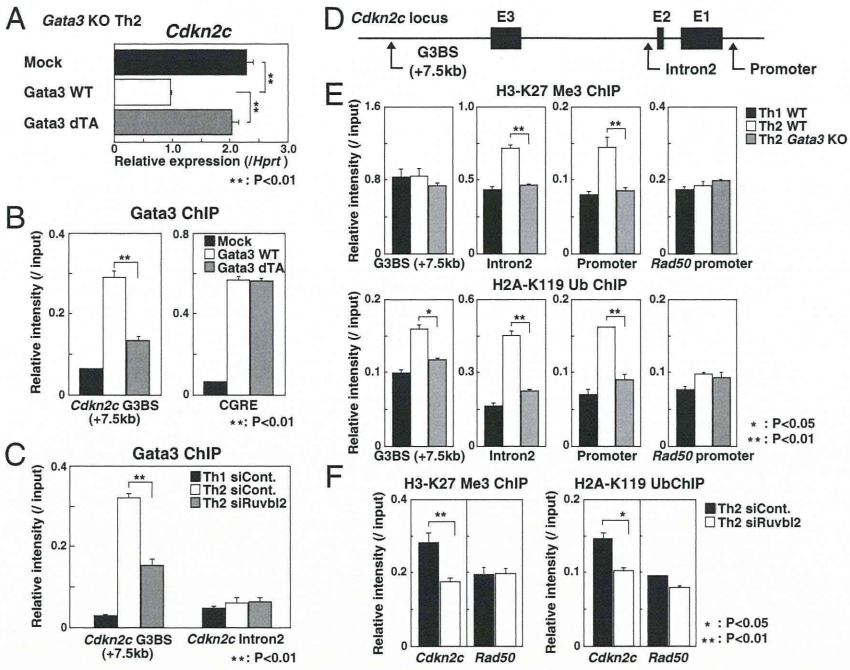

Fig. 4. Ruvbl2 is necessary for the recruitment of Gata3 at the *Cdkn2c* locus in developing Th2 cells. (*A*) Naive CD4 T cells from *Gata3*-deficient mice were stimulated under Th2 conditions for 2 d and then infected with a retroviral vector carrying WT or mutant (dTA) Gata3 cDNAs. Three days later, the retrovirus-infected GFP-expressing cells were purified, and the levels of mRNA of *Cdkn2c* were measured by RT-qPCR. $**P < 0.01$ by Student *t* test. (*B*) The binding of Gata3 WT or dTA to the *Cdkn2c* G3BS and Th2 cytokine loci (CGRE region) were determined by the ChIP assay with a qPCR analysis using the same cells shown in *A*. (*C*) A control or Ruvbl2 siRNA was transfected into naive CD4 T cells, and the cells were stimulated under Th1 or Th2 conditions for 3 d. The binding of Gata3 to the *Cdkn2c* locus was determined. (*D*) A schematic representation of the *Cdkn2c* locus. The locations of primers and exons are indicated. (*E*) Naive CD4 T cells from WT or *Gata3*-deficient mice were cultured under Th1 or Th2 conditions for 5 d. The status of H3-K27 Me3 and H2A-K119 Ub at the *Cdkn2c* and *Hprt* loci was determined by a ChIP assay, using specific primers to detect the indicated regions. $**P < 0.01$, $*P < 0.05$ by Student *t* test. (*F*) A control or Ruvbl2 siRNA was transfected into naive CD4 T cells, and the cells were stimulated under Th2 conditions for 4 d. The status of H3-K27 Me3 and H2A-K119 Ub at the *Cdkn2c* and *Hprt* promoters was determined by a ChIP assay. Four (*A*), three (*B* and *C*), and two (*E* and *F*) independent experiments were performed, and similar results were obtained.

Thus, our findings may support the latter hypothesis, Ruvbl2 might associate with Gata3 at the *Cdkn2c* locus to facilitate and stabilize the DNA binding of Gata3 (Fig. S7*B*).

The recent genome-wide analyses indicated that GATA family transcription factors mediate both activating and repressive gene regulation (10, 11, 34–36). Interestingly, most of the GATA

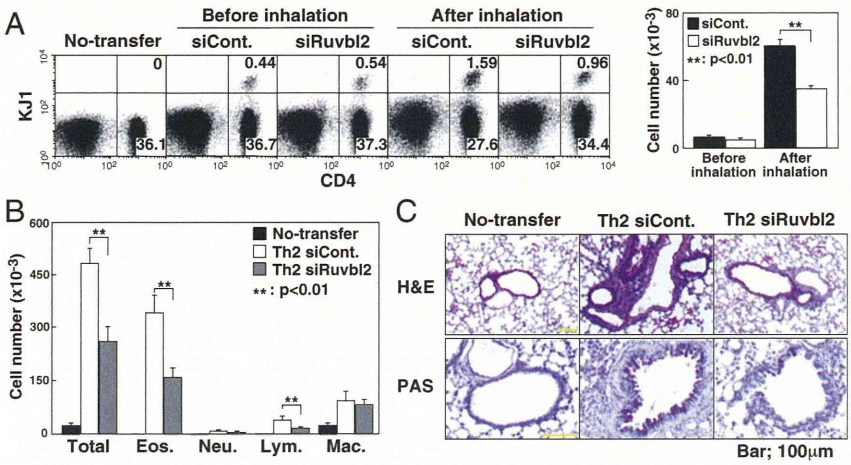

Fig. 5. Ruvbl2 regulates OVA-induced Th2 cell expansion and allergic airway inflammation in vivo. (*A*) A control or Ruvbl2 siRNA was transfected into naive CD4 T cells from DO11.10 Tg mice, and the cells were stimulated under Th2 conditions. On day 4, cells were i.v. transferred into BALB/c mice, and the mice were challenged with aerosolized OVA on days 5 and 7. Cells from lung were stained with antibodies against CD4 and KJ1.26 and assessed by FACS on days 5 (before inhalation) and 9 (after inhalation). A summary of the numbers of $KJ1^+$ cells in the lung is presented (*Right* graph). Four mice per group were used. $**P < 0.01$ by Student *t* test. (*B*) The number of inflammatory cells in the bronchioalveolar lavage (BAL) fluid was counted. The absolute cell numbers of eosinophils (Eos.), neutrophils (Neu.), lymphocytes (Lym.) and macrophages (Mac.) are shown with SDs. Four mice per group were used. (*C*) The lungs were fixed and stained with hematoxylin/eosin (H&E) or with PAS. Representative staining patterns are shown. (Scale bar: 100 μm.) Two independent experiments were performed, and similar results were obtained (*A*, *B*, and *C*).

binding sites are localized at intragenic or intergenic regions where they can function as distal enhancers or silencers (11, 34–36). We have recently reported that Gata3 organizes a Gata3/Chd4/p300 activating complex at the enhancer regions of Th2 cytokine loci and a Gata3/Chd4-NuRD repressive complex at the intron 1 of *Tbx21* locus in Th2 cells and, thus, simultaneously positively regulates the expression of the Th2 cytokine genes and negatively regulates the *Tbx21* locus (37). In the current study, we found that Gata3 binds to the downstream region of the *Cdkn2c* locus (+7.5 kb from the transcriptional start site) and down-regulates the expression of *Cdkn2c* (Fig. 3). The introduction of a fragment spanning the *Cdkn2c* G3BS specifically repressed the transcriptional activity of the *Cdkn2c* promoter (Fig. 3*D*). Thus, the G3BS downstream of the *Cdkn2c* may have a Gata3-dependent intergenic silencer activity that affects the expression of *Cdkn2c*.

In summary, our results indicate that the Gata3/Ruvbl2 complex plays a crucial role in the regulation of Th2-cell proliferation by repressing the expression of a CDK inhibitor, *Cdkn2c*. This finding highlights a unique molecular mechanism that controls the process of the proliferation during Th-cell differentiation.

Materials and methods

Mice. C57BL/6 and BALB/c mice were purchased from CLEA Co. Conditional *Gata3*-deficient mice and OX40-Cre transgenic mice were kindly provided by Dr. William E. Paul (National Institutes of Health, Bethesda, MD) and Dr. Nigel Killeen (University of California, San Franciso, CA), respectively. Anti-ovalbumin (OVA)-specific TCR-αβ (DO11.10) transgenic (Tg) mice were provided by Dr. Dennis Loh (Washington University, St. Louis, MO). All mice were maintained under specific pathogen-free conditions and were used at 6–8 wk of age. All animal experiments were approved by the Chiba University Review Board for Animal Care.

The Generation of Th1 and Th2 Cells. Th1 and Th2 cells were generated as described previously (38). In brief, CD4 T cells with a naive phenotype ($CD44^{low}$) were purified using a FACSAria instrument (Becton Dickinson) yielding a purity of >98% and were stimulated with 3 μg/mL immobilized anti-TCRβ mAb plus 1 μg/mL anti-CD28 mAb under the Th1 or Th2 conditions in vitro.

Detailed descriptions of all materials and methods are provided in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Kaoru Sugaya, Hikari Kato, Terumi Horiuchi, Kayo Suzuki, and Toshihiro Ito for expert technical assistance. We also thank Dr. Yuka Kanno for critical suggestions on the manuscript. The lentivirus system was kindly provided by Dr. Hiroyuki Miyoshi (RIKEN BioResource Center). This work was supported by Global Center for Education and Research in Immune System Regulation and Treatment (Ministry of Education, Culture, Sports, Science and Technology, Japan) Grants-in-Aid Innovative Areas "Genome Science" 221S0002, "Crosstalk Between Transcription Control and Energy Pathway" 24116506, Scientific Research (B) 21390147, 22300325, and 24390239, (C) 19659121, and Exploratory Research and Young Scientists (B) 20790367 and 23790522; the Takeda Science Foundation; the Sagawa Cancer Foundation; the Astellas Foundation for Research on Metabolic Disorders; the Naito Foundation Natural Science Scholarship; the Princess Takamatsu Cancer Research Foundation; NOVARTIS Foundation for Gerontologic Research; NOVARTIS Foundation for the Promotion of Science; the Uehara Memorial Foundation; and the Research Fund of the Mitsukoshi Health and Welfare Foundation.

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The Polycomb Protein Ezh2 Regulates Differentiation and Plasticity of CD4$^+$ T Helper Type 1 and Type 2 Cells

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http://dx.doi.org/10.1016/j.immuni.2013.09.012

SUMMARY

After antigen encounter by CD4$^+$ T cells, polarizing cytokines induce the expression of master regulators that control differentiation. Inactivation of the histone methyltransferase Ezh2 was found to specifically enhance T helper 1 (Th1) and Th2 cell differentiation and plasticity. Ezh2 directly bound and facilitated correct expression of *Tbx21* and *Gata3* in differentiating Th1 and Th2 cells, accompanied by substantial trimethylation at lysine 27 of histone 3 (H3K27me3). In addition, Ezh2 deficiency resulted in spontaneous generation of discrete IFN-γ and Th2 cytokine-producing populations in nonpolarizing cultures, and under these conditions IFN-γ expression was largely dependent on enhanced expression of the transcription factor Eomesodermin. In vivo, loss of Ezh2 caused increased pathology in a model of allergic asthma and resulted in progressive accumulation of memory phenotype Th2 cells. This study establishes a functional link between Ezh2 and transcriptional regulation of lineage-specifying genes in terminally differentiated CD4$^+$ T cells.

INTRODUCTION

Upon encountering antigen, naive CD4$^+$ T cells differentiate into discrete subsets of effector T helper (Th) cells, a process controlled by the cytokines present during activation. Th cell subsets including Th1, Th2, Th9, and Th17 then induce different types of immune responses (Reiner, 2007; Zhu et al., 2010). Interferon gamma (IFN-γ)-producing Th1 cells are essential for immunity to intracellular pathogens, and interleukin 4 (IL-4)-, IL-5-, and IL-13-producing Th2 cells promote immunity to parasitic worms and also allergic inflammation. Expression of lineage-specifying transcription factors is required for Th cell differentiation. T-bet (encoded by *Tbx21*) can induce Th1 cell differentiation (Szabo et al., 2000), and Gata3 is required and sufficient for differentiation of Th2 cells (Yamashita et al., 2004; Zheng and Flavell, 1997). In addition, a transcription factor related to T-bet called Eomesodermin (Eomes) can also induce IFN-γ production in Th cells under some conditions (Suto et al., 2006; Tofukuji et al., 2012; Yang et al., 2008). Appropriate expression of these transcription factors and the effector cytokines they control is therefore essential for proper immunoregulation. Th1 and Th2 cell differentiation programs possess strong feed-forward mechanisms to maintain polarization (Hosokawa et al., 2013; Hwang et al., 2005; Usui et al., 2003) and are generally considered to represent stable lineages of differentiated effector cells. However, it is also becoming apparent that appreciable levels of functional plasticity exist among the Th cell subsets (Kanno et al., 2012; O'Shea and Paul, 2010).

Epigenetic histone modifications provide cells with the ability to maintain specific phenotypic traits independently of changes to the primary DNA sequence. Several epigenetic modifications have been found to correlate with gene expression in Th cells (Ansel et al., 2006; Kanno et al., 2012; Löhning et al., 2002; Nakayama and Yamashita, 2008; Wei et al., 2009), with repressed genes frequently associated with increased levels of trimethylation of lysine 27 on histone 3 (H3K27me3). Polycomb proteins form two major repressive complexes: polycomb repressive complex 1 (PRC1) and PRC2. Interestingly, CD4$^+$ T cells lacking the PRC1 proteins Mel-18, Bmi1, and Ring1B exhibited impaired differentiation into Th2 cells, partially because of failure of these cells to inhibit cell death pathways (Kimura et al., 2001; Suzuki et al., 2010; Yamashita et al., 2008) and also because of the roles of Bmi1 (Hosokawa et al., 2006) and possibly Mel-18 (Kimura et al., 2001) in stabilization of the Gata3 protein. The polycomb proteins Enhancer of Zeste Homolog 1 (Ezh1) and Ezh2 form two closely related PRC2 complexes that can trimethylate H3K27 and are required for maintenance of cellular identity at several stages of development (Di Meglio et al., 2013; Ezhkova et al., 2009; Hirabayashi et al., 2009; O'Carroll et al., 2001; Shen et al., 2008; Su et al., 2003). Ezh2 is highly expressed in CD4$^+$ T cells and binds to the Gata3 locus prior to Th2 cell differentiation (Onodera

et al., 2010). In addition, the position of Ezh2 occupancy at the *Il4* and *Il13* loci was found to spatially correlate with the presence of H3K27me3 in Th1 cells (Koyanagi et al., 2005). In contrast, it has been suggested that Ezh2 is important for efficient actin polymerization and TCR-induced proliferation of peripheral $CD4^+$ T cells, and this study detected no evidence of decreased H3K27me3 in unstimulated Ezh2-deficient $CD4^+$ T cells (Su et al., 2005). Additionally, an unconventional role for Ezh2 in the positive regulation of cytokine genes in Th cells has been suggested (Jacob et al., 2008). Thus, it remains unresolved whether Ezh2 retains its canonical role as a genetic repressor in $CD4^+$ T cells.

Here we show that Ezh2 has important functions as a repressor of gene expression during Th cell differentiation. Ezh2 bound strongly to genes encoding the transcription factors T-bet, Eomes, and Gata3, controlled differentiation into Th1 and Th2 effector cells, and regulated plasticity of these subsets after differentiation. In vivo, Ezh2-deficient ($Ezh2^{\Delta SET/\Delta SET}$) Th2 cells caused enhanced pathology in a mouse model of allergic asthma, and $CD4^+$-specific Ezh2 deficiency resulted in progressive accumulation of memory phenotype Th2 cells. These data indicate that Ezh2 plays an essential role in regulating both differentiation and stability of effector function in $CD4^+$ T cells.

RESULTS

The Histone H3K27 Methyltransferase Ezh2 Controls Differentiation of Th1 and Th2 Cells

To directly assess the role of Ezh2 during Th cell differentiation, we used mice in which the Ezh2 SET domain (responsible for H3K27 methyltransferase activity of Ezh2) was conditionally deleted (Hirabayashi et al., 2009) in $CD4^+$ T cells by expression of Cre-recombinase under control of the *Cd4* promoter (herein referred to as $Ezh2^{\Delta SET/\Delta SET}$ or Ezh2-deficient cells). The truncated Ezh2 ΔSET protein could be detected in $Ezh2^{\Delta SET/\Delta SET}$ $CD4^+$ T cells, but expression was much lower compared to the levels of native Ezh2 in wild-type (WT) cells (Figure S1A available online). Mice with $CD4^+$ T cell-specific Ezh2 deficiency (6 weeks old, young adult) had normal proportions of $CD4^+$ and $CD8^+$ T cells in the spleen and thymus (Figure S1B) and normal expression of T cell-associated surface markers on $CD4^+$ T cells (Figure S1C). In addition, we did not observe any defects in IL-2 production (Figure S1D) or initial proliferation of $Ezh2^{\Delta SET/\Delta SET}$ $CD4^+$ T cells (Figures S1E and S1F). We first investigated whether loss of Ezh2 could affect Th cell differentiation. Ezh2-deficient Th cells produced markedly more IFN-γ in cultures with titrated doses of the Th1 cell-inducing cytokine IL-12 (Figures 1A and 1C). Likewise, more IL-4, IL-5, and IL-13 were produced in cultures with the Th2 cell-inducing cytokine IL-4 (Figures 1B and 1D). Interestingly, enhanced IFN-γ production by Ezh2-deficient cells was still observed without addition of IL-12, and this could be inhibited by IL-4 (Figure 1B). In addition, compared to WT cells, increased amounts of IFN-γ, IL-4, IL-5, and IL-13 were produced by Ezh2-deficient $CD4^+$ T cells activated via the TCR without any exogenously added cytokines (Figure 1E). Importantly, as shown by intracellular staining, the majority of IFN-γ- and IL-4-producing cells were mutually exclusive, indicating that loss of Ezh2 does not disable Th cell polarization (Figures 1A and 1B). We also investigated the effect of 3-Deazaneplanocin A (DZNep), a specific inhibitor of histone methyltransferase activity. This agent has been shown to inhibit Ezh2 and reactivate polycomb repressed genes in cancer cells (Tan et al., 2007) and to preferentially decrease H3K27me3 in T lymphocytes (He et al., 2012). Treatment of $CD4^+$ T cells with low concentrations of DZNep (7.5 and 15 nM) during differentiation resulted in a dose-dependent increase in the production of IFN-γ or of IL-4, IL-5, and IL-13 under Th1 or Th2 cell-inducing conditions, respectively (Figures 1F and 1G). These data indicate that inhibition of Ezh2 activity via genetic inactivation or chemical inhibition strongly enhances cytokine production and differentiation of Th1 and Th2 cells.

Loss of Ezh2 Results in Enhanced Th1 and Th2 Cell Polarization with Increased Expression of T-bet and Gata3

To determine whether cytokine expression in Ezh2-deficient cells coincided with expression of lineage-specifying transcription factors at the single-cell level, we analyzed expression of T-bet together with IFN-γ and of Gata3 together with IL-4 (Figures 2A and 2B). Ezh2-deficient cells again showed markedly increased cytokine production. Importantly, those cells that produced IFN-γ also expressed higher amounts of T-bet, and the cells that produced IL-4 expressed higher amounts of Gata3. We also found enhanced expression of *Tbx21* and *Gata3* mRNA at all of the concentrations of polarizing cytokines tested (Figures 2C and 2D). To further analyze the contribution of T-bet to the enhanced IFN-γ production by Ezh2-deficient cells, we compared WT, $Tbx21^{-/-}$, $Ezh2^{\Delta SET/\Delta SET}$, and cells doubly deficient for Ezh2 and T-bet ($Ezh2^{\Delta SET/\Delta SET}Tbx21^{-/-}$) differentiated under Th1 cell-inducing conditions. We detected an approximate 50% reduction in IFN-γ production by $Tbx21^{-/-}$ Th1 cells compared to WT cells, and again IFN-γ production by $Ezh2^{\Delta SET/\Delta SET}$ cells was markedly enhanced. In double-deficient $Ezh2^{\Delta SET/\Delta SET}Tbx21^{-/-}$ cells, the enhanced IFN-γ production induced by loss of Ezh2 was substantially reduced (Figure 2E), indicating that the increased expression of IFN-γ in Th1 polarized Ezh2-deficient cells is largely dependent on T-bet. To assess the contribution of Gata3 to Th2 cytokine production, we used Gata3 siRNA to knock down Gata3 expression in Ezh2-deficient cells differentiated under Th2 cell polarizing conditions. Gata3 siRNA abolished IL-4, IL-5, and IL-13 production by Ezh2-deficient Th2 cells (Figure 2F). These data indicate that Gata3 is required for enhanced Th2 cytokine production in cells lacking Ezh2 and confirm that Ezh2-deficient cells are unable to bypass the requirement for Gata3 in Th2 cytokine expression. In contrast to the large enhancement of Th1 and Th2 cell differentiation, we detected only a very small increase in IL-17 production when comparing WT and Ezh2-deficient cells differentiated under Th17 cell-inducing conditions, and the expression of Rorγt was also only slightly increased (Figures S2A and S2B). In addition, we found no enhancement, but rather a decrease in differentiation into Th9 and iTreg cells by Ezh2-deficient $CD4^+$ T cells (Figures S2C and S2D). Interestingly, under Th17, Th9, and also iTreg cell-inducing conditions, we again detected more IFN-γ production by Ezh2-deficient cells compared to WT cells.

Genome-wide Analysis of Ezh2 Binding in Th1 and Th2 Cells

To globally determine the genes most likely to regulate the enhanced Th1 and Th2 cytokine production by Ezh2-deficient

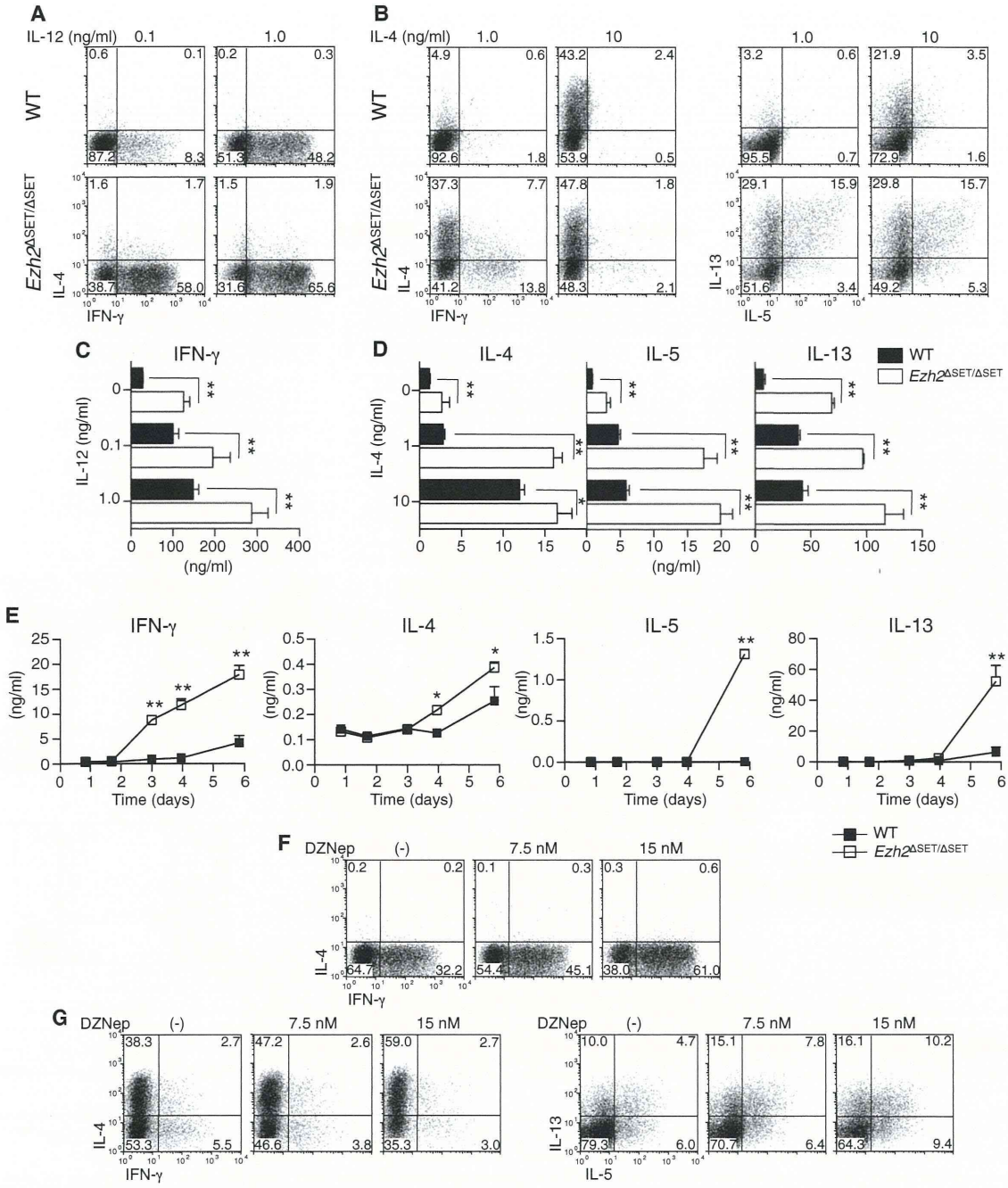

Figure 1. Loss of Ezh2 Function Results in Enhanced Th1 and Th2 Cell Differentiation

(A–D) Naive wild-type (WT) or Ezh2-deficient (*Ezh2*$^{\Delta SET/\Delta SET}$) CD4$^+$ T cells differentiated under Th1 (A and C) or Th2 (B and D) cell-inducing conditions with the indicated concentrations of IL-12 or IL-4, respectively.

(E) Naive CD4$^+$ T cells activated with anti-TCRβ alone for the indicated times.

(F and G) Naive CD4$^+$ T cells were cultured under Th1 cell-inducing conditions with 0.1 ng/ml IL-12 (F) or Th2 cell-inducing conditions with 1 ng/ml IL-4 (G) and the indicated concentrations of the Ezh2 inhibitor DZNep.

Cytokine production measured by cytoplasmic staining of IL-4 and IFN-γ or IL-5 and IL-13 after 5 hr restimulation with anti-TCRβ (A, B, F, and G) and ELISA after 24 hr restimulation with anti-TCRβ (C and D) or the indicated time of culture (E) (n = 3, mean ± SD). Data are representative of at least three independent experiments. *p < 0.05, **p < 0.01. See also Figure S1.

Th cells, we used transcriptional profiling of Ezh2-deficient Th cells combined with genome-wide mapping of Ezh2 target genes in WT Th1 and Th2 cells. Ezh2 deficiency resulted in 856 upregulated and 908 downregulated genes in nonpolarized Th cells. Gene ontology (GO) analysis (Huang et al., 2009) of the upregulated genes showed that the most statistically overrepresented

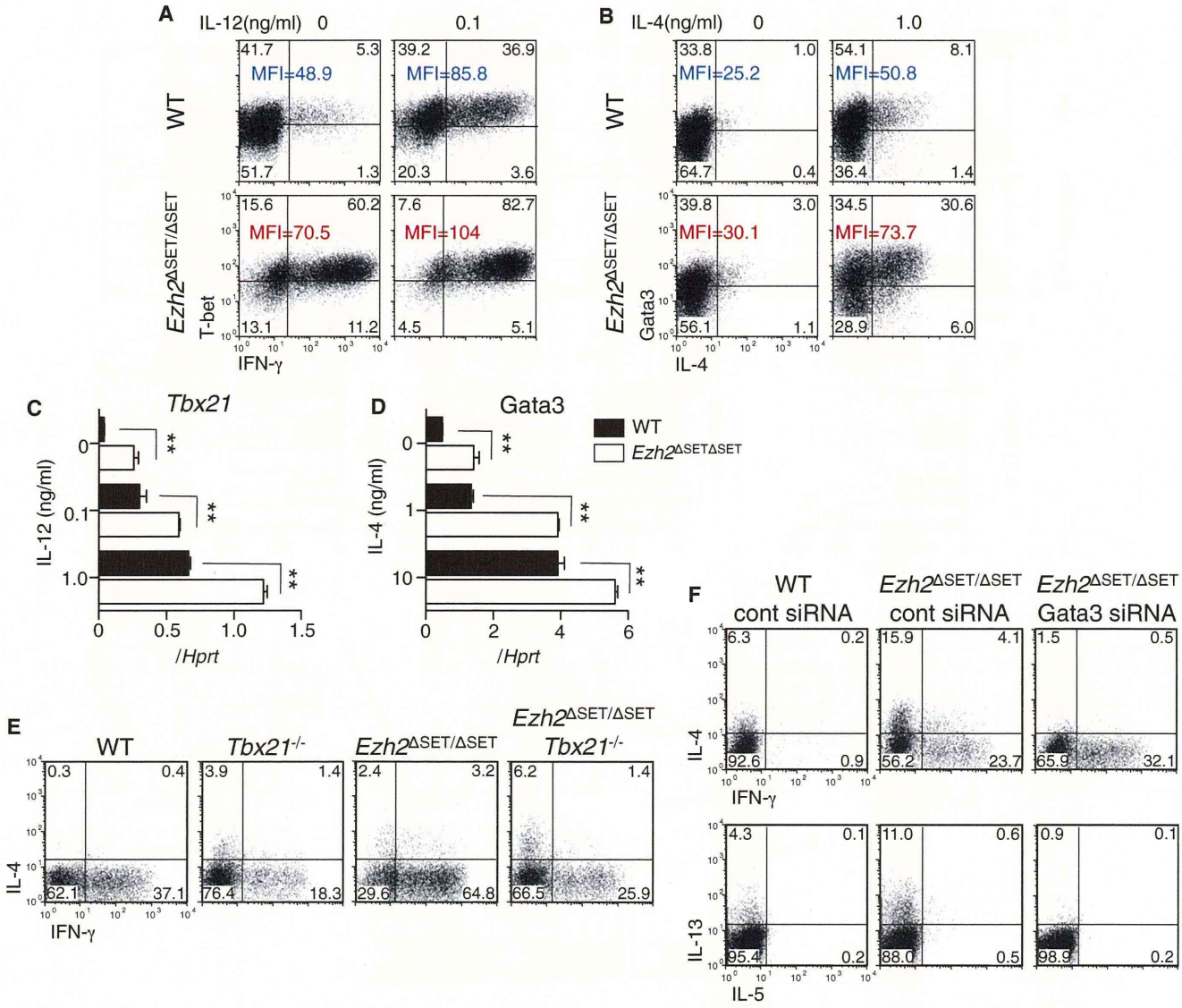

Figure 2. Loss of Ezh2 Results in Enhanced Expression of the Master Regulators T-bet and Gata3

(A–D) Naive WT or *Ezh2*$^{\Delta SET/\Delta SET}$ CD4$^+$ T cells differentiated under Th1 (A and C) or Th2 (B and D) cell-inducing conditions with the indicated concentrations of IL-12 or IL-4, respectively. mRNA expression of *Tbx21* (T-bet) (C) and *Gata3* (D), n = 3, mean ± SD. **p < 0.01.

(E) Naive WT, *Tbx21*$^{-/-}$, *Ezh2*$^{\Delta SET/\Delta SET}$, and *Ezh2*$^{\Delta SET/\Delta SET}$ *Tbx21*$^{-/-}$ double-deficient CD4$^+$ T cells differentiated under Th1 cell-inducing conditions with 0.1 ng/ml IL-12.

(F) Naive CD4$^+$ T cells differentiated under Th2 cell-inducing conditions with 1 ng/ml of IL-4 and electroporated with control siRNA (WT and *Ezh2*$^{\Delta SET/\Delta SET}$ cells) or Gata3 siRNA (*Ezh2*$^{\Delta SET/\Delta SET}$ cells).

Cytoplasmic staining of T-bet with IFN-γ (A) and Gata3 with IL-4 (B), mean fluorescence intensities (MFIs) of T-bet and Gata3 are shown (WT cells, blue; *Ezh2*$^{\Delta SET/\Delta SET}$ cells, red) and IL-4 with IFN-γ (E and F) and IL-5 with IL-13 (F). Data are representative of at least three independent experiments. See also Figure S2.

groups were involved in immune responses (immune system process; GOTERM_BP, p = 1.5 × 10^{-19}, 96 genes), with approximately half encoding cytokines and their receptors (cytokine-cytokine receptor interaction; KEGG pathway, p = 2.5 × 10^{-13}, 44 genes). This indicates that cellular context is important in determining which genes are derepressed when Ezh2 is absent, a phenomenon that has also been reported in other studies (Ezhkova et al., 2009; Hirabayashi et al., 2009). Of the genes defined in reference genome mm9 in the RefSeq database, 9.5% (1,998 genes) were bound by Ezh2 in both Th1 and Th2 cells, 1.1% (240 genes) were bound in Th1 cells only, and 2.0% (415 genes) were bound in Th2 cells only (Figure S3A). GO analysis of all bound genes showed very strong enrichment of genes involved in development (developmental process; GOTERM_BP, p = 5.1 × 10^{-117}, 743 genes) and transcription (transcription factor activity; GOTERM_MF, p = 1.1 × 10^{-87}, 308 genes), similar to that reported for polycomb proteins in embryonic stem cells (Boyer et al., 2006). Ezh2 binding was detected at 16.0% (137 of 856 genes) of the upregulated genes and also at 6.6% (60 of 908 genes) of the downregulated genes. Ezh2 binding was significantly more likely to be detected at genes upregulated in response to inactivation of Ezh2 (chi-square test,

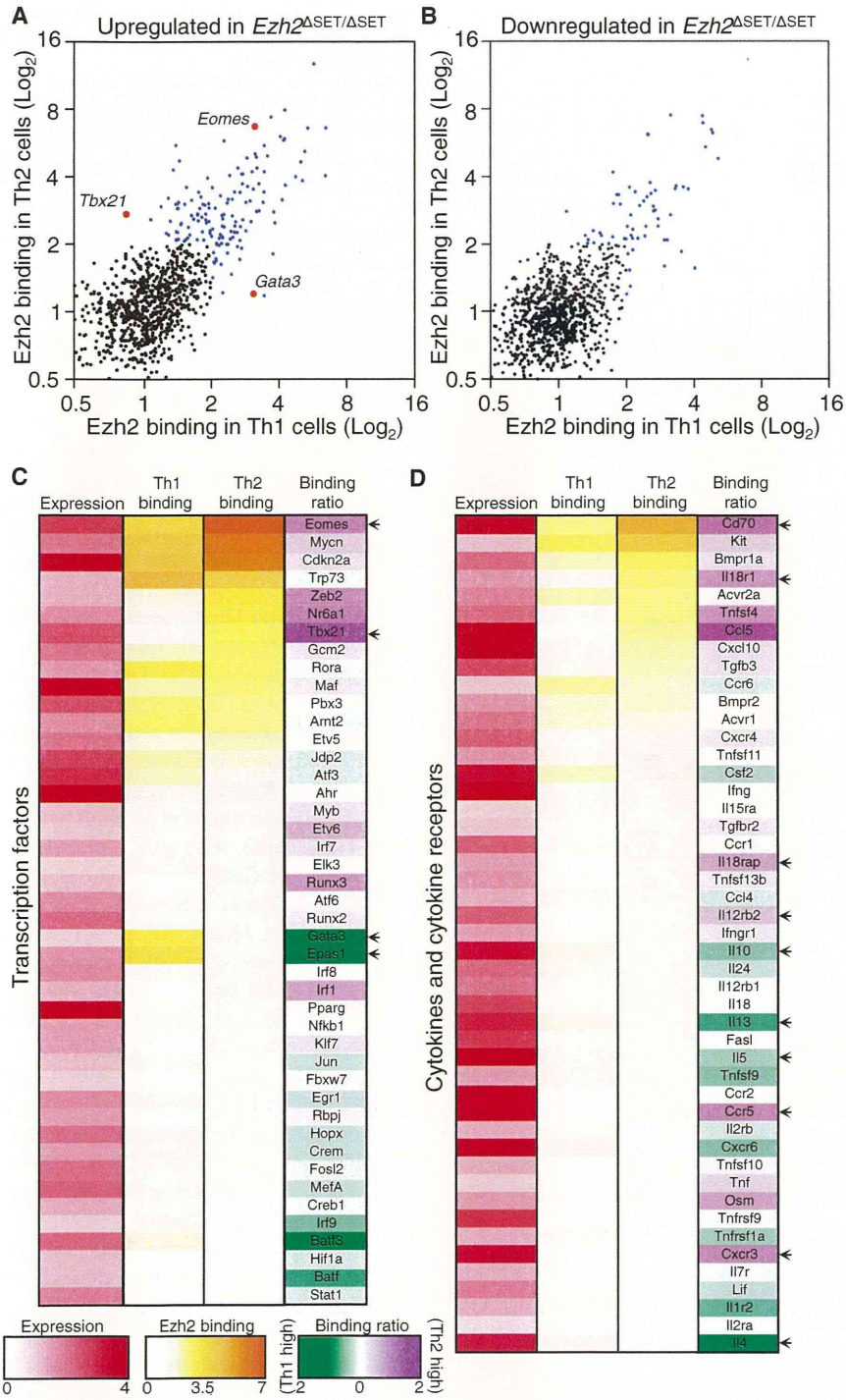

Figure 3. Global Analysis of Ezh2 Function and Localization by Gene Expression and ChIP-Seq Analysis

(A and B) Scatter plots depicting Ezh2 binding in Th1 cells (x axes) and Th2 cells (y axes) for all genes upregulated (A) and downregulated (B) in *Ezh2*$^{\Delta SET/\Delta SET}$ nonpolarized Th cells. Blue dots indicate greater than 2-fold enrichment of Ezh2 binding with *Tbx21*, *Eomes*, and *Gata3* (shown in red).

(C and D) Upregulated genes encoding transcription factors (C) and cytokines and cytokine receptors (D) in *Ezh2*$^{\Delta SET/\Delta SET}$ nonpolarized Th cells. Left column, fold upregulation (red); middle columns, Ezh2 binding in WT Th1 and Th2 cells (orange); right column, log2 ratio of Ezh2 binding between WT Th1 and Th2 cells (Th1 high, green; Th2 high, purple).

See also Figure S3.

$p = 7.3 \times 10^{-8}$); however, the presence of Ezh2 at a small subset of the downregulated genes also indicates that Ezh2 could play a more complex role in gene regulation at some loci. Ezh2 binding intensity in Th1 versus Th2 cells at all genes up- and downregulated by inactivation of Ezh2 are depicted; genes with more than 2-fold enrichment of Ezh2 are indicated in blue (Figures 3A and 3B and Table S1 and S2), with the transcription factors *Gata3*, *Tbx21*, and *Eomes* indicated in red. Based on our results by GO analysis, we then assessed the upregulated genes encoding transcription factors (44 genes) (Figure 3C) and cytokines and cytokine receptors (47 genes) (Figure 3D) in more detail. Overall, transcription factor genes showed significantly higher Ezh2 binding (Student's t test, $p = 0.003$). The lineage-specifying genes *Tbx21* and *Gata3*, and another gene with unknown function in Th1 or Th2 cells called *Epas1* (Figure 3C, arrows), showed the most dramatic changes in Ezh2 occupancy during differentiation. In Th1 cells, Ezh2 also preferentially bound genes encoding several cytokines produced by Th2 cells including IL-4, IL-5, IL-10, IL-13, and IL-24; however, Ezh2 occupancy at these loci was very low (Figure 3D, arrows). Loss of Ezh2 also resulted in increased expression of *Maf*, another transcription factor associated with regulation of Th2 cytokine expression; however, this gene did not appear to be a strong target of Ezh2, and there was no difference in Ezh2 binding at this loci in Th1 cells compared to Th2 cells (Figure S3B). As expected, no difference in Ezh2 binding between Th1 and Th2 cells was found at the *Ifng* gene; however, ChIP-seq analysis identified an area between 40 and 50 kb downstream of the *Ifng* gene that showed moderately higher Ezh2 binding in Th2 cells (Figure S3B), an area that may be functionally important for *Ifng* gene regulation (Schoenborn et al., 2007). We also found preferential Ezh2 binding in Th2 cells at genes encoding several surface receptors normally expressed on Th1 cells including *Cxcr3*, *Ccr5*, *Cd70*, *Il18r1*, *Il18rap*, and *Il12rb2* and the chemokine *Ccl5* (Figure 3D, arrows). Ezh2 ChIP-seq profiles for *Gata3*, *Epas1*, *Maf*, *Tbx21*, *Eomes*, Th2 cytokine, and *Ifng* loci are shown in Figure S3B. These data indicate that Ezh2 is dynamically regulated at several of the signature genes that define the Th1 and Th2 cell

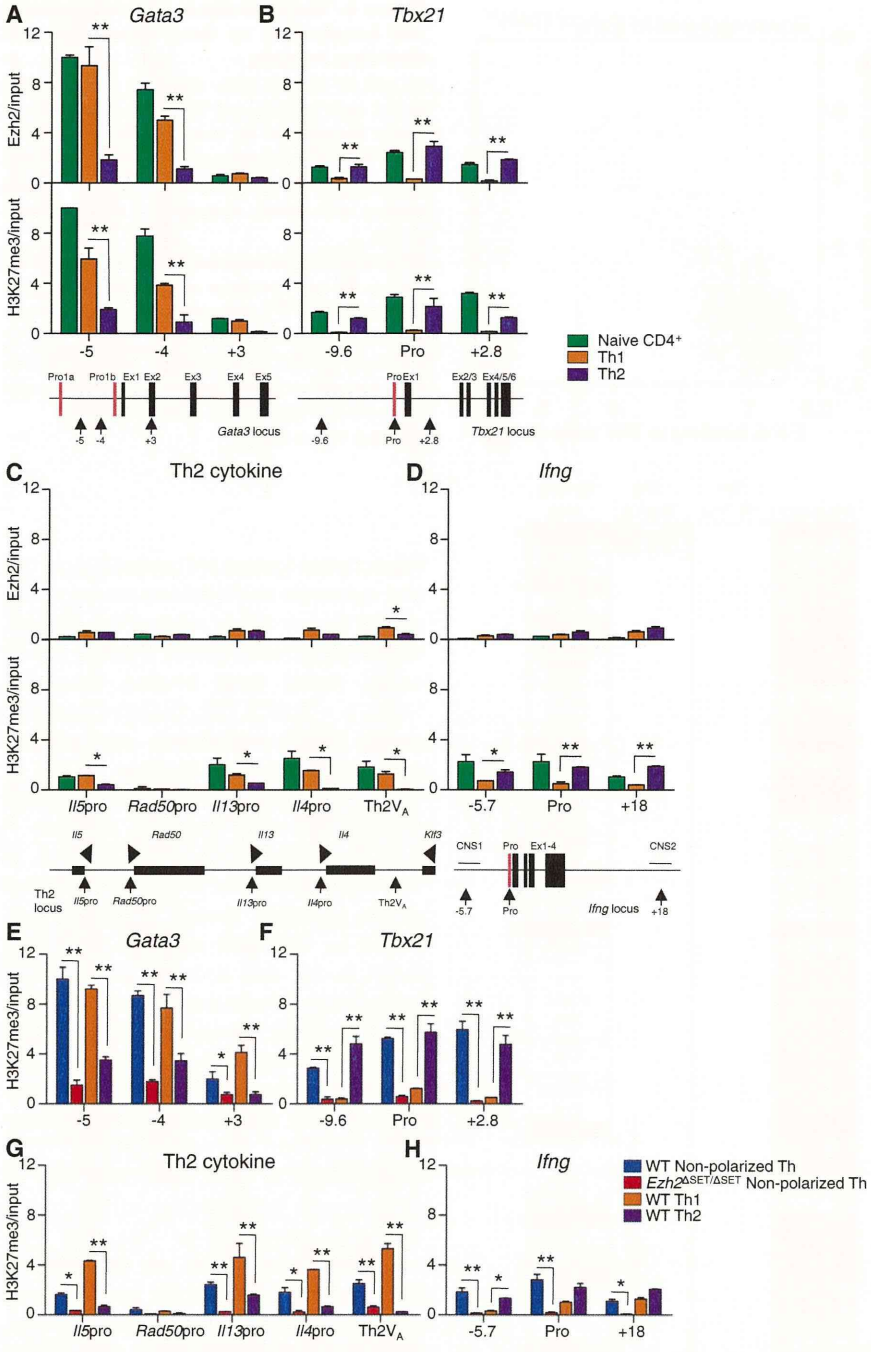

Figure 4. Dynamic Changes in Ezh2 Binding and H3K27me3 Occur during Th1 and Th2 Cell Differentiation

Ezh2-ChIP and H3K27me3-ChIP on WT CD4+ naive, Th1, and Th2 cells (A–D), and H3K27me3-ChIP on WT and *Ezh2*$^{\Delta SET/\Delta SET}$ nonpolarized Th cells with WT Th1 and Th2 cells for comparison (E–H), for *Gata3* (A and E), *Tbx21* (B and F), Th2 cytokine (C and G), and *Ifng* (D and H) loci measured by qPCR (n = 3, mean ± SD). Schematic representations of each gene loci show the positions of the primers used for detection relative to the transcriptional start site of each gene. Data are representative of three independent experiments. *p < 0.05, **p < 0.01; Abbreviation: pro., promoter. See also Figure S4.

differentiation programs. Moreover, it confirms that *Tbx21* and *Gata3* display comparatively stronger binding of Ezh2 and that these genes are among those that display the most dramatic differences in Ezh2 occupancy during differentiation.

Ezh2 Bound Directly to the Genes Encoding T-bet and Gata3 and Deficiency of Ezh2 Resulted in Loss of H3K27me3 at These Loci

To determine whether Ezh2 was lost after differentiation or recruited to *Gata3* and *Tbx21* loci, we performed ChIP on WT naive CD4+ T cells and differentiated Th1 and Th2 cells. Ezh2 binding was detected at the *Gata3* locus in naive and Th1 cells, which was dramatically diminished in Th2 cells (Figure 4A). A similar but reciprocal regulation was observed at the *Tbx21* locus where binding was detected in naive and Th2 cells but was absent in Th1 cells (Figure 4B). We also used H3K27me3-specific ChIP to assess the presence of the H3K27me3 modification at these loci in WT cells and found strong positive correlations between the level of Ezh2 binding and H3K27me3 (Pearson's correlation coefficient for *Gata3* locus r = 0.95, p < 0.0001 and *Tbx21* locus r = 0.77, p = 0.007) (Figures 4A and 4B). In agreement with the ChIP-seq data, we detected relatively little Ezh2 binding at the Th2 and *Ifng* cytokine loci compared to that observed for *Gata3* and *Tbx21* (Figures 4C and 4D). Interestingly, we did detect H3K27me3 associated with the Th2 and *Ifng* cytokine loci (Figures 4C and 4D), and H3K27me3 was lost at the Th2 cytokine loci in Th2 cells and at the *Ifng* locus in Th1 cells. At the *Rad50* promoter, an unrelated gene within the Th2 cytokine loci, we did not detect any differences in Ezh2 occupancy or H3K27me3 (Figure 4C), nor did we detect any differences in total H3 during differentiation at any of these genes (data not shown). Therefore, at the *Gata3* and *Tbx21* loci, differential binding of Ezh2 and the associated H3K27me3 modification in differentiated cells appears to be accomplished primarily via loss of high levels of pre-existing Ezh2 in naive CD4+ T cells. In contrast, both Ezh2 and H3K27me3 are maintained during the process of activation and cell division at lineage-specifying genes of the opposing Th cell subtype. Compared to the *Gata3* locus, Ezh2 occupancy and H3K27me3 at the *Il17a* and *Rorc* loci in Th cells activated without polarizing cytokines and in Th cells polarized under Th17 cell-inducing conditions were very low (Figure S4A). As

expected, association of Ezh2 with the *Gata3* and *Tbx21* loci was dramatically reduced in *Ezh2*$^{\Delta SET/\Delta SET}$ Th cells (Figures S4B and S4C). To determine whether a functional link exists between the presence of Ezh2 and H3K27me3, we assessed H3K27me3 at the *Gata3, Tbx21*, Th2, and *Ifng* cytokine loci. In nonpolarized Ezh2-deficient cells, we found that H3K27me3 was depleted at both transcription factor and cytokine loci, indicating that Ezh2 was required for the presence of H3K27me3 at both transcription factor and cytokine genes (Figures 4E–4H).

Ezh2-Mediated Repression of T-bet and Eomes Is Required to Control Spontaneous Generation of IFN-γ-Producing T Cells

Enhanced production of Th1 and Th2 cytokines was observed at varying levels, even in cultures without exogenously added polarizing cytokines (Figures 1 and 2). We next analyzed in more detail the mechanism through which Ezh2 controls cytokine production in nonpolarized Th cell cultures with neutralizing antibodies to both IL-4 and IFN-γ. WT nonpolarized Th cells did not produce appreciable levels of Th1 or Th2 cytokines, but a substantial percentage of Ezh2-deficient cells produced IFN-γ (42.1%) and smaller percentages produced IL-4 and IL-13 (3.4% and 17.3%, respectively) (Figure 5A). These data confirm that loss of Ezh2 results in deregulation of both Th1 and to a lesser extent Th2 effector cell subtypes in the absence of exogenous polarizing signals. In nonpolarized Ezh2-deficient Th cells, *Tbx21* mRNA was increased 11.3-fold, there was a large 49.5-fold increase in the expression of the related T-box transcription factor gene *Eomes*, and there was a more modest 3.1-fold increase in *Gata3* expression (Figure 5B). Corresponding increases in expression of T-bet, Eomes, and Gata3 proteins were also detected by intracellular staining and flow cytometry (Figure 5C).

To determine the relative contribution of Ezh2-mediated repression of *Tbx21* and *Eomes* to the deregulated IFN-γ expression by Ezh2 KO cells, we next compared Th cells doubly deficient in Ezh2 and T-bet with Th cells doubly deficient in Ezh2 and Eomes (*Ezh2*$^{\Delta SET/\Delta SET}$*Eomes*$^{\Delta Exon1/\Delta Exon1}$). Interestingly, cells doubly deficient in Ezh2 and T-bet produced only moderately less IFN-γ under nonpolarized conditions compared to Ezh2-deficient cells (Figures 5D and S5A), indicating that T-bet plays only a minor role in the spontaneous production of IFN-γ by Ezh2-deficient Th cells. In contrast, in cultures with exogenously added IL-12, the enhancement of IFN-γ production induced by loss of Ezh2 was more dependent on T-bet (Figures 2E and S5A). We next analyzed the contribution of Eomes to the enhanced production of IFN-γ by both nonpolarized and Th1 polarized Ezh2-deficient cells. Nonpolarized Ezh2-deficient Th cells again produced IFN-γ, but strikingly, the spontaneous production of IFN-γ was completely lost in Th cells doubly deficient in Ezh2 and Eomes (Figures 5E, top, and S5A), indicating that Ezh2-mediated repression of *Eomes* is essential to avoid spontaneous production of IFN-γ. In contrast, Ezh2 and Eomes double deficiency had only a mild effect on the production of IFN-γ induced by IL-12 (Figures 5E, bottom, and S5A). Therefore, Ezh2-mediated repression of both T-bet and Eomes is required to efficiently control IFN-γ production by activated Th cells. Ezh2 appears to modulate the sensitivity of Th cells to IL-12 via control of the *Tbx21* locus, and Ezh2 is also required to repress the *Eomes* locus to avoid spontaneous expression of IFN-γ.

In nonpolarized Th cells, we detected large amounts of H3K27me3 at the *Eomes* locus, and this modification was lost in the absence of Ezh2 (Figure 5F). As expected, the presence of Ezh2 at the *Eomes* locus was not detected in *Ezh2*$^{\Delta SET/\Delta SET}$ Th cells (Figure 5G). To further investigate possible mechanisms regulating the upregulation of *Eomes* in Ezh2-deficient Th cells, we next measured histone acetylation (ac), an epigenetic modification associated with accessible chromatin structure and active transcription. We found that both H3K9ac (Figure 5H) and H3K27ac (Figure 5I) were increased at the *Eomes* locus in cells lacking Ezh2. Both p300 and CBP contain a histone acetyltransferase (HAT) domain and form a coactivation complex that can recruit RNA polymerase and enhance gene expression. We found higher binding of both of these proteins to the *Eomes* locus in Ezh2-deficient cells (Figures 5J and 5K). We also detected modestly enhanced histone acetylation and accumulation of p300 and CBP at the *Tbx21* locus in Ezh2-deficient cells compared to WT cells (Figures S5B–S5E). It is therefore likely that the p300/CBP HAT complex plays a role in the spontaneous enhancement of *Eomes* and possibly *Tbx21* expression in the absence of Ezh2 function.

Ezh2-Deficient Th Cells Display Enhanced Plasticity

We next investigated whether Ezh2 was also involved in regulating the maintenance of CD4+ T cell phenotype after specialization into effector Th1 or Th2 cells. WT and Ezh2-deficient CD4+ T cells were differentiated into Th1 or Th2 cells for 3 days (primary culture) and then cells were cultured under the opposite conditions for a further 2 days (secondary culture) and assessed for their ability to produce effector cytokines. Very little production of IL-4 or IL-5 was detected in WT or Ezh2-deficient primary Th1 cell cultures, but Ezh2-deficient cells could still produce some IL-13 (Figure 6A). In contrast, when polarized Th1 cells were exposed to Th2 cell-inducing secondary culture, we observed much stronger induction of IL-4, IL-5, and IL-13 in Ezh2-deficient cells compared to WT cells (4.8, 7.6, and 8.8 times, respectively) (Figure 6A). In addition, compared to WT cells, we detected increased IFN-γ production by Th2 polarized Ezh2-deficient cells, and this was further enhanced in response to secondary culture under Th1 cell-inducing conditions (2.8 times) (Figure 6B). We next stained WT and Ezh2-deficient Th2 cells with both IFN-γ and IL-4. Polarized Ezh2-deficient Th1 cells could be readily induced to express IL-4, IL-5, and IL-13 when exposed to Th2 cell-inducing culture conditions (Figure 6C). Importantly, we detected both single- and double-positive populations in cells lacking Ezh2, indicating that IFN-γ-producing Th1 cells were induced to express IL-4 in the absence of Ezh2 function. Similar plasticity in cytokine production was observed in Ezh2-deficient Th2 cells; when exposed to Th1 cell-inducing conditions, Ezh2-deficient Th2 cells expressed IFN-γ and a large percentage of these cells produced both IFN-γ and IL-4 (Figure 6D). In contrast to the instability observed in both Th1 and Th2 cell differentiation programs, stable repression of Th17 cell differentiation by Th1 cells was not affected by loss of Ezh2 (Figure S6A).

WT and Ezh2-deficient Th1 cells expressed similar levels of Gata3; however, after exposure to Th2 cell-inducing conditions,

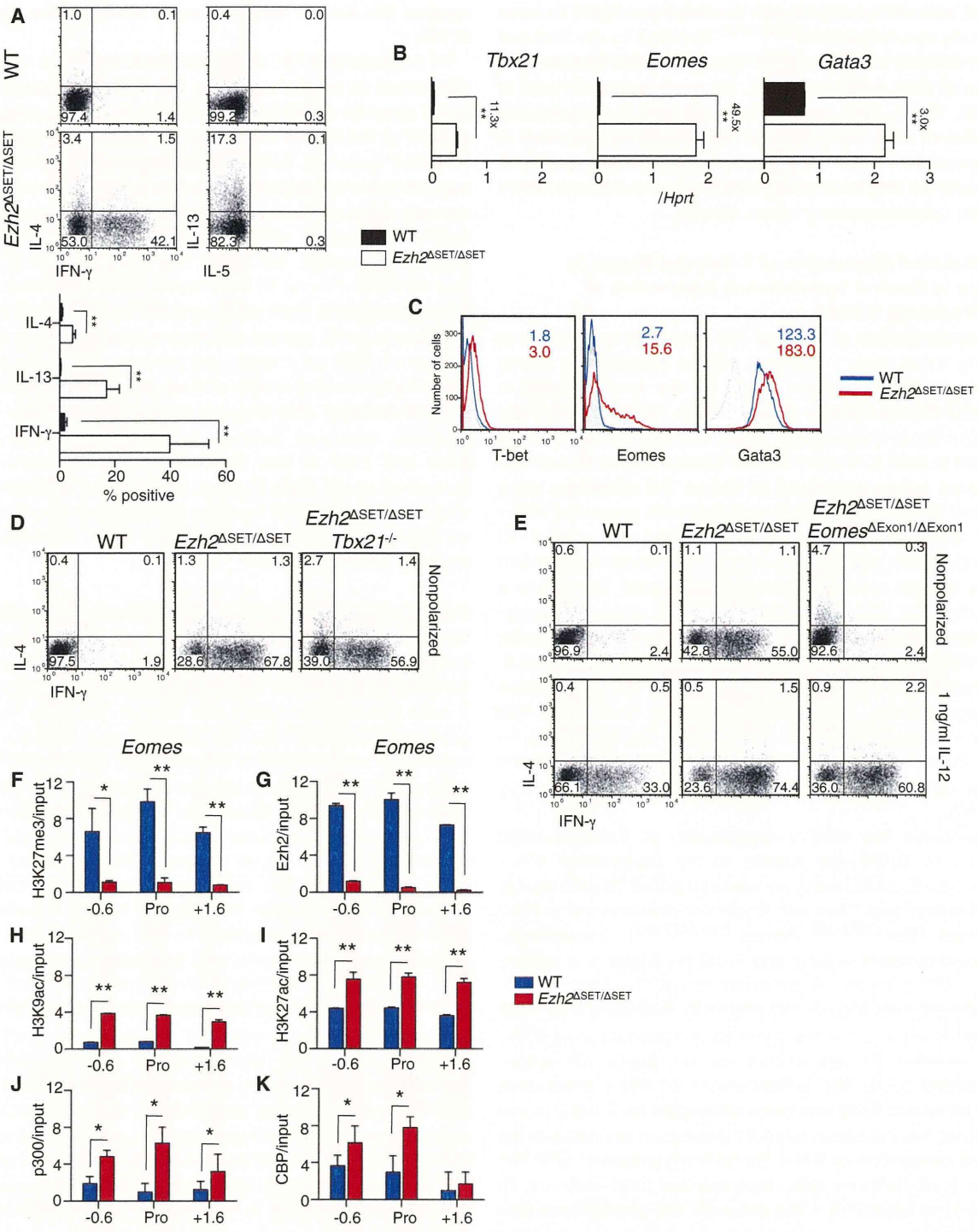

Figure 5. Ezh2-Mediated Repression of T-bet and Eomes Is Required to Control IFN-γ Production by Th Cells

(A–C) Naive WT and *Ezh2*$^{\Delta SET/\Delta SET}$ CD4$^+$ T cells differentiated under nonpolarizing conditions (with both anti-IL-4 and anti-IFN-γ neutralizing antibodies) followed by cytoplasmic staining of the indicated cytokines after 5 hr restimulation with anti-TCRβ (A, top) and combined results from five independent experiments (A, bottom), mRNA encoding *Tbx21*, *Eomes*, and *Gata3* (B), and protein expression of T-bet, Eomes, and Gata3 with MFIs shown (WT, blue; *Ezh2*$^{\Delta SET/\Delta SET}$, red) and 9,000 events displayed for each sample (C).

(D and E) Naive WT, *Ezh2*$^{\Delta SET/\Delta SET}$, and *Ezh2*$^{\Delta SET/\Delta SET}$*Tbx21*$^{-/-}$ double-deficient (D) or naive WT, *Ezh2*$^{\Delta SET/\Delta SET}$, and *Ezh2*$^{\Delta SET/\Delta SET}$*Eomes*$^{\Delta Exon1/\Delta Exon1}$ double-deficient (E) CD4$^+$ T cells differentiated under nonpolarizing (top) or Th1 cell-inducing conditions with 1 ng/ml IL-12 (bottom), and cytokine production measured by cytoplasmic staining of IL-4 and IFN-γ after 5 hr restimulation with anti-TCRβ.

(F–K) ChIP analysis for the indicated histone modifications and chromatin-modifying proteins at the *Eomes* locus in WT and *Ezh2*$^{\Delta SET/\Delta SET}$ nonpolarized Th cells. n = 3, mean ± SD. Data are representative of at least three independent experiments, *p < 0.05. **p < 0.01. See also Figure S5.