Published in final edited form as:

Sci Transl Med. 2010 June 23; 2(37): 37ra44. doi:10.1126/scitranslmed.3000813.

Nuclear Role of WASp in the Pathogenesis of Dysregulated T_H1 Immunity in Human Wiskott-Aldrich Syndrome

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Abstract

The clinical symptomatology in the X-linked Wiskott-Aldrich syndrome (WAS), a combined immunodeficiency and autoimmune disease resulting from WAS protein (WASp) deficiency, reflects the underlying coexistence of an impaired T helper 1 (T_H1) immunity alongside intact T_H2 immunity. This suggests a role for WASp in patterning T_H subtype immunity, yet the molecular basis for the T_H1-T_H2 imbalance in human WAS is unknown. We have discovered a nuclear role for WASp in the transcriptional regulation of the T_H1 regulator gene *TBX21* at the chromatin level. In primary T_H1-differentiating cells, a fraction of WASp is found in the nucleus, where it is recruited to the proximal promoter locus of the *TBX21* gene, but not to the core promoter of *GATA3* (a T_H2 regulator gene) or *RORc* (a T_H17 regulator gene). Genome-wide mapping demonstrates association of WASp in vivo with the gene-regulatory network that orchestrates T_H1 cell fate choice in the human T_H cell genome. Functionally, nuclear WASp associates with H3K4 trimethyltransferase [RBBP5 (retinoblastoma-binding protein 5)] and H3K9/H3K36 tridemethylase [JMJD2A (Jumonji domain-containing protein 2A)] proteins, and their enzymatic activity in vitro and in vivo is required for achieving transcription-permissive chromatin dynamics at the *TBX21* proximal promoter in primary differentiating T_H1 cells. During T_H1 differentiation, the loss of WASp accompanies decreased enrichment of RBBP5 and, in a subset of WAS patients, also of filamentous actin at the *TBX21* proximal promoter locus. Accordingly, human WASp-deficient T_H cells, from natural mutation or RNA interference-mediated depletion, demonstrate repressed *TBX21* promoter dynamics when driven under T_H1-differentiating conditions. These chromatin derangements accompany deficient T-BET messenger RNA and protein expression and impaired T_H1 function, defects that are ameliorated by reintroducing WASp. Our findings reveal a

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Author contributions: M.D.T. performed the fluorescence imaging studies, ChIP-chip assays, methylation or demethylation enzymatic assays, coimmunoprecipitation and Western assays, and RNAi assays. S.S. performed conventional and μ ChIP-qPCR assays, sequential ChIP-qPCR assays, ChIP-chip assays, and RT-PCR assays for mRNA quantification and designed all primers and probes for ChIP assays. P.K. performed conventional ChIP assays, coimmunoprecipitation and Western assays, and optimized DHS qPCR-based assays. A.R. established and optimized the μ ChIP assay for human samples. K.S. performed coimmunoprecipitation and Western assays. A.S. performed the RNAi assay. S.D. performed coimmunoprecipitation and Western assays. F.C. generated the immortalized WAS T_H lines and provided patient samples. M.D.T., S.S., P.K., K.S., and S.D. performed the tissue culture. Y.M.V. conceived the study, designed the experiments, analyzed the data, and wrote the paper. All authors reviewed the manuscript.

Competing interests: The authors declare that they have no competing interests.

previously unappreciated role of WASp in the epigenetic control of T-BET transcription and provide a new mechanism for the pathogenesis of WAS by linking aberrant histone methylation at the *TBX21* promoter to dysregulated adaptive immunity.

INTRODUCTION

Wiskott-Aldrich syndrome (WAS) results from a panoply of mutations in the *WAS* gene, manifesting in loss of WAS protein (WASp) expression or expression of mutant WASp (1). In human disease and certain murine models of WAS, severe invasive infections from intracellular pathogens and predisposition to hematologic malignancies, together with hyperimmunoglobulin E, atopic eczema, and auto-immune colitis, indicate coexistence of impaired T helper 1 (T_H1) immunity alongside heightened T_H2 immunity (2). Accordingly, pharmacologic neutralization of the augmented T_H2 cytokine expression ameliorates autoimmune colitis in the murine model of WAS (3). In WAS T_H cells, these clinical manifestations are associated with a selective defect in the expression of T-BET (T_H1 master regulator) but not of GATA3 (T_H2 master regulator) (4). However, the molecular basis for these observed effects of loss of WASp on *T-BET* gene (*TBX21*) induction and T_H1 differentiation is unknown. Thus far, WASp, expressed primarily in hematopoietic cells, is known only for its cytoplasmic function in modifying the cortical cytoskeletal structures by promoting actin polymerization via its small VCA domain (5). However, disruption in such a broad and general architectural function resulting from loss of WASp cannot readily explain an isolated defect in the regulation of T_H1 differentiation and functions in WAS. Indeed, the cellular phenotype in Y293E (a constitutively active form of WASp) mutant mice, despite supporting enhanced cortical actin polymerization, resembles that of WASp-null mice (6). Similarly, certain patients with activating *WAS* mutations resulting in an increased level of cellular actin polymerization still exhibit the pathological T cell phenotype that resembles that of classical WAS patients (7). The Foxp3 defect in T regulatory (T_{reg}) cells described in WASp-null mice is not associated with T cell receptor (TCR)-induced defects in actin polymerization or remodeling (8). Using domain-deleted WASp mutants, it was previously shown that TCR-mediated transcriptional activation of NFAT occurred independently of actin polymerization (9). Similarly, in human natural killer cells, we demonstrated that WASp controls nuclear translocation of NFAT2 and nuclear factor κB (RelA) transcription factors independently of its role in filamentous actin (F-actin) polymerization (10). Collectively, these observations raise the possibility that the multiple domains of WASp may have unique biological functions in shaping T_H1 immunity, one that may extend beyond its structural role in maintaining the cortical F-actin cytoskeleton. In this connection, neuronal WASp (N-WASp), a widely expressed homolog of WASp, has been previously shown to support transcriptional activity in HeLa cells (11).

Here, we tested the hypothesis that WASp has a nuclear role in the transcriptional control of *TBX21*, the gene known to direct T_H1 lineage commitment (12). In primary T_H cells from normal donors, we demonstrate that WASp locates to the nucleus and there it engages many T_H-specific immune function genes in vivo under T_H1-differentiating conditions. Specifically, we show that WASp is an important component in the epigenetic transcriptional control of T-BET activation through its local effects on chromatin configuration and conformation. The study also identifies WASp target genes, in the genome of T_H1-differentiating cells, that are important regulators of both immune and general cellular functions.

RESULTS

WASp localizes to the nucleus in human primary T_H1-differentiating cells

Because many actin-binding proteins (ABPs), including N-WASp, have previously been shown to shuttle between the cytoplasm and the nucleus (13–15), we investigated whether WASp, so far a cytoplasmic ABP, is also identified in the nucleus of primary human T_H cells. Using quantitative three-dimensional (3D) deconvolution immunofluorescence imaging of the entire T_H cell volume, we demonstrate at the single-cell level that a fraction of WASp is identified in the nucleus of about 80 to 85% of human primary T_H1-differentiating cells compared to ~5% in steady-state, undifferentiated T_H0 cells derived from the same donor (Fig. 1A and figs. S1 to S5). Moreover, subcellular fractionation identified robust WASp signals in the nuclear fraction in T_H1 cells compared with those in T_H0 or T_H2 cells by Western blot analysis (Fig. 1B). Although in T_H2 cells the magnitude of WASp nuclear signals is only slightly above the steady-state level found in T_H0 cells, it remains to be determined whether other states of T_H differentiation [for example, inducible T_{reg} (iT_{reg}) or T_H17] can also drive substantial WASp nuclear transport.

In addition to its nuclear location, WASp immunofluorescence is also identified at the perimembranous or cytoplasmic location in the same T_H1-differentiating cells, which is consistent with the established role of WASp in remodeling the F-actin cortical cytoskeleton (5) (Fig. 1, A, B, and D, and figs. S6 and S7). As a control for reagent specificity and lack of cross-reactivity of WASp antibodies, we demonstrate an absence of immunoreactivity for WASp, but not for N-WASp, in the patient-derived WASp-deficient T_H cells (Fig. 1C). Similarly, WASp can be successfully immunoprecipitated from the nuclear fraction of primary T_H1 cells but not of T_H0 cells (Fig. 1E). The average efficiency of our WASp immunoprecipitation is $40 \pm 8\%$ ($n = 7$ experiments) of the total input derived from the T_H1 nuclear extract, determined by Western gel densitometry.

In primary T_H1-differentiating cells, nuclear WASp immunofluorescence staining corresponds largely with 4',6-diamidino-2-phenylindole (DAPI)–“dim” regions that are also extranucleolar (Fig. 1F and figs. S2 and S7), suggesting WASp accumulation within the putative multifocal “transcription factories.” Accordingly, nuclear WASp colocalizes with hyperphosphorylated RNAP2 (Ser²), a polymerase II modification associated with active gene transcription elongation, and components of the human Mediator complex [thyroid hormone receptor–associated protein 220 (TRAP220), TRAP100, and TRAP95]. Pearson’s correlation coefficient calculated the degree of colocalization of two fluorescent probes within the nucleus, and the obtained values were $r_p = 0.69$ for WASp and RNAP2 and $r_p = 0.64$ for WASp and TRAP220/MED1 (Fig. 1F and fig. S3). The endogenous association of WASp with RNAP2 and TRAP220/MED1 protein complexes was also verified in coimmunoprecipitation assays in the purified nuclear fractions of primary T_H1 cells (Fig. 1G).

Collectively, our findings in primary human T_H cells provide the first evidence for activation-driven nuclear localization of endogenous WASp in any cell type where WASp is expressed. Accordingly, like N-WASp, WASp joins a growing list of ABPs that maintain a dual-site location (cytoplasmic and nuclear) to support location-specific cellular functions (13–15).

Genome-wide mapping reveals WASp binds a subset of immune function genes in human primary T_H1-differentiating cells

To define a potential function for WASp in the nucleus, we next investigated the limited genome distribution of WASp-binding sites *in vivo* in human primary T_H1-differentiating cells pooled from at least 12 healthy donors (Fig. 2). This involved the immunoprecipitation

of endogenous WASp-bound DNA with specific antibody to WASp from formaldehyde cross-linked, sonicated chromatin [chromatin immunoprecipitation (ChIP)] followed by detection with genome tiling arrays (ChIP-on-chip). A customized ChIP-on-chip microarray was tiled with around ~50 to 60 genes considered to be important in the development of CD4⁺ T_H cell-specific adaptive immune responses. Analysis of the genomic locations of WASp interaction with DNA showed most WASp peaks in close proximity to introns (49.04%) and distal enhancers (>1 kb upstream or downstream from the 5' or 3' end of the gene) (34.5%). WASp binding was also noted at the exons (9.62%), 5' proximal promoters [within 1 kb upstream of the 5' transcription start site (TSS)] (2.04%), and immediate downstream cis regions (within 1 kb downstream of the 3' end of the gene) (4.72%) (Fig. 2D).

A bioinformatic search for conserved DNA sequence motifs among WASp-bound DNA fragments in this limited mapping of the T_H1 cell genome identified an enriched ($P < 0.0005$) CG-rich motif that corresponds to DNA recognition sequence for the zinc finger transcription factors of the Sp1- or Kruppel-like proteins (Fig. 2B) (see Materials and Methods). Physical interaction of WASp with Sp1 transcription factor was verified by immunoprecipitation–Western blotting (Fig. 2C). These data allow us to posit that Sp1 may bring WASp to the DNA, although this will have to be experimentally validated.

To investigate the putative nuclear functions of WASp in T_H1 differentiation, we next analyzed the ChIP-chip data to identify WASp target genes. This analysis revealed WASp binding to multiple immune function genes in primary T_H1 cells ($n = 35$ genes) (Fig. 2A and figs. S8 and S9). These included the genes that encode canonical T_H transcription factors, T_H signature cytokines, chemokines, antiapoptotic proteins, metabolic enzymes, and cytoskeletal and microtubular proteins (table S1). On the other hand, WASp did not engage many other gene loci, demonstrating the specificity of WASp targets in T_H1 cell genome. The locus-binding specificity of WASp was further shown by the association of WASp with a T_H1 signature cytokine gene (*IFNG*), a T_H1 cytokine receptor gene (*IL12Rβ2*), and T_H1 transcription factor genes [*RUNX3*, signal transducer and activator of transcription 1 (*STAT1*), and *STAT4*], but not with T_H2 cytokine genes (*IL4*, *IL5*, and *IL13*) or a T_H2 transcription gene (*STAT6*), in this snapshot view of WASp-DNA interaction in primary T_H1 cells (Fig. 2A). Strikingly, WASp binds to the *RAD50* genomic locus on chromosome 5 (3' region of *RAD50* gene at the intronic locus between exons 21 and 22), previously shown to function as a T_H1-T_H2 locus control region during T_H differentiation (16,17).

Collectively, these results and the in vivo binding of WASp to the genomic loci of *T-BET* (primary regulator of T_H1 cell fate), *RUNX3* (secondary regulator of T_H1 cell fate), and *IFNG* (T_H1 signature cytokine gene) favor the model of WASp targeting to the gene-regulatory network that orchestrates T_H1 cell fate choice in the human immune system, at least in the T_H1-differentiating cytokine milieu.

WASp is recruited to DNA at multiple cis sites within the *TBX21* locus in primary T_H1-differentiating cells

To study the chromatin function of WASp at the mechanistic level and to test its biological significance in T_H1 differentiation, we focused on the *T-BET* gene (*TBX21*) because WASp-deficient T_H cells are also deficient in *TBX21* messenger RNA (mRNA) (4). Multiple high-confidence WASp-binding sites [false discovery rate (FDR) ≤0.2] were captured at the *TBX21* locus within the proximal 5' and 3' cis regions (Fig. 2A and figs. S8 and S10). WASp enrichment (immunoprecipitation/input ratio), quantified at both the forward and the reverse strands of *TBX21* DNA, ranged from two- to fivefold above the baseline in this snapshot view of the pooled chromatin. The WASp-bound 5' *TBX21* locus spanning ~1 kb upstream of TSS (that is, proximal promoter) contains many important cis-regulatory sequences, such

as the high-affinity-activated sequence (GAS) (core motif: 5'-TTCAGGCAA-3'), T-BET-binding motif [5'-(G/C/T)TGTG(A/C)A-3'], TATA-like sequence (5'-TCATAA-3'), E-box sequence (5'-CAGAGGGTG-3'), Onecut2 (OC2) cis sequence (5'-ATCAATAAAGATCGAT-3'), and multiple Sp1-binding cis sequences (CG-rich). Accordingly, this 5' cis region bears the characteristics of a core proximal promoter for regulating T-BET induction and expression. On the other hand, the functions of 3' cis regions, to which WASp also binds (Fig. 2A and fig. S10), in T-BET gene activation are thus far unknown. Therefore, to test the specific hypothesis of this study, we focused the rest of our investigations on elucidating the effects of WASp on the dynamics of the 5' proximal promoter of the *TBX21* gene.

Importantly, although WASp engages the *TBX21* promoter locus, it does not interact with the two well-characterized *GATA3* promoter loci (promoter 1 at exon 1/2 and promoter 2 located ~10 kb upstream of promoter 1) in the same sample of primary T_H1-differentiating cells (Fig. 2A and figs. S8 and S11). However, WASp binds to the intragenic *GATA3* locus (mainly intronic), as well as to the distal 5' intergenic locus (about -17 kb from promoter 1), both cis regions with yet to be defined roles in the transactivation of the *GATA3* gene. Similarly, the ChIP-chip assay did not capture any high-confidence WASp-binding signals (that is, FDR < 0.2) at the putative 5' core promoters of *RORc* on chromosome 1, *GAPDH* on chromosome 12, or *FOXP3* on chromosome X (figs. S8, S12, and S13). Although multiple high-confidence WASp-binding signals were captured within the body of the *FOXP3* gene (including the exons 9 to 12) that encode the forkhead domain considered important in controlling the transactivation of several T cell-specific cytokine genes. Moreover, besides *TBX21*, WASp is also recruited to the genomic regions that correspond with the putative 5' proximal promoters (within 2 kb from TSS) of *IFNG*, *STAT1*, and *IL12Rβ2* genes (Fig. 2A and figs. S8 and S9). Overall, this snapshot view shows a selectivity of WASp in engaging the 5' core promoters of the network genes that specify T_H1 cell fate choice.

STAT1, T-BET, Sp1, and preinitiation complex components are dynamically recruited to the WASp-bound cis sites at the *TBX21* promoter locus in T_H1-differentiating cells

We next investigated whether these WASp-bound cis sites at the *TBX21* promoter locus are functionally active sites that also recruit STAT1, a classical regulator of *TBX21* activation (18), as well as other component proteins of the general transcriptional machinery [RNAP2 and Mediator TRAP220/MED1 preinitiation complexes (PICs)] (19). Therefore, we performed the ChIP-quantitative real-time polymerase chain reaction (PCR) assay on micrococcal nuclease-digested ChIP (MNase-ChIP) to obtain a mononucleosomal resolution of the protein-DNA interactome (Fig. 3B) (fig. S14B demonstrates high efficiency of chromatin digestion by MNase). We simultaneously interrogated multiple contiguous cis sites within the *TBX21* core promoter to minimize false-positive or false-negative results. Accordingly, we designed three quantitative PCR (qPCR) primer and probe sets within the 5' *TBX21* promoter locus, where the binding of WASp to DNA was captured by the ChIP-chip approach. These cis sites, dubbed WASp-binding region 1 (WBR1) [about -250 base pairs (bp) from TSS], WBR2 (about -500 bp from TSS), and STAT1-binding GAS region (about -750 bp from TSS), were comprehensively analyzed (Fig. 3A and fig. S14A).

Using the MNase-ChIP assay, we first validated the ChIP-chip results of WASp binding to the *TBX21* promoter. This assay demonstrated that, in addition to WASp binding to the regions of highest ChIP-chip WASp signals (that is, chromosome 17 coordinates 43,164,872 to 43,165,096, which correspond to WBR2 and GAS cis sites), WASp binding was also captured at the WBR1 site (43,165,310 to 43,165,364), which is ~1.5 nucleosomes (~200 bp) downstream to the WBR2 cis site (Fig. 3 and fig. S14A).

Given the high concordance (~70%) of STAT1 binding to the GAS sequences in the genome (20,21), we next tested the binding of STAT1 to the *TBX21* promoter locus because the canonical GAS sequence is also present within this genomic region. The MNase-ChIP demonstrated that, although the enrichment of STAT1 was highest at the GAS site, STAT1 binding was also noted at the nearby WASp-binding cis sites (WBR1 and WBR2). The T_H1-driven promoter enrichment of STAT1, like that of WASp, was markedly enhanced at all three cis elements, albeit not equally (Fig. 3B). Because this finding suggests, but does not prove, that WASp and STAT1 bind to the same promoter chromatin, we next performed quantitative sequential ChIP assay on MNase-digested chromatin in both forward (first IP: WASp + second IP: STAT1) and reverse (first IP: STAT1 + second IP: WASp) directions (Fig. 3D). This assay revealed co-occupancy of STAT1 and WASp on the same mononucleosomal chromatin in vivo, and the findings are consistent with the idea that the binding sites for these two proteins are spaced closely together (<150 to 200 bases apart). Together, these results, while pointing to the formation of higher-order protein complexes involving WASp and STAT1 at this promoter locus, consolidate the well-established role of STAT1 in *TBX21* activation (18). Although *IFNG* is a direct target of T-BET, we also found that T-BET bound to its own promoter (Fig. 3B), which supports the idea that T-BET, like GATA3, may autoactivate *TBX21* as previously suggested (22).

We next tested whether the T_H1-driven dynamic recruitment of WASp, STAT1, and T-BET to the *TBX21* promoter chromatin accompanies other trans-regulatory events that are indicative of transcription initiation at these WASp-bound cis sites. Therefore, we tested whether components of the PIC, RNAP2 (unmodified CTD) and the human TRAP-Mediator complex (TRAP220/MED1), are also identified at the *TBX21* promoter. We demonstrate that, like WASp and STAT1, the chromatin occupancy of RNAP2 and TRAP220/MED1 was contemporaneously enhanced at all three cis elements in the T_H1-differentiating cells (Fig. 3B). Furthermore, sequential ChIP assay demonstrated co-occupancy of WASp with RNAP2 on at least two cis elements (WBR1 and WBR2) of the same promoter DNA (Fig. 3D). Because WASp does not contain the classical DNA binding motifs, we posit that recruitment of WASp to *TBX21* DNA may occur through its physical association with any of the classical DNA binding partners that interact with WASp (T-BET, STAT1, RNAP2, and MED1). In addition, as discussed earlier, Sp1- or Kruppel-like DNA binding proteins may also participate in recruiting WASp to the promoter DNA. Supporting this last idea is our finding that recruitment of Sp1 to the *TBX21* promoter is enhanced coordinately with that of WASp in differentiating T_H1 cells (Fig. 3B). Nonetheless, further studies are required to definitively show that WASp does not bind chromatin directly and, if so, to identify protein partners that recruit WASp to DNA in differentiating T_H1 cells.

Given a role for T-BET, STAT1, and TRAP-Mediator complexes in the epigenetic reprogramming of protein-coding gene promoters (23–25), the recruitment of WASp, direct or indirect, to the same *TBX21* chromatin cis sites, where these key regulatory proteins are also recruited, places WASp at the dynamic interface of transcription initiation. Such a result, therefore, suggested some level of WASp participation in the epigenetic control of *TBX21* activation in primary differentiating T_H1 cells, which we further investigated in this study.

The T_H1-driven recruitment of WASp to the *TBX21* proximal promoter accompanies large-scale epigenetic reprogramming of the local chromatin

The process of transcription initiation is identified by a well-characterized set of changes in the configuration and conformation of the promoter chromatin of protein-coding genes that identifies “active” versus “poised” or repressed genes (26–30). We therefore chose to use these in vivo epigenetic readouts as opposed to the in vitro promoter reporter assays to identify endogenous and physiologic *TBX21* gene activity. Accordingly, using the

quantitative MNase-ChIP qPCR assay, we show that the T_H1-driven recruitment of WASp to the *TBX21* promoter accompanies the inscription of chromatin signatures that were previously shown to correlate with activating or enhancer function of the genomic regions (26–30) (Fig. 3B). The snapshot view representing the average composition of the *TBX21* promoter loci (up to ~750 bp upstream of TSS) from the pooled mononucleosomal chromatin demonstrated that the permissive histone marks H2A.Z and H3K4me3 become enriched (H3K4me3 more markedly than H2A.Z) in primary T_H1 cells compared with those in the corresponding sample of the freshly isolated, undifferentiated T_H0 cells derived from the same donor. The T_H1-driven dynamic acquisition of these histone modifications at promoter loci of *TBX21* is in accordance with the previous findings of H3K4me3 and H2A.Z enrichment identified within the –1-kb region from TSS of active promoters of most protein-coding genes in human cells (31,32). Furthermore, coexistence of H3K4me3 mark with STAT1 binding at the three cis sites of the proximal *TBX21* promoter (Fig. 3B) also aligns well with the reported strong correlation (~80%) between H3K4me3 enrichment and proximal STAT1-binding sites in HeLa cells (20,21).

Consistent with the dynamic enrichment of H3K4me3 at the *TBX21* promoter is our other finding of a coordinate increase in the occupancy of RBBP5 (retinoblastoma-binding protein 5) (a core component of methyltransferases that mediates H3K4 trimethylation) in primary T_H1 cells compared with that in the undifferentiated T_H0 cells (Fig. 3B), whereas the degree of SET7/9 (H3K4 monomethylase) enrichment in T_H1 cells remained mostly unchanged from that in the undifferentiated T_H0 cells. Such a result is consistent with the idea that *TBX21* remains poised for rapid activation because the H3K4me1 mark (a harbinger of subsequent H3K4me2/H3K4me3 activating modifications) is enriched on the promoters and enhancers of many poised mammalian genes (20,27–29). Surprisingly, we also observed an enrichment of SMCX (H3K4me2/H3K4me3 demethylase), at least at the WBR2 and GAS cis sites, in T_H1 cells. Given the augmented promoter occupancy of H3K4me3 in T_H1 cells, such a result is counterintuitive but suggests that the H3K4 methylase and demethylase enzymes coexist on the same cis elements to fine-tune transcriptional readiness of the *TBX21* promoter during the process of T_H1 differentiation. Indeed, sequential MNase-ChIP experiments demonstrated co-occupancy of RBBP5 (H3K4 methylase) with WASp and of JMJD2A (H3K9/H3K36 demethylase) with WASp on the same chromatin segments of the *TBX21* promoter region (Fig. 3D). Together, these findings demonstrate that higher-order protein complexes that contain WASp, STAT1, and chromatin modifiers are being formed at the *TBX21* locus during T_H1 differentiation.

Next, because a strong countercorrelation between the promoter occupancy of H3K4me3 (a permissive mark) and H3R2me2 (a repressive mark) is described (33), we tested whether JMJD6 (H3R2/H4R3 demethylase) is also dynamically recruited to the *TBX21* promoter. Strikingly, we find increased promoter occupancy of JMJD6 in the T_H1-differentiating cells (Fig. 3B), which is consistent with the established paradigm above. Furthermore, we find nuclear WASp in complex with JMJD6 by reciprocal coimmunoprecipitation, and the deconvolution immunofluorescence images display at least partial colocalization of the two proteins in the nucleus (Fig. 4, C and D). However, we could not verify that the formation of these higher-order chromatin modifier complexes that likely also involves JMJD6 results in a concomitant erasure of the repressive H3R2 methylation mark because of the nonavailability of ChIP-grade H3R2me2 (asymmetric) antibody. Furthermore, the enrichment of H3K9 acetylation, known to associate with active gene promoters (27), was also augmented in T_H1-differentiating cells. Together, our results in Fig. 3B demonstrate that different permissive histone modifications (H3K4me3, H3K9ac, and H2A.Z) become prominently expressed at the *TBX21* proximal promoter in T_H1-differentiating cells.

On the other hand, the repressive mark H3K9me3 identified in undifferentiated T_H0 cells was diminished in differentiating T_H1 cells. Correspondingly, the enrichment of JMJD2A (H3K9 demethylase), previously shown to also bind H3K4me3 mark (34), was accordingly enhanced in differentiating T_H1 cells. Such a result would mechanistically explain the erasure of H3K9me3 mark at the H3K4me3-laden promoter locus by JMJD2A. Because the activating H3K9ac mark is reciprocally enhanced at the same mononucleosomal cis sites where H3K9me3 mark is depleted (Fig. 3B), a possibility for nucleosomal loss as an additional cause for H3K9me3 depletion is unlikely, at least in our setting. However, sequential ChIP assays are required to definitively establish this point.

Like H3K9me3, the repressive mark H3K27me3 was also diminished in differentiating T_H1 cells compared to T_H0 cells (Fig. 3B). However, here, the occupancy of JMJD3 (H3K27me3 demethylase) at the *TBX21* promoter was decreased (not increased) in the differentiating T_H1 cells. Such a result is contrary to our prediction, given the reported biochemical activity of JMJD3 as the H3K27 demethylase. However, such counterintuitive relation between JMJD3 and H3K27me3 modification has been previously reported in a genome-wide analysis of histone modifications in lipopolysaccharide-activated murine macrophages. Here, despite the contiguity of JMJD3 and H3K27me3 ChIP peaks at the proximal promoters of protein-coding genes, a JMJD3-dependent decrease in H3K27me3 enrichment rarely occurs (35). Together, these observations are consistent with the idea that site-specific balance of enzymatic activities of methylases and the opposing demethylases rather than the degree of their enrichment determines the final epigenetic code at the *TBX21* locus.

Importantly, this epigenetic reprogramming at the *TBX21* promoter locus accompanied the enrichment of heterochromatin protein-1 γ (HP-1 γ), a marker of actively transcribed chromatin, coordinately with the eviction of HP-1 α , a marker of repressed chromatin (36) (Fig. 3B). Accordingly, quantifying deoxyribonuclease I (DNase I) hypersensitivity (DHS) at WASp-bound cis sites (with the qPCR-based approach, see Materials and Methods) demonstrated a DHS pattern consistent with the acquisition of “open” promoter chromatin in primary differentiating T_H1 cells, whereas the corresponding undifferentiated primary T_H0 cells, in which we also show that the *TBX21* promoter expresses both H3K4me3 and H3K27me3 marks (29), demonstrated an intermediate DHS pattern consistent with a continuous range of chromatin accessibility that is characteristic of a poised promoter (Fig. 3C).

Collectively, these findings demonstrate that WASp is recruited to the *TBX21* promoter at multiple contiguous cis sites that collectively undergo large-scale reprogramming to switch the chromatin from poised to active, as the primary T_H cells transition from undifferentiated to T_H1-differentiated state.

Endogenous nuclear WASp coassociates with H3K4-trimethylating and H3K9-tridemethylating enzymes and activity

We next tested the hypothesis that WASp may function in the nucleus to render *TBX21* promoter chromatin “ready” for transcription during T_H1 differentiation. Because, as noted above, WASp-bound cis regions undergo permissive epigenetic reprogramming ($\uparrow$ H3K4me3, $\downarrow$ H3K9me3, $\downarrow$ H3K27me3), we investigated whether WASp associates with histone enzymes and/or their respective enzymatic activity that can modify these specific histone marks in primary differentiating T_H1 cells.

We first show that WASp, immunoprecipitated from the nuclei of primary T_H1 cells but not of undifferentiated T_H0 cells, catalyzes in vitro methylation of H3K4 to ~40% activity of the positive control H3K4 methyltransferase enzyme (Fig. 4A and fig. S15A) and converts unmodified recombinant human histone octamers to trimethylated H3K4 products (Fig. 4B).

This result, validated in samples from multiple normal donors, implied the physical presence of H3K4 methyltransferase(s) in the endogenous WASp complexes in primary differentiating T_H1 cells. Therefore, as a logical next step, we tested the interaction of WASp with RBBP5, a core subunit of H3K4 trimethylases (37), and found that this enzyme physically associated with endogenous nuclear WASp, verified by reciprocal coimmunoprecipitation (Fig. 4C). As controls, neither the IgG antibody nor the irrelevant LFA-1 antibody–derived nuclear immunoprecipitates from the same T_H1 cells associated with quantifiable H3K4 methylation activity in vitro (Fig. 4, A and B). Furthermore, 3D deconvolution images of the intact primary T_H1 cells captured partial colocalization of WASp with RBBP5 in the DAPI-dim regions of the nucleus (Pearson $r_p = 0.46$) (Fig. 4D and fig. S4). Conversely, nuclear WASp did not associate with the H3K4 tridemethylase activity in vitro (fig. S15B). These results are demonstrative of the specificity of WASp involvement with mechanisms that bolster the activating H3K4me3 modification in primary differentiating T_H1 cells.

Next, we tested the association of WASp with H3K9 methylase and demethylase activities because recruitment of WASp to the *TBX21* locus accompanied striking diminution of H3K9me3 mark in T_H1 cells (Fig. 3B). Here, nuclear WASp associated with H3K9 tridemethylase activity but not with H3K9 methylase activity in vitro (Fig. 4, A and B, and fig. S15B). Accordingly, WASp associated with JMJD2A (H3K9 demethylase) evidenced in reciprocal coimmunoprecipitation from nuclear lysates of primary T_H1 cells (Fig. 4C). Furthermore, the 3D deconvolution images displayed scattered foci of the two proteins partially colocalized in the nucleus (Pearson $r_p = 0.37$) (Fig. 4D).

Finally, we tested the association of WASp with H3K27-demethylating enzyme and activity. Here, despite the marked diminution of H3K27me3 mark from the WASp-bound promoter cis sites, WASp did not associate with either H3K27 tridemethylase enzyme (JMJD3) (Fig. 4C) or H3K27 trimethylase activity (fig. S15B). To rule out the possibility that our antibodies to WASp disrupt the interaction between WASp and JMJD3, we also verified the lack of this interaction by performing the reverse coimmunoprecipitation (immunoprecipitation: JMJD3, immunoblotting: WASp) (Fig. 4C). Nevertheless, mass spectrometry and other biochemical approaches are required to further clarify physical and functional relationship between WASp and JMJD3, if any. Similarly, studies clarifying the ill-understood structural basis for the observed specificity of WASp interactions with only certain chromatin-modifying enzymes or activities are also required.

At the minimum, our findings demonstrate association of endogenous nuclear WASp with histone-modifying enzymatic activities that are well known to be instrumental in imparting transcriptional readiness. Indeed, like the WASp-enriched complexes, other multiprotein complexes, including the one enriched with T-BET, associate with multiple histone-modifying enzymes or activities to regulate gene expression (24,38). However, future studies are required to clarify whether the purified WASp in isolation of its nuclear binding partners can support endogenous histone methylation or demethylation activity.

WASp-deficient primary T_H cells exhibit a repressive combinatorial histone code at the *TBX21* promoter under T_H1-differentiating conditions

To directly test whether WASp is necessary for reprogramming the *TBX21* promoter chromatin during T_H1 differentiation, we examined WASp-deficient primary T_H cells derived from multiple WAS patients. We chose to perform these studies in human primary T_H cells but not in murine T_H cells because the epigenetic profiling of the cis-regulatory regions of *IFNG*, T_H2 cytokine, and *FOXP3* loci during T_H differentiation has already revealed striking interspecies differences (39). More importantly, milder clinical variants [including X-linked thrombocytopenia (XLT): clinical severity score 1] (2) resulting from

WAS missense mutations cannot be modeled yet in mice, collectively making human studies more appropriate for testing the specific hypothesis of this study.

Therefore, primary T_H cells from three classical WAS patients (MSK001, MSK002, and NIH001) and multiple normal donors were activated under T_H1-polarizing in vitro conditions, and their epigenetic profiles at the *TBX21* promoter were examined (Fig. 5). Compared to normal T_H cells, the *TBX21* promoter in primary WAS T_H cells from all the three WAS patients revealed a marked decrease in the occupancy of the activating H3K4me3 mark contemporaneously with augmented occupancy of the repressive H3K9me3 and H3K27me3 marks at the mononucleosomal level (Fig. 5, A and B). Specifically, the T_H1-driven promoter enrichment of H3K4me3 at the *TBX21* locus was either low (in MSK001 and NIH001) or only modestly increased (in MSK002) compared with the steady-state values in the undifferentiated T_H0 control cells from the same WAS patients. This is in sharp contrast to the robust increase in H3K4me3 promoter occupancy observed in normal differentiating T_H1 cells. The overall occupancy of H3K4me3 at the *TBX21* promoter (average of all three cis sites) decreased by about 40 to 60% in the WASp-deficient primary T_H cells compared to that in normal T_H cells, activated under complete T_H1-biasing conditions. Such a result aligns well with the expected loss of WASp-associated H3K4 methylation activities in vivo. Therefore, the loss of WASp can phenocopy the loss of WDR5, a core component of H3K4 methylases, as far as the magnitude of H3K4me3 reduction at the genomic loci is concerned (26).

Next, we investigated whether the development of the repressive epigenetic profile (↓H3K4me3, ↑H3K9me3, ↑H3K27me3) at the *TBX21* promoter locus accompanies a concomitant lack of WASp binding to the promoter chromatin. As expected, the MNase-ChIP analyses on the same samples used for the histone studies above revealed complete absence of WASp from all three cis sites of the *TBX21* promoter in MSK001 and MSK002 patients. However, in the NIH001 patient [where the residual mutant WASp (deletion 422 to 427 amino acids in the proline-rich domain) is expressed in ~20% T cells], WASp was detected only at the WBR2 locus but not at the WBR1 or GAS sites. Here, despite the presence of the mutant WASp at this single locus, the combinatorial histone code (↓H3K4me3, ↑H3K9me3, ↑H3K27me3) at the *TBX21* promoter remained nonpermissive for actuating T-BET transcription (Fig. 5B). Such a result underscores the requirement for cooperativity among multiple cis-active regions to collectively support epigenetic transcriptional events to actuate productive gene activation.

Accordingly, we note a selective deficiency in the expression of *TBX21* mRNA compared to that of *GATA3* or *RORc* in NIH001 T_H cells activated under T_H1-polarizing conditions (fig. S16A). Such a result aligns well with the ChIP-chip data demonstrating the selective binding of WASp to the *TBX21* promoter but not to the core promoters of *GATA3* or *RORc* in the normal T_H1 cells and could mechanistically explain the observed selective deficiency of T_H1 functions in WAS T_H cells. The cytokine profile, consistent with an impaired T_H1 activation, was also captured in the MSK002 patient, as well as in another WAS patient (SEA001) bearing the clinical severity score 5A (fig. S16B). Even under phorbol 12-myristate 13-acetate–ionomycin activation (which bypasses the TCR proximal signaling events to directly activate protein kinase C and calcium mobilization), WAS T_H cells from the MSK002 patient still displayed a marked imbalance in favor of T_H2 cytokine production. Such a result consolidates our hypothesis that besides its membrane-localized cytoskeletal effects on proximal TCR signaling events, WASp likely has unique downstream functions in other subcellular compartments (for example, nucleus) that regulate activation of its target genes.

Collectively, these findings demonstrate that loss of WASp accompanies aberrant histone methylation dynamics at the *TBX21* promoter locus that is mirrored in impaired T_H1 activation and function in WASp-deficient primary T_H cells in this subset of WAS patients.

Loss of WASp accompanies a concomitant decrease in the *TBX21* promoter-localized enrichment of RBBP5 and F-actin but not of JMJD2A or actin-related protein 2/3

Because WASp physically associates with RBBP5 (H3K4 trimethylase) and JMJD2A (H3K9 trimethylase), we asked whether the loss of WASp accompanies a concomitant defect in the promoter recruitment of these histone modifiers to mechanistically explain the locus-specific inscription of the repressive histone code ($\downarrow$ H3K4me3, $\uparrow$ H3K9me3) observed in the WAS T_H cells. These studies were designed to investigate the putative “adaptor” or “cargo” functions of WASp at the chromatin. Therefore, MNase- μ ChIP (micro-ChIP) assay (with ~1000 to ~5000 primary T_H cells) was performed with antibodies to RBBP5 and JMJD2A. This assay showed that whereas the promoter enrichment of RBBP5 was reproducibly reduced, that of JMJD2A remained largely unaffected in WAS T_H cells activated to differentiate down the T_H1 pathway (Fig. 5C). Although the magnitude of RBBP5 decrement at the promoter varied between the three WAS patients ranging from ~40 to ~55% reduction compared to normal, this result mechanistically links WASp to the recruitment of RBBP5-containing histone-modifying complexes on the *TBX21* promoter and offers an explanation for the resultant diminution of H3K4me3 mark at the *TBX21* promoter in WASp-deficient T cells. However, the role of WASp in this process appears to be contributory at best because residual RBBP5 is still identified at the *TBX21* promoter in WAS T_H cells, implying that other factors or mechanisms to recruit RBBP5 to DNA remain operational even in the absence of WASp. Indeed, the constitutive presence of RBBP5 at the *TBX21* locus in normal T_H0 cells, where WASp is mostly extranuclear, suggests other physiologic mechanisms for the recruitment of this enzyme to the steady-state poised chromatin. Certainly, for JMJD2A, WASp appears to be less important at least for its physical recruitment to the promoter in T_H0 or T_H1 cells (Fig. 5C).

We next investigated the effect of WASp on the chromatin-resident F-actin events during T_H1 differentiation. It is well established that WASp binds actin monomers and can polymerize actin in the cytoplasm through its association with the putative actin-related protein 2/3 (ARP2/3) complex (5). Recent studies have highlighted a role for nuclear actin, both monomeric and polymeric, in gene transcription (13,14). We therefore investigated the putative actin-organizing function of WASp in the nucleus, specifically in connection to *TBX21* activation and T_H1 differentiation.

First, we tested by the MNase-ChIP assay whether ARP2/3 and F-actin are enriched at the *TBX21* promoter in normal T_H1-differentiating cells. We show that the acquisition of permissive chromatin dynamics at the *TBX21* promoter, as depicted in Fig. 3B, also accompanies enhanced recruitment of both ARP2/3 complex and F-actin at this promoter in T_H1 cells. Although low-level ARP2/3 occupancy at the *TBX21* promoter was identified even in the undifferentiated T_H0 cells, the F-actin signals were not, and the latter appeared only in the differentiating T_H1 cells (Fig. 3B). This finding suggests that chromatin-resident actin polymerization at the promoter locus likely accompanies transcription of *TBX21*. These results, although hinting at WASp as a potential contributor of nuclear F-actin, consolidate the emerging role of nuclear ARP2/3 and F-actin in RNAP2-dependent gene transcription in general and for *TBX21* in particular (40).

However, besides WASp, because many other nuclear ABPs can potentially support nuclear actin polymerization, we next tested the degree to which WASp contributes to this process, if any. We used the WASp-deficient primary T_H cells from the three WAS patients to test whether a natural loss of WASp accompanies a concomitant decrease in the chromatin-

associated ARP2/3 and F-actin enrichment during T_H1 differentiation. The MNase-μChIP assay revealed that whereas the degree of promoter enrichment of F-actin was reduced in MSK001 and MSK002 T cells, it remained normal in NIH001 T cells activated under T_H1-differentiating conditions (Fig. 5C). This suggests that, at least in MSK001 and MSK002 T cells, the cytoplasmic actin-organizing function of WASp that may be co-opted in the nucleus is disturbed in the absence of WASp. However, independently of whether F-actin enrichment was normal or abnormal, the epigenetic profile at the *TBX21* promoter was abnormal in each of the three WAS patients.

On the other hand, loss of WASp had minimal (in MSK001) or no effect (in MSK002 and NIH001) on the promoter recruitment of ARP2/3. This result in WAS T_H cells (T_H1-activated), along with our findings of ARP2/3 enrichment at the *TBX21* promoter in normal undifferentiated T_H0 cells (where WASp is nonnuclear), allows us to hypothesize that the nuclear localization of ARP2/3 and its recruitment to the chromatin may occur independently of WASp. Clearly, this possibility needs to be directly tested with WASp mutants that cannot bind ARP2/3 and therefore elicit actin polymerization.

WASp is required to achieve transcription-permissive chromatin dynamics at the *TBX21* promoter locus in T_H1-differentiating cells

To ascertain that the abnormal epigenomics at the *TBX21* promoter in WAS primary T_H cells is a direct consequence of WASp deficiency and is not merely secondary to patient's disease milieu, we performed two sets of experiments: (i) tested whether normal T_H cells depleted of WASp by RNA interference (RNAi) recapitulate the deranged WAS T_H epigenotype at the *TBX21* locus, and (ii) tested whether reconstituting patient-derived, WASp-deficient T_H cells with normal WASp is sufficient to rectify the abnormal chromatin dynamics at the *TBX21* locus and restore T-BET mRNA and protein expression.

First, in a purified population of T_H1-activated Jurkat T cells depleted of WASp via RNAi [green fluorescent protein (GFP) enriched by fluorescence-activated cell sorting (FACS)] (Fig. 6A), we demonstrate that the *TBX21* promoter acquires the same repressed histone configuration (↓H3K4me3, ↑H3K9me3) as in primary WAS T_H cells (Fig. 6, E and F) and, accordingly, exhibits a failure to up-regulate the *TBX21* mRNA and the expression of both T-BET and its downstream target RUNX3 in response to T_H1-differentiating signals (Fig. 6, B to D). However, this acute depletion of WASp had no discernible effect on the mRNA expression of *GATA3* and *GAPDH* or on N-WASp protein expression. On the other hand, Jurkat T cells transfected with the control RNAi vector demonstrated normal permissive reprogramming of the *TBX21* promoter and the expected up-regulation of *TBX21* mRNA under T_H1 differentiating conditions (Fig. 6F). Notably, Jurkat T_H cells sampled from the same T_H1-polarizing culture on day 10 after short hairpin RNA (shRNA) introduction revealed spontaneous recovery of WASp mRNA expression concomitantly with that of both *TBX21* and *IFNG*, which were either comparable or marginally higher than those of their controls (fig. S16C). These knockdown studies demonstrate an intimate link between the expression of WASp and T-BET and strongly support our contention that the observed aberrant *TBX21* epigenomics and the resulting defect in T-BET expression in WAS T_H cells are causative and not a consequence of systemic immune deficiency in human WAS.

Second, for the WASp reconstitution studies, we chose to perform the experiments in human T cell leukemia virus (HTLV)–immortalized WAS T_H lines because the gene transfer study in primary WAS T_H cells is severely limited by the small number of cells that can be repeatedly obtained from the same child with WAS. Although a variety of immortalized cell lines, including Jurkat T_H cells, have been used in many reported epigenetic studies, we first ascertained and demonstrated in fig. S17 that (i) undifferentiated human CD4⁺ T_H lines require T_H1-polarizing cytokines to undergo T_H1 differentiation, demonstrating that

immortalization per se does not render T_H cells independent of growth factor requirements for T_H differentiation (fig. S17C); (ii) only *TBX21* but not *GATA3* mRNA expression is augmented under T_H1 polarization, demonstrating that the gene activation programs that reciprocally instruct T_H1 over T_H2 choices are intact in immortalized T_H lines; (iii) chromatin dynamics (histone modifications and DHS) at the *TBX21* promoter locus are remarkably similar in normal primary T_H cells and T_H lines undergoing T_H1 differentiation (fig. S17, A and B); and (iv) neither viral immortalization nor exogenous interleukin-2 (IL-2) in the culture is sufficient to allow identification of WASp in the nucleus of the undifferentiated T_H0 cells, suggesting that these stimuli by themselves are inadequate to affect WASp nuclear transport (Fig. 1B). These results indicate that for the parameters tested, our human T_H lines not only are physiologically suitable, but also provide a source for large numbers of cells with consistent properties.

With this validation of human T_H lines, we next compared the chromatin dynamics in normal T_H lines with patient T_H lines. In the WAS T_H line (WAS-5 T_H), where WASp is undetected at the promoter DNA by MNase-ChIP (Fig. 6E), the *TBX21* promoter displays a pronounced diminution of the activating H3K4me3 mark compared with that in normal T_H line, activated under T_H1-differentiating conditions (Fig. 6, E and F). In contrast, the occupancy of the repressive H3K9me3 and H3K27me3 marks was reciprocally augmented. This repressed epigenetic profile (↓H3K4me3, ↑H3K9me3, ↑H3K27me3), once again, is reminiscent of the one identified in primary human WAS T_H cells, activated under T_H1-differentiating conditions. On the other hand, the absence of WASp did not adversely affect the T_H1-driven enrichment of the histone variant H2A.Z (the other activating histone mark) or H4K91ac (a modification associated with chromatin assembly) (41), highlighting the specificity of WASp in affecting only certain histone modifications (fig. S18). Whether the presence of H2A.Z in this setting imparts permissive or repressive functions to the *TBX21* promoter DNA in WAS T_H cells remains to be ascertained. We, however, speculate that H2A.Z occupancy at the *TBX21* promoter in WAS T_H cells is unlikely to impart transcription-permissive functions because the coassociation of H2A.Z with H3K27me3, as in WAS T_H cells, has been previously shown to render the H2A.Z mark functionally repressive through ubiquitylation (42). Similarly, RNAP2 (unmodified CTD) occupancy at the *TBX21* promoter was not coordinately decreased by the loss of WASp (fig. S18), suggesting that RNAP2 and WASp are likely independently brought into nascent chromatin-resident complexes, in a model that supports “neoassembly” rather than “preassembly” of the trans-regulatory complexes. Such a finding also implies that despite the association of WASp with RNAP2 (Fig. 1, F and G), whose promoter occupancy is more widespread across the T cell genome than that of WASp, WASp effects are likely limited to the genomic loci it targets.

Therefore, in human primary T_H cells and T_H lines that are WASp-deficient from natural mutations or RNAi depletion, the ensuing failure to enrich the activating H3K4me3 and erase the repressive H3K9me3 and H3K27me3 marks at the *TBX21* promoter in response to T_H1-differentiating signals is identified as a key epigenetic aberration in human WAS T_H cells.

Indeed, either a decrement in H3K4me3 or an increment in H3K9me3 alone has been shown to induce locus-specific, facultative heterochromatinization that draws the promoter chromatin into an inaccessible structure (43–45). Accordingly, because we show that both these repressive epigenetic events occur at the *TBX21* promoter in T_H1-activated WAS T_H cells, the promoter is rendered nuclease (DNase I)–insensitive (Fig. 6G), whereas the *TBX21* promoter in the normal T_H line, as expected, displays a DHS pattern that is consistent with an “open” chromatin conformation under T_H1-differentiating conditions. These derangements in chromatin configuration and conformation are mirrored by deficient *T-BET*

mRNA and protein expression in WAS T_H cells compared to normal T_H cells, both activated under T_H1-differentiating conditions (Fig. 6, H and I). In contrast, the absence of WASp did not affect the expected up-regulation of GATA3 protein expression in the same WAS T_H line activated under T_H2-polarizing conditions (fig. S19). These findings demonstrate that WASp expression is required to establish an active chromatin state at the *TBX21* promoter to actuate the transcription of *T-BET* in T_H1-differentiating cells.

These results allowed us then to determine the effect of WASp reconstitution on the aberrant chromatin dynamics in WASp-deficient T_H cells. Accordingly, in the same WAS T_H line reconstituted with normal WASp (WAS-5 corr), which we show locates to the nucleus and binds *TBX21* promoter under T_H1 conditions (Fig. 6E), H3K4me3 is markedly enriched, whereas H3K9me3 and H3K27me3 marks are depleted in tandem with the return of exquisite DHS and resultant restoration of T-BET expression in the nucleus (Fig. 6, E to H). These results suggest that in the absence of WASp, labile and flexible mechanisms to repress transcription become operational at the *TBX21* promoter but are rapidly and completely reversed after WASp reconstitution. Such a finding is consistent with the idea that WASp affects epigenomics mainly through its effects on reversible histone modifications, as demonstrated in this study; however, we cannot rule out potential effects of WASp on DNA methylation, especially because the latter is now shown to also be reversible (46). Together, these findings establish an obligate nuclear role for WASp in the coordinated regulation of *TBX21* chromatin dynamics and transcription in response to T_H1-differentiating cytokine signals.

Mutant WASp in the mildest clinical variant of WAS (XLT) supports permissive chromatin dynamics and normal transcription of *TBX21*

After defining a profile of epigenetic derangement in T_H cells from WAS patients with clinically severe disease (score 5A), we tested the limited hypothesis that a WAS missense mutation that manifests clinically as thrombocytopenia without systemic immunodeficiency or autoimmunity (clinical severity score 1: WAS-1) results in the expression of a mutant protein that still can support the above chromatin functions in the patient-derived T cells.

Therefore, we examined the degree of chromatin derangement at the *TBX21* promoter in a T_H line generated from a patient with the mildest clinical disease (WAS-1). Here, the WAS genotype (missense mutation at His³⁰) allows the physiologic expression of mutant WASp of normal molecular mass (~62 kD) that locates to the nucleus and binds the *TBX21* promoter under T_H1-differentiating conditions (Figs. 6E and 7A). Strikingly, the *TBX21* promoter in T_H1-activated WAS-1 T_H cells displayed permissive chromatin dynamics (↑H3K4me3, ↓H3K9me3, ↓H3K27me3, and intermediate DHS profile) that were mostly similar to one in normal T_H line (Fig. 6, E to G). Accordingly, WAS-1 T_H line activated under T_H1-polarizing conditions demonstrated augmented expression of *T-BET* mRNA and protein, as well as of the downstream target gene of T-BET, *IFNG*, without much affecting the expression of *GATA3* or *IL13* (Fig. 7A). This result shows that the missense mutation in this patient does not perturb the tested chromatin activities of the residual mutant WASp.

To test directly the mechanistic hypothesis that the mutant WASp (in WAS-1, XLT patients) is still able to physically interact with the chromatin-modifying complexes to inscribe permissive epigenetic code, we examined the T_H1-differentiating cells from the above WAS-1 T_H line and primary T_H cells from another WAS-1 patient (NIH002). The WAS genotype (231–240 del10insC) in NIH002 results in an internal truncation of three amino acids (Asn⁷⁸-Glu⁸⁰ del) that still allows expression of the mutant WASp by FACS. Under T_H1-differentiating conditions, the mutant WASp in the T cells from the two WAS-1 patients locates successfully to the nucleus and associates with both RBBP5 (Fig. 7B) and *TBX21* promoter DNA (Fig. 7C). We show that the degree of enrichment of both RBBP5

and H3K4me3 mark at the *TBX21* promoter is comparable to those of the normal controls (Fig. 7, C and D). Therefore, the main defect of T_H cells completely lacking WASp (that is, paucity of H3K4me3 at *TBX21* promoter) is not present in T_H cells expressing this mutant WASp in both WAS-1 patients. Furthermore, we show that the mutant WASp (in WAS-1 T_H line) coimmunoprecipitates with JMJD2A and RNAP2; in addition to RBBP5 and this binding profile of mutant WASp corresponds to that of normal WASp (Fig. 7B).

However, the same missense mutations in these WAS-1 patients still impair important functions in other cell types of the hematopoietic lineage (that is, platelets) that result in nonimmunologic, life-threatening complications arising from microthrombocytopenia, a clinical hallmark of XLT. Such a result raises the possibility that the cytoplasmic and nuclear functions of WASp may be cell type-specific and/or WASp domain-specific. The collective evidence, therefore, highlights the plasticity that WASp possesses to support diverse cellular functions in multiple subcellular compartments within the context of T_H1 differentiation. Although further research is required to address this issue, our limited analysis of two extreme clinical grades of WAS (clinical severity scores of 1 and 5) demonstrates that the degree of epigenetic aberration at the *TBX21* locus is mirrored in the magnitude of T_H dysfunction and reflects the overall clinical severity of WAS for a given WAS genotype.

DISCUSSION

This study identifies a previously unsuspected regulatory role for WASp in the nucleus. Our data demonstrate that WASp, better known for its actin-polymerizing function in the cytoplasm, also resides in the nucleus in response to T_H1-differentiating extracellular signals, where it seems to have an intimate role in the transcription of the T_H1 master regulator gene *TBX21*. WASp contains a cluster of positively charged basic amino acids (²²⁵Lys-Lys-Arg-Ser-Gly-Lys-Lys-Lys²³²), which we speculate may serve as a nuclear localization sequence for the nuclear entry of WASp via the classical importin pathway. In the nucleus, WASp is located mostly in the extranucleolar euchromatic regions, where it colocalizes with hyperphosphorylated RNAP2 (Ser²), suggesting its physical presence within the transcription factories. Accordingly, nuclear WASp is found to associate at least with T_H1 transcription factor (STAT1), basal transcription complexes (RNAP2 and TRAP220/MED1), and chromatin modifiers (RBBP5, JMJD2A, and JMJD6). The composition of these WASp-enriched proteomes is well endowed as a unit to support transactivation of certain T_H1-specific genes. Accordingly, during the process of T_H1 differentiation, WASp and its above nuclear-binding partners are recruited to the 5' proximal promoter of the *TBX21* gene at multiple cis-regulatory sites clustered within ~750 bp upstream of TSS. Sequential MNase-ChIP assay demonstrated co-occupancy of WASp with STAT1-, RNAP2-, RBBP5-, or JMJD2A-containing higher-order protein-DNA interactomes at the promoter locus of *TBX21*. This dynamic combinatorial binding of multiple regulatory proteins to the same genomic region of *TBX21* is well suited to integrate T_H1-differentiating signals for rapid and robust T-BET-activating response (47).

Accordingly, the three tested cis sites at the *TBX21* promoter collectively undergo large-scale epigenetic reprogramming, as the primary T_H cell transitions from an undifferentiated state to a T_H1-differentiated state. During this process, the T_H1-driven association of WASp (and other regulatory factors) to the endogenous *TBX21* DNA is accompanied by locus-specific augmentation of the permissive H3K4me3 and H2A.Z marks, and eviction of the repressive H3K9me3 and H3K27me3 marks, coordinately with the lifting of focal heterochromatin (HP-1α). These histone modifications are well-known important recognition platforms for many chromatin-remodeling factors that collectively impart transcriptional readiness (48,49). Accordingly, *TBX21* mRNA expression is augmented in

normal differentiating T_H1 cells compared to their undifferentiated counterparts. On the other hand, WASp-deficient T_H1 cells, in which the *TBX21* promoter is devoid of WASp, fail to sufficiently up-regulate *T-BET* mRNA expression and achieve T_H1 effector function (low *IFNG* mRNA and protein expression), whereas the expression of GATA3 and T_H2 cytokines (*IL4*, *IL5*, *IL13* mRNA, and protein expression) remains unaffected or is even exaggerated in a subset of WAS patients (and certain WAS murine models) that we and others have studied (3,4). Collectively, our study identifies WASp as an important component protein in the control of T-BET gene activation at the chromatin level.

Chromatin effects of WASp in the nucleus

In this study, we provide an epigenetic molecular mechanism for the reported deficiency of *T-BET* mRNA and impaired T_H1 differentiation observed in a subset of WAS patients. Our findings in normal T_H1 cells of the engagement of WASp with the *TBX21* promoter in vivo suggested its participation in controlling *T-BET* gene activation at the chromatin level. This result led us to test the mechanistic hypothesis that loss of WASp nuclear function impairs *T-BET* gene activation. Here, we have demonstrated in the WAS T_H cells that loss of WASp perturbs the epigenetic dynamics at the *TBX21* promoter locus during the process of T_H1 differentiation. Specifically, WASp deficiency accompanies significant diminution of promoter-enriched H3K4me3, a histone mark considered to be a critical component in regulating gene expression through a variety of mechanisms including by facilitating histone acetylation events (26). This defect in WAS T_H cells accompanies a reciprocal increase in the enrichment of H3K9me3- and H3K27me3-repressive marks. Therefore, a pathophysiologically significant defect observed in WAS T_H cells is the impaired inscription of the permissive combinatorial histone code at the *TBX21* promoter during T_H1 differentiation.

The evidence that WASp is involved in the locus-specific, activating modifications of H3K4 trimethylation and H3K9 tridemethylation at the *TBX21* locus is provided by our findings that (i) WASp physically associates with nuclear RBBP5 (a core component of H3K4 trimethylases) and JMJD2A (H3K9 demethylase) proteins, as well as with their respective enzymatic activities; (ii) co-occupancy of WASp with both RBBP5 and JMJD2A at the same *TBX21* promoter locus is demonstrated by sequential ChIP-qPCR assay; (iii) RBBP5 recruitment to the *TBX21* promoter locus decreases in the absence of WASp; (iv) the loss of WASp because of mutation or RNAi-mediated depletion results in a striking decrease in the “activating” H3K4me3 mark in vivo, and a reciprocal increase in the repressive H3K9me3 and H3K27me3 marks in the pool of *TBX21* promoter mononucleosomes; and (v) there is reversal of the *TBX21* promoter epigenomics from repressive to permissive chromatin dynamics along with increased T-BET expression in patient-derived WAS T_H cells reconstituted with normal WASp. These results collectively demonstrate that endogenous WASp is integral to the chromatin activities that orchestrate productive transcription of the *T-BET* gene and links aberrant histone methylation at the proximal promoter of *TBX21* to dysregulated T_H1 immunity in the human WAS disease. However, whether WASp is a primary or sole driver for T-BET gene activation is not experimentally established in our study, although the likelihood for this to be the case is low.

Besides *TBX21*, WASp targets many other immune function genes that together form a gene network that collectively instructs T_H1 cell fate choice (for example, *T-BET*, *RUNX3*, *STAT1*, *STAT4*, *IL12Rβ2*, and *IFNG*). Although experimental validation is wanting, these chromatin effects of WASp on the *TBX21* gene may also manifest in other T_H1 network genes. If so, loss of WASp would impair transcriptional activation of the entire T_H1 gene network, resulting in loss of T_H1 functions.

In a subset of WAS patients and in some murine models of WAS, deficient T_H1 functions allow the heightened manifestation of the unopposed T_H2 functions (fig. S16) (3,4). Such unopposed T_H2-“ness” has the potential to further bolster the local repressive environment at the *TBX21* locus by promoting STAT6/GATA3-dependent enrichment of the repressive H3K27me3 mark, as shown previously for the *IFNG* locus in T cells activated under T_H2-biasing conditions (50). Accordingly, in WAS T_H cells that are futilely attempting T_H1 differentiation, we observe an increased occupancy of the repressive H3K27me3 mark at the *TBX21* promoter when compared to their normal T_H1-differentiating counterparts. Furthermore, the presence of H3K27me3 at the gene promoters that either lack or have a diminished expression of H3K4me3 (as in WAS T_H cells) not only impairs transcription initiation but also can adversely affect postinitiation phases of *T-BET* transcription that may already be in progress (45,51). Indeed, the ChIP-chip profile of WASp binding in vivo demonstrates WASp signals within the body of the gene, which allows us to speculate that WASp might affect transcription through its yet to be defined effects on transcription elongation. This idea is further supported by our findings of the association of WASp with hyperphosphorylated RNAP2 (Ser²), an elongation-specific modification of RNAP2. Certainly, future research is warranted to address the interaction of WASp with the process of gene transcription elongation and termination to investigate whether the abnormal epigenomics at the 5' *TBX21* promoter locus is a cause or a consequence of impaired gene activation.

Another significant finding is that WASp deficiency is accompanied by a reproducible decrease in the physical enrichment of RBBP5 (H3K4 trimethylase) at the *TBX21* promoter during T_H1 activation. This result mechanistically links WASp to the recruitment of RBBP5-containing histone-modifying complexes on the *TBX21* promoter and offers an explanation for the resultant diminution of H3K4me3 at this promoter in WASp-deficient T cells. Conversely, however, we did not find significant diminution of JMJD2A (H3K9 demethylase) enrichment to explain the observed high occupancy of H3K9me3 at this promoter in WAS T_H cells. Even in normal undifferentiated T_H0 cells, we do not find the expected reciprocal relation between the enrichment of JMJD2A and H3K9me3 at the GAS cis site (Fig. 3B). Given that JMJD2A can bind both H3K4me3 and H3K9me3 marks and associate with both co-repressor and co-activator chromatin complexes (52), we speculate that the final activity of JMJD2A at the *TBX21* promoter in vivo is determined not only by the magnitude of its enrichment but also by the type of cofactors it associates with in a function involved more in fine-tuning the local epigenetic code. Supporting this idea is our finding that despite comparable enrichment of JMJD2A at the *TBX21* promoter in T_H0 and T_H1 cells, H3K9me3 mark is markedly diminished only in T_H1 cells (Figs. 3 and 6). Like JMJD2A, another JmjC demethylase (JMJD3) has been reported not to diminish the degree of trimethylation of its known biochemical substrate (H3K27me3) despite the overlapping of JMJD3 and H3K27me3 enrichment peaks on the same genomic regions (35).

These and other published data indicate that different histone-modifying enzymes collaborate to inscribe the final combinatorial histone code, permissive or repressive, to affect gene transcription. In light of this, a mechanistic question that is relevant to further dissecting the role of WASp in chromatin function(s) is whether RNAi-mediated depletion of any one of the chromatin-modifying binding partners of WASp (that is, RBBP5, JMJD2A, or JMJD3) is sufficient to impair *TBX21* transcription. Although we have not performed these molecular studies, the current knowledge does not support prediction of such an outcome. Specifically, it has been shown that deletion of single genes encoding the MLL/SET H3K4 histone-modifying enzymes (53,54) or the polycomb group proteins (EZH2) (55) or the JmjC protein JMJD3 (35) had a modest, if at all any, effect on the mRNA expression of most of its target genes. Such in vivo findings are counterintuitive because they do not align with the corresponding in vitro biochemical evidence but suggest

that functional redundancy likely exists among histone modifiers to compensate for the locus-specific function of the deleted enzyme. In this connection, further research is required to characterize fully the entire proteome of histone modifiers that WASp might associate with, to determine which histone-modifying functions are most perturbed by the loss of WASp, and to verify whether this defect results in the altered transcript level of WASp target genes. Pending interrogation of these molecular details, at the minimum, our findings demonstrate that primary T_H cells lacking WASp display a chromatin configuration (histone modification profile) and conformation (DHS pattern) that are unsupportive of *TBX21* transcription, a defect that is reflected in a decrease in T-BET mRNA expression under T_H1-differentiating conditions.

Although not experimentally established, these chromatin effects of WASp on the *TBX21* gene may be manifested in other cell types, for example, CD4⁺CD25⁺ T_{reg} cell, where the epigenetic derangements uncovered by this study may underlie the impaired function and homeostasis of WASp-deficient T_{reg} cells through the effects of WASp on *TBX21* activation (8,56,57). Indeed, we show that WASp targets *FOXP3* and *CTLA4*, two genes important in T_{reg} differentiation and function. Clearly, future work will need to address whether the specificity of the association of WASp in vivo to the T_H-specific gene networks is determined by the state of T_H differentiation (that is, T_H1 versus T_H2 versus iT_{reg} versus T_H17). Such genome-wide protein-DNA studies will begin to explore the possibility that, depending on the context of T_H differentiation, WASp may associate with both permissive and repressive epigenetic mechanisms on the cis-regulatory elements of the alternatively expressed cluster of genes involved in patterning mutually exclusive T_H cell fates. Our customized ChIP-chip studies already reveal a host of WASp target genes important not only for T_H differentiation but also for other important cellular functions. For example, we show abundant WASp occupancy at the genomic loci of *FOXO1*, *NFκB*, and *STAT3*, the critical component genes in the control of survival and apoptotic mechanisms. This finding is tantalizing, since WAS lymphocytes are prone to accelerated apoptotic death, and offers early leads into uncovering the molecular underpinnings of imbalanced cell survival versus apoptosis and malignant transformation in WAS. It is therefore likely that loss of WASp may perturb activation of some or all of its target genes (and its designated functions), which could have critical effects in the pathogenesis of human WAS.

Epigenotype-phenotype correlation in human WAS

Our limited analyses of two distinct WAS mutations suggest that the chromatin effects of WASp are specified by the type of WAS mutation. Missense mutations, which manifest in a mild clinical disease [WAS clinical grade 1 (WAS-1), XLT], display *TBX21* dynamics that are almost similar to those in normal T_H cells undergoing T_H1 differentiation. Indeed, we find that such mutations still allow expression of the residual mutant protein that locates to the nucleus, binds trans-regulatory protein complexes (histone modifiers and RNAP2), and associates with the *TBX21* chromatin. As a result, epigenetic reprogramming of the promoter proceeds as expected to support normal transcription of T-BET under T_H1-differentiating conditions. Analysis of T_H cells from multiple WAS patients with mild to moderate clinical grade (WAS-1 to WAS-3) would likely reveal a spectrum of epigenetic defects. Such analyses, currently impossible to model in mice, could be valuable in the identification of patients likely to present with, or progress to, severe disease. At a minimum, therefore, our study offers a new readout to genetically and epigenetically characterize the different clinical forms of human WAS thereby generating a predictive correlation between molecular diagnostics and symptom severity.

Actin effects of WASp in the nucleus

Although these newly identified chromatin effects of WASp offer a nuclear mechanism for the dysregulated T cell immunity in human WAS, this defect may theoretically still relate, in part, to the loss of the canonical cytoplasmic role of WASp in regulating the dynamics of the T_H cell immunological synapse (58). It is at the T cell immunological synapse where the cytokine receptor signalosomes form to control inaugural membrane events that determine the mutually exclusive developmental pathways taken in T_H1 versus T_H2 differentiation (59). In addition to a structural role for actin in the cytoplasm, a role for this protein, as well as for ARPs and other ABPs, including nuclear myosins (for example, NM1 isoform), in signal-induced gene expression programs is rapidly emerging (13,14).

In this regard, our study addresses, in a limited fashion, a potential for the classic role of WASp as a modulator of actin polymerization to apply to chromatin dynamics. In primary T_H1 cells, we show by the MNase-ChIP assay that F-actin enrichment at the *TBX21* promoter occurs coordinately with that of WASp. This suggests that the actin-organizing function of cytoplasmic WASp may be co-opted by nuclear WASp for creating transcription-ready actin-rich complexes at the *TBX21* locus. We show that loss of WASp accompanies a notable decrease in the enrichment of F-actin at the *TBX21* promoter, but only in some (MSK001 and MSK002), not all, WAS patients. Indeed, the promoter enrichment of F-actin was normal in the T_H cells from the NIH001 patient despite the finding of abnormal promoter epigenomics at the *TBX21* locus and the clinical manifestations of severe disease (WAS-5A) in this patient.

The findings in the NIH001 patient, therefore, bolster the possibility that WASp may support a subset of nuclear functions that do not require its role as an actin modifier and yet are important for reprogramming the chromatin. These results also suggest that in the absence of WASp, other nuclear factors or mechanisms can contribute toward generating locus-specific F-actin complexes, as in the NIH001 patient. Such F-actin-enriched chromatin complexes containing NM1 have been shown to serve as molecular motors that propel the RNAP2 complexes through different stages of transcription, that is, initiation, elongation, and termination (60). In this connection, it remains to be clarified if the nuclear role of WASp is limited to supporting this motor function of overcoming the obligatory RNAP2 pausing at the 5' end of the transcribing genes or if it has additional chromatin functions that can occur independently of its actin role.

Indeed, our findings demonstrating impaired chromatin recruitment of RBBP5 in WASp-deficient T_H cells suggest a loss of the putative cargo function of WASp of recruiting this histone-modifying enzyme to the promoter locus of the *TBX21* gene. However, whether these two defects (chromatin and actin) identified at the *TBX21* promoter in WAS T_H cells of certain patients occur concomitantly or sequentially remain to be mechanistically clarified. Also, because WASp can bind β -actin monomers, an integral component of the SWI/SNF chromatin-remodeling complexes, what putative roles WASp may play in the adenosine triphosphatase activity of this complex, independent of its actin-polymerizing effect, also remain unknown.

Consequently, current knowledge does not allow us to exclude any molecular mechanism by which WASp could potentially function in the nucleus, that is, as an allosteric adaptor or scaffold protein or an actin-organizing protein, but the likelihood that these WASp functions are mutually exclusive, physically and temporally, we predict, is low. Therefore, research into these and other issues related to the roles of the various forms of nuclear actin (monomeric, oligomeric, polymeric, and ARP2/3-driven branched F-actin versus nuclear formin-driven linear F-actins) in eukaryotic gene transcription would greatly expand our ability to uncover new roles of WASp in the nucleus and possibly offer molecular targets

and therapeutic strategies to manage this complex childhood genetic disease with high mortality and morbidity.

MATERIALS AND METHODS

WAS genotypes and patient characteristics; cells and reagents

Human blood samples were obtained and handled under procedures approved by the Institutional Review Boards of University of Pittsburgh, University of Washington, Memorial Sloan-Kettering Cancer Center, and the National Human Genome Research Institute. Human primary CD4⁺ T cells (T_H) from multiple donors were isolated by negative selection (Miltenyi) from the unwanted buffy coat peripheral blood mononuclear cells (PBMCs) obtained from the Central Blood Bank of Pittsburgh. The undifferentiated primary T_H cells were used fresh, immediately after their separation, to avoid potential salutary effects of exogenous serum or growth factors on any steady-state physiologic events. To induce in vitro T_H1 differentiation, we activated primary T_H cells with recombinant human IL-12 (rhIL-12) (25 ng/ml) (Sigma-Aldrich), antibody to IL-4 (2.5 µg/ml) (R&D Systems), and rhIL-2 (AIDS Reagent Program, National Institutes of Health) on days 1 and 4 of culture. On day 6, differentiating T_H1 cells were activated for another 24 hours with CD3/CD28 beads (Invitrogen) and then used for experiments. Successful in vitro T_H1 differentiation was verified by checking up-regulation of *TBX21* and *IFNG* mRNA transcripts by real-time PCR. Human T_H lines were differentiated under similar T_H1-polarizing culture conditions as described for primary T_H cells.

The genotype of NIH001 (exon 10, G1305 insertion; frameshift or early stop codon) manifested in complete lack of WASp expression in lymphocytes and in clinically severe WAS (clinical score 5A; that is, WAS grade 5 with autoimmunity). The WAS genotype in NIH001 reverted spontaneously when an internal deletion of 19 amino acids removed the mutation G1305, rendering the sequence in frame. This revertant WAS genotype is predicted to encode mutant WASp that contains a new internal deletion of six amino acids (422 to 427) in the proline-rich domain. By FACS, 20% of peripheral T cells expressed this mutant WASp. Despite this reversion, the patient continues to manifest severe disease (WAS-5A) with viral skin infections and auto-immune joint disease. The PBMCs from this patient were obtained during this clinical stage of the disease.

For MSK001 and MSK002, although the WAS genotype is unknown, both patients presented with classic WAS symptoms requiring stem cell transplantation. In these two patients, all studies were performed on peripheral blood samples obtained before transplantation. In primary T cells from patient SEA001 (WAS genotype: T21 deletion in exon 2 resulting in frameshift and early stop at amino acid 75), WASp is undetected. This patient has severe disease with the clinical score 5A.

The NIH002 patient (WAS-1) carries the WAS genotype (231–240 del10insC, Asn⁷⁸-Glu⁸⁰ del), resulting in the expression of mutant WASp by FACS and mild XLT clinical phenotype, without the manifestations of systemic immune deficiency or autoimmunity.

The HTLV-immortalized WAS T_H cell line was established from the primary CD4⁺ T_H cells of a patient bearing the WAS genotype (G57 deletion) that results in frameshift and early termination codon, no WASp expression, and severe disease phenotype (clinical score 5A). This WAS-5A T_H line was corrected by retrovirally transducing LTR-WASP-IRES-NEO-LTR construct to stably express WASp at near-normal level. Another WAS T_H line was established from the patient bearing the WAS genotype [missense mutation: His³⁰ in exon 1 resulting in a three-nucleotide deletion (nucleotides 88 to 90)] that allows expression of mutant WASp and mild clinical XLT phenotype without systemic immune deficiency

(clinical score 1). As a control for the HTLV-immortalized WAS T_H lines, we also established HTLV-immortalized T_H line from a normal donor. For each T_H line, the expression of WASp (mRNA and protein) was verified to match their respective WAS genotypes. All T_H lines were differentiated under similar T_H1-polarizing culture conditions as described above for primary T_H cells.

The characteristics of the antibodies or reagents used for ChIP and coimmunoprecipitation assays are detailed in table S2.

Deconvolution microscopy and quantitative 3D immunofluorescence analysis

Deconvolution imaging of paraformaldehyde-fixed single T_H cells was performed with Zeiss inverted digital microscopy workstation integrated with SlideBook software, as previously described (10). All images were acquired at $\times 63$ oil immersion magnification. Pixel quantification of any two immunofluorescence signals was performed on the constrained iterative deconvolved images by creating a cylindrical mask on bright and dim DAPI staining regions traversing the entire 3D volume of the nucleus. Significant intermolecular nuclear colocalization was determined by calculating the Pearson's correlation coefficient between the pixel intensities of two simultaneously acquired channels. About 30 single T_H cells chosen randomly from multiple experiments and multiple donors were analyzed for each antibody combination.

Immunoprecipitation and immunoblotting

Nuclear lysates were prepared from primary T_H cells or T_H lines with the NE-PER Nuclear/Cytoplasmic Extraction kit (Pierce), and their purity was verified by immunoblotting with antibodies to lysosomal-associated membrane protein 1 (LAMP1) (cytoplasmic marker) and to histone H3 (nuclear marker). Coimmunoprecipitations were performed with the Universal Magnetic Coimmunoprecipitation kit (Active Motif), as per the manufacturer's specifications. Where possible, single blots were sequentially reprobed with multiple antibodies, and/or the same coimmunoprecipitation sample was probed in different gels for consistency. For each coimmunoprecipitation experiment, isotype IgG antibody served as a negative control and immunoblotting the corresponding nuclear lysate with the primary antibody served as a positive control (input), and most images presented are from the same experiment and same gel exposure.

ChIP quantitative real-time PCR and ChIP-chip assays

ChIP assay was performed with the Chip-IT Express kit (Active Motif) on the MNase-digested chromatin after fixing protein-DNA interactions with 1% formaldehyde, as per the standard protocol.

For the limited number of primary T cells that could be obtained from the WAS patients, we adapted and optimized the recently described μ ChIP assay, which allows ChIP studies on 1000 to 5000 cells (61). Conventional ChIP and μ ChIP samples were then analyzed by real-time PCR with primers that were custom-made by Applied Biosystems and are detailed in table S3. We first ascertained that the histone enrichment profiles in normal T_H1-differentiating cells captured by the conventional MNase-ChIP assay corresponded with that obtained by the MNase- μ ChIP assay before applying the μ ChIP assay to resolve patient samples. In every experiment, isotype IgG ChIP, performed alongside each ChIP assay, determined the nonspecific background binding to the *TBX21* chromatin, and such values were subtracted from the final values displayed in the figures. ChIP samples were then used as templates for real-time PCR analysis performed on 7900HT Fast Real-Time PCR System (Applied Biosystems). Taqman Universal PCR Mastermix (Applied Biosystems) was used in all reactions, and the derived C_t values were converted to absolute copy numbers with a

cloned DNA plasmid standard dilution curve that was run simultaneously with each tested sample. The generated DNA plasmids were specific for each cis-regulatory region interrogated, and the PCR product was initially sequenced to verify specificity.

For the ChIP-chip assay, we used a customized 385K (385,000 probes on a single glass slide) Roche NimbleGen ChIP-on-chip microarray. The genomic regions were tiled to encompass ~60 kb upstream and ~20 kb downstream of the TSS of the selected genes. Genes were tiled with 75-oligonucleotide probes positioned every 25 bp along the gene of interest with the HG18 build (University of California Santa Cruz Human Genome Assembly). For each array, immunoprecipitation-enriched probe clusters were defined as regions with a minimum of four probes separated by a maximum of 500 bp with filtered logarithmic ratio of >2.5 SDs from the mean log ratio. These ratio data were then randomized 20 times to evaluate the probability of false positives. Each WASp peak was then assigned an FDR score on the basis of the randomization. ChIP peaks with FDR <0.2 were considered as high-confidence WASp-binding sites and represented in the ChIP-chip profiles as red, orange, or yellow bars. Gray bars represent WASp peaks with an FDR score of >0.2 and considered low-probability WASp peaks.

All ChIP-chip samples were prepared by NimbleGen's recommended methods, starting with ~2 mg of sonicated, formaldehyde cross-linked chromatin from primary differentiating T_H1 cells pooled from 12 healthy donors. The samples were processed, and data were acquired and analyzed at NimbleGen under a full service contract. The data of the hybridized NimbleGen arrays were extracted according to standard operating procedures by NimbleGen Systems Inc. SignalMap software provided by NimbleGen was used to visualize the array peaks.

To detect cis-regulatory elements bound to WASp in the T cell genome by ChIP-chip assay, we used the Web-based bioinformatic cis-regulatory element annotation system (CEAS) (62).

Sequential ChIP and qPCR analysis

Sequential ChIP was done with Re-ChIP-IT Magnetic Chromatin Re-Immunoprecipitation kit (Active Motif) on the MNase-digested chromatin after fixing protein-DNA interactions with 1% formaldehyde, according to the manufacturer's instruction. T_H1-polarized cells from normal donors were used for the assay. Sequential ChIP assay was performed in both forward and reverse directions using both "bait" and "prey" proteins in the first ChIP round to mitigate false-negative results. In the first round, cross-linked chromatin was immunoprecipitated with antibodies to WASp, STAT1, RNAP2 (unmodified CTD), JMJD2A, RBBP5, or IgG. The eluate was then subjected to a second round of ChIP with different antibody combinations as shown in Fig. 3D. The eluted ChIP materials after the second ChIP step were analyzed by qPCR to study the simultaneous occupancy of the proteins on the same cis elements.

RNAi-mediated transient knockdown of WASp protein

For transient WASp depletion, Jurkat T cells were electroporated at a density of 3.3×10^7 /ml with 1 µg of GFP-tagged WASp-shRNA vector (oligo sequence: GAGTGGCTGAGTTACTTGC; plasmid name: pCMS3-H1p-EGFP; plasmid size: 4690 bp) or the control empty GFP vector with BTX-8300 Electroporation System (Fisher Scientific). The electroporated T cells were propagated in the T_H1-biasing conditions [rhIL-12 (25 ng/ml) and antibody to IL-4 (2.5 µg/ml)] for 4 days. Transfection efficiency was between 55 and 60% by GFP positivity. Transduced T cells were GFP-enriched by sorting (FACS Aria

II). Purified GFP⁺ T cells in the T_H1 culture conditions were then stimulated with CD3/CD28 beads for 24 hours before using in different assays.

Histone methylation and demethylation in vitro assays

Two complementary experimental approaches, colorimetric enzyme-linked immunosorbent assay–based assay and Western blot–based assay, were used to test the in vitro methylase or demethylase activity of endogenous WASp. In the first approach, quantification of histone H3 methylation and demethylation activity was performed with EpiQuik Histone Methyltransferase Activity/Inhibition Assay kits (Epigentek) specific for H3K4 and H3K9 modifications, and EpiQuik Histone Demethylase Activity/Inhibition Assay Kit specific for H3K4 and H3K9 modifications, as per the manufacturer's guidelines. Briefly, for capturing H3K4 and H3K9 methyltransferase activity, fresh coimmunoprecipitated nuclear complexes from primary T_H1 or undifferentiated T_H0 (with antibody to WASp and other control antibodies) were combined with the biotinylated peptide specific for the histone modification of interest in a streptavidin-coated plate and incubated along with S-adenosylmethionine for 1.25 hours at 37°C. To ensure minimal loss of protein activity, we completed immunoprecipitation reactions and histone activity assays on the same day. Plates were then washed and incubated for 1 hour with capture antibody, rewashed and incubated with a detection antibody for 30 min, developed, and subsequently read on a plate reader at 450 nm for the methyltransferase assay. For the H3K9 and H3K4 demethylase assay, immunoprecipitated complexes from nuclear lysates were combined with the peptide containing the histone modification of interest and incubated for 1.25 hours at 37°C. After the removal of the beads, the reaction was read on a fluorescent plate reader at 545-nm excitation and 590-nm emission (for H3K4 demethylase activity) or at 390-nm excitation and 455-nm emission (for H3K9 demethylase activity). For all methylation and demethylation in vitro assays, a blank sample was always included as a control to determine the spontaneous background. To identify maximum activity for the methylation reactions (denoted as 100%), we included pure recombinant control methylases (SET or MLL enzymes for H3K4 methylation and G9a for H3K9 methylation, provided with the kit) with each assay. Similarly, for the demethylation assays, simultaneous immunoprecipitations with antibodies to SMCX (as H3K4 demethylase control) or to JMJD2A (as H3K9 demethylase control) were performed alongside the test samples and accorded 100% demethylase activity. All assays were run with an isotype IgG antibody control to determine the specificity of our antibodies and their background activity in these experiments.

In the experiments using the Western approach to determine the methylation activity, as previously described (24), freshly isolated, non-denatured immunoprecipitated nuclear complexes (pulled down with antibody to WASp and other control antibodies) were incubated for 1.5 hours at room temperature with 5 µg of human recombinant unmodified H3 histone octamer subunits (Millipore) at a concentration of 0.1 µg/µl along with 20 µM S-adenosylmethionine (Sigma) in 20 mM Tris (pH 8.0), 200 mM NaCl, and 0.4 mM EDTA. The total reaction was then denatured in Laemmli buffer with 5% β-mercaptoethanol by boiling for 5 min at 100°C and run on Western blot. For demethylation assays, 5 µg of purified HeLa cell core histones (Millipore) was incubated with freshly isolated immunoprecipitated complexes in 50 mM Hepes (pH 8.0) with 1 mM α-ketoglutarate, 2 mM L-ascorbate, and 70 µM Fe(NH₄)₂(SO₄)₂ for 5 hours at 37°C. The total reaction was denatured in Laemmli buffer with 5% β-mercaptoethanol by boiling for 5 min at 100°C and run on an SDS–polyacrylamide gel for Western blot analysis to capture the degree of H3 lysine modifications. All assays were performed simultaneously with donor-matched IgG immunoprecipitates (negative control) and a relevant positive control.

DHS and qPCR analysis

The PCR-based DHS assay was performed as described previously (63) with few modifications. Briefly, T cell nuclei were isolated with the Nuclei Isolation kit (Sigma) and incubated with 0, 2, 4, 6, 8, 10, 15, and 20 U of DNase I enzyme per 1 µg of nuclei at 37°C for 10 min. The reaction was terminated with 1 µl of stop solution followed by incubation at 70°C for 10 min. All samples were analyzed by quantitative real-time PCR to determine the number of amplicons remaining after nuclease treatment. All C_t values were converted to absolute copy numbers with a cloned DNA plasmid standard dilution curve run in all plates, specific to each primer fragment, and sequenced to ensure specificity. Primers were obtained from Integrated DNA Technologies, and 6FAM probes were from Applied Biosystems. Primers or probes were designed with Primer Express v3.0 software (Applied Biosystems) and are displayed in table S3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank J. Kolls for the use of the deconvolution imaging system; J. Burkhardt for shRNA constructs; R. J. O'Reilly and H. Ochs for providing WAS patient samples; and A. Rao, G. Blobel, J. Lieb, A. Ray, and J. Parness for discussions and critical reading of the manuscript.

Funding: NIH grants AI073561 and AI0797621 (Y.M.V.) and National Human Genome Research Institute intramural funds (F.C.).

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Fig. 1.

Nuclear and cytoplasmic dual-site localization of WASp in primary differentiating T_H1 cells. **(A)** Nomarski (differential interference contrast) and their corresponding immunofluorescence images of normal primary T_H cells, undifferentiated (T_H0) or T_H1 -differentiating, dual- or triple-labeled with WASp (green) and DAPI (blue). All images were acquired by deconvolution microscope at $\times 63$ magnification. The data are representative of at least 30 single cells analyzed. **(B to E)** Western blot analysis probed with the indicated antibodies performed on the cytoplasmic (cyt) and nuclear (nu) extracts generated from primary T_H cells [(B) and (E)] or T_H lines [(C) and (D)]. The purity of the two cell fractions was verified with histone H3 and LAMP1 probing, and an example in the T_H1 sample is shown. Data in (E) are from the nuclear extracts. **(F)** Representative immunofluorescence images of normal primary T_H1 -differentiating cells, triple-labeled with WASp (green), DAPI (blue), and nucleolar mark B23 (red, top panel); hyperphosphorylated RNA polymerase II (Ser²) (red, middle panel); or TRAP220/MED1 (red, bottom panel). Also shown are images where the red or green color is removed with Photoshop (see figs. S2 and S3 for tiled images of the entire T cell 3D volume). **(G)** Coimmunoprecipitation with antibodies to WASp and IgG immunoblotted (IB) with the indicated antibodies performed on the nuclear extracts generated from the primary T_H1 -differentiated cells from normal donors. The same blot was sequentially reprobed with the indicated antibodies. The use of TrueBlot reagent resulted in the reduction of signals from the denatured heavy and light chains.

Fig. 2.

Profile of WASp interaction in vivo with the human T_H cell genome. **(A)** Some examples of WASp-bound genomic regions in primary human T_H1 -differentiating cells. Plots show ChIP enrichment ratios of WASp for all oligonucleotide probes within the indicated genomic loci analyzed on both forward and reverse chromatin strands. The data are for either forward or reverse chromatin strand. The exon-intron configuration of the indicated RefSeq genes (in red) derived from the Vista plot (in blue) is aligned with the corresponding chromosomal coordinates. The fold enrichment peaks for WASp (in gray) are derived from the raw data displayed in figs. S8 and S9. See table S1 for the list of WASp-binding and WASp-nonbinding target genes. **(B)** Conserved DNA sequence motif identified among WASp-bound genomic regions with the CEAS bioinformatic algorithm. **(C)** Coimmunoprecipitation assay demonstrating association of WASp with Sp1 in the nuclear fraction of primary T_H1 cells. **(D)** Pie chart demonstrating enrichment of WASp to specific genomic cis elements in primary human T_H1 -differentiating cells.

Fig. 3.

Endogenous WASp is recruited to the *TBX21* proximal promoter in vivo. **(A)** Chromosomal coordinates of the WASp-bound cis elements at the human *TBX21* promoter locus on chromosome 17. A, WBR1; B, WBR2; C, GAS (core motif: 5'-TTCAGGCAA-3'); TATA, TATA sequence (TCATAA). **(B)** Quantitative MNase-ChIP qPCR assay. Quantifying mononucleosomal enrichment of WASp and other trans-regulatory proteins at the *TBX21* DNA, as well as the changes in the epigenetic profile at these sites in primary undifferentiated T_H cells and their T_H1 -differentiating counterparts. For each primary antibody ChIP, a control ChIP performed with the corresponding isotype antibody was always included for specificity. IgG ChIP values are not shown to avoid crowding. The final results are displayed as percent amplicons normalized to nuclear input chromatin after subtracting background ChIP signals obtained with the corresponding isotype IgG antibody. Real-time PCR data were derived by converting the C_t values to absolute copy numbers with cloned DNA plasmid standard dilution curve. For each ChIP-qPCR assay, no DNA control samples were always included to negate potential human cell contamination. Data represent the average of triplicates from multiple experiments in purified T_H cells derived from at least seven normal donors, with bars indicating SE. See figs. S8, S10, and S14 for the complementary data mapping WASp-binding sites at the *TBX21* locus with the ChIP-chip approach. The efficiency of chromatin digestion by MNase was >90% (fig. S14B). Asterisk denotes ChIP signals at or below the background obtained with the corresponding IgG control antibody. **(C)** DHS pattern at the three sampled cis regions of *TBX21* promoter in primary undifferentiated T_H and differentiating T_H1 cells. T_H cell nuclei were treated with increasing doses of DNase I enzyme from 0 to 10 U, and the number of amplicons lost was quantified by real-time qPCR and is displayed as percent of DNase I-untreated control. Data represent the average of triplicates from four independent experiments, with bars indicating SE. **(D)** Quantitative sequential MNase-ChIP assay. Data for WASp/STAT1, WASp/RNAP2, WASp/RBBP5, and WASp/JMJD2A co-occupancy at the same cis elements of the *TBX21* promoter locus in primary T_H1 cells. Sequential rounds of conventional ChIP assay was performed to obtain the absolute copy numbers for each immunoprecipitation (IP)/input ratio. This primary value was then converted to fold enrichment value, where the SeqChIP value obtained with the control IgG antibody was normalized to 1.0. Data represent the averages of triplicates in purified T_H cells from two normal donors, with bars indicating SE.

Fig. 4.

Endogenous nuclear WASp in primary TH1-differentiating cells associates with H3K4 trimethylase and H3K9 tridimethylase activities in vitro. **(A)** Colorimetric methylation or demethylation assay. Purified nuclear lysates from primary differentiating TH1 cells were enriched for WASp, IgG, and the respective positive control histone methyltransferase or histone demethylase enzyme by immunoprecipitation with specific antibodies. The resultant eluates were then tested for the indicated histone methylation or demethylation activity as per the manufacturer's specifications. Additional controls in LFA-1- and N-WASp-enriched eluates were also included in the assay. The primary values acquired as optical density per hour per milliliter ($\text{OD hour}^{-1} \text{ ml}^{-1}$) were converted to the displayed percent methylase or demethylase activity normalized to the activity of their respective positive control (histone-modifying) enzymes provided in the kit. The data represent an average of at least five biological replicates, with bars indicating the SE, reflecting the interindividual variation. **(B)** The coimmunoprecipitation aliquots obtained from the above assay were separately coincubated with either the recombinant human unmodified H3 (for the methylation assay) or the endogenous mix of methylated histones derived from HeLa cells (for the demethylase assay) as substrates. The reaction was assessed by Western blot analysis probing with the specified antibodies to detect the formation of the indicated histone-modified products. Blots were stripped and reprobed for total H3 as a loading control. Immunocomplexes enriched with antibodies to RBBP5 and JMJD2A served as positive controls. A representative Western blot from two independent experiments is shown. The signal density was quantified, and results were graphically displayed as fold change in relation to total H3. **(C)** Nuclear lysates from normal TH1-differentiating primary cells derived from two representative normal donors were subjected to reciprocal coimmunoprecipitation assay with the indicated antibodies along with the corresponding IgG controls. The data are representative of at least three independent experiments in different donor samples. **(D)** 3D immunofluorescence images of primary TH1 cells triple-labeled with WASp (green), DAPI (blue), and RBBP5 (red, top panel); JMJD6 (red, middle panel); or JMJD2A (red, bottom panel). The degree of overlap between any two fluorochromes was quantified for the entire 3D volume of the cell (z stack) and is shown as Pearson's r_p values (mean $\pm$ SEM). Calculations to quantify protein colocalization were performed on the merged three-color images. The data are representative of at least 30 single TH1 cells analyzed.

Fig. 5. Critical role of WASp in the epigenetic reprogramming of the *TBX21* proximal promoter during T_H1 differentiation in primary T_H cells. (**A**, **C**, and **D**) Histone modification and protein binding patterns inscribed at the *TBX21* promoter in the indicated primary T_H cells (normal and three WAS patients), undifferentiated T_H0 and T_H1 -differentiating, resolved at the mononucleosomal level by MNase- μ ChIP assay with between 1000 and 5000 T_H cells. In all experiments, three to five independent ChIPs were performed for the three cis sites (WBR1, WBR2, and GAS) with each antibody (plus the corresponding isotype IgG antibody), and the data were analyzed by triplicate qPCR. Background enrichment obtained with the isotype IgG antibodies was subtracted from the final values shown. Data are expressed as percent immunoprecipitation relative to nuclear input chromatin (mean $\pm$ SEM) averaged from all three cis sites within the *TBX21* promoter. μ ChIP assay precipitates more DNA relative to input than does conventional ChIP, as reflected by enhanced precipitation values when compared with the corresponding values depicted in Fig. 3. Only those primary antibody μ ChIP enrichment values that were above the background obtained with the concomitantly performed IgG antibody μ ChIP were included in the analysis. (**B**) The stacked column representation of the primary μ ChIP values compares the percentage contribution of each histone modification to the total combinatorial histone profile at the *TBX21* promoter locus spanning <1 kb from TSS.

Fig. 6.

Histone profile, DHS pattern, and transcription factor expression profile in T_H lines lacking WASp from natural mutation or RNAi-mediated depletion. (A) FACS profile depicting the transfection efficiency of control and WASp-shRNA-GFP knockdown (k/d) constructs in Jurkat T cells with the BTX electroporator. (B) Western blot analysis of control and WASp knockdown Jurkat T cells enriched after sorting GFP⁺ T cells on days 2 to 4 (d2/d3/d4) after electroporation. (C) Corresponding densitometric quantification for day 2 or 3 blots. Individual immunoblot images were obtained by sequential reprobing of a single blot with the indicated antibodies. The displayed blot is representative of two independent experiments. (D) Absolute quantification of mRNA expression in the sorted GFP⁺ (control or WASp knockdown) Jurkat T cells (~95% purity) performed with real-time qPCR. “ T_H0 ” denotes Jurkat T cells propagated in T_H -neutral culture conditions (IL-2 alone), and “ T_H1 ” denotes activation under complete T_H1 -polarizing conditions. The displayed data are an average of triplicates from at least five experiments, with bars indicating SE. *P* values reporting on the significance of the observed differences in the mRNA copy numbers between control and WASp knockdown T cells were calculated with Wilcoxon nonparametric statistical test. ***P* ≤ 0.01. Unmarked bars denote non-significant differences. (F) Corresponding epigenetic profiles at the *TBX21* locus in the control and WASp knockdown Jurkat T cells. (E and F) Conventional MNase-ChIP analysis performed on the indicated HTLV-immortalized T_H lines [normal, WAS-1, WAS-5, and WAS-5 T_H line reconstituted with normal WASp (WAS-5 corr)] or Jurkat T_H cells depleted of WASp by RNAi that were activated under T_H1 -polarizing conditions. Primary ChIP values for the individual histone modifications (E) and the relative percent enrichment depicting the combinatorial histone profile (F) at the *TBX21* promoter are both shown. The background

ChIP values obtained with the corresponding isotype IgG antibody were deducted from the final values displayed in the figure. Data are expressed as percent immunoprecipitation relative to nuclear input chromatin (mean $\pm$ SEM) and represent an average of five to seven experiments for the HTLV T_H lines and two experiments for Jurkat T cells each performed in triplicates. (G) DHS pattern at the *TBX21* promoter in the indicated T_H lines activated under T_H1-polarizing conditions. The amplicon calculations are as described in the legend to Fig. 3C. The nuclease-insensitive DNA in WAS-5 T cells was rendered nuclease-sensitive at a DNase I dose of 20 U (data not shown). (H and I) Protein (H) and mRNA (I) expression profile in the nuclear and cytoplasmic fractions in normal, WAS-5, and WAS-5 corrected (WAS-5 corr) T_H lines resolved by Western blot analysis (H) and real-time PCR assays (I). The RT-PCR values are average of triplicates, with bars indicating SE. *** $P < 0.001$, Wilcoxon nonparametric statistical analysis that compares values in normal with those in WAS T_H cells. See fig. S19 to contrast this result with the result demonstrating an intact ability of the same WAS T_H lines to differentiate down the T_H2 pathway.

Fig. 7.

T_H cells from WAS-1 patients display functional characteristics similar to those in normal T_H cells. **(A)** Left panel: Western analysis performed on the nuclear lysates of T_H1-activated WAS-1 T_H cells using the indicated antibodies. This mutant WASp was only detected with the rabbit polyclonal antibody but not with the mouse monoclonal antibody. Middle panel: corresponding immunofluorescence images of WAS-1 T_H cells triple-labeled with antibodies to WASp (green), T-BET (red), and DAPI (blue). Right panel: mRNA expression profile of the same WAS-1 T_H cells, either T_H1-activated (T_H1) or undifferentiated (T_H0), is shown for the indicated genes. The primary values in T_H1 cells were adjusted for the β -actin mRNA expression and displayed as fold change over the steady-state T_H0 values (y axis). **(B)** Coimmunoprecipitation assay performed with the antibodies to WASp and isotype IgG on the nuclear lysate derived from T_H line generated from a patient with clinical grade 1 (WAS-1). The same gel was sequentially reprobed with the indicated antibodies. The use of TrueBlot reagent resulted in the reduction of signals from the denatured heavy and light chains. The blot is representative of two independent experiments in different donors. **(C and D)** MNase- μ ChIP assay was performed on the primary T_H cells derived from another WAS-1 patient, and the ChIP enrichment results averaged from three experiments are shown along with SEM (C). In (D), the stacked column representation of the primary ChIP values compares the percentage contribution of each histone modification to the total combinatorial histone profile at the *TBX21* promoter locus. The asterisk denotes undetectable ChIP enrichment (or enrichment at or below that obtained with control IgG antibody) for H3K27me3.