Chapter 10

The Interplay between Transcription Factors and Epigenetic Modifications in Th2 Cells

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Additional information is available at the end of the chapter

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Abstract

Functionally polarized CD4 T helper (Th) cells, such as Th1, Th2, and Th17 cells, are essential for the regulation of acquired immunity. Differentiation of naïve CD4 T cells into Th2 cells is characterized by chromatin remodeling and the induced expression of a set of Th2-specific genes, which include Th2 cytokine genes. In the first stage of this differentiation, a Th2-skewing cytokine environment, especially IL-4, induces STAT6 activation. Activated STAT6 increases the expression of GATA3, a master regulator of Th2 cell differentiation, via direct binding to the *Gata3* gene locus. This transcriptional induction of *Gata3* mRNA during Th2 cell differentiation is accompanied by dynamic changes in the binding patterns of two epigenetic modification proteins such as Polycomb and Trithorax complexes. Consequently, expressed GATA3 epigenetically modifies and upregulates Th2-specific genes to establish Th2 cell identity. This identity is maintained by high-level expression of the *Gata3* gene controlled by Menin, which is a member of the Trithorax proteins, after cycles of cultivation *in vitro* and a long-term resting state *in vivo*. Thus, the Menin-GATA3 axis handles the Th2-specific gene regulatory network.

Keywords: Th2, GATA3, STAT6, Menin

1. Introduction

Naïve CD4-positive (CD4+) T cells can differentiate into several effector T cell subsets, mainly known as Th1, Th2, and Th17 cells [1]. Th1 cells perform the crucial function of protecting against viruses and intracellular pathogens. Th17 cells similarly work against extracellular bacteria or fungi. Th2 cells are required for the removal of extracellular parasites. Each effector subset exerts its protective functions through the secretion of unique cytokines. Th1 cells mainly produce IFN-γ, which activates macrophages and CD8 T cells. Th17 cells secrete IL-17A, which

propagates cascades of events that lead to neutrophil recruitment, inflammation, and host defense [2]. Th2 cells activate B cells to induce immunoglobulin class switching through IL-4, and enhance mucus production from epithelial cells by IL-13. In addition, Th2 cells recruit eosinophils to induce an inflammatory response through IL-5. However, the responses caused by these subsets are sometimes excessive and result in immunological diseases. For example, an excess amount of Th2 cytokines is known to induce allergic disease, such as asthma [3].

Each subset-specific cytokine enhances differentiation toward the corresponding Th subset, and environmental cytokines decide the differentiation fate of CD4 T cells. For example, IL-12-induced STAT4 activation in Th1 cells and IL-4-induced STAT6 activation in Th2 cells are essential for their respective differentiation [4, 5]. These STAT signals are commonly used for CD4 T cell differentiation into each subset and induce the upregulation of master transcription factors, T-bet in Th1 and GATA3 in Th2 [6, 7]. The master transcription factors directly bind to DNA and regulate the expression of each subset-specific gene, causing epigenetic modification of the DNA, which stabilizes the differentiation program. Due to this epigenetic modification, fully differentiated effector T cells are rarely converted to other Th subsets and are able to maintain their identity during the transition from effector to memory cells.

The Th2 master transcription factor GATA3 collaborates with the epigenetic regulator Menin to induce and stabilize the complex gene regulatory network. Th2-specific genes, which have been identified by gene expression profiling [8, 9], participate in this regulatory network and are controlled by neither, either or both GATA3 and Menin [10]. In fact, GATA3 or Menin deletion results in the loss of Th2 identity [10, 11]. Clarifying the interplay between the transcription factors and epigenetic modifiers is required to comprehend the Th2 cell biology and to identify new therapeutic targets for Th2-mediated immunological diseases [3].

2. STAT6 and GATA3: important transcription factors for Th2 cells

2.1. STAT6 is activated by IL-4 signaling

The most essential pathway promoting the Th2 fate is the IL-4 signaling cascade, followed by activating the transcription factor STAT6 [12–14]. When IL-4 is recognized by its receptor (type-I IL-4R), which consists of IL-4 receptor alpha chain (IL-4Rα) and a common gamma chain (γc), IL-4 can transmit a signal into a cell. Binding of IL-4 induces dimerization of IL-4Rα and γc, resulting in the phosphorylation of tyrosine residues within the intracellular portion of IL-4Rα by Janus Kinases. This phosphorylated intracellular portion of IL-4Rα recruits and phosphorylates signal transducer and activator of transcription (STAT)6, which then forms a dimer and translocates into the nucleus where the dimerized STAT6 regulates the expression of IL-4 target genes. STAT6 recognizes the DNA sequence TTCNNNNGAA, whereas other STAT family proteins prefer the DNA sequence TTCNNNGAA [15].

Like other STAT proteins, a major role of STAT6 is to activate the expression of its target genes, which is how it received its name ("signal transducer and activator of transcription"). The best-known target gene of STAT6 is the *Gata3* gene, and the detailed mechanisms underlying the STAT6-dependent regulation of the *Gata3* gene are described in Section 4. However, some studies have

reported that STAT6 also exerts an inhibitory function by occupying overlapping binding sites of other transcription factors and blocking their binding [16, 17]. It is now well known that STAT-mediated repression is important for the lineage commitment of Th subsets [18]. For example, STAT6 binds to the genomic loci of Th1-associated genes and inhibits their expression, and STAT4, a key transcription factor of Th1, acts on Th2-associated genes in a similar way [19].

It has been proposed that the IL-4/STAT6 cascade is necessary for the Th2 phenotype. This fact is also demonstrated by a series of knockout studies. In these studies, IL-4 deficient mice showed impaired Th2 responses, attributed to a reduced Th2 effector cytokine production, loss of IgE class switching, and reduced eosinophilia upon infection with *Nippostrongylus brasiliensis* [20]. A similar but more significant phenotype is observed in STAT6 knockout mice. In addition, STAT6 appears to be highly specific to Th2 functions, as the phenotype of STAT6-deficient mice is largely related to the loss of the Th2 cell function, and deficient mice show normal development with ordinary numbers of T cells [21, 22]. Other STAT signaling cascades are also involved in Th2 polarization. STAT5A and STAT3, which are activated by IL-2 [23] and IL-6 [24], respectively, are also reported to induce the Th2 phenotype. However, STAT5 and STAT3 are activated not only in Th2 but also in other CD4+ T cell subsets. Therefore, only STAT6 exclusively promotes Th2 differentiation.

2.2. GATA3 plays roles in various tissues as well as the immune system

The GATA family proteins (GATA1–6) are conserved transcription factors that contain one or two C2-C2-type zinc-finger motif that recognize the consensus DNA sequence WGATAR [25–27]. Each member of the GATA family has different expression patterns in the body and can be grouped into hematopoietic factors (GATA1–3) and endodermal factors (GATA4–6). Among hematopoietic cells, immune cells, particularly developing and mature T cells, natural killer (NK) cells, and CD1-restricted NKT cells, mainly express GATA-binding protein 3 (GATA3) [6, 28, 29]. Mature mast cells express GATA1 and GATA2 but not GATA3 [30]. Outside of the immune system, GATA3 is also expressed in many embryonic and adult tissues, including the adrenal glands, kidneys, central nervous system, inner ear, hair follicles and skin, and breast tissue [27].

In the immune system, GATA3 is predominantly expressed in T lymphocytes and is essential for the development of CD4 single-positive (SP) cells in the thymus [31–33]. GATA3 exerts an important function at the β-selection checkpoint, which is involved in the CD4 versus CD8 lineage choice in the thymus [34]. It is continuously expressed in peripheral naïve CD4 T cells at a basal level, where the activation of STAT6 induced by the IL-4/IL-4 receptor signaling pathway upregulates *Gata3* mRNA expression during Th2 cell differentiation [35]. GATA3 is thought to be necessary as the master regulator of Th2 differentiation [6, 7], since enforced GATA3 expression induces Th2 differentiation even when the cells are cultured under Th1-skewing conditions [35]. Enforced expression of GATA3 has also been reported to endogenously upregulate GATA3 expression [36]. In addition, the amount of GATA3 protein in Th2 cells is regulated by various posttranscriptional mechanisms [37–39]. Furthermore, high-level expression of GATA3 is essential for the production of large amounts of Th2 cytokines in established Th2 cells [11, 40–42]. The detailed mechanisms underlying the GATA3-dependent regulation of its target genes are described in Section 5.

3. Polycomb and Trithorax proteins: fundamental epigenetic regulators for cell differentiation

3.1. Polycomb and Trithorax proteins epigenetically modify chromatin in a different way

Huge numbers of genes involved in epigenetic regulation have been identified. Many of them encode histone-modifying enzymatic proteins and their interaction partners. Among them, members of the Polycomb group (PcG) and Trithorax group (TrxG) complexes have been recognized as key epigenetic regulators [3, 43–46]. PcG and TrxG proteins were originally identified in *Drosophila*; however, they also play essential roles in controlling mammalian gene expression in various normal and tumor tissues. It has long been thought that PcG and TrxG proteins antagonize each other for turning target gene expression off or on, respectively. PcG proteins mediate gene silencing by controlling the repressive histone mark H3K27me3 (trimethylated histone H3 lysine 27), whereas TrxG proteins mediate gene activation by modifying the permissive histone mark H3K4me3. Both histone-modifying complexes are often found to regulate the same genes at different stages of development [47]. In addition, emerging evidence shows that PcG and TxrG proteins participate in complex regulatory mechanisms in mammalian tissues [48].

PcG complexes are classified into two canonical types such as Polycomb repressive complex 1 (PRC1) and PRC2. Both of them are involved in transcriptional repression. A sequential recruiting mechanism is proposed for the binding of PRC2 and PRC1 to genomic DNA. First, enhancer of zeste (EZH), the enzymatically active subunit of PRC2, methylates H3K27. Next, the PRC1 complex recognizes trimethylated H3K27, resulting in its co-localization with PRC2. In addition, the ring finger protein 1 (RING1), a subunit of PRC1, has a ubiquitin ligase activity for histone H2AK119 [49]. In CD4+ T cells, Ezh2 appeared to directly bind and facilitate the correct expression of the *Gata3* gene during differentiation into effector Th2 cells [50, 51]. In our previous study, Ezh2 bound much more strongly to transcription factor genes, including the *Gata3* gene, than to the cytokine or cytokine receptor genes. Genome-wide, in the genes encoding transcription factors, the Ezh2 binding levels appear to be higher in non-expressed genes than in expressed genes [52].

In contrast, mixed lineage leukemia (MLL) family proteins, which are major subunits of the TrxG complex, have H3K4 methyltransferase activity that induces a change in the chromatin structure to a form permissive for transcription. In mammals, six H3K4 methylases (MLL1–4, SET1A, and SET1B) have been discovered [53]. The H3K4 methylase complexes containing MLL1 or MLL2 are associated with a unique subunit named Menin (encoded by the *Men1* gene in mice). A mutation of *MEN1* has been found in patients with multiple endocrine neoplasia type 1 (MEN1) syndrome [54, 55]. Menin can act as a tumor suppressor and is required for TrxG complex binding to DNA [53]. Menin is also indicated to have essential roles in the immune system, as Menin has been shown to be important for the Th2 cell function both in mice and humans [51, 56]. The MLL3- or MLL4-containing complex associates with the H3K27 demethylase UTX (encoded by the *Kdm6a* gene in mice) and induces demethylation. H3K4 trimethylation appears to be mediated by these MLL-associated complexes in a gene-specific manner. The SET1A- or SET1B-containing complexes have the unique WD repeat-containing 82 (WDR82). TrxG proteins activate target gene expression and/or keep them active, indicating

that these proteins are associated with more than simple gene activation [53]. TrxG proteins have more diverse binding molecules than PcG proteins with which they form complexes.

3.2. Spatial interplay between Polycomb and Trithorax complexes

Although many studies have been performed on the nature of PcG proteins and TrxG proteins individually, few have successfully defined how transcriptional counter-regulation is organized by the PcG and TrxG complexes. One pioneering work demonstrated the dynamic transformations of histone modifications during T cell development [57]. In addition, in our previous study, we successfully analyzed how the global signature of PcG and TrxG co-occupied genes changed during the developmental process. This study showed that a binding pattern in which Ezh2 binds upstream and Menin binds downstream of the transcription start site was frequently found at highly expressed genes, and a binding pattern in which Ezh2 and Menin bind to opposite positions was frequently found at low-expressed genes in T lymphocytes. Interestingly, genes showing a binding pattern in which Ezh2 and Menin occupied the same position displayed greatly enhanced sensitivity to Ezh2 deletion [3, 58].

4. STAT6 induces dynamic changes in epigenetic states at the *Gata3* gene locus

4.1. The *Gata3* gene is epigenetically regulated during Th2 cell differentiation

Epigenetic changes at the *Gata3* gene locus in T cells are essential for the acquisition and maintenance of the Th2 cell identity [3, 51, 59]. During Th2 cell differentiation, PcG and TrxG proteins dynamically change their binding patterns at the *Gata3* gene locus. In addition, these epigenetic changes result in GATA3 protein upregulation that consequently induces chromatin remodeling at the Th2 cytokine gene loci, including *Il4*, *Il5*, and *Il13* [51, 59]. The *Gata3* gene is known to have distal and proximal promoters. Both basal transcription in naïve CD4 T cells and induced transcription in differentiated Th2 cells are controlled by the proximal promoter [51, 60]. In naïve CD4 T cells, PcG complexes bind upstream and TrxG complexes bind downstream of the *Gata3* proximal promoter [51]. During Th2 cell differentiation, PcG proteins dissociate upstream of the *Gata3* proximal promoter, and the binding of TrxG proteins spreads into this region. Consequently, rapid alterations in the binding patterns of PcG and TrxG proteins are observed in the region between the *Gata3* distal and proximal promoters in this period. Histone modification patterns basically exhibit the same behavior; H3K27me3 levels are decreased at the upstream region of the *Gata3* proximal promoter, and H3K4me3 spreads into this region. In contrast, changes in DNA methylation pattern are only observed at exon 2, in which DNA is methylated in naïve CD4 T cells and demethylated in Th2 cells [61]. At present, the mechanism underlying this demethylation process remains unclear.

4.2. STAT6 directly modifies epigenetic states at the *Gata3* gene locus

We identified two functional STAT6 binding sites within the intronic regions of the *Gata3* gene locus [51]. A chromatin immunoprecipitation followed by massively parallel sequencing (ChIP-seq)

analysis also identified one STAT6 binding site at the same region [62]. In the absence of STAT6, displacement of PcG by TrxG is not observed. These results indicate that STAT6 directly binds to the *Gata3* gene locus and induces PcG/TrxG displacement, although the precise mechanism is still unclear. A study of human Th2 cells indicated that STAT6 binding was hardly detected at the *GATA3* gene locus, although *STAT6* knockdown was effective for reducing the *GATA3* expression [18]. Interestingly, our ChIP-seq analysis detected one GATA3 binding peak close to one of the STAT6 binding sites at the *Gata3* gene locus [8, 51] and one of the strong peaks on the assay for transposase-accessible chromatin sequencing (ATAC-seq) [63]. This GATA3 binding site may be important for cis-regulation via GATA3-dependent auto activation of the *Gata3* gene [36]. Although STAT6 induces TrxG spreading into the promoter region, the T cell-specific deletion of Menin, a component of the TrxG complex, does not affect Th2 cell differentiation. This suggests that the induction of high-level expression of *Gata3* (i.e. the acquisition of the Th2 cell identity) is dependent on STAT6 and not the Menin/TrxG complex [51]. However, the maintenance of the *Gata3* expression is dependent on the Menin/TrxG complex and independent of IL-4 and STAT6 in Th2 cells. A similar molecular mechanism was found to underlie the *Gata3* expression *in vivo* [10, 64]. In human memory Th2 cells, MLL and Menin form a core transcriptional complex and regulate the *GATA3* expression [65]. Therefore, TrxG proteins represent an essential mechanism underlying transcriptional maintenance in the memory Th2 cell response [3].

4.3. PRC2 components prevent hyperactivation of the *Gata3* gene

In contrast to TrxG proteins, PcG proteins are proposed to maintain their *Gata3* expression at an appropriate level in CD4 T cells [3]. T cell-specific deletion of Ezh2 enhances the sensitivity of IL-4 and results in *Gata3* upregulation and hyper-production of Th2 cytokines [50]. A ChIP-seq analysis revealed that the Ezh2 binding levels were high at the *Gata3* gene locus but very low at the Th2 cytokine gene loci, indicating that Ezh2 controls the Th2 cytokine expression via direct binding to the *Gata3* gene locus. However, measurable levels of H3K27me3 were detected at the *Il4* and *Il13* genes loci, and direct regulation of H3K27me3 by Ezh2 at these genes has also been proposed as important for transcriptional silencing in Th1 cells [66]. In contrast, SUV39H1-dependent H3K9me3 has been found to maintain the silencing of Th1 cell-related genes in Th2 cells [67].

5. GATA3-dependent epigenetic and transcriptional regulation in the Th2 cytokine gene loci

5.1. Chromatin remodeling induced by GATA3 at the Th2 cytokine gene loci

Induction of changes in histone modifications has been reported at the *Il4*, *Il5*, and *Il13* gene loci (so-called the Th2 cytokine gene loci) during Th2 differentiation [12, 59, 68]. Particularly, histone H3K4 methylation and H3K9 acetylation play an important role in forming the open chromatin structure. Thus, the regions that acquire these histone modifications become accessible to transcription factors and are frequently associated with DNase I hypersensitive (HS) sites. Chromatin remodeling at the Th2 cytokine gene loci is necessary for the efficient expression of IL-4, IL-5, and

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IL-13 in Th2 cells, and GATA3 has been proposed to regulate chromatin remodeling at these genes. Notably, the H3K9 acetylation levels are higher around the GATA3 binding sites at the Th2 cytokine gene loci than the regions without GATA3 binding [8]. However, genome-wide surveys on GATA3 binding and histone modifications suggest that GATA3 binding do not perfectly coincide with changes in permissive histone modifications, which correlate highly with the states of transcription [62, 69]. In fact, some studies suggest that GATA3 acts not only as an activator but also as a repressor in both Th1 and Th2 cells [8]. Although GATA3 is recognized as a master regulator of Th2 cell differentiation, the transcription of many Th2-specific genes is not regulated by GATA3 itself; therefore, GATA3 is not the only essential factor for Th2 differentiation.

5.2. Interaction between GATA3 and regulatory elements

It has been reported that GATA3 interacts with some regulatory elements at Th2 cytokine gene loci, including conserved non-coding sequence (CNS)-1, HSVa, the conserved GATA response element (CGRE), and HSII in intron 2 of the *Il4* gene [12, 68, 70–74]. CNS-1 is located at the intergenic region between the *Il4* and *Il13* genes and was originally described as Th2-specific HS sites (HSS1 and HSS2) [75, 76]. To characterize the function of CNS-1, mice lacking this genomic region was generated [77]. Genetic deletion of the CNS-1 region resulted in a reduction of Th2 cells producing IL-4, IL-5, and IL-13. In this mutant mouse, IL-4 production *in vivo* was also abrogated [77]. However, CNS-1-deficieny had no effect on IL-4 production in bone marrow-derived mast cells [78]. This is consistent with the observation of no HS sites in CNS-1 of mast cells. Although an electrophoresis mobility shift assay showed that GATA3 binds to HSS2 *in vitro* [70, 71], two independent genome-wide GATA3 ChIP-seq data analyses failed to detect significant GATA3 binding peak in the CNS-1 region (**Figure 1**) [8, 79].

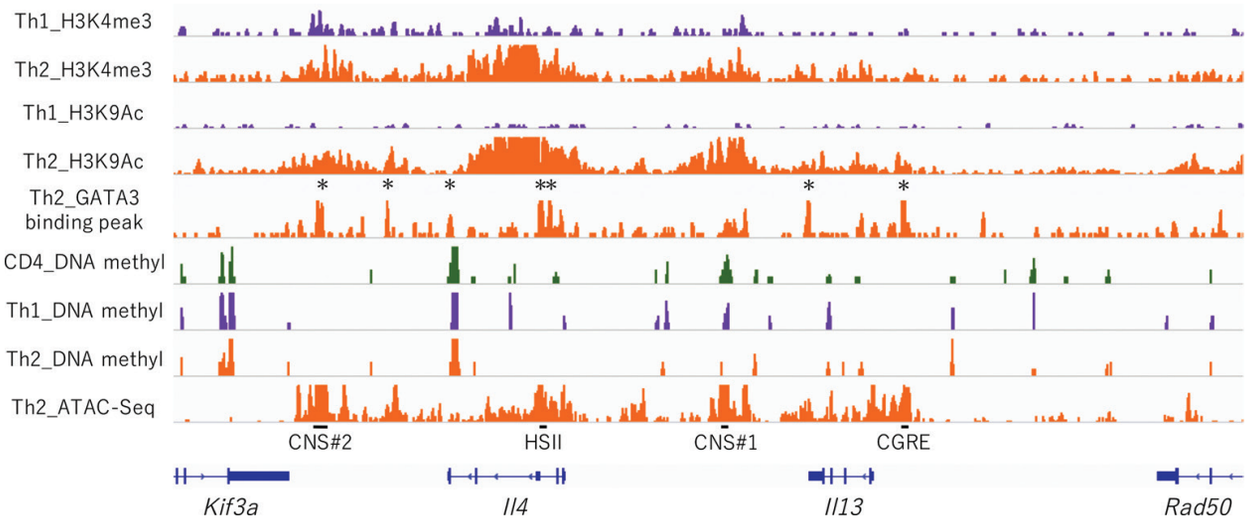

Figure 1. GATA3-dependent epigenetic and transcriptional regulation in the Th2 cytokine gene loci. Epigenetic permissive histone marks (H3K9 acetylation and H3K4 tri-methylation) in the Th2 cytokine loci are shown in Th1 and Th2 cells (GSE28292) using the IGV genome browser (http://www.broadinstitute.org/igv/). GATA3 ChIP-seq data with statistically significant peaks (asterisks) are also shown in Th2 cells. DNA methylation data are shown in CD4+ naïve T, Th1, and Th2 cells (GSE25688). Data from an assay for transposase-accessible chromatin sequencing (ATAC-Seq), which identifies opened chromatin regions, were obtained for lung Th2 cells (GSE77695).

Taken together with the fact that histone acetylation levels are increased with progressive DNA demethylation in the CNS-1 region [61, 80], this region may recruit other critical transcription factors that induce epigenetic modifications and promote IL-4 production in Th2 cells.

HSVa is a TCR re-stimulation-dependent HS site, whose DNase I hypersensitiveness is induced in Th2 cells upon stimulation [72]. HSVa is located 5 kbp downstream of the 3′ end of the *Il4* coding region. Th2 cells generated from the mice in which the genomic region containing both HSVa and HSV (CNS-2) has been deleted display a reduced IL-4 production [81]. Another study reported on the phenotypes of mice with genomes containing the specific deletion of the CNS-2 region [82]. Mice lacking CNS-2 display marked defects in Th2 humoral immune responses. However, the effector Th2 cells involved in tissue responses were not likely to be dependent on CNS-2. In this region, increased histone acetylation levels are observed. In contrast, changes in DNA methylation state are not induced, as DNA is demethylated even in naïve CD4 T cells [80]. By using a conventional ChIP technique, both GATA3 and nuclear factor of activated T cells 1 (NFAT1) have been shown to bind to HSVa in Th2 cells [72]. We and others have performed a GATA3 ChIP-seq analysis and detected GATA3 binding peaks at the HSVa [8, 79], implying that HSVa functions as an important regulatory element through which GATA3 and NFAT1 collaborate to induce IL-4 production in stimulated Th2 cells.

As we reported in 2002, CGRE was originally identified as a region with a 71-bp sequence located 1.6 kbp upstream of the *Il13* gene [73]. The location of CGRE corresponds approximately to the site of HSI. CGRE contains four putative GATA-binding sequences conserved across species [73]. Strong signals of GATA3 binding have been detected by both conventional ChIP assay and ChIP-seq analyses at the CGRE [8, 9, 79]. Interestingly, CGRE is also located at the 5′ edge of the region of histone hyperacetylation, suggesting that GATA3 binds to the CGRE and induces histone acetylation toward the 3′ region of the *Il13* gene. Indeed, GATA3 associates with RNA polymerase II and CBP/p300, which contain histone acetyltransferase activity at this region [73]. In addition, CGRE is located at the 5′ edge of the accessible DNA region detected by ATAC-seq [63]. Thus, the CGRE region may play an important role in *Il13* transcription and in chromatin remodeling at the *Il13* locus. Notably, the Th2 cells generated from CGRE-deficient mice exhibit diminished IL-13 but not IL-4 or IL-5 production [74].

Among several GATA3 binding sites found in the *Il4* gene locus, the strongest GATA3 binding signal was detected at the HSII site located in intron 2 of the *Il4* gene [8]. This region also contains binding sites for STAT5, which has been reported to be important for the maintenance of DNA accessibility of this region in Th2 cells [23, 83]. Correspondingly, a strong ATAC-seq peak was detected at this region in Th2 cells [63]. Recently, a group reported that genetic deletion of HSII resulted in a reduction in IL-4 but not IL-13 production, implying its role in regulating IL-4 production [74]. In addition to histone hyperacetylation induced in this region, H3K4me3 was strongly induced at HSII in Th2 but not Th1 cells [84], suggesting that GATA3 may work together with STAT5 and remodel the chromatin structure at this region. Parallel to changes in histone modifications, progressive DNA demethylation was observed across the *Il4* gene locus. In naïve CD4 T cells, only the promoter region of the *Il4* gene is demethylated, and DNA demethylation extends into the *Il4* gene body during Th2 cell differentiation [80].

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5.3. GATA3-dependent transcriptional regulation of Th2 signature genes

In addition to regulating chromatin remodeling, GATA3 may induce *Il5* and *Il13* transcription by directly binding to the promoters of these cytokine genes upon TCR re-stimulation [7, 85–87]. In fact, *Gata3* siRNA knockdown just before TCR re-stimulation resulted in reduced expression of *Il5* and *Il13* in established Th2 cells (**Figure 2**). The role of GATA3 in *Il5* and *Il13* transcription was also reported using genetic deletion of the *Gata3* gene. While GATA3 deletion during Th2 differentiation abolished the expression of all Th2 cytokines, GATA3 deletion in established Th2

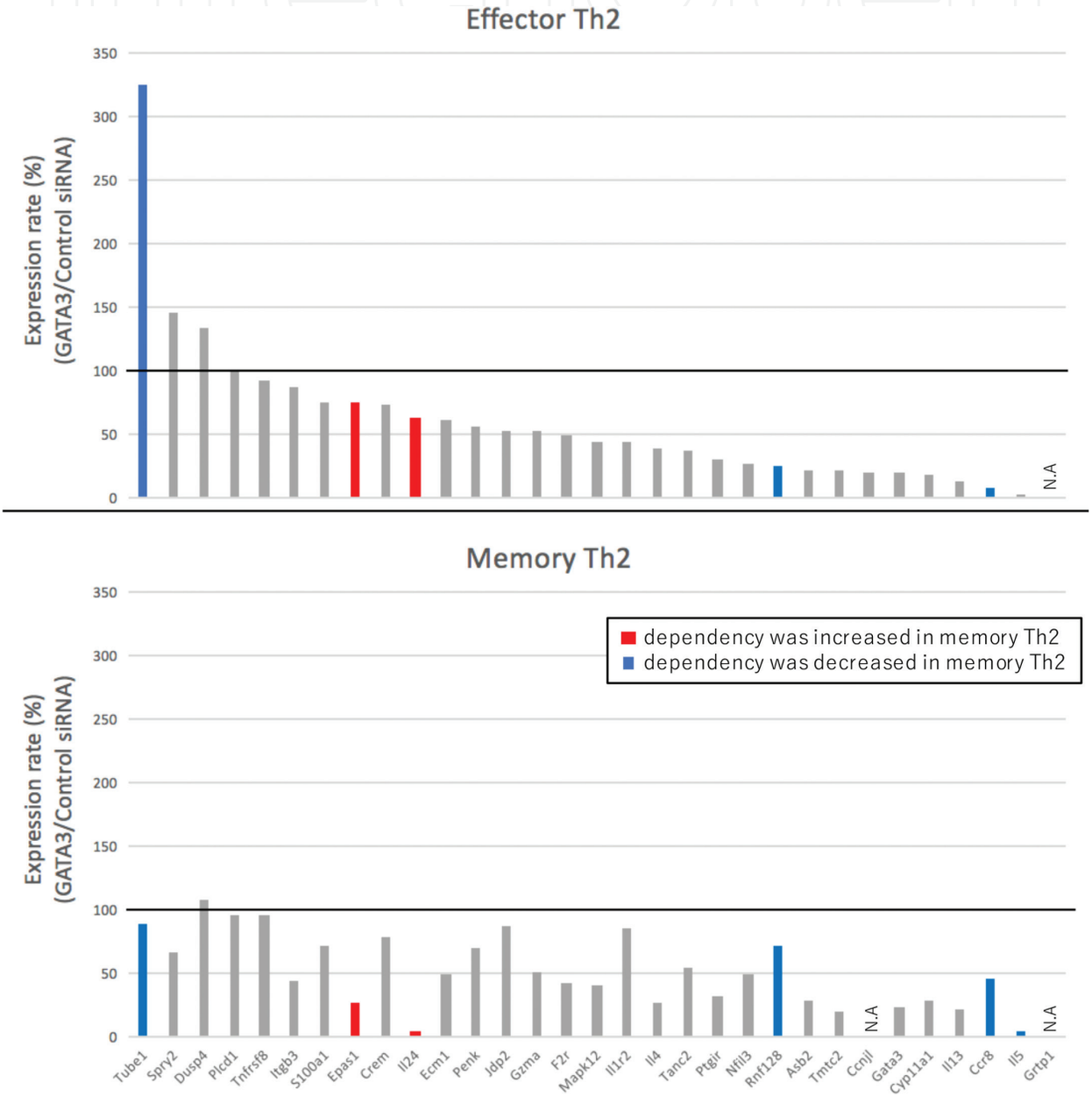

Figure 2. The effects of GATA3 knockdown on the Th2-specific genes in effector and memory Th2 cells. The effects of GATA3 knockdown on effector Th2 (upper) and memory Th2 (lower) were determined with qRT-PCR (originally published in *PLoS ONE*. Sasaki et al. [9]). The relative expression (GATA3/control siRNA) is rank-ordered and shown as a percentage. The genes indicated in the red bar showed increased GATA3 dependency in memory Th2 cells, while those in blue showed decreased GATA3 dependency.

cells strongly influenced the expression of both IL-5 and IL-13 and induced only a modest reduction in IL-4 production [42]. GATA3 is also crucial for the expression of the Th2 cytokine genes in memory Th2 cells, as *Gata3* siRNA knockdown reduces the transcription of those genes [9]. Furthermore, GATA3 is involved in the transcriptional regulation of other Th2 signature genes in both effector and memory Th2 cells (**Figure 2**). Approximately half of the Th2-specific genes (16 out of 31) showed a significant reduction in their expression in effector Th2 cells (*F2r*, *Mapk12*, *Il1r2*, *Il4*, *Tanc2*, *Ptgir*, *Nfil3*, *Rnf128*, *Asb2*, *Tmtc2*, *Ccnjl*, *Gata3*, *Cyp11a1*, *Il13*, *Ccr8*, and *Il5*) by GATA3 knockdown. In contrast, only the *Tube1* gene showed a significant increase in its expression. These results suggest that a major role of GATA3 is the activation of its target gene transcription.

Interestingly, changes in GATA3 dependency are observed during transition from effector to memory cells. In a previous study [9], we compared the GATA3 dependency in Th2-specific genes between effector Th2 cells and *in vivo*-generated memory Th2 cells by *Gata3* siRNA knockdown. GATA3 dependency increased by more than twofold in the *Epas1* and *Il24* genes in memory Th2 cells compared to the effector Th2 cells. In addition, for the *Tube1*, *Rnfl28*, *Ccr8*, and *Il5* genes, the GATA3 dependency decreased by more than twofold. These results indicate that each Th2-specific gene differentially changes its dependency on GATA3 during maturation to memory Th2 cells from effector Th2 cells. The changes in GATA3 dependency, however, do not correlate with dependency itself. For example, *Il5* is a gene with high dependency on GATA3 that shows a decreased dependency in memory Th2 cells. Taken together, these findings indicate that GATA3 is important for maintaining the transcriptional signatures in established Th2 cells.

6. A gene regulatory network in fully developed Th2 cells: the interplay between GATA3 and Menin, a component of the Trithorax complex

As described in Section 4.2, although Menin deficiency had little effect on the ordinary induction of Th2 differentiation, 'Th2 cells' lost their Th2 identity after several cycles of cultivation in the absence of Menin. Our study also revealed that Menin directly bound and epigenetically regulated the *Gata3* gene, suggesting that constant expression of Menin and its binding to the *Gata3* locus is necessary for the maintenance of the Th2 identity. Similar results were obtained with *in vivo*-generated memory Th2 cells, indicating that Menin maintains the memory Th2 cell function during the long-term resting phase. Indeed, Menin-deficient memory Th2 cells show an impaired ability to recruit eosinophils to the lung, causing the attenuation of airway inflammation induced by memory Th2 cells [52].

Since Th2 cells derived from Menin-deficient mice have defects in both Menin and GATA3 expression, whether the lack of Menin, decreased expression of GATA3, or both are responsible for the dysregulation of the Th2-specific gene expression in Menin-deficient cells remains unclear. In a recent study [52], we addressed this point using differentiated Th2 cells with two additional cycles of cultivation (Th2-3rd cells). Consequently, the gene expression profiles under three conditions (i.e. genetic deletion of Menin, *Gata3* siRNA treatment, and retroviral gene transduction of h*GATA3*) were used to classify the Th2-specific genes into four groups (**Figure 3**). *Asb2*, *Ccr8*, *Gzma*, *Il4*, *Il5*, *Il13*, *Il24*, *Mapk12*, *Tanc2*, and *Tube1* were assigned to Group 1, being

controlled by both GATA3 and Menin. Interestingly, only *Gzma* was negatively regulated by Menin, while the other nine genes were positively regulated. Although *Gata3* siRNA treatment downregulated the *Gzma* expression, the forced expression of h*GATA3* also reduced the *Gzma* expression for some unknown reason. Seven genes (*Crem*, *Cyp11a1*, *F2r*, *Nfil3*, *Ptgir*, *Rnf128*, and *Tmtc2*) were found to be positively controlled by GATA3 and not affected by Menin deficiency (Group 2). Group 3 consisted of *Spry2* and *S100a*, which were found to be controlled in a Menin-dependent and GATA3-independent manner. For the other 11 genes (*Ccnjl*, *Dusp4*, *Ecm1*, *Epas1*, *Grtp1*, *Il1r2*, *Itgb3*, *Jdp2*, *Penk*, *Plcd1*, and *Tnfrsf8*), neither *Gata3* knockdown nor Menin deficiency had a significant effect on the gene expression (Group 4).

In our ChIP-seq analysis, the direct binding of Menin was observed in most of the 31 Th2-specific genes, except for *Asb2*, *Mapk12*, *Ecm1*, *Grtp1*, and *Plcd1*. Nine of the Menin target genes (*Ccr8*, *Gata3*, *Il4*, *Il5*, *Il13*, *Il24*, *S100a1*, *Tanc2*, and *Tube1*) were positively regulated by Menin, whereas two targets (*Gzma* and *Spry2*) were negatively regulated. No significant effect of Menin deficiency was observed on the other 15 targets (*Ccnjl*, *Crem*, *Cyp11a1*, *Dusp4*, *Epas1*, *F2r*, *Il1r2*, *Itgb3*, *Jdp2*, *Nfil3*, *Ptgir*, *Penk*, *Rnf128*, *Tmtc2*, and *Tnfrsf8*). Several questions remain to be addressed regarding this regulatory network: Are any other factors involved? What recruits Menin to these gene loci? Why does Menin exert a suppressive effect on some target genes? (**Table 1**).

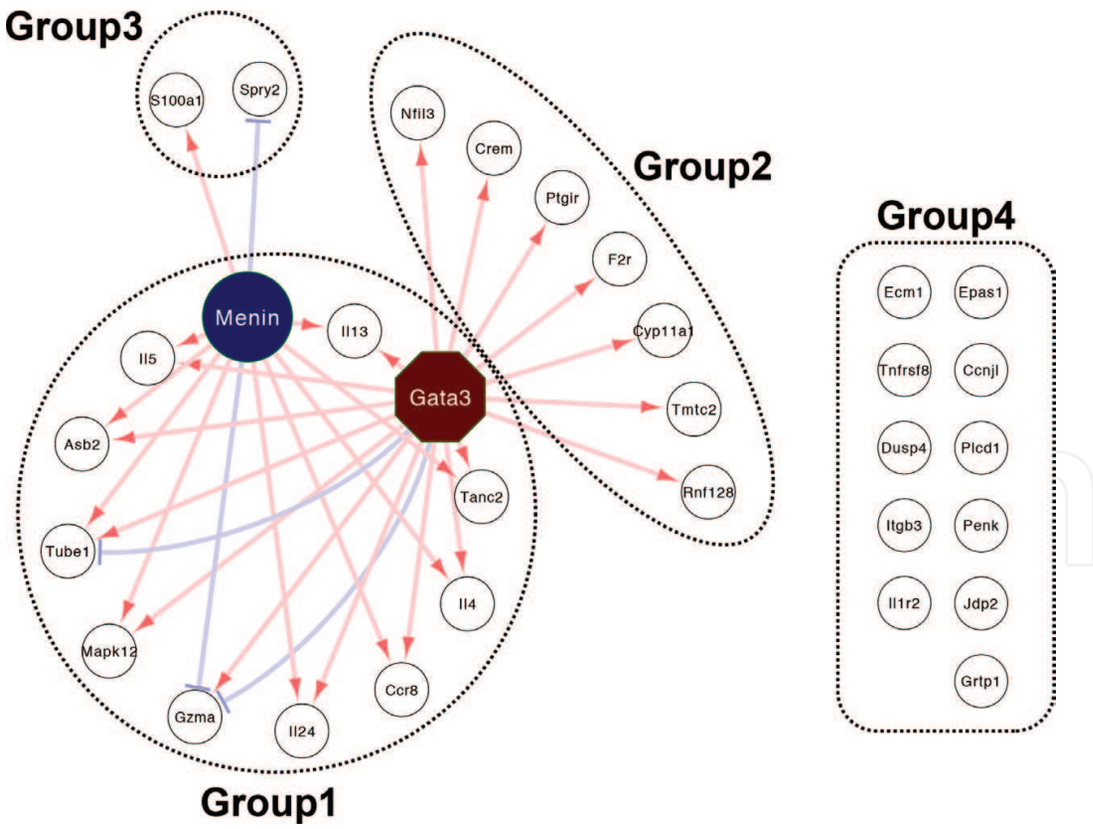

Figure 3. Th2-specific gene regulatory network. The regulatory network formed by Menin- and Th2-specific genes, including GATA3 (originally published in *The Journal of Immunology*. Onodera et al. [10]). Group 1 contains genes that are controlled by both GATA3 and Menin. Genes in Groups 2 and 3 are controlled by either GATA3 or Menin, respectively. Group 4 includes genes that are affected by neither GATA3 knockdown nor Menin knockout. Red arrows indicate the regulatory interactions that activate the target gene expression, whereas blue lines indicate the suppressive effects for targets.

RefSeq ID	Gene symbol	Group	GO term (function, process, or component)
NM_008355	Il13	1	Cytokine activity
NM_010558	Il5	1	Cytokine activity [88]
NM_023049	Asb2	1	Contributes to ubiquitin protein ligase activity [89]
NM_028006	Tube1	1	GTPase activity
NM_013871	Mapk12	1	MAP kinase activity
NM_010370	Gzma	1	Serine-type peptidase activity [90]
NM_053095	Il24	1	Cytokine activity [91]
NM_007720	Ccr8	1	C-C chemokine receptor activity [92]
NM_021283	Il4	1	Cytokine activity [93]
NM_181071	Tanc2	1	In utero embryonic development [94]
NM_017373	Nflil3	2	RNA polymerase II core promoter sequence-specific DNA binding [95]
NM_013498	Crem	2	Core promoter sequence-specific DNA binding [96]
NM_008967	Ptgir	2	G-protein coupled receptor activity
NM_010169	F2r	2	G-protein alpha-/beta-subunit binding [97]
NM_019779	Cyp11a1	2	Cholesterol monooxygenase (side-chain-cleaving) activity [98]
NM_177368	Tmtc2	2	Calcium ion homeostasis
NM_023270	Rnf128	2	Ubiquitin protein ligase activity [99]
NM_011309	S100a1	3	Protein binding [100]
NM_011897	Spry2	3	Negative regulation of ERK1 and ERK2 cascade [101]
NM_007899	Ecm1	4	Interleukin-2 receptor binding [102]
NM_009401	Tnfsf8	4	Tumor necrosis factor-activated receptor activity
NM_176933	Dusp4	4	MAP kinase tyrosine/serine/threonine phosphatase activity
NM_016780	Itgb3	4	Alpha9-beta1 integrin-ADAM8 complex [103]
NM_010555	Il1r2	4	Interleukin-1 receptor activity [104]
NM_010137	Epas1	4	DNA binding transcription factor activity [105]
NM_001045530	Ccnjl	4	Nucleus component
NM_019676	Plcd1	4	Phosphatidylinositol phosphate binding [106]
NM_001002927	Penk	4	Aggressive behavior [107]
NM_030887	Jdp2	4	RNA polymerase II proximal promoter sequence-specific DNA binding [108]
NM_025768	Grtp1	4	Rab GTPase binding

Table 1. Summary of the target genes of the GATA3 and Menin with functions of the encoded proteins (based on https://www.ncbi.nlm.nih.gov/gene).

7. Conclusions

Since the human genome project was completed in 2003, the human genomic DNA database has become accessible to researchers [109]. Open access to the reference genomes of humans, mice, and other organisms encourages scientists to develop elegant technologies, including ChIP-seq and high-throughput sequencing of RNA (RNA-seq) [110]. This technique enables us to analyze the epigenetic status of each population of cells on a genome-wide scale. Many scientists have tried to use this technique to clarify the functional roles of epigenetic modifications in gene expression, particularly in the fields of developmental biology and immunology [47].

Recently, we identified several important principles between the binding positions of PcG and TrxG proteins and the gene expression [52]; a binding pattern in which PcG binds upstream and TrxG binds downstream of the transcription start site is frequently found at highly expressed genes, and a binding pattern in which PcG and TrxG bind to opposite positions is frequently found at low-expressed genes in T lymphocytes. We hope that these findings will prove useful for understanding how CD4+ T cells acquire effector functions and identifying new therapeutic targets for treating allergic diseases, such as asthma, allergic rhinitis, food allergy, and atopic dermatitis. A recently developed epigenetic editing technique using the CRISPR/Cas9 system now allows us to modify epigenetic marks in a site-specific manner [111]. In the future, we may use this technique to treat various diseases cause by epigenetic alternations.

Abbreviations

ATAC-Seq	assay for transposase-accessible chromatin sequencing
ChIP-Seq	chromatin immunoprecipitation followed by massively parallel sequencing
CNS	conserved non-coding sequence
H3K27me3	trimethylated histone H3 lysine 27
H3K4me3	trimethylated histone H3 lysine 4
HS	DNase I hypersensitive site
IL	interleukin
PcG	Polycomb group
PRC	Polycomb repressive complex
STAT	signal transducer and activator of transcription
Th	helper T cell
TrxG	Trithorax group

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