**Zinc finger protein Zfp335 controls early T cell development and survival
through β -selection-dependent and -independent mechanisms**

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Abstract

T cell development in the thymus undergoes the process of differentiation, selective proliferation and survival from CD4⁻CD8⁻ double negative (DN) stage to CD4⁺CD8⁺ double positive (DP) stage prior to the formation of CD4⁺ helper and CD8⁺ cytolytic T cells ready for circulation. Each developmental stage is tightly regulated by sequentially-operating molecular networks, of which only limited numbers of transcription regulators have been deciphered. Here we identified Zfp335 transcription factor as a new player in the regulatory network controlling thymocyte development in mice. We demonstrate that *Zfp335* intrinsically controls DN to DP transition, as T cell-specific deficiency in *Zfp335* leads to a substantial accumulation of DN3 along with reduction of DP, CD4⁺ and CD8⁺ thymocytes. This developmental blockade at DN stage results from the impaired intracellular TCR β expression as well as increased susceptibility to apoptosis in thymocytes. Transcriptomic and ChIP-seq analyses revealed a direct regulation of transcription factors *Bcl6* and *Rorc* by Zfp335. Importantly, enhanced expression of TCR β and *Bcl6/Rorc* restores the developmental defect during DN3 to DN4 transition and improves thymocytes survival, respectively. These findings identify a critical role of *Zfp335* in controlling T cell development by maintaining intracellular TCR β expression-mediated β -selection and independently activating cell survival signaling.

Keywords: thymocyte; *Zfp335*; differentiation; apoptosis; β -selection

Research organism: Mouse

Introduction

T cell development proceeds in a series of developmental stages, which is precisely orchestrated by multiple signaling and molecular networks^{1, 2, 3}.

Prethymic progenitor cells originated from bone marrow migrate into the thymus and sequentially differentiate into CD4⁻CD8⁻(DN), CD4⁺CD8⁺(DP), and the CD4⁺ or CD8⁺ (SP) stage. Based on the expression of CD44 and CD25, DN thymocytes are divided into several phenotypically distinct stages, including DN1 to DN4^{4, 5, 6, 7}. In the presence of Notch signaling, early thymic

progenitor (ETP)-DN1 cells transit into DN2a stage, initiating the T cell lineage commitment, which is immediately accompanied by TCR β gene arrangement.

The majority of DN2 cells enter the DN3 stage with $\alpha\beta$ lineage potential⁸. Only DN3 cells with a complete pre-TCR complex, which consists of the functional TCR β protein, pre-T α (pT α) chain and CD3 molecule, can successfully trigger the subsequent maturation into DN4 and DP thymocytes. Further differentiation into mature CD4⁺ or CD8⁺ T cells requires positive and negative selection at DP stage before they migrate to peripheral lymphoid organs^{9, 10, 11,}

¹².

Pre-TCR signals regulate thymocyte differentiation by mediating protection from apoptosis, stimulating proliferation, and inducing allelic exclusion at the TCR β locus in post- β -selection DN3b cells and promoting DN to DP transition^{10, 13, 14}. Inactivation of pre-TCR components dampens T lymphocyte

development by arresting thymocytes at the DN3 stage and inducing apoptosis^{15, 16, 17, 18}. Multiple transcription factors downstream of pre-TCR signaling are involved in T cell differentiation and survival. The major pre-TCR signaling is conducted through the dose-dependent expression of Notch controlled by the Id3-E2A axis¹⁹. Abrogation of either Notch or E2A expression may lead to the developmental block of thymocytes at multiple stages^{20, 21, 22}. In addition, activation of NF- κ B²³, Ets1²⁴ and NFAT5²⁵ by pre-TCR signals also contributes to the developmental block. The transcription factor T cell factor 1 (TCF1), together with its downstream Bcl-11b, not only increases the potential to differentiate into T cells^{26, 27}, but also positively regulates thymocyte development via promoting TCR β recombination and expression, as well as DP cell survival^{28, 29}. Apart from the essential role in the T follicular helper cell lineage commitment³⁰, Bcl6 induced by pre-TCR signals is also involved in the DN to DP transition and protection of DN4 cells from apoptosis³¹. Additionally, abrogation of Rorc expression in thymocytes leads to a decreased DP proportion and impaired DP survival in a Bcl-xl-dependent manner^{32, 33, 34}.

Although pre-TCR signaling is crucial for the β -selection checkpoint, it is not sufficient for progression to the DN3 stage. Other pathways or transcription factors coupled with or independent of conventional pre-TCR signaling are found to play important roles in the process³⁵. The developmental blockade in *Smarca5* or *Nkap*-deficient thymocytes is confirmed by intact pre-TCR signals

in these mice ^{36, 37}. Overall, it remains largely unknown which factors are crucial for T cell development through mechanisms independent of pre-TCR signaling.

Zfp335, also known as the nuclear hormone receptor coregulator (NRC) – interacting Factor 1 (NIF-1), is a zinc finger protein with a 13 C2H2 zinc finger repeating structure consisting of 1337 amino acids ³⁸. The C2H2-ZF family encodes more than 700 proteins in the human genome, some of which play important roles in ontogenesis, immune cell differentiation, and disease occurrence ^{26, 39}, yet the biological characteristics and functions of most members are unclear ^{40, 41}. Zfp335 regulates gene transcription by recruiting H3K4 methyltransferase complexes, interacting with co-activators, or directly binding to certain gene promoters ^{38, 42, 43, 44}. Zfp335 plays important regulatory roles in early embryonic development and neurogenesis ⁴². Germline knockout of Zfp335 is embryonic lethal, while deletion of Zfp335 gene in nerve cells impairs the proliferation and differentiation of nerve progenitor cells in mice, eventually leading to severe microcephaly ⁴². Function of Zfp335 in T cell development has been observed in the study of the *Zfp335bloto* allele, a missense mutation derived from ENU mutagenesis. While thymocyte development is not significantly affected by this hypomorph mutation, there is a significant reduction in the number of peripheral T cells due to defects in the maturation and migration of thymocytes ⁴⁵. Without loss of function studies, it

remains to be determined whether Zfp335 is required for intrathymic T cell development.

In this study, we investigated Zfp335 expression during different thymocyte stages, as well as its function at the β -selection checkpoint and during DN to DP transition. We found that in the thymus, Zfp335 has the highest expression in DN3 thymocytes. Zfp335 is indispensable for thymocyte β -selection and supports the transition from DN to DP stage by maintaining intracellular TCR β expression, as well as by promoting DN and DP thymocyte survival via directly regulating *Bcl6* and *Rorc* expression.

Results

Impaired thymic $\alpha\beta$ T cell development in *Zfp335*-deficient mice

To study the role of *Zfp335* in T cell development, we first assessed the expression of *Zfp335* among different thymocyte subsets, including DN3, DN4, DP, CD4, CD8, NKT and $\gamma\delta$ T cells. We found that DN3 cells displayed a relatively high level of *Zfp335* mRNA expression (Figure 1A). Flow cytometry analysis also revealed that *Zfp335* protein had the highest expression in DN3 thymocytes (Figure 1—figure supplement 1A, B). Consistently, microarray data from ImmGen showed higher expression of *Zfp335* specifically at the DN3a stage during T cell development from ETP to CD4/CD8 SP (Figure 1—figure supplement 2A). Although RNA-seq data from ImmGen exhibited the highest expression of *Zfp335* in DP thymocytes, a gradually increased expression was observed from ETP to DN3 (Figure 1—figure supplement 2B). Given the importance of DN3 stage during β -selection checkpoint, we obtained T cell-specific *Zfp335* mice by crossing *Zfp335^{fl/fl}* strain with Lck-Cre strain (Figure 1—figure supplement 3A). *Zfp335* deletion was confirmed by real-time PCR (qPCR) analysis in DN4 cells (Figure 1—figure supplement 3B). Strikingly, *LckCre⁺Zfp335^{fl/fl}* (KO) mice exhibited significantly smaller thymi and drastically decreased thymocyte numbers than WT control (Figure 1B). Further analysis showed that both percentages and numbers of DP cells, as well as CD4 SP and CD8 SP cells, were considerably reduced in KO mice (Figure 1C-E). Conversely, the percentage of DN cells was increased by nearly 10-15 folds,

although the total number was decreased (Figure 1C-E). In secondary lymphoid organs, we also observed reduced CD4⁺ and CD8⁺ cells in the spleen and lymph nodes (Figure 1F-J). Thus, *Zfp335* is essential for the development of $\alpha\beta$ T cells in the thymus.

***Zfp335* intrinsically regulates T cell development in the thymus**

To address whether *Zfp335* deletion intrinsically affects T cell development, Lin⁻CD25⁺ CD44⁻ DN3 cells from WT or KO mice were harvested and plated with OP9-DL1 cells in the presence of Flt3L and IL-7, an *in vitro* model for T-cell development (Figure 2A)⁴⁶. On both day 2 and day 4, KO group produced fewer DP cells than WT control (Figure 2B-D, Figure 2—figure supplement 1A). When DN3 cells from WT (CD45.1⁺) and KO (CD45.2⁺) mice were mixed and co-cultured at a 1:4 ratio (KO cells were rapidly competed out when used at a 1:1 ratio), significantly lower percentage and cell proportion of DP cells was also observed in KO group (Figure 2E-G, Figure 2—figure supplement 1B). Furthermore, *in vivo* T cell development was investigated by adoptively transferring T and B cells-depleted bone marrow cells from WT (CD45.1⁺) and KO (CD45.2⁺) mice with a 1:4 ratio into WT (CD45.1⁺CD45.2⁺) recipients (Figure 2H). When thymocytes were analyzed 6 weeks post transfer, both the percentages and cell numbers of DP, CD4 and CD8 cells were significantly reduced while DN percentage was increased in the mice that received KO cells (Figure 2I-J, Figure 2—figure supplement 1C). Consistently,

when WT and KO bone marrow cells were mixed at a 1:4 ratio for co-transfer experiments, fewer DP cell proportion detected in KO compared to WT group (Figure 2K, L; Figure 2—figure supplement 1D). In addition, DN3 cells from KO mice also generated fewer $\gamma\delta$ T cells, despite a higher percentage, in comparison to WT controls after culturing for 4 days (Figure 2—figure supplement 2A-C). Together, we demonstrate that *Zfp335* intrinsically regulates thymocyte development from DN to DP stage.

Loss of *Zfp335* blocks the transition from DN3 to DN4 stage

We further examined the impact of *Zfp335* deletion on DN thymocyte development. Staining of CD44 and CD25 on pre-gated lineage (CD4/CD8/TCR β /TCR δ /NK1.1/CD19/CD11b/CD11c)-negative thymocytes was performed in WT and KO mice. The results showed that a higher percentage of CD44⁺CD25⁺ DN3 cells but a lower percentage of CD44⁺CD25⁺ DN4 cells were found in KO group compared to WT group (Figure 3A, B), in which the numbers of both DN3 and DN4 thymocytes were decreased (Figure 3C). The developmental blockade from DN3 to DN4 in *Zfp335*-deficient cells was verified by *in vitro* co-culture assays on day 2 using OP9-DL1 cultured with WT and KO DN3 cells respectively (Figure 3D, E) or mixed at a 1:4 ratio (Figure 3F, G) as described above. The *in vivo* bone marrow chimera models further confirmed the development block at the DN3 stage (Figure 3H-K), indicating that *Zfp335* is indispensable for DN3 to DN4 transition during

early-stage differentiation.

Ablation of Zfp335 promotes apoptosis in thymocytes

During the β -selection, efficient proliferation of pre-T cells is necessary for DN to DP progression⁴⁷, during which the pre-TCR signal functions as a positive regulator of thymocyte survival, allowing for differentiation from pre-T cells into DP thymocytes. We sought to examine whether the defect in *Zfp335* KO thymocyte development is due to impaired proliferation or survival. The *in vivo* BrdU incorporation assay showed comparable or even higher percentages of BrdU⁺ DN3 and DN4 cells (Figure 4A, B) as well as Ki67⁺ DN3 and DN4 cells (Figure 4—figure supplement 1). Nevertheless, when we examined thymocyte apoptosis, KO mice showed a remarkably higher percentages of Annexin V⁺ DN3 and DN4 cells compared to WT cells (Figure 4C-4D). After co-culture with OP9-DL1 for 4 days, both *Zfp335*-deficient DN3 and DN4 cells showed an increased percentage of Annexin V⁺ cells (Figure 4E, F). Further examination of the transition between the DN3a and DN3b stages found that the frequencies of both DN3a and DN3b were comparable between WT and *Zfp335*-deficient groups (Figure 4—figure supplement 2A-C). However, both cell populations showed enhanced cell apoptosis in *Zfp335*-deficient cells (Figure 4 — figure supplement 2D-F), indicating that *Zfp335* regulates thymocyte apoptosis in a TCR-independent manner. Moreover, in mixed bone marrow chimeras, *Zfp335*-deficient DN3 and DN4 cells also displayed

significantly higher Annexin V⁺ cells (Figure 4G, H), indicating an intrinsic role of *Zfp355* in regulating thymocyte survival.

Ectopic expression of TCR $\alpha\beta$ overcomes DN3 stage block in *Zfp335*-deficient mice

The pre-TCR signal-controlled β -selection is essential for thymocyte differentiation from DN3 to DN4 stage⁴⁸. We then examined the expression of genes involved in the pre-TCR complex in DN3 and DN4 cell populations. The expression of *Ptcra* (encoding pT α) was slightly increased while *Trbc1*, *Trbc2* and *Cd3e* in DN3 and DN4 cells were comparable in WT and KO group (Figure 5—figure supplement 1A, B). Additionally, the expression of intracellular CD3 (iCD3) was also unaffected in both *Zfp335*-deficient DN3 and DN4 cells (Figure 5—figure supplement 2A, B). Interestingly, flow cytometry analysis showed no substantial difference in intracellular TCR β (iTCR β) expression between WT and KO DN3 cells, whereas *Zfp335*-deficient mice displayed a significant decrease in the percentage of iTCR β ⁺ DN4 cells (Figure 5A, B). To further address whether *Zfp335* deficiency affects TCR β expression, we compared differential usage of TCR β in DN4 cells between WT and KO mice, in which the expression of TCR V β 5, V β 6, V β 8 and V β 12 were decreased concomitantly (Figure 5C-F). However, genomic DNA analysis for V-DJ β 5 and V-DJ β 8 rearrangements showed no difference between WT and KO DN3 cells (Figure 5—figure supplement 3). Thus, the reduced iTCR β expression may be

a consequence of protein degradation.

Given that *Zfp335* deficiency led to diminished $\alpha\beta$ TCR expression in DN4 cells, we next investigated the effect of TCR overexpression on aberrant thymocyte development caused by *Zfp335* deficiency. Offspring (*LckCre⁺Zfp335^{fl/fl}*OT1⁺) of *LckCre⁺Zfp335^{fl/fl}* mice crossed to OT1 transgenic (Tg) mice was generated to constitutively express *Tcra-V2* and *Tcrb-V5* Tg gene (OT1^{Tg}KO). Notably, forced expression of $\alpha\beta$ TCR successfully restored the decreased percentage of $\alpha\beta$ TCR⁺ DN4 cells in the KO mice, despite with little impact on the number of $\alpha\beta$ TCR⁺ DN4 cells (Figure 5G-I). Importantly, developmental arrest at the DN3 stage in *Zfp335*-deficient mice was fully rescued by OT1 transgene (Figure 5J-L). Unfortunately, DN3 and DN4 cells from OT1^{Tg}KO mice still exhibited a similar degree of apoptosis with *Zfp335*-deficient cells (Figure 5—figure supplement 4), suggesting *Zfp335* affects thymocyte apoptosis in a TCR-independent manner. Consistently, the proportions of DN, DP, CD4 and CD8 were still comparable in KO and OT1^{Tg}KO mice (Figure 5M, N; Figure 5—figure supplement 5), demonstrating that the aberrant DP development has not yet been restored by $\alpha\beta$ TCR overexpression.

***Zfp335* directly targets *Bcl6* and *Rorc* in DN thymocytes**

To address the underlying molecular mechanisms of *Zfp335*-mediated thymocyte development, we performed comprehensive RNA-seq analysis

253 comparing DN4 cells from WT and KO mice. Volcano plot showed that
254 *Zfp335*-deficient DN4 cells had a total 566 downregulated and 899
255 upregulated genes (Fold change>1.25, $P < 0.05$) (Figure 6A, Supplementary
256 file 1). Gene ontology (GO) analysis highlighted a large fraction of genes
257 downregulated in KO group belonging to lymphocyte differentiation and
258 apoptotic pathways (Figure 6B). Prominently down-regulated genes
259 associated with lymphocyte differentiation and apoptosis were summarized in
260 the heatmap (Figure 6C). To further determine genes directly regulated by
261 *Zfp335*, we analyzed *Zfp335* by chromatin immunoprecipitation followed by
262 deep sequencing (ChIP-seq). We screened a total of 2797 *Zfp335*-binding
263 sites (Supplementary file 2) and the prevalence of binding peaks across
264 genomic regions was displayed as a pie chart (Figure 6—figure supplement 1).
265 To identify the *Zfp335* targeting candidates, 119 profoundly downregulated
266 genes were further selected out by a cutoff of 2-fold change, and 22 genes
267 were overlapped with *Zfp335*-targeting genes from ChIP-seq data (Figure
268 6—figure supplement 2A, Supplementary file 3). Among these genes, top 10
269 genes were listed based upon their expression level from RNA-seq result
270 (Figure 6—figure supplement 2B). qPCR analysis was performed to confirm
271 their downregulation in KO DN4 cells (Figure 6—figure supplement 2C). Next,
272 we focused on genes related to lymphocyte differentiation and apoptosis. In
273 line with RNA-seq results (Figure 6C), qPCR analysis verified that *Bcl6* and
274 *Rorc* were significantly downregulated in KO DN4 cells (Figure 6D).

Importantly, *Zfp335* directly targeted the promoter regions of *Bcl6* and *Rorc* in ChIP-seq analysis (Figure 6E), which were further verified by luciferase assay (Figure 6F). Taken together, in depth genomic analysis of DN thymocytes supports that *Zfp335* directly regulates the transcription of *Bcl6* and *Rorc*.

Defects in thymocyte development caused by *Zfp335* deficiency can be rescued by *Bcl6* and *Rorc*

To determine whether *Bcl6* and *Rorc* participate in the regulation of thymocyte development downstream of *Zfp335*, overexpression of *Bcl6* and *Rorc* was conducted *in vitro* in the thymocyte development model (Figure 7A). DN3 cells from KO mice were co-cultured with OP9-DL1 cells and transduced with retrovirus encoding *Mock*-GFP, *Zfp335*-GFP, *Bcl6*-GFP or *Rorc*-GFP. After 3.5 days, overexpression of *Bcl6* and *Rorc* resulted in a substantial DP generation, particularly in the *Bcl6* group, resulting in a similar DP proportion to that in *Zfp335*-overexpressing cells (Figure 7B, C). Of note, overexpression of *Psmg2*, *Dctn1*, *Ankle2*, *Cep76*, *Fgf13* and *Ddx31* identified in the qPCR results (Figure 6—figure supplement 2B, C) in KO DN3 cells did not restore DP generation *in vitro* (Figure 7—figure supplement 1). Importantly, enhanced expression of *Bcl6* in DN3 cells rescued DN thymocyte apoptosis (Figure 7D, E), while overexpression of both *Bcl6* and *Rorc* rescued DP thymocytes from enhanced apoptosis (Figure 7F, G). p53, negatively regulated by *Bcl6*, is involved in lymphocyte apoptosis^{49, 50, 51}. Thus, we crossed *Zfp335* KO strain with *Trp53*

KO strain to obtain double knockout mice, in which *Trp53* deletion resulted in a partial recovery of DP percentage, but not cell number (Figure 7—figure supplement 2), further supporting the role of *Bcl6* in *Zfp335*-controlled thymocyte survival. Together, these data demonstrate that *Zfp335* controls DN thymocyte survival through direct regulation of *Bcl6* and *Rorc* expression.

Discussion

In this study, we reveal that *Zfp335* is essential for thymocyte development, particularly during DN to DP transition. *Zfp335* deficiency in T cells led to a significant loss of DP, CD4 SP and CD8 SP cells while an accumulation of DN3 cells. Mechanistically, the developmental blockade is attributed to both impaired pre-TCR signal and increased susceptibility to apoptosis. Serving as a transcription factor, *Zfp335* directly promotes *Bcl6* and *Rorc* expression in DN thymocytes to ensure their survival during early development.

Zfp335 was previously demonstrated to be crucial for early embryonic development as homozygous deletion of this gene resulted in neonatal death⁵². Conditional knockout of *Zfp335* in neural system led to severely reduced cortical size and impaired neurogenesis. Mechanistically, *Zfp335* was required for neural progenitor cell self-renewal and proliferation, and neuronal differentiation⁴² and neuronal morphogenesis⁵³. Besides, deficiency of naïve T cells in mice carrying a hypomorph allele of *Zfp335* (*Zfp335^{bloto}*) uncovered its role in immune system⁴⁵. So far, there is still very limited information about

the functions of Zfp335 in other aspects of immune system. Here we found that Zfp335 is absolutely required for multiple steps of early T cell development. Of note, it will be worth investigating whether and how Zfp335 is involved in the regulation of mature T cell differentiation and functions under static and immunized conditions in future.

We have shown that Zfp335 expression was upregulated specifically in DN3 thymocytes and significantly decreased in the subsequent stages, suggesting a critical role at the DN3 stage. Of note, loss of Zfp335 led to a dramatic reduction in both thymus size and thymocyte number. The accumulation of DN3 cells results from an intrinsic mechanism that hinders the transition from DN3 to DN4 stage, leading to the reduction of DP thymocytes and mature T cells in the periphery. These data are in line with another recent study reporting that *Zfp335* mutation led to a reduction in peripheral T cells as a result of defective naïve T cells and SP thymocytes⁴⁵. However, given the intact thymic selection with *Zfp335* mutation, the report was inconsistent with our observation of decreased β -selection with Zfp335 deficiency. The discrepancy is likely due to the different approaches used to disrupt Zfp335 function since a single-nucleotide missense mutation of Zfp335 may affect its function differently. Nevertheless, by knocking out the entire Zfp335 protein, we provide evidence that Zfp335 is indispensable for early thymocyte development.

342

343 Thymocyte β -selection is a critical developmental checkpoint allowing for the
344 progression from DN3 to DN4 stage and the maintenance of DP cell numbers,
345 which is primarily dependent on TCR β and pre-TCR signals constituted with a
346 functional iTCR β paired with a pre-T α chain ⁵⁵. Pre-TCR signaling regulates
347 thymocytes differentiation, proliferation, and survival in the full developmental
348 process ⁵⁶. In addition, there are reports that other pathways or transcription
349 factors coupled with or independent of conventional pre-TCR signaling are
350 found to play important roles in the process ^{35, 36, 37}. While *Zfp335*-deficient
351 DN4 cells exhibited no defects in the rearrangement of TCR β chain genes and
352 pT α gene expression, our results clearly demonstrated that *Zfp335* deficiency
353 markedly impaired intracellular TCR β expression and led to an unbiased
354 reduction of the majority of V β genes in DN4 populations. Future studies will
355 investigate the mechanisms how *Zfp335* regulates iTCR β expression.
356 Importantly, forced iTCR β expression in DN3 and DN4 cells by transduction of
357 OT1-TCR completely rescued the developmental impairment during the
358 DN3-DN4 transition in *Zfp335*-deficient mice despite the failure to rescue the
359 DN3, DN4 and DP population size. This suggests that *Zfp335* controls the
360 DN3-DN4 transition dependent on pre-TCR signals, but other mechanisms
361 may also regulate the DP population size.

362

363 The large population of DP thymocytes is maintained by both cell proliferation

and survival mechanisms. *Zfp335*-deficient DN3 and DN4 cells showed slightly higher or unchanged incorporation of BrdU, suggesting that cell proliferation was not affected. However, our data revealed a significant increase in apoptosis in *Zfp335*-deficient DN3 and DN4 thymocytes. Transcriptomic analysis (RNA-Seq and qPCR) unveiled the downregulation of *Bcl6* and *Rorc* signaling which are critically involved in thymocyte apoptosis^{31, 34}. Indeed, we have demonstrated that *Zfp335*, a transcription factor, directly bound to the promoter regions of *Bcl6* and *Rorc* genes. More importantly, enhanced expression of *Bcl6* and *Rorc* could improve thymocyte survival and substantially restore the DP thymocyte population. *Trp53* deletion resulted in a partial recovery of DP cells, further supporting the role of *Bcl6* in *Zfp335*-controlled thymocyte survival. Of note, *Zfp335* may also control thymocyte survival through directly regulating other targets.

In our study, *Zfp335* is indispensable for thymocyte β -selection given that T cell-specific deficiency in *Zfp335* leads to impaired intracellular TCR β expression, blockade of thymocytes at DN stage as well as a substantial DN cell apoptosis. Though enhanced expression of TCR β restores the developmental defect during DN3 to DN4 transition, it had little impact on the population size of DN3, DN4 and DP cells, suggesting the regulation of *Zfp335* on DN cell apoptosis through mechanisms more than β -selection. Indeed, we provided the evidence that *Zfp335* controlled DN cell survival through

regulating *Bcl6* and *Rorc* expression. Moreover, *Zfp335* regulates TCR β expression independent on *Bcl6* and *Rorc* since overexpression of neither *Bcl6* or *Rorc* in *Zfp335*-deficient DN3 thymocytes could restore the decreased intracellular TCR β expression (Figure 7—figure supplement 3).

Several key factors have been shown to involve in the regulation of T cell developmental process from DN3 to DP stages. Notch signaling is required for early T cell commitment and β -selection^{61, 62, 63}, which is subsequently weakened by *Bcl6* repression for the differentiation from DN to DP stage development³¹. Consistently, our results found that *Zfp335* could directly target *Bcl6*, and *Zfp335* deficiency led to decreased expression of *Bcl6* and upregulation of Notch target genes such as *Dtx1* and *Notch1* (data not shown), which collectively contributing to the developmental block from DN to DP stage. In addition, *Tcf1* plays a vital role in T cell lineage commitment since *Tcf1*^{-/-} DN3 thymocytes failed to progress to DN4 and subsequent DP stage through regulating *Bcl11b* and *Gata3* expression⁶⁴. *Bcl11b* and *Gata3* also differentially regulate the differentiation and survival of thymocytes at DN3, DN4 and subsequent ISP stages via TCR or/and survival signals^{65, 66}. However, we did not detect reduced expression of *Tcf1*, *Bcl11b* or *Gata3* in *Zfp335*-deficient DN4 cells, suggesting that *Zfp335* regulates DN3 to DP thymocyte development independent on these molecules.

In conclusion, our study reveals that the C2H2 zinc finger protein Zfp335 plays a novel and crucial role during thymocyte development, specifically during the transition from DN to DP stage. Mechanistically, Zfp335 promotes *Bcl6* and *Rorc* signaling to prevent thymocytes apoptosis and ensure the survival and differentiation of thymocytes. Collectively, we provide evidence that Zfp335 is essential for thymocyte development through both pre-TCR dependent and independent mechanisms.

419 **Materials and Methods**

420 Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Genetic reagent (<i>M. musculus</i>)	C57BL/6J background	Jackson	Stock No:	
		Laboratory	000664	
Genetic reagent (<i>M. musculus</i>)	C57BL/6- <i>Zfp335tm1Caw</i>	Jackson	Stock No:	
		Laboratory	022413	
Genetic reagent (<i>M. musculus</i>)	C57BL/6-Tg(<i>TcraTcrb</i>)1100Mj	Jackson	Stock No:	Common
	b/J	Laboratory	003831	Name: OT-1
Genetic reagent (<i>M. musculus</i>)	B6.Cg-Tg(Lck-cre)548Jxm/J	Jackson	Stock No:	
		Laboratory	003802	
Genetic reagent (<i>M. musculus</i>)	<u>C3Ou.129S2(B6)-Trp53tm1Tyj</u>	Jackson	Stock No:	
	<u>/J</u>	Laboratory	002547	
Antibody	anti-mouse CD4	Biolegend	Cat#10041	cell surface
	APC/Cyanine7 (Rat		4;RRID:AB	staining
	monoclonal)		_312699	1:400

Antibody	anti-mouse CD8a PE (Rat monoclonal)	Biolegend	Cat#10070 8;RRID:AB _312747	cell surface staining 1:400
Antibody	anti-mouse CD8a Pacific Blue™ (Rat monoclonal)	Biolegend	Cat# 100725, RRID:AB_4 93425	cell surface staining 1:400
Antibody	anti-mouse CD8a PE/Cyanine7 (Rat monoclonal)	Biolegend	Cat# 100722, RRID:AB_3 12761	cell surface staining 1:400
Antibody	anti-mouse TCR Vβ5.1, 5.2 PE (Mouse monoclonal)	Biolegend	Cat# 139504, RRID:AB_1 0613279	cell surface staining 1:400
Antibody	anti-mouse TCR Vβ6 PE (Rat monoclonal)	Biolegend	Cat# 140004, RRID:AB_1 0643583	cell surface staining 1:400
Antibody	anti-mouse TCR Vβ8.1, 8.2 PE (Mouse monoclonal)	Biolegend	Cat# 140104, RRID:AB_1	cell surface staining 1:400

0639942				
Antibody	anti-mouse TCR V β 12 PE (Mouse monoclonal)	Biolegend	Cat# 139704, RRID:AB_1	cell surface staining 1:400
0639729				
Antibody	anti-mouse TCR β chain APC/Cyanine7 (Armenian Hamster monoclonal)	Biolegend	Cat# 109220, RRID:AB_8	cell surface staining 1:400
93624				
Antibody	anti-mouse TCR β chain PE/Cyanine5 (Armenian Hamster monoclonal)	Biolegend	Cat# 109210, RRID:AB_3	cell surface staining 1:400
13433				
Antibody	anti-mouse TCR β chain PE/Cyanine7 (Armenian Hamster monoclonal)	Biolegend	Cat#10922 2;RRID:AB _893625	cell surface staining 1:400
Antibody	anti-mouse TCR γ/δ FITC (Armenian Hamster monoclonal)	Biolegend	Cat#11810 6;RRID:AB _313830	cell surface staining 1:400
Antibody	anti-mouse TCR γ/δ PerCP/Cyaninne5.5 (Armenian Hamster monoclonal)	Biolegend	Cat# 118118, RRID:AB_1	cell surface staining 1:400

0612756				
Antibody	anti-mouse TCR γ/δ APC (Armenian Hamster monoclonal)	Biolegend	Cat# 118116, RRID:AB_1 731813	cell surface staining 1:400
Antibody	anti-mouse/human CD44 PE/Cyanine7 (Rat monoclonal)	Biolegend	Cat#10303 0;RRID:AB _830787	cell surface staining 1:400
Antibody	anti-mouse CD25 PE (Rat monoclonal)	Biolegend	Cat#10200 8;RRID:AB _312857	cell surface staining 1:400
Antibody	anti-mouse CD25 PE/Cyanine5 (Rat monoclonal)	Biolegend	Cat# 102010, RRID:AB_3 12859	cell surface staining 1:400
Antibody	anti-mouse CD4 FITC (Rat monoclonal)	Biolegend	Cat# 100510, RRID:AB_3 12713	cell surface staining 1:400
Antibody	anti-mouse CD8a FITC (Rat monoclonal)	Biolegend	Cat# 100706, RRID:AB_3	cell surface staining 1:400

12745				
Antibody	anti-mouse TCR β chain FITC (Armenian Hamster monoclonal)	Biolegend	Cat# 109206, RRID:AB_3 13429	cell surface staining 1:400
Antibody	anti-mouse NK-1.1 FITC (Mouse monoclonal)	Biolegend	Cat#10870 6;RRID:AB _313393	cell surface staining 1:400
Antibody	anti-mouse CD19 FITC (Rat monoclonal)	Biolegend	Cat#11550 6;RRID:AB _313641	cell surface staining 1:400
Antibody	anti-mouse CD11b FITC (Rat monoclonal)	Biolegend	Cat#10120 6;RRID:AB _312789	cell surface staining 1:400
Antibody	anti-mouse CD11c FITC (Armenian Hamster monoclonal)	Biolegend	Cat#11730 6;RRID:AB _313775	cell surface staining 1:400
Antibody	anti-mouse TER-119/Erythroid Cells FITC (Rat monoclonal)	Biolegend	Cat#116206;RRID:AB_313 707	
Antibody	anti-mouse TCR β chain Pacific Blue™ (Armenian Hamster monoclonal)	Biolegend	Cat# 109226, RRID:AB_1027649	

Antibody	anti-mouse CD45.1 APC/Cy7 (Mouse monoclonal)	Biolegend	Cat#11071 6;RRID:AB_313505	cell surface staining 1:400
Antibody	anti-mouse CD45.2 PE (Mouse monoclonal)	Biolegend	Cat#109808 ;RRID:AB_313 445	
Antibody	anti-mouse CD45.2 APC (Mouse monoclonal)	Biolegend	Cat# 109814, RRID:AB_389211	
Antibody	anti-mouse CD3 Pacific Blue™ (Armenian Hamster monoclonal)	Biolegend	Cat#640918 ;RRID:AB_493 645	
Antibody	anti-mouse CD27 FITC (Armenian Hamster monoclonal)	Biolegend	Cat# 124208, RRID:AB_1236466	
Antibody	anti-BrdU FITC (Mouse monoclonal)	Biolegend	Cat# 364104, RRID:AB_2564481	
Antibody	anti-BrdU FITC (3D4)	Biolegend	Cat# 364104, RRID:AB_2564481	
Antibody	anti-mouse Ki-67 PE (16A8)	Biolegend	Cat#652404 ;RRID:AB_25 61525	
Antibody	Anti-Zfp335 antibody	Novus	Cat#NB100-2579	
Peptide, recombinant	Annexin V Pacific Blue™	Biolegend	Cat#640918 ;RRID:AB_127 9044	

protein			
Peptide, recombinant protein	DNaseI	Solarbio	Cat#D8071
Commercial assay or kit	Fixation Buffer	Biolegend	Cat#420801
Commercial assay or kit	Intracellular Staining Permeabilization Wash Buffer (10X)	Biolegend	Cat#421002
Commercial assay or kit	MojoSort™ Streptavidin Nanobeads	Biolegend	Cat#480016
Commercial assay or kit	Fixation/Permeabilization Solution Kit with BD GolgiPlug™	BD bioscience s	Cat#555028
Commercial assay or kit	One-Day Chromatin Immunoprecipitation Kits	MILLIPOR E	Cat#17-10085
Commercial assay or kit	Quick-RNA™ MicroPrep Kit	QIAGEN	Cat# R1051
Commercial assay or kit	Fixation Buffer	Biolegend	Cat#420801
Software, algorithm	FlowJo software v10.7	FlowJo LLC	https://www.flowjo.com/ ; RRID:SCR_008520

Software, algorithm	GSEA	(Subraman ian et al., 2005)	https://www.broadinstitute.org/gsea ; RRID:SCR_003199
Software, algorithm	Prism8 (v8.1.0)	GraphPad Software	https://www.graphpad.com/
Software, algorithm	RStudio	RStudio	https://rstudio.com/ ; RRID: SCR_000432

421

422 Mice

423 *Zfp335^{fl/fl}*, *LckCre*, *Trp53^{DKO}* and *OT-1* strains were purchased from The
424 Jackson Laboratory (Bar Harbor, ME, USA). *LckCre* mice were crossed with
425 *Zfp335^{fl/fl}* mice to generate *LckCre⁺Zfp335^{fl/fl}* (KO) mice and *LckCre⁺Zfp335^{+/+}*
426 (WT) mice. Mice aged 6-8 weeks were used for analyses in the study. All mice
427 were housed in specific-pathogen free conditions by the Xi'an Jiaotong
428 University Division of Laboratory Animal Research. All animal procedures were
429 approved by the Institutional Animal Care and Use Committee of Xi'an
430 Jiaotong University.

431

432 Antibodies and reagents

433 The following antibodies and kits were purchased from Biolegend (San Diego,
434 CA, USA): APC/Cy7 anti-CD4 (clone GK1.5), PE anti-CD8α (clone 53-6.7),

435 Pacific BlueTM anti-CD8 α (clone 53-6.7), PE/Cyanine7 anti-CD8 α (clone
436 53-6.7), PE anti-TCRV β 5.1, 5.2 (clone MR9-4), PE anti-TCRV β 6 (clone
437 RR4-7), PE anti-TCRV β 8.1, 8.2 (clone MR5-2), PE anti-TCRV β 12 (clone
438 MR11-1), APC/Cyanine7 anti-TCR β (clone H57-597), PE/Cyanine5 anti-TCR β
439 (clone H57-597), PE/Cy7 anti-TCR β (clone H57-597), FITC anti-TCR $\gamma\delta$ (clone
440 GL3), PE/Cyanine5 anti-TCR $\gamma\delta$ (clone GL3), APC anti-TCR $\gamma\delta$ (clone GL3),
441 PE/Cy7 anti-CD44 (clone IM7), PE anti-CD25 (clone PC61), PE/Cyanine5
442 anti-CD25 (clone PC61), FITC anti-CD4 (clone GK1.5), FITC anti-CD8 (clone
443 53-6.7), FITC anti-TCR β (clone H57-597), FITC anti-NK1.1 (clone PK136),
444 FITC anti-CD19 (clone 6D5), FITC anti-CD11b (clone M1/70), FITC
445 anti-CD11c (clone N418), FITC anti-TER-119/Erythroid Cells (TER-119),
446 Pacific BlueTM anti-TCR β (clone H57-597), APC/Cy7 anti-CD45.1 (clone A20),
447 PE anti-CD45.2 (clone 104), APC anti-CD45.2 (clone 104), Pacific BlueTM
448 anti-Annexin V (Cat # 640918), Pacific BlueTM anti-CD3 (clone 17A2), FITC
449 anti-CD27 (clone LG.3A10), FITC anti-BrdU (clone Bu20a) and the
450 Fixation/Permeabilization Solution Kit (Cat # 554722). PE anti-Ki67
451 monoclonal antibody (clone SolA15) was purchased from eBioscience (San
452 Diego, CA, USA). Quick-RNA Microprep Kit (Cat # R1051) was obtained from
453 Zymo Research (Irvine, CA, USA).

454

455 FACS analysis and sorting

Lymphocytes from *LckCre⁺Zfp335^{+/+}* and *LckCre⁺Zfp335^{fl/fl}* mice were isolated. For surface staining, single cell suspension was prepared. A total of 1×10^6 cells were stained in the dark at 4°C for 30 min with indicated antibodies. The analysis was performed on a CytoFLEX flow cytometer (Beckman Coulter; Brea, CA, USA). DN3 (Lin⁻CD25⁺CD44⁻) and DN4 (Lin⁻CD25⁻CD44⁻) cells were collected by BD FACS Aria™ II cell sorter (BD Biosciences, San Jose, CA, USA). FACS data were recorded and analysed using CytExpert software (Version 2.3.0.84; Beckman Coulter; Indianapolis, IN, USA).

Intracellular staining

DN and DP thymocytes were phenotyped using a combination of surface antibodies against lineage markers (CD4, CD8α, TCRβ, TCRγδ, NK1.1, CD19, CD11b, CD11c, and Ter119), together with CD44 and CD25 antibodies. For intracellular cytokine staining, the thymocytes were fixed and permeabilized using a Fixation/Permeabilization Solution Kit (Biolegend), followed by staining using indicated antibodies. The cells were analyzed on a CytoFLEX flow cytometer (Beckman Coulter).

Quantitative RT-PCR

Cell lysis was performed with RNA extraction and cDNA synthesis using Quick-RNA Microprep Kit (Zymo Research) and ReverTra Ace qPCR RT Master Mix Kit (TOYOBO), respectively. The qRT-PCR reactions were carried

out using StepOnePlus™ Real-Time PCR Systems (ABI) with SYBR mixture (Genstar) to determine relative gene expression. The sequences for the primers are list in the Supplementary file 4.

Bone marrow transplantation

Lineage-negative BM cells from CD45.1⁺ mice and *LckCre⁺Zfp335^{fl/fl}* mice (CD45.2⁺) were sorted, and mixed at a 1:4 ratio and co-transferred into lethally irradiated (8.5Gy) recipient mice (CD45.1⁺CD45.2⁺). Six weeks after bone marrow transplantation (BMT), thymocytes from recipient mice were harvested for FACS analysis.

In vitro OP9-DL1 Cell Co-culture

Both Lin⁻CD25⁺CD44⁻ DN3 cells and Lin⁻CD25⁻CD44⁻ DN4 cells were sorted from the thymi of *LckCre⁺Zfp335^{+/+}* and *LckCre⁺Zfp335^{fl/fl}* mice and co-cultured with OP9-DL1 feeder cells in α -MEM medium in the presence of IL-7 (1 ng/ml, PeproTech) and Flt3-L (5 ng/ml, PeproTech). On day 2 and day 4, total cells were collected and stained with indicated antibodies for FACS analysis.

Retroviral transduction of DN3 thymocytes

Retroviruses were produced from BOSC cells transfected with *Mock-GFP*,

497 *Zfp335*-GFP, *Bcl6*-GFP and *Rorc*-GFP retroviral plasmids. For retroviral
498 transduction, Lin⁻CD25⁺CD44⁻ DN3 thymocytes were sorted by FACS Aria flow
499 cytometry (BD) and co-cultured with OP9-DL1 feeder cells overnight in the
500 presence of 1ng/ml IL-7 and 5ng/ml Flt3L. Retroviral infection was performed
501 16h later by centrifugation (2500 rpm for 90 min at 37°C) in the presence of
502 retroviral supernatants and 8ug/ml polybrene. After spinning, supernatants
503 were replaced by α-MEM medium with 10% FCS supplemented with 1ng/ml
504 IL-7 and 5ng/ml Flt3L. 3.5 days later, GFP⁺ cells were examined using flow
505 cytometry analysis.

506

507 Luciferase Assay

508 To assess whether *Zfp335* regulates *Bcl6* and *Rorc* by directly binding to their
509 promoter regions, the DNA fragments were cloned into the pGL4.16
510 (luc2CP/Hgro) vector (Promega) which contains the luciferase reporter gene
511 luc2CP. The pGL4.16 plasmid, control vector pGL4.74 (hRluc/TK) encoding
512 the luciferase reporter gene hRluc (*Renilla reniformis*), along with plasmids
513 expressing candidate genes were transfected separately into 293T cell line
514 (ATCC). 48 hours post-transfection, the luciferase activity of both Firefly and
515 Renilla luciferase was measured using a Dual-Luciferase Reporter kit
516 (Promega) on SYNERGY Neo2 multi-mode reader (BioTek).

517

518 RNA-Seq library preparation and sequencing

519 Lin⁻CD25⁻CD44⁻ DN4 cells were sorted from the thymi of *LckCre⁺Zfp335^{+/-}* and
520 *LckCre⁺Zfp335^{fl/fl}* mice. The DN4 cell numbers in each group were as followed:
521 WT1, 8x10⁵ cells pulled from 4 mice; WT2, 9x10⁵ cells from 5 mice; KO1,
522 6.7x10⁵ cells from 13 mice; KO2, 7x10⁵ cells from 15 mice. RNA isolation was
523 performed using the RNeasy Mini Kit (Qiagen) according to the manufacturer's
524 protocol. RNA quality and quantity were detected by the Qubit RNA broad
525 range assay in the Qubit Fluorometer (Invitrogen). After quality control using
526 RNase-free agarose gel and Agilent 2100 (Agilent Technologies, Palo Alto, CA,
527 USA), RNA-Seq libraries were prepared by using 200ng total RNA with TruSeq
528 RNA sample prep kit (Illumina). Oligo(dT)-enriched mRNAs were fragmented
529 randomly with fragmentation buffer, followed by first- and second-strand cDNA
530 synthesis. After a series of terminal repair, the double-stranded cDNA library
531 was obtained through PCR enrichment and size selection. cDNA libraries were
532 sequenced with the Illumina HiSeq 2000 sequencer (Illumina HiSeq 2000 v4
533 Single-Read 50 bp) after pooling according to its expected data volume and
534 effective concentration.

535 Two biological replicates were performed in the RNA-Seq analysis. Raw reads
536 were then aligned to the mouse genome (GRCm38) using Tophat2 RNA-Seq
537 alignment software, and unique reads were retained to quantify gene
538 expression counts from Tophat2 alignment files. Data were analyzed and
539 preprocessed in the R environment. Differential expression analysis was
540 performed using R package DESeq2 (Adjusted P value < 0.05 and

fold-change > 1.25). Heat maps and volcano plots were visualized using the R package.

ChIP-Seq library preparation and sequencing

Both Lin⁻CD25⁺CD44⁻ DN3 cells and Lin⁻CD25⁻CD44⁻ DN4 cells were sorted from the thymi of 100 *WT* mice by FACS Aria flow cytometry (BD). Anti-Zfp335 antibody (Novus) and Millipore 17–10085 ChIP kit were used in the ChIP assay. Immunoprecipitated DNA was used for Illumina ChIP-Seq sample preparation. In brief, 5×10^7 cells were crosslinked to chromatin with 1% formaldehyde. Reaction was stopped with 0.125M glycine. Cells were then resuspended in cold nuclear lysis buffer and sonicated to obtain DNA with ~300-500bp size, followed by precipitation by incubation with immunoprecipitation-grade anti-Zfp335 antibody and Magnetic Protein A/G Beads overnight. The following day, beads were sequentially washed by low-salt, high-salt, LiCl, and TE buffers. Bound complexes were eluted in 150µl of elution buffer at 62°C for 2h with shaking, followed by reversal of formaldehyde crosslinking at 95°C for 10 minutes. DNA was eventually purified with spin columns.

The concentration of immunoprecipitated DNA was detected by the Qubit DNA broad range assay in the Qubit Fluorometer (Invitrogen). 10ng immunoprecipitated DNA was prepared for sequencing using the Illumina ChIP-Seq sample preparation protocol. Blunt-end DNA fragments were ligated

to Illumina adaptors, amplified, and sequenced using the SE150 model. Raw reads were filtered firstly to remove low-quality or adaptor sequences by SOAPnuke (version 1.5.6). Clean reads were mapped to the reference genome of GRCm39 with SOAPaligner/soap2 (version 2.21t) using default settings. The MACS2 software (Version 2.1.1) was used to process peak calling. MACS (Model-based Analysis of ChIP-Seq) is a commonly used computational method which is designed to identify peaks from ChIP-seq data. MACS assigns every candidate region an enrichment p-value, and targeted genes are identified as final peaks passing the threshold $p\text{-value} < 1e-5$ ^{67, 68}. The different enrichment peaks from different samples were plotted by MANorm (version 1.1). Genomic graphs were generated and viewed with the IGV (Integrative Genomics Viewer).

Statistical analysis

Data were presented as mean $\pm$ SEM. Statistical analyses were applied to biologically independent mice or technical replicates for each experiment which was independently repeated at least three times. Two-tailed Student's t-test was used for all statistical calculations using GraphPad Prism 7 software. All bar graphs include means with error bars to show the distribution of the data. The level of significance is indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Data availability

The sequencing data presented in this paper are available for download on GEO data repository with accession numbers GEO: GSE184532 and GSE184705.

Acknowledgements

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Additional files

Supplementary files

Supplementary file 1. Summary for downregulated and upregulated genes induced by Zfp335 deficiency.

Supplementary file 2. Chip-Seq analysis of Zfp335 binding peaks in DN thymocytes.

Supplementary file 3. Overlay analysis between downregulated genes from RNA-seq data and Zfp335 binding genes from Chip-Seq data.

Supplementary file 4. Primers used for quantitative PCR.

Source data files

Figure 1—source data 1

Figure 1D. The percentages of DN, DP, CD4, CD8 thymocytes from WT and

607 KO mice.

608 Figure 1E. The numbers of DN, DP, CD4, CD8 thymocytes from WT and KO
609 mice.

610 Figure 1G, H. The percentages and numbers of CD4⁺ and CD8⁺ T cells in
611 spleen from WT and KO mice.

612 Figure 1I, J. The percentages and numbers of CD4⁺ and CD8⁺ T cells in lymph
613 nodes from WT and KO mice.

614 Figure 1—figure supplement 1—source data 1

615 Figure 1—figure supplement 1B. Zfp335 protein expression in thymocytes.

616 Figure 1—figure supplement 3 –source data 1

617 The gel of *Zfp335* DNA in WT and KO mice. Line 1-8: 1: marker; 2:H₂O; 3:WT;
618 4: KO1; 5: KO2; 6: KO3; 7: KO4; 8: KO5.

619

620 Figure 2—source data 1

621 Figure 2C, D. The percentages of DN, DP, CD4⁺ and CD8⁺ thymocytes 2 and 4
622 days post separate culture in vitro.

623 Figure 2F, G. The percentages of DN, DP, CD4⁺ and CD8⁺ thymocytes 2 and 4
624 days post co-culture in vitro.

625 Figure 2J. The percentages of DN, DP, CD4⁺ and CD8⁺ thymocytes in chimeric
626 mice separately transferred with WT and KO bone marrow cells.

627 Figure 2L. The percentages of DN, DP, CD4⁺ and CD8⁺ thymocytes in chimeric
628 mice co-transferred with WT and KO bone marrow cells.

629 Figure 2—figure supplement 1—source data 1

630 Figure 2—figure supplement 1A. The numbers of DN, DP, CD4⁺ and CD8⁺
631 thymocytes 2 and 4 days post separate culture in vitro.

632 Figure 2—figure supplement 1B. The numbers of DN, DP, CD4⁺ and CD8⁺
633 thymocytes 2 and 4 days post co-culture in vitro.

634 Figure 2—figure supplement 1C. The numbers of DN, DP, CD4⁺ and CD8⁺
635 thymocytes in chimeric mice separately transferred with WT and KO bone
636 marrow cells.

637 Figure 2—figure supplement 1D. The numbers of DN, DP, CD4⁺ and CD8⁺
638 thymocytes in chimeric mice co-transferred with WT and KO bone marrow
639 cells.

640 Figure 2—figure supplement 2—source data 1

641 Figure 2—figure supplement 2B, C. The percentages and numbers of $\gamma\delta$ T
642 cells in WT and KO mice 2 and 4 days post co-culture in vitro.

643

644 Figure 3—source data 1

645 Figure 3B, C. The percentages and numbers of DN3 and DN4 thymocytes
646 from WT and KO mice.

647 Figure 3E, G. The ratio of DN3 to DN4 thymocytes 2 days post culture in vitro.

648 Figure 3I, K. The ratio of DN3 to DN4 thymocytes in chimeric mice transferred
649 with WT and KO bone marrow cells.

650

651 Figure 4—source data 1

652 Figure 4B. The percentages of BrdU⁺ thymocytes in DN3 and DN4 cells from
653 WT and KO mice.

654 Figure 4D. The percentages of Annexin V⁺ DN3 and DN4 cells from WT and
655 KO mice.

656 Figure 4F. The percentage of Annexin V⁺ DN3 and DN4 cells 4 days post
657 co-culture in vitro.

658 Figure 4H. The percentage of Annexin V⁺ DN3 and DN4 cells in chimeric mice
659 co-transferred with WT and KO bone marrow cells.

660 Figure 4—figure supplement 1—source data 1

661 Figure 4—figure supplement 1B. The percentages of Ki67⁺ thymocytes in DN3
662 and DN4 cells from WT and KO mice.

663 Figure 4—figure supplement 2—source data 1

664 Figure 4—figure supplement 2E,F. The percentages of Annexin V⁺ DN3a and
665 DN3b cells from WT and KO mice.

666

667 Figure 5—source data 1

668 Figure 5B. The percentages and numbers of iTCRβ⁺ thymocytes in DN3 and
669 DN4 cells from WT and KO mice.

670 Figure 5H, I. The percentages of iTCRβ⁺ DN3 and DN4 cells in WT, KO, OT1^{Tg}
671 and OT1^{Tg} KO mice.

672 Figure 5K, L. The percentages and numbers of DN3 and DN4 thymocytes in

673 WT, KO, OT1^{Tg} and OT1^{Tg} KO mice.

674 Figure 5N. The percentages of DN, DP, CD4⁺ and CD8⁺ thymocytes in WT, KO,
675 OT1^{Tg} and OT1^{Tg} KO mice.

676 Figure 5—figure supplement 2—source data 1

677 Figure 5—figure supplement 2B. Intercellular CD3 expression in
678 *Zfp335*-deficient DN3 and DN4 thymocytes.

679 Figure 5—figure supplement 3—source data 1

680 The gel of *CD14* gene. Line 1-8: 1:marker; 2:H₂O; 3:WT; 4: *Rag2*^{-/-}; 5: WT DN3;
681 6: KO DN3; 7: WT DN4; 8: KO DN4.

682 Figure 5—figure supplement 3—source data 2

683 The gel of TCR *Vb5*. Line 1-6: 1:marker; 2:H₂O; 3:WT; 4: *Rag2*^{-/-}; 5: WT DN3; 6:
684 KO DN3.

685 Figure 5—figure supplement 3—source data 3

686 The gel of TCR *Vb8*. Line 1-6: 1:marker; 2:H₂O; 3:WT; 4: *Rag2*^{-/-}; 5: WT DN3; 6:
687 KO DN3.

688 Figure 5—figure supplement 4—source data 1

689 Figure 5—figure supplement 4B,C. The percentages of Annexin V⁺ DN3 and
690 DN4 cells in WT, KO, OT1^{Tg} and OT1^{Tg}KO mice.

691 Figure 5—figure supplement 5—source data 1

692 Figure 5—figure supplement 5. The numbers of DN, DP, CD4⁺ and CD8⁺
693 thymocytes in WT, KO, OT1^{Tg} and OT1^{Tg}KO mice.

694

Figure 7—source data 1

Figure 7C. The percentages of DP cells differentiated from KO DN3 thymocytes transduced with *Mock*, or *Zfp335*, *Bcl6* and *Rorc* genes.

Figure 7E. The percentages of Annexin V⁺ DN cells differentiated from KO DN3 thymocytes transduced with *Mock*, or *Zfp335*, *Bcl6* and *Rorc* genes.

Figure 7G. The percentages of Annexin V⁺ DP cells differentiated from KO DN3 thymocytes transduced with *Mock*, or *Zfp335*, *Bcl6* and *Rorc* genes.

Figure 7—figure supplement 2—source data 1

Figure 7—figure supplement 2B, C. The percentages and numbers of DN, DP, CD4⁺ and CD8⁺ thymocytes from WT, KO and *Trp53*^{DKO} mice.

Figure 7—figure supplement 3—source data 1

Figure 7—figure supplement 3. iTCR β expression in DN4 cells infected by *Zfp335*, *Bcl6* and *Rorc* overexpression retrovirus.

Major datasets

The following datasets were generated :

Author(s)	Year	Dataset title	Dataset ID and/or URL	Database, license, and accessibility information
Wang X, Jiao AJ, Sun LN, Li WH, Yang B, Su YH, Ding RY, Zhang CG, Liu HY, Yang XF, Sun CM, Zhang BJ	2021	Zinc finger protein Zfp335 controls thymocyte differentiation and survival through b-selection-dependent and -independent mechanisms	GSE184532; https://www.ncbi.nlm.nih.gov/gds/?term=GSE184532	Publicly available at NCBI GEO DataSet.
Wang X, Jiao AJ, Sun LN, Li WH, Yang B, Su	2021	Zinc finger protein Zfp335 controls thymocyte	GSE184705; https://www.ncbi.nlm.nih.gov/gds/?term=GSE184705	publicly available at

YH, Ding RY, Zhang CG, Liu HY, Yang XF, Sun CM, Zhang BJ	differentiation and survival through b-selection-dependent and -independent mechanisms	m.nih.gov/gds/?ter m=GSE184705	NCBI GEO DataSet.
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Figure legends

Figure 1. Impaired thymocyte development in *Zfp335*-deficient mice

(A) DN3, DN4, DP, CD4, CD8, NKT and $\gamma\delta$ T cells were sorted from C57BL/6 thymocytes by flow cytometry. The mRNA levels of *Zfp335* were measured by qPCR. (B) Thymi from *LckCre⁺Zfp335^{+/+}* (WT) and *LckCre⁺Zfp335^{fl/fl}* (KO) mice. Representative thymi and total cell number of thymocytes. Scale bar, 1 cm. (C-E) The different stages of thymocyte development in WT and KO mice were measured by flow cytometry. (C) Representative FACS plots of DN, DP, CD4, CD8 thymocytes; (D) The percentages of DN, DP, CD4, CD8 thymocytes; (E) The numbers of DN, DP, CD4, CD8 thymocytes. (F-J) CD4⁺ and CD8⁺ cells in spleen and lymph nodes from WT and KO mice were measured by flow cytometry. (F) Representative FACS plots of CD4⁺ and CD8⁺ cells in spleen and lymph nodes; (G-H) The percentage and number of CD4⁺ T cells (G) and CD8⁺ T cells (H) in the spleen from WT and KO mice; (I-J) The percentage and number of CD4⁺ T cells (I) and CD8⁺ T cells (J) in the lymph nodes from WT and KO mice. Results represent three independent experiments. n=4 mice per group. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Figure 2. An intrinsic block from DN to DP stage in *Zfp335*-deficient mice

(A) Schematic overview of the *in vitro* OP9-DL1 stromal co-culture assay for T cell differentiation from WT (CD45.1⁺) and KO (CD45.2⁺) DN3 thymocytes to DP and SP thymocytes. (B-D) WT and KO DN3 thymocytes were cultured with

961 OP9-DL1 feeder cells *in vitro* in the presence of IL-7 (1ng/ml) and Flt3L
962 (5ng/ml) for 2 and 4 days. The DN and DP thymocytes were measured by flow
963 cytometry (n=3). **(B)** Representative FACS plots of DN, DP, CD4⁺ and CD8⁺
964 thymocytes; **(C)** The percentages of DN, DP, CD4⁺ and CD8⁺ thymocytes 2
965 days post culture *in vitro*; **(D)** The percentage of DN, DP, CD4⁺ and CD8⁺
966 thymocytes 4 days post culture *in vitro*. **(E-G)** A mixed population of WT and
967 KO DN3 thymocytes at a 1:4 ratio was co-cultured with OP9-DL1 feeder cells
968 *in vitro* in the presence of IL-7 (1ng/ml) and Flt3L (5ng/ml) for 2 and 4 days.
969 The DN and DP thymocytes were phenotyped by flow cytometry (n=3). **(E)**
970 Representative FACS plots of DN, DP, CD4⁺ and CD8⁺ thymocytes; **(F)**
971 Percentages of DN, DP, CD4⁺ and CD8⁺ thymocytes 2 days post culture *in*
972 *vitro*; **(G)** Percentages of DN, DP, CD4⁺ and CD8⁺ thymocytes 4 days post
973 culture *in vitro*. **(H)** Schematic overview of the *in vivo* bone marrow chimeric
974 mice model for T cell differentiation from WT (CD45.1⁺) and KO (CD45.2⁺)
975 progenitors cells to DP and SP thymocytes. **(I-J)** Full chimeric mice were
976 generated by transplanting WT (CD45.1⁺) or KO (CD45.2⁺) bone marrow
977 progenitor cells into lethally irradiated (8.5 Gy) WT recipient mice
978 (CD45.1⁺CD45.2⁺). Six weeks after transplantation, thymi from recipient mice
979 were harvested. **(I)** Representative FACS plots of DN, DP, CD4⁺ and CD8⁺
980 thymocytes; **(J)** The percentages of DN, DP, CD4⁺ and CD8⁺ thymocytes. **(K-L)**
981 Full chimeric mice were generated by transplanting a mixed population of WT
982 (CD45.1⁺) and KO (CD45.2⁺) bone marrow progenitor cells at a 1:4 ratio into

lethally irradiated WT recipients (CD45.1⁺CD45.2⁺) with 8.5 Gy. Six weeks after transplantation, thymi from recipient mice were harvested. **(K)** Representative FACS plots of DN, DP, CD4⁺ and CD8⁺ thymocytes; **(L)** Percentages of DN, DP, CD4⁺ and CD8⁺ thymocytes. Results represent three independent experiments. n=4 mice per group. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Figure 3. *Zfp335*-deficient thymocytes undergo a developmental block during DN3 to DN4 transition

(A-C) Thymi were harvested from 6- to 8-week-old WT and KO mice. The different stages of DN thymocytes in WT and KO mice were measured by flow cytometry (n=4). **(A)** Representative FACS plots of DN1 (CD25⁻CD44⁺), DN2 (CD25⁺CD44⁺), DN3 (CD25⁺CD44⁻), and DN4 (CD25⁻CD44⁻) thymocytes; **(B)** The percentages of DN3 and DN4 thymocytes; **(C)** The numbers of DN3 and DN4 thymocytes. **(D-E)** WT and KO DN3 thymocytes were cultured with OP9-DL1 feeder cells *in vitro* in the presence of IL-7 (1ng/ml) and Flt3L (5ng/ml) for 2 days. The expression of CD44 versus CD25 were measured by flow cytometry (n=3). **(D)** Representative FACS plots of DN3 and DN4 thymocytes; **(E)** The ratio of DN3 to DN4 thymocytes 2 days post culture *in vitro*. **(F-G)** A mixed population of WT and KO DN3 thymocytes at a 1:4 ratio was co-cultured with OP9-DL1 feeder cells *in vitro* in the presence of IL-7 (1ng/ml) and Flt3L (5ng/ml) for 2 days. The expression of CD44 versus CD25

were measured by flow cytometry (n=3). **(F)** Representative FACS plots of DN3 and DN4 thymocytes; **(G)** The ratio of DN3 to DN4 thymocytes 2 days post culture *in vitro*. **(H-I)** Full chimeric mice were generated by transplanting WT (CD45.1⁺) or KO (CD45.2⁺) bone marrow progenitor cells into lethally irradiated (8.5 Gy) WT recipients (CD45.1⁺CD45.2⁺). Six weeks after transplantation, thymi from recipient mice were harvested. The expression of CD44 versus CD25 were measured by flow cytometry (n=4). **(H)** Representative FACS plots of DN3 and DN4 cells; **(I)** The ratio of DN3 to DN4 thymocytes. **(J-K)** Full chimeric mice were generated by transplanting a mixed population of WT (CD45.1⁺) and KO (CD45.2⁺) bone marrow progenitor cells at a 1:4 ratio into lethally irradiated (8.5 Gy) WT recipient mice (CD45.1⁺CD45.2⁺). Six weeks after transplantation, thymi from recipient mice were harvested. The expression of CD44 versus CD25 were measured by flow cytometry (n=4). **(J)** Representative FACS plots of DN3 and DN4 thymocytes; **(K)** The ratio of DN3 to DN4 thymocytes. Results represent three independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001.

Figure 4. Zfp335 deficiency promotes thymocyte apoptosis *in vivo* and *in vitro*

(A and B) Thymi were harvested from 6- to 8-week-old WT and KO mice. The expression of BrdU in DN3 and DN4 thymocytes from WT and KO thymi was measured by flow cytometry (n=3). **(A)** Representative FACS plots of BrdU

expression in DN3 and DN4 thymocytes; **(B)** The percentages of BrdU⁺ thymocytes in DN3 and DN4 cells. **(C and D)** The binding of Annexin V in DN3 and DN4 thymocytes from WT and KO thymi were measured by flow cytometry (n=3). **(C)** Representative FACS plots of Annexin V binding in DN3 and DN4 thymocytes; **(D)** The percentages of Annexin V⁺DN3 and DN4 cells. **(E-F)** A mixed population of WT and KO DN3 thymocytes at a 1:4 ratio was co-cultured with OP9-DL1 feeder cells *in vitro* in the presence of IL-7 (1ng/ml) and Flt3L (5ng/ml) for 4 days. The binding of Annexin V was measured by flow cytometry (n=3). **(E)** Representative FACS plots of Annexin V⁺ DN3 and DN4 cells; **(F)** The percentage of Annexin V⁺DN3 and DN4 cells. **(G-H)** Full chimeric mice were generated by transplanting a mixed population of WT (CD45.1⁺) and KO (CD45.2⁺) bone marrow progenitor cells at a 1:4 ratio into lethally irradiated WT recipient mice (CD45.1⁺CD45.2⁺) with 8.5 Gy. Five weeks after transplantation, thymi from recipient mice were harvested. The binding of Annexin V was measured by flow cytometry (n=4). **(G)** Representative FACS plots of Annexin V⁺ DN3 and DN4 cells; **(H)** The percentage of Annexin V binding in DN3 and DN4 thymocytes. Results represent three independent experiments. **p < 0.01, and ****p < 0.0001.

Figure 5. OT1 transgenic TCR overexpression rescued Zfp335 deficiency-induced defect during DN3 to DN4 transition

(A-B) Thymi were harvested from 6-8 week-old WT and KO mice. The

1049 expression of iTCR β in DN3 and DN4 thymocytes was measured by flow
1050 cytometry (WT n=3; KO n=4). **(A)** Representative FACS plots of iTCR β
1051 expression in DN3 and DN4 thymocytes; **(B)** Percentages and numbers of
1052 iTCR β ⁺ thymocytes in DN3 and DN4 cells. **(C-F)** Representative FACS plots of
1053 intercellular TCR V β 5 (**C**), V β 6 (**D**), V β 8 (**E**) and V β 12 (**F**) expression in
1054 DN4 cells from WT and KO mice. **(G-I)** Thymi from WT, KO, OT1^{Tg} and
1055 OT1^{Tg}*LckCre⁺Zfp335^{fl/fl}* (OT1^{Tg} KO) mice were harvested. The expressions of
1056 iTCR β in DN3 (up) and DN4 (down) thymocytes were measured by flow
1057 cytometry (WT n=3; KO n=5; OT1^{Tg} n=3; OT1^{Tg}KO n=5). **(G)** Representative
1058 FACS plots of iTCR β expression in DN3 and DN4 thymocytes; **(H)** The
1059 percentage of iTCR β ⁺ DN3 and DN4 cells in WT, KO, OT1^{Tg} and OT1^{Tg} KO
1060 mice; **(I)** The numbers of iTCR β ⁺ DN3 and DN4 cells in WT, KO, OT1^{Tg} and
1061 OT1^{Tg} KO mice. **(J-L)** The different stages of DN thymocytes in WT, KO, OT1^{Tg}
1062 and OT1^{Tg} KO mice were measured by flow cytometry (WT n=3; KO n=5;
1063 OT1^{Tg} n=3; OT1^{Tg}KO n=5). **(J)** Representative FACS plots of DN3 and DN4
1064 thymocytes; **(K)** The percentages of DN3 and DN4 thymocytes; **(L)** The
1065 numbers of DN3 and DN4 thymocytes. **(M-N)** The different stages of
1066 thymocyte development in WT, KO, OT1^{Tg} and OT1^{Tg} KO mice were measured
1067 by flow cytometry (WT n=3; KO n=5; OT1^{Tg} n=3; OT1^{Tg}KO n=5). **(M)**
1068 Representative FACS plots of thymocytes. **(N)** The percentages of DN, DP,
1069 CD4⁺CD8⁻, and CD4⁻CD8⁺ thymocytes. Results represent three independent
1070 experiments. n=3 per group. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p <

0.0001.

Figure 6. Zfp335 downstream target analysis in DN4 thymocytes

(A) Volcano plot depicting log₂ (Fold Change) (x-axis) and -log₁₀ (P value) (y-axis) for differentially expressed genes (FC>1.25, P<0.05) in DN4 thymocytes sorted from WT and KO mice; up-regulated (red) and down-regulated (blue). n=2 per group. **(B)** Gene-ontology (GO) analysis of genes that down-regulated in *Zfp335*-deficient DN4 thymocytes, showing the GO terms related to lymphocyte differentiation and apoptosis (left), the number of genes overlapped with database from the indicated terms (middle left column), P values (middle column) and Q values (middle right column) and genes annotated to the indicated term (right). **(C)** Heatmap of representative genes related to lymphocyte differentiation and apoptosis. The scale ranges from minimum (green boxes) to medium (black boxes) to maximum (red boxes) relative expression. **(D)** The mRNA level of *Bcl6* (top) and *Rorc* (bottom) in DN4 thymocytes from WT and KO mice (n=3). **(E)** ChIP-seq analysis for binding of Zfp335 to the *Bcl6* and *Rorc* loci in wild-type DN4 cells. **(F)** Luciferase assay for the binding of different domains of Zfp335 to the promoter regions of *Bcl6* and *Rorc*. The pGL4.16 plasmid was transfected into 293T cells together with MSCV vector carrying different domains (n=3). Data represent 3 independent experiments. *p < 0.05, **p < 0.01 and ****p < 0.0001.

Figure 7. Identification of *Bcl6* and *Rorc* as functional targets of *Zfp335* for regulating thymocyte development

(A) Schematic overview of the *in vitro* gene overexpression in KO DN3 thymocytes and following T cell differentiation in OP9-DL1 co-culture system.

(B-C) *Zfp335*-deficient DN3 thymocytes (KO) were cultured with OP9-DL1 feeder cells *in vitro*, then transduced with either *Mock*, *Zfp335*-overexpressing, *Bcl6*-overexpressing, or *Rorc*-overexpressing vector for 3.5 days. The different stages of thymocyte development from GFP positive cells were measured by flow cytometry (n=3). **(B)** Representative FACS plots of DN and DP thymocytes from the indicated groups; **(C)** The percentage of DP thymocytes from GFP positive cells. **(D-G)** KO DN3 thymocytes were cultured with OP9-DL1 feeder cells *in vitro*, then transduced with either *Mock*, *Zfp335*-overexpressing, *Bcl6*-overexpressing, or *Rorc*-overexpressing vector for 3.5 days. The expressions of Annexin V in DN and DP thymocytes were measured by flow cytometry (n=3). **(D)** Representative FACS plots of Annexin V⁺ DN thymocytes from the indicated groups; **(E)** The percentage of Annexin V⁺ DN thymocytes from GFP positive cells; **(F)** Representative FACS plots of Annexin V⁺ DP thymocytes from the indicated groups; **(G)** The percentage of Annexin V⁺ DP thymocytes from GFP positive cells. Results shown represent three independent experiments. **p < 0.01, ***p < 0.001 and ****p < 0.0001.

Supplementary figure legends

Figure 1—figure supplement 1. Zfp335 protein expression in thymocytes.

(A) Representative FACS plots of Zfp335 protein expression in DN3, DN4, DP, CD4SP and CD8SP thymocytes. **(B)** Mean fluorescence intensity (MFI) of Zfp335 in **(A)**. Results shown represent three independent experiments. n=3 per group. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 1—figure supplement 2. The transcriptional profiling of Zfp335 expression in various subsets of thymocytes.

(A-B) The RNA profiling data sets of Zfp335 generated by microarray **(A)** or RNA-seq **(B)** data on the ImmGen database.

Figure 1—figure supplement 3. Verification of Zfp335 conditional knock-out mouse strain.

(A-B) *Zfp335^{fl/fl}* mice were crossed with LckCre mice to generate LckCre⁺*Zfp335^{fl/fl}* mice. **(A)** The size of *Zfp335* DNA in WT and KO mice; **(B)** The mRNA level of *Zfp335* in DN4 cells from WT and KO mice. n=3, ****p < 0.0001.

Figure 2—figure supplement 1. Zfp335 deletion caused defects of thymocyte development.

(A) WT and KO DN3 thymocytes were cultured with OP9-DL1 feeder cells *in*

vitro in the presence of IL-7 (1ng/ml) and Flt3L (5ng/ml) for 2 and 4 days. Thymocytes were analyzed by flow cytometry. The numbers of DN, DP, CD4⁺ and CD8⁺ thymocytes 2 days (Left) and 4 days (Right) post culture *in vitro* (n=3). **(B)** WT and KO DN3 thymocytes were mixed and co-cultured in the *in vitro* development model. The numbers of DN, DP, CD4⁺ and CD8⁺ thymocytes 2 days and 4 days post culture (n=3). **(C)** Full chimeric mice were generated by transplanting WT (CD45.1⁺) or KO (CD45.2⁺) bone marrow progenitor cells into lethally irradiated (8.5 Gy) WT recipient mice (CD45.1⁺CD45.2⁺). Six weeks after transplantation, thymi from recipient mice were harvested. The numbers of DN, DP, CD4⁺ and CD8⁺ thymocytes are indicated (n=4). **(D)** WT and KO mixed bone marrow chimeric mice were generated and thymi from recipient mice were harvested. The numbers of DN, DP, CD4⁺ and CD8⁺ thymocytes are indicated (n=4). Results shown represent three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

Figure 2—figure supplement 2. Effect of *Zfp335* deletion on $\gamma\delta$ T cell percentages and cellularity *in vitro*.

WT and KO DN3 thymocytes were cultured with OP9-DL1 feeder cells *in vitro* in the presence of IL-7 (1ng/ml) and Flt3L (5ng/ml) for 2 and 4 days. The $\gamma\delta$ T cells were measured by flow cytometry (n=3). **(A)** Representative FACS plots of $\gamma\delta$ T cells; **(B and C)** The percentages of $\gamma\delta$ T cells on day2 **(B)** and day4 **(C)**. Data are representative of at least three independent experiments shown

as the mean $\pm$ s.e.m. Two-tailed, unpaired t-tests were used for statistical analyses; **P < 0.01, ***P < 0.001.

Figure 4—figure supplement 1. *Zfp335* deficiency has no effect on the proliferation of DN3 and DN4 cells.

(A and B) Thymi from WT and KO mice were harvested. The expressions of Ki67 in DN3 and DN4 thymocytes from WT and KO thymi were measured by flow cytometry (n=3). **(A)** Representative FACS plots of Ki67 expression in DN3 and DN4 thymocytes; **(B)** The percentages of Ki67⁺ thymocytes in DN3 and DN4 cells. Data are representative of at least three independent experiments shown as the mean $\pm$ s.e.m. Two-tailed, unpaired t-tests were used for statistical analyses; *p < 0.05.

Figure 4—figure supplement 2. Effect of *Zfp335* deletion on DN3a and DN3b Cells.

(A) Representative FACS plots of WT and *Zfp335* KO DN3a and DN3b cells gated in DN3 thymocytes from 6-8-week-old mice. **(B-C)** The percentages of DN3a **(B)** and DN3b **(C)** cells in **(A)**. **(D)** Histograms showing the Annexin V expression in WT and *Zfp335* KO DN3a and DN3b thymocytes. **(E-F)** The percentages of Annexin V⁺ DN3a **(E)** and DN3b **(F)** cells in **(D)**. Results represent three independent experiments. n=3 per group. **p < 0.01.

1182

1183 **Figure 5—figure supplement 1. Effect of *Zfp335* deletion on pre-TCR**
1184 **complex expression in DN3 and DN4 Cells.**

1185 qPCR analysis of *Ptcra*, *Trbc1*, *Trbc2* and *CD3e* expression in DN3 **(A)** and
1186 DN4 **(B)** cells from WT and KO mice.

1187

1188 **Figure 5—figure supplement 2. Intercellular CD3 expression in**
1189 ***Zfp335*-deficient DN3 and DN4 thymocytes.**

1190 **(A)** Representative FACS plots of intercellular CD3 (iCD3) expression in DN3
1191 and DN4 thymocytes. **(B)** The percentages of iCD3⁺ DN3 and DN4 cells in WT
1192 and KO mice. Data are representative of at least three independent
1193 experiments shown as the mean ± s.e.m. Two-tailed, unpaired t-tests were
1194 used for statistical analyses, WT n=3; KO n=4.

1195

1196 **Figure 5—figure supplement 3. Effect of *Zfp335* deletion on TCRβ**
1197 **rearrangement in DN3 thymocytes.**

1198 PCR analysis of TCRβ gene rearrangements. Genomic DNA isolated from
1199 thymocytes of B6, recombination-activating gene-2-deficient (*Rag2*^{-/-}), WT, and
1200 KO mice was amplified with primers that detect rearrangements both Vβ5 and
1201 Vβ8. Data are representative of three replicates.

1202

1203 **Figure 5—figure supplement 4. TCRβ overexpression could not rescue**

the increased DN cell apoptosis caused by Zfp335 deficiency.

(A) Representative FACS plots of Annexin V expression in DN3 and DN4 thymocytes from WT, KO, OT1^{Tg} and OT1^{Tg}KO mice. **(B-C)** The percentages of Annexin V⁺ DN3 and DN4 cells in each group in **(A)**. Results shown represent three independent experiments. **p < 0.01, ***p < 0.001 and ****p < 0.0001.

Figure 5—figure supplement 5. OT1^{Tg} failed to rescue thymocyte numbers.

The numbers of DN, DP, CD4⁺CD8⁻, and CD4⁻CD8⁺ thymocytes in WT, KO, OT1^{Tg} and OT1^{Tg}KO mice were measured (WT n=3; KO n=5; OT1^{Tg} n=3; OT1^{Tg}KO n=5). **p < 0.01, ***p < 0.001 and ****p < 0.0001.

Figure 6—figure supplement 1. ChIP-Seq analysis of Zfp335 binding sites.

The distribution of Zfp335 binding peaks in various regions of the genome was calculated.

Figure 6—figure supplement 2. The intersection analysis of Zfp335 by RNA-Seq and ChIP-Seq.

(A) Venn diagram depicting the overlapping portion of differentially expressed genes in RNA-Seq (red) and Zfp335 binding regions in ChIP-Seq (blue). 119

profoundly downregulated genes from RNA-Seq were selected out by a cutoff of 2-fold change. **(B)** The shared top 10 overlapped genes from the RNA-Seq and ChIP-Seq. **(C)** qPCR analysis of the top 10 overlapped genes depicted in **(B)**.

Figure 7—figure supplement 1. Identification of the top 10 overlapped genes in KO DN3 thymocytes for the regulation of thymocyte development.

Zfp335 deficiency DN3 thymocytes (KO) were cultured with OP9-DL1 feeder cells *in vitro*, then transduced with the top 10 overlapped genes overexpression vector for 3.5 days. The different stages of thymocyte development were measured by flow cytometry.

Figure 7—figure supplement 2. Examination of *Trp53* as the target of Zfp335 during the regulation of thymocyte development.

Thymi from WT, KO, and *Trp53*^{KO}*LckCre*⁺*Zfp335*^{fl/fl} (*Trp53*^{DKO}) mice were harvested. The different stages of thymocyte development in WT, KO, and *Trp53*^{DKO} mice were measured by flow cytometry (WT n=4; KO n=4; *Trp53*^{DKO} n=3). **(A)** Representative FACS plots of thymocytes. **(B)** The percentages of DN, DP, CD4⁺CD8⁻, and CD4⁻CD8⁺ thymocytes. **(C)** The numbers of DN, DP, CD4⁺CD8⁻, and CD4⁻CD8⁺ thymocytes. Data are representative of at least three independent experiments shown as the mean ± s.e.m. Two-tailed, unpaired t-tests were used for statistical analyses; *p < 0.05, **p < 0.01, ***p <

0.001, and ****p < 0.0001.

Figure 7—figure supplement 3. iTCR β expression in DN4 cells infected by *Zfp335*, *Bcl6* and *Rorc* overexpression retrovirus.

WT and *Zfp335* KO DN3 cells were isolated and infected with *Mock* (WT and KO), *Zfp335*, *Bcl6* and *Rorc* (KO) overexpression retrovirus. 3 days later, iTCR β expression was examined. Histogram plot **(A)** and MFI **(B)** of iTCR β expression in each group (n=3). *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 1

Figure 1—figure supplement 1

Figure 1—figure supplement 2

Figure 1—figure supplement 3

Figure 2

A

B

C

E

F

■ WT
■ KO

H

1

J

K

L

Figure 2—figure supplement 1

Figure 2—figure supplement 2

Figure 3

Figure 4

Figure 4—figure supplement 1

Figure 4—figure supplement 2

Figure 5

Figure 5—figure supplement 1

Figure 5—figure supplement 2

Figure 5—figure supplement 3

Figure 5—figure supplement 4

Figure 5—figure supplement 5

Figure 6

Figure 6—figure supplement 1

Figure 6—figure supplement 2

Figure 7

Figure 7—figure supplement 1

Figure 7—figure supplement 2

Figure 7—figure supplement 3