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BTB-ZF Protein Znf131 Regulates Cell Growth of Developing and Mature T Cells

Tomohiro Iguchi,*,† Kazuhisa Aoki,* Tomokatsu Ikawa,‡ Masato Taoka,‡ Choji Taya,*, Hiroshi Yoshitani,* Makiko Toma-Hirano,§ Osamu Koiwai,† Toshiaki Isobe,§ Hiroshi Kawamoto,§ Hisao Masai,** and Shoichiro Miyatake*

Many members of the BTB-ZF family have been shown to play important roles in lymphocyte development and function. The role of zinc finger Znf131 (also known as Zbtb35) in T cell lineage was elucidated through the production of mice with floxed allele to disrupt at different stages of development. In this article, we present that Znf131 is critical for T cell development during double-negative to double-positive stage, with which significant cell expansion triggered by the pre-TCR signal is coupled. In mature T cells, Znf131 is required for the activation of effector genes, as well as robust proliferation induced upon TCR signal. One of the cyclin-dependent kinase inhibitors, p21Cip1 encoded by cdkna1 gene, is one of the targets of Znf131. The regulation of T cell proliferation by Znf131 is in part attributed to its suppression on the expression of p21Cip1.

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Znf131 was deleted in the DP stage, there is no effect on T cell development within thymus, but the number of peripheral T cells was reduced. Upon stimulation of mature SP T cells via TCR, the proliferation was impaired. We identified p21Cip1 encoded by cdkn1a gene, one of the cdk inhibitors playing a role in cell proliferation, autoimmunity, and tumorigenesis as a target gene of Znf131 (18, 19). Znf131 suppressed the promoter activity of cdkn1a through a novel regulatory region.

Materials and Methods

Mice

The targeting construct of the Znf131 locus is shown in Supplemental Fig. 1A. The exon 4 encoding a part of the BTB domain of the Znf131 was flanked with loxP sites; therefore, the deletion of exon 4 leads to joining of the exons 3 and 5, resulting in a frameshift. The targeting construct plasmid was introduced into ES line RENKA derived from C57BL/6N strain by electroporation; targeted clones were isolated by Southern blot (20). The ES clone with targeted allele was mixed with eight cell-stage embryo cells to produce chimeric mice. Chimeric males carrying Znf131 floxed allele. The disruption of the Znf131 allele in germelines was established by crossing to Zp3Cre mice expressing cre recombinase in the female germline (21). The Znf131 floxed mice were crossed to LckCre or Cd4Cre mice (16, 22). Mice were bred and housed in specific pathogen-free conditions. All the mice including either littermates or age-matched controls were examined at the age of 8–12 wk. The experimental protocols were approved by the Animal Use and Care Committee of the Tokyo Metropolitan Institute of Medical Science (permit no. 14054).

Flow cytometry and sorting

Single-cell suspensions from thymus and spleen were prepared and stained with specific combinations of Abs (see Supplemental Table I) purchased from Affymetrix and Biolegend. Lineage Abs were biotinylated (revealed with streptavidin-Brilliant violet 421), and remaining Abs were conjugated to FITC, PE, PerCP-Cy5.5, or allophycocyanin. Cells were analyzed with a FACS CantoII (BD) and the data were analyzed by FlowJo software (Tree Star). All the data were doublet excluded using FSC-A/FSC-H. For sorting, lineage+ cells were first depleted by magnetic cell separation with streptavidin-MicroBeads (Miltenyi Biotec) according to the manufacturer’s instructions. DN3 cells were sorted as Lin− CD25− CD44− cells and CD4 SP T cells were sorted as CD4+ CD8α− CD44+ cells. Dead cells were excluded by staining with propidium iodide or 7-aminoactinomycin D. For intracellular TCRb staining, cells were stained for surface Ags, fixed with IC Fixation Buffer and Permeabilization Buffer (Affymetrix), and stained with anti-TCRb Ab diluted with human Actb promoter to delete neomycin resistance cassette and to obtain Actb promoter to delete neomycin resistance cassette and to obtain

Cell culture

CD4 SP thymocytes were sorted from Znf131Δ/Δ (control) or Znf131Δ/ΔCd4cre mice. Sorted cells were stimulated by plates coated with anti-CD3ε Ab and soluble anti-CD28 Ab with or without IL-2. For proliferation assay, cells were labeled with CFSE and stimulated for 3 d. Proliferation was assessed by dilution of CFSE by flow cytometry. For the analysis of IL-7 response, cells were labeled with CFSE and stimulated for 3 d. Proliferation was assessed by dilution of CFSE by flow cytometry. For the analysis of IL-7 response, cells were labeled with CFSE and stimulated for 3 d. Proliferation was assessed by dilution of CFSE by flow cytometry.

Measurement of RNA

Total RNA was extracted with RNeasy Plus (Takara) and converted to cDNA using random primers and PrimeScript RT reagent Kit (Takara), according to the manufacturer’s instructions. Expression of the indicated genes was measured by quantitative real-time PCR (qRT-PCR) with SYBR Premix Ex TaqII (Takara) and LightCycler 480 system (Roche). Relative expression levels were calculated for each gene using Hprt, Actb, or L32 for normalization. Primers used for the experiments are available upon request.

 Western blot analysis

Protein extracts were resolved by 5–20% SDS-PAGE (ATTO), then transferred to a polyvinylidene fluoride membrane (Millipore) and analyzed by Western blot with the following Abs: anti-phospho-p44/42 (Thr202/Tyr204; D13.14.4E; Cell Signaling Technology), anti-p44/42 (137F5; Cell Signaling Technology), anti–phospho-Akt (Ser473; D9E; Cell Signaling Technology), anti-Akt (Cell Signaling Technology), and anti-Actin (Santa Cruz Biotechnology).

BrdU incorporation and flow cytometry

For determination of BrdU incorporation in vivo, mice were injected i.p. with 1 mg BrdU (Sigma-Aldrich) 4 and 2 h before the analysis. Single-cell suspensions from thymus were stained for cell-surface markers, treated with IC Fixation Buffer and Permeabilization Buffer, then treated with DNs (Roche) and stained with anti-BrdU Ab (Bu20a) from BioLegend.

p21Cre1− luciferase reporter assay

NIH/3T3 cells were transfected with p21Cre1− luciferase reporter construct (pGL3 carrying −2.9 Kb 5′ upstream region of the Znf131a gene) along with Znf131 or c-Myc expression plasmid and pRL-TK Renilla expression plasmid. Luciferase activity was measured 48 h after transfection with the Dual-Luciferase Reporter Assay Kit (Promega). Luciferase activity was normalized with Renilla luciferase activity.

Detection of rearrangement by PCR

We performed PCR-based analysis of TCRb-chain rearrangement using an assay modified from that described by Anderson et al. (23). High m.w. thymocyte DNA was extracted (2× phenol/chloroform, 1.1× chloroform), ethanol-precipitated, and resuspended in TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) before quantification and use. PCR amplifications were performed in 20 µl reaction buffer containing 0.05 µg template DNA, 0.2 µM 5′ and 3′ primers, and Tks Gflex DNA polymerase (Takara). The amplification cycle (10 s at 98°C, 15 s at 60°C, and 2 min at 68°C) was repeated 30 times. A total of 10 µl of the PCR was fractionated on a 1.2% agarose gel.

Statistical analysis

Unpaired two-tailed Student t tests were carried out using GraphPad Prism 4 software (GraphPad Software) to determine the statistical relation of different groups. In all the experiments, the results are representative of at least two independent experiments.

Online supplemental material

Supplemental Table I lists Abs used for surface staining of progenitor populations.

Results

Deletion of Znf131 during DN2 and DN3 stages results in the developmental arrest before the transition to the DP compartment

The mRNA expression of Znf131 in various subsets of thymocytes was examined by qRT-PCR. The expression of Znf131 did not change much during early developmental stages with the increase at the DP stage (Supplemental Fig. 1B). To address the function of Znf131 in various stages of T cell development, we have produced mice in which the exon 4 was flanked with loxP sites, allowing the conditional deletion of exon 4 (Supplemental Fig. 1A). Znf131 floxed mice were crossed to LckCre mice that start to express cre recombinase during DN2 to DN3 developmental stages in thymus (16). To evaluate the extent of the gene disruption in the DN stage, DN2, DN3, and DN4 populations of Znf131Δ/Δ or Znf131Δ/ΔLckCre mouse were sorted and qRT-PCR for Znf131 mRNA was performed (Supplemental Fig. 2). Although Znf131 expression was almost intact in DN2, it was significantly suppressed in DN3 and DN4 compartments. The comparison of Znf131 mRNA expression of DN3 from Znf131Δ/Δ, Znf131Δ/ΔLckCre, or Znf131Δ/ΔLckCre clearly showed that there is a good correlation with the gene dosage of Znf131, indicating that the extent of the deletion in DN3 is very high (Supplemental Fig. 2). Therefore, the deletion of the target region takes place when β-selection proceeds. In Znf131Δ/ΔLckCre mice, severe block of the transition from DN to DP compartment was observed (Fig. 1A, top panels). Total number of thymocytes was reduced by ∼25-fold compared with control Znf131Δ/ΔLckCre mice (Fig. 1B, left panel).
Most cells accumulated at the DN3 (CD25+ CD117\(^2\)) stage where β-selection took place (Fig. 1A, middle panels). The cell number was reduced by ~2.5- and 20-fold in DN3 and DN4, respectively (Fig. 1B, right panel). In addition, the CD8 immature SP population, a transitional stage between DN and DP, was reduced significantly, suggesting that the developmental arrest takes place mainly within the DN compartment (Supplemental Fig. 3). In contrast, disruption of Znf131 during DN2 and DN3 stages did not affect γδT cell development (Fig. 1A, bottom panels).

**Notch signal is not impaired in the DN3 stage**

Successful DNA rearrangement induced at DN2 and DN3 stages leads to the production of TCRβ-chain that pairs with the surrogate ρTx-chain triggering stimulation signal autonomously without ligand (24). The pre-TCR signal induces the differentiation into CD4/CD8 DP stage with a concomitant proliferation. Notch signaling is also crucial for T cells to proceed to β-selection step and the survival and expansion of post-β-selected cells by regulating cellular metabolism and inducing c-Myc (3, 25–28). In addition, the pre-TCR signal downregulates Notch expression to circumvent excessive stimulation that may lead to leukemic transformation.

Quantitative gene expression analysis of the DN3 subset revealed that the expression of Notch target genes such as ρTx, Hes1, and Deltex1 were not impaired in the absence of Znf131, rather they were upregulated (Fig. 1C). Thus, the arrest of differentiation at the DN3 stage is not due to the interference of Notch signaling.

**Znf131 is required for pre-TCR signaling**

The critical role of pre-TCR signaling is revealed through the typical phenotype of the arrest of differentiation before the DP stage and the accumulation of the DN3 subpopulation observed in mice deficient in genes that are critical in generating pre-TCR signal (29–31). DN3 compartment is further divided into DN3a and DN3b subpopulations. DN3b subpopulation is defined by CD27\(^{hi}\) and increased cell size representing the cells responding to the pre-TCR signal because pre-TCR signal upregulates CD27 and supports cells to exit from the resting state of cell cycle (32).

DN3b was less frequent in the absence of Znf131 than in control and the upregulation of CD27 expression was reduced in the Znf131-deficient cells, suggesting defects in pre-TCR signaling (Fig. 2A, 2B). To dissect the effect of Znf131 ablation on β-selection, at first we analyzed the rearrangement of TCRβ locus by genomic PCR with primers for Dβ2, Vβ5, Vβ8, Vβ11, and Jβ2 (Fig. 2C) (23). Recombined fragments were detected similarly in both samples. Those samples indicate that DNA rearrangement was not impaired in Znf131-deficient mice. Thus, the arrest of differentiation at the DN3 stage is not due to the interference of Notch signaling.
rearrangement of the TCRβ locus undergoes normally in the absence of Znf131.

Next, DN cells were separated according to their icTCRβ and CD25 expressions. It was shown that CD25+ DN3 cells can express icTCRβ, whereas the downregulation of CD25 accompanied by the differentiation toward DN4 was impaired in the absence of Znf131, resulting in the reduction of the cellularity of the DN4 population (Figs. 2D, top panel, 1B, right panel). The percentage of icTCRβ+ cells in DN3 was not affected, whereas that in DN4 was reduced in mutant mice (Fig. 2D, 2E).

Cell proliferation accompanied with DN-to-DP transition fails with enhanced expression of p21Cip1 in the absence of Znf131. Anti-CD3ε Ab is able to cross-link CD3 complex and mimic pre-TCR signaling to facilitate DP differentiation and accompanied proliferation. Znf131fl/2LckCre and Znf131fl/+LckCre Rag22/2 mice were produced and injected with anti-CD3ε Ab or PBS. Pre-TCR signal induced by the anti-CD3ε Ab failed to induce the DP differentiation and the concomitant cell expansion in the absence of Znf131, whereas substantial increase of the DP cell number was observed in the control, indicating that Znf131 plays a crucial role in the downstream of pre-TCR signaling (Fig. 3A, 3B).

To further evaluate cell proliferation upon pre-TCR signaling in vivo, we performed BrdU pulse label analysis (Fig. 3C). BrdU incorporation of DN was significantly suppressed, indicating that post-β-selection step including differentiation through DN4 stage with robust cell cycling was abrogated upon Znf131 deficiency.

The induction of c-Myc is a prerequisite for the cell proliferation and DP differentiation after β-selection (27, 28). One of the targets of c-Myc to facilitate cell proliferation is one of the cdk inhibitors, p21Cip1. c-Myc suppresses the expression of p21Cip1 via the association of Miz1. Nonetheless, the expression of c-Myc mRNA

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Znf131 plays important roles in the differentiation from DN3a to DN3b and DN4 compartments, but DNA rearrangement of TCRβ locus and the expression of icTCR are not affected. (A) Flow cytometry was performed for analysis of DN3a (lin− CD25+ CD117− CD27med FSCmed) and DN3b subsets (triangular gate, lin− CD25+ CD117− CD27bhigh FSChigh) in Znf131fl−/LckCre and control mice. Data are representative of at least three experiments. (B) Average DN3b percentages from control and Znf131fl/2LckCre mice were calculated (n = 7 for control and n = 9 for Znf131fl/2LckCre mice from at least three independent experiments). Data are the mean ± SD. (C) DN3 population was sorted from thymus of Znf131fl/2LckCre or Znf131fl/+LckCre (control) mice and DNA was prepared. PCR analyses to detect some Dβ-Jβ and Vβ-Jβ rearrangements were performed. Combinations of primers used are shown on the top. Data are representative of two experiments. (D) DN cells were separated according to their icTCRβ and CD25 expression to show the downregulation of CD25 of the icTCRβ+ population when differentiation toward DP proceeds (top). Histograms showing icTCRβ staining of gated DN3 (middle) and DN4 (bottom) populations from Znf131fl−/LckCre (control), Znf131fl−/LckCre, and Rag22/2 mice. Data are representative of three experiments. (E) The percentage of icTCRβ cells detected in DN3 and DN4 populations are shown (n = 6 for control and n = 6 for Znf131fl−/LckCre mice from three independent experiments). Data are the mean ± SD. ***p < 0.0001, **p < 0.01 (unpaired two-tailed Student t tests).
Znf131 is important for cell proliferation after β-selection, but expression of c-Myc is not affected. (A) FACS profiles for CD4 and CD8 expression 5 d after i.p. injection of 100 μg anti-CD3ε mAb or PBS into Znf131fl/fl, LckCreRag2fl/fl and Znf131fl/fl, LckCreRag2fl/fl (control) mice. Data are representative of three experiments. (B) Cellularity of total thymocytes 5 d after anti-CD3ε mAb injection. Results shown are the average absolute numbers (n = 3 for each samples from two independent experiments). Data are the mean ± SD. (C) BrdU incorporation of DN cells was analyzed by surface expression of CD25 and intracellular staining of anti-BrdU Ab. Data are representative of two independent experiments. (D) DN3 (Lin− CD25−CD117+) thymocytes were sorted from Znf131fl/fl, LckCre (control) and Znf131fl/fl, LckCre mice and analyzed for c-Myc protein expression by Western blot. Data are representative of two independent experiments. (E) Thymocytes from Znf131fl/fl, LckCre (control) and Znf131fl/fl, LckCre mice were investigated by flow cytometry for the presence of Annexin V+ cells. The cells were gated for DN3 (Lin− CD25−CD117+) and DN4 (Lin− CD25+CD117+). The percentage of Annexin V+ cells is shown. Data are the mean ± SD. (F) RNA was extracted from DN3 thymocytes of Znf131fl/fl, LckCre (control) and Znf131fl/fl, LckCre mice. mRNA expression for c-Myc and p53 target genes was examined by qRT-PCR. Data are the mean ± SD from three independent sorted samples. Data are representative of two independent experiments. **p < 0.01 (unpaired two-tailed Student t tests).

and as well as c-Myc protein in DN3 were not affected by Znf131 deficiency, whereas p21Cip1 mRNA was elevated in DN3 populations when Znf131 was removed (Fig. 3D, 3F). The major activity of p21Cip1 is to arrest cell-cycle progression through its binding and inhibition of the kinase activity of cyclin cdk complex. Accordingly, this indicates that Znf131 regulates p21Cip1 independently of c-Myc.

Another possibility is the requirement of Znf131 for the viability of T cells. Cell death activates p53 and its target genes such as p21Cip1, Noxa, and Bax. Although the enhancement of cell death and p53 mRNA expression upon Znf131 deficiency were not observed, the mRNA of p53 target genes p21Cip1, Noxa, and Bax were increased, suggesting that p53 might be activated (Fig. 3E, 3F).

Znf131 is not involved in the regulation of thymic selection and T cell maturation in thymus

To analyze the function of Znf131 after DP stage and in mature T cells, we crossed Znf131 floxed mice to CD4cre mice that start to express Cre recombinase during the DP stage in which positive and negative selection, as well as CD4 or CD8 lineage choice, take place (22). Cellularity and a proportion of DP, CD4 SP, and CD8 SP populations were comparable with those of litter control (Fig. 4A, 4B). The proceedings of T cell selection process can be monitored by TCR and CD69 expression (33). As shown in Fig. 4C, there are no alterations of TCR and CD69 expression, suggesting that the selection process proceeds without Znf131.

Maturation of SP populations in terms of functional competence is coupled with the upregulation of Qa2 and a reduction of CD24 (HSA) (34, 35). Maturation of SP cells deduced by the Qa2 and CD24 expression was not affected (Fig. 4D). These data indicate that the thymic selection process of the DP and the maturation of the SP within thymus do not require Znf131.

Znf131 is required for the maintenance of T cell pool in periphery through homeostatic expansion

The cellularity of peripheral CD4 and CD8 T cells was significantly reduced despite no effects on DP and SP thymocytes in the absence of Znf131 (Fig. 5A, 5B). The reduction of the cellularity of peripheral T cells could be interpreted by the impairment of several mechanisms. First, T cell egress from thymus regulated through sphingosine-1-phosphate receptor 1 (S1PR1) signaling is suppressed (36, 37). Second, the maintenance of T cell homeostasis regulated through the signal transduction pathways, such as TCR and cytokine signals, are impaired (38).

G protein–coupled S1PR1 is induced via transcription factor KLF2 (39). KLF2 is induced by the active dephosphorylated transcription factor FoxO1 (40). Cessation of TCR signal for thymic selection leads to the induction of S1PR1 detecting elevated concentration of sphingosine-1-phosphate in the blood and the egress of mature thymocytes into the circulation. When the signaling pathway required for T cell egress from thymus is impaired, the accumulation of SP
were calculated (least three independent experiments). Data are the mean
Znf131 and CD8 SP cells from
examined for surface expression of CD4 and CD8
Total thymocytes were analyzed for CD24 and Qa-2 expression. Data are
thymocytes from
populations in thymus are not affected in the absence of Znf131. (FIGURE 4.
Differentiation and maturation of CD4 SP and CD8 SP
A
B
C
D

FIGURE 4. Differentiation and maturation of CD4 SP and CD8 SP
populations in thymus are not affected in the absence of Znf131. (A) Total
thymocytes from Znf131+/-CD4cre and Znf131+/- (control) mice were
examined for surface expression of CD4 and CD8α. Data are representative
of at least three experiments. (B) Absolute numbers of total, DP, CD4 SP,
and CD8 SP cells from Znf131+/-CD4cre and Znf131+/- (control) mice were
were calculated (n = 8 for control and n = 8 for Znf131+/-CD4cre from
were the mean ± SD from at least three independent experiments). Data are the mean
CD69 and TCRβ. Data are representative of three experiments. (C) Total thymocytes from Znf131+/-
and Znf131+/- (control) mice were analyzed for surface expression of
CD69 and TCRβ. Data are representative of three experiments. (D) Total thymocytes were analyzed for CD24 and Qa-2 expression. Data are
representative of three experiments.

thymocytes is observed. However, the increase of proportion or cellularity of SP was not observed in the absence of Znf131 (Fig. 4A, 4B). In addition, the expression of S1PR1, KLF2, or FoxO1 was not affected in the Znf131-deficient SP population (Fig. 5C). These suggest that the reduction of peripheral T cell cellularity is not caused by the impairment of T cell egress from thymus.

IL-7 is required for the homeostasis of the peripheral resting T cells. For the maintenance of T cells, IL-7/IL-7R signaling regulates genes such as antiapoptotic Bcl2 and the regulators of the JAK-STAT signaling pathway SOCS1 and SOCS3. CD4 SP population was isolated and incubated with or without IL-7. Anti-apoptotic effect of IL-7 was not affected in the absence of Znf131 (Fig. 5D). The level of the expression and the extent of induction of the IL-7–regulated genes were comparable (Fig. 5E), which suggests that IL-7/IL-7R signaling does not require Znf131.

The expression pattern of CD44/CD62L revealed that memory-phenotype cells were increased, presumably because of the lymphopenic environment (Fig. 5F). It turned out that many of the peripheral memory-phenotype T cells retain undeleted floxed Znf131 allele (Fig. 5G). This indicates that Znf131-deficient naive T cells released from thymus are not able to proliferate to maintain naive T cell pool in periphery, whereas homeostatic expansion occurs with those that harbor intact Znf131 gene. This indicates that the mechanisms to maintain T cell homeostasis in periphery are impaired, resulting in the environment in which only undeleted T cells are selected to survive (41, 42).

Znf131 is required for the proliferation of activated T cells through the modulation of cdk inhibitor p21Cip1

To evaluate the role of Znf131 in the antigenic stimulation through TCR, we stimulated CD4 SP T cells isolated from the thymi of Znf131+/-CD4cre or control mice with anti-CD3ε and anti-CD28 Abs in vitro. The induction of the cell-surface expression of the immediate early genes such as CD25 and CD69 were unaffected in the absence of Znf131 (Fig. 6A). However, the robust cell proliferation and the activation of many genes for effector cell induction such as IL-2 and Tbx21 were almost completely abrogated in the absence of Znf131 (Figs. 6B, 7). Abrogated proliferation was not rescued in the presence of exogenous IL-2 (Fig. 6C). Those suggest that the TCR signaling pathways that lead to most of the effector functions were impaired. Activation of key signaling molecules for cell proliferation, MAPK and Akt, and their phosphorylation after TCR stimulation were analyzed (Fig. 6D). The time course of the phosphorylation of those kinases was not altered in the absence of Znf131. The analysis of the expression profile revealed that the induction of the proto-oncogene c-Myc absolutely required for the proliferative expansion of activated T cells was not affected (43) (Fig. 7). Among the cell-cycle regulators, the induction of cdk inhibitor p21Cip1 was further upregulated in T cells from Znf131+/-CD4cre mice (Fig. 7). This might be one of the causes of the cell-cycle arrest upon TCR stimulation. p21Cip1 encoded by cdkn1a has been extensively studied as a suppressive target of c-Myc. c-Myc requires BTB-ZF protein Miz1 to form a complex and bind to the promoter region of cdkn1a gene. It has been reported that the fine-tuning of T cell proliferation by the expression of p21Cip1 plays an important role in suppressing excessive proliferation of the effector/memory T cells necessary to avoid the development of autoimmunity (44). This indicates that one of the mechanisms in which Znf131 regulates peripheral T cell proliferation is its suppressive effect on p21Cip1 expression.

It is possible that the elevation of p21Cip1 was the result of enhanced cell death and activation of p53. However, the extent of cell death upon stimulation was comparable with that of the control (data not shown), and the expression of p53 target genes except p21Cip1 was not upregulated in the absence of Znf131 (Fig. 7). Because the upregulation of p21Cip1 in the absence of Znf131 was a common outcome of the Znf131 deficiency in both DN thymocytes and the peripheral T cells, we decided to pursue the possibility of the direct regulation of the p21Cip1 gene expression by Znf131.

To determine the region required for the suppressive effect of Znf131, we assessed reporter constructs carrying different length of...
the promoter of cdkn1a by transfection of NIH/3T3 cells in the presence of Znf131 or c-Myc (Fig. 8A). As reported earlier, c-Myc suppressed the promoter of cdkn1a through its interaction with Miz1 that binds to the initiator region of the promoter (Fig. 8A). This region was present in all the reporter constructs analyzed and thereby the suppression was observed with all the reporter constructs, whereas the suppressive effect of Znf131 was not detected with p21-Luc ΔB, suggesting that the regions required for the suppression by Znf131 and c-Myc/Miz1 are different (Fig. 8B).

Discussion
In this study, we show that Znf131 is a novel transcription factor required for the DN-to-DP differentiation, homeostasis of T cells in periphery, and the activation and robust proliferation of T cells by...
antigenic signal. Znf131 supports all of those processes coupled with proliferation of T cells through its role in cell-cycle regulation.

β-Selection checkpoint ensures the survival and expansion of cells that productively rearrange and express TCRβ locus. Two important signaling pathways, Notch and pre-TCR signals, are crucial for the β-selection checkpoint (3, 25, 26). These two pathways interact with each other for the appropriate cell expansion and differentiation to DP stage. Several lines of proximal LckCre mice are used in many reports. The LckCre line used in this article deletes floxed allele in a very small portion of DN1/2, CD4Cre line used in this study deletes floxed allele in T cell precursors that productively rearrange TCRβ-chain in association with the invariant surrogate α-chain, pTα detected as icTCRβ at DN3/4 stages. Pre-TCR produces critical signal autonomously for DN3 to progress differentiation forward. The cell size and the magnitude of CD27 expression allow the distinction between DN3a and DN3b populations (32). Pre-TCR is expressed and delivers stimulation signal in DN3a to proceed to DN3b of elevated expression of CD27 and larger cell size indicating the beginning of cell proliferation. DNA rearrangement of the TCRβ locus indicated no defect in the absence of Znf131. icTCRβ in DN3 of Znf131fl/fl –LckCre mice is comparable with that of litter control. However, the cellularity of DN3b, as well as the percentage of the icTCRβ+ population of DN4, was reduced in Znf131fl/–LckCre mice. This indicates that TCRβ locus rearrangement and the initial expression of pre-TCR occur appropriately. As a result, the DN3a population receives pre-TCR signaling in the absence of Znf131. Nevertheless, initiation of cell cycling leading to DNA synthesis of DN3 was significantly impaired and the number of DN4/DP cells was reduced. Moreover, anti-CD3e Ab treatment mimicking pre-TCR signal in the absence of pre-TCR complex was not able to rescue DP differentiation with cell proliferation efficiently in the absence of Znf131.

FIGURE 6. Znf131 is required for the proliferation after the antigenic stimulation through TCR. (A) CD69 and CD25 expression and forward scatter (FSC) of the sorted CD4 SP thymocytes from Znf131fl/flCD4Cre and Znf131fl/fl (control) mice before and 24 h after anti-CD3ε and anti-CD28 mAbs treatment. Data are representative of two experiments. (B) Fold increase of CD4SP thymocytes cell number isolated from Znf131fl/flCD4Cre and control mice at the indicated time points after stimulation with anti-CD3ε and anti-CD28 mAbs in vitro. Data are representative of two experiments. (C) CD4SP thymocytes sorted from Znf131fl/flCD4Cre and control mice were labeled with CFSE and stimulated with anti-CD3ε and anti-CD28 mAbs either in the absence or in the presence of IL-2. After 3 d of incubation, cells were analyzed by FACS. Data are representative of three experiments. (D) CD4SP thymocytes sorted from Znf131fl/flCD4Cre and Znf131fl/fl (control) mice were stimulated with anti-CD3ε and anti-CD28 mAbs for the indicated time periods. Phosphorylated ERK and AKT were measured by Western blot.

DNA rearrangement of TCRβ locus begins during DN2 stage. T cell precursors that productively rearrange TCRβ locus can express TCRβ-chain in association with the invariant surrogate α-chain, pTα detected as icTCRβ at DN3/4 stages. Pre-TCR produces critical signal autonomously for DN3 to progress differentiation forward. The cell size and the magnitude of CD27 expression allow the distinction between DN3a and DN3b populations (32). Pre-TCR is expressed and delivers stimulation signal in DN3a to proceed to DN3b of elevated expression of CD27 and larger cell size indicating the beginning of cell proliferation. DNA rearrangement of the TCRβ locus indicated no defect in the absence of Znf131. icTCRβ in DN3 of Znf131fl/fl –LckCre mice is comparable with that of litter control. However, the cellularity of DN3b, as well as the percentage of the icTCRβ+ population of DN4, was reduced in Znf131fl/–LckCre mice. This indicates that TCRβ locus rearrangement and the initial expression of pre-TCR occur appropriately. As a result, the DN3a population receives pre-TCR signaling in the absence of Znf131. Nevertheless, initiation of cell cycling leading to DNA synthesis of DN3 was significantly impaired and the number of DN4/DP cells was reduced. Moreover, anti-CD3ε Ab treatment mimicking pre-TCR signal in the absence of pre-TCR complex was not able to rescue DP differentiation with cell proliferation efficiently in the absence or in the presence of IL-2. After 3 d of incubation, cells were analyzed by FACS. Data are representative of three experiments. (C) CD4SP thymocytes sorted from Znf131fl/flCD4Cre and control mice were labeled with CFSE and stimulated with anti-CD3ε and anti-CD28 mAbs either in the absence or in the presence of IL-2. After 3 d of incubation, cells were analyzed by FACS. Data are representative of three experiments. (D) CD4SP thymocytes sorted from Znf131fl/flCD4Cre and Znf131fl/fl (control) mice were stimulated with anti-CD3ε and anti-CD28 mAbs for the indicated time periods. Phosphorylated ERK and AKT were measured by Western blot.
There is a possibility that DNA damage is induced in the absence of Znf131, leading to the increase of cell death and the activation of p53 and its target genes such as p21Cip1, Bax, and Noxa. However, the elevation of apoptosis of thymocytes was not detected in the absence of Znf131. In addition, cell death and the expression of p53 target genes except p21Cip1 were not enhanced in the absence of Znf131 in mature Znf131-deficient CD4+ T cells. As discussed later, Znf131 is a novel negative regulator of p21Cip1 gene.

Notch signal is another critical developmental cue for DN-to-DP transition. Notch signal activates cell metabolism through PI3K-Akt kinase axis. In addition, Notch signal induces transcription factor c-Myc required for the expansion of DN3/4 compartments, as well as differentiation into the DP population (46, 47). The expression of Hes1 and Deltex1, Notch target genes, were maintained or even upregulated in Znf131-deficient DN3 cells. In addition, c-Myc induction in the DN3 of Znf131-deficient mice was comparable with that in the litter control. These data indicate that Znf131 is not involved in Notch signaling and the downstream c-Myc induction that are critical processes to facilitate the differentiation and the accompanied proliferation of DN-to-DP transition. Von Boehmer’s group has reported that various strategies to stimulate the cell proliferation of DN3/4 compartments force them to expand and differentiate into DP cells, indicating that cycling of cells is the driving force for DP differentiation, although pTo signaling and the presence of E47 encoded by Tcfβ gene are prerequisites, and this concept is under debate (47, 48). It is conceivable that Znf131 is required for cell proliferation independent of Notch–c-Myc axis or in the downstream of c-Myc during DN-to-DP transition.

The expansion of T cells coupled with DP differentiation is followed by the process of positive and negative selections (7, 8). The survived cells become either CD4+ SP or CD8+ SP to mature in thymus. After the robust proliferative expansion of cells traversing DN-to-DP differentiation, cells rarely proliferate up to the egress from the thymus. Conditional inactivation of Znf131 during the DP compartment with CD4Cre transgene revealed that the selection processes in DP compartment and the maturation steps of SP populations in thymus were not perturbed. However, the number of T cells in periphery was reduced significantly. There are several possible mechanisms to explicate the reduction of peripheral T cells despite the normal differentiation of T cells within thymus. First is the impairment of the maturation of T cells in the SP compartment. In NKAP-deficient mice, the reduction of the peripheral pool of naive T cells is due to the impaired maturation of SP populations resulting in the failure to acquire functional competency and to fulfill naive T cell pool (49). As mentioned earlier, the maturation of SP populations in thymus is intact in Znf131 ablated mice. Second is the impairment of the mechanism to allow mature T cells in thymus to egress into circulation (36, 37). However, the accumulation of SP in thymus due to the impairment of the transit from thymus to circulation was not observed. As expected, the expression of genes involved in the regulation of T cell egress from thymus was not altered (40, 50). The third mechanism is the defect of the mechanisms to maintain...
Peripheral T cell pool. The homeostasis of naive T cells continuously supplied from the thymus requires moderate expansion triggered by more than two signaling pathways, such as IL-7/IL-7R, self-peptide MHC/TCR, and other unknown environmental cues (38, 41, 42). The ratio of memory-phenotype versus naive cells of either CD4 or CD8 cells in periphery is increased in Znf131-deficient mice. Further, memory-phenotype T cells of Znf131-deficient mice retain nondeleted floxed Znf131 allele, suggesting that Znf131-deficient naive T cells are unable to expand to maintain the homeostasis in periphery. As a consequence, lymphopenic environment drives the expansion of cells that failed to excise the Znf131 gene. Taking these data into consideration, it is conceivable that the homeostasis in periphery drives the expansion of cells that failed to excise the Znf131 gene. Therefore, we identified the enzyme driving the expansion of cells that failed to excise the Znf131 gene. Taking these data into consideration, it is conceivable that the homeostasis in periphery drives the expansion of cells that failed to excise the Znf131 gene.

Regulation of T cell proliferation is ingeniously integrated into the development and manifestation of effector functions of immune cells. Our data indicate that Znf131 is required for pre-TCR checkpoint, homeostasis of peripheral naive T cells, and activation of T cells by foreign Ags, all of which associate with proliferation. One of the candidate genes of Znf131 targets turned out to be one of the cdk inhibitors, p21cip1, encoded by the cdkn1a gene that is involved in the regulation of cell proliferation, autoimmunity, and tumorigenesis (18, 19). p21 has to be appropriately regulated for the immune response; therefore, the proliferative potency of T cells gets uncontrolled in the absence of this gene and eventually develop fatal autoimmune disease (44, 51–53). In contrast, the enhanced expression in the presence of exogenous p21 in T cells leads to the suppression of the proliferation capacity (54). The expression of p21cip1 was shown to be upregulated in Znf131-deficient DN3/4 compartments. In addition, although p21cip1 is induced upon TCR signaling in mature T cells, enhanced induction was observed in the absence of Znf131. These data support the idea that Znf131 is a direct or indirect repressor for p21cip1 expression.

Cdkn1a is regulated by various signaling pathways and transcription factors such as p53 (18, 19). Proto-oncogene c-Myc plays essential roles in differentiation, proliferation, cell survival, and tumorigenesis of various cell lineages including T cells. As mentioned earlier, the proliferative expansion during the progression from DN3/4 to DP stage absolutely requires c-Myc (46, 48). Moreover, the deletion of c-Myc results in the upregulation of p21cip1 in DN3/4 populations (46). Proliferation of mature T cells upon antigenic stimulation is also absolutely dependent on c-Myc expression (43). c-Myc suppresses the expression of p21cip1 through its interaction with Miz1, one of BTB-ZF proteins (55–57). Inactivation of Miz1 by the removal of the BTB domain in all the hematopoietic cells with Vav-Cre transgene results in the severe defects in the development of T and B lineages (58–60). In T lineage development, the expansion of early T cell precursors such as ETP and DN2 is severely impaired (59). In addition, the developmental arrest before the differentiation into DP with a concomitant upregulation of p21cip1 is observed. However, when inactivation of Miz1 is induced by Lck-Cre transgene that starts to express Cre recombinase during DN2 to DN3, impairment of DN3/4-to-DP transit and the differentiation thereafter is not observed (59). This may indicate that Miz1 has to be expressed earlier than Znf131 to facilitate DN-to-DP transition. Alternatively, Miz1 protein is more stable than Znf131; therefore, Miz1 protein is present even if the Miz1(Zbtb17) gene is deleted during DN2/3 compartments.

The substitution mutation of Val394 with Asp in c-Myc protein (MycV394D) has been shown to abolish its interaction with Miz1; therefore, the suppression of p21cip1 expression by c-Myc is inactivated in some cell types (56). However, T cell differentiation in mice with MycV394D knocked-in allele is not perturbed, suggesting that Miz1 functions independently of c-Myc during T cell development including DN-to-DP transition (59). Accordingly, although c-Myc, Miz1, and Znf131 are all critical proteins for the differentiation induced by the pre-TCR signal and the ablation of these genes leads to the enhancement of cdk inhibitor p21cip1, there is a possibility that those genes function in separate pathways.

Reporter assay performed in NIH/3T3 cells showed that Znf131 is inhibitory to the promoter of cdkn1a, consistent with the idea that Znf131 is a repressor of this promoter. Some members of BTB-ZF protein family function as a suppressor of transcription through forming a complex with epigenetic modifiers with repressive activities such as histone deacetylase 1/2. Binding motif of Znf131 has been reported (13). Znf131 acts as a positive transcription factor for the artificial promoter harboring several copies of this binding motif. In addition, Znf131 suppresses E2 (estrogen)-dependent ERα-mediated transcriptional activation through its interaction with the ERα (12). Therefore, we identified the endogenous promoter to which Znf131 functions as a repressor. The rough mapping of the regulatory region does not contain the
earlier-mentioned binding motif. It is possible that Znf131 exerts its function as either a positive factor or a negative factor depending on the recognition motifs. Otherwise, Znf131 may interact with some transcription factors or epigenetic regulators already present on the promoter of cdkn1a to suppress transcription. We have confirmed that c-Myc suppresses the promoter of cdkn1a. As reported earlier, the initiator region to which Mzi1 binds is sufficient for the suppression, and this region is distinct from the region required for Znf131-mediated suppression. Therefore, c-Myc/Mzi1 and Znf131 are in a separate pathway for the regulation of cdkn1a transcription. The precise mechanisms by which Znf131 regulates transcription, especially in T cells, remain to be explored in future studies.

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Disclosures

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References