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The Transcription Factor Zbtb7b Promotes CD4 Expression by Antagonizing Runx-Mediated Activation of the CD4 Silencer

Kathryn F. Wildt,† Guangping Sun,‡ Baerbel Grueter,‡ Maria Fischer,‡ Monica Zamisch,* Marc Ehlers,† and Rémy Bosselut*†

The persistence of CD4 expression is a key event distinguishing the differentiation of MHC class II-restricted thymocytes into CD4 T cells from that of MHC class I-restricted thymocytes into CD8 T cells. The zinc finger transcription factor Zbtb7b (also known as cKrox or Thpok) is normally expressed in MHC class II-restricted thymocytes and promotes CD4 lineage choice. When expressed in MHC class I-restricted cells, Zbtb7b redirects these cells from their normal CD8 fate to CD4 differentiation, implying that it promotes, directly or not, sustained CD4 expression; the present study has investigated the mechanism of this effect. We demonstrate that, although Zbtb7b does not enhance CD4 expression on its own, it antagonizes the CD4 repression mediated by the transcription factor Runx3, which is normally up-regulated during CD8 differentiation and promotes CD4 silencing. Zbtb7b also antagonizes CD4 repression by the related protein Runx1, which is expressed in CD4 lineage cells. This antagonism is observed both in vitro and in vivo, is transcriptional, and requires domains of Zbtb7b that are essential to its ability to promote CD4 differentiation in vivo. Furthermore, Zbtb7b fails to antagonize Runx in cells treated with histone deacetylase inhibitors, suggesting that Zbtb7b acts by reducing the expression of thus far unknown factors that cooperate with Runx molecules to repress CD4. These findings demonstrate that the transcription factor Zbtb7b promotes CD4 expression by antagonizing Runx-mediated CD4 repression. The Journal of Immunology, 2007, 179: 4405–4414.

The vast majority of thymus-derived αβ T cells belong to either of two lineages defined by the mutually exclusive expression of CD4 and CD8, two surface glycoproteins that bind MHC class II (MHC-II) or MHC class I (MHC-I) molecules, respectively. CD4 T cells are MHC-II-restricted and have helper function, whereas CD8 T cells are MHC-I-restricted and are cytotoxic. The divergence between CD4 and CD8 lineages occurs as double-positive (DP) thymocytes that express both CD4 and CD8 are rescued from programmed cell death upon engagement of their Ag TCR by intrathymic self-MHC-peptide complexes, a process referred to as positive selection (1–3).

The differentiation of CD4 and CD8 lineages involves specific effector transcription factors, including the Runx family member Runx3, and the zinc finger proteins Zbtb7b (also known as cKrox or Thpok) (4, 5) and Gata3 (6–8). Runx3 is up-regulated in CD8 lineage thymocytes and promotes the termination of CD4 expression during CD8 differentiation (9–11). The control of CD4 expression relies on both positive and negative cis-regulatory elements. An upstream CD4 enhancer is active in thymocytes and in both CD4 and CD8 T cells (12), whereas a downstream enhancer contributes to the expression of CD4 reporter transgenes in DP thymocytes (13). However, neither of these enhancers is CD4 lineage-specific. Rather, a 434-bp silencer located in the first intron of the CD4 gene is required to prevent CD4 expression in double negative (DN) thymocytes and CD8 T cells (14–17). Genetic evidence demonstrates that Runx3 binding to this silencer is required for the normal cessation of CD4 expression in CD8 lineage cells (9, 10, 12). Current models propose that Runx3 initially represses CD4 gene transcription by binding the CD4 silencer (9–11, 16, 18–20), and that such reversible repression is followed by the establishment of an irreversible, epigenetically-maintained silencing of the CD4 locus in CD8 T cells (12, 16). In addition to its role in CD8-differentiating cells, the same CD4 silencer element recruits the related protein Runx1 in early CD4−CD8− DN thymocytes, resulting in the reversible CD4 repression that ceases as thymocytes initiate CD4 (and CD8) expression as a result of pre-TCR signaling (9, 21).

The zinc finger transcription factor Zbtb7b is up-regulated by MHC-II-restricted thymocytes during their CD4 differentiation and is a major determinant of CD4 lineage choice: loss of Zbtb7b function redirects MHC-II-restricted thymocytes into the CD8 lineage (5), whereas constitutive Zbtb7b expression redirects MHC-I-restricted thymocytes into the CD4 lineage (4, 5). MHC-I-restricted thymocytes redirected by Zbtb7b to a CD4 fate maintain CD4 expression instead of down-regulating this gene as MHC-I-restricted thymocytes normally do during CD8 differentiation. This
observation indicates that Zbtb7b, directly or not, promotes CD4 expression in thymocytes. The present study has examined the mechanism of this effect. Although we found no evidence for a direct effect of Zbtb7b on CD4 expression, we demonstrate that Zbtb7b impairs CD4 repression by Runx3 and Runx1, that this effect is transcriptional and that it requires the same Zbtb7b domains as those needed to promote CD4 differentiation. Our findings further suggest that Zbtb7b antagonism of Runx3 is indirect, possibly through the repression by Zbtb7b of cofactors required for Runx3-mediated CD4 repression.

Materials and Methods

Mice
The following mice were described previously and were bred in our colony to generate offsprings of the appropriate genotype: mice carrying a Zbtb7b transgene driven by human CD2 enhancer elements (C8 line) (4), mice carrying a Runx3 transgene driven by the CD4 promoter and enhancer (line 3) (19), and Zap70 -/- mice (22). All mice were housed and maintained according to animal study protocols approved by the National Cancer Institute Animal Care and Use Committee. Experiments were conducted on 4- to 8-wk-old mice, except those performed on Runx3-Zbtb7b double-transgenic mice and appropriate controls that were performed on 1- to 3-wk-old litters.

Plasmid constructs and transient transfections
The CD4ES and CD4E GFP reporters were previously described (11) under the names of E/P2EGFP-2xins and E/P2Z/2EGFP-sil-2xins, respectively. CD4ESΔR-GFP was generated from CD4ES-GFP by mutating all potential Runx3 binding sites (11). CMV promoter-driven expression vectors for Runx1, Runx3, Cbfβ, and Cdxα were previously described (11, 19, 23, 24). Expression vectors for wild-type or A/TBT Zbtb7b were constructed by inserting the corresponding murine cDNA (4) into pcDNA3 (Invitrogen Life Technologies). pcDNA3 Zbtb7bHD was generated from pcDNA3 Zbtb7bHD by in vitro mutagenesis using the QuikChange II XL Mutagenesis kit (Stratagene). Detailed cloning procedures are available on request. The sequence of all oligonucleotide-encoded or PCR-amplified DNA segments was verified by Dideoxy DNA sequencing.

RLM-11 cells were electroporated using a BTX square wave electroporator, according to the manufacturer’s instructions and the following specific conditions: 300 V, 10 ms, 4 × 10⁶ cells, 200 μL. Except where indicated, transfections included 5 μg of each reporter and expression vector. Within each experiment, the total amount of transfected DNA was kept constant by adjusting the amount of empty pcDNA3 expression vector. Cells were analyzed 20–24 h after transfection. Trichostatin A (Tsa; Upstate Biotechnology) was used at 100 nM where indicated. For Zbtb7b protein expression analyses, 1% Triton X-100 cell lysates were immunoprecipitated and immunoblotted with an anti-Zbtb7b antiserum as previously described (4).

Abs, staining procedures, and analyses of GFP expression
Monoclonal Abs against mouse TCRβ (H57-597), CD4 (GK1.5), CD8α (53-6.7), CD25 (7D4), CD44 (IM7), and B220 (RA3-6B2) were from BD Pharmingen or Caltag Laboratories. Thymocytes and spleenocytes were prepared and stained as described (25). Transfected RLM-11 cells were stained with aliphophycocyanin-conjugated anti-CD8α and assessed for GFP and aliphophycocyanin fluorescence. Fluorescence was acquired on four-color FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star). Dead cells were excluded from analyses using forward light scatter (FSC) and propidium iodide gating. For analyses of reporter expression in transfected RLM-11 cells, the mean GFP fluorescence intensity was recorded on CD8low cells (see in Fig. 1B) for each transfection condition and was used to calculate average and SEM values shown (see Figs. 1D, 2B, 3, and 6A). Statistical analyses were performed using the t test function of Microsoft Excel (two-tailed, same variance).

Results

Zbtb7b impairs Runx3-mediated silencer activation
To evaluate Zbtb7b effects on CD4 expression, we first used an in vitro transient transfection approach to examine whether Zbtb7b activates CD4 transcription. Previous studies (11, 19) have shown that the activity of the cis-regulatory elements that control CD4 transcription can be evaluated by transiently transfecting the thy-moma cell line RLM-11 with a GFP-based reporter-driven by the enhancer, silencer and promoter of the mouse CD4 gene (referred to as CD4ES-GFP). RLM-11 cells express CD4 but not CD8 (19 and data not shown), and neither Runx3 (19) nor Zbtb7b (Fig. 1A). We transfected RLM-11 cells with the CD4ES-GFP construct, together with an expression vector for mouse CD8 driven by the heterologous CMV promoter-enhancer combination to serve as a reporter for transfection efficiency. Flow cytometry analyses identified a CD8high transfected population in which expression of the CD4ES-GFP reporter was easily detected (Fig. 1B). To confirm that transfection efficiency was high enough in this population to measure the response of CD4 cis-regulatory elements to transcription factors, we cotransfected RLM-11 cells with CD4ES-GFP, CMV CD8, and either a CMV-based Runx3 expression vector or the same amount of a CMV-based empty vector (control) (Fig. 1B). Expression of Runx3 reduced by 75% the expression of the CD4ES-GFP reporter in CD8low cells, thereby reproducing in vitro Runx3-mediated CD4 repression. In contrast, cotransfection of Runx3 did not affect expression of the CMV-driven CD8 reporter (data not shown), indicating that Runx3 had no effect on the CMV promoter-enhancer cassette driving all expression vectors used in the present study. Note that GFP expression was at near-background levels, and was not detectably affected by Runx3 co-transfection, in the CD8low subsets that include untransfected or inefficiently transfected cells (Fig. 1B); consequently, all subsequent analyses were performed on gated CD8low cells.

Cotransfection of Zbtb7b with the CD4ES-GFP reporter had little or no effect on GFP expression, suggesting that Zbtb7b does not activate CD4 cis-regulatory elements on its own (Fig. 1, C and D). We next considered that Zbtb7b could contribute to CD4 expression by counteracting the repression of CD4 by Runx3. That was indeed the case, as the expression of the CD4ES-GFP reporter in cells cotransfected with Runx3 and Zbtb7b was more than three times higher than in the presence of Runx3 alone, and almost as high as in cells expressing neither protein (Fig. 1, C and D). In parallel experiments, we used a “silencerless” GFP reporter driven by the CD4 enhancer and promoter elements only (referred to as CD4E-GFP). Although GFP expression from this construct was similar to that from the CD4ES-GFP plasmid, its activity was not affected by Runx3 or by Zbtb7b (Fig. 1D). This indicated that the effect of Zbtb7b was silencer-dependent. We further documented that the Zbtb7b-Runx3 combination had little or no effect on a reporter with mutated Runx3 binding sites (CD4ESAR), demonstrating that Zbtb7b antagonized Runx3-mediated silencer activation rather than acting on the silencer through a Runx-dependent but indirect mechanism (Fig. 1D).

Zbtb7b antagonism of Runx3 requires its BTB and zinc finger domains
As the BTB/POZ and the zinc finger motifs of Zbtb7b are important for its function during lineage differentiation (4, 5), we examined whether these domains are required for Zbtb7b to antagonize Runx3 in RLM-11 cells. We assessed the function of the BTB domain by using a version of Zbtb7b lacking its BTB domain (ΔBTB) (4) and the role of the zinc finger domain by generating a version of Zbtb7b carrying the R389G mutation (Zbtb7bR389G) previously shown to disrupt CD4 differentiation in vivo (5). Although both mutant proteins were expressed at levels similar to wild-type Zbtb7b in transfected 293T epithelial cells (data not shown), reduced expression was observed in transfected RLM-11 cells (Fig. 2A). Consequently, we compared the effect of ΔBTB and HD mutants in RLM-11 cells transfected with amounts of wild-type and mutant plasmids resulting in similar expression levels. Neither the ΔBTB nor the HD mutant protein antagonized Runx3-mediated
mediated repression of a reporter for CD4 transcription. RLM-11 cells had no effect on GFP levels in CD8low and CD8low to undetectable in CD8low and CD8high, CD8low, and CD8high 24 h after transfection. Transfected cells were electronically gated (FIGURE 1. were transfected with pcDNA3 Zbtb7b (\text{+/H11002}) or with empty pcDNA3 (\text{-/H9251}), RLM-11 thymoma cells do not express Zbtb7b. RLM-11 thymoma cells (filled histograms), GFP expression was detected in CD8high cells, but was scatter vs CD8. In cells transfected with CD4ES-GFP and control vector (control), Expression of mCD8\text{+/H9251} either a Runx3 expression vector or the same amount of empty vector CMV promoter-enhancer, as an indicator of transfection efficiency; and \text{CD4E-GFP} expression in cells transfected with the indicated vectors (thick line trace) to that in cells transfected with the reporters and control vector only (gray filled histograms). D, Zbtb7b antagonizes Runx3-mediated activation of the CD4 silencer. RLM-11 cells were transfected as in C with the indicated expression vectors and either the CD4ES-GFP (left), the “silencerless” CD4E-GFP (middle) or the Runx-mutated CD4ESAR-GFP (right) reporter. Fluorescence was analyzed as in B and C. Within a given experiment, the mean GFP fluorescence on CD8\text{high} cells for each sample was expressed relative to that of CD8\text{low} cells transfected with the same reporter, CMV-CD8\text{+/H9251} and control vector only (vector), arbitrarily set to 1. Average relative expressions were computed for each vector combination and data are shown as bar graphs. Errors bars, the SEM. Data are from eight (CD4ES-GFP), three (CD4E-GFP), or two (CD4ESAR-GFP) separate experiments. Student’s t test analyses showed significant differences in CD4ES-GFP transcription between cells transfected with control vs Runx3 only (*) \(p < 10^{-12}\) and between cells transfected with Runx3 only vs Runx3 and Zbtb7b \((**, p < 10^{-4}\) ), GFP expression in the absence of exogenously expressed proteins (“vector” samples) was similar for all three reporters (data not shown).

The Zbtb7b-Runx3 antagonism and Zbtb7b repressor function

That Zbtb7b\text{+/H11002} failed to antagonize Runx3 argued against a mechanism whereby Zbtb7b would sequester Runx3 away from its DNA targets through protein-protein interactions that do not involve DNA binding. Rather, this result suggested two possible mechanisms for Zbtb7b’s antagonism of Runx3. It was possible that this antagonism resulted from Zbtb7b binding the CD4 locus and inhibiting Runx3 recruitment or function, even though such a direct positive effect of Zbtb7b on CD4 transcription would be unexpected because Zbtb7b belongs to a family of proteins that generally act as transcriptional repressors. Alternatively, it was possible that Zbtb7b acted by repressing additional genes, not associated with the CD4 locus and encoding silencer-binding proteins or cofactors required for Runx3-mediated CD4 repression; we refer to this possibility as the repressor-of-repressor hypothesis. To distinguish between these alternatives, we noted that the repressor-of-repressor hypothesis predicted that impairing Zbtb7b repressor function should impair Zbtb7b antagonism of Runx3 and paradoxically result in intact repression of the CD4ES-GFP construct by Runx3 despite Zbtb7b expression. In contrast, if Zbtb7b acted directly on the CD4 locus to prevent Runx3 binding or function, Zbtb7b antagonism of Runx3 should not be affected by inhibition of Zbtb7b repressor function.

As transcriptional repression by BTB-POZ proteins is mediated in part by the recruitment of histone deacetylase (HDAC)-containing complexes, we tested whether inhibition of HDAC activity would affect Zbtb7b’s antagonism of Runx3 function. We used TsA, an inhibitor of class I and class II HDAC molecules (27). As we recently showed that retroviral expression of Zbtb7b in mature CD8 T cells inhibited CD8 expression (28), we verified that this inhibition was sensitive to TsA and therefore that Zbtb7b-mediated repression was at least in part HDAC-dependent (data not shown). Interestingly, Zbtb7b failed to relieve Runx3-mediated CD4 repression in the presence of TsA (Fig. 3A). Although TsA reduced repression of the CD4ES-GFP construct in RLM-11 cells (Fig. 2B), indicating that both the BTB and zinc finger domains of Zbtb7b are required for its ability to counteract Runx3. As Arg\text{389} is predicted to directly contact DNA and contribute to binding specificity (5, 26), this result strongly suggests that Zbtb7b antagonism of Runx3 requires Zbtb7b to bind specific DNA targets.
the repression by Runx3 of the CD4 reporter (Fig. 3A), the lack of reversion by Zbtb7b in TsA treated cells suggests that Zbtb7b antagonism of Runx3 is at least in part HDAC-dependent. This observation supported the hypothesis that Zbtb7b uses HDAC-dependent repression of endogenous repressors to antagonize Runx3-mediated silencer activation.

Because Runx transcription factors act in association with the structurally unrelated Cbfβ protein (29), which participates in Runx-mediated CD4 repression (24, 30), it was possible that Zbtb7b might antagonize Runx function by reducing the expression of endogenous Cbfβ. To address this possibility, we examined whether Zbtb7b would antagonize Runx3-mediated silencer activation in RLM-11 cells cotransfected with Cbfβ. Although cotransfection of Cbfβ minimally enhanced Runx3-mediated repression of the CD4ES-GFP reporter, it did not prevent Zbtb7b from countering Runx3 (Fig. 3B). These analyses indicate that Zbtb7b did not antagonize Runx3 by repressing Cbfβ expression.

Zbtb7b antagonizes Runx3 function in thymocytes

We next examined whether Zbtb7b counteracted Runx3 function in vivo. To this end, we generated mice that express either or both proteins in DP thymocytes, which in wild-type mice express the repression by Runx3 of the CD4 reporter (Fig. 3A), the lack of reversion by Zbtb7b in TsA treated cells suggests that Zbtb7b antagonism of Runx3 is at least in part HDAC-dependent. This observation supported the hypothesis that Zbtb7b uses HDAC-dependent repression of endogenous repressors to antagonize Runx3-mediated silencer activation.

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neither Zbtb7b nor Runx3 (4, 5, 10, 11, 31). We used a transgenic line expressing Runx3 under the control of the CD4 promoter and enhancer elements. Because it lacks the CD4 silencer, this combination is active in the thymus in DN and DP thymocytes, and in both CD4 and CD8 lineage T cells (19). This transgene expresses Runx3 at near-physiological levels and causes preselection thymocytes (normally CD4+CD8-) to appear CD4-CD8+. In the line used in the present study, 25–35% of CD8+ cells displayed a CD4low phenotype typical of CD8 single positive (SP) thymocytes (19) (Fig. 4, top panels). These CD4lowCD8+ cells were of small size (as assessed by FSC) and expressed levels of CD24 (HSA, a marker of thymocyte maturation) similar to their CD4+CD8- counterparts (data not shown). This indicated that these cells were developmental equivalents of DP thymocytes with low CD4 expression, rather than being “immature CD8 SP” (ISP) cells in transit from the DN to DP stage. The transgene did not affect CD4 expression in DP thymocytes of mice carrying a disrupted CD4 silencer allele (19), demonstrating that the repression of CD4 by the Runx3 transgene depends on Runx3 recruitment to the CD4 silencer. As previously documented using an in vitro retroviral expression system (18), the effect of Runx3 did not require any additional TCR-induced factor, as it was observed in Zap70−/− thymocytes (22) that are defective for TCR signal transduction (Fig. 4, bottom panels).

To evaluate whether Zbtb7b affects Runx3-mediated CD4 repression, we bred the Runx3 transgenic line to mice expressing Zbtb7b from a human CD2-based cassette active throughout T cell development from the DN2-DN3 stage (4). Thymomas were found at high frequency in adult Runx3-Zbtb7b double-transgenic animals (data not shown); consequently, all analyses reported below were performed in 7–21 day old mice that were tumor-free and had normal thymic cellularity (data not shown). Because of the effect of Runx3 on CD4 expression, analyses on double-transgenic mice used FSC and surface TCR expression to distinguish thymocyte developmental stages (Fig. 5A, top panels). In wild-type mice, this combination separates a TCRhigh subset (subset 3), mainly composed of CD4 and CD8 SP thymocytes undergoing selection (Fig. 5A, bottom panels), from a preselection TCRlow population that includes both FSClow (small) and FSChigh (large) cells. FSClow TCRlow cells (subset 2) constitute the most abundant thymocyte population and comprise almost exclusively CD4loCD8hi thymocytes in wild-type mice; in contrast, FSChigh TCRlow cells (subset 1) are less numerous and include earlier thymocytes that express neither coreceptor (DN) or express CD8 only (ISP) or both CD8 and CD4. Expression of the Zbtb7b or Runx3 transgenes had little or no effect on thymocyte size and TCR expression, so that the distribution of thymocytes based on FSC and TCR expression was similar in all three mutant strains to that in wild-type mice (Fig. 5A, top). Consequently, we examined the expression of CD4 in these mutant mice in each of the three subsets defined by FSC and TCR expression.

We first examined how the Runx3 and Zbtb7b transgenes, separately or in combination, affected CD4 expression in the FSClow TCRlow subset (Fig. 5B, subset 2). Expression of the Runx3 transgene alone resulted in the down-regulation of CD4 in 20–25% of these cells, whereas expression of the Zbtb7b transgene had no effect on CD4 expression. The CD4low population seen in Runx3 transgenic mice was absent in mice coexpressing the Runx3 and Zbtb7b transgenes (Fig. 5B, lower right plot and bottom graph), indicating that Zbtb7b largely reversed the down-regulation of CD4 caused by Runx3. Similar observations were made in the FSChigh TCRlow subset (subset 1), in which most CD8+ cells were CD4− in Runx3 transgenic mice but CD4+ in Runx3-Zbtb7b double-transgenic mice (Fig. 5C, top, compare plots in right column and bottom, compare graphs at right), indicating a reversal by Zbtb7b of the CD4 repression caused by Runx3.

To account for these observations, we considered the unlikely possibility that transgenic Zbtb7b would reduce the expression of the CD4-driven Runx3 transgene. Immunoblot analyses of Runx protein expression found that this was not the case, as the amount of transgenic Runx3 protein was not lower in Runx3-Zbtb7b double-transgenic than in Runx3 transgenic thymocytes (Fig. 5E). We conclude from these findings in Zbtb7b-Runx3 double-transgenic mice that Zbtb7b antagonizes the repression of CD4 by Runx3 in vivo.

Because Runx3 had been proposed to promote CD8 cell differentiation (32), we examined whether constitutive Runx3 expression would overcome the block in CD8 differentiation imposed by the Zbtb7b transgene. Analyses of the TCRhigh subset (subset 3) in Runx3-Zbtb7b double-transgenic mice showed that this was not the case, as these mice lacked true CD8 SP (TCRhigh CD4+CD8+) thymocytes (Fig. 5D). Further supporting this conclusion, these double-transgenic mice lacked spleen CD8 T cell populations at all ages examined (data not shown).

Zbtb7b antagonizes CD4 repression by Runx1

The distinct roles of Runx1 and Runx3 proteins in CD4 expression during T cell development are at least in part dictated by their expression pattern: Runx1 expression peaks in early DN thymocytes, whereas Runx3 is expressed in CD8-differentiating cells. Interestingly, Runx1 is also up-regulated during the DP to CD4 SP transition (32, 33) and is therefore coexpressed with endogenous Zbtb7b. Thus, it was of interest to examine whether Zbtb7b would antagonize Runx1-mediated CD4 repression. Transient transfection in RLM-11 cells showed this was indeed the case (Fig. 6A). As the Zbtb7b transgene is expressed in late DN and ISP thymocytes in which CD4 expression is normally repressed by Runx1, we examined whether the Zbtb7b transgene affected CD4 expression in these thymocyte subsets. Comparison of early thymocytes (FSChigh TCRlow) showed higher CD4 expression in Zbtb7b transgenic than in wild-type littermates, illustrated by the reduction in the size of the CD4lowCD8+ ISP subset (<50% wild type) (Fig. 6B) and the increased representation of a converse CD4+CD8low population. A similar shift from CD4lowCD8+ to CD4+CD8low was found in the younger mice analyzed in Fig. 5C (compare left plots top to bottom). More detailed analyses of CD4 expression...
FIGURE 5. Zbtb7b antagonizes Runx3-mediated CD4 repression in thymocytes. A–D, Single-cell thymocyte suspensions from 1-wk-old mice transgenic for Zbtb7b, Runx3, or both were surface-stained for CD4, CD8, and TCRβ and analyzed by three-color flow cytometry. A, Dot plots of FSC and TCRβ expression gated on all live cells (top) define three thymocyte subsets independently of CD4 and CD8 expression (subsets 1–3). Contour plots (bottom) show CD4 and CD8 expression on each of these subsets in wild-type mice. The TCRβhigh (subset 3) includes thymocytes undergoing selection.

B, FSClowTCRβlow thymocytes

C, FSCβTCRβ high thymocytes

D, TCRβ high thymocytes

E, Thymocytes

Runx1

Runx3

anti-Runx immunoblot

(+: p<0.025; ++: p<0.001)
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were conducted on TCR^-CD8^- thymocytes, an early subset that by definition excludes ISP thymocytes, which are CD8^+.

These experiments showed increased CD4 expression in early TCR^-CD8^-CD44^- thymocytes (roughly equivalent to the conventional DN4 subset) (Fig. 6C), whereas there was no difference in CD4 expression in earlier (CD25^+ or CD44^+) DN

the FSC^low TCR^low (subset 2) includes the vast majority of preselection thymocytes, which are almost entirely CD4^-CD8^- cells in wild-type mice, and the more heterogeneous FSC^high TCR^low thymocytes. B–D, Thymocytes from mice expressing the Zbtb7b transgene or Runx3 transgene (tg), both transgenes, or neither were analyzed from the same mice as shown in A; contour plots of CD4 and CD8 expression are shown on FSC^low TCR^low thymocytes (subset 2) (B), FSC^high TCR^low (subset 1) (C), and TCR^high (subset 3) (D). Total thymic cellularity was not affected by expression of the Zbtb7b or Runx transgenes (data not shown). A summary of these results, from three distinct litters ages 6–14 days, is shown at the bottom of (B and C). Data shown are the average percentage of the indicated population in wild-type mice (n = 6) (●), or in mice transgenic for Zbtb7b (n = 9) (dark gray), Runx3 (n = 6) (□), or both (n = 5) (◆). Error bars, SEM. In B and C, p values obtained by Student’s t test are indicated. In D, the average percentages ± SEM of TCR^high CD4^-lowCD8^- cells were 9.5 ± 1%; 0.34 ± 0.07%; 25 ± 3.2%, and 4.0 ± 1.22% in wild-type, Zbtb7b, Runx3, and double-transgenic mice, respectively. E, Expression of Runx proteins was assessed on thymocyte lysates by immunoblotting with an Ab recognizing both Runx1 and Runx3 proteins (11, 47). Arrows, −56-kDa Runx1 and −50-kDa Runx3 bands.
subsets (data not shown), possibly a reflect of the expression pattern of the Zbtb7b transgene. We verified that this effect was specific of CD4, as CD8 expression was not increased in the reciprocal TCR-CD4-CD25-CD44+ population from Zbtb7b transgenic mice (Fig. 6D). These experiments support the conclusion that Zbtb7b can antagonize CD4 repression by endogenous Runx proteins.

Discussion

The present study identifies a novel function of Zbtb7b, namely that it antagonizes the CD4 repressive function of Runx1 and Runx3 and thereby promotes CD4 expression. The cessation of CD8 and the maintenance of CD4 coreceptor expression are two key events characteristic of CD4 cell differentiation. That Zbtb7b belongs to a family of proteins that generally act as transcriptional repressors (34, 35) is in line with it repressing CD8 expression by targeting CD8 cis-regulatory elements, as we recently showed of the CD8 E8(I) enhancer (28, 36, 37). In contrast to its effect on CD8, the ability of Zbtb7b to promote CD4 expression obviously does not fit with a direct transcriptional repression effect. It was conceivable that Zbtb7b directly activates the transcription of the CD4 gene (Fig. 7, c). Many transcriptional regulators, including Runx proteins (38) and the BTB zinc finger protein MAZR (39), which was recently shown to control CD8 transcription (40), act as either activators or repressors presumably depending on the set of cofactors with which they associate. However, Zbtb7b failed to activate CD4 transcription directly in transient transfection experiments, and overexpression of Zbtb7b (either as a transgene or by retroviral transduction) did not increase CD4 levels in primary CD4+ thymocytes or T cells (4, 28 and our unpublished data). Thus, we do not favor the possibility that Zbtb7b promotes CD4 expression through a direct activation mechanism.

Alternatively, Zbtb7b could sustain CD4 expression by preventing the activation of the CD4 silencer, that is by counteracting CD4 repression. The present study supports this second hypothesis, by demonstrating that Zbtb7b counteracts CD4 silencer activation by Runx1 and Runx3 transcription factors. The putative repressor function of Zbtb7b raises the possibility that it would act through a repressor of repressor mechanism whereby Zbtb7b promotes CD4 expression by repressing the expression of cofactors required for Runx3-mediated CD4 repression (Fig. 7, a). As transcriptional repression by BTB-POZ proteins is at least in part mediated by recruitment of HDAC-containing complexes, our observation that the HDAC inhibitor TsA prevents the effects of Zbtb7b on CD4 transcription brings initial, albeit indirect, support to such a repressor of repressor model.

Which CD4 corepressors would Zbtb7b repress? Among the silencer sites important for its activity, only two bind Runx proteins, whereas at least two others are thought to bind so far unknown factors (12, 21). It is possible that Zbtb7b maintains CD4 expression in thymocytes by repressing the expression of such silencer-binding factors. Of note, as Runx3 represses CD4 expression in Zap70−/− thymocytes that fail to generate positive selection signals, such presumptive factors cooperating with Runx3 would not be CD8 lineage specific. It remains to be determined whether the absence of these factors would prevent Runx3 recruitment to the silencer, or would impair its ability to repress CD4 despite being recruited to its binding sites. By repressing the expression of these factors, Zbtb7b would make CD4 lineage cells, which express Zbtb7b, not permissive to Runx3-mediated silencer activation. Indeed, expression of CD4 in CD4 T cells is not sensitive to Runx: enforced expression of Runx1 or Runx3 does not repress CD4 in CD4 SP thymocytes or CD4 T cells (18–20 and our study), whereas the re-expression of endogenous Runx3 in CD4 T cells undergoing type 1 effector differentiation (41) does not silence CD4 in these cells.

A variation on the repressor-of-repressor theme is that Zbtb7b represses the expression of proteins that serve as corepressors or effectors of Runx3, rather than directly binding to the silencer. Candidates within this category include members of the Groucho/TLE family, of which five members have been identified in mammals (42) and which bind a VWRPY motif located at the C terminus of Runx proteins (43). As this motif was recently shown to be required for Runx3-dependent CD4 silencing (44), it is possible that Zbtb7b would prevent Runx-mediated CD4 repression by impairing expression of Groucho proteins. In addition, Runx molecules interact with multiple proteins involved in histone modifications, including HDACs, mSin3 corepressors, and the histone methyl transferase SUV39H1 (38, 45, 46). However, the role of these proteins in CD4 repression or silencing has yet to be established; furthermore, the target- and stage-specificity of Zbtb7b during T cell development do not favor a model whereby Zbtb7b would affect the expression of general activators or repressors of transcription.

The present data do not exclude alternatives to a repressor-of-repressor mechanism, notably because experiments using TsA are subject to general reservations on the specificity and possible indirect effects of inhibitors. It is conceivable that Zbtb7b directly inhibits Runx function by sequestering Runx proteins away from the CD4 silencer (Fig. 7, b). Our finding that the Zbtb7bΔID mutant fails to antagonize Runx3 argues against this possibility: structural analyses of related zinc finger domains predict that the arginine residue mutated in Zbtb7bΔID contacts DNA (5, 26), which supports the idea that Zbtb7b antagonizes Runx3 by binding to specific DNA targets rather than by a mechanism independent from DNA binding. Zbtb7b transgenic expression did not reproduce these effects. Although it could also be envisioned that Zbtb7b is recruited to the silencer and competes with Runx protein for silencer binding, such a mechanism would not explain that the Zbtb7b-mediated Runx3 antagonism is TsA-sensitive.

Of the three Runx proteins, both Runx3 and Runx1 are expressed during positive selection. Runx3 is expressed during CD8 lineage differentiation (9–11), whereas Runx1 is up-regulated during the DP to CD4 SP transition (33). Although Runx3 is characteristic of CD8 differentiation and Zbtb7b of CD4 differentiation, experimental evidence is consistent with both proteins being coexpressed in “transitional” cells undergoing lineage differentiation (4, 11, 31, 32). Notably, population-based analyses detect expression of both proteins in
the CD4⁺CD8⁻ subset in which lineage choice is thought to occur. Because Zbtb7b gene expression can be detected in most cells within this subset (L. Wang and R. Bosselut, unpublished results), we favor the possibility that Zbtb7b and Runx3 proteins are coexpressed, possibly at low levels, in CD4⁺CD8⁻ cells poised to undergo lineage choice; analyses by single cell approaches will be required to demonstrate this. However, because expression of Runx3 and Zbtb7b are mutually exclusive in mature SP thymocytes (4, 5, 9, 10), it is conceivable that Zbtb7b, in addition to repressing Runx3-mediated CD4 repression, would directly repress Runx3 expression. Although future analyses may shed light on that question, a direct repression of Runx3 by Zbtb7b would necessarily be cell-type-specific, as Runx3 is re-expressed during the differentiation of type 1 effector CD4⁺ T cells that continue to express Zbtb7b (41 and our unpublished observations).

Importantly, Zbtb7b antagonism of Runx-mediated CD4 silencing would also prevent CD4 repression by Runx1, which is up-regulated during CD4 SP thymocyte differentiation, remains expressed in mature CD4 cells and is therefore coexpressed with Zbtb7b in CD4 lineage cells (32, 33). Endogenous Runx1 molecules are competent to repress CD4 beyond the DN stage and notably contribute to CD4 silencing in Runx3-deficient thymocytes (10); we propose that Zbtb7b inhibition of Runx1 CD4-repressing activity is important to maintain CD4 expression in CD4-differentiating thymocytes.

In summary, the present study demonstrates that the zinc finger transcription factor Zbtb7b antagonizes the repression of CD4 transcription by Runx3 and Runx1 molecules. We document that this effect is transcriptional and requires the functional domains of Zbtb7b involved in CD4 differentiation. Our findings further suggest that such antagonism of Runx by Zbtb7b results from the repression by Zbtb7b of thus far unknown genes encoding cofactors required for Runx-mediated CD4 repression.

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Disclosures

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References


