

T helper type 1–specific Brg1 recruitment and remodeling of nucleosomes positioned at the IFN- γ promoter are Stat4 dependent

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Transcriptional competence of the interferon- γ (IFN- γ) locus is enhanced as Th1 effectors develop from naive CD4 T lymphocytes; conversely, this gene is repressed during Th2 differentiation. We now show that the Switch (Swi)–sucrose nonfermenter (SNF) component Brahma-related gene 1 (Brg1) is recruited, and positioned nucleosomes are remodeled, in a Th1–specific manner that is dependent on the transcription factor Stat4 and calcineurin phosphatase activity. Interference with specific components of mammalian Swi–SNF complexes decreased CD4 T cell differentiation into IFN- γ –positive Th1 cells. These findings reveal a collaborative mechanism of IFN- γ gene regulation during Th1 differentiation and suggest that a Th1–specific chromatin structure is created by early recruitment of Swi–SNF complexes and nucleosome remodeling dependent on Stat4 and calcineurin activation.

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Abbreviations used: BAF, Brg1-associated factor; Brg1, Brahma-related gene 1; ChIP, chromatin IP; CsA, cyclosporin A; IP, immunoprecipitation; LM-PCR, ligation-mediated PCR; MNase, micrococcal nuclease; RE, restriction enzyme; REA, RE accessibility; RNAi, RNA interference; SNF, sucrose nonfermenter; Swi, Switch.

Activated CD4 T cells have the potential to develop into functionally distinct lineages marked by their commitment to produce a specific profile of cytokines (1, 2). Th1 cells produce large amounts of IFN- γ , of which an adequate level is crucial for immunity against intracellular pathogens; Th2 cells produce IL-4, -5, and -13 to mediate responses against extracellular parasites or cause allergic disease (2). Early after their activation, naive CD4 T cells transcribe low levels of mRNAs encoding both IFN- γ and IL-4 irrespective of whether the cells are under conditions promoting Th1 or Th2 differentiation (3–5). The capacity to support high level subset-specific cytokine gene expression upon secondary stimulation of activated CD4 T cells arises later. This capacity is programmed by a collaboration of signals from TCR and receptors for cytokines or similar ligands (6, 7). IL-12/-27 or IL-2/-4 strongly drive Th1 or Th2 differentiation, respectively, whereas CD28, inducible costimulator, and Notch ligands acting on their receptors also influence development of the helper T cell subsets (6–8). These receptors activate ubiquitously expressed transcription factors present in latent form in the cytosol of T cells, including the cytokine-activated Stat proteins, complexes of the NF- κ B/Rel/I κ B families, and calcineurin

phosphatase-regulated NFATs (9–11). Such subset-independent factors lead to the selective enhancement of subset-specific activators, of which the most critical are T-bet and GATA-3 (12–16). The subset-independent protein Stat4, which is activated by IL-12 receptor signaling, and T-bet are crucial for the efficient differentiation of activated CD4 T cells into the Th1 lineage (7, 15). These mechanisms of Th1 development are countered by the factors driving Th2 differentiation, which restrict the fate potential of CD4 T cells by silencing the IFN- γ gene (3–5). Thus, polarization into the restricted Th2 program of cytokine gene expression involves the repression of transcriptional competence at the IFN- γ locus.

Epigenetic changes play crucial roles in controlling the gene expression of many developmentally regulated genes. Changes in the packaging of DNA into nucleosomes, such as nucleosome position, conformation, or histone composition, mediate key aspects of epigenetic regulation (17–21). However, relatively little is known about epigenetic regulation of the IFN- γ locus or other aspects of the Th1 gene expression program (17, 22, 23). DNase I hypersensitive sites surrounding the IFN- γ gene have been identified, some of which appear after activation and 6 d of Th1 differentiation (22, 24, 25), but the specific molecular basis for these changes is not known. Nucleosomes play

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a central role in the control of gene expression because they present a barrier to multiple aspects of transcription (the binding of transcriptional activators and the loading or progress of RNA polymerase along nucleosome-clad DNA). For the subset of genes at which nucleosomes occupy defined positions relative to the transcription start site, two mechanisms can potentially mitigate this inhibitory role. In some cases, a nucleosome is removed or repositioned, which may create new access to a transcription factor (20, 26). Alternatively, changes in the packaging of DNA on the core histone octamer can render DNA more accessible without any change in nucleosome position (20, 21, 27). Evidence from cell-free *in vitro* systems suggest that the remodeling complexes mediating repositioning differ from those that affect changes in packaging without moving the nucleosome (20, 28). However, it remains unknown whether any Th1 or Th2 cytokine gene is packaged in a positioned nucleosomal array in naive or effector T cells or how packaging of these promoters might change during differentiation.

Because of the topological challenges involved in transcribing chromatinized DNA, the remodeling of promoter chromatin by multimolecular machines may be a necessary, if not sufficient, mechanism for enhancing the competence of a gene to be expressed (18, 20, 21). Similarly, a diverse array of homologues to these complexes may be essential for aspects of gene repression or silencing (29, 30). So far, three biochemically and functionally distinct chromatin remodeling complexes have been defined in mammals (19, 20, 24). These include a mammalian Switch (Swi)–sucrose nonfermenter (SNF) homologue, Mi-2/NuRD, and ISWI complexes (18–20). Each multiprotein machine contains an essential ATPase component, but this ATPase differs for each class of complex. The mammalian Swi–SNF complex contains either a homologue of Brahma or Brahma-related gene 1 (Brg1; references 19, 20, 31) and includes Brahma/Brg1-associated factors (BAFs) such as BAF57, a component capable of binding DNA that is important for a subset of Swi–SNF-related functions (32). Recent work provides evidence of distinct regulatory roles and gene-specific functions for each chromatin remodeling complex, and a given protein or complex can lead to either activation or repression

depending on the target (33, 34). For instance, the SNF2 homologue Brg1 (31) can interact either with corepressor proteins and mediate gene silencing (35–38) or participate in activating Swi–SNF complexes that promote increased gene expression (39–41). However, there is no information as to whether any remodeling factors are recruited to the IFN- γ locus or IL-4/-5/-13 locus specifically in developing Th1 or Th2 cells. Similarly, molecular components of the Th1 differentiation process, whose activity is necessary for any such recruitment of remodeling factors, are unknown. In this study, we present analyses of nucleosomes at the IFN- γ promoter and Th1-specific changes in its chromatin structure together with an investigation of remodeling components and transcription factors that participate in the observed changes early in Th1 differentiation.

RESULTS

Kinetics of changing IFN- γ locus competence

Naive CD4 T lymphocytes activate a burst of both IFN- γ and Th2 cytokine mRNA expression in the hours after TCR stimulation, which was followed later by much higher levels of IFN- γ mRNA only under Th1 conditions (3, 4). Furthermore, the IFN- γ locus in a Th1 clone is better able to support transcription after TCR stimulation than that of a naive cell, whereas the competence of the locus in a Th2 clone is far less than that of the naive cell (4). To extend these findings, we quantitated the transcriptional competence across a time course of a first cycle of differentiation. IFN- γ mRNA levels were measured at a constant time after TCR stimulation: primary stimulation for naive CD4 T cells (0.25 d under Th1 or Th2 conditions) or a secondary stimulation 2, 4, or 6 d after initial activation. TCR-stimulated IFN- γ expression increased when cells had grown 2 d in Th1-differentiating conditions followed by a dramatic and progressive increase occurring from days 2 to 6 (Fig. 1). Thus, an increase in the transcriptional competence of the IFN- γ locus starts by 2 d after the initiation of Th1 differentiation and accelerates thereafter.

A positioned array of nucleosomes at the IFN- γ promoter

Altered transcriptional competence of the IFN- γ locus must be mediated, in part, through its promoter, and a limited

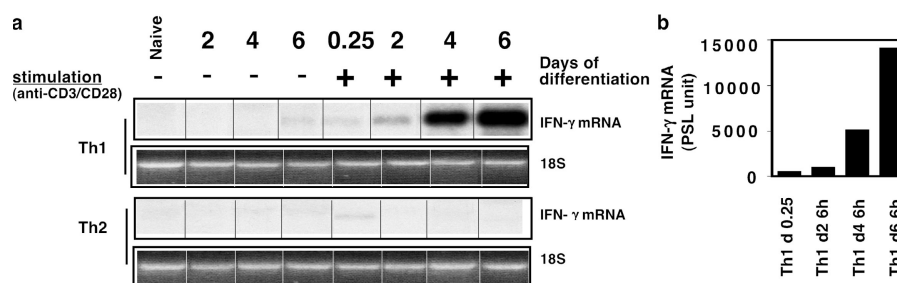


Figure 1. Progressive increase in IFN- γ locus competence to support transcription. Naive CD4 T cells were either analyzed directly or activated (anti-CD3 + anti-CD28) and differentiated for 0.25, 2, 4, or 6 d under Th1 or Th2 conditions. Samples differentiated for 2, 4, or 6 d were

irradiated, divided equally, and restimulated (+) or not stimulated (-). (a) IFN- γ mRNA levels were detected with Northern blots. (b) Quantitation of IFN- γ mRNA levels in cells from panel a (naive and Th1 conditions). PSL, pixel standard level.

portion (~600 bp) of this promoter containing highly conserved sequences sufficed to direct Th1-specific gene expression in transgenic mice (42). We investigated whether nucleosomes are present across the promoter in its transcriptionally inactive state and, if so, whether they are positioned randomly or instead are arrayed at defined positions. Nuclei from naive CD4⁺ T cells underwent limited digestion with micrococcal nuclease (MNase) and analysis by an indirect end-labeling method using restriction endonuclease sites flanking the promoter (27). With each of three restriction enzymes (REs), using a probe abutting the cleavage site for two of them (HincII and EcoRI), a nucleosome ladder of discrete bands was evident with spacing of 160–200 nucleotides (Fig. 2, a–c). Although this low resolution method does not pinpoint nucleosome positions to the nucleotide, the results indicated that at least three nucleosomes are positioned between 1 and –600 bp.

Ligation-mediated PCR (LM-PCR) was performed on MNase-treated genomic DNA to map more precisely the borders of nucleosomes positioned at the proximal IFN- γ promoter. One mapped to a position spanning nucleotides –120 to 30 relative to the transcription start site (nucleosome 1; Fig. 2, c and d; Nuc 1 in the schematic). A second nucleosome, nucleosome 2, was mapped to boundaries between nucleotides 70 and 220; nucleosome –2 was found between nucleotides –370 and –520 of the promoter region by indirect end labeling. These data indicate that the IFN- γ promoter is packaged in a positioned array of nucleosomes in naive CD4 T cells. Assays comparing naive resting CD4 T cells with a Th1-polarized population revealed no difference in the position of nucleosome 1 covering the conserved proximal regulatory element of the promoter, nor did a comparison of Th1 with Th2 populations (unpublished data).

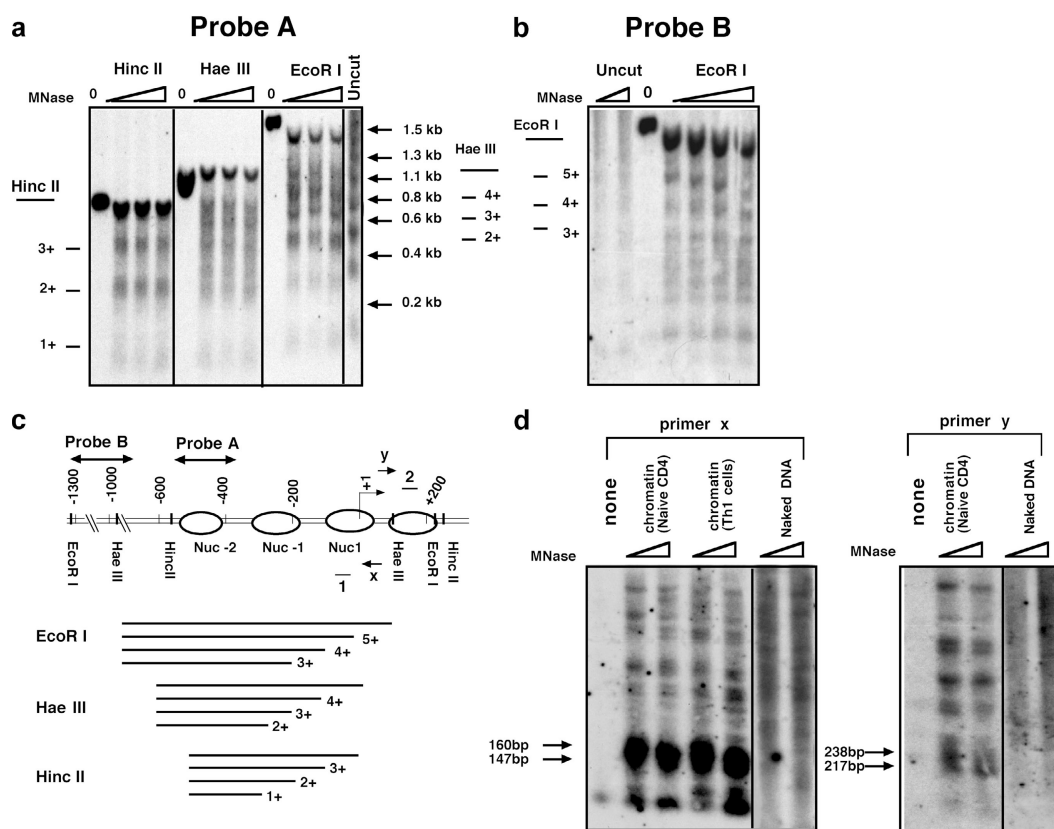


Figure 2. Nucleosomes positioned in IFN- γ promoter chromatin in CD4 T cells. (a and b) Naive CD4 T cells were cross-linked with formaldehyde, and their chromatin was treated with MNase (5, 7.5, and 10 enzyme units). DNAs were then purified, digested with the indicated RE (or left undigested; 0), and analyzed by Southern blots using a probe indicated in panel c (directly adjacent to the RE site for HincII-cut DNAs [a] or EcoRI digests [b]). (c) Diagram of RE sites and inferred nucleosome positions at the IFN- γ promoter. The fragment used as a probe in Southern blotting and primers used for LM-PCR are shown above the gene; the transcription start site (+1) is indicated by an

arrow. (d) LM-PCR mapping of nucleosome boundaries in the IFN- γ promoter. DNA purified from MNase (2.5 and 1 enzyme unit)-cleaved chromatin of naive or Th1 (6-d culture) CD4 T cells was analyzed by LM-PCR using primer x (nucleosome 1) or y (nucleosome 2) and a linker primer and was analyzed by Southern blot probed with an internal oligonucleotide (1 and 2 for nucleosomes 1 and 2). As a control for MNase cleavage preferences in DNA, pure cellular DNA was analyzed using the same preparation of MNase, linker ligation, and PCR (naked DNA). Shown is an autoradiograph representative of five independent experiments.

Increased accessibility of nucleosomal DNA at the IFN- γ promoter during Th1 differentiation

To further assess whether chromatin of the IFN- γ promoter is altered upon cell activation and differentiation, a RE accessibility (REA) assay was used. Nuclei were prepared from naive CD4 T cells, which have a low competence to transcribe IFN- γ after activation (Fig. 1; references 3, 4). Naive cells also were activated and grown under Th1 conditions, after which the Th1 population was rested or restimulated. The accessibility of IFN- γ promoter DNA in nuclei from each set of cells was measured by determining the relative efficiency of cleavage by RE (Fig. 3). Nucleosomal DNA of the most proximal portion of the IFN- γ promoter became substantially more accessible in rested Th1 cells compared with naive cells. The chromatin at a subset of these sites changed further after secondary stimulation of resting Th1 cells (Fig. 3 b). Together, these changes reveal that precise physical changes in the packaging of the IFN- γ promoter take place after T cell activation, which were more compatible with nucleosome remodeling rather than removal. Th1 cells that had not been reactivated exhibited degrees of remodeling at most sites similar to what was observed after restimulation (Fig. 3 b) and the elicitation of highly active gene transcription (4, 22).

Rapid, progressive Th1 subset-specific chromatin remodeling at the IFN- γ promoter

We next determined whether chromatin remodeling at the IFN- γ promoter is a Th1-specific process or instead is simply activation dependent. We analyzed an array of RE sites across the promoter to compare the chromatin of naive CD4 T cells with rested or reactivated Th1 and Th2 cell populations. IFN- γ promoter chromatin developed greater REA only in Th1 cells, whereas the cleavage efficiency in activated Th2 cells did not differ from that of naive CD4 cells (Fig. 4 a). This finding applied both to the nucleosome 1 MseI site and an adjacent, unique SnaBI site situated within a highly conserved transcriptional element that combines a composite ATF/AP-1/C/EBP-like site and a T-box half-site (Fig. 3 a; references 7, 43–45). In addition to changes at nucleosomes 1 and 2, another cleavage event was identified at a second MseI site within the fragment used to anchor these assays. Consistent with cleavage at a site positioned in a nucleosome packaging the -500 site in the IFN- γ promoter, the accessibility of chromatin to cleavage by XmnI at its unique site also dramatically increased in a Th1-specific manner and increased further upon secondary stimulation. To assess whether these changes represented the remodeling of nucleosomes in particular versus a general change equally affecting all of the chromatin in this region, cleavage by PvuII (cutting between nucleosomes 1 and 2) was examined in naive, Th1, and Th2 populations. Remodeling was more prominent for DNA within the positioned nucleosomes than at the PvuII site in the spacer, suggesting that this Th1-specific remodeling process targets nucleosomal DNA of the IFN- γ promoter. Intriguingly, however, the two sites

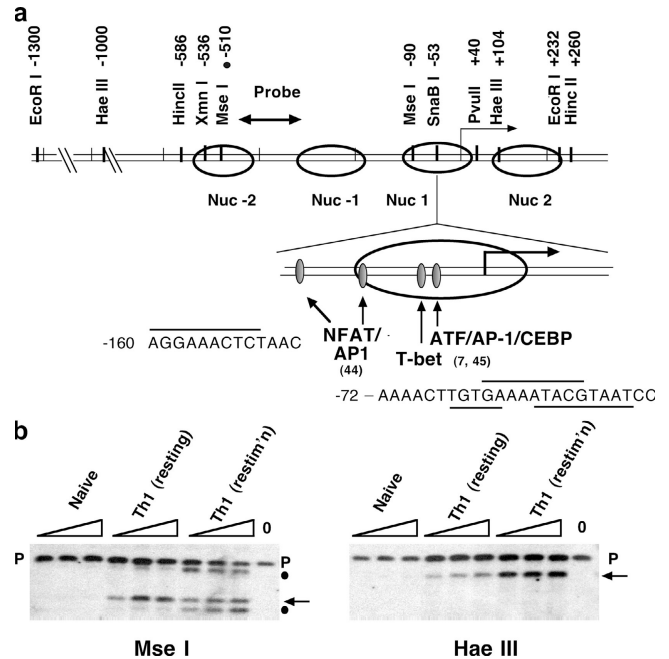


Figure 3. Chromatin remodeling at the IFN- γ promoter. (a) Diagram of the IFN- γ promoter with nucleosome positions identified in Fig. 2. Sites analyzed in the conserved IFN- γ promoter region are indicated by black bars; the positions of nucleosomes are indicated by open ovals; and the transcription start site is indicated by an arrow. Below the line, an expanded view of nucleosome 1 indicates the position of binding sites that comprise the proximal conserved element (46, 47). Further below, nucleotide sequences in pertinent sites are indicated, with lines designating binding motifs; papers identifying these sites (7, 45, 44) are noted. (b) Naive CD4 T cells were analyzed directly or activated (α CD3 + α CD28), differentiated under Th1 conditions for 6 d, and rested or restimulated as described previously (18). Nuclei from each cell population were digested with MseI (10, 25, and 50 enzyme units), HaeIII (10, 25, and 50 enzyme units), or no enzyme (0 in the right lanes) as indicated. Purified genomic DNAs were then cut to completion with HincII and analyzed using Southern blots. Black dots note the positions of bands generated from an upstream MseI site (nucleotide -510). Arrows point to the bands that are indicative of accessibility to the restriction endonuclease being tested. P, parental band.

mapping over nucleosome -2 (XmnI and the secondary MseI site) appeared preferentially to undergo further changes upon secondary stimulation of the T cells, whereas sites over the more proximal nucleosomes 1 and 2 (first MseI, SnaBI, and HaeIII) were relatively unaffected by restimulation of the cells.

We investigated the early kinetics of increased accessibility to determine whether the Th1-specific remodeling of IFN- γ promoter chromatin is a late event or whether it starts early in differentiation instead. CD4 T cells were polarized in Th1 and Th2 conditions for 18 and 48 h and were analyzed by REA assay. The change in IFN- γ promoter nucleosomes was absent at 18 h after activation but became evident 48 h after TCR stimulation during growth under Th1 conditions (Fig. 4 b; reference 46). Together, these findings

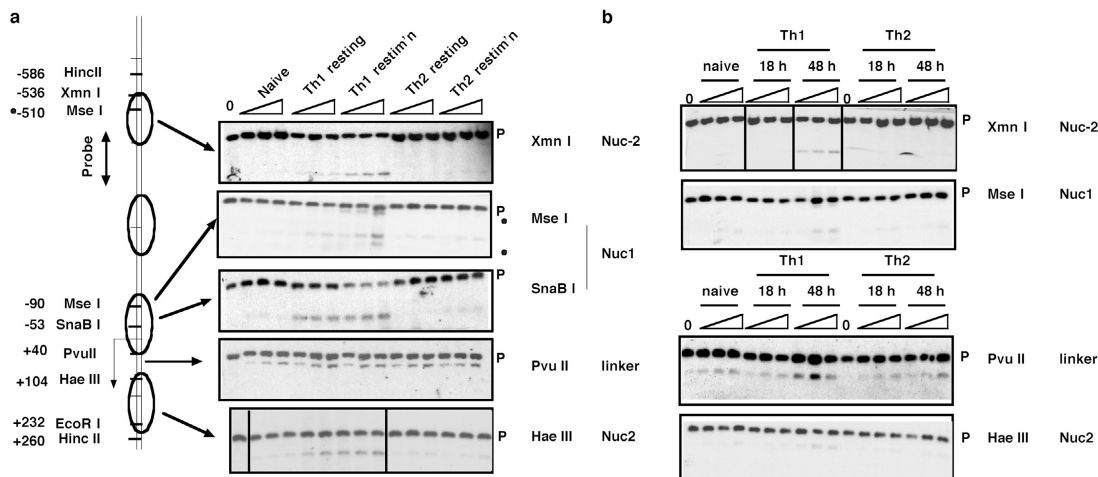


Figure 4. Early Th1 subset-specific chromatin remodeling at the IFN- γ promoter. (a) IFN- γ promoter chromatin remodeling is Th1 specific. The indicated CD4 T cell populations were prepared as in Fig. 3. Nuclei from these cells and naive CD4 T cells were incubated with no enzyme (0 at the left lanes) or increasing amounts (10, 25, and 50 enzyme units for each enzyme) of the indicated REs. Purified genomic DNA was then digested to completion with HincII (for MseI, SnaBI, PvuII, and HaeIII analyses) or EcoRI (for XmnI accessibility) and analyzed by Southern blotting using the probe indicated in Fig. 3 a. Results are representative of six

independent experiments. Arrows connect the map site in the promoter (vertical diagram of promoter region at left) with the fragment representing cleavage of chromatin by the indicated enzyme. Bent arrow represents the position of the transcription start site. Black dots note the positions of bands generated from an upstream MseI site (nucleotide -510). (b) Naive CD4 T cells were analyzed directly or were activated and differentiated for 18 or 48 h under Th1 or Th2 conditions. Samples were then analyzed as in panel a using the indicated REs. Shown is one set of autoradiographs representative of four independent experiments. P, parental band.

indicate that Th1-specific remodeling is targeted to nucleosomal chromatin in cells with relatively low IFN- γ gene transcription (22, 46). This remodeling is concurrent with

onset of the progressive increase in the competence of the locus to support high levels of transcription after TCR re-stimulation (Fig. 1).

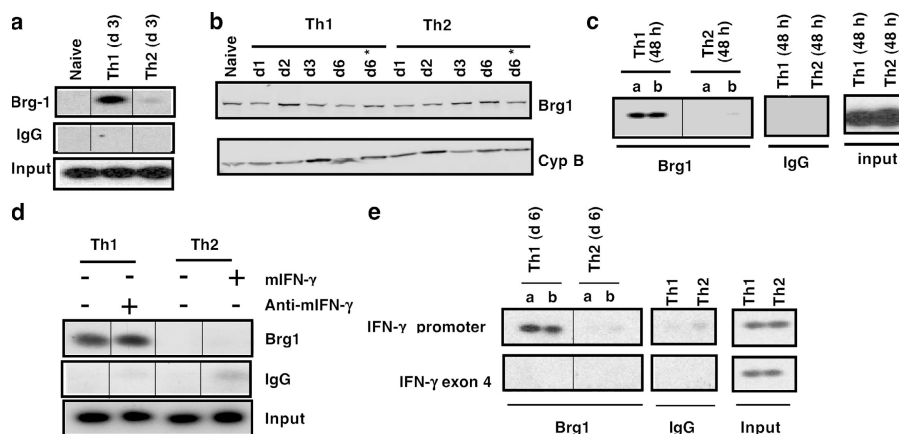


Figure 5. Th1-specific Brg1 recruitment to the IFN- γ promoter. (a) T cell activation leads to Brg1 association with the IFN- γ promoter specifically under Th1 conditions. Naive CD4 T cells were analyzed directly or differentiated under Th1 or Th2 conditions for 3 d followed by ChIPs using normal rabbit IgG or anti-Brg1 antibodies as indicated. IFN- γ promoter DNA was detected in the precipitate or in nonfractionated sample (input) by PCR in a linear range of amplification and Southern blot hybridization with an internal oligonucleotide probe. An autoradiograph from one of six independent experiments with the same result is shown. (b) Brg1 levels in CD4 T cell subsets. Naive, Th1, and Th2 populations of CD4 T cells (asterisks indicate restimulation) were assayed by Western blotting using anti-Brg1 antibodies and a loading control. (c) As in panel a, except that duplicate samples

were analyzed 48 h after T cell activation. (d) IFN- γ independence of Brg1 association with the IFN- γ promoter. Purified naive CD4 T cells were activated and cultured under Th1 conditions, \pm saturating concentrations of blocking antibody anti-IFN- γ , or were cultured under Th2 conditions (\pm IFN- γ). Anti-Brg1 ChIP analyses were then performed using these cells. (e) Stable Th1-specific Brg1 association with the IFN- γ promoter. Growth of cells from panel c was continued under polarizing conditions until day 6, at which time Brg1 ChIPs were performed. Each sample was divided equally for performance of independent duplicate IPs (a and b). Precipitates were used for PCR detection of DNA sequences of the IFN- γ promoter or exon 4, as indicated. Shown are autoradiographs from one experiment that is representative of results from three independent experiments.

Recruitment of Brg1 to the IFN- γ promoter and Swi-SNF function in developing Th1 cells

Changes in the accessibility of nucleosome-packaged DNA can be mediated by Swi-SNF, Mi-2/NuRD, or ISWI remodeling machines, but the Brg1-containing Swi-SNF complex appears best at enhancing endonuclease sensitivity without displacing or sliding the nucleosome (20, 28). However, nothing is known as to the requirements for or the constitution or localization of remodeling components at Th1 or Th2 cytokine genes during their activation or repression. To investigate whether the core Swi-SNF component Brg1 associates with the IFN- γ promoter during Th1 differentiation (locus activation) or in Th2 conditions (IFN- γ gene repression), we performed chromatin immunoprecipitations (IPs [ChIPs]). Chromatin from naive cells was compared with samples from recently activated CD4 T cells cultured under Th1 and Th2 conditions. Brg1 associated with the IFN- γ promoter in developing Th1 cells but not in their Th2 counterparts (Fig. 5 a) yet was expressed at similar levels in naive, Th1, and Th2 cells (Fig. 5 b). In comparisons of naive CD4 T cells activated and cultured 48 h in Th1 or Th2 conditions, binding had occurred at this early time (Fig. 5 c). Recruitment was not caused by IFN- γ production; recruitment was maintained despite neutralizing anti-IFN- γ in cultures with IL-12 present, and the addition of IFN- γ to Th2 cultures did not cause it (Fig. 5 d). Brg1 was still enriched at the IFN- γ promoter in Th1 cells when T cell populations were tested at 6 d of culture (Fig. 5 e), indicating that the recruitment of Brg1 is stably maintained at the accessible chromatin of Th1 cells.

Amplification of the same samples with primers specific for exon 4 of the IFN- γ gene detected no enrichment of Brg1 (Fig. 5 e), showing that binding was a localized process rather than a global association with chromatin or transcribed sequences.

We next investigated the functional significance of Swi-SNF components in Th1 differentiation. CD4 T cells were activated to permit retrovirus-mediated transduction and grown initially under conditions preventing Th1 differentiation. Cells were then switched to Th1 conditions after infection, grown 2 d, and scored for IFN- γ gene expression in cells transduced with molecules targeting Swi-SNF complexes or controls (Fig. 6 a). In experiments using RNA interference (RNAi) to decrease expression of the core ATPase component of the Swi-SNF complex, a substantial and reproducible decrease in the efficiency of Th1 differentiation was observed in the GFP⁺ CD4 T cells transduced to express short hairpin RNA compared with the GFP⁻ populations (not depicted) and GFP⁺ cells with empty vector (Fig. 6 b). Little information is available as to what would constitute a control gene in this setting, but CD4 expression appeared normal both in the GFP⁺ samples of these RNAi experiments (Fig. 6 b) and prior analyses of the mature T cells resulting from abnormal thymic differentiation in a lineage-specific Brg1 knockout (47). Stat4 and T-bet levels also appeared normal in flow cytometric analyses of the Swi-SNF RNAi cells (Fig. S1, available at <http://jem.org/cgi/content/full/jem.20060066/DC1>). Although it proved not to be feasible to obtain enough purified primary cells to

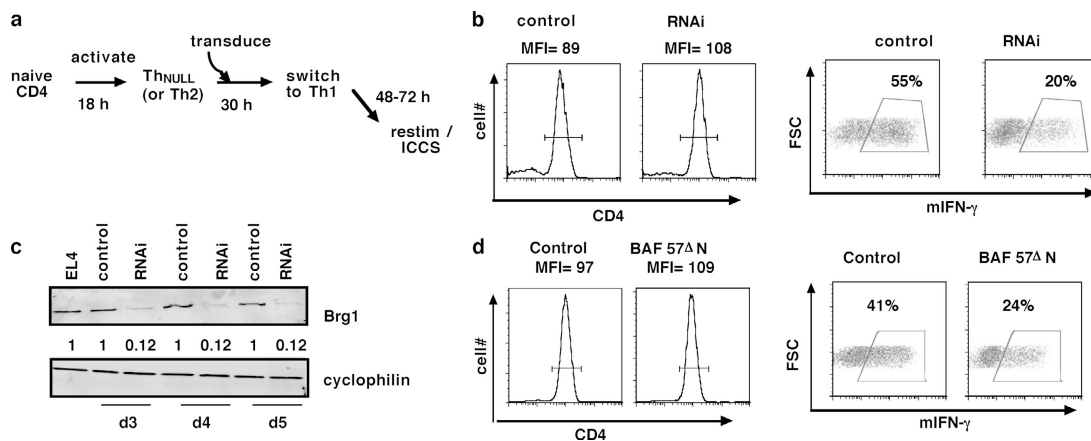


Figure 6. Role of Swi-SNF components in Th1 development.

(a) Schematic of experimental design using ThU (α IL-4, α IL-12 p40, and α IFN- γ) or Th2 conditions to defer the onset of Th1 differentiation until after retrovirus transduction and a later switch to Th1 conditions.

(b) T cells were transduced with empty vector or retrovirus encoding a short hairpin sequence targeting mouse Brg1 (RNAi), switched to Th1 conditions as in panel a, and analyzed by flow cytometry after staining for IFN- γ after secondary stimulation (α CD3 + α CD28). Histograms of the CD4 signal in the GFP⁺ population are shown as well as the IFN- γ signal of cells in the GFP⁺, CD4⁺ gates. Controls (Fig. S1, available at <http://jem.org/cgi/content/full/jem.20060066/DC1>; and not depicted)

showed normal cell viability and CD44, Stat4, P-Stat4, and T-bet levels in the RNAi samples. ELISA of supernatants from restimulated GFP⁺ cells confirmed a substantial decrease in IFN- γ production. (c) Brg1 levels in T cells transduced with the short hairpin-encoding or control virus for the indicated periods were measured by Western blotting. (d) As in panel b, except that cells were transduced with empty MiT vector (retrovirus) or virions encoding the dominant-negative BAF57 Δ N and the IFN- γ signal was measured in the Thy1a⁺, CD4⁺ gate. For both RNAi and BAF57 Δ N, results shown are representative of those reproduced in at least four independent experiments. MFI, mean fluorescence intensity; ICCS, intracellular cytokine staining; FSC, forward scatter.

analyze the knockdown, immunoblots of EL-4 T cells transduced with the short hairpin RNA showed substantial depletion of Brg1 compared with controls (Fig. 6 c). Because Brg1 expression was not eliminated completely, it seems likely that the residual Th1 development in these experiments is caused, in part, by the incomplete ablation of Swi-SNF complexes. A dominant-negative form of the noncatalytic BAF57 component of the Swi-SNF complex, BAF57 Δ N (32), lacks its DNA-binding moiety. When BAF57 Δ N was used in transduction experiments, we observed normal CD4 expression but a substantial decrease in Th1 differentiation among the cells expressing high levels of the bicistronic Thy1a tag as compared with controls (Fig. 6 d). Thus, the initiation of Th1 differentiation triggers localized Brg1 recruitment and chromatin remodeling; these temporally linked processes both precede secondary TCR stimulation. Furthermore, although Brg1-containing complexes are often associated with the repression of gene expression (36–39), the Brg1-containing Swi-SNF complex appears vital for normal Th1 differentiation. The lack of Brg1 recruitment to the IFN- γ promoter in developing Th2 cells suggests that this protein does not affect IFN- γ locus silencing as cells proceed from the naive to Th2 state.

Signaling Brg1 recruitment and chromatin remodeling

The activation of calcineurin phosphatase signals the induction of NFAT transcription factors, and a key role for NFATc2 (NFAT1) in regulating Th1 differentiation has been identified using mice selectively deficient in this protein (48). Moreover, the IFN- γ promoter contains a functional site for NFAT, which binds to IFN- γ chromatin in differentiating Th1 cells (43, 44, 46). We tested whether calcineurin phosphatase activity was required for the remodeling of IFN- γ promoter chromatin by activating naive CD4 T cells in the presence or absence of cyclosporin A (CsA). To bypass the requirement for NFATs in IL-2 induction, IL-2 was included in the cultures. CsA abrogated the increase in IFN- γ promoter chromatin accessibility in REA analyses of cells under Th1 conditions (Fig. 7 a). Furthermore, ChIP assays of activated CD4 T cells in the same conditions (Th1 conditions \pm CsA) showed that Brg1 recruitment was blocked by this inhibitor (Fig. 7 b).

This requirement for calcineurin phosphatase activity might arise because T cells failed to receive any activating signals or because few cells could enter S phase (49). Alternatively, these functions might still be inducible as a result of the collaboration of the CsA-insensitive components of TCR/CD28 signaling. Analyses of IL-2-supplemented

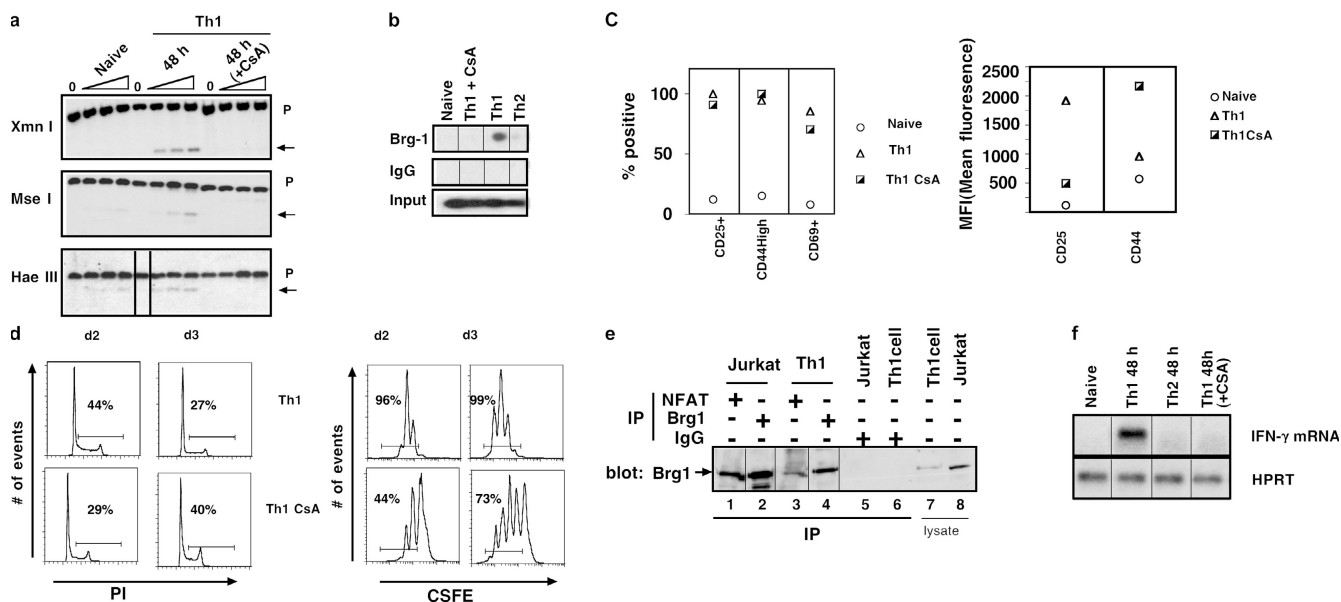


Figure 7. Essential role for calcineurin phosphatase activation in Brg1 recruitment and chromatin remodeling of Th1 cells. (a) IFN- γ promoter chromatin remodeling is abrogated by cyclosporin A (CsA). Naive CD4 T cells were purified, activated (or not; naive), and cultured under Th1 conditions (including supplemental IL-2) in the presence or absence of CsA for 48 h. Nuclei were digested with the indicated enzymes and processed as in Fig. 2. Arrows point to the bands that are indicative of accessibility to the restriction endonuclease being tested. P, parental band. (b) Brg1 recruitment to the IFN- γ promoter blocked by CsA. Samples generated as in panel a were analyzed by ChIPs as in Fig. 6. (c and d) T cells were activated in the presence or absence of CsA, with IL-2 added to all cultures. (c) Frequencies (percent positive) of CD4 cells positive for CD25 and CD44 were determined by flow cytometry, as were the mean fluores-

cence intensities for these parameters within the positive gate. (d) Cell cycle progression of CD4 lymphocytes was measured by flow cytometry of permeabilized propidium iodide-stained cells in the CD4⁺ gate (left) or with samples loaded with CFSE before activation (right). Inset numbers represent the fraction of cells in cycle or cells that divided at least once. (e) Association of Brg1 and NFAT. Extracts from the indicated cells were precipitated using the indicated antibodies; these precipitates were analyzed by anti-Brg1 immunoblotting. Lanes 7 and 8: extracts without IP; one immunoblot representative of three independent experiments is shown. Lanes 1 and 3 are from a longer exposure of the chemiluminescent membrane than lanes 2 and 4–9, all of which were from the same exposure of the same filter. (f) CsA inhibition of IFN- γ expression. Northern blot analysis of RNA from cells activated and differentiated as in panel a.

cultures of CD4 lymphocytes stimulated in the presence or absence of CsA revealed ample induction of multiple markers of T cell activation (Fig. 7 c) and showed ~50% efficiency of CD4 cell entry into S phase and completion of at least one division as compared with controls (Fig. 7 d). These findings indicate that the calcineurin-dependent pathway was required for Brg1 recruitment to and remodeling of the IFN- γ promoter even though the induction of other genes and cell cycle progression downstream from TCR signaling were observed under our experimental conditions. Consistent with a potential role for NFAT in initiating the recruitment of Swi-SNF complexes, Brg1 and NFAT coimmunoprecipitated from nuclear extracts of T cells (Fig. 7 e). As expected, IFN- γ gene expression was not induced (Fig. 7 f) when cells were cultured 48 h under Th1 conditions with CsA and supplemental IL-2. These data show the calcium signaling pathway to be a key mediator of Brg1 recruitment and nucleosome remodeling, suggesting that these are mechanisms by which NFAT regulates Th1 differentiation (48).

Nucleosome remodeling in *stat4*^{-/-} and *T-bet*^{-/-} developing Th1 cells

Because NFAT transcription factors are activated in developing Th2 cells as well as in Th1 differentiation, we hypothesized that a transcriptional regulator active only in Th1 cells may be crucial for Brg1 recruitment or remodeling of the IFN- γ promoter. Stat4 and T-bet are activated early in devel-

oping Th1 and not Th2 cells, and each is essential for normal Th1 differentiation. When we measured Brg1 recruitment and chromatin remodeling using Stat4-deficient CD4 T cells activated and cultured under Th1 conditions, the increase in REA was dramatically attenuated in the absence of Stat4 (Fig. 8 a), and Brg1 recruitment was considerably reduced (Fig. 8 b). Controls showed that T-bet was induced in the knockout samples (Fig. 8 c), Brg1 expression was normal, and Th1 differentiation was dramatically reduced in the absence of Stat4 (Fig. 8 d). These findings show that Brg1 recruitment and remodeling of the IFN- γ promoter are Stat4 dependent.

In contrast to the degree of dependence on Stat4, Brg1 recruitment and REA assays comparing wild-type to T-bet-deficient CD4 T cells indicated that each process proceeded in T-bet^{-/-} samples. Although quantitative analyses of the fraction of DNA cut by the RE indicate that the absence of T-bet reduced the nucleosome accessibility to REs (MseI and HaeIII), this decrease was <50% (Fig. 9 a). Consistent with prior work on Th1 development (15, 24), we observed that the frequency of Th1 cells (Fig. 9 c) and levels of IFN- γ mRNA were substantially decreased in the T-bet-deficient CD4 lymphocytes (Fig. 9 d). Stat4 activation requires IL-12R β 2, and other work has suggested that IL-12R β 2 induction is downstream from T-bet (50, 51). Because the data showed a greater dependence of remodeling on Stat4 than T-bet, we analyzed whether IL-12R β 2 induction or the activation of Stat4 can be affected in T-bet^{-/-} cells. IL-12R β 2

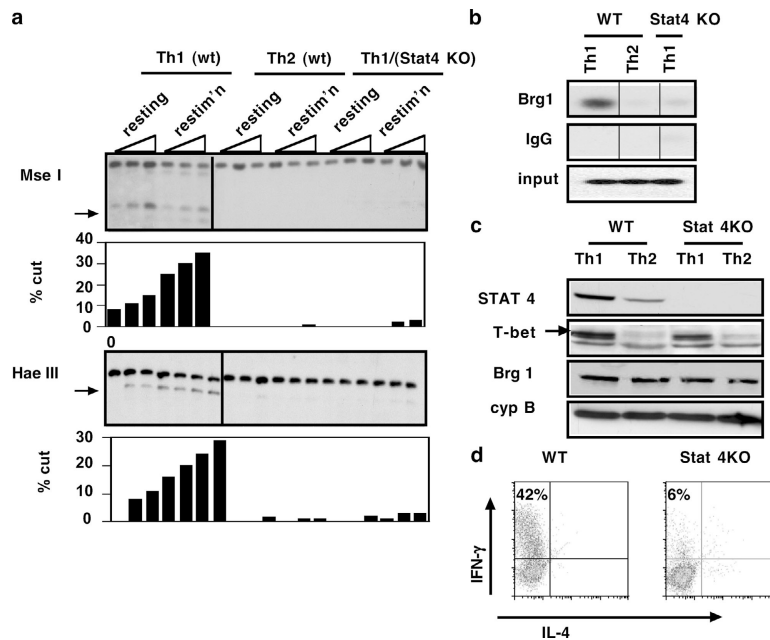


Figure 8. Remodeling and Brg1 recruitment depend on Stat4. (a and b) Wild-type (WT) and Stat4-null CD4 T cells were purified, activated, cultured under the indicated conditions (Th1 vs. Th2), and analyzed by REA assays (a) or ChIPs (b); the percent cleavage at the indicated sites was quantitated by phosphorimaging of the Southern blot signals for each sample. Arrows point to the bands that are indicative of accessibility to the restriction endonuclease being tested. (c) Levels of T-bet, Stat4, Brg1, and cyclophilin B in extracts of the wild-type and Stat4 knockout (KO) Th cells used in panels a and b were measured by Western blotting. (d) Th1 cells generated from naive CD4 T cells of wild-type and Stat4 knockout mice were subjected to secondary stimulation (α CD3 plus α CD28) analyzed by staining for intracellular IFN- γ in CD4⁺ cells. Each result is an experiment representative of at least three independent repeats (except Brg1 levels; n = 2).

Brg1, and cyclophilin B in extracts of the wild-type and Stat4 knockout (KO) Th cells used in panels a and b were measured by Western blotting. (d) Th1 cells generated from naive CD4 T cells of wild-type and Stat4 knockout mice were subjected to secondary stimulation (α CD3 plus α CD28) analyzed by staining for intracellular IFN- γ in CD4⁺ cells. Each result is an experiment representative of at least three independent repeats (except Brg1 levels; n = 2).

mRNA induction was readily detectable (Fig. 9 e), and antiphospho-Stat4 Western blots showed robust Stat4 activation in T-bet-deficient CD4 T cells (Fig. 9 f). This evidence suggests that Stat4 activation in T-bet-deficient cells could mediate their Brg1 recruitment, remodeling, and residual capacity to differentiate into Th1 cells (15, 52). Collectively, the results lead to a collaborative model of how Stat4 and a calcineurin phosphatase-induced component regulate the generation of transcriptional competence in IFN- γ -encoding chromatin during Th1 differentiation.

DISCUSSION

The choreography of transcription factors and mechanisms that modulate chromatin structure is central to regulating

differentiation and levels of cytokine gene expression. At a time before achieving the highest levels of transcriptional competence, IFN- γ promoter chromatin undergoes a Th1-specific structural transition. This remodeling is manifested as enhanced DNA accessibility over positioned nucleosomes with no apparent change in their ordered array (within the limits of the resolution of borders mapped in the present analyses). The data identify IFN- γ promoter-specific recruitment of a Swi-SNF component, Brg1, and nucleosomal remodeling as early Th1-specific events. Although the remodeling process can proceed in the absence of T-bet, the calcium signaling pathway, acting via calcineurin phosphatase, is crucial for recruitment of the Swi-SNF ATPase and induction of nucleosome remodeling. Our findings indicate

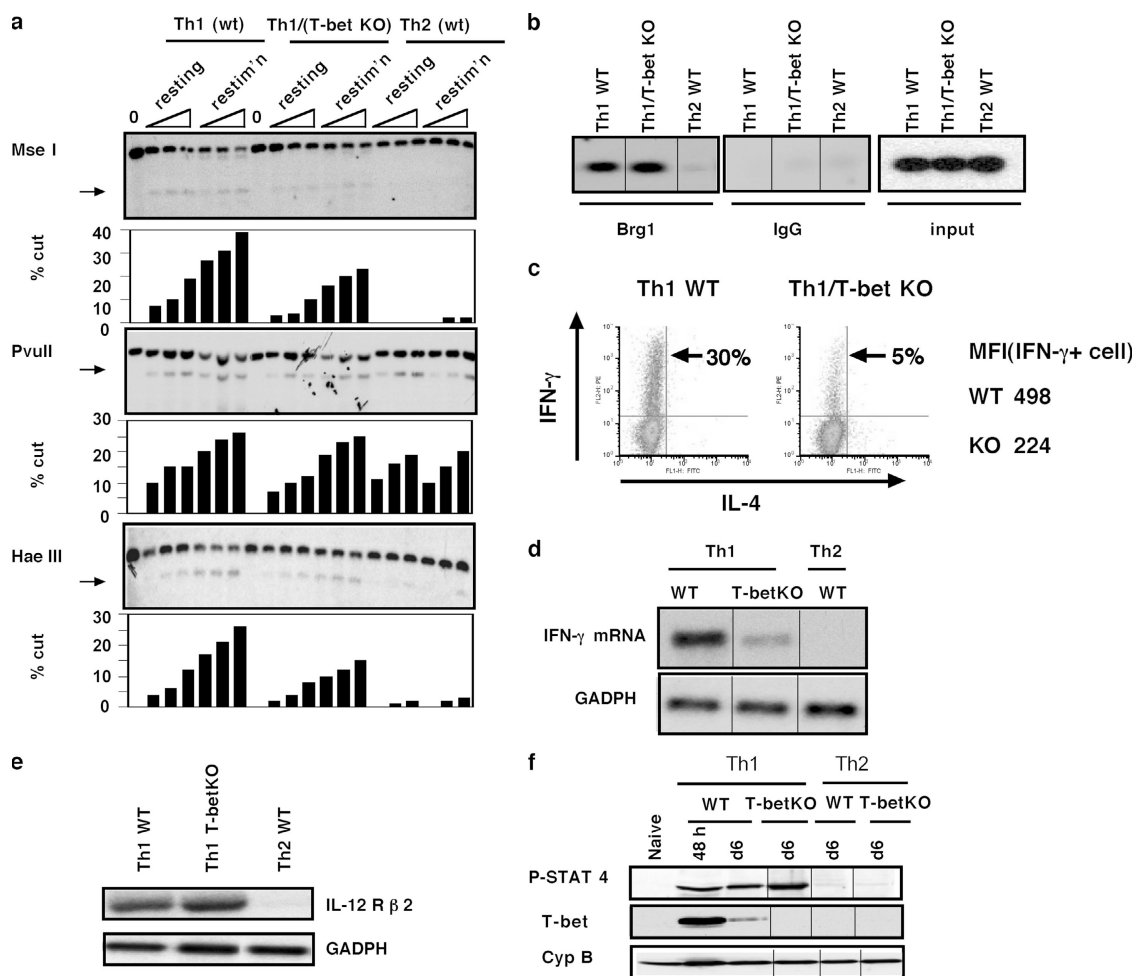


Figure 9. Role of T-bet in Brg1 recruitment and chromatin remodeling in developing Th1 cells. (a–d) Purified naive CD4 T cells from T-bet-deficient and wild-type (WT) mice were activated and grown 6 d under Th1 or Th2 conditions. Nuclei from these samples were then subjected to REA analyses (a) or ChIP assays (b) for Brg1, as in Fig. 8. Arrows point to the bands that are indicative of accessibility to the restriction endonuclease being tested. (c and d) IFN- γ gene expression in T-bet-deficient CD4 cells. (c and d) The frequency of IFN- γ -producing CD4 T cells after Th1 differentiation of naive CD4 T cells in wild-type and T-bet-

null samples was measured as in Fig. 8 d (c), or IFN- γ was measured by Northern blotting (d). (e and f) Normal levels of IL-12R β 2 and phospho-Stat4 induction in T-bet-deficient CD4 T cells developing under Th1 conditions. Purified naive CD4 T cells from wild-type and T-bet knockout (KO) mice were activated and grown in Th1 or Th2 conditions. Samples were then analyzed by Northern blots to measure IL-12R β 2 mRNA levels (e) and immunoblots probed with antiphospho-Stat4, -T-bet, and a loading control (f). Each result is from one experiment that is representative of at least three independent repeats.

that these steps in Th1 differentiation depend on Stat4 inasmuch as IL-12R β 2 expression and P-Stat4 levels in T-bet-deficient CD4 T cells under Th1 conditions were normal, whereas Brg1 recruitment and chromatin remodeling were decreased in Stat4-null CD4 T cells. Together, the results support a model in which collaborative contributions of NFAT and Stat4 lead to a functionally important recruitment of Swi-SNF complexes to the IFN- γ gene and show that Swi-SNF function is vital for achieving a normal efficiency of Th1 differentiation.

Two mechanisms for the recruitment of Brg1 and the associated Swi-SNF complex have been identified (20, 53, 54). In one, bromodomains of the complex recognize acetylated histones. Activation of naive CD4 T cells induces a transient burst of early IFN- γ gene expression (Fig. 1; references 4, 5) and, within the first 2 d, more histone acetylation in Th1 than Th2 conditions (46). Although it is reasonable to infer that this mechanism contributes to Brg1 targeting to the IFN- γ promoter, it may not be the only basis for the Th1 specificity of Brg1 recruitment. The second documented mechanism of Brg1 recruitment is direct interaction with transcription factors. The ability to coprecipitate NFAT along with Brg1 suggests that this second mechanism is also important for Th1-specific Swi-SNF association with the IFN- γ promoter. Our data show that Brg1 recruitment is an early step for which calcineurin-dependent transcription factors such as NFAT are essential. However, the nuclear translocation of NFAT is induced in developing Th2 cells, yet Brg1 recruitment to the IFN- γ promoter was not observed under Th2 conditions. We infer that the Brg1-NFAT complex would need to collaborate with a Th1-specific component; as discussed in the next paragraph, Stat4 appears crucial for this Th1 specificity. Furthermore, it is likely that early remodeling enhances the access of DNA-binding transcription factors and associated coactivators to their cognate sites in the IFN- γ gene. Because the density of histone acetylation at the IFN- γ promoter increases from day 2 to 6 in differentiating Th1 cells (55), remodeling may create a permissive environment that promotes this progressive increase.

Brg1 recruitment and a considerable extent of remodeling occurred in the absence of T-bet in developing Th1 cells. Three points suggest that this outcome is mediated by Stat4 activation. First, Stat4 promoted Th2 to Th1 conversion (i.e., IFN- γ production) in T-bet-deficient CD4 T cells switched from Th2 to Th1 conditions after the enforced expression of Stat4 (52). Second, P-Stat4 is activated in T-bet-null developing Th1 cells, and both Brg1 recruitment and the increases in REA depend on Stat4. Finally, T-bet was induced in Stat4-null CD4 cells under Th1 conditions, yet remodeling was dramatically reduced. Still, it is likely that T-bet can make contributions to the remodeling process. T-bet transduction into developing Th2 cells (i.e., with neutralizing anti-IL-12 to block activation of the Stat4 pathway) was able to force substantial amounts of Brg1 recruitment and increased REA at the IFN- γ promoter (unpublished data). Moreover, the deletion of either T-bet or Stat4 had a some-

what greater effect on remodeling (increased REA) than on Brg1 recruitment (Figs. 8 and 9). This point suggests that each of these transcription factors may collaborate with the Swi-SNF complex in executing remodeling after recruitment of the complex.

One potential obstacle to a model in which Stat4 accounts for Swi-SNF recruitment in the absence of T-bet is that experiments with the ectopic expression of T-bet indicated that IL-12R β 2 induction can be downstream T-bet (50, 51, 56). However, Stat4 would need to be induced in *tbx21* knockout CD4 cells under Th1 conditions. Our data show that P-Stat4 is readily induced by IL-12 in the absence of T-bet. How might the induction of Stat4 phosphorylation be executed under Th1 conditions in T-bet-null helper cells? We have previously shown that a Stat factor can be induced normally despite a 10-fold decrease in levels of its activating receptor (57) so that levels of IL-12R β 2, which at first were decreased, might mediate initial Stat4 activation followed by Stat4-dependent increases in receptor expression. In any event, IL-12R β 2 mRNA was induced in activated T-bet-deficient CD4 T cells under Th1 conditions (Fig. 9). Thus, T-bet may be sufficient but is not necessary for IL-12R β 2 and P-Stat4 induction in IL-12-treated cells.

Because T-bet was clearly essential for normal Th1 differentiation and IFN- γ production in our experiments (Fig. 9) as in previous studies (15, 24, 55), the findings on Stat4 and T-bet roles in remodeling the IFN- γ promoter chromatin suggest a need to account for the steps at which T-bet is essential. T-bet is present before secondary stimulation and directly transactivates both the IFN- γ promoter in a manner dependent on a highly conserved T-box half-site packaged into nucleosome 1 of the IFN- γ promoter (14, 24, 42, 45) and distant elements such as the IFN- γ CNS-1 sequence (25). Because T-bet-deficient CD4 T cells exhibit attenuated rates of gene expression despite achieving Brg1 recruitment and substantial nucleosome remodeling, we infer that T-bet may exert its vital functions after establishment of the Th1-specific chromatin structure revealed by REA. Remodeling renders nucleosome-clad DNA more accessible to further transcription factor binding (19, 20, 26). Swi-SNF might also promote long-range interactions with distant regulatory elements such as CNS-1 (ifng) that is transactivated by T-bet (24, 25, 58–60). For instance, changes in higher order arrangement of the regulatory segments of the IFN- γ locus appear to occur after day 2 under Th1 conditions (58). These steps would then establish the basis for achieving the full level of gene transcription characteristic of the Th1 cell. In this regard, it is also notable that the REA data may reveal two separable types of remodeling at the IFN- γ promoter. The first of these phases, before secondary stimulation, influenced each of the nucleosomes analyzed, whereas most additional increases in REA after reactivation were in the -500 region over the nucleosome mapped as nucleosome -2. Notwithstanding these inferences about the stages at which T-bet regulates IFN- γ transcription, the central points supported by our data are that (1) a Stat4-dependent, Th1-specific recruitment of

the Swi–SNF component and chromatin remodeling occur in IFN- γ chromatin in coordination with increasing transcriptional competence at this locus, and (2) the Swi–SNF complex is a mediator of efficient Th1 differentiation.

MATERIALS AND METHODS

Mice. BALB/cJ, BALB/c-T-bet^{-/-}, and BALB/c-Stat4^{-/-} mice (Jackson ImmunoResearch Laboratories) were maintained in microisolators in specific pathogen-free conditions in the Vanderbilt University mouse facility and used at 4–6 wk of age under Institutional Animal Care and Use Committee–approved protocols.

Cell preparation, culture, and T cell differentiation. Antibody and cytokine reagents were obtained from BD Biosciences unless otherwise noted. Naive CD4⁺ T cells were purified from the lymphoid organs of young mice with magnetic beads (Miltenyi Biotec) first with negative (anti-CD8 and MHC II beads) and then with positive selection of CD4⁺ CD62L^{hi} cells with CD62L-conjugated beads. Typical cell purities were $\geq 90\%$ CD4⁺ CD44^{lo}. Naive T cells were stimulated with 2 $\mu\text{g/ml}$ anti-CD3 ϵ and 1 $\mu\text{g/ml}$ anti-CD28 as described previously (61, 62). For Th1 differentiation, cells were stimulated in the presence of 10 ng/ml of recombinant mouse IL-12 (Leinco) and 1 $\mu\text{g/ml}$ of purified anti-IL-4 (11B11). For Th2 differentiation, cells were cultured in 5 ng/ml of mouse IL-4, 0.5 $\mu\text{g/ml}$ anti-IFN- γ , and 1 $\mu\text{g/ml}$ anti-IL-12. To block each form of differentiation (Th_{NULL} conditions), cells were cultured in IL-2, anti-IL-4, anti-IL-12 p40, and anti-IFN- γ . After 2–6 d, the cells were left unstimulated or were restimulated for 6 h with anti-CD3 plus anti-CD28 or with PMA plus ionomycin (14, 22). For cell cycle analyses, CD4 cells were activated and grown in Th1 conditions (\pm 0.5 $\mu\text{g/ml}$ CsA) as above, stained for CD4, permeabilized, and stained with propidium iodide. For measurements of division, cells were first stained with CFSE (Invitrogen) and then were activated and grown in Th1 conditions (\pm CsA).

Retroviral transduction of primary T cells. Retroviral vector transfection and infection were performed as described previously (61, 62). In brief, naive CD4⁺ T cells were purified by magnet-activated cell sorting, after which the cells were activated with anti-CD3/CD28 and cultured under Th_{NULL} or Th2 conditions. Developing Th_{NULL} or Th2 cells were transduced with empty vector (RVGP or MiT; reference 63) versus short hairpin cDNA in RVGP (gift of S. Smale, University of California, Los Angeles, Los Angeles, CA; reference 64) or MiT-BAF57 Δ N 18 h after activation and were cultured an additional 30 h under the same conditions. Infected populations were then switched to Th1 conditions, cultured for 3 d, restimulated (anti-CD3 plus anti-CD28), and analyzed by staining for intracellular IFN- γ . EL4 T cells were transduced with RVGP or short hairpin vector as were the primary CD4 T cells, except that 3 $\mu\text{g/ml}$ puromycin selection was started 24 h after infection, and GFP expression was monitored by flow cytometry. Western blots for Brg1 were performed on extracts of these selected EL4 cells ($>95\%$ GFP⁺).

Preparation and digestion of nuclei and chromatin. CD4⁺ T cells were subjected to cross-linking (20 min on ice) with one-tenth volume of 11% formaldehyde solution in 0.1 M NaCl, 1 mM EGTA, and 50 mM Hepes, pH 8.0 (1% vol/vol final concentration). Glycine was then added (final concentration of 0.125 M; 10 min). Cells were rinsed three times with ice-cold PBS, resuspended in lysis buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% NP-40, 0.15 mM spermine, and 0.5 mM spermidine), and incubated on ice for 10 min. Nuclei were pelleted, washed in MNase digestion buffer (10 mM Tris, pH 7.4, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, and 0.5 mM spermidine) without CaCl₂, and resuspended in MNase digestion buffer containing 3 mM CaCl₂. For nucleosome mapping, MNase (Roche) was added, and reactions (5 min at 20°C in 200 μl) were terminated by the addition of 200 μl proteinase K buffer (100 mM Tris-Cl, pH 8.5, 5 mM EDTA, 200 mM NaCl, 0.2% SDS, and 100 $\mu\text{g/ml}$

proteinase K). Samples were incubated overnight at 56°C, after which DNA was purified by isopropanol precipitation.

REA assay. Cells were washed once with 1 \times PBS, resuspended in lysis buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% NP-40, 0.15 mM spermine, and 0.5 mM spermidine) for 5 min on ice, and washed once with RE buffer (10 mM Tris, pH 7.4, 50 mM NaCl, 10 mM MgCl₂, 0.2 mM EDTA, 0.2 mM EGTA, 0.15 mM spermine, and 0.5 mM spermidine). Nuclei were resuspended in 100 μl of the recommended buffer (New England Biolabs, Inc.) followed by the addition of RE, and reactions (20 min at 20°C) were stopped by the addition of an equal volume of 100 mM Tris, pH 8.5, 5 mM EDTA, 200 mM NaCl, 0.2% SDS, and 100 $\mu\text{g/ml}$ proteinase K incubated overnight at 56°C. DNA purification was performed as described for MNase experiments.

Southern, Northern, and Western blot analyses. 15 or 10 μg MNase-treated or RE-digested cellular DNAs were digested to completion with flanking REs. Samples were resolved by agarose gel electrophoresis (1.2% for MNase-treated DNAs and 1% for RE-digested DNA) followed by transfer and hybridization with a radiolabeled probe specific for the IFN- γ promoter (–550 to –250). For Northern blots, naive CD4 T cells were activated and grown under Th1 or Th2 differentiating conditions in the presence or absence of 0.5 $\mu\text{g/ml}$ CsA. Total cellular RNA was resolved by denaturing agarose gel electrophoresis and was probed with cDNAs encoding IFN- γ , hypoxanthine phosphoribosyl transferase, IL-12R β 2, or GADPH as described previously (59). For Western blot assays, cell extracts were resolved by SDS-PAGE followed by immunoblots probed with anti-T-bet (4B10; Santa Cruz Biotechnology, Inc.), –p-STAT 4 (Y-693 STAT 4; Zymed Laboratories), –Brg1 (Upstate Biochemicals), or –cyclophilin B as described previously (62).

LM-PCR for mapping nucleosomes. LM-PCR was performed as described previously (65, 66) with minor modifications. 4 μg MNase-treated DNA was phosphorylated with polynucleotide kinase and ligated with unidirectional linker. After ligation, an equal volume of PCRL buffer (10 mM Tris-Cl, pH 8.8, 50 mM KCl, 0.25% Tween 20, and 0.25% NP-40) was added. One tenth of the ligated sample was used for PCR (22 cycles with an extension time starting at 5 min, adding 30 s for each additional three cycles). Products were resolved on 2% agarose gels and probed with an internal oligonucleotide. Approximate locations of primers and the internal oligonucleotide probe are shown in Fig. 2.

IP. For ChIP assays, T cells were briefly fixed with formaldehyde, rinsed, and lysed as for nucleosome mapping with MNase. Nuclei were partially digested with MNase, after which an equal volume of ChIP dilution buffer (15 mM Tris, pH 8.0, 150 mM NaCl, and 1% Triton X-100) was added, and samples were sonicated to mean DNA lengths of 100–600 bp. Sheared chromatin was centrifuged to pellet debris, and the supernatants were adjusted to 1% Triton X-100, 0.1% sodium deoxycholate, and 0.1% SDS (IP buffer) with 0.15 M NaCl. Protein A beads were incubated with normal rabbit IgG (30 min at 4°C), after which the bead–antibody complexes were used to preclear the chromatin (1 h at 4°C). IPs were then performed at 4°C overnight with 2 μg of normal rabbit IgG or anti-Brg1 rabbit polyclonal antibodies (Upstate Biochemicals). Immune complexes were collected with DNA-saturated protein A, washed four times with IP buffer with 0.3 M NaCl, twice with IP buffer, and once with TE buffer. Beads were then resuspended in TE buffer, adjusted to 0.5% SDS and 200 $\mu\text{g/ml}$ proteinase K, and incubated at 56°C for 3 h followed by 65°C overnight to reverse the cross-links. DNA was purified by phenol-chloroform extraction, precipitated in the presence of 10 μg glycogen, and resuspended in 50 μl TE buffer. IP products were amplified using primers within the IFN- γ promoter region (sense, 5′-CCACCTATCTGTCACCATCTTA-3′; antisense, 5′-GCCAAGATGCAGTGTAGCGTTCATT-3′). For co-IP, Th1 cells were prepared and restimulated for 6 h (PMA plus ionomycin, as described in Cell preparation, culture, and T cell differentiation), or Jurkat T cells were

activated (PMA + ionomycin; 4 h). Nuclear extracts (0.5 mg for Th1 cells and 1 mg of protein for Jurkat cells) were precleared with 1 μ g of nonimmune rabbit IgG in the presence of protein A beads for 2 h at 4°C. 5 μ g anti-NFAT or 1 μ g anti-Brg1 antibodies (Upstate Biochemicals) were added to precleared supernatants, incubated at 4°C overnight, and immune complexes were recovered as for ChIP assays. Eluted proteins were analyzed by Western blotting.

Figures. Northern, Southern, and Western blot data in figures in all instances represent images acquired after a single analysis of membranes within a single representative experiment. Dividing lines within figure panels for such blot data represent the existence of cut lines for a reordering of lanes to achieve greater simplicity or clarity. Unless indicated in the legends, all lanes are of equal exposure length.

Online supplemental material. Fig. S1 shows transcription factor expression that was unaffected by interference with Swi-SNF complexes. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20060066/DC1>.

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