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Distinct functions of the transcription factors Gata3 and ThPOK during intrathymic CD4 T cell differentiation*

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Abstract

The transcription factors Gata3 and Zbtb7b are required for intrathymic CD4 T cell differentiation, but their precise roles in this process remain unclear. Here we show that, contrary to previous findings, Gata3 disruption blocked CD4 T cell lineage differentiation before CD4 lineage commitment, and in some contexts permitted 'redirection' of MHC class II-restricted thymocytes into the CD8 lineage. We found that Gata3 promotes Zbtb7b expression, and binds within a region of the *Zbtb7b* locus established as critical for Zbtb7b expression. Finally, Zbtb7b promoted CD4 lineage differentiation in a manner dependent on Gata3, but inhibited CD8 lineage differentiation independently of Gata3. We propose that Gata3 acts as a specification factor for the CD4 lineage, "upstream" of the Zbtb7b-controlled CD4 commitment checkpoint.

Introduction

Most T cells expressing antigen receptors (TCR) specific for complexes of peptides and classical class Ia or class II MHC molecules (pMHCI and pMHCII, respectively) belong to either of two lineages defined by the mutually exclusive expression of CD4 and CD8 surface glycoproteins. CD8 T cells are typically MHCI-restricted and differentiate into cytotoxic effectors upon antigenic stimulation. In contrast, CD4 T cells are typically MHCII-restricted and either positively (T helper cells) or negatively (T regulatory cells) influence immune responses. CD4 and CD8 lineage cells differentiate from $CD4^+CD8^+$ double positive (DP) thymocyte precursors that, as a result of TCR engagement by intrathymic pMHC complexes, undergo positive selection[1-3]

Two transcription factors—Zbtb7b and Gata3—are specifically required for CD4 T cell development. Zbtb7b (also called cKrox or Thpok) is expressed in CD4 but not CD8 T cells[4,5] and belongs to a subset of zinc finger transcription factors that share an additional

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*The following report investigates the function of the gene officially designated as *Zbtb7b*, previously known as *cKrox* or *Thpok*. In the peer-reviewed version, the gene was referred to as *Zbtb7b* and its product as Zbtb7b. The corresponding publication in Nature Immunology refers to the gene and its product as *ThPOK* and ThPOK, respectively.

amino-terminal region of homology known as the BTB/POZ domain[6]. A spontaneous missense mutation of Zbtb7b in the helper deficient (HD) mouse (Zbtb7b^{hd}) disrupts CD4 T cell development and 'redirects' MHCII-restricted thymocytes into the CD8 lineage[4]. Reciprocally, enforced expression of Zbtb7b in MHCI-restricted thymocytes (where it is normally not expressed) results in redirection towards a CD4 lineage fate[4,5]. Thus, Zbtb7b controls a key lineage commitment checkpoint. It is unclear whether the mutated Zbtb7b^{hd} protein lacks any biological activity, or whether it participates in early CD4 differentiation or harbors aberrant activities that contribute to lineage redirection.

The zinc finger transcription factor Gata3 is expressed during and required for multiple steps of T cell differentiation, both in developing thymocytes and effector T cells[7-9]. During positive selection, Gata3 expression peaks during the DP to CD4 SP transition, and decreases during the DP to CD8 SP transition[10,11]. Targeted disruption of Gata3 in DP thymocytes demonstrated that this factor is necessary for the development of CD4 but not CD8 T cells[12-14]. However, unlike the Zbtb7b^{hd} mutation, Gata3 disruption arrested the development of MHCII-restricted thymocytes during the DP to CD4 SP transition but did not result in redirection to the CD8 lineage[12]. Conversely, enhanced Gata3 expression failed to redirect MHCI-restricted thymocytes into the CD4 lineage[11,14]. These observations led to the conclusion that Gata3 controls a post-commitment differentiation or survival step during CD4 T cell development[9,12].

Thus, the current perspective is that Zbtb7b directs CD4 lineage choice whereas Gata3 is needed for the terminal differentiation or survival of CD4 lineage-committed cells. Here, we re-assessed this model, using mouse lines carrying loss-of-function alleles of Gata3 or Zbtb7b. We demonstrated that, contrary to this current perspective, both transcription factors are required prior to CD4 commitment. Gata3 was required for Zbtb7b expression, whereas Zbtb7b was not necessary for Gata3 expression. Thus, the block caused by Gata3 disruption occurred upstream of that caused by Zbtb7b disruption. Enforced Zbtb7b expression failed to rescue the CD4 differentiation of Gata3-deficient thymocytes, suggesting that Gata3 functions as a key CD4 lineage specification factor, independently from its effects on Zbtb7b expression.

Results

Zbtb7b disruption blocks CD4 lineage commitment

The Zbtb7b^{hd} protein carries an arginine to glycine substitution (R389G) at a position within the second zinc finger that is predicted to contact DNA and contribute to site recognition specificity[4,15]. This raises the possibility that the Zbtb7b^{hd} allele is hypomorphic rather than null, and that the phenotype of HD mice does not reveal the full extent of Zbtb7b functions. In addition, the mutant Zbtb7b^{hd} protein could possibly function in earlier steps of T cell differentiation, or might harbor an aberrant activity (e.g. DNA binding specificity) that contributes to the redirection of MHCII-restricted thymocytes into the CD8 lineage. To evaluate these possibilities, we generated a null allele of *Zbtb7b* by disrupting the entire coding region of this gene (Supplementary Fig. 1, online), thereby preventing the expression of the Zbtb7b protein (Supplementary Fig. 2, online). *Zbtb7b*$^{-/-}$ mice were obtained from heterozygous crosses, developed until adulthood without detectable developmental abnormalities, and were propagated by homozygous *Zbtb7b*$^{-/-}$ matings. Thus, despite the broad expression pattern of Zbtb7b[4,16], its disruption did not affect organogenesis or early survival.

Zbtb7b$^{-/-}$ mice showed impaired CD4 T cell development similar to Zbtb7b^{hd} mice[4], with substantially reduced CD4 T cell numbers and corresponding increased CD8 T cell numbers, in lymph nodes and spleen (Fig. 1a,b and data not shown). *Zbtb7b*$^{-/-}$ thymi contained a large subset of CD4^{+}CD8^{lo} cells with high surface TCR expression characteristic of cells undergoing

positive selection (Fig. 1a); however, these cells expressed high amounts of CD24, a cell surface protein whose down-regulation is contemporaneous with the latest stages of thymocyte maturation. *Zbtb7b*$^{-/-}$ mice expressing the MHCII-restricted AND TCR transgene showed a diversion of MHCII-restricted TCR tg thymocytes and splenocytes from the CD4 to the CD8 lineage (Fig. 1c,d), similar to that in Zbtb7b^{hd} mice. Thus, Zbtb7b is not required during T cell development prior to the CD4-CD8 lineage commitment checkpoint, and the redirection of MHCII-restricted thymocytes to the CD8 lineage in HD mice is not the result of any aberrant activity of the Zbtb7b^{hd} mutant polypeptide.

Gata3 controls a pre-CD4 commitment checkpoint

We next compared the CD4 developmental blocks caused by Zbtb7b and Gata3 disruption. We used mice, hereafter referred to as Gata3$^{\Delta DP}$ mice, carrying conditional 'floxed' Gata3 alleles[13] (Gata3$^{fl/fl}$) and a CD4-Cre transgene that on its own had no influence on thymocyte development (Supplementary Fig. 3, online). The CD4-Cre transgene inactivates the floxed *Gata3* alleles after the early thymocyte developmental stages during which Gata3 is required, allowing the development of Gata3-deficient DP thymocytes[12,13] (Fig. 2a). Gata3$^{\Delta DP}$ thymi contained cells with high expression of TCR and CD69, indicative of intrathymic TCR signal transduction (Fig. 2b). However, TCR^{hi} CD4 SP thymocytes were severely decreased in number ($0.2 \pm 0.04 \times 10^6$ in Gata3$^{\Delta DP}$ vs. $10.9 \pm 1.4 \times 10^6$ in their wild-type counterparts), as was the TCR^{hi} CD4$^+$CD8^{lo} subset found within *Zbtb7b*$^{-/-}$ thymi (Fig. 2a). In contrast, the number of TCR^{hi} CD8 SP thymocytes was not significantly reduced in Gata3$^{\Delta DP}$ thymi (2.12 ± 0.52 vs. $2.62 \pm 0.43 \times 10^6$ in wild-type thymi). Unlike their *Zbtb7b*$^{-/-}$ counterparts, and in agreement with previous findings, Gata3-deficient CD8 SP and CD4$^+$CD8^{int} thymocytes displayed slightly reduced expression of TCR and CD69 (Fig. 2B and Refs. 12-14). Although Gata3$^{\Delta DP}$ mice had small peripheral CD4 populations, these CD4 T cells displayed an activated-memory phenotype (data not shown and Ref. 13), suggesting they arose by amplification of a small number of thymocytes that completed CD4 differentiation as a result of delayed Cre-mediated deletion of the conditional allele. Thus, Gata3 disruption in DP thymocytes had strikingly asymmetric consequences on CD4 and CD8 lineages; CD4 lineage development was arrested before or at the onset of CD8 down-regulation, whereas CD8 lineage development was only minimally affected.

The absence of CD4$^+$CD8^{lo} cells in Gata3$^{\Delta DP}$ thymi, unlike Zbtb7b$^{-/-}$ thymi, raised the possibility that Gata3 is required for the positive selection of MHCII-restricted thymocytes. To explore this possibility, we crossed Gata3$^{\Delta DP}$ mice with *Rag2*$^{-/-}$ mice expressing the I-E^k-restricted 5CC7 TCR transgene[17]. On an H-2$^{k/k}$ Gata3-sufficient background, this TCR promotes the generation of CD4 SP thymocytes that express high amounts of the $V_\alpha 11$ transgenic TCRα chain (Fig. 3a). Although Gata3 inactivation resulted in a major disruption of CD4 cell development, $V_\alpha 11^{hi}$ cells were present in Gata3$^{\Delta DP}$ 5CC7 TCR thymi, indicating that the lack of Gata3 did not prevent MHCII-induced signaling. Unexpectedly, in Gata3$^{\Delta DP}$ thymi, a large subset of $V_\alpha 11^{hi}$ cells expressed a CD4$^-$CD8$^+$ surface phenotype and gave rise to peripheral CD8 T cell populations (Fig. 3a,b and data not shown), indicating redirection to the CD8 lineage. Expression of maturation markers CD5 and CD24 on these CD8 SP thymocytes was comparable to that on Gata3-sufficient 5CC7 thymocytes (Supplementary Fig. 4, online), and they did not express CD122 (IL-2Rβ receptor, data not shown).

The presence of 'CD8-redirected' MHCII-restricted thymocytes in Gata3$^{\Delta DP}$ mice conflicts with previous observations indicating that Gata3-deficient thymocytes expressing the DO11.10 or AND MHCII-restricted TCRs did not differentiate into CD8 cells[9,12], and suggested that the developmental block in Gata3$^{\Delta DP}$ thymi preceded CD4 lineage commitment. It was possible that this discrepancy resulted from a higher avidity of the 5CC7 than of the AND or DO11.10 TCRs for intrathymic pMHC ligands, or a lesser dependence on CD4 for signaling.

To bypass such idiosyncrasies, we used bone marrow chimera experiments to assess the fate of MHCII-restricted Gata3$^{\Delta DP}$ thymocytes expressing a diverse TCR repertoire. We adoptively transferred T cell-depleted Gata3-sufficient (Gata3$^{fl/fl}$) or deficient (Gata3$^{\Delta DP}$) bone marrow into lethally irradiated β2-microglobulin (β2m)-deficient hosts, which lack surface MHCI expression. In these experiments, donor-derived thymocytes developing in β2m-deficient thymi only encounter pMHCII and thus undergo only MHCII-driven positive selection. As a result, Gata3-sufficient cells are positively selected and give rise to CD4 but not CD8 TCR^{hi} SP thymocytes (Fig. 3c,d); similar results were observed whether donor bone marrow was obtained from Gata3$^{fl/fl}$ mice or from Gata3$^{+/fl}$ mice carrying the CD4-Cre transgene (Fig. 3c,d, and Supplementary Fig. 5, online). Gata3-deficient thymocytes also received MHCII-driven TCR signals, as shown by the presence of donor-derived CD69^{+} TCR^{int} and TCR^{hi} thymocytes in Gata3$^{\Delta DP}$-donor chimeras (Fig. 3c); as expected, these cells failed to give rise to CD4 SP populations. However, Gata3$^{\Delta DP}$-donor chimeras contained small numbers of TCR^{hi} CD8 SP thymocytes (Fig. 3c,d), consistent with the observations in 5CC7 mice, and indicative of CD8 redirection. These CD8 SP thymocytes expressed levels of CD5 and CD24 similar to those of wild-type CD8 cells (Supplementary Fig. 6, online), and were selected by MHCII molecules expressed on the thymic epithelium, as no T cell developed when MHCI-MHCII double deficient recipients were reconstituted with Gata3$^{\Delta DP}$ bone marrow (data not shown). Together, these observations demonstrate that Gata3-deficient MHCII-restricted thymocytes can undergo positive selection, and indicate that these cells are arrested early in their differentiation, before CD4 lineage commitment.

Gata3 is required for Zbtb7b expression

To further examine the relationships between the Gata3- and Zbtb7b-controlled lineage checkpoints, we assessed whether either factor affected expression of the other. We first evaluated if Zbtb7b was required for Gata3 expression, which is normally up-regulated as MHCII-restricted thymocytes initiate positive selection[11]. We compared Gata3 mRNA and protein expression in the CD4^{+}CD8^{int} subset, in which Gata3 expression is normally highest, and which is present in both wild-type and *Zbtb7b*$^{-/-}$ mice. Gata3 mRNA expression in these cells, and upregulation of Gata3 protein expression in CD4^{+}CD8^{int} cells relative to DP thymocytes, were not affected by Zbtb7b disruption (Fig. 4a,b). Thus, Zbtb7b is not required for the expression of Gata3 in the thymus.

To evaluate the role of Gata3 in Zbtb7b expression, we compared Zbtb7b protein expression in wild-type and Gata3$^{\Delta DP}$ CD69^{+} thymocytes (Supplementary Fig. 7, online). Zbtb7b protein was detected in wild-type but not Gata3$^{\Delta DP}$ CD69^{+} cells (Fig. 4c). Titration of the wild-type input down to the detection limit showed that expression of Zbtb7b was at least 20-fold greater in wild-type than in Gata3$^{\Delta DP}$ thymocytes (Fig. 4d). We conclude from these analyses that Gata3 is required for the proper expression of Zbtb7b, and thus that Gata3 acts at least in part upstream of Zbtb7b.

Gata3-dependency separates two Zbtb7b functions

As Gata3 is required for proper Zbtb7b expression but not for MHCII-induced TCR signaling, we considered the possibility that intrathymically signaled Gata3$^{\Delta DP}$ thymocytes failed CD4 differentiation because they failed to up-regulate Zbtb7b. If that were the case, enforced expression of Zbtb7b should restore CD4 lineage differentiation in Gata3$^{\Delta DP}$ mice. To evaluate this possibility, we complemented Gata3$^{\Delta DP}$ mice with a Zbtb7b transgene[5] expressed in DP thymocytes in amounts similar to endogenous Zbtb7b expression in CD4 T cells (Supplementary Fig. 8, online).

As previously observed[5], expression of the Zbtb7b transgene in Gata3-sufficient thymocytes resulted in a complete loss of the CD8 SP thymocyte population but did not detectably affect

CD4 SP thymocyte numbers (Fig. 5a,b). However, expression of the Zbtb7b transgene in Gata3$^{\Delta DP}$ mice did not restore TCR^{hi} CD4^{+}CD8^{-} thymocyte numbers (Fig. 5a,b), suggesting that Zbtb7b expression fails to promote the CD4 differentiation of Gata3-deficient thymocytes. Although the presence of substantial numbers of peripheral CD4 T cells in Zbtb7b-transgenic Gata3$^{\Delta DP}$ mice challenged this conclusion (Supplementary Fig. 9, online), these peripheral cells were of large size (FSC^{hi}) and expressed the memory marker CD44, as in Gata3$^{\Delta DP}$ mice[13], suggesting that they emerged from the lymphopenia-driven expansion of an undetectable contingent of CD4-differentiating thymocytes that arose as a result of delayed Gata3 gene inactivation. This hypothesis predicted that no or few peripheral CD4 cells would be present in the spleen of one week-old mice, in which mature T cell numbers more accurately reflect thymic output than in adult animals. That was indeed the case, as few cells, all of which displayed an FSC^{hi}CD44^{hi} surface phenotype, were present in one week-old Gata3$^{\Delta DP}$ mice; in contrast, Gata3-sufficient one week-old spleens contained predominantly FSC^{lo}CD44^{lo} cells (Fig. 5c and Supplementary Fig. 10, online). As in older Gata3$^{\Delta DP}$ mice, one week-old Gata3$^{\Delta DP}$ mice had no detectable CD4 SP thymic populations (Supplementary Fig. 10, online). We conclude from these findings that Zbtb7b fails to promote the CD4 differentiation of thymocytes lacking Gata3.

In sharp contrast with its inability to restore CD4 lineage differentiation, the Zbtb7b transgene in Gata3$^{\Delta DP}$ mice caused a complete loss of CD8 SP thymocyte and peripheral CD8 T cell populations (Fig. 5a), indicating that Zbtb7b-mediated inhibition of CD8 lineage differentiation did not require Gata3. Of note, the absence of both CD4 and CD8 SP populations in the thymus suggested that Zbtb7b failed to promote the CD4-redirection of MHC I-restricted cells in the absence of Gata3.

Gata3 binds the *Zbtb7b* locus

That Gata3 acts upstream of Zbtb7b and is required for Zbtb7b to promote CD4 differentiation raises the possibility that Gata3 serves as a specification factor[18] for the CD4 lineage by binding to and promoting expression of CD4 lineage-specific genes. As a first assessment of this possibility, we examined whether Gata3 molecules were recruited to the *Zbtb7b* locus in thymocytes.

The *Zbtb7b* locus spans over 20 kilobases (kb) of mouse chromosome 3 (Fig. 6a) and contains more than 15 motifs matching the Gata3 binding consensus sequence (WGATAR, where W stands for A or T and R for A or G)[19,20]. Consequently, we selected candidate regions based on their location in functionally important gene regions and their conservation across species. This led us to focus on two motifs, hereafter referred to as site A and site B (Fig. 6a and Supplementary Fig. 11, online). Both motifs are included in a 3.7 kb region required for Zbtb7b expression, as shown by analyses of BAC reporter transgenes (Fig. 6a,b), and more specifically within two areas of DNase I hypersensitivity (L.W. et al, in preparation and sites E and F in Ref. 21), a hallmark characteristic of transcription factor binding sites. Both motifs are conserved among multiple mammalian species (Supplementary Fig. 11, online). Their location and their relationships with known *Zbtb7b* cis-regulatory elements are schematically depicted on Supplementary Figure 11, online: site B colocalizes with a recently identified regulatory element called PRE (Proximal Regulatory Element) or enhancer[21,22], whereas site A is within a highly conserved downstream 150 base pair (bp) conserved sequence.

Chromatin immunoprecipitation (ChIP) assays were performed using cross-linked thymocyte chromatin to evaluate Gata3 recruitment to sites A and B. These experiments detected consistent enrichment of both sites in Gata3 immunoprecipitates relative to immunoprecipitates prepared using an isotype control antibody (Fig. 6c,d). This enrichment was specific as it was not observed in chromatin prepared from Gata3-deficient thymocytes, and as no Gata3 enrichment was found on an unrelated sequence present in the *Cd8* gene

enhancer E8(I)[23,24]. In contrast, we found little or no specific Gata3 recruitment to a highly conserved GATA motif located 1.6 kb upstream of *Zbtb7b* exon 1 (data not shown). However, given the multiplicity of GATA motifs in the *Zbtb7b* sequence and the limited sensitivity of the ChIP procedure, our search was necessarily not exhaustive and it is quite conceivable that Gata3 is recruited at additional sites within the *Zbtb7b* locus. We conclude from these experiments that the transcription factor Gata3 is recruited to at least two distinct sites within a region of the *Zbtb7b* locus required for Zbtb7b expression. These findings, together with the Gata3 requirement for Zbtb7b expression, support the possibility that Gata3 directly activates *Zbtb7b* transcription.

In summary, the present study demonstrates that Gata3 and Zbtb7b play essential and non-redundant roles during early CD4 cell differentiation prior to lineage commitment. At this developmental stage, Gata3 is required both for Zbtb7b expression and for additional, thus far unknown, functions. In contrast, Gata3 is not needed for Zbtb7b to inhibit CD8 differentiation.

Discussion

The present study positions the requirement for the zinc finger transcription factors Zbtb7b and Gata3 prior to the CD4 lineage commitment checkpoint. We report that Gata3 is required early during CD4 differentiation and, notably but not only, for the proper expression of Zbtb7b; in contrast, Zbtb7b disruption does not impair Gata3 expression.

As previously demonstrated[12,14], the requirement for Gata3 is selective; Gata3 disruption does not prevent CD8 lineage differentiation and was reported not to impair MHCI-induced positive selection, consistent with the fact that this factor is downregulated in CD8 lineage thymocytes[10,11]. Here we showed that MHCII-restricted Gata3-deficient thymocytes can, albeit inefficiently, develop into CD8 cells. Thus, the requirement for Gata3 identifies an early, pre-commitment branch point during which gene expression requirements for each lineage begin to diverge. Additional studies are needed to position this branch point relative to the steps controlled by the HMG box protein Tox[25] and the transcription factor c-Myb[26,27]. However, the redirection of Toxdeficient MHCII-restricted thymocytes into the CD8 lineage was less efficient that that of Gata3-deficient thymocytes, and c-Myb deficiency impairs CD4 differentiation, but to an extent less severe than that caused by Gata3 disruption, and does not affect Zbtb7b expression[27]. Thus Tox, c-Myb and Gata3 seem to have distinct functions during CD4 differentiation. Analyses of hematopoietic cell differentiation have led to the distinction between lineage specification, understood as the activation of lineage-specific expression, and lineage commitment, defined as the loss of alternate developmental fate[18]. One illustration of this dichotomy is found during B cell differentiation where the specification factors E2A and EBF promote expression of the B cell commitment factor Pax5[28,29]. We propose that Gata3 is required for CD4 lineage specification and that it may serve this function through two non-mutually exclusive mechanisms.

First, it is possible that Gata3 directly acts as a specification factor to promote the expression of CD4 lineage genes, and our finding that Gata3 is recruited to Zbtb7b cis-regulatory elements supports this possibility. As Zbtb7b fails to promote the CD4 differentiation of Gata3-deficient cells, future studies will focus on identifying additional CD4 lineage-specific Gata3 target genes.

A second possibility is that Gata3 acts indirectly to specify the CD4 lineage, by promoting the expression of one or more TCR signaling intermediate(s) required for CD4 but not CD8 differentiation. Current models of lineage choice[2] propose that TCR signaling is of longer duration[30-32] or greater intensity[33,34] in MHCII- than in MHCI-restricted thymocytes, thereby promoting CD4 over CD8 choice and ensuring the correspondence in mature T cells between

MHC specificity and coreceptor expression. Thus, it is conceivable that Gata3 indirectly contributes to CD4 lineage specification by sustaining the expression of signaling intermediates that are required for CD4 but not for CD8 differentiation, even though their expression may not be CD4 lineage-specific. Although Gata3 disruption was reported not to affect TCR signaling in DP thymocytes[12], we noted that Gata3-deficient thymocytes express lower amounts of surface CD69 and TCR, both of which are up-regulated by TCR signaling ([Ref. 12,14] and this study). In addition, Gata3 negatively regulates expression of CD5, a surface molecule that participates in a negative feed-back control of TCR signal transduction[14,35].

Both possible mechanisms are consistent with the earlier developmental block and the smaller size of 'redirected' MHCII-restricted CD8 SP thymocyte populations in Gata3-deficient compared to Zbtb7b-deficient thymi. (See Supplementary Discussion, on line). Of note, the MHCII-restricted thymocytes that were 'redirected' to the CD8 lineage in the absence of Gata3 were dependent on epithelial pMHCII and did not express CD122, unlike CD8 cells selected on pMHCI expressed on hematopoietic thymic elements and that display an 'innate' effector phenotype[36-38].

During positive selection, Zbtb7b both inhibits CD8 and promotes CD4 differentiation[4,5]; thus, the question arises whether Zbtb7b contributes to both CD4 specification and commitment or to commitment only. In a simple perspective, specification factors turn on lineage-specific genes, whereas commitment factors turn off genes characteristic of alternate fates. As Zbtb7b belongs to a family of proteins that generally act as transcriptional repressors, it is conceivable that it indirectly promotes CD4 differentiation by repressing CD8 lineage genes induced by intrathymic signals[39]; such targets could include Runx3 and thus far unidentified repressors of CD4 differentiation. This simple model places Zbtb7b as a key commitment factor that lacks specification activity, and suggests that CD4 lineage specification would rely on transcriptional activators, including Gata3, that promote the expression of CD4 lineage-specific genes. The absence (or insufficient expression) of Zbtb7b (e.g. in MHCI-restricted or Zbtb7b-deficient thymocytes) would allow the up-regulation of CD8 specification factors and of the CD8 commitment factor Runx3[22,40,41]. Enforced expression of Zbtb7b in MHCI-restricted thymocytes prevents CD8 differentiation and promotes the CD4 differentiation of those cells in which expression of CD4 specifying factors (including Gata3) is sufficient to turn on CD4 lineage gene expression.

Several lines of evidence support this model. Consistent with a commitment function, Zbtb7b inhibits the expression of CD8 and of cytotoxic genes in post thymic CD8 cells[42], and antagonizes the repression of CD4 expression by Runx molecules[43]. In contrast, Zbtb7b has a more modest effect on promoting CD4 lineage gene expression in mature CD8 cells[42]. Conversely, there is evidence that Gata3 lacks commitment activity. Gain-of-function experiments show that enforced Gata3 expression does not prevent CD8 choice[11,14,44], whereas our finding that Gata3 is not required for Zbtb7b to inhibit CD8 differentiation supports the possibility that the committing function of Zbtb7b is Gata3-independent.

Variations on the model outlined above include the possibilities that both Gata3 and Zbtb7b participate in commitment (e.g. because Gata3 would be required for Zbtb7b to repress a subset of CD8 lineage genes), or that both Gata3 and Zbtb7b participate in the specification of the CD4 lineage, by directly promoting the expression of a common set of CD4 lineage-specific genes, or by triggering distinct target genes. Although there is no experimental evidence to support these possibilities at present, future analyses of Zbtb7b and Gata3 gene targets are necessary to exclude these possibilities.

The *Zbtb7b* Distal Regulatory Element (DRE [21], also referred to as silencer[22]) inhibits Zbtb7b expression in CD8-lineage cells and in DP thymocytes (silencing function), and also has

enhancer activity in transgenic reporter assays[21]. The DRE includes a motif (CGATTAG) distantly related to the WGATAR Gata3 binding sequence, and notably atypical by the presence of a T instead of an A at position +4 of the GATA sequence[19,20]. Whether this motif recruits Gata3 or contributes to the putative enhancer function of the DRE was not evaluated[21]. Experiments using conventional reporter transgenes indicate that the CGATTAG motif is dispensable for the silencing function of the DRE[21], in agreement with analyses by homologous recombination[22].

The connections between transcription factors and TCR signal attributes that ensure the matching of lineage differentiation to MHC specificity, and notably how Zbtb7b expression is restricted to MHCII-signaled cells during positive selection[4,5], are not understood. Whereas Runx proteins repress Zbtb7b expression[22], neither Runx1 nor Runx3 are known targets of TCR signals. Furthermore, Runx repression of Zbtb7b is cell context-dependent, as Runx1 is expressed in and required for the survival of CD4 lineage cells[45], and as Zbtb7b gene expression does not correlate with the recruitment of Runx complexes to the Zbtb7b silencer[22]. Finally, relieving Runx-mediated repression of Zbtb7b, or deleting the Zbtb7b silencer that recruits Runx complexes, results in an only partial up-regulation of Zbtb7b in pre-selection DP thymocytes[22].

These observations suggest that other transcription factors, recruited to additional Zbtb7b cis-regulatory elements, serve as relays between TCR signaling and Zbtb7b up-regulation. Our findings, together with previous work, indicate that Gata3 might act as such a “link” between TCR signaling and Zbtb7b expression. Gata3 has been proposed to serve as a sensor of TCR signaling during T cell lineage differentiation[11], because it is expressed in greater amounts in MHCII- than in MHCI-signaled cells[32], and because thymocytes up-regulate Gata3 upon TCR stimulation *in vitro*. If Gata3 also promotes TCR signaling, it may furthermore serve as the “engine” of a self-reinforcing loop that promotes Zbtb7b expression and CD4 choice. However, as enforced Gata3 expression does not redirect MHCI-restricted thymocytes into the CD4 lineage[11], it is likely that additional transcription factors recruited to positive regulatory elements within the Zbtb7b locus[21,22] also participate in TCR-induced Zbtb7b up-regulation.

Methods

Targeted disruption of *Zbtb7b* and transgenic constructs

A *Zbtb7b* gene targeting vector was generated using the recombineering technology and vectors (http://recombineering.ncifcrf.gov/) from BAC clone RP23-64L17 (BACPAC). The targeting vector included *Zbtb7b* sequences from nucleotide (nt) −7585 to +5907, where +1 is the A residue in the *Zbtb7b* initiation codon, a neomycin resistance cassette derived from pLTM330 inserted at position −212, and a loxP site derived from pLTM332 inserted at position +2263. See Supplementary Methods, online for detailed cloning procedures. Recombinant ES cells were selected after transfection with the targeting vector using G418 and guancyclovir, as previously described[46], and screened by Southern blotting using the strategy outlined in Supplementary Figure 1, online. Of 31 recombinant clones obtained, two were used for blastocyst injection, of which one gave rise to the line used in the present study. Deletion of the loxP-flanked segments was performed by crossing mice heterozygous for the recombinant allele with mice expressing Cre under the control of a β-actin promoter. The genotype of recombinant animals was verified by Southern blotting for the first two generations, and subsequently by PCR on tail DNA. GFP BAC reporter constructs were generated as described in Supplementary Methods, online.

Mice

Mice carrying a floxed Gata3 allele and a CD4-Cre transgene[13,47], a β-actin-Cre[46] or a Zbtb7b transgene[5] (C8 line) were previously described. AND TCR[48] and Rag2-deficient 5CC7 TCR[17] transgenic mice were obtained from Jax and Taconic, respectively. β2m-deficient mice were from Taconic, and MHCII-deficient mice were from Jax. Mice carrying the indicated genotypes were generated by appropriate intercrosses or backcrosses. All mice were housed in Specific Pathogen Free facilities. Animals were genotyped by PCR on tail DNA or phenotyped by staining of peripheral blood cells, and were analyzed between 4 and 12 weeks of age except when indicated otherwise. All transgenic animals were heterozygous for the transgenic allele. For bone marrow (BM) chimera experiments, BM suspensions were depleted of mature T cells with antibodies to Thy1.2 (J1J) and CD5 (C3PO) plus low-toxicity rabbit complement (Cedarlane). Irradiated MHC-deficient recipients (800 rads, cesium source) were reconstituted with T cell-depleted BM cells and maintained on antibiotic water until analysis at 4-6 wk post-reconstitution. Animal procedures used in the present study were approved by the NCI or NIAID Animal Care and Use committees, as appropriate.

Gene and protein expression

Cells were prepared from thymus, spleen or lymph nodes, enumerated and analyzed by flow cytometry according to previously described procedures[49], using either an LSR II flow cytometer (BD Biosciences) or a modified (Cytek) FacsCalibur cytometer (BD Biosciences). Cell sorting was performed as described[32] using Facs Vantage or Facs Aria instruments (BD Biosciences). $CD69^+$ cells were purified using anti-FITC beads (Miltenyi) after staining with FITC-conjugated anti-CD69. Analyses of gene expression by RT-PCR were performed as described[42]. Briefly, RNA was extracted with RNAqueous (Ambion) and reverse-transcribed with Oligo dT priming (Superscript III, Invitrogen). PCR was performed using Taqman reagents, probes (Gata3 probe Mm00484683_m1) and an ABI PRISM 7500HT sequence detection system, all from Applied Biosystems. Gene expression values were normalized to *Actb* in the same sample. For protein analyses, cells were lyzed in 1% Triton-containing buffer; total cell extracts or anti-Zbtb7b immunoprecipitates were separated on 10% SDS-PAGE gels under reducing conditions, transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore), and analyzed by immunoblotting and chemiluminescence as previously described[5].

Antibodies

A rabbit antibody directed against a GST fusion protein including aa 167-335 of mouse Zbtb7b was derived using conventional procedures; its specificity was verified by transient transfection assays in 293T cells (Supplementary Figure 2a, online). Mouse anti-GATA-3 (HG3-31) and control IgG1 were from Santa Cruz Biotechnology. The anti-β-actin was from Sigma (Clone AC-15). Antibodies used for staining are listed in Supplementary Table I, online.

ChIP

ChIP analyses were performed from AND TCR thymocytes or Gata3-deficient thymocytes using the Chromatin Immunoprecipitation Assay kit (Upstate Biotechnology), according to the manufacturer's instructions with minor modifications as described in Supplementary Methods, online and using primers listed in Supplementary Table II, online.

Statistical analyses

Statistical analyses performed in Fig. 5b used the two-tailed Rank Sum Wilcoxon test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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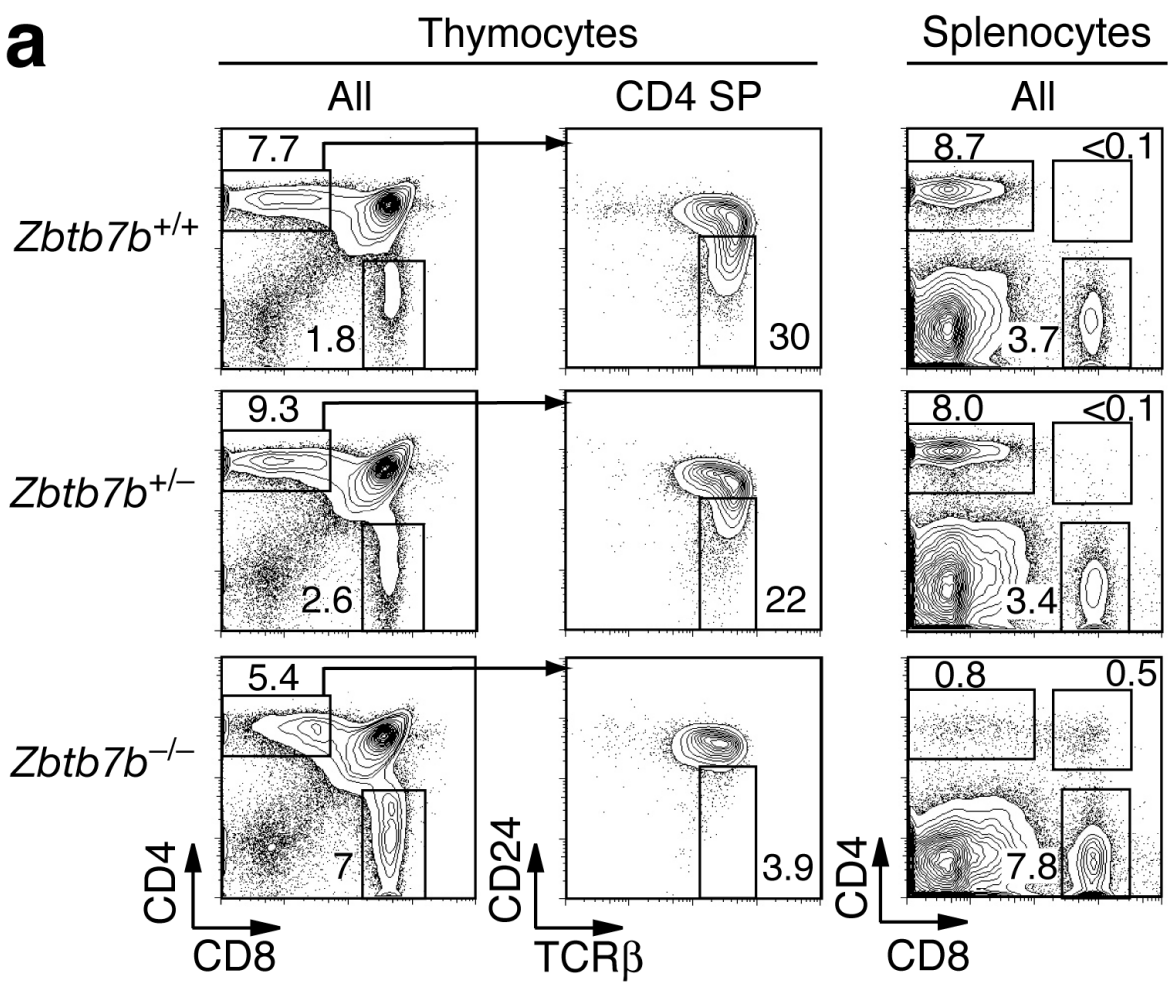
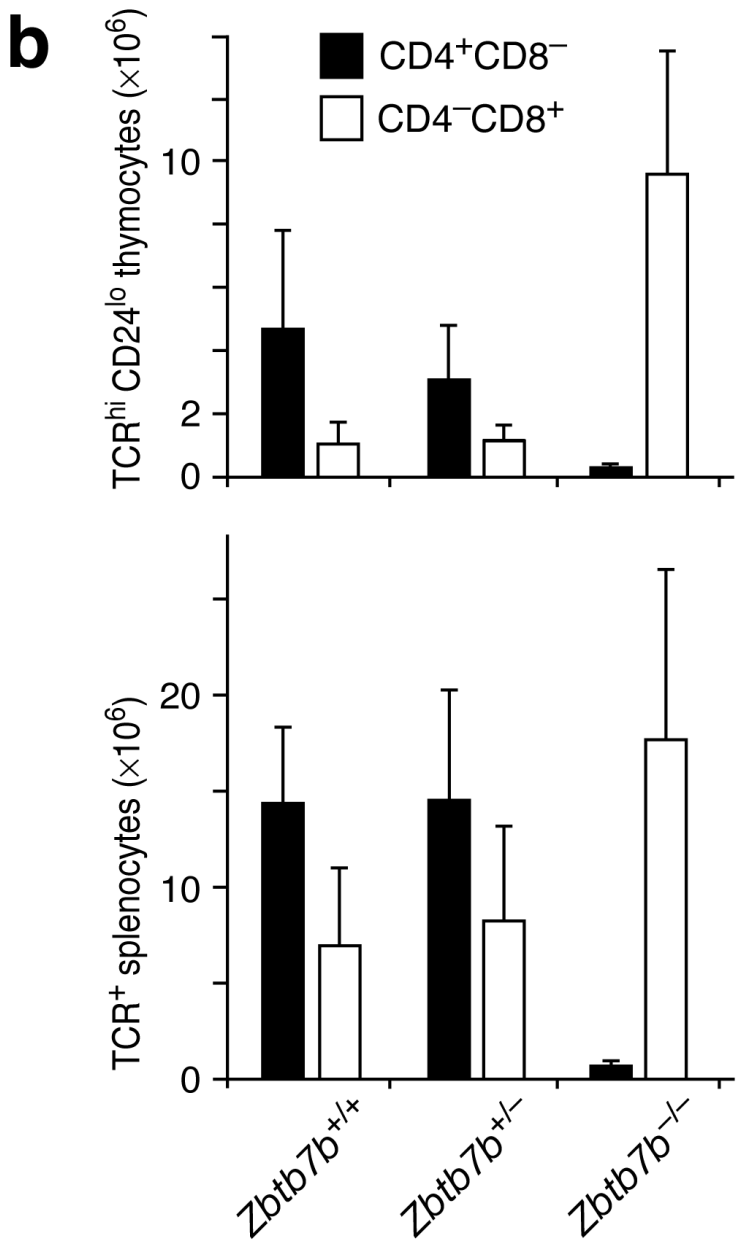
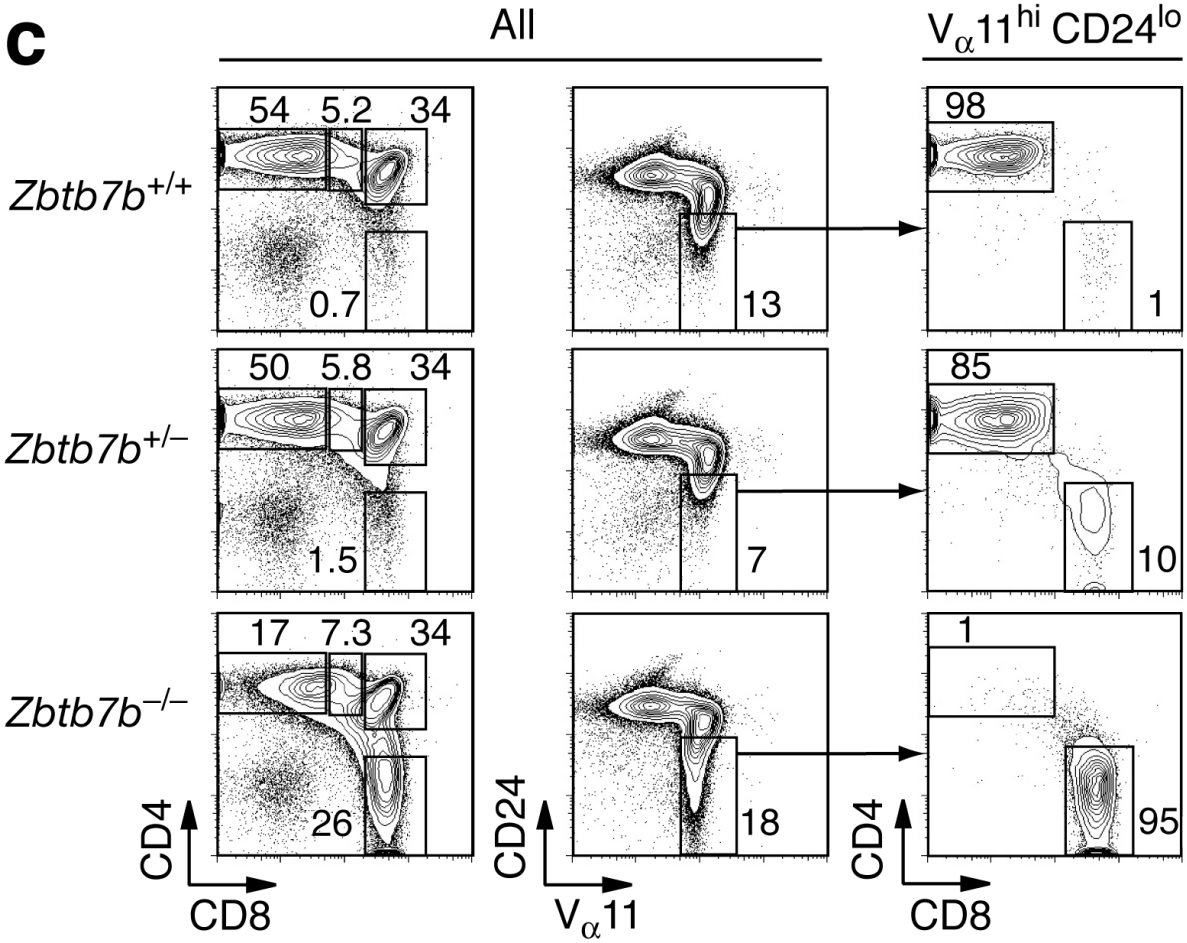






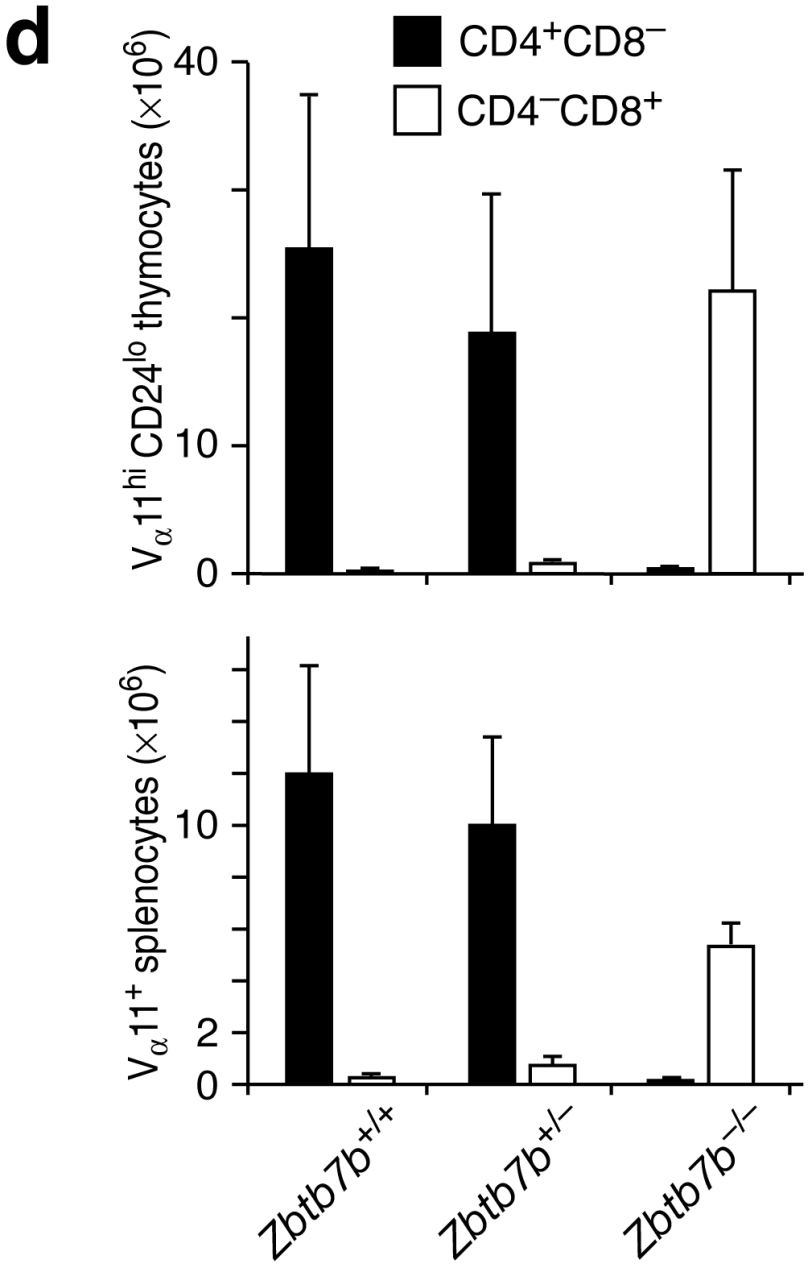


Figure 1. Zbtb7b disruption does not affect CD4 differentiation prior to the CD4-CD8 lineage commitment checkpoint

(**a,b**). Zbtb7b disruption selectively prevents CD4 T cell differentiation. (**a**) Thymocytes and splenocytes from the indicated mice were analyzed by 4-color flow cytometry. Numbers indicate the percentage of cells within boxes. (**b**) Bar graphs display the absolute numbers of mature CD4 SP (filled bars) and CD8 SP (open bars) thymocytes (top) and splenocytes (bottom). Note the distinct x-axis scales. Error bars indicate s.d. Data is from six mice of each genotype analyzed in six experiments.

(**c,d**). Zbtb7b disruption redirects MHCII-restricted thymocytes to the CD8 lineage. (**a**) 4-color flow cytometry analyses were carried out on thymocytes from *Zbtb7b*$^{+/+}$, *Zbtb7b*$^{+/-}$ and

Zbtb7b$^{-/-}$ mice carrying the AND TCR transgene that, in I-A^{b}-expressing mice, promotes the positive selection of large numbers of CD4 SP cells expressing the transgenic $V_{\alpha}11$ TCRα chain. Numbers indicate the percentage of cells within boxes. (**d**) Absolute numbers of mature thymocytes and splenocytes are displayed as in (**b**). Data is from 3 or 4 mice of each genotype analyzed in three experiments.

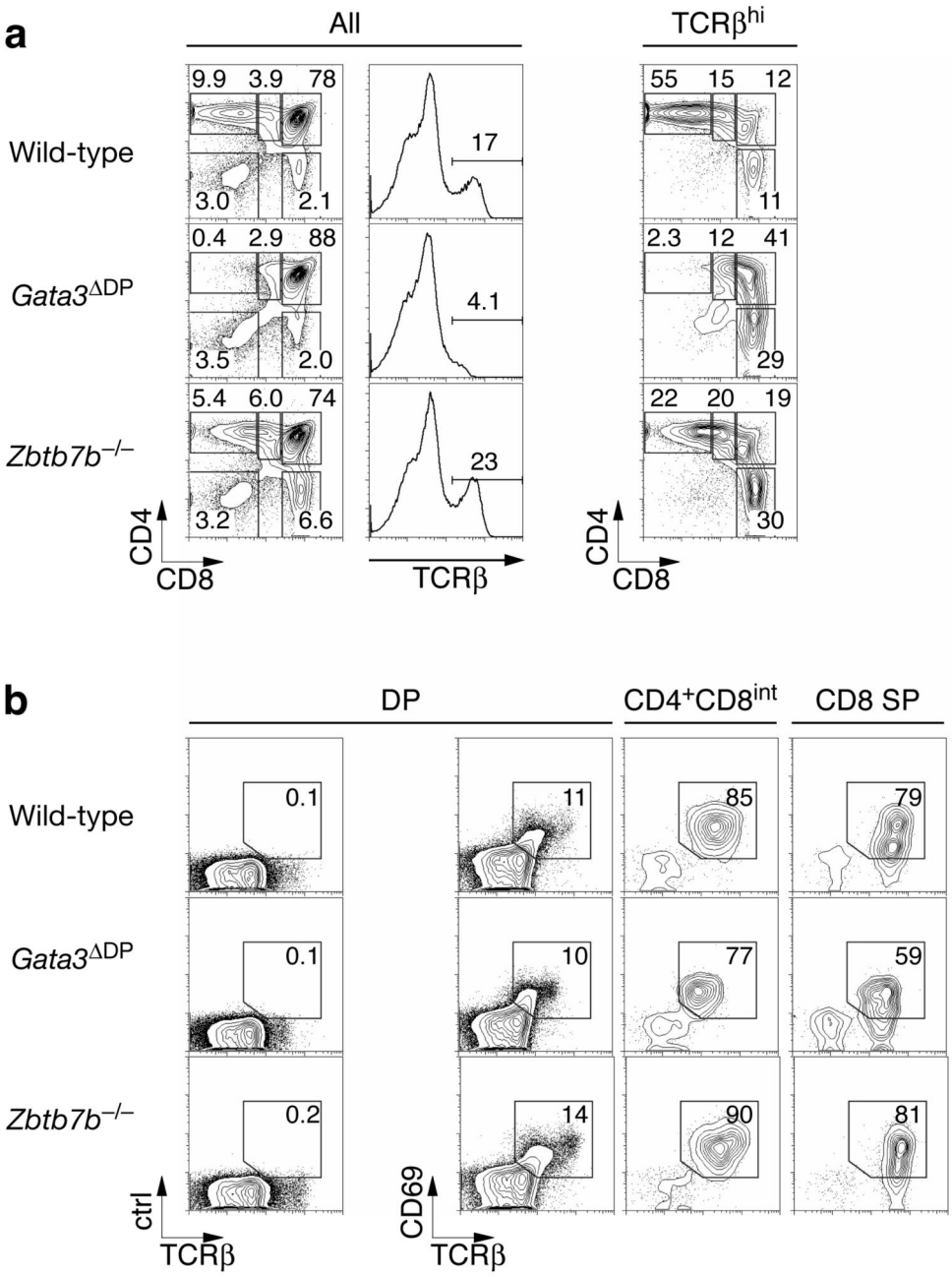
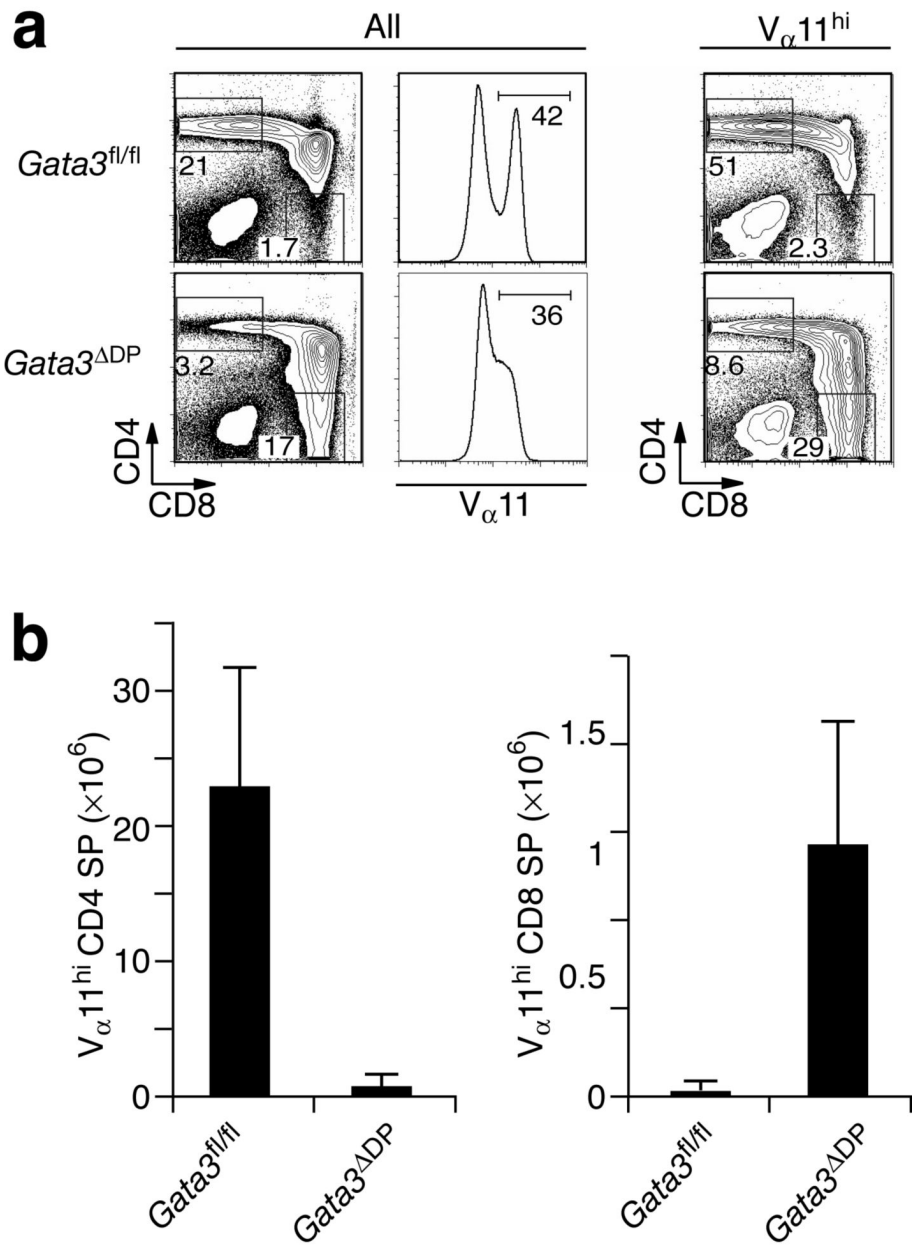


Figure 2. Gata3 disruption prevents CD4 differentiation but not positive selection

(**a**) Thymocytes from wild-type (*Zbtb7b*$^{+/+}$ *Gata3*$^{+/+}$), *Zbtb7b*$^{-/-}$ and *Gata3*$^{\Delta DP}$ mice were analyzed by 4-color flow cytometry. (**b**) Expression of TCRβ and CD69, an indicator of TCR signaling[50], on DP, CD4^{+}CD8^{int} and CD8 SP thymocytes gated as in (**a**). Parallel experiments using an isotype control antibody (ctrl, left column) verify the specificity of anti-CD69 staining. Numbers near or within boxes, or above brackets, indicate the percentage of enclosed cells. Data is from three independent experiments comparing both genotypes.



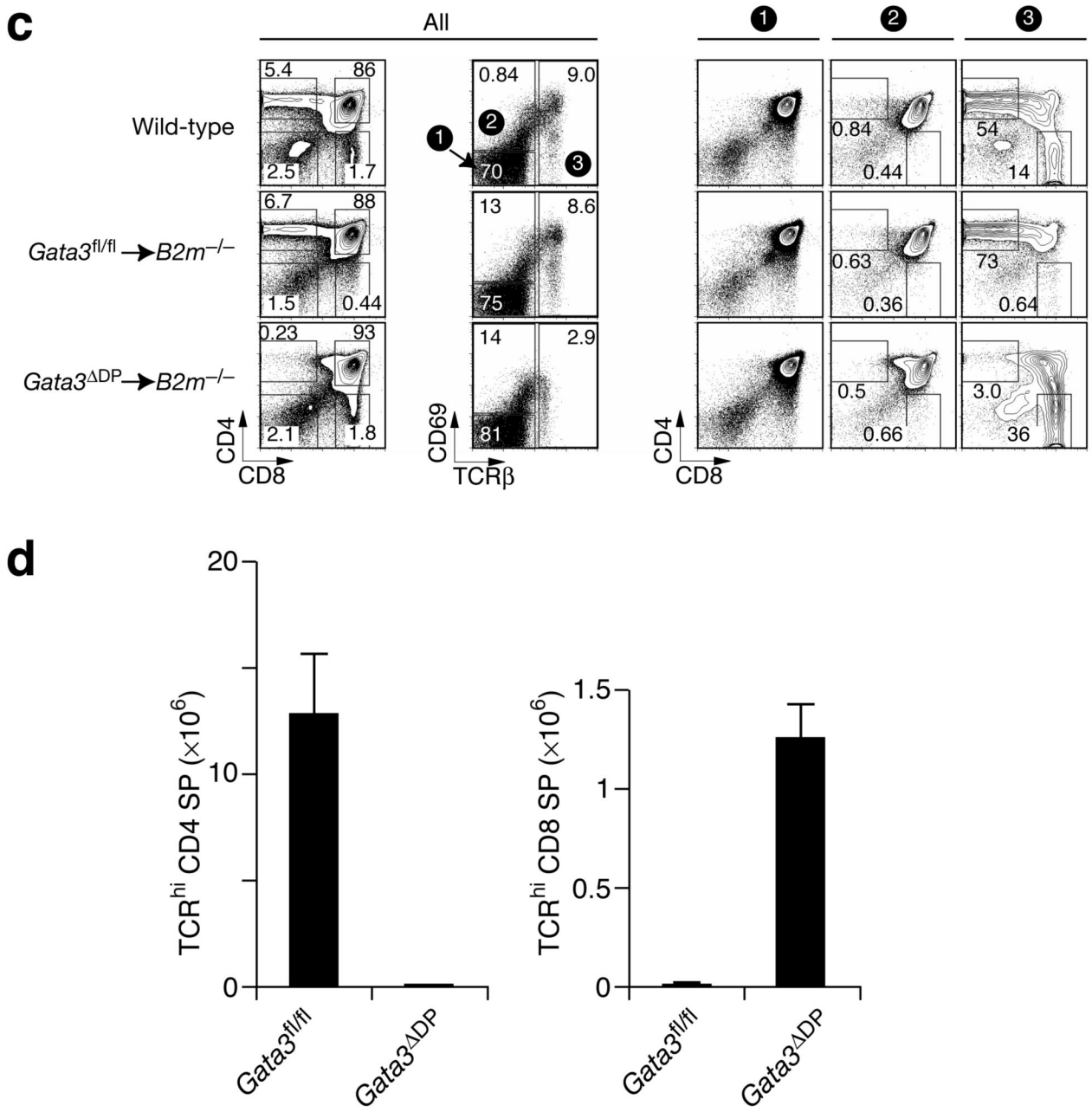


Figure 3. Gata3-deficient MHCII-restricted thymocytes undergo intrathymic signaling and are inefficiently redirected into the CD8 lineage

(**a,b**). Thymocytes from *Rag2*$^{-/-}$*Gata3*$^{\Delta DP}$ or *Rag2*$^{-/-}$*Gata3*$^{fl/fl}$ mice carrying the 5CC7 TCR transgene were analyzed by 3-color flow cytometry. Numbers in plots indicate the percentage of cells within boxes or brackets. (**b**) Numbers of Vα11^{hi} CD4 SP and CD8 SP thymocytes in *Rag2*$^{-/-}$*Gata3*$^{\Delta DP}$ or *Rag2*$^{-/-}$*Gata3*$^{fl/fl}$ mice carrying the 5CC7 TCR transgene. Error bars indicate s.d. Data is from four separate experiments, each including three or more mice of each genotype.

(**c,d**). Irradiated β2m-deficient hosts were reconstituted with bone marrow from Gata3$^{fl/fl}$ or Gata3$^{\Delta DP}$ mice and analyzed 6 weeks after reconstitution. (**c**) Flow cytometry analyses of

thymocytes in Gata3$^{fl/fl}$- and Gata3$^{\Delta DP}$-derived chimeras (middle and bottom rows) and in an unmanipulated wild-type thymus (top row). Middle: dot plots depict gating on CD69^{-} TCR^{lo} (subset 1), CD69^{+} TCR^{int} (subset 2); and TCR^{hi} (subset 3) populations. (**d**) Absolute numbers of TCR^{hi} CD4 SP and CD8 SP thymocytes (average ± s.d.) for each series of chimeras. Data is representative of two separate experiments, each including three or more chimeras of each genotype.

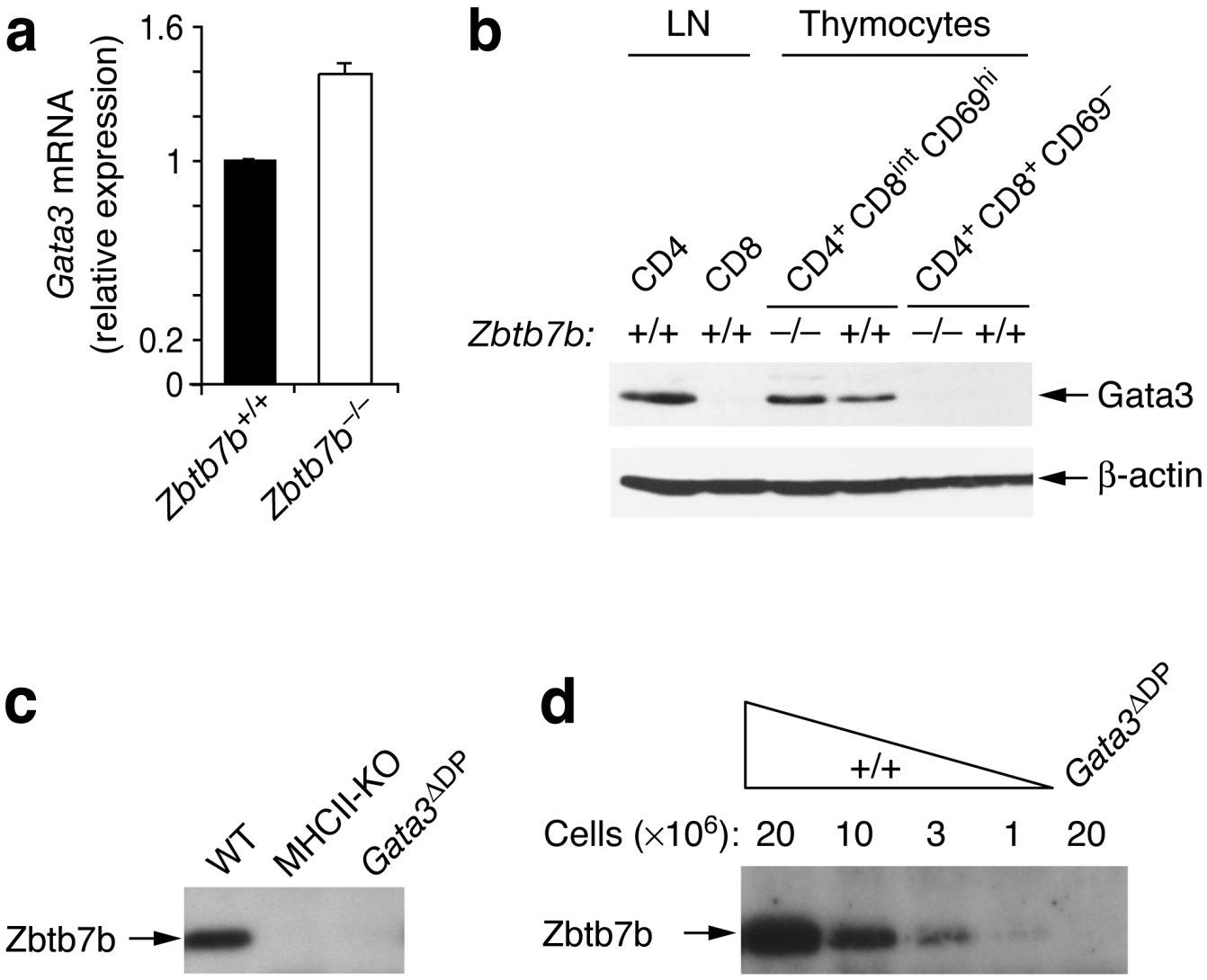
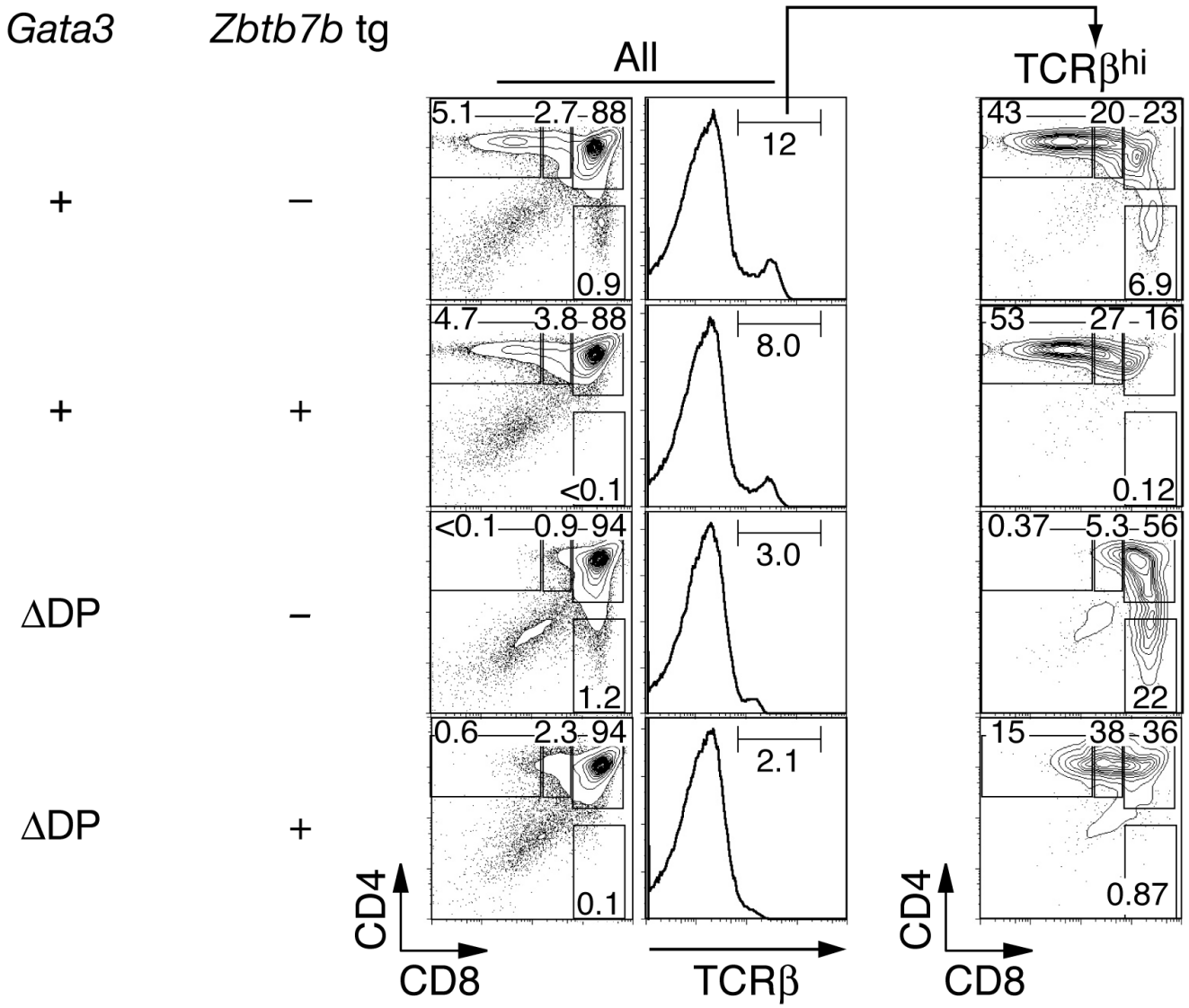
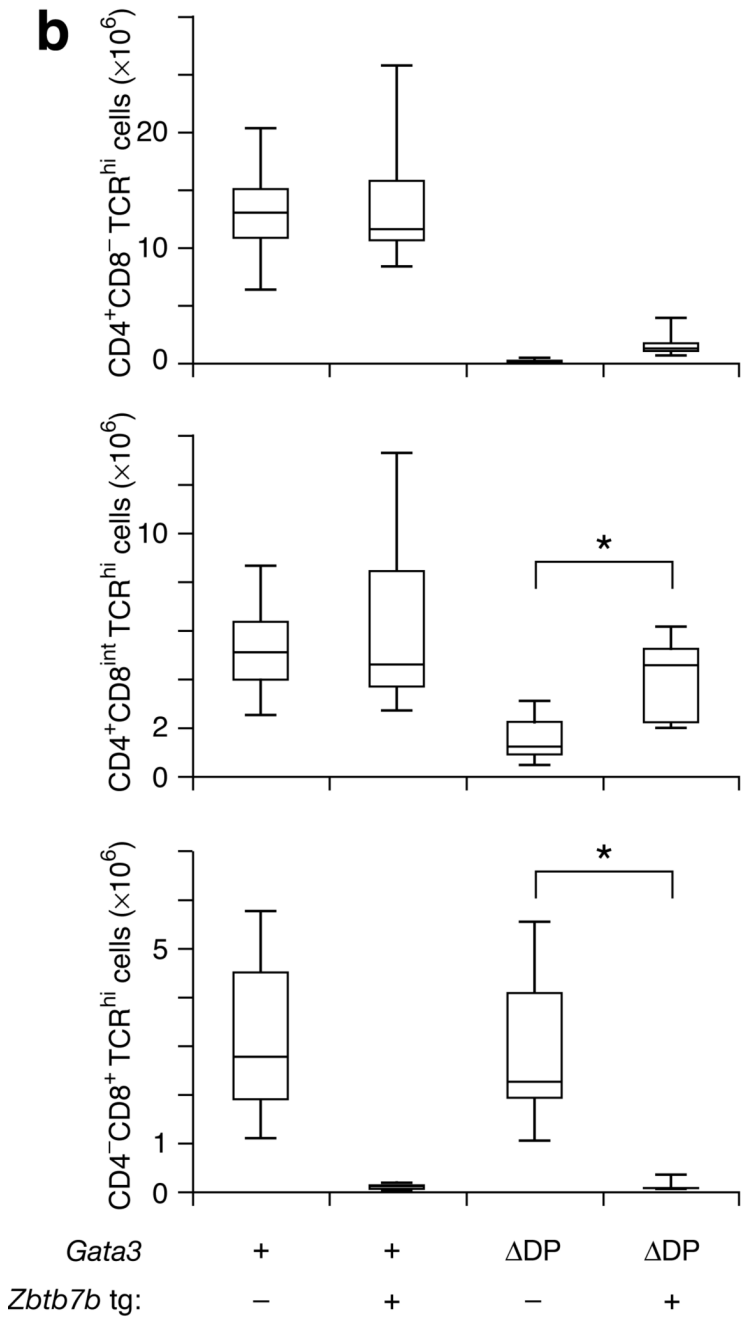


Figure 4. Gata3 is required upstream of Zbtb7b

(**a,b**). Comparison of Gata3 mRNA and protein expression in purified thymocyte subsets from *Zbtb7b*$^{+/+}$ and *Zbtb7b*$^{-/-}$ mice. (**a**) Expression of Gata3 mRNA in CD69^{+} CD4^{+}CD8^{int} thymocytes, normalized to *Actb* mRNA and shown relative to *Zbtb7b*$^{+/+}$ cells. (**b**) Gata3 protein expression, analyzed by immunoblotting of lysates prepared from the indicated populations; equal lane loading was verified by subsequent probing of the same membrane with anti-β-actin. Mature (lymph node, LN) T helper type 2 activated CD4 cells (lane 1) and activated CD8 cells (lane 2) were used as specificity controls. Data in (**a**) and (**b**) are representative of two separate experiments, from distinct sorted cell preparations, for each panel. The slight but reproducible increase in Gata3 expression in *Zbtb7b*$^{-/-}$ cells is probably caused by the greater representation of immature cells in the CD4^{+}CD8^{int} gate, due to the block caused by Zbtb7b disruption (Gata3 is normally down-regulated in the later stages of CD4 SP cell maturation[11]).

(**c,d**). Immunoprecipitation and immunoblotting analyses of Zbtb7b protein expression in CD69^{+} thymocytes from the indicated mice. (**c**) MHCII-deficient thymocytes were used as a negative control. (**d**) Wedge denotes decreasing numbers of *Gata3*$^{+/+}$ CD69^{+} thymocytes (respectively 2, 1, 0.3 and 0.1 × 10^{7}). Data is representative of three distinct experiments.





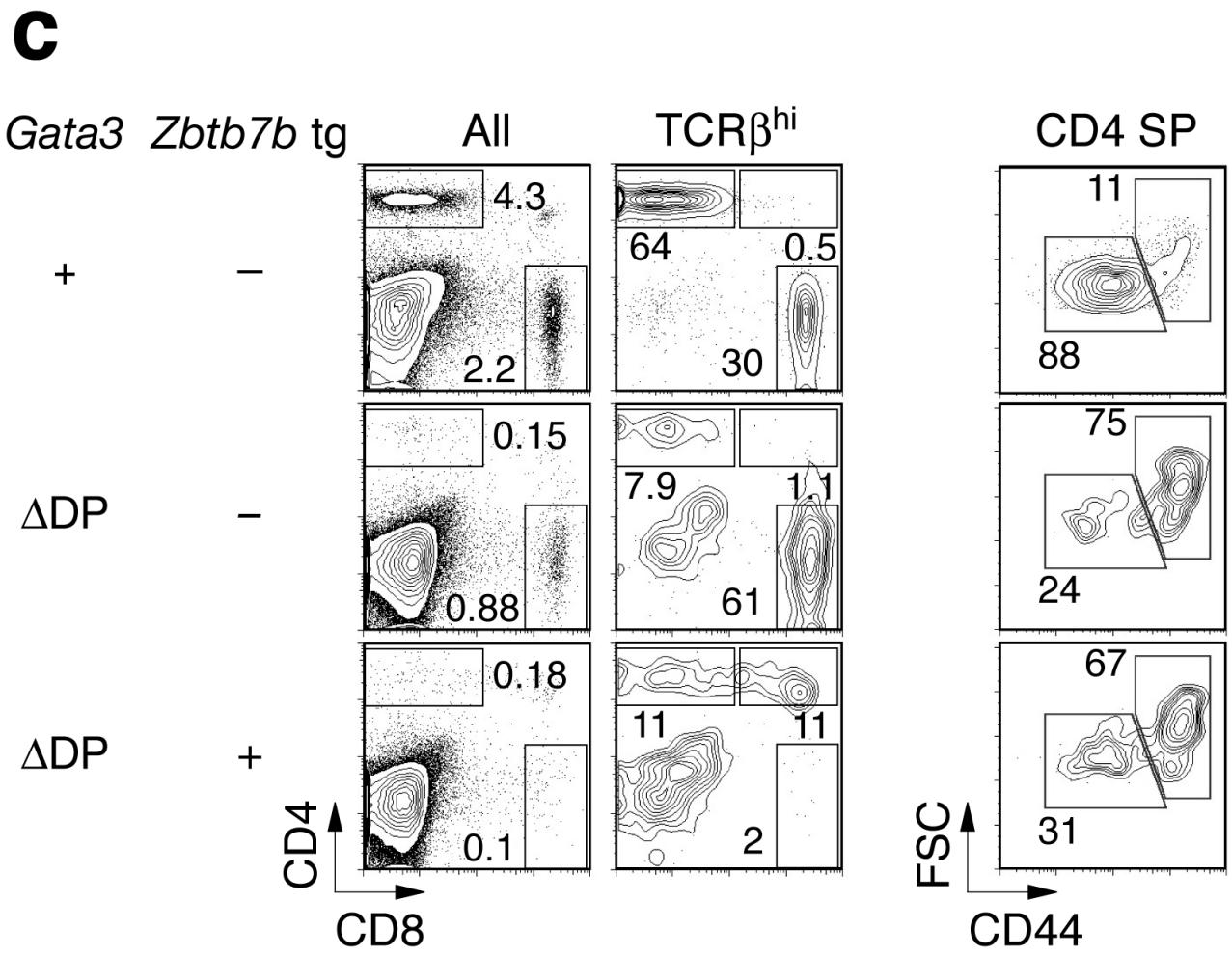
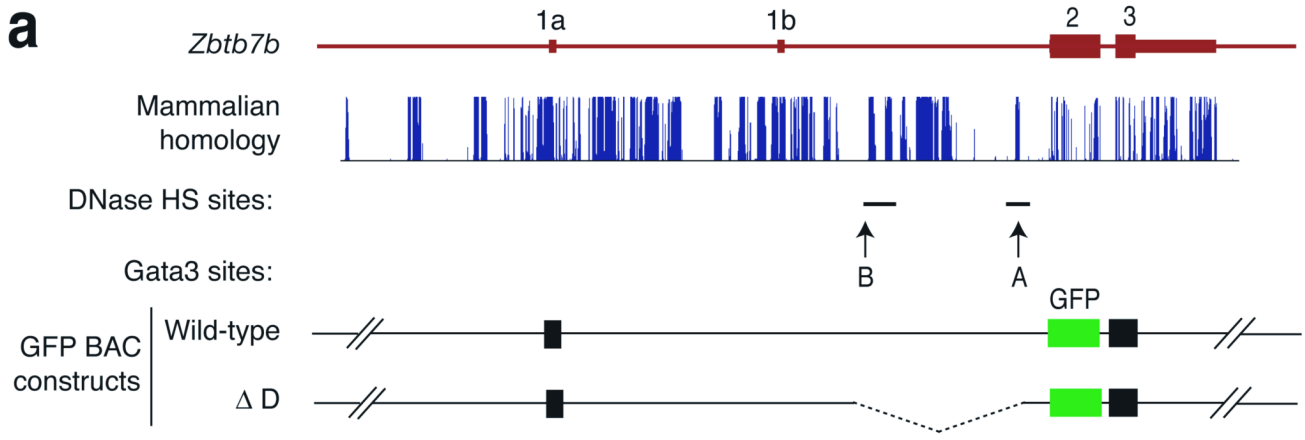
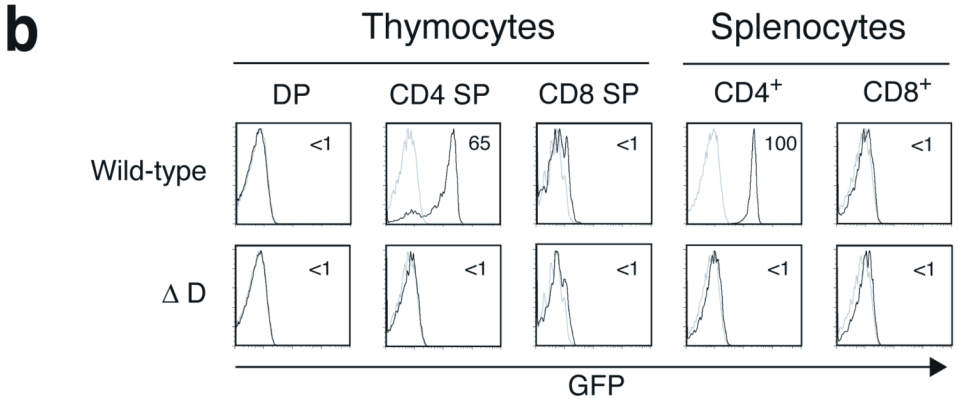


Figure 5. Zbtb7b requires Gata3 to promote CD4 but not to inhibit CD8 differentiation

(**a,b**). Thymocytes from mice expressing or lacking Gata3 and the Zbtb7b transgene as indicated (left) were analyzed by 3-color flow cytometry. (**a**) Numbers indicate the percentage of cells in boxes or within brackets. (**b**) Box and whiskers plots show absolute numbers of TCR^{hi} thymocytes within $CD4^{+}CD8^{-}$, $CD4^{+}CD8^{int}$ and $CD4^{-}CD8^{+}$ gates in each mouse strain. Data are representative of at least five mice of each genotype analyzed in more than five separate experiments. *, $P < 0.05$ by the exact two-tailed Wilcoxon rank sum test. We noted no detectable difference between *Gata3*$^{+/+}$ and *Gata3*$^{fl/fl}$ genotypes and they were used interchangeably and considered as a single class for statistical analyses.

(**c**). Spleen T cell populations were analyzed in one week-old mice of the indicated genotype by 3-color flow cytometry.





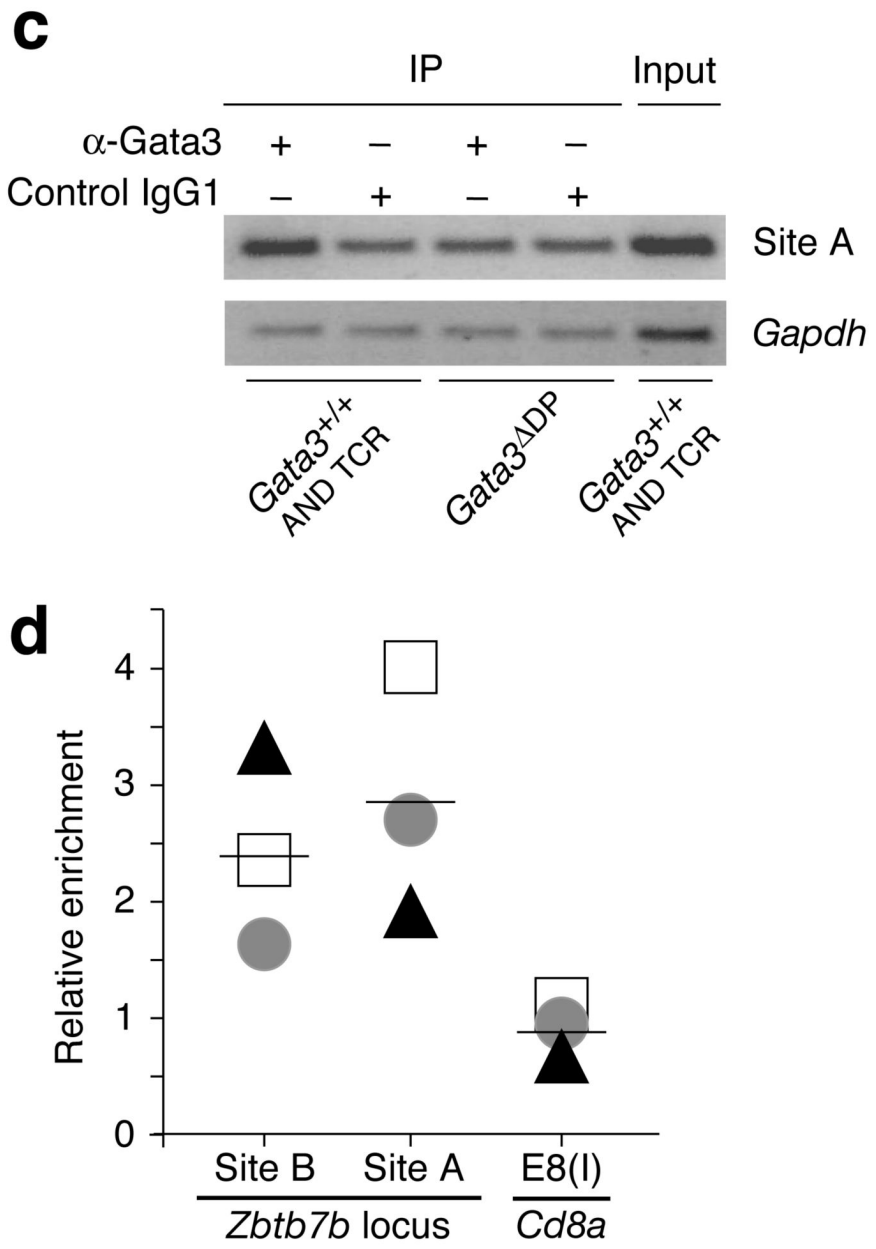


Figure 6. Gata3 is recruited to a region of the *Zbtb7b* locus critical for Zbtb7b expression

(**a**). Schematic of the *Zbtb7b* locus indicating regions of high sequence conservations in mammals (from the Mus musculus July 2007 assembly consulted on the UCSC Genome website, http://genome.ucsc.edu). The approximate position of two DNase I hypersensitivity (HS) sites are indicated by black horizontal bars. Wild-type and mutant BACs used in (**b**), in which the first coding exon of Zbtb7b was replaced by a GFP cDNA, are depicted; the modified *Zbtb7b* locus includes a translation stop codon at the end of the GFP ORF. The dashed lines indicate the deletion in construct ΔD. The location of sites A and B identified in (**c,d**) are indicated by vertical arrows.

(**b**). Analyses of Zbtb7b expression in the indicated cell populations using GFP BAC reporters depicted in (**a**). Numbers in histograms indicate the mean GFP fluorescence intensity, expressed relative to that in CD4 T cells from mice carrying the wild-type construct arbitrarily set to 100. Expression of the ΔD deleted construct remained below detection in all T cell subsets analyzed. GFP expression was detected in three out of seven founders injected with the wild-type construct, but none of 11 founders injected with the ΔD construct.
(**c,d**). ChIP analyses of Gata3 binding to the *Zbtb7b* locus. (**c**) Thymocytes from $Gata3^{+/+}$ AND TCR transgenic mice or from non TCR-transgenic $Gata3^{\Delta DP}$ mice were subjected to ChIP with a monoclonal antibody against Gata3 or an isotype-matched control antibody. Immunoprecipitates were analyzed by PCR using primers specific for Zbtb7b sites A and B, for the CD8α E8(I) enhancer, and for an irrelevant sequence from the *Gapdh* gene. The specificity of amplification was verified by agarose gel electrophoresis. (**d**) Amounts of immunoprecipitated DNA were quantified using Sybr Green qPCR and are expressed as fold enrichment relative to *Gapdh*, evaluated for each site *x* as $2^{-\left|\left(C^{x}_{Gata3}-C^{x}_{IgG1}\right)-\left(C^{Gapdh}_{Gata3}-C^{Gapdh}_{IgG1}\right)\right|}$, where C^{x}_{Gata3} and C^{x}_{IgG1} are the number of cycles for detection of target *x* in anti-Gata3 or control immunoprecipitates, respectively. The graph shows results from three distinct immunoprecipitations from $Gata3^{+/+}$ AND TCR transgenic thymocytes, each from a distinct chromatin preparation; each symbol refers to data acquired within a single experiment. Horizontal bars indicate mean values for each site.