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Tet1 is required for Rb phosphorylation during G1/S phase transition

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ABSTRACT

DNA methylation plays an important role in many biological processes, including regulation of gene expression, maintenance of chromatin conformation and genomic stability. TET-family proteins convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), which indicates that these enzymes may participate in DNA demethylation. The function of TET1 has not yet been well characterized in somatic cells. Here, we show that depletion of Tet1 in NIH3T3 cells inhibits cell growth. Furthermore, Tet1 knockdown blocks cyclin D1 accumulation in G1 phase, inhibits Rb phosphorylation and consequently delays entrance to G1/S phase. Taken together, this study demonstrates that Tet1 is required for cell proliferation and that this process is mediated through the Rb pathway.

1. Introduction

DNA methylation is an important epigenetic regulatory mechanism. Position 5 in the cytosine ring is the most common methylation site in mammalian cells. However, cytosine residues are not uniformly methylated throughout the human genome. Most cytosine methylation occurs at CpG dinucleotides. DNA methylation regulates genomic stability, chromosome structure and gene expression. Increased levels of methylation at a gene promoter indicate transcriptional inhibition [1,2]. Many tumor cells have an aberrant methylation profile. Loss of global methylation or increase in promoter methylation has been found in various types of cancers. Methylation changes are involved in a series of gene repression events, including tumor suppressor genes such as $p16^{ink4a}$, p21, p53 and RASSF1A [3–5]. DNA methylation has also been used as a novel prognostic marker and drug target [6–9]. However, it is unclear whether disruption of DNA methylation leads to tumorigenesis.

Although DNA methylation has been well studied, the demethylation process remains elusive. There are two types of DNA demethylation: passive and active. Passive demethylation refers to the maintenance of methylation in the daughter strand that is inherited during DNA replication. Active demethylation refers to the enzymatic removal of methyl groups [10,11]. Recently, Rao et al. reported that TET-family proteins could hydroxylate 5mC to 5hmC [12]. Tet1 is a member of the TET family of proteins and is highly expressed in mouse embryonic stem (ES) cells. Depletion of Tet1 in both ES and somatic cells increases DNA methylation levels [13–16]. These data indicate that Tet1 may be involved in active DNA demethylation. TET family members have also been implicated in tumorigenesis. TET2 mutations have been found in myeloid cancers, and loss of Tet2 inhibits blood stem cell differentiation [17–23]. TET1 forms a fusion protein with the product of the MLL gene in AML and is thus thought to be an oncogene [24,25]. However, its biological function remains unknown.

2. Materials and methods

2.1. Cell culture and synchronization

293T and NIH3T3 cells were purchased from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal bovine serum (FBS; HyClone) and penicillin–streptomycin (Invitrogen). Serum depletion was carried out by replacing the complete culture medium with serum-free DMEM for 48 h. Afterwards, the cells were released into fresh complete medium. A double thymidine block was performed in the presence of 2 mM thymidine (Sigma) for 14 h, with a 6-h incubation in thymidine-free medium between the two exposures to thymidine. Cells were harvested and analyzed at various time points after the final release from synchronization.

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2.2. shRNA design, lentivirus production and cell line generation

To knock down Tet1 expression, two specific shRNAs [shRNA3387 (GCAGCTAGCTATAGAGTATAG) and shRNA4915 (GCAGATGGCCGTGACACAAAT)], as well as a scrambled shRNA used as a control (AATTCTCCGAACGTGTCACGT), were subcloned into the lentiviral vector PLKO.1-TRC (Addgene) [26]. To package the lentivirus, 293T cells were co-transfected with the packaging plasmid VSVG, γ8.91 and the PLKO.1 plasmid containing the specific or scrambled shRNA sequence, separately, using Lipofectamine 2000 (Invitrogen). The medium was harvested 48 h after transfection and filtered through a 0.45 μm filter (Pall). Cells were infected with virus and selected in puromycin 48 h after infection (Sigma–Aldrich).

2.3. Cell proliferation assay

For growth curve assays, control and knockdown cells were seeded on 12-well plates at a density of 4×10^4 cells/well. Cells were counted at the indicated times. For soft agar assays, control and knockdown cells suspended in 0.35% agar were layered on 0.8% solidified agar in 3.5 mm dishes at a density of 20,000 cells/dish. After 21 days of incubation, colonies were photographed.

2.4. Western blot

Cells were harvested using trypsin (Invitrogen), according to the manufacturer's instructions. Whole-cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by blotting with specific antibodies. We used the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) for detection. Primary antibodies and dilutions used were as follows: Tet1 (Millipore, 1:500), p-Rb (Bioworld, 1:1000), Rb (Santa Cruz Biotechnology, 1:300), cyclin D1 (Cell Signaling, 1:500), Cdk4 (Cell Signaling, 1:500), p16 (Santa Cruz Biotechnology, 1:500), p27 (Cell Signaling, 1:500), and β-actin (Sigma–Aldrich, 1:5000).

2.5. RNA extraction and real-time PCR

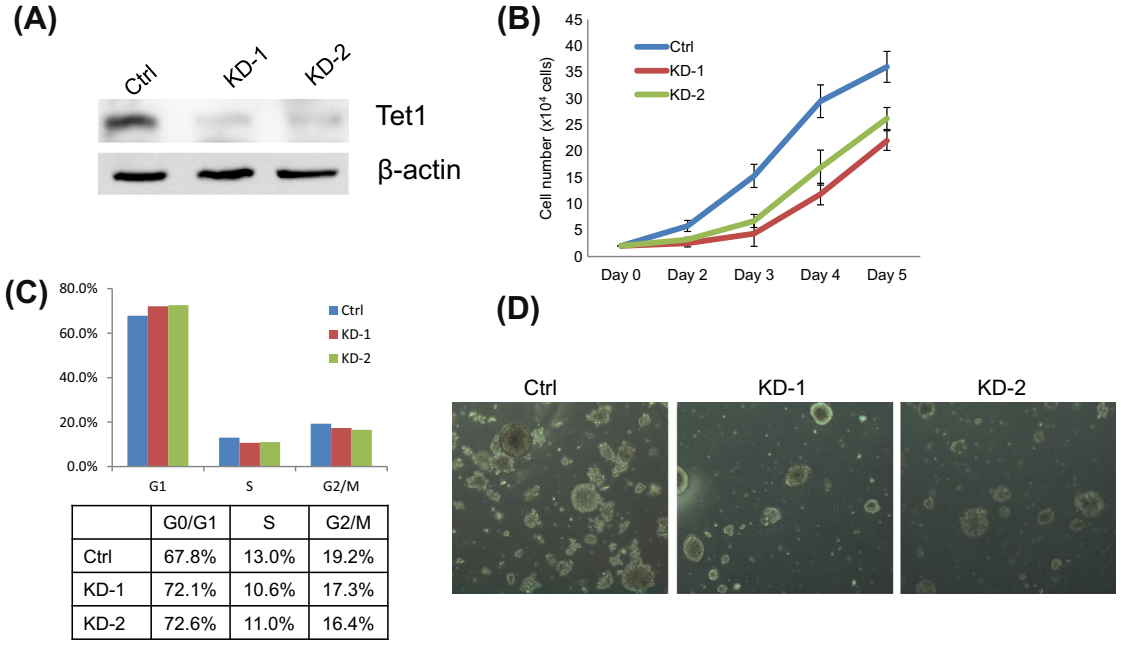
Total RNA was extracted using the RNAprep pure kit (Tiangen). First-strand cDNA was synthesized from 1 μg of RNA using the ReverTra Ace Synthesis kit (Toyobo). For real-time PCR, the *GAPDH* primer sequences were 5′-CTCCTGGAAGATGGTGATGG-3′ and 5′-GTGGCAAAGTGGAGATTGTTG-3′. The *cyclin D1* primer sequences were 5′-AGGAGCAGAAGTGCGAAGAG-3′ and 5′-CACAACTTCTCGGCAGTCAAG-3′.

3. Results

3.1. Tet1 knockdown inhibits NIH3T3 cell proliferation

To deplete Tet1 expression in NIH3T3 cells, we used a stable shRNA knockdown strategy. Two different lentivirus-based shRNA vectors were selected, and the knockdown cell lines were generated. Western blot analysis showed that Tet1 expression was significantly reduced in both knockdown cell lines (Fig. 1A). real-time PCR was performed to ensure Tet2 and Tet3 expression was not affected (Fig. S1).

To determine whether Tet1 is required for NIH3T3 cell proliferation, we compared the growth curves of knockdown and control cell lines. Both knockdown lines had a slower growth rate than the control line (Fig. 1B), indicating that Tet1 expression was required for NIH3T3 cell proliferation. Consistent with this observation, soft agar assays showed that Tet1 knockdown inhibited colony formation of NIH3T3 cells transformed with H-Ras (Fig. 1C). To identify the proliferation defect in knockdown cells, we performed cell cycle and apoptosis analyses. The apoptotic rate was comparable between knockdown and control cell lines (data not shown), whereas the cell populations in S and G2/M phase



	G0/G1	S	G2/M
Ctrl	67.8%	13.0%	19.2%
KD-1	72.1%	10.6%	17.3%
KD-2	72.6%	11.0%	16.4%

Fig. 1. Tet1 knockdown inhibits NIH3T3 cell proliferation. (A) Two Tet1 knockdown cell lines were generated by lentiviral infection. Tet1 expression was tested 72 h after infection by Western blot. (B) The growth rates of Tet1 knockdown cells were compared to control cells. (C) Cell cycle distribution was analyzed by flow cytometry. Unsynchronized control or knockdown cells were stained with PI and analyzed by flow cytometry. (D) Control and knockdown cells were transformed with H-Ras and assayed for their colony formation ability in soft agar.

were increased after knockdown. Based on these results, we concluded that Tet1 was required for NIH3T3 cell proliferation.

3.2. Tet1. Knockdown inhibits G1/S phase entry

The G1/S phase transition is determined by passage through the restriction point (R-point). In G1 phase, cells require cellular growth factors to pass the R-point. After the R-point, cells irreversibly commit to DNA replication. Phosphorylation of the retinoblastoma protein (Rb) is the key regulator of this process. R-point control blocks cell cycle progression by inhibiting Rb phosphorylation [27,28].

To find whether the observed proliferation inhibition is due to a G1/S phase transition defect, we synchronized cells using 48-h serum starvation. Afterwards, cells were released into complete medium, collected at the indicated times and subjected to cell cycle analysis. More than 50% of the control cells had entered S phase, compared to 30% of the knockdown cells (Fig. 2). These data demonstrated that Tet1 knockdown resulted in an increase in quiescent cells. We also performed a double thymidine block assay to find out whether knockdown cells had a slower S-to-M phase transition. We found that more control cells entered the cell cycle, but the time in each phase of the cell cycle was comparable (Fig. S2).

3.3. Tet1. knockdown results in Rb hypophosphorylation

Because Rb regulates the transition from G0 into G1 phase, and from G1 into S phase, we examined Rb expression and its phosphorylation levels. Similar to our cell cycle results, both knockdown cell lines had lower Rb phosphorylation levels compared to control cells (Fig. 3A). To study the cause of Rb protein hypophosphorylation in knockdown cells, we analyzed the expression of the Cdk4-cyclin D1 phosphorylation complex, as well as its inhibitors (p16, p27). Even though cyclin D1 mRNA levels decreased dramatically (Fig. 3B), the protein levels were comparable to control cells. It is possible that degradation of cyclin D1 protein during the cell cycle accounts for these changes. To determine whether the protein level of cyclin D1 was decreased in early G1 phase, cell synchronization through serum-starvation was required. The protein levels of Cdk4, p16, and p27 did not change after knockdown.

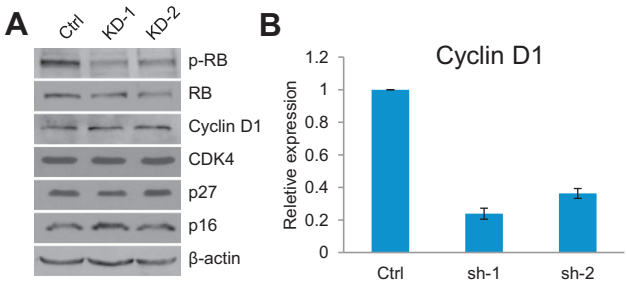


Fig. 3. Tet1 knockdown results in Rb hypophosphorylation. (A) Unsynchronized control or knockdown cells were subjected to Western blot analysis using the indicated antibodies. β-actin served as loading control. (B) cyclin D1 expression levels were examined by real-time PCR.

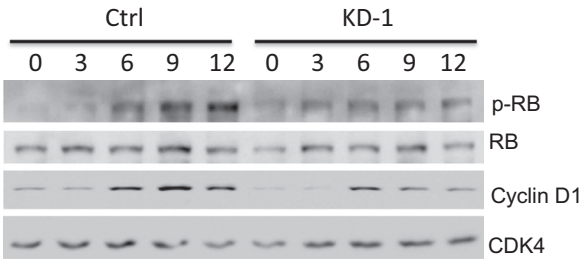


Fig. 4. Tet1 knockdown inhibits cyclin D1 accumulation and Rb phosphorylation in G0/G1 phase. Synchronized control or knockdown cells were released and subjected to Western blot analysis using the indicated antibodies. Cdk4 served as loading control.

3.4. Tet1 knockdown inhibits cyclin D1 accumulation and Rb phosphorylation in G0/G1 phase

Synchronized cells were released and harvested at the indicated times, and Rb phosphorylation levels were examined. As expected, total Rb levels remained constant in both control and knockdown cells. Rb phosphorylation increased 6 h after release in control cells, but was inhibited in knockdown cells. In contrast to unsynchronized cells, cyclin D1 accumulation was inhibited after Tet1 knockdown. CDK4, the enzymatic complex partner of cyclin D1, served as a loading control (Fig. 4).

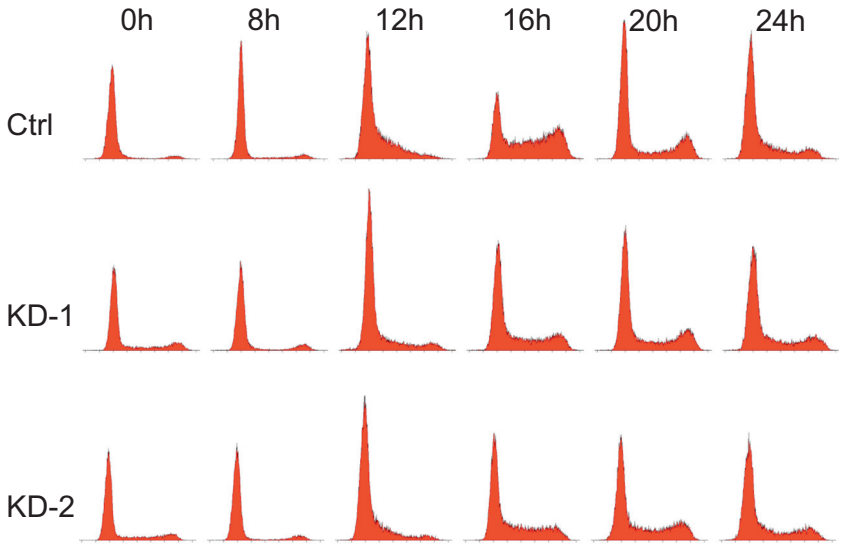


Fig. 2. Tet1 knockdown inhibits G1/S phase entry. Synchronized control or knockdown cells were released and analyzed at the indicated time by flow cytometry.

4. Discussion

Overall, we found that Tet1 knockdown blocked cyclin D1 accumulation in G1 phase. Decreased cyclin D1 levels resulted in Rb hypophosphorylation and inhibited cell proliferation. Whether Tet1 regulates cyclin D1 transcription remains an open question. Recently, it was reported that TET2-overexpressing melanoma cells had decreased 5mC and elevated 5hmC levels in cyclin D1 [29]. Therefore, we speculated that Tet1 might regulate cyclin D1 transcription, which would depend on Tet1's hydroxylase activity.

We demonstrated that Tet1 is required for NIH3T3 cell proliferation. However, results from our group and others showed that Tet1 depletion did not inhibit ES cell proliferation, even though ES cells express very high levels of Tet1. We believe that this is because ES cell proliferation is not regulated by the Rb pathway [30]. Jaenish group reported that Tet1 knockout did not impair mouse development [31]. We hypothesize that constitutive Tet1 knockout may cause a compensatory effect by other TET family members, such as Tet2 or Tet3. We have generated a conditional knockout mouse to avoid any potential compensation. This mouse can be used to test our hypothesis in future studies. Meanwhile, conditional inhibition of Tet1 expression in different organs is required to elucidate its role in organ development and tumorigenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.110.

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