Involvement of cdks and cyclins in muscle differentiation

M. De Falco,[1] A. De Luca[2]

[1]Department of Biological Sciences, Section of Evolutionary and Comparative Biology, University of Naples "Federico II"; [2]Department of Medicine and Public Health, Section of Clinical Anatomy, Second University of Naples, Italy

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Myocyte differentiation is due to transcription of genes that characterize the phenotypic and biochemical identity of differentiated muscle cells. These are the myogenic regulatory factors (MRFs) MyoD, Myf5, myogenin and MRF4. Overexpression of cdk/cyclins has been reported to inhibit the activity of MyoD and prevent myogenic differentiation by different modalities. Unlike other cdk/cyclin complexes, overexpression of cdk9/cyclin T2a, enhances MyoD function and promotes myogenic differentiation. In addition, cyclin T2a interacting with a novel partner, PKNα, is able to strongly enhance the expression of myogenic differentiation markers, such as myogenin and Myosin Heavy Chain. So, cyclin T2a could stimulate myogenic differentiation interacting with different kinase partners Cdk9 or PKNα in a synergistic or antagonistic way.

Key words: cell cycle, muscle differentiation, cyclin T2, PKN.

Correspondence: Antonio De Luca,
Department of Medicine and Public Health,
Section of Clinical Anatomy, Second University of Naples,
Via L. Armanni 5, 80138 Naples, Italy.
Fax: +39.081.458225
E-mail: antonio.deluca@unina2.it

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The cell cycle is formed by a number of different complex biochemical pathways ensuring that the start of a particular event depends upon the successful and right end of previous steps in the pathway (De Luca *et al.,* 2003). During the cell-division cycle, an important role is played by cyclins, a family of proteins named for their cyclical expression and degradation. These proteins are synthesized immediately before being used, and their levels fall abruptly after their action because of degradation through ubiquitination (King *et al.,* 1996). Cyclins act as regulatory subunits of complexes together with a family of related protein kinases called cyclin-dependent kinases (CDKs) that function as catalytic subunits. Interaction between the cyclins and the CDKs occurs at specific stages of the cell cycle, and the progression through the cell cycle requires their activities. Unlike the cyclins, the protein levels of the CDKs do not oscillate throughout the cell cycle, suggesting the cyclins are an important regulative element of the complexes. In fact CDKs are often present in an inactive form until the synthesis of the cyclins (De Falco and Giordano 1998). Intriguingly, CDK/cyclin complexes not only regulate cell division by phosphorylating different substrates, but are able also to control several cellular pathways such as signal transduction, differentiation and apoptosis (De Luca *et al.,* 2003). Among these, some CDKs/cyclin couples, such as CDK7/cyclin H, CDK8/cyclin C, and CDK9/cyclin T, seem to direct their activity in a cell cycle independent manner and appear to be involved in transcription during the initiation or the elongation steps (Dynlacht 1997). In particular, the Cdk9-cyclin T complex is known as P-TEFb and was originally identified as a positive transcription elongation factor in Drosophila transcription extracts (Marshall and Price 1995;

Herrmann and Mancini 2001). Peng *et al.,* (1998) and Wei *et al.,* (1998) showed that human P-TEF-b contains multiple cyclin subunits: cyclin T1 and T2. Cyclin T contains an amino terminal box motif that is highly conserved from Drosophila to human (De Luca *et al.,* 2003). The "cyclin homology box", formed by 290 amino acids, is the most conserved region among different members of the cyclin-family and serves to bind CDK9. More precisely, the region of cyclinT1 from amino acid 1 to 188 is necessary and sufficient to interact with CDK9 *in vivo*. Cyclin T2 has two forms termed T2a and T2b, that likely arise by an alternative splicing of the primary transcript, a process commonly used to produce variety in other cyclins. Cyclins T2a and T2b share the first 642 amino acids but have different carboxyl termini. All three cyclins T are expressed in a wide variety of human tissues and are found complexed with CDK9 in HeLa nuclear extracts. Cyclin T1 is not a typical cell cycle regulatory since its levels do not oscillate at any phase during the cell cycle (De Luca *et al.,* 2003). Immunohistochemical analysis of cyclin T2a expression in a large human tissue bank shows that cyclin T2a is widely expressed in all cell types, even if higher levels are found in some terminally differentiated tissues such as muscle, blood, lymphoid tissue, and connective cells (De Luca *et al.,* 2001a). This expression pattern is very similar to that described above for cyclin T1 (De Luca *et al.,* 2001b) and in agreement with mRNA distribution for cyclin T2a (Peng *et al.,* 1998). Differences between CDK9/cyclin T1-T2 complexes and the other CDKs/cyclin complexes involved in regulation of the cell cycle may reflect a different function during terminal differentiation in muscle cells. The induction of myocyte differentiation on partial serum withdrawal, as occurs with C2C7 cells, is a good *in vitro* system in which to look at the CDK9/cyclin T1-T2 functions during terminal differentiation since it has been investigated the cdk9 acitivy in these cells (Bagella *et al.,* 1998). Moreover, cyclin T2a protein level is high in human adult skeletal muscle cells (De Luca *et al.,* 2001b). The high immunohistochemical expression level of cyclin T2a in adult muscle cells (De Luca *et al.,* 2001b), as well as the peak of expression reached during *in vitro* muscle differentiation, suggests that CDK9/cyclin T2a complex might promote myogenic differentiation eliciting the expression of muscle-specific genes (Simone *et al.,* 2002a). Human cyclin T2a gene maps on chromosome locus 2q21 (De Luca *et al.,* 2001b), a locus involved in different forms of autosomal dominant myopathies (Nicolao *et al.,* 1999; Pelin *et al.,* 1999; Xiang *et al.,* 1999). Interestingly, the immunohistochemical expression of cyclin T2 is undetectable in the skeletal muscle cells from two patients with centronuclear myopathy, in contrast with its very high expression in normal skeletal muscle tissue. These findings could indicate an involvement of the CDK9/cyclin T2a complex in the centronuclear myopathy (De Luca *et al.,* 2001b). Skeletal muscle formation during development is a multistep process that involves the determination of multipotential, mesodermal cells to give rise to myoblasts, withdrawal of the myoblasts from the cell cycle, and differentiation into muscle fibers (Stockdale 1992; Miller *et al.,* 1999; Shen *et al.,* 2003). These processes are controlled by muscle-specific transcriptional regulators that determine cell fate and differentiation and by external signals that couple myogenesis to development and growth of the organism (Miller *et al.,* 1999; Bailey *et al.,* 2001; McKinsey *et al.,* 2001). At the molecular level, myogenic determination and muscle-specific gene expression involve myogenic regulatory factors (MRFs) as well as muscle-specific helix-loop-helix factors (MyoD family) and the MEF2 family of MADS-box myocyte enhancer-binding factors (Lassar *et al.,* 1994; Molkentin and Olson 1996). MRFs include MyoD, Myf5, myogenin, and MRF4. Expression of MRFs in several nonmyogenic cell lines is sufficient to induce myogenic differentiation (Davis *et al.,* 1987; Wright *et al.,* 1989; Braun et al. 1989). In last years, it has been hypothesized that myogenic factors can be subdivided into primary and secondary MRFs (Ishibashi *et al.,* 2005) and it has been demonstrated that in the course of muscle differentiation *in vivo*, MRFs are expressed in a defined sequence (Figure 1): MyoD and Myf-5 are expressed in proliferating, undifferentiated cells, whereas myogenin expression is only induced upon muscle differentiation (Smith *et al.,* 1993, 1994). Particularly, both MyoD and Myf 5 are active transciption factors in proliferating myoblasts (Wyzykowski *et al.,* 2002), but whereas whereas MyoD levels peak at the differentiation checkpoint in G1 of the cell cycle, Myf5 levels are high in S/G2 and G0 in association with

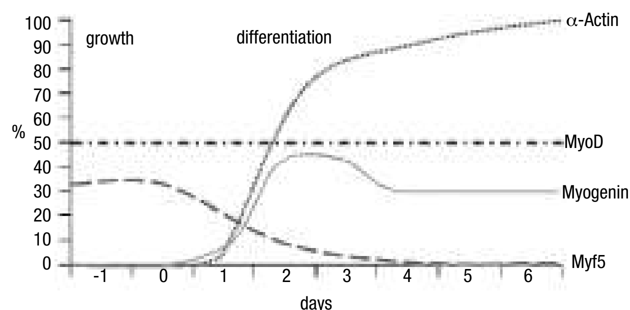

Figure 1. Expression of myogenic regulatory factors (MRFs) during cell growth and differentiation.

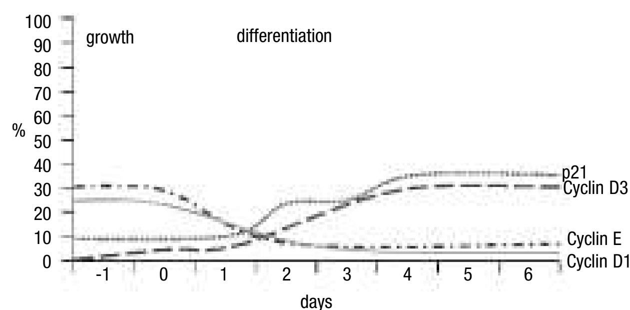

Figure 2. Differential expression of some cell cycle-related proteins during cell growth and differention.

proliferation and a failure to differentiate (Kitzmann *et al.,* 1998). These data strongly reinforce the concept of a role for Myf5 in myoblast proliferation versus MyoD instigating myogenic differentiation (Ishibashi *et al.,* 2005). MRF4 is unique since it is expressed both in early stages of myogenesis, and later during muscle development and in adult muscle tissue (Hinterberger *et al.,* 1991; Shen *et al.,* 2003). MRFs operate by heterodimerizing with the E2A gene products (E12 and E47) via the HLH domain and by binding to certain recognition sites in the regulatory regions of muscle-specific genes (Murre *et al.,* 1989; Lassar *et al.,* 1991; Weintraub *et al.,* 1991). Full-lenght cyclin T2a and N-terminal region of CDK9, interacting with bHLH domain of MyoD, allow the formation of a complex able to stimulate the transcription of specific muscle genes (Simone *et al.,* 2002a). In this complex, cyclin T2a interacts physically with MyoD that is phosphorylated by CDK9. CDK9/cyclin T2a-MyoD complex has several positive and negative regulating signals (Simone and Giordano 2001). Therefore, CK9/cyclin T2a binds and phosphorilates the C-terminus of pRb (Simone *et al.,* 2002b), an essential cofactor during muscle differentiation. At this stage, pRb is present in the active hypophosphorylated form, especially due to downregulation of cyclins A, E and D1 and the upregulation of cdk inhibitors (Puri and Sartorelli 2000) (Figure 2). It is possible that CDK9/CycT2 kinase activity is involved in the basal phosphorylation of the retinoblastoma protein and that pRb and CDK9/CycT2 cooperate to support MyoD-mediated myogenic transcription (Simone and Giordano 2001). In the last years several studies seem to confirm that whereas cyclin T1 has CDK9 as the most important intra-cellular partner, cyclin T2a could have one or more "Cdk9-like" partners (Cottone *et al.,* 2005). In order to demonstrate this hypothesis, we performed a two-hybrid screening in yeast using the full-length human cyclin T2a as bait, and a human heart cDNA library as a prey source (Cottone *et al.,* 2005). Upon several interesting genes selected for interaction, our attention has been focused on the one coding for PKNα, a fatty acid- and Rho-activated serine/threonine protein kinase, having a catalytic domain homologous to protein kinase C family (Mukai 2003). Several functions of this kinase are thought to be involved in cytoskeleton rearrangement, because many PKNα targets are structural or regulative components of the micro-filaments-microtubules-intermediate filaments network; however some published experimental data also suggest a nuclear role for this kinase. In fact, it has been shown that PKNα is translocated from cytoplasm to nucleus of *in vitro* cultured cells under stress conditions such as heat shock or serum starvation (Mukai *et al.,* 1996), and that this kinase can interact with a neuron-specific basic Helix-Loop-Helix transcription factor (NDRF/NeuroD2) (Shibata *et al.,* 1999). Moreover, PKNα is able to regulate ANF gene transcription in cardiomyocytes through a serum response element (Morissette *et al.,* 2000). In addition, it has been demonstrated that PKN is able to interact with skeletal muscle α-actinin (Mukai *et al.,* 1997), which is the major component of the Z-band in myofibrils. PKN phosphorylates α-actinin to maintain the integrity of sarcomere structure in skeletal muscle. Mutations in the PKN protein could therefore disturb the dynamics of sarcomeric proteins in skeletal muscle (Bartsch *et al.,* 1998). In particular, we have shown that the Ser/Thr kinase domain sub-region

of PKN, spanning from aa 782 to 873 was sufficient, but not necessary, for a weak interaction with cyclin T2a *in vitro* (Cottone *et al.,* 2005). This suggests that different portions of the full-length PKN protein may be somehow involved in the binding to cyclin T2a, or more probably that the whole serine/threonine kinase domain is required for the interaction *in vivo*. Moreover, by luciferase assays performed using a MyoD-responsive promoter, we pointed out that PKNα alone enhanced MyoD-mediated transcriptional activity, and that cyclin T2a co-overexpression caused a further increase in luciferase-reporter expression. Finally, we have shown that in C2C12 cells the overexpression of both cyclin T2a and PKNα increased and anticipated the expression of myogenic differentiation markers like myogenin and MHC during starvation-induced differentiation. In confirmation of our studies, it has been demonstrated that PKNα is able to regulate the gene transcription of the atrial natriuretic factor (ANF) gene, one of the few currently studied hypertrophic marker (Morisette *et al.,* 2000; Cottone *et al.,* 2005). These results suggest that cyclin T2a could strengthen MyoD-dependent transcription and stimulate myogenic differentiation having as kinase partner either Cdk9 or PKNα that could act in synergism or antagonism (Cottone *et al.,* 2005).

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References

Bagella L, MacLachlan TK, Buono RJ, Pisano MM, Giordano A, De Luca A. Cloning of murine CDK9/PITALE and its tissue-specific expression in development. J Cell Physiol 1998; 177:206-13.

Bailey P, Holowacz T, Lassar AB. The origin of skeletal muscle stem cells in the embryo and the adult. Curr Opin Cell Biol 2001; 13:679-89.

Bartsch JW, Mukai H, Takahashi N, Ronsiek M, Fuchs S, Jockusch H, Ono Y The Protein Kinase N (PKN) gene PRKCL1/Prkcl1 maps to human chromosome 19p12–p13.1 and mouse chromosome 8 with close linkage to the myodystrophy (myd) mutation. GENOMICS 1998; 49:129-32.

Braun T, Buschhausen-Denker G, Bober E, Tannich E, Arnold HH. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. EMBO J 1989; 8:701-9.

Cottone G, Baldi A, Palescandolo E, Manente L, Penta R, Paggi MG, De Luca A. PKN is a novel partner of cyclin T2a in muscle differentiation. J Cell Physiol 2005 in press.

Davis RL, Weintraub H, Lassar AB. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 1987; 51: 987-1000.

De Falco G, Giordano A. CDK9 (PITALRE): A multifunctional cdc-2 related kinase. J Cell Physiol 1998; 177:501-6.

De Luca A, Russo P, Severino A, Baldi A, Battista T, Cavallotti I, et al. Pattern of expression of cyclin T1 in human tissues. J Histochem Cytochem 2001a; 49:685-92.

De Luca A, Tosolini A, Russo P, Severino A, Baldi A, De Luca L, et al. Cyclin T2a gene maps on human chromosome 2q21. J Histochem Cytochem 2001b; 49:693-98.

De Luca A, De Falco M, Baldi A, Paggi MG. Cyclin T. Three forms for different roles in physiological and pathological functions. J Cell Physiol 2003; 194:101-7.

Dynlacht BD. Regulation of transcription by protein that control the cell cycle. Nature 1997; 389:149-52.

Hermann CH, Mancini MA. The Cdk9 and cyclin T subunits of TAK/P-TEFb localize to splicing factor-rich nuclear speckle regions. J Cell Sci 2001;114:1491-503.

Hinterberger TJ, Sassoon DA, Rhodes SJ, Konieczny SF. Expression of the muscle regulatory factor MRF4 during somite and skeletal myofiber development. Dev Biol 1991; 147:144-56.

King RW, Deshaies RJ, Peters JM, Kirschner MW. How proteolisis drives the cell cycle. Science 1996; 274:1652-8.

Kitzmann M, Carnac G, Vandromme M, Primig M, Lamb NJ, Fernandez A. The muscle regulatory factors MyoD and myf-5 undergo distinct cell cycle-specific expression in muscle cells. J Cell Biol 1998; 142:1447-59.

Ishibashi J, Perry RL, Asakura A, Rudnicki MA. MyoD induces myogenic differentiation through cooperation of its NH2- and COOH-terminal regions. J Cell Biol 2005; 171:471-82.

Lassar AB, Davis RL, Wright WE, Kadesch T, Murre C, Voronova A, Baltimore D, Weintraub H. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins *in vivo*. Cell 1991;66:305-15.

Lassar AB, Skapek SX, Novitch B. Regulatory mechanisms that coordinate skeletal muscle differentiation and cell cycle withdrawal. Curr Opin Cell Biol 1994; 6:788-94.

Marshall NF, Price DH. Purification of P-TEFb, a transcription factor required for the transition into productive elongation. J Biol Chem 1995; 270:12335-8.

McKinsey TA, Zhang CL, Olson EN. Control of muscle development by duelling HATs and HDACs. Curr Opin Genet Dev 2001; 11:497-504.

Miller JB, Schaefer L, Dominov JA. Seeking muscle stem cells. Curr Top Dev Biol 1999; 43:191-219.

Molkentin JD, Olson EN. Combinatorial control of muscle development by basic helix-loop-helix and MADSbox transcription factors. Proc Natl Acad Sci U S A 1996; 93:9366-73.

Morissette MR, Sah VP, Glembotski CC, Brown JH. The Rho effector, PKN, regulates ANF gene transcription in cardiomyocytes through a serum response element. Am J Physiol Heart Circ Physiol 2000; 278:H1769-74.

Mukai H, Miyahara M, Sunakawa H, Shibata H, Toshimori M, Kitagawa M, et al. Translocation of PKN from the cytosol to the nucleus induced by stresses. Proc Nat Acad Sci USA 1996; 93:10195-9.

Mukai H, Toshimori M, Shibata H, Takanaga H, Kitagawa M, Miyahara M, et al. Interaction of PKN with alpha-actinin. J Biol Chem 1997; 272:4740-6.

Mukai H. The structure and function of PKN, a protein kinase Having a catalytic homologous to that of PKC. J Biochem 2003; 133:17-27.

Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, et al. Interaction between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 1989; 58:537-44.

Nicolao P, Xiang F, Gunnarsson LG, Giometto B, Edstrom L, Anvret M, Zhang Z. Autosomal dominant myopathy with proximal weakness and early respiratory muscle involvement maps to chromosome 2q. Am J Hum Genet 1999; 64:788-92.

Pelin K, Hilpela P, Donner K, Sewry C, Akkari PA, Wilton SD, et al. Mutations in the nebulin gene associated with autosomal recessive nemaline myopathy. Proc Natl Acad Sci USA 1999; 96:2305-10.

Peng J, Zhu Y, Milton JT, Price DH. Identification of multiple cyclin

subunits of human P-TEFb. Genes Dev 1998; 12:755-62.

Puri PL, Santorelli V. Regulation of muscle regulatory factors by DNA-binding, interacting proteins, and post-transcriptional modifications. J Cell Physiol 2000; 185:155-73.

Shen X, Collier JM, Hlaing M, Zhang L, Delshad EH, Bristow J, Bernstein HS. Genome-wide examination of myoblast cell cycle withdrawal during differentiation. Dev Dynam 2003; 226:128-38.

Shibata H, Oda H, Mukai H, Oishi K, Misaki K, Ohkubo H, Ono Y. Interaction of PKN with a neuron-specific basic Helix-Loop-Helix transcription factor, NDRF/NeuroD2. Mol Brain Res 1999; 74: 126-34.

Simone C, Giordano A. New insighy in CDK9 function: from Tat to MyoD. Front Biosci 2001; 6:d1073-82.

Simone C, Stiegler P, Bagella L, Pucci B, Bellan C, De Falco G, et al. Activation of MyoD-dependent transcription by cdk9/cyclin T2. Oncogene 2002a; 21:4137-48.

Simone C, Bagella L, Bellan C, Giordano A. Physical interaction between pRb and cdk9/cyclin T2 complex. Oncogene 2002b; 21:4158-65.

Smith TH, Block NE, Rhodes SJ, Konieczny SF, Miller JB. A unique pattern of expression of the four muscle regulatory factor proteins distinguishes somitic from embryonic, fetal and newborn mouse myogenic cells. Development 1993; 117:1125–33.

Smith CK Jr, Janney MJ, Allen RE. Temporal expression of myogenic regulatory genes during activation, proliferation, and differentiation of rat skeletal muscle satellite cells. J Cell Physiol 1994; 159:379–85.

Stockdale FE. Myogenic cell lineages. Dev Biol 1992; 154:284–98.

Wei P, Garber ME, Fang S-M, Fischer WH, Jones KA. A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. Cell 1998; 92:451-62.

Weintraub H, Davis R, Tapscott S, Thayer M, Krause M, Benezra R, et al. The myoD gene family: nodal point during specification of the muscle cell lineage. Science 1991; 251:761-6.

Wyzykowski JC, Winata TI, Mitin N, Taparowsky EJ, Konieczny SF. Identification of novel MyoD gene targets in proliferating myogenic stem cells. Mol Cell Biol 2002; 22:6199–208.

Wright WE, Sassoon DA, Lin VK. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. Cell 1989; 56: 607–17.

Xiang F, Nicolao P, Chapon F, Edstrom L, Anvret M, Zhang Z. A second locus for autosomal dominant myopathy with proximal muscle weakness and early respiratory muscle involvement: a likely chromosomal locus on 2q21. Neuromusc Disord 1999; 9:308-12.