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ROLE OF CDK9 IN SKELETAL MUSCLE

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THE THESIS EXPLAINED

Cdk9 is a member of cyclin-dependent kinases and it is expressed in human and murine tissue with high levels in terminally differentiated cells. The elevated levels of Cdk9 and its regulatory subunits in terminally differentiated cells, together with the fact that it is implicated in the regulation of transcriptional elongation via phosphorylation of CTD of RNA polII, distinguishes Cdk9 from the other cdks.

Cyclin partners of CDK9 are cyclin T1, T2a, T2b and K, with a formation of a complex, called p-TEF-B. In Hela cells about 80% of cellular P-TEFb is composed of cyclin T1 and the cyclin – dependent kinase 9 (CDK9), and about 20% of cellular CDK9 is complexed to other cyclins, such as cyclin T2a, T2b and with cyclin K.

It was demonstrated that CdK9 complexed with CyT2a plays a role in the activation of myogenic program and Cdk9-CyT2a activity is not down-regulated in myotube formation, but its activation contributes to the transcriptional activity MyoD-mediated during myogenic program.

The formation of a multimeric complex, containing cdk9/cyclin T2a and MyoD is present in muscle cells during the activation of the differentiation program and the N-terminal region of Cdk9 directly interacts with the b-HLH region of MyoD, allowing the formation of a complex also containing cyclin T2a.

Recently a 55 kDa protein called CDK9-55 has been identified. This isoform presents 117 additional amminoacids residues at the N-terminal portion of Cdk9-42 and it conserves all molecular features of 42 isoform, indeed it associates with CycT phosphorylates CTD of RNA polII.

Cdk9-55 is significantly upregulated in cells induced to differentiate, either in C2C12 cells or in satellite isolated cells, and it was demonstrated that there is a clear induction of cdk9-55 expression in injured skeletal muscles.

Analysis in vivo on limbs at different times of development demostrated a different correlation between two CDK9 isoforms: CDK9-55 is involved during foetal myogenesis, whereas the CDK9-

42 is expressed during embrional myogenesis. Study in vitro demonstrated that CDK9-55 is the isoform more expressed during differentiation both embrional and fetal myoblasts. A new antibody, p-CDK9, permitted us to define CDK9-55 as the active isoform during the differentiation. CDK9-55 is also the isoform more expressed during the re-innervation process.

The analysis of different cyclins that complex with different members of kinase proteins showed that in vivo only cyclin K and D3 are involved during foetal myogenesis, suggesting their interaction with CDK9-55, whereas Cyclin T1, T2, L and H are implicated during primary myogenesis, in concomitance with CDK9-42 expression. Study in vitro demonstrated that only cyclin T2 is involved during the differentiation process for embrional and fetal myoblasts.

Between inhibitory post transcriptional regulator mechanism of CDK9 there is the interaction with 7SKsnRNA and HEXIM1, our studies, on C2C12 cells, by RIP assay demonstrated that CDK9 isn't associated with 7SKsnRNA/Hexim1 during differentiative phase, supporting that in differentiation CDK9 is active.

1. INTRODUCTION

1.THE SKELETAL MUSCLE

Skeletal muscle is the most abundant tissue in the body of vertebrates. The primary functions of skeletal muscle are the locomotor activity, the breathing and postural behaviour. Each muscles composed of bundles of fascicles, containing multinucleated cells called muscle fibers. Every muscle fiber contains numerous protein filaments, myofibrils, which are the contractile elements of skeletal muscle. Contractile segments of myofibrils, termed sarcomeres, are composed of two types of myofilaments. Thin filaments are mainly composed of actin molecules and thick filaments are made up of myosin molecules. It is the movement of the thick and thin filaments in relation to one another that produce contraction.

Skeletal muscle is composed of different fiber types with varying anatomical, molecular, metabolic, structural and contractile properties.

Fast contracting fibers have few mitochondria, low myoglobin content, high glycolytic and low oxidative metabolism and are highly fatigable. Conversely, slow contracting fibers have many mitochondria, a high content of myoglobin, high oxidative metabolism and fatigue slow.

Skeletal muscle is a dynamic tissue capable of responding to physiological stimuli such as intense physical activity, as well as various types of damage through a regenerative response which is able to reform the architecture of the muscle cells in a period of two weeks (Cossu et al., 2008).

The ability of skeletal muscle to mediate a regenerative response is mainly due to population consisting of mononucleated satellite cells. In response to damage or destruction of basal lamina, satellite cells are activated and show a significant proliferative activity. The satellite cells then fuse to form multinucleated myotubes and some of them may be to restore the pool of remaining quiescent satellite cells are able to support further process of regeneration.

1.2 MURINE MYOGENESIS AND SKELETAL MUSCLE PROGENITOR CELLS

Embryonic myogenesis is a multistep process that begins with commitment of an embryonic precursors to the myogenic lineage, followed by the proliferation of these committed myoblasts that differentiated into postmiotic myocytes and finally fusion of myocytes to form a multinucleated myotube. As the myotube matures, the syncytial cell becomes specialized for its particular function, with the bulk of the cytoplasm occupied by the contractile apparatus, and where the myotube/myofibre can further grow or hypertrophy in response to appropriate stimuli. Postnatal myogenesis is a similar process, except that fusion occurs primarily between myoblasts and preexisting myotubes, and where the role of the embryonic precursor is played by the quiescent satellite cell.

In the vertebrate body all the skeletal muscles derive from progenitors present in the somites, which are established as paraxial mesoderm beside the neural tube and nothocord. The somites are generated in rostro-caudial direction by segmentation of paraxial mesoderm on both sides of the neural tube. Each newly formed somite rapidly differentiates into a ventral sclerotome and a dorsal dermomyotome. The sclerotome gives rise to the ribs and accompanying cartilage, while the dermomyotome contributes to both the hepaxial musculature (back and intercostals muscles) and the hypaxial musculature (body wall and limb muscles). The hypaxial dermomyotome is specified by signals from the dorsal ectoderm (Wnt pathway) and the lateral plate mesoderm (Bmp4), and produces the hypaxial myotome that forms the limbs, diaphragm and body wall muscles.

Skeletal muscle is formed in different steps, involving different myoblasts populations.

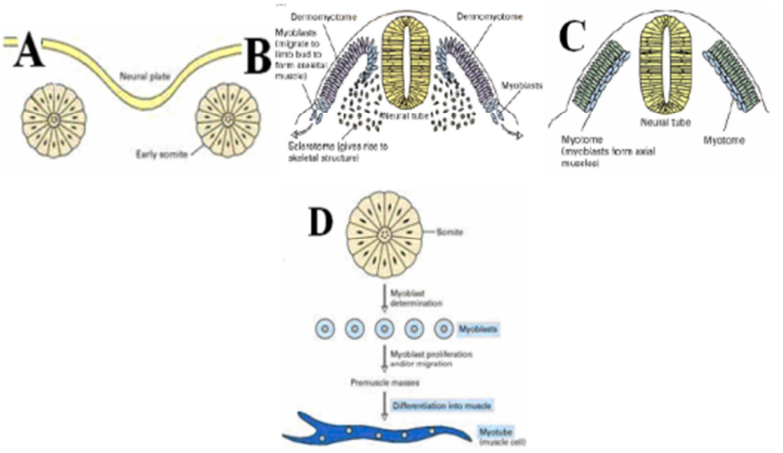


Fig.1*: From A to C is indicated the formation and the differentiation of somites. In D is reported the differentiation process, where the somite cell differentiated in myotubes (Lodish et al.,2002).*

In mice, somitogenesis begins during the eighth day of embryonic day (E8 dpc, days post coitum), with the segmentation of somites. After segmentation, somites give rise to epithelial dermomyotome on the dorsal side and mesenchymal sclerotome on the ventral side.
The myotome is formed by involution of cells from dermyotome. At around E11 in the mouse, embryonic myoblasts invade the myotome and fuse into myotubes. During this phase, called primary or embrional myogenesis, myogenic progenitors, which have migrated from the dermomyotome to the limb, start to differentiate into multinucleated muscle fibers, known as primary fibers. Embryonic myoblasts are elongated cells that differentiate into mononucletaed or oligonucleated myotubes.
A new phase of myogenesys takes place between E14.5 and E17,5, this phase is called secondary or fetal myogensis and involves fusion of fetal myoblasts either with each other to give rise to secondary fibers and also with primary fibers. Fetal myoblasts are triangular shape and proliferate to a limited extent in response to growth factors, differentiate into large multinucleated myotubes.

It is only at the end of secondary myogenesis that there is a formation of the satellite cells, that are mononucleated cells lying between the basal lamina and the fiber plasma membrane. Satellite cells are normally quiescent and they are activated in post natal life after injury, indeed when the fiber is damaged these cells become activated, replicate and then differentiate to form new fibers, thus permitting muscle repair.

Embryonic and fetal myoblasts, thought to generate primary and secondary fibers respectively, differ in the morphology of the myotubes they generate *in vitro* and in the myosin heavy chains isoforms and muscle enzyme that they express (Barbieri et al., 1990; Zappelli et al., 1996). They also differ with respect to media requirements (White et al., 1975), integrin-extracellular matrix interactions, resistance to inhibitors of myogenesis such as phobol esters (Cossu et al., 1988) and TGFβ (Causella-De Angelis et al., 1994).

The innervation of muscle starts while fibers are still forming. Each muscle fiber is initially innervated by multiple axons, all but one of which are subsequently eliminated.

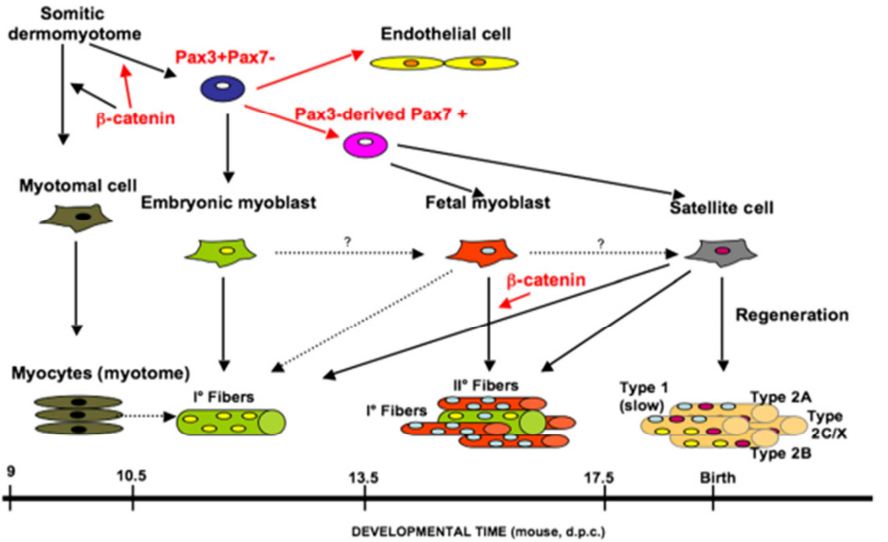


***Fig. 2**: Scheme of lineage of murine development (Biressi et al., 2007)*

The process of myogenesis is controlled by several myogenic transcription factors (MRFs: Myod, Myf5, myogenin and MRF4) that act as terminal effectors of signaling cascades and produce appropriate developmental stage-specific transcripts.
The MRF proteins contain the conserved basic domain that is essential for the binding of specific DNA sequence and a helix-loop-helix motif required for heterodimerize. Each of the MRFs has shown the ability to heterodimerize both *in vitro* and *in vivo* with E proteins and to bind DNA in a sequence- specific manner to particular canonical sites know as an E-box (CANNTG). This DNA motif is present in the promoter of many muscle specific genes and is able to mediated the activation of genes in a MRF dependent manner.
Myf 5 and MyoD are myogenic determinator factors contributing to myoblasts specification, while myogenin and MRF4 are myogenic differentiation factors contributing to the induction of terminal differentiation.
Recently it was observed that in the mouse embryo Myf 5 is the first MRF to be expressed (E8.0). Myogenin is expressed after Myf5 (E8.5) and MRF4 at E9.0; MyoD is the last to be expressed in the somite at E10.5 (Francetic et al., 2011).
Myoblast determination protein (MyoD) is believed to determine the differentiation potential of an activated myoblast, and acts together with myogenin and myocyte enhancer factor 2 (MEF2) to drive differentiation. It is known that Myf5 and MyoD are critical for myoblasts determination, while myogenin is a transcription factor that plays an essential role during muscle differentiation.
Double mutants for MyoD and Myf5 are not able to form skeletal muscle because the muscle precursor population is absent (Rudnicki et al., 1993). In absence of these factors the cells in the somite, which would normally become myoblast, are not able to correctly locate in the site of myogenesis and then adopt a different cell fate.
In addition to activating skeletal muscle-specific gene, MyoD expression can also lead to cell cycle arrest. The importance of MyoD as an inhibitor of cell cycle progression can also be seen during muscle repair. One potential mechanism by which MyoD

can arrest cell cycle progression is through the transcriptional activity on the CIP1 gene, which encodes the cyclin-dependent kinase-inhibitory protein p21.
Obviously these transcription factors do not act alone, but a part of complex signaling cascades that control every stage of myogenesis exists.
The expression of MRFs is regulated by Pax3 and Pax7 (paired box protein 3 and 7). The paired box (PAX) transcription factor family is encoded by development control genes and it is characterized by a highly conserved DNA-binding domain (PD). Pax3+/Pax7-positive muscle progenitor cells are located in the dermomyotome; these cells enter the myotome, the first skeletal muscle to form, in the central compartment of the somite, as the dermomyotome disaggregates, from embryonic day 10.5 (E10.5) in the mouse embryo.
Pax3 is involved in the development of both epaxial and hypaxial muscle and it has a role in the survival of muscle precursors in hypaxial dermomyotome. Pax3 contributes also to regulate MRF, indeed Pax3 regulates Myf5 expression indirectly through the epaxial enhancer (DMRT2). In C2C12 myoblasts cells Pax3 directly regulates expression of MyoD.
Pax 7, indeed, is required for maintenance of adult satellite cells.
In the Pax3/Pax7 double mutant mice, which are lacking in both Pax3 and Pax7, only the primary myotome and its derivatives are formed, thus compromising all the subsequent stages of myogenesis. It is therefore proposed that all cells of the myogenic lineage should arise from Pax3/Pax7 positive population (Messina et al., 2009).
Moreover it appears that Pax3 and Pax7 have partially overlapping and unique functions in myogenic progenitors. Both the pair- box genes are down regulated during myogenic differentiation, following differentiation of myogenic regulator factors.
In Pax7-/- mice, adult myogenesis and regeneration are severely compromised whereas is not in fetal myogenesis. Based on these results Hutcheson and colleagues suggested that during foetal myogenesis the function of Pax7 is not essential and its activity

could be compensated by other proteins, and the candidate is its paralogue Pax3.
In summary Pax3 would be essential for embryonic myogenesis and Pax7 for adult myogenesis, while during the fetal myogenesis both genes should show redundant functions (Messina and Cossu, 2007).

1.3 CDK9 PROTEIN

Cyclin- dependent kinase 9 (CDK9) is a cdc-2 like serine/threonine kinase. The cyclin-dependent kinases (CDKs) take their name from a catalytic dependence on the cyclin family of regulatory proteins.
Cdks are involved in cell cycle control and/or regulation of transcription. Their activity is coordinated by association with specific type of cyclins: this association forms a heterodimer, in which the cdk function as catalytic domain, whereas the cyclin function as regulatory subunits. The majority of cdks are involved in the control of cell cycle progression, only for CDK9 and CDK8 the main function is the regulation of transcription via phosphorylation of RNA polII carboxyl terminal domain.

Cdk	Main cyclin partner (other cyclins are listed between parentheses)	Other interacting factors	Cellular functions
Cdk1	A1, A2, B1, B2, (E, B3)	Cks (Cdc28-dependent kinase subunit)	G_2-M (cell cycle)
Cdk2	A1, A2, E1, E2 (D1, D2, B1, B3)		G_1-S (cell cycle)
Cdk3	E1, E2, A1, A2, C	E2F/DP (dimerization partner)	G_0-G_1-S (cell cycle)
Cdk4	D1, D2, D3	MyoD	G_1-S (cell cycle)
Cdk5	p35, p39 (D-, E-, and G-type cyclins)		Senescence, post-mitotic neurons
Cdk6	D1, D2, D3		G_1-S (cell cycle)
Cdk7	H		Cdk-activating kinase, transcription
Cdk8	C (K?)	RNA pol II	Transcription
Cdk9	T1, T2a, T2b, K	RNA pol II, MyoD	Transcription
Cdk10	Unknown	Ets2	Transcription, G_2-M (cell cycle)
Cdk11	L1, L2 (D)	14-3-3, 9G8, CK2, eIF3, RanBPM, RNPS1, RNA pol II	Transcription, M (cell cycle)

Fig. 3 *: List of CDKs and cyclin partners (Romano et al., 2008)*

The cellular function of CDK9 has been unknown until 1997, when Zue and colleagues identified in Drosophila CDk9 as a small subunit of the positive transcription and elongation factor (p-TEFb). CDK9 was initially named PITALRE.
CDK9 is distributed in all type of human and murine tissue with high levels of expression in terminally differentiate cells.
The regulatory units of CDK9 are the T family cyclins (T1, T2a and T2b) and K. In Hela nuclear extracts CDK9 complexes with cyclin T1 for 80%, and for 10% with cyclin T2. The chaperone proteins HSP70, HSP90, and Cdc37 bind transiently and, therefore, stabilize the monomeric CDK9 preceding the association with one of its cyclin partners.

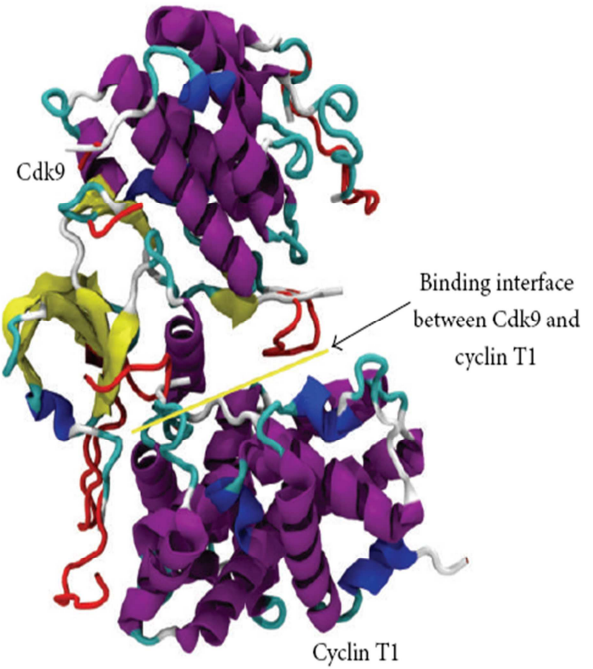


***Fig. 4**: The CDK9/cyclin T1 complex. (Romano, 2013)*

The elevated levels of CDK9 and its regulatory subunits in terminally differentiated cells, together with the fact that CDK9/cyclinT complexes are not cell cycle regulated, distinguish CDK9 from the other cdks. CDK9, at difference with other cdks that regulate cell cycle progression and phoshorylate histone H1, fails to phosphorylate H1.

Together with cyclin T, CDK9 forms the positive transcription elongation factor b (P-TEFb), which allows transcriptional elongation through phosphorylation of the carboxyl-terminal domain (CTD) of the RNA polymerase II.

The CDK9-related pathway regulates a wide range of functions in mammalian cell biology and replication program of numerous viral agents, such as immunodeficienty virus type I (HIV-1) and HIV2, human cytomegalovirus, herpes simplex virus 1, human adenovirus. Moreover, dysfunctions in the CDK9-related pathway are related with several forms of human tumors and cardiac hypertrophy.

In structural studies CDK9 shows a typical kinase fold, comprising the N-terminal lobe (residue 16-108), which consist mainly of a bet-sheet with one alpha-helix and a C terminal lobe (residue 109-230). The protein contains a phosporylated T loop structure (amino acids 168-97), that is conserved among CDK protein and control access of ATP and substrate to the enzyme.

In mammalian cells CDK9 is present in two isoforms: CDk9-42 and CDK9-55. CDK9-42 is a 42 kDa protein that is ubiquitously expressed and autophosphorylates itself. CDK9-55 originates from a promoter upstream of the 42k promoter and presents 117 additional amino acids in the portion amino terminal, which is fused in frame with CDK9-42 sequence. The 13KDa in addition to CDK9-55 contain a region rich in praline and a region rich in glycine. The messengers of CDK9-42 are transcribed from the promoter rich in G/C but TATA less (Liu and Rice, 2000), while the messengers of CDK9-55 originate from the promoter containing the TATA sequence and placed approximately 500bp upstream (Shore et al., 2003).

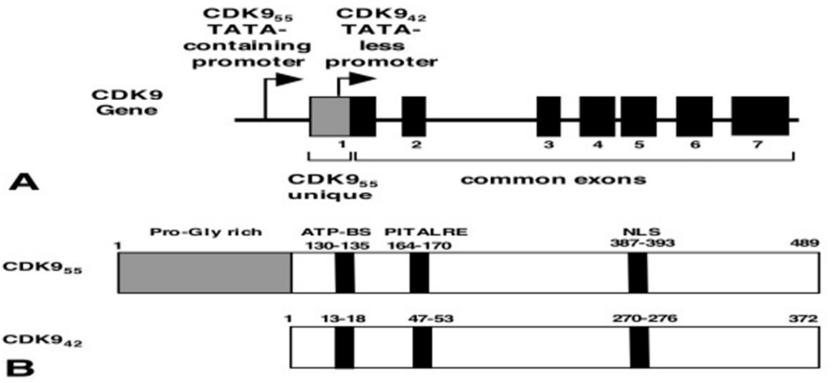


Fig. 5*: A) P55 and P42 indicate the start of transcription for the Cdk9-42 and CDK9-55. B) Representation of CDK9 molecular organization (Shore et al., 2005).*

In Hela cells, CDK9-55 is present at approximately 10-20% of the level of CDK9-42.

Both CDK9 isoforms generate heterodimers with cyclin T1,T2 and K, and they regulate the transcription via phosphorylation in the portion N-terminal of RNA polymerase II and their kinase activity is inhibited by 5,6- dichloro- l-b-D-ribofuranosylbenzimidazole (DRB).

Two CDK9 isoforms present a different cellular distribution, CDK9-42 is localized in nucleus and in cytoplasm, whereas CDK9-55 is essentially localized in cell nucleus. A different distribution of two isoforms it was observed in different type of human and mouse tissue: CDK9-42 is predominantly expressed in testis and spleen, whereas CDK9-55 is expressed in lung, liver and brain tissue.

A differential expression of two CDK9 isoforms is also observed in various human and mouse cell culture system. For example CDK9-42 is expressed in human cervical carcinoma Hela cells and in primary undifferentiated monocytes. CDK9-55 become predominant upon induced differentiation of human primary

monocytes into macrophages. Rat hepatocytes is expressed CDK9-55, but in primary cell cultures there is an increase of CDK9-42. These findings indicate that two CDK9 isoforms expression is regulated in a cell type-specific fashion and in a signal-dependent manner.

1.4 ROLE OF CDK9 IN SKELETAL MUSCLE

Biressi and colleagues demonstrated that CDK9 in association with cyclin T2a plays an important role during myogenic program. In the same study it was also demonstrated that CDK9 and its partners cyclin T2a isn't down regulated during the formation of myotubes, but rather their activation is required for MyoD activation. The formation of multimeric complex containing CDK9, cyclin T2a and MyoD is detectable in cells during differentiation program, and the portion N-terminal of CDK9 interacts directly with bHLH region of MyoD, thus allowing the formation of a complex containing also cyclin T2a. As a result of muscle differentiation, the complex CDK9/cyclin T2 promotes the MyoD dependent transcription and accelerates the myogenic program.

The main mechanism by which chromatin bound by CDK9/cyclin T2a complex activates the MyoD specific transcription is through the phosphorylation of the RNA polymerase II CTD by promoting the elongation (Hyacinths et al.,2006).

CDK9 binds directly to MyoD *in vitro* (Simone et al., 2002), and takes part in the formation of multimeric complex containing MyoD, cyclin T2a, p300, PCAF and BrgI in muscle cells (Giacinti et al., 2006). This transcriptional complex binds to the regulatory region of muscle specific genes to induce the acetylation of lysines on histones H3 and H4, the chromatin remodeling and the phosphorylation of specific serine targets for CDK9 at the level of RNA polymerase II CTD, thus promoting the gene expression (Simone and Giordano, 2007).

Giacinti and colleagues analyzed the expression of two CDK9 isoforms during differentiation of C2C12 cells, that are an

established cell line originate from mouse satellite cells. The reported results demonstrated that the expression levels of CDK9-42 displayed similar levels between proliferative and differentiative phase, while the expression of CDK9-55 isoforms increased during cells induced to differentiate. In the same study it was also observed that Cdk9-55 is induced during the regeneration program of damage fibers.

1.5 THE CYCLINS PARTNERS OF CDK9

Cyclins, a family of protein named for their cyclical expression and degradation, play an important role in the cell-division cycle. They act as a catalytic domain for CDK family protein: interaction between CDKs and cyclins occurs at specific stages of the cell cycle, and their activities are required for the progression of cell cycle.
The core form of P-TEFb is a heterodimer between the CDK9 and its regulatory subunit cyclin T1 or the minor forms T2 or K. These complexes have been implicated in stimulating elongation upon initiation, of otherwise paused transcripts, by phosphorylating the C-terminal domain of RNA polymerase II.

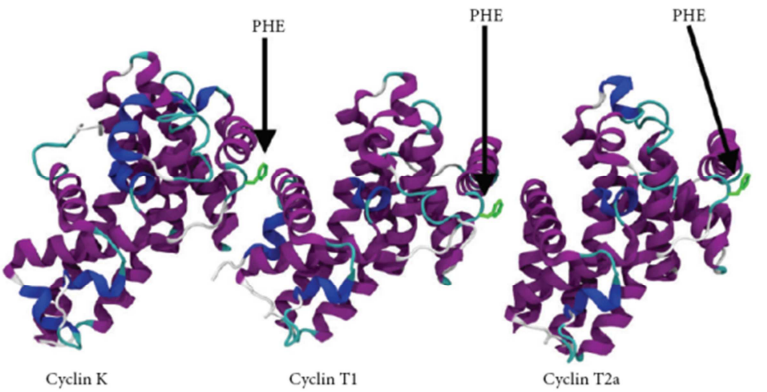


Fig. 6*: Cyclin partners of CDK9: Cyclin K, cyclin T1 and Cyclin T2a. PHE: phenylalanine. (Romano, 2013)*

Cyclin T1 and T2 are expressed widely in human adult tissues with high levels in muscle, the spleen, thymus, testes, ovaries and in peripheral blood lymphocytes. Cyclin K is expressed in adult mouse and in human tissue, but it most abundant in developing germ cells of the adult testes and ovaries.

Between all cyclins partners of CDK9 only the complex CDK9/cyclin T1 can interact with the HIV transcriptional activator, Tat, to promote HIV replication and only CDK9/cyclin T2a can bind to MyoD to promote myogenic transcription.

The cyclin T2 has two forms T2a and T2b, that derive from splice variants of the same transcript. These two cyclins share the first 642 amino acids in common, but cyclin T2b contains in addition a large C terminal domain (CTD).

The domain N-terminal of cyclin T1 contains a coiled coil motif, a His-rich motif and a PEST sequence carboxyl terminal. C-terminal PEST sequences are commonly found in G1 cyclins and serve to regulate protein turnover by the cellular ubiquitination.

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. The region of cyclin T1 from amino acids 1-188 is necessary to interact with CDK9 *in vivo*.

Peng and colleagues observed that removing the carboxyl terminal domain of cyclin T1 and T2 the ability of CDK9/cyclin T to phosphorylate the CTD of RNA polII and function in transcription was significantly reduced.

The protein T2a shows high level of expression in human adult skeletal muscle cells and study *in vitro* suggest that CDK9/cyclin T2a complex might promote myogenic differentiation eliciting the expression of muscle-specific genes.

The function of CDK9/cyclin K has been less clear. Cyclin K interacts with CDK9 *in vitro* and *in vivo,* and the CDK9/cyclin K complex can activate transcription only when tethered to RNA but not DNA. Cyclin K expression is also activated transcriptonally by the p53 tumor suppressor in response to DNA damage by adriamycin, ultraviolet (UV) light and ionizing radiation.

In addition to CDK9, cyclin K associates with CDK12 and CDK13: these heterodimers are both implicated in the control of RNA-pol II-mediated transcription.

1.6 7SKsnRNA and HEXIM1: THEIR ROLE IN REGULATING TRANSCRIPTION

The positive transcription elongation factor b (P-TEFb) plays an important role in elongation of nascent RNA molecules by RNA polymerase II.
In Hela cells there are two distinct P-TEFb complexes, which differ in size, composition and activity. A smaller P-TEF b complex has kinase activity and is composed of CDK9-42 or CDK9-55 and a cyclin partner T1,T2 or K. A large P-TEFb complex with reduced kinase activity composed by 7SKsnRNA and HEXIM1, in addition to CDK9 and cyclin T1, T2 or K. Independently 7SKsnRNA and HEXIM1 have limited inhibitory effects in P-TEFb complex, but together they inhibits the P-TEFb kinase activity. HEXIM1 is composed by two regions, the region comprised between amino acids 152-155 is involved in binding of 7SK, and a second region, amino acids 202-205 is involved in interaction with P-TEFb. Hexim 1 was first identified from vascular smooth muscle cells treated with hexamethylene bisacetamide (HMBA), a proliferation-inhibiting and differentiation-inducing compound. Treatment of HMBA led to increase in both mRNA and protein levels of HEXIM1.
The N-terminal portion of Hexim1 was identified as inhibitory domain, a model proposed that upon binding to 7SKsnRNA, Hexim1 undergoes a conformational change, thereby exposing its C-terminal domain for binding to CDK9/cyclinT1.
Once binding to Hexim1/7SKsnRNA complexes, the kinase activity of p-TEFb is inhibited. About 50% of P-TEFb is found to associate with Hexim1 in cells, suggesting the importance of Hexim1 in the regulation of P-TEFb.
In cells there is a delicate and dynamic balance of the two P-TEFb complexes in cells. The dissociation of P-TEFb from 7SK and

HEXIM1 is rapid and reversible. Treatment of cells with UV irradiation, actinomycin D or DRB results in the dissociation of large P-TEFb complexes, and when cells are allowed to recover from those treatments, large P-TEFb complexes reform. Physiological signals leading to cardiac hypertrophy converge on activation of P-TEFb, through dissociation of 7SK, resulting in increased transcription and an increase in size of cardiomyocytes.

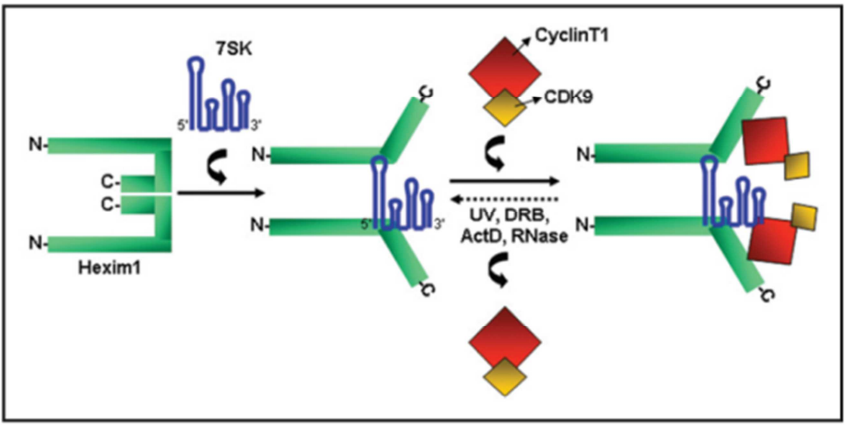


Fig.7*: Regulation of Hexim1: the conformational change of Hexim1 in C terminal portion and the binding with 7SKsnRNA and CDK9/Cyclin T1 (Dey et al., 2007).*

It was observed that phosphorylation of the T-loop of CDK9 has been implicated in the activation of P-TEFb, and is required for the formation of the 7SK/HEXIM1/P-TEFb complex.
CDK9 acetylation, indeed, did not affect the association of P-TEFb with Hexim1 or 7SKsnRNA.
Recently it was demonstrated that the skeletal muscle regeneration, which is achieved by a formation of new myofibers from the satellite cell pool, is controlled by the HEXIM1/P-TEFb pathway, that regulates satellite expansion after injury.

2. AIMS OF THE RESEARCH PROJECT

This research project has analyzed and characterized the molecular and cellular mechanisms during development of the skeletal muscle, in particular investigating the involvement of the protein serine kinase CDK9. In the last years, my research group demonstrated that the CDK9 kinase protein, in association with the cyclin T2a, has an important role during the activation of the myogenic program (Giacinti et al., 2006). The complex Cdk9/Cyclin T2a isn't down regulated during myogenis, but its activation contributes to the transcriptional activity mediated by MyoD during the differentiation (Simone et al., 2002; Giacinti et al., 2006). In myoblasts, induced to differentiate, CDK9 complexes with Myod and Cyclin T2a, is recluted on the promoter of specific muscle genes and promotes the phosphorylation of CTD of RNA polII (Giacinti et al., 2006).

Recently the identification of new CDK9 isoform, CDK9-55 (Shore et al., 2003), and its expression in different tissues and cells, led us to analyze its role during myogenesis. It was demonstrated that the expression of CDK9-55 isoform increases during differentiation both in muscle stable lines and in myoblasts derived from primary cultures (Giacinti et al., 2006), allowed us to hypothesize the role of this isoform during myogenic program. The same research group analyzed the role of CDK9-55 isoform in damaged fibers and the obtained data demonstrated that CDK9-55 is highly expressed during regeneration process, while the CDK9-42 is not involved in this process (Giacinti et al 2008).

Later on they start to investigate the role of two isoforms of CDK9 during the differentiation process in myogenesis both *in vivo* and *in vitro*. Preliminary results show that CDK9-42 and CDK9-55 have a different timing expression during development (between embryonic and fetal periods) (Background).

The aim of this project is to get inside the specific mechanisms by which the two isoforms of cdk9 participate in the muscle differentiative mechanisms both *in vitro* and *in vivo*.

To this purpose we will further analyze the mechanisms by which the two isoforms are activated during myogenesis looking at the co-expression of cdk9 activators (as different members of cyclin family) and inhibitors (as 7SKsnRNA and Hexim1) at different stages of development. The same study will be performed on embrynal and fetal myoblasts *in vitro*.
This innovative study try to discern among multiple possible combinations of cdk9 isoforms and activators/inhibitors so that to individuate the selective combination in correlation with a specific function during the different steps of myogenesis.

3. RESULTS AND DISCUSSIONS

Background

3.1 ANALYSIS OF CDK9-42 AND CDK9-55 EXPRESSION AND THEIR RELATIONSHIP WITH PAX3/7 AND MRFs DURING LIMB DEVELOPMENT

Skeletal muscle differentiation is a multistep process in which muscle precursor cells initially express early differentiation markers, exit the cell cycle, then express muscle-specific structural genes and fuse to form multinucleated myotubes (Andrès and Walsh, 1996).
In precedent works it was demonstrated the importance of CDK9 during myogenic program, and it was demonstrated that the proteic levels of CDK9-42 are stable while the expression of CDK9-55 increases during differentiation both in C2C12 cells, an established cell line originated from mouse satellite cells, that in myoblast derived from primary coltures *in vitro* (Giacinti et al., 2008).
Starting from this data the impact of CDK9 on myogenesis was characterized, the two isoform expressions were set up during muscle development. Western blotting analysis on total proteic extracts of limbs at different time of development E10,5, E11,5, E12,5, E14,5, E16,5, E17,5, shows a different expression between two isoforms (Figure 1).

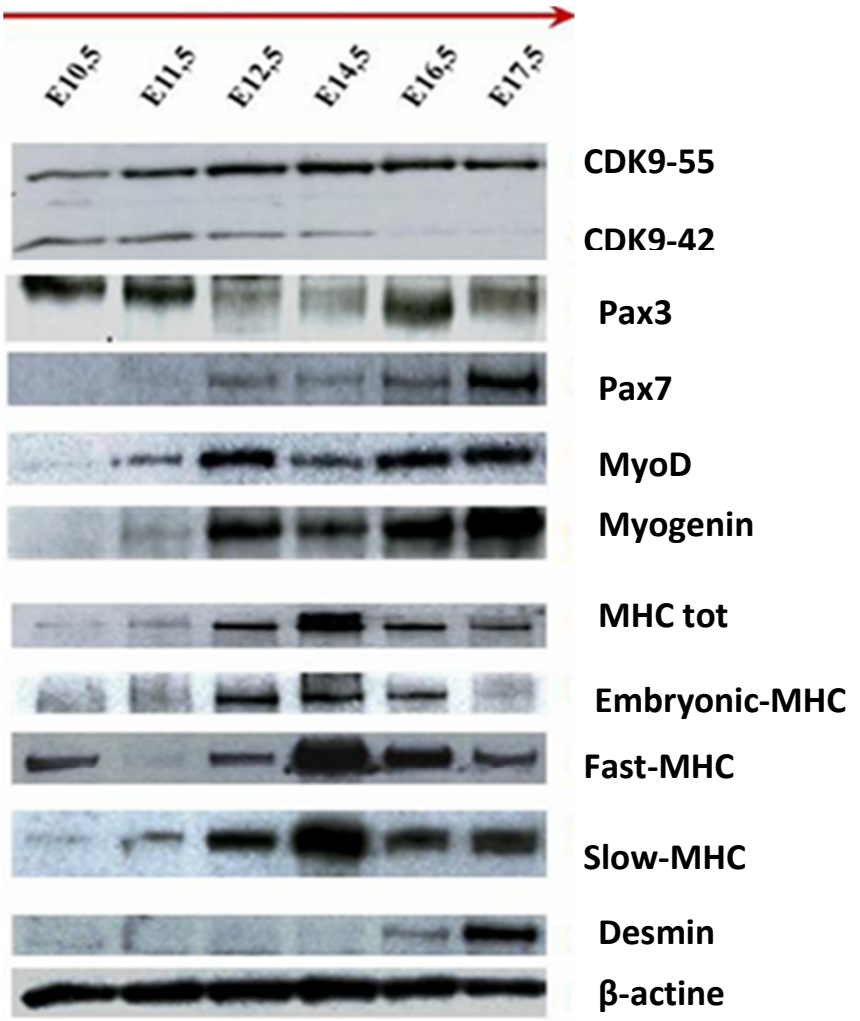


Figure 8: *Western blotting analysis in mouse embryo limbs since E10.5 to E17.5 of the marker of myogenic lineage, Pax3 and Pax7, and some MRFs, MyoD and Myogenin, and other genes involved in muscle differentiation in order to better characterize the impact of two CDK9 isoforms on the myogenic program during limb development.*

Western blotting analysis shows that there is an inverse correlation between two isoforms: CDK9-42 decreases, whereas CDK9-55 increases during development. In particular at the time E14,5, the transition stage between the embryonic and fetal development phases, there is a switch in CDK9-42 and CDK9-55 expression, supporting the hypothesis that the two isoforms have different roles during myogenesis. CDK9-55 expression comes before the presence of terminal markers of muscle differentiation, as myosin and its isoforms.

It has been reported that embryonic MHC and slow MHCIβ isoforms are expressed during early myoblast differentiation, whereas the fast isoforms (MHCIIa, MHCIIx, MHCIIb) are up regulated later in differentiation. The transition between the slow and fast isoforms coincides with myotube hypertrophy; thus, the different expression of MHC isoforms defines a specific phase and phenotype during muscle development (Brown et al., 2011).

From the analysis of the proteic extract it was observed a maximum of slow and fast MHC isoform expressions, which followed a bell trend between the late phase of embryonic development (E12,5) and the first steps of fetal myogenesis (E16,5), although fast MHC was already expressed at E10,5.

In order to characterize the role of CDK9-55 and CDK9-42 on the myogenic program the determinators of myogenic lineage are analyzed, Pax3 and Pax7, and some MRFs, as MyoD and myogenin. Pax3 is the only one between all factors analyzed to anticipate the expression of CDK9-55, showing an oscillatory profile which determines the terminal differentiation. To allow for terminal differentiation it is necessary that Pax3 is down regulated in response to a co-expression of the primary MRF, MyoD (Relaix et al., 2005). Pax7 shows a different expression level, that increases gradually during fetal myogenesis. Pax7 seems to be required to allow the secondary myogenesis and its increase coincides with the satellite cell formation, during the time E16,5-E17,5.

Later the CDK9 expression profile was correlated with MRFs: Western blotting analysis for MyoD confirmed an initial expression at E11.5, followed by an increase at E12.5 up to steady levels at the

late stages of limb development (E16.5-E17.5). Worthy of attention was the transient decrease at stage E14,5 dpc, this decrease coincides with the peak in expression of the myosin heavy chains and with their "bell" progress. The initial expression of MyoD is enough to activate the myogenic program, then its down regulation is necessary for terminal differentiation and to allow for the expression of sarcomere filaments (Choi et al., 1990). Furthermore the expression of MyoD followed a pattern similar to that of CDK9-55, suggesting a correlation between MyoD and CDK9-55. For Myogenin, MRF involved in the late differentiation of muscle cells and is activated following the expression of MyoD and Myf5 to promote the formation of myocites and myotubes, it was observed a progressive increase from E12.5, with a performance similar to MyoD, although Myogenin showed a particular increase at stage E17.5.

Desmin protein levels were not detected at early stages of limb development (since E10.5 to E14.5), whereas its expression levels were higher during fetal myogenesis (E16.5-E17.5). The Desmin maximum at E17.5 coincides with the Pax7 maximum, according to the fact that they are two important markers of satellite cells, highly expressed at this development phase.

The molecular characterization of two CDK9 isoforms during myogenic program, the genic expression, was analyzed by qRT-PCR. Embryonic limbs at E10.5 were used as a control, and mRNA levels were normalized to the housekeeping GAPDH. The obtained results (Fig. 9, B.) showed that CDK9-55 was significantly up-regulated throughout muscle differentiation, peaking in fetal limbs (E14.5-E17.5), whereas CDK9-42 displayed a progressive decrease in its expression levels during development (Fig. 9, A).

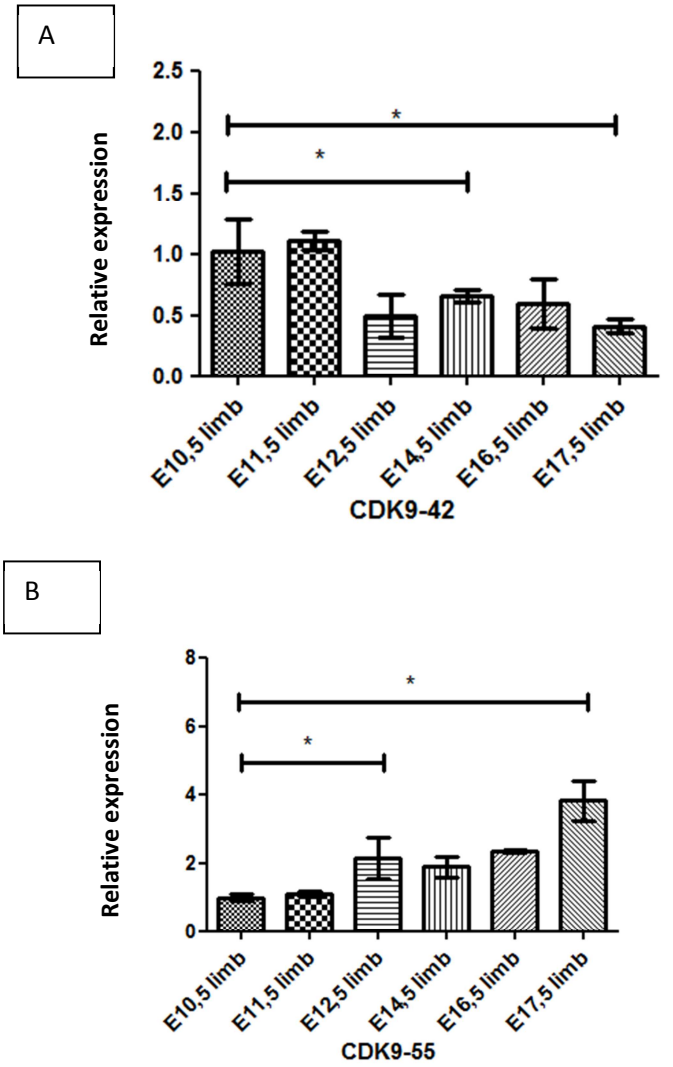


***Figure 9**: A)mRNA levels of CDK9-42 in mouse embryo limbs from embryonic day (E10.5) to fetal day (E17.5).*
** E10,5 limbs (ctr) vs E14,5 limbs P <0,05*
** E10,5 limbs (ctr) vs E17,5 limbs P <0,05*
B)mRNA levels of CDK9-55 in mouse embryo limbs from embryonic day (E10.5) to fetal day (E17.5).
** E10,5 limbs (ctr) vs E12 limbs P <0,05*
** E10,5 limbs (ctr) vs E17 limbs P<0,05*

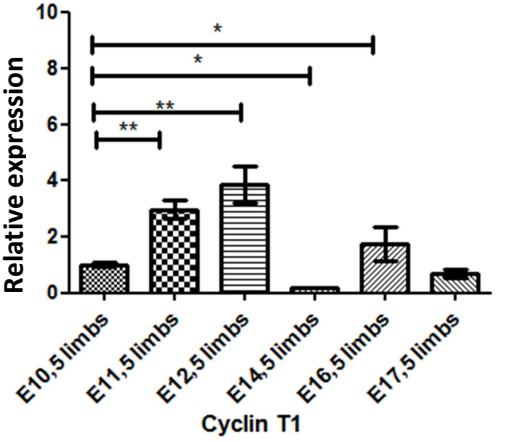
4. RESULTS AND DISCUSSIONS

4. 1 ANALYSIS OF THE CDK9 REGULATOR DURING MUSCLE DEVELOPMENT

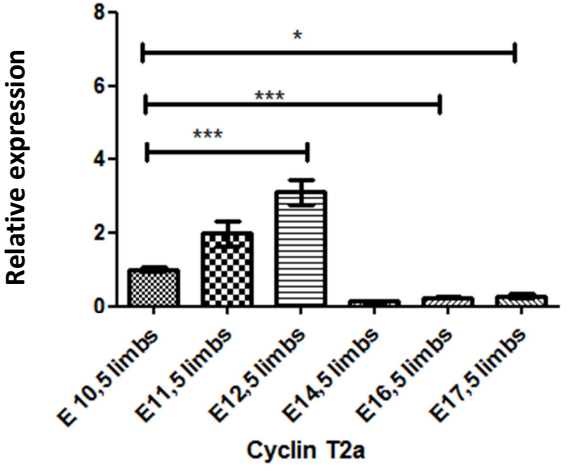
4.1. 1 CYCLINS T1, T2a AND K EXPRESSION

The different functions of CDK9 on distinct promoters depend on their ability to associate with different regulatory subunits.
In adult tissues the members of Cyclins T family are the first regulators of CDK9 kinase activity. The interaction between Cyclin T and CDK9 is necessary to control the transcription of muscle specific genes. It is known that the complex CDK9/CycT2a increases the transcriptional activity of MyoD and promotes myogenic differentiation (Simone et al., 2002).
Therefore, in mouse embryonic and fetal limbs RNA extracts the expression of canonical CDK9 partners, cyclins T (T1 and T2a) and K, are examined.
qRT-PCR results showed that cyclins T1 and T2a were clearly expressed at E11,5 and at E12,5 stages, suggesting their major involvement in embryonic myogenesis rather than in fetal myogenesis, although a partial recovery of cyclin T1 expression was obtained at stage E16.5 (Fig. 10).
As for cyclin K, a maximum of its expression was observed at E16,5, whereas it was poorly expressed during embryonic development (from E10,5 to E12,5). Cyclin K was significantly up-regulated during fetal myogenesis, therefore we supposed its involvement in this process in association with CDK9-55, that is mainly involved in muscle differentiation.
Conversely, cyclin T1 and T2a were expressed in concomitance with CDK9-42 expression, suggesting their preferential association with this CDK9 isoform.

A


Relative expression
10
8
6
4
2
0
*
*
**
**
E10,5 limbs
E11,5 limbs
E12,5 limbs
E14,5 limbs
E16,5 limbs
E17,5 limbs
Cyclin T1

B


Relative expression
8
6
4
2
0
*
***
***
E 10,5 limbs
E11,5 limbs
E12,5 limbs
E14,5 limbs
E16,5 limbs
E17,5 limbs
Cyclin T2a

C

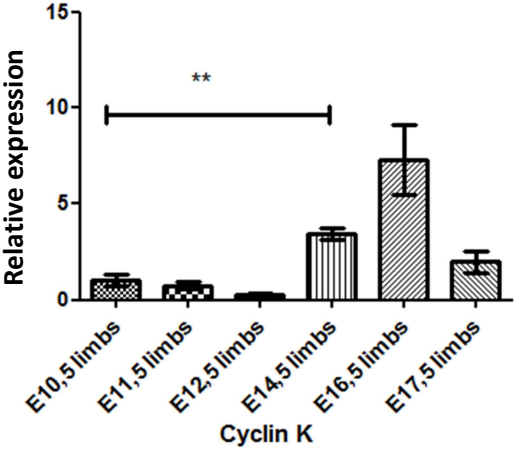


Figure 10: *mRNA levels of Cyclin T1 (A), T2a (B) and K (C) in mouse embryo limbs from E10,5 to E17,5. Cyclin T1 and T2a are mainly expressed during embryonic myogenesis, whereas cyclin K is significantly up-regulated during fetal myogenesis.*
*A)** E10,5 limbs (ctr) vs E11,5 limbs P<0,005*
*** E10,5 limbs (ctr) vs E12,5 limbs P< 0,005*
**E10,5 limbs (ctr) vs E14,5 limbs P<0,05*
**E10,5 limbs (ctr) vs E16,5 limbs P< 0,05*
*B)***E10,5 limbs (ctr) vs E12,5 limbs P<0,0005*
**** E10,5 limbs (ctr) vs E16,5 limbs P<0,0005*
**E10,5 limbs (ctr) vs E17,5 limbs P <0,05*
*C) **E10,5 limbs (ctr) vs E14,5 limbs P <0,005*

qRT-PCR results were confirmed by Western Blotting analysis of embryonic and fetal limb protein extracts.
For Cyclin T1 a marked expression was observed at early stages of limb development (E11,5-E12,5), whereas it was not detected

during the late phase (from E14,5 to E17,5) (Fig. 11). It isn't possible to analyze the cyclin T2a by Western blotting due to the absence of a good antibody.
Conversely, cyclin K was clearly expressed from E16,5, confirming its main function during secondary myogenesis, which is characterized by cell cycle withdrawal and terminal differentiation.
It is well known in literature that the induction of differentiation in cultured myoblasts results in up-regulation of cell cycle inhibitors such as p21 and p16 (Guo et al., 1995; Schneider et al., 1994). Indeed p21 is expressed when the differentiation process is ongoing and in concomitance with cyclin K, at stage E16,5 and E17,5, supporting the correlation between p21 induction and cell cycle arrest of muscle progenitors, thus inducing differentiation.

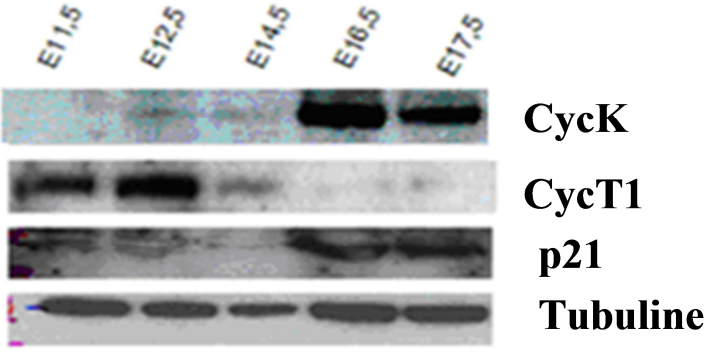


Figure 11*: Western blotting analysis showing cyclins T1, K and p21, normalized with tubuline, in mouse embryo limbs from E11,5 to E17,5.*

This data obtained with RT-PCR and Western blotting analysis allowed us to hypothesize an association of cyclin T with CDK9-42 and cyclin K with CDK9-55.

4.1.2 CYCLINS D3, H AND L *IN VIVO* ANALISYS

Cyclin D3 is expressed at very low levels in myoblasts due to GSK3β-mediated phosphorylation and consequent degradation, which is prevented in differentiating cells, where pRb stabilizes cyclin D3 by directly binding to it, thus allowing up-regulation of cyclin D3 during myogenesis (De Santa et al., 2007).
The qRT-PCR analysis showed low expression levels of cyclin D3 in primary myogenesis (from E10,5 to E 12,5), whereas it became up-regulated during secondary myogenesis (from E14,5 to E 17,5), suggesting its involvement in the induction and/or establishment of skeletal muscle differentiation, according to scientific reports.

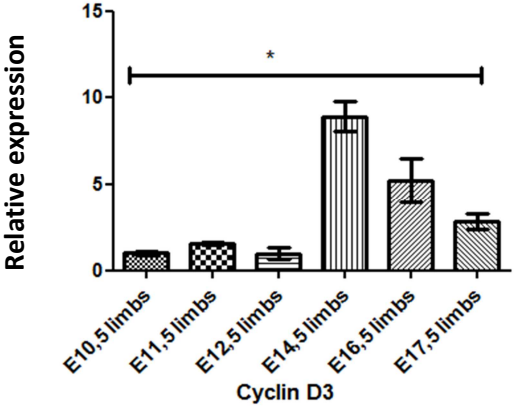


Figure 12: *mRNA levels of Cyclin D3 in mouse embryo limbs from E10,5 to E17,5. Cyclin D3 is up-regulated during fetal limb development (from E 14,5 to E17,5).*
** E10,5 limbs (ctr) vs E17,5 limbs P< 0,05*

In scientific articles it is reported that cyclin H, complexed with CDK7, has a role in transcriptional regulation mediated by its phosphorylation of specific sites on RNA polymerase II, so we

investigated the expression of cyclin H during embrional and fetal myogenesis.
The data obtained by RT-PCR analysis demonstrated that there isn't an important difference of expression between two myogenesis. It is possible to observe that the expression levels of cyclin H are stable enough during primary myogenesis, while for secondary myogenesis it was observed a decrease of expression at the time E17,5, when there is cell cycle arrest.

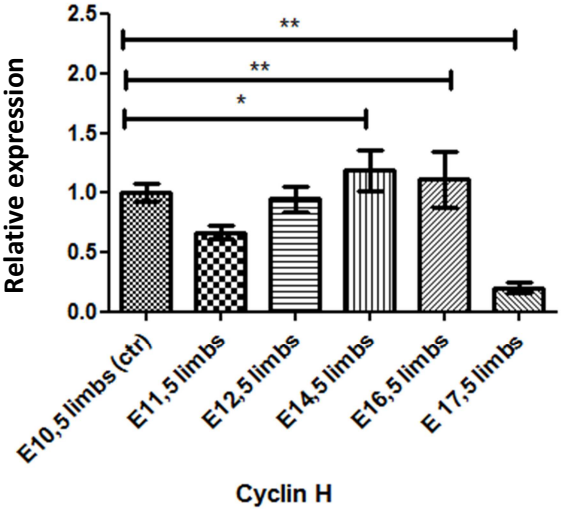


***Figure 13**: mRNA levels of cyclin H in mouse embryo limbs from E10,5 to E17,5. The expression levels of cyclin H are stable for primary myogenesis (from E10,5 to E12,5), while during secondary myogenesis there is a decrease at the time E17,5.*
** E10,5 limbs (ctr) vs E14,5 limbs P <0,05*
*** E10,5 limbs (ctr) vs E16,5 limbs P<0,005*
*** E10,5 limbs (ctr) vs E17,5 limbs P<0,005*

Precedent works demonstrated that CDK-11, a kinase that as CDK9 regulates the transcription through the phosphorylation of CTD of RNA polII, complex with cyclin L, this complex is involved in splicing.

In this experiment the expression of cyclin L was tested, and we observed that this cyclin isn't involved in the muscle development, indeed it is expressed weakly only at the time E12,5, during primary myogenesis, while during fetal myogenesis it decreases rapidly, above all at time E17,5.

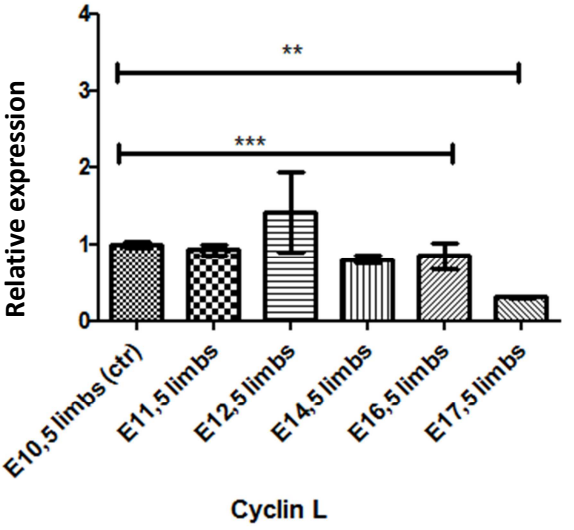


***Figure 14**: mRNA levels of cyclin L in mouse embryo from E10,5 to E17,5.*
**** E10,5 limbs (ctr) vs E16,5 limbs P<0,0005*
***E10,5 limbs (ctr) vs E17,5 limbs P <0,005*

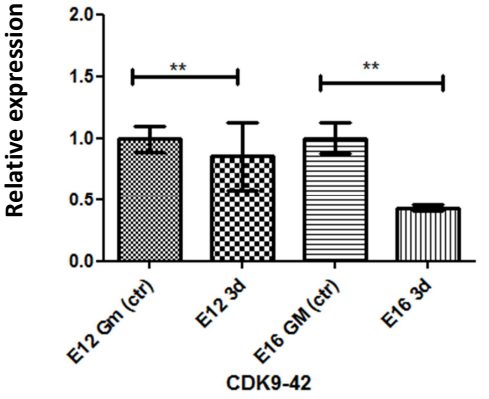
4.2 ISOLATION OF MYOBLASTS AT DIFFERENT TIMEs OF DEVELOPMENT

Precedent works demonstrated that development of the embryo is coordinated by sequential and different responsiveness to external signals. When embrional and fetal myoblasts are planted they present different characteristics. Embrional myoblasts are elongated cells that differentiate in multinucleated myotube, while fetal myoblasts show triangular shapes, proliferate more and differentiate in multinucleated myotubes (Biressi et al., 2007). Studies on primary cultures allow us to discriminate the intrinsic properties of the population of myoblasts isolated from the draft of the limb. These cells were cultured and induced to differentiate for 3 and 5 days.

4.2.1 ANALYSIS OF CDK9 ISOFORMS EXPRESSION *IN VITRO*

Data obtained *in vivo* demonstrated that two CDK9 isoforms show an inverse correlation in genic expression levels and in transcript levels during muscle development. This different expression profile suggests a different role of two CDK9 isoforms: CDK9-42 seems to be involved during the early phase of muscle development, whereas CDK9-55 is required in the final phase of muscle formation.
Preliminary study on stable cell line C2C12 demonstrated that expression levels of CDK9-55 were increased during differentiation, whereas the CDK9-42 levels were stable.
In order to characterize the specific role of two CDK9 isoforms on myogenesis and its relationship with the other factors involved in muscle formation , mouse embryonic (E12) and fetal (E16) CD1 mice limb primary cultures were set up. Then the expression *in vitro* of CDK9-42 and CDK9-55 were analyzed.

A



B

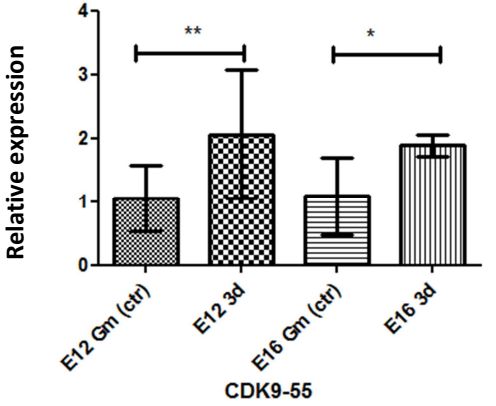


Figure 15: *mRNA levels of CDK9-42 (A) and CDK9-55 (B) in mouse embryonic (E12) and fetal (E16) limb primary cultures. GM refers to proliferating myoblasts, while 3d to differentiating myoblasts, harvested 3 days after the induction of differentiation.*
*A)**E12 GM (ctr) vs E12 3d P<0,005*
*** E16 GM (ctr) vs E16 3d P <0,005*
*B) **E12 GM (ctr) vs E12 3d P <0,005*
** E16 GM (ctr) vs E16 3d P<0,05*

The RT-PCR analysis demonstrated a significant increase of CDK9-55 isoform in cells induced to differentiate both in embrional phase (E12) then in fetal phase (E16), whereas transcript levels of CDK9-42 are stable.
Also the analysis of Western blotting analysis (Fig.16) showed an increase of CDK9-55 during differentiation.

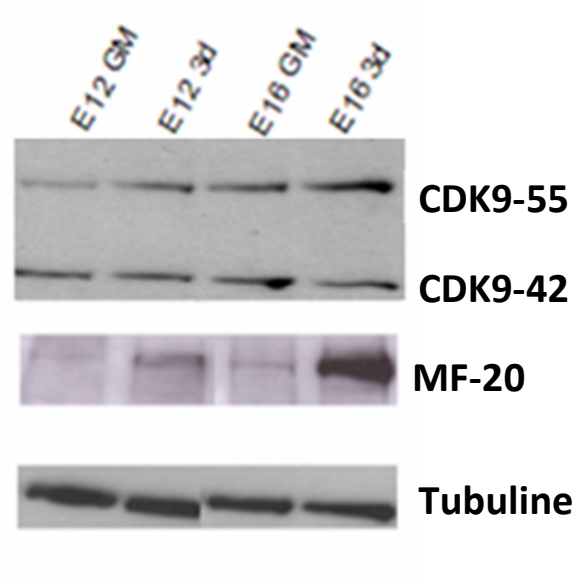


Figure 16: *Analysis of Western blotting analysis on proteic extracts of embrional myoblasts (E12) and fetal myoblasts (E16) .*

This data supports the hypothesis that there is a specific activation for CDK9-55 isoform during the differentiation phase of muscle cell.
Embrional and fetal cultures have, another, a different profile of differentiation, showing a different expression profile during the expression time of sarcomeric myosin. It was observed that fetal myoblasts (isolated at the time E16,5) expressed sarcomeric filaments on the third day of differentiation (Fig. 16).
The new antibody, pospho CDK9, that detects endogenous levels of CDK9 only when phosphorylated at Thr186, permitted to verify

which of the two CDK9 isoform is active during differentiation. Preliminary data obtained on proteic extract of embryonic myoblasts (E12) demonstrate that CDK9-55 is the active isoform during differentiation.

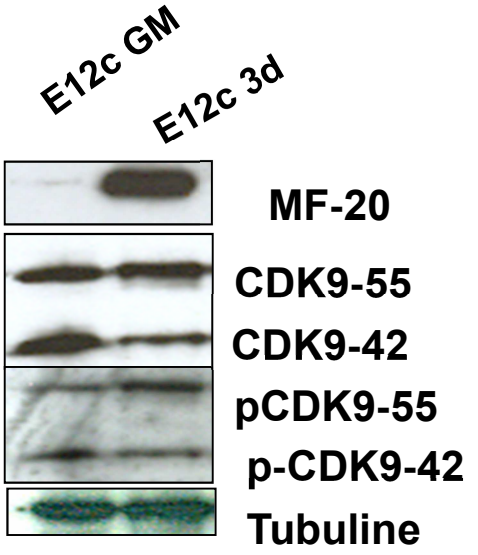


Figure 17: *Western blotting analysis on embryonic myoblasts (E12) in proliferation and differentiation phase, using the phospho CDK9 antibody, that permits the active isoform to be verified during differentiation.*

4.2.2 CYCLINS T1, T2a AND K

In mouse embryonic and fetal limbs RNA were extracted and the expression of canonical CDK9 partner was analyzed.
For cyclinT1 the results showed a progressive decrease of its expression levels in differentiating myoblasts (E12 3d and 5d); a similar trend was observed also in fetal myoblasts (E17), although cyclin T1 was slightly up-regulated in 3d differentiating cells, suggesting its marginal involvement in myogenesis.

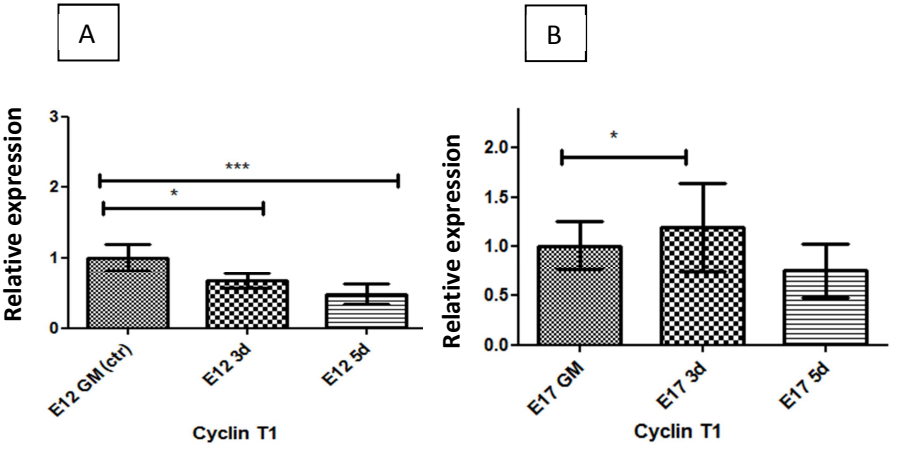


Figure 18: *mRNA levels of cyclin T1 in mouse embryonic E12 (A) and fetal E17 (B) limb primary cultures. Cyclin T1 expression levels decrease in differentiating embryonic and fetal myoblasts, although a slight increase in E17 3d differentiating cells.*
*A)*E12 GM (ctr) vs E12 3d P<0,05*
**** E12 GM (ctr) vs E12 5d P<0,0005*
B) E17 GM (ctr) vs E17 3d P<0,05*

On the other hand, cyclin T2a was progressively over expressed in 3d and 5d embryonic myoblasts, and only in 3d fetal myoblasts, in concomitance with the CDK9-55 expression. This supports the hypothesis that cyclin T2a *in vitro* might complex with CDK9-55, thus promoting myogenic differentiation and muscle specific gene expression. On the contrary, the fact that cyclin T1 does not significantly increase in both embryonic and fetal myoblasts induced to differentiate, may indicate a different role from differentiation.

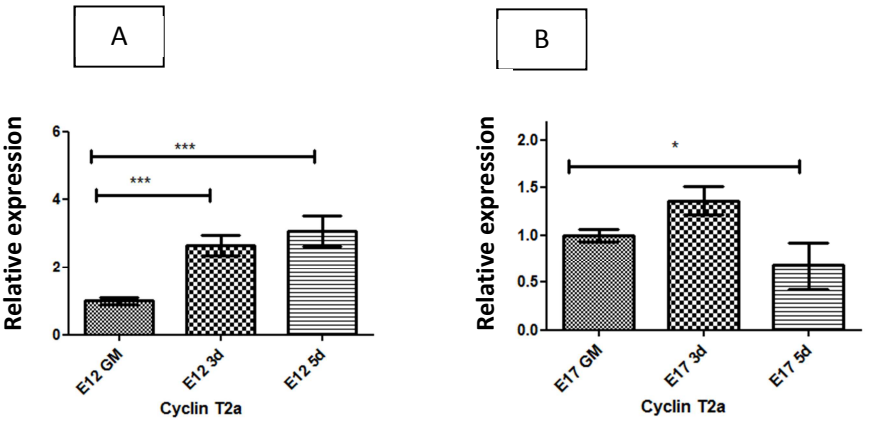


Figure 19**:** *mRNA levels of cyclin T2a in mouse embryonic E12(A) and fetal E17(B) limb primary cultures. Cyclin T2a is progressively over expressed in 3d and 5d embryonic myoblasts, and only in 3d fetal myoblasts.*
*A)***E12 GM (ctr) vs E12 3d P <0,0005*
****E12 GM (ctr) vs E12 5d P<0,0005*
*B) * E17 GM (ctr) vs E17 3d P <0,05*

Cyclin k was shown to be the regulatory cyclin of CDK9 during the fetal myogenesis in *in vivo* experiments carried out on developing limbs.
In vitro this finding does not seem to occur, indeed the gene analysis revealed that cyclin K expression decreases progressively in both embryonic than fetal myoblasts after 3 and 5 days of differentiation.

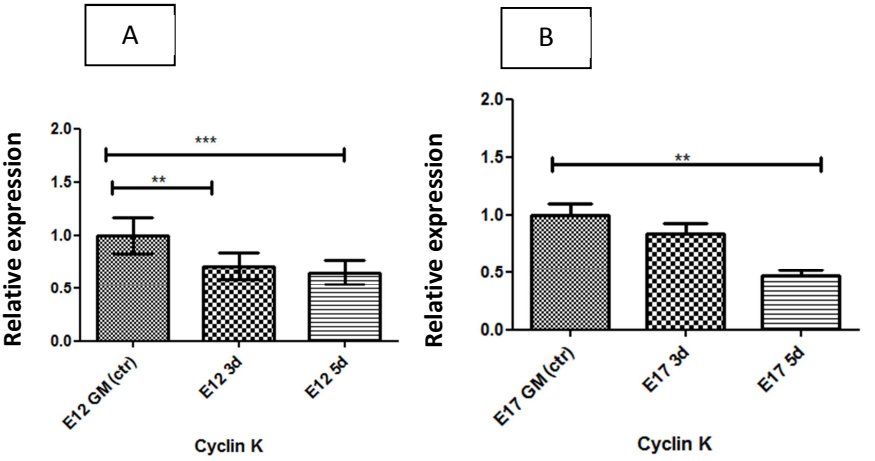


Figure 20: *mRNA levels of cyclin K in mouse embryonic E12 (A) and fetal E17 (B) limb primary cultures. Cyclin K undergoes a gradual decrease of its expression in embryonic and fetal myoblasts.*
*A)**E12 GM (ctr) vs E12 3d P< 0,005*
**** E12 GM (ctr) vs E12 5d P< 0,0005*
*B) ** E17 GM (ctr) vs E17 5d P <0,005*

Cyclin K, as well Cyclin T1, does not seem to be involved in the differentiation *in vitro*, while *in vivo* cyclin k was expressed during fetal myogenesis; whereas *in vitro* only for the cyclin T2a it was observed an increase of expression during the differentiation phase both for embrional and fetal myoblasts.

4.2.3 CYCLIN D3, H AND L IN EMBRIONAL AND FETAL MYOBLASTS

The obtained results *in vivo* showed that cyclin D3 is expressed during fetal myogenesis, supporting the hypothesis that it is involved in the induction of the muscle differentiation. *In vitro* cyclin D3 shows a different profile of expression, indeed our qRT-PCR analysis showed steady expression level in embryonic

myoblasts (E12, 3d and 5d), whereas it was down regulated in differentiating fetal myoblasts (E17, 3d and 5d), suggesting its involvement only in embryonic myogenesis.

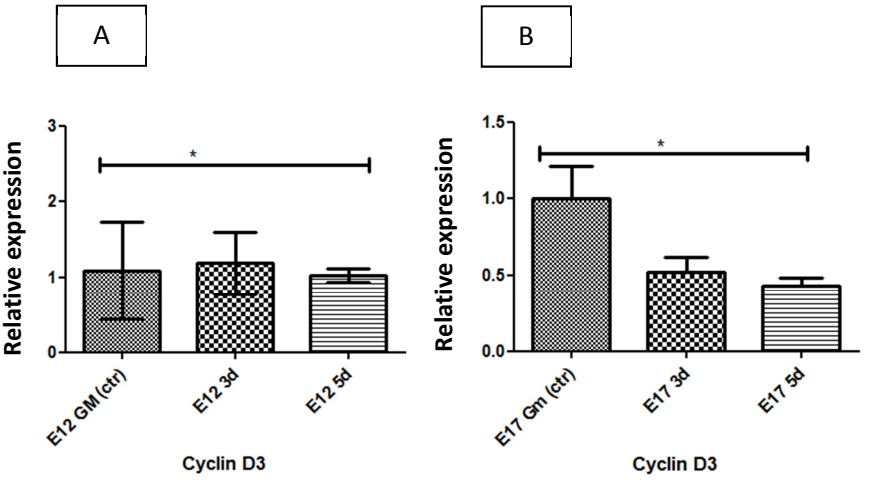


Figure 21: *mRNA levels of cyclin D3 in mouse embryonic E12 (A) and fetal E17(B) limb primary cultures. Cyclin D3 shows steady expression levels in embryonic myoblasts, whereas it is down regulated in fetal myoblasts.*
A) E12 GM (ctr) vs E12 5d P <0,05*
B) E17 GM (ctr) vs E17 5d P <0,05*

In vivo we analyzed also cyclin H and L, and we didn't observe significant profile of expression both in embrional and fetal myogenesis. Cyclin L is expressed, at low levels, during differentiation of embryonic myoblasts, indeed its expression increases progressively, while in fetal myoblasts its expression decreases gradually during differentiation.

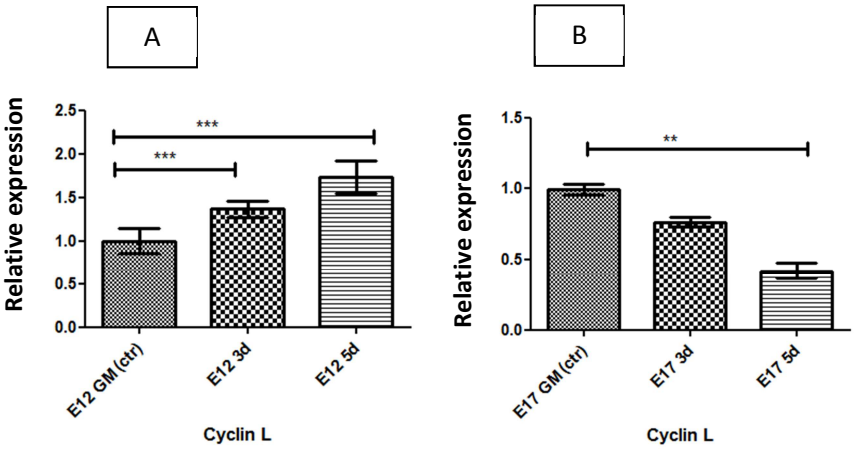


Figure 22*: mRNA levels of Cyclin L in mouse embryonic E12 (A) and fetal E17 (B) limb primary cultures.*
*A)*** E12 GM (ctr) vs E12 3d P <0,0005*
*****E12 GM (ctr) vs E12 5d P <0,0005*
*B)** E17 GM (ctr) E17 3d P <0,005*

For cyclin H we observed a profile of expression similar to cyclin L, also this cyclin increases during differentiation of embryonic myoblasts, while during differentiation of fetal myoblasts it decreases.

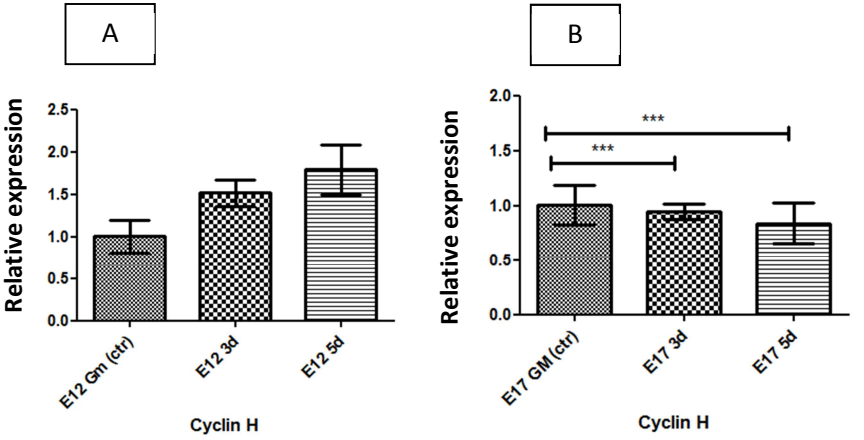


Figure 23**:** *mRNA levels of cyclin H in mouse embryonic E12 (A) and fetal E17 (B) limb primary cultures.*
*B)*** E17 GM (ctr) vs E17 3d P< 0,0005*
****E17 GM (ctr) vs E17 5d P<0,0005*

4.3 THE EXPRESSION OF 7SKsnRNA and HEXIM1 DURING DIFFERENTIATION

In Hela cells there are two distinct P-TEFb complexes, which differ in size, composition and activity. The smaller P-TEFb complex has kinase activity and is composed of CDK9-42 or CDK9-55 and a cyclin partner T1,T2 and K. A large P-TEFb complex with reduced kinase activity was found to contain the small nuclear RNA 7SK and HEXIM1, in addition to P-TEFb subunit. There is a delicate and dynamic balance between the two P-TEFb complexes in the cells. The disassociation of P-TEFb from 7SKsnRNA and Hexim1 is reversible.
In order to verify the modulation of CDK9 expression during muscle differentiation of C2C12 cells the RNA

immunoprecipitation assays were done. In this assay, similar to chromatin immunoprecipitation (ChIP), cells are treated with formaldehyde to generate protein-protein, protein-DNA and protein-RNA cross links between molecules. A whole-cells extract is prepared in the presence of RNase inhibitors to maintain the integrity of RNA, and the cross-linked nucleic acids are sheared by sonication to enable their solubilization. The extract is then enzymatically treated to remove DNA and the resulting material is immunoprecipitated with an antibody against the protein of interest.
In the first step of this assay we analyzed the expression of 7SKsnRNA on total extract during a proliferative phase and a differentiative phase of C2C12 cells; we observed that 7SKsnRNA is expressed in C2C12 cells only during proliferation, while it isn't present in cells induced to differentiation.

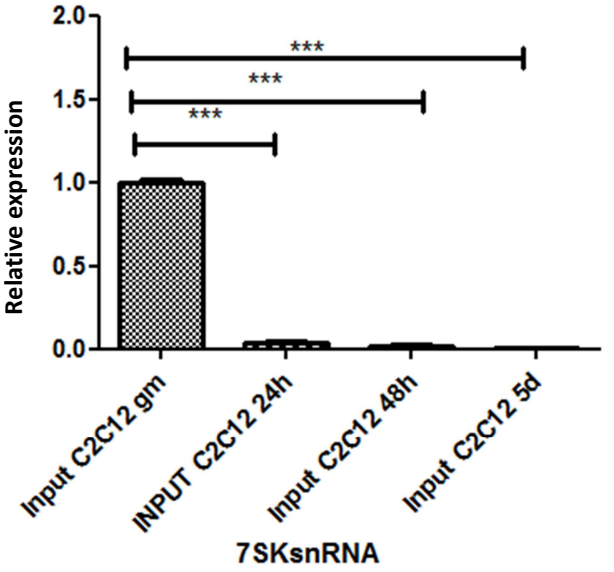


Figure 24: *mRNA levels of 7SKsnRNA in total extract of C2C12 cells during proliferative phase and differentiative phase.*
**** Input C2C12 Gm (ctr) vs Input C2C12 24h P<0,0005*
****Input C2C12 Gm (ctr) vs Input C2C12 48h P<0,0005*
****Input C2C12 Gm (ctr) vs Input C2C12 5d P<0,0005*

On total proteic extracts of C2C12 cells it was analyzed also the HEXIM 1 expression and it was observed that it presents a peak of expression after 5 days of differentiation, when there is the formation of myotubes.

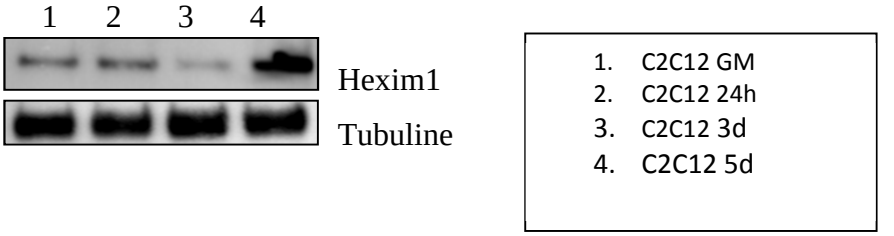


Figure 25**:** *Western blotting analysis on total extract of C2C12 cells to evaluate the expression of HEXIM1.*

By using RNA immunoprecipitation (RIP) it is possible to detect the association of individual proteins with specific RNA.
In this experiment we isolated the RNA associated to CDK9 and then it was observed the expression of 7SKsnRNA. Data obtained with RT-PCR demonstrated that 7SKsnRNA is associated with CDK9 in the proliferative phase, but its expression decreases during differentiation.

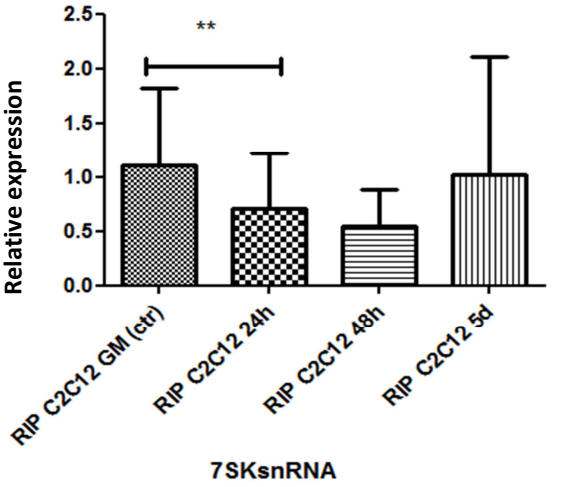


Figure 26**:** *mRNA levels of 7SKsnRNA associated to CDK9 in C2C12 cells during proliferative and differentiative phase.*
*** RIP C2C12 GM (ctr) vs RIP C2C12 24h P< 0,005*

A different approach of expression for HEXIM1 it is possible to observe when it was immunoprecipitated with CDK9, therefore it is associated with CDK9 during the proliferative phase and in the early phase of differentiation, its expression decreases during advanced differentiation.

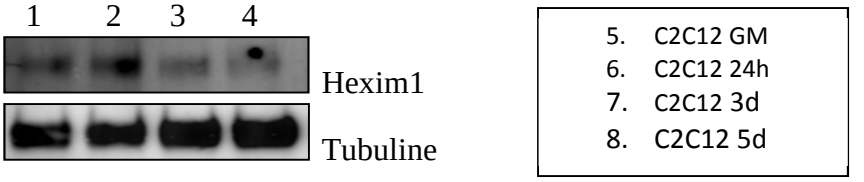


Figure 27: *Western blotting analysis on extract after immunoprecipitation with CDK9 to verify the expression of Hexim1 associated with CDK9.*

4.4 THE ROLE OF TWO CDK9 ISOFORMS IN DENERVATED MUSCLE

Skeletal muscle is susceptible to injury after direct trauma and or indirect trauma, in order to maintain a functional skeletal musculature this tissue has a notable ability to initiate the repair of the damage muscle. In fact, after muscle injury, a complex set of cellular responses is activated, leading to the regeneration of a well-innervated, totally vascularized and contractile muscle apparatus (Chargè et al., 2004).

A precedent work demonstrated that the CDK9-55 is the isoform involved in regeneration of muscle after injury, our studies analyzed the expression of CDK9 isoforms in muscle after denervation, in order to evaluate the importance of CDK9- 55 in the innervation process. In scientific articles is known that in myogenesis the process of innervation occurs during fetal myogenesis, where we observed an increase of expression of CDK9-55, while the CDK9-42 decreases.

In our experiments we analyzed the expression of two isoforms in denervated muscle: we caused a trauma in sciatic nerve of mice and then tibial and quadriceps were extracted after a week and a month by denervation.

We analyzed the muscle exctracted after denervation at proteic level, by Western blotting analysis and the obtained data demonstrated that two isoforms are expressed more in denervated

muscle, with a peak of expression in quadriceps after a month of denervation.

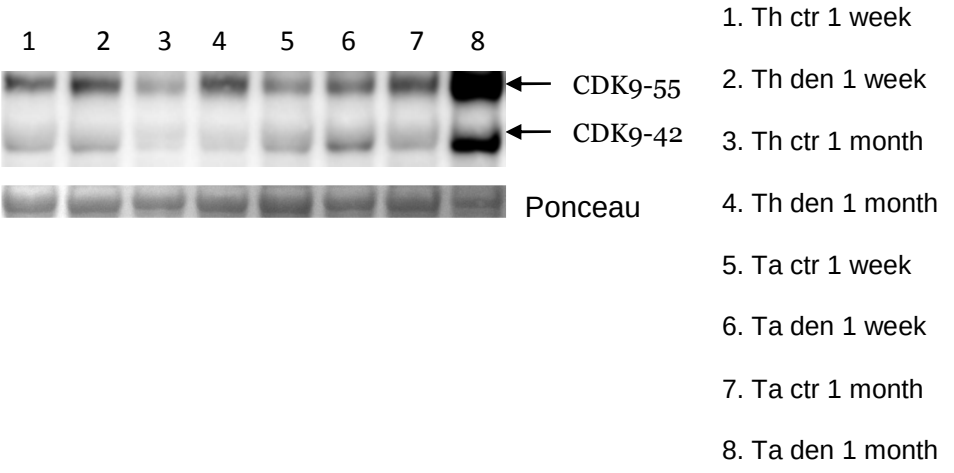


Figure 28**:** *Western blotting analysis in tibial and quadriceps after a week and a month of denervation, to evaluate CDK9-55 and CDK9-42 expression.*

By using densitometry analysis we compared the expression of CDK9-55 and CDK9-42 and it was observed that CDK9-55 is the isoform expressed more in denervated muscle both in the tibial and quadriceps, after a week and a month of denervation.

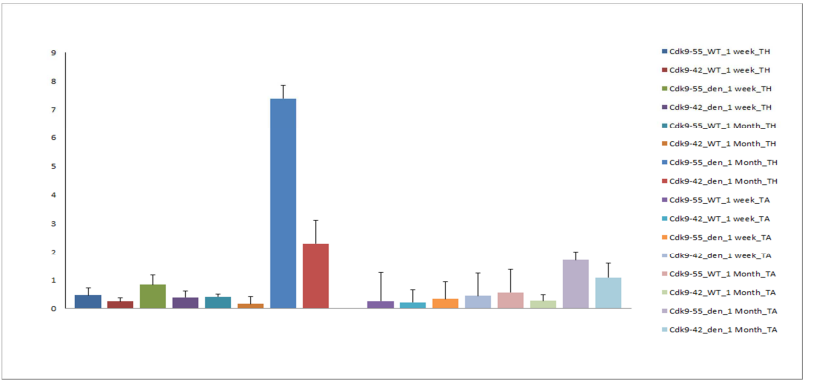


***Figure 29**: Densitometry analysis comparing the two CDK9 isoform expressions in proteic exctract in denervated muscle (tibial and quadriceps), after a month and a week of denervation. Protein levels of CDK9-42 and CDK9-55 were normalized respect to β-actine, which was used as a control.*

After denervation in muscle there is an increase of the transcription factors, above all there is an increase of myogenin, that induces an increase of transcription of genes as an Ach receptor.
In our experiments we found that CDK9-55 is the isoform expressed more in muscle after a month of denervation, and this phase is characterized by regeneration and innervation of the muscle, we hypothesize that this isoform is involved in the innervation process, in concomitance with an increase of expression of myogenin.

4.5 DISCUSSION

Skeletal muscle is a highly complex and heterogeneous tissue serving a multitude of functions in the organism, in particular movement, postural behaviour and breathing.
The process of generating muscle, myogenesis, can be divided into several distinct phases. During embryonic myogenesis (E10,5-E12,5), mesoderm derived structure generates the first fibers of the body, and during the subsequent fetal (E14,5-E17,5) and post natal development, additional fibers form along these template fibers (Parker et al., 2003). The continued growth of the muscle is due to a population of muscle progenitors, which co-express Pax3 and Pax7 and can enter the myogenic program with the activation of the myogenic regulatory factors Myf5 and MyoD (Relaix et al., 2003). In the perinatal phase, muscle progenitors initially proliferate extensively but later on decrease as the number of myonuclei reaches a steady state and myofibriller protein synthesis peaks (Davis and Fiorotto, 2009). Once the muscle has matured, these myogenic precursors cells, known as satellite cells, maintain the tissue homeostasis. This type of myogenesis occurs in response to different types of stimuli, including direct trauma, further growth demands on genetic defects (Huard et al., 2002), and it depends on the activation and differentiation on satellite cells in new fibers.
The obtained data permitted to conclude that the expression of CDK9 kinase is induced during differentiation of muscle cell *in vitro*, in culture of embryonic and fetal myoblasts, and *in vivo*. Scientific articles reported that CDK9 binds directly MyoD *in vitro* (Simone et al., 2002) and takes part in the formation of multimeric complex cointaining MyoD, cyclin T2a, p300, PCAF and BrgI in muscle cells (Giacinti et al., 2006). This transcriptional complex binds to the regulatory regions of muscle-specific genes to induce the acetylation of specific lysines on histones H3 and H4, the chromatin remodelling and the phosphorylation of serines target-specific for CDK9 at the levels of the RNA polymerase II CTD and

promotes the gene transcription (Giacinti et al., 2006; Simone et al., 2007).
The identification of new CDK9 isoform, called CDK9-55 (Shore et al., 2003) and its involvement in differentiated cells and tissues, led us to evaluate the role and the possible impact of this isoform during myogenesis.
In additions the results of Giacinti and colleagues demonstrated that CDK9-55 expression increases significantly during differentiation in stable muscle cell line (Giacinti et al., 2008); in the same study it was observed that CDK9-55 is highly expressed during regeneration process of damage myofibers.
Subsequently the research group start to characterized the profile of expression of CDK9 isoforms during muscle development. In this preliminary study the two CDK9 isoforms was analyzed during mouse embryogenesis starting at the stage E10,5 and they observed that there is a different correlation between two CDK9 isoforms, both in protein extracts and at transcript levels, during muscle development. This study *in vivo* demonstrated that CDK9-55 increases progressively during muscular differentiation, while CDK9-42 isoform decreases gradually. These data have suggested a different role of two isoforms. It is interesting to observe that at E14,5 dpc, key point of transition between embrional and fetal myogenesis, the CDK9-55 increases while CDK9-42 decreases.
In order to characterize the role of CDK9 isoforms it was also tested the expression of some genes involved in myogenic program.
The determiners of myogenic lineage, Pax3 and Pax7, play an important role in the formation and in maintenance of muscle tissue both during its development and after its formation. Both genes are supposed to be involved in the myogenic cell specification and are known as early markers of the forming dermomyotome, which is the source of future myoblasts (Goulding et al., 1991). It is known that at E11,5 Pax3 positive cells in the somites constituted the dermomyotome and delaminating myotome, this data was confirmed also by Western blotting analysis, Pax3 is indeed expressed at the stage E10,5 and E11,5, then it must be down regulated, for a co-expression of MyoD (Relaix et al., 2005). This

phase was present at the stage E11,5 and E12,5 dpc, in concomitance we observed an increase of CDK9-55 isoform. Moreover, the presence of satellite cells in the late fetal phase of muscle development is confirmed by high levels, from E16,5 dpc to E17,5 dpc, of both desmin, known marker of active satellite cells, and Pax7.

The obtained data are in agreement with what is known in the scientific reports, indeed precedent works demonstrated that an expression profile Pax3+/Pax7- is characteristic of embryonic myoblasts, while a profile Pax3-/Pax7+ is characteristic of fetal myoblasts (Biressi et al., 2007).

Muscle regulatory factors play a critical role in myogenesis, and their expression is tightly regulated, according their physiological functions. In this work we analyzed the expression of MyoD and Myogenin during muscle differentiation. In scientific articles is reported that MyoD is a marker of determined myoblasts, whereas myogenin-positive cells were judged as myoblasts that are more advanced in their differentiation. The results, according to the literature, have highlighted that the two protein are expressed at different stages of muscle development, being MyoD expressed during embryonic and Myogenin during foetal myogenesis.

The data obtained demonstrated that the more differentiation in muscle development is present between the stage E14,5 and E17,5, this phase is characterized by an increase of muscular mass. This period coincides with the increase of CDK9-55 isoform expression, whereas the CDK9-42 expression decreases.

By the Western blotting analysis we observed that the myosins expression started at the stage E14,5, time of switch of embrional and foetal myogenesis, but for the isoform fast of myosin we observed that was expressed at the time E10,5. This data permitted to suppose that the process, present at the stage E10,5, for the presence of the isoform fast of myosin, is the result of precendent inductive mechanisms present in somites.

It is known that activation of MyoD is in turn depend on the CDK9/cyclin T2a complex, which plays a critical role in the activation of myogenic program (Giacinti et al., 2006). CDK9 has

been reported to participate to the differentiation program of several cell type, as monocytes, B and T-lymphocytes, mainly complexed to cyclin T1, suggesting that the CDK9/cyclin T1 complex may be specifically required for the differentiation process of T lymphocytes.
In muscle differentiation, CDK9 binds to cyclin T2a, which is not down-regulated during myotube formation; rather their activity contributes to the activation of MyoD (Simone et al., 2002). In particular, the N-terminal region of CDK9 directly interacts with the bHLH region of MyoD, allowing the formation of multimeric complex also containing cyclin T2a (Giacinti et al., 2006; Simone et al., 2002). It is detectable in muscle cells during the activation of differentiation program, when it can provide a functional link between MyoD and the basal transcription machinery. In fact, it binds to the chromatin of muscle specific gene regulatory regions, including the acetylation of histones H3 and H4 specific lysines, chromatin remodelling, stimulation of transcription elongation through the ability of CDK9 to phosphorylate target serines at the RNApolII CTD, and finally muscle-specific gene expression (Simone et al., 2006; Giacinti et al., 2006; Simone and Giordano 2007).
The kinase activity of CDK9 is tightly regulated: association with a cyclin partner and phosphorlation of the T-loop is needed for activation of CDK9.
Roughly 80% of the CDK9 is complexed with cyclinT1, 10% with cyclin T2a and 10% with cyclin K: each of the cyclin proteins complexed with CDK9, form an active P-TEFb molecule. This molecule is able to phosphorylate the carboxyl-terminal domain of RNA polymerase II (RNA polII).
Recent study show that CDK9/cyclin T complex may be recruited to specific promoters by cellular transcription factors that bind to the promoter sequences of genes regulated at the level of transcriptional elongation (Simone and Giordano,2001). It has been shown also that CDK9/ cyclin T is a very stable complex, with a half-life of about 36h, while free CDK9 appears to be degraded rapidly, with a half-life of about 6h (Majello and Napolitano, 2001).

According to scientific articles, cyclins T1, T2a and K bind to CDK9, prompting us to investigate their involvement during myogenesis.
In vivo results have shown a different expression of these CDK9 cyclin partners along the two phases of myogenesis. Indeed cyclin T1 and cyclin T2 are expressed during primary myogenesis, while cyclin K is expressed during fetal myogenesis. We also observed a further correlation between two CDK9 isoforms and cyclins: cyclin T1 and T2 are probably associated with CDK9-42, while cyclin K is associated with CDK9-55, that is expressed principally during fetal myogenesis.
We observed also that the expression of cyclin k coincides with the increase of expression of p21, an inhibitor of cellular cycle, that determines cellular arrest in concomitance with the cellular differentiative program. In a recent study it was demonstrated that CDK9 coordinates the transcription of p21, in collaboration with BRG1 (a factor of remodelling of cromatine) and STAT3 (Giraud et al., 2004). It is known that Cyclin K is a target of p53 and it is activated in response to a signal of cellular arrest, or in response to stress p53-mediated. The cyclin K complexed with CDK9 seems to be the specific P-TEFb activated for the transcription of genes induced by p53, as p21. It was also demonstrated that while the complex CDK9/Cyclin T1 induces the replication of HIV-1, the complex CDK9-Cyclin K stops the replication of HIV-1, because cyclin K doesn't bind to TAT sequence (Fu et al., 1999).
In precedent work it was demonstrated that CDK9 together to MyoD is on the promoter of p21 gene in C2C12 cells induced to differentiate.
We can suppose that cyclin K is the specific regulator, *in vivo*, of the CDK9-55 kinase and the complex CDK9-55/cyclin K regulates pathways of arrest and cellular differentiation, that are present during fetal development phase, that is characterized by synthesis process and muscular growth.
Being involved in the transition between cell proliferation and growth arrest in the myogenic program (Gurung and Parnaik, 2012), we have examined the *in vivo* expression of cyclin D3 during

muscle differentiation. Our results have displayed high expression levels of cyclin D3 throughout fetal myogenesis, in concomitance with the expression of p21 gene, arguing for a role in terminally differentiated myotubes.
The fact that the different cyclin genes (cyclin T1, T2, K, D3) can function as CDK9 regulatory subunits suggest that CDK9 might associate with functionally different complexes and thereby participate in different cellular process.
In order to discriminate the properties of isolated myoblasts during limbs development *in vitro* experiments were conduced.
In vitro we observed that the expression of CDK9-55 isoform increases during differentiation both embrional and fetal myoblasts, while for CDK9-42 isoform it possible to observe that its levels are stable during proliferative and differentiative phase, begins to decrease with the progress of differentiation program.
These data permit us to conclude that the expression of two isoforms of CDK9 is present in embrional and fetal myoblasts, and their timing of expression defines their likely role: CDK9-42 isoform prevails during proliferative phase and/or during the myogenic determination, while the CDK9-55 isoform has a specific role during differentiation.
On embrional and foetal myoblasts the expression of different cyclins was analyzed, obtained results demonstrated that only cyclin T2 is expressed during differentiation of embrional and fetal myoblasts.
Data reported in scientific reports demonstrated that cyclin T2 and N-terminal region of CDK9, interacting with bHLH domain of MyoD, allow the formation of a complex ale to stimulate the transcription of specific genes (Simone et al., 2002). In this complex, cyclin T2 interacts physically with MyoD that is phosphorylated by CDK9.
Liu and Hermann demonstrated that, in Hela cells, was present in immunoprecipitate containing both CDK9-42 and CDK9-55, although the levels of associated cyclinT1 was significantly lower in the 55k complexes. In our experiments we observed that cyclin T1 is expressed during proliferative phase in embrional and fetal

myoblasts suggesting its interaction with CDK9-42, the isoform more expressed in this phase.
Cyclin T1 and cyclin T2 differ for the carboxy terminal domains and Peng et al. (1998) have found that removal of this region of the cyclin T2 significantly reduced the ability of the CDK9/cyclin T2 to phosphorylate the CTD of RNA polII and function in transcription. The different carboxy terminal domains of two cyclins could explain the different expression level of these cyclins in these experiments.
We analyzed also the cyclin H expression both *in vivo* and *in vitro* experiments: cyclin H complexed with CDK7 appear to be involved in transcription regulation, rather than cell cycle control and they are commonly found in zebrafish embryo cells. In zebrafish it was observed that cyclin H is present in ovary, raising the possibility that cyclin H mRNA might be maternally inherited. The importance of cyclin H during embryogenesis is supported by the fact that the injection of cyclin H ^{DN} mRNA into embryos disturbed normal cell cycle or transcription, inducing apoptosis. The transcription of cyclin H in rat neurons was increased primarily after ischemia, suggesting additional roles for cyclin H in neurons, other than cell cycle regulation and DNA repair (Liu et al., 2007).
In our experiments the levels of cyclin H expression are stable during embrional and foetal myogenesis (*in vivo* analysis), while *in vitro* it is expressed at low levels only during differentiative phase of embrional myoblasts.
A similar profile of expression it was observed for cyclin L, that complexed with CDK-11 is involved in splicing process.
The different expression pattern of these cyclin partners between *in vitro* and *in vivo* analysis maybe due to the lack, in *in vitro* system, of stimuli such as innervation and circulating hormones, determining homeostasis in the organism. Thus, further investigations about external factors influencing their expression are required.
In scientific articles is reported that CDK9 is activated by interaction with some members of cyclins family, by autophosphorylation in different residues of threonine and serine,

T186, S347, S362, S363, and acetylation of histones (the acetylation of CDK9 enhances its ability phophorylate the CTD of polII and promotes transcription elongation), while it inactivated by autophosphorylation in T29 and by interaction with 7SKsnRNA and HEXIM1.

In this work we started to study an inhibitory transcriptional factor, the interaction between 7SKsnRNA and HEXIM1 with CDK9.

In the cells p-TEFb exists in two forms, an active form composed by CDK9 and its cyclin partner and an inactive form composed by 7SKsnRNA and Hexim1 complexed with CDK9/cyclin. P-TEFb is uniquely regulated by the reversible association with 7SKsnRNA and Hexim proteins. The relative partitioning into active an inactive complexes is a highly dynamic process that is central to the global regulation of cells growth and differentiation (Zhou et al., 2006).

It is known that 7SKsnRNA is stable when it is dissociated by Hexim1, while when it binds to Hexim1 promotes a major conformational change allowing the C-terminal domains of the proteins to interact with N-terminal domains of cyclin T.

In the first step of this analysis we studied the expression of 7SKsnRNA on total extract of C2C12, and we observed that 7SKsnRNA is expressed in C2C12 cells during proliferative phase, while it isn't expressed during differentiation. For Hexim1, indeed, we observed that is expressed in C2C12 at the 5th day of differentiation, when there is the presence of some myotubes.

The study on 7SKsnRNA complexed with CDK9 demonstrates that 7SK is present during proliferative phase, so as HEXIM1, that it is present during proliferative phase and in the early phase of differentiation of C2C12 demonstrating that in cells induced to differentiate CDK9 it isn't associated with 7SKsnRNA/HEXIM1.

The fact that Hexim1 it is associated with Cdk9 during proliferative phase and in the early differentiative phase (24h) could significant that HEXIM1 mediate the regulation of P-TEFb activity in the transition of C2C12 cells from growth to differentiation.

Galatioto and colleagues demonstrated that by reducing expression of HEXIM1 in C2C12 cells there is an arrest in the transition of skeletal myoblast to myotubes, implying that Hexim1 and possibly

its association with components of P-TEFb are obligatory to the transition. In the same study it was observed that MyoD is associated with Hexim1 in the early phase of differentiation, suggesting that hexim1 is recruited by MyoD to offer an environment optimal for repression of proliferative genes and allowing the transition of myoblasts to myotubes (Galatioto et al., 2010).

In order to verify the role of two CDK9 isoforms in the innervation process sciatic nerve transaction was performed. The tibialis and quadriceps, muscle innervated by sciatic nerve, were collected and used for analysis.

During development of muscle neurons grow out towards the muscle fibers, which play an attractant role in axon guidance and the establishment of innervation. This includes both sensory innervation through the intrafusal fiber of muscle spindles, which retain a more embryonic contractile protein phenotype even in the adult and motor innervation of muscle fibers which drives contraction. The establishment of definitive neuromuscular junctions and excitation- contraction coupling is associated with secondary or fetal myogenesis.

The innervation of the muscles in development begins shortly after the fibers begin to form. On the newly- formed muscle fibers can be observed widespread expression of the acetylcholine receptor (AchR) between developmental stages E12 and E14 dpc, an organization of AchR clusters to guide the ramifications of the motor nerves to the future synaptic sites (Witzemann, 2006). During the following days of development are witnessing a sequential elimination of multiple axons, with the exception of one, which innervate a single muscle fiber (Biressi et al ., 2007). During this time there is the maturation of neuromuscular junctions that ends around the stadium E17 dpc, the AchRs are progressively focused on the post- synaptic membrane and stabilize the neuromuscular junction (Witzemann, 2006)

Innervation is critical for growth and maintenance of muscle fibers, and denervation is well known to cause muscle atrophy. A loss of trophic support to the muscle following denervation, in absence of

neural activity muscle mass decreases, along with a reduction in specific force, fiber diameter and fibers number.
In our experiments we analyzed the expression of two CDK9 isoforms after a week and a month by denervation, and we observed that in regeneration process there is an increase of CDK9-55 expression, compared to CDK9-42, suggesting its involvement in the innervation process. After the denervation in the muscle there is an increase of transcriptional factors expression, with an increase of myogenin. The myogenin increase gradually after few days from denervation, stimulating the transcription and the expression of some genes, as Ach receptor.
Because *in vivo* analysis on limbs at different time of development it was observed that at the time E14,5, point of switch between embrional and fetal myogenesis, there is an inverse correlation of two CDK9 isoforms. In the specific CDK9-42 starts to decrease, while CDK9-55 increases gradually, in this phase there is, also, the innervation of the muscle and the passage between slow and fast fibers. Those preliminary data, together to the fact that CDK9-55 is the isoform more expressed in muscle after a month by denervation, permit us to speculate that the CDK9-55 isoform is involved in the recovery phase of muscle and in the new innervation process.
Those data permit us to conclude that the expression of two CDK9 expression is associated at a specific cellular phenotype and is correlated at a specific function.
The expression of CDK9-42 and CDK9-55 is associated with embrional and foetal myoblasts *in vitro*, and their expression profile defines the specific role. CDK9-42 is correlated with proliferative phase and/or in the first step of myogenic determination, whereas CDK9-55 has a predominant role during differentiation process.
Also *in vivo* CDK9-55 is expressed during the differentiation of the muscle, at the end of embrional phase and at the start of foetal phase. In this period the muscle is characterized by an intense growth, by the innervation and by all processes that permit the formation of the arts.
During embrional myogenesis there is a prevalence of CDK9-42 expression, during this phase there is expansion and determination

of muscular phenotype. The difference of expressions timing of two isoforms, in embrional and in fetal myoblasts, probably is the consequence of extra-cellular signals, that change during the development of the muscle. Another the combination between CDK9 and specific regulators, as the cyclins, determine a different function of the CDK9 isoforms during the different phase of myogenic development.

5. MATERIALS AND METHODS

5.1 EMBRYO COLLECTION

CD1 mice, obtained from the department of Histology and Medical Embryology of “Sapienza” University of Rome, were used for the following experiments. They were docile albino mice, easy to work with, and with a rapid growth and a high reproduction rate.
To obtain staged embryos, pairs were set up as natural (1:1) matings and females checked every morning for vaginal coitum. On the day of coitum detection, embryos were counted as E0.5 days (12h post-fertilization). Once vaginal coitum was detected, males were removed from the cage to ensure accuracy of embryonic staging. When the desired embryonic stage was reached (E11 to E17), mothers were sacrificed by cervical dislocation, the abdomen was shaved, the skin and surrounding areas were swabbed with 70% alcohol and the uterus was removed via horizontal abdominal incision performed using sterile dissecting instruments. The uterus was then washed in prewarmed (37°C) PBS supplemented with Ca2+/Mg2+ (Dulbecco’s Phosphate Buffered Saline with CaCl2 and MgCl2; Sigma-Aldrich, St. Louis, MO, USA) before being placed into a small Petri dish containing PBS with Ca2+/Mg2+ prior to dissection. E11 to E17 embryos were dissected from the uterus using a dissecting microscope and placed individually into Petri dishes containing PBS with Ca2+/Mg2+ ready to be microdissected.

5.2 EMBRYO AND FETAL LIMBS PREPARATION FOR *IN VIVO* ANALYSIS

Individual embryos were further dissected to isolate skeletal muscles. The embryo head was then removed via an incision along the length of the neck. Then, hind and forelimbs were dissected out. The limb muscle dissection was quicker for E10.5-E12.5 mouse

embryos, although embryo limbs were small in size and they had to be removed carefully, making an incision on the back at the level of the limb attachment points and then taking them off with insulin needles. It was instead more difficult for embryos in fetal phase (E14.5-E17.5), where limb muscles had been already coated by the epidermis and connective tissue, and cartilage/bone had already developed. For this reason, in older embryos (E14.5-E17.5) it was necessary to remove cartilage/bone and peel off the dermis prior limb muscle dissection.
Once dissected out, embryo or fetal limb muscles were placed into an eppendorf containing prewarmed (37°C) PBS supplemented with Ca2+/Mg2+ and centrifuged at 1200 rpm for 7 min. The supernatant was removed and the pellet was washed in prewarmed (37°C) CMF-PBS (Dulbecco's Calcium and Magnesium Free Phosphate Buffered Saline; Sigma-Aldrich) to remove salt residues due to PBS washing, followed by centrifugation at 1200 rpm for 7 min. The supernatant was removed and the pellet was submitted to a second CMF-PBS washing. Eventually, CMF-PBS was discarded and the pellet was immediately stored at -80°C or submitted to RNA/proteins extraction.

5.3 PREPARATION OF EMBRYONIC LIMB (E11, E12) PRIMARY CULTURES

Skeletal muscle cells obtained by the above described procedure can be used to set up embryonic limb primary cultures. The mentioned procedure was performed under sterile conditions, using sterile materials and reagents and working in class II vertical laminar flow biological cabinet.
The pellet was resuspended four times with different amounts of medium [Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich), 10% Fetal Bovine Serum (FBS; Sigma-Aldrich), 2% L-Glutamine (L-Glut; Sigma-Aldrich), 5ml/l Penicillin/Streptomycin (P/S; Sigma-Aldrich)], then left to settle in the incubator at 37°C for

5 min and each time the surnatant containing cells was collected into a falcon.
Cells were counted using the Burker chamber and resuspended in an adequate medium volume considering 300.000 cells/200 µl. At this point, the bubble method was applied. Only 200 µl medium containing 300.000 cells were cultured in the middle of a 60 mm plate pre-coated with collagen. Cells were left to incubate at 37°C for 3 hours. At the end, the remaining medium (2.8 ml) was gradually added with a 1000 µl pipette.
24h post plating, cells were washed with CMF-PBS. Some proliferating cells were stopped, whereas other cells were allowed to differentiate in the same cell-medium reported above, and stopped at the pre-established time (3 or 5 days post plating) removing the medium, washing cells with CMF-PBS twice and detaching them with a scraper. Cells were then collected in a microcentrifuge tube and centrifuged at 1200 rpm for 10 min. The supernatant was removed, whereas the pellet was stored at -80°C or submitted to RNA/proteins extraction.

5.4 PREPARATION OF FETAL LIMB (E16, E17) PRIMARY CULTURES

Skeletal muscle cells obtained with the procedure described above can be used to set up fetal limb primary cultures.
The pellet was placed on a 100 mm plate cover and triturated until a homogeneous slurry was obtained (Mechanical Digestion). It was transferred into a 15 ml falcon and 0.05% trypsin in CMF-PBS was added up to a 10 ml final volume. The falcon was placed under shaking in a thermostatic bath at 37°C for 45 min (First Enzymatic Digestion). At the end, suspended muscle cells were left to settle at room temperature (occasionally a centrifugation step was required). The supernatant was filtered through a 40 µm filter and transferred into a 50 ml falcon containing 5 ml horse serum (HS; Sigma-Aldrich) necessary to supply cells with nutrients, to reduce cell stress and to inactivate trypsin. 0.05% trypsin in CMF-PBS was

added to the sediment up to a 10 ml final volume. The sediment was resuspended and placed under shaking at 37°C for 10 min (Second Enzymatic Digestion), and then to settle at room temperature for other 10 min. The supernatant was filtered and transferred into the same previous falcon. This procedure was repetead for one more time (Third Enzymatic Digestion).
The supernatant resulting from the three enzymatic digestions was centrifuged at 1200 rpm for 12 min. The supernatant was removed and the pellet was resuspended in 3 ml medium [Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich), 2% L-Glutamine (L-Glut; Sigma-Aldrich), 10% Horse Serum (Sigma-Aldrich), 3% Chick Embryo Extract (produced in-house)], which were cultured in a 60 mm plate and left to incubate at 37°C for 3 min. This pre-plating step was necessary to remove non-muscle cells, such as blood cells. At the end, the medium volume was recovered and cell-counting was performed using the Burker chamber. Cells were resuspended in an adequate medium volume considering 300.000 cells/200 µl. At this point, the bubble method was applied. Only 200 µl medium containing 300.000 cells were cultured in the middle of a 60 mm plate pre-coated with collagen, and they were left to incubate at 37°C for 3 hours. At the end, the remaining medium (2.8 ml) was gradually added with a 1000 µl pipette.
24h post plating, cells were washed with CMF-PBS. Some proliferating cells were stopped, whereas other cells were allowed to differentiate in the same cell-medium reported above, and stopped at the pre-established time (3 or 5 days post plating) removing the medium, washing cells with CMF-PBS twice and detaching them with a scraper. Cells were then collected in a microcentrifuge tube and centrifuged at 1200 rpm for 10 min. The surnatant was removed, whereas the pellet was stored at -80°C or submitted to RNA/proteins extraction.

5.5 TOTAL RNA EXTRACTION FROM MOUSE EMBRYONIC AND FETAL LIMB MUSCLE TISSUES

The RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA, USA) was used for total RNA extraction from mouse embryonic and fetal limb muscle tissues (30-50 mg) at different stages of development. RNeasy Fibrous Tissue Mini Kit is designed for optimal lysis of fibre-rich tissues (such as skeletal muscle) and purification of high-quality total RNA, which can be difficult due to the abundance of contractile proteins, connective tissues and collagen, which can be removed thanks to proteinase K supplied by the RNeasy Fibrous Tissue Mini Kit.

Limb muscle tissues were lysed and homogenized in 300 µl Buffer RTL containing β-Mercaptoethanol (10 µl β-ME per 1 ml Buffer RTL). Lysates were carefully pipetted into new microcentrifuge tubes and diluted with 590 µl RNase-free water. 10 µl proteinase K solution were added to lysates, mixed throughly by pipetting and incubated at 55°C for 10 min. Then, lysates were centrifuged at 10.000 x g for 3 min at 20-25°C. The surnatant was carefully removed and pipetted into a new 1.5/2 ml microcentrifuge tube. 450 µl of 96-100% ethanol were added to cleared lysates and mixed well by pipetting. Samples were transferred onto the RNeasy Mini spin column (where RNA bound to the silica membrane) placed in a 2 ml collection tube, and centrifuged at 8.000 x g for 15 sec at 20-25°C. The flow-through was discarded.

350 µl Buffer RW1 were added to the RNeasy spin column and centrifuged at 8.000 x g for 15 sec at 20-25°C to wash the membrane. The flow-through was discarded.

10 µl DNase I stock solution were added to 70 µl Buffer RDD, mixed by gently inverting the tube (DNase I is especially sensitive to physical denaturation), and spinned to collect residual liquid from the sides of the tube. The DNase I incubation mix (80 µl) was added directly to the RNeasy spin column membrane, and left at room temperature for 15 min.

350 µl Buffer RW1 were added to the RNeasy spin column and centrifuged at 8.000 x g for 15 sec at 20-25°C. The flow-through was discarded.
500 µl Buffer RPE were pipetted onto the RNeasy spin column and centrifuged at 8.000 x g for 15 sec at 20-25°C to wash the membrane. The flow-through was discarded. Other 500 µl Buffer RPE were added onto the RNeasy spin column and centrifuged at 8.000 x g for 2 min at 20-25°C to wash and to dry the RNeasy spin column membrane, ensuring that no ethanol was carried over during RNA elution. Residual ethanol might interfere with downstream reactions; for this reason, following centrifugation, the RNeasy Mini spin column was removed from the collection tube carefully in order not to get in contact with the flow-through.
The RNeasy Mini spin column was transferred into a new 1.5 ml collection tube. 30-50 µl RNase-free water were directly added to the RNeasy Mini spin column membrane and centrifuged at 8.000 x g for 1 min at 20–25°C to elute the RNA.
Eventually, extracted total RNA was stored at -80°C or analyzed by ND-1000 Spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA), a full-spectrum (220-750nm) spectrophotometer that measures 1 µl sample with high accuracy and reproducibility. It is based on a technology that employs surface tension alone to hold the sample in place. In addition, the ND-1000 has the capability to measure highly concentrated samples without dilution (50X higher concentration than the samples measured by a standard cuvette spectrophotometer). It is able to read the RNA concentration expressed in ng/µl using the nucleic acids and purified proteins wavelength ratio (260/280). In particular, the ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as “pure” for DNA; a ratio of ~2.0 is generally accepted as “pure” for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

5.6 TOTAL RNA EXTRACTION FROM MOUSE EMBRYONIC AND FETAL LIMB PRIMARY CULTURES

Total RNA was extracted from mouse embryonic and fetal limb primary cultures following the miRNeasy Mini Kit protocol instructions (Qiagen).
The miRNeasy Mini Kit is designed for purification of total RNA, including miRNA and other small RNA molecules, from cultured cells and various animal and human tissues. It combines phenol/guanidine-based lysis of samples and silica membrane-based purification of total RNA. QIAzol Lysis Reagent is a monophasic solution of phenol and guanidine thiocyanate, designed to facilitate cells and tissues lysis, to inhibit RNases, and also to remove most of the cellular DNA and proteins from the lysate by organic extraction.
To lyse cells directly, the cell-culture medium was discarded and cells were washed with PBS. 700 µl QIAzol Lysis Reagent were added directly to cells, and the lysate was collected with a scraper into a microcentrifuge tube and homogenized by vortexing for 1 min. The homogenate was left at 15-25°C for 5 min to promote dissociation of nucleoprotein complexes. Then, 140 µl chloroform were added to the homogenate, the tube was vortexed vigorously for 15 sec, left at room temperature for 2-3 min and then centrifuged for 15 min at 12.000 x g at 4°C. After centrifugation, the sample was separated into 3 phases: an upper, colorless, aqueous phase; a white interphase; and a lower, red, organic phase. RNA partitioned to the upper, aqueous phase, while DNA partitioned to the interphase and proteins to the lower, organic phase or the interphase.
The upper, aqueous phase was recovered and transferred into a new collection tube. 525 µl 100% ethanol were added and mixed throughly by pipetting up and down several times. The sample was then applied to the RNeasy Mini spin column (where the total RNA bound to the membrane, and phenol and other contaminants were efficiently washed away), and centrifuged at 8.000 x g for 15 sec at 15-25°C. The flow-through was discarded and 500 µl Buffer RPE were pipetted onto the RNeasy Mini spin column and centrifuged at 8.000 x g for 15 sec to wash the column. The flow-through was

discarded. Other 500 µl Buffer RPE were added onto the RNeasy Mini spin column and centrifuged at 8.000 x g for 2 min to dry the RNeasy Mini spin column membrane, ensuring that no ethanol was carried over during RNA elution.
The RNeasy Mini spin column was transferred into a new 1.5 ml collection tube. 30-50 µl RNase-free water were directly added onto the RNeasy Mini spin column membrane and centrifuged at 8.000 x g for 1 min at 20-25°C to elute the RNA.
Extracted total RNA was stored at -80°C or analyzed by NanoDrop.

5.7 RNA IMMUNOPRECIPITATION ASSAY

The C2C12 cells were grown in DMEM supplemented with 20% FBS (Growth medium, GM) and differentiated in DMEM supplemented with 2% HS (Differentiation Medium, DM). 900000 cells for each time of development are collected. The cells are treated with trypsin, the digestion is stopped with the addition of medium and the cells are spin at 400g for 5 minutes. The cell pellet is washed for two times with 10 ml of PBS and the cells are spin again.
The cell pelelt is resuspended in 10 ml of PBS and 270 µl of 37% formaldehyde (Sigma Aldrich). The cells are incubat for 30 minutes at 37°C on rocking platform. To stop cross-linking 1.4 ml of 2M sterile glycine are added, the cells are incubate 5minutes at 37°C; then they are spinned and the pelelt is washed for two times with PBS. The pellet is resuspende in 1ml of ice-cold 2x Cell Lysis buffer, containing 1x Protease inibitor coktail (20mM Na-PB pH7, 300mM NaCl, 5mM MgCl2, 4mM EDTA, 2mM DTT, 10 mg/ml Heparin, 10% glycerol, 2%Na-deoxycholat, 0,2% SDS, water). The cells are placed on ice for 1 hours, the cell lysate is spnned at 10000 g for 15 minutes at 4°C. The supernatant is collected. The samples are sonicated at 50% amplitude for 15 sec. in ice, to 1 ml cell lysate is added 100 µl of 10x Dnase buffer, 5 µl RNasin and 10 µl of RNase-free DNase. The lysate is incubated at 37°C for 15 minutes, then the 40 µl of 0,5 M EDTA are added to stop the reaction, and

the samples are centrifugated 5 minutes at 14000 rpm; the supernatant is collected. To cell lysate 5µg of antibody (a sample is used as negative control, using IgG) are added, thelysate I incuaed at 4°C O/N. 50 µl of washed Protein A/G are added, and the lysate is centrifugated at 10000g for 30 seconds at 4°C. The obtained pellet is washed 5 times with 500 µl of 1x Cell Lysis buffer. After the last wash the supernatant is aspirated, and 85µl of protein-RNA elution buffer (100mM Tris-HCl pH8, 10mM Na2-EDTA, 1% SDS, wathr) are added. The samples are incubated 10 inutes at 37°C: 20 µl of eluate are conserved for Western blotting analysis.
To 150 µl eluates are added 6 µl of 5 M NaCl and 20 µg proteinase K. Incubation at 42°C for 1hour to digest cross-linked polypeptides, and successive incubation at 65°C for 1 hour to reverse the formaldeyde cross-links.
At the sample 100 µl DEPC-H2O, 25 µl 3M Na-acetate and 20 µg glycogen are added.
The RNA is extracted with the method Phenol/ CHCl3 (1:1), 625 µl ethanol are added and the RNA is precipitated at -80°C O/N.
The extracted RNA is analyzed with RT-PCR assay.

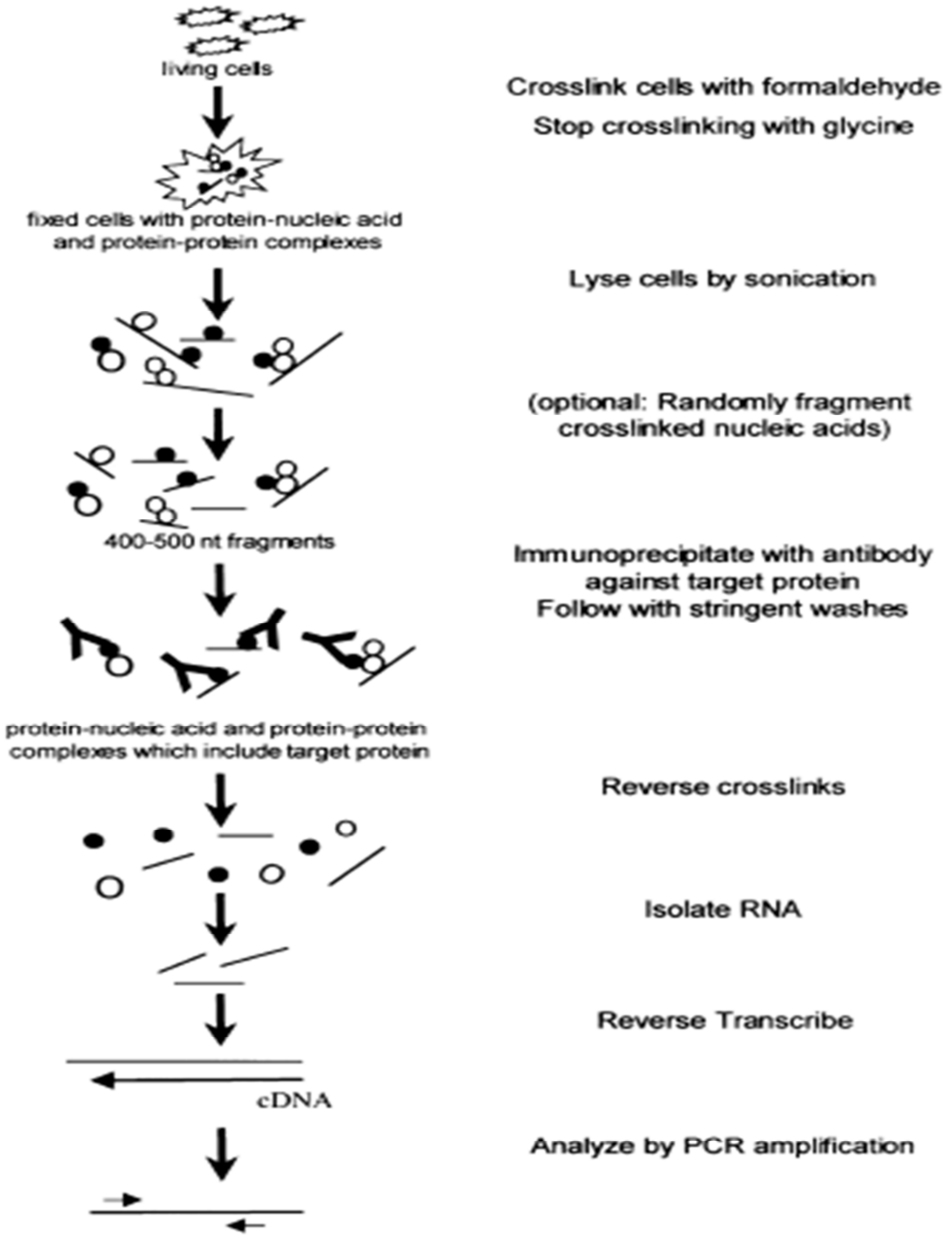


Figure 30**:** *Schematic representation of RNA-immunoprecipitation assay.*

5.8 RNA RETRO-TRANSCRIPTION

The iScriptTM complementary DNA (cDNA) Synthesis Kit (Bio-Rad Laboratories) was used for the reverse transcription of total RNA extracted from mouse limb muscles and embryonic/fetal primary cultures. This kit allows to obtain high-quality single-strand cDNA in a simple and efficient manner thanks to the presence of a single reaction mixture that reduces the reaction time, increases the experimental reproducibility and minimizes the cross contamination risk.
The cDNA synthesis requires the preparation of a mixture composed of 4 µl 5X iScriptTM Reaction Mix [oligo(dT) and random hexamer primers1, MgCl2, DTT, dNTPs, RNase inhibitor, buffer], 1 µl iScriptTM Reverse Transcriptase, 500 ng RNA sample, and Nuclease-free water up to a 20 µl final volume.
The mixture was placed in the thermocycler PTC-200 (Bio-Rad Laboratories) and submitted to following steps: 5 min at 25°C; 30 min at 42°C; 5 min at 85°C and finally held at 4°C.
The cDNA was stored at -20°C or analyzed by Real-Time quantitative Reverse Polymerase Chain Reaction (Real-Time qRT-PCR).

5.9 REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (REAL-TIME QRT-PCR)

The cDNA was analyzed by Real-Time qRT-PCR using the Opticon 2 real-time PCR cycler (Bio-Rad Laboratories) and the program Opticon 3.2 (Bio-Rad Laboratories).
The qRT-PCR is used to amplify and simultaneously quantify a targeted DNA molecule, enabling both detection as the reaction

[1] The presence of both oligo(dT) and random hexamer primers ensures the reverse transcription of all RNA sequences including those that do not have poly(A)-tails, and the generation of full-length cDNA, respectively. In fact, random hexamers bind along the entire length of the RNA, while oligo(dT) primers are designed to bind at the beginning of the poly(A)-tail; therefore they are not specific for mRNAs.

progresses in real time and quantification (as absolute number of copies or relative amount when it is normalized to additional normalizing genes). Two common methods used for products detection in real-time PCR are non-specific fluorescent dyes that intercalate with any double-stranded DNA, and sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which allows detection only after hybridization of the probe with its complementary DNA target.

In particular, Syber Green (Bio-Rad Laboratories) or TaqMan probes (Applied Biosystems, Foster City, CA, USA) were used for the sample cDNA amplification following manufacturer's instructions.

The reaction mixture for cDNA amplification using TaqMan probes was made up of 1 µl Taqman probe (Life Technologies, Carlsbad, CA, USA), 9 µl RNase-free water (Life Technologies), 10 µl SsoFastTM Probes Supermix (Bio-Rad Laboratories) and 1 µl cDNA.

The qRT-PCR steps were: 2 min at 95°C (activation), 40 cycles of 10 sec at 95°C (denaturation) and 20 sec at 60°C (annealing and elongation).

The reaction mixture for cDNA amplification using Syber Green was composed of 2.5 µl forward primer, 2.5 µl reverse primer, 10 µl SsoFastTM EvaGreen Supermix, 4 µl RNase-free water (Life Technologies) and 1 µl cDNA.

The qRT-PCR steps were: 2 min at 95°C (activation), 40 cycles of 10 sec at 95°C (denaturation) and 20 sec at the pre-established annealing temperature (annealing and elongation).

Primers of GAPDH and T1, T2a, H, L, D3 cyclins (Table 2) were designed on the base of gene sequences available on the NCBI website (www.ncbi.nlm.nih.gov). The remaining sequences are custom TaqMan Probes pre-designed by Applied Biosystems.

The cDNA levels were normalized to those of the housekeeping GAPDH gene and calculated relatively to controls by the 2-ΔΔCt method (Live and Schmittgen, 2001).

GAPDH: 5'-TCGGAGTCAACGGATTTG-3'
5'-GGATCTCGCTCCTGGAAG-3'

CYCLIN T1: 5'-GAATGAGAGTGCTTGTGTGAG-3'
5'-AAACCAGAGGAGATAAAAATG-3'

CYCLIN T2a: 5'-GACATGTCCGTAGCCCACCTG-3'
5'-GCATCCTCGGAAAGACACAGC-3'

CYCLIN H: 5'-CCTCGGATAATAATGCTTAC-3'
5'-TCATAGCCTTTCCTCTTC-3'

CYCLIN L: 5'-CGAGATTGTTGCTATGGC-3'
5'-CACTGGCTTCTCCTTGGG-3'

CYCLIN D3: 5'-CAGCGTGTCCTGCAGAGTT-3' For
5'-CCTTTTGCACGCACTGGAA-3' Rev

7SKsnRNA: 5'- ATCTGTCACCCCATTGATCG-3'
5'- GCGCAGCTACTCGTATACCC-3'

CDK9-55 Sonda Taqman (Applied Byosistems): CDK9M55KDA-ANY 445370 20X MIX

CDK9-42 Sonda Taqman (Applied Byosistem) : CDK9M42KDA-ANY 445370 20X MIX

The statistical test calculates the p-value of every detector compared in the analysis.
Comparison of group was performed using ANOVA test. The control group was compared to the individual experimental group; differences were considered significant at $P<0,05$.
Results were analyzed using GraphPad PRISM (version 5,00, March 2007).

5.10 PROTEIN EXTRACTION

For protein extraction, limb muscle tissues and embryonic/fetal primary cultures pellets were washed in PBS and then lysed in RIPA BUFFER [50 mM Tris-HCl pH 7.5, 50 mM EDTA pH 8, 150 mM NaCl, 1% (v/v) Nonidet P-40] supplemented with protease inhibitors (0.3 mM PMSF and Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets; Roche, Basel, Switzerland) and phosphatase inhibitors (2 mM NaOV, 10 mM NaF). Lysates were transferred into new 1.5/2 ml microcentrifuge tubes, left to incubate on ice for at least 30 min and then centrifuged at 12.000 x g for 20 min at 4°C. The surnatant containing the protein extract was transferred into a new eppendorf and stored at -80°C or submitted to Bradford protein assay.

5.11 DETERMINATION OF PROTEIN CONCENTRATION

The Bradford protein assay (Bio-Rad Laboratories) was used to determine the extracts protein concentration. It is a colorimetric assay based on the proportional binding of the Coomassie Brilliant Blue G-250 dye to proteins (Bradford, 1976). Coomassie absorbs at 465 nm, but this value shifts to 595 nm when it binds to proteins. The absorbance increase at 595 nm is proportional to the amount of bound dye and, thus, to the concentration of proteins present in the sample.

The protein concentration was determined using a standard curve in which the increasing concentration values of the BSA (bovin serum albumine) standard protein solutions were reported in x-axis, whereas the corresponding absorbance values in y-axis. By calculating the BSA absorbance in concentration-known solutions, it was possible to determine the samples protein concentration.

Based on the results, a relative normalization was made versus the sample with the lowest protein concentration, in order to use the same amount of total proteins in the following protein analysis.

5.12 POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The protein separation was performed in polyacrylamide gels following Laemmli instructions (Laemmli, 1970), using a Mini Protean 3 Cell (Bio-Rad Laboratories). The gels were made at an acrylamide/bis-acrylamide concentration of 10% for the separation gel, and 4% for the stacking gel, whose composition is reported below:

COMPONENTS OF 10% SEPARATION GEL	VOLUM
H2O	4 ml
1.5 M Tris-HCl pH 8.8	2.5 ml
30% (w/v) Acr/Bis	3.3 ml
10% (w/v) SDS (Sodium Dodecyl Sulfate)	0.1 ml
10% (w/v) APS (Ammonium Per-Sulfate)	0.1 ml
TEMED (N,N,N′,N′-Tetramethylethylenediamine) stock	0.01 ml

COMPONENTS OF 4% STACKING GEL	VOLUM
H2O	3.4 ml
1.5 M Tris-HCl pH 6.8	0.63 ml
30% (w/v) Acr/Bis	0.83 ml
10% (w/v) SDS (Sodium Dodecyl Sulfate)	0.05 ml
10% (w/v) APS (Ammonium Per-Sulfate)	0.05 ml
TEMED (N,N,N′,N′-Tetramethylethylenediamine) stock	0.005 ml

In order to denature proteins to promote their separation in gels, loading buffer (LB) [50 mM Tris-HCl pH 6.8, 10% (w/v) glycerol, 0.5% (w/v) SDS, 0.025% (w/v) bromophenol blue, 10% (v/v) β-mercaptoethanol] was added to protein extracts. Molecular weight standards from 12 to 225 kDa (Precision Plus ProteinTM All Blueb Standards, Bio-Rad Laboratories) and protein extracts were then boiled for 5 min at 95°C, transferred on ice, and finally loaded on the polyacrylamide gel. In particular, equal amounts of protein extracts (25-50 μg) for each sample were loaded onto the gel and separated at 90V in running buffer [25 mM Tris-Base, 192 mM glycine, 0.1% (w/v) SDS]. At the end, they were transferred onto a PVDF membrane (Bio-Rad Laboratories) as described by Towbin et al., 1979. The electrotransfer was perfomed in transfer buffer [25 mM Tris-Base, 192 mM glycine, 20% (v/v) methanol] at 20V/60mA overnight at 4°C, using the Bio-Rad Transblot system. In order to confirm the protein transfer from the polyacrylamide gel to the PVDF membrane, proteins were reversibly stained with Ponceau Red, which was subsequently washed away with TBST [0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.1% Tween 20 pH 7.4].

5.13 IMMUNO-BLOTTING

To block non-specific binding sites, the PVDF membrane was incubated with 5% non-fat powdered dry milk in TBST for 1h at room temperature. The membrane was then incubated with primary antibody diluted in 5% non-fat powdered dry milk in TBST

overnight at 4°C (Table 3). It was then washed three times for 10 min in TBST and incubated with secondary antibody conjugated with peroxidase diluted in 1% non-fat powdered dry milk in TBST for 1h at room temperature. Primary antibody: CDK9 (1:200; Santa Cruz, CA), MyoD (1:100; 5.8A, BD), Myogenin (1:100; F5D, Dako), MHC (1:20; MF20, Fishman), Desmin (1:100; Sigma), Embyonic myosin (1:100; BF-G6, Schiaffino, 1989), Fast myosin (1:300; Sigma), Slow myosin (1:300; Sigma), Pax3 (1:100; Hybridoma Bank), Pax7 (1:100; Hybridoma Bank), Cyclin T1 (1:100; Santa Cruz, CA), Hexim 1, Tubuline(1:250; Sigma), β-Actine (1:300; Sigma). Three more washings in TTBS were performed and the membrane was then incubated with the peroxidase substrate (SuperSignal® West Pico Chemiluminescent Substrate; Thermo Scientific, Rockford, USA), whose chemiluminescent product allowed the detection of proteins bound by the antibody.

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