Coordinated but physically separable interaction with H3K27-demethylase and H3K4-methyltransferase activities are required for T-box protein-mediated activation of developmental gene expression

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During cellular differentiation, both permissive and repressive epigenetic modifications must be negotiated to create cell-type-specific gene expression patterns. The T-box transcription factor family is important in numerous developmental systems ranging from embryogenesis to the differentiation of adult tissues. By analyzing point mutations in conserved sequences in the T-box DNA-binding domain, we found that two overlapping, but physically separable regions are required for the physical and functional interaction with H3K27-demethylase and H3K4-methyltransferase activities. Importantly, the ability to associate with these histone-modifying complexes is a conserved function for the T-box family. These novel mechanisms for T-box-mediated epigenetic regulation are essential, because point mutations that disrupt these interactions are found in a diverse array of human developmental genetic diseases.

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During development, there is a dynamic regulation of gene expression patterns that allows multipotential progenitor cells to differentiate toward a defined and committed endpoint lineage. The process of lineage commitment from a self-renewing stem cell starts at the outset of the organism, as embryonic stem cells begin with the potential to become any cell type of the body, but progressive decisions during development define each lineage (Boyer et al. 2006). The complement of lineage-determinant transcription factors present within the cell is responsible for determining the committed cell’s phenotype by creating a cascade of gene expression changes that define its biological capability. Even a minor alteration in the activity of developmental transcription factors can severely compromise this process and result in pathogenic consequences (Speck and Gilliland 2002; Naiche et al. 2005; Davis et al. 2006).

During lineage-specification choices, it is clear that modulating the chromatin environment is one important way that cell-fate-specific gene expression patterns are established (Mikkelsen et al. 2007). Epigenetic changes not only alter the transcriptional profile at that moment in time, but they also can be inherited so that the lineage identity is perpetuated to the daughter cells after a cell division (Bantignies and Cavalli 2006). There has been much speculation that lineage-determinant transcription factors will play some role in this process because the factors responsible for regulating epigenetic changes have the potential to influence cellular fate long past the time they are found at an individual promoter. However, in many cases, definitive evidence for the exact role an individual factor plays in these processes has been lacking.

The T-box transcription factor family is known to be intimately involved in defining gene expression networks in developmental processes and lineage specification. The homozygous deletion of numerous T-box proteins results in an embryonic lethal phenotype in mice.
T-box-mediated epigenetic regulation

[Shi et al. 2004; Klose et al. 2006; Russ et al. 2000; Naiche et al. 2005]. Genetic studies of human disease also have established the importance of the T-box family in development. The mutation of T-box family members Tbx5 and Tbx3 result in Holt-Oram and Ulnar-Mammary syndromes, respectively [Packham and Brook 2003; Mori and Bruneau 2004]. In addition, mutations in Tbx19 (T-pit) have been associated with adrenocorticotropic [ACTH] deficiency, and mutations in Tbx22 result in cleft palate [Pulichino et al. 2003; Andreou et al. 2007; Vallette-Kasic et al. 2007]. In the hematopoietic system, the T-box transcription factor T-bet is required for the differentiation of naive CD4+ T helper cells into the Th1 phenotype [Szabo et al. 2000, 2002]. Th1 cells are important for the clearance of pathogenically altered self-cells. Dysregulated T-bet expression has been noted in several T- and B-cell lymphoproliferative diseases including Hodgkin’s lymphoma [Dorfman et al. 2004, 2005]. Therefore, the T-box family regulates cell fate decisions, and the dysregulation of T-box family member activity can result in human disease states.

Previously, we performed a chromatin immunoprecipitation [ChIP]-chip study to explore the mechanisms by which T-bet establishes cell-type-specific gene expression patterns in distinct hematopoietic lineages. In an examination of B, Th1, and NK cell populations, T-bet binding to target promoters was cell-type-independent, but there was a cell-type-specific component to the functional regulation of target genes that occurred downstream from the DNA-binding event [Beima et al. 2006]. A structure-function analysis revealed that separable chromatin remodeling and transactivation activities are needed for T-bet to activate target promoters. Interestingly, the transactivation potential localized to the N- and C-terminal regions of the protein, while the ability to recruit a permissive histone H3 Lys 4 dimethyl (H3K4me2)-specific methyltransferase complex localized to the T-box DNA-binding domain [Lewis et al. 2007].

The T-box transcription factor family is defined by a highly conserved DNA-binding domain termed the “T-box” [Naiche et al. 2005]. The T-box domain is ~180 amino acids and is located in the middle portion of the protein. In addition to the DNA contact residues, there are several regions of high identity or similarity between subfamily members. Interestingly, numerous human genetic disease mutations are found within these highly conserved regions of the T-box [Bamshad et al. 1999; Packham and Brook 2003; Reamon-Buettner and Borlak 2004; Andreou et al. 2007]. This suggests that in addition to DNA binding, there are important, conserved activities contained within the T-box domain that are required to establish developmental gene expression networks. Our previous studies suggest that epigenetic mediated activities are likely to be among these essential functions [Lewis et al. 2007].

With the recent discovery of histone demethylases, including the jumonji [JMJD] family and LSD1, it has been appreciated that the status of histone methylation can be dynamically regulated [Shi et al. 2004; Klose et al. 2006; De Santa et al. 2007]. Biochemical studies have identified the existence of protein complexes that contain both H3K27-demethylase and H3K4-methyltransferase activities, which has led to speculation that at least in some circumstances, there may be a simultaneous reorganization of both repressive and permissive chromatin states at target promoters [Agger et al. 2008]. In fact, when examining the status of methylation modifications on nucleosomes, a mutually exclusive series of nonpermissive—such as H3K9me3 and H3K27me3—versus permissive—such as H3K4me2 and H3K4me3—marks has been observed in locus-specific studies of differentiated cell types [Koyanagi et al. 2005; Mikkelsen et al. 2007; Schoenborn et al. 2007]. However, these histone methylation patterns are not strictly created in an “all or none” fashion, as bivalent chromatin domains that contain both repressive and permissive modifications are found in pluripotent cells [Mikkelsen et al. 2007]. Thus, epigenetic states at differentiation checkpoints may require the separation of H3K27-demethylase and H3K4-methyltransferase activities, in either a promoter- or cell-type-specific manner, to establish tightly regulated and complex gene expression networks that are required for the identity of the cell lineage. To date, it is unclear whether developmentally important transcription factors can regulate epigenetic events in such a finely tuned manner.

In this study, we define two overlapping, but physically separable regions within the conserved T-box DNA-binding domain that are required for the coordinated interaction with enzymatic activities that both remove nonpermissive H3K27me2/3 modifications and establish the permissive H3K4me2 epigenetic state. A mutagenesis analysis examining the T-box domain demonstrated the essential nature for these epigenetic-mediated events because mutations associated with several different T-box protein-dependent human developmental genetic diseases, when placed within the context of T-bet, abolish these activities and the functional induction of target gene expression. The novel mechanisms defined in this study provide multiple points of regulation for defining both promoter- and cell-type-specific epigenetic patterns.

Results

T-box proteins induce the permissive H3K4me2 state at target promoters

We showed previously that T-bet’s ability to functionally recruit permissive H3K4me2 methyltransferase activity to target promoters localizes to the T-box DNA-binding domain [Lewis et al. 2007]. From an evolutionary perspective, coupling the DNA-binding domain with the ability to alter epigenetic states presents a powerful mechanism for the T-box family to regulate gene expression patterns in a targeted fashion at developmental checkpoints. In fact, this is a conserved activity for the T-box family because the overexpression of T-box pro-
proteins from the subfamilies T, Tbr1, Tbx6, Tbx2, and Tbx1 is able to functionally induce the permissive H3K4me2 modification (Supplemental Fig. 1A). Consistent with previous results, the ability to establish a permissive chromatin state is not sufficient to induce transcription, as family member-specific transactivation potential is also required to activate target gene expression (Supplemental Fig. 1B; Lewis et al. 2007).

Cluster of human genetic disease mutations in T-box found on non-DNA contact surface

The conserved ability of the T-box family to recruit H3K4me2-specific methyltransferase activity led us to examine the commonalities in the T-box domain outside of the DNA contact residues. We examined both the published crystal structure for the T-box domain from Tbx3 co-crystallized with DNA as well as the primary amino acid sequence alignment from multiple T-box family members (Fig. 1, Supplemental Fig. 2; Coll et al. 2002). The model extrapolated for T-bet’s T-box domain in Figure 1A is presented from an overhead view, with the DNA contacting the T-box at residues on the opposite face. Highlighted are two conserved pockets of amino acids, here termed “T-box domain 1” and “T-box domain 2.” T-box domain 1 contains a cluster of point mutations from several different human genetic diseases, and T-box domain 2 contains a stretch of conserved amino acids forming another pocket-like structure. The location of these regions on the opposite face from the DNA contact points make them good candidates for an activity that is independent from DNA binding. In addition, the clustering of human genetic disease point mutations found in patients with diverse developmental defects mediated by distinct T-box family members highly suggests that an essential activity is located in this region.

T-box domain 1 is required for T-bet’s activity

To begin to test the functional significance of these two regions, we made amino acid substitution mutations within the conserved residues in T-box domains 1 and 2 in the context of T-bet and tested their ability to transactivate endogenous target promoters [Figs. 2, 4 [below]]. This experimental system allows us to assess if the mutant proteins retain the required ability to create a permissive chromatin environment as well as downstream general transactivation potential. For these studies, we examined activity at Cxcr3, Ccl3, and Ifnγ, which we, and others, have shown previously to be functionally regulated by T-bet (Szabo et al. 2000; Lord et al. 2005; Beima et al. 2006; Lewis et al. 2007). In addition, we monitored Calm2 as a control promoter that is bound by T-bet, but where no functional consequence has been detected (Lewis et al. 2007). Substitution mutations of either an alanine or an amino acid found in a T-box protein-mediated genetic disease were created and tested.

Protein expression levels for all constructs were monitored in each individual experiment to ensure equal expression of the wild-type and mutant proteins. Representative experiments are shown for each series of mutant proteins. An independent experiment with the disease mutations is also provided in Supplemental Figure 3.

We first performed an analysis examining the T-box-mediated genetic disease mutations found within T-box
The mutation of amino acid Y182 to either the serine disease mutation found at the same relative position in Tbx3 from a patient with Ulnar-mammary syndrome (Packham and Brook 2003) or an alanine substitution mutant completely abolished T-bet’s ability to activate endogenous target promoters (Fig. 2A,B). A cysteine mutation in this same position found in Tbx5 from a patient with congenital heart defects (CHD) (Reamon-Buettner and Borlak 2004) also significantly diminished activity.

We next examined the leucine at amino acid position 176 in T-box domain 1. A proline substitution mutation in Tbx3 was found in five family members with Ulnar-mammary syndrome (Bamshad et al. 1999). The proline mutation (L176P) severely compromised T-bet’s activity at Cxcr3, Ccl3, and Ifngr (Fig. 2C,D). Interestingly, a double alanine substitution mutation in residues G175A + L176A also lost activity, whereas the mutation of L176A + E177A retained some function. This may suggest that the proline creates a structural disruption that influences the surrounding amino acid structure more in the direction of residue G175, but that the L176 residue alone is important for the functional activity of the T-box protein.

We also examined disease mutations corresponding to positions P225, N226, and P178. The proline at position 225 is mutated to a leucine, and the asparagine at position 226 is mutated to a phenylalanine in the same relative position in Tbx22 from patients with cleft palate (Andreou et al. 2007). The proline corresponding to position 178 is mutated to a leucine in a Tbx5 allele found in a CHD patient (Reamon-Buettner and Borlak 2004). We also examined alanine mutations in residues I271 and E273. In Tbx19 (T-pit), the amino acid position equivalent to 271 in T-bet is mutated from an isoleucine to a threonine, resulting in ACTH deficiency in the pituitary (Packham and Brook 2003; Pulichino et al. 2003). Interestingly, in the three-dimensional crystal structure, this amino acid is located adjacent to the tyrosine at position 182 (Fig. 1A). In addition, amino acid I271 is located within a conserved motif in the T-box family that forms a consensus sequence for a sumoylated protein interaction site (Lin et al. 2006).
Interestingly, the N226F and I271A + E273A mutant proteins had significantly reduced transcriptional activity at the endogenous target promoters, although both retained more activity than the Y182S/A and L176P mutants [Fig. 2E–H]. The mutations of P178L/A and P225L/A are much less severe. Significantly, the patient with the P178L mutation also had a second mutation, L184P (Reamon-Buettner and Borlak 2004), and indeed, a double mutant in these residues has no activity [Supplemental Fig. 3]. Taken together, the data from Figure 2 demonstrate that the T-box mutations that result in human genetic disease states, when placed in the background of T-bet, severely affect the induction of endogenous target gene expression.

**T-box domain 1 mutations impair the functional induction of the permissive H3K4me2 chromatin state**

Our previous studies suggest that T-bet participates in both chromatin-mediated events as well as subsequent transactivation steps at target promoters (Lewis et al. 2007). Therefore, the inability of these mutant constructs to up-regulate endogenous target genes could be due to either the failure to recruit a chromatin remodeling complex or the loss of transactivation potential. In order to determine which event(s) are affected by these mutations, we examined the activity of the most severely affected mutants in two different systems. First, we examined their ability to up-regulate activity of an Ifnγ-promoter-reporter construct. Transiently transfected plasmids do not have normal chromatin structure and are considered to be permissive templates that are valuable for assessing transcriptional regulation activities that are largely independent of chromatin events (Smith and Hager 1997). Interestingly, most mutations in T-box domain 1 do not affect T-bet’s ability to transactivate the Ifnγ-promoter-reporter construct [Fig. 3A–F], suggesting that the general transactivation potential of these mutants in a pre-established permissive chromatin setting is intact. In addition, these data also indicate that the mutants retain the capacity to bind to DNA because a T-bet DNA-binding mutant completely abolished activity [data not shown]. It should be noted that the mutation in amino acid L176P can transactivate the Ifnγ-promoter-reporter construct above the pcDNA3.1 or DNA-binding mutant control background; however, its activity is impaired approximately threefold compared with wild-type T-bet [Fig. 3C,D; data not shown]. This reduction is not as severe as the complete loss of activity that occurs in the context of endogenous promoters. Therefore, it appears that both nonchromatin- and chromatin-mediated activities are impaired in the L176P mutant.

The data from Figure 3 indicate that the majority of T-box domain 1 mutations do not affect transactivation potential in a nonchromatin setting. Therefore, we next assessed the ability of these mutants to perform chromatin-mediated functions. Strikingly, the Y182S and L176P mutations severely impair T-bet’s ability to functionally induce the H3K4me2 modification at both the endogenous Cxcr3 and Ifnγ promoters. EL4 T-cells were transfected with either a pcDNA control, wild-type T-bet, or the T-box domain 1 mutant constructs as indicated. Cell aliquots from the same transfections were harvested either for luciferase analysis [A,C,E] or Western analysis [B,D,F] to monitor construct expression levels. Representative experiments are shown. [A,C,E] Graphical representation of the luciferase data with the Y-axis representing the ratio of the relative luciferase units compared with the relative transfection efficiency control Renilla units. [G] Shown are the quantitations from representative ChIP experiments examining the ability of the T-box domain 1 mutants to induce the H3K4me2 modification at the endogenous Cxcr3 and Ifnγ promoters. EL4 T cells were transfected with either a pcDNA control, wild-type T-bet, or the indicated T-box domain 1 mutant proteins and processed for standard ChIP analysis as indicated in Supplemental Figure 1. The ChIP samples were analyzed by qPCR using Cxcr3, Ifnγ, or Il4 promoter-specific primers.

![Image](https://genesdev.cshlp.org/article/content/31/7/2984/F3.large.jpg)
wild-type protein (Fig. 3G). Collectively, the data suggest that the inability of the disease mutations to activate endogenous target gene expression is due to a defect in initiating the permissive chromatin-mediated events, specifically the induction of the H3K4me2 modification.

T-box domain 2 is also required for chromatin-mediated events

In the T-box domain, there is a motif of conserved amino acids with a consensus sequence HKYQPR starting at position H263 of T-bet. There are some natural variants in the consensus between different T-box subfamily members, but these variants contain closely related amino acids (Supplemental Fig. 2). To interrogate the functional significance for this motif, here termed “T-box domain 2,” we created both alanine substitution mutations as well as mutations corresponding to the natural T-box subfamily member variants in the background of T-bet. We made the mutations in residues that are predicted to be on the surface of the T-box structure (Supplemental Fig. 2C). We first examined the ability of these mutant proteins to activate endogenous target gene expression (Fig. 4A–D). Natural T-box variants retain more activity than single alanine mutations, suggesting that there is conservation in the functional activity for this motif. We also mutated combinations of two conserved residues in T-box domain 2 and found that the double alanine mutations abolished T-bet’s activity at the endogenous Cxcr3, Ccl3, and Ifnγ promoters (Fig. 4C,D). Thus, T-box domain 2 is required for activity.

Similar to the analysis performed with T-box domain 1, we next examined the ability of the most severe double alanine mutations to activate an Ifnγ-promoter-reporter construct. As shown in Figure 4E, T-box domain 2 mutants activate transcription in this permissive chromatin setting, with K264A + Q266A displaying wild-type activity. These data suggest that the transactivation and DNA-binding potential are intact. In contrast, the mutant proteins with diminished transcriptional activity at the endogenous promoters also were severely impaired in their ability to induce the H3K4me2 modification, with the double alanine mutations completely abolishing this activity (Fig. 4G). These data localize the defect in T-box domain 2 mutations to an inability to establish a permissive chromatin state.

Figure 4. T-box domain 2 is required in chromatin-mediated events. EL4 T cells were transfected with either a pcDNA3.1 control, wild-type T-bet, or single or double mutants in T-box domain 2 as indicated. Representative experiments are shown. Transfections were harvested with one cell aliquot used for RNA and qRT–PCR analysis of endogenous gene expression (A,C) and the second cell aliquot subjected to a Western analysis to examine the level of the transfected protein expression construct (B,D) as described for Suplemental Figure 1. (E,F) Shown is an experiment examining the ability of the double alanine mutants in T-box domain 2 to up-regulate the activity of an Ifnγ-promoter-reporter construct as described in Figure 3. (G) Shown are the quantitations from representative ChIP experiments from EL4 T cells transfected with either single or double alanine mutant proteins in T-box domain 2. An antibody to the H3K4me2 modification or a nonspecific IgG control was used. Quantitative analysis of the ChIP samples with either Cxcr3 or Ifnγ promoter-specific primers was performed as described in Supplemental Figure 1.
T-box domain 1, but not T-box domain 2, is required for the physical interaction with the H3K4-methyltransferase complex subunit RbBp5

We previously demonstrated that T-bet can physically interact with H3K4me2-specific methyltransferase activity in coimmunoprecipitation (co-IP) experiments (Lewis et al. 2007). To further explore this mechanism, we examined the functional relationship between T-bet and RbBp5, a known, common core component found in H3K4-methyltransferase complexes [Dou et al. 2006; Cho et al. 2007; De Santa et al. 2007; J.H. Lee et al. 2007]. As shown in Figure 5A, we first established that RbBp5 recruitment to the Cxcr3 and Ifnγ promoters is T-bet-dependent. RbBp5 is enriched at these promoters in primary wild-type Th1 cells, but is significantly reduced in T-bet−/− Th1 cells. Importantly, the H3K4me2 modification coincides with the T-bet-dependent recruitment of RbBp5 to the target promoters [Fig. 5A]. We next examined whether there is a physical interaction between T-bet and RbBp5. Co-IP experiments were performed with extracts from EL4 T-cells transfected with either a pcDNA3.1 vector control or wild-type T-bet and immunoprecipitated with a T-bet-specific antibody. Significantly, RbBp5 is enriched in the T-bet-transfected sample relative to the negative control [Fig. 5B]. To confirm that these results were not solely due to the overexpression system, we performed similar experiments examining endogenous protein levels in primary CD4+ Th1 cells. Th1 cell extracts precipitated with a T-bet-specific antibody contained significantly more RbBp5 than samples immunoprecipitated with a negative control antibody [Fig. 5C]. These data indicate that T-bet physically interacts with RbBp5.

To determine if T-box domains 1 and 2 are required for the physical interaction with an RbBp5-containing H3K4-methyltransferase complex, we performed co-IP experiments with the mutant proteins and assessed their ability to interact with RbBp5. Consistent with a required role for T-box domain 1 in the induction of the permissive H3K4me2 modification, two T-box domain 1 mutants cannot physically interact with RbBp5 [Fig. 5D]. Surprisingly, however, a mutant in T-box domain 2...
retains the ability to associate with RbBp5 despite the fact that this mutant cannot functionally induce the H3K4me2 modification at target promoters [Figs. 5E and 4G]. Interestingly, in most experiments, we actually observed an increased level of RbBp5 and H3K4me2-methyltransferase activity in T-box domain 2 mutant co-IPs compared with wild-type T-bet [Fig. 5, Supplemental Fig. 5; data not shown]. These data suggest that in the absence of T-box domain 2 contacts, the H3K4-methyltransferase complex still is able to interact with T-bet.

The conserved nature of T-box domain 1 suggests that the ability to interact with an RbBp5 containing H3K4-methyltransferase complex may be a common function for the T-box family. To examine this possibility, we performed co-IP experiments with extracts from EL4 cells transiently transfected with T-box proteins. We observed enrichment of RbBp5 in cells transfected with three additional T-box subfamily members—Brachyury, Tbx5, and Eomesodermin—in comparison with the vector control cells [Fig. 5F; Supplemental Fig. 4]. These data indicate that T-box proteins have a common ability to interact with an RbBp5-containing H3K4-methyltransferase complex.

We also wanted to determine the identity for the enzymatic component in the H3K4-methyltransferase complex that interacts with T-bet. To accomplish this, we performed co-IP experiments with two independent antibodies to the H3K4-methyltransferase SET7/9 [also known as Setd7]. Significantly, wild-type T-bet, but not a mutant in T-box domain 1, is coprecipitated with SET7/9 [Fig. 5G; Supplemental Fig. 4]. These data suggest that T-bet can physically associate with SET7/9 or a highly related family member.

**T-box domains 1 and 2 are required for functionally alleviating the repressive H3K27me3 state**

T-box domains 1 and 2 are both required for the functional induction of the permissive H3K4me2 modification at target promoters, but only T-box domain 1 is necessary for the physical interaction with an RbBp5-containing H3K4-methyltransferase complex. This led us to investigate whether T-box domain 2 is required for chromatin-mediated events that potentially precede the induction of the H3K4me2 modification. During cellular differentiation, a complex series of events must occur to establish the appropriate gene expression patterns for that cell. To establish a permissive chromatin environment, in many cases, the first step that occurs is the resolution of repressive chromatin states that were present to prevent inappropriate gene activation during a prior developmental state. The repressive H3K27me3 modification is present at many inactive genes, including Ifnγ when it is inactive in Th2 cells [Schoenborn et al. 2007]. Therefore, we hypothesized that one step that may be required prior to the T-bet-dependent induction of the permissive chromatin modifications is the resolution of this repressive mark.

To begin to address this question, we examined whether T-bet is required and sufficient to functionally resolve a repressive H3K27me3 modification at target promoters. In primary Th1 cells, there is a higher level of the H3K27me3 modification at the Cxcr3 and Ifnγ promoters in T-bet−/− Th1 cells compared with wild-type Th1 cells [Fig. 6A]. This suggests that T-bet is required for the reduction in the H3K27me3 modification in Th1 cells. To determine if T-bet is sufficient to functionally resolve the H3K27me3 state, we overexpressed T-bet in EL4 cells and assessed whether this results in the reduction of the repressive modification. Indeed, the overexpression of T-bet caused a decrease in the H3K27me3 mark at the endogenous Cxcr3 and Ifnγ promoters [Fig. 6B]. Significantly, mutations in both T-box domains 1 and 2 were unable to mediate the reduction in H3K27me3 levels [Fig. 6C,D]. Collectively, these data suggest that T-bet is functionally required for alleviating the repressive H3K27me3 state at target promoters, and this activity is dependent on both T-box domains 1 and 2.

**T-bet physically associates with H3K27-demethylase activity**

To assess whether the ability to resolve the repressive H3K27me3 state is due to a physical interaction between T-bet and an H3K27-demethylase complex, we examined whether T-bet associates with H3K27-demethylase activity in co-IP experiments. We transfected EL4 cells with either a pcDNA3.1 vector control or wild-type T-bet and immunoprecipitated the samples with a T-bet-specific antibody before subjecting the samples to a demethylase assay. H3K27me3-demethylase activity is enriched in the T-bet-transfected sample, but not in the control [Fig. 6E]. Importantly, the physical interaction with the H3K27-demethylase activity is abolished with mutations in either T-box domains 1 or 2 [Fig. 6F,G]. These data indicate that T-bet can physically interact with an H3K27-demethylase and this association requires conserved residues in T-box domains 1 and 2.

**The physical interaction with H3K27-demethylase activity is a conserved function for the T-box family**

We next wanted to characterize the nature of the H3K27-demethylase interaction. Interestingly, the H3K27-demethylase activity that coprecipitates with T-bet can remove the H3K27me3 and H3K27me2 marks, but not the H3K27me1 modification [Fig. 7A]. In addition, we hypothesized that this is a common function for the T-box family due to the high degree of conservation within the residues of the T-box domain that are required for the interaction with the H3K27-demethylase activity. Indeed, Brachyury, Tbx5, and Eomesodermin all physically interact with H3K27-demethylase activity [Fig. 7B–D]. Therefore, the association with both H3K4-methyltransferase and H3K27-demethylase activity is common for the T-box family.
**T-bet physically interacts with the H3K27-demethylase JMJD3**

At this time, there are three known H3K27-demethylases: JMJD3, UTX, and UTY (Agger et al. 2007; De Santa et al. 2007; M.G. Lee et al. 2007). The mRNAs for UTX and JMJD3 are both highly expressed in EL4 cells as well as in differentiating primary Th1 cells (data not shown). JMJD3 is known to be specifically up-regulated in activated macrophages, whereas UTX is more ubiquitously expressed (De Santa et al. 2007). Due to the cell-type-restricted expression profile and known role for JMJD3 in immune cells, we assessed whether JMJD3 can physically interact with T-bet in primary Th1 cells. Consistent with the physical interaction between T-bet and an H3K27me2/3-specific demethylase, JMJD3 is present in T-bet immunoprecipitated samples, but is not present in control antibody IPs (Fig. 7E). These data demonstrate that T-bet interacts with JMJD3.

**JMJD3 functionally regulates T-bet-dependent target gene expression**

To determine the role for JMJD3 in the activation of T-bet-dependent target gene expression, we performed siRNA experiments to knock down JMJD3 in either pcDNA3.1 control or T-bet-transfected EL4 cells. T-bet’s ability to activate Cxcr3, Ifng, and Ccl3 was significantly impaired in JMJD3 siRNA transfected samples relative to a siGFP control (Fig. 7F-G and Supplemental Fig. 6). The magnitude of these effects is variable between target genes, suggesting that the role for JMJD3 may differ between individual genes. In the future, it will be interesting to determine if JMJD3 is solely acting as an H3K27-demethylase, or, rather, it plays additional roles independent of its demethylase activity, perhaps in a context-specific manner. Due to the large size of JMJD3, it is not unreasonable to suggest that it is likely to contain several functional activities. Nevertheless, these data indicate that JMJD3 is important in T-bet-dependent target gene expression and that the interaction between the T-box domain and the H3K27-demethylase is functionally relevant.

**Discussion**

In this study, we identify two overlapping, but physically separable regions within the T-box DNA-binding domain that are required for the interaction with H3K27-demethylase and H3K4-methyltransferase activities. By examining T-box-mediated genetic disease mutations, we localized a domain, here termed “T-box domain 1,”...
that is required for the physical interaction with an RbBP5-containing H3K4-methyltransferase complex. Another conserved region in the T-box, termed “T-box domain 2,” also is required to functionally induce the permissive H3K4me2 modification, but surprisingly, this domain is not required for the physical interaction with the permissive H3K4-methyltransferase complex. Instead, T-box domain 2, in conjunction with T-box domain 1, are required for the association with H3K27-demethylase activity. Taken together, our data suggest a novel mechanism by which the T-box transcription factor family recruits physically separable H3K27-demethylase and H3K4-methyltransferase complexes to target promoters to establish developmentally appropriate epigenetic states. Significantly, point mutations that disrupt the interaction between the T-box domain and these epigenetic-modifying activities are found in several human genetic diseases.

Lineage-determinant transcription factors define cellular fates during differentiation. It has long been speculated that these factors will play a role in establishing chromatin environments, but to date, the mechanisms by which they negotiate both repressive and permissive methylation states at a given promoter have been largely unknown. The results in this study highly suggest that the T-box family possesses both the capability to alleviate repressive as well as establish permissive epigenetic states through their interaction with H3K27-demethylase and H3K4-methyltransferase activities, respectively. Although both of these activities appear to be required for the activation of at least a portion of target genes, importantly, they can be physically separated. Recent biochemical studies examining demethylase complexes have suggested that methyltransferase/demethylase complexes exist in cells, and it has been speculated that such an individual dual complex will coordinately regulate the induction of gene expression in an “all or none” fashion (De Santa et al. 2007; Issaeva et al. 2007; Rivenbark and Strahl 2007; Agger et al. 2008). It is highly probable that in some cases, a dual specificity complex will be recruited to target genes, but our study clearly suggests that the two functions can be physically separated in their interaction with the T-box transcription factor family. Thus, T-box target genes have the potential to be regulated in a precise fashion, either functionally using H3K27me2/3-demethylase, H3K4me2-methyltransferase, or both activities.

Importantly, the uncoupling of the H3K27-demethylase and H3K4-methyltransferase complexes gives rise to the potential for a multistep model for the mechanism by which the T-box family alters epigenetic states at cell fate decision checkpoints, and it provides the opportunity to finely tune the epigenetic status of individual target genes in a context-specific manner. Our results are
compatible with three potential models [Fig. 8]. In one model, first T-box domains 1 and 2 recruit H3K27-demethylase activity to target promoters to remove the H3K27me2/3 repressive modification. It is possible that at some target genes the process stops here and leaves the target gene in a derepressed, but not yet poised state until a signaling event occurs to release the checkpoint.

Next, the H3K27-demethylase complex is released, exposing T-box domain 1 to physically interact with an RbBp5-containing H3K4-methyltransferase complex. The data that T-box domain 2 mutants have an enhanced capacity to interact with H3K4me2-methyltransferase activity suggest that the T-box domain’s interaction with the H3K27-demethylase and H3K4-methyltransferase complexes may actually be mutually exclusive, at least in some circumstances. Alternatively, RbBp5 may be a common component that bridges points of contact with both H3K27-demethylase and H3K4-methyltransferase complexes. If this is the case, a point of contact in T-box domain 2 is required for the stabilization of the physically separable interaction with the H3K27-demethylase complex that is not absolutely required for the interaction with H3K4-methyltransferase activity. In this scenario, two other models of recruitment to the target promoters can be envisioned. Here, the H3K27-demethylase and H3K4-methyltransferase activities would have the potential to either be recruited separately to target promoters or in a single complex, possibly in a promoter-dependent manner.

Significantly, in our multistep models, individual promoters may be subject to either one activity or both, providing a point of regulation for the epigenetic status at individual targets. This adds a greater level of complexity to the epigenetic possibilities for individual target promoters in the context of regulating the overall gene expression cascades at developmental checkpoints. In support of the possibility for this level of precise regulation, it is worth noting that we do see a context- and family-member-dependent quality to the induction of the epigenetic events at the Cxcr3 versus Ifnγ promoter [Supplemental Fig. 1]. Thus, the data observed to date suggest that there are multiple epigenetic events that are regulated by the T-box domain and there may be context-dependent utilization of these activities.

The fact that a cluster of mutations from T-box protein-dependent genetic diseases serves to define T-box domain 1 demonstrates the essential nature for these newly uncovered events in human development. In addition to the T-box domain 1 mutations, there are also clinical mutations found within the intervening variable region and another in the T-box domain 2 demethylase recruitment domain [Packham and Brook 2003; Reamond-Buettner and Borlak 2004; Kirk et al. 2007]. To date, human disease mutations localized to the T-box domain have been suggested to influence DNA-binding capability, however, in many cases, this hypothesis was not experimentally interrogated. Indeed, it makes a certain amount of sense that an essential, epigenetic-related function resides in close proximity to the DNA-binding region. It is not unreasonable to speculate that the tran-
scription factors that are changing the fate of a cell will not be limited to binding to only regions of open chromatin structure, but rather, they will have the ability to access and alter nonpermissive gene states in a more generalized manner. Our previous studies support this type of role for the T-box family because binding to target genes does not appear to be the key regulated event for T-box protein functional activity at individual loci (Beima et al. 2006; Lewis et al. 2007). Therefore, the placement of these epigenetic-mediated events in the T-box may aid in allowing the family to access DNA in any chromatin state that may be present at the cell fate decision checkpoint. Overall, the mechanisms uncovered in this study provide a previously unappreciated mode for T-box transcription factors to precisely regulate epigenetic states at developmental checkpoints. The essential nature of these tightly controlled events is highlighted by the dramatic developmental defects that are found in patient populations with T-box domain mutations that disrupt these interactions.

Materials and methods

Generation of T-box and mutant protein expression constructs

T-box protein expression constructs were made with gene-specific primers used to amplify cDNA generated from various cell types that express each protein. These products were cloned as previously described using the TOPO-TA cloning kit with the pcDNA3.1/V5-HIS tagged vector [Invitrogen | Lewis et al. 2007]. All T-box protein expression constructs contained a deletion in the natural stop codon to allow for the addition of a C-terminal V5 epitope tag. T-bet substitution mutation expression constructs were generated with the QuickChange PCR mutagenesis kit (Stratagene). All constructs were sequenced to ensure that only the desired mutations were found within the constructs. The amino acid position indicated for the mutations is from the start of murine T-bet.

EL4 cell transfections

The EL4 T-cell line was transfected with the AMAXA nucleofection method as previously described |Lewis et al. 2007|. Program O17 and nucleofection solution V were used. Cells were harvested between 16 and 22 h post-transfection and processed for one of several different downstream assay systems including standard ChIP analysis to monitor chromatin modifications and RbBp5 association at select target promoters, examination of endogenous gene transcriptional activity by quantitative RT–PCR (qRT–PCR) analysis, co-IP assays, or luciferase promoter-reporter assay. For the luciferase experiments, the 5′CNS Ifry promoter reporter construct was used |Shnyreva et al. 2004|. In all cases, representative experiments are shown. For the qRT–PCR and luciferase analyses, a Western analysis from the same experiment used to monitor the mutant protein expression levels was also performed.

Endogenous gene expression analysis

The ability of wild-type and mutant T-box proteins to activate endogenous target gene expression was analyzed by qRT–PCR analysis as described previously |Lewis et al. 2007|. Briefly, RNA was harvested using the Qiagen RNA purification system. cDNA was prepared with the First Strand Synthesis System [Invitrogen] and resuspended to a final concentration of 10 ng/µL. A qPCR reaction was then performed with gene-specific primers using the Brilliant Sybr Green Core PCR reagents [Stratagene]. Final expression levels are represented as the relative level compared with the wild-type T-bet protein expression construct.

ChIP assay

The ChIP assay and primary CD4+ T-cell isolation were performed as described previously |Beima et al. 2006; Lewis et al. 2007|. Antibodies to H3K4me2 and H3K27me3 were from Millipore [formerly Upstate Biotechnologies]. For the quantitation of the ChIP samples, promoter-specific primers were used for qPCR as described above. Using a standard curve to determine the relative amount of the specific promoter product in each sample, the sample was first normalized to the total input control followed by the subtraction of the background level that was present in the nonspecific IgG control.

Co-IP and demethylase assay

Whole-cell extracts were prepared and incubated with primary antibody overnight at 4°C. Two independent T-bet-specific antibodies [39D and 4B10, Santa Cruz Biotechnologies], a V5 antibody [R960-25, Invitrogen], or antibodies to SET7/9 [V-20 and N-20, Santa Cruz Biotechnologies] were used in these studies. The immunocomplexes were then incubated with protein A and/or G agarose beads for 1–2 h. After the samples were washed with RIPA I and/or RIPA II, the collected immunocomplexes were either run on an SDS-PAGE gel for Western analysis to determine the identity of the interacting proteins, or were subjected to a demethylase reaction. For the demethylase reaction, following the wash step with RIPA I, the beads containing the immunoprecipitated complexes were incubated with 5 µg of purified core histones [Millipore] and incubated in demethylase reaction buffer [50 mM HEPES at pH 8, 1 mM α-ketoglutarate, 2 mM L-ascorbate, and 70 µM Fe[NH4]2(SO4)2] for 4–5 h at 37°C. The reactions were then terminated by the addition of SDS-PAGE loading buffer and run in a Western analysis to examine the level of H3K27me3/2/1.

Crystal structure modeling

A predicted structure for the T-box domain of T-bet was generated by threading the protein sequence into the solved structure of Tbx3 |Coll et al. 2002| using the Geno 3D program |Comb et al. 2002| available at http://pbil.ibcp.fr/htm/index.php. After submitting the amino acid sequence corresponding to the T-box of T-bet into Geno 3D, the pdb template 1h6f was selected. The resulting structure was visualized and modified using the Swiss Pdb viewer |Guex and Peitsch 1997| available from ExPASy at http://www.expasy.org/spdbv.

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