Published in final edited form as:

Trends Immunol. 2012 February ; 33(2): 78–83. doi:10.1016/j.it.2011.10.005.

T-bet employs diverse regulatory mechanisms to repress transcription

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Abstract

Lineage-defining transcription factors are responsible for activating the signature genes required for a given cell fate. They are also needed to repress the genetic programs associated with alternative lineage decisions. The T-box transcription factor T-bet is required for CD4⁺ T helper 1 cell differentiation. Numerous studies have explored the mechanisms by which T-bet activates the Th1-gene profile, but until recently, not much was known about the mechanisms T-bet utilizes to negatively regulate alternative T helper cell differentiation pathways such as the Th2 and Th17 fates. Here, we discuss new advances in the field that highlight the diverse mechanisms that T-bet employs to antagonize the gene programs for alternative T helper cell fates.

Lineage-defining transcription factors

Cellular differentiation requires that a precise series of events occur in a tightly regulated fashion. Unique genetic programs need to be activated, and others repressed, in response to environmental cues that signal the cell to proceed towards a specific cell fate decision. Numerous regulatory proteins are needed for a cell to establish its gene expression profile and the sheer number of factors that participate in this process should not be trivialized. However, a few transcription factors have been singled out and termed “lineage-defining” (or “master regulator”) because they orchestrate the cell fate choice, and without them, a particular cell-fate specific gene expression program cannot be established. Significantly, these factors are not inhibited by the current epigenetic state of the cell that normally dictates gene expression potential. Instead, they have the functional capacity to dramatically alter the chromatin environment and ultimately the expression of the genome [1]. Therefore, the lineage-defining transcription factors are so named because they have the mechanistic capability to create cell-type specific gene profiles to program a unique cell fate. In this review, we will discuss new insights into the diverse mechanisms that the T helper cell lineage-defining transcription factor T-bet, classically known for its role in activating Th1 signature genes, uses to functionally repress the gene expression programs for alternative T helper cell fates. These mechanisms highlight the emerging appreciation for the concept that there is a complex interplay between T helper cell lineage-defining factors that is required to establish the specific T helper cell subtypes.

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Helper T cell differentiation

Naïve helper T cells have the capacity to develop into a number of different subtypes based upon the cytokine environment that they are exposed to at the time of activation [2]. Cytokine-signaling events are responsible for the initial decision to induce expression of one of the T helper cell lineage-defining transcription factors. The expression of T-bet is required for T helper 1 (Th1) cell development, GATA3 for Th2 cells, Ror γ t for Th17 cell differentiation, and Bcl-6 for T follicular helper (Tfh) cell development [3–8]. Importantly, these T helper cell lineage-defining transcription factors coordinate the series of events that activate the genes required for establishing a given T helper cell phenotype, while simultaneously repressing the genes necessary for the development of alternative subtypes.

One intriguing, but difficult to reconcile, concept is that each individual T helper cell lineage-defining transcription factor has been shown only to have the intrinsic ability to either activate or repress gene transcription. Specifically, T-bet, GATA3 and Ror γ t are inherently transcriptional activators that in isolation will induce transcription, but cannot on their own repress it [3, 5, 9]. In contrast, Bcl-6, the Tfh-lineage-defining transcription factor, is inherently a transcriptional repressor that does not possess the intrinsic ability to activate gene transcription [10, 11]. As discussed, the factors that are capable of defining a lineage must by definition have the ability to direct the entire underlying gene expression program, which includes both the activation and repression of genes. Therefore, mechanisms must exist outside of our current understanding that allow these factors to accomplish the seemingly opposing functions of gene activation and gene repression in a tightly regulated manner.

T-bet as the lineage-defining transcription factor for Th1 cell differentiation

Studies examining the mechanisms by which the T-box transcription factor T-bet regulates the Th1 gene program are starting to provide insight into this important conundrum. The role for T-bet in activating Th1-signature genes has been extensively analyzed [4, 12–14]. One mechanism by which T-bet regulates the decision to express Th1-specific genes is by altering the epigenetic or chromatin environment of the T helper cell [15–17]. To accomplish this, T-bet physically interacts with, and functionally recruits, several chromatin remodeling complexes to its target genes ([9, 16, 17], Figure 1A). Importantly, the T-bet-dependent enhancement of chromatin accessibility at Th1 signature genes creates an epigenetic environment that is now primed for the binding of other transcriptional activators such as NFAT, AP-1, STAT4, and NF- κ B [15, 18–20]. These more ubiquitously expressed regulatory proteins are then able to access their exposed DNA binding elements and induce gene transcription. In addition, T-bet also physically interacts with some regulatory factors, such as Runx3, to aid in their recruitment to gene loci [21]. Of note, the physical interaction and cooperative binding of T-bet and Runx3 to the *Ifng* and *Il4* loci results in context-dependent gene activation or repression, respectively [21].

Dating back to the first studies examining T-bet-deficient mice, it has also been clear that T-bet functionally opposes alternative T helper cell fate decisions [22, 23]. In the absence of T-bet, T helper cells are more likely to adopt the functional characteristics of Th2 or Th17 cells because they fail to repress opposing genetic programs [22, 24, 25]. These studies provided strong evidence that T-bet both promotes Th1 cell differentiation as well as prevents the development of alternative T helper cell fates. Importantly, the propensity of T-bet-deficient cells to express alternative T helper cell genetic programs represents the underlying mechanism that is responsible for causing airway inflammation in asthma and vascular inflammation that results in cardiac allograft rejection in *Tbx21*-(the gene that encodes T-bet)-deficient mice [22, 26–29]. Therefore, disrupting the ability of T-bet to mediate the

repression of alternative T helper cell gene programs plays a pathogenic role in autoimmune and inflammatory diseases [26].

At the cellular level, it has long been known that IFN γ production by Th1 cells inhibits the development of alternative T helper cell fates [30]. Therefore, the ability of T-bet to activate *Ifng* gene expression plays a cell extrinsic role in repressing opposing T helper cell programs. Recently, several unique mechanisms by which T-bet functionally represses alternative T helper cell fate genetic programs in a cell intrinsic manner have been elucidated. These run the gamut from indirectly sequestering or blocking the critical activators for opposing lineage-specific genes to directly interacting with the transcriptional repressor Bcl-6 to hijack its inherent repressive capabilities [24, 25, 31, 32]. Here, we will discuss each of these repression mechanisms in more detail as well as their implications for both establishing and maintaining gene expression patterns in Th1 cells.

Sequestering the Th2-lineage-defining factor GATA3

The discovery that T-bet can physically interact with the Th2-lineage-defining transcription factor GATA3 provided the first insight into the role of T-bet in the repression of alternative T helper cell gene programs [24]. The model for how a T-bet–GATA3 complex represses the Th2 genetic program invokes the idea that at the early stages during naïve helper T cell commitment decisions, very low amounts of the T helper cell lineage-defining transcription factors are present. As the naïve helper T cell becomes activated, a competition begins to select the T helper cell lineage-defining transcription factor that will be the dominant factor to enforce a specific developmental gene expression program. Part of the competition between the opposing T helper cell specific factors takes place during the time period preceding the dramatic induction of their expression by cytokine signaling events. In the case of the competition between T-bet and GATA3, TCR signaling through Itk phosphorylates T-bet to promote its interaction with GATA3 [24]. The interaction between T-bet and GATA3 then prevents GATA3 from binding to and activating its direct target genes to establish the Th2 signature genetic program (Figure 1B-I). Therefore, in this model, an initial TCR signaling event tips the cell away from the Th2 gene program, subsequently followed by positive-feedback loops that enhance the expression of T-bet and repress the expression of GATA3 to reinforce the Th1-gene program. Thus, the ability of T-bet to sequester the required Th2-activator away from its target sites creates a situation where T-bet can indirectly repress Th2 signature genes at the early stages of the T helper cell commitment decision.

Preventing the expression of the Th17-lineage-defining factor Ror γ t

A recent study suggests that T-bet functionally represses the Th17 genetic program by physically interacting with Runx1 and blocking it from activating *Rorc* (the gene that encodes Ror γ t) transcription (Figure 1B-II) [25]. Ror γ t is required to activate the Th17 signature cytokine IL-17 [3]. Thus, unlike the case with GATA3 where there is a direct competition between T-bet and the Th2-lineage-defining transcription factor, in this scenario, T-bet indirectly represses the Th17 program by actually preventing the expression of the Th17-lineage-defining transcription factor Ror γ t.

The mechanism by which T-bet sequesters Runx1 from activating the *Rorc* promoter is intriguing [25]. Similar to the mechanism for GATA3, T-bet and Runx1 physically interact with each other at the early stages of the naïve helper T cell commitment decision, which allows T-bet to effectively prevent Runx1 from activating its target genes. However, what makes this interaction different, and possibly changes its functional implications, is that the interaction interface for the Runx1–T-bet complex includes amino acids that are required for T-bet's ability to bind to DNA [25]. Thus, a T-bet–Runx1 complex will likely hinder T-bet

from binding to its target sites as well. Therefore, if the concentration of the two proteins are equal, the competition will result in a canceling out of each protein's activity. This means that for the T-bet–Runx1 interaction to promote Th1 over Th17 differentiation, excess T-bet will need to be present to overcome a likely reciprocal inhibition.

Another observation from the study defining T-bet's role in indirectly repressing Th17 differentiation was that the *Rorc* promoter contains adjacent Runx1 and T-bet binding elements [25]. The binding of T-bet and Runx1 to these elements is mutually exclusive [25]. Specifically, if T-bet is bound to the element, Runx1 cannot bind, and conversely, if Runx1 is bound, T-bet cannot bind. This mutually exclusive binding pattern was postulated to occur because the physical interaction between the two proteins prevents the other from accessing its DNA binding element, with only the protein found in excess able to eventually associate with the DNA. However, the location of the binding elements also makes it possible that the binding of either T-bet or Runx1 to the *Rorc* promoter precludes the other from associating with its site because the bound factor may prevent the recognition of the adjacent DNA binding element. Future research examining whether all Runx1 target genes are inhibited by T-bet expression, or rather only a subset with adjacent DNA binding elements are repressed, will address the specificity and overall functional impact for the T-bet–Runx1 repression mechanism. Nevertheless, in either scenario, the inhibition of the Th17 program involves a mechanism by which T-bet represses the Th17-lineage-defining transcription factor Ror γ t by competing with Runx1, a required activator for its expression.

T-bet is converted into a site-specific repressor by interacting with the Tfh-lineage-defining transcriptional repressor Bcl-6

The role of T-bet role in sequestering GATA3 and Runx1 represent indirect mechanisms to repress the gene expression signatures for opposing T helper cell subtypes. That is, T-bet prevents the functional activity of the required activators for the Th2 and Th17 gene programs, but T-bet itself does not directly participate in the site-specific repression of these programs. Evidence that T-bet plays a direct role in repressing gene expression came in a study examining the T-bet-dependent repression of *Socs1*, *Socs3*, and *Tcf7* in committed Th1 cells [32]. T-bet represses these genes by directly recruiting the transcriptional repressor Bcl-6 to their promoters (Figure 1C–D). T-bet accomplishes this by physically interacting with Bcl-6 and targeting it to T-bet DNA binding elements in a subset of promoters in Th1 cells. Thus, T-bet is effectively transformed into a site-specific transcriptional repressor when in complex with Bcl-6.

Several aspects of this T-bet-dependent repression mechanism are worth highlighting. T-bet utilizes Bcl-6 to directly repress gene expression in fully committed Th1 cells. This contrasts to T-bet–GATA3 or T-bet–Runx1 interactions, which take place in naïve helper T cells at the earliest stages of the commitment decision [24, 25]. Thus, the ability of T-bet to sequester GATA3 and Runx1 is only functionally important during the initial commitment process because GATA3 and Runx1 expression are downregulated in polarized Th1 cells [24, 25]. However, T-bet still needs to repress genes in fully committed Th1 cells and its interaction with Bcl-6 provides a direct mechanism to accomplish this task. Bcl-6 is expressed at low concentrations throughout Th1 differentiation and the T-bet–Bcl-6-containing complex is targeted to a subset of T-bet DNA binding elements in committed Th1 cells [32]. As Bcl-6 is the Tfh-lineage-defining transcription factor [6–8], there must be mechanisms in place in Th1 cells to limit both the expression levels and functional activity of Bcl-6, otherwise, high Bcl-6 activity will result in the expression of a Tfh-gene program. Current data suggest that the T-bet–Bcl-6 complex found in Th1 cells may be specifically targeted to T-bet DNA binding elements because the T-bet, but not the Bcl-6, DNA binding sites in the *Socs1* promoter were required for the T-bet–Bcl-6 complex to functionally

repress its transcription [32]. Defining the mechanisms that precisely regulate the T-bet–Bcl-6 complex might provide insight into the stable versus dynamic nature of T helper cell gene programs.

How T-bet targets Bcl-6 to regulatory regions in Th1 cells offers a mechanism by which a T helper cell lineage-defining transcription factor can alter its intrinsic functional activity. Specifically, the classical activator T-bet can function as a site-specific transcriptional repressor when it forms a complex with Bcl-6. T-bet needs to activate Th1 signature genes at the same time that it must repress the genes for alternative lineage fate decisions [21, 24, 25, 28, 32]. How T-bet DNA binding sites are selected in a target gene specific manner to either interact with the T-bet–Bcl-6 repressive complex or the more classically defined T-bet–chromatin remodeling complexes, or the T-bet–general activation complexes is unresolved. Is there a preference for a particular DNA binding element to recruit the T-bet–Bcl-6 repressive complex to a highly specific subset of promoters? It is reasonable to hypothesize that there will be a mechanism to precisely regulate which T-bet complex is targeted to an individual regulatory region to either promote gene activation or repression. Perhaps a bioinformatics analysis of the composition of the T-bet DNA binding elements from the different classes of genes will provide insight into this important question.

The implication of a T-bet–Bcl-6 repressive complex selectively regulating a subset of target genes extends beyond the paradigm of T helper cell differentiation. In CD8⁺ T cells, T-bet and its related T-box family member Eomes are required for the development of effector and memory gene programs [33–35]. Significantly, Bcl-6 also plays a role in this process [36–39]. Thus, both T-bet and Bcl-6 regulate the functional characteristics of CD8⁺ T cells, but to date, the mechanisms by which they accomplish this are mostly unknown. Will a T-bet–Bcl-6 repressive complex be targeted to a similar subset of genes in the context of all cell types or are there competing influences in each context that mediate cell-type specificity for the T-bet–Bcl-6 interaction and/or targeting? One can imagine that there may be signaling events that regulate the T-bet–Bcl-6 interaction and/or targeting similar to what is observed with the T-bet–GATA3 or T-bet–Runx1 complex formation [24, 25]. In addition, there may be other competing regulatory factors in different cell types that change the probability of whether a T-bet–Bcl-6 repressive complex forms.

T-bet directly represses *Pdcd1* (PD-1) transcription in CD8⁺ T cells

A recent study found that T-bet directly represses *Pdcd1* (the gene that encodes PD-1) transcription in CD8⁺ T cells [31]. Mechanistically, the data point to a direct role for T-bet in the repression of *Pdcd1*. Specifically, T-bet directly binds to the endogenous *Pdcd1* promoter in CD8⁺ T cells and the overexpression of wild-type T-bet, but not a T-bet DNA binding mutant, represses *Pdcd1* promoter-reporter activity [31]. These data strongly indicate that T-bet directly represses the *Pdcd1* promoter in a site-specific manner. However, what is currently unclear is the exact nature of the T-bet-dependent repression mechanism. One hypothesis is that T-bet binding to the *Pdcd1* promoter functionally interferes with the DNA binding site for a required transcriptional activator (Figure 1C-II). As discussed above for the adjacent Runx1 and T-bet elements in the *Rorc* promoter, effectively competing for an overlapping or adjacent binding element will result in site-specific gene repression if the competition displaces a required activator. At present, it is unknown whether this, or an as yet to be discovered, mechanism regulates *Pdcd1* expression. It is notable that PD-1 is one of several inhibitory receptors that can be negatively regulated by T-bet in CD8⁺ T cells [31]. It will be of significant interest to determine whether a common T-bet-mediated repression mechanism enforces the similar expression patterns for this class of inhibitory receptors during chronic infections.

Implications for T-bet DNA binding site-specific versus site-independent repression mechanisms

The wave of recent research examining how the Th1-lineage-defining transcription factor T-bet functionally represses gene expression highlights the diverse mechanisms that a classically defined transcriptional activator can employ to negatively regulate the gene programs for opposing fate choices. Not surprisingly, a number of these repression mechanisms are indirect methods to effectively impede the required activators for opposing genetic programs. Competition between activators can be directed in either a DNA site-specific or site-independent manner. Site-specific competition occurs when a T-bet DNA binding element is adjacent to, or overlapping with, another required transcriptional regulatory protein element (Figure 1C-II). In this scenario, the expression of a select subset of genes will be altered as T-bet levels reach a competitive equilibrium with another transcriptional activator. One inherent assumption in this model is that the mechanisms that T-bet utilizes for regulating transcription are not compatible with the mechanistic requirements for the activation of that particular gene subset. That is, the regulatory mechanisms of T-bet cannot be functionally redundant with the activities of the transcriptional activator that it is displacing. Otherwise, T-bet binding to that promoter would have the same effect as the binding of the displaced activator. This suggests that gene subsets require a highly specific composition of regulatory factors that are only compatible with gene activation in precisely controlled circumstances.

The other mechanism for competitive interference does not require the presence of the T-bet DNA binding element in the repressed gene program. Rather, in this model, T-bet physically interacts with another required activator to prevent it from targeting to its own DNA binding elements (Figure 1B). For this mechanism to be effective, several assumptions have to be true. First and foremost, competitively sequestering another factor will impact all genes that rely on that factor, making this somewhat of a “sledgehammer” approach to repressing transcription. In the case of T-bet and GATA3, this may be advantageous because they regulate opposing T helper cell lineage gene expression programs in a mutually exclusive manner [2, 40]. Another significant hurdle in the competitive interference model is that to effectively sequester a factor from its target genes, conditions within the cell must significantly favor one factor over the other, if not, both gene programs will be cancelled out. Regulating the absolute expression levels for the competing factors must be involved to some degree if a physical interaction is expected to saturate the activity of one factor, while maintaining enough of the other to freely access its own target genes. It is also possible that selective protein modifications that either enhance or impede the formation of a sequestering complex will allow environmental signaling events to regulate the timing and nature of the competition to tip the balance in favor of one factor.

Notably, whether the complexes that form are unidirectional (the interaction only impacts the target genes of one factor) or bidirectional (it impacts the target genes of both factors) will have significant implications on the fate of the cell. What we refer to here as a unidirectional complex creates a situation where the DNA binding capability of one factor is disrupted by the interaction, while the DNA binding domain for the other factor is exposed and free to access its target genes. This means the factor that retains its DNA binding capability has a competitive advantage for maintaining the ability to regulate its own gene program, even when the other factor is expressed in the cell. However, the factor with its DNA binding domain masked by the interaction will not be able to associate with its direct target genes whenever it is found in the complex. This creates a setting where the factor with the masked DNA binding domain is at a competitive disadvantage whenever the other interacting protein is expressed in the same cell. In contrast, a bidirectional complex blocks the DNA binding potential of both factors and will prevent each one from accessing its

target genes. In this scenario, neither factor has a competitive advantage and their will be a mutual inhibition of their activities when they are sequestered in a complex. Thus, one factor will need to be expressed in significant excess, otherwise the interacting factors will cancel out each other's activity and keep the cell in an uncommitted state. It will be important to determine whether the T-bet complexes that are formed with the other lineage-defining transcription factors are uni- or bidirectional in nature.

Concluding remarks

New research defining the mechanisms by which T-bet represses the gene programs for alternative T helper cell lineages has brought with it an appreciation for the diverse methods that are employed by a single lineage-defining transcription factor with classical activator functions to negatively regulate gene expression events. At present, studies are also underway defining the mechanisms of action for other T helper cell lineage-defining factors such as GATA3, Ror γ t, and Foxp3 [41, 42]. In some cases, similar mechanisms to those described here for T-bet, such as competitive inhibition and sequestering, are utilized by these factors as well. However, the specificity of their binding partners and the DNA elements that they recognize provide a unique context for the regulatory activities of each factor. Collectively, the emerging research in this field is providing significant insight into how the co-expression of key T helper cell lineage-defining transcription factors can impact each other's functional capabilities and alter the genetic profile and activity of the cell.

Acknowledgments

We would like to thank colleagues and members of the Weinmann lab for insightful discussions. Research in the authors' lab is supported by grants from the NIAID (AI061061 and AI07272) and the American Cancer Society (RSG-09-045-01-DDC) to A.S.W.

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A. Direct activation of Th1 target genes

B. Indirect repression of alternative Th gene expression

I. Inhibits Th2 differentiation

II. Inhibits Th17 differentiation

C. Direct repression of alternative Th gene expression

I. Inhibits Th2 differentiation

II. Inhibits Tfh differentiation(?), CD8⁺ T cell exhaustion

Figure 1. Models representing the mechanisms by which T-bet positively and negatively regulates gene expression

(A) T-bet directly activates the Th1-signature gene program. T-bet induces transcription by recruiting chromatin remodeling complexes (including the histone H3K4-methyltransferase, Set7/9, as well as the histone H3K27-demethylase, Jmjd3) to its target genes. (B) T-bet indirectly represses alternative T helper cell genetic programs. In scenario (I), T-bet physically associates with the Th2-lineage-defining transcription factor GATA3 to prevent it from binding to and directly activating Th2-signature genes. In the second scenario (II), T-bet physically interacts with Runx1, which prevents Runx1 from activating *Rorc* (the gene that encodes the Th17-lineage-defining transcription factor Ror γ t). (C) T-bet can directly repress gene transcription by two mechanisms. In the first mechanism (I), T-bet directly recruits the transcriptional repressor, Bcl-6, to repress the promoter activity of genes preferentially expressed in alternative T helper cell subtypes. In the second mechanism (II), T-bet binding to a promoter prevents a required activator (activator X: Act. X) from recognizing its DNA binding element because there is a partial overlap between the T-bet and Act. X binding sites (represented by the multicolored DNA-binding element). Thus, T-bet DNA-binding to the promoter displaces a required activator to prevent gene

transcription. It is possible that the T-bet-dependent repression of *Pdcd1* may represent an example of this mechanism.