HHS Public Access

Author manuscript

Eur J Immunol. Author manuscript; available in PMC 2019 January 04.

Published in final edited form as:

Eur J Immunol. 2013 April ; 43(4): 918–928. doi:10.1002/eji.201242944.

Thpok-independent repression of *Runx3* by Gata3 during CD4+ T-cell differentiation in the thymus

Yumei Xiong[1], **Ehydel Castro**[1,2], **Ryoji Yagi**[3], **Jinfang Zhu**[3], **Renaud Lesourne**[4], **Paul E. Love**[4], **Lionel Feigenbaum**[2], and **Rémy Bosselut**[1]

[1]Laboratory of Immune Cell Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA.

[2]NCI-SAIC, Frederick, Maryland, USA

[3]Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA.

[4]Section on Cellular and Developmental Biology, Program in Genomics of Differentiation, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, USA.

Summary

CD4+ helper T cells are essential for immune responses and differentiate in the thymus from CD4+CD8+ 'double-positive' (DP) thymocytes. The transcription factor Runx3 inhibits CD4+ T-cell differentiation by repressing *Cd4* gene expression; accordingly, Runx3 is not expressed in DP thymocytes or developing CD4+ T cells. The transcription factor Thpok is up-regulated in CD4-differentiating thymocytes and required to repress *Runx3*. However, how *Runx3* is controlled at early stages of CD4+ T-cell differentiation, before the onset of Thpok expression, remains unknown. Here we show that Gata3, a transcription factor preferentially and transiently up-regulated by CD4+ T-cell precursors, represses *Runx3* and binds the *Runx3* locus in vivo. Accordingly, we show that high-level Gata3 expression and expression of *Runx3* are mutually exclusive. Furthermore, whereas Runx3 represses *Cd4*, we show that Gata3 promotes *Cd4* expression in Thpok-deficient thymocytes. Thus, in addition to its previously documented role in promoting CD4-lineage gene-expression, Gata3 represses CD8-lineage gene expression. These findings identify Gata3 as a critical pivot of CD4-CD8 lineage differentiation.

Keywords

CD4-CD8 differentiation; Gata3; Runx3; T-cell development; transcription

Address for correspondence: Rémy Bosselut, Laboratory of Immune Cell Biology, NCI, NIH, Building 37, Room 3032, 37 Convent Drive, Bethesda, MD 20892-4259, USA, phone 301-402-4849, fax 301-402-4844, remy@helix.nih.gov.

The authors declare no financial or commercial conflict of interest.

1. Introduction

Helper T cells are essential for immune responses. They recognize peptide antigens bound to class II major histocompatibility complex (MHC-II) molecules and express the CD4 'coreceptor' [1]. These attributes distinguish helper T cells from cytotoxic T cells, which are MHC-I restricted and express the CD8 coreceptor. Helper and cytotoxic cells differentiate in the thymus from precursors that express both CD4 and CD8 (double positive, DP) [2, 3]. The divergence of these two lineages takes place in thymocytes that have undergone positive selection, i.e. that have been rescued from programmed cell death upon engagement of their T-cell antigen receptor (TCR) by MHC-peptide complexes expressed on the thymic stroma.

MHC II-signaled thymocytes must maintain CD4 expression during positive selection, because CD4 helps MHC-II recognition and signaling by the T-cell receptor (TCR) [4, 5]. Accordingly, the *Cd4* gene is tightly regulated, and transcriptional repression is critical for such regulation. Most notably, the transcription factors Runx1 and Runx3 limit *Cd4* expression to DP and MHC II-restricted cells [6, 7]. Runx1 represses *Cd4* in early thymocytes, before the DP stage. In contrast, Runx3 represses *Cd4* in CD8-differentiating cells, in which it is specifically expressed, and thereby contributes to CD8-lineage commitment [8, 9]. Runx3 is also important for expression of cytotoxic genes, a hallmark of the CD8 lineage [10, 11] and therefore control multiple aspects of CD8-lineage differentiation. Because ectopic *Runx3* expression represses *Cd4* and impairs CD4CD4$^+$ T cell differentiation [12, 13], the differentiation of CD4$^+$ T cells requires expression of *Runx3* to be limited to thymocytes undergoing MHC I-induced positive selection. How this is achieved remains poorly understood.

Two transcription factors, Ets1 and Stat5, have been proposed to promote *Runx3* expression [14, 15]. However, both are expressed throughout T-cell development, raising the question of how they could limit *Runx3* expression to MHC I-restricted thymocytes. Stat5 is activated in thymocytes in response to signaling by IL-7, and is therefore inactive in DP thymocytes which do not express the IL-7 receptor (IL-7R). However, IL-7R is expressed in both MHC-I and MHC II-selected thymocytes [16], and it is unclear how Stat5 could activate *Runx3* in the former but not the latter. Reciprocally, the transcription factor Thpok, specifically expressed in MHC II-restricted cells and required for CD4$^+$ T cell differentiation, represses *Runx3* [10, 17–20]. However, Thpok is not expressed in DP cells and is expressed at low levels in CD4$^+$CD8^{int} 'transitional' cells, the precursors of CD4$^+$-lineage thymocytes. Thus, the transcriptional control of *Runx3* expression in early CD4$^+$-lineage precursor cells remains unclear.

Here, we show that a Thpok-independent mechanism represses *Runx3* in MHC II-restricted thymocytes, and we present evidence that it involves the transcription factor Gata3, previously shown to promote CD4$^+$-lineage differentiation [21–23]. These studies identify a novel, repressive, function of Gata3 during CD4$^+$-lineage differentiation in the thymus.

2. Results

Thpok-independent Runx3 repression during CD4+ cell differentiation in the thymus

To study the kinetics of *Thpok* and *Runx3* up-regulation in the thymus, we set up an experimental system using a GFP-based BAC reporter for the gene expressing Thpok (*Zbtb7b*, thereafter called *Thpok*), and a tRFP-based reporter for *Runx3*. Both reporters, *Thpok*^{GFP} and *Runx3*^{tRFP} respectively, have been previously shown to appropriately track expression of the respective gene [10, 14]. In wild-type mice, DP thymocytes express neither *Thpok*^{GFP} nor *Runx3*^{tRFP} (Fig. 1A, top, column 1); in contrast, thymocytes that have undergone positive selection (CD4^{+}CD8^{-} and CD4^{-}CD8^{+} single positive (SP)) express *Thpok*^{GFP} if CD4 SP and *Runx3*^{tRFP} if CD8 SP (Fig. 1A, top, columns 3 and 5). Most CD4^{+}CD8^{int} 'transitional' thymocytes, which are either MHC-I or MHC II-restricted cells undergoing positive selection, failed to express either reporter (Fig. 1A, top, column 2), consistent with the fact that most of them are not yet committed to either lineage [4]. In contrast, the CD4^{int}CD8^{+} subset was enriched in CD8-committed *Runx3*-expressing cells (Fig. 1A, top, column 4).

While Thpok disruption impairs the development of mature CD4 SP cells, it does not prevent the positive selection of MHC II-restricted cells [17]. In *Thpok*$^{-/-}$ mice, MHC II-restricted DP thymocytes initially down-regulate CD8 expression and adopt a 'CD4 SP-like' surface phenotype (CD4^{+}CD8$^{int/lo}$) (Supporting Information Fig. 1A). Consistent with previous results [19], most *Thpok*$^{-/-}$ CD4 SP-like cells expressed *Thpok*^{GFP} (Fig. 1A, bottom, column 3), indicating that Thpok protein is not needed to initiate *Thpok* gene expression. However, unlike *Thpok*$^{+/+}$ CD4 SP thymocytes which differentiate into CD4^{+} T cells, *Thpok*$^{-/-}$ CD4 SP-like thymocytes terminate CD4 and reinitiate CD8 expression, thereby being 'redirected' to the CD8-lineage [24]. Their persistent GFP expression identifies such redirected cells in the CD8 SP compartment of *Thpok*$^{-/-}$ mice (Fig. 1A, bottom, columns 4 and 5).

Because Thpok was previously shown to repress *Runx3* [10, 19, 20], we predicted that *Thpok*$^{-/-}$ CD4 SP-like thymocytes, which make no Thpok protein, would express the *Runx3* reporter. Unexpectedly, while a few CD4 SP-like thymocytes expressed *Runx3*^{tRFP}, most of them failed to do so (Fig. 1A, bottom, column 3, shaded area). Similar observations were made in *Thpok*$^{-/-}$ mice carrying the MHC II-restricted AND TCR transgene (Fig. 1B, bottom left, shaded area), indicating that such a *Thpok*$^{GFP+}$ *Runx3*$^{tRFP-}$ population indeed resulted from MHC-II signaling.

It was possible that MHC II-signaled *Thpok*$^{-/-}$ cells failed to express *Runx3* because *Runx3* up-regulation is a late event in thymocyte maturation, requiring signals that these cells had not yet received. A non mutually exclusive possibility was that *Runx3* was repressed by Thpok-independent intrathymic signals. The latter but not the former hypothesis predicted that removing *Thpok*$^{-/-}$ CD4 SP-like thymocytes from their intrathymic environment would relieve such repression and therefore enhance, rather than reduce, *Runx3* expression. Experimental evidence supported this conclusion (Fig. 1C, bottom): whereas a substantial subset of *Thpok*$^{-/-}$CD4 SP-like thymocytes expressed little or no *Runx3* (tRFP) *ex vivo*, they all did after overnight single-cell suspension culture to disrupt thymocyte-stroma

interactions. Accordingly, mutant cells also down-regulated the Runx3 target CD4 (Fig. 1C). In contrast, Thpok-sufficient CD4 SP cells remained tRFP^{-} and CD4^{hi} in these same conditions. These findings supported the idea that intrathymic signals, not mediated by Thpok, restrained *Runx3* expression in MHC II-signaled thymocytes. We therefore decided to explore this possibility.

Gata3 represses Runx3

The Thpok-deficient cells that expressed *Thpok*^{GFP} but not *Runx3*^{tRFP} expressed CD69 (Fig. 1D, gray shaded), a molecule up-regulated by intrathymic TCR engagement [25]. This raised the possibility that intrathymic TCR signaling repressed *Runx3* in a Thpok-independent manner. The transcription factor Gata3 is up-regulated by TCR signaling in thymocytes [26, 27], whereas its expression is down-regulated when thymocytes are removed from their intrathymic environment (Supporting Information Fig. 1B). This pattern of expression was reciprocal to that of *Runx3*, prompting us to consider the possibility that high-level Gata3 expression would repress *Runx3*. Previous analyses have shown that Gata3 is required for CD4 but not CD8-lineage differentiation, and that in the absence of Gata3 MHC II-restricted thymocytes are 'redirected' into CD8 SP cells [21, 22]. These previous findings are consistent with the hypothesis that Gata3 represses *Runx3*. Indeed, this hypothesis predicts that, in the absence of Gata3, MHC II-restricted thymocytes would express *Runx3*, repress *Cd4* and be 'redirected' to a CD8-lineage fate. While it was not possible to directly evaluate the hypothesis by inactivating *Gata3* specifically in cells with high Gata3 expression (CD4^{+}CD8^{int} thymocytes, see below), we reasoned that ectopic *Gata3* expression should impair *Runx3* up-regulation. To assess this prediction, we used a *Gata3* transgene that expresses Gata3 protein at the 'high' physiological set point (the peak level during positive selection) in all thymocytes (Fig. 2A and S2A) [28]. At this level, the *Gata3* transgene had little or no effect on the differentiation of wild-type (Thpok-sufficient) MHC II-restricted thymocytes (Supporting Information Fig. 2B).

To evaluate Gata3 repression of *Runx3*, we examined how the *Gata3* transgene affected *Runx3*^{tRFP} expression in *Thpok*$^{-/-}$ thymocytes. Because the predicted effects on *Runx3* might affect *Cd4* and *Cd8*, it was essential not to restrict these analyses to particular subsets defined by CD4 and CD8 expression, but rather to examine the whole population of TCR^{hi} post-selection *Thpok*$^{-/-}$ cells. In that subset, the *Gata3* transgene substantially impaired *Runx3* up-regulation, although it did not prevent it (Fig. 2B). Most TCR^{hi} *Gata3*-transgenic cells failed to express tRFP, whereas 60% of their non-transgenic counterparts did so; furthermore, the *Gata3* transgene reduced tRFP fluorescence intensity in reporter-expressing cells by almost 60% (Fig. 2B, bottom). Both effects were most pronounced in CD69^{hi} cells (Fig. 2B, C), suggesting that *Runx3* repression by Gata3, whether transgenic or endogenous, was specific of TCR-signaled cells. We conclude from these experiments that Gata3 represses *Runx3* in *Thpok*$^{-/-}$ thymocytes.

In *Thpok*$^{-/-}$ mice, both MHC II- and MHC I-restricted thymocytes express *Runx3*. To determine if the *Gata3* transgene repressed *Runx3* in MHC I-restricted cells, we examined its effects on *Runx3*^{tRFP} in *Thpok*$^{+/+}$ mice, in which only MHC I-restricted cells express *Runx3*. We found that the *Gata3* transgene had little if any effect on *Runx3*^{tRFP} expression in

these cells (Supporting Information Fig. 2C): both the frequency of RFP-expressing cells and RFP fluorescence intensity were similar in transgenic and control cells. We had similar results when analyzing *Runx3*^{tRFP} expression on gated CD8 SP thymocytes (data not shown). These analyses support the conclusion that *Runx3* repression by Gata3 is specific of MHC II-signaled thymocytes. Of note, this indicates that the effects shown in *Thpok*$^{-/-}$ cells (Fig. 2B, C), which are a mix of MHC-I and MHC II-specific cells, probably underestimate the actual Gata3-mediated *Runx3* repression in the latter.

We next examined whether Gata3-mediated *Runx3* repression had physiological consequences. Because Runx3 represses *Cd4*, we predicted that repression of *Runx3* would result in sustained CD4 expression by *Gata3*-transgenic cells. We evaluated this prediction in the TCR^{hi} CD69^{+} subset from *Thpok*$^{-/-}$ mice, in which Gata3-mediated *Runx3* repression was the most prominent. Indeed, the fraction of CD4^{lo} cells in this subset was reduced in *Thpok*$^{-/-}$ *Gata3* transgenic mice compared with that in their non-transgenic counterparts (Fig. 2D), demonstrating that Gata3-mediated *Runx3* repression improved CD4 expression. We also noted that the *Gata3* transgene impaired CD8 expression in CD8 SP cells (Figs. 3 and S2D). However, this effect is unlikely to be *Runx3*-dependent, because it was observed in MHC I-restricted cells, in which Gata3 had little or no effect on *Runx3* (Supporting Information Fig. 2C), as well as in DP thymocytes, which do not express *Runx3* [29]. Accordingly, previous studies had shown that *Runx3* has little effect on CD8 expression in the thymus, unlike in effector T cells [29, 30].

High-level Gata3 and Runx3 expression are mutually exclusive

If the conclusion that Gata3 represses *Runx3* is correct, high-level Gata3 and Runx3 expression should be mutually exclusive. To evaluate this prediction, we used intra-cellular staining to assess Gata3 expression. We preferred this procedure to GFP reporter mice [27] because GFP, due to its long half-life [31], does not accurately reflect transient changes in Gata3 protein expression. We compared Gata3 expression in MHC-II and MHC-I restricted thymocytes obtained from *B2m*$^{-/-}$ and I-A$^{b-/-}$ mice respectively. Consistent with previous studies [26, 32], Gata3 expression was highest in CD69^{+} CD4^{+}CD8^{int} MHC II-restricted cells (Fig. 4A, compare top and bottom rows). In *Thpok*$^{+/+}$ mice these cells do not express *Runx3* (Fig. 1A), consistent with the idea of Gata3 repression of *Runx3*. In *Thpok*$^{-/-}$ mice however, both CD4^{+}CD8^{int} and CD4 SP-like subsets contained *Runx3*$^{tRFP+}$ and Gata3^{hi} cells (compare Figs. 1A and 4B respectively). Unfortunately, we could not use the same staining procedure to directly compare Gata3 and *Runx3*^{tRFP} expression at the single-cell level, because the cell fixation required to measure intra-cellular Gata3 abolishes RFP fluorescence.

To overcome this obstacle, we used the MHC-I molecule H-2K^{b} as a surrogate for *Runx3* expression. This marker is normally up-regulated in immature post-selection cells (TCR^{hi} CD69^{+}) and remains expressed in more mature cells (Supporting Information Fig. 3), and its expression is not affected by the *Gata3* transgene (data not shown). In wild-type CD4 SP thymocytes, which do not express *Runx3*, H-2K^{b} and *Runx3* expression are not correlated (Fig. 4C, middle column, top). In contrast, expression of H-2K^{b} parallels that of *Runx3* in wild-type CD8-lineage cells (Fig. 4C, top right). Importantly, there was an excellent

correlation between H-2K^{b} and *Runx3*^{tRFP} expression in all subsets of *Thpok*$^{-/-}$ thymocytes, CD4^{+}CD8^{int}, CD4 SP and CD8 SP (Fig. 4C, bottom), and among all post-selection (TCR^{hi}) cells (Fig. 4D). Thus, H-2K^{b} can be used as a surrogate for *Runx3* expression in cells that do not express Thpok, i.e. wild-type CD8-lineage cells and all post-selection cells in *Thpok*$^{-/-}$ mice.

Consequently, we plotted H-2K^{b} vs. intra-cellular Gata3 expression in thymocytes (Fig. 4E). In *Thpok*$^{-/-}$ thymocytes, there was no substantial population in the top right quadrant of each plot, where would be cells with high Gata3 and H-2K^{b} expression. Thus, Gata3 protein levels are low in H-2K^{b}-expressing cells, from which we conclude that Gata3^{hi} cells do not express *Runx3,* consistent with our finding that Gata3 represses *Runx3*. In summary, these studies document that Gata3 expression peaks in CD69^{hi} MHC II-restricted thymocytes that are H-2K^{b-}, whereas *Runx3* is expressed in H-2K^{b+} cells.

Gata3 delays the CD8-lineage redirection of Thpok-deficient cells

Previous studies had shown that Gata3 is needed for CD4 T cell development [21, 26], and notably for *Thpok* expression [22, 33]. The repression of *Runx3* by Gata3, together with previous findings that Gata3 antagonizes Runx3 protein function in mature T cells [34], raised the possibility that unrestrained *Runx3* expression was responsible for the impaired CD4-differentiation of *Gata3*-deficient thymocytes. If that were the case, disruption of *Runx3* should restore the CD4-differentiation in *Gata3*-deficient mice. To evaluate this possibility, we generated mice conditionally deleting both *Gata3* and *Runx3* in DP cells. As expected because of *Runx3* inactivation, a large fraction of double-deficient CD8-lineage thymocytes failed to terminate CD4 expression (Fig. 5A, right), resulting in the appearance of CD4^{+}CD8^{+} post-selection thymocytes. This indicated that *Cd4* silencing in *Gata3*-deficient thymocytes remained largely Runx3-dependent. However, we found no restoration of CD4 SP cell development (Fig. 5A), indicating that Gata3 is required for CD4^{+} T-cell development independently of its effect on Runx3.

Conversely, it was possible that, by repressing *Runx3* and CD8, sustained *Gata3* expression would rescue in part the CD4-developmental block of Thpok-deficient thymocytes and prevent their redirection to the CD8 lineage. To evaluate this possibility, we examined the most mature thymocytes, which have both up-regulated TCR and down-regulated the maturation marker CD24 (Heat stable antigen, HSA). Whereas these cells normally are either CD4 or CD8 SP, the CD4 SP component is nearly absent in *Thpok*$^{-/-}$ mice, because the CD8-redirection of MHC II-restricted cells occurs before their terminal maturation (Fig. 5B) [10, 17, 19, 20]. The *Gata3* transgene greatly increased the number of TCR^{hi} CD24^{lo} thymocytes with a CD4 SP-like phenotype (Fig. 5B). However, despite their large numbers (half that of mature CD4 SP cells in Thpok-sufficient mice, Fig. 5C), these CD4 SP-like cells did not terminate CD8 expression and there was no reconstitution of the peripheral CD4^{+} cell population (data not shown). Thus, as expected from its repression of *Runx3*, enforced Gata3 expression delays the CD8-lineage redirection of *Thpok*$^{-/-}$ thymocytes; however, it does not restore their differentiation into CD4^{+} T cells, presumably because its repression of *Runx3* is transient only.

Gata3 molecules bind the Runx3 locus

Genome-wide analyses of Gata3 DNA binding have identified a Gata3 site within the *Runx3* locus, occupied in $CD4^{-}CD8^{-}$ double negative (DN) thymocytes (Fig. 6A) [35, 36]. While binding to this site was not detected in DP or naïve $CD4^{+}$ T cells, this could be because Gata3 levels in these cells are lower than in DN or MHC II-signaled thymocytes. Consequently, we performed Chromatin Immunoprecipitation (ChIP) from AND TCR transgenic thymocytes, most of which are MHC II-signaled. We detected the target *Runx3* sequence in Gata3 immunoprecipitates from AND thymocytes (Fig. 6B, top row, lanes 7–9), but not from Gata3-deficient cells (lanes 1–3), or in immunoprecipitates prepared with an isotype control antibody (lane 8). Of note, Gata3 also bound a site downstream of the *Cd8b* gene, consistent with the reduced expression of CD8 in *Gata3*-transgenic cells (Fig. 6A and B, middle row). Similar results were obtained from *Gata3* transgenic thymocytes (Fig. 6B, lanes 4–6). These findings support the idea that Gata3 binding to *Runx3* and *Cd8* contribute to its repressive effect.

In summary, our study provides evidence for a novel function of Gata3 during the differentiation of CD4 lineage T cells in the thymus, namely transiently repressing *Runx3* expression, that comes in addition to the previously reported functions of this factor in promoting CD4-lineage differentiation (schematized in Fig. 6C).

3. Discussion

Our study identifies a novel function of Gata3, whereby this factor represses *Runx3* before *Thpok* up-regulation in MHC II-signaled thymocytes. It was previously proposed that MHC II-induced TCR signals repress *Runx3* in cells that do not yet express *Thpok* [4], and our findings identify Gata3 as an important contributor to this effect.

We have previously shown that Gata3 is required in MHC II-restricted thymocytes for CD4-lineage specification, including for the expression of Thpok [22]. The current study points to a distinct function of Gata3. While it participates in the logic of CD4-lineage differentiation, it involves repressing a CD8-lineage specific gene, *Runx3*. Runx3 activity is critical to CD8-lineage differentiation [7, 37]. Runx3 promotes both the cessation of *Cd4* and *Thpok* expression (therefore ensuring CD8-lineage commitment) and the initiation of CD8-lineage gene expression, even though such functions are in part masked by the redundancy between Runx1 and Runx3. Thus, by repressing *Runx3*, Gata3 controls a key factor in CD8-lineage differentiation.

Unlike *Runx3* repression by Thpok, which persists in mature thymocytes and T cells and is a key component of CD4-lineage commitment [10, 20, 29], the repression of *Runx3* by Gata3 is transient and therefore does not induce CD4-lineage commitment. From a developmental perspective, Gata3 repression of *Runx3* would serve to preserve the CD4-CD8 ‘bi-potency’ of thymocytes while they undergo TCR signaling; this would notably be effected by ensuring the sustained *Cd4* expression required for proper $CD4^{+}$ cell development [5]. Bi-potency is resolved if *Thpok* is up-regulated, resulting in CD4-lineage commitment, or if TCR signaling ceases without *Thpok* up-regulation, resulting in *Runx3* up-regulation and CD8-lineage commitment.

The repression of *Runx3* by Gata3 was specific of MHC II-signaled cells in the thymus. Similarly, we did not observe any specific *Runx3* repression in peripheral CD8 cells of *Gata3* transgenic mice (Y.X. and R.B., unpublished observation). What accounts for this specificity remains to be determined. Because Gata3 functions are exquisitely dependent on its expression level [38], it is possible that expression of the *Gata3* transgene is insufficient to bring Gata3 levels in MHC I-restricted cells to the peak seen in MHC II-specific transitional cells (see Fig. 2A). A non-mutually exclusive possibility is that *Runx3* repression requires stage-specific post-translational modifications of Gata3, or its cooperation with other factors, such as IRF4 [39]. Indeed, genome-wide studies of Gata3 binding suggest that the outcomes of Gata3 recruitment to DNA are context-dependent [35]. Of note, our observations do not exclude that high-level Gata3 expression represses *Runx3* in other contexts. Previous studies have pointed to such a possibility during the differentiation of mature $CD4^+$ T cells into Th2 effectors [34].

Regardless of its mechanism, the fact that Gata3-mediated repression of *Runx3* is specific of MHC II-signaled cells has two separate and important correlates. First, Gata3 repression of *Runx3* is not caused by Gata3 repression of *Cd8*. Indeed, MHC II-restricted cells, in which Gata3 represses *Runx3*, do not need CD8 molecules to signal. Second, it explains the apparent paradox that the *Gata3* transgene did not inhibit the development of wild-type CD8-lineage cells, which are MHC I-restricted and in which Gata3 does repress *Runx3*.

Our findings suggest a possible mechanism underpinning the repression of *Runx3* by Gata3, by showing direct binding of Gata3 molecules to the *Runx3* locus. However, Gata3 recruitment to *Runx3* does not imply a direct regulatory function, and determining the role of such binding in *Runx3* repression will require the identification of the cis-regulatory elements that control *Runx3*, which are not yet known. In addition, given the pleiotropic effects of Gata3 during positive selection, it is possible that additional mechanisms are involved in Gata3-mediated *Runx3* repression. In particular, Gata3 has been proposed to activate expression of the transcription factor E2A [40], an E-box binding protein that serves as a ‘gate-keeper’ of positive selection redundantly with the related factor HEB [41, 42]. Inactivation of both E2A and HEB promotes the differentiation of CD8-lineage cells, and there is evidence that these factors repress *Runx3*, directly or indirectly [41, 42]. Thus, Gata3 could repress *Runx3* through E2A.

We also noted that the Gata3 transgene repressed IL-7Rα expression (data not shown), an effect more pronounced in CD8 than CD4 SP cells. While it had been proposed that IL-7 promoted *Runx3* expression in CD8-differentiating thymocytes, a recent report has shown IL-7, and γ_c-cytokines in general, to be dispensable for *Runx3* up-regulation in these cells [43]. Thus, the effect of Gata3 on IL-7Rα expression does not account for repression of *Runx3* by Gata3.

The inhibition of CD8 expression by the *Gata3* transgene mirrored the slightly increased CD8 expression of *Gata3*-deficient thymocytes [22, and Y.X. and R.B., unpublished observations]. In contrast, the conclusion on *Runx3* repression relies on transgenic Gata3 expression only. As with all gain-of-function approaches, this raises the question of whether it also applies to endogenous Gata3. In support of our conclusion, the CD8 redirection’ of

Gata3-deficient MHC II-restricted thymocytes [22] is fully consistent with the idea that Gata3 represses *Runx3*. That is, our current results predict that Gata3 inactivation, by promoting expression of *Runx3*, would redirect MHC II-restricted cells into the CD8 lineage, which is indeed the case [22]. The fact that *Runx3* disruption fails to restore the CD4-lineage differentiation of *Gata3*-deficient thymocytes supports the concept that Gata3 is required for the specification of the CD4 lineage, in addition to its effects on *Runx3*.

This novel repressive role of Gata3 adds to the panoply of functions this factor performs during CD4-lineage differentiation. We previously reported that Gata3 is required for Thpok expression both in conventional MHC II-restricted thymocytes and in iNK T cells [22, 33]. Gata3 promotes other aspects of CD4-lineage differentiation, as enforced Thpok expression does not rescue the CD4-differentiation of Gata3-deficient cells [22]. What these functions include remains to be determined. Gata3 binds to genes encoding the CD3 subunits of TCR complexes or TCR signaling intermediates [35, 40], and may therefore be required for efficient TCR signal transduction. This could explain a stronger requirement for Gata3 in CD4- than CD8-lineage differentiation, as sustained TCR signaling is required for the former but not for the latter [4]. It is also possible that Gata3 promotes the expression of other CD4-lineage specific transcription factors, whose identity would have remained elusive. The recent observation that *Gata3* expression is targeted by the Ras-Erk and calcium-calcineurin signaling pathways [27], both downstream of the TCR, further emphasizes its function as a critical pivot in the choice between CD4 and CD8 lineages.

4. Materials and Methods

Mice

Thpok$^{-/-}$, Gata3$^{f/f}$, *Thpok*^{GFP} and *Runx3*^{tRFP} mice were previously described [14, 22, 44]. *B2m*$^{-/-}$ and I-A$^{b-/-}$ mice and mice carrying the AND or P14 TCR transgenes were obtained from Jax. *Gata3* transgenic mice were generated in our laboratory [28]. Briefly, a *Gata3* cDNA was inserted into the transgenic expression vector p29Δ2, driven by the promoter of human *CD2* gene [45]; the resulting DNA was microinjected to generate *Gata3* transgenic mice using previously described procedures [46]. Transgenic founders were identified by Southern blotting; transgenic animals were subsequently identified by PCR from tail DNA using the following primers: 5’ CTC GAC TTA CAT CCG AAC CCG GTA 3’ and 5’ CGC TCT TGC TCT CTG TGT ATG 3’. Animal procedures used in this study were approved by the National Cancer Institute Animal Care and Use Committee.

Antibodies

Flow cytometry antibodies against CD4 (RM4.5), CD8 (53–6.7), TCRβ (H57–597), CD24 (M1/69), CD44 (IM7), CD69 (H1.2F3), H-2K^{b} (AF6–120.1) and anti-Gata3 (L50–823) were from BD Biosciences or eBioscience.

Cell preparation and staining

Thymocytes and spleen cells were prepared and stained as described [22], and analyzed by flow cytometry on LSRII or LSR Fortessa cytometers (BD Biosciences). Dead cells were excluded by forward light-scatter gating and DAPI staining. Data was analyzed with Flowjo

software. Gata3 intracellular staining was performed on cells fixed and permeabilized (using eBioscience kit 00–5523-00) after surface staining; dead cells were excluded using the live/dead fixable staining (Invitrogen L-23105).

Thymocytes were sorted as described [22] and placed (5×10^6/ml) in RPMI 1640 medium, supplemented with 10% FCS, Glutamine and antibiotics. Cells were analyzed by immunofluorescence and flow cytometry after 18 hours at 37°C.

Gata3 chromatin precipitation (ChIP)

Anti-Gata3 ChIP was performed using the EZChIP kit (Millipore 17–371) as described [22], with modifications. Briefly, thymocytes from Gata3$^{f/f}$ *Cd4*-Cre mice, Gata3 transgenic mice and AND TCR transgenic mice were fixed with 1% formaldehyde and lysed in 1% SDS lysis buffer. The crosslinked DNA was sonicated to 200–500bp (Ultrasonic processor XL, Misonix Inc), and then diluted with the kit 'dilution buffer' to a concentration of 10^7 cell-equivalents per ml. 20 μl of sonicated chromatin (~2×10^5 cells) was set aside as input. For each ChIP reaction, 3 ml chromatin (~3×10^7 cells) were incubated overnight with protein G Dynalbeads (Invitrogen) coupled antibodies (5μg): anti-Gata3 (BD Bioscience 558686) or control mouse IgG. The immunoprecipitated DNA was retrieved and washed according to the manufacturer's recommendations. Input and ChIP DNA were then subject in parallel to cross-linking reversal, phenol/chloroform extraction and ethanol precipitation. 1% of ChIP DNA (~3×10^5 cells) or input DNA (~2×10^3 cells) was used for conventional PCR and analyzed on 1.5% agarose gel. The primer pairs for *Cd8, Runx3* and *Gapdh* for ChIP-PCR were as follows: *Cd8*: 5'CAACTTCCACTGGTTGGATTTACG3'; 5' TTGATGCCCCGCTTTTGAAG3';

Runx3: 5' CTCCAGGCAGGCAGGATCTG 3'; 5' GGTCTGGGTAGCTGAGCCCTG 3'

Gapdh: 5' GAGGACAATAAGGCTCAAGG 3'; 5' CTCTCGGCTGGGTGGAGTG-3'.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank E. Rothenberg for insightful discussions and for sharing unpublished results, B. Taylor and S. Banerjee for cell sorting, T.-A. Lewis for mouse technical support, and Andrea C. Carpenter, B.J. Fowlkes and Melanie Vacchio for reading the manuscript.. Supported by the Intramural Research Programs of the National Cancer Institute, Center for Cancer Research, and of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Abbreviations:

DP:	double positive
SP:	single positive

References

1. Paul WE, (ed). Fundamental Immunology, 6th Edition. Lippincott Williams & Wilkins, Philadelphia 2008.
2. Bosselut R, CD4/CD8-lineage differentiation in the thymus: from nuclear effectors to membrane signals. Nat Rev Immunol 2004 4:529–540. [PubMed: 15229472]
3. Carpenter AC and Bosselut R, Decision checkpoints in the thymus. Nat Immunol 2010 11:666–673. [PubMed: 20644572]
4. Singer A, Adoro S and Park JH, Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. Nat Rev Immunol 2008 8:788–801. [PubMed: 18802443]
5. Adoro S, McCaughtry T, Erman B, Alag A, Van Laethem F, Park JH, Tai X et al., Coreceptor gene imprinting governs thymocyte lineage fate. EMBO J 2011 31:366–377. [PubMed: 22036949]
6. Taniuchi I, Ellmeier W and Littman DR, The CD4/CD8 lineage choice: new insights into epigenetic regulation during T cell development. Adv Immunol 2004 83:55–89. [PubMed: 15135628]
7. Collins A, Littman DR and Taniuchi I, RUNX proteins in transcription factor networks that regulate T-cell lineage choice. Nat Rev Immunol 2009 9:106–115. [PubMed: 19165227]
8. Taniuchi I, Osato M, Egawa T, Sunshine MJ, Bae SC, Komori T, Ito Y et al., Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. Cell 2002 111:621–633. [PubMed: 12464175]
9. Woolf E, Xiao C, Fainaru O, Lotem J, Rosen D, Negreanu V, Bernstein Y et al., Runx3 and Runx1 are required for CD8 T cell development during thymopoiesis. Proc Natl Acad Sci U S A 2003 100:7731–7736. [PubMed: 12796513]
10. Wang L, Wildt KF, Castro E, Xiong Y, Feigenbaum L, Tessarollo L and Bosselut R, The zinc finger transcription factor Zbtb7b represses CD8-lineage gene expression in peripheral CD4+ T cells. Immunity 2008 29:876–887. [PubMed: 19062319]
11. Cruz-Guilloty F, Pipkin ME, Djuretic IM, Levanon D, Lotem J, Lichtenheld MG, Groner Y et al., Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs. J Exp Med 2009 206:51–59. [PubMed: 19139168]
12. Grueter B, Petter M, Egawa T, Laule-Kilian K, Aldrian CJ, Wuerch A, Ludwig Y et al., Runx3 Regulates Integrin {alpha}E/CD103 and CD4 Expression during Development of CD4-/CD8+ T Cells. J Immunol 2005 175:1694–1705. [PubMed: 16034110]
13. Kohu K, Sato T, Ohno S, Hayashi K, Uchino R, Abe N, Nakazato M et al., Overexpression of the Runx3 transcription factor increases the proportion of mature thymocytes of the CD8 single-positive lineage. J Immunol 2005 174:2627–2636. [PubMed: 15728469]
14. Zamisch M, Tian L, Grenningloh R, Xiong Y, Wildt KF, Ehlers M, Ho IC et al., The transcription factor Ets1 is important for CD4 repression and Runx3 up-regulation during CD8 T cell differentiation in the thymus. J Exp Med 2009 206:2685–2699. [PubMed: 19917777]
15. Park JH, Adoro S, Guinter T, Erman B, Alag AS, Catalfamo M, Kimura MY et al., Signaling by intrathymic cytokines, not T cell antigen receptors, specifies CD8 lineage choice and promotes the differentiation of cytotoxic-lineage T cells. Nat Immunol 2010 11:257–264. [PubMed: 20118929]
16. Sudo T, Nishikawa S, Ohno N, Akiyama N, Tamakoshi M, Yoshida H and Nishikawa S, Expression and function of the interleukin 7 receptor in murine lymphocytes. Proc Natl Acad Sci U S A 1993 90:9125–9129. [PubMed: 8415665]
17. He X, He X, Dave VP, Zhang Y, Hua X, Nicolas E, Xu W et al., The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. Nature 2005 433:826–833. [PubMed: 15729333]
18. Sun G, Liu X, Mercado P, Jenkinson SR, Kypriotou M, Feigenbaum L, Galera P et al., The zinc finger protein cKrox directs CD4 lineage differentiation during intrathymic T cell positive selection. Nat Immunol 2005 6:373–381. [PubMed: 15750595]
19. Egawa T and Littman DR, ThPOK acts late in specification of the helper T cell lineage and suppresses Runx-mediated commitment to the cytotoxic T cell lineage. Nat Immunol 2008 9:1131–1139. [PubMed: 18776905]

20. Muroi S, Naoe Y, Miyamoto C, Akiyama K, Ikawa T, Masuda K, Kawamoto H et al., Cascading suppression of transcriptional silencers by ThPOK seals helper T cell fate. Nat Immunol 2008 9:1113–1121. [PubMed: 18776907]
21. Pai SY, Truitt ML, Ting CN, Leiden JM, Glimcher LH and Ho IC, Critical roles for transcription factor GATA-3 in thymocyte development. Immunity 2003 19:863–875. [PubMed: 14670303]
22. Wang L, Wildt KF, Zhu J, Zhang X, Feigenbaum L, Tessarollo L, Paul WE et al., Distinct functions for the transcription factors GATA-3 and ThPOK during intrathymic differentiation of CD4(+) T cells. Nat Immunol 2008 9:1122–1130. [PubMed: 18776904]
23. Ho IC, Tai TS and Pai SY, GATA3 and the T-cell lineage: essential functions before and after T-helper-2-cell differentiation. Nat Rev Immunol 2009 9:125–135. [PubMed: 19151747]
24. He X, Park K, Wang H, He X, Zhang Y, Hua X, Li Y et al., CD4-CD8 lineage commitment is regulated by a silencer element at the ThPOK transcription-factor locus. Immunity 2008 28:346–358. [PubMed: 18342007]
25. Swat W, Dessing M, von BH and Kisielow P, CD69 expression during selection and maturation of CD4+8+ thymocytes. Eur J Immunol 1993 23:739–746. [PubMed: 8095460]
26. Hernandez-Hoyos G, Anderson MK, Wang C, Rothenberg EV and Alberola-Ila J, GATA-3 expression is controlled by TCR signals and regulates CD4/CD8 differentiation. Immunity 2003 19:83–94. [PubMed: 12871641]
27. Gimferrer I, Hu T, Simmons A, Wang C, Souabni A, Busslinger M, Bender TP et al., Regulation of GATA-3 Expression during CD4 Lineage Differentiation. J Immunol 2011 186:3892–3898. [PubMed: 21357543]
28. Lesourne R, Uehara S, Lee J, Song KD, Li L, Pinkhasov J, Zhang Y et al., Themis, a T cell-specific protein important for late thymocyte development. Nat Immunol 2009 10:840–847. [PubMed: 19597498]
29. Egawa T, Tillman RE, Naoe Y, Taniuchi I and Littman DR, The role of the Runx transcription factors in thymocyte differentiation and in homeostasis of naive T cells. J Exp Med 2007 204:1945–1957. [PubMed: 17646406]
30. Hassan H, Sakaguchi S, Tenno M, Kopf A, Boucheron N, Carpenter AC, Egawa T et al., Cd8 enhancer E8I and Runx factors regulate CD8alpha expression in activated CD8+ T cells. Proc Natl Acad Sci U S A 2011 108:18330–18335. [PubMed: 22025728]
31. McCaughtry TM, Wilken MS and Hogquist KA, Thymic emigration revisited. J Exp Med 2007 204:2513–2520. [PubMed: 17908937]
32. Hendriks RW, Nawijn MC, Engel JD, van DH, Grosveld F and Karis A, Expression of the transcription factor GATA-3 is required for the development of the earliest T cell progenitors and correlates with stages of cellular proliferation in the thymus. Eur J Immunol 1999 29:1912–1918. [PubMed: 10382753]
33. Wang L, Carr T, Xiong Y, Wildt KF, Zhu J, Feigenbaum L, Bendelac A et al., The sequential activity of Gata3 and Thpok is required for the differentiation of CD1d-restricted CD4(+) NKT cells. Eur J Immunol 2010 40:2385–2390. [PubMed: 20706986]
34. Yagi R, Junttila IS, Wei G, Urban JFJ, Zhao K, Paul WE and Zhu J, The transcription factor GATA3 actively represses RUNX3 protein-regulated production of interferon-gamma. Immunity 2010 32:507–517. [PubMed: 20399120]
35. Wei G, Abraham BJ, Yagi R, Jothi R, Cui K, Sharma S, Narlikar L et al., Genome-wide analyses of transcription factor GATA3-mediated gene regulation in distinct T cell types. Immunity 2011 35:299–311. [PubMed: 21867929]
36. Zhang JA, Mortazavi A, Williams BA, Wold BJ and Rothenberg EV, Dynamic Transformations of Genome-wide Epigenetic Marking and Transcriptional Control Establish T Cell Identity. Cell 2012 149:467–482. [PubMed: 22500808]
37. Wang L and Bosselut R, CD4-CD8 lineage differentiation: Thpok-ing into the nucleus. J Immunol 2009 183:2903–2910. [PubMed: 19696430]
38. Taghon T, Yui MA and Rothenberg EV, Mast cell lineage diversion of T lineage precursors by the essential T cell transcription factor GATA-3. Nat Immunol 2007 8:845–855. [PubMed: 17603486]

39. Cao Y, Li H, Sun Y, Chen X, Liu H, Gao X and Liu X, Interferon regulatory factor 4 regulates thymocyte differentiation by repressing Runx3 expression. Eur J Immunol 2010 40:3198–3209. [PubMed: 21061442]

40. Ling KW, van Hamburg JP, de Bruijn MJ, Kurek D, Dingjan GM and Hendriks RW, GATA3 controls the expression of CD5 and the T cell receptor during CD4 T cell lineage development. Eur J Immunol 2007 37:1043–1052. [PubMed: 17357106]

41. Jones ME and Zhuang Y, Acquisition of a functional T cell receptor during T lymphocyte development is enforced by HEB and E2A transcription factors. Immunity 2007 27:860–870. [PubMed: 18093538]

42. Jones-Mason ME, Zhao X, Kappes D, Lasorella A, Iavarone A and Zhuang Y, E Protein Transcription Factors Are Required for the Development of CD4(+) Lineage T Cells. Immunity 2012 36:348–361. [PubMed: 22425249]

43. McCaughtry TM, Etzensperger R, Alag A, Tai X, Kurtulus S, Park JH, Grinberg A et al., Conditional deletion of cytokine receptor chains reveals that IL-7 and IL-15 specify CD8 cytotoxic lineage fate in the thymus. J Exp Med 2012 209:2263–2276. [PubMed: 23109710]

44. Zhu J, Min B, Hu-Li J, Watson CJ, Grinberg A, Wang Q, Killeen N et al., Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. Nat Immunol 2004 5:1157–1165. [PubMed: 15475959]

45. Shores EW, Huang K, Tran T, Lee E, Grinberg A and Love PE, Role of TCR zeta chain in T cell development and selection. Science 1994 266:1047–1050. [PubMed: 7526464]

46. Liu X, Adams A, Wildt KF, Aronow B, Feigenbaum L and Bosselut R, Restricting Zap70 expression to CD4+CD8+ thymocytes reveals a T cell receptor-dependent proofreading mechanism controlling the completion of positive selection. J Exp Med 2003 197:363–373. [PubMed: 12566420]

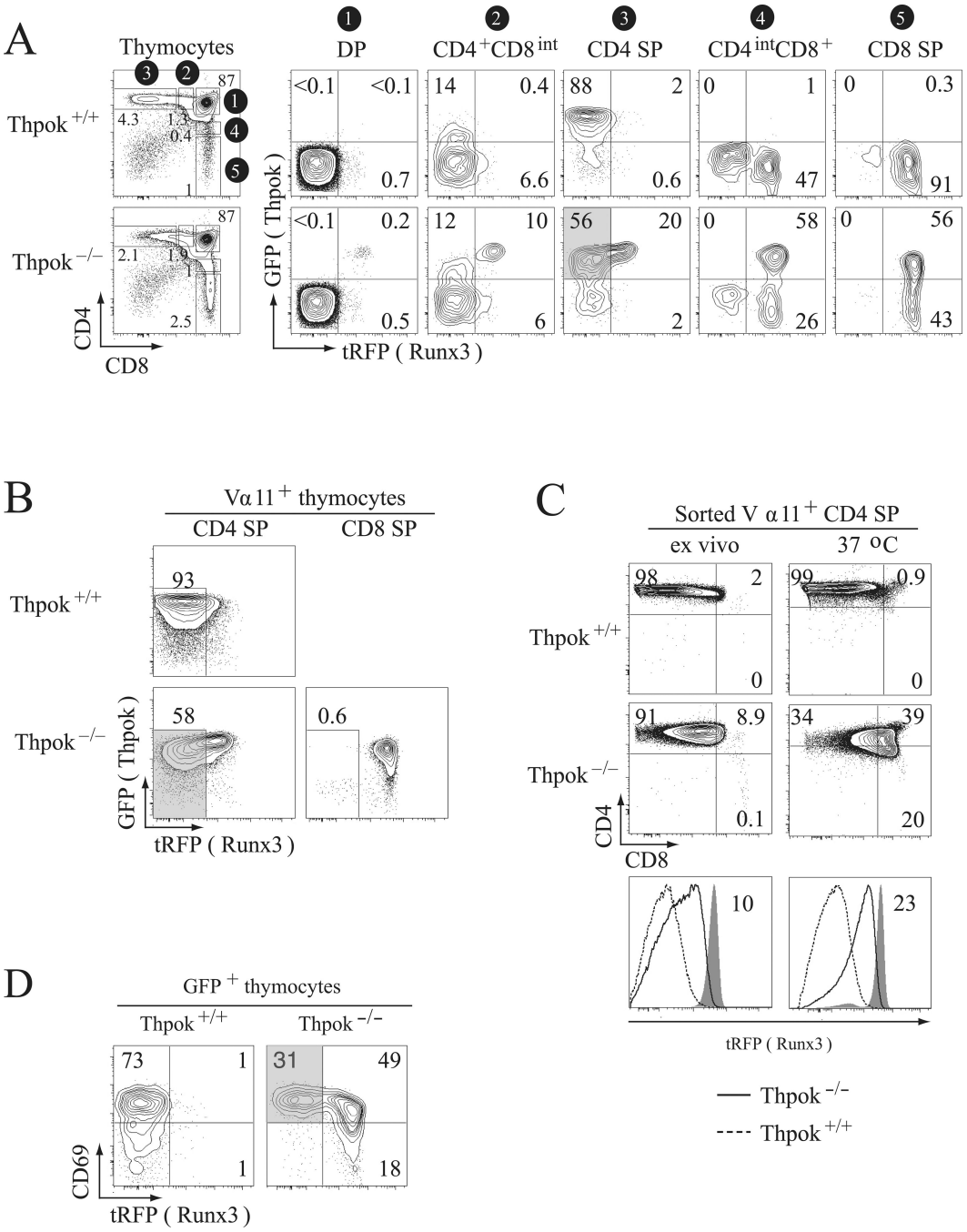

Figure 1. *Runx3* repression during CD4-lineage differentiation.

(**A**) Contour plots show expression of *Thpok*^{GFP} and *Runx3*^{tRFP} reporters in the indicated thymocyte subsets from *Thpok*$^{+/+}$ and *Thpok*$^{-/-}$ mice (gating on the left, gate numbers shown on a black background). Note the expression of *Thpok*^{GFP} in the CD4^{int}CD8^{+} and CD8 SP subsets in *Thpok*$^{-/-}$ mice, identifying MHC II-restricted 'redirected' thymocytes. The gray-shaded box in column 3 (bottom) contains *Thpok*$^{GFP+}$ *Runx3*$^{tRFP-}$ cells. (**B**) Contour plots of *Thpok*^{GFP} and *Runx3*^{tRFP} expression in Vα11^{+} CD4 or CD8 SP thymocytes from *Thpok*$^{-/-}$ and *Thpok*$^{+/+}$ mice carrying the MHC-II-restricted AND TCR

transgene; gates are defined in Supporting Information Fig. 1. The gray-shaded area identifies *Thpok*$^{GFP+}$ *Runx3*$^{tRFP-}$ cells. (**C**) Sorted CD4 SP thymocytes from *Thpok*$^{+/+}$ or *Thpok*$^{-/-}$ AND mice carrying the *Runx3*^{tRFP} reporter were placed in single cell suspension culture overnight and analyzed for expression of surface CD4 and CD8 (top) and tRFP (bottom) before (left) and after (right) culture. Overlaid histograms (bottom) show *Runx3*^{tRFP} expression in *Thpok*$^{-/-}$ (solid line histogram) or *Thpok*$^{+/+}$ (dashed line histogram) cells. Gray-filled histograms show tRFP fluorescence in CD8 SP cells from AND *Thpok*$^{-/-}$ mice analyzed in parallel. Numbers in bottom panels indicate tRFP fluorescence relative to CD8 SP controls (gray-shaded histograms) set to 100. (A-C) In each panel, data shown are representative of at least three experiments. (**D**) Contour plots of CD69 vs. tRFP expression gated on GFP^{+} thymocytes from *Thpok*$^{+/+}$ or *Thpok*$^{-/-}$ mice carrying both the *Thpok*^{GFP} and *Runx3*^{tRFP} reporters. The gray-shaded area identifies the CD69^{hi} GFP^{+} tRFP^{-} subset in *Thpok*$^{-/-}$ mice.

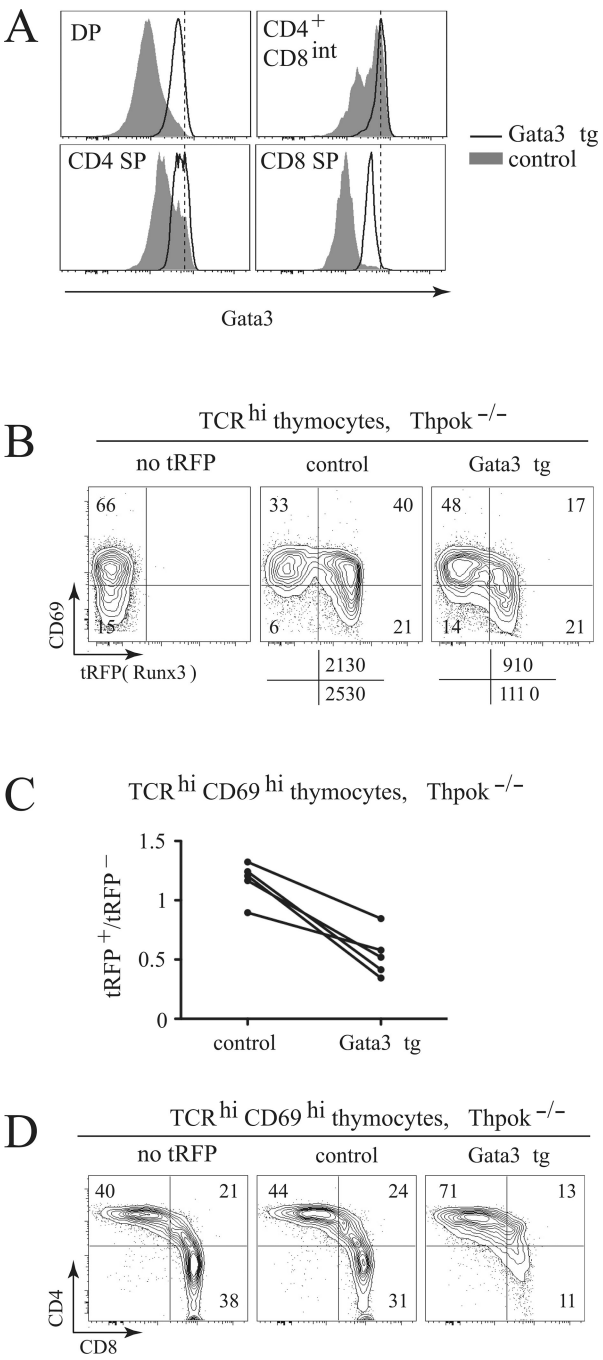

Figure 2. Enforced Gata3 expression represses *Runx3* in MHC II-restricted thymocytes.

(**A**) Expression of intra-cellular Gata3 was analyzed by flow cytometry in *Gata3* transgenic thymocyte subsets (solid line histogram) or their non-transgenic counterparts (gray-shaded histograms). The vertical dotted line indicates the peak of Gata3 expression in wild type $CD4^{+}CD8^{int}$ thymocytes. Data are from two mice analyzed in a single experiment, and representative of three independent determinations. (**B**) Plots depict expression of $Runx3^{tRFP}$ vs. CD69 in post-selection (TCR^{hi}) thymocytes from *Gata3* transgenic and control $Thpok^{-/-}$ mice. Numbers below plots show the mean tRFP fluorescence intensity (arbitrary units) in

the top and bottom right quadrants; the leftmost plot shows background fluorescence in mice that do not carry the *Runx3*^{tRFP} reporter. (**C**) The ratio of tRFP^{+}/tRFP^{-} cells among TCR^{hi} CD69^{hi} thymocytes, either *Gata3*-transgenic (right) or non-transgenic controls (left) is shown. Lines link mice analyzed within the same experiment. (**D**) Plots show CD4 and CD8 expression in TCR^{hi} CD69^{hi} *Thpok*$^{-/-}$ thymocytes from the same mice as in (B). (B-D) Data shown are from five pairs of mice analyzed in five distinct experiments.

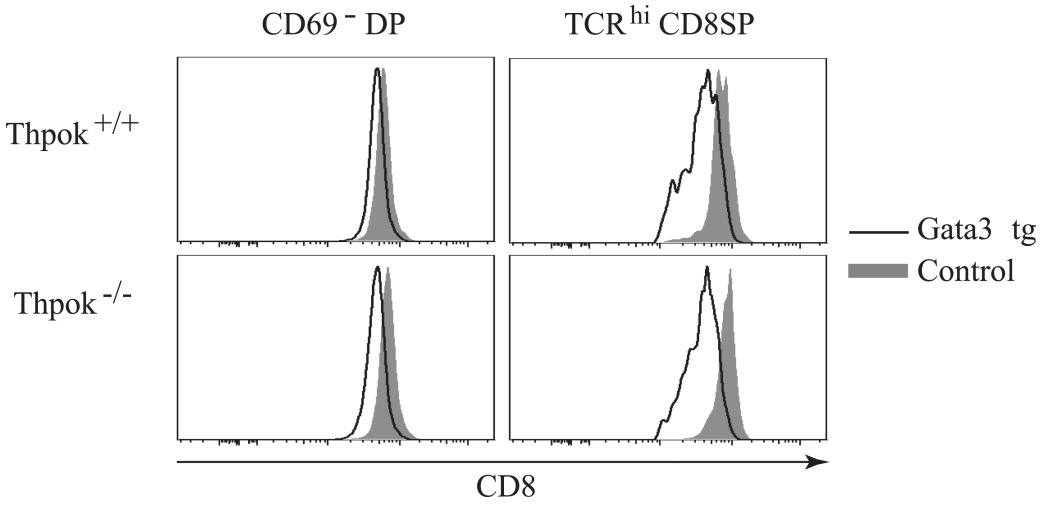

Figure 3. Gata3 represses expression of CD8.

Overlaid histograms show surface CD8 expression on gated pre-selection DP and TCR^{hi} CD8 SP thymocytes from *Gata3*-transgenic (solid line histogram) and control (gray-filled histogram) thymocytes. Data shown are representative of five experiments.

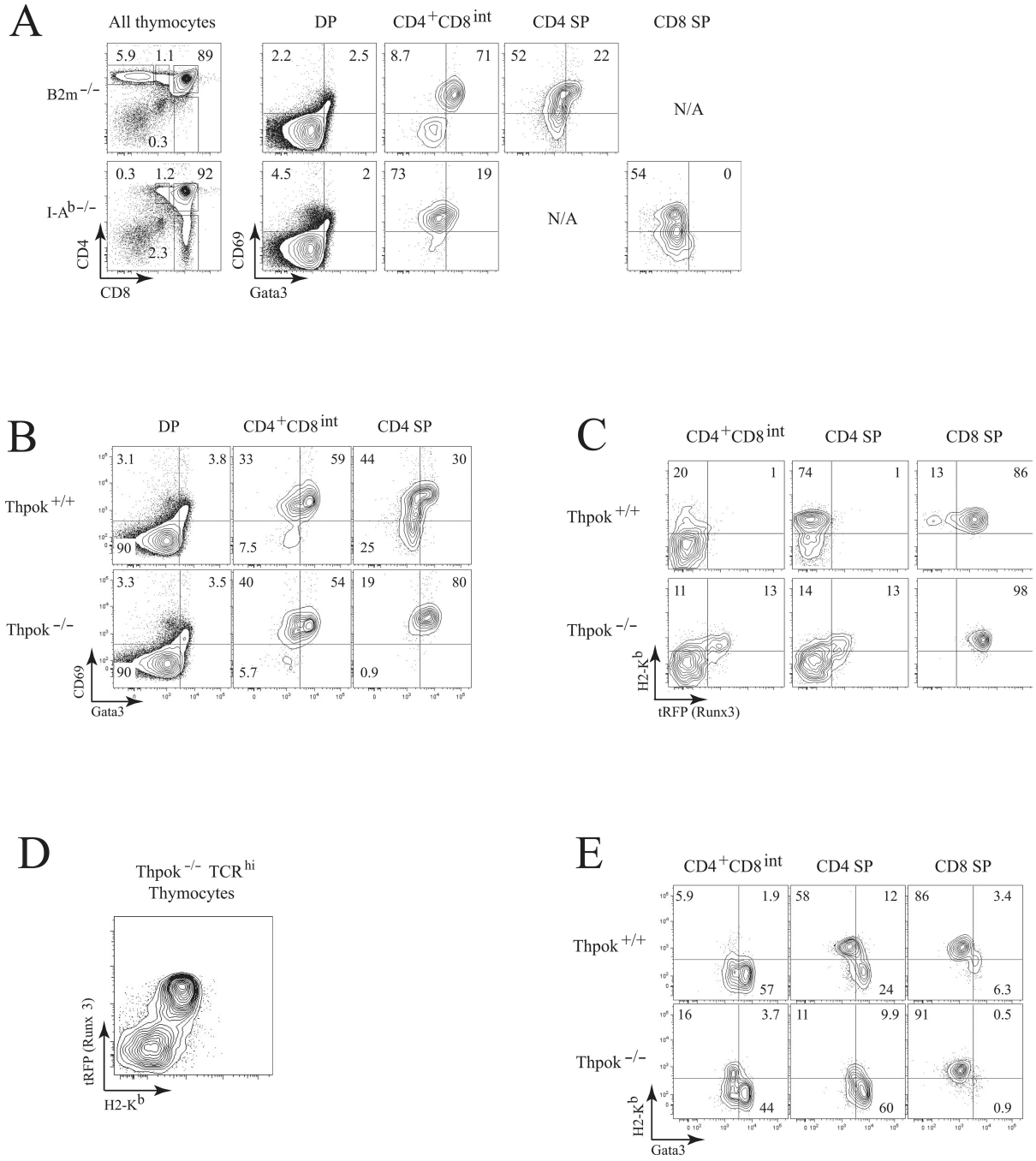

Figure 4. Runx3 and high-level Gata3 expression are mutually exclusive in- thymocytes.

(**A**) Contour plots (right) show intra-cellular Gata3 and surface CD69 expression in MHC II- (top, from *B2m*$^{-/-}$ mice) and MHC I-restricted (bottom, from MHC-II-deficient mice) thymocyte subsets as gated on the left. (**B**) Contour plots show intracellular Gata3 and surface CD69 expression in *Thpok*$^{+/+}$ (top) and *Thpok*$^{-/-}$ (bottom) thymocyte subsets gated as in (A). (**C**) Expression of H-2K^{b} and *Runx3*^{tRFP} in *Thpok*$^{+/+}$ and *Thpok*$^{-/-}$ thymocytes gated as indicated in (A). (**D**) Plots show surface H-2K^{b} vs. *Runx3*^{tRFP} expression in gated TCR^{hi} thymocytes from *Thpok*$^{-/-}$ mice. (**E**) Plots show intracellular Gata3 vs. surface

H-2K^{b} expression in CD4^{+}CD8^{int}, CD4 SP and CD8 SP thymocytes from *Thpok*$^{+/+}$ and *Thpok*$^{-/-}$ mice. Data shown are representative of two or more experiments.

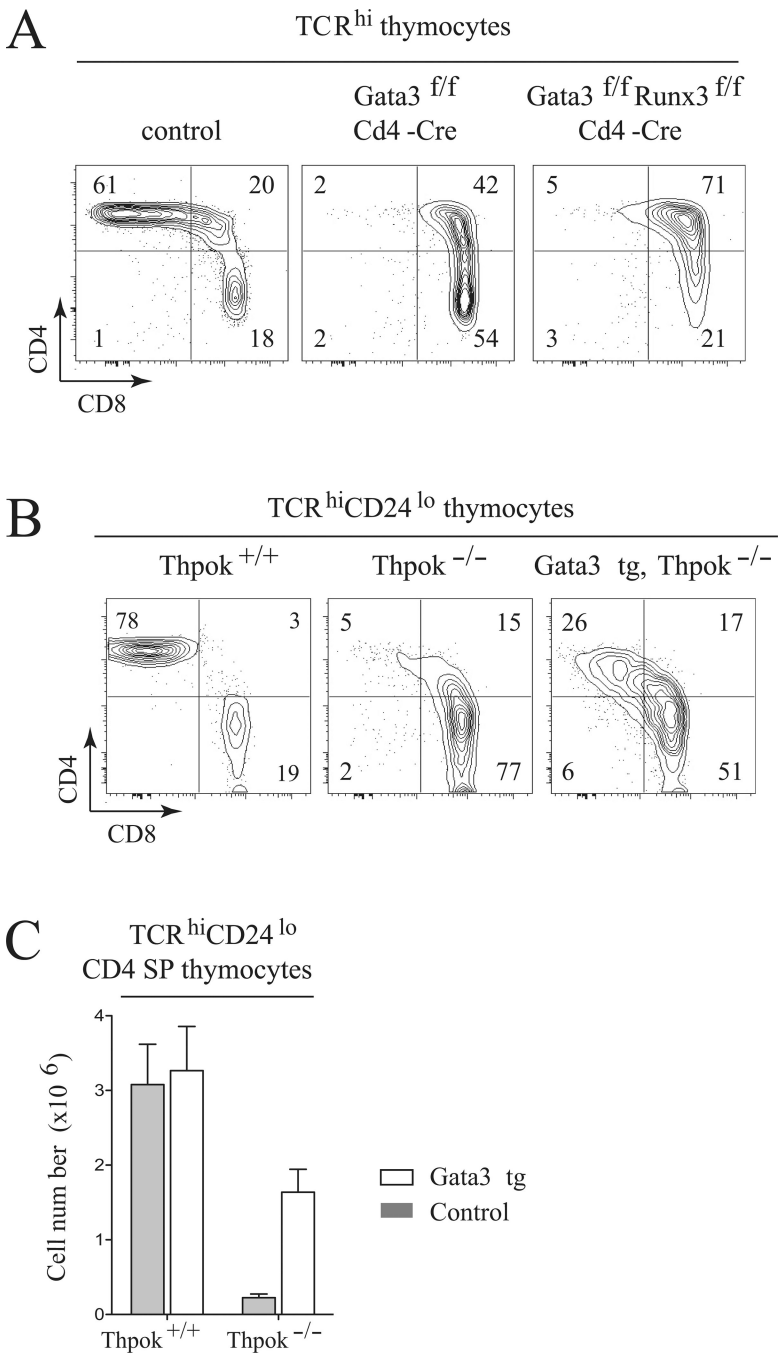

Figure 5. Gata3 delays the CD8-redirection of Thpok-deficient thymocytes.

(**A**) Plots show CD4 and CD8 surface expression in TCR^{hi} thymocytes from wild-type (control) mice, *Gata3*$^{f/f}$ or *Runx3*$^{f/f}$ *Gata3*$^{f/f}$ *Cd4*-Cre mice; data shown are from one experiment representative of three performed. (**B**) CD4 and CD8 expression in TCR^{hi} CD24^{lo} thymocytes from *Thpok*$^{+/+}$ mice, and from *Gata3*-transgenic and control *Thpok*$^{-/-}$ mice. Data shown are from one experiment representative of three performed. (**C**) The number of TCR^{hi} CD24^{lo} CD4 SP thymocytes from the indicated mice is shown as mean +

SD. Data pooled from five independent experiments analyzing 14 *Thpok*$^{-/-}$ mice (7 each *Gata3* transgenic and control) and 8 *Thpok*$^{+/+}$ mice (4 each *Gata3* transgenic and control).

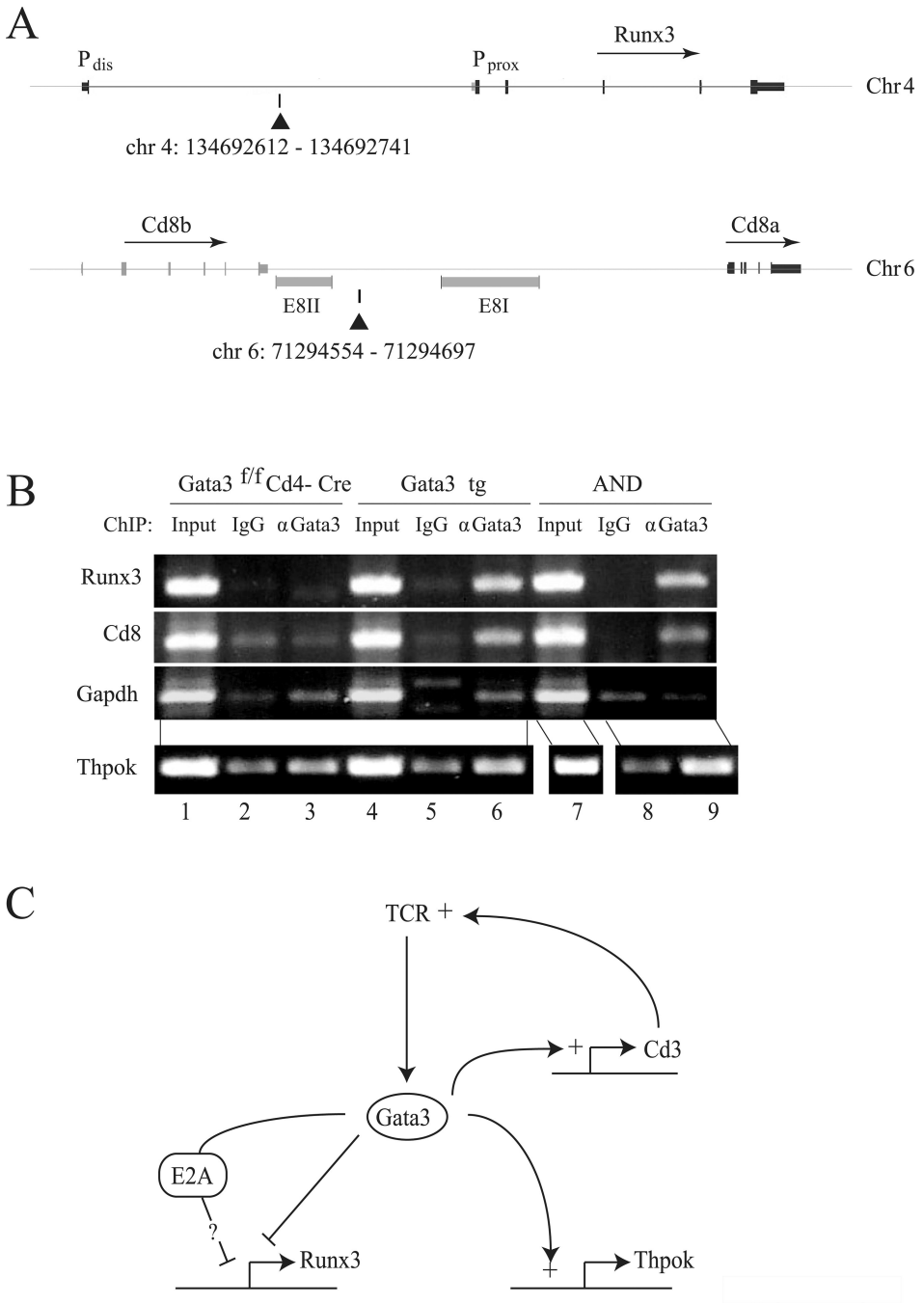

Figure 6. Gata3 binds to *Runx3* and *Cd8* loci

(**A**) Chromosomal coordinates and schematic representations of *Runx3* and *Cd8* loci were obtained from the UCSC genome browser (mm9 mouse genome assembly). Location of PCR amplicons identifying Gata3 binding sites in each locus are shown by black triangles. P_{dis} and P_{prox} refer to the distal and proximal *Runx3* promoters respectively. Note the Gata3 binding site location immediately downstream of the *Cd8* E8(II) enhancer active throughout T-cell development [6]. (**B**) ChIP analyses of Gata3 binding in unseparated thymocytes from AND TCR transgenic, *Gata3* transgenic or *Gata3*-deficient mice. Agarose-gel bands show

PCR amplicons on the *Cd8*, *Runx3*, *Gapdh* (as a control for background) and *Thpok* (as a positive control, site A in Ref. [22]) loci from anti-Gata3 or control IgG immunoprecipitates, or from unfractionated chromatin (input). Data shown are representative of at least two separate experiments (two distinct mice of each genotype). (**C**) Central position of Gata3 in the circuitry of CD4-lineage differentiation. Gata3 (i) preserves bi-potency of MHC II-signaled thymocytes by repressing *Runx3* (this study, left), (ii) promotes TCR signaling, possibly through binding to genes encoding CD3 subunits (top right, [35, 40]), and (iii) promotes *Thpok* expression (bottom right, [22]).