

# IL-2 production in developing Th1 cells is regulated by heterodimerization of RelA and T-bet and requires T-bet serine residue 508

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**Interleukin (IL)-2 is the predominant cytokine that is produced by naive Th cells in a primary response. It is required for proliferation and differentiation of Th precursor cells into effector cells. Initial high-level IL-2 production is followed by its decline, and the concomitant induction of cytokines that are typical of the differentiated state. Although the factors that are responsible for the early induction of IL-2 are well defined, the mechanisms that are responsible for its down-regulation in later stages of Th development have not been studied as much. Previous work from our laboratory revealed a repressor function for the T-box transcription factor, T-bet, in *IL-2* gene transcription. Here, we report that T-bet<sup>S508</sup> is required for the optimal repression of IL-2 production in developing Th1 cells. Phosphorylation of T-bet<sup>S508</sup> by casein kinase I and glycogen synthase kinase-3 kinases accompanies T-bet's interaction with the RelA nuclear factor- $\kappa$ B transcription factor. Heterodimerization of T-bet and RelA interferes with the binding of RelA to the IL-2 promoter, and hence, transcriptional activation of the *IL-2* gene by RelA.**

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Abbreviations used: ChIP, chromatin immunoprecipitation; CKI, casein kinase I; CREB, cyclic AMP response element binding protein; CREM, cyclic AMP responsive element modulator gene; GSK, glycogen synthase kinase; MS, mass spectrometry; PKA, protein kinase A; S508, serine 508; Thp, Th precursor.

The T cell growth factor, IL-2, is the major cytokine that is produced during the primary response of Th cells. Upon differentiation into one of the two types of Th effector cells, Th1 and Th2, IL-2 production declines and is replaced by production of Th1-like (IFN- $\gamma$ ) or Th2-like (IL-4) cytokines. IL-2 acts through its receptor (IL-2R) to activate signaling molecules that are involved in cell proliferation; defects in the ligand or the receptor result in autoimmunity (1). Although IL-2 has been characterized as a Th1-like cytokine, increasing evidence indicates that IL-2 and its downstream signaling molecule, Stat5, also are vital for the induction of anti-inflammatory Th2 cytokines during a primary response (2).

IL-2 expression is controlled tightly at the transcriptional level, although posttranscriptional control through coding sequences also occurs (3). Extensive analysis of the *IL-2* gene established a minimal promoter region, which extends -300 bp relative to the transcription start site, that is known to be sufficient for IL-2 induction upon T cell activation in vitro (4, 5) (for reviews see references 6-9). Multiple cis regulatory elements have been identified within this region that

bind antigen-inducible factors, such as NFATs, OCT-1, AP-1, HMG I(Y), and NF- $\kappa$ B family members, p65 and c-Rel. These factors were shown to transactivate an IL-2 promoter in transient reporter assays (for reviews see references 6-9), and some of them are required for IL-2 expression in vivo (10-12). NF- $\kappa$ B family members regulate the transcription of the *IL-2* gene (6-9). Whereas p50/p50 homodimers are present in large amounts in unstimulated cells, they are inhibitory and are replaced by p50/p65 or p50/c-Rel heterodimers upon T cell activation. c-Rel nucleates chromatin remodeling across the IL-2 promoter (13-20). Interestingly, increased amounts of the NF- $\kappa$ B p65 (RelA) factor in the nucleus of Th1 than in the nuclei of Th2 cells has been reported, which is consistent with the preferential secretion of IL-2 by Th1 cells (21, 22).

Lines of transgenic mice revealed a requirement for an additional IL-2 upstream sequence to achieve expression in vivo that faithfully mirrors endogenous IL-2 expression (23). The contribution of regions beyond the minimal promoter also is evident from studies which showed that selective demethylation of a 600-bp

region of an IL-2 enhancer occurred rapidly upon T cell activation (24). The function of individual factors that bind IL-2 promoter DNA and the initiation of chromatin remodeling of the *IL-2* gene in response to T cell activation has been the subject of several reports (25–29). The NF- $\kappa$ B subunit, c-Rel, is required for chromatin remodeling across the proximal promoter, and c-Rel binds with high mobility group I(Y) to the CD28 response element (19, 30). Mice lacking c-Rel exhibit impaired IL-2 expression, and treatment with the c-Rel inhibitor, pentoxifylline, reduces IL-2 mRNA levels (11, 12, 31).

Negative regulation of *IL-2* gene transcription also is an important mechanism for controlling its expression. During primary Th1 cell differentiation, IL-2 is induced rapidly and peaks between days 2 and 3 after TCR stimulation, then decreases gradually. Homodimers of the NF- $\kappa$ B member, p50, are believed to repress *IL-2* gene transcription in resting Th cells (13, 32), and expression of a dominant negative cyclic AMP response element binding protein (CREB) transgene resulted in impaired IL-2 production *in vivo* (33). The cyclic AMP responsive element modulator gene (CREM) transcriptional repressor is activated by CaMKIV to bind to a CRE at position –180 to suppress IL-2 production in patients who have systemic lupus erythematosus (34, 35); CREM also is involved in establishing the anergic state (36). A zinc finger protein, ZEB, is believed to be a transcriptional repressor of the *IL-2* gene, but its function in primary Th cells has not been established (37). The antiproliferative factor, Tob, represses IL-2 through enhancing Smad binding to the –105 negative regulatory element of the IL-2 promoter (38).

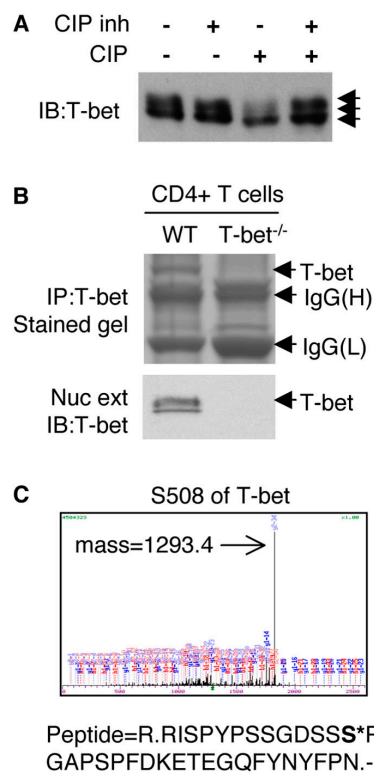
Another transcriptional repressor candidate for IL-2 in Th cells is T-bet, a T-box transcription factor. T-bet has three separate functions: (a) it is required for Th1 development from the Th precursor (Thp), (b) it represses Thp differentiation along the Th2 pathway by inhibiting GATA-3 activity through the physical interaction of tyrosine phosphorylated T-bet and GATA-3, and (3) it represses *IL-2* gene activation (39–42). Consequently, T-bet<sup>-/-</sup> mice exhibit impaired Th1 cell development, increased Th2 cytokine production, and interestingly, increased IL-2 production in CD4 and CD8 cells (40, 43, 44). Originally, T-bet was isolated in a yeast one hybrid screen that used the 400-bp IL-2 promoter as substrate, and subsequently was shown to repress IL-2 promoter activation (39). Further, overexpression of T-bet in T-bet<sup>-/-</sup> Th cells repressed IL-2 production (39, 42). Although we now understand the first two functions of T-bet at a molecular level, the mechanism by which T-bet controls production of IL-2 has not been apparent.

Here we report that T-bet acts as a repressor of *IL-2* gene transcription in developing Th1 cells by an interaction with the RelA NF- $\kappa$ B transcription factor that requires T-bet<sup>S508</sup> and is associated with T-bet<sup>S508</sup> phosphorylation. RelA/T-bet heterodimers regulate the binding of RelA to IL-2 promoter DNA, and hence, its transactivation of *IL-2* gene expression.

## RESULTS

### T-bet is phosphorylated at serine residue 508 *in vivo*

T-bet is expressed at high levels in AE7, a Th1 cell clone. Interestingly, there were three immunoreactive species of T-bet protein in AE7 extracts, which suggests that T-bet might be posttranslationally modified. Although T-bet is tyrosine phosphorylated at residue 525 (42), it was unlikely that this single phosphorylated tyrosine could account for the multiple species that we detected. To test whether phosphorylation was responsible for the triple complex that was observed, we treated AE7 cells with calf intestinal phosphatase (CIP) in the presence or absence of phosphatase inhibitors. The upper two bands disappeared upon CIP treatment in the absence, but not the presence, of phosphatase inhibitors (Fig. 1 A), which prompted us to identify specific phosphorylation sites. Endogenous T-bet in primary Th1 cells was



**Figure 1. T-bet is phosphorylated at serine 508.** (A) The Th1 (AE7) clone maintained in RPMI 1640 with recombinant human IL-2 (200 U/ml) was stimulated with anti-CD3 (2  $\mu$ g/ml) overnight and treated with calf intestinal phosphatase (CIP) in the absence or presence of phosphatase inhibitors. Immunoblot analysis was performed using T-bet mAb, 4B10. (B) CD4<sup>+</sup> LN and spleen Thp cells were stimulated with plate-bound anti-CD3 (2  $\mu$ g/ml), anti-CD28 (2  $\mu$ g/ml), and IL-2 (100 U/ml) with IL-12 (2 ng/ml) and anti-IL-4 (5  $\mu$ g/ml) for 60 h. Nuclear extracts were prepared and used for immunoprecipitation with T-bet mAb, 4B10. Immune complexes resolved by SDS-PAGE were stained with gel code blue. T-bet expression in activated CD4<sup>+</sup> T cells was assessed by immunoblotting. (C) Specific T-bet bands from the stained gel in B were excised for peptide identification and phosphorylation mapping. IM, immunoblot.

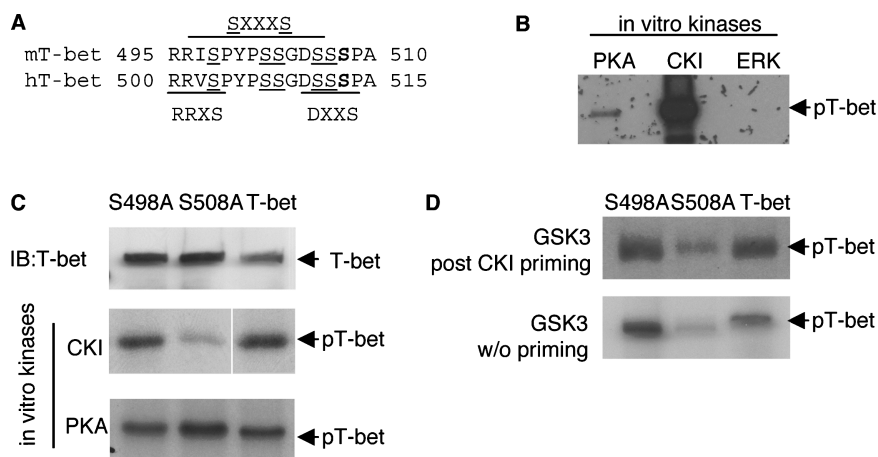
immunoprecipitated using anti-T-bet antibody, resolved by SDS-PAGE, and the gels were stained (Fig. 1 B). T-bet<sup>-/-</sup> Th1 cells were used as a negative control. Specific T-bet protein bands were detected by Western blot and excised for mass spectrometry (MS) to analyze phosphorylated peptides (Fig. 1 B). Mass spectrometry identified a specific phosphorylated peptide, which was phosphorylated at serine (S) 508 of T-bet (Fig. 1 C). We also observed S508 phosphorylation by MS analysis of overexpressed T-bet in 293T cells (unpublished data). These results indicate that T-bet is serine phosphorylated in primary Th1 cells by a kinase that also is expressed in non-T cells.

### Serine phosphorylation of T-bet is mediated by CKI and GSK-3 kinases

To identify the specific upstream serine/threonine kinase that phosphorylates T-bet<sup>S508</sup>, we analyzed the T-bet COOH-terminal sequence with the scansite program (<http://scansite.mit.edu>), which predicts kinases/phosphorylation sites. The T-bet COOH-terminal peptide, well conserved between human and mouse, contains several serine residues (Fig. 2 A); the scansite program predicted S508 as a phosphorylation site for casein kinase I (CKI). Therefore, we assayed *in vitro* phosphorylation of T-bet using a panel of recombinant protein kinases. CKI, but not active extracellular signal-regulated kinase, phosphorylated T-bet protein *in vitro* (Fig. 2 B). T-bet also was phosphorylated by protein kinase A (PKA), but with a 1,000-fold lesser efficiency than CKI. All recombinant kinases phosphorylated control substrates efficiently (unpublished data). To test whether CKI specifically phosphorylated S508, we constructed a serine to

alanine mutant T-bet (S508A) as well as an S498A mutant as a control, and then we compared the *in vitro* phosphorylation of these proteins. T-bet proteins were overexpressed in 293T cells, immunoprecipitated, comparable expression levels were confirmed by Western blot, and then lysates were used as substrates for further studies (Fig. 2 C). Although PKA-induced phosphorylation was not different among the WT, S508A, and S498A T-bet proteins, CKI-mediated phosphorylation of the S508A mutant, but not the S498A control mutant, was reduced dramatically compared with WT proteins (Fig. 2 C). These data suggest that T-bet<sup>S508</sup> is a specific phosphorylation site for CKI, but that there are likely to be additional CKI phosphorylation sites in T-bet because residual phosphorylation of T-bet<sup>S508</sup> by CKI was present at very low levels.

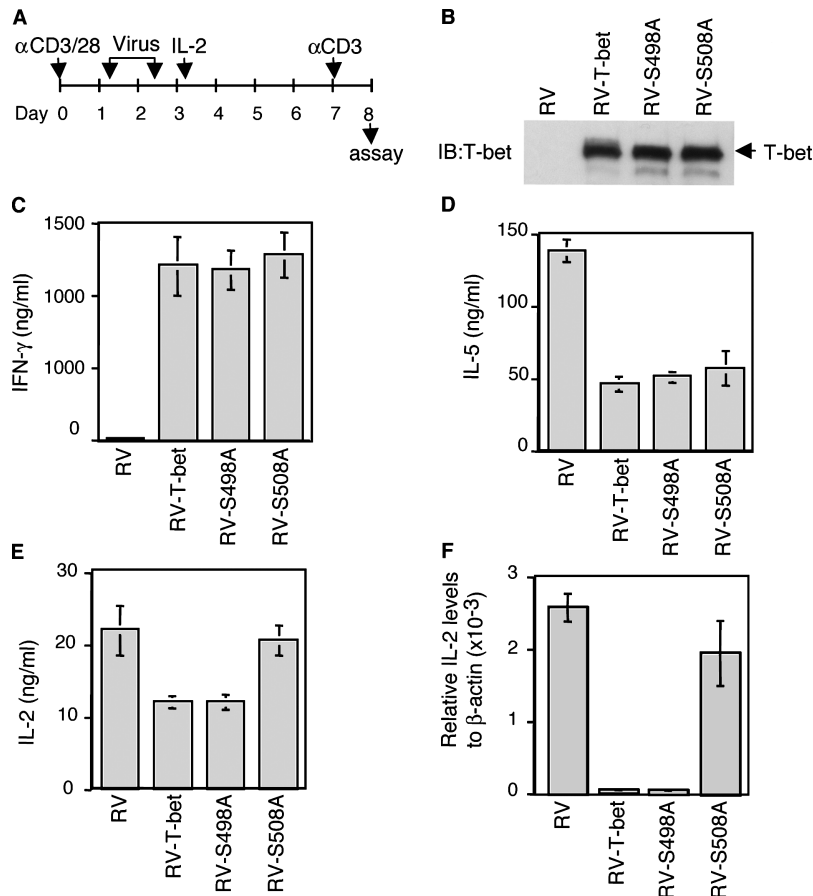
CKI-mediated phosphorylation induces additional phosphorylation by kinases, such as glycogen synthase kinase (GSK)-3. GSK-3 is a proline-directed serine/threonine kinase that recognizes prephosphorylated substrates and processively hyperphosphorylates substrates with ser/thr pentad repeats (SXXXS) (45, 46). Interestingly, there are conserved GSK-3 phosphorylation sites in T-bet that are located close to S508 (Fig. 2 A). We asked whether T-bet could be hyperphosphorylated by GSK-3. Similar amounts of T-bet proteins were preincubated with CKI in the presence of ATP for prephosphorylation, washed with PBS to remove excess ATP and CKI, and then reacted with GSK-3 and  $\gamma$ -[<sup>32</sup>P]ATP. Hyperphosphorylation of T-bet by GSK-3 was apparent (Fig. 2 D). Although GSK-3 induced WT levels of phosphorylation in the S498A mutant, GSK-3-mediated phosphorylation was decreased markedly in the S508A mutant (Fig. 2 D). Fur-



**Figure 2. CKI/GSK-3 mediate serine phosphorylation of T-bet.**

(A) The conserved amino acid sequences between mouse and human T-bet. S508 is in bold; consensus phosphorylation sites, RRXS for PKA, DXXS for CKI, and SXXXS for GSK-3 are underlined. (B) T-bet protein was purified by immunoprecipitation from 293T cells that were transfected with a T-bet expression vector. Recombinant protein kinases (10 U) were incubated with precipitated T-bet and 10  $\mu$ Ci of  $\gamma$ -[<sup>32</sup>P]ATP (6,000 Ci/mM) at 37°C for 1 h. Reaction mixtures were resolved by SDS-PAGE, and the resulting

gels were dried and subjected to autoradiography. (C) T-bet and serine mutants S508A and S498A were transfected into 293T cells and purified for further phosphorylation assay. The input amount of T-bet proteins was detected by immunoblotting and equivalent amounts of proteins were used for *in vitro* kinase assays as described in B. (D) T-bet proteins used in C were preincubated with or without 10 U of CKI in the presence of  $\gamma$ -ATP, followed by washing, and were incubated with GSK-3 kinase for phosphorylation assays as in B.



**Figure 3. T-bet<sup>S508</sup> is required for repression of IL-2 production.** (A) Retroviruses producing WT and serine mutant T-bets were transduced into T-bet<sup>-/-</sup> Thp cells, and cells were sorted for GFP on day 3 and were expanded for an additional 3 d. Cells were restimulated overnight with plate-bound anti-CD3 (2  $\mu$ g/ml). (B) Whole cell extracts were prepared

from the restimulated cells. Comparable expression of T-bet proteins was verified by immunoblot analysis. (C–E) ELISA on supernatants from the restimulated cells. (F) IL-2 transcripts were assessed by real time RT-PCR using specific primers as follows: IL-2-FWD, 5'-CTCCTGAGCAGGATGGAGAATT-3', IL-2-REV, 5'-CGCAGAGTCCAAGTGAGCT-3'. RV, retrovirus.

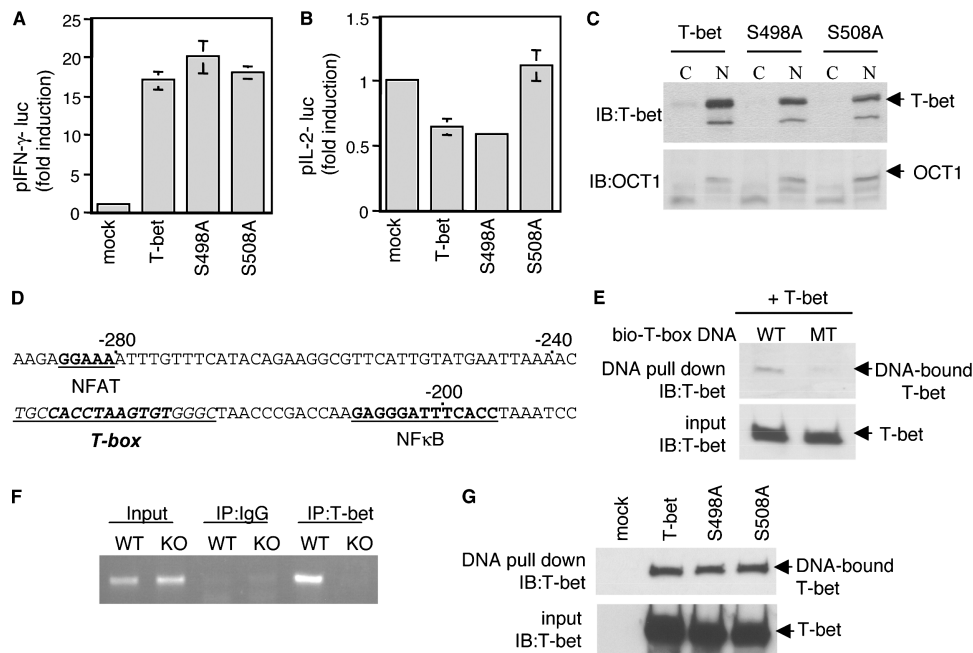
thermore, T-bet was phosphorylated by GSK-3 without pre-phosphorylation by CKI in vitro, which suggested that T-bet was phosphorylated by endogenous CKI in 293T cells. This is consistent with our detection of T-bet<sup>S508</sup> phosphorylation in 293T cells by MS. We conclude that endogenous or exogenous CKI-mediated phosphorylation of T-bet<sup>S508</sup> precedes its subsequent phosphorylation by GSK-3 in vitro. However, we cannot be sure that T-bet is phosphorylated in vivo in Th1 cells by CKI/GSK-3, because we do not have an antibody that recognizes only the serine phosphorylated form of T-bet and attempts to block in vivo phosphorylation with CKI or GSK-3 inhibitors were not successful.

#### T-bet<sup>S508</sup> is required for its function as a repressor of IL-2 gene transcription

To establish the function of serine phosphorylated T-bet in vivo, we introduced WT, S498A control mutant, and S508A mutant T-bet GFP retroviruses into T-bet<sup>-/-</sup> primary CD4<sup>+</sup> Th cells, and stimulated them as shown (Fig. 3

A). Western blot analysis confirmed that the expression levels of the three transduced T-bet proteins were similar (Fig. 3 B). T-bet controls the expression of multiple cytokines in Th cells. It directly activates the transcription of the IFN- $\gamma$  gene; indirectly represses the transcription of Th2 cytokines IL-4, -5, and -13; and represses the expression of IL-2 through unknown mechanisms. Upon TCR activation, all three T-bet retroviruses increased IFN- $\gamma$  production comparably (Fig. 3 C) and efficiently repressed Th2 cytokine production (Fig. 3 D). However, the mutation of S508 to alanine abolished the ability of T-bet to repress the expression of both mRNA transcripts encoding IL-2 and IL-2 protein (Fig. 3, E and F). These data demonstrate that T-bet<sup>S508</sup> is required selectively for its function in repressing IL-2 production.

**T-bet specifically binds to a T-box site within the proximal promoter of the IL-2 gene and binding does not require S508**  
To investigate the mechanism of such repression, we asked whether T-bet directly bound to and transactivated or re-



**Figure 4.** T-bet specifically binds to a T-box site within the proximal promoter of the IL-2 gene. T-bet expression vectors were transfected with IFN- $\gamma$  (A) or IL-2 (B) promoter reporter genes into EL4 cells. Luciferase activities were normalized by  $\beta$ -galactosidase activity and indicated as fold induction. (C) Nuclear and cytosolic proteins were prepared from T-bet-transduced T-bet<sup>-/-</sup> Thp cells that were used in Fig. 3. The protein blot was probed with anti-T-bet mAb 4B10 and reprobed with anti-OCT1 antibody, a control antibody. (D) The proximal promoter region of the IL-2 gene includes NFAT, NF- $\kappa$ B, and AP-1 binding sites. The T-bet binding site (T-box, -230 bp) is positioned between NFAT and NF- $\kappa$ B binding sites.

(E) WT or mutant (MT) T-box sites in the IL-2 promoter as below were labeled with biotin at the 5' end and incubated with T-bet proteins. Complexes were precipitated by incubation with streptavidin-agarose beads and were subjected to T-bet immunoblotting. WT T-box site: 5'-bio-ATTA AAACTGCCACCTAAGTGTGGGCTAACCCG-3'; MT T-box site: 5'-bio-ATTA AAACTGCTCTCTACTAAGGGCTAACCCG-3'. (F) ChIP of T-bet in WT and T-bet<sup>-/-</sup> Th1 cells reveals specific binding of T-bet to IL-2 promoter DNA. (G) WT and serine mutant T-bets were incubated with biotinylated DNA (-188 to -254 in D) for DNA binding pull-down assays as described in E. IM, immunoblot.

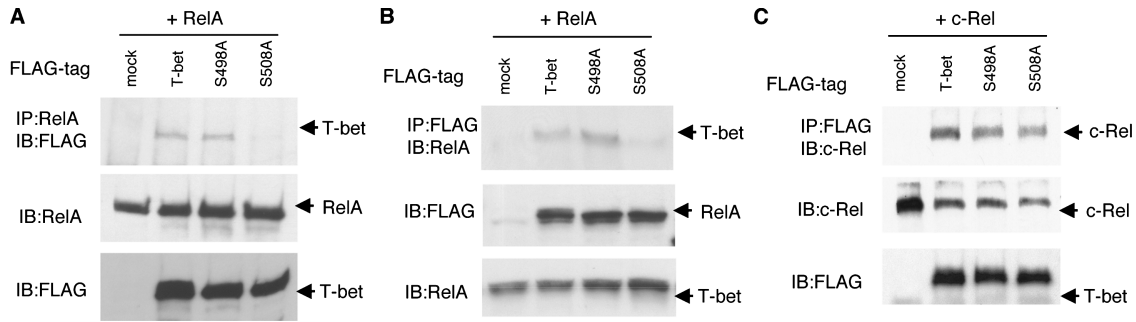
pressed IFN- $\gamma$  and IL-2 promoter reporters that contain T-bet binding sites. Consistent with its ability to drive endogenous IFN- $\gamma$  production, the T-bet S508A mutant transactivated an IFN- $\gamma$  promoter reporter comparably to WT and S498A control T-bet (Fig. 4 A). In contrast, whereas T-bet and the S498A control mutant repressed IL-2 promoter activity, the S508A mutant T-bet failed to do so (Fig. 4 B). This failure was not due to changes in T-bet subcellular localization; T-bet and its mutants were expressed exclusively in the nucleus (Fig. 4 c). A search for T-bet binding sites within the IL-2 gene promoter yielded putative T-box binding sequences between NFAT and NF- $\kappa$ B binding sites in the proximal IL-2 gene promoter (Fig. 4 D). Therefore, we performed DNA pull-down assays to examine the DNA binding activity of T-bet. Protein extracts expressing T-bet were incubated with biotinylated WT or mutant T-bet binding site DNA (T-box). The WT T-box-containing DNA pulled down T-bet, but T-box-mutated DNA did not (Fig. 4 E), which demonstrated sequence-specific binding of T-bet to the IL-2 promoter. Chromatin immunoprecipitation (ChIP) assays confirmed that T-bet binds to IL-2 promoter DNA (Fig. 4 F). S508A and S498A T-bet mutants

had DNA binding activity that was equivalent to WT T-bet (Fig. 4 G). This result indicated that T-bet<sup>S508</sup> was not required for T-bet's ability to bind DNA, a result that is consistent with the comparable induction of IFN- $\gamma$  gene transcription by the S508A mutant (Figs. 3 C and 4 A).

#### T-bet heterodimerizes with RelA and this interaction correlates with S508 phosphorylation

Because alterations in DNA binding activity could not explain the failure of the S508A mutant T-bet to repress IL-2 gene expression, it was possible that T-bet controlled the activity of other factors that regulated IL-2 gene expression. We first asked whether a physical interaction between T-bet and IL-2 activating transcription factors, such as NFAT and NF- $\kappa$ B, occurred. NFAT is a critical activator of IL-2 gene transcription, and five distinct essential NFAT binding sites have been mapped to the IL-2 proximal promoter (47). A recent study demonstrated that T-bet does associate physically with NFATc2 (48); however, that interaction is not dependent on T-bet<sup>S508</sup> (unpublished data). NF- $\kappa$ B family members also participate in activation of the IL-2 gene. Coimmunoprecipitation assays of overexpressed T-bet and RelA proteins re-





**Figure 5. NF-κB RelA interacts with T-bet, but not with S508A mutant T-bet.** FLAG peptide tagged T-bets were coexpressed with NF-κB RelA into 293T cells. Immunoprecipitation using RelA mAb (A) or FLAG-M2 agarose (B) was performed with whole cell extracts, and

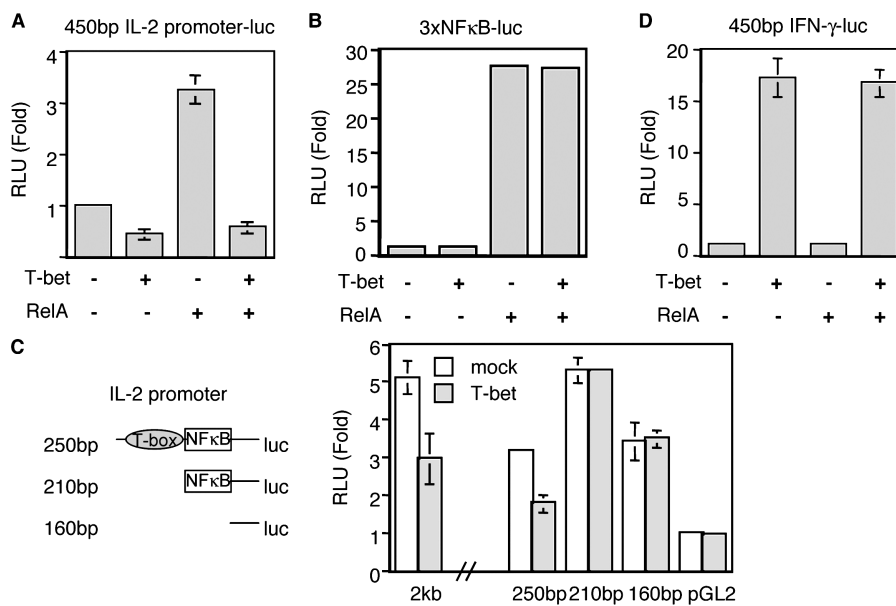
protein blots were probed with anti-FLAG polyclonal antibody or anti-RelA antibody. (C) T-bet proteins coexpressed with c-Rel were immunoprecipitated with FLAG-M2 and resolved protein blots were probed with c-Rel polyclonal antibody.

revealed that T-bet interacted physically with RelA. Notably, this interaction required T-bet<sup>S508</sup> as revealed by the failure of the S508A mutant T-bet to coimmunoprecipitate RelA (Fig. 5, A and B). c-Rel, another NF-κB family member that is known to regulate *IL-2* gene expression, also interacted with T-bet; however, this interaction did not require T-bet<sup>S508</sup> (Fig. 5 C). Endogenous interaction of T-bet and RelA also was detected in Th1 cells as described below (see Fig. 7 D).

**T-bet requires the T-bet binding (T-box) site to repress RelA-mediated *IL-2* gene transcription**

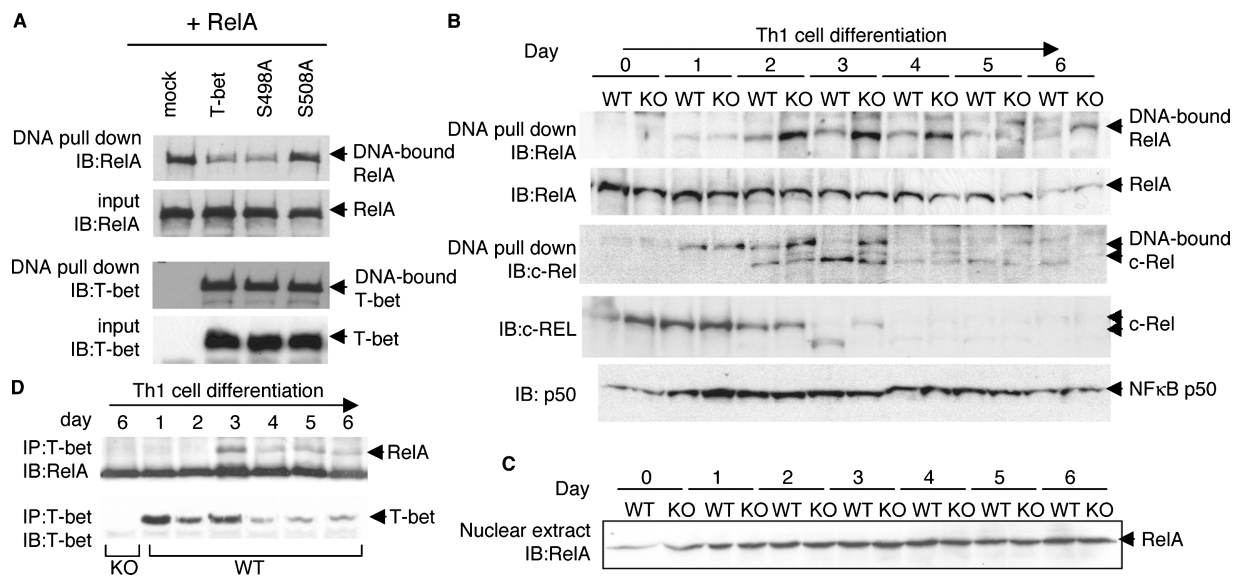
We asked whether the physical association of T-bet with RelA regulated *IL-2* gene transactivation by RelA. Coex-

pression of T-bet and RelA with a 450-bp *IL-2* promoter reporter revealed that T-bet interfered with *IL-2* promoter transactivation by RelA (Fig. 6 A). We next asked whether T-bet repression of RelA-dependent *IL-2* promoter activation required T-bet binding to the T-box site and RelA binding to its target site. T-bet was not able to repress NF-κB-mediated gene activation when three copies of an NF-κB binding site-linked reporter construct was used (Fig. 6 B). This result suggests that T-bet required additional sequences, likely the T-box site, to interfere with NF-κB-dependent gene activation. We tested this by using 5' deletion constructs of the *IL-2* promoter. T-bet overexpression inhibited the exogenous (Fig. 4 B) and endogenous (Fig. 6 C)



**Figure 6. T-bet (T-box) and NF-κB binding sites are required for the repression of NF-κB-mediated *IL-2* gene transcription by T-bet.** T-bet and RelA expression vectors were cotransfected with an *IL-2* promoter reporter (A), a multimerized (three copies) NF-κB binding site-linked reporter

(B), or a 450-bp *IFN-γ* promoter reporter gene (D) into EL4 cells. (C) *IL-2* promoter truncations as shown in the left panel were constructed in pGL2-luc and transfected with or without T-bet. Relative luciferase activities were normalized by β-galactosidase activities and indicated as fold induction.



**Figure 7. T-bet regulates the DNA binding activity of NF- $\kappa$ B RelA.** (A) Whole cell extracts were harvested from 293T cells, transfected with T-bet and RelA expression vectors, and incubated with biotinylated DNA. DNA-protein complexes were resolved by SDS-PAGE and were probed with RelA and T-bet mAbs. (B) CD4<sup>+</sup> LN and spleen Thp cells were isolated from WT B6 and T-bet<sup>-/-</sup>/B6 mice, stimulated as in Fig. 1 B. Whole cell extracts were prepared from developing Th1 cells at the indicated time points and were

used for DNA pull-down assays. Blots were probed sequentially with RelA, c-Rel, and NF $\kappa$ B p50 antibodies. (C) Nuclear extracts from WT and T-bet<sup>-/-</sup> Th1 cells were used in immunoblot assays with RelA antibody. (D) Whole cell extracts were prepared from developing WT and T-bet<sup>-/-</sup> Th1 cells at the indicated time points. Endogenous T-bet was precipitated with anti-T-bet mAb 4B10, applied to SDS-PAGE, and immunoblotted. The blot was probed with RelA mAb and reprobbed with T-bet polyclonal antibody after stripping.

RelA-mediated transactivation of the 2-kb and 250-bp IL-2 promoter constructs that contain T-box and NF- $\kappa$ B sites. However, activation of the 210-bp IL-2 promoter construct, from which the T-box site had been deleted, was not repressed by T-bet expression. We also wondered whether RelA might inhibit T-bet activity similarly. Coexpression of T-bet and RelA with a T-box site-linked reporter construct (not depicted) or with a 450-bp IFN- $\gamma$  promoter reporter in EL4 cells revealed no effect of RelA on T-bet-induced IFN- $\gamma$  promoter activity (Fig. 6 D). We conclude that T-bet requires the T-box binding site to repress NF- $\kappa$ B-mediated gene transcription. Further, the T-bet/RelA heterodimer functions only to regulate RelA, and not T-bet-mediated, gene activation, at least for the genes that were studied here

#### T-bet regulates NF- $\kappa$ B DNA binding activity to the IL-2 gene: RelA DNA binding activity is increased in T-bet<sup>-/-</sup> Th1 cells

We questioned whether the physical interaction of T-bet with RelA and its repression of RelA-mediated IL-2 gene activation might affect the binding of RelA to DNA. Indeed, the repressor function of T-bet for IL-21 gene transcription was secondary to an interaction of T-bet with NFATc2 that interfered with its binding to DNA (48). Although NFAT binding activity to the IL-2 promoter was not affected by concomitant T-bet expression (unpublished data), DNA binding activity of RelA to the IL-2 promoter was reduced markedly in the presence of T-bet (Fig. 7 A).

Of note, in contrast to WT T-bet, S508A mutant T-bet did not inhibit RelA DNA binding to the IL-2 promoter (Fig. 7 A). Comparable expression levels and DNA binding activities of T-bet proteins were confirmed by Western blot analysis (Fig. 7 A). We conclude that S508 of T-bet is not required for its own interaction with DNA. Instead, S508 is required for T-bet interference with the binding of RelA to the IL-2 promoter.

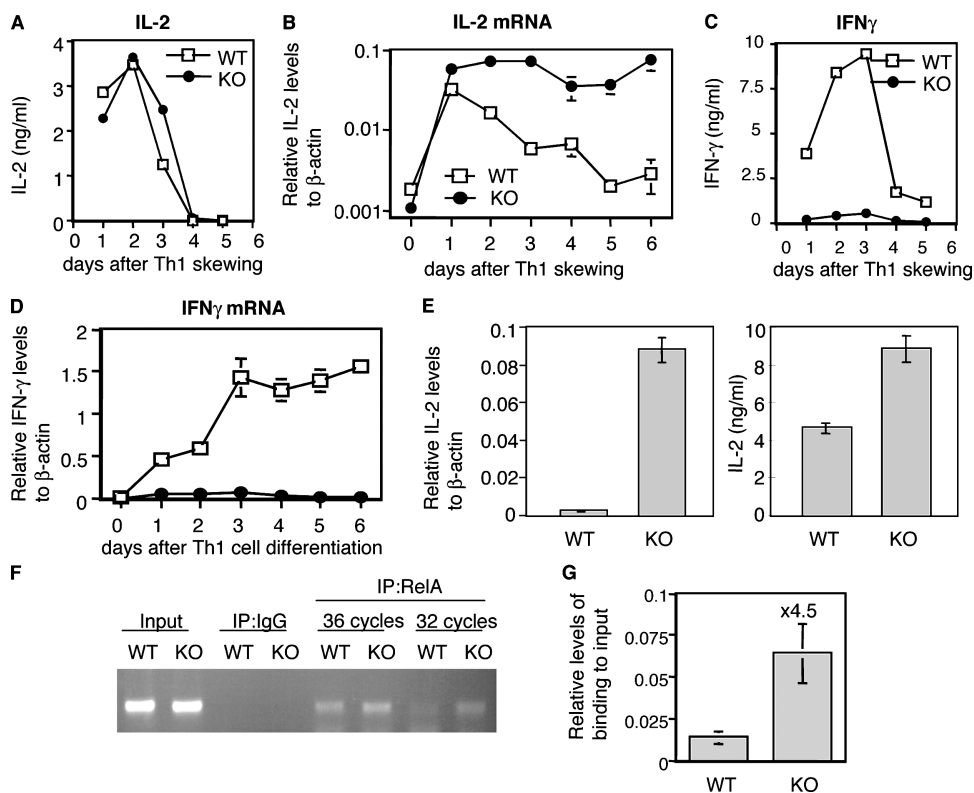
To establish the physiologic relevance of this phenomenon, RelA DNA binding activity was measured over time in T-bet<sup>-/-</sup> and WT Th1 cells. There was no obvious difference in protein expression levels of RelA between WT and T-bet<sup>-/-</sup> Th1 cells, and RelA was expressed continuously at all stages of Th1 cell differentiation (Fig. 7 B, top panel). However, the DNA binding activity of RelA was increased substantially in T-bet<sup>-/-</sup> Th1 cells by day 2 after TCR stimulation. In contrast, c-Rel was expressed in early developing Th1 cells and decreased after day 3 in Th1 cell differentiation. Similarly, c-Rel DNA binding activity continued to increase up to day 3, but was not detected after day 3 in developing Th1 cells, although two species of c-Rel with different DNA-binding activities were present (Fig. 7 B, bottom panel). The NF- $\kappa$ B p50 subunit was expressed continuously over time in developing Th1 cells, similar to RelA. Increased DNA binding of RelA could not be attributed to increased amounts of nuclear RelA protein, because Western blot analysis of nuclear extracts that were prepared from WT and T-bet<sup>-/-</sup> Th1 cells revealed equal expression

of RelA (Fig. 7 C). To investigate the physiologic relationship between RelA and T-bet, we performed experiments to detect an endogenous interaction of T-bet and RelA in developing Th1 cells (Fig. 7 D). Preliminary experiments that were done at day 6 of Th1 differentiation revealed an association of endogenous T-bet and RelA. A time-course analysis of this association revealed that it did not occur until day 3 and persisted through day 6, which correlated with the decreased IL-2 expression that was observed in late developing Th1 cells (Fig. 8, A and B). Together, these results are consistent with the notion that the interaction of RelA and T-bet down-regulates IL-2 production in late, but not early, Th1 cell differentiation.

**RelA DNA binding activity correlates with endogenous IL-2 mRNA transcripts and protein**

Next, we investigated the relationship between T-bet-controlled RelA DNA-binding activity and *IL-2* gene transcription during Th1 differentiation. We compared levels of IL-2 protein and transcripts during Th1 cell differentiation of WT and T-bet<sup>-/-</sup> Th cells. There was no difference in levels of IL-2 produced by WT and T-bet<sup>-/-</sup> Th cells at day 2. This

is consistent with a report that reconstitution of mice with fetal liver from RelA<sup>-/-</sup> embryos revealed no defect in IL-2 production at 18 h, and strongly suggests a primary role for c-Rel, but not RelA, at early time points (49). However, by day 3, substantially more IL-2 was secreted by T-bet<sup>-/-</sup> Th1 cells as compared with WT Th1 cells (Fig. 8 A). Because IL-2 protein was undetectable at later time points by ELISA—probably as the result of increased consumption of it by activated Th cells—we measured the kinetics of IL-2 mRNA expression during Th1 cell differentiation. IL-2 transcripts were induced by TCR stimulation, but decreased gradually in developing WT Th1 cells, which was consistent with a repression of *IL-2* gene expression (Fig. 8 B). In contrast, IL-2 transcripts persisted in T-bet<sup>-/-</sup> Th1 cells, which was consistent with a repressor role of T-bet at that stage of differentiation (Fig. 8 B). Th1 polarization was confirmed by measuring IFN-γ protein, which increased in WT Th1 cells, peaked at day 3, and decreased gradually (Fig. 8 C), whereas IFN-γ transcripts increased continuously over time (Fig. 8 D). As expected, protein and mRNA levels of IFN-γ were almost completely absent in T-bet<sup>-/-</sup> Th1 cells (40) (Fig. 8, C and D). Further, we observed substantially increased levels



**Figure 8. The DNA-binding activity of RelA correlates with increased IL-2 expression in T-bet<sup>-/-</sup> Th1 cells.** Cells and supernatants harvested from WT and T-bet<sup>-/-</sup> Th1 cells in Fig. 7 B were used for ELISA (A and C) and real time RT-PCR (B and D). The restimulated Th1 cells from WT and T-bet<sup>-/-</sup> mice also were harvested for assays for IL-2 transcripts and protein (E). Endogenous mRNA levels were analyzed by real time RT-PCR and nor-

malized by β-actin levels. Specific primers used were: IFN-γ-FWD 5'-AGCAACAGCAAGGCGAAAA-3', IFN-γ-REV, 5'-CTGGACCTGTGGGTGTGA-3'. (F and G) ChIP of RelA in WT and T-bet<sup>-/-</sup> CD4 Th1 cells reveals increased binding of RelA to IL-2 promoter DNA in the absence of T-bet. PCR was performed with specific primers for the IL-2 promoter and DNA-bound RelA was quantitated using real time PCR.



of transcripts encoding IL-2 as well as increased IL-2 protein in restimulated T-bet<sup>-/-</sup> Th1 cells as compared with WT Th1 cells (Fig. 8 E). This is consistent with the repression of IL-2 mRNA and protein that we observed with T-bet overexpression (Fig. 3 E).

We used ChIP assays to test directly whether T-bet expression controlled binding of RelA to IL-2 promoter DNA. DNA/protein complexes in late-stage developing WT and T-bet<sup>-/-</sup> Th1 cells were immunoprecipitated with anti-RelA polyclonal antibody, and were detected by PCR and real-time PCR. Increased IL-2 promoter binding of RelA in T-bet<sup>-/-</sup> Th1 cells was observed in PCR reactions (Fig. 8 F). Real-time PCR quantitatively measured IL-2 promoter binding of RelA compared with input DNA, and revealed a 4.5-fold increase in T-bet<sup>-/-</sup> Th1 cells (Fig. 8 G). We conclude that RelA DNA-binding activity is increased in Th1 cells lacking T-bet, and correlates with an increase in endogenous IL-2 mRNA transcripts and protein. Together, our studies provide strong evidence that the interaction of T-bet with RelA interferes with the binding of RelA to IL-2 promoter DNA, and hence, the coactivation of *IL-2* gene expression by RelA. Further, this process depends upon S508 of T-bet and is correlated closely with phosphorylation of that residue.

## DISCUSSION

Our studies present a novel mechanism for the control of the major T cell growth hormone, IL-2, during Th cell development. Previous work established that the transcription factor, T-bet, had three functions, one of which was to repress the production of IL-2 (39, 40). Here we delineate the mechanism by which T-bet silences *IL-2* gene transcription in developing Th1 cells. We show that T-bet heterodimerizes with the IL-2 activating NF- $\kappa$ B transcription factor, RelA, in a manner that requires T-bet<sup>S508</sup>. The effect of this association is to interfere with the binding of RelA to the IL-2 promoter, and hence, transcriptional activation of the *IL-2* gene by RelA.

Transcription factors are well known to function as activators and repressors. Pit-1, a pituitary POU-domain factor activates growth hormone gene expression in somatotropes, but represses its expression in lactotropes by recruiting a corepressor complex (50). TCF/LEF-1 is a T cell-specific transcription factor that can function as an activator and a repressor (51, 52). The transcription factor, T-bet, also can function as an activator and a repressor of gene expression. We and other investigators showed that T-bet is a positive regulator of IFN- $\gamma$  gene expression through direct interaction with IFN- $\gamma$  promoter DNA (53–57). We also demonstrated that tyrosine phosphorylation of T-bet regulates its interaction with the transcription factor, GATA-3, and hence, its repression of Th2 cytokine gene expression (42). Here we have demonstrated another mechanism by which T-bet can function as a repressor of gene activation. A precedent for such an interaction is the heterodimerization of the transcription factor,

Nur77, with RelA that interferes with the DNA binding activity of the NF- $\kappa$ B complex, and results in repression of RelA-mediated gene transcription (58, 59).

We do not know how the T-bet/RelA interaction acts at the level of the IL-2 locus. Why, for example, are the T-bet and NF- $\kappa$ B elements apposed to one another at the promoter, and why is the T-box element necessary for IL-2 inhibition if the function of T-bet is to sequester RelA away from NF- $\kappa$ B sites? We know that there are at least two posttranslationally modified forms of T-bet (serine- and tyrosine-phosphorylated T-bet), as well as unmodified T-bet, in Th1 cells and speculate that the modified forms probably perform different functions and have different targets than do the unmodified forms. The primary function of the serine-phosphorylated form may be to interact with proteins, such as NF $\kappa$ B RelA, whereas the unmodified T-bet may bind directly to DNA. Antibodies that specifically recognize the serine phosphorylated or tyrosine phosphorylated forms of T-bet will be useful to distinguish these functions.

Posttranslational modification of transcription factors by phosphorylation, ubiquitination, acetylation, or methylation may contribute to dual activating and silencing functions in specifying tissue-specific gene expression (60). T-bet<sup>S508</sup> is phosphorylated in vivo and in vitro, and in vitro phosphorylation of T-bet<sup>S508</sup> is mediated by CKI and GSK-3 kinases. Phosphorylation of serines regulates many fundamental physiologic processes, such as cell cycle control, growth and differentiation, and gene transcription, as exemplified by the critical function of CREB<sup>S133</sup> phosphorylation and the serine phosphorylation of most vertebrate Stat proteins (61, 62). In T cells, stimulation through the TCR results in serine phosphorylation of many cellular proteins and leads to activation and IL-2 production (63–66). In certain instances, serine phosphorylation can be required for homodimerization and heterodimerization of transcription factors. For example, serine phosphorylation of UBF is required for its interaction with RNA polymerase 1 (67), PU.1 phosphorylation regulates its interaction with NF-EM5 (68) and the dimerization of IRF-3 and -7 is activated by phosphorylation of their COOH-terminal domains by the IKK kinase complex (69). Phosphorylation by CKI regulates critical processes, such as Wnt signaling, circadian rhythm, nuclear import, and Alzheimer's disease progression (70–74). In the absence of a Wnt signal, hyperphosphorylation of CKI-prephosphorylated  $\beta$ -catenin by GSK-3 results in ubiquitination and degradation of  $\beta$ -catenin. Therefore, serine phosphorylation of  $\beta$ -catenin ultimately results in decreased gene transcription.

Although our data strongly suggest that the association of T-bet and RelA depends on serine phosphorylation, we cannot conclude firmly that it is required. We were not able to test the contribution of endogenous CKI and GSK-3 phosphorylated T-bet to IL-2 production specifically, because CKI and GSK-3 also phosphorylate and control the function of NFAT transcription factors in IL-2 production (75, 76).

We also cannot rule out the possibility that GSK-3-mediated phosphorylation of T-bet may serve a different function, such as protein stabilization or regulation of T-bet tyrosine phosphorylation. Although not definitive, our data may provide another instance where heterodimerization of two transcription factors is controlled by serine phosphorylation.

The gradual decline of IL-2 production in developing Th cells is an appropriate response to T cell activation and differentiation to ensure that an immune response can be terminated. Many factors have been shown to activate *IL-2* gene expression, but very few have been identified that down-regulate it. T-bet drives the differentiation step by initiating the expression of IFN- $\gamma$  and simultaneously acts to ensure that the production of the early factor IL-2 does not persist. These two functions are physically separable because S508 of T-bet is not required for IFN- $\gamma$  production, but is essential for T-bet-mediated IL-2 repression.

## MATERIALS AND METHODS

**Mice, cell lines, and reagents.** T-bet<sup>-/-</sup> mice backcrossed more than seven generations onto a C57BL/6 genetic background were used with WT control C57BL/6 mice. The mouse Th1 cell clone, AE7, and mouse thymoma, EL4 cells were cultured in RPMI 1640 complete medium. Recombinant mouse cytokines were purchased from BD Biosciences. Human recombinant IL-2, which is not recognized by mouse anti-IL-2 antibody, was obtained from Chiron Corp. All capture and biotin-labeled anti-cytokine antibodies for ELISA were obtained from BD Biosciences. All mice were maintained in a pathogen-free biosafety level 3 facility at the Harvard School of Public Health and were provided with water and mouse chow. The mice were negative for all pathogens as indicated by testing of sentinel animals for mouse pathogens. Handling of mice and experimental procedures were in accordance with the institutional and National Institutes of Health guidelines for animal care and use.

**Isolation of CD4<sup>+</sup> Th cells and in vitro differentiation.** CD4<sup>+</sup> T cells were isolated by magnetic bead purification (MACS, Miltenyi Biotec) from the LNs of 6–8-wk-old T-bet<sup>-/-</sup> and WT C57BL/6 mice, and were stimulated with plate-bound anti-CD3 (2  $\mu$ g/ml), and anti-CD28 (2  $\mu$ g/ml) with recombinant human IL-2 (100 U/ml). For Th1 cell differentiation, anti-IL-4 (5  $\mu$ g/ml) and IL-12 (2 ng/ml) were added at day 0.

**Phosphorylation mapping.** Th1 cells that were stimulated for 72 h were harvested, and nuclear extracts were used for immunoprecipitation of T-bet proteins. Resolved T-bet proteins were stained by GelCode blue staining solution (Pierce Chemical Co.) and excised for MS. Gel pieces were digested in gel with trypsin, and were analyzed by reverse-phase LC-MS/MS in the Taplin Biological Mass Spectrometry Facility.

**Retroviral transduction and ELISA.** Retroviral transduction was performed as described in Fig. 3 A. Supernatants were incubated with cytokine-capturing antibodies, and then incubated sequentially with biotinylated secondary antibodies, avidin-horse radish peroxidase, and phosphatase substrate (Sigma-Aldrich) for detection.

**In vitro kinase assay.** FLAG-tagged T-bet proteins were overexpressed in 293T cells, then immunoprecipitated using FLAG-M2 agarose. Recombinant protein kinases were incubated with T-bet proteins for 1–4 h in the presence or absence of  $\gamma$ -[<sup>32</sup>P]ATP. Reactions were resolved by SDS-PAGE and phosphorylated T-bet was detected by radiography.

**DNA pull-down assay.** Whole cell extracts were prepared with lysis buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 5 mM MgCl<sub>2</sub>, 10% glycerol,

0.1% NP-40, and 1 mM DTT) and incubated with biotinylated double-stranded DNA and streptavidin-agarose for precipitation. Precipitates were washed with HKMG buffer three times and applied onto SDS-PAGE for Immunoblot assay.

**ChIP assays.** ChIP assays were performed according to the manufacturer's instructions (Upstate Biotechnology). Cells ( $6 \times 10^7$ ) were cross-linked with 1.1% formaldehyde, rinsed with ice-cold PBS, and resuspended in lysis buffer (10 mM Tris HCl pH 8.0, 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100, and protease inhibitors). Nuclei were pelleted and sonicated to yield chromatin fragments of 500 bp. The sonicated extracts were incubated with anti-RelA and anti-T-bet polyclonal antibodies. Immune complexes were washed with wash buffer containing lithium chloride. Following the last wash, antibody/protein/DNA complexes were eluted and incubated at 67°C overnight to reverse formaldehyde cross-links. DNA was purified using the QIAGEN PCR Purification kit (QIAGEN), eluted, and used for PCR. The following primer set was used to amplify the IL-2 promoter; IL2p-FWD: 5'-GTTTCATACAGCAGGCGTTCATTG-3'. IL2p-REV: 5'-TTTCTCTTCTGATGACTCTCTGG-3'.

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L.H. Glimcher has equity in, and is on the Corporate Board of, the Bristol-Myers Squibb Company; and has equity in, and is a paid consultant for, Mannkind Corporation, a biopharmaceutical company that owns the rights with Harvard University to the T-bet technology. The authors have no other conflicting financial interests.

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