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A novel Role of Cdk9/CyclinT2 complexes in skeletal muscle and Rhabdomyosarcoma cells

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CONTENTS

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1. ABSTRACT

Cyclin dependent kinase 9 (Cdk9) is a member of the cyclin dependent kinase family. This protein is a seorine-threonine kinase, involved in many cellular processes. The regulatory units of Cdk9 are the T family Cyclins (T1, T2) and Cyclin K1. Cyclin T2 has two forms termed CycT2a and CycT2b that arise by an alternative splicing of the primary transcript. Human Cyclins T2a and T2b share the first 642 amino acids but have different carboxyl termini. Previous studies underscored a crucial role for Cdk9 in association of Cyclin T2 during skeletal myogenesis. Upon induction of muscle differentiation, MyoD recruits Cdk9/CycT2 on muscle-specific gene promoter sequences. This complex is able to phosphorylate the C-terminal domain of RNA polymerase II, enhancing Myod function and promoting myogenic differentiation.

Rhabdomyosarcoma (RMS), one of the most common childhood solid tumor, arises from muscle precursor cells and fails to complete both the differentiation program both the irreversibly cell cycle exit, resulting in uncontrolled proliferation and incomplete myogenesis. In RMS, Cdk9 fails to phosphorylate MyoD and the ability of MyoD to arrest cell proliferation and to activate the myogenic program is repressed. The result of this study confirmed the involvement of Cdk9/ CyclinT2 complexes during the myogenesis. Both isoforms of Cyclin T2 are able to activate the myogenic program at different stages of differentiation but CycT2b have a predominant role in particular during the latest stages. Moreover we demonstred that EZH2 is probably responsible to inhibition of Cdk9 in RMS cells and her overexpression contribuite to inhibition of muscle differentiation program.

2. INTRODUCTION

2.1 Differentiation of skeletal muscle

The identity, proliferation and terminal differentiation of skeletal muscle cells is controlled by combinatorial activities of several transcription factors (Sartorelli and Caretti, 2005).

In particular, an important "modulatory" role in the development of skeletal muscle tissue is performed by a family of transcription factors, which have in common a basic helix-loop-helix DNA binding domain, called myogenic bHLH family. This transcription factors family includes MyoD (reviewed by Weintraub *et al*., 1991), myogenin (Braun *et al*., 1989a; Edmondson and Olson, 1989; Wright *et al*., 1989), Myf-5 (Braun *et al*., 1989b), and MRF4, or Myf-6/herculin, factors. (Rhodes and Konieczny, 1989; Braun *et al*., 1990; Miner and Wold, 1990; Rudnicki *et al*., 1993).

During the skeletal muscle differentiation process, this family of muscle-restricted bHLH proteins activate the differentiation program by binding to sequence-specific DNA elements, E box sites (CANNTG), located in enhancer and promoter sequences of muscle specific genes (Lassar *et al*., 1989), and by inducing the transcription of regulatory and structural muscle specific genes (Lassar and Munsterberg, 1994; Molkentin and Olson, 1996; Yun and Wold, 1996; Arnold and Winter, 1998). Notably, efficient MyoD DNA-protein-binding is achieved by heterodimerization with other non-myogenic bHLH proteins, which include the products of the E2A gene (E12, E47) and HEB, also referred as E proteins (Murre *et al*., 1989; Lassar *et al*., 1991; Puri and Sartorelli, 2000).

The cardinal role of MyoD in skeletal myogenesis is evinced by the amount of its target genes. In fact, MyoD regulates more than 300 genes that could be grouped into at least eight categories:

1. adhesion/matrix
2. cell cycle/DNA replication
3. grow factors/ligand
4. metabolism
5. nuclear regulatory factors

6. proteolysis/apoptosis/chaperone
7. receptors/signaling
8. structural/cytoskeletal

(Bergstrom *et al*., 2002; Giacinti *et al*., 2006).

Each MyoD monomer forms two α helics interrupted by a short stretch of aminoacids modeled as a loop. The first α helix (H1) includes the basic and the helix1 domains; the second helix (H2) starts immediately after the loop and ends at aminoacid 166. The basic domain fits in the major groove of the DNA, establishes most of the DNA-protein interactions and is involved in the activation of transcription (Davis and Weintraub, 1992; Puri and Sartorelli, 2000). Instead, the two α helics organize the dimerization interface for the formation of the heterodimers (Figure 1).

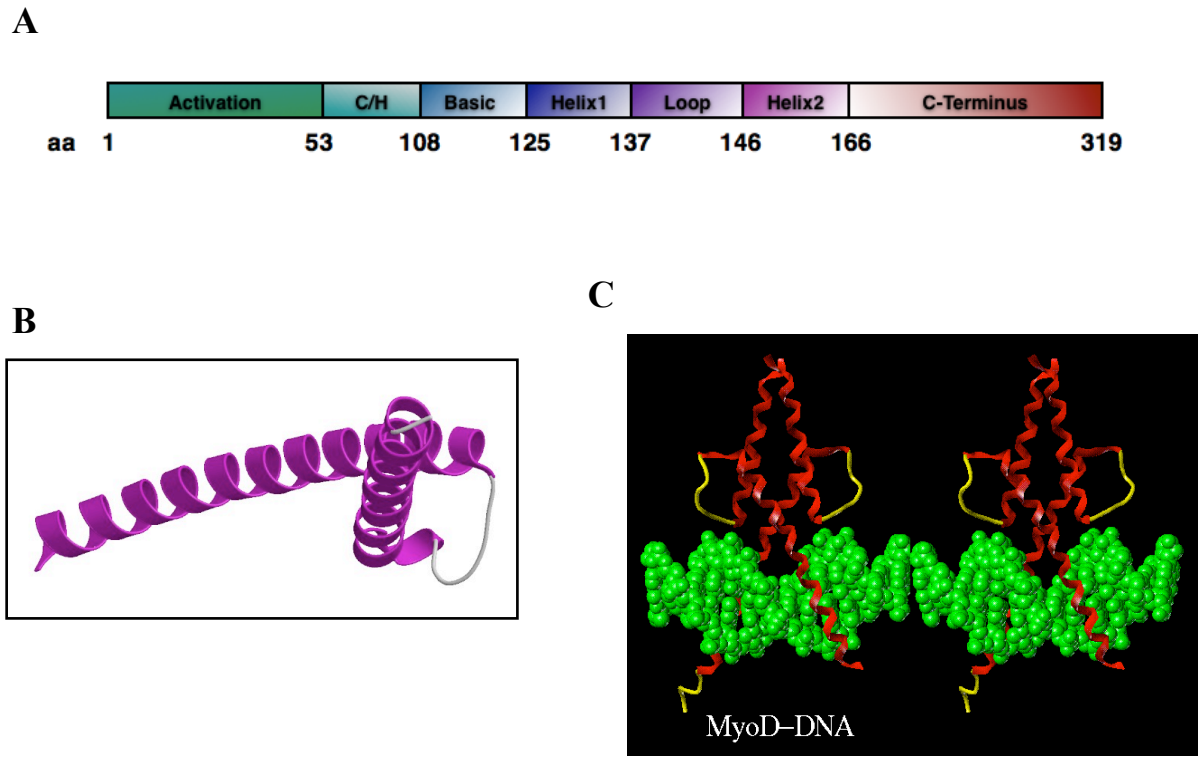


Figure 1: A) Domains organization of Myod. B) bHLH domain C) Schematic representation of MyoD-DNA binding.

Interestingly, the bHLH proteins MyoD, its interacting partners E12, E47 and c-myc recognize similar, yet distinct, E-boxes. In fact, in vitro experiments have established that MyoD prefers the CAGCTC sequence, whereas E47 selects the CACCTG, and c-Myc the CACGTG motif, respectively (Blackwell *et al*., 1990; Blackwell and Weintraub, 1990; Blackwell *et al*., 1993; Puri and Sartorelli, 2000).

Full activation of muscle gene expression by MRFs is also dependent by their association with members of the MEF2 transcription factors family. In fact, it has been reported that MEF2 factors cannot activate muscle genes on their own, but they potentiate the activity of MRFs. (Sartorelli and Caretti 2005; Lluìs *et al*., 2006).

Other than bHLH non-myogenic factors, MyoD can recruit transcriptional co-activators p300 and PCAF, two histone acetyltransferases (HATs) that promote muscle gene transcription by inducing acetylation of both chromatin and sequence-specific transcription factors, as for MyoD (Eckner *et al*., 1994; Eckner *et al*., 1996; Yuan *et al*., 1996; Sartorelli *et al*., 1997; Puri *et al*., 1997a,c; Puri *et al*., 1997c; Struhl, 1998; Giordano and Avantaggiati, 1999; Sartorelli *et al*., 1999; Puri and Sartorelli, 2000; Iezzi *et al*., 2002). Significantly, p300/CBP directly interacts with MyoD and conveys transcriptional competency by contacting proteins present in the TFIID complex and the basal transcription machinery (Eckner *et al*., 1996; Yuan *et al*., 1996; Sartorelli *et al*., 1997; Puri *et al*., 1997c; Giacinti *et al*., 2006).

2.2 Interplay between proliferation, differentiation and rhabdomyosarcoma

A clear and effective statement asserts that cell proliferation and differentiation are mutually exclusive events. In muscle cells, as in other cell types, the decision to divide or differentiate is determined by a balance of opposing cellular signals (Olson, 1992).

Several HLH proteins have been characterized as inhibitors of myogenesis which exert their activity by distinct mechanisms:

1. Inhibition of myogenic bHLH proteins by direct protein-protein interaction: Id (Benezra *et al*., 1990), Twist (Hamamori *et al*., 1997), I-mfa (Chen *et al*., 1996), Cdk4-CyclinD1 (Zhang *et al*., 1999a)

2. Displacement of E12/47: Id- E12/47 complex (Jen *et al*., 1992)

3. Competition for DNA binding sites: MyoR (Lu *et al*., 1999), Mist (Lemercier *et al*., 1998) and ZEB (Postigo and Dean, 1997; Postigo *et al*., 1999)

4. Cytoplasmic retention of myogenic bHLH proteins: I-mfa (Chen *et al*., 1996)

Numerous proteins either facilitate or are required in order to enhance myogenic bHLH transcriptional activity. Some of these are transcriptional activators themselves, whereas others do not directly interact with sequence-specific DNA targets (coactivators).

For instance, as alredy explained, MyoD forms a multiprotein complex with its heterodimeric partners, E12 or E47, and with acetyltransferases p300 and PCAF, whose histone acetylation alters nucleosomal conformation and increase accessibility of transcription factors to DNA. Furthermore, PCAF-dependent MyoD acetylation stabilizes MyoD binding to DNA, presumably by inducing conformational changes in MyoD protein structure. Moreover, additional acetylations of the basal transcription machinery are also involved in the activation of transcription (Puri and Sartorelli, 2000).

It is essential to stress that other proteins influence the myogenic program through several mechanisms. For instance, thyroid hormone (TH) and retinoic acid receptors (RAR and RXR) are functionally related to the activation of muscle-specific promoters (Carnac *et al*., 1992; Albagli-Curiel *et al.*, 1993; Downes *et al*., 1993; Halevy and Lerman, 1993; Downes *et al*., 1994; Alric *et al*., 1998). Moreover, pRb is able to significantly upregulate MyoD activity and is involved in the hexpression of late muscle differentiation markers, MHC and MCK (Gu *et al*., 1993).

Another crucial mechanism in the activation of myogenic program is established by the multi-protein chromatin-remodeling complex SWI/SNF, recruited on myogenic loci by a p38-dependent mechanism. In fact, upon MKK6-dependent enzymatic activation, p38 localizes on the chromatin of muscle-gene regulatory elements promoting the recruitment of SWI/ SNF (Simone *et al*., 2004b). Thus, cofactor binding sites might help to expose a crucial E box or substitute for an E box facilitating the formation of stable and functional MyoD transcriptioncomplexes (Lluìs et *al*., 2006).

Significantly, Rhabdomyosarcoma (RMS), one of the most common childhood solid tumor, arises from muscle precursor cells and fails to complete both the differentiation program and the irreversibly cell cycle exit, resulting in uncontrolled proliferation and incomplete myogenesis (Merlino and Helman, 1999). Surprisingly, RMS cells express MyoD and myogenin to varying degrees, but they show only limited expression of genes associated with terminal differentiation (Hiti *et al*., 1989; Dias *et al*., 1991). Interestingly, the ability of MyoD to arrest cell proliferation and to activate the myogenic program is repressed in RMS, hypothesizing a MyoD activity-dependent inhibition of myogenesis (Tapscott *et al*., 1993; Otten *et al*., 1997).

2.3 Cdk9, Cyclin T and role in muscle differentiation.

Cyclin-dependent kinase 9 (cdk9), previously named PITALRE (Grana *et al.*, 1994), is a cdk2-related serine/threonine kinase, widely expressed in human and murine tissues with high protein levels in terminally differentiated cells (De Luca *et al*., 1997a; Bagella *et al.*, 1998, 2000; Simone *et al*., 2002).

Cdk9 regulation and activity strictly differs from other CDKs. Cdk9 activity is not cell cycle-dependent and it does not appear to be required in cell cycle progression (MacLachlan *et al*., 1995; De Falco and Giordano, 1998). In addition, unlike the other cdks, Cdk9 fails to phosphorylate histone H1. In fact, it is involved in the promotion of transcription elongation via phosphorylation of the carboxyl-terminal domain (CTD) of RNA polymerase II, converting the inactive unphosphorylated, pre-initiation complex into the phosphorylated and active form (Grana *et al*., 1994; Dahmus, 1996; Marshall *et al*., 1996; Zhu *et al*., 1997; Wei *et al*., 1998 Simone *et al*., 2002; Soutoglou and Talianidis, 2002). The essential residues in transcription elongation are serine 2 and 5 (Zhou *et al*., 2001; Sano *et al*., 2002; Soutoglou and Talianidis, 2002).

CDK9 activity is regulated by cyclins T (T1, T2) and cyclin K (Fu *et al*., 1999).

Cyclin T1 and T2 share a highly conserved amino terminal motif (cyclin box region, 81% identity in human T-cyclins), a putative coiled-coil motif, a His-rich motif (responsible of the protein-protein interactions with the CTD of RNA polymerase II) and a carboxy- terminal PEST sequence (less conserved than cyclin box region, 46% identity in human T-cyclins) (Peng *et al*.,

1998b; De Luca *et al*., 2003). The ‘‘cyclin homology box,’’ formed by 290 amino acids, is the most conserved region among different members of the cyclin-family and serves to bind CDK9.

Cyclin T2 has two isoforms, T2a and T2b, that likely arise by an alternative splicing of the primary transcript, which share the first 642 amino acids but have different carboxyl termini (De Luca *et al*., 2003) (Figure 2). Interestingly, CycT2 bears a leucine-rich stretch next to its cyclin box capable to bind to CTD of RNA polymerase II, thus providing an extra domain capable of targeting RNAPII (Peng *et al*., 1998; Kurosu *et al*., 2004).

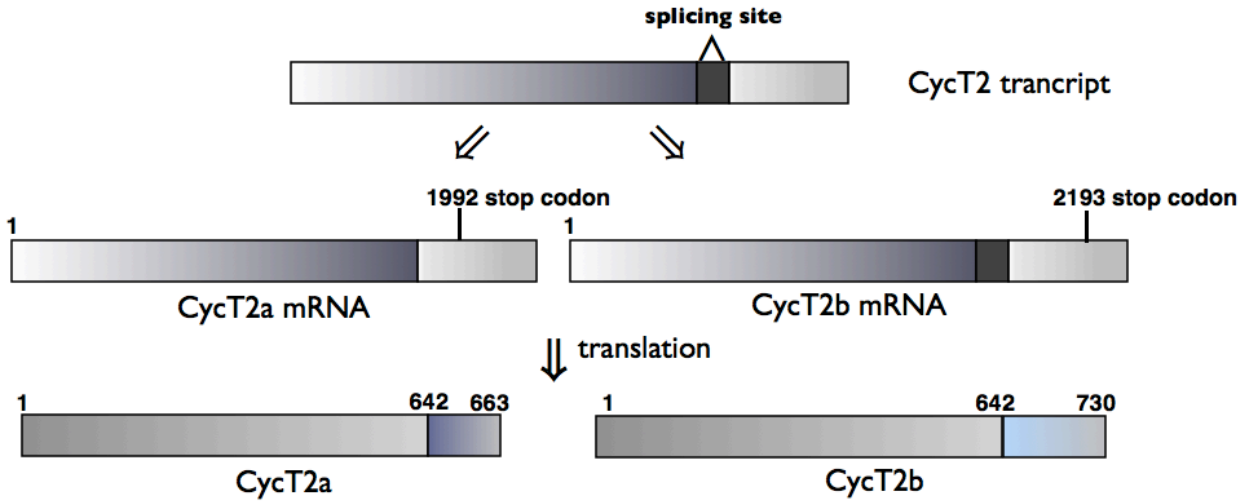


Figure 2: Schematic representation of mRNA and protein structure of the two isoforms of cyclin T2.

In muscle differentiation, Cdk9 is one of the co-activators of MyoD necessary for the completion of the myogenic program (Simone and Giordano, 2001, 2007; Simone *et al*., 2002). Indeed, it has been demonstrated that Cdk9 directly interacts with MyoD *in vitro* (Simone *et al*., 2002). Moreover, recently it has been shown that Cdk9, in muscle cells, takes part of a multimeric complex containing MyoD, cyclin T2, p300, PCAF and Brg1 (Giacinti *et al*., 2006). This complex binds to muscle-specific gene promoter regions and promote gene expression by

inducing chromatin remodeling, through acetylation of specific lysine residues of histones H3 and H4 and phosphorylation of RNA polymerase II CTD through Cdk9 (Simone *et al*., 2004; Giacinti *et al*., 2006; Simone and Giordano, 2007; Giacinti *et al*., 2008) (Figure 3).

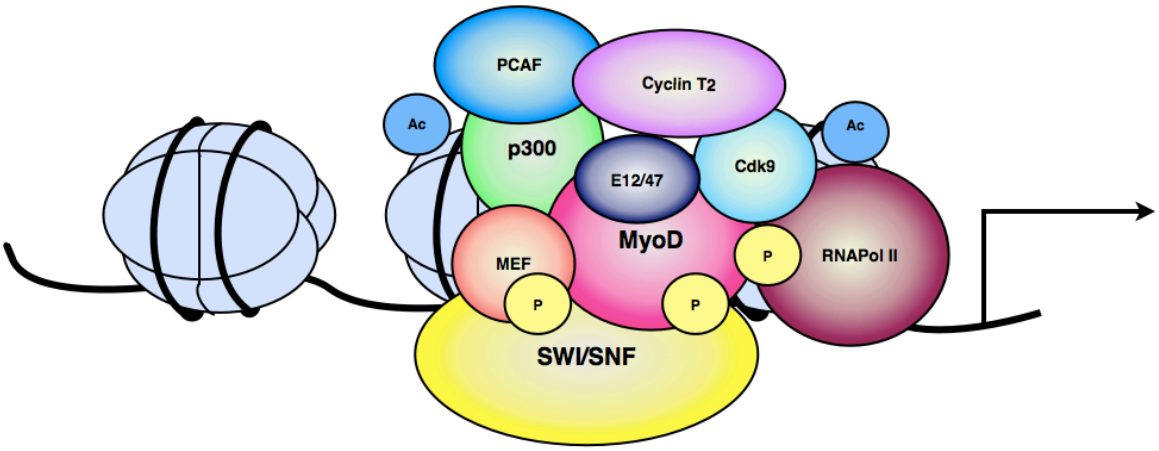


Figure 3: Schematic representation of the transcriptional complexes regulating gene expression in differentiation muscle cells.

Significantly, cyclin T1 was not detected on the same regions, suggesting cyclin T2-depedent Cdk9 activation (Giacinti e*t al*., 2006).

Surprisingly, Rhabdomyosarcoma cells showed upregulation of both Cdk9 and Cyclin T2 and a strongest Cdk9-cyclinT2 interaction when compared to myoblasts, although Cdk9 fails to phosphorylate MyoD. It is important to stress that no mutations were detected in the coding sequences of Cdk9 and Cyclin T2 genes and no significant values muscle-specific gene expression was detected in presence of overexpressed MyoD in RD cells (Simone and Giordano 2007).

2.4 EZH2 and role in the regulation of muscle differentiation

Polycomb repressive complex 2 (PRC2) is a histone methyltransferase which is responsible of the tri-methylation of lysine-27 of histone H3 (H3-K27). (Cao *et al*., 2002; Czermin *et al*., 2002; Kuzmichev *et al*., 2002; Muller *et al*., 2002).

PRC2 was initially purified and characterized from human cells and Drosophila embryos (Muller *et al*., 2002; Kuzmichev, *et al*., 2002; R. Cao *et al*., 2002; Czermin, *et al*., 2002); it contains a conserved catalytic subunit, termed EZH2 in human, that include the signature SET domain, which provides the methyltransferase active site (Rea *et al*., 2000). The SET domains have an unusual "thread-the-needle" structure, called pseudoknot (Cheng and Zhang, 2007; Dillon *et al*., 2005 for reviews), formed by juxtaposition of two conserved peptide motifs with one peptide inserted through the loop created by the other. These structures show that the substrate lysine and methyl donor cofactor bind opposite sides of the SET domain with their binding pockets connected by an interior channel that aligns the reactive groups for methyl transfer. To attain robust histone methyltransferase activity, EZH2 must be complexed with at least two of its non catalytic partners, EED/ESC and SUZ12 (Cao and Zhang 2004; Pasini *et al*., 2004; Ketel *et al*., 2005; Nekrasov *et al*., 2005; Montgomery *et al*., 2005). Both the C-terminal SET domain and the adjacent cysteine-rich (CXC) domain are required for histone methyltransferase activity (Muller *et al*., 2002; Kuzmichev, *et al*., 2002; R. Cao *et al*., 2002; Cao and Zhang 2004). Instead, N-terminal domains provide binding sites for assembly with the required partner subunits (Figure 4).

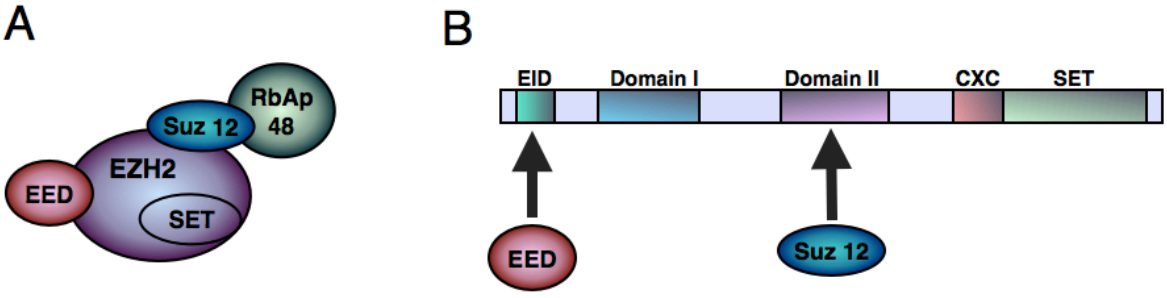


Figure 4: Composition of PRC2 and domain organization of EZH2. (A) The four core subunits of human PRC2 are EZH2, EED, SUZ12 and RbAp48 (B) Five functional domains in EZH2.

PRC2 enzyme function can also be influenced by another associated component, called PHF1 (PCL in flies). Although PHF1 is not a core subunit of PRC2, its association with the complex can stimulate PRC2 enzyme activity and/or influence its recruitment to target genes in vivo (Nekrasov *et al*., 2007; Savla *et al*., 2008; Sarma *et al*., 2008; Cao *et al.,* 2008).

Polycomb (PcG)-mediated modifications of histones is an essential mechanism that ensures the establishment and maintenance of gene expression pattern during mammalian development. (Sparmann and van Lohuizen 2006). Gene expression silencing by PcG proteins, characterized by the trimethylation of lysine 27 on histone 3 (H3K27me3) (Czermin *et al*., 2002; Muller *et al*., 2002), is required at specific stages of development for the timely expression of genes involved in stem cell fate and lineage commitment upon differentiation (Boyer *et al*., 2006; Lee *et al*., 2006; Bracken *et al*., 2006; Pietersen and van Lohuizen, 2008) and for mammalian X-inactivation and imprinting (Plath et al., 2003; Umlauf *et al*., 2004).

Polycomb silencing and DNA methylation have often been considered biochemically independent gene silencing systems. However, recent studies show that EZH2 and DNA methyltransferases (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 an hypothesis is that target genes are initially silenced through histone H3-K27 methylation by PRC2. PRC2 recruits DNA methyltransferases (DNMTs) 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 a*l., 2007).

Moreover, in human cells, PRC2 can physically associate with HDAC1 and HDAC2. HDACs are not core subunits of PRC2 but transient interactions likely still provide functional synergy between these silencing enzymes *in vivo*. The precise mechanism of this synergy at target gene chromatin 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) (Figure 5).

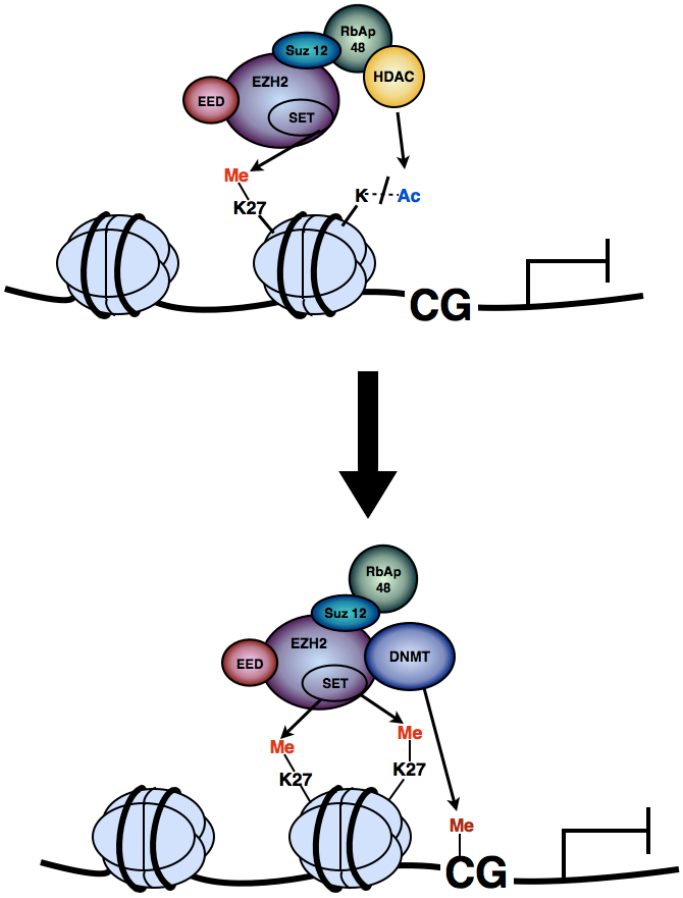


Figure 5: Model for collaboration of epigenetic silencing enzymes. Target genes are initially silenced through histone H3-K27 methylation by PRC2. If the lysines are acetylated, may first require deacetylation by a histone deacetylase (HDAC). PRC2 may also recruit DNA methyltransferases (DNMTs) which methylate CpG DNA of target genes, leading to a more permanently or deeply silenced chromatin state

Polycomb-mediated gene silencing and DNA methylation underlie many epigenetic processes important in normal development as well as in cancer.

Typically, EZH2 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 roles 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). Furthermore, even when detected

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).

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

Significantly, numerous MyoD-target sequences in muscle-restricted gene promoter regions, silent in embryonic staminal cells, are occupied by PcG proteins marked by H3K27me3 (Lee *et al*., 2006). Moreover, in skeletal muscle cells (SMC), PcG proteins and H3K27me3 are no longer present at MyoD target-sequences, allowing for their transcriptional activation. Importantly, although MyoD is expressed in undifferentiated SMC, PcG proteins continue to bind certain MyoD target genes, which continue to be marked by H3K27me3 and silenced. After additional molecular signals that promote the complete myogenic program, initiate PcG binding and H3K27me3 are lost at MyoD target loci, resulting in appropriate muscle gene expression and SMC differentiation (Caretti *et al*., 2004; Juan *et al*., 2009) (Figure 6).

Furthermore, recent studies showed that levels of Ezh2 transcript and protein in RMS compared to normal myoblasts are consistently higher (Ciarapica *et al*., 2009).

These data suggest that EZH2 plays a key role in skeletal-muscle differentiation, specifically in the maintenance of the undifferentiated state of muscle cell precursors. We hyphotesize that EZH2 overexpression may participate in Rhabdomyosarcoma formation and progression.

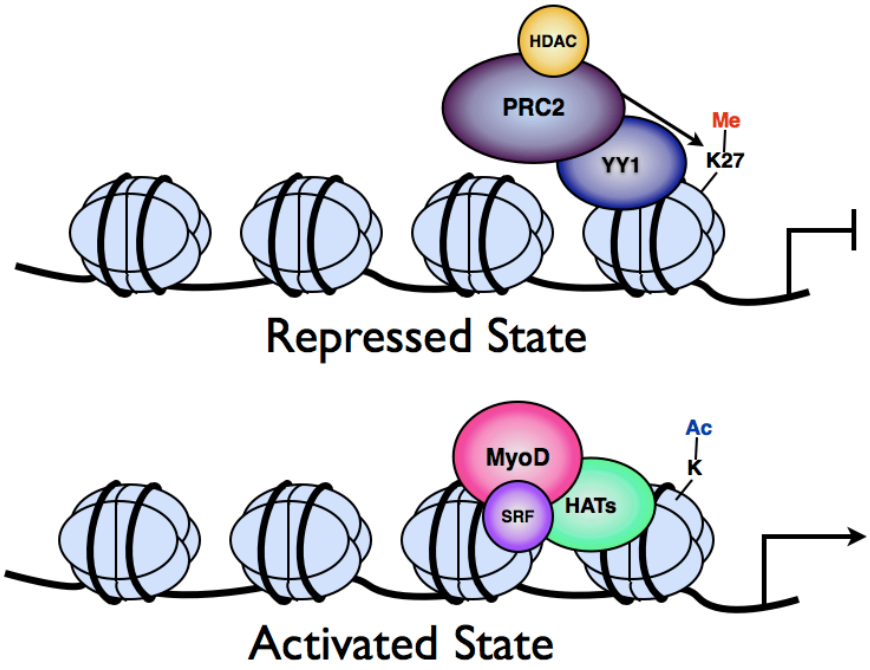


Figure 6: Two-step activation model of muscle gene expression. Regulatory regions of certain muscle-specific genes are occupied by a protein complex containing the DNA-binding protein YY1, the methyltransferase Ezh2, and the deacetylase HDAC1. Deacetylation of lysine residues by HDAC1 and trimethylation of H3-K27 by Ezh2 actively prevent transcription (repressed state). At the triggering of transcriptional activation, YY1 is displaced from the chromatin, Ezh2 and HDAC1 are replaced by SRF. H3-K27 becomes hypomethylated, and MyoD and HATs are recruited to the regulatory regions and allowing initiation of transcription (activated state).

3. AIM OF THE PROJECT

Skeletal muscle differentiation is influenced by multiple pathways which regulate the activity of myogenic regulatory factors (MRFs) and the MEF2 family members, in positive or negative ways.

Elucidating the mechanisms governing muscle-specific transcription will provide important insight to better understand the embryonic development of muscle at the molecular level and will have important implications in setting out new therapeutic strategy.

Rhabdomyosarcoma is a highly malignant pediatric tumor that derive from mesenchymal cells already committed to become skeletal muscle cells. In this tumor, the activity of MRFs are compromised. Furthermore, recent studies showed that levels of EZH2, a protein involved in the regulation of muscle differentiation process, in rhabdomyosarcoma are consistently higher compared to normal myoblasts.

The purpose of this study is the characterization of the two murine Cyclin T2 isoforms, CycT2a and CycT2b and the evaluation of their role in muscle differentiation program. Moreover the project focus on the modulation by PRC2 of specific skeletal muscle differentiation-related gene promoters and its involvement in rhabdomyosarcoma formation. In particular the study focus on the possible interaction between Cdk9/Cyclin T2 complexes and EZH2.

This study will help to clarify the function of these proteins and the molecular regulation during the myogenic program tumor formation.

4. MATERIALS AND METHODS

4.1 Cloning and Sequencing

Rapid amplication of cDNA ends (RACE) was employed to generate complete cDNA sequence encoding the Cyclin T2 isoforms. Mouse skeletal muscle Marathon-ready cDNAs (Clontech) were used as templates in RACE polymerase chain reaction (PCR) to obtain the 5‘and 3’-end cDNA fragment according with the manufacture’s protocol

The PCR for 5’-end was carried out using Adaptor primer 1, included in the kit, as sense primer and CycT2rev1 (GCTTGCAAATGGTCCAATTGGG) as antisense primer.

The PCR for 3’-ends was carried out using CycT2afor and CycT2bfor (CCACGGTGCTCAGGAGTCCT; CAGCGGATGGAATGCCTCCC respectively) as sense primers and Adaptor primer 1 as antisense primer.

PCR products were cloned into pGEM-T Easy Vector System II (Promega) and the sequenced using T7 and SP6 primers. The sequences are analyzed on the 3730 DNA Analyzer from Applied Biosystems.

The sequence was used to design the primers for full lenght amplification:

CycT2for1: GGATCCATGGCGTCGGGCCGTGGA

CycT2arev: GGATCCCTGGAGTCAGGACCGTGGGGCTCC

CycT2brev: GGATCCTTACATATTCATTCCTTG

4.2 Plasmids

The plasmids Myogenin-luciferase Myh-luciferase promoter were constructed by PCR. The genomic DNA was extract by DNeasy blood and tissue kit (Qiagen) following the manufacture’s protocol. The PCR were performed with specific primers:

Myogenin promoter for CAAACGCTAGCCAGCTCTCACGGCTGCTATGA

Myogenin promoter rev GGGAGATCTGGTAGAAATAGGGGGATGTCTC

Myh promoter for CTCCCGGGCTGTATTTCCTCATCTGTGAGGA

Myh4 promoter rev: CTACAAGCTTAGACCAGTTGCTCCTATGCCC

The amplifed products were cloned in the NheI-BglII site and XmaCI-HindIII of the pGL3 basic vector (Promega) for Myogenin promoter and Myh promoter respectively.

The plasmids pcDNA3-CycT2a and pcDNA3-CycT2b were constructed by PCR using pGEM-T Easy-CycT2a and pGEM-T Easy-CycT2b. The PCR were performed with the primers:

mBamHICycT2for: GGATCCACCATGGCGTCGG

mBamHICycT2arev: GGATCCCTGGAGTCAGGACC

mBamHICycT2brev: GGATCCTTACATATTCATTCTTG

The amplifed products were cloned in the BamHI site of the pcDNA3 vector (Invitrogen)

The correct sequences of all these constructs were confirmed by sequencing using the 3730 DNA Analyzer from Applied Biosystems. The constructs pcDNA3-cdk9wt expressing full-length and Gst-cdk9 have been previously described (De Falco *et al*., 2000). The constructs expressing, MyoD and EZH2 have been described previously. (Simone *et al*., 2002; Tonini *et al*., 2004 respectively).

4.3 Lentivirus production and infection

293T packaging cell were seed at $1.5x10^5$ cells/ml (6 ml per plate) in low-antibiotic growth media (DMEM + 10% FBS) in 6 cm tissue culture plates.

After 24 hours, the packaging cells were transfected with 3 lentivirus plasmids. 1µg of Hairpin-pLKO.1 vector (shRNA-EZH2 from Sigma), 0.9µg of packaging plasmid psPAX2, 0.1µg of envelope plasmid pMD2G were diluted in OPTI-MEM to total volume of 250µl. 24µl of FuGene HD were added to plasmids mix and incubated 20 minutes at room temperature. The transfection mix was transfered to the the packaging cells. The cells were incubated at 37 °C, 5% CO_2. 18 hours post-transfection the medium was replaced with fresh high-serum medium. After 24 hours the virus in the medium were harvested and the replaced with high-serum media. 24 hours after the first harvest, the virus were harvested and the packaging cells were discarded. The media containing virus were filtered with 0.45µm filter. The eluate was transferred to a sterile polypropylene storage tube.

RD cells were infected with 1ml (1 moi) of virus solution. After 18-24h of incubation the media were replaced with growth media. After 48h the media were replaced with growh media added with puromicine (final concentration 2μg/ml).

4.4 Cell culture and differentiation

Myoblasts (C2C12) were grown in DMEM supplemented with 20% FBS 1% L-glutamine and antibiotics (Growth Medium, GM). Rhabdomyosarcoma cells RD were grown in DMEM supplemented with 10% FBS and antibiotics. The differentiation was induced by serum withdrawal in the presence of 2% horse serum (Differentiation Medium, DM). All cell lines were obtained from ATCC. Pellets were collected every 24h for 144h or 96h.

4.5 Immunoblotting

Cell were lysated in lysis buffer (20 mM Tris HCl pH 8; 137 mM NaCl; 10% glycerol 1% Nonidet P-40; 2 mM EDTA; Protease Inhibitor Cocktails)

The protein concentration was determined by Bradford assay (Biorad, CA), following the manufacturer's instructions and by using BSA as a standard.

The protein extract (50μg) was resolved in 8% SDS/PAA gel and transferred to a nitrocellulose membrane at 4°C and at 100V for 1h.The blots were blocked with TBS-T containing 5% non-fat dry milk.

The protein levels were detected with the followed antibody anti-Cdk9 (Rockland), Anti-CyclinT2, anti-MyoD anti-Myogenin, anti-MYH (SANTA CRUZ) anti EZH2 (Invitrogen). Equal loading was controlled with anti-Gapdh and anti-Hsp70 (SANTA CRUZ).

Antibody were used in TBS-T containing 3% non-fat dry milk. Anti-mouse, rabbit (1:10000), goat (1:2500) peroxidase conjugated (Pierce) and ECL detection system (PerkinElmer) were used for detection.

4.6 Total RNA extraction, cDNA synthesis and Real Time PCR

Total RNA was extracted from 5–10 x 10^6 cells using High Pure RNA Isolation Kit (Roche). 1µg of RNA was used for cDNA production with random primers, using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), following the manufacture's protocol. 20µl of reaction was diluited in 200µl of sterile water.

Real Time was performed using 4,5µl of cDNA and 250 nM primers diluited in FastStart Universal SYBR Green Master (ROX), (Roche), to a final volume of 10µl. Amplification conditions for all amplicons were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15s, 60°C for 30s, 72°C for 30s.

Accumulation of fluorescent products was monitored using an Applied Biosystem 7300 system. Each data point was obtained from at least three independent experiments. Transcripts for glyceraldehyde-3-phosphate dehydrogenase were used as a reference. To ensure specific PCR amplification, every real time PCR run was followed by a dissociation phase analysis (denaturation curve) and by gel electrophoresis. ΔΔCT method was used to calculate relative changes in gene expression; primer efficiency was calculated for every target using five x 10-fold serial dilutions of PCR products. Specific primer sequences are reported in the table below

	For	Rev
mGAPDH	AGAAGGTGGTGAAGCAGGCATC	CGAAGGTGGAAGAGTGGGAGTTG
mCycT2a	CGTCTCCTCCGCCTCCAGTG	AGATGTCCGTAGCCCACCTGC
mCycT2b	AGCGAAGCCTCCCACAACC	GTCCGTAGCCCACCTGGTATG
mMyoD	GATGGCATGATGGATTACAGCG	GGAGATGCGCTCCACTATGCT
mMyogenin	CAATGCACTGGAGTTCGGTCC	AGTGATGGCTTTTGACACCAAC
mMYH	CAGAGCTTATTGAGATGCTTCTG	ATCACAGCGCCTGTGAGCTTG
hGAPDH	GAAGGTGAAGGTCGGAGT	CATGGGTGGAATCATATTGGA
hCycT2a	CAGGACTCCTCAGAACAGTGG	TGTCCGTAGCCCACCTGAACT
hCycT2b	CAACCACCACTCCAAAATGAGC	GAGGAGGGGGTAAGGGATGG
hMyoD	GACGGCATGATGGACTACAGC	GGAGATGCGCTCCACGATGC
hMyogenin	CCTGCTCAGCTCCCTCAACC	AGGGTCAGCCGTGAGCAGATG

4.7 Transient transfections and luciferase assay

Transient transfections were performed using FuGene HD (Roche applied). 2µg of total DNA diluted in 100µl Opti-MEM (CellGro) was incubated with 8µl of FUGeneHD for 20 minutes to let the transfection complex form. The transfection complex was added in a ratio 1:16 to the volume of the incubation medium of the cells (6µl of transfection complex was added to 100µl of complete medium in 96 wells plate).

Dual luciferase reporter assay (Promega) was used to measure the firefly luciferase and renilla luciferase activity within the transfected cells. Each experiment was conducted as suggested by the manifacturer. Luciferase assay was conducted on C2C12 myoblast transfected with the Myogenin-luc reporter, Myh-luc reporter, RL-TK renilla and expression vectors for CycT2a, CycT2b, Cdk9 and MyoD. The transfected cells were cultured in DM for 24h and 48h. Luciferase activity was normalized to TK-directed Renilla expression, in a ratio 1:20 respect to firefly luciferase vector, to control for variability in transfection efficiencies. The assay were performed with Sirius Luminometer (Berthold detection systems).

The results are expressed in arbitrary units relative to the activity of the basic luciferase vector (pGL3-myogenin/Myh promoter).

4.8 Nuclear extraction and Co-immunoprecipitation

For nuclei isolation cells were lysated with NP40 buffer (10mM Tris-HCl; 10mM NaCl; 3mM $MgCl_2$; 30mM Sucrose; 0.5% NP40; Protease Inhibitor Cocktails) for 30 minutes and centrifuged for 10 minutes at 3000 g. The pellet with the nuclei was washed with NP40 buffer and resuspended in non denturing lysis buffer (20 mM Tris HCl pH 8; 137 mM NaCl; 10% glycerol 1% Nonidet P-40; 2 mM EDTA; Protease Inhibitor Cocktails). Lysates were then sonicated at amplitude 30% in twice cycles of 30 sec, spaced out 15 sec, using a Fisher Model 550 Sonic Dismembrator (Fisher, Pittsburgh, PA).

2mg of nuclear extract was precipitated with 2µg of anti-Cdk9 antibody (Rockland) or normal rabbit IgG. The immunoprecipitates were purified by the addition of protein A-agarose (Roche) following the manufacturer's instructions. Immunoblot were performed with anti-EZH2 (Invitrogen).

4.9 Espression and Purification of GST-fusion proteins, Translation and Binding *in vitro*

GST-Cdk9 and GST were generated by growing 400ml of recombinant *E. coli* BL21 culture at 37°C to an A_{600} of 0.4. Cultures were induced for 4 hours with 1 mM IPTG. After induction, cultures were pelleted, resuspended in NETN buffer (20 mM Tris HCl Ph 8; 100 mM NaCl; 1 mM EDTA; 0.5% NP-40) containing 1mM PMSF and 1mM DTT and sonicated at amplitude 30% in six cycles of 1 minute, spaced out 30 seconds, using a Fisher Model 550 Sonic Dismembrator (Fisher, Pittsburgh, PA). The bacterial lysates were cleared of cellular debris by centrifugation and incubated with gluthatione agarose beads over night at 4°C. The complexes beads-GST and beads GST-Cdk9 were washed twice in NETN buffer 0.2M once with RIPA buffer (50 mM Tris HCl pH 8; 150 mM NaCl; 1% NP-40; 0.5% Sodium deoxycholate) and once with NETN buffer 1mM PMSF and 1mM DTT. The complexes were resuspended in 150 µl of NETN buffer.

The TNT coupled reticulocyte kit was used for in vitro translation (Promega, WI, USA), according the manufacturer's instructions. All the samples were labeled using 35S- Methionine

10 µl of labeled samples were incubated with 10 µg of GST-CDK9 or GST as a negative control, in 150 µl of Buffer A (20 mM Tris HCl pH 8; 150 mM KCl; 5 mM $MgCl_2$; 0.2 mM EDTA; 10% Glycerol; 0.1% NP-40) containing DTT 1mM and PMSF 1mM. The samples then were washed in buffer A ,c and were resolved on 8% SDS-PAGE. The gels then were fixed with a fixing solution (50% methanol 10% glacial acetic acid) in slowly agitation for 30 minutes. Finally the gels were dried for 30 minutes at 80°C and subjected to autoradiography.

5. RESULTS

5.1 Isolation and characterization of the murine Cyclin T2 cDNA

Murine cDNA of Cyclin T2 isoforms were cloned into pGEM-T Easy Vector System II and sequenced. The murine CycT2a and CycT2b, show 86% and 87% of similarity respect the human counteparts. Instead, the comparison between the amino acidic sequences show 85% and 86% of similarity (Figure 7). The predicted molecular weights of CycT2a and CycT2b are 73 kDa and 80 kDa respectively.

A

	Similarity between human and murine cycT2	
	cDNA	Amino acidic sequences
CycT2a	86%	87%
CycT2b	85%	86%

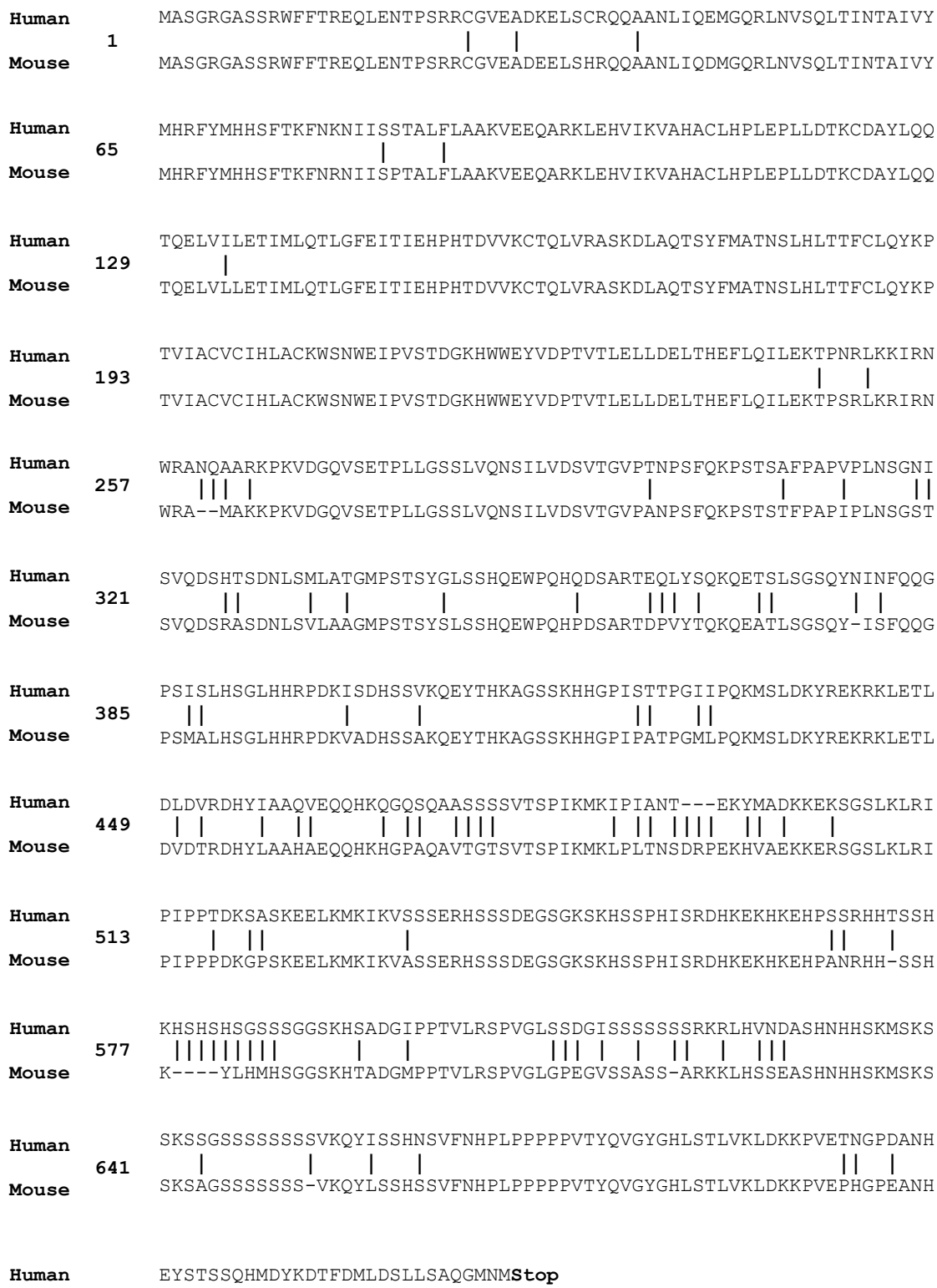
Figure 7: **Cloning and sequencing of murine Cyclin T2 cDNA.** (A) Similarity between human and murine isoforms. (B) Amino acid sequence of the murine Cyclin T2. The difference between the mouse and human protein sequence is denoted by a bar between the two.

A

Cyclin T2a

```
Human      MASGRGASSRWFFTREQLENTPSRRCGVEADKELSCRQQAANLIQEMGQRLNVSQLTINTAIVY
         1                              |   |         |
Mouse      MASGRGASSRWFFTREQLENTPSRRCGVEADEELSHRQQAANLIQDMGQRLNVSQLTINTAIVY

Human      MHRFYMHHSFTKFNKNIISSTALFLAAKVEEQARKLEHVIKVAHACLHPLEPLLDTKCDAYLQQ
        65                     |    |
Mouse      MHRFYMHHSFTKFNRNIISPTALFLAAKVEEQARKLEHVIKVAHACLHPLEPLLDTKCDAYLQQ

Human      TQELVILETIMLQTLGFEITIEHPHTDVVKCTQLVRASKDLAQTSYFMATNSLHLTTFCLQYKP
       129      |
Mouse      TQELVLLETIMLQTLGFEITIEHPHTDVVKCTQLVRASKDLAQTSYFMATNSLHLTTFCLQYKP

Human      TVIACVCIHLACKWSNWEIPVSTDGKHWWEYVDPTVTLELLDELTHEFLQILEKTPNRLKKIRN
       193                                                        |   |
Mouse      TVIACVCIHLACKWSNWEIPVSTDGKHWWEYVDPTVTLELLDELTHEFLQILEKTPSRLKRIRN

Human      WRANQAARKPKVDGQVSETPLLGSSLVQNSILVDSVTGVPTNPSFQKPSTSAFPAPVPLNSGNI
       257    ||| |                          |         |    |     ||
Mouse      WRA--MAKKPKVDGQVSETPLLGSSLVQNSILVDSVTGVPANPSFQKPSTSTFPAPIPLNSGST

Human      SVQDSHTSDNLSMLATGMPSTSYGLSSHQEWPQHQDSARTEQLYSQKQETSLSGSQYNINFQQG
       321      ||     |  |       |          |     ||| |    ||      | |
Mouse      SVQDSRASDNLSVLAAGMPSTSYSLSSHQEWPQHPDSARTDPVYTQKQEATLSGSQY-ISFQQG

Human      PSISLHSGLHHRPDKISDHSSVKQEYTHKAGSSKHHGPISTTPGIIPQKMSLDKYREKRKLETL
       385   ||           |     |                 ||   ||
Mouse      PSMALHSGLHHRPDKVADHSSAKQEYTHKAGSSKHHGPIPATPGMLPQKMSLDKYREKRKLETL

Human      DLDVRDHYIAAQVEQQHKQGQSQAASSSSVTSPIKMKIPIANT---EKYMADKKEKSGSLKLRI
       449  | |    |  ||     | ||  ||||         | || ||||  || |   |
Mouse      DVDTRDHYLAAHAEQQHKHGPAQAVTGTSVTSPIKMKLPLTNSDRPEKHVAEKKERSGSLKLRI

Human      PIPPTDKSASKEELKMKIKVSSSERHSSSDEGSGKSKHSSPHISRDHKEKHKEHPSSRHHTSSH
       513     |  ||           |                                    ||   |
Mouse      PIPPPDKGPSKEELKMKIKVASSERHSSSDEGSGKSKHSSPHISRDHKEKHKEHPANRHH-SSH

Human      KHSHSHSGSSSGGSKHSADGIPPTVLRSPVGLSSDGISSSSSSSRKRLHVNDASHNHHSKMSKS
       577  |||||||||      |   |          ||| |  |  ||  |  |||
Mouse      K----YLHMHSGGSKHTADGMPPTVLRSPVGLGPEGVSSASS-ARKKLHSSEASHNHHSKMSKS

Human      SKSSGGLRTSQHPRETGQEASGDQRS**Stop**
       641    |             |  ||
Mouse      SKSAGGLRTSQHPRETGQETSGAPRS**Stop**
```

Cyclin T2b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1
Mouse
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Human
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65
Mouse
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Human
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129
Mouse
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Human
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193
Mouse
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Human
WRANQAARKPKVDGQVSETPLLGSSLVQNSILVDSVTGVPTNPSFQKPSTSAFPAPVPLNSGNI
257
Mouse
WRA--MAKKPKVDGQVSETPLLGSSLVQNSILVDSVTGVPANPSFQKPSTSTFPAPIPLNSGST
Human
SVQDSHTSDNLSMLATGMPSTSYGLSSHQEWPQHQDSARTEQLYSQKQETSLSGSQYNINFQQG
321
Mouse
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Human
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385
Mouse
PSMALHSGLHHRPDKVADHSSAKQEYTHKAGSSKHHGPIPATPGMLPQKMSLDKYREKRKLETL
Human
DLDVRDHYIAAQVEQQHKQGQSQAASSSSVTSPIKMKIPIANT---EKYMADKKEKSGSLKLRI
449
Mouse
DVDTRDHYLAAHAEQQHKHGPAQAVTGTSVTSPIKMKLPLTNSDRPEKHVAEKKERSGSLKLRI
Human
PIPPTDKSASKEELKMKIKVSSSERHSSSDEGSGKSKHSSPHISRDHKEKHKEHPSSRHHTSSH
513
Mouse
PIPPPDKGPSKEELKMKIKVASSERHSSSDEGSGKSKHSSPHISRDHKEKHKEHPANRHH-SSH
Human
KHSHSHSGSSSGGSKHSADGIPPTVLRSPVGLSSDGISSSSSSSRKRLHVNDASHNHHSKMSKS
577
Mouse
K----YLHMHSGGSKHTADGMPPTVLRSPVGLGPEGVSSASS-ARKKLHSSEASHNHHSKMSKS
Human
SKSSGSSSSSSSSVKQYISSHNSVFNHPLPPPPPVTYQVGYGHLSTLVKLDKKPVETNGPDANH
641
Mouse
SKSAGSSSSSSS-VKQYLSSHSSVFNHPLPPPPPVTYQVGYGHLSTLVKLDKKPVEPHGPEANH
Human
EYSTSSQHMDYKDTFDMLDSLLSAQGMNMStop
705
Mouse
EYSTSSQHMDYKDTFDMLDSLLSAQGMNMStop

5.2 During the myogenesis, mRNA and protein levels of CycT2b are significantly higher respect to CycT2a levels

The muscle differentiation process was induced in murine myoblasts (C2C12) through serum-deprivation triggering in presence of horse serum, for 144 hours (Figure 8). The CycT2a and CycT2b transcripts and proteins levels were analysed by Real-Time PCR and immunoblotting at several stages of differentiation (every 24 hours).

Immunoblot show that protein levels of CycT2b, in comparison to CycT2a are markedly higher in all the stages of differentiation. The levels of Cyclin T2 increase during the myogenic program, (figure 9B). This results were confirmed by Real-Time PCR analysis that show that the levels of CycT2b cDNA are more or less 3.5 times higher respect the CycT2a cDNA levels (Figure 9A).

The myogenesis was verified by the evaluation of cells phenotype (figure 8) and the detemination of mRNA and protein espression of MyoD, Myogenin and MYH (Figure 9).

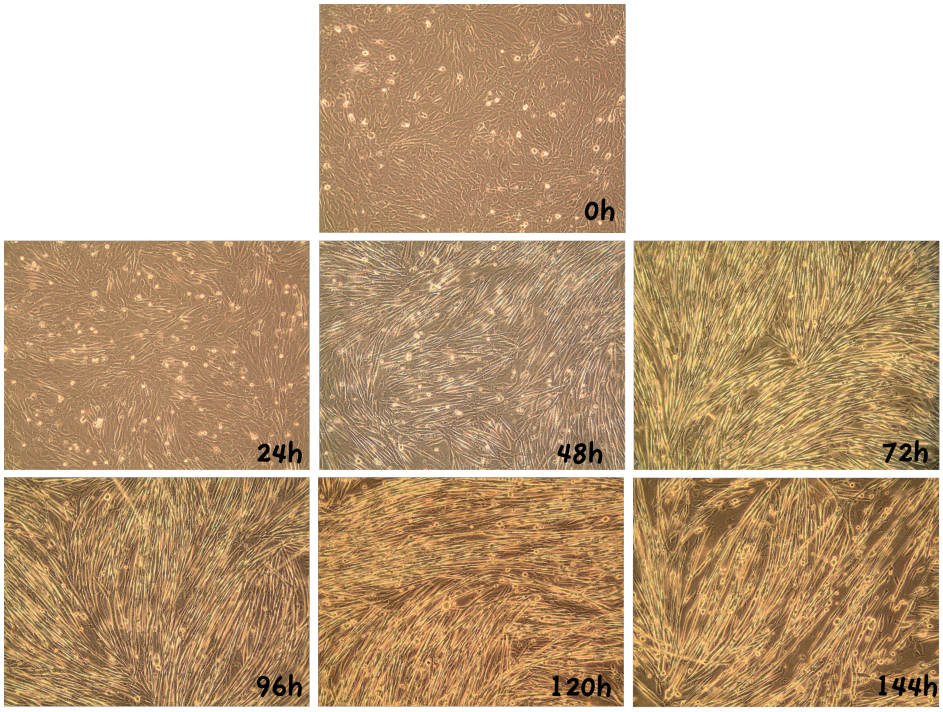

Figure 8: **C2C12 differentiation time course**. Myoblasts (C2C12) were grown to 90% confluence in complete medium (GM, 20% FBS) and induced to differentiate by serum withdrawal in the presence of 2% horse serum (DM) for 144h. Pellets were collected every 24h.

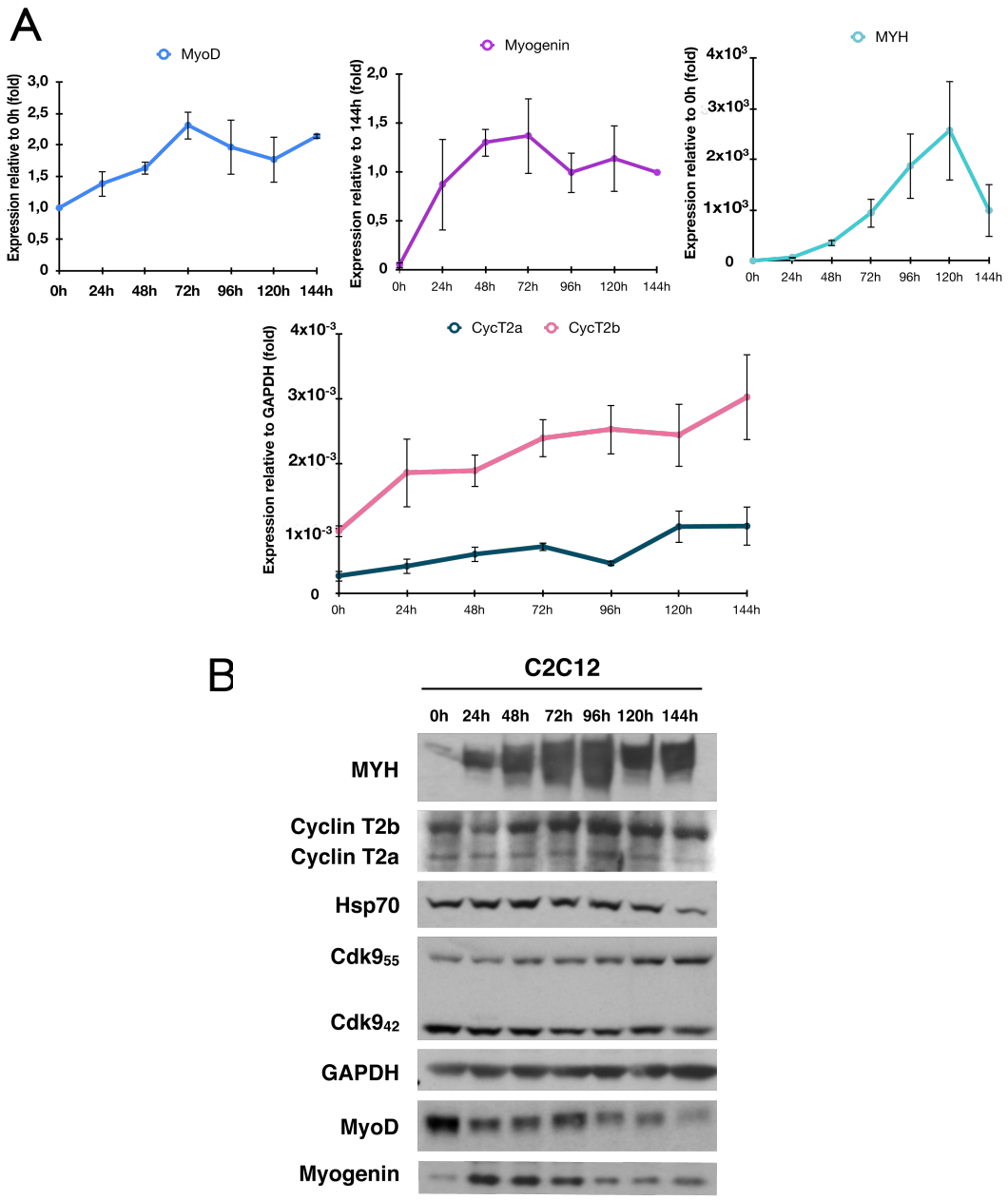

Figure 9: **Espression of Cyclin T2 in C2C12 during the muscle differentiation.** (A) Real-time quantitative PCR (qPCR) was used to determine relative mRNA expression levels upon induction of myogenesis. qPCR was performed using cDNA from myoblasts at several stage of differentiation (24h-144h). To distinguish the two Cyclin T2 isoforms, specific primers were designed in the splicing site region. The myogenesis was verified by detemination of mRNA espression of MyoD, Myogenin and MYH. The reported data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. (B) Immunoblot analysis The protein levels were detected with the followed antybody Anti-Cyclin T2, anti-Cdk9, anti-MyoD anti-Myogenin, anti-MYH. Equal loading was controlled with anti-Gapdh and anti-Hsp70.

5.3 Cyclins T2 activate the muscle specific genes promoters; CycT2b have a predominat role in the latest stages of the myogenesis

To establish a functional difference between CycT2a and CycT2b, C2C12 myoblast were transiently transfected with Myogenin promoter luciferase reporter (Myogenin-luc) and Myh promoter luciferase reporter (Myh-luc), and expression vectors for CycT2a, CycT2b, Cdk9, MyoD. The myoblasts were cultured in DM for 24h and 48h.

Transfection of either individual or pairwise combinations of Cdk9, CycT2a and CycT2b expression vectors had no effect on Myogenin-luc and Myh-luc in the absence of MyoD. Conversely, MyoD-dependent transactivation of promoters was increased, by coexpression of both complexes Cdk9/CyclinT2 (Figure 10). At 24h, CycT2a and CycT2b had the same effect on the myogenin-luc (Figure 10A); at 48h the effect of CycT2b in comparison to the effect of CycT2a was stronger (Figure 10C). On the Myh-luc the effect of CycT2b was stronger at both 24h and 48h (Figure10 B,D). This suggest a predominant role of Cyct2b in latest stages of differentiation process.

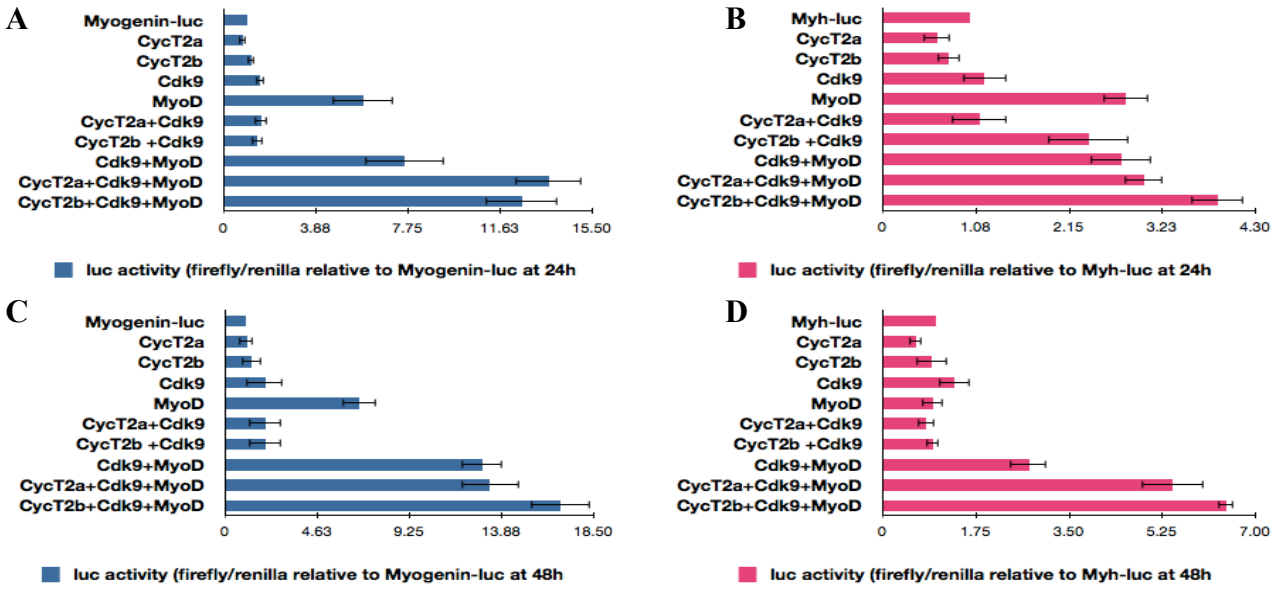

Figure 10: Regulation of Myogenin promoters by Cyclin T2. Luciferase assay was conducted on C2C12 myoblast transfected with the Myogenin-luc (A, C), Myh-Luc (B, D), RL-TK renilla and expression vectors for CycT2a, CycT2b, Cdk9 and MyoD. The transfected cells were cultured in DM for 24h and 48h. Luciferase activity was normalized to TK-directed Renilla expression. The results are expressed in arbitrary units relative to the activity of the basic luciferase vector (pGL3-myogenin promoter, pGL3 Myh promoter).

5.4 RD cells show mRNA and protein levels of muscle specific genes lower and EZH2 levels significantly higher respect to C2C12

To better understand the skeletal muscle differentiation process and the rhabdomyosarcoma formation, RD cells were grow 90% confluence in complete medium and induced to differentiate by serum withdrawal in the presence of 2% horse serum (DM) for 96h. Pellet were collected every 24h.

After 96h unlike C2C12, RD cells failed to complete the differentiation program and they continued to proliferate (Figure 11A). Immunoblot showed that protein levels of all markers of myogenesis (MyoD, Myogenin and Myh) are markedly lower respect C2C12 differentiation program. Interestingly, the levels of EZH2, a protein frequently overexpressed in several tumors, were considerably higher (Figure 11B).

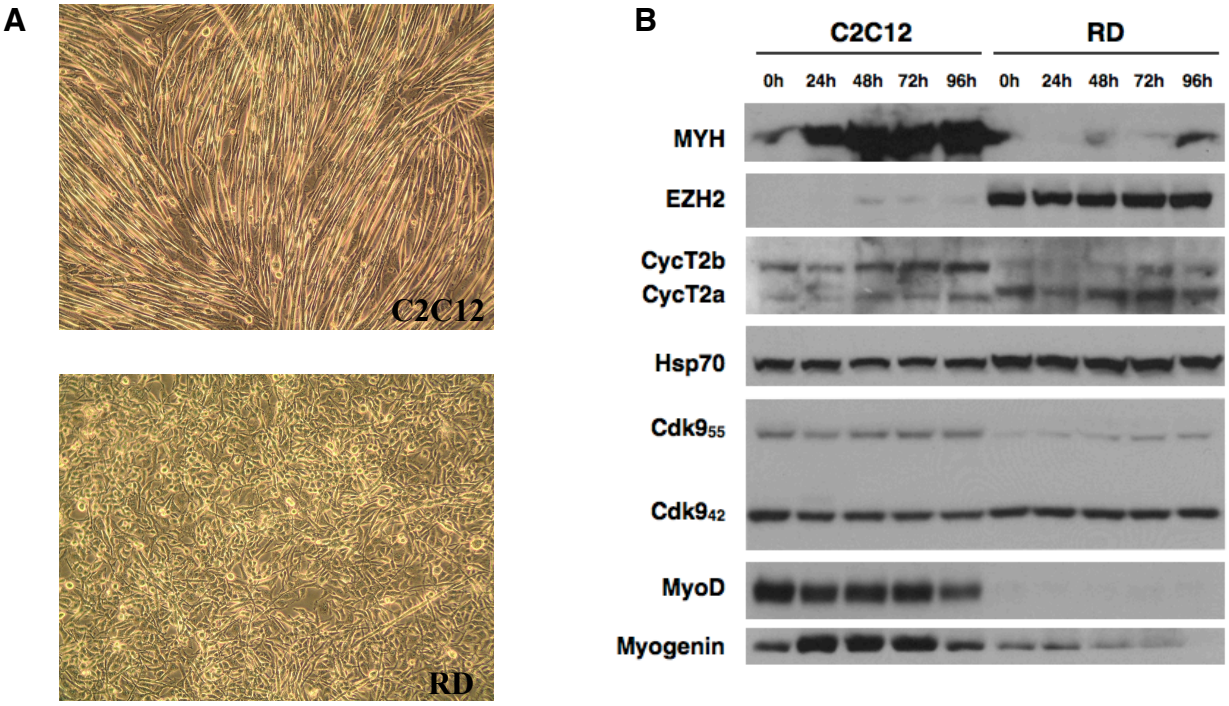

Figure11: **C2C12 and RD differentiation time course.** (A) Comparison between C2C12 and RD phenotype after 96h in DM. (B) Immunoblot analysis. The protein levels were detected with the followed antybody anti-Cyclin T2, anti-Cdk9, anti-EZH2 anti-MyoD anti-Myogenin, anti-MYH. Equal loading was controlled with anti-Hsp70.

5.5 EZH2 inhibit the promoters of several muscle specific gene

In order to understand the role of PCR2 in the rhabdomyosarcoma formation process a stable EZH2 knockdown RD cell line was generated using a vector-based shRNA. EZH2 knockdown cells did not show changes in cellular morphology.

The cells were induced to differentiate by serum withdrawal in the presence of 2% horse serum for 96h. Pellet were collected every 24h.

Interestingly, analysis of mRNA and protein expression showed that EZH2 knockdown resulted in a significant increase on the MyoD, Myogenin and MYH levels (Figure 12). The partial reactivation of muscle specific genes, suggests a role of EZH2 in the inhibition of muscle differentiation program.

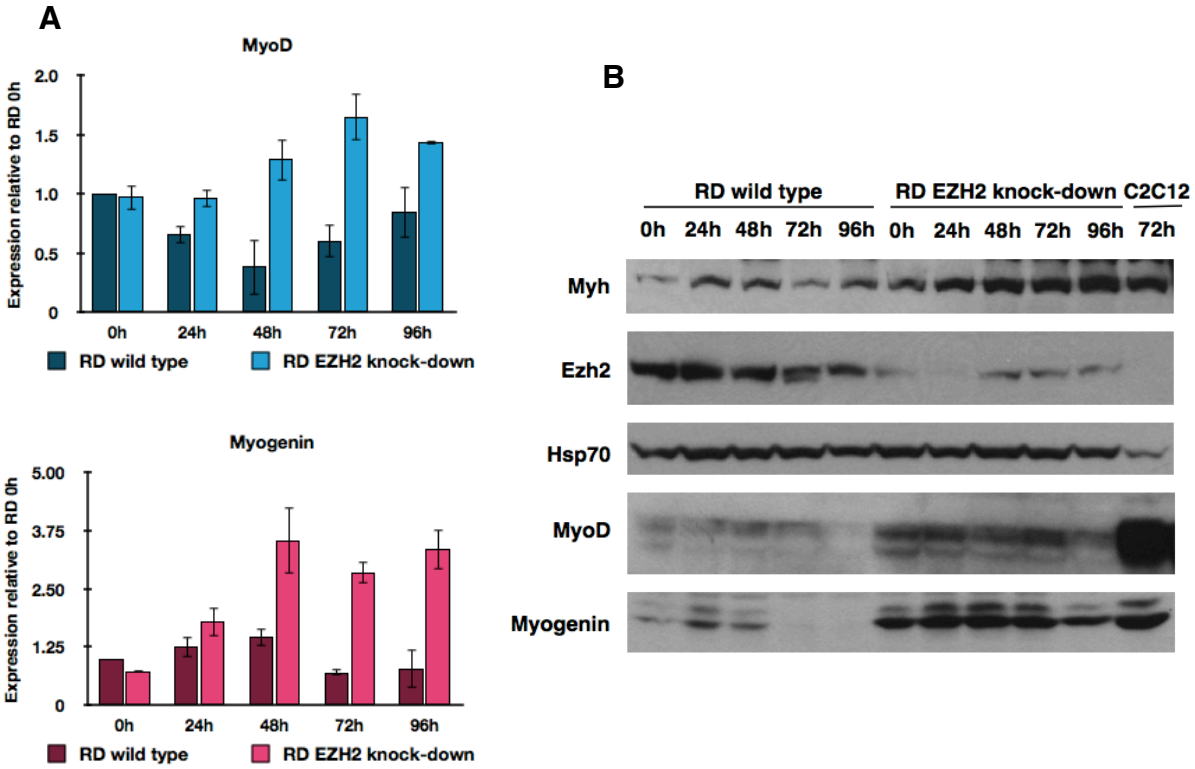

Figure 12: **Comparison between mRNA and protein espression in RD and RD EZH2 knockdown .** (A) Real-time quantitative PCR with specific primers for MyoD and Myogenin. (B) Immunoblot analysis. The protein levels were detected with the followed antybody: anti-MyoD, anti-Myogenin, anti-MYH and anti-EZH2. Equal loading was controlled with anti-Hsp70.

5.6 EZH2 binds to Cdk9 and Cyclins T2 both in *vitro* and in *vivo*

To investigate whether EZH2 was able to interact with Cdk9 and with *in vitro*, Gst pull-down experiments were performed using Gst-Cdk9 and GST-EZH2 purified from BL21 E. Coli cells and four in vitro translated product [^{35}S]-EZH2 full-length, [^{35}S]-Cdk9, [^{35}S]-CycT2a and [^{35}S]-CycT2b. As shown in figure 13A, Gst-Cdk9 was able to pull down full-length EZH2, while this was not the case for the Gst fragment alone. In addition, Gst-EZH2 was able to pull down both Cyclin T2 isoforms, showing a direct interaction between EZH2 and Cyclins T2 (Figure 13B,C). Moreover in order to test if EZH2 and Cdk9 were able to interact in Rhabdomyosarcoma cells, RD cells lysates were subjected to Co-immunoprecipitation with anti-Cdk9 and immunoprecipitation were then probed with anti-EZH2 (Figure 14). Normal Rabbit IgGs have been used as negative control.

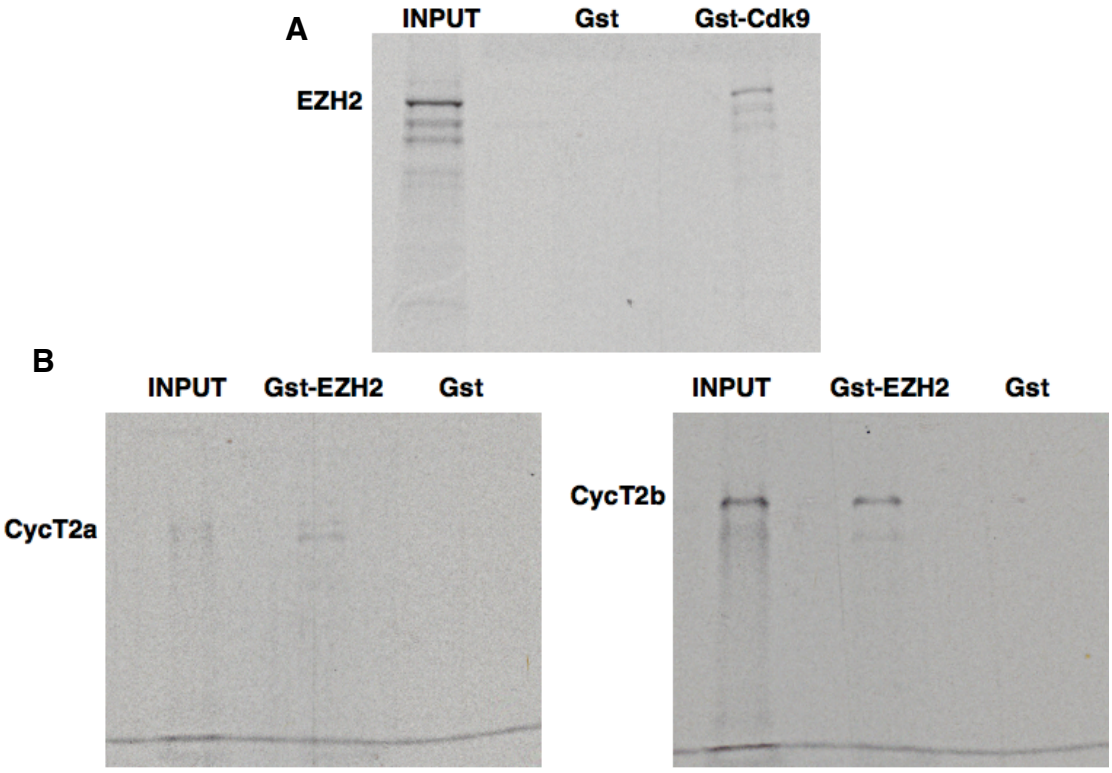

Figure 13: Binding in vitro assay: EZH2 binds to Cdk9, CycT2a and CycT2b in vitro. (A) Physical interaction between Gst-Cdk9 and in vitro translated EZH2. (B) Physical interaction between Gst-EZH2 and in vitro translated CycT2a and CycT2b.

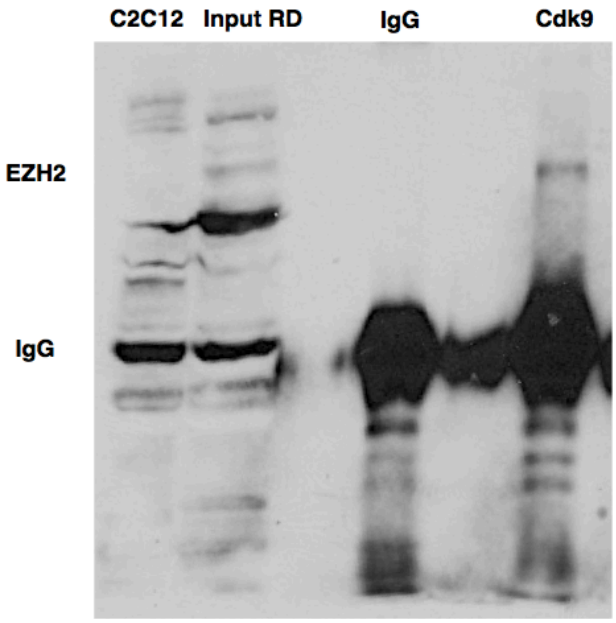

Figure 14: Co-immunoprecipitation. RD cells lysates were co-immunoprecipitated with anti-Cdk9 and Normal Rabbit IgG as negative control. Immunoblotting was performed with anti-EZH2.

6. DISCUSSION

During cell division, cyclins play an essential role being subjected to cyclical expression and ubiquitin-dependent degradation, and acting as regulatory subunits of complexes with the cyclin-dependent kinases (CDKs) (Sherr, 1996; Grana and Reddy, 1995).

Some CDKs/cyclin, as CDK7/cyclin H, CDK8/ cyclin C, and CDK9/cyclin T, seem to direct their activity in a cell cycle independent manner and appear to be involved in other processes as signal transduction, apoptosis, differentiation and transcription during the initiation or the elongation steps (Dynlacht, 1997; De Luca *et al*., 2003).

A Previous study showed that Cdk9, in association with Cyclin T2, plays an important role in the activation of the myogenic program (Simone *et al*., 2002b). Upon induction of muscle differentiation, MyoD recruits Cdk9/CycT2 on muscle-specific gene promoter sequences. This complex is able to phosphorylate the C-terminal domain (CTD) of RNA polymerase II, enhancing MyoD function and promoting myogenic differentiation (Giacinti *et al*., 2006). The transcriptional activity of MyoD is deficient in rhabdomyosarcoma cells and in these cells, Cdk9 fails to phosphorylate MyoD (Simone and Giordano, 2007).

EZH2 is the catalytic subunit of PRC2 and is responsible of the lysine-27 tri-methylation of histone H3.

Gene expression silencing by PcG proteins, is required at specific stages of development and is down-regulated in adult differentiated tissues (Varambally *et al*., 2002; Bracken *et al*., 2003; Kleer *et al*., 2003). PcG is able to regulate embryonic development of muscle inhibiting the homeobox gene expression (Caretti *et al*., 2004). Interestingly, EZH2 is overexpressed in a variety of different tumors as in the rhabdomyosarcoma.

This work focuses on the evaluation of role of Cdk9/Cyclin T2 complexes during the skeletal muscle differentiation and in rhadbomyosarcoma cells.

In order to understand the role of Cyclin T2 isoforms in muscle differentiation, myogenesis was induced in murine myoblast and mRNA and protein levels were analyzed at several stage of differentiation process. In addition, luciferase assay performed allowed to identify functional differences between two complexes.

The results demonstrated that CycT2b levels, both mRNA and protein expressions, in comparison to CycT2a are markedly higher in all the stages of differentiation. These results assumed a major role for CycT2b in muscle differentiation process. Moreover the luciferase assay showed that both cyclins T2 isoforms was able to increased MyoD-dependent transactivation of Myogenin and Myh promoters. During the first stages of differentiation both Cyclins T2 activate the muscle differentiation program but during the latest stages the activity of CycT2b is stronger.

The higher espression and the differences of functional activity show by CycT2b suggest a predominant role of this protein in the differentiation process, in particular during the latest stages.

Rhabdomyosarcoma (RMS) is one of the most common childhood solid tumor that arises from muscle precursor cells. In RMS cells, the ability of MyoD to arrest cell proliferation and to activate the myogenic program is repressed and the myoblasts fail to complete the differentiation program (Tapscott *et al*. 1993; Otten *et al*. 1997; Merlino and Helman, 1999). Moreover, Cdk9 fails to phosphorylate MyoD but no mutations were detected in the coding sequences of Cdk9 and Cyclin T2 (Simone and Giordano 2007). This allow to hypothesize that the MyoD inhibition arise from inhibition of Cdk9 activity.

The comparison between normal myoblast (C2C12) and RMS cells (RD) proteins levels confirmes that the expression of muscle specific genes is inhibited in RD cells whereas Cdk9 is strongly expressed. Interestingly, EZH2 a protein involved in the regulation of differentiation process of several tissue as in skeletal muscle differentiation, is overexpressed. EZH2 is the catalytic subunit of Polycomb repressive complex 2 (PRC2), which is histone methyltransferase that targets lysine-27 of histone H3 responsible of silencing ot target genes. Typically, EZH2 is down-regulated in adult differentiated tissues but over-expressed in a wide variety of cancerous tissue types (for review Simon and Lange 2008). Previous study showed that PRC2 plays a key role in the maintenance of the undifferentiated state of muscle cell precursors (Caretti *et al*., 2004).

In order to understand if the overexpression of EZH2 was the cause of rabdomyosarcoma formation, a stable EZH2 knockdown RD cell line was generated and induced to differentiate.

Interestingly, EZH2 knockdown allow a significant increase on the MyoD, Myogenin and MYH levels. The partial reactivation of muscle specific genes confirm the hypothesis that EZH2 overexpression contribuite to inhibition of muscle differentiation program.

To investigate whether the inhibition of Cdk9 in Rabdomyosarcoma cells and overexpression of EZH2 was interrelated, we assumed an interaction between Cdk9 and EZH2. We confirmed the interaction between the two proteins by alternative methods, co-immunoprecipitation and *in vitro* pull-down assays. Moreover we demonstrate that EZH2 is able to interact with both CycT2a and CycT2b.

These studies highlight a critical role for Cdk9/CycT2b complex in controlling skeletal muscle growth and differentiation. Our observation point toward the cooperation with MRFs such as MyoD and Myogenin suggesting that CycT2b has a crucial role in maintaining cells at the differentiation terminal stage. In addition, the physical interaction between Cdk9/Cyclin T2 complex and EZH2 emphasizes its commitment to the skeletal muscle lineage. It is reasonable to assume that during myogenesis Cdk9/Cyclin T2 complex might be implicated in the downregulation of EZH2, a step that is functionally required for the process of myotube formation.

The understanding of the functional properties of the Cdk9/Cyclin T2 complexes and the interaction with specific associated partners will help us to clarify the complex mechanisms that regulate the myogenic program. Moreover, the characterization of the binding between Cdk9 and myogenic regulatory factors like EZH2 may contribuite to explain the most important pathways involved in muscle program regulation and rhabdomyosarcoma formation.

Further studies are on going in our laboratory to define more accurately the molecular mechanisms underlying the myogenic function of Cdk9/CyclinT2 complexes with EZH2, and to verify its biological relevance in rhabomyosarcoma formation.

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