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T-bet represses T_H17 differentiation by preventing Runx1-mediated activation of the gene encoding RORγt

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Overactive responses by interleukin 17 (IL-17)-producing helper T cells (T_H17 cells) are tightly linked to the development of autoimmunity, yet the factors that negatively regulate the differentiation of this lineage remain unknown. Here we report that the transcription factor T-bet suppressed development of the T_H17 cell lineage by inhibiting transcription of *Rorc* (which encodes the transcription factor RORγt). T-bet interacted with the transcription factor Runx1, and this interaction blocked Runx1-mediated transactivation of *Rorc*. T-bet Tyr304 was required for formation of the T-bet–Runx1 complex, for blockade of Runx1 activity and for inhibition of the T_H17 differentiation program. Our data reinforce the idea of master regulators that shape immune responses by simultaneously activating one genetic program while silencing the activity of competing regulators in a common progenitor cell.

The signals received during an infection trigger a strong adaptive immune response tailored to combat a particular class of pathogen. In the presence of cytokines produced by cells of innate immunity, naive $CD4^+$ T cells differentiate into a helper T cell subset with distinct functions and cytokine profile. Two main helper T cell subsets, T_H1 and T_H2, were the first described[1]. T_H1 cells, which secrete mainly interferon-γ (IFN-γ), are essential for immunity against intracellular microorganisms, whereas T_H2 cells, which secrete interleukin 4 (IL-4), IL-5 and IL-13, are important for protection against parasites and extracellular pathogens. A third subset of helper T cells, T_H17, has also been described[2–7]. T_H17 cells produce IL-17A, IL-17F, IL-21 and IL-22, which protect the host against bacterial and fungal infections encountered at mucosal surfaces[8]. In mice, T_H17 differentiation is initiated by the combination of transforming growth factor-β (TGF-β) and IL-6 or IL-21, which induces expression of the T_H17 cell–specific transcription factor RORγt and the receptor for IL-23 (IL-23R)[7,9–12]. This acquisition of responsiveness to IL-23 is necessary for terminal differentiation of the T_H17 cell lineage and for the maintenance of T_H17 function *in vivo*[13].

In general, lineage-specific transcription factors and cytokines can inhibit the differentiation of other helper T cell subsets. T-bet suppresses the generation of T_H2 cells by blocking expression of the T_H2-polarizing cytokine IL-4 and by interfering with the activity of the T_H2-cell–specific transcription factor GATA-3 (refs. 14,15). The results of several studies have indicated that T_H17 responses are stronger in T-bet-deficient animals, although the mechanism underlying this phenomenon has not been described[16–19]. This raises the question of whether, analogous to its role in inhibiting the T_H2 pathway, T-bet also actively suppresses T_H17 differentiation. Furthermore, several studies have reported that the reprogramming of committed T_H17 cells and T_H2 cells into effector cells with a T_H17-T_H1 or T_H2-T_H1 phenotype is driven by T-bet in response to inflammatory cytokines such as IL-12 and interferons[20,21].

In this study we sought to determine whether T-bet has a regulatory role in the development of the T_H17 lineage. Here we investigate the T_H17 differentiation of T-bet-deficient (*Tbx21*$^{-/-}$) and wild-type $CD4^+$ T cells *in vitro* and *in vivo* during experimental autoimmune encephalomyelitis (EAE). We found that T-bet had a negative effect on expression of the gene encoding RORγt (*Rorc*) and genes encoding T_H17 cytokines. Ectopic expression of T-bet in naive helper T cell precursors or in committed T_H17 cells was sufficient to repress the expression of *Rorc* and genes encoding T_H17 signature cytokines under T_H17-polarizing conditions. Mechanistic studies showed that interaction of T-bet with the transcription factor Runx1 via the T-bet residue Tyr304 was critical for blocking Runx1-mediated transactivation of the *Rorc* promoter and for inhibiting commitment to the T_H17 lineage.

RESULTS

T-bet deficiency promotes IL-17A production *in vitro*

T-bet is a transcriptional activator of IFN-γ and is the key regulator of the T_H1 differentiation program[22]. In addition to promoting the differentiation of naive $CD4^+$ T cells into the T_H1 subset, T-bet actively suppresses development of the T_H2 lineage[14,15]. To investigate whether T-bet expression has a similar antagonistic effect on the development of IL-17A-producing helper T cells, we cultured

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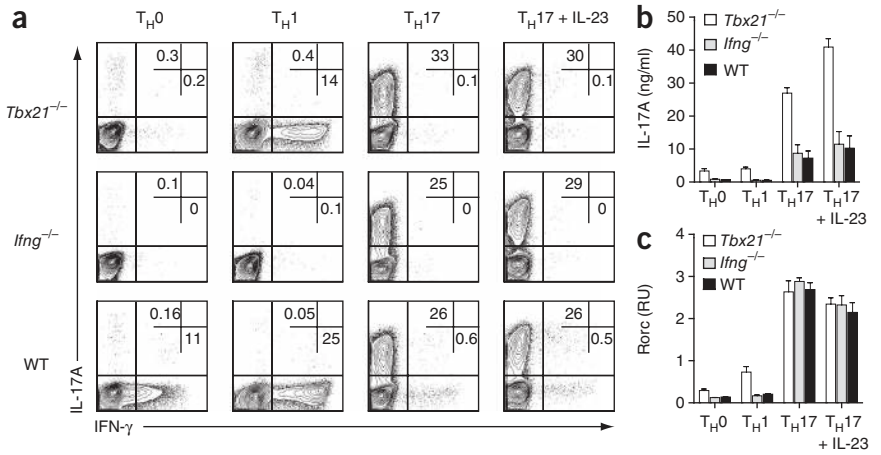

Figure 1 T-bet deficiency promotes IL-17A production *in vitro* independently of IFN-γ. (**a**) Flow cytometry of *Tbx21*$^{-/-}$, *Ifng*$^{-/-}$ and wild-type (WT) CD4$^+$ T cells cultured for 5 d in the presence of IL-2 (T_H0), IL-2, IL-12 and antibody to IL-4 (anti-IL-4; T_H1), or TGF-β, IL-6, anti-IL-4 and anti-IFN-γ with (T_H17 + IL-23) or without (T_H17) subsequent IL-23 treatment, then stimulated for 4 h with PMA and ionomycin, followed by intracellular cytokine staining with anti-IL-17A and anti-IFN-γ. Numbers in plots indicate percent IL-17A$^+$IFN-γ$^-$ cells (top left) or IL-17A$^-$IFN-γ$^+$ cells (bottom right). (**b**) Enzyme-linked immunosorbent assay (ELISA) of IL-17A in supernatants of cells differentiated and stimulated as in **a**. (**c**) RT-PCR analysis of *Rorc* mRNA expression in *Tbx21*$^{-/-}$ and wild-type cells differentiated and stimulated as in **a**; results are presented in relative units (RU) relative to the expression of the housekeeping gene *Hprt1*. Data are representative of four independent experiments (**a**,**b**) or are from two independent experiments (**c**; mean and s.e.m. in **b**,**c**).

Tbx21$^{-/-}$ and wild-type CD4$^+$ T cells under nonskewing conditions or differentiated them into T_H1 cells or into T_H17 cells grown in the presence or absence of IL-23. As IFN-γ has a negative effect on the polarization of T_H17 cells and *Tbx21*$^{-/-}$ T cells produce much less IFN-γ than do wild-type CD4$^+$ T cells, we also tested IFN-γ-deficient (*Ifng*$^{-/-}$) helper T cells to delineate effects of T-bet versus IFN-γ on T_H17 development. After differentiating *Tbx21*$^{-/-}$, *Ifng*$^{-/-}$ and wild-type cells for 5 d *in vitro* under T_H0, T_H1 or T_H17 conditions or under T_H17 conditions with IL-23, we briefly stimulated them with phorbol myristate acetate (PMA) and ionomycin. We observed a higher percentage of IL-17A-producing cells in T-bet-deficient T_H0 and T_H1 cultures than in *Ifng*$^{-/-}$ and wild-type cultures (**Fig. 1a**). Although we detected a similar percentage of IL-17A-producing cells under T_H17-polarizing conditions, more IL-17A was secreted by *Tbx21*$^{-/-}$ helper T cells than by *Ifng*$^{-/-}$ and wild-type helper T cells under all differentiating conditions (**Fig. 1b**).We did not observe a substantial difference among the helper T cell subsets in *Rorc* mRNA expression at 24 h after activation (data not shown). However, the enhanced IL-17A production by *Tbx21*$^{-/-}$ T_H0 and T_H1 cultures correlated with their twofold higher expression of *Rorc* mRNA after 5 d of culture. In contrast, *Tbx21*$^{-/-}$, *Ifng*$^{-/-}$ and wild-type T_H17 cells had similar expression of *Rorc* mRNA (**Fig. 1c**). These results show that T-bet deficiency promotes the development of IL-17A-producing cells under all polarizing conditions independently of IFN-γ and suggest that T-bet-mediated effects on the generation of IL-17A-producing cells *in vitro* may be achieved through the transcriptional regulation of *Rorc* and/or *Il17a* in T_H0-T_H1 and T_H17 cells.

T_H17 responses in *Tbx21*$^{-/-}$ and wild-type mice during EAE

Tbx21$^{-/-}$ mice are protected from the development of EAE[23]. When the results of that study[23] were first reported, T_H17 cells were yet to be discovered, and the resistance of *Tbx21*$^{-/-}$ mice to central nervous system (CNS)-specific autoimmune attack was ascribed to a polarization shift of CD4$^+$ T cells from a pathogenic T_H1 response to a protective T_H2 response[23]. Given the propensity of T-bet-deficient CD4$^+$ T cells to develop into IL-17A-producing cells *in vitro*, we investigated whether *Tbx21*$^{-/-}$ mice generate T_H17 responses during EAE, whose pathology is widely accepted to be dependent on T_H17 cells. To determine the types of cytokines produced by CNS-infiltrating CD4$^+$ T cells, we stained intracellular cytokines in mononuclear cells isolated from the CNS of *Tbx21*$^{-/-}$ and wild-type mice during the peak of disease (day 17 after immunization with a myelin oligodendrocyte glycoprotein peptide of amino acids 35–55 (MOG(35–55)) plus complete Freund's adjuvant (CFA)). In wild-type mice, three different cytokine-producing populations entered the CNS: those that produced IFN-γ alone (most CD4$^+$ T cells), those that produced only IL-17A and those that produced both cytokines (**Fig. 2a**). In contrast, in the CNS of *Tbx21*$^{-/-}$ mice, IL-17A-producing CD4$^+$ T cells represented most of the cytokine-producing cells at day 17 after immunization (**Fig. 2a**). Consistent with the idea of role for T-bet in controlling expression of *Ifng*, there was a deficiency in IFN-γ-producing CD4$^+$ T cells in the CNS of *Tbx21*$^{-/-}$ mice (**Fig. 2a**). Collectively, there was a shift in the T_H1-T_H17 balance in the CNS of *Tbx21*$^{-/-}$ mice during EAE characterized by a tendency toward the recruitment of T_H17 cells and a significantly lower frequency and absolute number of IFN-γ-producing CD4$^+$ T cells (**Fig. 2b**). Moreover, CD4$^+$ T cells isolated from the CNS of *Tbx21*$^{-/-}$ mice secreted significantly more IL-17A than did wild-type CD4$^+$ T cells at day 17 after immunization (**Fig. 2c**). Thus, there was recruitment of IL-17A-secreting T_H17 cells into the CNS of *Tbx21*$^{-/-}$ mice.

IL-23R signaling drives the pathogenic potential of CNS-infiltrating T_H17 cells by promoting the expression of proinflammatory chemokines and by suppressing IL-10 expression[4,24]. In addition, IL-23R signaling is essential for the terminal differentiation of T_H17 cells and their long-term survival[13,25]. To address the role of T-bet in controlling IL-23R expression in T_H17 cells, we crossed reporter mice that express green fluorescent protein (GFP)-tagged IL-23R (IL-23R.GFP) onto a T-bet-deficient background. On day 17 after immunizing *Tbx21*$^{-/-}$ and wild-type IL-23R.GFP reporter mice with MOG(35–55) plus CFA, we isolated mononuclear cells from the draining lymph nodes, spleen and CNS and determined the percentage of IL-23R$^+$ cells in the CD4$^+$ population by flow cytometry. The *Tbx21*$^{-/-}$ mice had higher percentage of IL-23R$^+$ CD4$^+$ cells in the lymph nodes and the CNS than did the control wild-type mice, whereas in spleen the frequency of IL-23$^+$ CD4$^+$ T cells was similar (**Fig. 2d**). In addition, there was a significantly higher percentage of CD4$^+$ cells expressing the T_H17-specific chemokine receptor CCR6 in the lymph nodes and CNS of *Tbx21*$^{-/-}$ mice (**Fig. 2d**). We assessed the expression of genes encoding other T_H17 signature molecules in purified CD4$^+$ T cells isolated from the CNS of *Tbx21*$^{-/-}$ and wild-type mice during the disease peak and observed more transcripts of *Rorc*, *Il23r*, *Il17a* and *Il17f* in *Tbx21*$^{-/-}$ CD4$^+$ T cells, whereas the amount of *Il21* and *Il22* transcripts was more variable in *Tbx21*$^{-/-}$ versus wild-type mice (**Fig. 2e**). The enhanced T_H17 response in *Tbx21*$^{-/-}$ mice could have

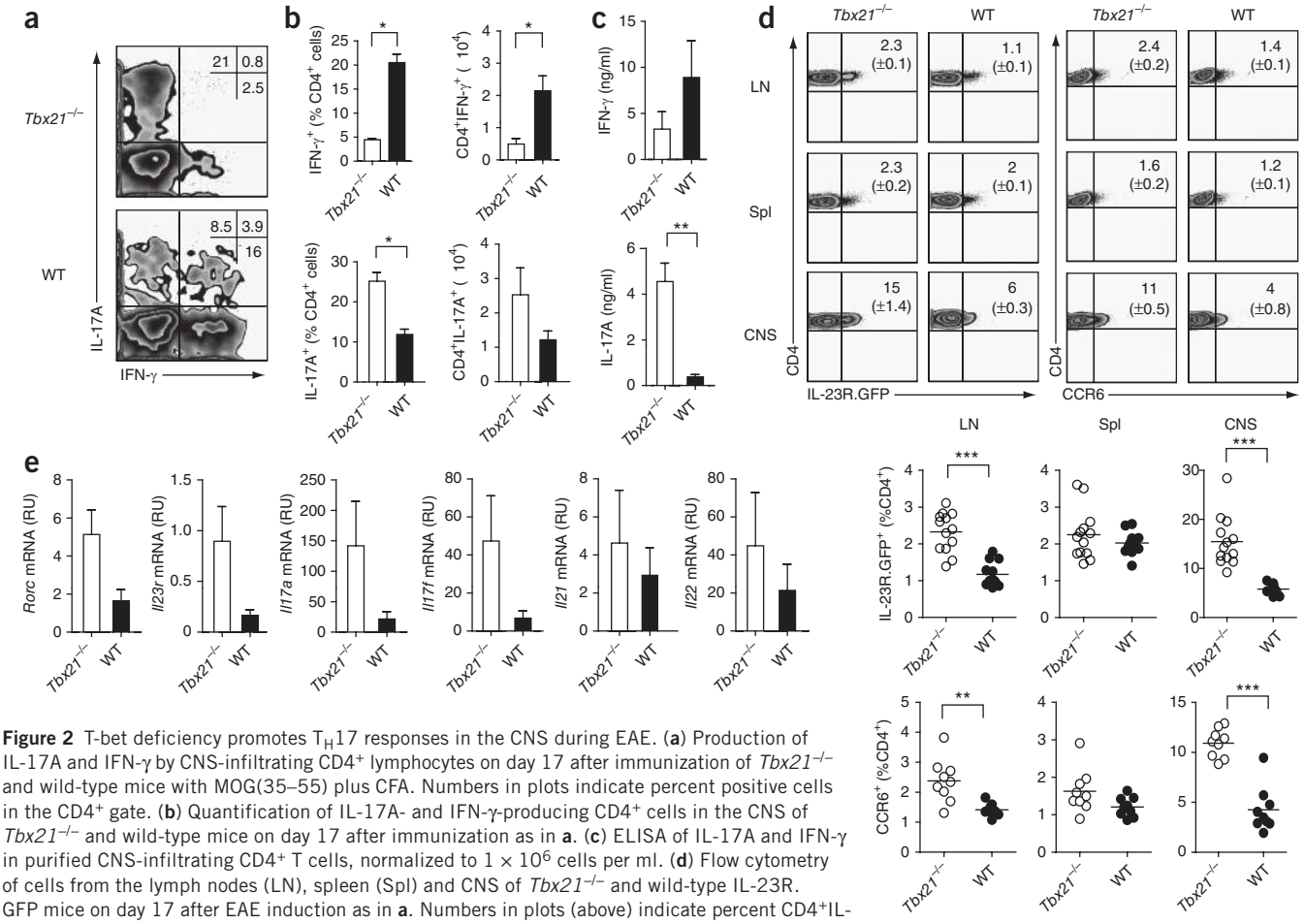

Figure 2 T-bet deficiency promotes T_H17 responses in the CNS during EAE. (**a**) Production of IL-17A and IFN-γ by CNS-infiltrating $CD4^+$ lymphocytes on day 17 after immunization of *Tbx21*$^{-/-}$ and wild-type mice with MOG(35–55) plus CFA. Numbers in plots indicate percent positive cells in the $CD4^+$ gate. (**b**) Quantification of IL-17A- and IFN-γ-producing $CD4^+$ cells in the CNS of *Tbx21*$^{-/-}$ and wild-type mice on day 17 after immunization as in **a**. (**c**) ELISA of IL-17A and IFN-γ in purified CNS-infiltrating $CD4^+$ T cells, normalized to 1×10^6 cells per ml. (**d**) Flow cytometry of cells from the lymph nodes (LN), spleen (Spl) and CNS of *Tbx21*$^{-/-}$ and wild-type IL-23R.GFP mice on day 17 after EAE induction as in **a**. Numbers in plots (above) indicate percent $CD4^+$IL-23R.GFP$^+$ cells (left group) or $CD4^+$CCR6$^+$ cells (right group) in the $CD4^+$ gate (mean and s.e.m.); each symbol (below) represents an individual mouse and small horizontal lines indicate the mean. (**e**) Expression of genes encoding T_H17 signature cytokines in $CD4^+$ T cells purified from the CNS of *Tbx21*$^{-/-}$ and wild-type mice on day 17 after immunization as in **a**, then stimulated for 4 h with PMA and ionomycin before RNA extraction (cells pooled from four mice per group); results are presented relative to *Hprt1* expression. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.0001$ (Student's *t*-test). Data are representative of three independent experiments (**a**) or are from three independent experiments (**b**–**e**; mean and s.e.m.) with three to four mice per group in each.

been intrinsic to the T cell or could have reflected differences in cytokine production by innate immune cells that could create a milieu more conducive to the polarization of T_H17 cells *in vivo*. To differentiate between those two possibilities, we did functional analysis of $CD4^+$ T cells in the CNS 14 d after EAE induction using cells from mice with and without T cell–specific deletion of *lox*P-flanked *Tbx21* alleles by Cre recombinase driven by the *Cd4* promoter (**Supplementary Fig. 1**). We found that T cell–specific deletion of T-bet resulted in an augmented T_H17 response in the CNS during EAE, which suggested that this is a T cell–intrinsic phenomenon. Thus, a dominant T_H17 response is generated in *Tbx21*$^{-/-}$ mice after immunization with MOG(35–55) plus CFA, which suggests that T-bet expression limits the magnitude of T_H17 responses in the CNS during EAE.

T-bet expression blocks T_H17 differentiation

The results reported above suggested that T-bet negatively regulates commitment to the T_H17 lineage. To directly assess whether T-bet has a negative role in T_H17 differentiation, we activated naive $CD4^+$ T cells ($CD62L^{hi}CD25^{lo}$) under T_H17-polarizing conditions. After 24 h, we transduced activated $CD4^+$ cells with empty retrovirus or expressing GFP alone or retrovirus expressing GFP and T-bet in cultures containing T_H17-skewing cytokines plus neutralizing antibodies to IL-4 and IFN-γ. After culturing cells for 5 d under T_H17-polarizing conditions, we assessed the cytokine production of sorted GFP$^+$ cells by intracellular cytokine staining. Transduction of either *Tbx21*$^{-/-}$ or wild-type naive $CD4^+$ T cells with T-bet resulted in a much lower frequency of IL-17A-producing cells and a much greater frequency of cells producing both IFN-γ and IL-17A or IFN-γ alone (**Fig. 3a**). T-bet expression resulted in much lower expression of *Rorc*, RORγt target genes (*Il17a* and *Il17f*), *Il21* and *Il23r* (**Fig. 3b**). Although we observed no substantial difference between *Tbx21*$^{-/-}$ and wild-type T_H17 cells in the amount of *Il22* transcripts, T-bet overexpression augmented *Il22* mRNA expression in both *Tbx21*$^{-/-}$ and wild-type T_H17 cells (**Fig. 3b**). Ectopic expression of T-bet therefore prevents the differentiation of helper T cell precursors into T_H17 cells under T_H17-polarizing conditions by blocking expression of *Rorc* and, consequently, RORγt target genes.

In a separate series of experiments, we used a transgenic inducible T-bet expression system in which T-bet is induced in naive helper T cell precursors in response to treatment with doxycycline. We activated naive $CD4^+$ T cells for 24 h under T_H17-polarizing conditions. On day 2, we induced T-bet expression by administering 0.5 μg/ml of doxycycline in the presence or absence of neutralizing antibody to IFN-γ. We measured *Rorc* and *Tbx21* mRNA and RORγt and T-bet protein by real-time PCR and immunoblot analysis, respectively.

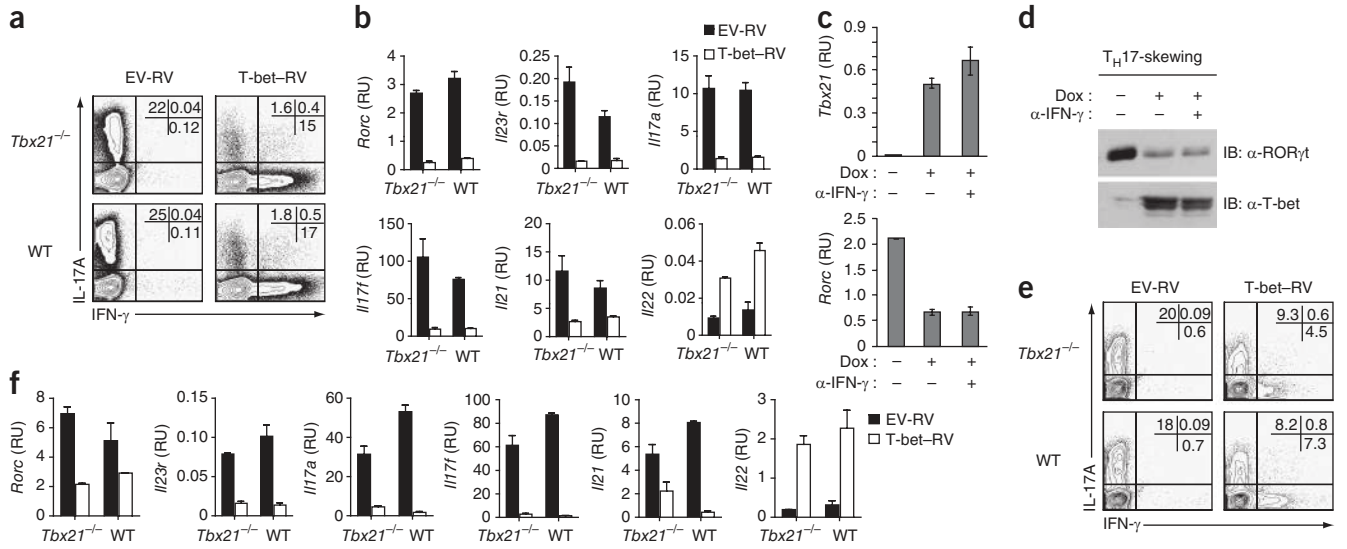

Figure 3 T-bet expression in naive helper T cell precursors and fully differentiated T_H17 cells inhibits the T_H17 response. (**a**) Flow cytometry of the expression of IL-17A and IFN-γ by naive $CD4^+$ T cells transduced with empty retrovirus expressing GFP alone (EV-RV) or retrovirus expressing T-bet and GFP (T-bet–RV) under T_H17-polarizing conditions, then stimulated for 4 h with PMA and ionomycin, followed by intracellular cytokine staining of sorted GFP^+ cells. (**b**) Real-time PCR analysis of *Rorc*, *Il23r*, *Il17a*, *Il17f*, *Il21* and *Il22* mRNA in naive $CD4^+$ T cells transduced with empty or T-bet-expressing retrovirus under T_H17-polarizing conditions. (**c**) Expression of *Tbx21* and *Rorc* mRNA in cells obtained from *Tbx21*$^{-/-}$ mice transgenic for T-bet expression induced in response to doxycycline treatment, left untreated (–) or treated (+) with doxycycline (Dox), then activated under T_H17-polarizing conditions in the presence (+) or absence (–) of anti-IFN-γ (α-IFN-γ). (**d**) Immunoblot analysis (IB) of T-bet and RORγt in the cells in **c**, probed with anti-RORγt (α-RORγt) or anti-T-bet (α-T-bet). (**e**) Flow cytometry analysis of the expression of IL-17A and IFN-γ by fully differentiated T_H17 cells transduced with empty or T-bet-expressing retrovirus under T_H17-polarizing conditions, then stimulated for 4 h with PMA and ionomycin, followed by intracellular cytokine staining. (**f**) Real-time PCR analysis of genes encoding T_H17 signature cytokines in fully differentiated T_H17 cells transduced with empty or T-bet-expressing retrovirus in the presence of T_H17-polarizing cytokines. Numbers in plots (**a**,**e**) indicate percent positive cells in each quadrant; gene or mRNA results (**b**,**c**,**f**) are presented relative to *Hprt1* expression (**b**,**f**) or expression of the housekeeping gene *Actb* (**c**). Data are representative of three independent experiments (mean and s.e.m. in **b**,**c**,**f**).

Induction of T-bet expression in transgenic helper T cell precursors by doxycycline treatment resulted in a much lower abundance of *Rorc* transcripts and RORγt protein under T_H17-polarizing conditions (**Fig. 3c**,**d**). We observed suppression of RORγt by T-bet even in the presence of neutralizing antibody to IFN-γ, which demonstrated that this mode of RORγt suppression is independent of IFN-γ.

T-bet can redirect fully differentiated T_H2 cells into the T_H1 pathway[22]. To determine whether T-bet could similarly reprogram committed T_H17 cells, we differentiated naive $CD4^+$ T cells ($CD62L^{hi}CD25^{lo}$) under T_H17-polarizing conditions for 6 d, then reactivated *Tbx21*$^{-/-}$ and wild-type T_H17 cells for 24 h and transduced them with empty retrovirus expressing GFP alone or retrovirus expressing GFP and T-bet under T_H17-polarizing conditions. We sorted GFP^+ cells 48 h after retroviral transduction and examined intracellular cytokines. We found 50% fewer IL-17A-producing cells and a greater frequency of IFN-γ-producing cells after transduction with T-bet-expressing retrovirus (**Fig. 3e**). Similar to the results reported above, enforced T-bet expression in fully differentiated T_H17 cells resulted in fewer transcripts of *Rorc*, *Il17a*, *Il17f*, *Il21* and *IL23r* and higher expression of *Il22* (**Fig. 3f**). These results indicate that ectopic expression of T-bet is sufficient to suppress expression of genes encoding T_H17 signature cytokines in committed T_H17 cells even in the presence of T_H17-polarizing cytokines.

T-bet inhibits Runx1 activity

Our results above supported the idea that the negative effect of T-bet on T_H17 differentiation could be mediated by inhibition of RORγt. To determine whether T-bet binds to the *Rorc* promoter, we did chromatin-immunoprecipitation (ChIP) assays of T-bet-bound chromatin from nuclear lysates of nonpolarized T_H0 cells and differentiated T_H1 and T_H17 cells. We detected modest but reproducible binding of T-bet to a site located approximately 2 kilobases (kb) upstream of the first exon of *Rorc* (the thymus-specific isoform) in nonskewed T_H0 cells and found considerable enrichment of T-bet bound to that same site in T_H1 cells (**Fig. 4a**,**b**). We did not detect binding of T-bet to the *Rorc* or *Ifng* promoter in T_H17 cells (data not shown). To test whether T-bet directly inhibits *Rorc* expression, we did reporter luciferase assays in HEK293 human embryonic kidney cells with a luciferase reporter spanning 2 kb upstream of *Rorc* exon 1. Transfection of T-bet had no effect on the activity of the *Rorc* luciferase reporter (**Fig. 4c**), which indicated that T-bet might not directly suppress *Rorc* transcription. T-bet generally does not act as a direct transcriptional repressor[15,26,27]. Instead, T-bet exerts its negative effect on gene expression by binding to and sequestering transcriptional activators away from regulatory regions[15,26–29]. Runx1 induces *Rorc* expression[30], and there are two Runx1-binding sites immediately upstream of the T-bet-binding site (2 kb upstream of *Rorc* exon 1). That prompted us to investigate whether T-bet could inhibit *Rorc* expression by blocking the transcriptional activity of Runx1. To analyze the regulation of *Rorc*, we transfected HEK293 cells with the *Rorc* luciferase reporter construct described above in the presence of increasing concentrations of Runx1 plasmid with or without a T-bet expression vector. Runx1 expression increased luciferase activity in a dose-dependent manner, and this was blocked by coexpression of T-bet (**Fig. 4c**). Furthermore, T-bet blocked Runx1-mediated transactivation of the *Rorc* promoter in a dose-dependent manner (**Fig. 4d**). Several other transcription factors (IRF4, BATF and STAT3) control T_H17 differentiation by positively regulating *Rorc* expression[31–33]. The expression

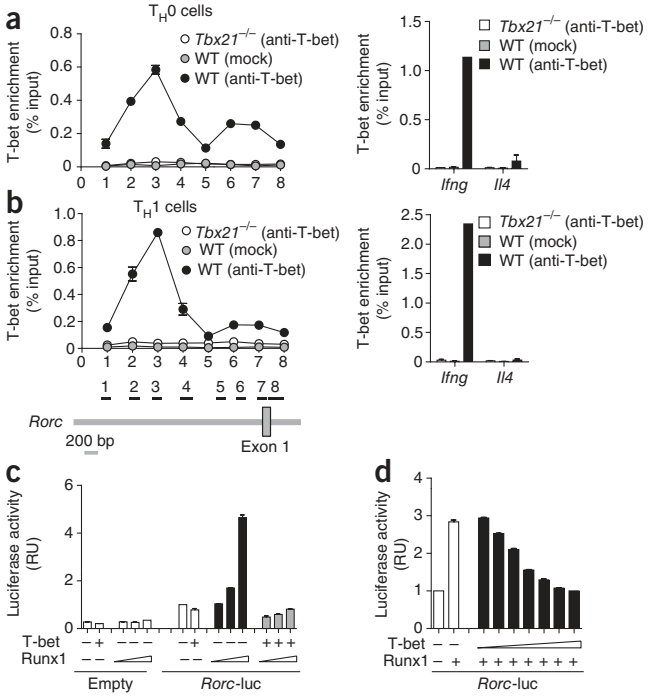

Figure 4 T-bet blocks Runx1-mediated transactivation of the *Rorc* promoter. (**a**,**b**) ChIP analysis of the binding of T-bet to the *Rorc* promoter (numbers along horizontal axis correspond to *Rorc* at bottom left) in wild-type (WT (anti-T-bet)) and *Tbx21*$^{-/-}$ (anti-T-bet) T_H0 cells (**a**) and differentiated T_H1 cells (**b**) after 6 h of stimulation with PMA and ionomycin, as well as in preimmune serum (WT (mock)); the *Ifng* and *Il4* promoters (right) serve as positive and negative controls, respectively. Results are presented relative to input DNA. (**c**) Luciferase activity in HEK293 cells transfected with empty or *Rorc* firefly luciferase reporter (*Rorc*-luc; constructed from a 2-kb fragment of the mouse *Rorc* promoter), plus renilla luciferase reporter, along with increasing concentrations of Runx1 (wedges) in the presence or absence of T-bet; activity was normalized to that of renilla luciferase for transfection efficiency and is presented relative to that of cells transfected with empty vector, set as 1. (**d**) Luciferase activity in HEK293 cells transfected with the *Rorc* luciferase reporter and Runx1 in the presence or absence of increasing concentrations of T-bet (wedge), presented as in **c**. Data are representative of three experiments (**a**,**b**; mean ± s.e.m.) or three (**c**) and two (**d**) independent experiments (mean and s.e.m. of duplicate samples).

of *Irf4* and *Batf* was similar in *Tbx21*$^{-/-}$ and wild-type helper T cells, as determined by RT-PCR (**Supplementary Fig. 2**). It is unknown at present whether regulation of *Rorc* expression by IRF4 and BATF is mediated by direct binding of these transcription factors to the *Rorc* locus, but the binding sites for STAT3 in the *Rorc* and *Il17a* loci have been defined[33]. To assess the binding of STAT3 to its target sequences in the *Rorc* and *Il17a* loci, we did ChIP of STAT3-bound chromatin from nuclear lysates of nonpolarized T_H0 and T_H17 cells. The binding of STAT3 to its target sites in the intergenic and intragenic regions of the *Rorc* locus and *Il17a* locus was similar in *Tbx21*$^{-/-}$ and wild-type T_H0 and T_H17 cells (**Supplementary Fig. 3**). These data suggest that interference with the transcriptional activity of Runx1 is the likely mechanism by which T-bet blocks *Rorc* expression, but interference with STAT3 is not.

T-bet interacts with Runx1

To examine further the mechanism of T-bet-mediated repression of *Rorc* expression, we investigated whether T-bet interacted with Runx1. First, we overexpressed T-bet with Myc-tagged Runx1, Runx2 or Runx3 in HEK293 cells and did coimmunoprecipitation experiments. T-bet interacted with both Runx1 and Runx3, but not with Runx2, in HEK293 cells (**Fig. 5a**). To determine in which helper T cell subset T-bet and Runx1 interact, we did immunoblot analysis of the expression of Runx1 and T-bet in unskewed T_H0 and differentiated T_H1 and T_H17 cells. We detected expression of T-bet and Runx1 in nonpolarized T_H0 cells (**Fig. 5b**). There was much lower expression of Runx1 in T_H1 cells differentiated *in vitro* and, conversely, much less T-bet in T_H17 cells differentiated *in vitro* (**Fig. 5b**). These data suggested that T-bet and Runx1 could interact in

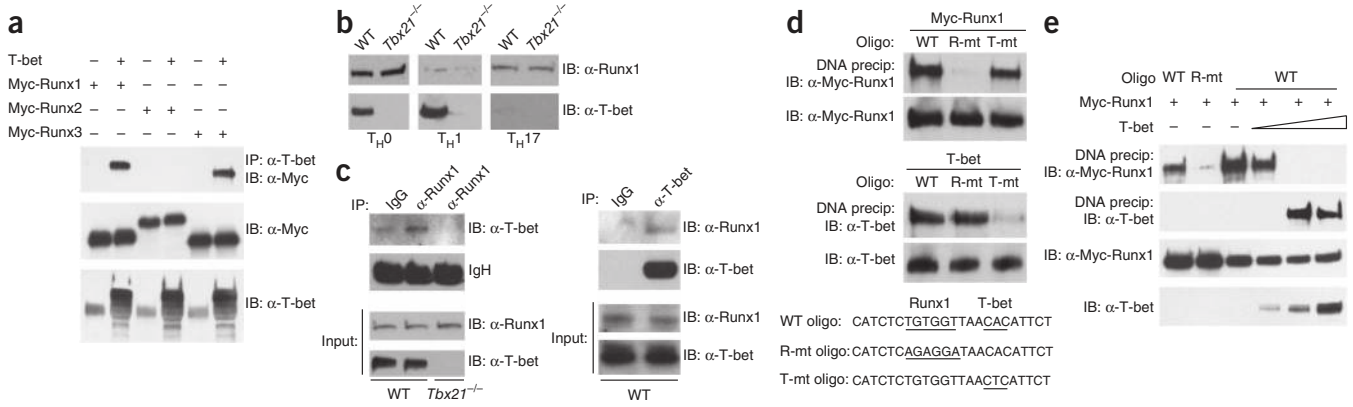

Figure 5 Interaction of T-bet with Runx1. (**a**) Immunoprecipitation (IP; with anti-T-bet) of lysates of HEK293 cells transfected with various combinations of empty vector or expression vector for T-bet or Myc-tagged Runx1, Runx2 or Runx3 (above blot), followed by immunoblot analysis (IB) with anti-Myc or anti-T-bet. (**b**) Expression of Runx1 and T-bet protein in *Tbx21*$^{-/-}$ and wild-type T_H0, T_H1 and T_H17 cells after 6 h of stimulation with PMA and ionomycin. (**c**) Immunoprecipitation (with control immunoglobulin G (IgG), anti-Runx1 or anti-T-bet) of lysates of *Tbx21*$^{-/-}$ and wild-type T_H0 cells, followed by immunoblot analysis immunoprecipitates (top) or input protein (bottom) with anti-T-bet or anti-Runx1. IgH, additional loading control. (**d**) DNA-precipitation assay (DNA precip) and immunoblot analysis of HEK293 cells transfected with oligonucleotide containing wild-type Runx1- and T-bet-binding sites (WT oligo) or a mutated Runx1-binding site (R-mt oligo) or T-bet-binding site (T-mt oligo) at a site 2 kb upstream of *Rorc* exon 1, in the presence of Myc-tagged Runx1 or T-bet. Below, oligonucleotide sequence; underlining indicates binding sites. (**e**) DNA-precipitation assay and immunoblot analysis (top two blots) of HEK293 cells transfected with oligonucleotide with a wild-type or mutated Runx1-binding site (as in **d**), plus Myc-tagged Runx1, in the presence or absence of increasing doses of T-bet (wedge; 0.1, 0.5 and 1 μg). Bottom two blots, immunoblot analysis of input DNA (samples without precipitation). Data are representative of one to two independent experiments.

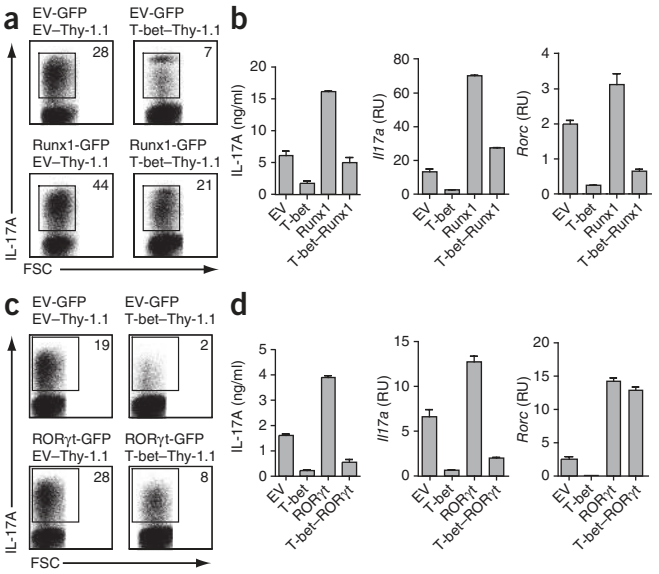

Figure 6 Runx1 overexpression restores IL-17A production in T_H17 cells expressing T-bet. (**a**) Flow cytometry of CD4$^+$ T cells transduced with various combinations of retrovirus expressing GFP, Thy-1.1, Runx1-GFP or T-bet–Thy-1.1 (above plots) within 24 h of activation, then cultured for 5 d under T_H17-polarizing conditions and stimulated for 4 h with PMA and ionomycin before intracellular cytokine staining for IL-17A. Numbers adjacent to outlined areas indicate percent IL-17A-producing cells in the GFP$^+$Thy-1.1$^+$ gate. FSC, forward scatter. (**b**) Production of IL-17A and expression of *Il17a* and *Rorc* by CD4$^+$ T cells transduced with various combinations of retroviruses as in **a**, then differentiated for 5 d under T_H17-polarizing conditions, followed by sorting of GFP$^+$Thy-1.1$^+$ cells and stimulation for 4 h with PMA and ionomycin. (**c**) Flow cytometry of activated CD4$^+$ T cells transduced with various combinations of retrovirus expressing GFP, Thy-1.1, RORγt-GFP or T-bet–Thy-1.1 (above plots), then cultured for 5 d under T_H17 conditions and stimulated for 4 h with PMA and ionomycin before intracellular cytokine staining for IL-17A. (**d**) ELISA of IL-17A production and RT-PCR analysis of *Il17a* and *Rorc* mRNA transcripts of cells transduced under T_H17 conditions as described in **c**. Numbers adjacent to outlined areas (**a**,**c**) indicate percent IL-17A-producing cells in the GFP$^+$Thy-1.1$^+$ gate; gene or mRNA expression (**b**,**d**) is presented relative to *Hprt1* expression. Data are representative of two independent experiments (error bars (**b**,**d**), s.e.m.).

nonpolarized T_H0 cells in which both proteins were coexpressed. Indeed, Runx1-immunoprecipitation confirmed the presence of an endogenous T-bet–Runx1 interaction in nonpolarized wild-type T_H0 cells but not in differentiated T_H1 or T_H17 cells (**Fig. 5c** and data not shown). We also confirmed interaction between T-bet and Runx1 in uncommitted T_H0 cells in reverse coimmunoprecipitation assays (**Fig. 5c**). These data suggest that a functionally important interaction between T-bet and Runx1 most probably occurs in uncommitted helper T cells but not in fully differentiated T_H1 and T_H17 cells because of restrictive expression of Runx1 and T-bet, respectively, in those helper T cells.

On the basis of our luciferase data and coimmunoprecipitation results, we hypothesized that interaction between T-bet and Runx1 could block the binding of Runx1 to its consensus sites located 2 kb upstream of the *Rorc* exon 1. We detected binding of Runx1 to an oligonucleotide containing wild-type Runx1 target sequence but not to an oligonucleotide in which the Runx1 target sequence was mutated by a T-to-A substitution (**Fig. 5d**). T-bet bound to a wild-type oligonucleotide containing a T-bet-specific half-site but not to an oligonucleotide in which the T-bet half-site was mutated (**Fig. 5d**). After confirming that the binding of Runx1 and T-bet to the site 2 kb upstream of the *Rorc* exon 1 was sequence specific, we did a DNA-precipitation assay with the wild-type oligonucleotide and Runx1 in the presence or absence of increasing concentrations of T-bet. In the absence of T-bet, Runx1 bound strongly to the oligonucleotide containing the wild-type Runx1-binding site (**Fig. 5e**). Increasing concentrations of T-bet ablated the ability of Runx1 to bind to its target sequence in a dose-dependent manner (**Fig. 5e**), which showed that interaction of T-bet with Runx1 interferes with the binding of Runx1 to its site 2 kb upstream of the *Rorc* exon 1.

Runx1 reverses the effect of T-bet on T_H17 polarization

Next we investigated whether Runx1 overexpression could block the inhibitory effects of T-bet on T_H17 differentiation. We transduced purified CD4$^+$ T cells with various combinations of retroviruses expressing GFP alone, Thy-1.1 alone, GFP-tagged Runx1 and/or T-bet–Thy-1.1. We grew the transduced cells for 5 d under T_H17 conditions, then sorted double-positive GFP$^+$Thy-1.1$^+$ cells and assessed their T_H17 commitment. Retroviral transduction of helper T cells with T-bet had a negative effect on T_H17 commitment under T_H17-polarizing conditions, whereas transduction of helper T cells with Runx1 augmented the differentiation of T_H17 cells (**Fig. 6a**,**b**). Overexpression of Runx1 reversed the inhibitory effect of T-bet and fully restored T_H17 polarization in cells coexpressing Runx1 and T-bet. However, *Rorc* expression was only partially upregulated (**Fig. 6b**), which suggested that there may be additional mechanisms by which T-bet inhibits *Rorc* transcription in T_H17 cells. Conversely, transduction of purified *Tbx21*$^{-/-}$ CD4$^+$ T cells with a retrovirus expressing dominant negative Runx1 during T_H17 differentiation reversed the effects of T-bet deficiency on IL-17A production by T_H17 cells (**Supplementary Fig. 4**).

In addition to directly promoting *Rorc* expression, Runx1 also acts as a coactivator, and together with RORγt, it induces expression of *Il17a* and *Il17f*[30]. T-bet suppressed T_H17 differentiation by inhibiting *Rorc* expression (**Fig. 3**). However, it is possible that the interaction of T-bet with Runx1 serves to sequester this transcriptional coactivator and blocks the expression of genes encoding T_H17 signature cytokines in this manner. To investigate that possibility, we sought to determine whether RORγt was able to restore a T_H17 developmental program in helper T cells coexpressing T-bet and RORγt under T_H17-polarizing conditions. Expression of RORγt independently of a T-bet transcriptional block (from retroviral long terminal repeat control elements) in developing T_H17 cells was unable to fully reverse the T-bet-mediated inhibition of T_H17 differentiation (**Fig. 6c**,**d**). We were unable to coimmunoprecipitate T-bet and RORγt in HEK293 cells, which suggested that sequestration of RORγt from its target genes by T-bet is unlikely. These data demonstrate that in addition to inhibiting *Rorc* transcription, the interaction of T-bet with Runx1 depletes the pool of free Runx1 available for the formation of transcriptionally active Runx1-RORγt complexes in T_H17 cells.

T-bet Tyr304 is crucial for the suppression of T_H17 cells

To investigate which amino acid residue is important for formation of the T-bet–Runx1 complex, we tested the ability of a series of T-bet mutants to interact with Runx1 in HEK293 cells. Two of these mutants with point substitution, T-bet(S508A) and T-bet(Y525F), have been shown before to be functionally important in the repression of *Il2* transcription in T_H1 cells and in T_H2 lineage suppression, respectively[15,27]. Coimmunoprecipitation studies showed that Runx1 interacted with the mutants T-bet(Y265F), T-bet(S508A)

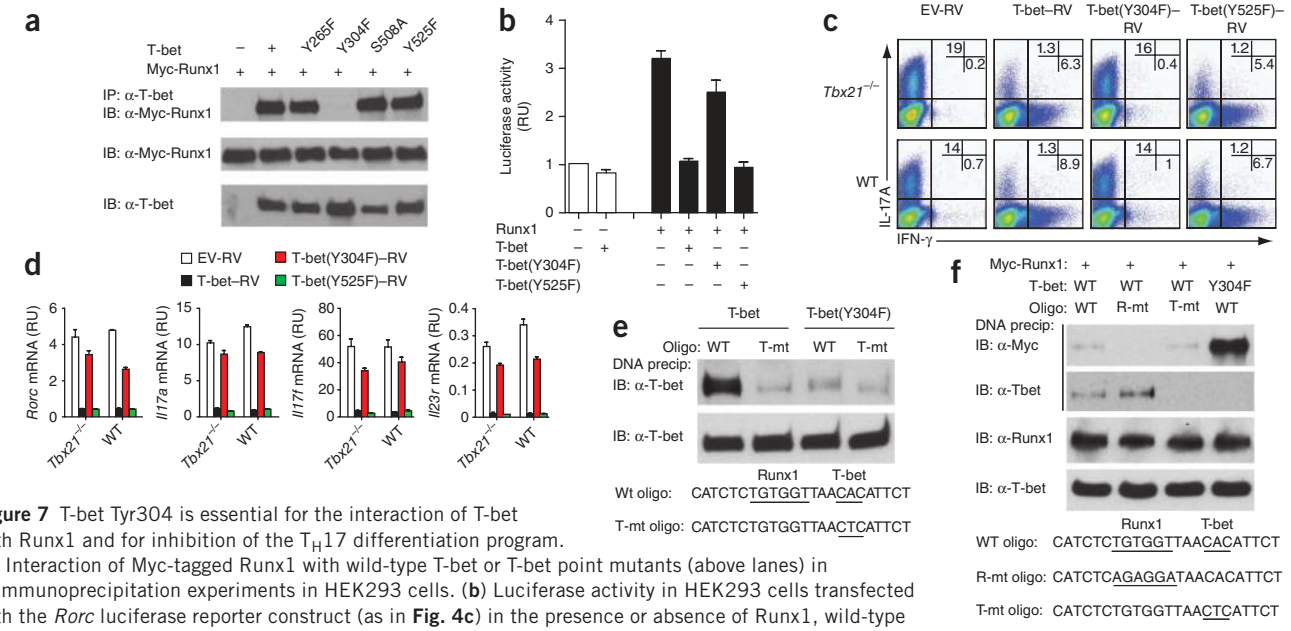

Figure 7 T-bet Tyr304 is essential for the interaction of T-bet with Runx1 and for inhibition of the T_H17 differentiation program. (**a**) Interaction of Myc-tagged Runx1 with wild-type T-bet or T-bet point mutants (above lanes) in coimmunoprecipitation experiments in HEK293 cells. (**b**) Luciferase activity in HEK293 cells transfected with the *Rorc* luciferase reporter construct (as in **Fig. 4c**) in the presence or absence of Runx1, wild-type T-bet or T-bet(Y304F) or T-bet(Y525F) (below graph), assessed as in **Figure 4c**. (**c**) Flow cytometry analysis of the expression of IL-17A and IFN-γ by naive $CD4^+$ T cells transduced with control (empty) retrovirus (EV-RV) or retrovirus encoding wild-type T-bet (T-bet–RV), T-bet(Y304F) (T-bet(Y304F)–RV) or T-bet(Y525F) (T-bet(Y525F)–RV) under T_H17-polarizing conditions. Numbers in plots indicate percent $IL\text{-}17A^+IFN\text{-}\gamma^-$ cells (top left) or $IL\text{-}17A^-IFN\text{-}\gamma^+$ cells (bottom right) in the $CD4^+$ gate. (**d**) RT-PCR analysis of genes encoding T_H17 signature cytokines by $CD4^+$ T cells transduced with as in **c**; results are presented relative to *Hprt1* expression. (**e**) DNA-precipitation assay and immunoblot analysis of HEK293 cells transfected with wild-type T-bet or T-bet(Y304F) in the presence of oligonucleotide containing a wild-type or mutated T-bet-binding site (as in **Fig. 5d**). (**f**) DNA-precipitation assay and immunoblot analysis of HEK293 cells transfected with Myc-tagged Runx1, wild-type T-bet or T-bet(Y304F) in the presence of oligonucleotide containing wild-type or mutated Runx1- or T-bet-binding sites (as in **Fig. 5d**), probed with anti-Myc and anti-T-bet. Data are representative of three independent experiments (**a**–**d**; mean and s.e.m. in **b**,**d** or one experiment (**e**,**f**).

and T-bet(Y525F) but not with the T-bet(Y304F) mutant (**Fig. 7a**). Furthermore, T-bet(Y304F) was unable to suppress Runx1 transcriptional activity in the luciferase assay described above (luciferase reporter ~2 kb upstream of *Rorc* exon 1; **Fig. 7b**), which suggested that T-bet Tyr304 may be important for the suppression of commitment to the T_H17 lineage. To clarify this, we activated sorted naive helper T cells for 24 h under T_H17-polarizing conditions and transduced them with control retrovirus or retrovirus encoding wild-type T-bet, T-bet(Y304F) or T-bet(Y525F) (control mutant). We cultured the transduced cells under T_H17-polarizing conditions for an additional 5 d and determined the frequency of IFN-γ- and IL-17-producing cells by intracellular cytokine staining. Wild-type T-bet and the T-bet(Y525F) control mutant suppressed T_H17 differentiation under T_H17-polarizing conditions. In contrast, the Y304F substitution abrogated the ability of T-bet to repress T_H17 lineage commitment (**Fig. 7c**). In contrast to wild-type T-bet and the T-bet(Y525F) mutant, T-bet(Y304F) was unable to suppress expression of *Rorc*, *Il17a*, *Il17f* and *Il23r* in developing T_H17 cells (**Fig. 7d**). These data indicate that T-bet Tyr304 is important for the formation of the T-bet–Runx1 complex, for the inhibition of Runx1 transcriptional activity and for the suppression of T_H17 lineage development.

In DNA-precipitation assays, the T-bet(Y304F) mutant failed to bind to the T-bet-binding site in the region 2 kb upstream of the *Rorc* exon 1 (**Fig. 7e**), which suggested that this residue is also important for the binding of T-bet to DNA. To delineate whether T-bet-mediated inhibition of Runx1 activity was dependent on binding to DNA or the T-bet–Runx1 (protein-protein) interaction, we investigated whether wild-type T-bet inhibited the binding of Runx1 to its target site; for this, we used oligonucleotides in which the T-bet-binding site was mutated. T-bet blocked the binding of Runx1 to the Runx1-specific sequence independently of the ability of T-bet to bind to DNA (**Fig. 7f**). Collectively, these data suggest that the T-bet–Runx1 (protein-protein) interaction is mainly responsible for the inhibition of Runx1 activity, with the T-bet (protein)–DNA interaction having a minor (if any) role.

DISCUSSION

Lineage-specific transcription factors can both activate and repress differentiation programs. T-bet simultaneously promotes T_H1 differentiation and represses T_H2 differentiation[3,6]. Although several studies have reported an enhanced T_H17 response in $Tbx21^{-/-}$ mice in various disease models[16–19], no mechanistic explanation for this was provided. Here we have demonstrated that T-bet suppressed commitment to the T_H17 lineage by inhibiting transcription of the gene encoding the T_H17 cell–specific transcription factor RORγt and its target genes. T-bet did not directly repress the *Rorc* promoter. Instead, T-bet interacted with Runx1 and blocked Runx1-mediated transactivation of *Rorc*. Overexpression of Runx1 was sufficient to reverse the inhibitory effects of T-bet on IL-17A production by T_H17 cells. Furthermore, T-bet Tyr304 was crucial not only for formation of the T-bet–Runx1 complex but also for blocking Runx1 activity and inhibiting the T_H17 differentiation program. Thus, our data have identified a molecular mechanism to explain the exaggerated T_H17 responses observed in T-bet-deficient hosts.

In addition to activating a set of genes that promote the differentiation of helper T cells toward a specific lineage, a master regulator can also suppress the developmental program of the opposing T cell lineages[34]. Among the helper T cell–specific transcription factors, T-bet seems to be particularly active in this. The ability of T-bet to negatively regulate T_H2 differentiation[15,22], IL-2 production by T_H1 cells[27] and the production of tumor necrosis factor in

dendritic cells[26] prompted us to investigate the contribution of T-bet to regulation of the T_H17 response. Our data have shown that T-bet deficiency resulted in an augmented T_H17 response *in vitro* and *in vivo* during CNS inflammation in the EAE model of multiple sclerosis. Our results differ from those of published studies reporting that mice injected with T-bet-specific small interfering RNA have lower expression of IL-23R and lack T_H17 cells after immunization with myelin basic protein plus CFA or after immunization with MOG(35–55) plus CFA[35,36]. We found instead that T_H17 cells were present in the CNS of *Tbx21*$^{-/-}$ mice in greater numbers and had high expression of genes encoding T_H17 signature cytokines after induction of EAE via MOG(35–55) plus CFA. The disparity between those studies[35,36] and our results here may arise from differences in experimental conditions, such as the use of T-bet-specific small interfering RNA rather than complete genetic deletion *in vivo*. In support of our results, one of the studies noted above also detected a greater frequency of myelin-specific T_H17 cells in *Tbx21*$^{-/-}$ mice than wild-type mice after immunization with MOG(35–55) plus CFA[36]. Notably, despite their strong T_H17 response, *Tbx21*$^{-/-}$ mice were largely protected from the development of EAE[36]. To explain that observation, we postulate that in the inflammatory milieu of the CNS, T-bet controls the expression of a newly identified set of genes important for the pathogenicity of T_H17 cells but not for the development of T_H17 cells. In support of that hypothesis, a study has reported that T-bet is expressed in IL-23-treated T_H17 cells and that these T-bet-expressing T_H17 cells are pathogenic during CNS inflammation, which indicates the important function of T-bet in a subset of T_H17 cells[37]. Alternatively, the presence of both T_H1 cells and T_H17 cells might be required for CNS pathology. Finally, T-bet expression in other cell types might be important for driving disease development.

Here we focused on the function of T-bet in the commitment of CD4$^+$ T cells to the T_H17 lineage. We found that T-bet overexpression in naive helper T cell precursors or committed T_H17 cells had a negative effect on *Rorc* transcription and consequently on the expression of RORγt target genes. In addition, we observed downregulation of *Il21* expression. T-bet suppresses IL-21 in T_H1 cells by interacting with the transcription factor NFATc2, thus preventing NFATc2 from binding to the *Il21* promoter[29]. As IL-21 promotes IL-23R expression in T_H17 cells[10], T-bet-mediated suppression of *Il21* could also contribute to the lower expression of *Il23r* in T_H17 cells after transduction with a retrovirus expressing T-bet. In contrast to the expression of *Rorc*, *Il17a*, *Il17f* and *Il23r*, which was suppressed by T-bet, *Il22* expression was augmented by ectopic expression of T-bet. The aryl hydrocarbon receptor (AhR) controls IL-22 production by T_H17 cells, as CD4$^+$ T_H17 cells from AhR-deficient mice fail to produce IL-22 when exposed to AhR ligands[38,39]. Thus, it is plausible that T-bet expression has a synergistic effect on the AhR-mediated induction of IL-22.

We detected binding of endogenous T-bet at a region 2 kb upstream of the first *Rorc* exon in nonskewed and T_H1 cells but not in T_H17 cells. Notably, differentiation of helper T cells down the T_H1 pathway resulted in much lower expression of Runx1. Conversely, culture of helper T cells in the presence of T_H17-polarizing cytokines resulted in suppression of the expression of T-bet protein. This was not unexpected, as TGF-β has a negative effect on T-bet expression, and IFN-γ, the most potent inducer of T-bet expression, is neutralized under *in vitro* T_H17-polarizing conditions. Thus, the lack of binding of T-bet to the *Rorc* promoter in committed T_H17 cells could be explained by much lower expression of T-bet in this helper T cell subset.

We did not detect any substantial effect of T-bet overexpression on *Rorc* promoter activity in luciferase assays. Evidence that T-bet acts as a direct transcriptional repressor or can recruit corepressors to promoters is lacking at present. However, T-bet can exert a negative regulatory effect on gene expression by blocking the activity of competing transcription factors[15,26,27]. Understanding of the transcriptional regulation of *Rorc* is still incomplete. It has been reported that *Rorc* expression is much lower in *Irf4*$^{-/-}$ and *Stat3*$^{-/-}$ CD4$^+$ cells[31,40–42]. Runx1 induces *Rorc* expression, and BATF is important for the maintenance of *Rorc* expression in stimulated T cells[30,32]. It is unknown at present whether regulation of *Rorc* expression by IRF4 and BATF is mediated by direct binding of these transcription factors to the *Rorc* locus, but the binding sites for STAT3 in the *Rorc* and *Il17a* loci have been identified[33]. The binding of STAT3 to both *Rorc* and *Il17a* was similar in *Tbx21*$^{-/-}$ and wild-type helper T cells. Hence, we focused on the transcriptional activity of Runx1, as we detected two Runx1 consensus sites in the proximity of the site for peak binding of T-bet, 2 kb upstream of the first exon of *Rorc*, as shown by ChIP.

On the basis of our protein-expression data and coimmunoprecipitation experiments, we propose that the functionally important interaction of T-bet and Runx1 occurs in uncommitted helper T cells. We mapped the function of T-bet in its interaction with Runx1, repression of Runx1 activity and suppression of the T_H17 differentiation program to Tyr304. Our preliminary data indicated that this tyrosine residue was not phosphorylated and that the protein was stably expressed in HEK293 cells and primary T cells. In DNA-precipitation assays, the T-bet(Y304F) mutant failed to bind to DNA. Although Tyr304 was important for the interaction of T-bet with DNA, our results have indicated that this interaction was not essential for the ability of T-bet to block Runx1. Wild-type T-bet was still able to block the binding of Runx1 to its target site in a *Rorc* promoter bearing a mutated T-bet half-site. Thus, the T-bet–Runx1 (protein-protein) interaction is the main mechanism by which T-bet blocks Runx1 activity by sequestering Runx1 away from the *Rorc* promoter.

In both infectious and autoimmune diseases, T cell–mediated responses are characterized by the presence of cells coexpressing IL-17A and IFN-γ, the so-called 'IFN-γ$^+$ T_H17 cells'. T cell clones from mice immunized with a peptide of myelin basic protein are either T-bet$^+$RORγt$^-$ or T-bet$^+$RORγt$^+$ (ref. 28). T-bet$^+$RORγt$^+$ cells are very responsive to exogenous cytokines such as IL-12 and IL-23, which influence the relative amounts of T-bet and RORγt and shift cytokine production toward IFN-γ or IL-17A, respectively[28]. The unstable phenotype of T_H17 cells is not restricted to CNS-specific autoimmunity. Studies of IL-17F reporter mice in a transfer model of colitis have demonstrated that IFN-γ-producing CD4$^+$ T cells can emerge from T_H17-committed cells during T cell–driven inflammation. This transition of T_H17 cells into IFN-γ-producing cells is dependent on STAT4 and T-bet[21]. In the context of those findings, we are tempted to propose that this T-bet-mediated transition of T_H17 cells into a 'T_H1-like' subset is controlled partly by T-bet-mediated interference with the transcriptional activity of Runx1. Ectopic expression of T-bet in T_H17 cells results in suppression of *Rorc*. However, in fully differentiated T_H17 cells, which already express RORγt, T-bet could still interfere with transcriptional activity of RORγt by sequestering its coactivator, Runx1. Overexpression of Runx1 in T_H17 cells overcame the inhibitory effect of T-bet and completely restored IL-17A production. Although Runx1 fully restored IL-17A production, *Rorc* expression was only slightly higher. These results indicate that there are additional mechanisms by which T-bet inhibits *Rorc* expression. One potential mechanism could be the induction of repressive epigenetic changes in the *Rorc* locus by IL-12 signaling,

which are dependent on STAT4 and T-bet[43]. Thus, T-bet re-expression in T_H17 cells turns off *Rorc* expression through the sequestration of Runx1 and through the introduction of epigenetic changes that result in the expression of genes encoding T_H1 signature molecules and acquisition of the 'T_H1-like' phenotype by T_H17 cells.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

V.L. designed and did experiments and prepared the manuscript; X.C. did ChIP assays; J.-H.S. did DNA-precipitation and coimmunoprecipitation assays; E.-S.H. created T-bet mutant retroviral constructs; E.J. did doxycycline transgenic T cell experiments; M.O. generated IL-23R.GFP mice; V.K.K. contributed to discussions and manuscript preparation; A.N.B. provided technical assistance; and L.H.G. supervised the research, designed experiments and participated in preparing the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/natureimmunology/.

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ONLINE METHODS

Mice. *Tbx21*$^{-/-}$ mice and wild-type and *Tbx21*$^{-/-}$ IL-23R.GFP mice (all on a C57BL/6 background) were housed at Harvard School of Public Health and were handled in accordance with guidelines from the Center for Animal Resources and Comparative Medicine of Harvard Medical School. *Tbx21*$^{-/-}$ mice in which T-bet is induced in naive helper T cell precursors in response to treatment with doxycycline were housed at College of Pharmacy of Ewha Womans University. *Ifng*$^{-/-}$ mice were from Jackson Laboratories. Mice with *lox*P-flanked *Tbx21* alleles and mice with expression of Cre recombinase driven by the *Cd4* promoter were provided by S.L. Reiner and C.B. Wilson, respectively.

Plasmids. Constitutively active STAT3 (pRc-CMV STAT3-C) was from Addgene (submitted by J.E. Darnell)[44]. The retroviral plasmids pMCsIg-EV, pMCsIg-Runx1 and pMCsIg-Runx1–dominant negative were provided by W. Strober.

CD4$^+$ T helper differentiation *in vitro*. *Tbx21*$^{-/-}$, *Ifng*$^{-/-}$ and wild-type CD4$^+$ T cells were stimulated for 48 h with anti-CD3 (2 μg/ml; 145-2C11; eBioscience) in the presence of irradiated splenocytes at a ratio of 5:1. CD4$^+$ T cells were cultured under T_H0 conditions (200 U/ml of human IL-2; National Cancer Institute Biological Resources Branch Preclinical Repository) or were differentiated into T_H1 cells by culture for 5 d with human IL-2 (200 U/ml), mouse IL-12 (10 ng/ml; Peprotech) and anti–mouse IL-4 (10 μg/ml; 11B11; BioXCell) or into T_H17 cells by culture for 5 d with human TGF-β (2 ng/ml; R&D Systems), mouse IL-6 (20 ng/ml; R&D Systems), anti–mouse IL-4 (10 μg/ml) and anti–mouse IFN-γ (10 μg/ml; XMG1.2; BioXCell). Cells differentiated under T_H17 conditions with IL-23 were cultured in the presence of human TGF-β, mouse IL-6, anti-IL-4 and anti-IFN-γ for the first 48 h of activation, then were cultured in the presence of IL-23 (10 ng/ml; R&D Systems).

Isolation and functional analysis of CNS mononuclear cells. EAE was induced in 8- to 10-week-old mice as described[23]. CNS-infiltrating cells were restimulated for 4 h with PMA and ionomycin before intracellular cytokine staining with anti-IFN-γ and anti-IL-17A (TC11-18H10; BD Pharmingen). For ELISA and RT-PCR analysis, CNS-derived CD4$^+$ T cells were purified with a CD4$^+$ T Cell Enrichment kit (negative selection; Stem Cell Technologies). Purified CD4$^+$ T cells (>95% purity) were pooled from four to five mice per group and cells were stimulated for 4 h with PMA and ionomycin. The RT-PCR primer sequences are in **Supplementary Table 1**.

Retroviral transduction. Naive (CD62L^{hi}CD25^{lo}) CD4$^+$ T cells were transduced with retroviruses at 24 h after activation. Transduced cells were cultured for 5 d under T_H17 conditions, then sorted GFP$^+$ cells were tested for cytokine production after 4 h of stimulation with PMA and ionomycin. For transduction of committed T_H17 cells, naive CD4$^+$ T cells were cultured for 6 d under T_H17 conditions. T_H17 cells were activated on plates coated with plate-bound anti-CD3 (145-2C11; eBioscience) and anti-CD28 (37.51; BD Pharmingen) and were transduced for 24 h with retrovirus under T_H17 conditions. Transduced cells were cultured for an additional 48 h under T_H17 conditions. Sorted GFP$^+$ were stimulated for 4 h with PMA and ionomycin before functional analysis.

***In vitro* culture of doxycycline-inducible T-bet-transgenic T_H17 cells.** At 24 h after activation with anti-CD3 and anti-CD28, T-bet was induced in helper T cells by the addition of 0.5 μg/ml of doxycycline to the T_H17 culture media. Then, 24 h after induction, lysates were analyzed by RT-PCR for expression of *Tbx21* and *Rorc*. In addition, cells were analyzed by immunoblot with polyclonal anti-T-bet (9856; prepared in-house) and anti-RORγt (B2D; eBioscience).

ChIP. Naive cells were sorted from spleens and lymph nodes of *Tbx21*$^{-/-}$ and wild-type mice and were differentiated under T_H0-, T_H1- and T_H17-polarizing conditions as described above. Differentiated helper T cells were analyzed by ChIP as described[45] after 6 h of stimulation with PMA and ionomycin, followed by real-time PCR for quantification of ChIP-enriched DNA (primers sets, **Supplementary Table 2**). The antibodies used for ChIP were polyclonal rabbit anti-T-bet and anti-STAT3 (C-20; Santa Cruz).

Luciferase assay. The mouse *Rorc* promoter was cloned into the pGL3-Basic plasmid upstream of the firefly luciferase gene (Promega). For analysis of the effects of T-bet and Runx1 on the *Rorc* promoter activity, HEK293 cells were transfected via Fugene (Roche) with the *Rorc* luciferase reporter plasmid and increasing concentrations of empty pCDNA3.1 vector (control) or pCDNA3.1 expressing Runx1 or T-bet, plus the pRL-TK renilla luciferase plasmid (Promega). For analysis of the dose-dependent effect of T-bet on Runx1 activity, HEK293 cells were transfected with the *Rorc* luciferase reporter plasmid, Runx1, increasing concentration of T-bet, and pRL-TK. Firefly and renilla luciferase activity was measured 48 h after transfection with the Dual-Luciferase system (Promega).

Coimmunoprecipitation. HEK293 cells were transiently transfected via Fugene (Roche) with wild-type T-bet or T-bet point mutants, Myc-Runx1 or empty vector. Then, 48 h after transfection, cells were lysed in lysis buffer (1% Triton X-100, 50 mM Tris, pH 7.5, 200 mM NaCl, 1 mM dithiothreitol, and protease and phosphatase inhibitors). For coimmunoprecipitation of endogenous Runx1 and T-bet, differentiated T_H0, T_H1 and T_H17 cells (3×10^8 to 4×10^8) were stimulated for 6 h with PMA and ionomycin, then were lysed in the lysis buffer described above. Whole-cell lysates were immunoprecipitated at 4 °C with polyclonal rabbit anti-Runx1 (ab23980; Abcam) or rabbit polyclonal IgG (ab27472; Abcam) and beads coupled to protein A–sepharose. Immune complexes were analyzed by immunoblot with polyclonal rabbit anti-T-bet. In the reverse-coimmunoprecipitation assay, T-bet was immunoprecipitated with polyclonal rabbit anti-T-bet (H-210; Santa Cruz), and separated immune complexes were analyzed by immunoblot with polyclonal rabbit anti-Runx1. To minimize interference in the detection of Runx1 or T-bet by the heavy chains of the anti-T-bet used for immunoprecipitation, a TrueBlot ULTRA kit (eBioscience) was used for analysis of the Runx1–T-bet interaction in this reverse assay.

DNA-binding assay. DNA binding was assayed as described[46]. HEK293 cells were transfected with expression plasmid for Myc-tagged Runx1 in the presence or absence of increasing concentrations of T-bet expression vector. Nuclear protein (150 μg) was incubated for 1 h at 4 °C with a 50-nucleotide biotinylated probe containing wild-type or mutated T-bet- or Runx1-binding sites (at a site 2 kb upstream of exon 1 of *Rorc*) plus streptavidin-agarose (Invitrogen) in binding buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 1 mg/ml of BSA and 20 μg/ml of poly(dI:dC), plus protease inhibitors). Streptavidin-beads were washed in binding buffer, and bound proteins were analyzed by immunoblot for overexpressed Myc-tagged Runx1 and T-bet.

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