



Research Article

The master regulator gene *PRDM2* controls C2C12 myoblasts proliferation and Differentiation switch and *PRDM4* and *PRDM10* expression

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Abstract

The Positive Regulatory Domain (PRDM) protein family gene is involved in a spectrum variety of biological processes, including proliferation, differentiation and apoptosis: its member seem to be transcriptional regulators highly cell type and tissue peculiar, towards histones modifications or recruitment of specific interaction patters to modify the expression of target genes. In this study we analyzed the expression profile of different member of PRDM gene family focusing our attention on the role of *PRDM2*, *PRDM4* and *PRDM10* genes in mouse C2C12 cell line, during the differentiation of myoblasts into myotubes and speculate about the role of the protein Retinoblastoma protein-interacting zinc finger protein 1-RIZ1, coded by *PRDM2* gene, as a regulator of the proliferation/differentiation switch.

Results showed a reduction of *PRDM2*, *PRDM4* and *PRDM10* expression level during the commitment of the differentiation of myoblasts into myotubes. The RIZ1 silencing stimulated myoblasts differentiation, similar to the effect of serum deprivation on these cells, associated with an increase of Myogenin expression level, which is considered to be involved in the differentiation of myoblasts into multinucleated myotubes. As demonstrated by chromatin immunoprecipitation experiments, RIZ1 is associated with Myogenin promoter in proliferation condition and after 24h from differentiation induction, negatively controlling therefore Myogenin expression. Moreover RIZ1 silencing induced a reduction in *PRDM4* and *PRDM10* expression levels leaving us to speculate that the PRDM genes have a redundant role and they are hierarchically organized.

Introduction

The myogenesis of mammalian skeletal muscle cells is regulated by myogenic regulatory factors (MRFs), related in a hierarchical relationship [1], which are considered to be members of a superfamily of the highly conserved variant of the basic helix-loop-helix (bHLH) domain [2], that confers their peculiar myogenic potential [3]. The phylogenetic analysis of the sequences of these genes indicates that MRF genes have evolved from a single ancestral MRF gene progenitor, by gene duplication events followed by divergent mutations [4]. The four main basic helix-loop-helix myogenic regulatory factors, which exhibit a pivotal role in skeletal muscle development and which are responsible for coordinating muscle-specific gene expression in the developing embryo [5], are MyoD (Myf-3) [3], which coordinates an open chromatin structure at muscle-specific genes [1]; Myf-5 [6], which is required for the specification and proliferation of myoblasts [7-10], enhances myogenesis by promoting myoblast proliferation [11-13], because it is also the earliest to be expressed during myogenesis process, acting as a transcription factor in muscle progenitor cells (satellite cells) and myocytes [14]; Myogenin (Myf-1) [15], which is involved in the differentiation of myoblasts into multinucleated myotubes [16] and MRF4 [17], which is implicated



in the latest phase of differentiation that includes myofiber maintenance [18]. The Myogenic regulatory factor (MRF) genes are expressed with a peculiar pattern [19,20] and they can auto- and cross-regulate the expression of each other and interact with the myocyte enhancer factor-2 (MEF2) family of transcription factors, to activate the transcription of muscle-specific genes [21]: currently, these MEF2 transcription factors are considered to drive the development of muscle, cardiac, skeletal, vascular, neural and blood cells, because of their pivotal effects on cell differentiation, proliferation, apoptosis, migration, shape and metabolism [22].

The activity of MRF proteins require heterodimerization with a member of the ubiquitously expressed E-protein family of bHLH proteins. This event leads the binding to the regulatory regions of muscle-specific genes on the E-box consensus sequence (CANNTG) [5]. Currently, only a small fraction of 14 million potential sites are available for MRF binding [23,24]. MyoD, the master regulator of the skeletal muscle gene expression program, activates genes which display the consensus E-box sequence VCASCTG (where V is A, C or G while S is C or G) within their promoter/enhancer regions [23,25-27] and it leads a gene expression program by heterodimerizations with E-proteins and giving rise to multinucleated myotubes [28]. The binding of MyoD on the E-box sequence (CANNTG) and the recruitment of factors, which are involved in remodelling the chromatin, are considered the crucial event for transcription [29,30] because MyoD activates pRb and p21 gene expression [31,32] to shoot down the cell cycle machinery [29]. The skeletal muscle differentiation is a strongly coupled event to the cell cycle exit [33] by an upregulation of cyclin-dependent kinase inhibitors (CDKIs), which inhibit cyclin-CDK complexes driving a downregulation of the activity of cyclin D1,E,A, and B-CDK complexes [34] and by an induction of the permanent cell cycle exit. This is a pivotal step because of the overexpression of cyclin/CDKs has been reported to inhibit the activity of MyoD by different mechanisms [29]. In particular, p21cip1/waf1, and p57/kip2, encoded respectively by *Cdkn1a* and *Cdkn1c* genes, control differentiation of skeletal muscle and their loss affects fiber formation: they have a key role both for cell cycle exit, both in triggering a muscle-specific transcriptional program [35]. The PRDM (Positive Regulatory Domain) gene family which consists of 17 orthologs in primates and 16 orthologs in rodents, birds and amphibians [36], encodes transcription factors with a PR domain and a variable number of zinc finger motifs [37], with the exception of PRDM11 [36,38]. The PR domain displays a 20-30% amino acid homology sequence to the catalytic SET (*Suvar3-9*, *Enhancer-of-zeste*, *Trithorax*) domain with histone lysine methyltransferase (HMTs) activity [39]. In contrast to the SET domain proteins, currently only three PRDM proteins have been demonstrated to possess intrinsic HMTase activity [40] and a number of PRDMs have not endowed with catalytic activity towards histones/nucleosomes [41-44]. In particular the HMT's activity has been found only in the PR domains of PRDM2/RIZ1, Prdm8 and Prdm9 [45-47]. In fact, the PR domain has diverged significantly from the SET domain [48-51] and most PR domains lack the H/RxxNHxC motif required for methyltransferase activity [52,53]. Generally, the PR domain is localized at the N-terminus of the protein, whereas the SET domain is often localized to the C-terminus [39]. A common characteristic of PRDM genes is the expression of molecular variants by alternative splicing or by alternative use of promoters. The *PRDM1*, *PRDM2* and *PRDM3*, which is also called *MECOM* (*MDS1-EVI1 complex locus*) genes are expressed as two alternative forms, by intergenic splicing, which produce the PR *plus* and PR *minus* forms of these genes [54-57]. PR *plus* and PR *minus* forms are expressed at equimolar concentration and their ratio is maintained in a fine equilibrium [58]: an imbalance in the amounts of the two products, through either disruption or underexpression of the PR *plus* form or overexpression of the PR *minus* form commonly occurs in human cancers through genetic and epigenetic mechanisms [59-66]. *PRDM2* gene gives two alternative products: RIZ1, the PR *plus* form, implicated in tumor suppressor function, and RIZ2, the PR *minus* form. This PR domain (PRDI-BF1 and RIZ homologous) [67] which is endowed with histone H3 K9 methyltransferase activity, is targeted by inactivating mutations in human cancers [68].



PRDM proteins mediate transcriptional activation or repression depending on the nature of their intrinsic HMT activity: PRDM proteins appear to function by modulating gene expression states either directly (via intrinsic HMTase activity), or indirectly (via recruitment of various cofactors), controlling critical aspects of cell integrity, spanning from cell differentiation to cell growth and apoptosis [58]. These genes also play a role in human cancer, where they mainly act as tumor suppressors: for example, PRDM1 is a tumor suppressor of diffuse large B cell lymphoma (DLBCL); PRDM3 and PRDM16 show different isoforms with separate functions in leukemia [44,69]. PRDM16 (PRD1-BF1-RIZ1 homologous domain containing 16) controls a bidirectional cell fate switch between skeletal myoblasts and brown fat cells [70], the targeting of 3'UTR of *Prdm16* is involved in the choice between myogenic and brown adipose determination of the adult skeletal muscle stem cells (satellite cells) [71], PRDM5 acts with a potential tumor suppressor role for gastrointestinal carcinogenesis [72-74].

The RIZ proteins regulate cell proliferation in a *yin-yang* manner [57,58,75]: gene silencing of the RIZ1 form, by genetic or epigenetic mechanisms, has been described in a variety of human tumors [14], whereas the RIZ2 form, lacking of PR domain, is always present or overexpressed [76]: this finding suggests a positive selection for RIZ2 in cancer progression. Further evidence indicates that forced expression of RIZ1 in tumor cell cultures induces growth arrest and apoptosis, possessing an anticancer activity in the PR domain [77], and the silencing of RIZ1 expression can stimulate breast cancer cell proliferation [78]. In addition, forced expression of the Zn-finger domain present in both RIZ forms increases the growth rate of breast cancer cells [79]. Based on these findings, RIZ1 could be considered as a crucial tumor suppressor gene candidate and the Zn-finger domain could be responsible for the putative oncogenic activity of the RIZ2 gene product. Such an effect might be more relevant in estrogen target tissues, where RIZ gene products are reported to directly interact with the estrogen receptor in a hormone-dependent manner through a LXXLL motif [80,81], promoting optimal estrogen response; conversely, in osteosarcoma cancer cell line (SAOS2), RIZ1 is expressed at high level in proliferating cell compared to serum-free culture conditions [82]. Murine models studies demonstrated that PRDM1 regulates commitment, as well PRDM14, PRDM9 is involved in meiosis [83], PRDM16 is involved in the switch controlling of myoblast differentiation to brown adipocytes [70,84]; PRDM3 and PRDM16 are also required for the maintenance of brown adipocyte identity [85], but currently the PRDM2 role in the development and in myoblast differentiation is still unclear. The purpose of this study therefore was to investigate the expression profile and the role of *PRDM* genes in the molecular mechanism responsible of the proliferation-differentiation *switch*, paying particular focus on the *PRDM2* gene expression in skeletal muscle C2C12 cell line, an established cell model for skeletal muscle differentiation studies [86,87], that has been successfully used to study differentiation and proliferation-differentiation switch [88].

Materials and Methods

Cell culture and transfection

C2C12 cells (kindly provided by Professor Fabio Naro from University "La Sapienza"-Rome) were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, FBS (Invitrogen), referred as GM, *i.e.* growth medium, in humidified 95% air and 5% CO₂. As the cells reached confluence, the medium was replaced with DMEM supplemented with 10 µg/ml insulin (Invitrogen). And 5 µg/ml transferrin (Invitrogen) referred as DM, *i.e.* differentiation medium, to induce differentiation of myoblasts into myotubes. Cell transfection with plasmid DNA was performed using Lipofectamine® 2000 Reagent (GIBCO BRL, Life Technologies, Rockville, MD, USA) in OptiMem I Reduced Serum Medium (Invitrogen) for 6 hours, according to the manufacturer's instructions. Transfection medium was then removed,

and cells were grown in DMEM supplemented with 5% FBS or DM, as indicated in the legend to figures, for additional 24-48-72 hours. *Reverse transcriptase-polymerase chain reaction (RT-PCR)*-Total RNA (1 µg) was extracted from C2C12 cells using Trizol reagent (Invitrogen), according to the manufacturer's instructions. Gel electrophoresis in denaturing conditions was performed to evaluate the integrity of extracted RNA; the quality of RNA extracted was evaluated by the measure of 260/280 nm and 260/230 nm absorbance ratios (the threshold acceptance was 1.9 for absorbance ratio 260/280 nm and 2.2 for absorbance ratio 260/230). To remove contaminant DNA, RNA samples were treated with 40 U of RNase-free DNase-I (Boehringer Mannheim, Indianapolis, IN, USA) for 45 minutes at 37°C. The absence of contaminant genomic/plasmid DNA was checked by PCR of not reverse transcribed RNA samples. Total RNA was reverse transcribed using cDNA Synthesis Kit Transcriptor High Fidelity (Roche, Basilea, Switzerland). The cDNAs amplifications were performed by RT-PCR with specific primers set for glyceraldehyde-3-phosphate dehydrogenase (GAPDH)s, for MRF and for the detection of the PRDM plus transcript (PRDM PR as reported in table 1) and also for all PRDM transcripts (PRDM TOT as reported in table 1) using JF buffer (30mM Tris base, 8mM HEPES base, 20mM K glutamate, 60mM NH₄ acetate, 2mM DTT, 8% glycerol, 1.5mM MgCl₂, 0.2mM dNTPs) [79]. The reaction was performed using a thermal cycler (Eppendorf, Milan, Italy). Analysis of amplified products was done by electrophoresis on 2% agarose gel. The gel images were acquired by the Gel DOC XR System platform (Bio-Rad laboratories, Hercules, CA).

Table 1: Sequences of primers used for RT-PCR analysis ZA .

PRDM2 PR F	ACTGGCTCCGCTATGTGAAC
PRDM2 PR R	CGCGATTGGCTTTAAGGTT
PRDM2 TOT F	CCGGAGAGGGAAGAAGAAAT
PRDM2 TOT R	TCATGTTTGCAGAGGTGGAG
PRDM10 PR R	TCACGAACTCAGCGTAGGATG
PRDM10 PR F	TGGTCCTCTACATAGATAGGTT
PRDM10 TOT F	TGAATGGACTAGATCAGCCAG
PRDM10 TOT R	AGCCCTTGTACAGAGATCAC
PRDM4 PR F	AGCAGCTTGTCTCCGCCAGTCC
PRDM4 PR R	GCACTCCTTGCCACAGTTACAGAG
PRDM4/PFM F	TCTCCCTCTCACAGTGCCAT
PRDM4/PFM R	GATCTAGTGCTGAAGGGTTGTTGG
PRDM1 PR F	GACGGGGTACTTCTGTTC
PRDM1 PR R	GCATTCTTGGGAACTGTGT
PRDM1 TOT F	ACACCGGACTCCTACTCCT
PRDM1 TOT R	GTACGGTGGCAACAGGAACT
PRDM5 PR F	GAGCCGAGCTCATTTCCCTC
PRDM5 PR R	GTACGTACATGCCAGCATC
PRDM5 TOT F	GCCATTCAAACACACACAGG
PRDM5 TOT R	TTTCACAGTACGGGCATTGA
PRDM14 PR F	TTGGTGATGTGCCACACTTT
PRDM14 PR R	TCCAGTCCCAGAACCTTTG
PRDM14 TOT F	CACTCCCGAAGTACCACGAT
PRDM14 TOT R	CCCACCTCTGACCACTGATT
PRDM16 PR F	TTCCAATCCCACCAGACTTC
PRDM16 PR R	CATCTGCTCCCATCCAAAGT
PRDM16 TOT F	TGGGCTCACTACCCTACCAC
PRDM16 TOT R	GACTTTGGCTCAGCCTTGAC
MyoD F	TGGGATATGGAGCTTCTATCGC
MyoD R	GGTGAGTCGAAACACGGATCAT
Myf-5 F	TGAAGGATGGACATGACGGACG
Myf-5 R	TTGTGTGCTCCGAAGCTGCTA
Myogenin F	AGGAGAGAAAGATGGAGTCCAGAG
Myogenin R	TAACAAAAGAAGTACCCCCAAGAG
c-Myc F	ATGCCCTCAACGTGAACTTC
c-Myc R	CGCAACATAGGATGGAGAGCA
GAPDH F	TGTGTCCGTCGTGGATCTGA
GAPDH R	CCTGCTTACCACCTTCTTGA



Quantitative Reverse-Transcription PCR (qRT-PCR) Analysis-Aliquots of cDNA were subjected to quantitative analysis by real-time PCR (qRT-PCR) using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a Mastercycler ep Realplex (Eppendorf), as previously described [89]. The amplification was performed using the same primer sets of semi-quantitative RT-PCR. GAPDH was used as housekeeping gene for normalization. The dissociation curves showed a single peak and the agarose gel analysis of PCR amplicons showed a single DNA band of the expected molecular size. The relative quantification (RQ) was performed using the $\Delta\Delta C_t$ method [90]. Data were presented as RQ (Relative Quantification) by comparing the threshold cycle of PCR products to the threshold cycle of standard cDNA. The linearity and the efficiency of the PCR reaction were analyzed by serial cDNA dilutions. Each amplification was done in triplicate and the reaction specificity was assessed by melting curves analysis.

***PRDM2* PR silencing by RNA interference** -The gene silencing of *PRDM2* PR gene product was achieved using the BLOCK-iT™ Pol II miR RNAi with emGFP expression vector (Invitrogen), according to the manufacturer's instructions. The oligonucleotides, Hmi418948 and Hmi418951, which target *PRDM2* PR gene product (Invitrogen Co.), were cloned into the vector (pcDNA™ 6.2-GW/EmGFP-miR). C2C12 cells were transfected with recombinant vector or with kit control vector (β -galactosidase, CTRL).

Protein assay -The protein concentration was measured with BIO-RAD Protein Assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Western blot analysis- C2C12 cells were washed with PBS and lysed in RIPA buffer 1X [50 mM Tris HCl pH 7.5, 150 mM NaCl, 0.25 % sodium deoxycholate, 1% NP-40 supplemented with protease inhibitor cocktail (Roche, Basilea, Svizzera). The clarified lysates were processed for Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis, SDS- PAGE (10% polyacrylamide gel) according to Laemmli procedure [91]; subsequently the proteins were transferred to PVDF membrane (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) using transfer buffer. Blots were blocked with 20 mM Tris pH 7.8, 100 mM NaCl, 0.1% Nonidet P40/Tween 20(1:1), 5% non-fat milk and incubated with primary antibodies diluted in the same buffer (0.1ml/cm²). The following antibodies were used at the indicated concentrations: mouse monoclonal anti-MyoD: sc-32758 (Santa Cruz Biotechnology) at the following concentration 0.2 μ g/ml; mouse monoclonal anti- Myc: sc-40 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at the following dilution 1:500; mouse monoclonal anti-Myogenin: sc12732 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at the following dilution 1:500; rabbit polyclonal antibodies to RIZ1, RIZ 9710 (Abcam Ltd., Cambridge, UK) at the following dilution 1:1000, rabbit polyclonal antibodies against *PRDM4* and *PRDM10* (EPIGENTEK) at the following dilution 1:1000. Thereafter the same filter was stripped and reprobed with mouse monoclonal antibody against Histone H1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at the following dilution 1:1000. Peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG secondary antibodies were used at 1:5000 dilution. Peroxidase activity was detected using an Amersham ECL™ Advance Western Blotting Detection Kit (GE Healthcare, Little Chalfold, UK), according to the manufacturer's instructions. Membranes were exposed with Amersham Hyperfilm ECL film (GE Healthcare, Little Chalfold, UK) and images were acquired with the Gel DOC XR Platform (Bio-Rad Laboratories, Hercules, CA).

Cell growth analysis- Cell proliferation was evaluated by cell counting, by MTT (3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma-Aldrich, Co. St. Louis, MO, USA) and by FACS (Fluorescence Adsorbed Cell Sorting, Becton Dickinson, New Jersey, USA) analysis as previously indicated [58].

Immunofluorescence analysis

Myoblasts were plated on poly-L-lysine (3x)-coated coverslip and cultured in DM for 72h. Subsequently DM was discarded and cells washed three times with PBS and



incubated at room temperature with freshly-made paraformaldehyde (4% w/v) for 10 min. The cells were washed with glycine 0.1% for 5 min, followed by three washing in PBS, subsequently incubated for 2 hours with the following primary antibodies: (a) mouse monoclonal anti-MyHC (Sigma-Aldrich, Co. St.Louis, MO,USA) at the following dilution 1:500; (b) RIZ 9710 (Abcam Ltd, Cambridge, UK) at the following dilution 1:100. The primary antibodies were diluted in PBS containing 10% (v/v) fetal bovine serum (FBS) and 0.1% Triton X-100. Cells were then washed in PBS and incubated at room temperature for 1 h with fluorescent secondary antibodies (Jackson Laboratories, USA): (a) anti-mouse or anti-rabbit IgG conjugated to Texas red were diluted 1:100 in PBS containing 10%(v/v) fetal bovine serum (FBS) and 0.1% Triton X-100. Subsequently, coverslips were placed onto untreated glass slides and allowed to air dry. Coverslips were analyzed using a Zeiss LSM 510 Meta argon/krypton laser scanning confocal microscope. Each image was acquired 4 times and the signal averaged to improve the signal to noise ratio. Computer-assisted quantitative evaluation of channel distribution in cell compartment was done using the Image-J software.

Chromatin immunoprecipitation (ChIP)

C2C12 cells (approximately 5×10^6 cells/plate) were cultured in GM or in DM for 24, 48 and 72 hours. One-tenth aliquots were immunoprecipitated using the following antibodies: 1 μ g of purified IgG control antibody (Sigma-Aldrich), 1mg of rabbit polyclonal anti-RIZ1 (Abcam Ltd, Cambridge, UK) or 1 μ g of mouse monoclonal anti-MyoD (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Secondary immunoprecipitation was performed with Sepharose coupled to protein A (Sigma-Aldrich, Co. St. Louis, MO, USA). One-twenty-fifth of the DNA extracted from each immunoprecipitation was amplified using primers complementary to the Myogenin promoter region E2_E1 Forward-GAATCACATGTAATCCACTGGA E2_E1 Reverse- ACGCCAACTGCTGGGTGCCA). Amplifications were performed for an empirically determined number of PCR cycles producing a linear correlation between amplified band signals and template dilutions. AmpliTaq polymerase in AB1 buffer (30mM Tris base, 10mM HEPES base, 25mM KCl, 20mM K glutamate, 20mM NH₄ acetate, 1.25mM DTT, 5% glycerol, 1.25mM MgCl₂, 0.2mM dNTP) (Applied Biosystems, Foster City, CA) was used for all PCR analyses. Amplification products were analyzed by 2% agarose gel electrophoresis and bands were visualized with the Gel DOC XR System (Bio-Rad laboratories, Hercules, CA); densitometric analysis was performed using Total Lab 1D software. *Statistical analysis* -Statistical significance was determined with a paired t-test with Graphpad prism 5.0 software (La Jolla, CA, USA).

Results

Analysis of the expression levels of MRF genes and PRDM genes in myoblasts and myotubes

To induce differentiation of myoblasts into myotubes, confluent C2C12 cells were incubated in low mitogenic media (DM) and after 72 hours, differentiation is complete as observed by morphological changes. To verify the fidelity of the model used, an immunofluorescence analysis (Figure 1S a) of C2C12 cells differentiated in the same experiment was performed. The results revealed that after 72 h in DM medium cells formed myotubes expressing the myosin heavy chain (MyHC), a marker of myoblasts differentiation (Figure 1S b-c) [92]. The expression level of *PRDM* genes (*PRDM1, 2, 4, 5, 10, 14* and *16*) and MRF genes: *Myc* and *Myogenin* was evaluated by qRT-PCR analysis. The amplification of PR *plus* product was performed with a set of primer amplifying a transcript region encoding for the PR domain (PRDM PR F/R) and another primer set was used for amplification of a region common to both PR *plus* and PR *minus* forms (PRDMtot F/R). As expected, the low mitogenic environment increased the expression level of differentiation markers Myogenin and reduced the proliferation markers c-Myc (Figure 1). In our experimental conditions, myotubes showed no significant variation

in the *PRDM1*, *PRDM5*, *PRDM14* and *PRDM16* expression level than myoblasts. On the other hand, *PRDM2*, *PRDM4* and *PRDM10* expression levels falls down into myotubes than myoblasts. So we decided to further investigate the variation in the expression level of *PRDM* genes that showed a significantly difference in the expression level between myoblasts and myotubes.

Analysis of the expression levels of MRF genes and *PRDM* genes during differentiation of myoblasts in myotubes

To induce differentiation of myoblasts into myocytes, confluent C2C12 cells were incubated in low mitogenic media (DM) and at different time (0, 24, 48, 72 hours) was evaluated by qRT-PCR the expression level of MRF genes: *Myc*, *Myf5*, *MyoD* and *Myogenin*. As expected, the low mitogenic environment increased quickly the expression

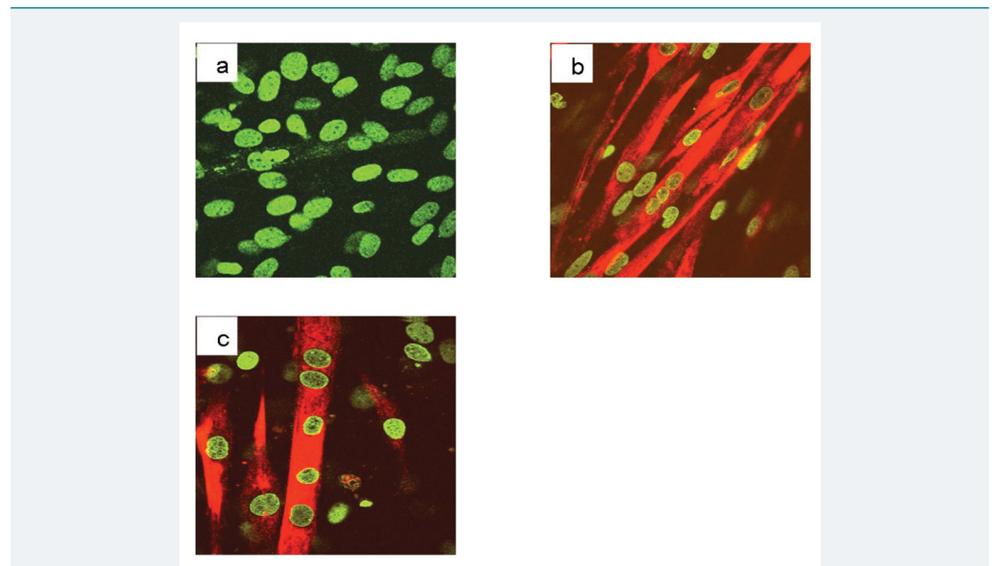


Figure 1S: Immunofluorescence analysis of the differentiation marker, myosin heavy chain, MyHC, in C2C12 cell line cultured in GM or DM. C2C12 cells were plated on glass slides for immunofluorescence and cultured for additional 120 hours in DM. After treatment, the cells were fixed, permeabilized and incubated with an antibody against the differentiation marker, myosin heavy chain, MyHC. Nuclei were stained with the chromomycin dye. The slides were analyzed by confocal fluorescence microscopy and significant images were acquired and displayed.

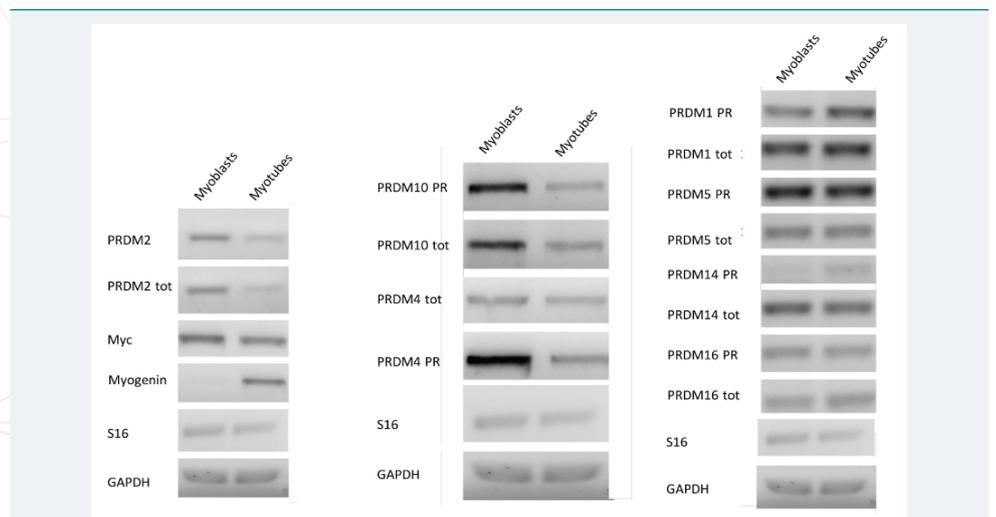


Figure 1: MRF and *PRDM* gene expression in myoblasts and myotubes. The electrophoretic analysis shows the fragments obtained by RT-PCR of total mRNA/cDNA extracted from C2C12 cell. The amplification of PR plus product was performed with a set of primer amplifying a transcript region encoding for the PR domain (PRDM PR F/R) and another primer set was used for amplification of a region common to both PR plus and PR minus forms (PRDMtot F/R).

of differentiation markers MyoD and Myogenin and reduced the proliferation markers Myc and Myf5 expression level (Figure 2). It has been extensively demonstrated by Cheedipudi et al. [87], that *PRDM2* gene expression levels is lower in myotubes than myocytes but no evidence are available on the variation of other member of PRDM family gene expression level during differentiation induction. In order to determine the precise temporal gap in which occurred *PRDM* expression level variation, qRT-PCR analysis of total C2C12 RNA extracted at different time (24, 48 and 72 hours) from differentiation induction was performed. As shown in figure 3A and B, at 24 h from differentiation induction *PRDM2*, *PRDM4* and *PRDM10* genes were expressed at lower level then myoblasts. This reduction was more evident at 72 h for *PRDM2* PR plus product. Interestingly and according to Cheedipudi [87], the maximal expression of Myogenin at 72 h from differentiation induction corresponds to the reduction of band intensity of *PRDM2* PR. The particular behaviour of *PRDM2* gene prompted us to investigate the relationship between *PRDM2* gene and myoblasts differentiation. Immunofluorescence analysis (Figure 2S) of C2C12 in GM or after culture in DM for 24

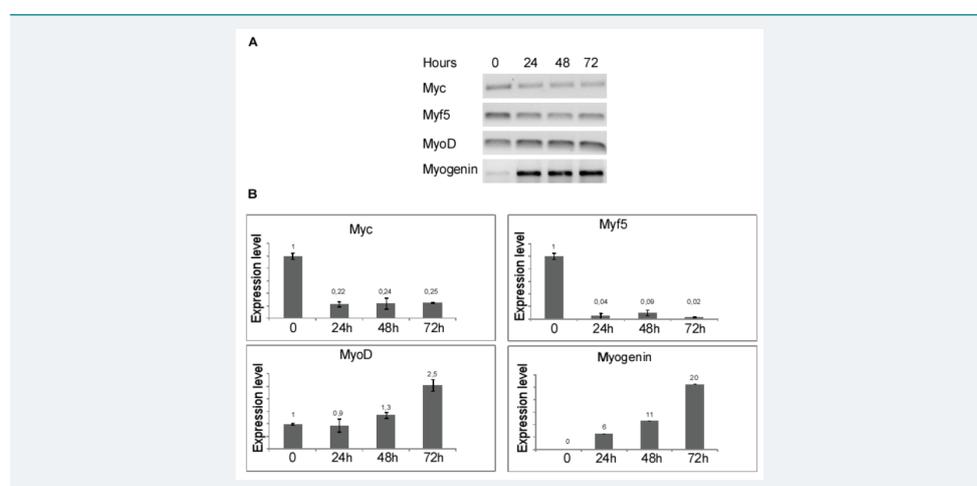


Figure 2: MRF genes expression after induction of differentiation. The transcripts encoded by MRF genes was measured by RT-PCR and qRT-PCR after 24, 48, 72 hours of differentiation induction (see Materials and methods section). The expression level is indicated as fold changes from basal conditions. A) Electrophoretic analysis of the fragments obtained by RTPCR of total mRNA/cDNA extracted from C2C12 cells; B) qRT-PCR of mRNA/cDNA extracted from C2C12 cells. Histograms represent the averages (+/- standard error) from at least three independent experiments, normalized for the expression of the control housekeeping gene GAPDH, reported in figure 2 (# indicates $p < 0.05$ for each gene versus control).

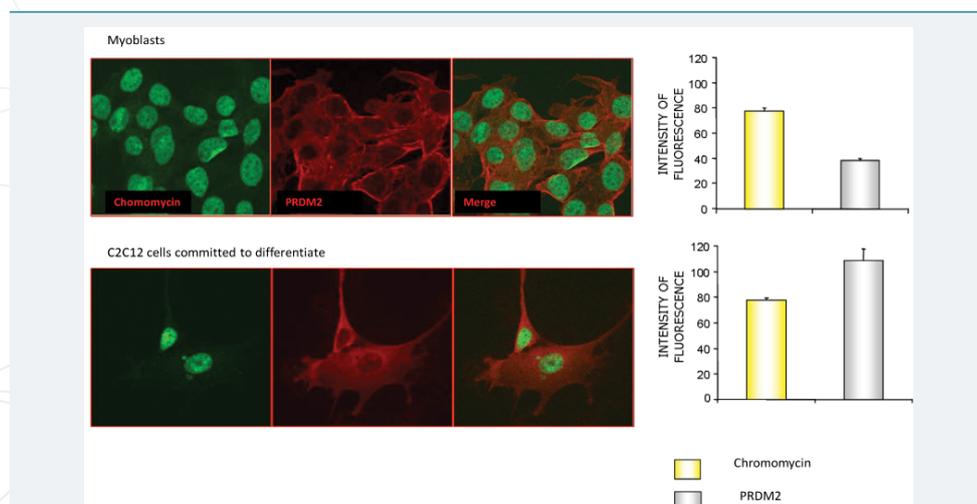


Figure 2S: Immunofluorescence analysis of the RIZ1 protein in the C2C12 cell line cultured in GM or DM. C2C12 cells were plated on glass slides for immunofluorescence and cultured for additional 24 hours in GM or DM. After treatment, the cells were fixed, permeabilized and incubated with an antibody against the RIZ1 protein. Nuclei were stained with the chromomycin dye. The slides were analyzed by confocal fluorescence microscopy and significant images were acquired and displayed.

h revealed that RIZ1 in proliferating condition is predominantly localized in the nucleus and translocates into the cytoplasm when C2C12 cells are committed to differentiate.

Effect of *PRDM2* silencing on C2C12 myoblasts differentiation

As observed by qRT-PCR analysis of *PRDM2*/*RIZ1* (60%) forced suppression with the plasmid coding the miRNAPRDM2 spanning a sequence coding for the aa 97-103 of *RIZ1* protein, induced an increase in expression level of the differentiation markers *MyoD* and *Myogenin* that was more evident for the last one (Figure 4). This evidence confirms that *RIZ1* protein negatively controls the induction of differentiation mediated by MRF factor. Western blot analysis of whole cell lysates from C2C12 transiently transfected with a control plasmid or with the plasmid coding the miRNAPRDM2,

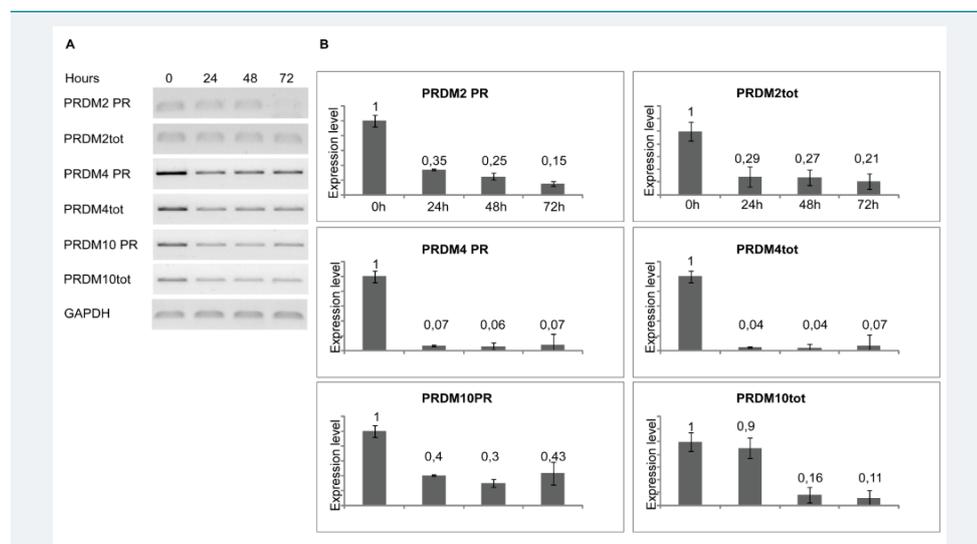


Figure 3: Modulation of PRDM genes expression during differentiation. The transcripts encoded by PRDM genes was measured by RT-PCR and qRT-PCR after 24, 48, 72 hours of differentiation induction (see Materials and methods section). The expression level is indicated as fold changes from basal conditions. The PRDM PR and PRDM2tot sets of primers recognize sequences on the region coding PR domain of PRDM genes or on a region common to both PRDM PR plus and minus, respectively. A) Electrophoretic analysis of the fragments obtained by RT-PCR of total mRNA/cDNA extracted from C2C12 cells; B) qRT-PCR of total mRNA/cDNA extracted from C2C12 cells. Histograms represent the averages (+/- standard error) from at least three independent experiments, normalized for the expression of the control housekeeping gene GAPDH (# indicates $p < 0.05$ for each form of PRDM genes versus their untreated control).

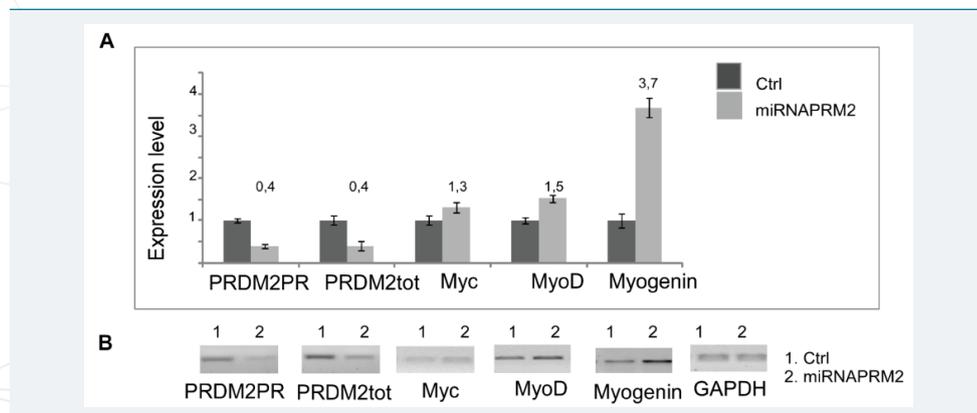


Figure 4: Modulation of MRF genes expression during differentiation. The transcripts encoded by PRDM genes was measured by RT-PCR and qRT-PCR after 24, 48, 72 hours of differentiation induction (see Materials and methods section). The expression level is indicated as fold changes from basal conditions. The PRDM PR and PRDM2tot sets of primers recognize sequences on the region coding PR domain of PRDM genes or on a region common to both PRDM PR plus and minus, respectively. A) Electrophoretic analysis of the fragments obtained by RT-PCR of total mRNA/cDNA extracted from C2C12 cells; B) qRT-PCR of total mRNA/cDNA extracted from C2C12 cells. Histograms represent the averages (+/- standard error) from at least three independent experiments, normalized for the expression of the control housekeeping gene GAPDH (# indicates $p < 0.05$ for each form of PRDM genes versus their untreated control).

showed a strong reduction in the RIZ1 expression level, revealing the efficacy of the interference (Figure 5). The PRDM2/RIZ1 silencing induced a decrease in the PRDM4 and PRDM10 expression levels. These evidence support the hypothesis that different genes of PRDM family control proliferation/differentiation *switch* and are probably organized in a hierarchical manner.

Interaction of RIZ with E2/E1 Myogenin promoter region

ChIP experiments were performed to detect the presence of *MyoD*, a master regulator of the skeletal muscle gene expression program [28], and RIZ on E2/E1 Myogenin promoter region during differentiation induction. Therefore, equivalent amounts of cross-linked chromatin from myoblasts cultured in GM or DM (24, 48 and 72 hours) were immunoprecipitated in parallel with two antibodies that recognize PRDM2/RIZ1 or MyoD. The precipitated DNA then was subjected to PCR amplification with the use of a primers set, spanning two of the E-box sites (E1 and E2) located in the proximal region of Myogenin promoter. Results shown in Fig. 6B, revealed that MyoD was ever associated with Myogenin promoter according to Mal et al., [93]; conversely, as shown in figure 6A, RIZ1 was associated with the E2/E1 Myogenin promoter region

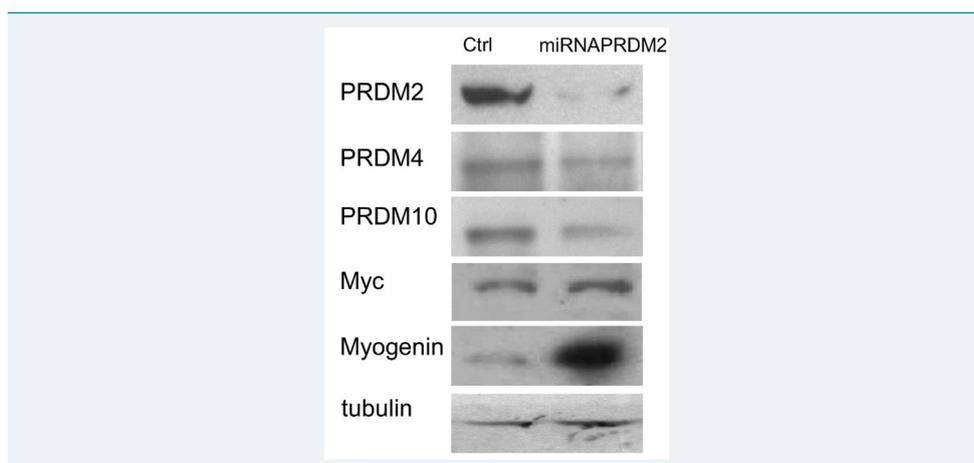


Figure 5: Modulation of PRDM and MRFs proteins expression into PRDM2 silenced C2C12 myoblasts. The expression level of protein encoded by PRDM2, PRDM4 and PRDM10 genes and MRFs was evaluated by SDS-PAGE and Western blot analysis of total protein cell extracts from C2C12 myoblasts and PRDM2 silenced C2C12 myoblasts (see Materials and methods section). The blots are representative of three independent experiments.

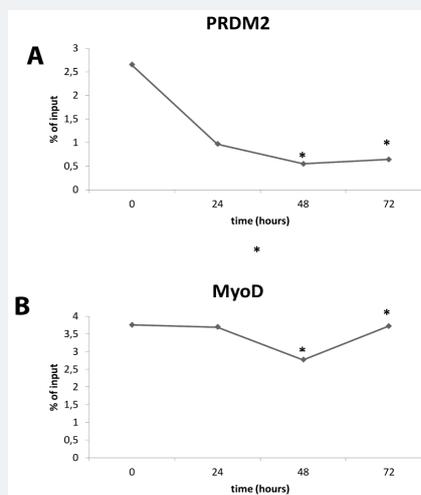


Figure 6: Interaction of RIZ with E1/E2 promoter. ChIP analysis of E2/E1 myogenin promoter region. Densitometric analysis of bands amplified from ChIP samples with antibodies to RIZ1 (upper panel) or to MyoD (lower panel), expressed as percentage of the input after background subtraction. The graph is representative of three independent experiment (* indicates $p < 0.05$ vs control).

in myoblasts at 24h whereas at 48h and 72h no association of RIZ1 was detected. The specificity of this assay is demonstrated by the observation that the binding of MyoD or RIZ1 was not observed when a normal rabbit IgG was used in the analysis.

RIZ1 silencing induced cell cycle arrest

In order to investigate the effect of RIZ silencing on proliferation rate, we performed a cell cycle analysis evaluating propidium iodide incorporation by FACS. As shown in figure RIZ1 silencing by miRNA induced a G1 phase delay. Likewise MTT assays of C2C12 cells transfected with the miRNAPRDM2 plasmid significantly reduced the cells number (Figure 7).

Discussion

PRDM family gene plays a key role in the control of a plethora of cell life processes, like cell cycle progression, homeostasis maintenance of immune system and control of early stage of development. *PRDM1* and *PRDM14* for example guide the epigenetic reprogramming, necessary to determinate the progenitors of germ cells during embryonic development; *PRDM9* also is indispensable for meiotic prophase progression during gametogenesis; *PRDM16* controls the cell fate switch between myoblasts and brown adipocytes: recently studies, conducted by Li et colleagues, demonstrated that C2C12 myoblasts, stably transfected with *PRDM16*, showed a repression of myogenic genes and an upregulation of adipogenic genes at proliferation and differentiation genes, probably due to CpG methylation of MyoD [84]. To this behaviour, the study of molecular basis of *PRDM* action mechanism appears very important. Data obtained by RT-PCR and Western blot analysis in C2C12 cell line showed that the expression of *PRDM2* gene was selectively modulated during differentiation, because of the differentiated cells showed reduced levels of expression of RIZ1 than myoblasts. This trend is in disagreement with the experimental evidence obtained in epithelial cell line models [78] and lymphocytes [94] in which treatment with differentiating agents, or

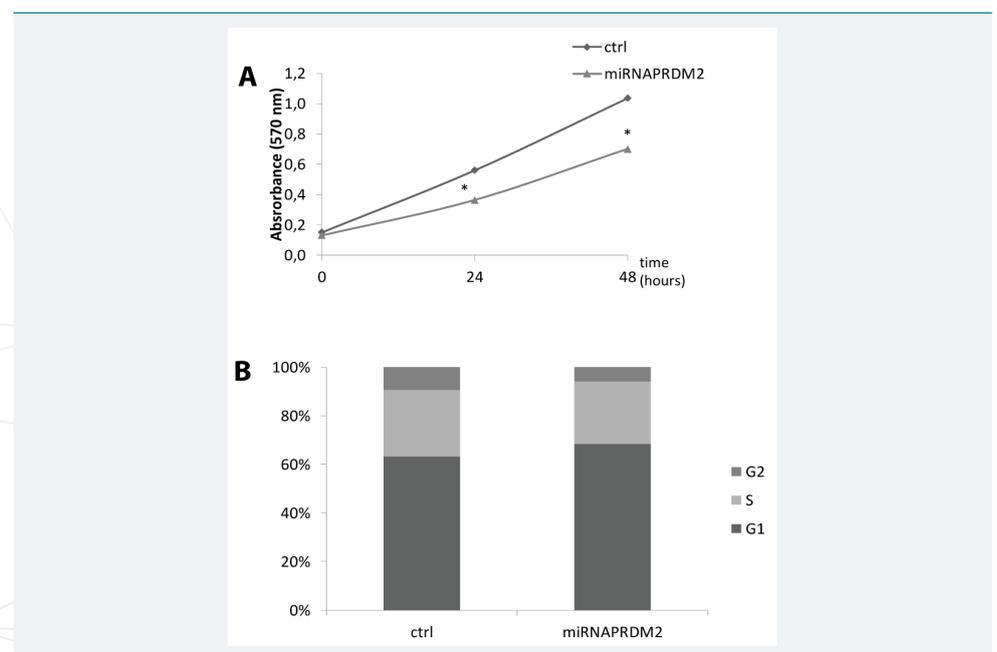


Figure 7: Effect on cell proliferation and survival upon RIZ1 knock down in C2C12 cell line. (A) For the colorimetric MTT assay, C2C12 cells transiently transfected with a plasmid encoding a miRNA against RIZ1 or a not-targeting plasmid (ctrl) were plated at the same density and allowed to grow for 0, 24 or 48 h. MTT was added in the last 2 h, formazan precipitates were dissolved with dimethyl sulfoxide reagent and absorbance read at 570 nm [31]. Values are the mean (\pm SE) of three analyses from three independent experiments # $p < 0.05$ vs. control; (B) C2C12 cells transiently transfected with a plasmid encoding a miRNA against RIZ1 or a not-targeting plasmid (ctrl) were cultured in 60-mm dishes for 48 h. Cells were processed following manufacturer's instruction and finally analyzed by flow cytometer to determine the percentage of cells in the different phases of cell cycle. The data are the mean of three independent experiments performed in triplicate ($n = 9$) # $p < 0.05$ vs control.

the stimulation of cell proliferation, respectively, caused the increase and decreased of *PRDM2*/*RIZ1* expression. On the contrary, there are some similarities with the experimental evidence obtained with the osteosarcoma cell line (SAOS2), in which *PRDM2*/*RIZ1* showed an elevated expression in proliferating cells, compared to cells that had temporarily left the cell cycle [95]. These suggestions left us to speculate that *RIZ* can be related to the cell type and in the mesenchymal cells perhaps cell cycle escape and subsequent differentiation coincide with *RIZ1* expression level reduction: immunocytochemistry experiments, performed in myoblasts and myotubes, showed that during differentiation there is an intracellular redistribution of *RIZ* protein, predominantly localized in the perinuclear and nuclear area. This phenomenon, as myeloid leukemia cell line HL60, could be interpreted as an intranuclear confinement of *RIZ* protein. Expression level analysis by qRT-PCR of other member of *PRDM* family gene *PRDM4* and *PRDM10* showed that the differentiation induction was characterized by a reduction of its expression levels. In order to confirm the involvement of *PRDM2* gene in the proliferation-differentiation switch, we performed knockdown experiments. Interestingly, the *PRDM2* gene silencing was characterized by a Myogenin expression level increasing, suggesting its peculiar role in the transcriptional control of myogenin expression. ChIP experiments performed with MyoD and *RIZ* antibodies on E2/E1 Myogenin promoter region in proliferation condition and after 24 h from differentiation induction, confirmed a binding on the Myogenin promoter. As demonstrated by Mal A. [93], Lys-9 of H3 histones were methylated by SUV39H1 [96], surrounding the myogenin promoter in undifferentiated myoblasts. This marking was dramatically reduced in myoblasts that had undergone differentiation. We hypothesize that another member of SET family protein, *RIZ1*, can modulate myogenin expression, and *RIZ1* interference reduced the expression level of *PRDM4* and *PRDM10* genes, lead us to speculate that the members of the family have a redundant *PRDM* behavior and/or cooperate in the transition proliferation-differentiation. Furthermore *PRDM2* silencing induced a G1 phase delay and a reduction of proliferation.

Conclusion

In conclusion, our studies suggest that *PRDM2*, *PRDM4* and *PRDM10* play a pivotal role in the proliferation-differentiation switch of myoblast in myotubes.

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