

The lineage-defining factors T-bet and Bcl-6 collaborate to regulate Th1 gene expression patterns

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The T-box transcription factor T-bet is important for the differentiation of naive CD4⁺ T helper cells (Th cells) into the Th1 phenotype. Much is known about T-bet's role as a transcriptional activator, but less is known about the mechanisms by which T-bet functionally represses alternative Th cell genetic programs. In this study, we first identify *Socs1*, *Socs3*, and *Tcf7* (TCF-1) as gene targets that are negatively regulated by T-bet. Significantly, T-bet's role in the repression of these genes is through a direct interaction with their promoters. Consistent with this, we identified two T-bet DNA-binding elements in the *Socs1* promoter that are functionally used to down-regulate transcription in primary Th1 cells. Importantly, T-bet's novel role in transcriptional repression is because of its ability to physically associate with, and functionally recruit, the transcriptional repressor Bcl-6 to a subset of promoters. Furthermore, T-bet functionally recruits Bcl-6 to the *Ifng* locus in late stages of Th1 differentiation to repress its activity, possibly to prevent the overproduction of IFN- γ , which could result in autoimmunity. Collectively, these data establish a novel mechanism for T-bet-mediated gene repression in which two lineage-defining transcription factors, one a classical activator and one a repressor, collaborate to promote and properly regulate Th1 development.

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Abbreviations used: ChIP chromatin IP; H3K4me2, histone 3-lysine 4 dimethyl; IP, immunoprecipitation; P/I, PMA and ionomycin; qPCR, quantitative PCR; siRNA, small interfering RNA; Tfh cell, T follicular helper cell.

CD4⁺ T cells are central to the adaptive immune response. Naive CD4⁺ T cells can differentiate into several distinct effector cell lineages (Murphy and Reiner, 2002). These include, but are not limited to, the original Th cell subsets, Th1 and Th2, and the more recently defined Th17, regulatory T cell (T reg cell), and T follicular helper cell (Tfh cell) populations. Not surprisingly, each of these cell types harbors a somewhat unique gene expression profile. These distinct profiles are in part regulated by lineage-defining transcription factors, sometimes deemed master regulators. These include T-bet for Th1 cells, GATA-3 for Th2 cells, Foxp3 for T reg cells, ROR- γ t for Th17 cells, and more recently, the transcriptional repressor Bcl-6 for Tfh cells (Zheng and Flavell, 1997; Szabo et al., 2000; Hori et al., 2003; Ivanov et al., 2006; Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009a). The diversity of expression profiles allows each of these cell types to play roles in a variety of immune responses ranging from immune tolerance to promoting antibody generation (Zhu et al., 2010).

The prevailing concept has been that each Th cell type is separate from the other lineages

and strictly defined by the expression of lineage-defining master regulator transcription factors. However, recent data suggest that determining whether these cell types represent a plastic subset versus an endpoint lineage may not be that straightforward because of the fact that many of the lineage-defining factors are expressed in multiple subsets of Th cells as well as more divergent cell types (Zhu and Paul, 2010a,b). For example, the Th1 cell lineage-defining factor T-bet is expressed at varying levels in Th1, T reg, Th17, and Tfh cells (Szabo et al., 2000; Nurieva et al., 2009; Oldenhove et al., 2009; Wei et al., 2009). These studies suggest that instead of a single master regulator for each lineage, the expression levels of, and potential interactions between, lineage-defining transcription factors may drive the overall development and function of a given naive CD4⁺ T cell. This leads to the intriguing possibility that the simultaneous expression of two or

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more lineage-defining factors may promote plasticity between cell subsets dependent on the levels of each factor present in a given condition (Zhou et al., 2009; O'Shea and Paul, 2010). Therefore, more comprehensive studies addressing the molecular mechanisms by which these factors regulate gene expression both cooperatively and independently from one another are important for understanding the true capability of the cell.

The interplay between the expression, localization, and activity of specific transcription factors as well as their ability to bind to and influence the local chromatin structure determines the overall mechanisms of gene control during Th cell development. A subset of these transcription factors, including the Th1 cell lineage-defining factor T-bet, are able to regulate both the activation and repression of genetic loci to promote the development of lineage-specific gene expression patterns (Finotto et al., 2002; Szabo et al., 2002; Djuretic et al., 2007). Although much is known about how T-bet directly activates a few prototypic Th1 target genes, surprisingly little is known about the identity of the genes silenced by T-bet and the mechanisms by which it down-regulates gene expression. In addition, it is still unclear whether T-bet is able to directly repress target genes or rather T-bet's ability to functionally repress gene expression is strictly indirect (Hwang et al., 2005). Therefore, identifying genes that are repressed by T-bet is long overdue and will aid in understanding the mechanisms involved in establishing Th cell genetic programs.

To begin to address these unanswered questions, we identified three biologically important genes, *Socs1*, *Socs3*, and *Tcf7*, that were expressed at higher levels in T-bet-deficient CD4⁺ Th1 cells as compared with their WT counterparts. We show that the promoters of these genes are directly bound and functionally regulated by T-bet. The mechanisms by which T-bet functionally represses these genes are related to its physical association with the Tfh cell lineage-defining transcriptional repressor, Bcl-6. Importantly, T-bet's ability to associate with Bcl-6 allows T-bet, which normally serves as a transcriptional activator, to instead effectively direct a specifically targeted gene repression program. Indeed, we demonstrate in primary Th1 cells that a Bcl-6 repressive complex is targeted by T-bet to the promoters of *Socs1*, *Socs3*, and *Tcf7*, which leads to their T-bet-dependent transcriptional repression during Th1 cell development. Finally, we show that Bcl-6 also is recruited to the *Ifng* locus in a T-bet-dependent manner during late time points of Th1 differentiation correlating with the loss of *Ifng* expression. Collectively, our study provides evidence for a mechanism of transcriptional repression in which two lineage-defining factors collaborate to promote the development and proper functioning of a single Th cell lineage.

RESULTS

T-bet binds to the *Socs1*, *Socs3*, and *Tcf7* promoter regions and represses their transcription

We first wanted to identify gene targets repressed by T-bet in Th1 cells for the purpose of subjecting them to comprehensive transcriptional experiments. This will provide a model

system to start to illuminate the detailed transcriptional regulation mechanisms that T-bet utilizes to repress alternative Th cell genetic programs and ultimately promote Th1 cell lineage differentiation. First, similar to a previous study (Jenner et al., 2009), we performed a microarray analysis comparing WT with T-bet^{-/-} CD4⁺ T cells skewed for 7 d in Th1 conditions to identify candidate targets for the in depth molecular experiments. We identified several genes that had a higher expression level in T-bet^{-/-} as compared with WT Th1 cells, and we selected three for further analysis. Interestingly, the suppressor of cytokine signaling family members *Socs1* and *Socs3* were functionally repressed by T-bet expression in Th1

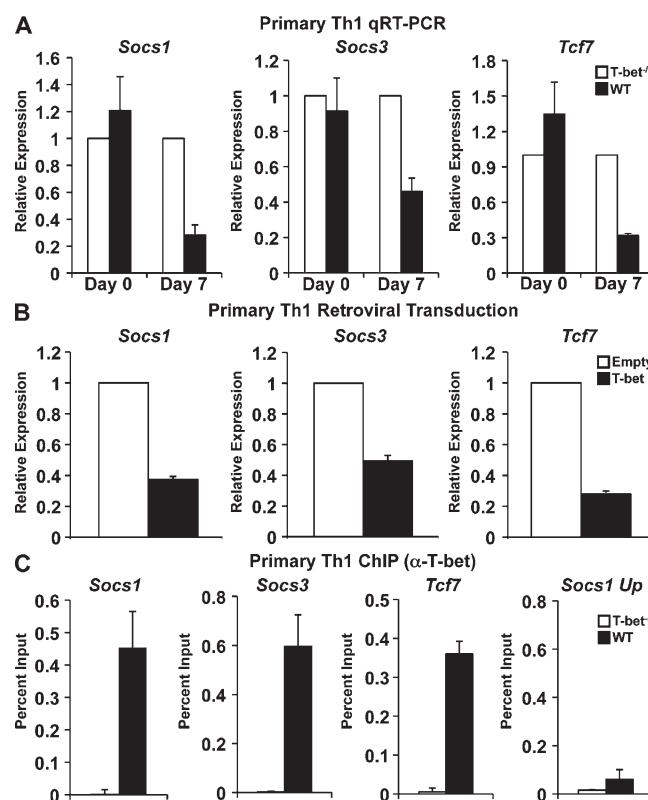


Figure 1. T-bet binds to and represses the transcription of *Socs1*, *Socs3*, and *Tcf7*. (A) RNA was isolated from WT and T-bet-deficient primary CD4⁺ T cells either directly ex vivo or after polarization in Th1 conditions for 7 d. *Socs1*, *Socs3*, and *Tcf7* transcript levels were analyzed by quantitative RT-PCR. Results were normalized to the values obtained for *Actb* and are expressed as a ratio relative to the T-bet^{-/-} sample. (B) RNA was isolated from T-bet^{-/-} CD4⁺ T cells transduced with an empty expression vector or one containing T-bet. Transcript levels were determined by quantitative RT-PCR, and the data were normalized and represented as in A. (C) T-bet association with the promoters of *Socs1*, *Socs3*, and *Tcf7* was assessed by ChIP. Chromatin was isolated from either WT or T-bet^{-/-} CD4⁺ T cells stimulated under Th1 conditions for 7 d. Chromatin samples were immunoprecipitated with antibodies to T-bet or a nonspecific antibody control. After purification, immunoprecipitated DNA was quantitated by qPCR. qPCR signals were normalized to the nonspecific antibody control as well as a standardized aliquot of the input chromatin. (A–C) Data represent the mean of three (A and B) or five (C) independent experiments (error bars indicate SEM).

cells (Fig. 1 A). As their names suggest, Socs proteins are involved in the inhibition of cytokine signaling pathways, and both *Socs1* and *Socs3* have been linked to the negative regulation of the canonical Th1 cytokine, IFN- γ (Alexander et al., 1999; Eyles et al., 2002; Seki et al., 2003; Yamamoto et al., 2003; Harada et al., 2007; Palmer and Restifo, 2009). In addition to *Socs1* and *Socs3*, the gene *Tcf7* was more highly expressed in T-bet^{−/−} Th1 cells (Fig. 1 A). Significantly, the protein product of the *Tcf7* gene, TCF-1, is a transcription factor that was recently shown to promote the development of the Th2 lineage (Yu et al., 2009b). Thus, the biological roles for *Socs1*, *Socs3*, and *Tcf7* make them logical targets to be repressed by T-bet to promote Th1 differentiation, and they provide a good model for determining the mechanisms by which T-bet mediates the repression of alternative Th cell fates.

To confirm that *Socs1*, *Socs3*, and *Tcf7* were functionally repressed by T-bet, we performed a quantitative RT-PCR analysis comparing RNA isolated from WT or T-bet^{−/−} CD4⁺ T cells skewed under Th1 conditions for 7 d (Fig. 1 A). Consistently higher transcript levels for *Socs1*, *Socs3*, and *Tcf7* were detected in the T-bet-deficient cells as compared with their WT counterparts at day 7 of Th1 differentiation. As a control, we also examined gene expression directly ex vivo (day 0) in CD4⁺ T cells that did not yet express high levels of T-bet. Importantly, in these freshly isolated naive CD4⁺ T cells, we observed no difference in *Socs1*, *Socs3*, or *Tcf7* transcript levels between WT and T-bet^{−/−} T cells. These results suggest that the expression of *Socs1*, *Socs3*, and *Tcf7* is functionally repressed by T-bet during the course of Th1 differentiation.

To further confirm that the T-bet-dependent transcriptional repression of these genes is cell intrinsic and not caused by another secondary defect in the T-bet^{−/−} cells, we performed retroviral transduction experiments to express WT T-bet in T-bet^{−/−} CD4⁺ T cells. Consistent with a role for T-bet in repressing *Socs1*, *Socs3*, and *Tcf7* gene transcription, retroviral transduction of T-bet into T-bet^{−/−} CD4⁺ T cells skewed in Th1 conditions caused a decrease in their expression as compared with an empty vector control (Fig. 1 B). Collectively, these data suggest that T-bet has the ability to functionally repress *Socs1*, *Socs3*, and *Tcf7* transcription.

We next performed chromatin immunoprecipitation (IP [ChIP]) assays to examine whether T-bet associates with the *Socs1*, *Socs3*, and *Tcf7* promoters to determine whether the T-bet-mediated functional repression may be the result of a direct role for T-bet. Importantly, T-bet associated with the promoter regions of *Socs1*, *Socs3*, and *Tcf7* in primary CD4⁺ Th1 cells (Fig. 1 C). As negative controls, there were no detectable signals in the T-bet-precipitated chromatin at these promoters in T-bet^{−/−} Th1 cells, and there was no enrichment at a region upstream of the *Socs1* promoter in the T-bet-precipitated sample from the WT Th1 cells (Fig. 1 C). These data suggest that the repression of *Socs1*, *Socs3*, and *Tcf7* has the potential to be, at least in part, caused by direct T-bet binding at each locus.

T-bet recruits permissive chromatin-remodeling complexes to target promoters

Previous studies have demonstrated that T-bet binding to positively regulated target promoters leads to altered histone modifications that are consistent with a permissive chromatin environment. Specifically, T-bet physically recruits chromatin-modifying complexes to induce the permissive histone 3-lysine 4 dimethyl (H3K4me2) mark and an SWI/SNF-dependent general chromatin-remodeling event (Lewis et al., 2007; Miller et al., 2008, 2010). It is possible that T-bet may recruit the same permissive chromatin-remodeling complexes to all target genes, with a downstream event accounting for target gene-specific activation versus repression. Alternatively, T-bet may functionally recruit a unique, repressive chromatin-remodeling complex to the repressed target genes to effectively close the chromatin structure. To explore these possibilities, we performed ChIP assays in the presence and absence of T-bet in primary Th1 cells to determine the relative levels of the permissive H3K4me2 modification. Importantly, at the negatively regulated *Socs1*, *Socs3*, and *Tcf7* loci, as well as the positively regulated *Ifng* locus, there were higher levels of the permissive H3K4me2 modification in WT Th1 cells in comparison with the T-bet^{−/−} Th1 cells (Fig. 2 A). Additionally, we used a restriction enzyme accessibility assay to measure the

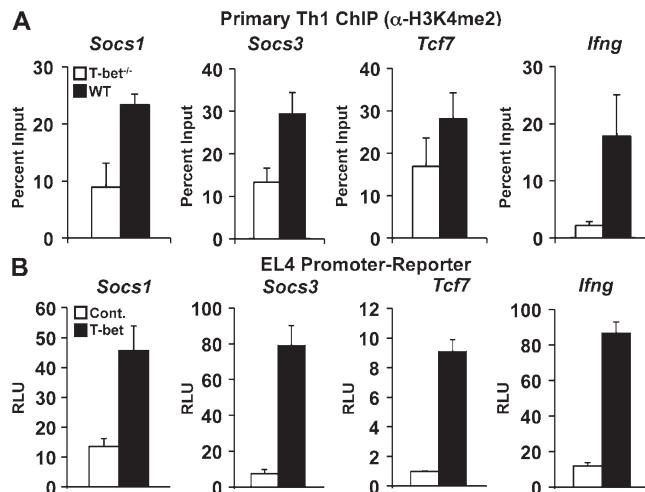


Figure 2. The T-bet-dependent repression of *Socs1*, *Socs3*, and *Tcf7* is independent of the chromatin environment and requires a corepressor. (A) H3K4me2 levels at the promoters of *Socs1*, *Socs3*, *Tcf7*, and *Ifng* were assessed by ChIP. Chromatin was isolated from either WT or T-bet^{−/−} CD4⁺ T cells skewed in Th1 conditions. Chromatin samples were immunoprecipitated with antibodies specific to the H3K4me2 mark or a nonspecific antibody control and quantitated as described in Fig. 1. (B) T-bet-dependent promoter-luciferase reporter activity was examined for *Socs1*, *Socs3*, *Tcf7*, and *Ifng*. EL4 T cells were transfected with the indicated pGL3 promoter-reporter construct and either an empty vector control (Cont.) or a T-bet expression plasmid. After P/I stimulation, luciferase values were measured and normalized to the activity obtained for a cotransfected *renilla* control. (A and B) Data represent the mean of four (A) or six (B) independent experiments (error bars indicate SEM). RLU, relative light units.

T-bet and SWI/SNF-dependent general chromatin remodeling at the promoters (Miller et al., 2010). In both primary Th1 cells that express T-bet and EL4 T cells transfected with a T-bet expression vector, we observed increased accessibility at the promoters of both the positively (*Ifng*) and negatively (*Socs1*, *Socs3*, and *Tgf7*) regulated target genes when compared with their T-bet-deficient counterpart cells (Fig. S1). Collectively, these data suggest that T-bet functionally induces a more accessible chromatin environment independent from the final outcome on the target gene expression.

Overexpression of T-bet alone does not functionally repress *Socs1*, *Socs3*, and *Tcf7* promoter activity

Another mechanism for T-bet-mediated gene repression could involve competition for an overlapping binding site. In this case, T-bet binding would displace a transcriptional activator by partially or completely blocking its binding site in the promoter. In this scenario, T-bet expression alone would be sufficient to repress the negatively regulated target genes. To test this mechanism, we cloned the *Socs1*, *Socs3*, and *Tcf7* promoters upstream of a luciferase reporter vector (pGL3) and performed promoter-reporter assays. For comparison, we also examined an *Ifng* promoter-reporter construct, which is activated by T-bet (Shnyreva et al., 2004). The reporter constructs were cotransfected into EL4 T cells with either a T-bet expression plasmid or a control empty expression vector. Interestingly, T-bet overexpression resulted in enhanced promoter-reporter activity for all promoters tested (Fig. 2 B).

It is important to note that the overexpression of T-bet causes the up-regulation of several endogenous T-bet target genes in EL4 cells including other transcription factors (Beima et al., 2006). Therefore, we hypothesized that the activation of the promoter-reporter constructs for the negatively regulated genes was most likely caused by the up-regulation of another secondary endogenous regulatory factor rather than the direct transactivation capabilities of T-bet at their promoters. To test this possibility, we examined promoter-reporter activity in response to a T-bet mutant that cannot activate endogenous target genes. Importantly, this T-bet mutant is deficient in its ability to interact with chromatin-remodeling complexes but is still capable of binding to DNA and performing general chromatin-independent transactivation events (Miller et al., 2008). The T-bet mutant construct did not activate the *Socs1*, *Socs3*, or *Tgf7* promoters but was still able to induce activity of the *Ifng* promoter-reporter as a control (Fig. S2). These data suggest that the T-bet-dependent up-regulation of a secondary endogenous factor is responsible for the activation observed for the *Socs1*, *Socs3*, and *Tgf7* promoter-reporter constructs. Significantly, the T-bet mutant did not further repress the negatively regulated promoter-reporters, suggesting that T-bet alone does not inherently repress these promoters by blocking the binding of an essential activator (Fig. S2).

The data presented thus far strongly suggest that T-bet does not independently repress the transcription of these negatively regulated gene targets. Rather, the data support a model in which T-bet may act in conjunction with a transcriptional

corepressor to inhibit gene expression. We next wanted to identify putative candidates that might contribute to the T-bet-dependent repression of these target genes. To address this question, we examined the composition of potential DNA-binding motifs for T-bet and transcriptional repressors in the *Socs1* promoter using a computer software program (Genomatix). Interestingly, there are two putative T-bet binding sites as well as two potential binding elements for the transcriptional repressor Bcl-6 in the *Socs1* promoter (Fig. 3 A and Fig. S3). Significantly, both the *Socs3* and *Tgf7* promoters also contain T-bet and Bcl-6 binding sites (Fig. S3 A).

To ascertain the role, if any, for these sites in *Socs1* regulation, we engineered truncation mutant *Socs1* promoter-reporter constructs progressively deleting both the T-bet and Bcl-6 binding sites (Fig. 3 A). Intriguingly, progressive truncation of these sites enhanced promoter-reporter activity in comparison with the full-length *Socs1* construct in both the EL4 cell line and primary Th1 cells (Fig. 3, B and C). These data indicate that a potential negative regulatory element may be located within this region (−1 kb to −280 bp) of the *Socs1* promoter.

Bcl-6 represses promoter activity of *Socs1*, *Socs3*, and *Tcf7*

Given the failure of T-bet overexpression alone to repress promoter activity, we examined the functional consequence that Bcl-6 has on *Socs1* promoter-reporter activity in the presence of T-bet (Fig. 3, D–F). We first examined *Socs1* promoter-reporter activity in response to Bcl-6 knockdown. Importantly, when a small interfering RNA (siRNA) specific to Bcl-6 was transfected in combination with T-bet, there was a modest enhancement of the *Socs1* promoter-reporter activity when compared with cotransfection with a control siRNA (Fig. 3 D). Although the observed effect was modest, it was proportional to the knockdown in Bcl-6 levels in these cells (Fig. 3 E). Importantly, an increase in *Tgf7* promoter-reporter activity was also observed upon Bcl-6 siRNA knockdown (Fig. S4 A). These data are consistent with a role for Bcl-6 in the repression of these genes.

To complement the knockdown results, we also performed overexpression experiments. Strikingly, simultaneous expression of Bcl-6 and T-bet substantially inhibited *Socs1* promoter activity in comparison with T-bet transfection alone (Fig. 3 F). Furthermore, Bcl-6 and T-bet overexpression also inhibited the activity of the *Socs3* and *Tgf7* promoter-reporter constructs. Importantly, expression of Bcl-6 alone only minimally inhibited overall promoter activity in the absence of T-bet. Collectively, these data suggest that a combination of T-bet and Bcl-6 represses the promoter activity of *Socs1*, *Socs3*, and *Tgf7*.

T-bet-dependent repression is independent of Bcl-6 DNA-binding activity

To ascertain whether the Bcl-6-mediated repression is caused by direct binding of Bcl-6 to the *Socs1* promoter, we made a *Socs1* promoter-reporter construct with mutations in the Bcl-6 DNA-binding elements (Fig. 4 A and Fig. S3 B). Surprisingly, this mutant *Socs1* promoter was still repressed by the simultaneous overexpression of T-bet and Bcl-6 (Fig. 4 A).

These data suggest that Bcl-6 may be functionally recruited to the *Socs1* promoter by another factor. To confirm this unexpected result and to rule out the possibility that a cryptic Bcl-6 DNA-binding element is found in the *Socs1* promoter, we next created a Bcl-6 construct with a point mutation in its DNA-binding domain. This mutation disrupts the DNA-binding activity of Bcl-6 but keeps its transcriptional repression capacity intact (Mascle et al., 2003). Significantly, in the presence of T-bet, both WT Bcl-6 and the Bcl-6 DNA-binding mutant (Bcl6^{DBmut}) repressed the T-bet-dependent activity of the *Socs1* promoter to similar levels (Fig. 4 B and Fig. S5 B). In addition, both the *Socs3* and *Tcf7* promoter-reporters were repressed to an equivalent degree by the WT Bcl-6 and Bcl-6 DNA binding-deficient proteins (Fig. 4 B). To further confirm these results, we also examined a second independent Bcl-6 DNA-binding point mutant construct and observed similar results (Figs. S5 and S6). Importantly, as a control, WT Bcl-6 but not the two Bcl6^{DBmut} proteins repressed the promoter activity of *Gzmb*, a known direct Bcl-6 gene target (Fig. S6; Yoshida et al., 2006). These data indicate that in contrast to *Gzmb*, the repressive capability of Bcl-6 at the *Socs1*, *Socs3*, and *Tcf7* promoters does not require its direct DNA-binding activity despite the presence of Bcl-6 DNA-binding elements. Rather, these data highly suggest that Bcl-6 is recruited to these promoters by another factor. Because of the

data presented thus far demonstrating a functional role for T-bet in the repression of *Socs1*, *Socs3*, and *Tcf7* in primary Th1 cells, we hypothesized that T-bet is required for the recruitment of Bcl-6 to these loci.

To start to test this hypothesis, we first examined whether the putative T-bet DNA-binding elements are required for the negative regulation of the *Socs1* promoter. Significantly, as the data from Fig. 3 indicate, there is an increase in *Socs1* promoter activity in the truncation constructs that do not contain the T-bet binding elements. We next made a series of *Socs1* reporter constructs mutating the most distal T-bet site (S1mut1), the most proximal T-bet site (S1mut2), or both in combination (S1mut1,2; Fig. S3 C). We transfected these T-bet point mutant constructs into EL4 cells in either the presence or absence of T-bet. Interestingly, the elimination of both T-bet binding sites in the S1mut1,2 construct resulted in a striking increase in *Socs1* promoter activity, whereas the mutation of either T-bet site individually (S1mut1 or S1mut2) had little to no effect on promoter activity (Fig. 4 C). Importantly, we also examined the same *Socs1* point mutant constructs in the more physiological setting of primary Th1 cells. Once again, the simultaneous mutation of both the proximal and distal T-bet sites led to a significant increase in promoter activity in comparison with the WT *Socs1* promoter-reporter construct (Fig. 4 D). Consistent with the conclusion that the

repression mediated through these T-box DNA-binding elements is T-bet dependent, we did not observe any enhancement of the *Socs1* T-bet binding site mutant construct in comparison with the WT control in T-bet^{−/−} primary Th1 cells (Fig. 4 D vs. Fig. S7). These results suggest that the two T-bet binding elements are critical for the repression of the *Socs1* promoter.

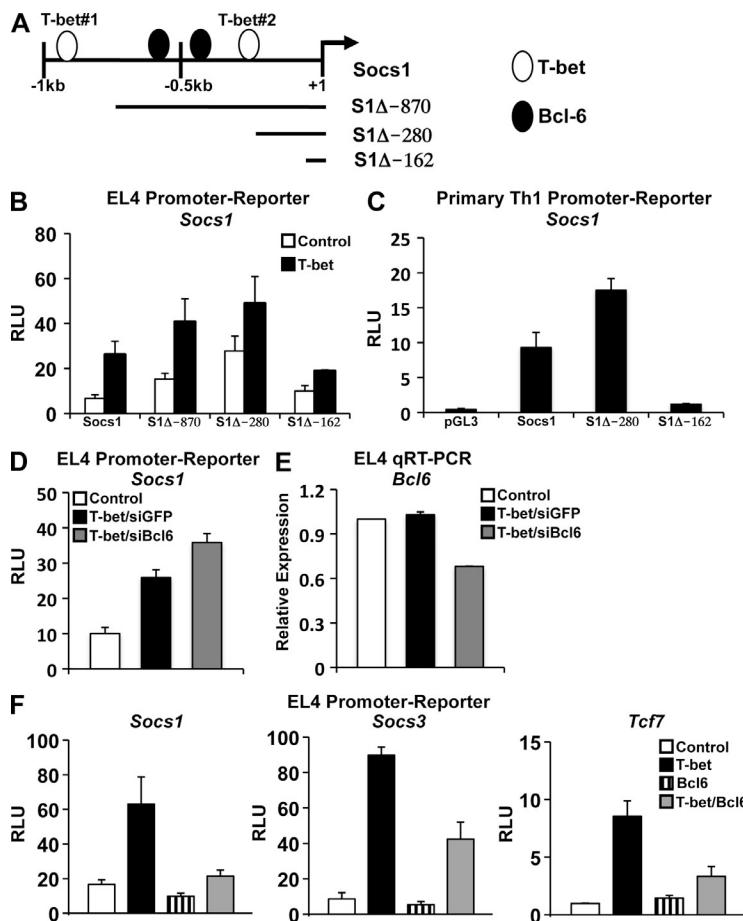
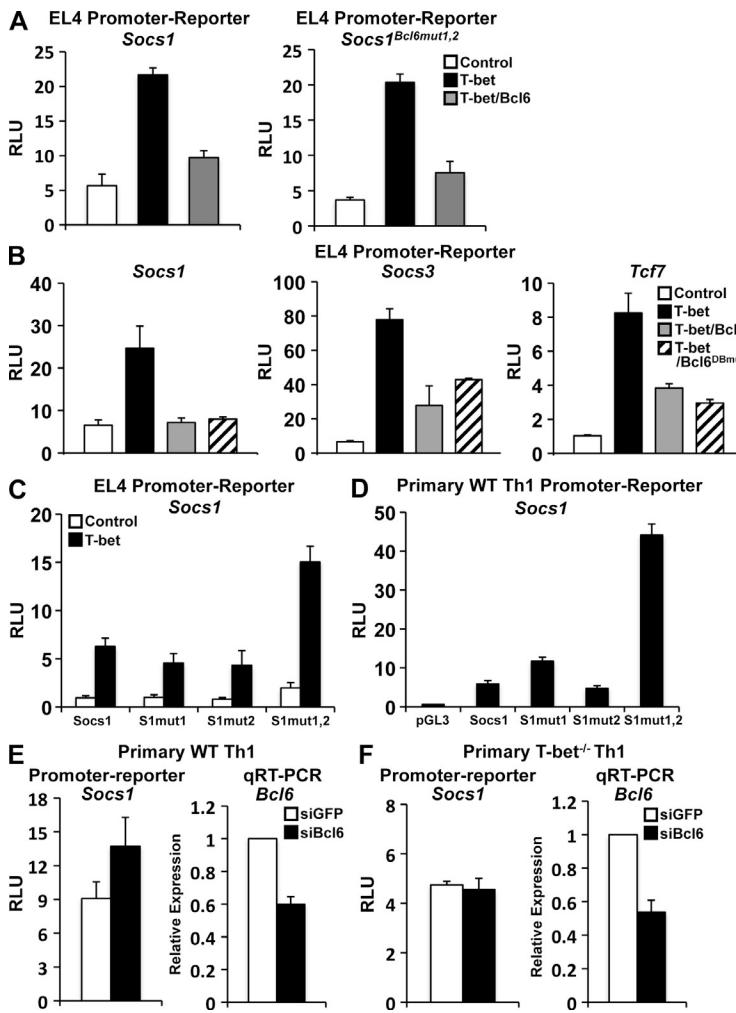


Figure 3. Bcl-6 represses the promoter activity of *Socs1*, *Socs3*, and *Tcf7*. (A) The schematic indicates the location of predicted T-bet binding sites in the *Socs1* promoter as well as the *Socs1* truncation mutant constructs (see Fig. S3 for precise location). (B and C) EL4 (B) or primary WT Th1 (C) cells were transfected with the indicated *Socs1* truncation or full-length promoter-reporter construct. In addition, EL4 cells were cotransfected with either an empty expression vector (control) or one expressing T-bet. Luciferase reporter activity was normalized to the activity obtained for the cotransfected *renilla* control. (D) EL4 cells were transfected with the *Socs1* promoter-reporter in conjunction with a control or T-bet expression vector and a control siRNA (siGFP) or one specific to Bcl-6 (siBcl6). After P/I stimulation, luciferase promoter-reporter values were normalized to the *renilla* control. (E) EL4 cells were transfected and treated as in D. RNA was isolated, and *Bcl6* transcript levels were analyzed by quantitative RT-PCR. Results were normalized to the values obtained for *Actb* and are expressed as a ratio relative to the control sample. (F) EL4 T cells were cotransfected with the indicated pGL3 promoter-reporter construct and an empty vector control, T-bet, Bcl-6, or T-bet and Bcl-6 in combination. After P/I stimulation, luciferase promoter-reporter values were normalized to the *renilla* control. (B–F) Data represent the mean of three (B–E) or four (F) independent experiments (error bars indicate SEM). RLU, relative light units.



We next wanted to determine whether the repressive capability of Bcl-6 requires a T-bet-dependent activity. To test this possibility, we transfected WT versus T-bet^{-/-} primary Th1 cells with either a control siRNA or one specific to Bcl-6. In WT Th1 cells, the knockdown of Bcl-6 enhanced *Socs1* promoter-reporter activity (Fig. 4 E). Significantly, Bcl-6 knockdown had no effect on *Socs1* activity in the absence of T-bet in the T-bet^{-/-} Th1 cells (Fig. 4 F). Importantly, the Bcl-6 knockdown levels were equivalent in WT and T-bet^{-/-} Th1 cells, with the degree of Bcl-6 reduction proportional to the enhancement of *Socs1* promoter-reporter activity in the WT cells (Fig. 4 E and F). Collectively, these data suggest that the Bcl-6-mediated repression of the *Socs1* promoter is dependent on T-bet.

T-bet recruits Bcl-6 to the *Socs1*, *Socs3*, and *Tcf7* promoters in Th1 cells

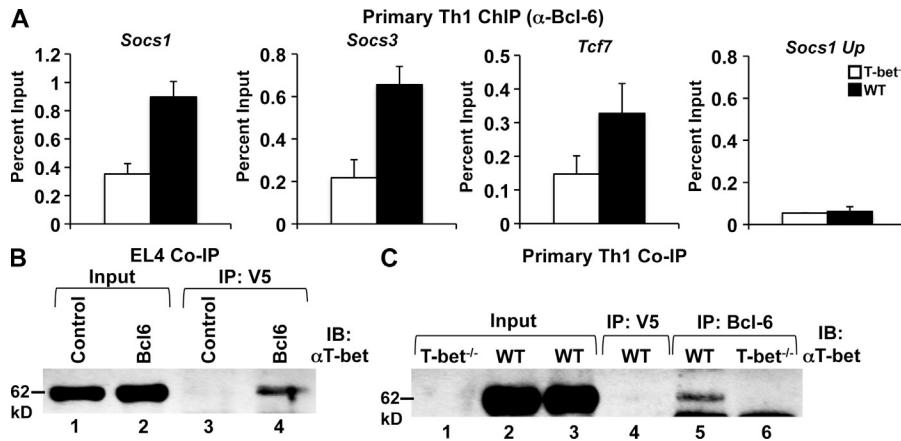
The data we have presented thus far strongly imply that both a T-bet DNA binding-dependent and a Bcl-6 DNA binding-independent activity are necessary for the repression of the *Socs1*, *Socs3*, and *Tcf7* genes. To test whether T-bet is required

Figure 4. Bcl-6-dependent repression of *Socs1*, *Socs3*, and *Tcf7* is independent of Bcl-6 DNA binding. (A) The *Socs1^{Bcl6mut1,2}* or WT promoter-reporter constructs were cotransfected with an empty expression vector (control), T-bet, or T-bet and Bcl-6. (See Fig. S3 for precise location of the Bcl-6 binding site mutations in the *Socs1^{Bcl6mut1,2}* construct.) (B) EL4 T cells were transfected with the indicated pGL3 promoter construct and an empty vector control, T-bet, T-bet and Bcl-6, or T-bet and the Bcl-6 DNA-binding mutant (Bcl6^{DBmut}). (C) The indicated *Socs1* point mutants (in T-bet binding sites) or WT promoter-reporter constructs were cotransfected with either an empty expression vector (control) or one expressing T-bet. (A–C) The samples were stimulated with P/I, and the reporter activity was measured and normalized to the activity of a *renilla* control. (D) The indicated *Socs1* point mutant (in T-bet binding sites) or WT promoter-reporter constructs were transfected into primary WT Th1 cells. After overnight incubation, reporter activity was measured and normalized to a *renilla* control. (E and F) Primary WT (E) or T-bet^{-/-} (F) Th1 cells were transfected with the *Socs1* promoter-reporter and either a control siRNA (siGFP) or one specific to Bcl-6 (siBcl6). Luciferase promoter-reporter values were normalized to the *renilla* control. RNA was isolated from the primary Th1 cells, and *Bcl6* transcript levels were analyzed by quantitative RT-PCR. Results were normalized to the values obtained for *Actb* and are expressed as a ratio relative to the control sample. (A–F) Data represent the mean of three (A and D) or four (B, C, E, and F) independent experiments (error bars indicate SEM). RLU, relative light units.

for the recruitment of Bcl-6 to these promoters in Th1 cells, we performed ChIP experiments to examine Bcl-6 association with the *Socs1*, *Socs3*, and *Tcf7* promoters in WT versus T-bet^{-/-} primary Th1 cells. Significantly, there was a higher level of Bcl-6 association with all three promoters in the WT versus T-bet^{-/-} Th1 cells (Fig. 5 A). As a negative control, we did not detect Bcl-6 at a location upstream of the *Socs1* promoter (which also is not bound by T-bet; Fig. 1 C), indicating that the targeting of Bcl-6 is specific to the T-bet-bound area in the promoter region. As an additional control, the protein and RNA expression levels of Bcl-6 were similar in WT and T-bet^{-/-} Th1 cells (Fig. S8), indicating that alterations in the amount of Bcl-6 present in each cell type cannot explain the observed differences. Collectively, these data strongly suggest that the recruitment of Bcl-6 to the *Socs1*, *Socs3*, and *Tcf7* promoters is dependent on the presence of T-bet.

T-bet and Bcl-6 physically interact to repress transcription

Collectively, the aforementioned data suggest that T-bet is required to recruit Bcl-6 to the negatively regulated promoters. We next wanted to determine whether T-bet targets Bcl-6 to these promoters by physically interacting with Bcl-6. To start to test this possibility, we performed a co-IP experiment in which we cotransfected EL4 T cells with T-bet in combination with either an empty vector construct or one expressing Bcl-6. Importantly, T-bet specifically precipitated with Bcl-6, indicating that T-bet and Bcl-6 can be found in the same complex in this overexpression system (Fig. 5 B). We then



empty vector control. Lysates were prepared and immunoprecipitated with an antibody for the V5 epitope tag and then probed with a T-bet-specific antibody. (C) Lysates from either WT or T-bet^{-/-} primary Th1 cells were immunoprecipitated with either a Bcl-6 (lanes 5 and 6) or control antibody (lane 4). After IP, a Western blot analysis was performed with a T-bet-specific antibody. (B and C) Data are representative of three (B) or five (C) independent experiments. IB, immunoblot.

wanted to explore whether T-bet and Bcl-6 form a stable complex at their endogenous expression levels in primary CD4⁺ Th1 cells. In co-IP experiments, T-bet coprecipitated with Bcl-6 in WT Th1 cells (Fig. 5 C). As a negative control, the T-bet signal was not detected in either WT Th1 cells immunoprecipitated with a nonspecific antibody control or in T-bet^{-/-} CD4⁺ Th1 cells (Fig. 5 C). Collectively, these experiments suggest that T-bet and Bcl-6 have the ability to

Figure 5. Bcl-6 is recruited to the promoters of *Socs1*, *Socs3*, and *Tcf7* through a physical interaction with T-bet in Th1 cells. (A) Chromatin was isolated from either WT or T-bet^{-/-} CD4⁺ T cells stimulated in Th1 conditions for 7 d. Chromatin samples were immunoprecipitated with either an antibody to Bcl-6 or a nonspecific antibody control. Immunoprecipitated DNA was quantitated by qPCR and normalized to the nonspecific antibody control as well as a standardized aliquot of the input chromatin. Data represent the mean of five independent experiments (error bars indicate SEM). (B) EL4 T cells were transfected with an untagged T-bet expression construct in combination with either a V5 epitope-tagged Bcl-6 expression vector or

form a repressive complex to inhibit the transcription of *Socs1*, *Socs3*, and *Tcf7*.

The C-terminal domain of Bcl-6, which contains six zinc fingers, is required for its association with T-bet

We next wanted to determine which domain or domains within Bcl-6 mediate its interaction with T-bet. To accomplish this, we created Bcl-6 truncation constructs and tested their ability to form a complex with T-bet in EL4 co-IP experiments (Fig. 6 A). A Bcl-6 construct lacking the repressive BTB/POZ domain still associated with T-bet, with only a modest reduction in its interaction as compared with WT Bcl-6 (Fig. 6 B). Interestingly, Bcl-6 mutant constructs with a C-terminal truncation deleting its six zinc fingers were no longer able to

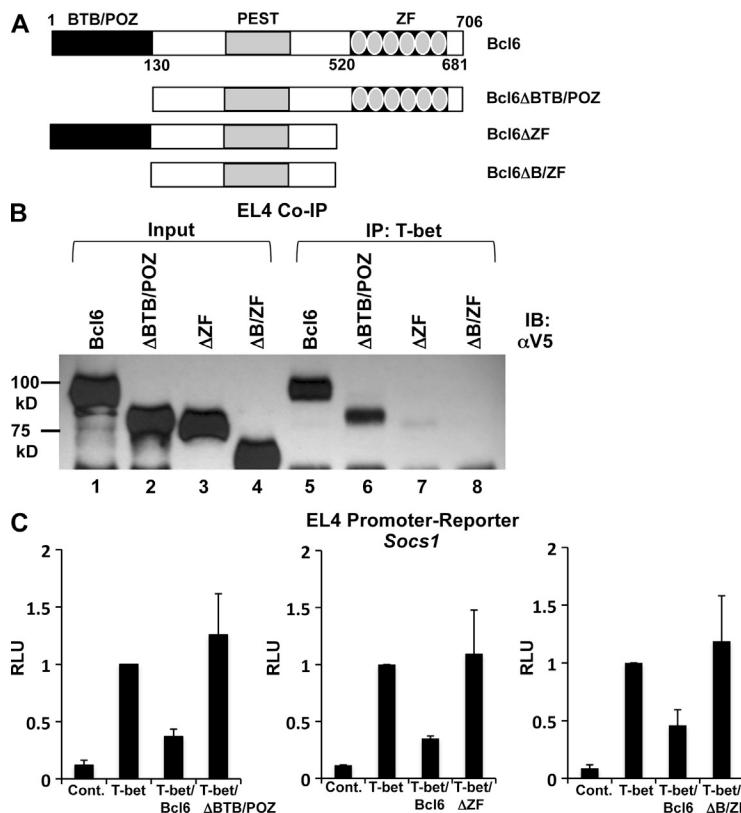


Figure 6. The C-terminal zinc finger domain of Bcl-6 is required for its association with T-bet. (A) The schematic indicates the location of the primary functional domains contained within Bcl-6. These include an N-terminal BTB/POZ domain, a C-terminal domain containing six individual zinc fingers (displayed as gray ovals), and a centrally located PEST domain. Diagrams of the Bcl-6 truncation mutant constructs are shown below the WT schematic. (B) EL4 T cells were transfected with an untagged T-bet expression construct in combination with V5 epitope-tagged WT Bcl-6 (lanes 1 and 5) or the Bcl-6 truncation mutant expression vectors as indicated (lanes 2–4 and 6–8). Lysates were prepared and immunoprecipitated with an antibody to T-bet (lanes 5–8). The blot was then probed with a V5 epitope tag-specific antibody. Data are representative of three independent experiments. (C) EL4 T cells were transfected with the full-length *Socs1* promoter-reporter construct and an empty vector control, T-bet, T-bet and Bcl-6, or T-bet and the indicated Bcl-6 truncation mutants. After P/I stimulation, luciferase promoter-reporter values were normalized to the *renilla* control. Data represent the mean of three independent experiments (error bars indicate SEM). IB, immunoblot; RLU, relative light units.

associate with T-bet (Fig. 6 B). These data suggest that the C-terminal domain of Bcl-6 is required for its interaction with T-bet. This is an intriguing result because the six zinc fingers within this domain are known to mediate both DNA binding as well as other protein–protein interactions (Mascle et al., 2003). It will be important to determine in future experiments whether the association between T-bet and Bcl-6 impedes the DNA-binding activity of Bcl-6, which could preferentially redirect its targeting to T-bet binding elements.

We next assessed the functional role for each domain in mediating T-bet–dependent repression. Significantly, the Bcl-6 C-terminal zinc finger truncation constructs defective in their ability to associate with T-bet no longer repressed *Socs1* promoter-reporter activity (Fig. 6 C). In addition, the Bcl-6 BTB/POZ domain truncation also could not repress *Socs1* promoter-reporter activity (Fig. 6 C). This is consistent with the required role for the BTB/POZ domain in the repressive capability of Bcl-6 (Basso and Dalla-Favera, 2010). We also observed similar losses of repressive capabilities for the Bcl-6 truncations at the *Tgf7* promoter (Fig. S9). Collectively, these data are consistent with the hypothesis that Bcl-6 is functionally recruited by T-bet to repress the activity of these promoters.

Bcl-6 also represses *Ifng*, a target gene positively regulated by T-bet

We next sought to determine whether Bcl-6 plays a role in regulating genes that are normally activated by T-bet in Th1 cells. To accomplish this goal, we first examined the effect of Bcl-6 overexpression on T-bet–dependent *Ifng* promoter-reporter activity. Interestingly, Bcl-6 repressed the T-bet–dependent activation of the *Ifng* promoter similar to its functional effect at the *Socs1*, *Socs3*, and *Tgf7* promoters (Fig. 7 A). In addition, the knockdown of Bcl-6 also enhanced the T-bet–dependent activation of the *Ifng* promoter-reporter construct (Fig. S4). To assess whether Bcl-6 has the same effect on *Ifng* transcription in the context of the chromatin environment, we overexpressed T-bet either alone or in combination with Bcl-6 and assessed endogenous *Ifng* gene transcripts by quantitative RT-PCR. Significantly, Bcl-6 also repressed the T-bet–dependent activation of endogenous *Ifng* gene expression (Fig. 7 B). These results suggest that Bcl-6 plays a role in the functional repression of both the genes that are positively and negatively regulated by T-bet.

These intriguing observations led us to examine whether Bcl-6 may play a role in regulating *Ifng* expression during primary Th1 cell differentiation. We first wanted to determine whether Bcl-6 is present at the *Ifng* promoter in primary Th1 cells. Interestingly, in a ChIP analysis comparing WT with T-bet^{−/−} Th1 cells (day 7), similar to the negatively regulated targets, Bcl-6 associated with the *Ifng* promoter in a T-bet–dependent manner (Fig. 7 C). Given these results, we hypothesized that the Bcl-6 association with the *Ifng* locus may temper its expression. If this is the case, Bcl-6 may be mechanistically down-regulating *Ifng* in the late stages of the Th1 effector cell to prevent the overproduction of IFN- γ , which could result in potential T cell–mediated autoimmune diseases.

To begin to test this possibility, we analyzed the time course of *Ifng* expression during Th1 differentiation. We observed high levels of *Ifng* transcripts at day 3 and a subsequent decrease in transcript levels by day 7 of Th1 differentiation (Fig. 7 D).

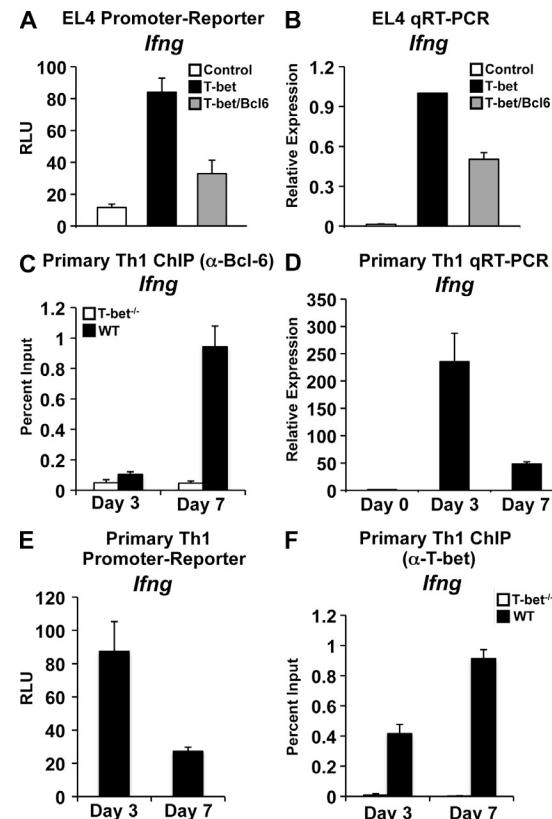


Figure 7. T-bet-dependent recruitment of Bcl-6 to the *Ifng* promoter in late time points of Th1 culture correlates with a reduction in *Ifng* gene transcription. (A) EL4 T cells were cotransfected with the indicated pGL3 promoter-reporter construct and an empty expression vector control, T-bet, or T-bet and Bcl-6. After P/I stimulation, luciferase values were measured and normalized to a *renilla* control. (B) EL4 T cells were transfected and treated as in A followed by RNA isolation. A quantitative RT-PCR analysis to determine endogenous *Ifng* transcript levels was performed and normalized to *Actb*. (C) Chromatin was isolated from either WT or T-bet^{−/−} CD4⁺ T cells at day 3 or 7 of Th1 differentiation. Chromatin samples were immunoprecipitated with either an antibody to Bcl-6 or a nonspecific antibody control. Immunoprecipitated DNA was quantitated by qPCR with signals for the *Ifng* promoter normalized to the nonspecific antibody control as well as a standardized aliquot of the input chromatin. (D) RNA samples were isolated from WT CD4⁺ T cells at days 3 and 7 of Th1 differentiation. A quantitative RT-PCR analysis was performed to determine *Ifng* transcript levels, and results were normalized to *Actb*. The data are represented as fold change over the *Ifng* transcript levels at day 0. (E) The *Ifng* promoter-reporter construct was transfected into primary Th1 cells at days 3 and 7 of Th1 differentiation. After overnight incubation, luciferase values were measured and normalized to both empty vector (pGL3) and *renilla* controls. (F) ChIP samples were treated and analyzed as in C with the exception that a T-bet antibody was used for IP. (A–F) Data represent the mean of four (A, C, and F) or three (B, D, and E) independent experiments (error bars indicate SEM). RLU, relative light units.

Additionally, *Ifng* promoter-reporter activity was higher in day 3 versus day 7 primary Th1 cells (Fig. 7 E). Importantly, Bcl-6 was not associated with the *Ifng* promoter at day 3 when *Ifng* expression was approximately fivefold higher (Fig. 7 C). Therefore, the decrease in *Ifng* expression from day 3 to 7 correlates with the T-bet-dependent recruitment of Bcl-6 to the *Ifng* promoter specifically at this late stage of Th1 differentiation.

To rule out the possibility that the lower levels of *Ifng* transcripts in the late stages of Th1 differentiation are caused by a reduction in T-bet binding to the *Ifng* promoter, we performed a ChIP analysis on day 3 versus day 7 from WT and T-bet^{-/-} Th1 cells. In contrast to the decrease observed in *Ifng* transcripts at day 7, there was actually an increase in the amount of T-bet bound to the promoter at day 7 (Fig. 7 F). Thus, the amount of T-bet bound to the *Ifng* promoter does not explain the decrease in transcription. Collectively, these data suggest the mechanism by which Bcl-6 represses T-bet-dependent promoter activity may be important for inhibiting both genes that are activated and repressed by T-bet in Th1 cells.

DISCUSSION

In this study, we have identified three novel gene targets that are negatively regulated by T-bet in Th1 cells. Interestingly, the mechanism by which T-bet functionally represses these genes involves a physical association with the Tfh cell lineage-defining factor, Bcl-6. Bcl-6 is targeted by T-bet to the promoters of *Socs1*, *Socs3*, and *Tcf7* and, once associated, represses the transcriptional activation of these genes. Interestingly, Bcl-6 also antagonizes the T-bet-dependent activation of *Ifng*, a gene positively regulated by T-bet. Collectively, the data suggest that the interplay between T-bet and Bcl-6 is important for establishing appropriate gene expression patterns in Th1 cell development (Fig. 8).

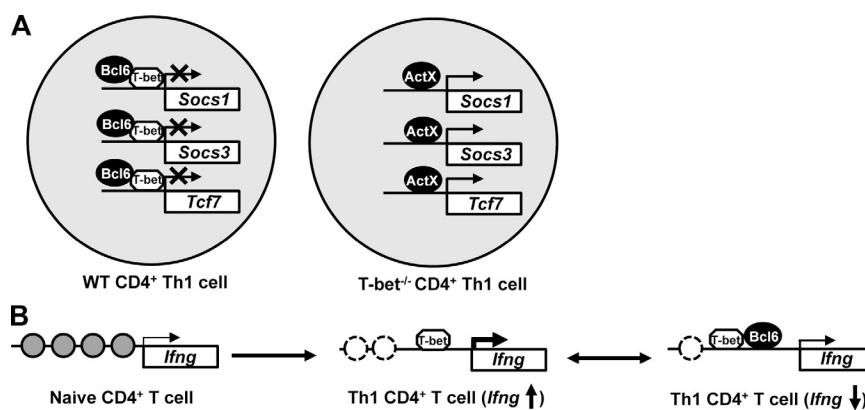
Recently, the transcriptional repressor Bcl-6 was identified as a key factor that promotes Tfh cell differentiation (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009a). In this study, we demonstrate that in differentiated Th1 cells, T-bet and Bcl-6 collaborate to repress the transcription of several genetic loci. Our findings, as well as others that demonstrate the simultaneous expression of multiple lineage-defining

factors in a single Th cell type, now strongly suggest that the notion of one unique master regulator per Th cell type may not be entirely accurate (Zhou et al., 2009; O'Shea and Paul, 2010; Zhu and Paul, 2010b). Instead, the regulation of critical lineage-defining factors by each other and the interactions between them are clearly important aspects in determining Th cell fate. For example, it is known that Th1 cells express high levels of T-bet and low levels of Bcl-6, whereas the reverse scenario is found in Tfh cells (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009a). Our data suggest that when T-bet is in excess, it will target the available Bcl-6 to a subset of promoters that are preferentially repressed during the later stages of Th1 development. Furthermore, these data argue that a simple change in the expression levels of either of these two lineage-defining factors could result in dramatic changes in gene expression networks and possibly have implications for plasticity between the Th1 and Tfh cell types.

In addition to Tfh cells, Bcl-6 has been implicated in the formation of both CD4⁺ and CD8⁺ memory T cells. Central memory T cells are characterized by reduced expression of Blimp1 as compared with effector memory T cells, which express high levels of Blimp1 (Kallies et al., 2009; Rutishauser et al., 2009). Importantly, Blimp1 and Bcl-6 negatively regulate each other's expression, which results in an inverse correlation between the two factors. Thus, central memory T cells with low Blimp1 should express higher levels of Bcl-6 in comparison with effector memory T cells. Interestingly, the cytokine production from a reactivated central memory T cell is indeed subdued in comparison with that of an effector memory T cell response (Kaech and Wherry, 2007). Therefore, it is possible that the mechanisms described in this study could aid in tempering T-box protein-dependent cytokine production in central memory T cells.

It is also interesting to speculate about how the DNA binding-independent targeting of Bcl-6 to gene loci may differentially affect gene expression patterns in different cell types. To date, microarray data sets examining gene expression patterns between

Figure 8. Models representing the mechanisms by which T-bet and Bcl-6 cooperate to regulate transcription. (A) Schematic portraying the differences in the regulation of *Socs1*, *Socs3*, and *Tcf7* genes in WT versus T-bet-deficient CD4⁺ T cells. In WT Th1 cells, T-bet-dependent targeting of Bcl-6 to the *Socs1*, *Socs3*, and *Tcf7* promoters results in transcriptional repression. Conversely, the absence of T-bet in the T-bet^{-/-} Th1 cells results in a loss of Bcl-6 targeting to the *Socs1*, *Socs3*, and *Tcf7* promoters and gene activation. (B) A schematic representation of Bcl-6 repression at the *Ifng* locus. In the naive CD4⁺ T cells, gray circles represent closed chromatin. By day 3 of Th1 differentiation, T-bet binds to the promoter and functionally induces chromatin remodeling, resulting in enhanced *Ifng* transcription. By day 7 of Th1 differentiation, the reduced level of *Ifng* transcription correlates with the T-bet-dependent recruitment of Bcl-6 to the *Ifng* promoter.



immune cell types that harbor high Bcl-6 expression levels contain few similarities (Johnston et al., 2009; Rutishauser et al., 2009). Our study demonstrates that some gene targets repressed by Bcl-6 are determined by its recruitment to T-bet binding sites rather than the DNA-binding capabilities of Bcl-6 itself. Therefore, if the mechanisms described in this study are conserved in other cell types, Bcl-6 could be targeted to genetic loci based on the predominance of a particular transcription factor, in this case, T-bet. This will then alter both T-bet and Bcl-6 target gene expression patterns to create a specialized profile for the cell.

The identification of *Socs1*, *Socs3*, and *Tgf7* as genes that are negatively regulated by T-bet provides new insight into the gene expression network that is established during Th1 development. Importantly, the repression of the Socs family members *Socs1* and *Socs3* is necessary to create a functional Th1 cell. Numerous studies have shown that *Socs1* and *Socs3* repress the Th1 cell lineage and promote other Th cell types such as Th2 cells (Diehl et al., 2000; Egwuagu et al., 2002; Eyles et al., 2002; Seki et al., 2003). For *Socs1*, this is because of its role in inhibiting the IFN- γ and STAT1 signaling pathway, a signaling module critical for the development and function of a Th1 cell (Sakamoto et al., 1998; Alexander et al., 1999). Significantly, *Socs1*-deficient CD4 $^{+}$ T cells have a propensity to develop into Th1 cells, whereas overexpression of *Socs1* inhibits the Th1 cell lineage (Diehl et al., 2000; Eyles et al., 2002). Similar to the role for *Socs1* in antagonizing IFN- γ signaling, *Socs3* plays an analogous role in disrupting STAT4 and IL-12 signaling, which is another pathway crucial for Th1 cell development (Seki et al., 2003; Yamamoto et al., 2003; Takatori et al., 2005). Furthermore, *Socs3* is preferentially expressed in the CD4 $^{+}$ T cells of the Th2 cell lineage (Egwuagu et al., 2002). Importantly, the dysregulation of *Socs1* and *Socs3* has been linked to the onset of autoimmune diseases and some types of cancer, providing strong evidence for their biological significance in the proper regulation of the immune response (Cottet et al., 2001; Karlsen et al., 2001; He et al., 2003; Seki et al., 2003; Chong et al., 2004; Weniger et al., 2006). Collectively, the impact of *Socs1* and *Socs3* on immune cell function and its involvement in human disease highlights the importance in determining the mechanisms governing their proper regulation, including the role of T-bet and Bcl-6 in this process.

Our experiments also identified *Tgf7* as a target of T-bet-mediated repression. Recently, the protein product of the *Tgf7* gene, TCF-1, has been linked to the transcriptional regulation of GATA-3, the lineage-defining factor for the Th2 lineage (Yu et al., 2009b). TCF-1 promotes the development of the Th2 cell fate by promoting GATA-3 expression and repressing IFN- γ production. Therefore, the repression of *Tgf7* by T-bet will inhibit the Th2 fate. To underscore the biological significance of this, *Tgf7* has been linked to the onset of Type 1 diabetes, a disease which has also been associated with dysregulation of T-bet and Th1 cells (Noble et al., 2003; Erlich et al., 2009).

Interestingly, our data also suggest that Bcl-6 is directly involved in the regulation of the canonical Th1 cytokine,

IFN- γ . Previous studies examining Thf cells found that when Bcl-6 is in excess during Thf cell development, *Ifng* expression is significantly impaired (Nurieva et al., 2009; Yu et al., 2009a). In addition, overexpression of Bcl-6 in primary T cells results in a modest decrease in *Ifng* transcription, whereas the absence of Bcl-6 results in a dramatic increase (Nurieva et al., 2009). These observations were previously attributed to an indirect mechanism related to the ability of Bcl-6 to repress *Tbx21* (the gene encoding T-bet). However, the absolute levels of T-bet did not always correlate with *Ifng* expression in these experiments, and this explanation also could not account for an initial reduction in *Ifng* before the down-regulation of T-bet (Nurieva et al., 2009). Our data now extend these findings by identifying *Ifng* as a direct target of Bcl-6 repression. We demonstrate that Bcl-6 associates with the *Ifng* locus in a T-bet-dependent manner and inhibits its transcription at late time points in Th1 differentiation. Collectively, these data suggest that the interplay between T-bet and Bcl-6 may help to ensure that the expression of *Ifng* remains at a moderate level so that there is neither too little, which would impede the initiation of an appropriate immune response for pathogen clearance, nor too much, which could result in autoimmunity.

It is interesting to note the timing for the T-bet-dependent association of Bcl-6 with the *Ifng* promoter. *Ifng* transcript levels inversely correlate with the recruitment of Bcl-6 at later time points in Th1 differentiation. However, the environmental or intracellular cues that prompt the targeting of Bcl-6 to the *Ifng* locus are currently unknown. The answers to these questions await a better understanding of the additional regulatory signaling pathways and complexes involved in this process. A comprehensive understanding of these mechanisms will provide insight into Th1 and Thf cell fate choices and the potential for plasticity between these two Th cell fates. Collectively, the study presented here provides evidence for a model of gene regulation in which two lineage-defining transcription factors collaborate to promote the development of the Th1 cell fate by repressing genes that contribute to the differentiation of other Th cell types, as well as regulating the expression of genes necessary for the proper functioning of the Th1 cell.

MATERIALS AND METHODS

Cell culture and transfection

Primary T cells. Primary CD4 $^{+}$ T cells were isolated from the spleen and lymph nodes of WT and T-bet $^{-/-}$ mice using the Mag Cellect kit (R&D Systems) as previously described (Beima et al., 2006). After isolation, cells were grown on plate-bound α -CD3/ α -CD28 and treated with Th1 polarizing cytokines: 5 μ g/ml α -IL-4, 5 ng/ml IL-12, and 10 ng/ml IL-2 (National Cancer Institute preclinical repository). On day 3, cells were split and treated under Th1 conditions for an additional 3 d. All experiments involving mice were conducted in accordance with Institutional Animal Care and Use Committee approval. For transfections involving primary cells, the Amaxa nucleofection system (Lonza) was used. Manufacturer protocols were followed using mouse primary T cell solutions and program X-01.

EL4 T cells. Mouse EL4 T cells (American Type Culture Collection) were grown in RPMI with 10% FBS and penicillin/streptomycin. Cells were transfected using the Amaxa nucleofection system using program 0-17 and solution V as previously described (Beima et al., 2006). After transfection, the

cells were left untreated or treated overnight with PMA (Sigma-Aldrich) and ionomycin (P/I; Sigma-Aldrich) as indicated. Western blot analysis was performed to ensure equal protein expression.

Retroviral constructs and transduction

The T-bet–GFP–MSCV construct was generated by subcloning T-bet into the GFP–MSCV vector. T-bet–MSCV was transfected into Phoenix cells according to protocols from G. Nolan (Stanford University, Stanford, CA). Primary CD4⁺ T cells from T-bet^{−/−} mice were spin infected with T-bet–GFP–MSCV or GFP–MSCV viral supernatant in the presence of 5 mg/ml polybrene at 37°C for 90 min at 2,000 rpm and then left overnight. After transduction, cells were cultured in Th1 polarizing conditions. GFP-positive cells were sorted on day 7, and RNA was harvested for analysis.

RNA and RT-PCR

RNA was isolated using the Nucleospin (Macherey-Nagel) RNA purification protocol. For quantitation, cDNA was prepared using the First Strand Superscript II Synthesis System protocol (Invitrogen). cDNA was then used as a template for quantitative PCR (qPCR) reactions using the qPCR Sybr Green mix (Thermo Fisher Scientific) and gene-specific primers (Table S1). Data obtained were normalized to *Actb* levels for each sample.

ChIP assay

The ChIP assay was performed as described previously (Beima et al., 2006; Lewis et al., 2007; Miller et al., 2008). Antibodies to T-bet (H-210) and Bcl-6 (C-19) were obtained from Santa Cruz Biotechnology, Inc., whereas the antibody to the H3K4me2 mark was obtained from Millipore. In brief, chromatin was harvested from primary WT and T-bet^{−/−} CD4⁺ T cells stimulated under Th1 skewing conditions. Chromatin was incubated overnight with the indicated antibody. After a series of washes, DNA was isolated, and qPCR was conducted (for primers see Table S1). Samples were normalized to a standardized total input DNA control and nonspecific antibody control (IgG).

Restriction enzyme accessibility assay

This protocol has been described previously (Weinmann et al., 1999; Oestreich et al., 2006; Miller et al., 2010). In brief, nuclei from 5 × 10⁶ cells were isolated and treated with the restriction enzyme BanII for 2.5 min at 37°C. After digestion, DNA was purified, and samples were ligated to a BanII-specific linker using T4 DNA ligase (New England Biolabs, Inc.). Nested ligation-mediated PCR was performed with primers specific for both the linker and the promoter of the gene analyzed (Table S1). DNA input was controlled for by amplifying *Actb*, which is independent of the restriction enzyme digestion.

Promoter-reporter assay

The *Socs1*, *Socs3*, and *Tgf7* promoters were cloned into the pGL3 luciferase reporter construct (Promega). *Socs1* mutant promoter-reporters were generated by either PCR, in the case of the truncation mutants, or QuikChange (Agilent Technologies) for the T-bet and Bcl-6 DNA-binding element mutant constructs. The *Ifng* promoter-reporter construct has been described previously (Shnyreva et al., 2004). EL4 cells were transfected with the indicated promoter-reporter constructs in combination with T-bet, Bcl-6, Bcl-6 mutant V5-tagged expression vectors, or an empty vector control as indicated. A TK-renilla control plasmid was used to normalize transfection efficiency. Cell aliquots were collected for analysis 16–22 h after transfection. For luciferase analysis, the cells were treated and analyzed according to the Dual-Luciferase Reporter assay protocol (Promega). The siRNA experiments included a siBcl6 smart-pool (Thermo Fisher Scientific) and siGFP (Invitrogen) and were performed as described previously (Miller et al., 2008, 2010).

Co-IP

The co-IP assay was performed as described previously (Miller et al., 2008, 2010). AV5 antibody (Invitrogen) and a T-bet-specific antibody (H-210) were used for the experiments in the EL4 cells. For endogenous protein expression in the primary Th1 cells, an antibody for Bcl-6 (C-19) was used for IP, with the presence of T-bet detected using the 4B10 antibody (Santa Cruz Biotechnology, Inc.).

Online supplemental material

Fig. S1 uses a chromatin accessibility assay to show that the T-bet–dependent repression of *Socs1*, *Socs3*, and *Tgf7* is independent of the chromatin environment. Fig. S2 demonstrates that a T-bet mutant deficient in chromatin-remodeling capabilities is unable to activate the *Socs1*, *Socs3*, and *Tgf7* promoters. Fig. S3 is a schematic representation of the *Socs1*, *Socs3*, and *Tgf7* promoters displaying the locations of the T-bet and Bcl-6 binding sites. Fig. S4 is a promoter-reporter assay demonstrating an increase in *Tgf7* and *Ifng* promoter activity when Bcl-6 levels are reduced. Fig. S5 is a Western blot analysis of the expression levels for the WT Bcl-6 and Bcl6^{DBmut} constructs. Fig. S6 demonstrates that the overexpression of two unique Bcl6^{DBmut} constructs represses *Socs1* and *Tgf7* promoter-reporter activity but not that of *Gzmb*. Fig. S7 shows that there is no alteration of *Socs1* promoter activity in T-bet^{−/−} primary Th1 cells when T-bet binding sites are mutated. Fig. S8 demonstrates that the expression levels of Bcl-6 are similar in WT and T-bet^{−/−} primary Th1 cells. Fig. S9 is a promoter-reporter assay demonstrating the requirement for both the BTB/POZ and C-terminal zinc finger domains in the repression of *Tgf7* promoter activity by Bcl-6. Table S1 lists the primer sequences used in this study. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20102144/DC1>.

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The authors declare that they have no financial conflicts of interest.

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