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## Twist1 regulates *Ifng* expression in T helper 1 cells by interfering with Runx3 function

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### Abstract

A transcription factor network that includes STAT4, T-bet and Runx3 promotes the differentiation of T helper type 1 (Th1) cells and inflammatory immune responses. How additional transcription factors regulate the function of Th1 cells has not been defined. In this report we show that the negative regulatory factor Twist1 decreases expression of T-bet, Runx3 and IL-12R $\beta$ 2 as it inhibits IFN- $\gamma$  production. Ectopic expression of Runx3, but not T-bet or IL-12R $\beta$ 2 compensates for the effects of Twist1 on IFN- $\gamma$  production, and Twist1 regulation of *Ifng* depends upon complex formation with Runx3. Twist1 decreases Runx3 and T-bet binding at the *Ifng* locus, and 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 provides further insight into regulation of Th1 differentiation.

### Keywords

T helper 1 cells; transcription factors; differentiation; interferon-gamma; Twist1; Runx3; STAT4

### Introduction

A network of transcription factors regulates the development of Type 1 helper T cells (Th1), cells that play a crucial role in anti-bacterial and inflammatory immunity. IL-12 and IFN- $\gamma$  promote Th1 differentiation by the respective activation of STAT4 and STAT1 leading to the induction of additional transcription factors including T-bet and Runx3. Th1 differentiation requires both T-bet and STAT4 for complete development of the Th1 genetic program (1) and is mediated by the interlinked and sequential effects of TCR-IFN- $\gamma$ -STAT1-T-bet and IL-12-STAT4-T-bet signaling pathways (2). Runx3 cooperates with T-bet to activate *Ifng* and silence *Il4* in Th1 cells, but can regulate *Ifng* expression independently of T-bet and STAT4 (3, 4). Although some factors that impact this network, including GATA3 (5–7), have been described, regulation of these pathways is still not clearly defined.

Twist1 is a transcriptional repressor and a member of the basic helix-loop-helix (bHLH) family of proteins that plays a positive role in dorso-ventral patterning in *Drosophila* embryos (8). Although the role of Twist1 in developmental and cancer biology has been explored (9), the function of Twist1 in the immune response is only beginning to be understood. Twist1 regulates cytokine production in macrophages through a negative feedback loop by targeting NF- $\kappa$ B activation resulting in decreased TNF- $\alpha$  and IL-1 $\beta$  (10,

11). The repressive mechanism may involve binding of Twist1 to E-boxes in cytokine promoters to inhibit the transcriptional activity of NF- $\kappa$ B (10, 11). In Th1 cells, NF- $\kappa$ B, NFAT and IL-12-STAT4 signaling can induce *Twist1* expression, and Twist1 limits inflammation by suppressing IFN- $\gamma$  and TNF- $\alpha$  production (12). However, a detailed mechanism of how Twist1 regulates cytokine production in Th1 cells has not been described.

In this report, we define the role of *Twist1* in the Th1 cell transcriptional network. Using retroviral transduction studies, we demonstrate that Twist1 negatively regulates Th1 gene expression by decreasing expression and function of transcription factors including T-bet, STAT4, and Runx3. Twist1 functions through several mechanisms including physical interaction with Runx3 and interfering with the association of T-bet and Runx3 at the *Ifng* locus. The results suggest that Twist1 acts as a modulator that regulates multiple transcription factors in Th1 cells to limit potentially harmful inflammatory responses.

## Materials and Methods

### Mice

C57BL/6 mice were purchased from Harlan Sprague Dawley (Indianapolis, IN, USA). *Stat4*<sup>-/-</sup> mice were previously described (13). *Twist1*<sup>fl/fl</sup> mice (14) were crossed with CD4-Cre transgene mice to generate *Twist1*<sup>fl/fl</sup> CD4-Cre<sup>+</sup> mice with Cre-negative littermates as WT mice. *Twist1*<sup>cc/wt</sup> mice (15) were crossed with *Twist1*<sup>fl/fl</sup> CD4-Cre<sup>+</sup> mice to generate *Twist1*<sup>fl/cc</sup> CD4-Cre<sup>+</sup> and *Twist1*<sup>fl/wt</sup> CD4-Cre<sup>+</sup> 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<sup>+</sup>CD62L<sup>+</sup> T cells were isolated from spleen and lymph nodes using a MACS isolation system (Miltenyi Biotec). CD4<sup>+</sup> T cells were activated with plate-bound anti-CD3 (2 ug/ml 145-2C11) and soluble anti-CD28 (0.5 ug/ml BD Pharmingen for Th0, Th1, Th2, and Th17 or 1 ug/ml for Th9 and Treg) with additional cytokines (all from PeproTech) and antibodies to generate Th1 (5 ng/ml IL-12; and 10 ug/ml anti-IL-4 11B11), Th2 (10ng/ml IL-4; and 10ug/ml anti-IFN- $\gamma$  XMG), Th9 (20 ng/ml IL-4; 2 ng/ml TGF- $\beta$ ; and 10 ug/ml anti-IFN- $\gamma$  XMG) or Th17 (100 ng/ml IL-6; 10 ng/ml IL-23; 10 ng/ml IL-1 $\beta$ ; 2 ng/ml TGF- $\beta$ ; 10 ug/ml anti-IL-4, 11B11; and 10 ug/ml anti-IFN- $\gamma$ , XMG) or Treg (2ng/ml TGF- $\beta$ , and 10ug/ml anti-IL-4, 11B11) culture conditions. Cells were expanded after 3 days without additional cytokines (Th0, Th1 and Th2), with 50 U/ml human-IL-2 (Treg), 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 EGFP only (MIEG) and T-bet and EGFP (T-bet) were previously described (16, 17). PBMN-IRES-GFP-IL-12Rb2c was a kind gift from Dr. Takashi Usui (Kyoto University). *Iil2rb2c* and *Twist1* (Open Biosystems) cDNAs were digested and sub-cloned into MIEG3-EGFP or MIEG3-hCD4. Flag-tagged Twist1 (18) and Flag-tagged Twist1cc pCDNA3.1 that was made using QuikChange Site-Directed Mutagenesis Kit (Stratagene) with primer pair Forward 5' - TCAGCTACGCCTTCCCCGTCTGGAGGATG-3' and Reverse 5' - CATCTCCAGACGGGGAAGGCGTAGCTGA-3', were digested, and subcloned into MIEG3-EGFP. *Runx3* cDNA (Open Biosystems) was amplified, digested and sub-cloned into MSCV-Thy1.1. *Twist1*-targeting shRNA oligo was designed as described (12) and introduced into RNAi-Ready pSIREN-RetroQ-ZsGreen according to the manufacture

manual (Clonetechn). Retroviral stocks were prepared by calcium phosphate transfection of Phoenix GP cells. The medium was replaced after 12 h, and viral supernatants were collected after 24 h and 48 h later. Purified CD4<sup>+</sup> T cells were cultured under Th1 cell differentiation condition. 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 human IL-2. After spin infection, cells were expanded on day 3 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) and anti-rat CD90/mouse CD90.1 FITC Abs (Biolegend), and sorted using a FACSVantageSE cell sorter (BD Biosciences). Sorted cells were rested or re-stimulated with 2ug/ml anti-CD3 for RT-qPCR (6 h) and ELISA (24 h). Quantitative RT-PCR and ELISA were performed as previously described (17). For surface staining, resting T cells were stained with anti-IL12Rβ2-PE (R&D) or anti-IL-18Rα-Alexa Fluor 647 (Biolegend) and fixed with 2% paraformaldehyde for 10min before analysis. For phospho-STAT4 and T-bet analyses, cells were fixed, permeabilized using 100% ice cold methanol, and stained for anti-phospho-Stat4-PE (BD Pharmingen) and anti-T-bet Alexa Fluor 647 (Biolegend) before analysis.

### Immunoblot, Immunoprecipitation and DNA affinity precipitation assay

Whole-cell protein lysates were extracted from sorted T cells and immunoblotted with Runx3 (6821C3a), Stat4 (C-40), Twist1 (Twist2C1a), T-bet (4B10) or β-Actin (C4) (Santa Cruz) as a control. For immunoprecipitation, whole-cell protein lysates were generated from Th1 cells, or 293T cells transfected with constructs expressing Twist1, Runx3, T-bet, Flag-tagged Twist1, or Flag-tagged- Twist1cc using calcium phosphate. Whole-cell protein lysates were pre-cleared with Protein A agarose (Thermo Scientific), before immunoprecipitation of equal amounts of protein with Runx3, T-bet (Santa Cruz) or Flag (M2, Sigma Aldrich) Abs overnight at 4°C. Immunocomplexes were captured by Protein A agarose and eluted at 100°C for 5 min in Laemmli's sample buffer. Immunoprecipitates were separated by 10% SDS-PAGE and immunoblotted with Twist1, Runx3 and T-bet Abs. DAPA assay was performed as described (19). Oligonucleotides containing a Twist1 binding site were described previously (20). Additional oligonucleotides (biotinylated at 5') containing wild type or mutated (bold) Runx3 and T-bet binding sites (underline) are: wild type Runx3 and T-bet 5'-

ACCTATGTGGTCTGCCTTTTCTTCTTTCTGGGCACGTTGA-3', mutant Runx3 5'-ACCTA**AGAGG**ACTGCCTTTTCTTCTTTCTGGGCACGTTGA-3', mutant T-bet 5'-ACCTATGTGGTCTGCCTTTTCTTCTTTCTGGG**CTCG**TTGA-3', and mutant Runx3-T-bet 5'-ACCTA**AGAGG**ACTGCCTTTTCTTCTTTCTGGG**CTCG**TTGA-3'. Biotinylated oligonucleotides were incubated with streptavidin-agarose beads for 30min at 4°C. The complex was washed with pull-down buffer (25mM HEPES, 15mM NaCl, 0.5mM DTT, 0.5% NP-40, 0.1mM EDTA pH 7.5, 10% glycerol). Nuclear extracts were added and incubated at 4°C for 2 h. The complex was washed with pull-down buffer, eluted, and separated with 10% SDS-PAGE.

### Chromatin Immunoprecipitation (ChIP)

ChIP assay was performed as described (1). In brief, resting Th1 cells were cross-linked for 10 min with 1% formaldehyde and lysed by sonication. After pre-clearing with salmon sperm DNA, bovine serum albumin, and Protein A agarose bead slurry (50%), cell extracts were incubated with either rabbit polyclonal T-bet (4B10), PEBP2β (FL-182) (Santa Cruz)

or normal rabbit IgG (12–370, Milipore) overnight at 4°C. The immunocomplexes were precipitated with protein A agarose beads at 4°C for 2 h, washed, eluted, and reversed cross-links at 65°C overnight. DNA was purified, resuspended in H<sub>2</sub>O and analyzed by quantitative PCR with Taqman or SYBR primers as previously described (19, 21, 22)

### Chromosome conformation capture assay (3C)

3C assay was performed as described (23–25) with some modifications. 10<sup>7</sup> cells were cross-linked with 2% formaldehyde for 10 min at room temperature and quenched with 0.125M Glycine for 5 min. Cells were lysed with ice-cold lysis buffer (10mM Tris-HCl pH 8, 10mM NaCl, 0.2% NP-40, Protease inhibitor cocktail) for 30 min. Nuclei were resuspended in 0.5 ml of restriction enzyme buffer (NEB3) containing 0.3% SDS and shaken for 1h at 37°C. Triton X-100 (final concentration 1.8%) was added and shaken for 1h at 37°C to sequester the SDS. Crosslinked DNA was digested overnight with 400–800U BglIII containing 1mM ATP that has been shown to enhance digestion efficiency (26). Enzyme was inactivated by addition of SDS (final concentration 1.6%) and samples were shaken for 20min at 65°C. The reaction was diluted with 7ml of ligation buffer (50mM Tris-HCl pH 8, 10mM MgCl<sub>2</sub>, 10mM DTT, 1mM ATP, 1mg/ml BSA), and Triton X-100 (final concentration 1%) was added and shaken for 1h at 37°C. DNA fragments were ligated with 4000U T4 ligase (NEB) for 4 h at 16°C followed by 40 min at room temperature. Crosslinks were reversed by incubation with 300ug proteinase K overnight at 65°C. The samples were further incubated with 300ug RNase for 30–45min at 37°C, and the DNA 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 *Ifng* transcription start site in the parenthesis are as follows: (+35) 5'-GGAGACACAGAAGTTCGAAGTTAGAA-3', (+46) 5'-AAAAACCAACCTGTGTTATTTTG-3', (-35) 6FAM-CCTCTGCAAGCCTCACAGAGCA-TAMRA, and (+51) 6FAM-CCATCTACTGCAAAAAGAAGCT-TAMRA. To generate 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 amounts of three BAC clones spanning the mouse *Ifng* locus (RP23-353P23, RP23-138P22, and RP23-55O21) and BAC spanning the mouse *Gapdh* locus (RP23-410F11) from CHORI were mixed, digested, phenol chloroform extracted and ethanol precipitated, and ligated at a DNA concentration of 300ng/ul. This sample was used as the DNA reference standard. Relative crosslinking frequencies between the analyzed pairs 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 to other T cell subsets (Figure 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*<sup>-/-</sup> than wild type Th1 cells (Figure 1B). Stimulation of Th1 cells with anti-CD3 resulted in the induction of Twist1 mRNA and protein (Figure 1C, D). Thus, *Twist1* is expressed in the greatest amounts in Th1 cells compared to 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 (Figure 1E–F). In agreement with a previous report (12), ectopic Twist1 expression in Th1 cells reduced IFN- $\gamma$  and TNF- $\alpha$  mRNA and protein levels, and decreasing *Twist1* expression resulted in increased IFN- $\gamma$  and TNF- $\alpha$  production (Figure 1G–J). The difference in IFN- $\gamma$  and TNF- $\alpha$  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 (Figure 1G–H and data not shown).

Since 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 to vector control, following re-stimulation with increasing doses of IL-12 (Figure 2A). Decreased induction of pSTAT4 correlated with reduction in IL-18 $\alpha$  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 (Figure 2B). We next examined the expression of genes that contribute to STAT4 activation. STAT4 mRNA and protein expression, and mRNA of suppressors of cytokine signaling (SOCS) and protein tyrosine phosphatase-Basophil like (PTP-BL) that negatively regulate STAT4 activation (28, 29) were not altered. However, *Il12rb2* mRNA expression was decreased approximately 50% by ectopic Twist1 expression compared to vector control (Figure 2C–E and data not shown). In parallel, transduction of Th1 cells with retroviral Twist1 shRNA resulted in increased *Il12rb2* expression (Figure 2F).

### Runx3 rescues the inhibitory effect of Twist1 independent of T-bet and STAT4

Since Twist1 regulates multiple genes in Th1 cells, we wanted to determine if a single factor could rescue the inhibitory effect of Twist1. Initially, we asked whether ectopic expression of IL-12R $\beta$ 2 could compensate for the repressive effect of Twist1 by co-transducing Th1 cells using transduction of both IL-12R $\beta$ 2 and Twist1. IL-12R $\beta$ 2 surface expression was decreased in cells with ectopic Twist1 expression but was higher in double transduced cells, compared to vector control (Figure 3A). Recovery of IL-12R $\beta$ 2 expression resulted in increased IL-12-induced phospho-STAT4 (Figure 3B). Despite recovery of T-bet expression and phospho-STAT4 (Figure 3B and data not shown), ectopic IL-12R $\beta$ 2 expression was unable to rescue IFN- $\gamma$  production, although there was recovery of TNF- $\alpha$  production (Figure 3C). Co-expression of T-bet and Twist1 resulted in higher T-bet expression compared to vector control but failed to recover IFN- $\gamma$  and TNF- $\alpha$  production (Figure 3D, E). Thus, although recovery of IL-12 signaling was able to induce TNF- $\alpha$  in the presence of Twist1, neither recovery of IL-12 signaling nor T-bet expression was able to compensate for the effects of Twist1 on IFN- $\gamma$  production.

Since Runx3 regulates IFN- $\gamma$  independently of T-bet and STAT4 (4), we hypothesized that regulation of Runx3 by Twist1 might be a critical target. Ectopic Twist1 expression resulted in decreased Runx3, and retroviral expression of Runx3 resulted in higher Runx3 level in double transduced cells compared to control cells (Figure 3F). IFN- $\gamma$  and TNF- $\alpha$  production in Runx3/Twist1-transduced cells was comparable to that of control transduced cells (Figure 3G). The recovery of IFN- $\gamma$  and TNF- $\alpha$  production by Runx3 in Th1 cells was independent of T-bet and STAT4 since there were no recovery of T-bet expression or phospho-STAT4-positive cells (data not shown). Thus, although Twist1 can negatively regulate many Th1 genes, the recovery of T-bet and IL-12R $\beta$ 2 expression (and as a consequence, STAT4

phosphorylation) 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- $\gamma$ .

### 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 co-purified with Runx3, and 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 antibody (Figure 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 (Figure S1). Twist1-deficiency did not alter normal lymphocyte development in thymus, spleen and lymph nodes (Figure S1). *Twist1*-deficient Th1 cells produced significantly greater amounts of IFN- $\gamma$  on a per cell basis and in frequency of IFN- $\gamma$ -positive cells compared to wild type cells (Figure 4B–E). We also confirmed the increase in gene expression of *Tbx21*, *Runx3*, and *Il12rb2* in *Twist1*-deficient Th1 cells, compared to 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 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 (Figure 4F). We observed that *Runx3* and *Tbx21* mRNA expression increased in *Twist1*-deficient Th1 cells compared to WT cells, although there was a modest increase at protein level, suggesting the importance of protein complex formation in the function of Twist1. Since there is an association between Twist1, Runx3, and T-bet, we tested if, 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 to WT samples (Figure 4G, H, lane 2 vs. 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 (Figure 4G). We then tested if 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 (Figure 4I). These results suggested that the decrease in IFN- $\gamma$  production in Th1 cells might not only be due to the reduction in *Runx3* gene expression but also due 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

Since 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 non-coding sequences (CNS) (4). To examine T-bet and Runx3 binding in the absence of Twist1, we performed chromatin Immunoprecipitation (ChIP) assay using wild type 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 (Figure 5A)(4, 22), using ChIP for T-bet or CBF- $\beta$ , the binding partner of Runx3 (4). Our data showed that more T-bet was bound in *Twist1*-deficient Th1 cells than in wild type cells at the *Ifng* promoter and CNS+46 while there were no difference at CNS-34 and CNS-6 (Figure 5B). In contrast, more CBF- $\beta$  was bound at CNS-34 and CNS-6 in *Twist1*-deficient Th1 cells compared to littermate controls (Figure 5B). This result suggested that Twist1 interferes with T-bet and CBF- $\beta$ -Runx3 complex binding at specific regulatory regions of *Ifng* gene. Since T-bet and Runx3 bind to many CNS regions of the *Ifng* locus, and the association between T-bet and Runx3 is required for optimal *Ifng* expression (3, 4), it is likely that Runx3 binding to the *Ifng* locus enhances T-bet binding to regulatory elements. This is consistent with the recovery of IFN- $\gamma$  production in the Runx3 transduction experiment (Figure 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 *Ifng* locus (24, 25). To determine whether Twist1 interferes with chromatin looping at the *Ifng* locus, a chromosome conformation capture (3C) assay was performed with wild type and *Twist1*-deficient Th1 cells that examined the interactions among *Ifng* CNS regions using an established assay (23, 25)(Figure 5C, D). Using three different anchor points, our results showed increased crosslinking frequency between CNS-34, CNS+46, and the *Ifng* promoter, and increased crosslinking of CNS-6 with CNS-34 and CNS+46 (Figure 5E–G). The distance between the *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. Together, these results suggest that Twist1 regulates *Ifng* expression by altering the binding of the Th1 transcription factors T-bet and Runx3, and altering the conformation of the *Ifng* locus.

### Twist1-Runx3 interactions are required to regulate *Ifng*

A mutation in Twist1 at amino acid 192 from Serine to Proline (Twist1<sup>cc</sup>) results in diminished interaction between Twist1 and Runx2 (15). Thus we wanted to examine whether Twist1 S192P (Twist1<sup>cc</sup>) could interact with Runx3. DAPA using extracts from cells transfected with Runx3, WT Twist1 (Twist1), and Twist1<sup>cc</sup> expressing vectors that were incubated with biotinylated oligonucleotides containing a Twist1-binding sequence demonstrated decreased interaction between Runx3 and Twist1<sup>cc</sup> (Figure 6A, B). The mutation in Twist1<sup>cc</sup> did not affect the association of Twist1 with T-bet (data not shown).

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 Twist1<sup>cc</sup> was introduced into Th1 cells, and cytokine production and gene expression were analyzed. Introduction of Twist1<sup>cc</sup> did not repress IFN- $\gamma$  production in Th1 cells compared to vector control and wild type Twist1-transduced cells (Figure 6D–H). Importantly, Twist1<sup>cc</sup> was able to repress expression of several Th1 genes including *Il12rb2*, *Runx3* and *Tbx21* as effectively as wild type Twist1 (Figure 6H). To further define the function of Twist1<sup>cc</sup> in Th1 cells, we utilized 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>fl/cc</sup>* CD4-Cre<sup>+</sup> (*Twist1<sup>fl/cc</sup>*) mice that have T cells expressing one wild type or one mutated allele of *Twist1*, respectively. IFN- $\gamma$  production in *Twist1<sup>fl/cc</sup>* Th1 cells was increased compared to control cells (Figure 6I–K). These results demonstrate although Twist1<sup>cc</sup> retains some repressive

function in Th1 cells independent of Runx3, Twist1 control of IFN- $\gamma$  production is primarily through association with Runx3 (Figure 7).

## Discussion

*Twist1* is expressed in Th1 effector memory cells and limits Th1-mediated inflammation in mouse models of delayed-type hypersensitivity and antigen-induced arthritis (12). In this report, 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- $\gamma$  production in Th1 cells. The formation of the Twist1-Runx3 complex is required for Twist1 to decrease IFN- $\gamma$  production. In the absence of Twist1, there is increased transcription factor binding and increased looping at the *Ifng* locus resulting in increased IFN- $\gamma$  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- $\alpha$  and IL-1 $\beta$  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 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*. Yet, 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 report, we showed that *Twist1* is expressed highest in resting Th1 cells compared to 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 and gradually decreased, though *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 T helper lineages. In breast cancer cell lines, STAT3 binds directly to the Twist1 promoter, and amounts of phospho-STAT3 correlate with expression of Twist1 (31). Since STAT3 has a critical role in Th2 and Th17 differentiation, and STAT3-deficient Th2 cultures show increased IFN- $\gamma$  production compared to wild-type cultures, Twist1 might play a role in regulating cytokine production in these lineages as well (32–35). Twist2 promotes IL-10 production by activating the transcription factor c-Maf in myeloid cells, and since c-maf is an important transcription factor for Th2 and Th17 cells Twist1 might play an analogous role in these cells (36, 37). The function of Twist1 in other Th subsets will be the focus of further study.

Twist1 requires dimerization to bind DNA (38). In *Drosophila*, dimerization partners determine the activity of Twist1 (39). Twist1 homodimers have opposing effects on mesoderm gene expression as heterodimers of Twist1 and the *Drosophila* E protein homologue, Daughterless (39). The *Daughterless* gene has 79% identity to E12 and E47, two alternative splice products of *E2a* gene (40, 41). Th1 cells differentiated from *E2a*-deficient mice showed a decrease in IFN- $\gamma$  production compared to WT cells (42). 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 (43). 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 Twist1 homodimers (44, 45). Understanding how this balance is regulated in T helper cells will lead to greater insight into the networks of transcription factors that regulate differentiation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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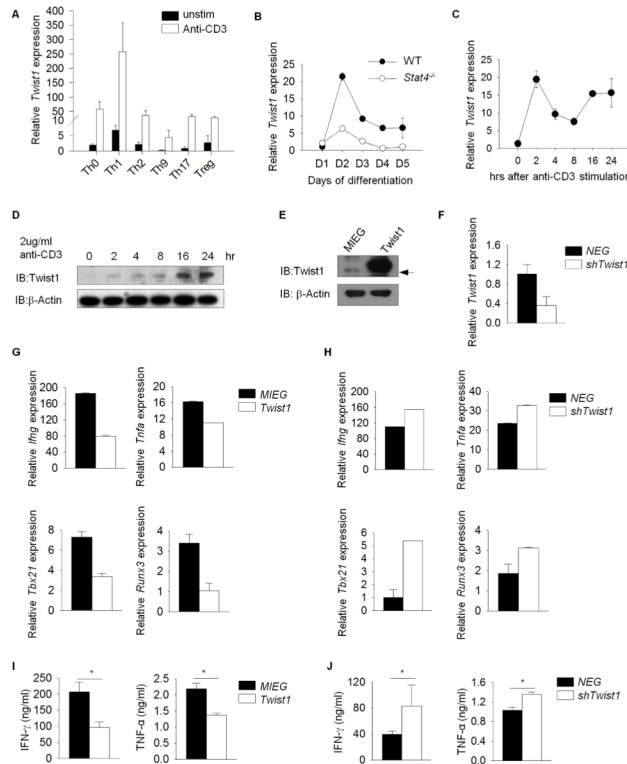
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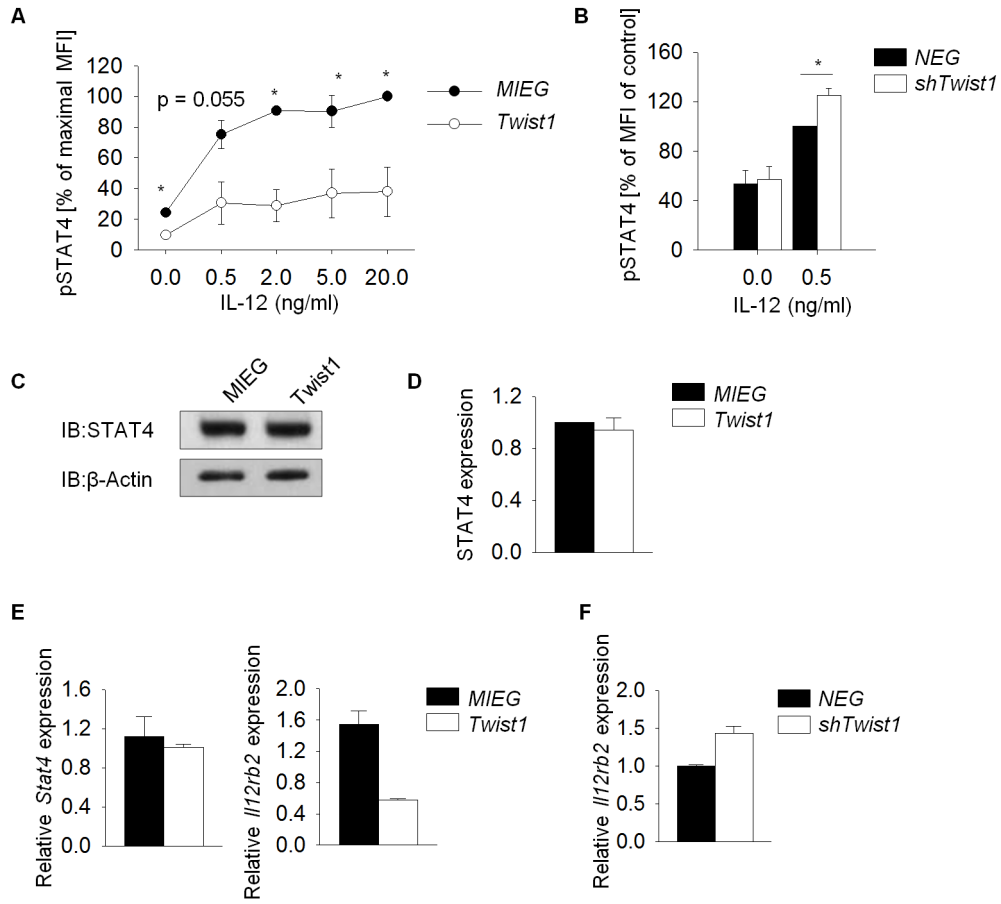
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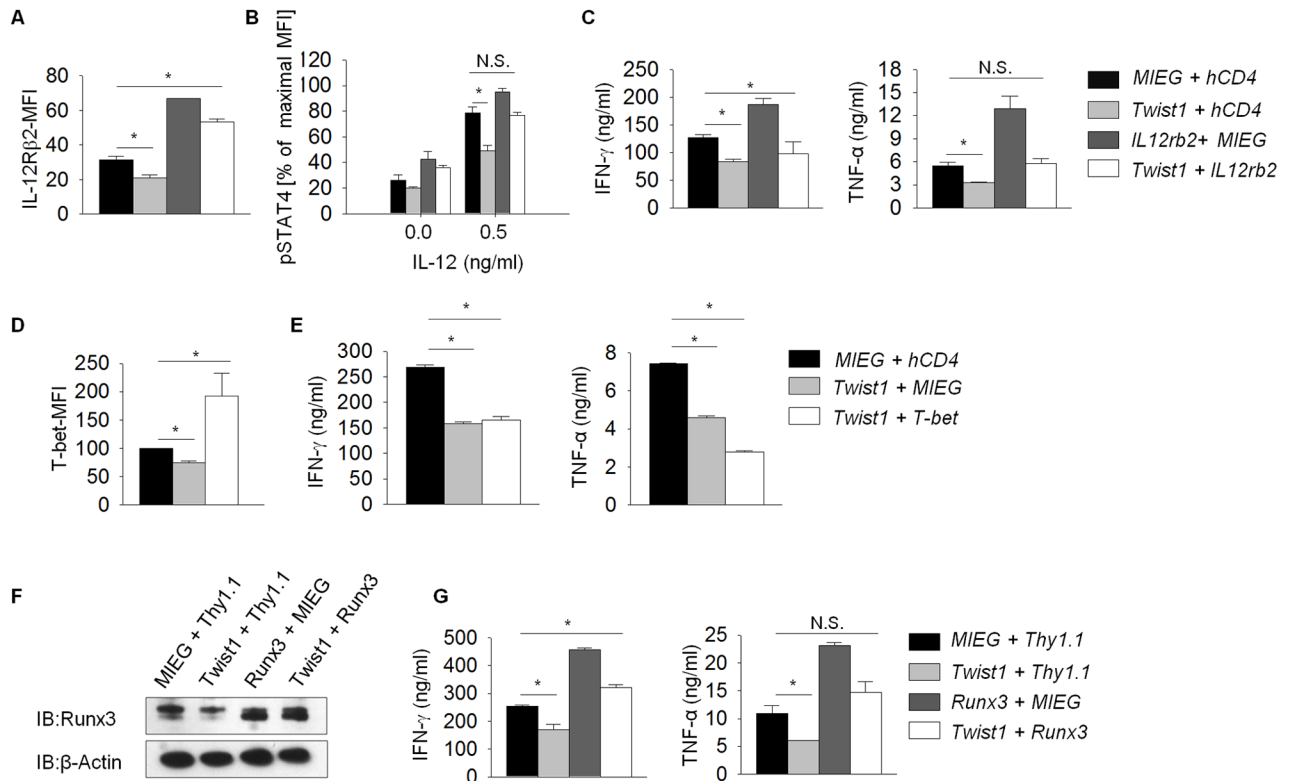


**Figure 1. Twist1 negatively regulates Th1 gene expression and cytokine production**  
 A, Naive wild-type (WT) lymphocytes were differentiated under neutral conditions (Th0) or Th1 (IL-12 + anti-IL-4), Th2 (IL-4 + anti-IFN- $\gamma$ ), Th9 (IL-4 + TGF- $\beta$ ), Th17 (IL-6 + TGF- $\beta$ ) or Treg (TGF- $\beta$ )-polarizing conditions *in vitro*. *Twist1* mRNA expression in resting or anti-CD3 activated Th1 cells was determined by RT-qPCR. Data represent mean  $\pm$  S.D. of 3 mice; and representative of two independent experiments. B, Kinetics of *Twist1* mRNA expression during Th1 differentiation in WT and *Stat4*<sup>-/-</sup> cells. Average of replicate samples  $\pm$  S.D. (C–D) Kinetics of *Twist1* mRNA expression (C) and Twist1 protein (D) in Th1 cells after stimulation with 2ug/ml anti-CD3 for 2, 4, 8, 16 and 24 h. Results are representative of two to three independent experiments with similar results. (E–J) Naïve WT T cells were stimulated under Th1-polarizing condition. On day 2, cells were transduced with control EGFP (MIEG) or retroviral vector expressing Twist1 and EGFP (Twist1)(E, G, I), or control or short hairpin Twist1 (shTwist1)(F, H, J). On day 5, GFP<sup>+</sup> cells were sorted for analysis. (E–F) Altered Twist1 protein expression in cells transduced with Twist1 (E) and altered *Twist1* expression in shRNA transduced Th1 cells (F). (G–H) Th1 gene expression in ectopic Twist1- (G) or short hairpin- (H) transduced cells was assessed by RT-qPCR before (*Tbx21* and *Runx3*) or after (*Ifng* and *Tnfa*) 6 h re-stimulation with 2ug/ml anti-CD3. (I, J) Sorted GFP<sup>+</sup> cells were stimulated with 2ug/ml anti-CD3 for 24 h, supernatants were collected and analyzed for IFN- $\gamma$  and TNF- $\alpha$  by ELISA. Data are (I) mean  $\pm$  S.D. of three independent experiments or (J) mean of replicate samples  $\pm$  S.D. and representative of three independent experiments with similar results. \*p<0.05

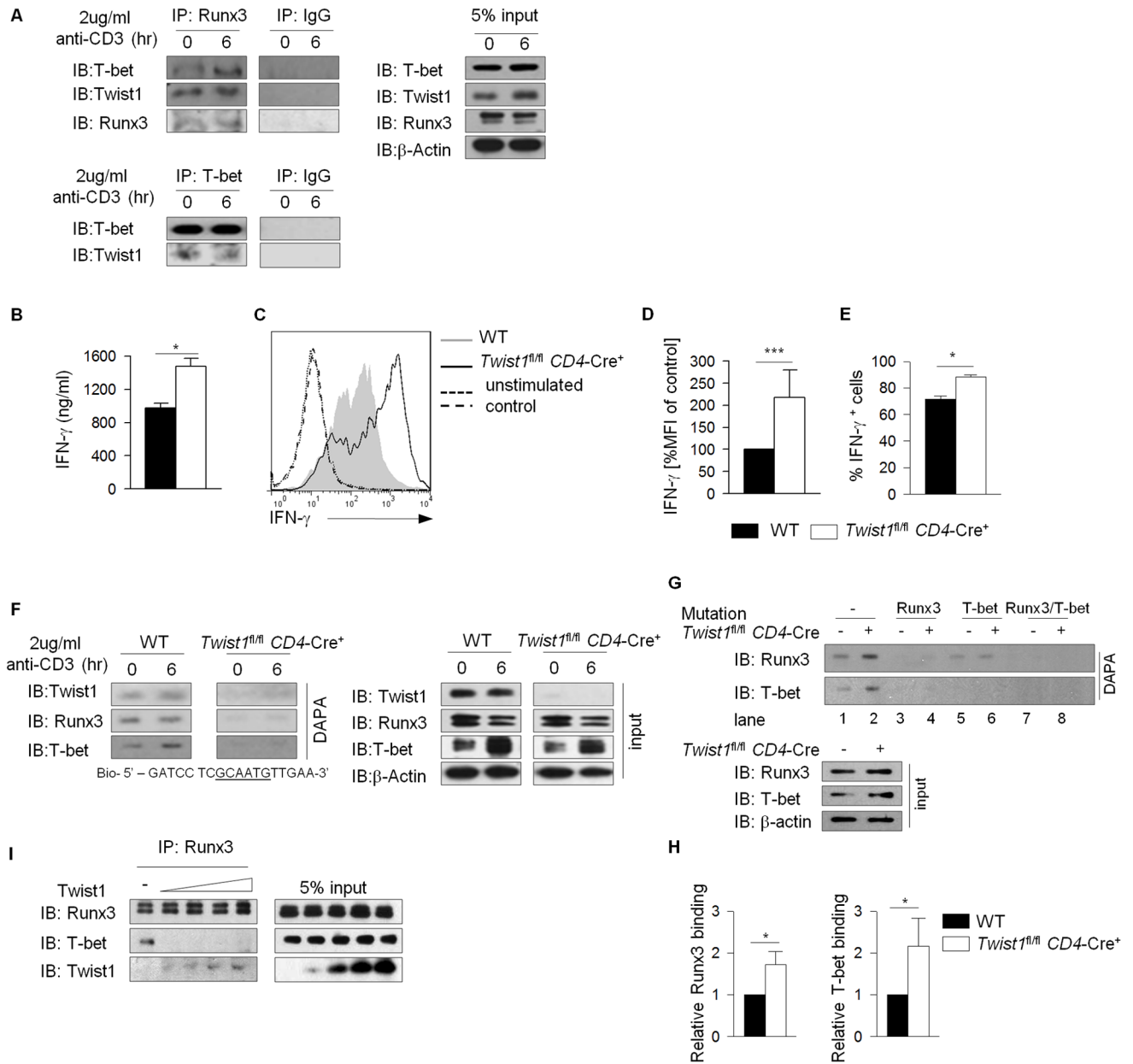


**Figure 2. *Twist1* regulates *Il12rb2* expression and STAT4 activation**

A, Naïve WT T cells were stimulated and transduced as described in Figure 1. Transduced Th1 cells were stimulated with IL-12 for 4 h and GFP<sup>+</sup> cells were analyzed for phosphorylated STAT4 (pSTAT4). B, Phospho-STAT4 were determined in control or shRNA transduced Th1 cells following sorting for GFP expression. (C–D) Total cell extracts were immunoblotted for STAT4 and β-Actin as a control (C), and densitometry analysis of STAT4 protein is indicated (D). (E–F) *Stat4* and *Il12rb2* gene expression in *Twist1* (E) or sh*Twist1* (F) transduced Th1 cells sorted for GFP expression. Data are mean of three to four independent experiments ± S.D. (A, B) or the average of replicate samples ± S.D. and representative of two to three independent experiments with similar results (C–F). \*p<0.05



**Figure 3. Ectopic Runx3 expression compensates for the repressive activity of Twist1 in Th1 cells**  
 Naïve 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 (F, G) Twist1-GFP and Runx3-Thy1.1. Th1 cells were stained for IL-12Rβ2 by surface staining (A) or phospho-STAT4 with or without IL-12 stimulation by ICS (B), or T-bet by ICS (D). Analysis was performed by gating on doubly transduced cells. (E, G) Double positive cells were sorted and re-stimulated with 2ug/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 mean of three to four independent experiments ± S.D (A, B) or averages of replicate samples ± S.D. and representative of two to three independent experiments with similar results (C–G). \*p<0.05

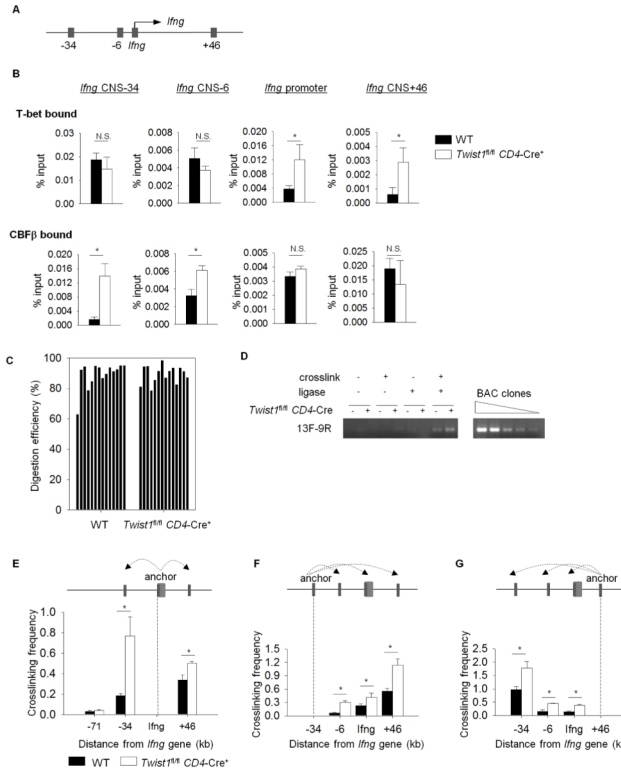


**Figure 4. Twist1 physically interacts with Runx3 and T-bet**

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 antibodies as indicated. Input for immunoprecipitation is indicated on the right. (B–E) Naïve 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- $\gamma$  production was measured by ELISA (B) after reactivating with 2ug/ml anti-CD3 for 24h or ICS (C–E) after reactivating with PMA/IONO for 6 h. (DE) Graphs indicate the mean fluorescence intensity (D) or average percent IFN $\gamma$ -positive 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 (wild type or mutant as indicated) (G). H, Immunoblots of precipitated proteins, with

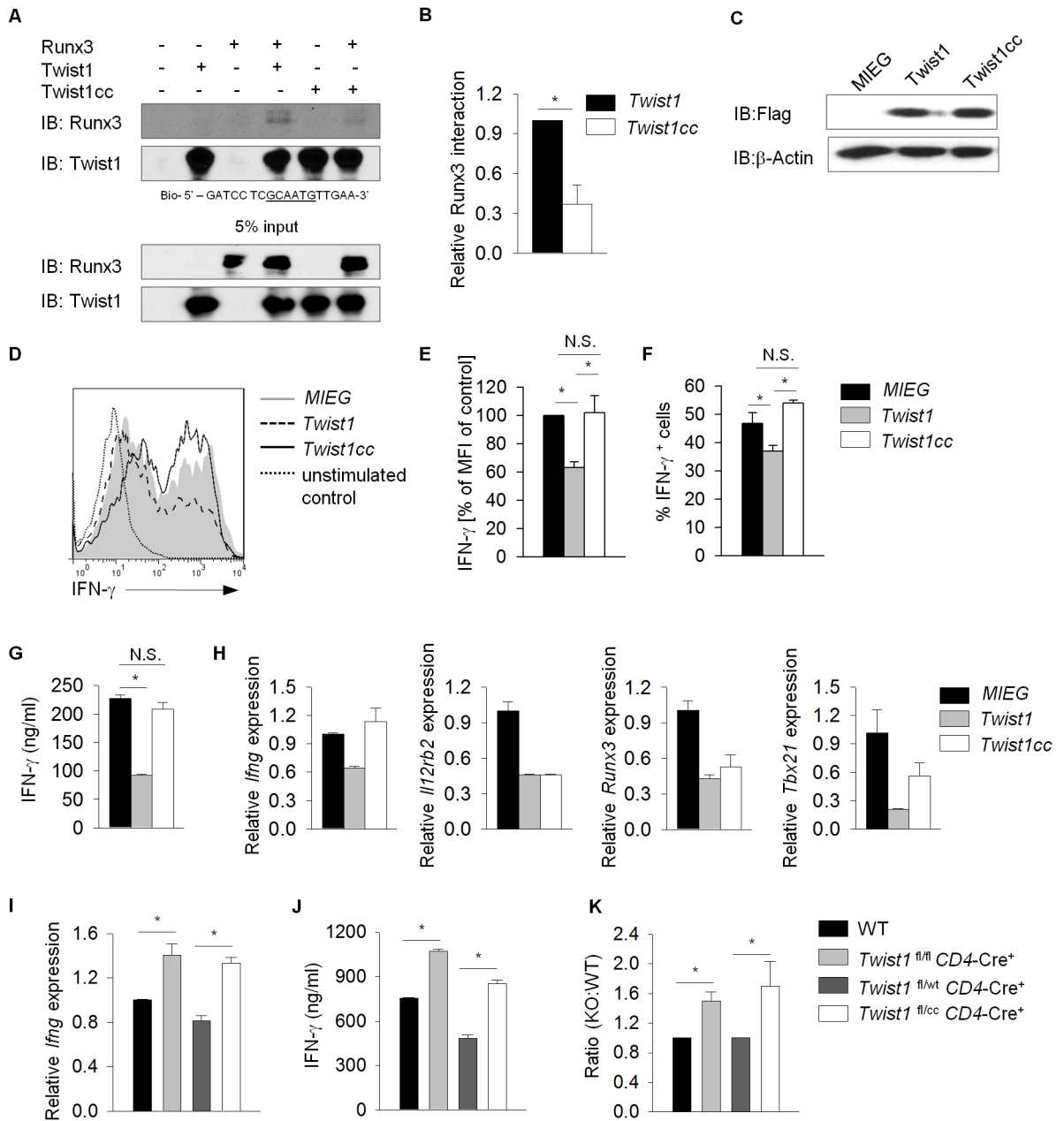
densitometry measurements for (G). I, Mixture of whole cell lysates collected from 293T cells transfected with constructs expressing Runx3, T-bet or Twist1 were incubated at 4°C overnight. Immunoblots indicate proteins immunoprecipitated with Runx3. Results are average  $\pm$  S.E.M (B) or  $\pm$  S.D. (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).





**Figure 5. Twist1 interferes with transcription factor binding and chromatin conformation at the *Ifng* locus in Th1 cells**

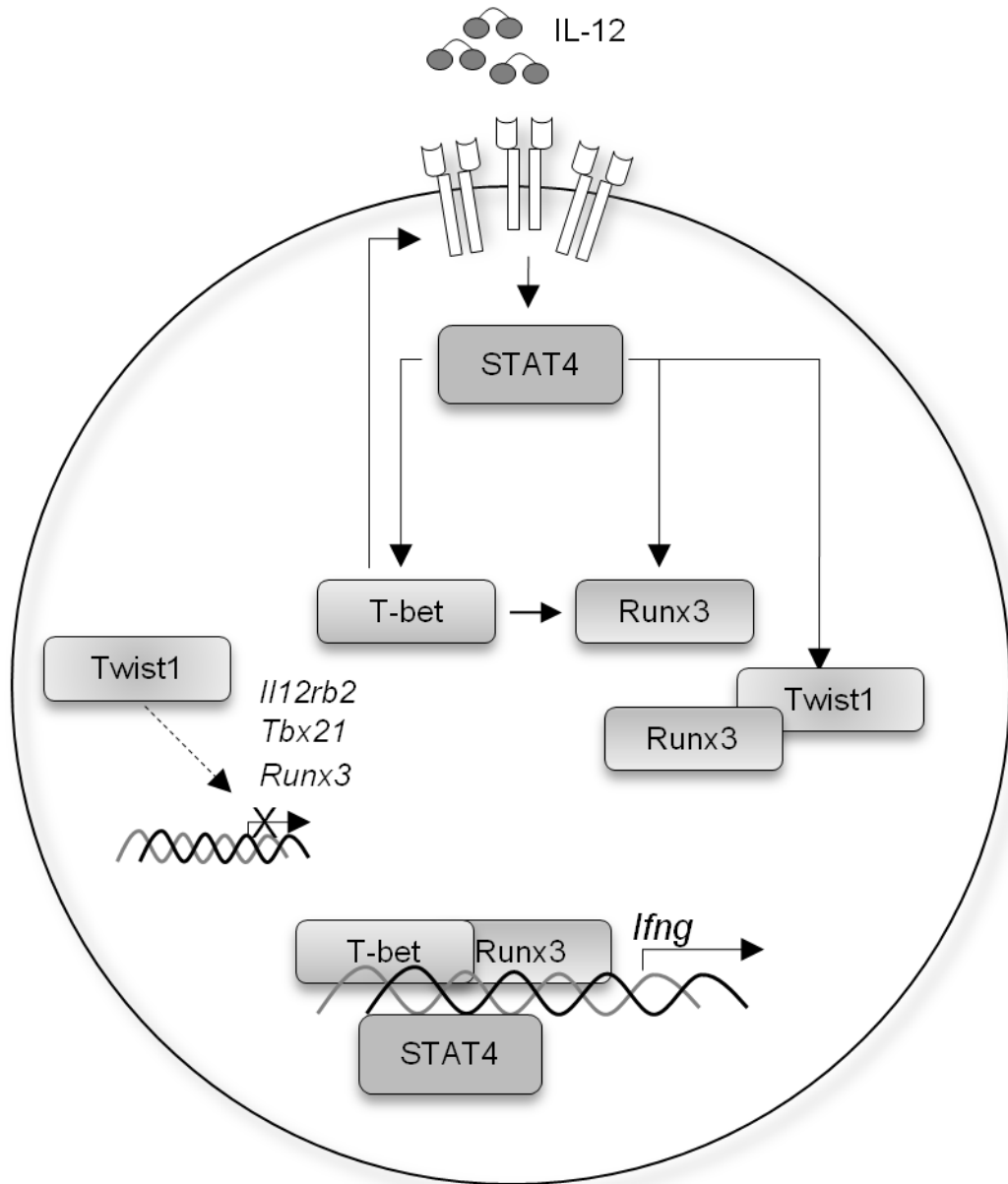
A, Representation of *Ifng* locus indicating conserved non-coding sequences (CNS) used for analysis. (B–G) Naïve CD4<sup>+</sup>CD62<sup>hi</sup> T cells from WT and *Twist1*<sup>fl/fl</sup> CD4-Cre<sup>+</sup> were stimulated under Th1-polarizing conditions. B, ChIP assays were performed on Th1 cells using T-bet and CBFβ antibodies. C, Th1 cells were fixed and digested with *Bgl*III enzyme. Undigested and digested samples were subjected for qPCR using primer pairs spanning the restriction sites. % Digestion was calculated using the formula:  $100 - 100/2((CtR - CtC)D - (CtR - CtC)UND)$ ; D, digested, UND: undigested, R: restriction site, C: internal control. D, Th1 cells as described above were fixed (crosslinked) and/or ligated as indicated. qPCR was performed using primer 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 cross-linking frequencies between the *Ifng* promoter (E), CNS-34 (F) or CNS+46 (G) as the fixed anchor fragments and other *Bgl*III fragments containing the indicated CNS regions. Results are the average ± S.D. of replicated samples and are representative of four independent experiments with similar results. \*p<0.05



**Figure 6. The Twist1-Runx3 interaction is required for regulation of *Ifng***

(A, B) Nuclear extracts from 293T cells co-transfected with either Flag-tagged Twist1 or Flag-tagged Twist1cc and 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) Naïve CD4<sup>+</sup>CD62<sup>hi</sup> 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<sup>+</sup> populations following 6h of activation with anti-CD3 (D–F) or by ELISA following 24 h stimulation with anti-CD3 of sorted GFP<sup>+</sup> populations (G). (E–F) Graphs indicate the mean fluorescence intensity (E) or average percent IFN- $\gamma$ -positive cells (F) of each transduced population. H, Th1 gene

expression in ectopic *Twist1* or *Twist1cc* expression was assessed by RT-qPCR before (*Il12rb2*, *Runx3* and *Tbx21*) or after (*Ifng*) 6 h re-stimulation with anti-CD3. (I–K), Naïve CD4<sup>+</sup>CD62<sup>hi</sup> T cells from mice of the indicated genotypes were cultured under Th1-polarizing conditions. Th1 cells were stimulated with anti-CD3 for 6 h or 24 h respectively for testing *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 mean of three to four independent experiments  $\pm$  S.D, \* $p$ <0.05 (A–F), or are mean of replicate samples  $\pm$  S.D. and representative of two independent experiments with similar results (G–K). \* $p$ <0.05



**Figure 7. Transcriptional regulatory network in Th1 cells**

IL-12-IL-12R $\beta$ 2-STAT4 induces T-bet expression that positively regulates IL-12R $\beta$ 2 expression. Both STAT4 and T-bet are required for the induction of Runx3 that contribute to the optimal IFN- $\gamma$  production. Twist1, a transcription repressor that is induced by IL-12-STAT4, suppresses IFN- $\gamma$  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* nor *Tbx21* expression did not rescue IFN- $\gamma$  production.