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Modular Utilization of Distal *cis*-Regulatory Elements Controls *Ifng* Gene Expression in T Cells Activated by Distinct Stimuli

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SUMMARY

Distal *cis*-regulatory elements play essential roles in the T lineage-specific expression of cytokine genes. We have mapped interactions of three transacting factors – NF-κB, STAT4 and T-bet – with *cis* elements in the *Ifng* locus. We find that RelA is critical for optimal *Ifng* expression and is differentially recruited to multiple elements contingent upon T cell receptor (TCR) or interleukin-12 (IL-12) plus IL-18 signaling. RelA recruitment to at least four elements is dependent on T-bet-dependent remodeling of the *Ifng* locus and co-recruitment of STAT4. STAT4 and NF-κB therefore cooperate at multiple *cis* elements to enable NF-κB–dependent enhancement of *Ifng* expression. RelA recruitment to distal elements was similar in Th1 and Tc1 effector cells, although T-bet was dispensable in CD8 effectors. These results support a model of *Ifng* regulation in which distal *cis*-regulatory elements differentially recruit key transcription factors in a modular fashion to initiate gene transcription induced by distinct activation signals.

INTRODUCTION

Specification of distinct effector T cell lineages from naïve T cell precursors is controlled by transcriptional networks that are activated by cytokine signals received in concert with antigen recognition. T-bet, Gata-3, and RORγt and RORα, and specific members of the STAT family, among others, are key transcription factors that are recruited by cytokine signals to regulate T helper 1 (Th1), Th2 and Th17 cell differentiation programs (Afkarian et al., 2002; Ivanov et al., 2006; Kaplan et al., 1996; Shimoda et al., 1996; Szabo et al., 2000; Yang et al., 2008; Zheng and Flavell, 1997). The generation of functionally mature effector T cells is contingent, in turn, upon lineage-specific remodeling of cytokine gene loci that is orchestrated by the interplay of *trans*-acting factors with *cis*-regulatory elements (Agarwal and Rao, 1998; Lee et al., 2006; Wilson et al., 2009). However, a detailed understanding of the interactions between these *trans*acting molecules and the *cis*-regulatory elements to which they bind to regulate transcription of effector cytokines remains to be defined.

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Production of IFN-γ in response to antigen re-encounter represents an innovation of Th1 cells of the adaptive immune system. However, Th1 cells can also secrete IFN-γ in a TCR–independent fashion in response to synergistic actions of the cytokines IL-12 and IL-18, akin to cytokine-driven production of IFN-γ by NK and NKT cells (Berenson et al., 2004). Although these pathways have evolved to complement each other in the generation of a successful Th1 cell immune response, they differ in several respects. The amplitude and kinetics of IFN-γ production induced by TCR versus cytokine signaling are distinct, and the utilization of several transcription factors, including NFAT, STAT4, and T-bet, differ in their contributions to these two pathways (Yang et al., 1999; Yang et al., 2001). More widely expressed transcriptional activators, such as AP-1 and NF-κB, appear to play roles in enhancing *Ifng* gene transcription in response to both TCR-dependent and TCR-independent activation (Robinson et al., 1997; Sica et al., 1997). Despite extensive characterization of the signaling cascades activated by these pathways, relatively little is known regarding interactions between these factors and *cis*-regulatory elements of the *Ifng* locus that might control stimulus-specific transcription of *Ifng* (Wilson et al., 2009).

Several functionally distinct distal regulatory elements have been identified and characterized in the Th2 cytokine gene cluster, including multiple enhancers, a silencer element (HSIV) and a locus control region that coordinate expression of the *Il4*, *Il5* and *Il13* genes (Ansel et al., 2006; Lee et al., 2006). Analogous studies to identify distal regulatory elements that impact *Ifng* gene transcription are relatively nascent. Initial DNase I mapping identified three hypersensitive sites within introns of the *Ifng* gene (Agarwal and Rao, 1998). Despite their intrinsic enhancer activities, transgenic analysis indicated that these introns were insufficient to confer lineage-specific expression of IFN-γ (Soutto et al., 2002). Subsequent analysis using a BAC transgene that contained ~191kb flanking the human *IFNG* gene successfully recapitulated lineage-specific expression of human IFN-γ in murine effector T cells (Soutto et al., 2002). Further, transgenic reporter mice that incorporated ~160kb surrounding the murine *Ifng* gene display lineage-specific transcription of a reporter molecule, Thy1.1 (Harrington et al., 2008; Hatton et al., 2006). Collectively, these studies have strongly affirmed essential roles for distal regulatory elements in regulating lineage-specific expression of IFN-γ.

Comparative genomics has emerged as a powerful tool to identify putative distal regulatory elements (Loots et al., 2000), and has advanced characterization of the *Ifng* locus (Hatton et al., 2006; Schoenborn et al., 2007; Sekimata et al., 2009; Shnyreva et al., 2004). To date, nine evolutionarily conserved non-coding sequences (CNS) have been identified within ~120kb flanking the murine *Ifng* locus (Hatton et al., 2006; Lee et al., 2004; Schoenborn et al., 2007; Shnyreva et al., 2004). Of these, CNSs -34, -22 and -6 have drawn attention as T-bet–responsive elements that markedly impact *Ifng* gene transcription (Hatton et al., 2006; Lee et al., 2004; Shnyreva et al., 2004). In a previous report, we used a BAC-transgenic model to demonstrate that one of these elements, CNS-22, plays an obligatory role in driving *Ifng* gene transcription in both effector T cells and NK cells (Hatton et al., 2006). Two recent studies identified CTCF-dependent boundary elements that insulate the *IFNG* and *Ifng* loci from neighboring gene loci (Hadjur et al., 2009; Sekimata et al., 2009). Using chromosome conformation capture, Th1-specific, T-bet–dependent interactions between multiple *Ifng* CNSs and the *Ifng* gene itself were identified, indicating that these distal elements employ chromosomal looping to transactivate promoter-driven gene expression (Sekimata et al., 2009). Although these recent studies have assigned broad functional attributes to other *Ifng* CNSs, their precise functions remain unknown (Chang and Aune, 2005, 2007; Schoenborn et al., 2007).

Here, we have mapped the chromatin state of the extended *Ifng* locus prior to and after Th1 and Th2 cell differentiation and have carried out analyses of multiple distal regulatory

elements that impact *Ifng* gene transcription under conditions of TCR versus cytokine induced signaling. We demonstrate that key distal *cis*-regulatory elements in the *Ifng* locus become permissive upon Th1 cell differentiation whereas repressive chromatin remodeling of this locus during Th2 cell differentiation limits accessibility to these elements. Th1 differentiation is accompanied by progressive recruitment of key transcription factors to distal elements that ultimately determine the transcriptional competence of the *Ifng* locus. Specifically, we show that *Ifng* CNSs -54, -34, -22, +40, +46 and +54 are NF-κB consensus sequence-containing elements that modulate *Ifng* gene transcription through differential recruitment of RelA in response to TCR versus cytokine induced signaling. Further, we have delineated specific roles for T-bet and STAT4 in positively modulating the functions of these NF-κB response elements. Taken together, our study provides new insights into the dynamics between distal *cis*-regulatory elements and the *trans*-acting factors they recruit to dictate lineage- and stimulus-specific *Ifng* gene transcription.

RESULTS

Long-range DNase-chip mapping of the *Ifng* locus in naïve and effector T cells

Recent studies have used global genome alignment tools to identify multiple conserved, non-coding sequences (CNSs) as candidate *cis*-regulatory elements in the *Ifng* locus (Frazer et al., 2004; Hatton et al., 2006; Schoenborn et al., 2007). To determine which CNSs correspond to functional regulatory elements in T cell subsets, we employed a microarray based approach (DNase-chip) (Crawford et al., 2006) to identify sites of DNase I hypersensitivity (HS) across an ~780kb region flanking the *Ifng* gene on mouse chromosome 10. Comparison of naïve $CD4^+$ T cells with polarized Th1 and Th2 cells indicated that the extended *Ifng* locus undergoes extensive lineage-specific remodeling during the course of effector T cell differentiation (Fig. 1). Notably, lineage-specific HS sites were not identified outside of a region bounded by common HS sites 70kb upstream (HS-70kb) and 66kb downstream (HS+66) of the start site of *Ifng* transcription, each of which contain consensus CCCTC-binding factor (CTCF) sites and appear to represent boundary sites that delimit *cis*-elements that control the *Ifng* locus (Hadjur et al., 2009; Sekimata et al., 2009).

In addition to the CTCF-containing boundary sites, two additional HS sites were common to each of the subsets. A major hypersensitivity peak correlated with CNS-22, which was previously identified as an upstream element required for *Ifng* expression from a BAC transgene (Hatton et al., 2006). An additional HS site was identified within intronic sequence of the *Ifng* gene, as previously reported (Agarwal and Rao, 1998), although it was more prominent in Th1 cells (Fig. 1). This intronic HS site contains an additional CTCF element, which appears to play role in structural organization of the locus through cooperation with the distal CTCF boundary elements (Sekimata et al., 2009). Hypersensitivity peaks at CNS+19, CNS+40, CNS+46 and immediately upstream of CNS +30 (HS+28) were present in naïve T cells, consistent with possible roles for these sites, in addition to CNS-22, as foci for initiation of lineage-specific remodeling of the *Ifng* locus during effector T cell differentiation.

Th1 cell differentiation was associated with induction of multiple additional HS sites, including those at the promoter and at CNSs -54, -34, +17, +30 and +54 as well as those at non-CNS sites, HS-40, HS-37 and HS+60. In addition, the intronic HS site and those at +17, +28, and +46 become more sensitive to DNase I after Th1 cell differentiation. At this stage of Th1 cell development, although the *Ifng* locus is poised for gene transcription, high-level transcription is activation-dependent. After TCR activation or cytokine driven signaling, CNS-34 became hypersensitive and other major Th1 cell-specific sites at CNSs -54 and +54 also became more hypersensitive to DNase I. The stimulus-dependent changes in chromatin

structure at these sites are consistent with their function as important enhancers of *Ifng* gene transcription.

While Th1 cell differentiation is accompanied by favorable epigenetic changes across the extended *Ifng* locus, the silencing of *Ifng* transcription that accompanies Th2 cell differentiation is reflected in acquired resistance to DNase I digestion at CNSs+19, +40 and +46, and HS+28, each of which is accessible in naïve $CD4^+$ T cells. Several sites became hypersensitive in Th2 cells, but to a lesser extent than observed in Th1 cells. Importantly, CNSs -54, -34 and +54 were completely resistant to DNase I digestion in Th2 cells. These results identify candidate *cis*-regulatory elements likely to play important roles in regulating *Ifng* gene transcription in effector T cells and other IFN-γ–producing cells.

RelA is required for optimal acute transcription of the *Ifng* gene

Activation of Th1 cells or effector $CD8^+$ T cells (Tc1) by either TCR or IL-12+IL-18 signaling induces *Ifng* transcription, albeit with different kinetics and recruitment of distinct signaling cascades (Sweetser et al., 1998; Yang et al., 1999; Yang et al., 2001). Activation of NF-κB occurs downstream of both TCR-dependent and -independent pathways (Robinson et al., 1997; Schoenborn et al., 2007; Schulze-Luehrmann and Ghosh, 2006). While several studies have examined the importance of NF-κB activation in *Ifng* gene transcription (Corn et al., 2003; Corn et al., 2005), the precise contribution of individual family members has remained unclear. Specifically, a possible role for RelA has not been critically examined, primarily due to embryonic lethality of RelA-deficient mice (Beg et al., 1995).

To address this, mice with a conditional deletion of RelA in T cells were generated through crosses of mice with a floxed *Rela* allele ($Rela^{fl/fl}$) with mice that express cre recombinase under control of the *Cd4* locus (*Cd4-cre*) (Lee et al., 2001; Steinbrecher et al., 2008). $Rela^{fl/fl}.Cd4\text{-}cre^+$ mice were produced at expected Mendelian ratios, and their deficiency of RelA was confirmed by both transcript analysis and by immunoblotting (Fig. S1). Consistent with previous studies (Steinbrecher et al., 2008), effector T cells that lacked RelA show increased activation of c-Rel (Fig. S1). $Rela^{fl/fl}.Cd4\text{-}cre^+$ mice showed no differences in numbers of $CD4^+$ and $CD8^+$ T cells in peripheral lymphoid tissues (data not shown), and their T cells had similar proliferative responses to those of littermate controls (Fig. 2A). However, production of IFN-γ by RelA-deficient Th1 and Tc1 cells was compromised in response to both TCR-dependent and IL-12+IL-18–dependent signaling, with substantially greater decrements of IFN-γ found in RelA-deficient Th1 cells. Notably, expression of other Th1 cell genes, including *Tbx21, Hlx, Runx3* and the receptors for IL-12 and IL-18 (*Il12rb1*, *Il12rb2* and *Il18r*), was not compromised by loss of RelA (Fig. S1). Further, repression of reciprocally expressed gene loci of non-Th1 cell lineages, including *Il4, Il17a and Foxp3* was intact in RelA-deficient Th1 cells, and there was no significant alteration in expression of *Il21* or *Il10* relative to WT controls (data not shown, and Fig. S1). Collectively, these results indicate that whereas RelA is dispensable for antigen-driven proliferation, it is indispensable for optimal induction of *Ifng* gene transcription, particularly in Th1 cells.

To determine whether impaired IFN-γ production by RelA-deficiency was due to impairment of effector cell development or impaired acute signaling, additional experiments were performed in which RelA signaling was specifically blocked after differentiation of WT Th1 cells. In the presence of a peptide that is a RelA phosphorylation decoy ((peptide Ser 276) (Derossi et al., 1994; Takada et al., 2004)), there was a dose-dependent inhibition of TCR-driven IFN-γ expression by Th1 cells (Fig. 2B), which was associated with inhibition of RelA nuclear translocation (Fig. 2C). Both TCR- and IL-12+IL-18-driven induction of IFN-γ were markedly compromised, as was IFN-γ induction by synergistic actions of IL-12+IL-18 and antigen receptor activation (Fig. 2D). Under all conditions

examined, pre-incubation with the RelA inhibitory peptide led to decreases in both the frequency of IFN-γ^+ cells and single-cell expression of IFN-γ (Fig. 2E), and could not be ascribed to cell toxicity (data not shown). Thus, RelA plays an important role in acute transcription of *Ifng* gene expression in Th1 cells whether induced by either TCR or IL-12+IL-18 signaling.

RelA is differentially recruited to *cis*-regulatory elements by TCR-dependent and -independent activation

Previous studies have demonstrated recruitment of NF-κB subunits to the *Ifng* promoter and intronic sites (Sica et al., 1997; Tato et al., 2006). However, analysis of NF-κB binding to more distal elements has not been reported, and promoter binding alone may not account for defects in *Ifng* transcription (Corn et al., 2003; Corn et al., 2005). We therefore evaluated whether distal elements defined by comparative genomic and DNase-chip analyses might bind NF-κB under conditions of active *Ifng* gene transcription. Sequence scans for consensus NF-κB binding sequences were performed and compared to CNSs and DNase HS sites (Fig. 1). Although numerous potential NF-κB consensus sites were identified across the *Ifng* locus, relatively few of these coincided with identified CNSs or HS sites (Fig. S2, and data not shown). Thus, in addition to NF-κB sites previously described (Sica et al., 1997; Tato et al., 2006), highly conserved NF-κB consensus sites were identified in multiple distal CNSs, including CNSs -54, -22, -6, +17, +40, +46 and +54, as well as a tandem site identified in CNS-34 (Fig. S2).

To screen for functional binding sites for RelA, we initially performed ChIP-chip analysis of Th1 cells without re-stimulation (“resting”), or after activation with IL-12+IL-18, anti-CD3 and anti-CD-28, or PMA plus ionomycin (Fig. S3, and data not shown). Binding sites identified from these analyses were restricted to previously identified CNS or HS sites (Fig. 1), so further studies were performed using conventional ChIP–RT-PCR analysis of these sites (Fig. 3). Upon TCR activation, significant RelA binding was detected at three CNSs: CNS-34, CNS-54 and CNS+40 (Fig. 3A). Comparable RelA recruitment was observed at each of these sites in response to stimulation by either anti-CD3 plus anti-CD28 or the TCR signaling surrogate, PMA plus ionomycin (Fig. 3A). In some experiments, RelA binding achieved significance at other sites (eg, CNSs +17–19 and +54), but this was less prominent and varied between experiments and activation conditions. Modest binding at the *Ifng* promoter was seen in some experiments, but did not achieve statistical significance.

Activation of Th1 cells with IL-12+IL-18 induced a different pattern of RelA recruitment (Fig. 3B). CNS-34 retained prominent RelA binding, and there was strong recruitment to three additional distal sites that were not consistently detected following TCR-dependent signaling: CNS-22, CNS+46 and CNS+54. No significant binding was detected at CNS-54. In agreement with the ChIP-chip data (Fig. S3), RelA bound two additional CNSs (ie, CNS +30 and CNS+40), albeit substantially weaker than observed for the four other binding sites. Again, modest, variable RelA binding was detected at the *Ifng* promoter, but did not achieve statistical significance, despite a significant peak of binding at the promoter in the ChIP-chip analyses. No RelA recruitment was detected outside of mapped DNase I HS sites (see Fig. 1). Collectively, these results establish that RelA is differentially recruited to distinct binding sites to drive *Ifng* gene transcription in response to distinct stimuli. Based on the high degree of cross-species conservation of NF-κB binding sites of the six distal *cis*-elements that bind RelA most prominently in Th1 cells (i.e., CNSs -54, -34, -22, +40, +46 and +54), it would appear that coordinated, inducible recruitment of RelA to multiple distal elements in the *Ifng* locus is an evolutionarily conserved mechanism to drive *Ifng* gene transcription.

RelA recruitment induced by IL-12 plus IL-18 is STAT4-dependent

Given the differential recruitment of RelA to distal CNS elements by TCR- versus cytokine-driven signaling, we speculated that other transcription factors that are differentially activated by these pathways might regulate usage of distinct NF-κB response elements in the *Ifng* locus. Because IL-12-induced STAT4 activation is critical to cytokine-driven, but not TCR-driven activation of *Ifng* transcription, it was plausible that STAT4 might cooperate with IL-18-induced NF-κB under conditions of cytokine-driven activation. This possibility was supported by the proximity of evolutionarily conserved NF-κB and STAT binding sites, particularly in CNSs -22, -34 and +46 (Fig. S2). We therefore evaluated whether the observed binding of RelA to *Ifng* CNSs was dependent on coordinate IL-12-induced STAT4 activation.

Although IL-18 induced NF-κB activation is independent of IL-12 (Yang et al., 1999), and the target NF-κB sites in Th1 cells are accessible (Fig. 1), IL-18 signaling alone failed to induce significant RelA recruitment compared to that induced by combined activation with IL-18+IL-12 (Fig. 4A). Thus, although there were modest increases in binding detected at several sites after stimulation with IL-18 alone (eg, CNS+46 and the *Ifng* promoter), these were not significant, and were substantially reduced relative to binding induced by IL-18+IL-12. Accordingly, RelA recruitment by IL-18+IL-12 was significantly impaired in Th1-polarized $CD4^+$ T cells from *Stat4*$^{-/-}$ mice (Fig. S4). These results are consistent with a role for IL-12-induced activation of STAT4 for recruitment of IL-18-induced RelA to the *Ifng* locus.

While signaling downstream of the IL-12 and IL-18 receptors may converge and synergize at multiple levels, the proximity of STAT and NF-κB binding sites in several of the CNSs led us to evaluate whether STAT4 and RelA might be coordinately recruited to these sites. We therefore surveyed the extended *Ifng* locus for STAT4 binding sites by ChIP-chip analysis of Th1 cells (Fig. S3), followed by confirmatory ChIP–RT-PCR analyses (Fig. 4B).

Upon activation of Th1 cells with IL-12+IL-18, we observed robust recruitment of STAT4 to both CNS-22 and CNS+46 (Fig. 4B), with lesser, but significant binding to CNSs -34, +30, +40 and +54, and the promoter. In contrast to STAT4–dependent recruitment of RelA, STAT4 recruitment to the *Ifng* locus in response to IL-12+IL-18 was not strictly RelA-independent (Fig. S4). Thus, with the exception of CNS-34 and CNS+54, STAT4 binding was independent of NF-κB, as comparable binding was observed in the absence of IL-18 signaling (Fig. S4A) and in RelA-deficient T cells (Fig. S4B). No significant STAT4 binding was detected at CNS-34 or CNS+54 in response to IL-12 signaling alone, despite the requirement for IL-12 for optimal RelA binding to these sites. CNS-54 lacks a STAT consensus sequence (data not shown), suggesting that localization of STAT4 to this site might be indirect and perhaps independent of direct STAT4-DNA interactions. Notably, we detected significant binding of STAT4 to CNS+46 in resting Th1 cells, prior to stimulation by cytokines (Fig. 4B), suggesting that STAT4 might be poised on this site even in the absence of on-going IL-12 signaling. These results establish that RelA binding to target elements in the *Ifng* locus is substantially enhanced by concurrent STAT4 binding, and suggest that NF-κB complexes that contain RelA might require STAT4 as a chaperone for binding to the majority of sites in the *Ifng* locus.

RelA recruitment to the *Ifng* locus is T-bet-dependent in Th1 but not Tc1 cells

The T-box family members, T-bet and Eomes, play redundant and non-redundant roles in effector T cell differentiation and cell fate (Intlekofer et al., 2008; Intlekofer et al., 2005; Pearce et al., 2003). Whereas production of IFN-γ by cytotoxic T cells is independent of T-bet, T-bet is required for optimal production of IFN-γ in Th1 cells (Szabo et al., 2000). T-bet

has been shown to regulate *Ifng* gene transcription in concert with several other transcription factors, including NFAT, Runx3, and Hlx (Djuretic et al., 2007; Lee et al., 2004; Mullen et al., 2001), and was more recently shown to cooperate with the architectural factor, CTCF, to restructure the *Ifng* locus for active transcription (Sekimata et al., 2009). In addition to its role in locus remodeling, T-bet also functions as an enhancer through its interactions with several CNS elements (Chang and Aune, 2005; Hatton et al., 2006; Shnyreva et al., 2004). In view of sequence scans that identify evolutionarily conserved composite T-box and NF-κB consensus sites in these elements (Fig. S2), and our findings that RelA interacts with these sites and additional CNS elements identified herein, we examined whether recruitment of RelA to CNSs in the *Ifng* locus is T-bet–dependent.

Th1 and Tc1 were derived by polarization of $CD4^+$ or $CD8^+$ T precursors from WT or *Tbx21*$^{-/-}$ (T-bet–deficient) mice, and RelA ChIP–RT-PCR analyses were performed after stimulation with PMA+ionomycin or IL-12+IL-18 (Fig. 5). Notably, the pattern of RelA recruitment to CNSs in Tc1 cells mirrored that of Th1 cells under both conditions of activation (Fig. 5B,D). Like Th1 cells, Tc1 cells also demonstrated differential utilization of CNSs contingent upon the stimulus. However, consistent with the disparate requirement for T-bet in Th1 and Tc1 cells, inducible recruitment of RelA was selectively impaired in T-bet–deficient Th1 cells (Fig. 5B,D); RelA recruitment to distal CNSs in Tc1 cells was independent of a requirement for T-bet, irrespective of the mode of activation. Thus, T-bet's role in the recruitment of RelA to the *Ifng* locus is $CD4^+$ T cell-specific (see also, Fig. S6).

Since RelA recruitment in T-bet–deficient Th1 cells was impaired in response to both TCR- and cytokine-dependent signals, we speculated that T-bet might play a Th1-specific role in the epigenetic remodeling of key distal elements that regulate *Ifng* gene transcription. Previous studies demonstrated that Th1 cell differentiation is associated with increased acetlyation of histone H4 and methylation of lysine 4 on histone H3 (H3K4), and decreased trimethylation of lysine 27 on histone H3 (H3K27) across the *Ifng* locus (Schoenborn et al., 2007; Wei et al., 2009). Consistent with previous findings (Chang and Aune, 2005), we observed that T-bet is dispensable for the changes in acetylation of histone H4 at sites across the *Ifng* locus, both in Th1 and Tc1 cells (Fig. S5). In contrast, methylation of H3K4, which is also associated with a permissive chromatin state, was different between Th1 and Tc1 cells. There was Th1 cell-specific impairment of H3K4 methylation at CNS-34 in the absence of T-bet (Figs. 6A). Additionally, *Tbx21*$^{-/-}$ Th1 cells showed significantly decreasd H3K4 methylation at CNS-6 and the *Ifng* promoter compared to WT controls (Fig. 6A, upper panel). Interestingly, there was increased H3K4 methylation at CNS-22 in the absence of T-bet. In contrast, T-bet was dispensable for H3K4 methylation at all sites examined in Tc1 cells, including CNS-34 (Figs. 6B, upper panel); no significant decreases in H3K4 methylation were evident at any of the *Ifng* CNSs examined in Tc1 cells, and, in general, methylation of H3K4 at most of the CNSs was substantially higher in Tc1 cells (Fig. S5).

Deficits in H3K4 methylation observed in *Tbx21*$^{-/-}$ Th1 cells, which were limited to the promoter and upstream CNS elements, were associated with more extensive increases in repressive H3K27 methylation, which extended broadly both upstream and downstream of the *Ifng* gene (Fig. 6A, lower panel). Thus, in comparison to wild-type controls, *Tbx21*$^{-/-}$ Th1 cells had significant increases in H3K27 trimethylation at CNSs -54, -34, -6, +30, +40 and +46, and at the promoter. In contrast, no significant changes in repressive H3K27 methylation were observed in Tc1 cells. These findings suggest that T-bet is specifically required to remodel *cis*-regulatory elements across the extended *Ifng* locus in Th1 cells, with removal of repressive histone modifications as a basis for remodeling downstream regulatory elements that are required for both TCR-dependent and -independent binding of RelA-containing NF-κB complexes.

Activation of STAT4 and RelA in Tbx21$^{-/-}$ T cells

In view of the critical role for T-bet in regulating expression of key receptors upstream of NF-κB and STAT4 signaling, we determined whether decreased expression of inducible components of the IL-12 and IL-18 receptors in *Tbx21*$^{-/-}$ Th1-polarized cells might explain the defective chromatin re-structuring of the *Ifng* locus in this population. Although transcript levels of *Il12rb2* and *Il18r1* were decreased in both Th1 and Tc1 cells derived from T-bet–deficient mice, there remained substantial expression of both receptors relative to WT naïve T cells (Figs. 7A,B, and data not shown). Accordingly, whereas nuclear translocation of RelA in response to IL-12+IL-18 stimulation in *Tbx21*$^{-/-}$ Th1-polarized cells was reduced, there remained considerable nuclear accumulation of RelA downstream of the IL-18 receptor (Fig. 7C). In response to TCR signaling in contrast to the *Ifng* locus, recruitment of RelA to the *Il2* promoter was substantially enhanced (Fig. 7D). Similarly, despite decreased expression of IL-12Rβ2 in *Tbx-21*$^{-/-}$ Th1-polarized cells, substantial IL-12-induced signaling was retained, as indicated by the significant induction of STAT4 phosphorylation (Fig. 7E). Nevertheless, while there was retention of some STAT4 binding to selected CNS elements in *Tbx21*$^{-/-}$ Th1 cells (i.e., CNS-22 and CNS+46), this was significantly suppressed at all STAT4 binding sites identified in the *Ifng* locus (Fig. 7F, and see Fig. 4, above). Thus, the defects in RelA recruitment that result from T-bet deficiency appear to be due more to diminished recruitment of STAT4 to the poorly remodeled *Ifng* locus than a deficit of activated RelA.

DISCUSSION

In this study, we have extended the functional map of the *Ifng* locus through delineation of interactions between evolutionarily conserved *cis*-regulatory elements and key *trans*acting factors that dictate lineage- and stimulus-specific transcription of *Ifng* in Th1 and Tc1 cells. We identify RelA as a central Rel-NF-κB family member that controls *Ifng* transcription through interactions with six major CNSs in the *Ifng* locus (CNSs -54, -34, -22, +40, +46 and +54), and show that these sites are differentially utilized in response to TCR- or cytokine-driven mechanisms. Recruitment of RelA to key *cis* elements induced by cytokine-driven *Ifng* transcription was found to be coincident with, and dependent upon, coordinate recruitment of IL-12–induced STAT4, whereas STAT4 binding to the same sites was largely independent of IL-18–induced NF-κB. Thus, acute STAT4 binding to accessible sites in the *Ifng* locus appears to enhance *Ifng* transcription, at least in part, by chaperoning the recruitment of RelA-containing NF-κB complexes.

In previous studies, it was established that NF-κB signaling is critical to Th1 cell development and function (Corn et al., 2003). The canonical, or classical pathway of NF-κB activation, which is activated by downstream of the TCR and IL-18 signaling, has been primarily linked to homo- or heterodimers of RelA, c-Rel and NF-κB p50 (Vallabhapurapu and Karin, 2009). While it has been speculated that the functions of c-Rel and RelA are at least partially redundant, studies of c-Rel–deficient mice have identified several instances where RelA cannot compensate for absence of c-Rel (Hayden et al., 2006). In particular, c-Rel plays an obligatory role in the induction of IL-2 and antigen-driven proliferation of T cells (Liou et al., 1999, Kontgen et al., 1995), establishing a requirement for c-Rel in the differentiation of naïve precursors, but also complicating analysis of its function in effector T cells by limiting their development (refs. (Hilliard et al., 2002; Kontgen et al., 1995; Liou et al., 1999; Mason et al., 2004), and data not shown). Herein, we find that T cells deficient for RelA had minimal impairment of proliferation, but instead demonstrated profound defects in *Ifng* transcription that could not be rescued by c-Rel, whether driven by the TCR-dependent or TCR-independent pathway. Thus, c-Rel and RelA have distinct, non-redundant roles in effector T cell biology.

In studies to identify NF-κB family members that complex with RelA in the *Ifng* locus, we found that binding of NF-κB p50 (NF-κB1), the most common partner for RelA (Vallabhapurapu and Karin, 2009), was primarily limited to CNS-22 — both in unactivated or activated Th1 cells (Fig. S6). Accordingly, RelA-p50 heterodimers are not the major CNS-binding NF-κB complex in the *Ifng* locus, in agreement with a previous study of *Nfkb1*$^{-/-}$ mice, in which Th1-polarized cells had unimpaired *Tbx21* and *Ifng* expression (Das et al., 2001). Although we were unable to identify binding of other NF-κB family members to the *Ifng* locus, this could reflect the unavailability of good antibodies for ChIP analysis of these factors (data not shown). Because RelA does not typically partner with NF-κB p52 (NF-κB2), we infer that with the exception of CNS-22, RelA might bind as a homodimer to most, if not all, of the other RelA sites in the *Ifng* locus, although a contribution of complexes with c-Rel and RelB cannot be absolutely excluded (Corn et al., 2005). Alternatively, RelA has been reported to heterodimerize with a phophorylated variant of T-bet (Hwang et al., 2005). Given previous findings that T-bet constitutively occupies several of the CNS elements identified as RelA binding sites herein (Hatton et al., 2006; Sekimata et al., 2009), RelA recruitment via this mechanism is also a possibility, and will require further study.

The unique occupancy of CNS-22 by NF-κB p50 in both naïve and Th1 cells is notable, in that selective deletion of CNS-22 in our previous study abrogated *Ifng* reporter expression in a BAC transgenic reporter model (Hatton et al., 2006). This suggests a particularly important role for this element in regulating *Ifng* transcription and raises the possibility that NF-κB p50 plays a central role in coordinating the function of this element, whether to repress it prior to effector T cell differentiation as a homodimer, or to activate it in the context of RelA recruitment and generation of p50-RelA heterodimers at this site.

Because TCR stimulation does not activate STAT4, RelA recruitment downstream of TCR signaling must occur by STAT4-independent mechanisms. In contrast, IL-12–induced STAT4 activation clearly impacts recruitment of RelA activated by IL-18; at each of the sites of RelA binding following IL-12+IL-18 co-signaling, STAT4 was also bound and was required for RelA binding. Indeed, ChIP-chip analysis revealed that there were few, if any, sites of that bound STAT4 without RelA. While this suggested a possible physical interaction between STAT4 and RelA–containing hetero- or homodimers, an interaction between STAT4 and RelA was not found in the absence of DNA in co-precipitation studies (data not shown). Nevertheless, the coordination of these two factors at multiple sites in the *Ifng* locus indicates that cooperative binding is essential for optimal *Ifng* expression and provides a basis for the requirement for coordinate IL-12 and IL-18 receptor signaling. This extends the range of cooperative interactions identified between the STAT and NF-κB transcription factor families downstream of immunomodulatory cytokine receptors (Shen and Stavnezer, 1998; Tran et al., 2010), and suggests that cooperative binding of STAT and NF-κB family members at conserved non-coding sequences might be a common feature of cytokine responsive genes.

An alternative mechanism by which IL-12 and IL-18 cooperate to induce *Ifng* is via a mechanism that requires *de novo* synthesis of GADD45β, which acts through MEKK4 to sustain activation of p38 MAPK (Yang et al., 1999; Yang et al., 2001). Taken together with the findings herein, this suggests that sustained activation of the p38 MAPK pathway downstream of IL-12+IL-18-induced expression of *Gadd45b* might cooperate with more transient recruitment of STAT4 and RelA complexes to generate the pattern of prolonged expression that characterizes *Ifng* induction by IL-12 and IL-18 (Berenson et al., 2006; Lu et al., 2004). It will be of interest to determine whether factors downstream of the p38 MAPK pathway are recruited to the same CNS elements defined for STAT4 and RelA herein, where

they might cooperate with and/or stabilize these complexes, or alternatively, might act at independent *cis*-regulatory sites to enhance *Ifng* transcription.

High-level transcription of *Ifng* is contingent upon epigenetic modifications that remodel the *Ifng* locus during Th1 differentiation (Mukasa et al., 2010; Wilson et al., 2009). STAT4 and T-bet cooperate in this process (Thieu et al., 2008), although details of the mechanisms by which this occurs, and the sites within the *Ifng* locus where they might directly interact are only beginning to emerge. A recent study found that both T-bet and STAT4 were necessary for removal of Sin3A-associated HDAC complexes that repress *Ifng* gene transcription (Chang et al., 2008). Other studies have identified conserved proximal and distal *cis*-regulatory elements to which T-bet binds (Hatton et al., 2006; Lee et al., 2004; Sekimata et al., 2009; Shnyreva et al., 2004), and with this report, we identify IL-12-dependent STAT4 binding at six CNS elements, and confirm its binding to the promoter (Chang and Aune, 2005; Thieu et al., 2008). With the exception of CNS-54, which binds T-bet in the absence of STAT4, each of the CNS sites that binds T-bet also binds STAT4, suggesting cooperativity between these factors following acute activation of STAT4 by IL-12. Remarkably, however, STAT4 was constitutively bound to CNS-22 and CNS+46, independently of acute Th1 cell activation, and the same sites were unique in their binding of STAT4 in Th1-polarized *Tbx21*$^{-/-}$ cells. This indicates that STAT4 recruitment to these sites during Th1 differentiation is sustained without a requirement for acute reactivation, and suggests that STAT4 might initially bind these sites without requirement for co-factors, such as T-bet. It is notable that both of these sites are DNase I hypersensitive in naïve T cells, supporting the possibility that CNS-22 and CNS+46 are critical in orchestrating early chromatin remodeling of the *Ifng* locus, perhaps as sites for recruitment of STAT4 as a "pioneer" factor that initiates this process.

Two recent reports identified orthologous CTCF-containing boundary elements that appear to define limits of the *Ifng* and *IFNG* loci (Hadjur et al., 2009; Sekimata et al., 2009), and Sekimata et al. defined an important role for T-bet in acting with these boundary elements and an intronic CTCF site to restructure the *Ifng* locus so as to approximate distal *cis*-regulatory elements with the *Ifng* promoter region (Sekimata et al., 2009). Interestingly, in addition to its reported binding of T-bet in Th1 cells, we find that the downstream CTCF site (HS +66) also binds STAT4 and RelA upon IL-12+IL-18 signaling, suggesting that this site might act as both boundary element and distal enhancer. Here, we provide additional evidence in support of a central role for T-bet in the generation of a transcriptionally competent *Ifng* locus in CD4$^+$, but not CD8$^+$, effector T cells. In the absence of T-bet, Th1-polarized CD4$^+$ T cells show impairment of H3K4 methylation at CNSs -34, -6 and the *Ifng* promoter, while exhibiting increases in H3K27 trimethylation at these same sites, in addition to increased repressive marks at CNSs -54, +30, +40, and +46. Notably, with the exception of CNS+40, which is also hypersensitive in activated Th2 cells, each of these sites demonstrates Th1-specific hypersensitivity, suggesting that T-bet regulates their H3K4 methylation and H3K27 demethylation. This is consistent with the recent identification of conserved regions within T-box domains that mediate interactions with H3K4 methyltransferase and H3K27-demethylase (Lewis et al., 2007; Miller et al., 2008). Whether T-bet is sufficient to orchestrate these changes independently of other Th1 lineage factors or effects these histone modifications via direct or indirect mechanisms is unclear, and will require further study. Our finding that T-bet is dispensable for the establishment of favorable H3K4 and H3K27 methylation marks at these sites in CD8$^+$ effector T cells suggests a similar function for the T-box family member Eomes in remodeling the *Ifng* locus in this lineage (Intlekofer et al., 2008; Pearce et al., 2003).

In conclusion, we find that coordinate and differential recruitment of transcription factors to multiple distal *cis*-acting elements plays an essential role in *Ifng* transcription initiated by

TCR- or cytokine-induced signaling, in essence defining a modular pattern of utilization of distal transcriptional "units" composed of complexes of *trans*-acting factors and the *cis*-acting elements to which they are recruited. Our findings suggest that CNSs -22, +40 and +46, which are accessible prior to Th1 differentiation, might act as multifunctional nodes that participate both in the initiation of early remodeling of the *Ifng* locus and in acute regulation of *Ifng* transcription, whereas other distal elements, including CNSs -54, -34, -6 and +54, may be functionally restricted to acute regulation of *Ifng* transcription subsequent to differentiation-driven locus remodeling. The multiplicity of sites to which STAT and RelA are coordinately recruited in the *Ifng* locus is remarkable, and suggests that there are advantages to having the option of recruiting graded multiples of distal STAT4/NF-κB units to the proximal promoter, perhaps as a mechanism for rheostat-like control of the rate of *Ifng* transcription by mass action, as has been found for repetitive *cis*-elements in simple promoters. Careful deletion analyses using transgenic and gene targeting approaches will be necessary to address these issues going forward.

EXPERIMENTAL PROCEDURES

Mice, antibodies and reagents

C57BL/6, BALB/c, DO11.RAG2$^{-/-}$ TCR transgenic mice and STAT4$^{-/-}$ BALB/c mice were purchased from Jackson laboratory and/or were bred at the University of Alabama at Birmingham. *Tbx21*$^{-/-}$ BALB/c mice were a gift from Dr. Dan Bullard (UAB). All mice were maintained in accordance with Institutional Animal Care and Use Committee regulations. Antibodies specific for p50 (sc-1150), p65 (sc-372), c-Rel (sc-71) and STAT4 (sc-486) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific for acetylated histone H4 (06–598), mono-, di-, or trimethyl H3K4 (04–791; detects all of the three possible methylation states) and trimethylated H3K27 (17–622) were purchased from Millipore (Billerica, MA). Cyclophilin B (ab16045) antibody was purchased from Abcam (Cambridge, MA). Anti-T-bet antibody has been described previously (Hatton et al., 2006). Peptides (IMG-2001, RelA peptide and control) used for inhibiting RelA were purchased from IMGENEX (San Diego, CA). All primers were custom-synthesized by IDT (Coralville, IA).

Isolation of CD4$^+$/CD8$^+$ T cells and generation of Th1/Tc1 effectors

CD4$^+$ T cells were isolated by positive selection using anti-CD4 magnetic DYNA beads (Invitrogen), as per manufacturer's instructions. For separation of CD8$^+$ T cells, following CD4$^+$ isolation, CD4 negative fraction was labeled with PE labeled anti-CD8 antibody followed by selection with anti-PE microbeads (Miltenyi Biotech; Bergisch Gladbach, Germany) and then fractionated over magnetic separation columns (Miltenyi Biotech). Both Th1 and Tc1 cells were generated by culturing CD4$^+$ and CD8$^+$ T cells with 2.5 μg/ml anti-CD3 antibody and 10 ng/ml recombinant IL-12 (R&D Systems: Minneapolis, MN) and irradiated feeder cells (3000 rads) at a ratio of 1:5. Th1 cultures were additionally supplemented with 10 μg/ml anti-IL-4. In case of OT-II and DO11.10 strains, CD4$^+$ T cells were isolated in a similar manner, but the cells were activated with 5 μg/ml OVA peptide 323–339 instead of anti-CD3. Th1 and Tc1 cultures were split on days 3 and 2 of culture and supplemented with 50 U/ml of recombinant IL-2 (R&D Systems).

Reactivation of Th1 and Tc1 cells

Th1 and Tc1 cultures were restimulated on days 5 and 3 of culture respectively. Restimulation was carried out with 50 ng/ml PMA and 750 ng/ml Ionomycin or 10 ng/ml rIL-12 and 25 ng/ml rIL-18. For TCR restimulation, anti-CD3 antibody was diluted to 10 μg/ml in phosphate buffered saline (PBS, 150mM NaCl, 0.02M Phosphate) and coated overnight at 4°C. The following day the plates were was washed twice with PBS and the

media was supplemented with 5 μg/ml of anti-CD28 antibody (eBioscience; San Diego, CA). All conditions involved reactivation of cell for 4 hours, except Tc1 cells, which were restimulated with PMA and Ionomycin for 1hour.

Intracellular cytokine staining

Cells were reactivated as described above for 4 hours in the presence of GolgiStop (BD Biosciences; San Jose, CA). Cells were stained with fluorescent-labeled antibodies against CD4, CD8, IL-4 and IFN-γ using the Cytofix/Cytperm kit (BD Biosciences). Intracellular staining for T-bet was done similarly using FoxP3 staining kit (eBioscience). In both cases, dead cells were excluded by staining with LIVE/DEAD® fixable stain kits (Invitrogen; Carlsbad, CA). Phospho-STAT4 stains were prepared using Phosflow kit (BD Biosciences) as per manufacturer's protocol. Samples were acquired on an LSRII flow cytometer and analyzed using FlowJo software (Treestar Inc.; Ashland, OR).

Chromatin Immunoprecipitation

All ChIP experiments were carried out using ChIP assay kit (Millipore) as previously described (Mukasa et al., 2010). Relative recruitment assessed by real-time PCR is expressed as $2^{\Delta\Delta Ct}$ or as a percentage of input DNA. Inputs were appropriately diluted to facilitate normalization against input DNA. Primer sequences are listed in the supplement.

DNase-chip

Samples were prepared as previously described (Crawford et al., 2006). Briefly, nuclei were isolated from 5×10^7 cells and subject to digestion with DNase I (Roche; Basel, Switzerland) concentrations ranging from 0–12 Units. Digestion was stopped using 100mM EDTA and nuclei were embedded in equal volumes of 1% InCert Agarose (Lonza; Basel, Switzerland). The plugs were incubated overnight at 37°C in LIDS buffer (1% lithium dodecyl sulfate, 10 mM Tris-HCl (pH 7.5), 100 mM EDTA) and then washed with 50mM EDTA. Following digestion with T4 DNA polymerase (New England Biolabs; Ipswich, MA), the blunted fragments were extracted, labeled with biotin-labeled linkers, captured and amplified using ligation mediated PCR and hybridized to microarrays. Data obtained from these experiments were visualized using IGB browser (Affymetrix; Santa Clara, CA).

Statistical analyses

Statistical significance was evaluated using two-tailed unpaired t-test. Unless specifically indicated, all p-values <0.05 are considered significant.

Highlights

- NF-κB family member RelA regulates *Ifng* gene expression in effector T cells.
- RelA cooperates with STAT-4 at multiple sites in the *Ifng* locus.
- T-bet plays an obligatory role in long-range remodeling of the *Ifng* locus.
- *Cis*-regulatory sites are modularly recruited in response to distinct stimuli.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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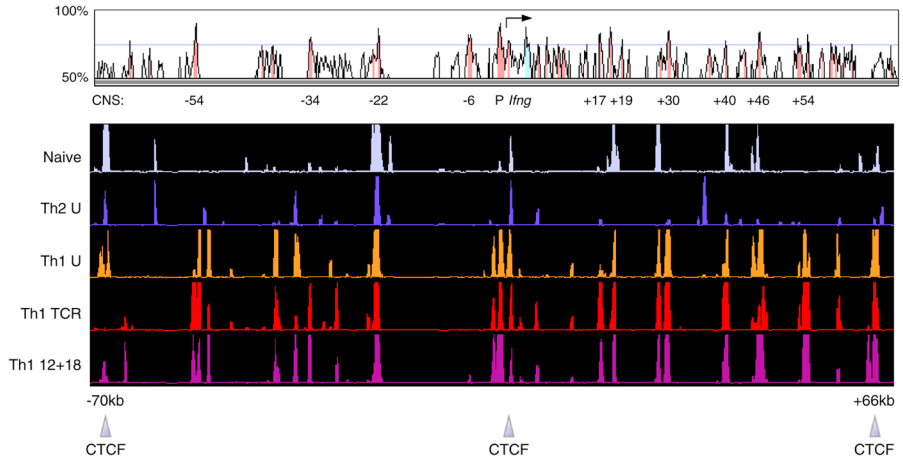

Figure 1. DNase I hypersensitivity map of extended *Ifng* locus in naïve, Th1 and Th2 cells

DNase I HS profiles of T helper subsets aligned with a VISTA plot of syntenic regions of the *Ifng and IFNG* gene loci. DNase–chip analyses were performed on $CD4^+$ T cells from OT-II transgenic mice that were naïve, differentiated under Th1 polarizing conditions (5 d), or differentiated under Th2 polarizing conditions (14 d). Prior to processing, cells were either left unstimulated ("U"), or were restimulated with anti-CD3 plus anti-CD28 ("TCR") or rIL-12 plus rIL-18 ("IL-12+IL-18"). P, *Ifng* promoter; CTCF, CCCTC-binding factor site.

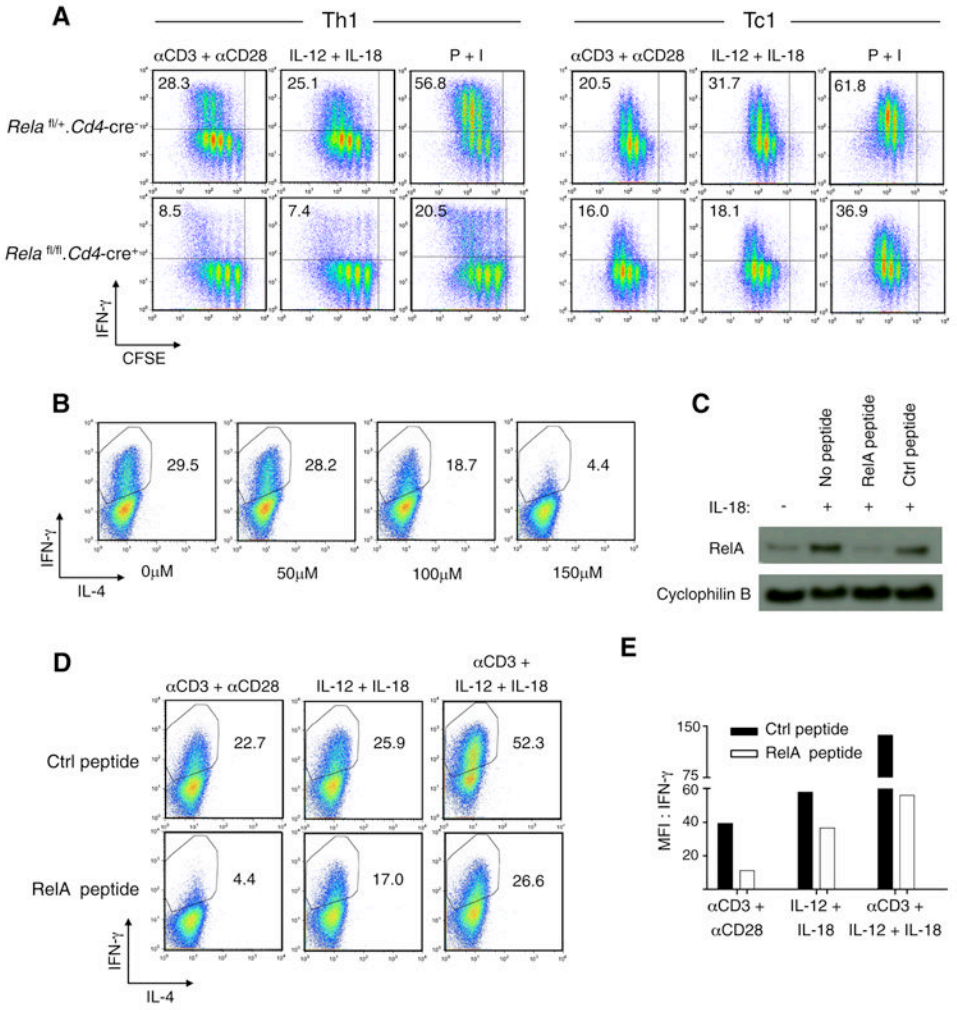

Figure 2. RelA is essential for acute induction of *Ifng* gene expression

(A) CD4$^+$ and CD8$^+$ T cells from *Rela* $^{fl/+}$.*Cd4-cre*$^-$ or *Rela* $^{fl/fl}$.*Cd4-cre*$^+$ mice were isolated and labeled with CFSE. CD4$^+$ T cells were cultured under Th1 cell conditions for 5 d (**upper panel**), and CD8$^+$ T cells were cultured under Tc1 condition for 3 d (**lower panel**). Recovered T cells were reactivated as indicated and intracellular IFN-γ was assessed by flow cytometry. Plots were gated on live CD4$^+$ or CD8$^+$ T cells. Numbers in each plot indicate the percentage of the gated cells in each quadrant. (**B**) Th1 cells were incubated for 1 hour with indicated concentrations of the RelA inhibitory peptide, (Ser 276), then activated with anti-CD3 plus anti-CD28 for 4 h and analyzed for intracellular IFN-γ. Dot plots are gated on live CD4$^+$ T cells, and numbers indicate the percentage of viable CD4$^+$ T cells in the indicated subgates. **(C)** Th1 cells were incubated for 1 hour with 150 μM of the inhibitory RelA peptide (lower panel) or control peptide (upper panel), then activated with the indicated stimuli for 4 h and analyzed for intracellular cytokines. Plots are gated on live CD4$^+$ T cells; numbers indicate the percentage of viable CD4$^+$ T cells in the indicated subgates. **(D)** Comparison of mean fluorescence intensities (MFI) of IFN-γ$^+$ cells from (C). **(E)** Th1 cells were activated with IL-12 with or without IL-18, following 1 h pre-incubation with RelA inhibitory peptide or the indicated controls, and nuclear translocation of RelA was assesed by immunoblotting. Cyclophilin B was used as loading control. All data are representative of at least two independent experiments.

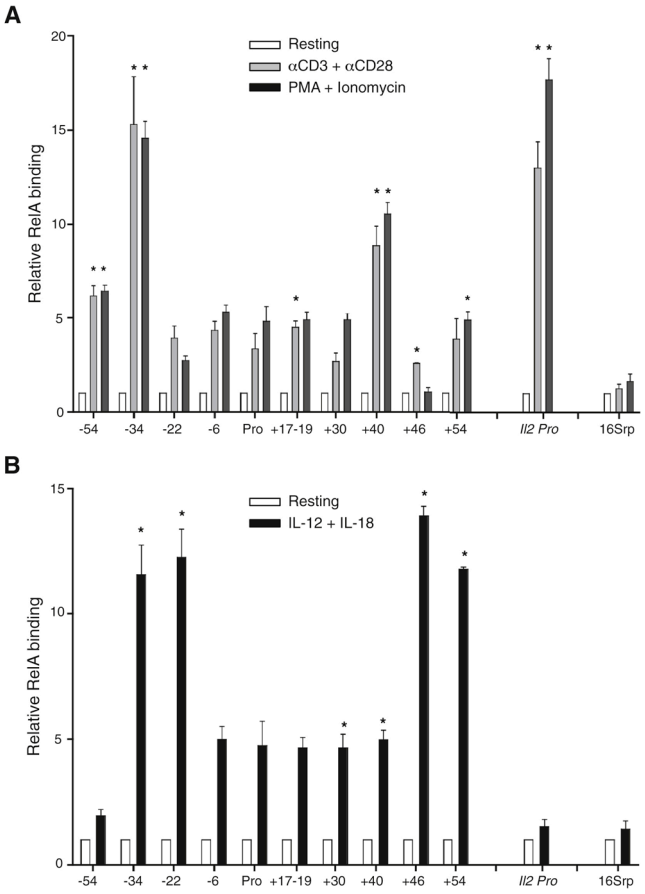

Figure 3. Differential recruitment of NF-κB p65 to distinct *Ifng* CNSs

Th1 cells that were either left unstimulated ("resting"), stimulated for 4 hours with 50ng/ml PMA plus 750 ng/ml Ionomycin or with anti-CD3 plus anti-CD28 (**A**), or restimulated with 10 ng/ml rIL-12 plus 25 ng/ml rIL-18 (**B**) were processed for ChIP using an NF-κB p65-specific antibody, and analysis of immunoprecipitated DNA was performed using real-time PCR with primer sets designed to detect DNA at the indicated CNS elements (-55, -34, -22, -5, +18–20, +29, +40, +46 and +55) and the *Ifng* promoter (Pro). The *Il2* promoter (*Il2* Pro) and 16S ribosomal protein (16Srp) promoter were used as positive and negative controls, respectively. Values were normalized against input DNA and expressed relative to resting Th1 cells, which was <0.05% of input DNA. Data represent Mean ± SEM from at least three independent experiments. Statistical significance is relative to binding at 16Srp under the same conditions (* $p<0.01$).

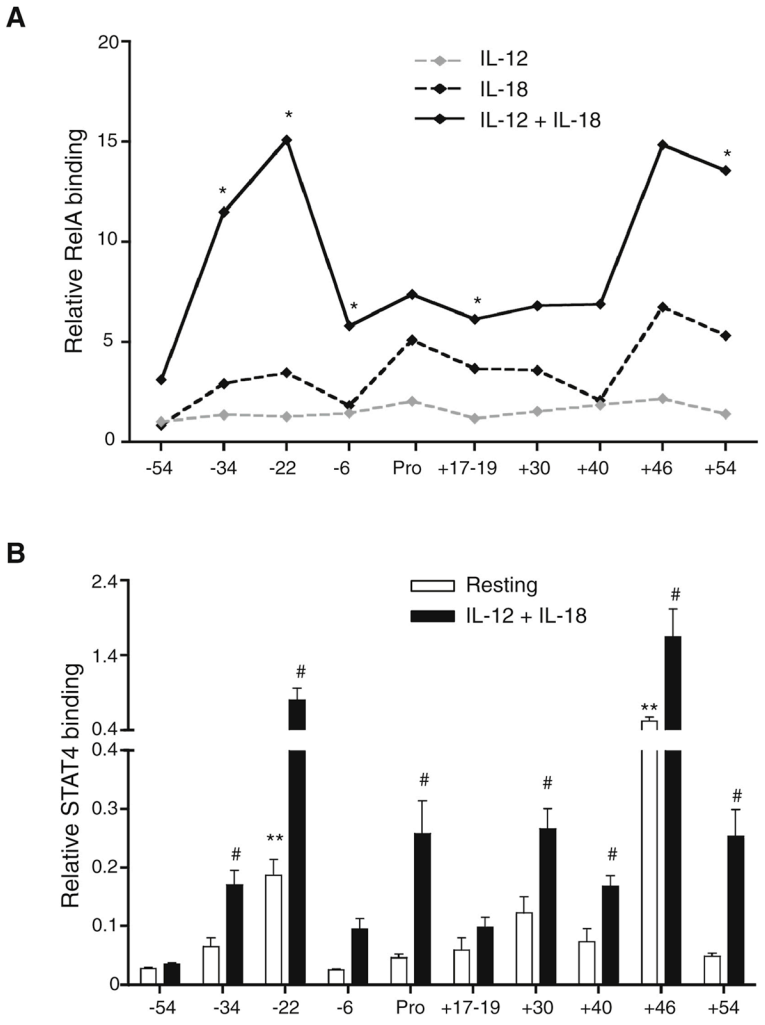

Figure 4. Co-recruitment of STAT4 and RelA at multiple *cis* elements that drive *Ifng* gene transcription

Th1 cells were either left unstimulated or stimulated as indicated for 4 h and ChIP was performed for the indicated CNS sites using antibodies specific for NF-κB p65 (**A**) or STAT4 (**B**). Relative RelA binding (**A**) was calculated as described in Fig. 3. Data are representative of at least three independent experiments (* $p<0.01$ for Th1 samples reactivated with IL-12+IL-18 versus IL-18 alone). **(B)** Recruitment of STAT4 was calculated as percentage of input DNA. Data represent mean ± SEM from at least three independent experiments. (# $p<0.05$ versus STAT4 recruitment in resting Th1 samples; ** $p<0.01$ versus recruitment of STAT4 to 16Srp in resting Th1 cells).

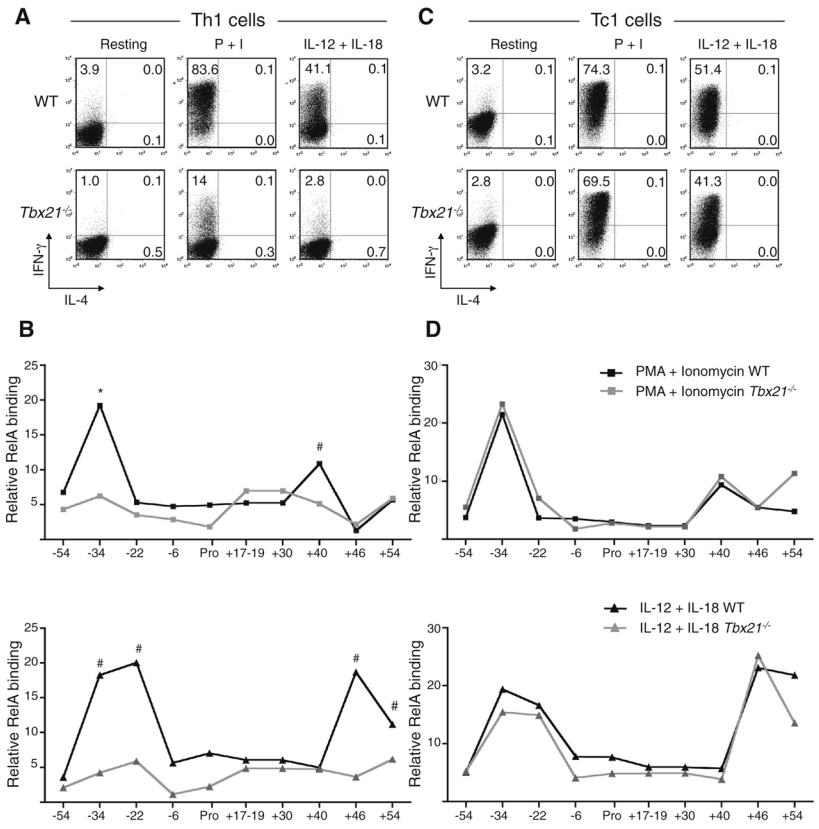

Figure 5. RelA recruitment is dependent on T-bet in Th1 cells but not in Tc1 cells

(A),(C) Cytokine production by Th1 and Tc1 cells generated from WT or *Tbx21*$^{-/-}$ mice was evaluated by intracellular cytokine staining. Flow cyometry plots are gated on live CD4^{+} (A) or CD8^{+} (B) T cells. Percentage values shown indicate frequencies of cells positive for the indicated cytokines. **(B),(D)** ChIP was performed for NF-κB p65 and enrichment was calculated as described in Fig. 3 following restimulation with PMA and ionomycin (top panel) or IL-12 plus IL-18 (bottom panel). Data are representative of at least three independent experiments. T cells from *Tbx21*$^{-/-}$ or WT mice were polarized under Th1 (B) or Tc1 (D) conditions and restimulated prior to ChIP. * $P < 0.01$, # $P<0.05$ versus *Tbx21*$^{-/-}$ samples.

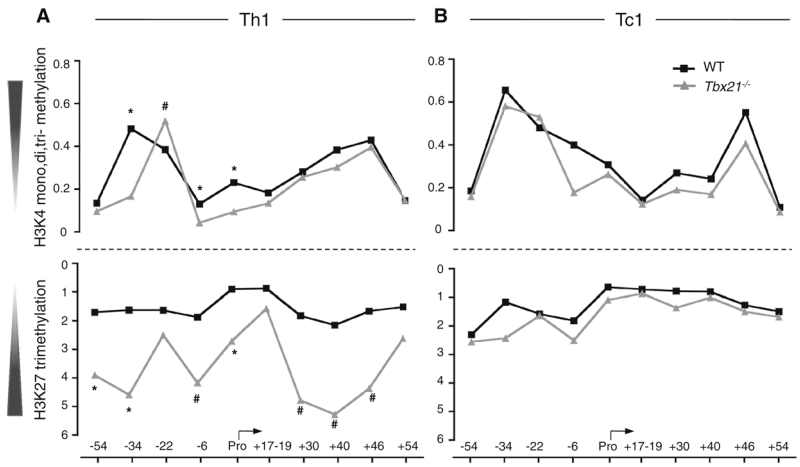

Figure 6. Remodeling of the *Ifng* locus in Th1 cells is T-bet–dependent

ChIP was performed on the indicated T cell effectors with antibodies specific for mono,di,tri-methylated H3K4 (top panels) or trimethylated H3K27 (bottom panels). $CD4^+$ T cells **(A)** or $CD8^+$ T cells (**B**) from wildtype or *Tbx21*$^{-/-}$ mice were activated under Th1- or Tc1 polarizing conditions respectively, then subject to ChIP. * $P < 0.01$, # $P<0.05$ versus *Tbx21*$^{-/-}$ Th1 samples. Values were normalized against the 16S ribosomal protein promoter, which was assigned a value of 1. Data are representative of at least three independent experiments.

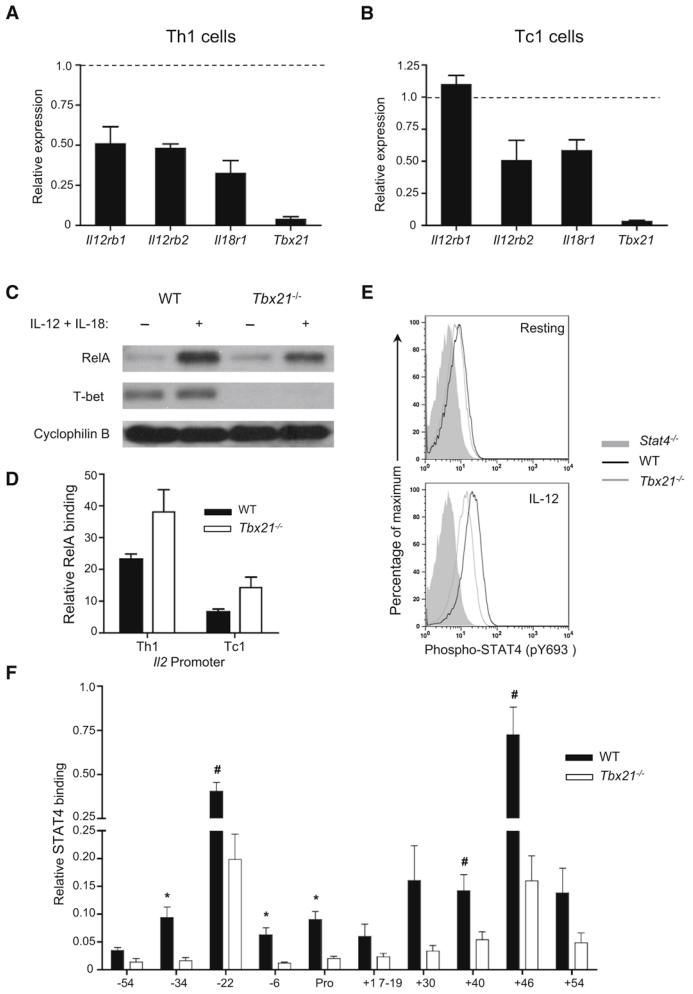

Figure 7. Impaired IL-12 and IL-18 signaling do not account for diminished RelA and STAT4 recruitment to the *Ifng* locus in T-bet–deficient Th1 cells

(A),(B) mRNA transcripts of the indicated genes were quantitated in Th1- and Tc1-polarized cells generated from WT or *Tbx21*$^{-/-}$ mice. Expression was normalized against β2-microglobulin, and relative expression compared to wildtype controls (dashed lines; assigned value of "1.00"). Data represent mean ± SEM from three independent experiments. (C) Th1-polarized cells derived from WT or *Tbx21*$^{-/-}$ mice were restimulated with IL-12 + IL-18 for 4 h and nuclear translocation of p65 evaluated by immunoblotting, using cyclophilin B as a loading control. Blots were re-probed for T-bet as a control for T-bet expression. (D) RelA ChIP was performed on indicated T cell populations and relative recruitment at the *Il2* promoter was assessed as in Fig 3. Data represent mean ± SEM from at least three independent experiments. (E) Th1-polarized cells from WT, *Tbx21*$^{-/-}$ or *Stat4*$^{-/-}$ mice were left untreated (resting) or reactivated (IL-12) and phosphorylation of tyrosine residue 693 (pY693) of STAT4 was evaluated by flow cytometry. Histograms are gated on viable CD4^{+} T cells. (F) STAT4 ChIP was performed following IL-12+IL-18 activation of *Tbx21*$^{-/-}$ and WT Th1-polarized cells, as in Fig. 4. Data are represented as fractions of input DNA and represent mean ± SEM from at least three independent experiments (*p<0.01, #P<0.05; stimulated WT versus stimulated *Tbx21*$^{-/-}$).