In addition ChIP analyses demonstrated the strong occupancy of Ezh2 that is known to maintain transcriptional repression of muscle specific genes in the early phase of differentiation, at the promoters of Myog and MyH genes in the RD-WT. These data suggest that Ezh2 impedes the normal transcriptional regulation in rhabdomyosarcoma.

The principal activity of the complex Cdk9/CycT2 during myogenesis is the phosphorylation of the CTD of RNapolII on myogenic regulatory regions that has been already demonstrated for MyoD target genes (Giacinti *et al.,* 2006; Marchesi 2010, unpublished results). Overexpression experiments of Cdk9 and CycT2 in the RD cell line don't reestablish the kinase activity of the complex suggesting the possible presence of a mechanism inhibiting the complex in rhabdomyosarcoma (Simone and Giordano 2007). The extended time course in both RD-WT and RD-EZH2-KD induced to myogenesis allows

us to support our assumption. In fact, the RD-EZH2-KD shows an evident increase in both RNApolIISer2P and RNApolIISER5P (whose presences indicate active transcription) at every time point investigated compared with the RD-WT while the expression levels of total RnapolII are comparable between the two cell lines (Fig.10).

Upon induction of muscle differentiation, Cdk9 and Cyclin T2 were recruited on regulatory regions of Myog and MyH in myoblasts and this event correlates with phosphorylation of the CTD of RnapolII (Simone *et al.,* 2002b; Giacinti *et al.,* 2006). In RD cells Cdk9/CycT2 kinase activity is abrogated. In order to evaluate if the same occurred in the RD-EZH2-KD cell line, we analyzed the phosphorylation state of the CTD of RNApolII on that genes, because it had been previously shown that Cdk9/CycT2 coactivates transcription from these genes. Significantly, ChIP analyses demonstrate that 72h RD-EZH2-KD differentiating cells exhibit an increase in the phosphorylation state of both Ser2 and Ser5 of the CTD at Myog and Myh promoters (Fig.9), which agrees with the previous results that shows partial reactivation of gene expression (Fig.6 and Fig.7). A greater augment is observed in the phosphorylation state of Ser5 at both Myogenin and Myh promoters. In addition, further analyses reveal that Ezh2 knockdown restores Cdk9 occupancy at Myog and Myh promoters in RD-EZH2-KD (Fig.12), with a more evident recovery on the myogenin promoter. This is in agreement with the assumption that Ezh2 interferes with Cdk9 recruitment and activity in rhabdomyosarcoma. In conclusion, our results indicate that Ezh2 inhibits transcriptional activation in rhabdomyosarcoma, that EZH2 depletion correlates with CTD phosphorylation of RNApolII and interferes with Cdk9/CycT2 recruitment and activity, thus impairing skeletal muscle differentiation in rhabdomyosarcoma.

Kinase assays are further needed to completely confirm this hypothesis. If Ezh2 inhibits Cdk9 through a direct mechanism or by competing with it is still to be elucidated. The comprehension of molecular mechanisms regulating displacement of Ezh2 from myogenic regulatory regions will provide further insights on the interplay between Cdk9 and EZh2.

These studies contribute to a better understanding of the mechanisms triggering the RMS carcinogenesis and can be useful in the future to design new therapeutic lines that can be combined with other drugs or conventional cancer therapies.

Chapter 6

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