

Functionally distinct Gata3/Chd4 complexes coordinately establish T helper 2 (Th2) cell identity

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GATA binding protein 3 (Gata3) is a GATA family transcription factor that controls differentiation of naïve CD4 T cells into T helper 2 (Th2) cells. However, it is unknown how Gata3 simultaneously activates Th2-specific genes while repressing those of other Th lineages. Here we show that chromodomain helicase DNA-binding protein 4 (Chd4) forms a complex with Gata3 in Th2 cells that both activates Th2 cytokine transcription and represses the Th1 cytokine IFN- γ . We define a Gata3/Chd4/p300 transcriptional activation complex at the Th2 cytokine loci and a Gata3/Chd4-nucleosome remodeling histone deacetylase repression complex at the *Tbx21* locus in Th2 cells. We also demonstrate a physiological role for Chd4 in Th2-dependent inflammation in an in vivo model of asthmatic inflammation. Thus, Gata3/Chd4 forms functionally distinct complexes, which mediate both positive and negative gene regulation to facilitate Th2 cell differentiation.

T helper 2 cell differentiation | transcriptional regulation

The regulation of gene expression is fundamental to cell fate decisions and lineage specification during developmental processes. Lineage commitment of T lymphocytes in the immune system involves the activation of specific gene programs and the concomitant suppression of multipotential and alternate lineage gene profiles. These events are regulated in large part by a limited set of lineage-specific master transcription factors that functionally cross-antagonize one another.

The GATA family transcriptional factors (Gata1–6) selectively bind GATA sites in vertebrate genomes to regulate specific gene expression programs. Each GATA family member possesses two highly conserved type IV zinc fingers, referred to as the N-terminal and C-terminal zinc fingers, both of which are involved in DNA binding and protein–protein interactions (1). Among the six vertebrate homologs, Gata1–3 play key roles in the development and maintenance of hematopoietic and immune cells (2). Gata3 plays a critical role in T-cell development in the thymus; it has roles in the CD4 vs. CD8 lineage choice and at the β -selection checkpoint (3), as well as in T helper 2 (Th2) cell differentiation through the activation and repression of transcription (4, 5). After antigenic stimulation in a particular cytokine milieu, naïve CD4 T cells differentiate into one of several Th cell subsets including Th1 and Th2 cells (6). Differentiation of Th2 cells requires IL-4 stimulation, which leads to Stat6 phosphorylation and the up-regulation of *Gata3* transcription (7, 8). The deletion of *Gata3* in peripheral CD4 T cells prevents their differentiation into the Th2 lineage, causing cells to differentiate toward a Th1 phenotype in the absence of polarizing cytokines (9, 10). Conversely, the overexpression of *Gata3* in Th1 cells switches their polarity to a Th2 phenotype (11). Recent genome-wide analyses using chromatin immunoprecipitation and microarray analysis (ChIP–chip), ChIP sequence, and RNA sequence (4, 5, 12) have indicated that *Gata3* can directly or indirectly control a large number of Th2 cell-specific genes, as well as other genes including transcription factors such as T-bet (encoded by *Tbx21*), a key regulator of Th1 cell fate determination (13). Despite its fundamental importance, little is known regarding how

the Gata3 transcriptional complex positively and negatively regulates T-cell lineage gene profiles in a cell type-specific manner.

Dynamic changes in chromatin structure induced through ATP-dependent remodeling and covalent histone modifications play important roles in regulating gene expression (14). Several lines of evidence identify Gata3 as a regulator of histone modifications, including histone H3-K9 acetylation (H3-K9Ac); H3-K4me1, 2, and 3; and H3-K27me3 (4, 5, 15). Such modifications influence transcription by modulating interactions of transcription factors with chromatin (16). Indeed, we previously identified CGRE, a conserved GATA response element, 1.6 kb upstream of the *Il13* gene, corresponding to the 5' border of the long-range histone hyperacetylation region, and Gata3 was shown to bind to CGRE with histone acetyltransferase (HAT) complexes including CREB-binding protein (CBP)/p300 (15).

Numerous ATP-dependent chromatin-remodeling and histone-modifying enzymes have been identified, including those important for T-cell development (17). Among them is the 2-MDa nucleosome remodeling histone deacetylase (NuRD) complex (18), which is highly expressed in the thymus and associates with the Ikaros family of lymphoid-lineage regulating factors in differentiating and mature T cells. Chromodomain helicase DNA-binding protein 4 (Chd4) is an ATP-dependent chromatin remodeler and a major subunit of the repressive NuRD complex (18, 19). The Chd4–NuRD complex plays pivotal roles in transcriptional regulation, reorganization, and maintenance of chromatin structures and has recently been implicated in DNA damage repair (20). Other components of the complex include a catalytic subunit Hdac1/2 and the nonenzymatic proteins methyl-CpG binding domain 2/3 (Mbd2/3), retinoblastoma-associated 46/48 (RbAp46/48), metastasis-associated 1/2/3 (Mta1/2/3), and p66 α/β (19). The subunit composition of NuRD can vary depending on the cell type, altering the activity and localization of the complex. To date, the NuRD complex has been shown to mediate both transcriptional activation and repression programs by several distinct transcriptional factors, including p53, Ikaros, Bcl-6, and friend of GATA 1 (Fog-1) (20). Chd4 is highly expressed in thymocytes and lymphocytes, and it exerts a positive role in gene expression at the *Cd4* locus through the recruitment of HATs—i.e., p300, Moz, and Taf1—to the *Cd4* enhancer and silencer regions (21, 22).

We herein identify Chd4 as a central component of two functionally distinct Gata3 complexes. Genome-wide analysis using ChIP sequence revealed that Gata3 together with Chd4 binds to both the Th2 cytokine gene loci and the *Tbx21* locus. We found

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that Gata3 organizes a Gata3/Chd4/p300 complex at the Th2 cytokine gene loci and a Gata3/Chd4–NuRD repression complex at the *Tbx21* locus in Th2 cells, thus simultaneously regulating Th2 cytokine gene activation and *Tbx21* repression. We also demonstrated a physiological role for Chd4 in Th2-dependent inflammation in an in vivo model of asthmatic inflammation. Together, our results support a model in which Gata3/Chd4 centrally regulates T-cell fate and Th2 cell differentiation by forming functionally distinct complexes.

Results

Identification of Chd4, a Major Subunit of the NuRD Complex, as a Gata3-Interacting Protein in Th2 Cells. Recent genome-wide analyses suggest that Gata3 mediates both activating and repressive gene regulation (4, 5). We therefore reasoned that Gata3 might interact with different cofactors to perform appropriate regulatory functions. To test this idea and isolate Gata3 complexes in Th2 cells, extracts from the Th2 cell clone D10G4.1, expressing Flag-tagged Gata3 at physiological levels (Fig. S1A), were subjected to two-step affinity purification by using anti-Flag and -Gata3 mAbs followed by SDS/PAGE and silver staining. Mass spectrometry identified multiple subunits of the NuRD complex, including Chd4, Mta1, Mta2, p66 α , and Mbd3, as Gata3-interacting proteins (Fig. 1A). To pursue this observation, we first examined the Gata3 association with Chd4. Flag-tagged Gata3 and Myc-tagged Chd4 were ectopically co-expressed in 293T cells, and then immunoprecipitation (IP) was performed with an anti-Flag mAb. A specific complex containing Gata3 and Chd4 was detected by immunoblotting (IB) with anti-Myc mAb (Fig. S1B). The association of Gata3 with Chd4 persisted in the presence of ethidium bromide, suggesting that the association is DNA-independent (Fig. S1C). To confirm that endogenous Gata3 and Chd4 interact, cell extracts from primary Th2 cells were subjected to IP with anti-Gata3 mAb. Endogenous Chd4 was reproducibly detected in Gata3 immunoprecipitates (Fig. S1D). Further, purified recombinant Gata3 and Chd4 interacted in GST pull-down assays (Fig. S1E), and partial colocalization of the two proteins was detected by confocal microscopy (Fig. S1F). These results indicate that Chd4 is a bona fide Gata3-interacting protein in Th2 cells.

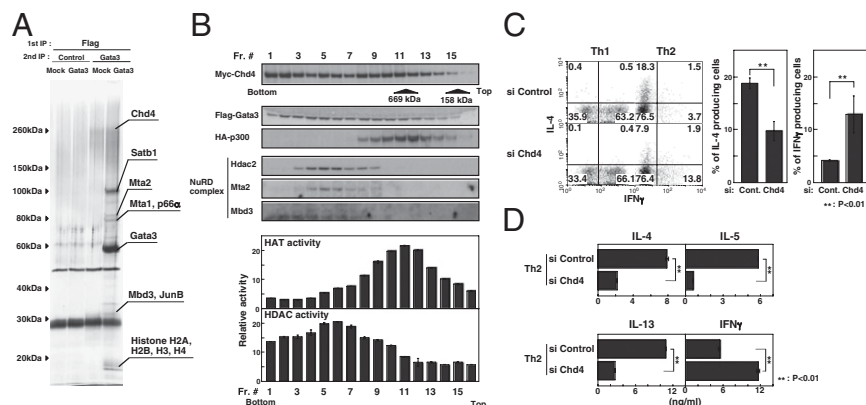
Gata3/Chd4 Complex Forms Functionally Distinct Assemblies with HAT or Histone Deacetylase Activity. To address the functional role of the Gata3/Chd4 complex in chromatin regulation, 293T cells were cotransfected with Myc-tagged Chd4, Flag-tagged Gata3, and HA-tagged p300, because we have previously shown that

Gata3 HAT complexes include p300 at the Th2 cytokine gene loci (15). Chd4 complexes were immunopurified, eluted by using a Myc-peptide, and then subjected to sucrose gradient ultracentrifugation and size fractionation (Fig. 1B). Chd4 and Gata3 were detected broadly throughout the gradient, indicating that Gata3/Chd4 are a part of multiple complexes of different sizes. Interestingly, HA-tagged p300 was detected in the slowly sedimenting fractions at a peak of ~670 kDa, whereas endogenous subunits of the NuRD complex, Hdac2, Mta2, and Mbd3, were detected in the rapidly sedimenting fractions eluting with an apparent mass of >1 MDa (Fig. 1B Upper). This finding suggests that Chd4 forms distinct types of multiprotein complexes, including Gata3/Chd4/p300 and Gata3/Chd4–NuRD. We then measured HAT and histone deacetylase (HDAC) activity in each fraction, and importantly the peak pattern of HAT and HDAC activity segregated with p300 and Hdac2, respectively (Fig. 1B Lower). These data suggest the existence of functionally distinct Gata3/Chd4 complexes, which may differentially regulate gene expression in Th2 cells.

Activating and Repressive Functions of Chd4 in Th2 Cell Differentiation.

To determine whether Chd4 is required for efficient Gata3-mediated regulation of Th1 and Th2 cytokine expression, we engineered Chd4-knockdown (KD) D10G4.1 cells stably expressing a shRNA against Chd4 via a lentivirus system. Chd4 KD had no effect on the expression level of *Gata3* mRNA (Fig. S2A). Interestingly, although Chd4 is known as a component of the NuRD repressor complex, its KD significantly inhibited the induction of mRNA for the Th2 cytokines *Il4* and *Il5*. In contrast, the expression of *Ifng* was up-regulated in the Chd4-KD D10G4.1 cells (Fig. S2B), suggesting that Chd4 may have biphasic functions—Th2 cytokine gene activation and IFN- γ repression in Th2 cells. To further examine the role of Chd4 in Th2 cell differentiation in a more physiological experimental setting, naive CD4 T cells from C57BL/6 mice were transfected with Chd4 siRNA and cultured under Th1 or Th2 conditions in vitro. Although *Chd4* mRNA expression was silenced efficiently (Fig. S2C), Chd4 KD had no impact on the differentiation of naive CD4 T cells into IFN- γ -producing Th1 cells (Fig. 1C). Intriguingly, however, the number of IL-4-producing cells cultured under Th2 conditions was decreased substantially by KD of Chd4 (18.3% vs. 7.9%), whereas the number of IFN- γ -producing cells was increased (3.7% vs. 13.8%) (Fig. 1C). Furthermore, quantitative RT-PCR (RT-qPCR) and ELISA analysis revealed that Chd4 KD during Th2 cell differentiation inhibited the expression of IL-4, -5, and -13, without altering *Gata3* expression, whereas the expression of IFN- γ was significantly increased (Fig. 2D and Fig. S2D). These results indicate

Fig. 1. Identification of Chd4 in functionally distinct Gata3 complexes. (A) Total extracts from Flag-Gata3-expressing D10G4.1 cells were subjected to tandem affinity purification by using anti-Flag and -Gata3 mAbs followed by SDS/PAGE and silver staining. Several specific polypeptides were identified by mass spectrometry as described in *SI Materials and Methods*. (B) The 293T cells were transfected with expression plasmids encoding Myc-tagged Chd4, Flag-tagged Gata3, and HA-tagged p300 for 2 d. Extracts were immunoprecipitated with anti-Myc mAb, and elutes were then subjected to ultracentrifugation through a 15–40% (wt/vol) sucrose gradient. The collected gradient fractions were subjected to IB with anti-Myc, -Flag, -HA, -Mta2, -Hdac2, and anti-Mbd3 antibodies. The specific HAT and HDAC activities in each fraction were determined as described in *SI Materials and Methods*. The relative activity is shown with SDs. (C) A control or Chd4 siRNA was transfected into naive CD4 T cells, and the cells were stimulated with immobilized anti-TCR- β and anti-CD28 mAbs under Th1 or Th2 conditions for 4 d. (Left) Representative IFN- γ /IL-4 profiles are shown with percentages of cells in each quadrant. (Right) A summary of the percentage of IL-4-producing and IFN- γ -producing cells in Chd4 KD Th2 cells is presented. $n = 5$. $^{**}P < 0.01$ (Student *t* test). (D) A portion of the same cultured cells used in C (Th2 conditions) were stimulated with immobilized anti-TCR- β mAb for 24 h, and the concentrations of cytokines in the culture supernatant were determined by ELISA. $^{**}P < 0.01$ (Student *t* test). All data are representative of two or more independent experiments.



that, together with Gata3, Chd4 participates in both up-regulation of Th2 cytokines and repression of IFN- γ in developing Th2 cells.

Gata3/Chd4 Complex Recruits p300 to Th2 Cytokine Gene Loci and Facilitates Th2 Cytokine Transcription. Next, we performed genome-wide analysis of Gata3 and Chd4 occupancy in developing Th1 and Th2 cells by ChIP followed by massive parallel sequencing (ChIP-Seq). We identified 611 target genes shared by Gata3 and Chd4 in Th2 cells (Fig. S3A), including several regions in the Th2 cytokine gene loci. Significant binding of Gata3 and Chd4 at the Th2 cytokine gene loci was observed specifically in Th2 cells (Fig. 2A, asterisks), and three out of six of significant peaks overlapped (Fig. 2A).

To determine whether the increased binding of Chd4 in Th2 cells was Gata3-dependent, wild-type (WT) and OX40-Cre-driven conditional *Gata3*-knockout (KO) (*Gata3*-deficient) CD4 T cells were cultured under Th1 or Th2 conditions for 3 d and then subjected to Chd4 ChIP. Chd4 binding at various regions in the Th2 cytokine gene loci was significantly increased in WT Th2 cells compared with WT Th1 cells, including the overlapping Gata3/Chd4 binding site (G3/C4BS), but this binding was abolished in *Gata3*-deficient Th2 cells (Fig. 2B Left). Interestingly, the increase in Th2-specific binding was not observed for other subunits of the NuRD complex Hdac2 and Mbd3 (Fig. 2B Right). Gata3 is thus required for accumulation of Chd4 at the Th2 cytokine gene loci, and the Gata3/Chd4 complex recruited to this loci does not appear to include the repressor components of the NuRD complex, such as Hdac2. Given that one of the highest enrichments of the Gata3 and Chd4 coassociation was observed at the CGRE region of the Th2 cytokine gene loci—and that

Gata3, Chd4, and p300 appear to form a complex with HAT activity (Fig. 1B)—we were prompted to examine Th2-specific histone hyperacetylation and recruitment of p300 to these sites. H3-K9Ac at the Th2 cytokine gene loci was lower in Chd4-KD cells compared with those of control Th2 cells (Fig. S3B). Among various HAT(s), Th2-specific binding of p300 to the CGRE, *Il13p*, and *Il4p* regions was observed, whereas others examined were either unaffected (CBP) or produced very low signal (Taf1 and Moz) in the assay (Fig. S3C). Moreover, the recruitment of p300 to the Th2 cytokine gene loci was abrogated in *Gata3*-deficient Th2 cells (Fig. 2C) and was significantly impaired in Chd4-KD Th2 cells (Fig. 2D), although the binding of Gata3 to the CGRE region was unaffected in Chd4-KD Th2 cells (Fig. S3D).

To determine whether the common binding regions for Gata3 and Chd4 around the Th2 cytokine gene loci play a functional role in transcriptional activation, 260-bp fragments spanning the Gata3 and Chd4 binding regions either upstream of the *Il13* gene (CGRE) or intron 2 of the *Il4* gene (HS2) were examined in reporter assays. Introduction of the CGRE and HS2 fragments significantly enhanced the transcriptional activity of the *Il4* promoter in Th2 cells, but not in Th1 cells (Fig. 2E). We also assessed the role of p300 in Th2 cell differentiation, using an adenovirus E1A protein that binds the acetyltransferase region of p300 and inhibits its activity (23). Introduction of WT E1A (E1A WT) dramatically decreased the number of IL-4-producing cells, whereas mutant E1A lacking the p300-binding sequence (E1A d2-36) had no effect (Fig. 2F). Together, these results indicate that the Gata3/Chd4 complex positively regulates Th2 cell differentiation through the recruitment of p300 and subsequent induction of histone H3-K9Ac at the Th2 cytokine gene loci.

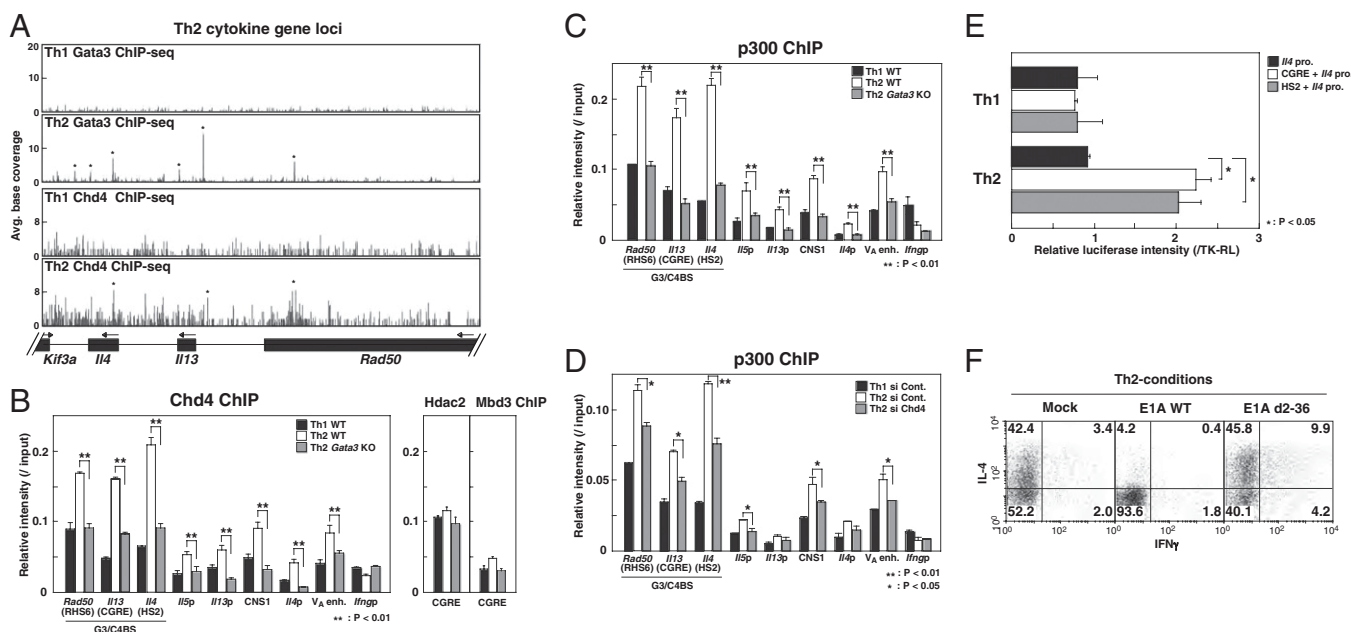


Fig. 2. Gata3 and Chd4 are necessary for the recruitment of p300 at the Th2 cytokine gene loci in developing Th2 cells. (A) Naïve CD4 T cells were stimulated under Th1 or Th2 conditions for 3 d, and then ChIP-seq for Gata3 and Chd4 was performed as described in *SI Materials and Methods*. The binding pattern of Gata3 and Chd4 at the Th2 cytokine gene loci is shown. Each bar represents the average base coverage at the corresponding position. The asterisk (*) indicates a fivefold greater peak value compared with input DNA. (B) CD4 T cells from WT or *Gata3*-deficient mice were cultured under Th1 or Th2 conditions for 3 d. The binding of Chd4, Hdac2, and Mbd3 to the Th2 cytokine gene loci including the CGRE or the *Ilfng* promoter (indicated at bottom) in Th1 WT (filled bars), Th2 WT (open bars), or Th2 *Gata3* KO (gray bars) cells was determined by ChIP assay with a qPCR analysis. The relative intensities (input) are shown with SDs. (C) The binding of p300 to the Th2 cytokine gene loci was determined and analyzed as in B. (D) A control or Chd4 siRNA was transfected into naïve CD4 T cells, and the cells were stimulated under Th1 or Th2 conditions for 3 d. The binding of p300 to the Th2 cytokine gene loci was determined and analyzed as in B. (E) Developing Th1 and Th2 cells were transfected with 3.5 μ g of the indicated reporter constructs in the presence of 0.5 μ g of the pRL-TK vector as an internal control on day 3 of the culture. One day after transfection, cells were stimulated with immobilized anti-TCR- β mAb for 12 h, followed by measurement of luciferase activity. The data indicate the mean results of three independent experiments with SDs. ** P < 0.01 (Student *t* test). (F) CD4 T cells were stimulated under Th2 conditions for 2 d, and then the cells were infected with a retrovirus vector containing E1A WT cDNA or E1A d2-36 cDNA. Three days later, the cells were stimulated with immobilized anti-TCR- β mAb for 6 h before IL-4/IFN- γ staining. All data are representative of two or more independent experiments.

Gata3 Recruits the NuRD Complex to the *Tbx21* Locus and Facilitates Repression of T-Bet-Dependent IFN- γ Expression in Developing Th2 Cells. To clarify the role of the Gata3/Chd4 complex in the repression of IFN- γ in Th2 cells, we investigated the effect of Chd4 silencing on various transcription factors important for IFN- γ expression or Th1 cell differentiation. Expression of *Tbx21* mRNA was increased in Chd4-KD cells, whereas there was little or no impact on *Eomes*, *Hlx*, or *Runx3* (Fig. 3A and Fig. S4A). Expression of *Tbx21* mRNA was increased in *Gata3*-deficient Th2 cells compared with naïve CD4 or WT Th2 cells (Fig. 3B), and T-bet protein was increased in *Gata3*-deficient Th2 cells compared with WT Th2 cells (Fig. S4B). These results indicate that both Chd4 and Gata3 are required for repression of T-bet in Th2 cells.

ChIP-seq analysis revealed that in Th2 cells, both Gata3 and Chd4 bind to intron1 of the *Tbx21* locus (Fig. 3C, asterisks); however, we detected no obvious binding of Chd4 at the *Ifng* locus in Th2 cells (Fig. S4C). In addition, the significant increase in *Ifng* expression detected in Chd4-KD Th2 cells was not observed when Chd4 was knocked down in *Tbx21* KO Th2 cells (Fig. S4D). These results suggest that the Gata3/Chd4 complex represses IFN- γ production by direct inhibition of *Tbx21* gene expression.

To assess whether the binding site of both Gata3 and Chd4 at intron1 of the *Tbx21* locus (*Tbx21* G3/C4BS) plays a functional role in transcriptional repression, a 250-bp fragment spanning the *Tbx21* G3/C4BS was placed between the *Tbx21* enhancer and promoter (−500) regions, and luciferase reporter assays were performed (Fig. S4E). As expected, the introduction of the *Tbx21* G3/C4BS fragment substantially repressed the transcriptional activity of the *Tbx21* enhancer–promoter in Th2 cells but not in Th1 cells (Fig. 3D and Fig. S4F).

Next, we sought to address whether Gata3 recruits the NuRD complex to the *Tbx21* locus. Subunits of the NuRD repressor complex, Chd4, Hdac2, and Mbd3, were associated with *Tbx21* G3/C4BS in a Gata3-dependent manner (Fig. 3E). Importantly, we found no preferential recruitment of p300 to *Tbx21* G3/C4BS

in Th2 cells. Because the NuRD complex represses transcription primarily via histone deacetylation, we examined the effect of a HDAC inhibitor, trichostatin A (TSA), on the Gata3-mediated repression of *Tbx21* expression in Th2 cells. CD4 T cells were cultured for 3 d in the absence or presence of TSA after stimulation for 2 d under Th2 conditions. The number of IFN- γ -producing cells was increased after TSA treatment (4.7% vs. 20.0%), whereas no effect on the number of IL-4-producing cells was observed (67.3% vs. 66.8%) (Fig. 3F). In accordance with induction of IFN- γ production by TSA treatment, *Tbx21* mRNA was up-regulated in TSA-treated Th2 cells (Fig. 3G). Thus, in Th2 cells, HDAC activity plays a pivotal role in the repression of *Tbx21* and subsequent inhibition of IFN- γ production, but it has little activity in regulating Th2 cytokines. These results suggest that direct binding of Gata3 to the *Tbx21* locus is required for recruitment of Chd4 and HDACs, to repress T-bet-dependent IFN- γ expression in Th2 cells.

Classification of Gata3/Chd4 Target Genes Identified by Using ChIP-Seq and Transcriptional Start Site Sequencing Analyses. Our results thus far suggested that Gata3 simultaneously organizes functionally distinct complexes in Th2 cells—the Gata3/Chd4/p300 complex at the Th2 cytokine gene loci and the Gata3/Chd4–NuRD complex at the *Tbx21* locus. To generalize this concept, we searched for additional genes that were bound by both Gata3 and Chd4 and that were differentially regulated in Th2 cells in a Gata3-dependent manner. Massive parallel sequencing of mRNA transcribed from transcriptional start sites (TSS-seq) (24) was used to identify genes that were specifically expressed in Th2 cells compared with both Th1 and *Gata3*-deficient Th2 cells. Of the 611 genes cobound by Gata3 and Chd4, we identified 18 candidate target genes that were positively regulated (higher expression in Th2 cells compared with both Th1 and *Gata3*-deficient Th2 cells) and 5 target genes that were negatively regulated (lower expression in Th2 cells compared with both Th1 and

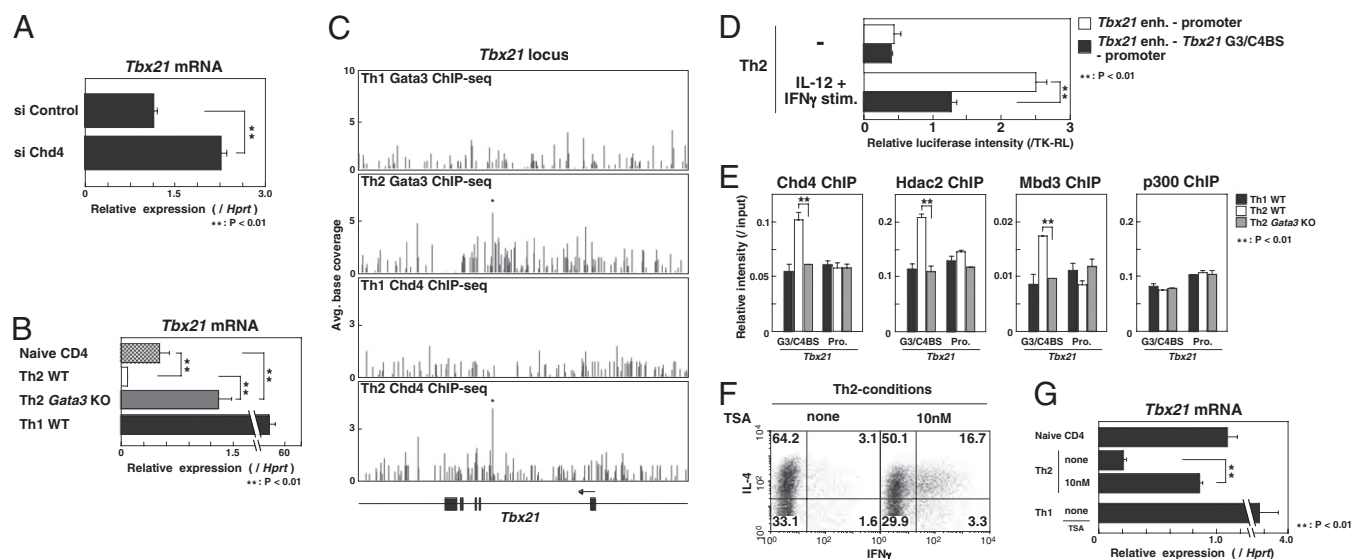


Fig. 3. Gata3 recruits the NuRD complex to the *Tbx21* locus and represses *Tbx21* expression in developing Th2 cells. (A) Naive CD4 T cells were transfected with control or Chd4 siRNA and cultured under Th2 conditions for 3 d, and then expression of *Tbx21* was determined by RT-qPCR. ** $P < 0.01$ (Student *t* test). (B) Naive CD4 T cells from either WT or *Gata3*-deficient mice were cultured under Th2 conditions for 5 d, and the expression levels of *Tbx21* mRNA were determined by RT-qPCR. ** $P < 0.01$ (Student *t* test). (C) ChIP-seq for Gata3 and Chd4 was performed as described in SI Materials and Methods. The binding patterns of Gata3 and Chd4 at the *Tbx21* locus are shown as Fig. 2A. (D) Developing Th2 cells were transfected with the indicated reporter constructs shown in Fig. S4E. Cells were then stimulated with or without 10 ng/mL IL-12 and 100 ng/mL IFN- γ for 36 h, followed by measurement of luciferase activity. The data indicate the mean results of three independent experiments with SDs. ** $P < 0.01$ (Student *t* test). (E) The binding of Chd4, Hdac2, Mbd3, and p300 at the *Tbx21* locus in Th1 WT, Th2 WT, and Th2 *Gata3* KO cells was determined by ChIP assay using qPCR analysis. (F) CD4 T cells were stimulated under Th2 conditions, and 10 ng/mL TSA was added on day 2 of the culture. Three days later, the cells were stimulated with immobilized anti-TCR- β mAb and subjected to IL-4/IFN- γ staining, followed by FACS analysis. (G) Naive CD4 T cells were stimulated under Th1 or Th2 conditions, and 10 ng/mL TSA was added on day 2 of the culture. Three days later, the *Tbx21* mRNA levels were assessed by RT-qPCR. All data are representative of two or more independent experiments.

Gata3-deficient Th2 cells) in Th2 cells (Fig. S5A). We then measured expression of these candidate genes by RT-qPCR in Th1, Th2, and *Gata3*-deficient Th2 cells (Fig. S5B) and in Th2 cells where Chd4 had been silenced by siRNA (Fig. S5C). This analysis showed that *Epas1*, *Ccr8*, and *Myl6b* were highly expressed and that *Mb21d1* was suppressed in Th2 cells in a *Gata3*- and Chd4-dependent manner. The other molecules were either *Gata3*-independent and/or Chd4-independent. We then performed ChIP assays to confirm the recruitment of the *Gata3*/Chd4/p300 activating complex or the *Gata3*/Chd4–NuRD repressive complex at these differentially regulated gene loci. As observed at the CGRE region of the Th2 cytokine gene loci, Chd4 and p300 were recruited to the *Gata3* binding sites of the *Epas1* and *Ccr8* loci in a *Gata3*-dependent manner (Fig. S5D Left), and Chd4 silencing by siRNA compromised the recruitment of p300 to the *Gata3* binding sites of the *Ccr8* locus (Fig. S5E Left). Conversely, like the *Tbx21* locus, Chd4 and Hdac2 were associated with the *Gata3* binding site of the *Mb21d1* locus in a *Gata3*-dependent manner, and Chd4 KD by siRNA disrupted Hdac2 recruitment to this region (Fig. S5D Right and E Right). Thus, our data support a hypothesis whereby functionally distinct *Gata3*/Chd4 complexes simultaneously activate or repress specific subsets of genes in Th2 cells through the recruitment of p300 or Hdac2.

Physiological Role of Chd4 in Th2-Mediated Allergic Responses in Vivo. Finally, we investigated the in vivo physiological role of Chd4 using a Th2-cell-dependent airway inflammation model (25). Chd4 KD Th2 cells or control cells generated by in vitro culture from DO11.10 transgenic [ovalbumin (OVA)-specific TCR (T-cell receptor)- $\alpha\beta$ Tg] mice were i.v. injected into normal BALB/c mice. The mice were challenged twice by inhalation with 1% OVA (Fig. 4A). A significant decrease in the infiltration of inflammatory cells, including eosinophils, in the bronchioalveolar lavage (BAL) fluid was observed in the Chd4 KD group in comparison with the control group (Fig. 4B). Histological analysis showed that mononuclear cell infiltration into the peribronchiolar regions of the lung was also modest in the Chd4 KD animals (Fig. 4C Upper). The levels of mucus hyperproduction and Goblet cell metaplasia assessed by PAS staining were lower in the bronchioles of the Chd4 KD group (Fig. 4C Lower). No obvious methacholine-induced airway hyperresponsiveness was induced in the Chd4 KD group (Fig. 4D). Together, these results indicate that Chd4 is involved in the regulation of Th2-cell-mediated allergic airway inflammation and airway hyperresponsiveness.

Discussion

We herein describe a mechanism whereby *Gata3* is able to mediate both positive and negative effects on gene expression in Th2 cells. Chd4 was found to be a central component of two functionally distinct *Gata3* complexes. The activating *Gata3*/Chd4 complex recruits p300 and binds to specific locus control regions in the Th2 cytokine gene loci, whereas the repressive *Gata3*/Chd4 complex interacts with Hdac2 and forms a *Gata3*/Chd4–NuRD complex at the *Tbx21* locus; the former induces Th2 cytokine gene activation, and the latter represses the *Tbx21* gene, thus suppressing Th1 differentiation and IFN- γ expression (Fig. S6). Because Chd4 binding to the target genes is abolished in the absence of *Gata3* (Figs. 2B and 3E), Chd4-mediated positive and negative regulation is dependent on Chd4–*Gata3* interaction. These two functionally distinct complexes exist in the same developing Th2 cells and coordinately establish Th2 cell identity.

Although a requirement for *Gata3*-mediated chromatin remodeling at the Th2 cytokine gene loci during Th2 cell differentiation has been well established (26), the molecular mechanisms underlying *Gata3*-mediated chromatin remodeling remain unknown. Discovery of the Chd4/*Gata3* interaction and the critical role of Chd4 in the initial step of *Gata3*-mediated chromatin remodeling, including H3-K9 hyperacetylation of the Th2 cytokine gene loci, provides key mechanistic insight into this process (Fig. 2). A similar mode of positive regulation by the *Gata3*/Chd4/

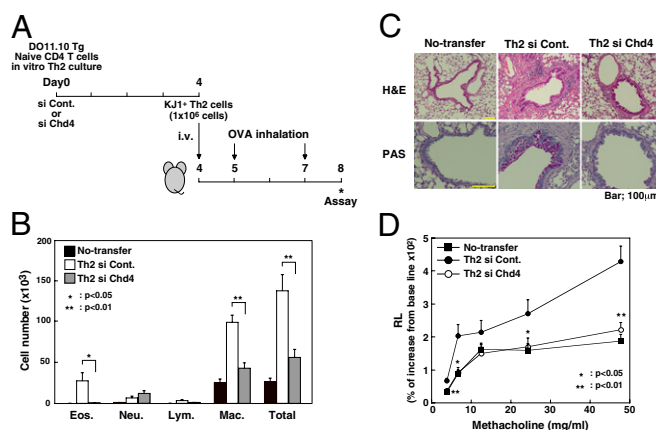


Fig. 4. Chd4 regulates OVA-induced allergic airway inflammation. (A) Schematic outline of in vivo model for Th2-mediated allergic responses. In brief, DO11.10 Tg Th2 cells (1×10^6 cells) were treated with control (si Cont.) or Chd4 (si Chd4) siRNA and i.v. transferred into BALB/c mice on day 4, before OVA inhalation twice on days 5 and 7. A no-cell-transfer group was also prepared as a negative control (No-transfer). All assays were performed on day 8. (B) The number of inflammatory cells in the BAL fluid was counted. The absolute cell numbers of eosinophils (Eos.), neutrophils (Neu.), lymphocytes (Lym.), and macrophages (Mac.) are shown with SEM. Five mice per group were used. ** $P < 0.01$; * $P < 0.05$ (ANOVA and Bonferroni posttest). (C) The lungs were fixed and stained with hematoxylin and eosin (H&E) or with PAS. Representative staining patterns are shown. (Bars, 100 μ m.) (D) Airway resistance (RL) was assessed as described in *SI Materials and Methods*. Mean values (five mice per group) are shown with SEM. ** $P < 0.01$; * $P < 0.05$ (ANOVA and Bonferroni posttest). All data are representative of two or more independent experiments.

p300 complex was shown for *Ccr8* providing evidence of the generalized nature of this process (Fig. S5). It is important to note, however, that two thirds of the identified *Gata3* target genes (1,040 out of 1,651 genes) were not bound by Chd4 in Th2 cells (Fig. S3A), and thus the expression of these *Gata3* target genes is likely controlled by Chd4-independent mechanisms.

Previously identified *Gata3*-regulators such as repressor of *GATA* (Rog), Fog, spleen focus forming virus proviral integration oncogene sp1 (PU.1), T-bet, lymphoid enhancer factor 1 (Lef1), Eomesodermin, and Sox4 appear to primarily regulate *Gata3* through inhibition of its DNA binding (27–33). However, in this study we show that the *Gata3*/Chd4–NuRD complex mediated direct repression of transcription through chromatin modulation of the *Gata3* target gene regulatory regions (Fig. 3).

Our data demonstrating activation or repression of transcription by functionally distinct *Gata3*/Chd4 complexes pose a question of binding-site selectivity. Given that, we found a palindromic *GATA* binding site with the GATC sequence (underlined) in the *Tbx21* G3/C4BS (TATCAGATC) but not in the CGRE region. Thus, the differences in the primary DNA sequence of each *Gata3* target site may contribute to the selection of the locus for *Gata3*/Chd4–NuRD complex-mediated repression. Further contribution to selectivity may come from recognition of histone modification by the two plant homeodomain (PHD) fingers and two chromodomains of Chd4. The Chd4 N-terminal PHD finger preferentially binds unmodified Lys4 on histone H3, and the C-terminal PHD finger specifically recognizes unmodified Lys4 and trimethylated Lys-9 (34). This bivalent recognition of nucleosomes by the two PHD fingers of Chd4 is required for Chd4-mediated transcriptional repression (35). Therefore, it is possible that the *Gata3*/Chd4–NuRD complex may be able to recognize differences in the epigenetic code at target regions.

Finally, we have demonstrated the importance of Chd4-mediated regulation of Th2 cell function in vivo. Allergic eosinophilic inflammation and airway hyperresponsiveness were significantly suppressed in mice transferred with Chd4 KD Th2

cells (Fig. 4). In our experimental system, any alterations in inflammation observed originate from defects in the transferred Th2 cells, and consequently as a result of the loss of Chd4 function. It is therefore likely that Chd4 controls asthmatic inflammation through the activation of Th2 cytokines and repression of IFN- γ expression in Th2 cells. Further detailed biochemical studies on the Gata3/Chd4/p300 and Gata3/Chd4–NuRD complexes may lead to the discovery of novel therapeutic targets for the treatment of allergic asthma.

In summary, we have herein demonstrated that Gata3 concomitantly forms the Gata3/Chd4/p300 transcriptional activation complex at the Th2 cytokine gene loci and the Gata3/Chd4–NuRD transcriptional repression complex at the *Tbx21* locus. Functioning cooperatively, these complexes orchestrate proper gene expression during Th2 cell differentiation by repressing subsets of genes while facilitating transcription at other loci.

Materials and Methods

Mice. 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.

Identification of Gata3 Complexes in the Th2 Cell Clone D10G4.1. Extracts from Flag-Gata3-expressing D10G4.1 cells were subjected to tandem affinity purification using anti-Flag and -Gata3 mAbs, followed by LC-MS/MS analysis as described in *SI Materials and Methods*.

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

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Supporting information

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SI Materials and Methods

Mice. C57BL/6 and BALB/c mice were purchased from CLEA Co. (Tokyo). OVA-specific TCR- $\alpha\beta$ (DO11.10) transgenic (Tg) mice were provided by D. Loh (Washington University School of Medicine, St. Louis, MO). Conditional *Gata3*-deficient mice and OX40-Cre transgenic mice were kindly provided by William E. Paul (National Institutes of Health, Bethesda) and Nigel Killeen (University of California, San Francisco), respectively. *Tbx21*-deficient mice were kindly provided by Laurie Glimcher (Cornell University, Ithaca, NY).

Identification of *Gata3* Complexes in the T Helper 2 Cell Clone D10G4.1

Flag-tagged cDNA for *Gata3* was inserted into a multicloning site of the CS-CDF-EG-internal ribosome entry site (IRES)-green fluorescent protein (GFP)-PRE vector. The lentivirus vector-containing culture supernatant was prepared as described (1). D10G4.1 cells were infected with either control mock (CS-CDF-E α -IRES-GFP-PRE) or Flag-*Gata3* (CS-CDF-E α -Flag-*Gata3*-IRES-GFP-PRE)-containing lentiviruses. Three days after infection, GFP-positive lentivirus-infected cells were sorted with a FACSaria (Becton Dickinson). Flag-tagged *Gata3* or mock-infected D10G4.1 cells were solubilized with a protease inhibitor-containing immunoprecipitation (IP) buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 0.1% Tween 20, 1 mM EDTA, 10 mM NaF, 1 mM DTT, and protease inhibitor mixture (Roche Applied Science)] and lysed on ice for 30 min with gentle shaking and sonication with a Misonix sonicator (Microson). The insoluble materials were removed by centrifugation, and IP with anti-Flag M2 agarose (Sigma-Aldrich) was performed overnight at 4 °C. Immune complexes were eluted from the agarose by 3 $\times$ Flag peptide, and the eluted *Gata3* complexes were immunoprecipitated with an anti-*Gata3* mAb (HG3-31; Santa Cruz Biotechnology) or control IgG coupled to protein A/G beads. Immune complexes were eluted from the beads with elution buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, 10 mM EDTA) and separated by SDS/PAGE. The bands were excised from the gel and subjected to mass spectrometric analysis to identify corresponding proteins. The gel pieces were washed twice with 100 mM bicarbonate in acetonitrile, and the proteins were digested with trypsin. After adding 0.1% formic acid to the supernatant, the peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a LTQ Mass Spectrometer (Thermo Scientific). The resulting MS/MS dataset was analyzed by using the Mascot search engine (Matrix Science).

IP and Immunoblotting. Anti-Flag mouse mAb (M2; Sigma-Aldrich), anti-Myc-Tag mouse mAb (PL14; MBL), and anti-*Gata3* mAb (HG3-31) were used for IP. For experiments assessing the physical association between chromodomain helicase DNA-binding protein 4 (Chd4) and *Gata3*, protein extracts from the transfected cells were prepared by using radioimmunoprecipitation assay buffer (1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μ g/mL aprotinin, leupeptin, and pepstatin, 1 mM Na₃VO₄, 1 mM NaF, and 50 mM Tris-HCl, pH 7.4). For IP analysis, the cell lysates were subjected to a preclear process with protein G-Sepharose at 4 °C for 1 h with rotation. The precleared extracts were subjected to IP with an anti-Flag, anti-Myc, or anti-*Gata3* mAb (HG3-31) at 4 °C for 2 h, and then the immunocomplexes were precipitated with protein G-Sepharose beads at 4 °C for 1 h. For immunoblot (IB) analysis, immunoprecipitates were eluted from beads in SDS-gel loading buffer and run on 10% or

8–16% gradient polyacrylamide gels. After electrophoresis, the proteins were subjected to IB as described (2). Nuclear extracts used for the detection of *Gata3* were prepared by using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Chemical; CAT#78833). The antibodies used for the analyses were anti-*Gata3* (HG3-31), anti-Chd4 (Bethyl Laboratories), anti-Mta2 (Abcam), anti-Mbd3 (C-17; Santa Cruz Biotechnology), anti-Hdac2 (CycLex Co.), anti-Flag (M2; Sigma-Aldrich), anti-Myc (PL14), and anti-HA (3F10; Roche).

Sucrose Gradient and Size Fractionation. For sucrose gradient fractionation, 293T cells were transfected with Myc-tagged Chd4, Flag-tagged *Gata3*, and HA-tagged p300. Total extract was immunoprecipitated with an anti-Myc antibody. Chd4 complexes were eluted from the agarose by a Myc peptide and layered directly onto a gradient of 15–40% (wt/vol) sucrose in 3 mL of IP buffer. Ultracentrifugation was performed with a Hitachi P55ST2 rotor at 29,100 rpm for 36 h. Fractions were collected in volumes of 200 μ L and subjected to IB, and their histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities were measured using a HATs Acetylase and HDACs Deacetylase Fluorometric Assay Kit (CycLex Co.) according to the manufacturer's instructions.

Quantitative RT-PCR. Total RNA was isolated by using TRIzol reagent (Invitrogen). Reverse transcription was performed by using SuperScript II (Invitrogen). For quantitative real-time PCR, a TaqMan universal PCR master mix was used for all reactions (Applied Biosystems) with an ABI Prism 7500 Sequence Detection System. Primers and probes used for the detection of mouse *Gata3*, *Tbx21*, *Il4*, *Il5*, *Il13*, *Ifng*, and *Hprt* were described (3). The new specific primers and TaqMan probes (Roche Universal Probe Library System) for the detection of mouse *Chd4*, *Epas1*, *Ccr8*, *Myl6b*, and *Mb21d1* are as follows:

Chd4 forward: 5' ctaagagcaagcgccaaaa 3'

Chd4 reverse: 5' tctctctccacaactctgg 3'

Universal probe 69

Epas1 forward: 5' ggagctcaaaaggtgtcagg 3'

Epas1 reverse: 5' caggttaaggtcgaacgatg 3'

Universal probe 3

Ccr8 forward: 5' agaagaaaggtcgtcaga 3'

Ccr8 reverse: 5' ggctccatggttaatccat 3'

Universal probe 4

Myl6b forward: 5' tcaaggtctctgggaacc 3'

Myl6b reverse: 5' ggcaggacgtctcaagctct 3'

Universal probe 17

Mb21d1 forward: 5' tggcagctactatgaacatgtga 3'

Mb21d1 reverse: 5' tctctggggacttccagtta 3'

Universal probe 68

The data are shown as relative expression normalized to *Hprt* signal.

Knockdown Assay. D10G4.1 cells were infected with a lentivirus vector containing control (pLKO.1-puro-shControl) or Chd4 shRNA (pLKO.1-puro-shChd4) (Sigma-Aldrich) and cultured in the presence of puromycin (10 μ g/mL) for 2 wk, and resistant cells were subjected to further analysis. To deplete cellular Chd4, Silencer Select siRNAs were purchased from Ambion. Naive CD4 T cells were transfected with the siRNA oligonucleotide by using a mouse T-cell Nucleofector kit and Nucleofector I (Amaxa) and were stimulated with immobilized anti-T-cell receptor

(TCR)- β and anti-CD28 mAbs under T helper 1 (Th1) and Th2 conditions. Th1/Th2 cell differentiation was assessed by intracellular cytokine staining with anti-IL-4 and anti-IFN- γ or by quantitative RT-PCR (RT-qPCR) and ELISA as described (3, 4).

Assessment of Ovalbumin-Induced Allergic Airway Inflammation and Hyperresponsiveness. Control or Chd4 siRNA was transfected into naive DO11.10 transgenic (Tg) CD4 T cells, and the cells were then stimulated under Th2 conditions for 4 d. The siRNA-transfected Th2 cells (1×10^6 cells) were then i.v. transferred into BALB/c mice, and the mice were challenged with aerosolized ovalbumin (OVA) in saline (10 mg/mL) on days 5 and 7. One day after the last OVA inhalation, bronchioalveolar lavage fluid cells and lung tissue samples for histological examination were prepared as described (5). Airway hyperresponsiveness was assessed by measuring the changes in lung resistance (RL) in response to increasing doses of inhaled methacholine, as described (6).

Chromatin IP Assay and ChIP-Sequence Analysis. ChIP assays were performed by using anti-Gata3 (D-16; Santa Cruz Biotechnology), anti-Chd4 (Bethyl Laboratories), anti-p300 (N-15; Santa Cruz Biotechnology), anti-Hdac2 (Abcam), anti-Mbd3 (C-17), and anti-acetylhistone H3-K9 (Millipore) antibodies as described (7). qPCR analyses were performed on an ABI Prism 7500 real-time PCR machine with probes from the Roche Universal Probe Library System. The new specific primers and TaqMan probes used for the detection are as follows:

Tbx21 G3/C4BS forward: 5' acagcctcactgccacag 3'
Tbx21 G3/C4BS reverse: 5' aggcagagctcagtcattgg 3'
 Universal probe 9

Tbx21 promoter forward: 5' gaggggtgtagaggatcagaa 3'
Tbx21 promoter reverse: 5' ctacttttgagataagatccctgt 3'
 Universal probe 64

RHS6 forward: 5' cagaacccattatgtcatcagc 3'
 RHS6 reverse: 5' tccaagccctgtacgactc 3'
 Universal probe 84

HS2 forward: 5' tgcttctgctggacatctct 3'
 HS2 reverse: 5' ctgtccctccactacaaa 3'
 Universal probe 40

Epas1 G3BS forward: 5' agattgcagctgtgtgtga 3'
Epas1 G3BS reverse: 5' gcattgcaccagattctgt 3'
 Universal probe 12

Ccr8 G3BS forward: 5' attccctccataaccacca 3'
Ccr8 G3BS reverse: 5' atctggggcagagataagca 3'
 Universal probe 6

Myl6b G3BS forward: 5' cttagcctccccgagtgctc 3'
Myl6b G3BS reverse: 5' ggcaagttgggctcacag 3'
 Universal probe 92

Mb21d1 G3BS forward: 5' ccagcgctgaaagtacgtg 3'
Mb21d1 G3BS reverse: 5' cttttgattggcgggttg 3'
 Universal probe 69

The other specific primers and TaqMan probes used in this report were described (7). For ChIP-seq analysis, the immunoprecipitate and input samples were prepared by using a ChIP-seq Sample Prep kit (Illumina) (8). Adaptor-ligated DNA fragments were size-fractionated on 12% acrylamide gels, and the 150–250 bp fraction was recovered. The DNA thus obtained was amplified by 18 cycles of PCR. A total of 1 ng of DNA was used for the sequencing reaction of the GAIIX (Illumina) according to the manufacturer's instructions. An estimated 150,000–250,000 clusters were generated per tile, and 36 cycles of the sequencing reactions were performed. Short-read sequences were aligned to the mouse genome sequences [mm9 from the University of California, Santa Cruz (UCSC) Genome Browser] by using the Eland program.

Sequences allowing no more than two mismatches per sequence were used for the analysis.

Transcriptional Start Site Sequence Analysis. Developing Th1, Th2, and *Gata3*-deficient Th2 cells were harvested, and total RNA was extracted by using the TRIzol reagent. Fifty micrograms of total RNA was subjected to oligo-capping with some modifications of the original protocol as described (8). Approximately 1 ng of the size-fractionated cDNA was used for sequencing using an Illumina GAIIX. The generated sequence tags were mapped onto the mouse genomic sequence (mm9 from the UCSC Genome Browser) by using the Eland program (Illumina). Unmapped or redundantly mapped sequences were removed from the dataset, and only uniquely mapped sequences without any mismatches were used for analyses.

Luciferase Reporter Assay. A single copy of the fragments spanning the *Gata3*/Chd4-binding site (*Tbx21*, CGRE, and HS2) was cloned at the 5' end of the *Tbx21* promoter (–500) or *Il4* promoter (–766) in the luciferase reporter plasmid pGL3 Basic (Promega). A single copy of the *Tbx21* enhancer (–11817~–11645) (9) was cloned at the 5' end of the *Tbx21* G3/C4BS–*Tbx21* promoter in the pGL3 Basic plasmid. Primary developing Th1 and Th2 cells were used for transfection with the mouse T-cell Nucleofector kit and Nucleofector I (Amaxa). A renilla luciferase reporter vector with a HSV thymidine kinase promoter (pRL-TK; Promega) was added into each transfection reaction as an internal control for transfection efficiency. The transfected cells were stimulated with immobilized anti-TCR- β mAb (*Il4* promoter reporter assay) or 10 ng/mL IL-12 and 100 ng/mL IFN- γ (*Tbx21* promoter reporter assay). Thirty-six hours later, cell extracts were prepared and subjected to a luciferase assay by using a Dual Luciferase Reporter System (Promega) according to the manufacturer's instructions.

Retroviral Vectors and Infection. The retrovirus vector pMXs-IRES-GFP was provided by T. Kitamura (University of Tokyo, Tokyo). The methods used to generate the virus supernatant and for infection were described (10). Infected cells were collected 5 d after stimulation and were subjected to RT-qPCR analysis and intracellular staining with anti-IL-4 and anti-IFN- γ .

Trichostatin A Treatment. Naive CD4 T cells were stimulated under Th2 conditions, and 10 nM trichostatin A (Sigma-Aldrich) was added to the culture on day 2. After a further 3-d culture, the CD4 T cells were collected for intracellular cytokine staining and RT-qPCR.

GST Pull-Down Assay. GST-tagged recombinant Gata3 protein (Abnova), control GST protein (ab70456; Abcam), and His-tagged recombinant Chd4 (1–355 aa; Bioclone) were used for the pull-down assays. Briefly, GST-tagged Gata3 protein (0.5 μ g) and His-tagged Chd4 protein (0.5 μ g) were mixed with glutathione Sepharose 4B (GE Health Care) in binding buffer (20 mM Tris-HCl (pH 7.4), 250 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate) and incubated at 4 °C for 1 h. The amount of coprecipitated Chd4 protein was separated by electrophoresis and visualized by IB with an anti-His antibody (OGHis; MBL Co.).

Immunostaining. Numbers of viable Th1 and Th2 cells were determined by trypan blue exclusion, and 10^5 viable cells were cytocentrifuged onto slides using a Cytospin4 (Shandon) and fixed with 4% paraformaldehyde (WAKO) after extensive washing. Slides were then refixed in 100% ethanol at –20 °C for 10 min, followed by incubation with blocking solution, then primary antibodies against Gata3 (mouse; HG3-31; Santa Cruz Biotechnology) and Chd4 (goat; Bethyl Laboratories). Signals were detected by using labeled rabbit anti-goat IgG and goat anti-mouse IgG secondary antibodies (Invitrogen). Nuclei were

visualized by DAPI staining. Stained cells were analyzed by using a confocal laser microscope (LSM710; Carl Zeiss).

Expression Plasmids. Flag-tagged Gata3 (pFlag-CMV2-Gata3) mutants and Myc-tagged Chd4 (pCMV Myc-Chd4) mutants were generated by a PCR-based method.

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4. Endo Y, et al. (2011) Eomesodermin controls interleukin-5 production in memory T helper 2 cells through inhibition of activity of the transcription factor GATA3. *Immunity* 35(5):733–745.
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Expression plasmids were transfected into 293T cells using Eugene reagent (Roche) according to the manufacturer's protocol.

Statistical Analysis. Student *t* test was used. Where indicated, ANOVA and Bonferroni test were used for multiple comparisons of different groups.

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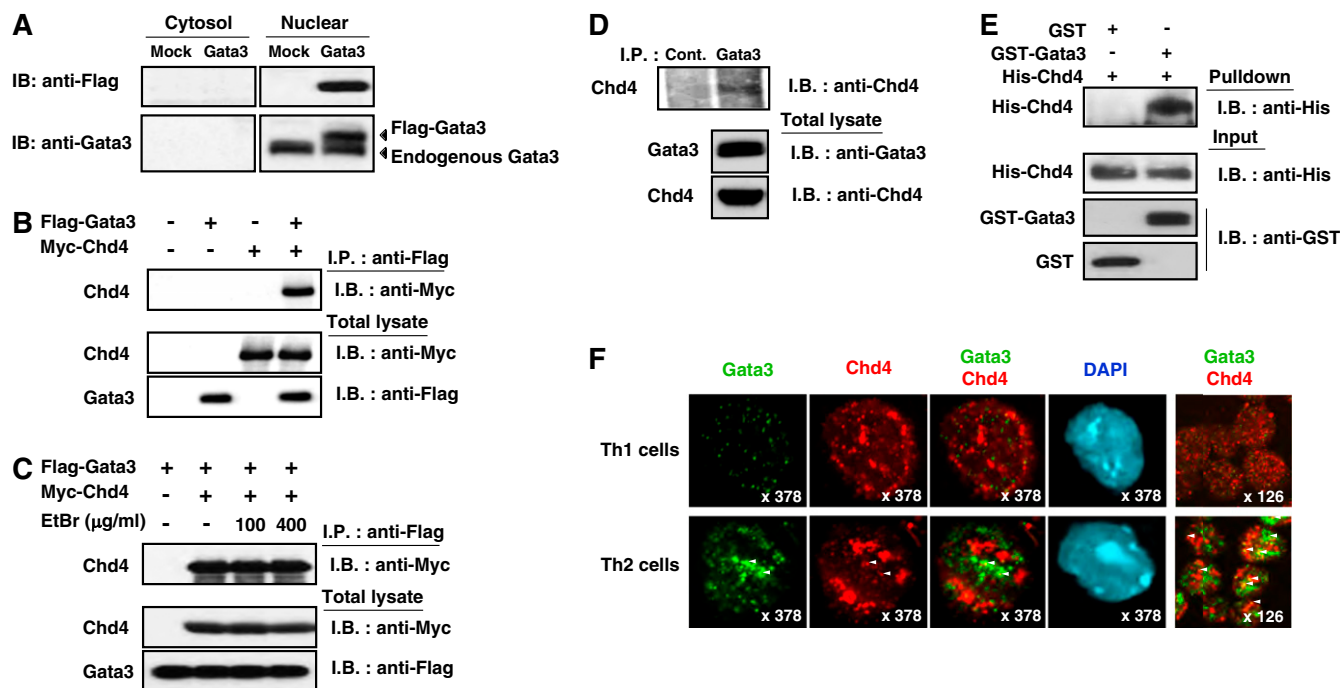


Fig. S1. Characterization of Gata3 association with Chd4. (A) A mouse Th2 cell clone, D10G4.1, was infected with a lentivirus encoding Flag-Gata3, and 3 d later the GFP-expressing infected cells were sorted. The amount of Flag-Gata3 protein was assessed by immunoblotting (IB) with anti-Flag and -Gata3 mAb. (B) Chd4 associates with Gata3 in 293T cells. The 293T cells were transfected with expression plasmids encoding Flag-tagged Gata3 and Myc-tagged Chd4. (Upper) Two days later, extracts were immunoprecipitated (IP) with anti-Flag mAb followed by IB with an anti-Myc mAb. (Lower) Total lysates were also subjected to IB in parallel. (C) DNA-independent interaction of Gata3 with Chd4. Cell extracts were pretreated with increasing concentrations of ethidium bromide before IP with anti-Flag mAb. The amount of Myc-tagged Chd4 associated with Flag-tagged Gata3 was assessed as in B. (D) Endogenously expressed Chd4 associates with Gata3 in Th2 cells. (Upper) Total lysates from developing Th2 cells were subjected to IP with anti-Gata3 mAb followed by IB with an anti-Chd4 Ab. (Lower) Total lysates were also subjected to IB in parallel. (E Upper) Chd4 interacts with Gata3 directly in GST pull-down assay. GST or GST-Gata3 proteins were bound to glutathione Sepharose 4B and incubated with recombinant His-Chd4 protein (1–355 aa). (Lower) Bound proteins were subjected to SDS/PAGE and IB with an anti-His mAb. Inputs represent 10% of the amount of protein used in the pull-down sample. (F) Partial colocalization of endogenous Gata3 and Chd4. Th1 and Th2 cells were fixed and stained by using an anti-Gata3 mAb and an anti-Chd4 polyclonal antibody and then visualized by using Alexa Fluor 488- and 594-conjugated secondary antibodies, respectively. Nuclei were counterstained with DAPI, and images were taken by using a confocal laser microscope (LSM710; Carl Zeiss).

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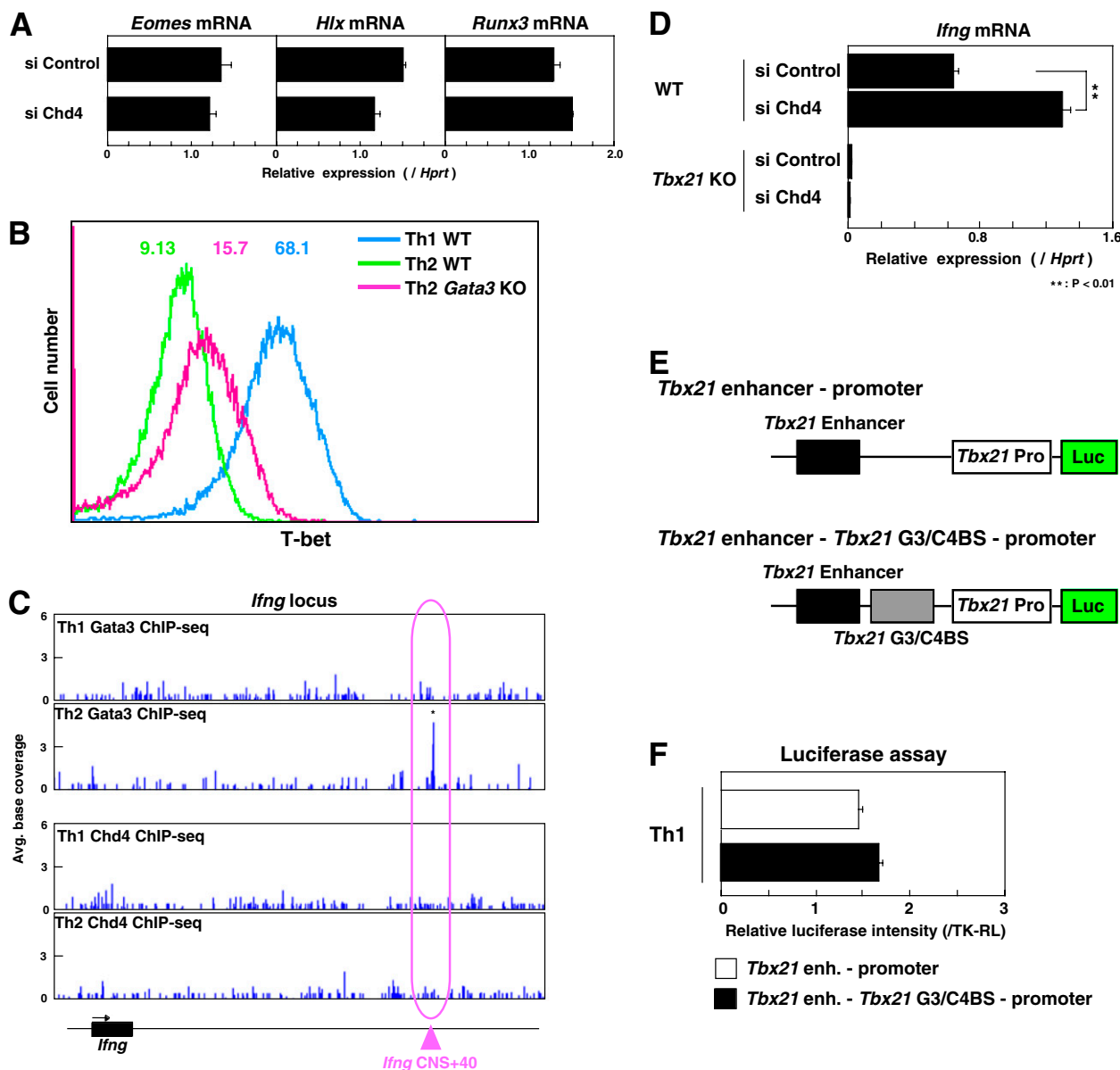


Fig. S4. Chd4 represses T-bet-dependent *Ifng* expression in Th2 cells. (A) Chd4 KD has little impact on the level of *Eomes*, *Hlx*, and *Runx3* mRNA in Th2 cells. Control (si Control) or Chd4 (si Chd4) siRNA was transfected into naive CD4 T cells, and the cells were stimulated with immobilized anti-TCR- β and anti-CD28 mAbs under Th2 conditions for 4 d. The expression levels of *Eomes*, *Hlx*, and *Runx3* mRNA were measured by RT-qPCR analysis. (B) Gata3 represses T-bet expression in Th2 cells. Naive CD4 T cells from WT or *Gata3*-deficient mice were stimulated under Th1 or Th2 conditions. T-bet protein expression was determined by intracellular staining followed by FACS analysis. Numbers represent mean fluorescent units. (C) Shown is a representative view of the *Ifng* gene locus from the Gata3 and Chd4 ChIP-seq analysis, which was performed in Fig. 2A, in developing Th1 and Th2 cells. Each blue bar represents the mean base coverage at the corresponding position. The gene structure is shown at the bottom. The *Ifng* CNS+40 region, a previously identified Gata3 binding site, is marked in magenta. The asterisk indicates the binding peak. (D) Chd4 KD up-regulates *Ifng* mRNA expression in a T-bet-dependent manner in Th2 cells. Control (si Control) or Chd4 (si Chd4) siRNA was transfected into naive CD4 T cells from WT or *Tbx21* KO mice, and the cells were then stimulated under Th2 conditions for 4 d, followed by stimulation with immobilized anti-TCR- β mAb for 4 h. The expression level of *Ifng* mRNA was determined by RT-qPCR analysis. (E) Schematic representations of the reporter constructs are shown. A 250-bp fragment spanning the *Tbx21* G3/C4BS (gray box), a common binding site of both Gata3 and Chd4 at intron1 of the *Tbx21* locus, was placed between the *Tbx21* enhancer (filled black box) and promoter region: -500 bp (open box) in the luciferase (green box) reporter construct. (F) Developing Th1 cells were transfected with the indicated reporter constructs as described in Fig. 3D. The luciferase activity was measured 36 h after transfection. The data indicate the mean results of three independent experiments with SDs.

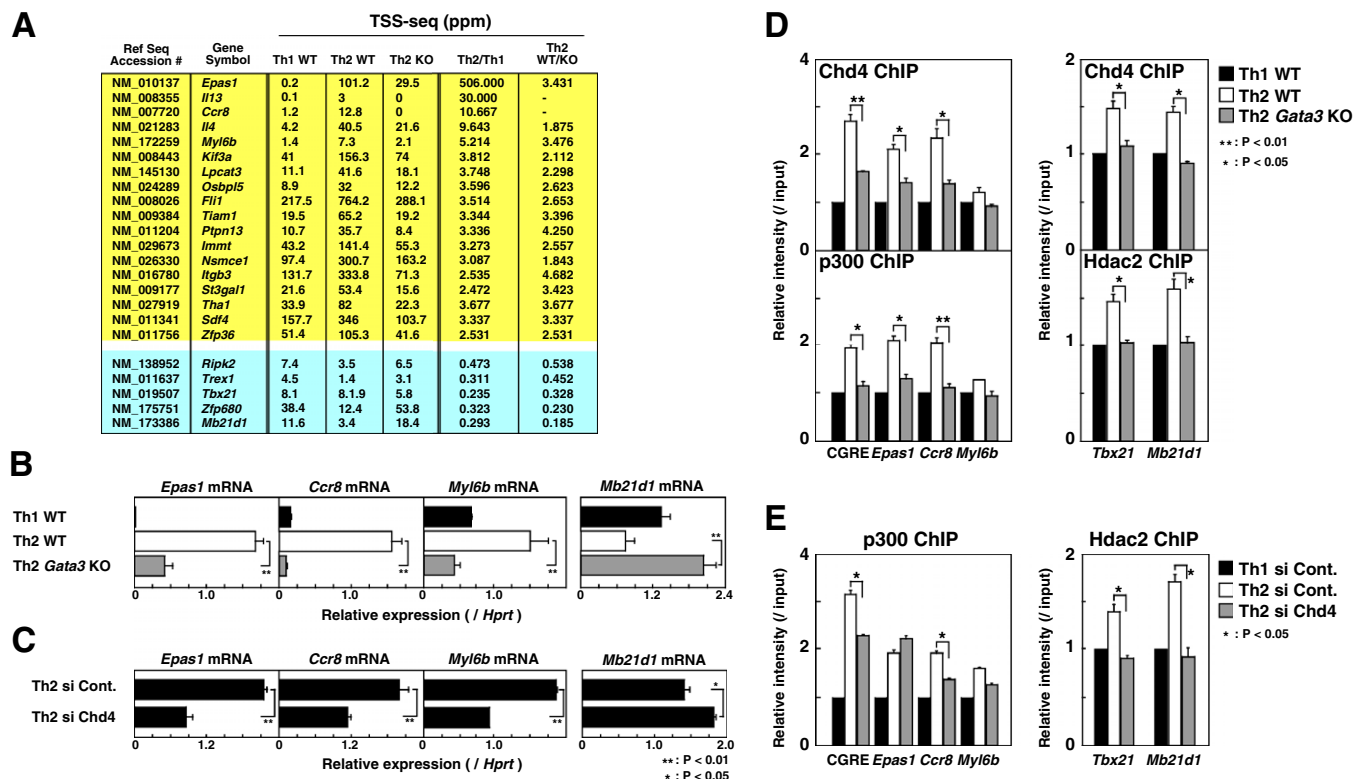


Fig. S5. Classification of Gata3/Chd4 target genes identified through the ChIP-seq and TSS-seq analysis. (A) List of candidate genes that are positively (Upper, highlighted in yellow) or negatively (Lower, highlighted in blue) regulated by Gata3/Chd4 complexes. Among Gata3-regulated genes, whose expression was up- or down-regulated in Th2 cells compared with Th1 cells (twofold) and *Gata3*-deficient Th2 cells (1.5-fold) in TSS-seq analysis, common targets of both Gata3 and Chd4 were selected by ChIP-seq. (B) Gata3-dependent active and repressive gene regulation. The expression levels of *Epas1*, *Ccr8*, *Myf6b*, and *Mb21d1* mRNA were determined by RT-qPCR. (C) Effects of Chd4 silencing on the expression of *Epas1*, *Ccr8*, *Myf6b*, and *Mb21d1* were determined by RT-qPCR. (D) Gata3-dependent recruitment of Chd4 and p300 to the positively regulated and of Chd4 and Hdac2 to the negatively regulated gene loci. The binding of Chd4 and p300 at CGRE, *Epas1*, *Ccr8*, and *Myf6b* loci and Chd4 and Hdac2 at the *Tbx21* and *Mb21d1* loci were determined by the ChIP assay using qPCR analysis. (E) Chd4 KD inhibited the recruitment of p300 to the positively regulated and of Hdac2 to the negatively regulated gene loci. The binding of p300 to the CGRE, *Epas1*, *Ccr8*, and *Myf6b* loci and the binding of Hdac2 to the *Tbx21* and *Mb21d1* loci were assessed by the ChIP assay using qPCR analysis.

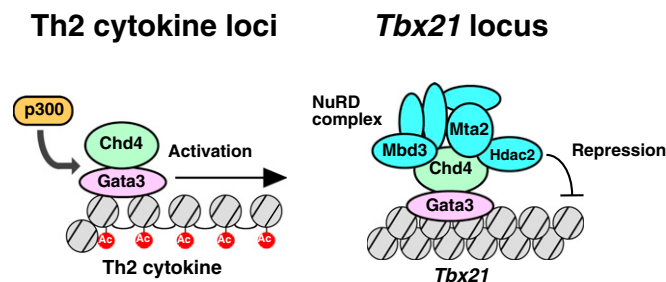


Fig. S6. Model for functionally distinct Gata3/Chd4 complexes to govern active and repressive gene regulation. The Gata3/Chd4 complex simultaneously activates Th2 cytokine transcription and represses Tbet-dependent Th1 cytokine (IFN- γ) expression in Th2 cells. Gata3 is present in functionally distinct protein complexes—a Gata3/Chd4/p300 transcriptional activation complex at the Th2 cytokine loci and a Gata3/Chd4-nucleosome remodeling HDAC repression complex at the *Tbx21* locus.