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FULL LENGTH ARTICLE

# Transient expression of inactive RB in mesenchymal stem cells impairs their adipogenic potential and is associated with hypermethylation of the PPARγ2 promoter

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**Abstract** The retinoblastoma gene product (pRb) is a chromatin-associated protein that can either suppress or promote activity of key regulators of tissue-specific differentiation. We found that twelve weeks after transfection of the exogenous active (ΔB/X and Δp34) or inactive (ΔS/N) forms of RB into the 10T1/2 mesenchymal stem cells and clonal selection not a single cell line did contain exogenous RB, despite being G-418 resistant. However, the consequences of the transient production of exogenous RB had different effects on the cell fate. The ΔB/X and Δp34 cells transfected with active form of RB showed elevated levels of inducible adipocyte differentiation (AD). On the contrary, the ΔS/N cells transfected with inactive RB mutant were insensitive to induction of AD associated with abolishing of expression of the PPARγ2. Additionally, the PPARγ2 promoter in undifferentiated ΔS/N cells was hypermethylated, but all except −60 position CpG became mostly demethylated after cells exposure to AD. We conclude that while transient expression of inactive exogenous RB induces long term epigenetic alterations that prevent adipogenesis, production of active exogenous RBs results in an AD-promoting epigenetic state. These results indicate that pRb is involved in the establishment of hereditary epigenetic memory at least by creating a methylation pattern of PPARγ2.

*Abbreviations:* AD, adipogenic differentiation; DNMT1, DNA methyltransferase 1; MSCs, mesenchymal stem cells; RB, retinoblastoma susceptibility gene; pRb, RB product.

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## Introduction

pRb is a multifunctional chromatin-associated protein that restricts transcription when associates with promoters through its target — E2f transcription factors.[1] This protein is inactivated in majority of tumours due to gene mutations, to phosphorylation, or to inactivation of its product by viral oncoproteins.[2–5] Additional lines of evidence show that pRb regulates activity of several transcription master inducers of tissue specific differentiation. Depending on the differentiation factor, pRb can either suppress or promote its transcriptional activity. For example, pRb binds to Runx2 and promotes its ability to osteogenic differentiation. In contrast, pRb through E2f suppresses PPAR$\gamma$2, the master factor of adipogenesis. Since osteocytes and adipocytes can both arise from mesenchymal stem cells (MSCs), pRb can regulate their choice of fate.[6] However, the mechanism of pRb-mediated regulation of the cell fate is still elusive. Adipocytes have been divided into the brown and the white ones, which differ in regulation of expenditure of energy excess in opposite ways: the white adipocytes accumulate the energy excess, and the brown ones convert it into heat.[7] Brown adipocytes originate from MSCs with myogenic abilities and their production is suppressed by pRb, so that formation of white adipocytes is promoted.[8] The terminal stage of adipocyte differentiation (AD) is under control of CCAAT/enhancer binding proteins (C/EBPs) and Ppar$\gamma$2. Loss of pRb is associated with the inability of mouse embryonic fibroblasts to AD due to disruption of the pRb interaction with C/EBPs.[9] Another pRb target, the transcription factor E2f4, mediates suppression of the PPAR$\gamma$2.[10] pRb-deficient adipocytes possess high levels of uncouple protein 1 (UCP1), expression of which governs differentiation towards adipocytes of the brown type.[11]

pRb is considered as a local chromatin organizer with global possibilities.[12] pRb interacts with enzymes that modify histones, nucleosomes, and promote the formation of heterochromatin. pRb represses transcription when associates with promoters through its targets — E2f transcription factors.[1] Several proteins have been found to mediate the pRb induced suppression, promote the formation and spreading of heterochromatin. These proteins include DNA methyltransferase 1 (DNMT1), histone deacetylases (HDACs), SWI/SNF chromatin complexes, histone methyltransferases, and histone demethylases. A member of pRb family p130 is present at most E2F-regulated promoters in arrested cells.[13]

We have studied the pRb role in regulation of the cell fate by using the polypotent embryonal fibroblasts of the C3H10T1/2 (10T1/2) cell line inducible to differentiation into adipocytes, myocytes and chondrocytes.[14–16] A stable transfection of the wild type mouse RB (ΔB/X), inactive mutant of RB (ΔS/N), or hyperactive RB encompassing mutations in eight phosphorylation sites (Δp34) into 10T1/2 cells have been made and yielded three cell lines with constitutive production of different types of exogenous RB. We found that the ΔS/N cells lose the ability to AD but differentiate more effectively into myocytes in contrast to the ΔB/X or Δp34 cells producing the active types of the exogenous pRb and showing high levels of inducible adipocyte differentiation.[14,15]

The goal of the present work was to study ability of the exogenous pRb to promote the AD in a long term culture of the 10T1/2 cells stably transfected with different types of exogenous pRb. We found that in 12 weeks after transfection and cloning all cell lines were resistant to G-418 due to presence of the pSV2-Neo plasmid. At the same time, not a single cell line did contain exogenous RB. In spite of the loss of exogenous RB, the ΔB/X and Δp34 cells transfected with active types of exogenous RB showed elevated levels of inducible AD. In contrast, the ΔS/N cells transfected with inactive RB mutant were insensitive to induction of adipocyte commitment associated with significant decrease in expression of PPAR$\gamma$2. And finally, the promoter of the PPAR$\gamma$2 in undifferentiated ΔS/N cells became hypermethylated. Our results suggest that transient expression of exogenous pRb may induce long term epigenetic alterations. AD is promoted in the cells with transient production of active types of exogenous RB and is prevented in the cells producing inactive protein. These indicate that pRb is involved in the establishment of hereditary epigenetic memory at least by creating a methylation pattern of PPAR$\gamma$2.

## Materials and methods

### Cell culture

Mouse embryonic polypotent fibroblasts C3H10T1/2 (10T1/2) were obtained from the American Type Culture Collection (ATCC). The cells were expanded in Eagle's Basal medium (Thermofisher Scientific Cat. 21010-046) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 50 μg/mL gentamicin in $CO_2$ incubator (5% $CO_2$ and 100% humidity).

### Plasmids and cell transfection

Plasmids with murine RB gene were kindly provided by Dr. P. Hamel (The Hospital for Sick Children, Toronto, Canada) and described previously (Popov et al, 2015). Shortly, full-length cDNA of murine RB gene was inserted into pECE vector containing SV40 promoter. The resultant plasmid served as the basis for all vectors containing RB. For preparing the construct with wild type RB (ΔB/X), non-translating 3′-terminal of RB and polylinker pECE were cleaved at BstXI and XbaI sites, respectively, with

subsequent completion of overhanging ends (Fig. 1). Plasmid ΔS/N was obtained by removal of a fragment between restriction sites SspI and EcoNI in 22 RB exon and subsequent ligation. This deletion removes 6 amino acid residues in the functional domain of pRb. Plasmid Δp34 encoding hyperfunctional pRb was obtained by substitution of coding triplets Thr246, Ser601, Ser605, Ser788, and Ser800 for Ala, Thr350 for Arg, Ser781 for Glu, and Ser804 for Asn using PCR-targeted mutation. 5′-end of RB was modified by the insertion of a fragment encoding an epitope of influenza virus hemagglutinin (HA) — CATGGGGTACCCATACGATGTTCCAGATTACGCTAG — into the initiating codon of cDNA.

## Stable transfection and cell cloning

Cell lines with constitutive production of exogenous pRb were obtained as follows: $2 \times 10^5$ 10T1/2 cells were placed into 60-mm culture dishes with Eagle's Basal medium one day before transfection. For transfection, 5 μg vector containing RB gene or initial pECE vector and 1 μg pSV2-Neo

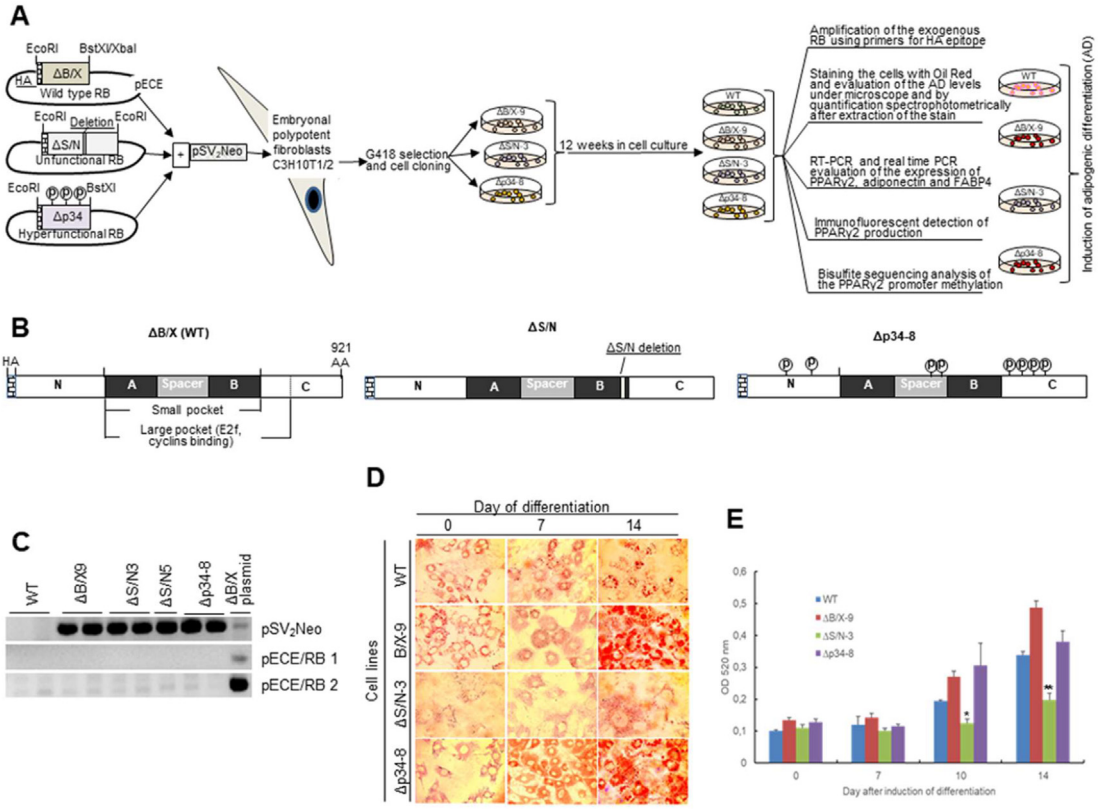


**Figure 1** 10T1/2 cells that were stably transfected with different types of murine exogenous RB, lose it in long term culture but retain the ability to adipocyte differentiation. **(A)** General experimental design. Plasmids with murine RB gene of the wild type (ΔB/X), modified by small deletion in T-antigen binding domain (ΔS/N) or mutation in 8 phosphorylation sites (Δp34) were transfected along with pSV2-Neo plasmid carrying resistance to G-418 into polypotent mouse embryonal 10T1/2 fibroblasts. The transfected cells were selected with G-418 for two weeks and cloned to obtain separate cell lines. In twelve weeks after selection all cell lines including the wild type cells (WT) were evaluated for the exogenous RB expression in RT-PCR using primers for HA epitope. The cells were also induced to adipogenic differentiation, and the level of which was quantified spectrometrically. Expression of PPARγ2, Adiponectin, FABP4 was detected in undifferentiated and differentiated cells by RT-PCR and real time PCR. Production of PPARγ2 was evaluated in the cells by immunofluorescent staining, while the methylation level of the PPARγ2 promoter by bisulfite sequencing. **(B)** Schematic diagram of the functional domains of the exogenous murine pRb (pRb). pRb consists of 921 AA that are divided into 4 functional domains (N, A, B, C). The most conservative part of pRb is the small pocket mediating interactions with many functional pRb partners like E2fs and cyclins. The 5′-end of the pRb is modified by fusion with HA epitope in all used plasmids. The ΔB/X plasmid contains unmodified mouse RB (WT), in the ΔS/N RB the B domain is modified by small deletion making this protein inactive, Δp34 is modified by the mutations of 8 phosphorylation sites — "p". C, D, E. 10T1/2 cells that were stably transfected with different types of exogenous RB, lose it in long term culture but retain the ability to adipocyte differentiation. **(C)** Exogenous RB was not detected in genomic DNA of long term culture of the 10T1/2 cell lines that contained NEO gene and were resistant to the G-418 treatment. **(D)** Representative images of the wild type, ΔB/X, ΔS/N, and Δp34 cell lines in 0, 7 and 14 days after induction of AD, Oil Red staining. The cells were observed under the microscope Pascal with transmitted light using a 40x objective. **(E)** The AD quantification was performed by the dye extraction and measuring its optical density at 520 nm with a Nanodrop spectrophotometer. *, ** — $P < 0.05$ in comparison with the levels of AD in all other groups on the 10 and 14 day, correspondingly. Experiments were repeated three times.

plasmid containing NEO gene providing resistance to G-418 antibiotic (obtained from Dr. R. Phillips, The Hospital for Sick Children, Toronto, Canada) were dissolved in 50 μl water and mixed with 30 μl lipofectamine (Lipofectamine 2000 Reagent, Cat. 11668030, ThermoFisher Scientific, USA) added with 20 μl water 15 min before transfection. The cells were washed with PBS; 3 ml Opti-MEM Reduced Serum Medium (Cat. 51985042, ThermoFisher Scientific, USA) without FCS and 100 μl lipofectamine 2000 (ThermoFisher Scientific, USA) with DNA were added to each dish. On the next day after transfection, the content of a 60-mm dish was transferred to 3 × 100-mm dishes containing Eagle's Basal medium with 500 μg/ml G-418 (Cat. A1720, Merck, USA) and cultured for 3 weeks.

## Induction of adipogenic differentiation (AD) and adipocytes staining

$1 \times 10^4$ 10T1/2 cells in the logarithmic growth phase were seeded on coverslips in 35 mm plates in 1.5 mL growth medium. The next day, the growth medium was replaced with a differentiation medium composed of 50 μg/mL gentamicin, 10% FBS, 5 μg/mL insulin, 50 μM indometacin, $10^{-5}$ M dexamethasone, and 0.5 μM 3-isobutyl-1-methylxanthine (Sigma, USA). The cells were fixed with 4% paraformaldehyde (Sigma, USA) in PBS for 1 h in 10 days after AD induction to assay lipid droplets. Fixed cells were washed twice with distilled water and once with 60% isopropanol (Vekton, Russia), air dried, stained with Oil Red (Sigma, USA), and washed four times with distilled water. Working dye solution was prepared by mixing six parts of the solution containing 25 μg/mL Oil Red in 100% isopropanol (Sigma, USA) with four parts of distilled water. Before cell treatment, the solution was filtered through a 0.45 μm filter. The cells were observed under the microscope Pascal (Carl Zeiss, Germany) with transmitted light using a 10 x objective. To quantify AD, the dye was extracted with 1 mL 100% isopropanol. The optical density was measured at 520 nm with a Nanodrop spectrophotometer (Thermo Scientific, USA). Experiments were repeated three times.

## Preparation and electrophoresis of RNA in denaturing gel and its quantitative evaluation

RNA was extracted from the cultured cells with acidic water-saturated phenol. The RNA integrity was monitored and RNA amount was normalized by electrophoresis in a denaturing agarose gel supplemented with formaldehyde. Upon the electrophoresis, RNA was visualized in the gel by luminescence of the 18S and 28S bands stained with ethidium bromide. The RNA amounts in different specimens were normalized upon the determination of the 28S band intensity on the corresponding lanes using the TotalLab Quant program product.

## Synthesis of cDNA on the RNA template and amplification of gene fragments using RT-PCR

To synthesize cDNA, 5 μg total RNA dissolved in 3—5 μl of deionized water were mixed with 0.5 μg oligo-dT18 and 2 μl of 10 mM dNTP, and the mixture volume was adjusted to 12 μl, incubated for 5 min at 65 °C, cooled on ice, supplemented with 2 μl of 0.1 M dithiothreitol, 2 μl of 10-fold buffer for reverse transcriptase, 20 U of ribonuclease inhibitor, incubated for 2 min at 37 °C, supplemented with 200 U of reverse transcriptase M-Mul V (Fermentas, Lithuania), incubated for 1 h, and the reaction was stopped by heating at 75 °C for 15 min. The gene fragments were amplified by PCR. The mixture for PCR was prepared on ice and included 2.5 μl of 10-fold buffer for Taq polymerase, 1 μl of 5 mM mixture of dNTPs, 0.5 μl of 100 μM forward and reverse primers, 0.5 μl of cDNA or 0.5 μl of the PCR product, 0.125 μl of Taq polymerase with the activity of 5 U/μl (Beagle, Russia), and 20 μl of water to the reaction mixture total volume of 25 μl. RT-PCR was performed on cycler T100 (BioRad, USA). The amplification regime was as follows: denaturation at 94° C, 1 min (1 cycle); 35 cycles of denaturation at 94° C, 15 s; annealing at 58° C, 30 s; synthesis at 72° C, 30 s; 1 cycle of elongation at 72° C, 10 min. GAPDH was used as a loading control. Amplified cDNA fragments were analyzed with electrophoresis using 1.2% agarose gel in TAE buffer containing 0.5 μg/ml EtBr. Electric field was 5 V/cm. PCR primers were synthesized by Beagle (Russia) (Table 1).

## Real time PCR

Total RNA was isolated from $2 \times 10^6$ cells with a GeneJET RNA kit (ThermoFisher Scientific, USA). Purified RNA was treated with DNase I (ThermoFisher Scientific, USA) (3 units/35 μL at 37 °C for 1 h). The reaction was stopped by addition of 3.5 μL 50 mM EDTA for 10 min at 65 °C. RNA was quantified using a spectrophotometer at 260 nm. Its integrity was verified by the presence of 28S and 18S bands after electrophoresis on denaturing agarose gel with formaldehyde. For cDNA synthesis 2 μg RNA was mixed with 0.5 μg oligo-dT18 primer. The volume was adjusted to 12.5 μL and incubated for 5 min. The reaction mixture was cooled, added with 4 μL five-times buffer, 1 μL (200 units) RevertAid reverse transcriptase (ThermoFisher Scientific, USA), 2 μL 10 mM dNTP mixture, and 0.5 μL RNase inhibitor (ThermoFisher Scientific, USA) and incubated at 42 °C for 1 h. The reaction was stopped by heating to 70 °C for 10 min. Real time PCR was performed on the Applied Biosystems 7300 real PCR system. The parameters were as follows: denaturation at 95 °C for 5 min, melting at 95 °C for 15 s, annealing at 58 °C for 30 s, synthesis at 72 °C for 20 s; 40 cycles. The reaction mixture was composed of 8 μL 2.5-times solution containing deoxynucleoside triphosphates (dNTP), PCR buffer, $MgCl_2$, Taq DNA polymerase SYBR Green I and ROX (Syntol, Russia), 0.2 μL forward and reverse primers (Beagle, Russia) (Table 1), and 0.2 μL cDNA and water to adjust the volume of 20 μL. Actin gene was used as a loading control. Gene relative expression was calculated using the formula $R = 2^{-\Delta\Delta C(T)}$. These experiments were repeated 3 times.

## Immunofluorescent staining

Coverslips with adhered cells in 35 mm plates were washed with PBS for 5 min and fixed with 4% paraformaldehyde

**Table 1** Primers for amplification of exogenous RB, pSV2-Neo, PPARγ2, FABP4, Adiponectin in RT-PCR, Real time PCR, and Bisulfite sequencing analysis.

| Gene | Forward primer (5′—3′) | Reverse primer (5′—3′) |
|---|---|---|
| *Exogenous RB (HA)* | GCTATTCCAGAAGTAGTGAGGAGGC | |
| *Exogenous/endogenous RB primer 1* | | TCAAACTCAAGCCTGGCCAG |
| *Exogenous/endogenous RB primer 2* | | TCTCTGACATGATCGGGTACCTTT |
| *pSV2-NEO* | TGAACAAGATGGATTGCACGCAGG | AACGTCGAGCACAGCTGCGCAA |
| *PPARγ2* | TGACCCAGAGCATGGTGCCTTC | TGTGGCATCCGCCCAAACC |
| *FABP4* | TGGAAGCTTGTCTCCAGTGA | TCGACTTTCCATCCCACTTC |
| *ADIPONECTINE* | GGAGAGAAAGGAGATGCAGGTCT | GGTAGTTGCAGTCAGTTGGTATCAT |
| *GAPDH* | CCATCTTCCAGGAGCGAGA | GGCAGTGATGGCATGGACTGT |
| *B-ACTIN* | ACAGAGCCTCGCCTTTGC | GGAATCCTTCTGACCCATGC |
| *PPARγ2 primers for bisulfite sequencing* | TTTTAGATGTGTGATTAGGAGTTTT | ACAATTTCACCCACACATAAA TA |

overnight. The samples were treated with 0.2% Triton X-100 for 10 min and washed with PBS for 5 min. Nonspecific antibody binding was blocked by the cell incubation for 1 h with 3% bovine serum albumin and 0.1% Tween-20. Then the cells were incubated with specific antibodies (dilution 200) in the blocking solution for 1 h at room temperature, washed with PBS for 5 min three times and treated with fluorescent-labeled secondary antibodies. The samples were washed three times with PBS for 5 min and mounted into Anti-Fade solution reducing unspecific fluorescence and containing DAPI as DNA stain (BioRad, USA). Images were captured with digital scanning electron scanning microscope Leica (Carl Zeiss, Germany) using 405 and 633-nm lasers.

### DNA extraction and bisulfite analysis

Genomic DNA was obtained by overnight cell incubation in TES buffer containing 0.1% SDS and 100 μg/ml proteinase K at 55 °C with subsequent phenol/chloroform extraction and isopropanol precipitation. 2 μg of DNA in 50 μl of TE buffer was denatured during 15 min in 0.3 M NaOH at 37 °C. The denatured DNA was mixed with 550 μl of freshly prepared solution of 10 mM hydroquinone and 3 M sodium bisulfite at pH 5.0, and incubated under mineral oil at 50 °C for 12 h. Bisulfite treated DNA was desalted by isopropanol precipitation, desulfonated with 0.3 M NaOH for 5 min at room temperature, and precipitated with ethanol. Converted DNA was dissolved in 100 μl of water and stored at −20 °C. 3 μl of precipitated DNA were used for each PCR. DNA of peripheral white blood cells (WBC) was used as a control for normal differentiated quiescent cells.

### Bisulfite sequencing analysis

The bisulfite treated DNA was used to amplify the promoter region of the PPARγ2 gene with primers specific for bisulfite converted DNA (Table 1). The PCR reaction was carried out using 2.5 units of homemade Taq polymerase in a final volume of 50 μl and the following cycling conditions: 5 min at 95 °C, followed by 35 cycles (30 s denaturation at 95 °C, annealing for 30 s at 62 °C and elongation at 72 °C for 1 min). The PCR products were gel purified and cloned using TOPO TA cloning kit. To prevent clonal amplification of sequences, the competent cells transformed were plated immediately after heat shock; excluding shaking bacteria for 1 h. 7—8 clones for each PCR product were sequenced and analysed. The efficiency of cytosine to uracil conversion was estimated as the ratio of cytosine in a non-CpG context to total number of cytosine in the region. The clones with the efficiency of cytosine conversion less than 98% were omitted from the analysis. DNA sequencing was performed using the ABI BigDyeTerminator Cycle Sequencing Kit v3.1 according to the manufacturer's instructions on a Gene Amp 9700 PCR machine and the sequences were detected on an ABI 3130XL Genetic Analyzer.

### Antibodies

Rabbit polyclonal antibodies to Pparγ2 (Cat. ab45036, Abcam, GB) was used as primary antibody. Alexa Fluor® 633 Fab-fragment of goat antibodies to rabbit light and heavy immunoglobulin chains (Cat. A-31576, ThermoFisher Scientific, USA) served as secondary antibody.

### Statistical analysis

The results are presented as mean values with standard deviations calculated with Microsoft Office Excel 2010 software. One-way ANOVA (Analysis of variance) with post-hoc Tukey HSD (Honestly significant difference) test was applied to calculate statistical significance. All experiments were performed in triplicate. Fluorescence intensity in captured images was quantified with Zeiss LSM software.

## Results

### Exogenous RB is lost in long term culture of the 10T1/2 cells

Earlier we showed that stable transfection of the exogenous RB, containing HA epitope, into polypotent mouse fibroblasts 10T1/2 resulted in production of the protein which was detected in immunoblot by an antibody to the HA epitope in short term culture after selection and cloning.[15] Production of active exogenous pRb in short term culture elevated the ability of these cells to AD. In contrast, production of inactive exogenous protein induced loss of

this ability.[15] Two pairs of primers were used in our work: the forward one was complemented to the HA sequence fused to the exogenous RB, while the reverse primer recognized sequences common for exogenous and endogenous RB (Fig. 1A, B, Table 1). These experiments showed that the exogenous RB was not amplified from the genomic DNA of the 10T1/2 transfected with this gene. Interestingly, genomic DNA of all transfected cell lines in long term culture contained NEO gene (Fig. 1C) and twelve weeks after stable transfection the cells were resistant to G-418.

## Loss of exogenous RB in a long-term culture of 10T1/2 polypotent fibroblasts does not negate their ability to adipocyte differentiation

The 10T1/2 cells transfected with different types of exogenous RB retained the ability to affect levels of AD depending on the type of RB mutant gene they were transfected with (Fig. 1D, E). In connection with the levels of AD, the ΔS/N-3 cells did not show expression of the PPARγ2 RNA, while RNA expression of other AD markers, FABP4 and Adiponectin, was decreased in these cells (Fig. 2A, B). While the ΔS/N-3 cells did not show appearance of Pparγ2, the ΔB/X-9 and Δp34-8 cells hyperproduced this protein as compared to the wild type cells (Fig. 2C). Quantitative evaluation of adipocytic differentiation also revealed significant difference in fat accumulation between ΔS/N-3 and all other cell lineages (Fig. 1D, E).

## Adipogenic differentiation of the 10T1/2 and their derivatives transfected with functional RB is associated with elevated expression of PPARγ2 and other markers of AD

Pparγ2 - master factor of AD as well as other markers of AD - Adiponectin and FABP4, were not detected in undifferentiated 10T1/2 cells, but their expression levels evaluated by RT-PCR were greatly elevated after induction of adipogenic differentiation in the 10T1/2, ΔB/X-9, Δp34-8 cell lines transfected with active types of RB. In contrast, the ΔS/N-3 cells transfected with inactive RB mutant did not show expression of PPARγ2 while expression of Adiponectin and FABP4 in this cell line was lower compared to the cells expressing active types of RB (Fig. 2A). Real time PCR supported results of RT-PCR. Undifferentiated cells did not express PPARγ2 and Adiponectin. Expression of these markers was evident in the wild type 10T1/2 cells and significantly elevated in the ΔB/X-9 and Δp34-8 cell lines after induction of AD (Fig. 2B).

## Immunofluorescent staining revealed an increase in the Pparγ2 production in the cell lines transfected with functional pRb

Immunofluorescent staining the cells of all 4th lines with antibody against Pparγ2 let us to reveal, that this protein is not produced in undifferentiated cells. In 10 days after

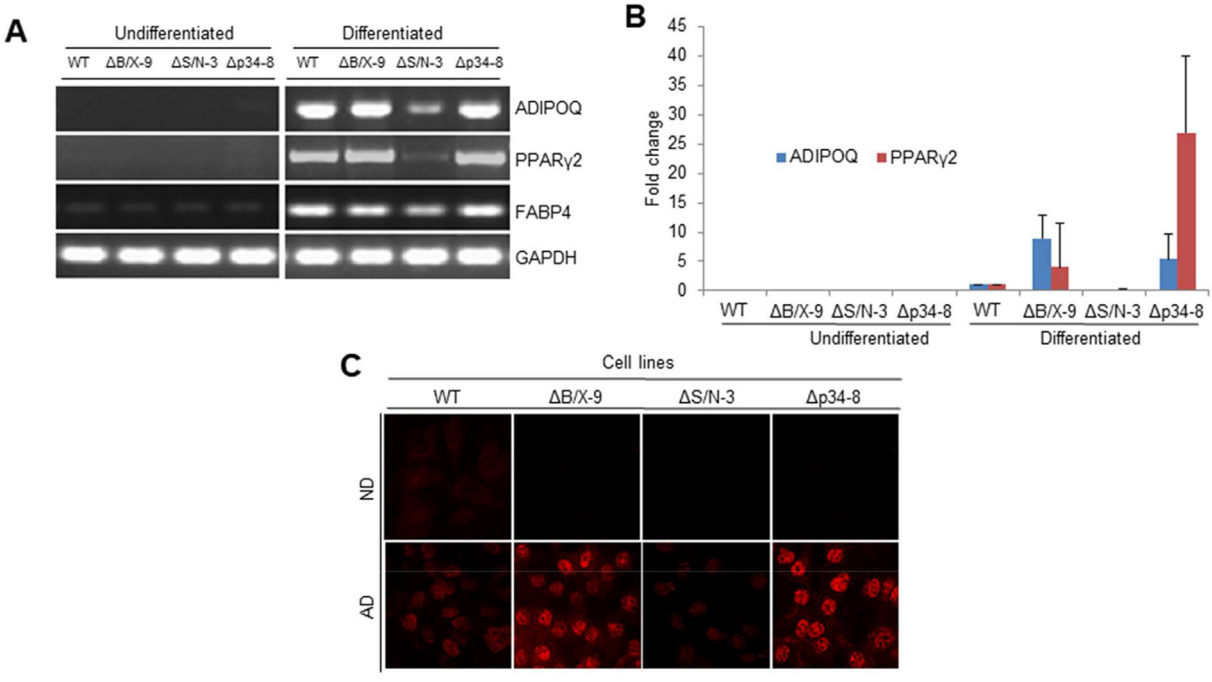


**Figure 2** Pparγ2 is not produced in long culture of the ΔS/N cells. **(A)** The S/N-3 cells induced to AD revealed the low levels or loss of the PPARγ2 and other adipocyte markers RNA expression in RT-PCR. **(B)** Real time PCR showed absence of expression of PPARγ2 and Adiponectin in long term culture of the ΔS/N cells. **(C)** The Pparγ2 is not detected in long term culture of the ΔS/N-3 cells by immunofluorescent staining. Images were captured with digital scanning electron scanning microscope Leica (Carl Zeiss, Germany) using 405 and 633-nm lasers, objective 63x.

induction of AD the wild type 10T1/2 cells showed low level of nuclear staining with antibody against Pparγ2, the ΔB/X-9 and Δp34-8 cells produced much more this protein compared to the wild type cells, while the ΔS/N-3 cells was negative for Pparγ2 staining (Fig. 2C).

## Promoter of the PPARγ2 is hypermethylated in undifferentiated ΔS/N cells

The ΔS/N cells were not sensitive to induction of the AD and did not express the master tissue specific inductor of this type of differentiation - the PPARγ2. We suggested that this might be caused by methylation status of the PPARγ2 promoter due to production of mutated inactive exogenous RB in the ΔS/N cells. To evaluate the idea we assessed the methylation status of the PPARγ2 5' flanking region containing 7 CpG sites localized near the transcription start site (Fig. 3A). We found the slightly increased methylation level of the upstream TSS PPARγ2 promoter under overexpression of ΔB/X and Δp34 RB mutants. In the case overexpressed ΔS/N inactive pRb, this region was heavily methylated (Fig. 3B). In the presence of differentiation stimulus, the CpG sites located upstream of the TSS in ΔB/X-9 and Δp34-8 cell lines adipocytes became mostly demethylated, however, the cells overexpressing the ΔS/N inactive pRb variant shown pronounced demethylation of the CpG at −437/−247 but continued the CpG methylation found in almost all plasmid clones analyzed at −60 position of PPARγ2 proximal promoter (Fig. 3B). The CpGs are located downstream of the TSS, at positions +89 and + 158, were methylated in the cells treated with all pRb variants even despite on the cells differentiation state. We suggest that these methylated CpG sites do not participate in PPARγ2 mRNA expression, rather might be involved in alternative splicing of PPARγ2 gene, due to proximity to the boundary of exon1/intron of PPARγ2 isoform (Fig. 4A). Interestingly, the CpG at −247 position mainly avoid the methylation. We propose that hypermethylated status of the PPARγ2 proximal promoter, resistance of the CpG at −60 position to demethylation, and inability the cells to differentiate, resulted from production of inactive ΔS/N-3 mutant form of exogenous pRb.

## Discussion

Having established the stably transfected cell line we found the absence of expression of exogenous pRb in 12 weeks. Despite this fact, the cells retained their traits in respect to AD. Keeping in mind the loss of ability of ΔS/N cells to differentiate, we decided to examine whether this phenomenon is linked with PPARγ2 promoter methylation and the gene expression silencing. In addition, taken into consideration that DNA methylation patterning is involved in establishing of the cellular memory on epigenetic level, we made the attempt to evaluate the proneness of do not expressing exogenous proteins cell lines to AD.

It is known that promoters of adipogenic genes including *Leptin*, *PPARγ2*, *fatty acid binding protein (FABP)* and *lipoprotein lipase (LPL)* are globally hypomethylated in humiption factoan ASCs (MSCs from fat tissue) with about 5−30% of CpGs being methylated.[17] An epigenetic mechanism of gene expression regulation is involved in Pparγ2 functioning and the expression of PPARγ2 was shown to be regulated by proximal promoter methylation, repressing PPARγ2 transcription in NIH/3T3 fibroblasts and to a lesser extent in 3T3-L1 preadipocytes.[18] We applied the PCR bisulphite sequencing approach to assess the methylation status of the *PPARγ2* promoter region in the 10T1/2 cells transfected with different RB mutant forms. Overexpression of ΔB/X and Δp34 did not have any significant impact on the level of upstream TSS 5' flanking region promoter methylation. The cells exposed to excessed ectopic expression of these modified pRb forms were prone to AD (Fig. 1D, E). The extensive CpG methylation under conditions of undifferentiation was observed in the cells transfected with ΔS/N RB construct. These cells also showed a pronounced decrease in the level of demethylation as compared to other cell lines under differentiation conditions (Fig. 3B).

DNA methyltransferase 1 (DNMT1) is the enzyme responsible for the transfer methyl group to the cytosine of CpG dinucleotides. It has been found that the absence of the pRb in $RB^{-/-}$ murine cells of epithelial or fibroblast origin resulted in elevation of the DNMT1 level and CpG hypermethylation of the gene promoters regulated through pRb/E2f signalling.[19] In the case of the ΔS/N pRb overexpression, the extensive CpG methylation of the pRb/E2f regulated *PPARγ2* promoter also has been observed. The effect is comparable with the $Rb^{-/-}$ cell state. Based on this fact we suggest that the ΔS/N pRb when overexpressed exerts a dominant negative effect on the activity of the wild-type gene in the ΔS/N-3 cells, eliciting the hypermethylation of the *PPARγ2* promoter.[20] The PPARγ2 is capable to direct the local demethylation surrounding the PPARγ response elements (PPREs) during AD.[21] PPARγ2 is able to autoregulate its own expression due to existing the binding sites for PPARγ2 found at the Pparg loci.[22] Thus, the transient exposure to adipogenesis triggers promotes local erasure of the CpG methylation thereby removing promoter-methylated repressive state. However, to our surprise, the CpG at −60 position was almost fully methylated (Fig. 3B). So, it appears, that an individual CpG methylation status might be crucial for transcription machinery to start initiation when being located in the proximity of the TSS. It is interesting to note, that adult rat offspring from a high-fat diet exhibited sustained hypermethylation of the two of four CpGs (−215/−199) of the *PPARγ2* promoter, which in a similar way was affected by maternal obesity. Both CpGs were hypermethylated at postnatal day 21 (PND21), whereas only the CpG at −199 remained hypermethylated at 9 months (9M).[23] The expression level of mRNA at PND21 and 9M correlated with hypermethylated status of CpG.

Notably, although both PPARγ1 and PPARγ2 are regulated through pRb/E2f signal pathway, the E2f binding site (GCGGGAAA) was found only in PPARγ1.[24,25] We suggest that conservative sequence GGCGACA (Fig. 4A, B) may play the role of the E2f binding site in the PPARγ2. This putative E2f site harbours the CpG dinucleotide that could be epigenetically affected and contributed to the PPARγ2 expression through the pRb/E2F pathway. However, the −60 CpG location is found only in mice. Another CCAAT transcription element is a putative CCAAT/

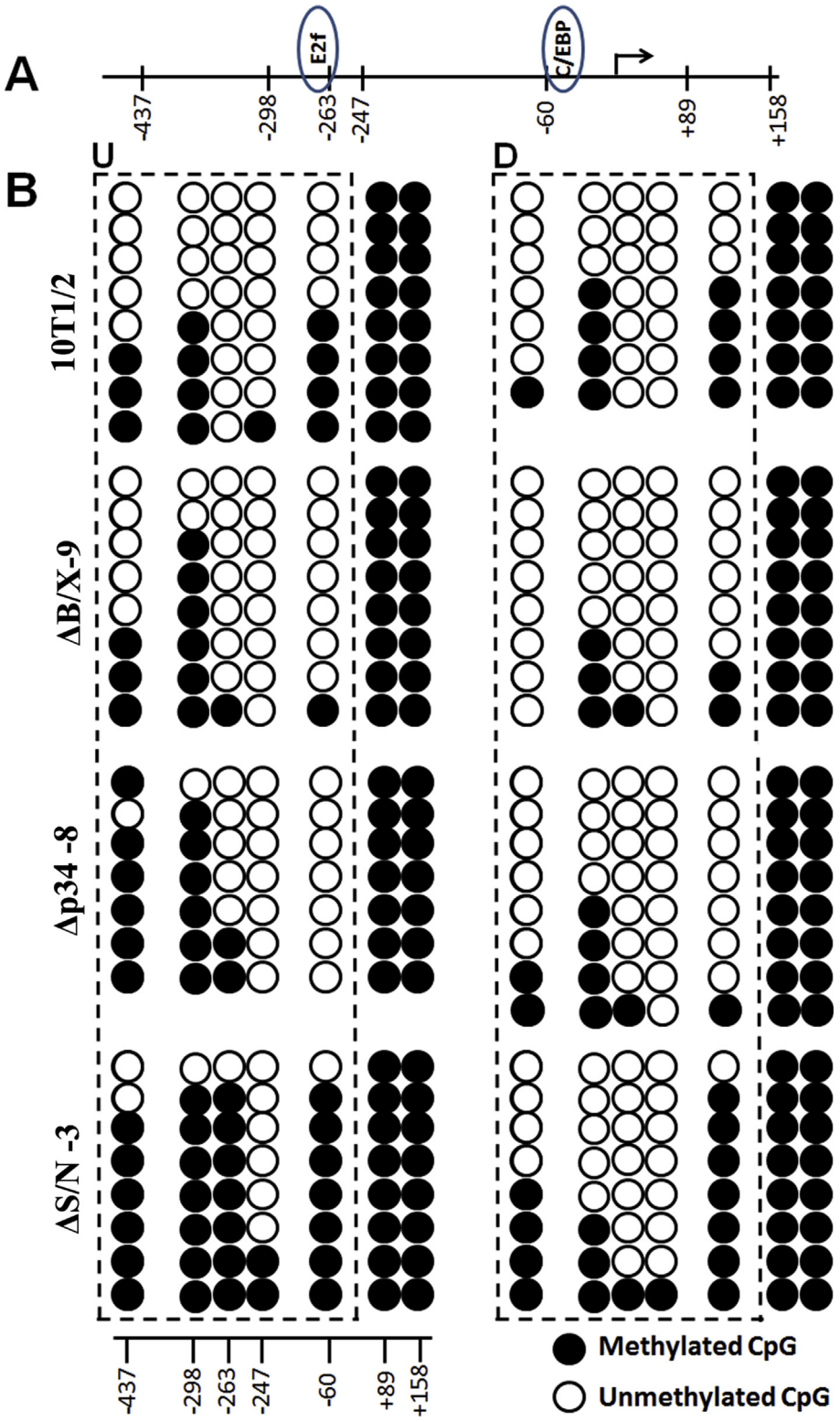


**Figure 3** Promoter of the PPARγ2 is highly hypermethylated in undifferentiated ΔS/N cells. **(A)** Schematic illustration of the PPARγ2 promoter. An arrow indicates the transcription start site (TSS) (+1 bp); short vertical lines indicate the positions of the CpG relative to the TSS. **(B)** Bisulfite sequencing analyses of the PPARγ2 promoter. Methylation status of the PPARγ2 in cells producing exogenous ΔB/X, Δp34 or ΔS/N was analyzed: (U) under undifferentiated conditions; (D) upon differentiation stimulus. The regions marked by dash lines represent the 5′upstream TSS region. At the bottom of the illustrations, the methylation status of the CpGs is shown.

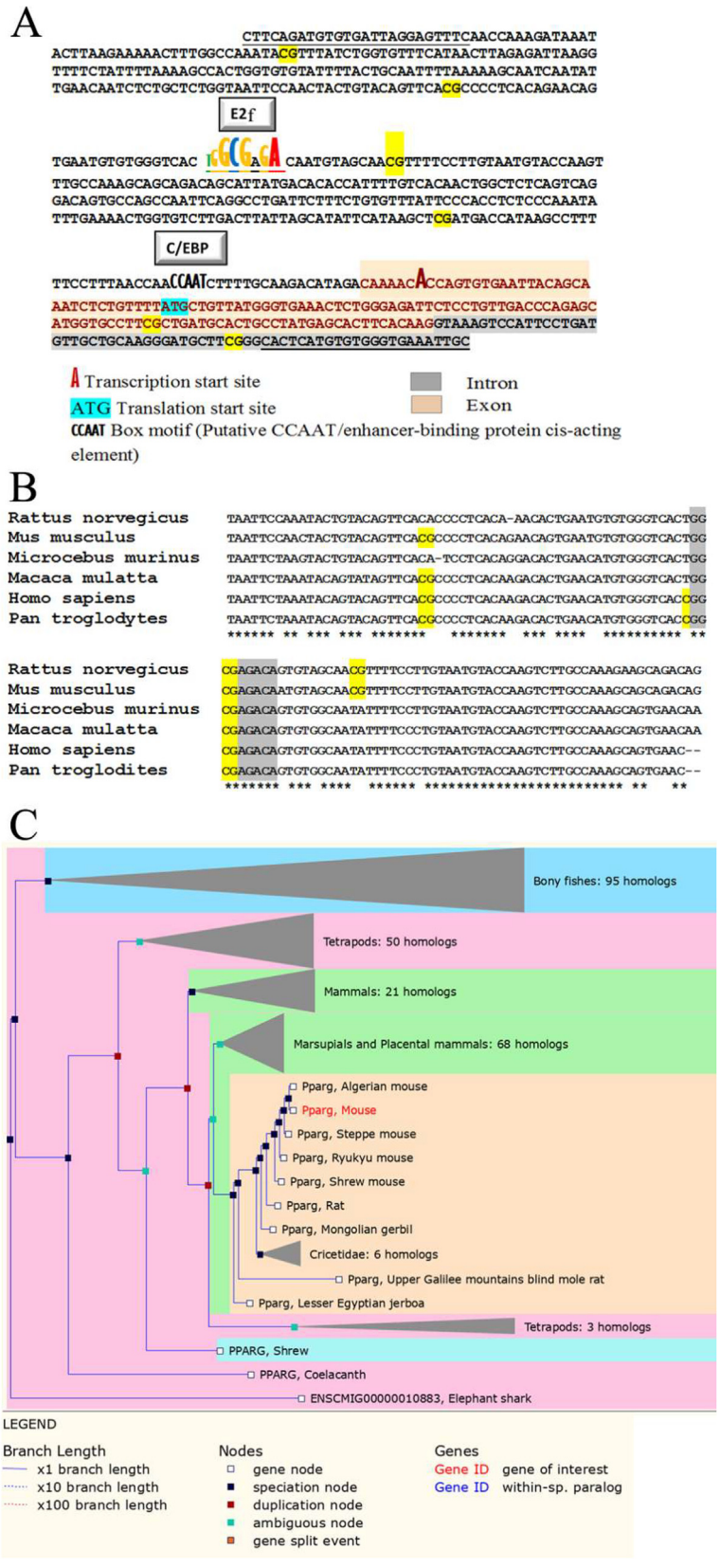


**Figure 4** PPARγ2 promoter region studied. (**A**) −500/+185 PPARγ2 5′flanking region. (**B**) A multiple sequence alignment. Conserved sequences of the putative E2f and CpGs in PPARγ2 promoter of different species are shown. The PPARγ2 promoter sequences of human (*Homo sapiens*), troglodyte (*Pan troglodytes*), monkey (*Macaca mulatta*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), the gray mouse lemur (*Microcebus murinus*), were aligned for conserved sequences analysis. All the PPARγ2 promoter sequences are from Genbank database. (**C**) Phylogenetic tree of the nuclear receptor class II superfamily of Pparγ transcription factor. Phylogenetic tree constructed based on the alignment of partial PPARγ2 gene sequences using the maximum likelihood method.

enhancer binding protein (C/EBP). The transcription factor binding site for this protein is located downstream of −60 position in mouse PPARγ2 but it is disrupted in the rat sequences. This CCAAT box might be essential for C/EBPs-driven upregulation of the PPARγ2 under AD, and in the case of its inaccessibility, AD might be blocked. Comparative genomic analysis was performed and showed that PPARγ2 is evolutionarily conserved across mammalian species (Fig. 4C). The rat CpG (−215), mouse (−263) as well as their counterparts of aligned species are located in putative E2f transcription factor binding motif (Fig. 4A, B).

DNA methylation as a part of epigenetic process may regulate alteration of chromatin conformation and cause transcriptional repression of genes in a context-dependent manner. The way these methylation marks interpreted into different functionalities is one of the principal mechanisms through which the genes are regulated. The mechanism typically involves different types of "reader" proteins that can recognize and bind to the methylated cytosine. In murine cells, Dnmt1 affects the histone deacetylase activity of Hdacs, suggesting that DNA methylation generate an altered chromatin state via histone deacetylase activity towards inheritance of the repressive heterochromatin state.

Introduction of the exogenous RB into 10T1/2 polypotent fibroblasts does not generate the stable system with exogenous pRb because the exogenous pRb was lost in the long term culture. However, the functional consequences of transient expression of the exogenous pRb do exist for a long time. Presumably, the cells transfected with different type of mutant RB retained the epigenetic mechanism that was turned on by the exogenous RB and continued to promote the methylation status of PPARγ2 and levels of AD in long term culture after loss of the exogenous RB. Based on the ability to induce the hypermethylation of *PPARγ2* proximal promoter under normal cell conditions and on the insensitivity the ΔS/N cells to differentiate upon adipogenic stimulus, we temp to speculate that the ΔS/N pRb acts as a dominant negative mutant and is able to promote the formation of DNA methylation-dependent stably repressed inherited chromatin state. This suggestion is supported by inaccessibility of demethylating enzymes to convert 5-methylcytosin to cytosine at −60 position. We propose that the ΔS/N pRb mutants may represent a useful model to investigate the interrelationship between DNA methylation and stably repressed inherited chromatin state in mouse 10T1/2 polypotent fibroblasts. Based on the ability of transient production of the active and inactive pRb forms to retain their promoting and suppressive effect on AD and to maintain the PPARγ2 promoter methylation status, for the first time, we provide evidence that pRb is involved in the establishment of hereditary epigenetic memory, at least by creating a methylation pattern of *PPARγ2*.

## Author contributions

M.B., N.P. and B.P. designed and performed experiments, analyzed and interpreted data; V.R. assisted with collecting data; M.B and B.P. wrote, revised and edited the manuscript, provided resources.

## Conflicts of interests

The authors declare no competing interests.

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## References

1. Weintraub SJ, Chow KN, Luo RX, Zhang SH, He S, Dean DC. Mechanism of active transcriptional repression by the retinoblastoma protein. *Nature*. 1995;375(6534):812—815.
2. Weinberg RA. The retinoblastoma protein and cell cycle control. *Cell*. 1995;81(3):323—330.
3. Sherr CJ. Cancer cell cycles. *Science*. 1996;274:1672—1677.
4. Nevins JR. The Rb/E2F pathway and cancer. *Hum Mol Genet*. 2001;10(7):699—703.
5. Dyson NJ. RB1: a prototype tumor suppressor and an enigma. *Genes Dev*. 2016;30(13):1492—1502.
6. Calo E, Quintero-Estades JA, Danielian PS, Nedelcu S, Berman SD, Lees JA. Rb regulates fate choice and lineage commitment in vivo. *Nature*. 2010;466(7310):1110—1114.
7. Hallenborg P, Feddersen S, Madsen L, Kristiansen K. The tumor suppressors pRB and p53 as regulators of adipocyte differentiation and function. *Expert Opin Ther Targets*. 2009;13(2):235—246.
8. Timmons JA, Wennmalm K, Larsson O, et al. Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. *Proc Natl Acad Sci U S A*. 2007;104(11):4401—4406.
9. Chen PL, Riley DJ, Chen Y, Lee WH. Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. *Genes Dev*. 1996;10(21):2794—2804.
10. Landsberg RL, Sero JE, Danielian PS, Yuan TL, Lee EY, Lees JA. The role of E2F4 in adipogenesis is independent of its cell cycle regulatory activity. *Proc Natl Acad Sci U S A*. 2003;100(5):2456—2461.
11. Hansen JB, Jørgensen C, Petersen RK, et al. Retinoblastoma protein functions as a molecular switch determining white versus brown adipocyte differentiation. *Proc Natl Acad Sci U S A*. 2004;101(12):4112—4117.
12. Longworth MS, Dyson NJ. pRb, a local chromatin organizer with global possibilities. *Chromosoma*. 2010;119(1):1—11.
13. Litovchick L, Sadasivam S, Florens L, et al. Evolutionarily conserved multisubunit RBL2/p130 and E2F4 protein complex represses human cell cycle-dependent genes in quiescence. *Mol Cell*. 2007;26(4):539—551.
14. Popov BV, Watt SM, Rozanov IM, Chang LS. A pocket pRb mutation induces the increase in its affinity to E2F4 coupled with activation of muscle differentiation. *Mol Biol (Mosk)*. 2010;44(2):323—334.
15. Popov BV, Shilo PS, Zhidkova OV, Zaichik AM, Petrov NS. Experimental model to study the role of retinoblastoma gene product (pRb) for determination of adipocyte differentiation. *Bull Exp Biol Med*. 2015;159(2):289—292.
16. Pinney DF, Emerson CP Jr. 10T1/2 cells: an in vitro model for molecular genetic analysis of mesodermal determination and differentiation. *Environ Health Perspect*. 1989;80:221—227.

17. Collas P, Noer A, Sørensen AL. Epigenetic basis for the differentiation potential of mesenchymal and embryonic stem cells. *Transfus Med Hemother*. 2008;35(3):205—215.
18. Fujiki K, Kano F, Shiota K, Murata M. Expression of the peroxisome proliferator actived receptor gamma gene is repressed by DNA methylation in visceral adipose tissue of mouse models of diabetes. *BMC Biol*. 2009;7:38.
19. McCabe MT, Davis JN, Day ML. Regulation of DNA methyltransferase 1 by the pRb/E2F1 pathway. *Cancer Res*. 2005; 65(9):3624—3632.
20. Herskowitz I. Functional inactivation of genes by dominant negative mutations. *Nature*. 1987;329(6136):219—222.
21. Fujiki K, Shinoda A, Kano F, Sato R, Shirahige K, Murata M. PPARγ-induced PARylation promotes local DNA demethylation by production of 5-hydroxymethylcytosine. *Nat Commun*. 2013;4:2262.
22. Wu Z, Rosen ED, Brun R, et al. Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity. *Mol Cell*. 1999;3(2): 151—158.
23. Lecoutre S, Pourpe C, Butruille L, et al. Reduced PPARγ2 expression in adipose tissue of male rat offspring from obese dams is associated with epigenetic modifications. *FASEB J*. 2018;32(5):2768—2778.
24. Fajas L, Egler V, Reiter R, Miard S, Lefebvre AM, Auwerx J. PPARgamma controls cell proliferation and apoptosis in an RB-dependent manner. *Oncogene*. 2003; 22(27):4186—4193.
25. Fajas L, Landsberg RL, Huss-Garcia Y, Sardet C, Lees JA, Auwerx J. E2Fs regulate adipocyte differentiation. *Dev Cell*. 2002;3(1): 39—49.