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*J Immunol* 2012; 189:832-840; Prepublished online 8 June 2012;
doi: 10.4049/jimmunol.1200854
http://www.jimmunol.org/content/189/2/832

**Supplementary Material**

*http://www.jimmunol.org/content/suppl/2012/06/08/jimmunol.1200854.DC1*

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Twist1 Regulates Ifng Expression in Th1 Cells by Interfering with Runx3 Function

Duy Pham,*† Joshua W. Vincentz,* Anthony B. Firulli,* and Mark H. Kaplan*†

A transcription factor network that includes STAT4, T-bet, and Runx3 promotes the differentiation of Th1 cells and inflammatory immune responses. How additional transcription factors regulate the function of Th1 cells has not been defined. In this study we show that the negative regulatory factor Twist1 decreases expression of T-bet, Runx3, and IL-12Rβ2 as it inhibits IFN-γ production. Ectopic expression of Runx3, but not T-bet or IL-12Rβ2, compensates for the effects of Twist1 on IFN-γ production, and Twist1 regulation of Ifng depends on complex formation with Runx3. Twist1 decreases Runx3 and T-bet binding at the Ifng locus, and it decreases chromatin looping within the Ifng locus. These data define an IL-12/STAT4-induced negative regulatory loop that impacts multiple components of the Th1 transcriptional network and provide further insight into regulation of Th1 differentiation. The Journal of Immunology, 2012, 189: 832–840.

Received for publication March 19, 2012. Accepted for publication May 16, 2012.

The online version of this article contains supplemental material.

Materials and Methods

Mice

C57BL/6 mice were purchased from Harlan Sprague Dawley (Indianapolis). Stat4−/− mice were previously described (13). Twist1−/− mice (14) were crossed with CD4-Cre transgene mice to generate Twist1−/−CD4-Cre mice with Cre− littersmates as wild-type (WT) mice. Twist1+/− mice (15) were crossed with Twist1fl/+CD4-Cre+ and Twist1fl+CD4-Cre+ as WT control. Mice were maintained under specific pathogen-free conditions. All experiments were performed with the approval of the Indiana University Institutional Animal Care and Use Committee.

In vitro T cell differentiation

Naïve CD4+CD62L+ T cells were isolated from spleen and lymph nodes using a MACS isolation system (Miltenyi Biotec). CD4+ T cells were activated with plate-bound anti-CD3 (2 μg/ml; 145-2C11) and soluble anti-CD28 (0.5 μg/ml; BD Pharmingen for Th0, Th1, Th2, and Th17 cells or 1 μg/ml for Th9 and regulatory T cells [Tregs]) with additional cytokines (all from PeproTech) and Abs to generate Th1 (5 ng/ml IL-12 and 10 μg/ml anti-IFN-γ, IL-4), Th2 (10 ng/ml IL-4 and 10 μg/ml anti-IFN-γ), Th9 (10 ng/ml IL-4, 2 ng/ml TGF-β, and 10 μg/ml anti-IFN-γ), or Th17 (100 ng/ml IL-6, 10 ng/ml IL-23, 10 ng/ml IL-1β, 2 ng/ml TGF-β, 10 μg/ml anti-IFN-γ, IL-4 11B11, Th2 (10 ng/ml IL-4 and 10 μg/ml anti-IFN-γ XMG), Th9 (20 ng/ml IL-4, 2 ng/ml TGF-β, and 10 μg/ml anti-IFN-γ XMG), or Th17 (100 ng/ml IL-6, 10 ng/ml IL-23, 10 ng/ml IL-1β, 2 ng/ml TGF-β, 10 μg/ml anti-IFN-γ, IL-4 11B11, and 10 μg/ml anti-IFN-γ XMG) or Treg (2 ng/ml TGF-β, and 10 μg/ml anti-IL-4, 11B11) culture conditions. Cells were expanded after 3 d without additional cytokines (Th0, Th1, and Th2), with 50 U/ml human IL-2 (Tregs), or with full concentration (Th9) or half concentration of IL-6 (Th17) of the original cytokines in fresh medium. Cells were harvested on day 5 for analysis.
Retroviral expression vectors and retroviral transduction

Bicistronic retrovirus expressing enhanced GFP (EGFP) only (MIEG) and T-bet and EGFP (T-bet) were previously described (16, 17). pBMN-ires-EGFP-IL-12b2a was a gift from Dr. Takashi Usui (Kyoto University). Il12rb2c and Twist1 cDNAs (Open Biosystems) were digested and subcloned into pMIG-eCD4. Flag-tagged T-bet and Flag-tagged Twist1 cDNA pCDNA3.1 that was made using a QuickChange site-directed mutagenesis kit (Stratagene) with primer pair forward (5'-TCAGCTACGCCCTCCCGCTGAGGATG-3') and reverse (5'-CATCCTCCAGGCGGAGCCGGTCAGTCA-3') were digested and subcloned into MIEG-EGFP-Rumx3 cDNA (Open Biosystems) was amplified, digested, and subcloned into MSCV- Thy1.1. Twist1-targeting short hairpin RNA (shRNA) oligonucleotide was designed as described (12) and introduced into RNAi-Ready pSIREN-RetroQ-ZsGreen (Clontech) according to the manufacturer’s directions. Retroviral stocks were prepared by calcium phosphate transfection of Phoenix GP cells. The medium was replaced after 12 h, and viral supernatants were collected 24 and 48 h later. Purified CD4+ T cells were cultured under Th1 cell differentiation conditions. On day 2, cells were transduced with retrovirus-expressing vector control or gene of interest by centrifugation at 2000 rpm at 25˚C for 1 h in the presence of 8 μg/ml polybrene. Viral supernatant was replaced with the former culture supernatant supplemented with 50 U/ml IL-2. After spin infection, cells were expanded on day 3, 7 and analyzed on day 5.

Cell sorting, analysis of gene expression, and flow cytometry

Transduced cells were collected on day 5, stained with anti-human CD4 Alexa Fluor 647 (BD Biosciences) and anti-rat CD90/mouse CD90.1 FITC (BD Biosciences). Sorted cells were rest or re-stimulated with 2 μg/ml anti-CD3 for real-time quantitative PCR (RT-qPCR; 6 h) and ELISA (24 h). Quantitative RT-PCR and ELISA were performed as described previously (17). For surface staining, resting T cells were stained with anti-IL-2, anti–IL-4, anti–IFN-γ, and anti–T-bet Alexa Fluor 647 (BioLegend) and anti-rat CD90/mouse CD90.1 FITC (BD Biosciences) and analyzed on a FACSVantage SE cell sorter (BD Biosciences).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was performed as described (1). In brief, resting Th1 cells were crosslinked for 10 min with 1% formaldehyde and lysed by sonication. After pre-clearing with salmon sperm DNA, BSA, and protein A–agarose bead slurry (50%), cell extracts were incubated with either rabbit polyclonal T-bet (4B10), PEP2b2 (FL-182) (Santa Cruz Biotechnology), or normal rabbit IgG (12-370; Millipore) overnight at 4˚C. The immunocomplexes were precipitated with protein A–agarose beads at 4˚C for 2 h, washed, eluted, and reverse crosslinked at 65˚C overnight. DNA was purified, resuspended in H2O, and analyzed by qPCR with TaqMan or SYBR primers as previously described (19, 21, 22).

Chromosome conformation capture assay

A chromosome conformation capture (3C) assay was performed as described (23–25) with some modifications. Cells (106) were crosslinked with 2% formaldehyde for 10 min at room temperature and quenched with 0.125 M glycine for 5 min. Cells were lysed with ice-cold lysis buffer (10 mM Tris-HCl [pH 8], 10 mM NaCl, 0.2% Nonidet P-40, protease inhibitor mixture) for 30 min. Nuclei were resuspended in 0.5 ml restriction enzyme buffer (NEB) containing 0.3% SDS and shaken for 1 h at 37˚C. Triton X-100 (final concentration, 1%) was added and shaken for 1 h at 37˚C to sequester the SDS. Crosslinked DNA was digested overnight with 400–800 U BglII containing 1 mM ATP that has been shown to enhance digestion efficiency (26). Enzyme inactivation was achieved by addition of SDS (final concentration, 1%), and samples were shaken for 20 min at 65˚C. The reaction was diluted with 7 ml ligation buffer (50 mM Tris-HCl [pH 8], 10 mM MgCl2, 1 mM DTT, 1 mM ATP, 1 mg/ml BSA), and Triton X-100 (final concentration, 1%) was added and shaken for 1 h at 37˚C. DNA fragments were ligated with 4000 U T4 ligase (NEB) for 4 h at 16˚C followed by 40 min at room temperature. Crosslinks were reversed by heating at 37˚C for 30 μg proteinase K overnight at 65˚C. The samples were further incubated with 300 μg RNase for 30–45 min at 37˚C, and the DNAs were purified by phenol-chloroform extraction and ethanol precipitation. Ligation products were quantified by quantitative TaqMan real-time PCR using primers as described (23, 25). Additional primers and TaqMan probes (with the location in kb from the Ifng transcription start site in parenthesis) are as follows: (+35) 5' TGGAGGAACAGAGAGTGGGGGTTG A 3', (+46) 5' AAAAACACCTGGTTAATTTGTCGAAAAGGTGCTGAATC-3', (-35) 5' CCACGCTGCGAACCAAGAAGCTGGCAGAAGC-3', and (+51) 6FAM-CACATCTCGCCGCTCGCTGAGCTGAC-3' for control templates for the positive controls and to correct for differences in ligation and PCR efficiency between different templates, BAC clones were used to generate control template containing all possible ligation products. Equimolar copies of three BAC clones spanning the mouse Ifng locus (RP23-355P23, RP23-138P22, and RP23-55021) and BAC spanning the mouse Gapdh locus (RP23-410F11) from CHORI were mixed, digested, phenol-chloroform extracted, ethanol precipitated, and ligated at a DNA concentration of 300 ng/μl. This sample was used as the DNA reference standard. Relative crosslinking frequencies between the amplified and paired cuts were calculated as described (23) and were normalized to control interaction frequencies using primer pairs within the Gapdh locus.

Results

Twist1 is induced by Th1 cell activation

Twist1 regulates Th1 cytokine production through an unidentified mechanism (12). To begin to define that mechanism, we determined the expression of Twist1 in T cell subsets. Twist1 mRNA expression was highest in resting and activated Th1 cells, compared with other T cell subsets (Fig. 1A). Previous reports identified Twist1 as a STAT4 target gene (1, 12). Although Twist1 mRNA expression was detected during differentiation, expression was lower in Stat4-/- than in WT Th1 cells (Fig. 1B). Stimulation of Th1 cells with anti-CD3 resulted in the induction of Twist1 mRNA and protein (Fig. 1C, 1D). Thus, Twist1 is expressed in the greatest amounts in Th1 cells compared with other T cell subsets and is induced by TCR stimulation.

Twist1 negatively regulates the Th1 transcription factor network

To define how Twist1 regulates Th1 cell function, Twist1 was ectopically expressed or targeted by shRNA in Th1 cells using retroviral transduction (Fig. 1E, 1F). In agreement with a previous report (12), ectopic Twist1 expression in Th1 cells reduced IFN-γ and TNF-α mRNA and protein levels, and decreasing Twist1 ex-
pression resulted in increased IFN-γ and TNF-α production (Fig. 1G–J). The difference in IFN-γ and TNF-α production in Th1 cells was not due to altered expression of other cytokines, including Il4 and Il17a (data not shown). Coincident with decreased cytokine production, the expression of Th1-related transcription factors such as T-bet, Hlx1, and Runx3 were decreased upon ectopic Twist1 expression and increased upon Twist1 shRNA transduction (Fig. 1G, 1H and data not shown).

Because STAT4 is required for the expression of many Th1-specific genes (1), we examined whether Twist1 was a component in a feedback mechanism to control STAT4 activation. Phospho-STAT4, assessed by flow cytometry, was lower in Th1 cells transduced with Twist1-expressing retrovirus compared with vector control, following restimulation with increasing doses of IL-12 (Fig. 2A). Decreased induction of pSTAT4 was correlated with reduction in IL-18rα expression, a STAT4-target gene (data not shown) (19, 27). Consistent with this result, reduced Twist1 expression results in increased IL-12–induced STAT4 phosphorylation (Fig. 2B). We next examined the expression of genes that contribute to STAT4 activation. STAT4 mRNA and protein expression, as well as mRNA of suppressors of cytokine signaling and protein tyrosine phosphatase-basophil like that negatively regulate STAT4 activation (28, 29), were not altered. However, Il12rb2 mRNA expression was decreased ∼50% by ectopic Twist1...
Ectopic Runx3 expression compensates for the repressive activity of Twist1 in Th1 cells. Naive WT T cells were stimulated under Th1-polarizing conditions. On day 2, cells were transduced with control retrovirus vectors or (A–C) retroviral vector-expressing Twist1-GFP and IL-12Rβ2-hCD4. (D, E) Twist1-hCD4 and T-bet-GFP, or (F, G) Twist1-GFP and Runx3-Thyl.1. Th1 cells were stained for IL-12Rβ2 by surface staining (A) or phospho-STAT4 with or without IL-12 stimulation by intracellular cytokine staining (B), or T-bet by intracellular cytokine staining (D). Analysis was performed by gating on doubly transduced cells. (E and G) Double-positive cells were sorted and restimulated with 2 μg/ml anti-CD3 for 24 h. Supernatants were collected before IFN-γ and TNF-α production was measured by ELISA. (F) Whole-cell lysates were extracted from sorted double-positive Th1 cells and were immunoblotted for Runx3 and β-actin as a control. Data are means of three to four independent experiments ± SD (A, B) or averages of replicate samples ± SD and representative of two to three independent experiments with similar results (C–G). *p < 0.05.
tion) did not compensate for the effects of Twist1. These results suggested that Runx3, or a Runx3-induced gene, is at least one of the important Twist1 targets in the regulation of IFN-γ.

Twist1 exists in a complex with Runx3 or T-bet

Twist1 inhibits osteoblast differentiation by interacting with the Runt domain of Runx2, a highly conserved domain shared with related proteins, including Runx3 (15). We hypothesized that Twist1 physically interacts with Runx3 and inhibits its regulatory function. Using immunoprecipitation of extracts from resting and early activated Th1 cells, we observed that Twist1 copurified with Runx3, and we confirmed the association between Runx3 and T-bet in Th1 cells (3). We also found an association between T-bet and Twist1 following precipitation with T-bet Ab (Fig. 4A).

To examine the function of Runx3 and T-bet in the absence of Twist1, Twist1<sup>fl/fl</sup> mice (14) were mated with mice carrying a CD4-Cre transgene to generate mice with Twist1-deficient T cells (Twist1<sup>fl/fl</sup> CD4-Cre<sup>+</sup>). We confirmed the absence of Twist1 expression in Twist1-deficient Th1 cells (Supplemental Fig. 1). Twist1 deficiency did not alter normal lymphocyte development in thymus, spleen, and lymph nodes (Supplemental Fig. 1). Twist1-deficient Th1 cells produced significantly greater amounts of IFN-γ per cell and in frequency of IFN-γ<sup>+</sup> cells compared with WT cells (Fig. 4B–E). We also confirmed the increase in expression of Tbx21, Runx3, and Il12rb2 in Twist1-deficient Th1 cells, compared with WT Th1 cells. There was no significant difference in Eomes expression between WT and Twist1-deficient Th1 cells (data not shown).

We next tested the effect of Twist1 on Runx3 and T-bet DNA binding by DNA affinity precipitation assay using extracts from Twist1-deficient mice and littermate control (WT)-activated Th1 cells. Extracts from WT and Twist1-deficient Th1 cells were incubated with biotinylated oligonucleotides containing a Twist1-binding sequence. Although T-bet and Runx3 were precipitated

**FIGURE 4.** Twist1 physically interacts with Runx3 and T-bet. (A) Whole-cell lysates were extracted from activated WT Th1 cells. Immunoblots were performed for the indicated proteins following immunoprecipitation with Runx3 or T-bet or control Abs as indicated. Input for immunoprecipitation is indicated on the right. (B–E) Naive CD4<sup>+</sup>CD62L<sup>hi</sup> WT or Twist1<sup>fl/fl</sup> CD4-Cre<sup>+</sup> T cells were cultured under Th1-polarizing conditions. IFN-γ production was measured by ELISA (B) after reactivating with 2 μg/ml anti-CD3 for 24 h or intracellular cytokine staining (C–E) after reactivating with PMA/ionomycin for 6 h. (D and E) Graphs indicate the mean fluorescence intensity (D) or average percentage IFN-γ<sup>+</sup> cells (E) of Th1 cells from mice of the indicated genotype. (F–H) Nuclear extracts from activated (F) or resting (G) WT and Twist1<sup>fl/fl</sup> CD4-Cre<sup>+</sup> Th1 cells were incubated with biotinylated oligonucleotides containing Twist1-specific binding site (F) or Runx3/T-bet–specific binding sites (WT or mutant as indicated) (G). (H) Immunoblots of precipitated proteins, with densitometry measurements for (G). (I) Mixture of whole-cell lysates collected from 293T cells transfected with constructs expressing Runx3, T-bet, or Twist1 were incubated at 4˚C overnight. Immunoblots indicate proteins immunoprecipitated with Runx3. Results are average ± SEM (B and C) or ±SD (D, E, H) of three independent experiments (*p < 0.05, ***p < 0.001) or representative of three or more independent experiments with similar results (A, C, F, I).
with DNA-bound Twist1 in WT cell extracts, T-bet and Runx3 were absent from precipitates of Twist1-deficient extracts, further supporting the interaction of these transcription factors and suggesting that interactions can occur when Twist1 is bound to DNA (Fig. 4F). We observed that Runx3 and Tbx21 mRNA expression increased in Twist1-deficient Th1 cells compared with WT cells, although there was a modest increase at the protein level, suggesting the importance of protein complex formation in the function of Twist1. Because there is an association between Twist1, Runx3, and T-bet, we tested whether, in the absence of Twist1, there would be greater binding of Runx3 and T-bet to the oligonucleotides containing Runx3- and T-bet–specific binding sites. In the absence of Twist1, we detected greater binding of Runx3 and T-bet to the respective oligonucleotides compared with WT samples (Fig. 4G, 4H, lane 2 versus 1). When the binding sites of Runx3, T-bet, or both were mutated, the binding of Runx3 and T-bet was diminished, regardless of Twist1 expression (Fig. 4G). We then tested whether Twist1 mediates its inhibitory effect in Th1 cells by interfering with the T-bet/Runx3 interaction. Using Runx3 immunoprecipitation, we detected a Runx3 and T-bet interaction in the absence of Twist1 (first lane), although this interaction was diminished in the presence of Twist1 (Fig. 4I). These results suggested that the decrease in IFN-γ production in Th1 cells might not only be due to the reduction in Runx3 gene expression but also to diminished association and DNA binding activity of Runx3 and T-bet.

Twist1 interferes with the binding of Runx3 and T-bet to the Ifng locus

Because Twist1 interferes with T-bet and Runx3 DNA binding, and we observed only modest binding of Twist1 to the Tbx21, Ifng, or other Th1 gene promoters (data not shown), we hypothesized that the interaction of Twist1 with Th1 transcription factors decreased their binding to target genes, including Ifng. T-bet and Runx3 regulate Ifng gene expression by binding to several conserved noncoding sequences (CNS) (4). To examine T-bet and Runx3 binding in the absence of Twist1, we performed a ChIP assay using WT and Twist1-deficient Th1 cells and examined the binding at previously documented regions, including CNS−34, CNS−6, Ifng promoter, and CNS+46 of the Ifng gene (Fig. 5A) (4, 22), using ChIP for T-bet or CBF-β, the binding partner of Runx3 (4). Our data showed that more T-bet was bound in Twist1-deficient Th1 cells than in WT cells at the Ifng promoter and CNS+46 whereas there were no differences at CNS−34 and CNS−6 (Fig. 5B). In contrast, more CBF-β was bound at CNS−34 and CNS−6 in Twist1-deficient Th1 cells compared with littermate controls (Fig. 5B). This result suggested that Twist1 interferes with T-bet and CBF-β/Runx3

**FIGURE 5.** Twist1 interferes with transcription factor binding and chromatin conformation at the Ifng locus in Th1 cells. (A) Representation of Ifng locus indicating CNS used for analysis. (B–G) Naive CD4+CD62hi T cells from WT and Twist1fl/fl CD4-Cre+ were stimulated under Th1-polarizing conditions. (B) ChIP assays were performed on Th1 cells using T-bet and CBF-β Abs. (C) Th1 cells were fixed and digested with BglII enzyme. Undigested and digested samples were subjected for qPCR using primer pairs spanning the restriction sites. Percentage digestion was calculated using the formula: 100 – 100/2[(CtR−CtC)D−(CtR−CtC)UND]. (D) Th1 cells as described above were fixed (crosslinked) and/or ligated as indicated. qPCR was performed using primer pair pair 13F-9R. PCR products were run on 2% agarose gel. BAC clones were titrated for qPCR and used as control. (E–G) 3C assay showing the relative crosslinking frequencies between the Ifng promoter (E), CNS−34 (F), or CNS+46 (G) as the fixed anchor fragments and other BglII fragments containing the indicated CNS regions. Results are the average ± SD of replicated samples and are representative of four independent experiments with similar results. *p < 0.05. C, Internal control; D, digested; R, restriction site; UND, undigested.
complex binding at specific regulatory regions of the \textit{Ifng} gene. Because T-bet and Runx3 bind to many CNS regions of the \textit{Ifng} locus, and the association between T-bet and Runx3 is required for optimal \textit{Ifng} expression (3, 4), it is likely that Runx3 binding to the \textit{Ifng} locus enhances T-bet binding to regulatory elements. This is consistent with the recovery of IFN-\(\gamma\) production in the Runx3 transduction experiment (Fig. 3G).

The effect of Twist1 on T-bet and CBF-\(\beta\)/Runx3 binding at distinct elements suggested that interfering with T-bet/Runx3 interactions might also alter chromatin looping at the \textit{Ifng} locus (24, 25). To determine whether Twist1 interferes with chromatin looping at the \textit{Ifng} locus, a 3C assay was performed with WT and Twist1-deficient Th1 cells that examined the interactions among \textit{Ifng} CNS regions using an established assay (23, 25) (Fig. 5C, 5D). Using three different anchor points, our results showed increased crosslinking frequency between CNS\(\_34\), CNS\(\_46\), and the \textit{Ifng} promoter, and increased crosslinking of CNS\(\_6\) with CNS\(\_34\) and CNS\(\_46\) (Fig. 5E–G). The distance between the \textit{Ifng} promoter and CNS\(\_6\) is too short to provide consistent results in this assay. Crosslinking of the CNS region at \(\_71\) was not altered. Taken together, these results suggest that Twist1 regulates \textit{Ifng} expression by altering the binding of the Th1 transcription factors T-bet and Runx3, and altering the conformation of the \textit{Ifng} locus.

Twist1/Runx3 interactions are required to regulate \textit{Ifng}

A mutation in Twist1 at aa 192 from serine to proline (Twist1cc) results in diminished interaction between Twist1 and Runx2 (15). Thus we wanted to examine whether Twist1 S192P (Twist1cc) could interact with Runx3. A DNA affinity precipitation assay using extracts from cells transfected with Runx3, WT Twist1 (Twist1), and Twist1cc expressing vectors that were incubated with biotinylated oligonucleotides containing a Twist1-binding sequence demonstrated decreased interaction between Runx3 and Twist1cc (Fig. 6A, 6B). The mutation in Twist1cc did not affect the association of Twist1 with T-bet (data not shown).

**FIGURE 6.** The Twist1/Runx3 interaction is required for regulation of \textit{Ifng}. (A and B) Nuclear extracts from 293T cells cotransfected with either Flag-tagged Twist1 or Flag-tagged Twist1cc with or without Runx3 were incubated with biotinylated oligonucleotides containing Twist1-specific sequence. Immunoblot demonstrates protein expression of Twist1 and Runx3 (A) with densitometry measurements (B). (C–H) Naive CD4\(^+\)CD62\(hi\) T cells from WT were cultured under Th1-polarizing conditions and were infected with retrovirus expressing Twist1 or Twist1cc. (C) Twist1 protein was assessed in transduced Th1 cells using immunoblot, with \(\beta\)-actin as a control. (D–G) IFN-\(\gamma\) production was assessed by intracellular staining and gating on GFP\(^+\) populations following 6 h activation with anti-CD3 (D–F) or by ELISA following 24 h stimulation with anti-CD3 of sorted GFP\(^+\) populations (G). (E and F) Graphs indicate the mean fluorescence intensity (E) or average percentage IFN-\(\gamma\) cells (F) of each transduced population. (H) Th1 gene expression in ectopic Twist1 or Twist1cc expression was assessed by RT-qPCR before (\textit{Il12rb2}, \textit{Runx3}, and \textit{Tbx21}) or after (\textit{Ifng}) 6 h restimulation with anti-CD3. (I–K) Naive CD4\(^+\)CD62\(hi\) T cells from mice of the indicated genotypes were cultured under Th1-polarizing conditions. Th1 cells were stimulated with anti-CD3 for 6 or 24 h, respectively, for testing \textit{Ifng} expression by RT-qPCR (I) or IFN-\(\gamma\) production by ELISA with the ratio of production from Twist1-deficient or Twist1 mutant Th1 cells to the respective controls (J, K). Data are means of three to four independent experiments \pm SD (A–F) or are mean of replicate samples \pm SD and are representative of two independent experiments with similar results (G–K). *\(p < 0.05\).
The mutant Twist1 provided a tool to mechanistically distinguish the effects of Twist1 that rely on interactions with Runx3 at the protein level, versus effects independent of Runx3 binding. To test the effects of mutant Twist1, a retrovirus expressing Twist1cc was introduced into Th1 cells, and cytokine production and gene expression were analyzed. Introduction of Twist1cc did not repress IFN-γ production in Th1 cells compared with vector control and WT Twist1-transduced cells (Fig. 6D–H). Importantly, Twist1cc was able to repress expression of several Th1 genes, including Il12rb2, Runx3, and Tbx21, as effectively as WT Twist1 (Fig. 6H). To further define the function of Twist1cc in Th1 cells, we used a mouse mutant strain termed Charlie Chaplin (Twist1<sup>cc/wt</sup>) that encodes Twist1 S192P and results in hindlimb polydactyly (15, 30). We mated Twist1<sup>cc/wt</sup> with Twist1<sup>fl/fl</sup> CD4-Cre<sup>+</sup> mice generating Twist1<sup>fl/wt</sup> CD4-Cre<sup>+</sup> and Twist1<sup>cc/nc</sup> CD4-Cre<sup>+</sup> (Twist1<sup>cc/ncc</sup>) mice that have T cells expressing one WT or one mutated allele of Twist1, respectively. IFN-γ production in Twist1<sup>cc/ncc</sup> Th1 cells was increased compared with control cells (Fig. 6I–K). These results demonstrate that although Twist1cc retains some repressive function in Th1 cells independent of Runx3, Twist1 control of IFN-γ production is primarily through association with Runx3 (Fig. 7).

**Discussion**

*Twist1* is expressed in Th1 effector memory cells and limits Th1-mediated inflammation in mouse models of delayed-type hyper-sensitivity and Ag-induced arthritis (12). In this study, we have identified a mechanism by which Twist1 modulates inflammatory cytokine production in Th1 cells. We showed that Twist1 negatively regulates transcription factors, including T-bet, STAT4, and Runx3, leading to decreased IFN-γ production in Th1 cells. The formation of the Twist1/Runx3 complex is required for Twist1 to decrease IFN-γ production. In the absence of Twist1, there is increased transcription factor binding and increased looping at the *Ifng* locus, resulting in increased IFN-γ production. Thus, Twist1 impairs the activity of the Th1 transcription factor network.

Our data suggest that Twist1 regulates Th1 gene expression through several mechanisms. First, it negatively regulates the expression of Th1 genes. In macrophages, Twist1 has been shown to regulate TNF-α and IL-1β by binding to E-boxes in gene promoters (15). However, we did not observe Twist1 binding to the promoters of *Ifng*, *Tnfa*, or *Il12rb2*, and we found only minimal binding to the promoter of *Tbx21*. It is still possible that Twist1 binds directly to Th1 genes in regions other than the promoter that we tested. The second mechanism of Twist1-dependent gene regulation is through complex formation with Runx3. This mechanism appears to be the primary mechanism for regulating *Ifng*. However, the Twist1 S192P mutant that reduced interactions with Runx3 was still able to transcriptionally regulate other Th1 genes, suggesting that Twist1 has gene regulatory activity independent of Runx3. The direct regulation of genes other than *Ifng* might further impact the Th1 phenotype, and further analysis will be required to further refine this regulatory network.

In this study, we showed that Twist1 is expressed highest in resting Th1 cells compared with other T cell subsets, although its expression increased in all T cell subsets upon TCR stimulation. During differentiation, Twist1 expression peaked at day 2 in both Th1 and Th2 cells and gradually decreased, although Twist1 expression was lower in Th2 cells than Th1 cells after day 3. Thus, Twist1 might be a negative regulator of Th1 cytokine production in other T cell subsets.

Although Twist1 is a STAT4 target in Th1 cells, it might be targeted by other STATs in other Th lineages. In breast cancer cell lines, STAT3 binds directly to the Twist1 promoter, and amounts of phospho-STAT3 correlate with expression of Twist1 (31). Because STAT3 has a critical role in Th2 and Th17 differentiation, and STAT3-deficient Th2 cultures show increased IFN-γ production compared with WT cultures, Twist1 might play a role in regulating cytokine production in these lineages as well (32–34). Twist2 promotes IL-10 production by activating the transcription factor c-Maf in myeloid cells, and because c-Maf is an important transcription factor for Th2 and Th17 cells, Twist1 might play an analogous role in these cells (35, 36). The function of Twist1 in other Th subsets will be the focus of further study.

Twist1 requires dimerization to bind DNA (37). In *Drosophila*, dimerization partners determine the activity of Twist1 (38). Twist1 homodimers have opposing effects on mesoderm gene expression as heterodimers of Twist1 and the *Drosophila* E protein homolog, Daughterless (38). The *Daughterless* gene has 79% identity to E12 and E47, two alternative splice products of the *E2a* gene (39, 40). Th1 cells differentiated from E2a-deficient mice showed a decrease in IFN-γ production compared with WT cells (41). It is possible that E12/E47 sequesters Twist1 in heterodimer complexes to inhibit its repressive function in Th1 cells. In the absence of E2a gene products, there is more “free” Twist1 to form homodimers and thus inhibit cytokine production. E47 and the HLH inhibitor Id3 regulate the balance between Treg and Th17 cell differentiation (42). The balance among Twist1, Id, and E proteins is likely important in defining Twist1 activity, as Id proteins interacting and titrating E proteins would favor the formation of

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**FIGURE 7.** Transcriptional regulatory network in Th1 cells. IL-12/IL-12Rβ2/STAT4 induces T-bet expression that positively regulates IL-12Rβ2 expression. Both STAT4 and T-bet are required for the induction of Runx3, which contributes to the optimal IFN-γ production. Twist1, a transcription repressor that is induced by IL-12/STAT4, suppresses IFN-γ production by two mechanisms: the repression of *Il12rb2*, *Tbx21*, and *Runx3* expression potentially by directly binding to the conserved motif E-box (CANNTG) in gene regulatory regions, and the interference with the function of Runx3 through physical interaction. The latter mechanism is critical for regulation of *Ifng* since complementation of *Il12rb2* or *Tbx21* expression did not rescue IFN-γ production.
Twist1 homodimers (43, 44). Understanding how this balance is regulated in Th cells will lead to greater insight into the networks of transcription factors that regulate differentiation.

Acknowledgments
We thank A.L. Dent and L.D. Mayo for critical reading of the manuscript. We thank R. Berrington for providing Twist1Hmo mice and T. Usui for providing plasmids.

Disclosures
The authors have no financial conflicts of interest.

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