Biochemical and Biophysical Research Communications 462 (2015) 208–214

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

BTG/Tob family members Tob1 and Tob2 inhibit proliferation of mouse embryonic stem cells via Id3 mRNA degradation

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ARTICLE INFO

Article history:
Received 13 April 2015
Available online 5 May 2015

Keywords:
Embryonic stem cells
Tob subfamily
Double knockout ESCs
Anti-proliferation

ABSTRACT

The mammalian BTG/Tob family is a group of proteins with anti-proliferative ability, and there are six members including BTG1, BTG2/PC3/Tis21, BTG3/ANA, BTG4/PC3B, Tob1/Tob and Tob2. Among them, Tob subfamily members, specifically Tob1/Tob and Tob2, have the most extensive C-terminal regions. As previously reported, overexpression of BTG/Tob proteins is associated with the inhibition of G1 to S-phase cell cycle progression and decreased cell proliferation in a variety of cell types. Tob subfamily proteins have similar anti-proliferative effects on cell cycle progression in cultured tumor cells. An important unresolved question is whether or not they have function in rapidly proliferating cells, such as embryonic stem cells (ESCs). Tob1 and Tob2 were expressed ubiquitously in mouse ESCs (mESCs), suggesting a possible role in early embryonic development and mESCs. To address the above question and explore the possible functions of the Tob subfamily in ESCs, we established ESCs from different genotypic knockout inner cell mass (ICM). We found that Tob1$^{-/-}$, Tob2$^{-/-}$, and Tob1/2 double knockout (DKO, Tob1$^{-/-}$ & Tob2$^{-/-}$) ESCs grew faster than wild type (WT) ESCs without losing pluripotency, and we provide a possible mechanistic explanation for these observations: Tob1 and Tob2 inhibit the cell cycle via degradation of *Id3* mRNA, which is a set of directly targeted genes of BMP4 signaling in mESCs that play critical roles in the maintenance of ESC properties. Together, our data suggest that BTG/Tob family protein Tob1 and Tob2 regulation cell proliferation does not compromise the basic properties of mESCs.

1. Introduction

The mammalian BTG/Tob family is including BTG1, BTG2/PC3/Tis21, BTG3/ANA, BTG4/PC3B, Tob1/Tob and Tob2, a group of anti-proliferative proteins [1–3]. They are characterized by the presence of a conserved N-terminal anti-proliferative domain (APRO domain, also known as BTG domain). The APRO/BTG domain is a protein–protein interaction module, which contains two highly homologous regions, Box A and Box B. Previous structural analysis showed that these regions are critical for complex formation with Caf1a and Caf1b (the enzymatic subunits of the Ccr4-Not complex) [4–8], and this complex is important for anti-proliferative activities [9]. Unlike the conserved N-terminal APRO domain, the C-terminal moiety of these proteins is divergent among the family members, allowing the further classification of those members into three distinct subfamilies: the BTG1/BTG2 subfamily, the BTG3/BTG4 subfamily, and the Tob subfamily [2]. Among them, the Tob subfamily members, specifically Tob1 and Tob2, have the most extensive C-terminal regions, which contains PAM2 motifs that mediate interactions with proteins such as poly(A)-binding proteins [1]. These divergent regions appear to mediate protein–protein interactions that are unique to each family member and important for transcription, mRNA turnover, and other regulatory functions [1,4,6,10].

Orthologs of the human TOB1 and TOB2 genes have been identified in mouse [11,12], chicken [13], and frog [14]. However, only a single Tob-related gene has been identified in the invertebrate species *Caenorhabditis elegans*, fog-3 [15,16], *Drosophila melanogaster* [17], and *Branchiostoma floridae* [18]. Mouse Tob1 is expressed maternally and continuously throughout embryonic development [11]. Tob2 is also expressed ubiquitously in mouse

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http://dx.doi.org/10.1016/j.bbrc.2015.04.117
0006-291X/

embryos [12], suggesting a possible role in early embryonic development [14].

As previously reported, expression of BTG/Tob proteins results in the inhibition of the cell cycle progression [1,2,19,20]. Consistent with the anti-proliferative activity, loss of expression of BTG2, BTG3, and TOB1 is frequently observed in clinical samples of various cancers, including thyroid, breast and lung tumors [21–24]. Supporting a causal relationship between loss of expression and disease, gene inactivation of BTG3 and Tob1 is associated with predisposition to cancer in mouse models [23,25]. Together, all of these findings highlight an important role for the BTG/Tob family proteins as cell cycle regulators and tumor suppressors.

In agreement with their anti-proliferative activity, overexpression of BTG/Tob proteins is associated with the inhibition of G1 to S-phase cell cycle progression and decreased cell proliferation in a variety of cell types [1]. Meanwhile, loss of expression or low expression of TOB1 is frequently observed in clinical samples of lung, thyroid and breast tumor [21,22]. Overexpression of Tob2 in stromal cells repressed vitamin D3-induced osteoclasts formation [26], indicating the similar anti-proliferative effect of this gene. Although clear functional phenotypic expressions of Tob1 and Tob2 were observed in such cultured tumor cells, what were their function in this kind of rapid proliferating cells, such as embryonic stem cells (ESCs). Embryonic stem cells are originated from the inner cell mass of a blastocyst stage embryo. They can proliferate indefinitely, maintain an undifferentiated state (self-renewal), and differentiate into any cell type (pluripotency). The ability of ES cells to maintain the self-renewal and pluripotency is associated with their ability to remain in a proliferative condition, which requires a unique transcriptional profile. ES cells divide rapidly with a short generation time of approximately 8–10 h in mouse ES cells [27,28], and 8–16 h in human ES cells [29].

To address the above question and explore the possible functions of the Tob subfamily in mESCs, we established ESCs from WT, Tob1−/−, Tob2−/− and DKO ICM. In this study, we investigated BTG/Tob family protein Tob1 and Tob2 inhibit proliferation of mESCs and tested our hypothesis that Tob1 and Tob2 may be involved in the degradation of Id3 in mESCs.

2. Materials and methods

2.1. Mice

All animal handling and procedures were approved by the Institutional Animal Care and Use Committee at the Peking University Health Science Center.

2.2. Protein extraction, Western blot

Cells were lysed in cold lysis buffer containing protease inhibitors (Roche). Western blotting was carried out by standard procedures; primary antibodies used for detection were anti-Tob1 (Santa Cruz, sc-18549), anti-Tob2 (Abcam, ab179760), anti-Oct4 (Santa Cruz, sc-365509), anti-Nanog (Cosmo BioCo, REC-RCAB0001P), and anti-Sox2 (Santa Cruz, sc-20088). Proteins were visualized with an Odyssey Two-Color Infrared Imaging System (LI-COR Biosciences) according to the manufacturer's instructions.

2.3. Immunofluorescence microscopy

Cells on cover slips were fixed in cold methanol for 15 min. After three washes in phosphate buffered saline, cells were blocked in phosphate buffered saline containing 5% bovine serum albumin for 30 min and then incubated with primary antibodies in phosphate buffered saline containing 2.5% bovine serum albumin for 2–12 h at 37 °C, anti-SSEA1 (Millipore), anti-Oct4 (Santa Cruz, sc-365509), anti-Nanog (Cosmo BioCo, REC-RCAB0001P), or anti-Sox2 (Santa Cruz, sc-20088). After three washes, cells were incubated with FITC (fluorescein isothiocyanate)-conjugated α-mouse and PE-conjugated α-rabbit secondary antibodies for 1 h at 37 °C and then with DAPI (Roche) for 15 min. The cover slips were washed extensively and mounted onto slides. Imaging of the cells was carried out using a Zeiss LSM 510 Meta Confocal Microscope.

2.4. Statistics

The Student's t-test was used for a comparison of two independent treatments. The two-way ANOVA statistical test was used for analysis of CCK8 results. The paired samples t-test was used to analyze the teratoma data. For all tests, a p-value < 0.05 was considered significant (**).

3. Results

3.1. Derivation of Tob knockout mouse embryonic stem cells

Tob1 protein was detectable and localized mainly in the cytoplasm of mouse ESCs (Fig. 1A). In addition, Tob2 protein had the similar expression levels in mouse ESCs (Fig. 1B). Then we obtained blastocysts from four different genotypic mice, which are Tob1 null [25] and Tob2 null mice [26]. We obtained 4–8 ES cell lines of each genotype of knockout mice, and all of the results were based on at least three cell lines with independently reproducible results. The morphology of ES colonies was round and multilayered with definite boundaries, and there was no visible difference between the wild type ESCs and the other three knockout ESC cell lines (Fig. 1C). We performed immunoblotting analyses to measure whether Tob1 and Tob2 protein were successfully knocked out (Fig. 1D). Finally, The results indicate that the wild type ESCs and the other three knockout ESC cell lines are positive for Oct4, Nanog, Sox2 and SSEA1 (Fig. 1E). So far, we have successfully generated the single and double knockout ESCs.

3.2. Loss of Tob subfamily members had no effect on ESCs pluripotency

We performed alkaline phosphatase (AP) staining, an indicator assay for undifferentiated ESCs, and found that three knockout ESCs were almost 100% AP-positive (Fig. 2A). We also measured the expression of pluripotent factors including Oct4, Nanog, Sox2 and stage-specific embryonic antigen-1 (SSEA-1); we found that there was no obvious difference between them as observed through immunoblotting analysis (Fig. 2B) or immunofluorescence analysis (Fig. 1C). This data demonstrates that the expression of the pluripotent marker of Tob1$^{-/-}$, Tob2$^{-/-}$, and DKO ESCs was similar to that seen in WT ESCs.

We further examined the pluripotency of ESCs by teratoma formation. We obtained tumors with WT, Tob1$^{-/-}$, Tob2$^{-/-}$, and DKO ESCs after subcutaneous injection into SCID-nude mice (Fig. 2C). We measured the weight of every tumor formed from the ESCs, and found that the tumor size of three knockout ESCs was larger than that formed in WT cells (Fig. 2D). In our statistical analysis, we observed significant difference between the Tob2$^{-/-}$, DKO and control WT groups (paired samples t-test, $p < 0.05$); however no significant difference was observed between the single knockout and double knockout groups. Histological examination revealed that WT, Tob1$^{-/-}$, Tob2$^{-/-}$, and DKO clone differentiated

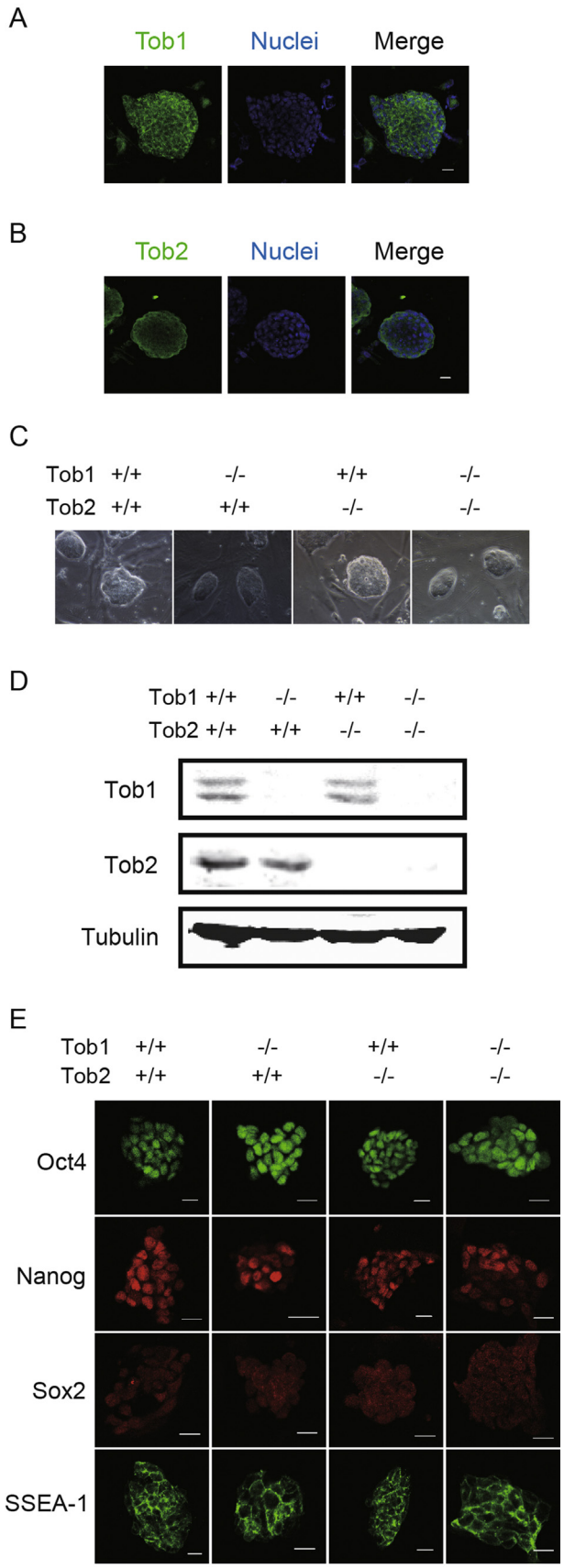

Fig. 1. Derivation of Tob knockout mouse embryonic stem cells (A) Immunofluorescence staining for Tob1 in mouse ESCs. Scale bars: 20 μm. (B) Immunofluorescence staining for Tob2 in mouse ESCs. Scale bars: 20 μm. (C) Phase-contrast images of WT, Tob1$^{-/-}$, Tob2$^{-/-}$, and DKO ESCs on MEFs. (D) Western blot analysis of Tob1 and Tob2 in WT, Tob1$^{-/-}$, Tob2$^{-/-}$, and DKO ESCs. Tubulin was used as a loading control. (E) Immunofluorescence staining for Oct4, Nanog and Sox2 in WT, Tob1$^{-/-}$, Tob2$^{-/-}$, and DKO ESCs. Scale bars: 20 μm.

into all three germ layers (Fig. 2E). This data confirmed that all of the knockout ESCs, including Tob1$^{-/-}$, Tob2$^{-/-}$, and DKO ESCs, did not lose pluripotency.

3.3. Tob subfamily members had an anti-proliferative effect on ESCs

Although all of the ESC lines showed the normal three germ layer differentiation, the size of teratoma increased in Tobs knockout lines as compared to the WT line. Meanwhile, different studies reported that overexpression of BTG/Tob proteins was associated with the inhibition of G1 to S-phase cell cycle progression and decreased cell proliferation in a variety of cell types [1]. To further examine the growth of those ESCs, we conducted a cell proliferation assay using the cell counting kit-8 (cck-8) assay. The results showed that the three knockout ESCs had higher cell counts than WT ESCs (Fig. 3A, ±SD, n = 3, two-way ANOVA statistical test, $^{a}p<0.001$ Tob1$^{-/-}$ ESCs vs. WT ESCs, $^{b}p < 0.001$ Tob2$^{-/-}$ ESCs vs. WT ESCs, and $^{c}p < 0.001$ DKO ESCs vs. WT ESCs). Since the BTG/Tob family protein can regulate cell cycle progression in a variety of cell types and cell cycle stages distributions, we tested whether the same situation occurred in ESCs by performing cell cycle analysis and found that there was 4–6% more S-phase cells in three knockout ESCs than in control WT ESCs (Fig. 3B). The percentage of S-phase cells had a significant difference between Tob1$^{-/-}$, Tob2$^{-/-}$, DKO and the control WT groups (student's t-test, $p < 0.05$). This data suggests that Tob1$^{-/-}$, Tob2$^{-/-}$, and DKO ESCs proliferate faster than control ESCs.

3.4. Tob1 and Tob2 repress production of Id3 via mRNA degradation

We used DNA microarrays to analyze the differential gene expression pattern of four mESCs. A total number of 45,037 probe sets corresponding to 33,994 known or predicted genes was detected using Affymetrix Mouse Genome 430 2.0 Array. There were 223 up-regulated and 146 down-regulated genes in Tob1 knockout mESCs, and 44 up-regulated and 89 down-regulated genes in Tob2 knockout mESCs. In addition, 51 up-regulated and 94 down-regulated genes were detected in DKO mESCs (data not shown). According to the results of the overlapping of gene expression in different mESCs, we found 4 genes that were up-regulated: *Mid1*, *Entpd4*, *Id3* and *Aplnr*. We also found the following six genes to be down-regulated: *Serglycin*, *Mup1*, *Cubilin*, *Cml2*, *Brn-3b* and *Arg2*. Combining the gene expression and the observed proliferation changes of different mESCs with results from other labs' reports, we finally focused on Id3, which was thought to affect the balance between cell growth and differentiation by negatively regulating the function of bHLH transcription factors [30]. We confirmed the Id3 protein level expression using immunoblotting analyses (Fig. 4A) and performed real-time PCR to measure *Id3* mRNA level (Fig. 4B). The results showed that Tob knockout ESCs had a significant increase in *Id3* expression compared with WT ESCs, supporting idea that Tob1 and Tob2 act as repressors of Id3 expression.

Indeed, Tob proteins can bind to both Caf1 and PABP [2], and form a TOB-CAF1-CCR4 complex to exert their anti-proliferative function by modulating mRNA turnover and enhancing deadenylation to degrade their targets. Therefore, *Id3* mRNA might be the target of the Tob1-Caf1-Ccr4 complex in mESCs. To determine

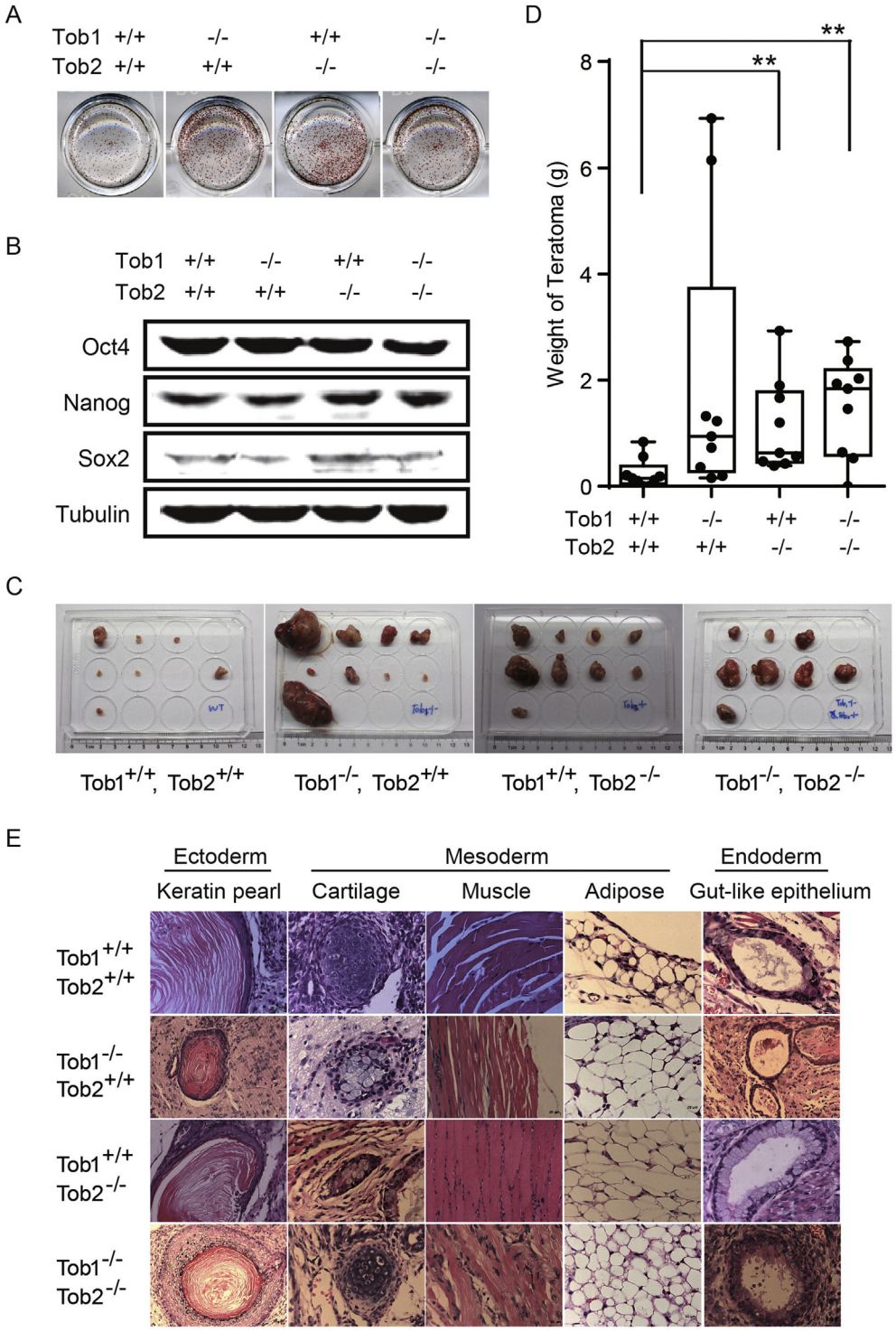

Fig. 2. Loss of Tob subfamily members had no effect on ESCs pluripotency (A) Alkaline phosphatase staining analysis of WT, Tob1$^{-/-}$, Tob2$^{-/-}$, and DKO colonies. (B) Western blot analysis of Oct4, Nanog and Sox2 in WT, Tob1$^{-/-}$, Tob2$^{-/-}$, and DKO ESCs, Tubulin was used as a loading control. (C) Teratoma isolated from the SCID-null mice transplanted with WT, Tob1$^{-/-}$, Tob2$^{-/-}$, and DKO ESCs. (D) Average weight of teratoma derived from WT, Tob1$^{-/-}$, Tob2$^{-/-}$, and DKO ESCs (Student's t-test, **$p < 0.05$). (E) H&E stain of tissue sections in teratomas formed from WT, Tob1$^{-/-}$, Tob2$^{-/-}$, and DKO ESCs, including keratin pearl (ectoderm), cartilage, muscle and adipose (mesoderm) and gut-like epithelium (endoderm).

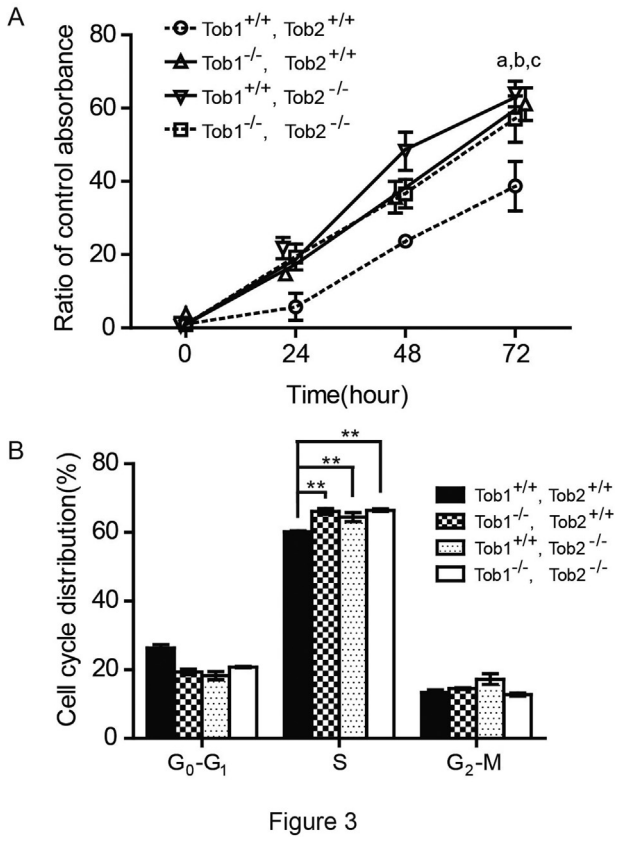

Fig. 3. Tob subfamily members had an anti-proliferative effect on ESCs. (A) CCK-8 analysis of WT, Tob1$^{-/-}$, Tob2$^{-/-}$, and DKO ESCs. Data was representative of three independent experiments (±SD, n = 3, two-way ANOVA statistical test, $^{a}p<0.001$ Tob1$^{-/-}$ ESCs vs. WT ESCs, $^{b}p < 0.001$ Tob2$^{-/-}$ ESCs vs. WT ESCs, and $^{c}p < 0.001$ DKO ESCs vs. WT ESCs). (B) Cell cycle profiles of WT, Tob1$^{-/-}$, Tob2$^{-/-}$, and DKO ESCs were examined by FACS with PI staining; cell numbers were calculated based on DNA content of G0/G1, S, and G2/M phases (student's t-test, $^{**}p < 0.05$).

the half-life of *Id3* mRNA, we performed actinomycin D chase experiments. As shown in Fig. 4C, after adding actinomycin D in the cells to inhibit de novo transcription, we observed an approximately 64.5% increase in Id3 mRNA stability (two-way ANOVA statistical test, $p < 0.05$) in the absence of Tob1. Similarly, an approximately 39.5% and 49.1% increase of *Id3* mRNA stability was observed, respectively, in the absence of Tob2 and two Tob proteins (two-way ANOVA statistical test, $p < 0.05$). The calculated *Id3* half-lives in WT, Tob1−/−, Tob2−/− and DKO ESCs were 1.24, 2.04, 1.73 and 1.82 h, respectively. These results indicated that Tob1 and Tob2 repressed *Id3* mRNA stability in Tob subfamily protein knockout ESCs. If so, over-expression of Tob in cells would lead to the repression of a reporter gene bearing an Id3 3′UTR. To test this hypothesis, Id3 3′UTR was inserted downstream of the Renilla luciferase coding region to get the pRL-Id3-3′UTR. As show in Fig. 4D, after Gapdh, unrelated gene, was expressed in cells, no difference in luciferase activities of pRL-Id3-3′UTR was observed compared with that of the control vector. By contrast, expression of either Tob1 or Tob2 decreased the luciferase activity of pRL-Id3-3′UTR in a dose-dependent (0, 0.5, 1, or 2 μg/ml) manner. To confirm that the response of Id3 3′UTR reporter activity was specific to Tob subfamily proteins, Tob1 and Tob2 silencing was performed for competition assays (Fig. 4E). Further study revealed that loss of the Tob1 or Tob2 increased the luciferase activity of pRL-Id3-3′UTR compared with the Gapdh control group. These results suggest that the luciferase activity of pRL-Id3-3′UTR is Tob-specific and that the Tob subfamily proteins, Tob1 and Tob2 repress production of Id3 via mRNA degradation.

4. Discussion

Over the past many years, compelling research work has provided evidence that Tob proteins function as important negative regulators of the cell cycle in different cells. Tob subfamily proteins interact with transcription factors altering the ultimate outcome of DNA binding as they function either as transcriptional repressors or transcriptional enhancers. The Tob members of the APRO family regulate development and replication of various cell types. In this present study, we used ESCs and gene knockout methods to address the function of Tob subfamily genes in mESCs and found that all of these knockout ESC lines including Tob1−/−, Tob2−/−, and DKO ESCs had more rapid cell proliferation than WT ESCs without losing self-renewal and pluripotency. Then, we provide a mechanistic explanation for these observations by demonstrating that Id3 was upregulated by mRNA stability process when the ESCs are loss of Tob subfamily protein.

Both Tob1 and Tob2 were expressed at almost the same level in mESCs, and knockout of either did similarly affect cell proliferation. Surprisingly, combined knockout of Tob1 and Tob2 did not result in further enhanced proliferation. Furthermore, knockout of Tob1 or Tob2 up-regulated Id3 and extended *Id3* half-life in Tob1−/−, Tob2−/− ESCs almost 1.5-fold than that in WT cells. It will be of interest to establish whether Tob1 and Tob2 regulate the abundance and translational efficiency of other distinct mRNA sets and regulate cell cycle progression by different mechanisms, which will be our future work.

As previously reported, Tob proteins bind to both Caf1 and PABP [2] and we verified this interaction by using ESCs. We found that both Tob1 and Tob2 interacted with Caf1 as well as the PABP protein in mESCs(data not shown). In other laboratories, TOB proteins was proved to exert their anti-proliferative function by modulating mRNA turnover and the ability of TOBs to enhance deadenylation and decay of their targets becomes independent of PABP binding when TOBs are tethered to the mRNAs. Meanwhile, TOB proteins were required for the TOB-CAF1-CCR4 complex [31]. Our results suggest that the target mRNA of Tob1 and Tob2 regulating proliferation in mESCs is different from other cell lines reported before, and it is not clear whether this occur in a cell type- or tissue-specific manner.

Mouse Tob1 is expressed maternally and continuously throughout embryonic development [11]. Tob2 is also expressed ubiquitously in mouse embryos [12], suggesting a possible role in early embryonic development. But Tob1 deletion mice had increased bone mass [14], and in contrast, Tob2 knockout decreased bone mass [26]. Unexpectedly, DKO mouse pups can survive after birth (data not shown). It becomes possible to understand that double knockout Tobs did not result in further enhanced proliferation than single knockout. It will be interesting to decipher the mechanisms controlling and/or coordinating the multiple actions of these two functionally important but may redundant proteins.

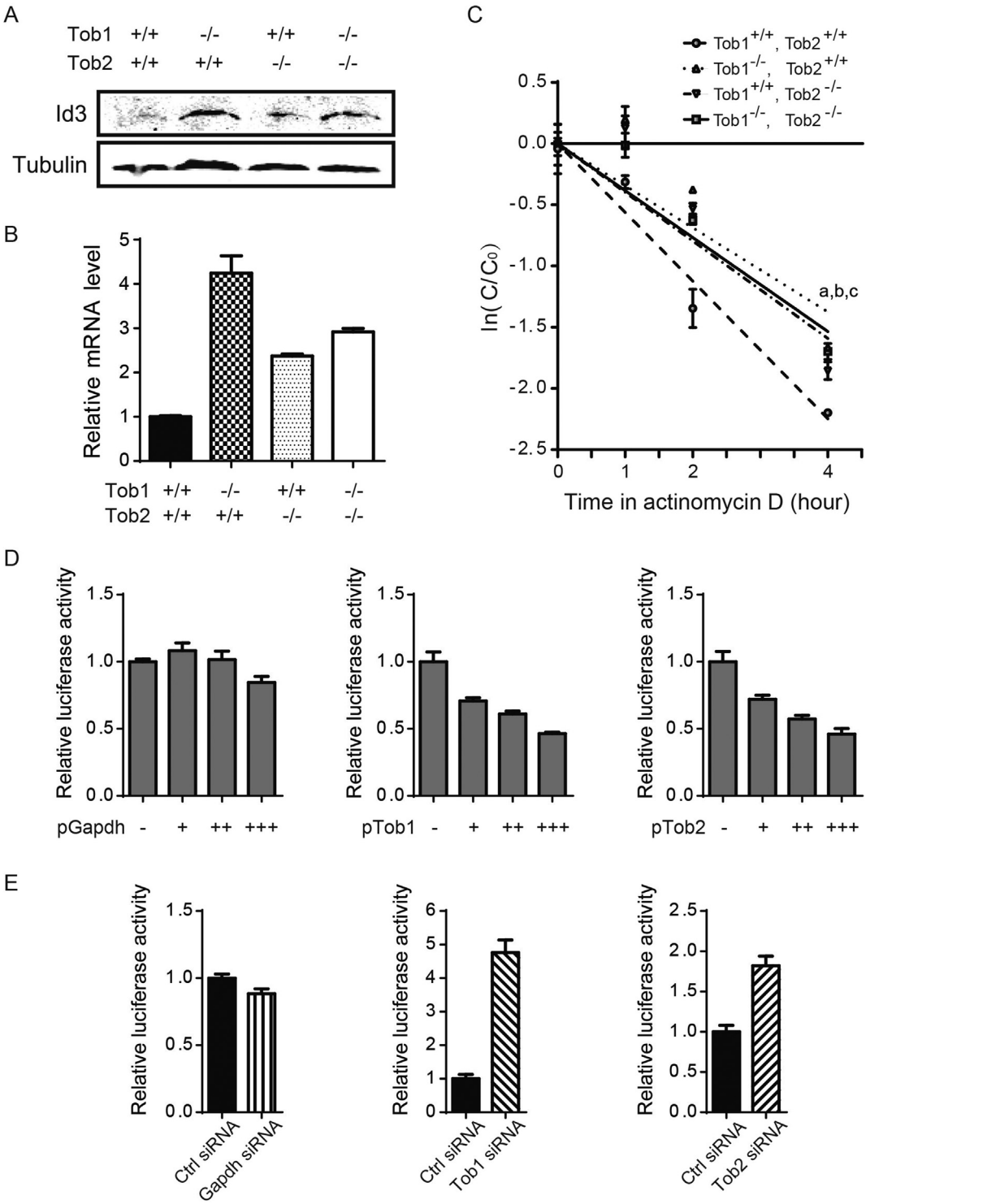

Fig. 4. Tob1 and Tob2 repress production of Id3 via mRNA degradation (A) Western blot analysis of Id3 in WT, Tob1$^{-/-}$, Tob2$^{-/-}$, and DKO ESCs. Tubulin was used as a loading control. (B) *Id3* mRNA expression levels in WT, Tob1$^{-/-}$, Tob2$^{-/-}$, and DKO ESCs. Data was normalized to Gapdh mRNA and the results were representative of three independent experiments (±SD, n = 3). (C) The decay time course for *Id3* mRNA in WT, Tob1$^{-/-}$, Tob2$^{-/-}$, and DKO ESCs. After actinomycin D treatment was administered for the indicated time, *Id3* mRNA level was measured by real-time PCR and normalized to Gapdh. The *Id3* mRNA decay kinetics in Tob1$^{-/-}$, Tob2$^{-/-}$, and DKO ESCs was significantly different from WT ESCs (two-way ANOVA statistical test, $P < 0.05$). Values measured at t = 0 were set to 0. Calculated half-lives are mean values, and the data is representative of three independent experiments (±SD, n = 3). (D) Tob1 and Tob2 mediates repression of a reporter that bears Id3 3′UTR. Renilla luciferase reporters containing Id3 3′UTR (pRL-Id3-3′UTR) were transfected together with the Firefly luciferase reporter, along with either a Gapdh expression vector (pGapdh), Tob1 vector (pTob1), or Tob2 vector (pTob2). Tob1 and Tob2 were transfected with different dosages [0 μg/ml (−), 0.5 μg/ml (+), 1 μg/ml (++), or 2 μg/m (+++)]. Firefly luciferase activities were determined and normalized against Renilla luciferase activity. Mean values are reported (±SD, n = 6). (E) Loss of the Tob1 or Tob2 increased the luciferase activity of pRL-Id3-3′UTR compared with the control Gapdh group. Either Tob1 siRNA or Tob2 siRNA was transfected together with pRL-Id3-3′UTR and the Firefly luciferase reporter, Gapdh siRNA was used as a negative control. Firefly luciferase activities were determined and normalized against Renilla luciferase activity. Mean values are reported (±SD, n = 6).

Conflict of interest

All authors declare no conflict of interest.

Acknowledgments

We thank Haibin Wang, for providing Tob1 null and Tob2 null mice, Hui Yao, for generation of mouse embryonic stem cells line, and Li Wang and Zhi Zhou, for editing the manuscript. This work was supported by the International S&T Cooperation Program [grant number 2011DFA31040] and the 863 Program [grant number 2011AA020106] from the Ministry of Science and Technology of China, The New Drug Innovation of China [grant number 2011ZX09102-010-02], a grant from Strategic Priority Research Program of the Chinese Academy of Sciences [grant number XDA01040000], a grant from National Nature Science Foundation [grant number 30800425] a grant from Shenzhen key laboratory for molecular biology of neural development [grant number ZDSY20120617112838879] and a special grant from Shanghai Bureau of Science and Technology [grant number 12DZ1910900].

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.117.

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