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Genome-Wide Regulatory Analysis Reveals That T-bet Controls Th17 Lineage Differentiation through Direct Suppression of IRF4

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The complex relationship between Th1 and Th17 cells is incompletely understood. The transcription factor T-bet is best known as the master regulator of Th1 lineage commitment. However, attention is now focused on the repression of alternate T cell subsets mediated by T-bet, particularly the Th17 lineage. It has recently been suggested that pathogenic Th17 cells express T-bet and are dependent on IL-23. However, T-bet has previously been shown to be a negative regulator of Th17 cells. We have taken an unbiased approach to determine the functional impact of T-bet on Th17 lineage commitment. Genome-wide analysis of functional T-bet binding sites provides an improved understanding of the transcriptional regulation mediated by T-bet, and suggests novel mechanisms by which T-bet regulates Th cell differentiation. Specifically, we show that T-bet negatively regulates Th17 lineage commitment via direct repression of the transcription factor IFN regulatory factor-4 (IRF4). An in vivo analysis of the pathogenicity of T-bet-deficient T cells demonstrated that mucosal Th17 responses were augmented in the absence of T-bet, and we have demonstrated that the roles of T-bet in enforcing Th1 responses and suppressing Th17 responses are separable. The interplay of the two key transcription factors T-bet and IRF4 during the determination of T cell fate choice significantly advances our understanding of the mechanisms underlying the development of pathogenic T cells. *The Journal of Immunology*, 2013, 191: 5925–5932.

The transcription factor T-bet plays an essential role in regulating both adaptive and innate immune responses. T-bet was first described in T cells as the key transcription factor that directs naive CD4⁺ T cells toward the Th1 lineage, which is characterized by the production of the hallmark cytokine

IFN- γ (1). T-bet directly activates the *Ifng* gene, and is required for CD4⁺ T cells to acquire the Th1 migratory program (1, 2). In addition, T-bet inhibits the differentiation of Th2 cells by a number of mechanisms (3, 4). The role of T-bet in regulating the Th17 lineage is less well characterized. Indeed, the relationship between Th1 and Th17 cells appears to be more complex than that between the Th1 and Th2 lineages. Cells expressing both IFN- γ and IL-17 are frequently isolated from humans and experimental animals with inflammatory conditions (5, 6). Recently the generation of T-bet-expressing Th17 cells in the absence of TGF- β has also been described (7), and *Candida albicans*-specific CD4⁺ T cells producing both IL-17 and IFN- γ have been shown to express both T-bet and the Th17 transcription factor ROR γ t (8). However, the absence of T-bet has also been shown to be associated with augmented Th17 responses in a number of settings, including cardiac allograft rejection, allergic airway inflammation, autoimmune myocarditis, and experimental autoimmune encephalomyelitis (9–14). T-bet has been shown to be a potent suppressor of Th17 development independently of STAT1, which is downstream of IFN- γ (15). The recent demonstration of the repression by T-bet of Runx1-mediated *Rorc* activation provides important mechanistic insight on how this phenomenon might occur (12). However, it is unclear whether a direct cell-intrinsic transcriptional mechanism exists to explain the significant effect T-bet exerts on Th17 lineage commitment. We sought to address this question by examining further the role of T-bet in directing the transcriptional program of CD4⁺ Th cells.

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G.M.L., R.G.J., and M.R.G. designed the research; M.R.G., R.D., N.P., E.P., and G.M.L. conducted the experiments; genomic data were analyzed by A.K. and R.G.J.; M.R.G. and R.G.J. wrote the manuscript with input from the other authors.

The sequences presented in this article have been submitted to Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) under accession number GSE40623 (mouse).

The views expressed are those of the author(s) and not necessarily those of the National Health Service, the National Institute for Health Research, or the Department of Health.

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Abbreviations used in this article: ChIP-seq, chromatin immunoprecipitation sequencing; ES cell, embryonic stem cell; IRF4, IFN regulatory factor-4; qPCR, quantitative PCR; RV, retrovirus; WT, wild-type.

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housed in specific pathogen-free facilities either at King's College London or at Charles River Laboratories. All experiments were performed according to King's College London and national guidelines under the U.K. Home Office project license PPL/70/6792.

Generation of *T-bet*^{fl/fl} mouse

Construction of targeting vector. The genomic region of interest containing the murine *Tbx21* locus was isolated by PCR from 129Sv/Pas ES cell genomic DNA. PCR fragments were subcloned into the pCR4-TOPO vector (Invitrogen). The genomic clones (containing intron 1 to exon 6) were used to construct the targeting vector. Briefly, a 5.6-kb fragment comprising *Tbx21* exon 2 and 6 and a 1.6-kb fragment located downstream of the *Tbx21* exon 6 STOP codon were used to flank an IRES-Flpe-ERT2 NEO cassette (*FRT* site-PGK promoter-Neo cDNA-*FRT* site-*LoxP* site); a distal *LoxP* site was introduced within *Tbx21* intron 3 to have access to the conditional knockout line by deleting exons 4–6 of the *Tbx21* gene (Fig. 4C).

Screening of *T-bet*-targeted embryonic stem cell clones. The *NruI*-linearized targeting vector was transfected into 129SvPas embryonic stem (ES). Positive selection was started 48 h after electroporation, by addition of 200 µg/ml G418 (150 µg/ml active component; Life Technologies). Then, 370 resistant clones were isolated, amplified, and screened by PCR and further confirmed by Southern blot. PCR and Southern blot screening conditions are available on request.

Generation of chimeric mice and breeding scheme. One floxed mutated *Tbx21* ES cell clone was microinjected into C57BL/6 blastocysts, and gave rise to male chimeras with a significant ES cell contribution (as determined by an agouti coat color). After mating with C57BL/6 CMV-Flp-expressing female mice to remove the *FRT*-flanked Neo cassette, offspring were genotyped by PCR and Southern blot to ensure removal of the Neo cassette. PCR and Southern blot screening conditions are available on request.

T cell isolation and differentiation

CD4⁺ T cells from spleens and lymph nodes of 4- to 10-wk-old mice were purified by CD4 positive selection (Miltenyi Biotec) followed by sorting of naive CD4⁺CD25[−]CD62L^{high}CD44^{low} cells using a FACS Aria II (BD Biosciences). Cells were activated by plate-bound anti-CD3 and anti-CD28 (both 10 µg/ml; clones 145-2C11 and 37.51, respectively; Bio X Cell). Th1 conditions comprised recombinant human IL-2 (20 ng/ml; R&D Systems) and mouse IL-12 (20 ng/ml; eBioscience, San Diego, CA), and anti-IL-4 (10 µg/ml; Bio X Cell). Th2 conditions comprised IL-2 (20 ng/ml) and IL-4 (20 ng/ml; eBioscience), and anti-IFN-γ (20 µg/ml; Bio X Cell). Th17 conditions comprised plate-bound anti-CD3 only, with soluble anti-CD28 (5 µg/ml) added to culture medium; recombinant human TGF-β (2 ng/ml; R&D Systems), IL-6 (20 ng/ml), IL-1β (10 ng/ml; both eBioscience), anti-IFN-γ (20 µg/ml), and anti-IL-4 (10 µg/ml) were also added.

Flow cytometry

In all experiments, freshly isolated or in vitro cultured cells were restimulated with PMA (50 ng/ml) and ionomycin (1 µg/ml) for 4 h, with the addition of monensin (2 µM; Sigma-Aldrich) for the final 2 h. A LIVE/DEAD fixable dead cell stain (Invitrogen) was used according to the manufacturer's instructions to discriminate live cells. Cells were fixed with freshly prepared 4% formaldehyde, and intracellular and intranuclear Ags were stained using eBioscience nuclear Permeabilization Buffer.

T cell transfer colitis model

Rag2^{−/−} mice received an i.p. injection of 5 × 10⁵ naive CD4⁺ T cells, isolated as described above. Mice were assessed regularly for signs of colitis, including diarrhea, rectal bleeding, and weight loss, and for signs of peritonism. Mice were culled at 1–6 wk after adoptive transfer of naive CD4⁺ T cells, to assess in vivo T cell differentiation and induction of colitis.

Chromatin immunoprecipitation sequencing

Following 7 d of culture, polarized Th1 cells from WT and *T-bet*^{−/−} mice were activated for 4 h with PMA (50 ng/ml) and ionomycin (1 µg/ml), crosslinked with 1% formaldehyde, lysed, and sonicated at 24 W for 10 × 30-s pulses using a Misonix Sonicator 3000. The resulting whole-cell extract was incubated overnight at 4°C with Dynal Protein G Beads preincubated with 10 µl purified rabbit anti-T-bet polyclonal antiserum (9856) (1). Beads were washed, bound complexes were eluted, and crosslinks were reversed by heating at 65°C. Immunoprecipitants and input DNA were then purified by treatment with RNase A, proteinase K, and

phenol:chloroform extraction. Libraries were constructed from immunoprecipitants and input DNA by standard Illumina protocols, except that DNA in the range 150–350 bp was gel purified after PCR amplification. The library was quantified using an Agilent bioanalyzer and subjected to 35-bp single-end read sequencing with an Illumina Genome Analyzer II.

Data analysis

Initial processing was performed with the CASAVA pipeline. Reads were aligned to the Mouse NCBI37/mm9 reference genome with ELAND, background corrected using whole-cell extract data, and converted to tags per million total reads. Significant peaks were identified with MACS (16), using a *p* value threshold of 10^{−6}.

Chromatin immunoprecipitation sequencing (ChIP-seq) experiments using human cells are described elsewhere (17). Gene-specific ChIP for the *Ifng* and *Irff4* promoters was performed on the whole-cell extracts, as described above using monoclonal anti-T-bet IgG1 (4B10, eBioscience) and mouse IgG1 isotype (eBioscience).

All raw and processed ChIP-seq data are available at Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) under accession numbers GSE40623 (mouse) and GSE31320 (human).

Real-time PCR and gene expression microarrays

All in vitro-cultured cells derived from naive precursors were cultured for 7 d; both freshly isolated and in vitro-cultured cells for real-time PCR and gene expression microarrays were restimulated with PMA (50 ng/ml) and ionomycin (1 µg/ml) for 4 h and then lysed in TRIzol (Invitrogen). For real-time PCR experiments, extracted RNA was reverse transcribed to cDNA, using an iScript Select cDNA Synthesis Kit (Bio-Rad laboratories) according to the manufacturer's instructions. All PCR experiments were performed using TaqMan gene expression assays (Applied Biosystems), using the mouse β-actin gene (*Actb*) as an internal control in each well. TaqMan primers were as follows: *Ifng* Mm01168134_m1; *Il4* Mm99999154_m1; *Il17a* Mm00439619_m1; *Tbx21* Mm00450960_m1; *Gata3* Mm01337569_m1; *Rora* Mm00443103_m1; *Rorc* Mm01261021_g1; *Irff4* Mm00516431_m1; *Actb* Mm00607939_s1.

Gene expression microarray analysis was performed using Affymetrix GeneChip Mouse Gene 1.0 ST arrays according to the manufacturer's instructions. Array images were analyzed using Microarray Analysis Suite 5.0 with the default settings, and normalization was performed by robust multiarray average. For comparison with T-bet binding results, data for multiple transcripts were averaged for each gene.

Mouse array data can be accessed at ArrayExpress (<http://www.ebi.ac.uk/arrayexpress/>) under accession number E-TABM-1187.

Luciferase assay

A 1489-bp promoter region upstream of *Irff4* exon 1 was amplified from BALB/c DNA, using forward primer 5'-CTCGAGTTGGCTGCTTC-TGTCCTTAG-3' and reverse primer 5'-AAGCTTAGAGGAACCTTTATAGAGCCGGAG-3'. TG to CA mutations were introduced into T-bet consensus sequences within this region, using primer 1 (5'-GAGGG-GAAAATGGGCATGACCAAAATTTCTTTG-3') for the 202–203-bp position and primer 2 (5'-GATTAAGAAAGAAACCAAGAAACATG-TGAAATG-3') for the 435–436 position within the promoter. All PCR products were verified by sequencing. These WT and mutant *Irff4* promoter sequences were digested using XhoI and HindIII and cloned into a pGL4 vector (Promega, Madison, WI). HEK293 cells were transfected by calcium chloride-mediated gene transfer: 4 × 10⁴ HEK293 were cotransfected with either 0.5 µg retrovirus (RV)-T-bet-GFP or 0.5 µg RV-GFP, together with 5 ng WT or mutant *Irff4* promoter-pGL4. Firefly luciferase activity was assayed 24 h after transfection, using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Results are presented as *Renilla* luciferase activity normalized to firefly luciferase activity.

Retroviral gene transduction

A plasmid encoding mouse IFN regulatory factor-4 (IRF4), pcDNA3 Flag-mIRF4, was kindly donated by Dr. Kiri Honma and Professor Katsuyuki Yui of Nagasaki University, Japan. The IRF4-encoding sequence was cloned into the MSCV-IRES-eGFP vector. MSCV-T-bet-Thy1.1 and MSCV-Thy1.1 were kindly donated by Dr. Alejandro Villarino and Professor Abul Abbas of the University of California, San Francisco. pMY-cre and pMY-GFP plasmids were donated by Prof. Adrian Hayday of King's College London. RV-T-bet and RV-GFP vectors were produced as previously described (1). HEK293T cells were transfected with the vector plasmids, along with gag-pol and eco-env, using calcium phosphate precipitation. Viral supernatants were collected 48 h later. Naive mouse

T cells were sorted and maintained as before. After activation for 24 h, cells were transduced in the presence of 8 $\mu\text{g}/\text{ml}$ polybrene. Cells were activated for an additional 48 h and then cultured for 3 d with cytokines, as described. Cells were stained for Thy1.1 expression and sorted using a FACSaria II. Total RNA isolation and real-time PCR analysis were performed as described above.

Results

T-bet directly represses the expression of Irf4

To determine novel mechanisms by which T-bet regulates Th cell differentiation, we sought to identify T-bet target genes in primary mouse T cells using ChIP coupled with massively parallel sequencing (ChIP-seq). We purified naive CD4^+ T cells from WT mice, polarized these in vitro into Th1 cells, and performed ChIP for T-bet. Efficacy of Th1 polarization was confirmed by flow cytometry, quantitative PCR (qPCR), and ELISA (data not shown). As a control to verify the specificity of the technique, we performed ChIP for T-bet in cells from $\text{T-bet}^{-/-}$ mice. Th1 cells were used for this experiment, as they are the only subset with T-bet expression. With this technique, 14,880 significant T-bet binding sites were identified in WT cells, with a false-positive rate of 0.7%. Assignment of T-bet binding sites to genes with transcription start sites located within 2 kb identified 3982 genes potentially regulated by T-bet. These T-bet targets included the key Th1 genes *Ifng*, *Tnf*, *Cxcr3*, and *Gzmb*, providing confidence in the dataset (Supplemental Fig. 1A). We also found that in the mouse, T-bet targets a number of genes involved in T cell trafficking, including *Ccr5*, *Itgal*, and *Icam1* (not shown), as previously demonstrated in human T cells using ChIP-Chip (18).

We next sought to identify genes both bound and regulated by T-bet. To identify genes regulated by T-bet, we profiled gene expression in Th1 cells generated in vitro from naive precursors from WT and $\text{T-bet}^{-/-}$ mice. As for the ChIP-seq experiment described above, efficacy of skewing was confirmed using intracellular staining and FACS analysis, qPCR, and ELISA (not shown).

Overlap of the binding and expression data yielded a subset of genes that were bound by T-bet within 2 kb of the transcription start site and also maximally differentially expressed in the absence of T-bet (Fig. 1A, Supplemental Table 1A).

To focus on the role of T-bet in Th17 differentiation, this list was examined for the presence of genes with well-described roles in Th17 differentiation. Crucially, although the Th17-related genes *Rorc*, *Rora*, *Batf*, and *Il23r* were found to be bound by T-bet, the expression of these genes was not significantly affected by loss of this transcription factor in the in vitro system, and the *Il17a* gene itself was not a target of T-bet (Supplemental Fig. 1B). This approach instead revealed that T-bet represses the gene encoding the transcription factor IRF4, which has previously been shown to be required for optimal Th17 development (19, 20). ChIP-seq shows that the *Irf4/IRF4* promoter is bound by T-bet, both in mouse and in human Th1 cells (Fig. 1B). In the mouse, T-bet binding colocalized with the transcription initiation marker H3K4me3 (21). Detailed comparison of binding sites in mouse and human showed that three of the binding peaks are conserved between mouse and human, whereas one binding peak unique to each species is also observed (Supplemental Fig. 2); such evolutionary divergence of transcription factor binding sites, despite conserved functional targets, has been described by other groups (22). T-bet binding to the *Irf4* promoter was confirmed using gene-specific ChIP, with binding to the *Ifng* promoter used a positive control (Fig. 1C).

The finding that *Irf4* expression was increased in the absence of T-bet on microarray was confirmed by qPCR (Fig. 2A). This increased expression of *Irf4* in in vitro-generated Th cell subsets in the absence of T-bet was confirmed by immunoblotting, (Fig. 2B). These data suggest that transcriptional repression of *Irf4* may be a mechanism by which T-bet regulates Th17 development.

Given that one of the key effects of T-bet expression on Th cell differentiation is the induction of IFN- γ expression, we next sought to determine whether T-bet directly suppressed *Irf4* expression

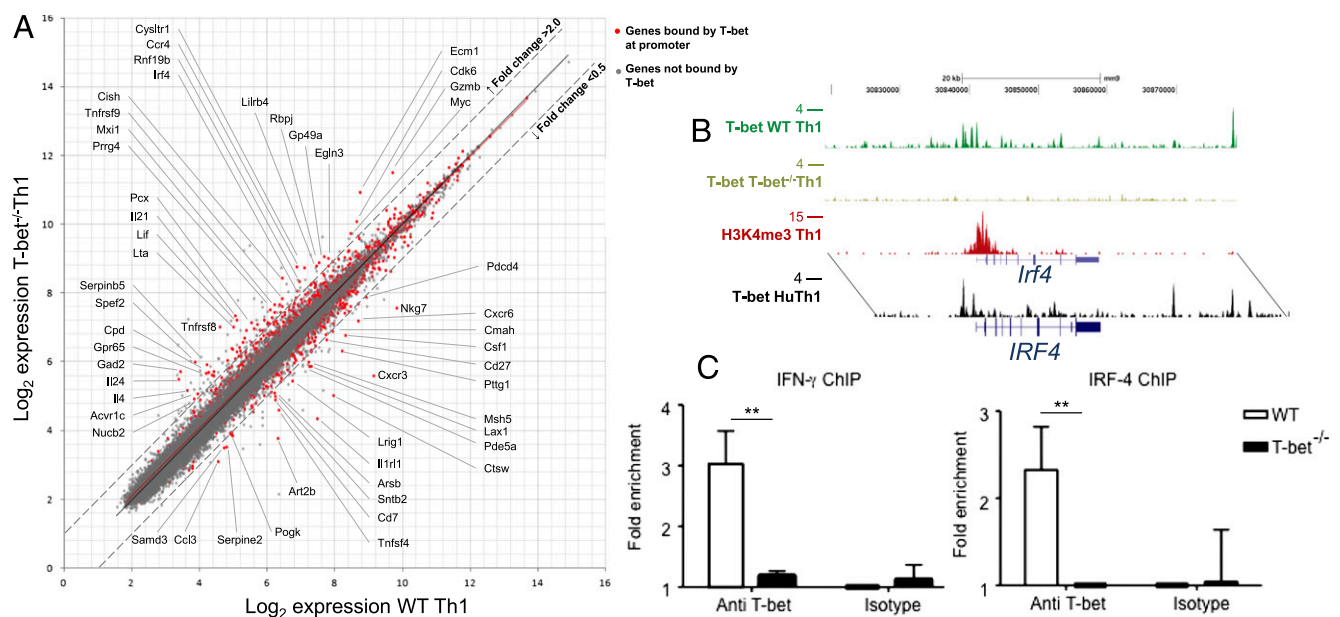


FIGURE 1. T-bet binds the promoter of *Irf4/IRF4*. **(A)** Scatter plot of genes up- and downregulated in $\text{T-bet}^{-/-}$ compared with WT Th1 cells, showing those genes determined by ChIP-seq to be bound by T-bet within 2 kb of the transcription start site (red). The full list of bound genes with fold change > 2.0 or < 0.5 is provided in Supplemental Table 1A. **(B)** T-bet binding at the *Irf4* locus in Th1 cells from WT and $\text{T-bet}^{-/-}$ mice, and the *IRF4* locus in human Th1 cells. The number of sequencing reads from T-bet ChIP-enriched DNA are plotted per million background-subtracted total reads and aligned with the mouse genome. Gene structures are marked at the bottom of the figure. The sites of H3K4me3 occupancy in mouse Th1 cells are also shown as reads per million. **(C)** T-bet binding of the *Ifng* and *Irf4* promoters demonstrated by gene-specific ChIP. ChIP was performed on chromatin derived from WT and $\text{T-bet}^{-/-}$ Th1 cells after 1 wk of in vitro culture and restimulation with PMA and ionomycin. $**p < 0.005$, paired Student *t* test.

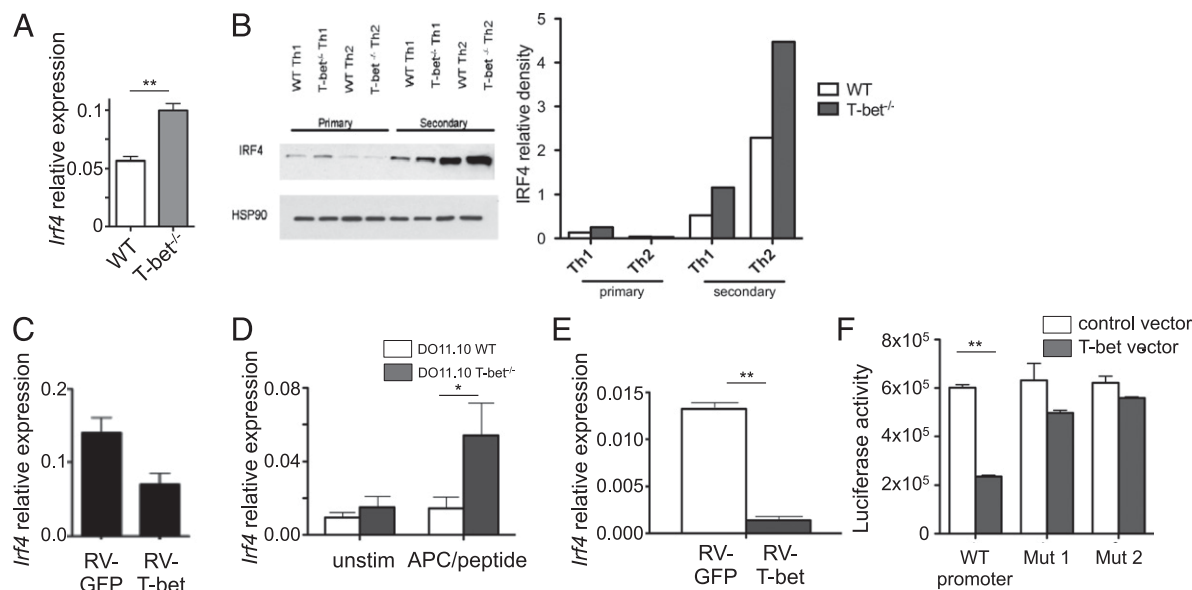


FIGURE 2. T-bet is a direct transcriptional repressor of *Irf4*. **(A)** *Irf4* mRNA relative to *Actb* mRNA in CD4⁺ T cells from BALB/c WT and T-bet^{-/-} mice cultured from CD4⁺CD25⁻CD62L^{high}CD44^{low} precursors in Th1 conditions for 1 wk and restimulated with PMA/ionomycin. Data are pooled from three independent experiments. ***p* < 0.005, paired Student *t* test. **(B)** Increased IRF4 protein in the absence of T-bet. Secondary stimulation involved reactivating washed cells with plate-bound anti-CD3 and anti-CD28 Abs for 24 h. **(C)** *Irf4* expression relative to *Actb* in GFP⁺ CD4⁺CD25⁻ T cells from T-bet^{-/-} × IFN-γ^{-/-} mice transduced with either RV-GFP or RV-T-bet. **(D)** *Irf4* expression relative to *Actb* in CD4⁺ cells from DO11.10 Rag2^{-/-} WT and DO11.10 Rag2^{-/-} × T-bet^{-/-} mice activated with APCs and OVA peptide and cultured in neutral conditions. **p* < 0.05, paired Student *t* test. **(E)** *Irf4* expression relative to *Actb* in GFP⁺ DO11.10 Rag2^{-/-} × T-bet^{-/-} cells transduced with RV-GFP or RV-T-bet. Data are representative of three experiments. Means ± SEM are depicted throughout. ***p* < 0.005, paired Student *t* test. **(F)** Suppression of *Irf4* expression by T-bet in a luciferase reporter system. HEK293 cells were cotransfected with either empty (control) or T-bet-encoding plasmids together with one of three different luciferase constructs: WT *Irf4* promoter and two constructs with mutated T-bet binding sites. Results are presented as *Renilla* luciferase activity normalized to firefly luciferase activity, and show mean ± SEM of three independent experiments. ***p* < 0.005; paired Student *t* test.

independently of IFN-γ. To this end, CD4⁺CD25⁻ T cells from T-bet^{-/-} × IFN-γ^{-/-} mice were transduced with either control RV encoding GFP only (RV-GFP), or RV encoding both T-bet and GFP (RV-T-bet). qPCR demonstrated that in this system lacking IFN-γ, *Irf4* mRNA levels were reduced upon expression of T-bet (Fig. 2C). This phenomenon was also observed in an Ag-specific setting, using the DO11.10 transgenic TCR system. When CD4⁺ T cells from DO11.10 Rag2^{-/-} and DO11.10 Rag2^{-/-} × T-bet^{-/-} mice were stimulated with dendritic cells and OVA peptide, those lacking T-bet expressed higher levels of *Irf4* mRNA (Fig. 2D). Furthermore, when Ag-APC-stimulated CD4⁺ T cells from DO11.10 Rag2^{-/-} × T-bet^{-/-} mice were transduced with RV encoding T-bet, *Irf4* transcripts levels were suppressed when compared with control RV (Fig. 2E).

These data demonstrate that T-bet binds to the *Irf4* gene, and that *Irf4* expression was reduced in the presence of T-bet through a mechanism genetically independent of IFN-γ. To assess whether a direct link existed between T-bet binding and transcriptional repression at the *Irf4* locus, we developed a luciferase reporter assay. We identified two T-bet consensus sequences (17) within a portion of the *Irf4* promoter ~1500–500 bp upstream of the transcription start site (Supplemental Table IB), and transfected T-bet together with luciferase constructs in which either of these T-bet consensus sequences had been mutated (Fig. 2F). This approach demonstrated that T-bet suppressed transcription initiated through the wild type *Irf4* promoter, and that this suppression was abrogated if either of the T-bet consensus sequences within this promoter was mutated. This observation suggests that T-bet binding to the *Irf4* promoter has a direct suppressive effect on *Irf4* gene transcription, in keeping with an emerging picture that one of the major functions of T-bet is as a transcriptional repressor (23).

The absence of T-bet is associated with augmented Th17 responses

Having determined that T-bet can act as a transcriptional repressor of the *Irf4* gene, we next sought to investigate the role of T-bet in Th17 differentiation in vivo, using the T cell transfer model of colitis (24, 25). Naive CD4⁺ T cells were purified from WT and T-bet^{-/-} mice and adoptively transferred into sex- and strain-matched Rag2^{-/-} recipients. Interestingly, adoptive transfer of T-bet^{-/-} naive CD4⁺ T cells resulted in more severe disease, as evidenced by the more profound weight loss and increased colon weight seen in the recipients of these cells (Fig. 3A, Fig. 3B). The adoptive transfer of WT naive CD4⁺ T cells was characterized by predominant differentiation toward a Th1 phenotype, with the production of large amounts of IFN-γ by CD4⁺ T cells recovered from the mesenteric lymph nodes of colitic mice. Importantly, however, T-bet^{-/-} naive CD4⁺ T cells were seen to differentiate predominantly into IL-17-secreting effectors (Fig. 3C, Supplemental Fig. 3A). The same phenomenon was evident in splenic CD4⁺ T cells from colitic mice assessed by qPCR (Fig. 3D) and in the supernatants of cultured colonic mucosa from mice that had received T-bet^{-/-} naive CD4⁺ T cells (Supplemental Fig. 3B). These data together suggest that augmented Th17 differentiation seen in the absence of T-bet also occurs in vivo, and that T-bet-deficient Th17 responses are sufficient to cause disease. Crucially, the increased *Irf4* transcript levels seen in the absence of T-bet in vitro were also observed in vivo in the T cell transfer colitis model (Fig. 3E).

T-bet inhibits Th17 development independently of IFN-γ

Having shown that Th17 differentiation is augmented in the absence of T-bet, we next sought to determine whether this suppressive effect of T-bet on Th17 differentiation is dependent on

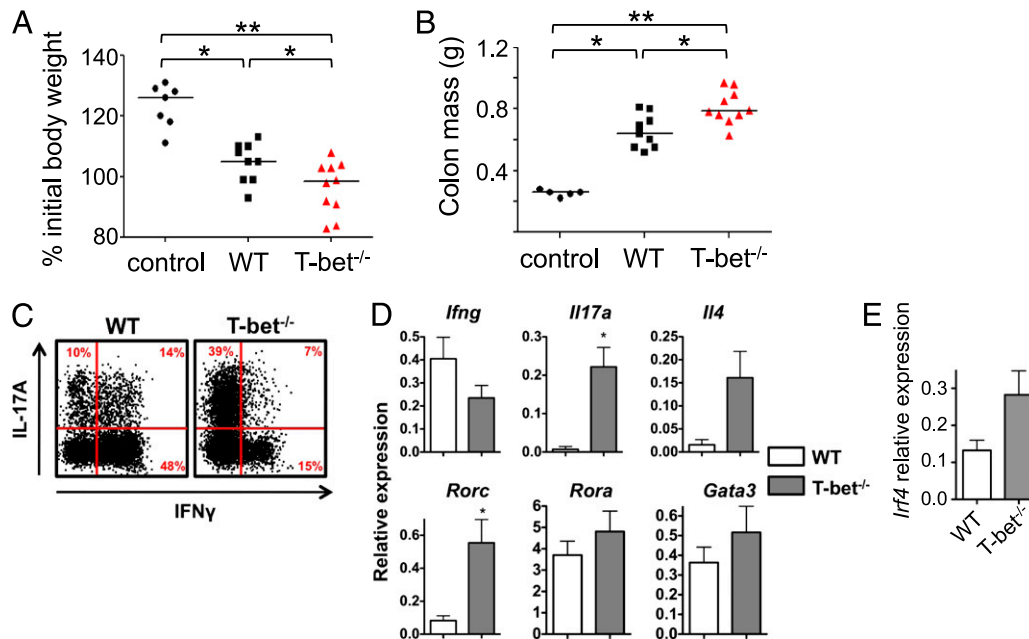


FIGURE 3. Adoptive transfer of T-bet^{-/-} naive CD4⁺ T cells into immunodeficient hosts results in Th17-predominant colitis. Rag2^{-/-} mice received i.p. injections of either PBS (control) or 5×10^5 freshly isolated naive sorted CD4⁺CD25⁻CD62L^{high}CD44^{low} T cells from WT or T-bet^{-/-} mice. (**A** and **B**) Adoptive transfer of T-bet^{-/-} naive CD4⁺ T cells results in more severe weight loss (**A**) and greater colonic infiltration (**B**) at 4 wk compared with the transfer of WT cells. * $p < 0.05$, ** $p < 0.005$; two-tailed unpaired Student *t* test. $n = 6$ –10 in each group. (**C**) IFN- γ and IL-17A expression in CD4⁺ T cells purified from mesenteric lymph nodes of Rag2^{-/-} mice 4 wk after adoptive transfer of either WT or T-bet^{-/-} naive CD4⁺ T cells. Cells were stimulated with PMA and ionomycin prior to staining; representative flow plots from one pair of animals is shown ($n = 6$ –10 in each group). (**D**) Expression of cytokines and transcription factors as determined by qPCR on RNA extracted from splenic CD4⁺ T cells from mice 4 wk after adoptive transfer of WT or T-bet^{-/-} naive CD4⁺ T cells. $n = 4$ in each group. Means \pm SEM are shown throughout. * $p < 0.05$; Mann–Whitney *U* test. (**E**) Increased *Irf4* transcript levels in CD4⁺ cells isolated from the spleens of Rag2^{-/-} mice that had received either WT or T-bet^{-/-} naive CD4⁺ T cells 4 wk previously.

IFN- γ , because IFN- γ itself has been proposed to be a direct negative regulator of Th17 differentiation (26, 27). To this end, the effects of ectopic expression of T-bet in CD4⁺ T cells from mice lacking both T-bet and IFN- γ were assessed. Naive CD4⁺ T cells were extracted from T-bet^{-/-} \times IFN- γ ^{-/-} mice, and transduced with either RV-T-bet or RV-GFP. In addition to suppressing the Th2 transcriptional program, ectopic expression of T-bet was associated with total suppression of *Il17a* expression, both at the mRNA and at the protein levels (Fig. 4A, 4B), demonstrating that T-bet can suppress Th17 development independently of IFN- γ and that the observed increase in IL-17–producing cells in the absence of T-bet is not simply due to reduced IFN- γ . Although it has been shown that T-bet–mediated suppression of IL-17 production is independent of STAT1 (15), and that T-bet^{-/-} CD4⁺ cells can be induced to express more IL-17A than IFN- γ ^{-/-} CD4⁺ cells (12), these results provide, to our knowledge, the first genetic proof that T-bet suppresses IL-17A production independently of IFN- γ .

T-bet–mediated suppression of Th17 cells is postdevelopmental and separable from Th1 commitment

A great deal of current data concerning the role of T-bet in Th cell lineage determination derive from constitutive T-bet knockout mice, in which the transcription factor is entirely absent throughout development. This situation makes it difficult to define at which stage of cell fate choice T-bet acts, although some insights have been gained from inducible ectopic expression of T-bet in mice constitutively lacking the transcription factor (28). To allow the experimental deletion of T-bet after activation of naive T cells, we generated a mouse line with *LoxP* sites flanking exons 4–6 of the *Tbx21* (T-bet) coding sequence (T-bet^{fl/fl}, Fig. 4C). Naive CD4⁺ T cells from T-bet^{fl/fl} mice were cultured in Th1 or Th17 conditions and transduced at 24 h post activation with either RV

encoding Cre recombinase or control RV. Inhibition of T-bet expression at 24 h in Th1 cells was associated with a marked reduction in IFN- γ production (Fig. 4D), suggesting that continued T-bet expression is required for maintenance of the Th1 phenotype. However, inhibition of T-bet expression at 24 h in developing Th17 cells was not associated with de-repression of IL-17A expression. This finding suggests that the effect of T-bet on the Th1/Th17 lineage choice is separable from IFN- γ transactivation. These data demonstrate that repression of Th17 commitment possibly occurs early post activation, whereas continuous T-bet expression is required to maintain Th1 polarity.

Expression of IRF4 rescues T-bet–mediated suppression of Th17 differentiation

Having established that T-bet binds the *Irf4* gene, and that T-bet binding represses *Irf4* transcription, we next sought to determine whether ectopic expression of IRF4 reverses the T-bet–mediated suppression of Th17 differentiation. As shown in Fig. 5, ectopic expression of T-bet alone was associated with reduced *Il17a* mRNA levels, whereas ectopic expression of IRF4 alone led to increased *Il17a* transcript levels. Crucially, when IRF4 and T-bet were coexpressed, there was an abrogation of the T-bet–mediated suppression of IL-17A expression, with *Il17a* transcript levels in the double-transduced cells being comparable with those in untransduced cells. These data suggest that optimal Th17 development is maintained in the face of T-bet expression when cells can express IRF4 in the absence of T-bet–mediated transcriptional repression.

Discussion

The mechanisms controlling Th cell lineage commitment have been the focus of much study over recent years. However, a paradox

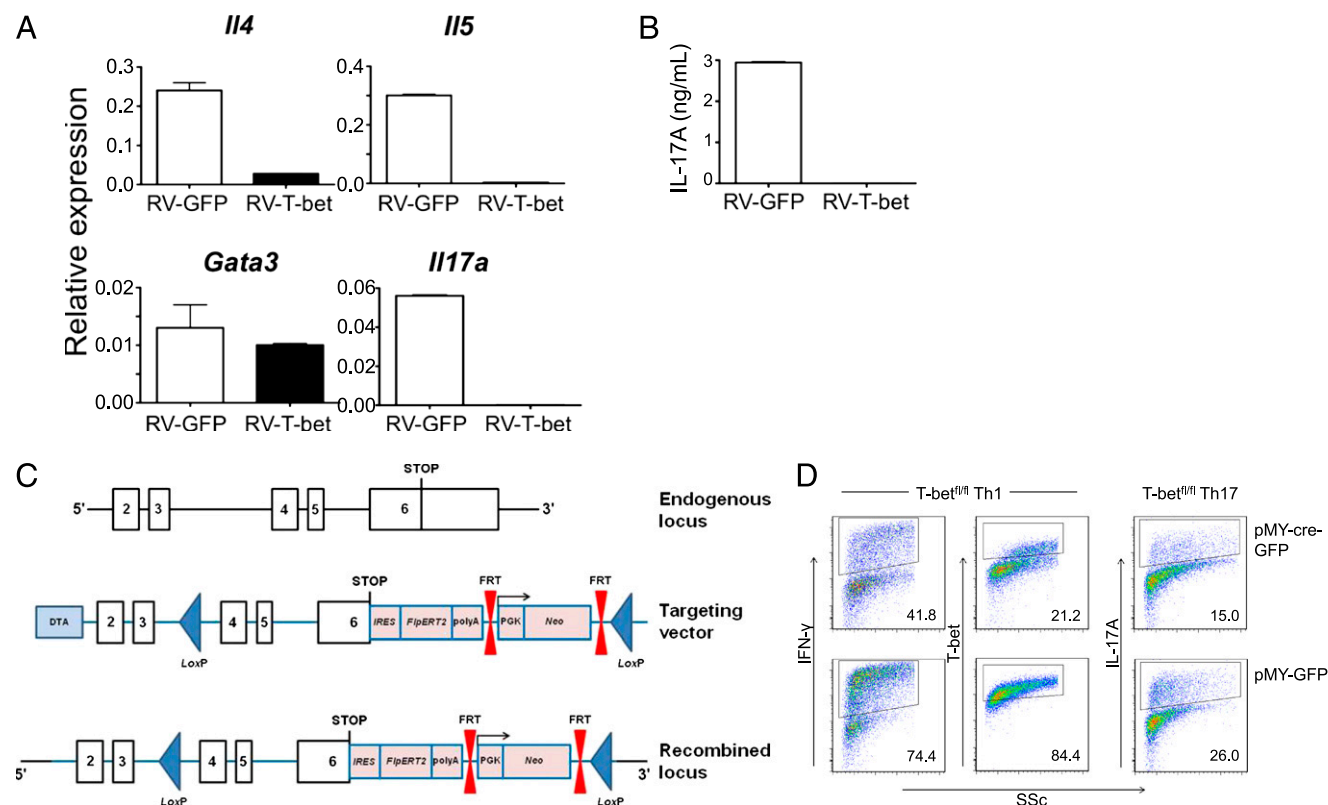


FIGURE 4. T-bet suppresses IL-17 production independently of IFN- γ , within the first 24 h following stimulation. CD4⁺ T cells from T-bet^{-/-} \times IFN- γ ^{-/-} mice were cultured in Th0 conditions (IL-2 only) and transduced with either RV encoding both T-bet and GFP (RV-T-bet) or control RV encoding GFP only (RV-GFP). **(A)** Expression of cytokines and the transcription factor *Gata3* relative to *Actb* in sorted GFP⁺ cells. **(B)** ELISA for IL-17A protein performed on the supernatants from 1×10^6 GFP⁺ cells restimulated for 24 h with plate-bound anti-CD3 and anti-CD28 Abs. Data are representative of three independent experiments. **(C)** Strategy for generation of T-bet^{fl/fl} mouse line, as described in *Materials and Methods*. **(D)** Naive CD4⁺ T cells from T-bet^{fl/fl} mice were cultured in either Th1 or Th17 conditions and transduced at 24 h with either RV encoding Cre recombinase (pMY-cre, top) or control RV (pMY-GFP, bottom). Cells were restimulated with PMA and ionomycin at day 4 prior to intracellular staining.

currently exists, whereby T-bet has been associated with both inhibition and promotion of Th17 cells. To resolve this at a mechanistic level, we have used multiple gene-targeted mice to dem-

onstrate that T-bet represses Th17 lineage commitment in a temporally defined manner that is genetically independent of IFN- γ . Using an unbiased approach, we have combined ChIP-seq and expression data to identify a key cell-intrinsic transcriptional mechanism by which T-bet represses Th17 lineage choice via direct *trans*-repression of IRF4 (Figs. 1, 2).

Our findings from the T cell transfer colitis model showing augmented Th17 responses in the absence of T-bet (Fig. 3, Supplemental Fig. 2) appear to conflict with previous reports describing a lack of colitis when T-bet^{-/-} CD4⁺CD62L⁺ T cells were transferred into immunodeficient hosts (24). This discrepancy is most likely explained by different patterns of colonization by commensal gut organisms in different animal facilities. Gut-residing segmented filamentous bacteria have been shown to be essential for the development of systemic Th17 responses (29); the presence of these bacteria in the mouse colonies used in the experiments described in this article has been confirmed by microbial sequencing analysis (not shown).

T-bet has been shown also to suppress Th17 responses through a physical interaction with the transcription factor Runx1, blocking Runx1-mediated transactivation of *Rorc* (12). This interaction was seen to be dependent on the DNA binding domain of the T-bet molecule, and was observed in uncommitted Th cells. Our data suggest an additional mechanism by which T-bet suppresses Th17 development; our experimental approach sought to determine novel mechanisms mediated through DNA binding. As the interaction between T-bet and Runx1 would seem to affect the ability of T-bet to bind target DNA sequences (12), it is likely that T-bet-mediated suppression of *Ir4* expression occurs in different set-

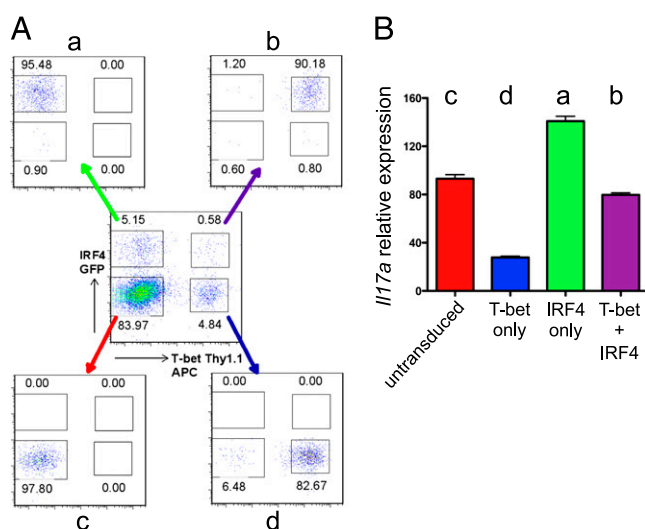


FIGURE 5. Ectopic expression of IRF4 rescues T-bet-mediated suppression of Th17 differentiation. Naive CD4⁺ T cells from T-bet^{-/-} mice were transduced with either both MSCV-T-bet-Thy1.1 and MSCV-IRF4-eGFP or both MSCV-Thy1.1 and MSCV-eGFP at 36 h, and cultured in Th17 conditions. **(A)** The cell-sorting strategy on the basis of eGFP and Thy1.1 expression at day 5. **(B)** qPCR for *Il17a* mRNA was performed on sorted populations. Representative of three experiments.

tings, or where T-bet is in excess. Further work is required to determine the relative contributions of these complementary mechanisms. It is likely that lineage-specifying transcription factors such as T-bet employ a range of mechanisms to suppress the gene programs of alternative lineages (30).

We show that T-bet acts as a direct transcriptional repressor at the *Irf4* promoter through sequence-specific DNA binding. The precise mechanism of this repression remains to be determined. It has been shown that T-bet can repress the genes *Socs1*, *Socs3*, and *Tcf7* in fully committed Th1 cells through recruiting the transcriptional repressor Bcl-6 to their promoters (31). It is possible that T-bet-mediated repression of *Irf4* expression is similarly dependent on a corepressor. Blocking of the DNA binding of an activator of *Irf4* expression would seem unlikely, given that the phenomenon was observed in HEK293 cells. Our ChIP-seq and gene expression data suggest that T-bet represses the expression of more of its genomic targets than it activates (data not shown); the mechanisms of T-bet-mediated repression at these loci are yet to be fully described.

The relationship between T-bet and IRF4 represents a new axis of regulation of Th cell fate. The importance of IRF4 in Th17 differentiation has been underlined by a study using an iterative systems approach to delineate the Th17 transcriptional regulatory network (32), in which IRF4 was shown to bind with BATF and thereby contribute to initial chromatin accessibility and the recruitment of ROR γ t.

Mucosal IRF4 levels are now known to correlate with proinflammatory cytokine production in patients with inflammatory bowel disease, whereas *Irf4*^{-/-} mice are protected from T cell transfer colitis (33). The demonstration that IRF4 is itself regulated by T-bet is further evidence of the role of T-bet in regulating mucosal immune responses (34). In addition, IRF4 has an important role in the differentiation and function of a number of Th cell subsets besides the Th17 lineage, including a function in promoting Th2 differentiation and preventing Th1 development (35, 36), and in the generation of IL-9-secreting Th9 cells (37). T-bet has been shown to repress IL-9 production in Th9 conditions, suggesting that T-bet might be acting via suppression of IRF4 in this cell lineage also (38). Although the expression of IRF4 in Treg cells has been shown to be essential for the suppression of Th2-mediated inflammation (39), any functional interaction between T-bet and IRF4 in Treg cells has yet to be determined. The transcriptional control of IRF4 by T-bet represents a further mechanism by which T-bet regulates inflammation that is likely to be relevant for understanding the pathogenesis of a variety of autoimmune conditions.

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Disclosures

The authors have no financial conflicts of interest.

References

- Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100: 655–669.
- Lord, G. M., R. M. Rao, H. Choe, B. M. Sullivan, A. H. Lichtman, F. W. Luscinskas, and L. H. Glimcher. 2005. T-bet is required for optimal proinflammatory CD4+ T-cell trafficking. *Blood* 106: 3432–3439.
- Djuretic, I. M., D. Levanon, V. Negreanu, Y. Groner, A. Rao, and K. M. Ansel. 2007. Transcription factors T-bet and Runx3 cooperate to activate Ifng and silence Il4 in Th helper type 1 cells. *Nat. Immunol.* 8: 145–153.
- Hwang, E. S., S. J. Szabo, P. L. Schwartzberg, and L. H. Glimcher. 2005. T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. *Science* 307: 430–433.
- Annunziato, F., L. Cosmi, V. Santarlasci, L. Maggi, F. Liotta, B. Mazzinghi, E. Parente, L. Fili, S. Ferri, F. Frosali, et al. 2007. Phenotypic and functional features of human Th17 cells. *J. Exp. Med.* 204: 1849–1861.
- Shi, G., C. A. Cox, B. P. Vistica, C. Tan, E. F. Wawrousek, and I. Gery. 2008. Phenotype switching by inflammation-inducing polarized Th17 cells, but not by Th1 cells. *J. Immunol.* 181: 7205–7213.
- Ghoreschi, K., A. Laurence, X.-P. Yang, C. M. Tato, M. J. McGeachy, J. E. Konkel, H. L. Ramos, L. Wei, T. S. Davidson, N. Bouladoux, et al. 2010. Generation of pathogenic T(H)17 cells in the absence of TGF- β signalling. *Nature* 467: 967–971.
- Zielinski, C. E., F. Mele, D. Aschenbrenner, D. Jarrossay, F. Ronchi, M. Gattorno, S. Monticelli, A. Lanzavecchia, and F. Sallusto. 2012. Pathogen-induced human TH17 cells produce IFN- γ or IL-10 and are regulated by IL-1 β . *Nature* 484: 514–518.
- Dalton, D. K., S. Pitts-Meek, S. Keshav, I. S. Figari, A. Bradley, and T. A. Stewart. 1993. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science* 259: 1739–1742.
- Durrant, D. M., S. L. Gaffen, E. P. Riesenfeld, C. G. Irvin, and D. W. Metzger. 2009. Development of allergen-induced airway inflammation in the absence of T-bet regulation is dependent on IL-17. *J. Immunol.* 183: 5293–5300.
- Fujiwara, M., K. Hirose, S.-I. Kagami, H. Takatori, H. Wakashin, T. Tamachi, N. Watanabe, Y. Saito, I. Iwamoto, and H. Nakajima. 2007. T-bet inhibits both TH2 cell-mediated eosinophil recruitment and TH17 cell-mediated neutrophil recruitment into the airways. *J. Allergy Clin. Immunol.* 119: 662–670.
- Lazarevic, V., X. Chen, J.-H. Shim, E. S. Hwang, E. Jang, A. N. Bolm, M. Oukka, V. K. Kuchroo, and L. H. Glimcher. 2011. T-bet represses T(H)17 differentiation by preventing Runx1-mediated activation of the gene encoding ROR γ t. *Nat. Immunol.* 12: 96–104.
- Mathur, A. N., H. C. Chang, D. G. Zisoulis, R. Kapur, M. L. Belladonna, G. S. Kansas, and M. H. Kaplan. 2006. T-bet is a critical determinant in the instability of the IL-17-secreting Th-helper phenotype. *Blood* 108: 1595–1601.
- Rangachari, M., N. Mauermann, R. R. Marty, S. Dirnhofer, M. O. Kurrer, V. Komnenovic, J. M. Penninger, and U. Eriksson. 2006. T-bet negatively regulates autoimmune myocarditis by suppressing local production of interleukin 17. *J. Exp. Med.* 203: 2009–2019.
- Villarino, A. V., E. Gallo, and A. K. Abbas. 2010. STAT1-activating cytokines limit Th17 responses through both T-bet-dependent and -independent mechanisms. *J. Immunol.* 185: 6461–6471.
- Zhang, Y., T. Liu, C. A. Meyer, J. Eeckhoutte, D. S. Johnson, B. E. Bernstein, C. Nussbaum, R. M. Myers, M. Brown, W. Li, and X. S. Liu. 2008. Model-based analysis of ChIP-Seq (MACS). *Genome Biol.* 9: R137.1–R137.9.
- Kanhere, A., A. Hertweck, U. Bhatia, M. R. Gökmen, E. Perucha, I. Jackson, G. M. Lord, and R. G. Jenner. 2012. T-bet and GATA3 orchestrate Th1 and Th2 differentiation through lineage-specific targeting of distal regulatory elements. *Nat Commun* 3: 1268.
- Jenner, R. G., M. J. Townsend, I. Jackson, K. Sun, R. D. Bouwman, R. A. Young, L. H. Glimcher, and G. M. Lord. 2009. The transcription factors T-bet and GATA-3 control alternative pathways of T-cell differentiation through a shared set of target genes. *Proc. Natl. Acad. Sci. USA* 106: 17876–17881.
- Brüstle, A., S. Heink, M. Huber, C. Rosenplänter, C. Stadelmann, P. Yu, E. Arpaia, T. W. Mak, T. Kamradt, and M. Lohoff. 2007. The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat. Immunol.* 8: 958–966.
- Huber, M., A. Brüstle, K. Reinhard, A. Guralnik, G. Walter, A. Mahiny, E. von Löw, and M. Lohoff. 2008. IRF4 is essential for IL-21-mediated induction, amplification, and stabilization of the Th17 phenotype. *Proc. Natl. Acad. Sci. USA* 105: 20846–20851.
- Wei, G., L. Wei, J. Zhu, C. Zang, J. Hu-Li, Z. Yao, K. Cui, Y. Kanno, T.-Y. Roh, W. T. Watford, et al. 2009. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Immunity* 30: 155–167.
- Schmidt, D., M. D. Wilson, B. Ballester, P. C. Schwalie, G. D. Brown, A. Marshall, C. Kutter, S. Watt, C. P. Martinez-Jimenez, S. Mackay, et al. 2010. Five-vertebrate ChIP-seq reveals the evolutionary dynamics of transcription factor binding. *Science* 328: 1036–1040.
- Oestreich, K. J., and A. S. Weinmann. 2012. T-bet employs diverse regulatory mechanisms to repress transcription. *Trends Immunol.* 33: 78–83.
- Neurath, M. F., B. Weigmann, S. Finotto, J. Glickman, E. Nieuwenhuis, H. Iijima, A. Mizoguchi, E. Mizoguchi, J. Mudter, P. R. Galle, et al. 2002. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *J. Exp. Med.* 195: 1129–1143.
- Powrie, F., M. W. Leach, S. Mauze, S. Menon, L. B. Caddle, and R. L. Coffman. 1994. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity* 1: 553–562.
- Harrington, L. E., R. D. Hatton, P. R. Mangan, H. Turner, T. L. Murphy, K. M. Murphy, and C. T. Weaver. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the Th helper type 1 and 2 lineages. *Nat. Immunol.* 6: 1123–1132.

27. Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y.-H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* 6: 1133–1141.
28. Matsuda, J. L., T. C. George, J. Hagman, and L. Gapin. 2007. Temporal dissection of T-bet functions. *J. Immunol.* 178: 3457–3465.
29. Wu, H.-J., I. I. Ivanov, J. Darce, K. Hattori, T. Shima, Y. Umesaki, D. R. Littman, C. Benoist, and D. Mathis. 2010. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity* 32: 815–827.
30. Oestreich, K. J., and A. S. Weinmann. 2012. Master regulators or lineage-specifying? Changing views on CD4+ T cell transcription factors. *Nat. Rev. Immunol.* 12: 799–804.
31. Oestreich, K. J., A. C. Huang, and A. S. Weinmann. 2011. The lineage-defining factors T-bet and Bcl-6 collaborate to regulate Th1 gene expression patterns. *J. Exp. Med.* 208: 1001–1013.
32. Ciofani, M., A. Madar, C. Galan, M. Sellars, K. Mace, F. Pauli, A. Agarwal, W. Huang, C. N. Parkurst, M. Muratet, et al. 2012. A validated regulatory network for Th17 cell specification. *Cell* 151: 289–303.
33. Mudter, J., J. Yu, C. Zufferey, A. Brüstle, S. Wirtz, B. Weigmann, A. Hoffman, M. Schenk, P. R. Galle, H. A. Lehr, et al. 2011. IRF4 regulates IL-17A promoter activity and controls ROR γ t-dependent Th17 colitis in vivo. *Inflamm. Bowel Dis.* 17: 1343–1358.
34. Powell, N., J. B. Canavan, T. T. MacDonald, and G. M. Lord. 2010. Transcriptional regulation of the mucosal immune system mediated by T-bet. *Mucosal Immunol.* 3: 567–577.
35. Lohoff, M., H. W. Mittrücker, S. Prechtel, S. Bischof, F. Sommer, S. Kock, D. A. Ferrick, G. S. Duncan, A. Gessner, and T. W. Mak. 2002. Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4. *Proc. Natl. Acad. Sci. USA* 99: 11808–11812.
36. Tominaga, N., K. Ohkusu-Tsukada, H. Udono, R. Abe, T. Matsuyama, and K. Yui. 2003. Development of Th1 and not Th2 immune responses in mice lacking IFN-regulatory factor-4. *Int. Immunol.* 15: 1–10.
37. Staudt, V., E. Bothur, M. Klein, K. Lingnau, S. Reuter, N. Grebe, B. Gerlitzki, M. Hoffmann, A. Ulges, C. Taube, et al. 2010. Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity* 33: 192–202.
38. Goswami, R., R. Jabeen, R. Yagi, D. Pham, J. Zhu, S. Goenka, and M. H. Kaplan. 2012. STAT6-dependent regulation of Th9 development. *J. Immunol.* 188: 968–975.
39. Zheng, Y., A. Chaudhry, A. Kas, P. deRoos, J. M. Kim, T.-T. Chu, L. Corcoran, P. Treuting, U. Klein, and A. Y. Rudensky. 2009. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature* 458: 351–356.