



Znhit1 causes cell cycle arrest and down-regulates CDK6 expression

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ABSTRACT

Cyclin-dependent kinase 6 (CDK6) is the key element of the D-type cyclin holoenzymes which has been found to function in the regulation of G1-phase of the cell cycle and is presumed to play important roles in T cell function. In this study, Znhit1, a member of a new zinc finger protein family defined by a conserved Zf-HIT domain, induced arrest in the G1-phase of the cell cycle in NIH/3T3 cells. Of the G1 cell cycle factors examined, the expression of CDK6 was found to be strongly down-regulated by Znhit1 via transcriptional repression. This effect may have correlations with the decreased acetylation level of histone H4 in the CDK6 promoter region. In addition, considering that CDK6 expression predominates in T cells, the negative regulatory role of Znhit1 in TCR-induced T cell proliferation was validated using transgenic mice. These findings identified Znhit1 as a CDK6 regulator that plays an important role in cell proliferation.

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Introduction

The zinc finger domain defines one of the largest protein superfamilies in eukaryotes. Zinc finger domains are believed to design a piece of protein surface to fit a particular segment of a DNA double helix [1]. Although different members of zinc finger families contribute to many distinct cellular processes, including transcriptional regulation, mRNA stability and processing [2], binding to specific DNA and participating in gene regulation is still the most important role of zinc finger proteins (Zfps).

The Zf-HIT domain is a new treble clef zinc finger domain with ~50 amino acids and binds two zinc atoms in a compact $\beta\beta\alpha$ core. Studies on this domain are so limited that the function of this domain is unknown, but the domain is mainly found in gene regulation and chromatin remodeling correlation proteins [3]. Only two members of Zf-HIT domain containing family have been studied up to now. Znhit3 was reported as a coactivator associated with hepatocyte nuclear factor-4 α (HNF-4 α) and was considered to be involved in Maturity-onset diabetes of the young (MODY) [4]. The other one, Znhit1 was described as a subunit of the SNF-2 related CBP activator protein (SRCAP) complex [5] and was reported to accumulate in response to genotoxic stresses and induce the transcriptional activation of several p53 target genes [6]. Importantly, it was also found that Znhit1 protein levels were controlled by cyclin G1 under normal growing conditions [6], which implied a potential role of Znhit1 in cell cycle regulation.

Cyclins and Cyclin-dependent kinases (CDKs) are the principle positive regulators of the cell cycle, among them D-type cyclins and their partners being the first cell cycle machinery reacting with extracellular signals. CDK6 and CDK4 are the catalytic partners of D-type cyclins and their kinetics of expression follow closely that of the D-type cyclins [7]. Both kinases are expressed in most cell types [8], but previous studies identified that CDK6 activity is dominative in T cells [9] and plays a pivotal role in development and proliferation. These CDKs are negatively regulated by cyclin-dependent kinase inhibitors (CKIs) via direct binding to themselves. A few genes were reported to directly regulate CDK6 transcription, including NANOG in embryonic stem cells, MEP50 in prostate cancer cell and Smad in Osteoblast [10–12].

In this study, we found that over-expression of Znhit1 led to a significant decrease of CDK6 expression as well as cell cycle arrest in NIH/3T3 cells. This decrease may be correlated with the decreased acetylation level of histone H4 in the CDK6 promoter region. Meanwhile, CDK6 is regarded as the predominant D-type cyclin-binding kinase in T cells [7], thus the function of Znhit1 in T cells was tested. Transgenic mice showed an inhibitory effect of Znhit1 on T cell proliferation in vivo.

Materials and methods

Antibodies. Stimulation and staining of T cells was performed using the following monoclonal antibodies from BD-Pharmingen: anti-TCR (H57–597), anti-CD4 (GK1.5), anti-CD8 (53–6.7), anti-CD28 (37.51). Anti-CDK6 was from NeoMarkers. Anti-HA was from Santa Cruz Biotechnology. Anti-FLAG was from Sigma. DNA

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fragment encoding Znhit1 (1–74 aa) was cloned into pGEX-4T-1 vector (Amersham Pharmacia Biotech), and the GST-tag fusion proteins of Znhit1 were expressed, purified and used as antigens to immunize mice for generation of anti-Znhit1 polyclonal antibody according to standard protocol.

Cells and plasmids. T cells were cultured in RPMI 1640 medium supplemented with 50 μ M 2-mercaptoethanol, 2 mM L-glutamine, and 10% FBS. The full-length cDNA of Znhit1 was amplified from mouse thymocyte cDNA and cloned into the tagged eukaryotic expression vector pcDNA3.0-HA, pcDNA3.1-FLAG and pEGFP-C1.

Immunoprecipitation and immunoblot analysis. Cells were lysed on ice for 30 min in lysis buffer (1% Triton X-100, 50 mM Tris-HCl (PH 7.4), 150 mM NaCl, 1 mM EDTA) containing protease inhibitors. The cell lysates were cleared and incubated with FLAG antibody beads (Sigma). Samples were subjected to western blotting with anti-HA antibody and HRP-conjugated secondary antibody (Santa Cruz Biotechnology).

PI (propidium iodide) staining and FACS analysis. Cells were trypsinized, washed with PBS, fixed with 70% ethanol at 4 °C for 30 min. After fixation, the cells were incubated in PBS containing 200 μ g/ml of RNase (Sigma) and 5 μ g/ml of PI (Sigma) for 30 min at 37 °C and run on a FACScalibur flow cytometer (Becton Dickinson). Cell cycle analysis was performed using FlowJo software (Treestar).

RNA interference. The RNAi experiment was performed using the pSilencer system (Ambion). Segments of siRNA targeting Znhit1 mRNA were designed by using siRNA-designing software. Two oligonucleotides, 5'-GATCCGATCCTGGAAGTTGTCATTCTCAAGAGA AATGACAACTTCCAGGATGACTTTTTTGGAAA-3' and 5'-AGCTTTTCCA AAAAAGTCATCCTGGAAGTTGTCATTCTCTTGAGAATGACAACTTCCA GGATGCG-3' were synthesized and annealed to produce a double-stranded DNA linker. The DNA linker was then inserted into vector pSilencer. Stable cell lines were generated by transfecting NIH/3T3 cells with pSilencer 3.0-Znhit1 siRNA plasmid or empty pSilencer (control) and selected with 500 ng/ml puromycin (Sigma).

ChIP analysis. NIH/3T3 cells were treated with 1% formaldehyde for 10 min at 37 °C. Chromatin immunoprecipitation was performed using anti-acetyl-histone H3, anti-acetyl-histone H4 (Millipore) and anti-FLAG as indicated. After immunoprecipitation, the eluted histone-DNA cross-links were washed and reversed by heating at 65 °C for 4 h, after which the DNA was quantified by semi-quantitative PCR.

Mice. Mouse Znhit1 cDNA was cloned and placed under the control of T-lineage cell specific human CD2 promoter and locus control region in the transgenic expression vector p29 Δ 2 (from Dr. Remy Bosselut). Transgenic mice were generated and maintained on the C57BL/6 background. Mice were genotyped by PCR

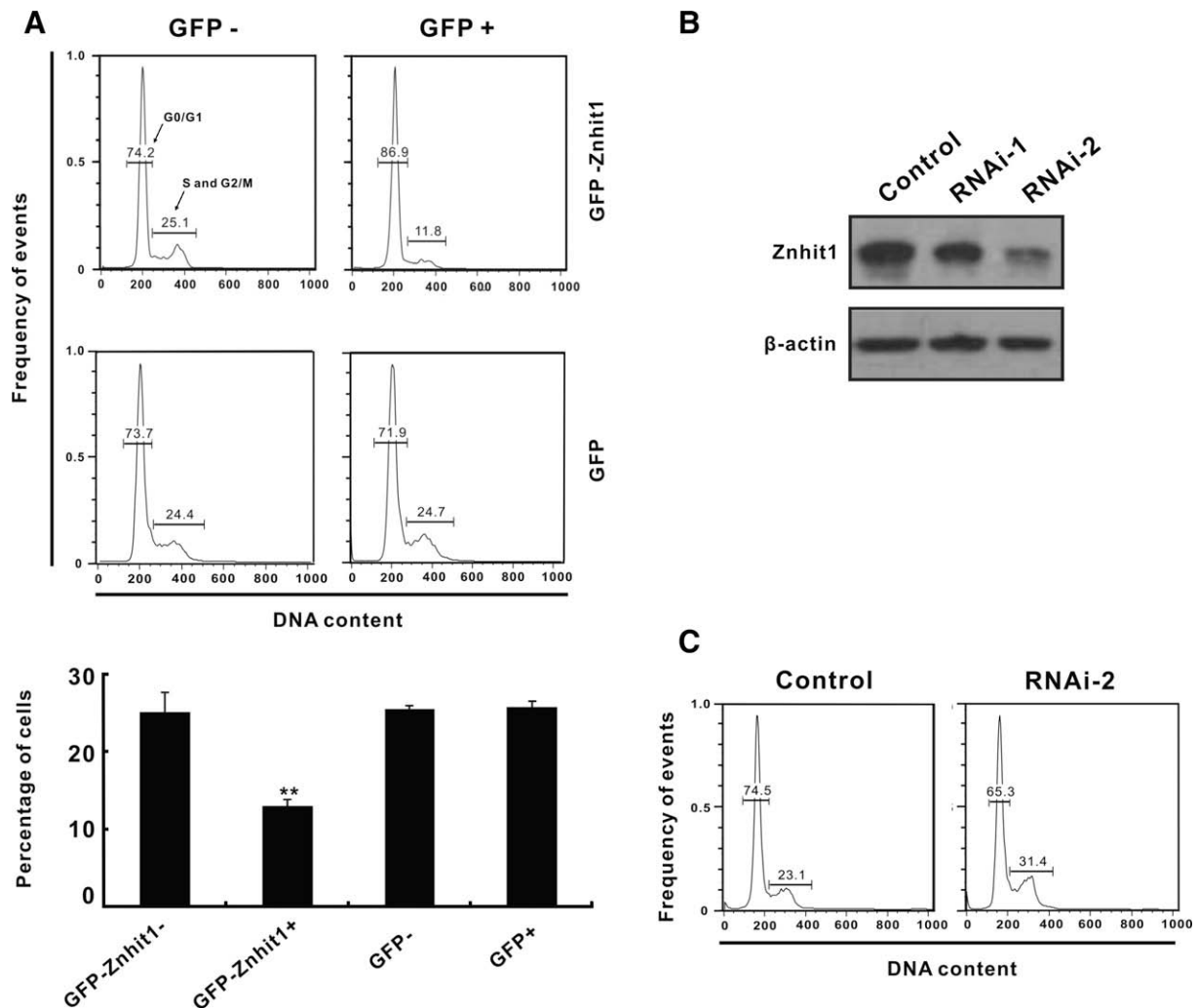


Fig. 1. Znhit1 causes a G0/G1 arrest in NIH/3T3 cells. (A) Flow cytometric analysis of DNA content in GFP-positive or GFP-negative NIH/3T3 cells over-expressing GFP-Znhit1 or GFP. The phases of the cell cycle are labeled and values given are proportions (%) of the cells analyzed. Columns mean proportions (%) of the cells. $^{**}P < 0.01$ versus GFP-Znhit1-. (B) Immunoblot analysis of Znhit1 expression in intact (control) and two stable RNAi NIH/3T3 cells. β -Actin served as a loading control. (C) Flow cytometric analysis of DNA content in intact (control) and stable RNAi NIH/3T3 cells. Values given are proportions (%) of the cells analyzed.

analysis of tail DNA. All animals were housed in a specific pathogen-free facility. Animal experimentation protocols were approved by Institutional Animal Care and Use Committee.

T cell proliferation assay. Lymphocytes (2×10^6 cells/ml) were labeled with 5 μ M CFSE (carboxyfluorescein diacetate succinimidyl ester) in labeling buffer [HBSS (Hanks balanced salt solution)] for 10 min at 37 °C in the dark, with occasional stirring. Labeling was stopped by HBSS supplemented with 2% FBS. Cells were resuspended at 2.5×10^6 cells/ml in complete medium and incubated at 37 °C for 48 h in plates previously coated with anti-TCR (2 μ g/ml) together with anti-CD28 (2 μ g/ml). For analysis, cells were stained with APC (allophycocyanin)-conjugated anti-CD4 or APC-conjugated anti-CD8 antibodies and analyzed by flow cytometry. Live cells were identified by forward light scatter and PI gating.

Results

Znhit1 inhibits cell growth in NIH/3T3 murine fibroblasts

Recently, it has been reported that in normal conditions, Znhit1 protein level is under control of cyclin G1 [6], indicating a role of Znhit1 in cell cycle regulation. Thus, we first examined whether Znhit1 has any effect on cell cycle progression. After verifying that over-expression of GFP alone had no effect on cell cycle, we over-expressed GFP-tagged Znhit1 in NIH/3T3 cells. The cell cycle period profiles in Znhit1-overexpressing NIH/3T3 cells were analyzed by flow cytometry, and Znhit1-overexpressing NIH/3T3 cells exhibited more than twofold reduction of S- and G2/M-phase populations compared with non-transfected (GFP-negative) cells in the same dish indicative of a G0/G1 arrest (Fig. 1A). Furthermore, we employed RNA interference to deplete endogenous Znhit1. A stable clone (RNAi-2) with knockdown Znhit1 to 1/3 was used to investigate the effect on the cell cycle (Fig. 1B). An increase of S- and G2/M-phase populations was detected in Znhit1-knockdown cells,

compared with intact NIH/3T3 cells (Fig. 1C). These results suggested an inhibitory effect of Znhit1 in cell cycle progression.

Over-expression of Znhit1 leads to the down-regulation of CDK6

The expression of cyclinD-CDK4/6 holoenzymes is the earliest and most important event in G1-phase progression and S-phase entry. Their expression is under control of inhibitors of cyclin-dependent kinases (CKIs) who play key roles in coordinating cell proliferation and development. To test the effect of Znhit1 on the expression of these pivotal molecules, the mRNA levels of D-type cyclins and CDKs were examined. The results showed that over-expression of Znhit1 led to more than threefold reduction of CDK6 mRNA while the mRNA levels of key CDKs were not changed (Fig. 2A). These observations indicated that Znhit1 may directly affect CDK6 expression instead of regulating through CKIs.

Consistent with the change of mRNA level, the expression of CDK6 protein in Znhit1 over-expressing cells greatly decreased (Fig. 2B). On the contrary, in Znhit1 knockdown cells, CDK6 expression was up-regulated (Fig. 2C). These results revealed that Znhit1 can regulate CDK6 expression in NIH/3T3 cells.

Znhit1 binds to CDK6 promoter and decreases its transcription

In most cases, Zfps function by binding to DNA in a sequence-specific fashion and are involved in gene transcription regulation. The fact that Znhit1 was reported as a coactivator [6] led to the presumption that if Znhit1 might directly bind to CDK6 promoter and inhibit its activity. To examine this possibility, Chromatin immunoprecipitation (ChIP) was performed with NIH/3T3 cells over-expressing FLAG-Znhit1. As shown in Fig. 3A, antibodies against FLAG co-precipitated the promoter region of CDK6 whereas no band was detected in the IgG control, which indicated the association of Znhit1 with CDK6 promoter.

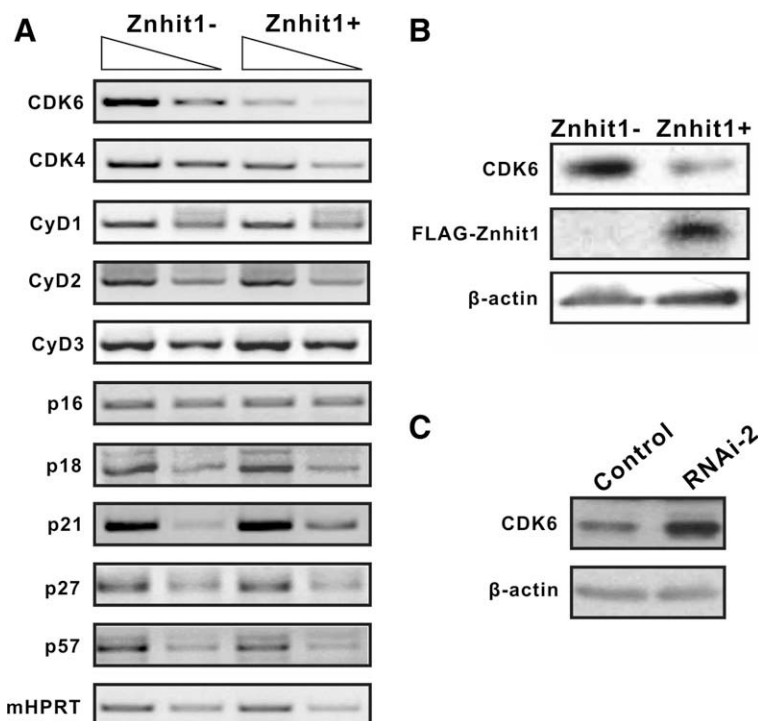


Fig. 2. Znhit1 down-regulates CDK6 expression in NIH/3T3 cells. (A) The mRNA levels of key regulators determined by RT-PCR analysis in NIH/3T3 cells transfected with pEGFP-C1 vector and pEGFP-Znhit1 separately. Wedges indicate 1:3 dilution of template. (B) Immunoblot analysis of CDK6 expression in NIH/3T3 cells transfected with FLAG vector and FLAG-Znhit1 separately. Znhit1 expression was tested by anti-FLAG antibodies. β-Actin was evaluated as a loading control. (C) Immunoblot analysis of CDK6 expression in wild type (control) and RNAi-2 NIH/3T3 cells.

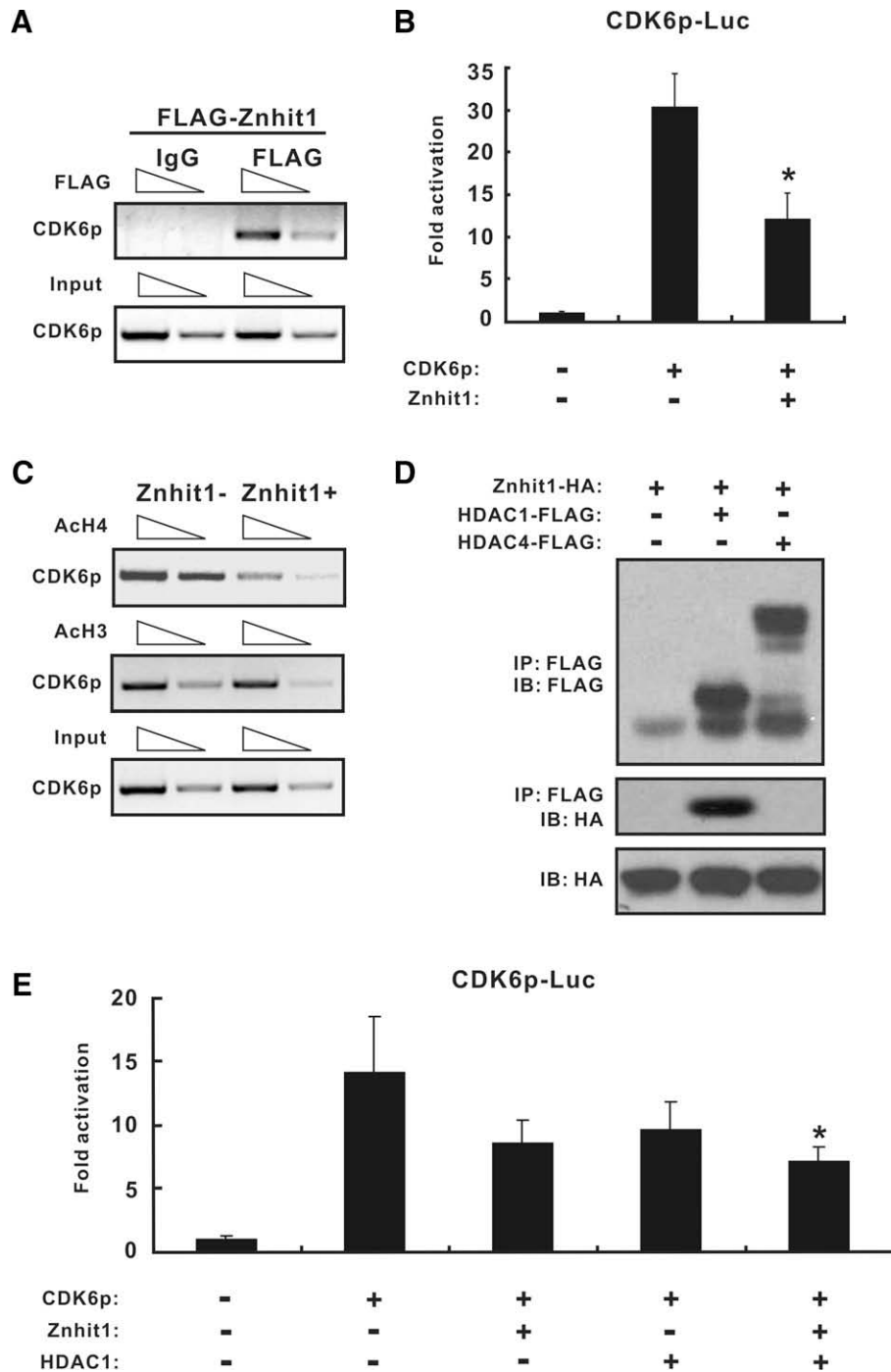


Fig. 3. Znhit1 binds to CDK6 promoter and decreases the acetylation of histone H4. (A) ChIP analysis for Znhit1 binding to the CDK6 promoter. NIH/3T3 cells transfected with pcDNA3.1-FLAG-Znhit1 were processed for ChIP analysis using the FLAG antibody. Wedges indicate 1:3 dilution of template. (B) Relative luciferase activity from HEK293 cells transfected with the CDK6-dependent luciferase reporter and Znhit1. $^*P < 0.05$ versus Znhit1-. (C) ChIP analysis for acetylated H4 (H4Ac) and H3 (H3Ac) binding to CDK6 promoter. Wedges indicate 1:3 dilution of template. (D) Co-immunoprecipitation in HEK293T cells co-transfected with HA-tagged and FLAG-tagged constructs as indicated. Equal amounts of lysates were immunoprecipitated (IP) with anti-FLAG beads. The immunoprecipitates were immunoblotted (IB) with the indicated antibodies. (E) Relative luciferase activity from HEK293 cells transfected with the CDK6-dependent luciferase reporter, Znhit1 and HDAC1. $^*P < 0.05$ versus Znhit1 or HDAC1.

Next, the effect of Znhit1 on CDK6 promoter transcription activation was evaluated on pGL3 luciferase reporter plasmid. To this aim, HEK293 cells were co-transfected with FLAG-Znhit1 or FLAG along with pGL3-CDK6 promoter, with a *Renilla* plasmid as a control for transfection efficiency. As shown in Fig. 3B, Znhit1 suppressed the luciferase reporter activity driven by CDK6 promoter.

Although Znhit1 functions as a CDK6 transcriptional repressor within the nucleus, the mechanism underlying this effect is unknown. Given the emerging paradigm that epigenetic regulation, especially chromatin remodeling plays a critical role in regulation of eukaryotic gene transcription and Zf-HIT domain has been found in chromatin remodeling related proteins, we assessed the possibility that Znhit1 may change the status of his-

tone acetylation within CDK6 promoter region. ChIP experiments showed that the acetylation levels of histone H4 but not H3 in the CDK6 promoter region were decreased by over-expression of Znhit1 in NIH/3T3 cells (Fig. 3C).

The observation that over-expression of Znhit1 results in deacetylation of histone H4 at the CDK6 promoter suggests that Znhit1 may have interaction with HDACs. Consequently, HEK293T cells were co-transfected with HA-tagged Znhit1 and FLAG-tagged HDAC1 or HDAC4. After immunoprecipitation with anti-FLAG antibodies, the interaction between Znhit1 and HDAC1 or HDAC4 was monitored by immunoblots using anti-HA antibodies. A significant amount of HDAC1 was associated with Znhit1. In contrast, no interaction between Znhit1 and HDAC4 was observed (Fig. 3D). Thus, Znhit1 appeared to specifically interact with class I but not class II HDACs. Znhit1 can interact with HDAC1 suggests that HDAC1 may participate in Znhit1-induced CDK6 decrease. Indeed, co-transfection of plasmids encoding Znhit1 and HDAC1 together promoted a greater than separate repression of CDK6-induced luciferase reporter activity (Fig. 3E). Collectively, these results suggested that Znhit1 can regulate acetylation level of histone H4 and may thus affected transcription of CDK6.

Znhit1 transgenic mice exhibit impaired T cell proliferation

Although CDK4 and CDK6 are both expressed in most cell types, T cells express mainly CDK6 [13]. Since Znhit1 has the

ability to regulate the expression of CDK6 and it is preferentially expressed in T cells, we were curious about its possible role in T cell proliferation. First we tested the expression of Znhit1 in T cell response. T cells were stimulated with plate-bound antibodies anti-TCR plus anti-CD28 for 24 h. The expression of Znhit1 was reduced to less than 1/6 compared with fresh T cells (Fig. 4A).

We subsequently investigated the role of Znhit1 in the regulation of cell cycle of T cells *in vivo* by generating Znhit1 transgenic mice. The expression of Znhit1 in T cells increased approximately 2.8-fold in transgenic animals compared with littermates (Fig. 4B). Consistent with the results in NIH/3T3 cells, CDK6 expression in Znhit1 transgenic T cells decreased compared with controls (Fig. 4C). To further examine the effect of transgenic Znhit1 on T cell proliferation, CFSE-labeled T cells were stimulated with plate-bound antibodies anti-TCR plus anti-CD28. Since each daughter cell inherits approximately half of the CFSE labeled, CFSE fluorescence was analyzed by flow cytometry to monitor cell division. As shown in Fig. 4D, T cells from either transgenic mouse or littermate underwent several rounds of division as is evident from the decreasing CFSE peaks. Strikingly, a significant portion of CD4/CD8 T cells from littermates went through two or three cell divisions in 48 h after stimulation whereas a less portion of CD4/CD8 T cells from transgenic mice had undergone two or three cell divisions. These results clearly demonstrated that transgenic Znhit1 suppressed T cell proliferation.

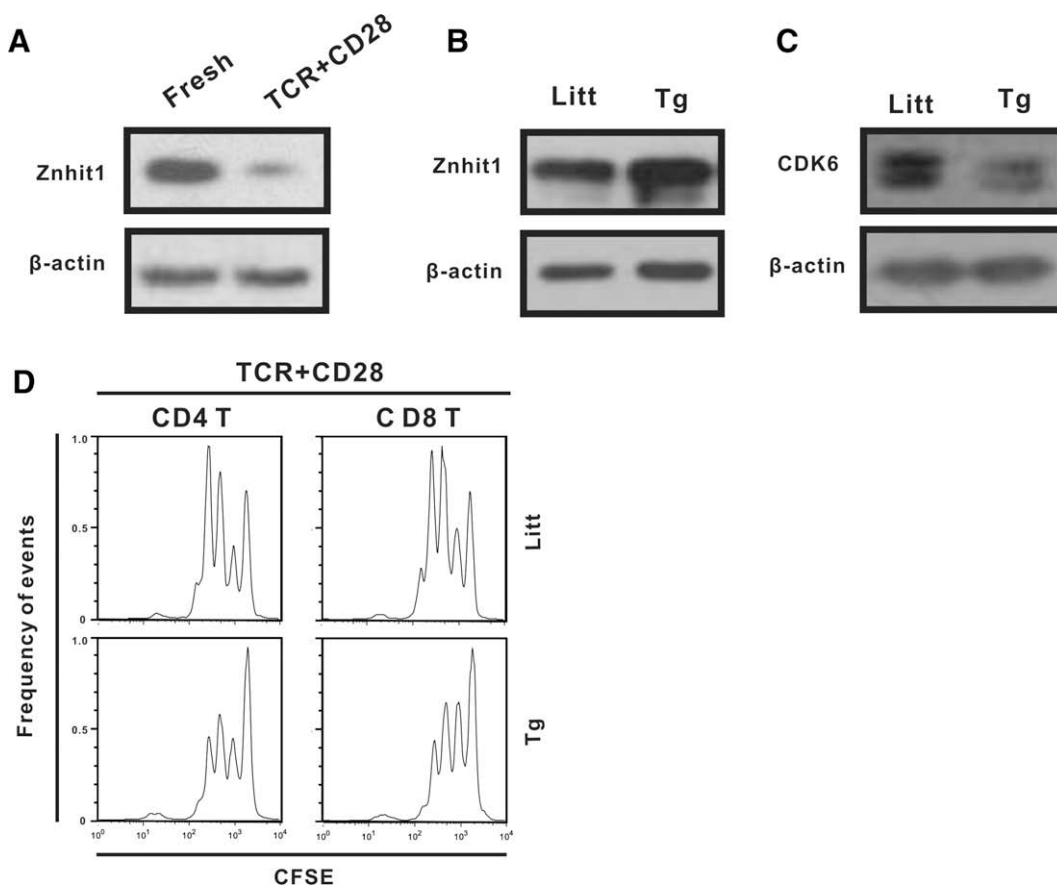


Fig. 4. Znhit1 transgenic mice exhibit impaired T cell proliferation. (A) Immunoblot analysis of Znhit1 expression in T cells freshly prepared (left) or stimulated with anti-TCR plus anti-CD28 antibodies (right). β-Actin served as a loading control. (B) Immunoblot analysis of Znhit1 expression in Znhit1 transgenic and littermate lymphocytes. (C) Immunoblot analysis of CDK6 expression in transgenic and littermate lymphocytes. (D) Flow cytometric analysis of fluorescence intensity in CFSE-labeled Znhit1 transgenic and littermate lymphocytes stimulated with anti-TCR plus anti-CD28 antibodies. Analyses were gated on CD4⁺ or CD8⁺ cells as indicated. (Litt, littermate; Tg, Znhit1 transgene).

Discussion

In this paper, we showed that *Znhit1* induced G0/G1 cell cycle arrest and down-regulated CDK6 expression in NIH/3T3 cells. By ChIP assay, we presented that *Znhit1* decreased the acetylation level of histone H4 in the CDK6 promoter region. To test if *Znhit1* had function in vivo, *Znhit1* transgenic mice were constructed. In *Znhit1* transgenic T cells, CDK6 expression was lower than the counterparts, and the TCR-induced proliferation was destroyed.

Cell cycle is strictly regulated by complex machinery composed of accelerator molecules cyclins and CDKs, and inhibited by brake molecules CKIs [14]. A lot of Zfps play roles in cell cycle regulation through regulating the expression of these genes. For example, GATA4 regulates cardiomyocyte proliferation by binding and activating cyclin D2 and CDK4 promoter [15]. KLF4 transactivates the CKI p27 (Kip1) expression and inhibits the growth and metastasis of human pancreatic cancer [16]. Cyclin D-CDK4/6 holoenzymes are the earliest cell cycle machinery integrating with extracellular signals. Moreover, CDK4 and CDK6 are the catalytic partners of D-type cyclins, with kinetics of expression following closely that of the D-type cyclins [17]. Targeted disruption of either of them results in delayed S-phase entry in fibroblasts [18], whereas over-expression of CDK6 or CDK4 accelerates S-phase [19,20]. Expression of these two CDKs is ubiquitous, but the relative importance of one over the other varies with cell type [7]. In this study, we reported a new Zfp *Znhit1* that could down-regulate the expression of CDK6, which was thought to be one of the most important parts of cell cycle machinery. Previous studies have shown that CDK6 could affect cell cycle in several cell types [20,21] and suggested that CDK6 may play a role in S-phase entry in fibroblasts [18], however, no study yet has verified that CDK6 was indispensable in fibroblasts proliferation [22], hence we cannot exclude the possibility that *Znhit1* had other targets in cell cycle regulation.

The function of zinc finger HIT domain remains largely unknown. The existing findings indicate that the domain exists in nuclear proteins involved in gene regulation and chromatin remodeling [3], which is in accordance with our finding. The function of *Znhit1* as a transcriptional coactivator to affect acetylation modification of histones is supported by recent work, which has identified *Znhit1* as a potential subunit of a complex that may have correlations with histone acetyltransferase [5]. These findings suggested that *Znhit1* may have contributions on acetylation regulation. We found that *Znhit1* physically interacted with histone deacetylase HDAC1. This can explain the way applied by *Znhit1* to regulate histones acetylation in some extent. How *Znhit1* functions with HDAC1 to regulate the acetylation of CDK6 promoter needs further investigation.

T cells are the basis of immunity but precipitate autoimmune disease at the same time [23]. Proper regulation of T cells is the guarantee of normal immunity. Recent studies have implicated CDKs as major regulators of T cell immunity and novel potential targets for therapy in autoimmune disease and organ transplantation [7]. These molecules are thought to be a potential link between the cell cycle and T cell function [23]. In T cells, CDK6 expresses predominantly and casts important roles in various stage of hematopoiesis, including T cell development [13]. Knockout of CDK6 in mice is followed by a lengthened G1-phase [24]. Gene ablation studies have not directly supported the requirement for CDK4 in T cell function [18]. Generally, CDK6 is regarded as the predominant D-type cyclin-binding kinase in T cells [7]. In this study, it has been shown that *Znhit1* can bind to CDK6 promoter and exert its inhibitory effect on the transcription of CDK6. Interestingly, *Znhit1* was degraded rapidly in T cells stimulated with

anti-TCR plus anti-CD28 antibodies and transgenic T cells exhibited impaired proliferation, indicating a negative regulatory role on T cells encountering TCR stimulation. Hence, it is possible that *Znhit1* affects cell cycle while in TCR signaling. However, further experimentation will be needed to confirm this assumption. Our study on the function of *Znhit1* in CDK6 expression regulation may shed light on the understanding of modulation of T cell immunity and tolerance.

Acknowledgments

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