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Pontin is required for pre-TCR signaling at the β-selection checkpoint in T cell development

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ABSTRACT

Pontin is a chromatin remodeling factor that possesses both ATPase and DNA helicase activities. Based on high expression in lymphoid tissues, we examined whether Pontin has a T cell-specific function. We generated *Pontin*$^{f/f}$;*Lck-Cre* mice, in which *Pontin* can be conditionally deleted in T cells and then explored T cell-specific function of Pontin *in vivo*. Here, we show that specific abrogation of Pontin expression in T cells almost completely blocked development of αβ T cells at the β-selection checkpoint by inducing cell apoptosis indicating that Pontin is essential for early T cell development. Pontin-deficient thymocytes show a comparable expression level of T cell receptor (TCR)β chain, but have enhanced activation of p53 and Notch signaling compared to wild-type thymocytes. Intriguingly, the developmental block of αβ T cells can be partially rescued by loss of p53. Together, our data demonstrate a novel role of Pontin as a crucial regulator in pre-TCR signaling during T cell development.

1. Introduction

T cells mature in the thymus through a tightly controlled series of steps, whereby uncommitted precursors become restricted to the T cell pathway, rearrange a functional TCR, and undergo positive and negative selection based on their TCR specificity. The least mature T cell precursors enter the thymus as double-negative (DN; CD4− and CD8−) thymocytes, which progress to the double-positive (DP; CD4+ and CD8+) stage before selectively silencing one of these coreceptors to become mature single-positive (SP; CD4+ or CD8+) cells [1]. During this process, one of the critical checkpoints is the DN3 (CD25+CD44−) to DP transition, also known as the β-selection checkpoint. At the DN3 stage of αβ T cell development, productive rearrangement of TCRβ genes leads to the expression of a functional pre-TCR on the cell surface, producing a signal that causes increased proliferation, expression of CD4 and CD8 on the cell surface, and subsequent rearrangement of the TCRα chain. Failure to produce functional TCRβ chain or to transmit pre-TCR signals results in cellular apoptosis and developmental arrest at the DN3 stage [2].

The pre-TCR signals that assure normal T cell survival and development are promoted by regulating the expression and activity of the p53 tumor suppressor protein, as well as by increasing the expression of anti-apoptotic proteins, such as Bcl2 and Bcl-x_L [3]. The p53 is crucial for the regulation of cell cycle progression and apoptosis in response to the physiological DNA damage signal generated by V(D)J recombination for rearrangement of TCRβ genes at the DN3 stage of pre-TCR cells. Only thymocytes that express a functional pre-TCR and transit its proper downstream signaling succeed in inactivating the p53-induced cell apoptosis pathway and go onto the DP stage. Developmental arrest caused by defective pre-TCR signaling at the DN3 stage, caused by loss of Rag-1/2, DNA–protein kinase (PK) (SCID), the CD3γ-chain, or Rpl22, can be rescued by the loss of p53, suggesting that the β-selection at the DN3 stage depends on a balance between pre-TCR signaling and p53 activation [4–9]. Therefore, it has been proposed that p53 may act as a sensor for β-selection. However, the mechanisms to control a p53-dependent apoptosis have yet to be fully elucidated.

Pontin is a member of AAA+ ATPase family and functions as a transcriptional coactivator for various transcription factors such as T cell factor (TCF) and androgen receptor [10–15]. In addition, Pontin has been shown to block p53-mediated apoptosis by repressing p53 expression and its target genes [16].

Various transcription factors have been shown to be important for early T cell development. The Rel-like transcription factors nuclear factor kappa B (NF-κB) and the calcineurin-dependent

Abbreviations: TCR, T cell receptor; DN, double-negative; DP, double-positive; SP, single-positive.

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nuclear factor of activated T cells (NFATc) are crucial for regulating early T cell development, downstream of the pre-TCR [17–25]. Transcription factors in Notch signaling pathway, such as Notch1 and NKAP, [26–31] and the oncoprotein c-Myc and its binding protein Miz-1 [32–34] also play essential roles in pre-TCR signaling pathway in T cell development.

Here, we generated mice carrying a conditional allele of *Pontin*, which can be deleted by Cre recombinase–*loxP* system. Using these mice, we found that loss of Pontin expression in thymocytes fails to inactivate the p53-induced apoptosis pathway followed by a blockade of T cell development at the DN3 stage. These results indicate that Pontin is essential for mature T cell development.

2. Materials and methods

2.1. Mice

To create a targeting vector in which exon 3 of the *Pontin* gene was flanked by *loxP* sites, a 13 kb region used to construct the targeting vector was first subcloned from a BAC clone (bMQ403n16, Source BioScience) into a pBluescript phagemid system. The *FRT*-flanked neomycin cassette containing a *loxP* sequence was inserted at the 3′ and the single *loxP* site was inserted at the 5′ of exon 3. The target region was ~2.5 kb and included exon 3. Twenty micrograms of the targeting vector was linearized by NotI restriction enzyme and then transfected to E14Tg2A ES cells (BayGenomics) by electroporation. After neomycin selection, surviving clones were expanded to identify recombinant ES clones by Southern blot analysis. For XbaI digestion, the bands representing WT and mutant alleles are 13.0 kb and 6.0 kb, respectively. The DNA probe used in Southern blotting was a 480-bp EcoRV–XbaI fragment containing exon 4. Targeted ES cells were microinjected into C57BL/6 blastocysts which were used to generate chimeras. The male chimeras were mated to C57BL/6 female mice to obtain F1 heterozygous offspring. Neomycin selection cassette was deleted by crossing targeted heterozygous F1 with Flp deleter strain (FLPeR mice, The Jackson Laboratory strain 003946). Genotypes were verified by PCR and Southern blots. PCR primers used in genotyping are as follows; primer A, 5′-TCGAGGCAGGAGTACCAGGC-3′; primer B, 5′-TTCAGGACAGCAGACTCTGG-3′; primer C,

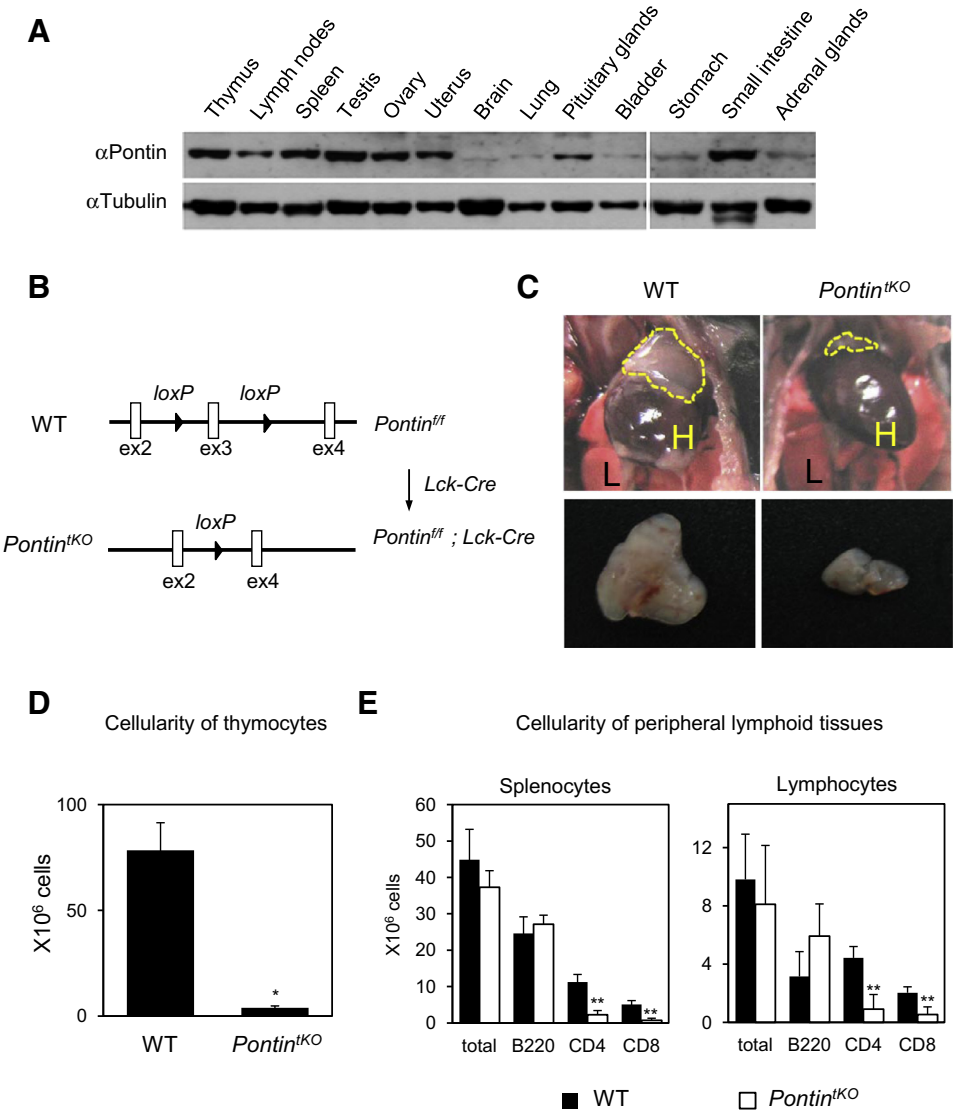

Fig. 1. Reduced number of thymocytes and peripheral T cells in *Pontin*^{tKO} mice. (A) Expression level of Pontin in various mouse tissues. Tubulin was used as a loading control. (B) The strategy for deletion of *Pontin* using *Pontin*$^{f/f}$; *Lck-Cre* mouse (*Pontin*^{tKO}). (C) Thymus size in WT and *Pontin*^{tKO} mice. Each thymus is surrounded by yellow dot lines. Heart, H; lung, L. (D) Cell numbers of thymocytes in WT and *Pontin*^{tKO} mice. Error bars are SD. $^{*}p < 0.001$. (E) Numbers of B220+, CD4+ and CD8+ cells in lymph nodes and spleens of WT and *Pontin*^{tKO} mice. B220 indicates B220+ B cells. CD4 and CD8 indicate CD4+ and CD8+ T cells, respectively. Error bars are SD. $^{**}p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

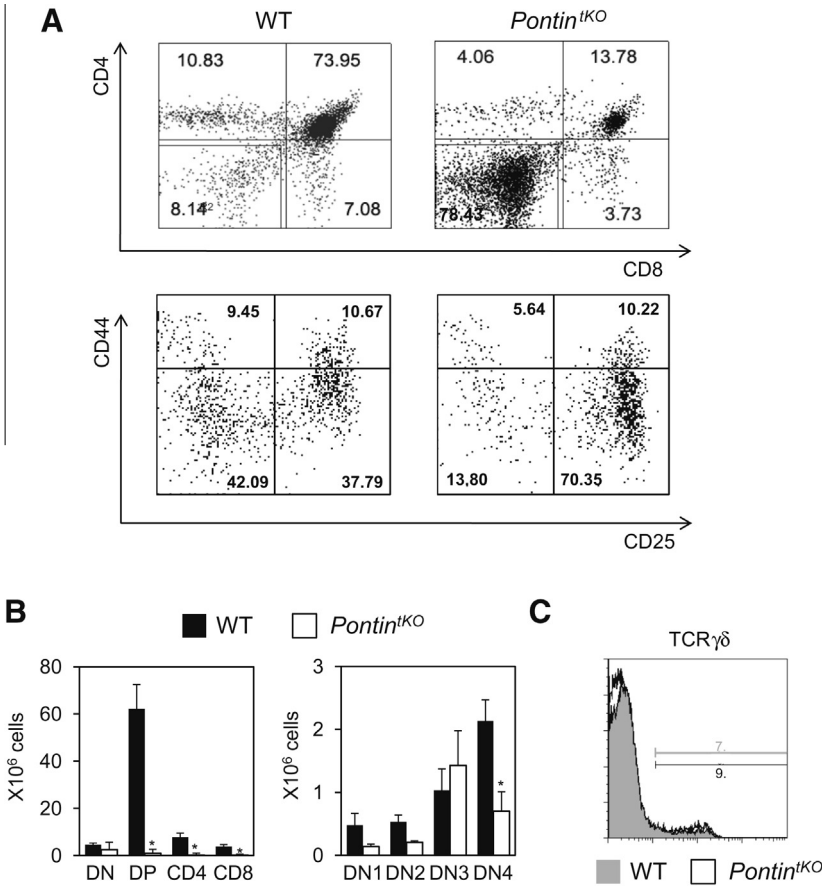

Fig. 2. Pontin is required for T cell development. (A) Total thymocytes from WT and *Pontin*^{tKO} mice at 6–8 weeks of age were examined for surface expression of CD4 and CD8. Gated DN cells from WT and *Pontin*^{tKO} mice were examined for surface expression of CD44 and CD25. Numbers indicate percentages to total thymocytes or DN cells. (B) Absolute cell numbers of thymocyte subsets in WT and *Pontin*^{tKO} mice were calculated. The average absolute numbers of the cells is shown from between three to five *Pontin*^{tKO} or WT mice. Error bars are SD. $^{*}p < 0.01$. (C) DN thymocytes from WT and *Pontin*^{tKO} mice were examined for surface expression of TCRγδ. Numbers indicate percentages to DN cells.

5′-CTCTGCCTGTGAAACCATACC-3′. All mice used for this work were backcrossed to C57BL/6 at least seven generations. *Lck-Cre* transgenic mice were purchased from Taconic. p53-deficient mice were received from NCI Mouse Repository. This study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of National Cancer Center Research Institute.

2.2. Antibodies and flow cytometry

Thymi were separated to single-cell suspensions using glass slides. 1–5 × 10^5 cells were used for each stain. Monoclonal antibodies against CD4, CD8, CD25, CD44, CD27, TCRγδ, and TCRβ were purchased from BD Biosciences and eBiosciences. For RNA purification, cellular subsets were sorted using FACSAria II (BD Biosciences). FACSCanto II (BD Biosciences) was used for flow cytometry analysis. Data were analyzed with FACSDiva software and FlowJo. Annexin V staining was performed using the manufacturer's protocol (BD Biosciences).

2.3. RNA isolation and RT-PCR

RT-PCR was performed using sorted fractions of thymocytes. Total RNA was extracted and purified using RNeasy mini kit according to the manufacturer's instructions (Qiagen). cDNA was synthesized using SuperScript III (Invitrogen). For these experiments, expression was normalized to β-*actin* expression.

2.4. Intracellular staining

Intracellular staining was performed with the Cytofix/Cytoperm kit (PharMingen). After incubation of antibodies against cell surface proteins, cells were gently resuspended in the Cytofix/Cytoperm solution for 20 min, washed with Cytoperm/Wash buffer and stained with TCRβ antibody.

3. Results

3.1. Reduction in number of thymocytes and peripheral T cells in *Pontin*^{tKO} mice

We performed immunoblot analysis with isolated mouse tissues to examine the expression pattern of Pontin in various tissues. Interestingly, we found that Pontin is highly expressed in lymphoid organ tissues, such as thymus, lymph nodes and spleen, as well as in reproductive organs including testis or ovary (Fig. 1A). To further examine whether Pontin has a role in thymocytes or T cells, we designed a strategy for the conditional deletion of *Pontin* by *Lck-Cre* (Fig. 1B and Supplementary Fig. S1A). *Pontin*$^{f/f}$ mice were bred to *Lck-Cre* transgenic mice that subsequently undergo Cre recombinase-mediated *Pontin* excision at the DN2 stage of T cell development (Supplementary Fig. S1B and C). *Pontin*$^{f/f}$; *Lck-Cre* (*Pontin*^{tKO}) mice had smaller thymus (Fig. 1C) and greatly reduced thymic cellularity. Wild-type (WT) mice had on average 78.4 × 10^6 ± 12.6 × 10^6 thymocytes, whereas *Pontin*^{tKO} mice had almost 20-fold fewer number of thymocytes (3.91 × 10^6 ± 0.6 × 10^6)

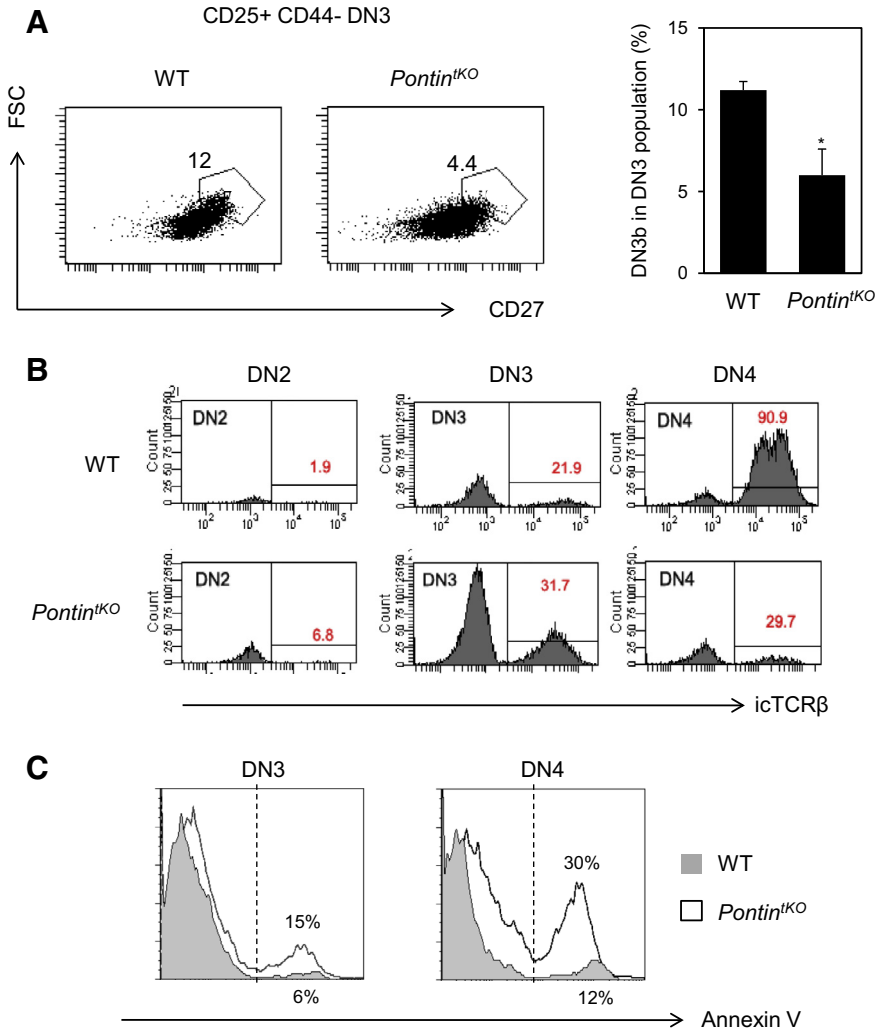

Fig. 3. *Pontin*-deficient DN thymocytes have a severe block at the β-selection checkpoint between DN3 and DN4 stage. (A) Gated DN3 subset was further fractionated into DN3a ($FSC^{low}CD27-$) and DN3b ($FSC^{high}CD27+$). The percentage of positive cells for each gate is indicated. Shown is a representative plot to show gating strategy, with the average DN3b percentage calculated from three WT and four *Pontin*^{tKO} mice also shown. Error bars are SD. $^{*}p < 0.01$. (B) Flow cytometry was performed for analysis of the expression of intracellular TCRβ (ic TCRβ) expression on the cell surface of DN2, DN3, and DN4 cells. Shown is representative data from three independent experiments. (C) Single-cell suspensions of thymocytes were stained with antibodies against lineage markers, CD44 and CD25, followed by Annexin V staining. Percentages of Annexin V^{+} cells are indicated for DN3 and DN4 cells. Shown is representative data from three independent experiments.

(Fig. 1D). Furthermore, *Pontin*^{tKO} mice had fewer numbers of mature CD4+ and CD8+ cells in lymph nodes and spleen, but normal numbers of B cells (B220+) (Fig. 1E). These data indicate that loss of Pontin in thymocytes affects T cell development.

3.2. Loss of Pontin blocks α T cell development at the DN3 stage

To gain insight into the nature of the developmental defect of T cell by loss of Pontin, flow cytometric analyses of stage-specific cell surface markers were performed on thymocytes from *Pontin*^{tKO} versus WT mice. Analysis of CD4 and CD8 expression revealed that *Pontin*^{tKO} mice had significantly fewer DP and SP thymocytes than WT with a relative increase in the DN population (Fig. 2A). In absolute cell numbers of thymocytes, DP thymocytes were reduced by 70-fold, CD4 SP by 40-fold, and CD8 by 20-fold, respectively, whereas the total DN cell population showed little or no change (Fig. 2B). Thus, Pontin deficiency appears to block thymocyte development at the DN-to-DP transition. Further, we analyzed DN thymocytes to determine if the increase in DN thymocytes reflected a block in T cell development. Analysis of CD44 and CD25 expression with DN-gated thymocytes revealed a disproportionate increase in the DN3 population and a relative reduction in the DN4 population in *Pontin*^{tKO} mice, indicating that loss of Pontin impedes T cell development at the DN3 stage (Fig. 2A and B). Although αβ T cell development was disrupted, γδ T cell development was not altered by *Pontin* deficiency (Fig. 2C). Therefore, we conclude that the lack of Pontin causes a block in αβ T cell development at the DN3-to-DP transition, whereas it has little or no effect on γδ T cell development.

3.3. Increased cell apoptosis in post- β-selection population in *Pontin*^{tKO} mice

In normal development, DN3 cells, that rearrange and express the TCRβ chain as part of the pre-TCR and transit its proper downstream signaling, undergo β-selection and maturation to the DN4 stage [1,2]. To investigate whether Pontin deficiency affects β-selection checkpoint, we performed flow cytometric analysis for post-selection DN3b cells on the basis of forward scatter and upregulation of CD27 [35]. DN3b cells, which upregulate CD27 and are larger in size than DN3a cells, were fewer in *Pontin*^{tKO} mice than in WT mice implying that DN3 cells in *Pontin*^{tKO} mice have a defect in passing the β-selection checkpoint (Fig. 3A). Next, we examined whether the loss of *Pontin* affects the expression of TCRβ chain,

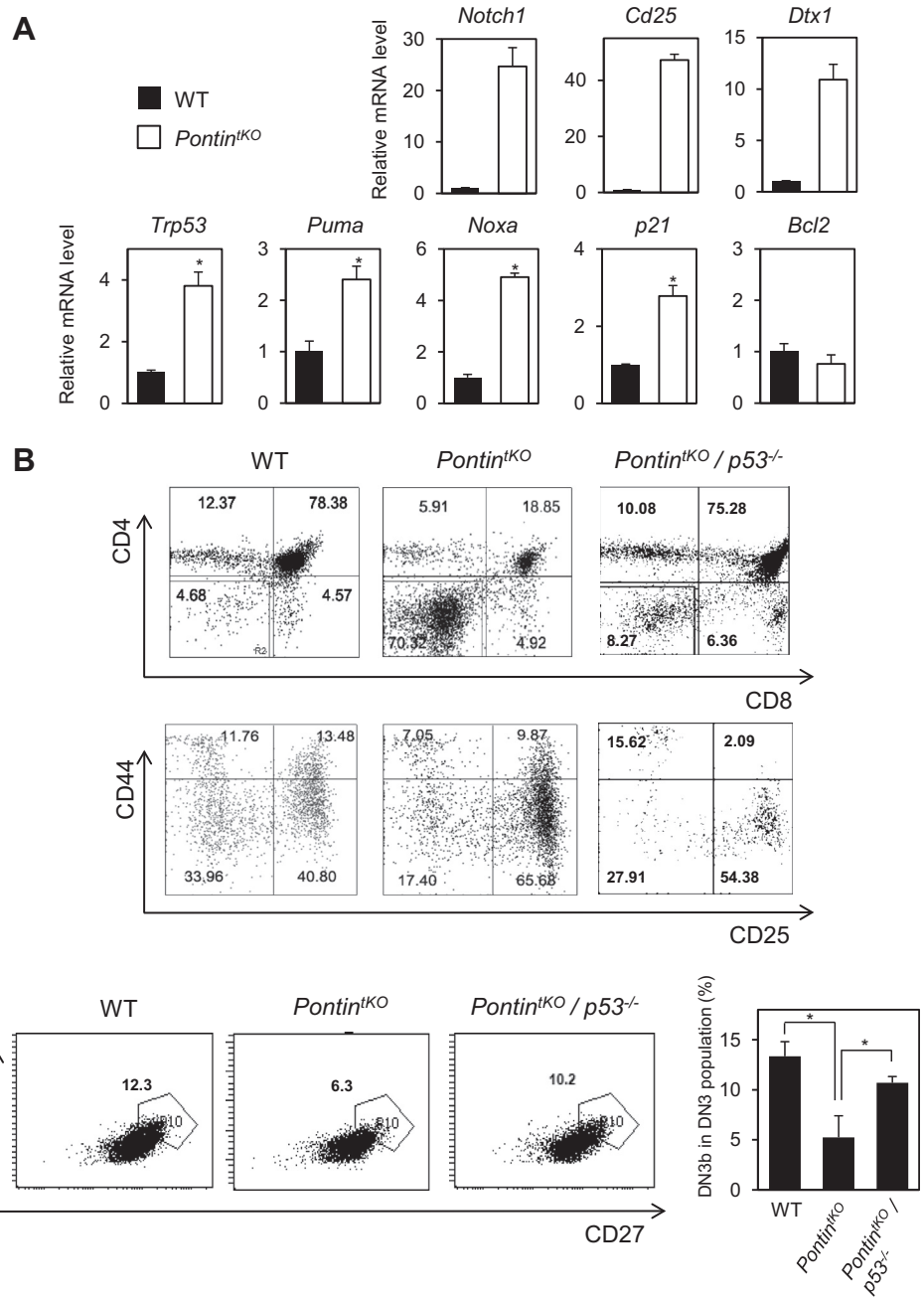

Fig. 4. Inactivation of p53 partially rescues the pre-T cell developmental block caused by loss of Pontin. (A) qRT-PCR was performed using sorted DN3 thymocytes. DN3 cells from *Pontin*^{tKO} mice show enhanced expression of Notch or p53 target genes. Expression was normalized to β-actin expression. Error bars are SD. $^{*}p < 0.01$. (B) Total thymocytes from 6-week-old WT, *Pontin*^{tKO} and *p53*-deficient *Pontin*^{tKO} (*Pontin*$^{f/f}$;*Lck-Cre*;*p53*$^{-/-}$) mice were monitored for the expression of CD4, CD8, CD25 and CD44. The percentage of cells within each quadrant is indicated. Shown is representative data from three independent experiments. (C) Gated DN3 subset was further fractionated into DN3a (FSC^{low}CD27−) and DN3b (FSC^{high}CD27+). The percentage of positive cells for each gate is indicated. Shown is a representative plot to show gating strategy, with the average DN3b percentage calculated from three independent experiments. Error bars are SD. $^{*}p < 0.01$.

which is expressed at the DN3 stage and more strongly activated by pre-TCR signaling at the DN4 stage in normal T cell development. Similar amounts of intracellular TCRβ chain was expressed in DN3 cells from WT and *Pontin*^{tKO} mice, suggesting that *Pontin* deletion impairs thymocyte developmental processes, downstream of pre-TCR assembly. Indeed, the expression of TCRβ chain was not upregulated in DN4 cells from *Pontin*^{tKO} mice (Fig. 3B).

DN thymocytes that do not receive the survival signal from the proper pre-TCR signaling at the β-selection checkpoint are removed by cell apoptosis [2,3]. Annexin V staining was therefore performed to evaluate the percentage of DN3 and DN4 cells undergoing apoptosis from *Pontin*^{tKO} mice. Our data showed a significant increase in the percentage of apoptotic DN3 and DN4 thymocytes in *Pontin*^{tKO} mice compared with WT (Fig. 3C). Together, these results indicate that *Pontin*-deficient DN thymocytes normally express TCRβ chain, but fail to survive past the β-selection checkpoint due to the defect in proper transit of downstream signals from pre-TCR.

3.4. Ablation of p53 partially rescues the pre-T cell developmental block caused by loss of Pontin

The appropriate signals transmitted from pre-TCR are important for inactivation of other signaling pathways, such as p53 and

Notch signaling, which are induced at the pre-selection stage [8,26]. To investigate the inactivation of p53 and Notch signaling by pre-TCR signaling in *Pontin*^{tKO} mice, we quantified Notch or p53 target gene transcript levels in DN3 cells of WT and *Pontin*^{tKO} mice. An enhanced activation of Notch target genes, such as *Notch*, *Dtx1* and *Cd25*, occurred from *Pontin*-deficient DN3 cells, compared to WT DN3 cells. In addition, p53 and its target genes, such as *Puma*, *Noxa*, and *p21*, were highly expressed in *Pontin*-deficient DN3 cells, indicating that Pontin is essential for inactivation of p53 and Notch signaling by pre-TCR signaling in T cell development (Fig. 4A). Aberrant activation of p53 might be a major cause of increased apoptosis of DN3 and DN4 cells in *Pontin*^{tKO} mice.

Some genetic mutant mouse models, which have a developmental block from DN to DP cells caused by defective pre-TCR signaling, can be rescued to some extent, by loss of p53 [4–6,8,9]. To test this possibility, we analyzed whether deletion of p53 can rescue the developmental defect of *Pontin*-deficient thymocytes. Indeed, loss of p53 in Pontin mutant background restored partially the cellularity of thymocytes and the development to the DP and SP stages to the levels similar to WT (Fig. 4B and Supplementary Fig. S2). To confirm that loss of p53 can restore the developmental block at the β-selection stage in *Pontin*^{tKO} mice, we examined the population of DN3b thymocytes, the post-selected DN3 subset by flow cytometric analysis. Our data showed that post-selected DN3b cells were restored to some extent, in *Pontin*^{tKO} mice by loss of p53 implying that a developmental block at the β-selection stage in *Pontin*^{tKO} mice is, at least in part, dependent on p53 activation (Fig. 4C).

4. Discussion

In this study, we show that Pontin is essential for pre-TCR signaling at the β-selection stage between DN3 and DP, which is the first critical checkpoint in T cell development. During the developmental process of thymocytes, *Pontin*-deficient thymocytes were arrested at the DN3 stage, when pre-TCR signaling was activated, and had increased apoptosis that reduced the population of post-selected thymocytes. Interestingly, thymocytes from *Pontin*^{tKO} mice normally expressed TCRβ chain, but showed defects in the transition of pre-TCR signaling, resulting in abnormal activation of p53 and Notch signaling. Indeed, *p53*-deficient *Pontin*^{tKO} mice were partially rescued from a developmental block at the β-selection.

At the β-selection checkpoint, inactivation of Notch and p53, is an important part of pre-TCR signaling. For instance, enhanced or prolonged p53 activation in thymocytes causes developmental block [4–9] and hyperactivation of Notch signaling may be oncogenic [26–30]. Given that the inactivating mechanisms are poorly understood, here, we propose a novel player, Pontin as a crucial factor for inactivation of p53 and Notch signaling by pre-TCR.

Previous reports show that some transcription factors or coregulators are involved in regulation of pre-TCR signaling that mediates inactivation of signaling [17–34]. Pontin is a chromatin remodeling factor and interacts with other DNA-binding transcription factors [10–16]. One plausible mechanism for the involvement of Pontin in this inactivation by pre-TCR signaling is that Pontin functions as a coregulator of the transcription factors that are critical to the pre-TCR signals. On the other hand, Pontin may play a role in the regulation of Notch and p53 target gene expression as a direct repressor. Indeed, recent studies report that Pontin blocks p53-mediated apoptosis by repressing the expression of p53 and its target genes in colon cancer cells, suggesting the possibility for Pontin as a repressor [16].

In conclusion, we report a previously unknown function of Pontin in T cell development process by using *Pontin*$^{f/f}$; *Lck-Cre* mouse models and demonstrate that Pontin is required for inactivation of p53 and Notch signaling in pre-TCR signaling pathway at the β-selection stage in T cell development.

Acknowledgments

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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.2014.03.092.

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