EZH2 and Histone 3 Trimethyl Lysine 27 Associated with Il4 and Il13 Gene Silencing in Th1 Cells*

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Madoka Koyanagi‡, Aurelie Baguet‡§, Joost Martens¶, Raphael Margueron†, Thomas Jenuwein‡, and Mark Bix**

From the ‡Department of Immunology, University of Washington, Seattle, Washington 98195-7650, the §Research Institute of Molecular Pathology, Dr. Bohrsgasse 7, A-1030 Vienna, Austria, and the ¶University of Medicine and Dentistry of New Jersey, Division of Nucleic Acids Enzymology, Department of Biochemistry, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

Differeṇtiation of naïve CD4 T cells toward the T helper 1 (Th1) and T helper 2 (Th2) fates involves the transcriptional repression and enhancement, respectively, of Il4 and Il13, adjacent chromosome 11 genes encoding the canonical Th2 cytokines interleukin-4 and interleukin-13, respectively. These genes are involved in the differentiation of CD4 T cells into Th1 and Th2 subsets, and their expression is controlled by a variety of transcriptional factors. Here, using chromatin immunoprecipitation and real time reverse transcription PCR we identify the Polycomb family histone methyltransferase EZH2 as the enzyme responsible for methylating lysine 27 of histone H3 at the Il4-Il13 locus in Th1 cells but not Th2 cells, implicating EZH2 in the mechanism of Il4 and Il13 transcriptional silencing.

Acting on instructions issued by cells of the innate immune system, naïve CD4 T cells commit to effector cell fates specifically tailored to address the confronting class of pathogens and the constraints of infected host tissue environments. The best characterized of these fates are called T helper 1 (Th1)1 and Th2, distinguished primarily by their non-overlapping patterns of cytokine gene expression potential. Il4 and Il13, adjacent genes on chromosome 11, encode the canonical Th2 cytokines responsible both for promoting immune responses against extracellular pathogens and, when misregulated, causing allergic and autoimmune diseases (1). Heritable states of transcriptional repression and enhancement of these genes during Th1 and Th2 development, respectively, are associated with developmentally programmed chromatin structural changes at the Il4-Il13 locus. The molecular nature of these changes, the cis-acting regulatory elements that coordinate them, and the relationship of these changes to Il4 and Il13 transcriptional potential are not well understood.

The flexible amino termini of nucleosomal histones harbor multiple residues that can undergo a variety of post-translational modifications, including acetylation, methylation, and phosphorylation. Combined with the octameric structure of the nucleosomal core, this flexible modification system harbors tremendous combinatorial diversity and information-coding potential (the so-called histone code) that can endow discrete genetic intervals with specific functional properties (2). Along a given interval, factors recruited to specific regulatory elements can create heritable patterns of histone modification that, in turn, can influence the transcriptional behavior of associated genes by creating or destroying binding platforms for transcriptional activators and repressors, nucleosome remodeling, and chromatin-packaging machinery (3). Histone H3, for example, can undergo a variety of post-translational modifications that can be classified as transcription-associated (acylated lysine 9/lysine 14 (H3K9/14ac), dimethylated lysine 4 (H3K4me2), and phosphorylated serine 10) and silence-associated (dimethylated and trimethylated lysine 9 (H3K9me2 and H3K9me3) and trimethylated lysine 27 (H3K27me3)) (for review see Ref. 4).

Thirteen clusters of DNase I hypersensitive sites (HSs) have been mapped at the Il4-Il13 locus of Th2 cells. Based upon lineage specificity and activation dependence, these can be classified as Th2-specific/constitutive (HSv, HS1, HS2, HS3, HS4, HS5, HS6, HS7, HS8, HS9, HS10, TH12-2-specific/activation-dependent (HSva), and naive/Th1-2-shared/constitutive (HSv and HSs) (see Fig. 2, top). Although useful as a roadmap of potential regulatory elements at the Il4-Il13 locus, DNase I hypersensitivity alone is insufficient to reveal functional regulatory elements and their modes of action at each stage of effector T cell development. For example, the transcriptional states of Il4 and Il13 in naïve CD4 T cells and Th1 cells are permisive and silent, respectively, despite sharing identical patterns of DNase I hypersensitivity at the Il4-Il13 locus (5, 6).

Analysis of locus-wide patterns of histone modification provides an avenue to discern the developmental stages at which specific HS-containing regions act to modulate Il4 and Il13 transcriptional permissiveness. A recent survey of transcription-associated HS modifications revealed the occurrence of H3K4me2 near the Th12-2-specific/constitutive HSv in naïve CD4 T cells but not in Th1 cells. Thus, the HSv-containing region is implicated in the maintenance of Il4 and Il13 transcriptional permissiveness.
at the naive CD4 T cell stage, even though hypersensitivity at HS8 is not yet detectable in these cells (5, 6). Missing still from this emerging picture of the sequence of chromatin structural events at the Th2 cytokine locus in developing Th1 and Th2 cells is the nature and dynamic distribution of transcriptional silence-associated histone modifications.

EZH2 and SUV39 are histone methyltransferases (HMTs) with in vivo specificity for H3 lysine 27 and H3 lysine 9, respectively (7). SUV39, together with heterochromatin protein 1 with binding specificity for methylated H3 lysine 9, has been shown to be critical in the formation of heterochromatin (7). EZH2, a member of the Polycomb group, occurs in a number of multisubunit complexes with other Polycomb group members including EED (embryonic ectoderm development). A Polycomb group complex containing the eponymous Polycomb has binding affinity for methylated H3 lysine 27 and appears to be involved in the repression of euchromatic genes (4). EZH2 is expressed in lymphocytes and has been shown to play a critical role in B lymphopoesis (8). Its role in T cell biology is not known.

Here, we report the analysis of repressive H3 modifications at the Il4-Ii13 locus during effector T cell development. Our data demonstrate that, in naive CD4 T cells and Th1-primed but not Th2-primed cells, lysine 27-methylated H3 occurs selectively at HS1v and HS3v, the only two lineage-specific hypersensitive sites at the Il4-Ii13 locus. Th1-specific silencing of Il4 and Ii13 correlates with elevated levels of H3K27me3 at HS1v and the subsequent spreading of H3K27me3 into flanking regions. Furthermore, we show that EZH2 associates with HS1v and HS3v in naive, Th1, and, unexpectedly, Th2 cells. Together, our results suggest that developmentally regulated H3 lysine 27 methyltransferase activity of EZH2 constitutively bound at HS1v and HS3v is responsible for specifying/maintaining Il4 and Ii13 transcriptional silence in Th1 cells.

MATERIALS AND METHODS

**Cell Lines—**NIH3T3 cells were grown in Dulbecco's modified Eagle's medium 10 (Dulbecco's modified Eagle's medium supplemented with 4 mM l-glutamine, 4.5 g/liter glucose, 1.5 g/liter sodium bicarbonate, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum).

**Naïve T Cell Purification—**Combined lymph node and spleen cell suspensions generated from 4–6 week BALB/c mice (Jackson Laboratories, Bar Harbor, ME; housed under specific pathogen-free conditions at the University of Washington, Seattle, WA) were subjected to two sequential AutoMACS purification steps. First, CD4 T cells were isolated by positive selection from CD4 T cells stained with anti-CD62L MicroBeads (Miltenyi Biotec, catalog number130-049-701). The purity of this cell population was at least 97%. CD4+ cells isolated by negative selection using a CD4 T cell isolation kit (Miltenyi Biotec, catalog number 130-049-806). Next, CD4+ CD62Lhi cells were isolated by positive selection from CD4 T cells stained with anti-CD62L MicroBeads (Miltenyi Biotec, catalog number130-049-701). The purity of this cell population was at least 97%.

**T1+ and T2+ Cultures—**Primary Th1 cultures were initiated with naive CD4 T cells prepared as described above. Splenic antigen-presenting cells, prepared by complement-mediated lysis using anti-Thy1.1 (Jj; American Type Culture Collection) and a combination of rabbit and guinea pig sera, were given 3000 rads prior to use. Th1 cultures were seeded at a ratio of 1:5 (T cell/antigen-presenting cell) with anti-TCR (H57-597; 2.5 μg/ml), anti-CD28 (37N; 1 μg/ml), and recombinant human IL2 (10 units/ml). In addition, Th1 cultures contain recombinant mouse IL12 (40 ng/ml) and IL-10 (11B11; 40 μg/ml), whereas Th2 cultures contain recombinant mouse IL4 (300 units/ml; kindly provided by William Paul), anti-IL12 (C17.8; 10 μg/ml) and anti-interferon-γ (Xmg; 10 μg/ml). Cultures were harvested on day 4 and rested in IL2 (10 units/ml) for 2 days prior to recovery for analysis or initiation of a second round of priming. Rest同仁 were activated by a 4-h culture with pokeweed 12-myristate 13-acetate (5 ng/ml) and ionomycin (250 ng/ml).

**Real Time Reverse Transcription-PCR (RT-PCR)—**RNA was isolated free of contaminating genomic DNA using the RNAeasy mini kit (Qiagen) Random hexamer-primed cDNA was generated using the Superscript II RNase H reverse Transcriptase kit (Invitrogen; catalog number 18064-014). Real time PCR reactions were performed and analyzed on a Stratagene MX4000. The PCR reaction buffer contained 10 mm Tris (pH8.3), 50 mM KCI, 4.5 mM MgCl2, 0.01% Tween 20, 0.3% MeSO, 0.0025% SYBR Green 1 solution (Molecular Probes; catalog number S-7563), 50 ng each primer (Supplemental Fig. 1 in the on-line version of this article), and 2 units/reaction Hot Start Taq polymerase (Qiagen) per 25-μl reaction. Cycling conditions were 94 °C for 15 min followed by 40 cycles of 94 °C for 20 s, 61 °C for 1 min, and 72 °C for 40 s. Ct values for no reverse transcriptase controls were at least four times higher than experimental samples, corresponding to ~6% background. More often, no reverse transcriptase background was <0.8%. For a given cDNA, relative abundance of each target was normalized to Hprt according to the formula 2−ΔΔCT, where ΔΔCT = CTARGET−CTHPR

**Chromatin Immunoprecipitation (ChIP) Assays—**DNA recovered from an aliquot of sheared chromatin was used as the “input” sample. The remaining chromatin was pre-cleared with protein A- and protein G agarose (Upstate Biotechnology; catalog numbers 16-156 and 16-266) and then incubated with antibody overnight at 4 °C (anti-H3K9/14ac, catalog number 06-599 and anti-H3K4me2, catalog number 07-030 from Upstate Biotechnology), both used at 2 μg/ml; anti-H3K9me3 (sera 4861) and anti-H3K27me3 (sera 6523, number 3290; Ref. 15), both used at 2 μg/ml; anti-H3K4me2 (9), used at 1 μg/ml; and anti-H3K9me3 (2), used at 5 μg/ml. Input DNA and DNA recovered after immunoprecipitation (IP) were quantified using PicoGreen fluorescence (Molecular Probes, Eugene, OR). Equivalent masses of IP and input DNA were compared by real time PCR as described above for RT-PCR with the following modifications. Tag polymerase was from Qiagen (Hot Start), and cycling conditions were 94 °C for 15 min followed by 45 cycles of 94 °C for 20 s, 61 °C for 1 min, and 72 °C for 40 s. Data are presented as the ratio of IP to input, Cq values. For analysis of cromatin repeats, end point PCR analysis was performed to compare equivalent masses of input and IP DNA. PCR products were subjected to 3- or 4-fold serial dilution and separated by gel electrophoresis.

**RESULTS**

**Transcriptional Response of Th1- and Th2-primed Naïve CD4 T Cells—**Using real time RT-PCR, we characterized the 4-hour phorbol 12-myristate 13-acetate/isonomycin-induced transcriptional response of the clustered Kif3a, Il4, Ii13, and Rad50 genes in one-round and two-round Th1- and Th2-primed (Th1R1, Th1R2, Th2R1, and Th2R2) CD4 T cells (Fig. 1). For developing Th1R2 cells, transcription of Il4 and Ii13 increased whereas that of Ifnγ decreased with each successive round of priming. The reciprocal pattern was observed for developing Th2R1 cells. Interestingly, Il13 expression consistently decreased in Th1R1 cells and then reappeared at low levels in Th1R2 cells. Though still, to date, unexplained, this Il13 effect was highly reproducible and consistent with observations made by others.2 Previously, we demonstrated that Il4 transcriptional permissiveness was maintained at naive CD4 T cell levels 48 h after the onset of Th1 priming (6). Together with our current results demonstrating transcriptional silence of Il4 in one-round Th1-primed cells (Fig. 1), we conclude that transcriptional repression of Il4 requires between 2 and 4 days of Th1-priming. Consistent with a previous report (5), expression of Kif3a and Rad50 was ~2–3-fold higher in Th2-primed versus Th1-primed cells (Fig. 1). However, unlike Il4 and Il13, Kif3a and Rad50 expression did not increase further in Th2R2 cells, demonstrating, as shown previously (6), that the genes flanking Il4 and Il13 are insulated from the regulatory events governing the Il4-Ii13 locus.

**Repressive H3 Modifications in Th1- and Th2-primed Cells—**One previous study has described the occurrence of H3K9me at the Il4-Ii13 locus of Th1 cells, suggesting that this modification might be involved in the transcriptional silencing of Il4 and Ii13 (10). To test this possibility, we purified naive CD4 T cells from young BALB/c mice and cultured them for two rounds in Th1-priming conditions. Primed cells were harvested and pro-
cessed for ChIP using an antibody highly specific for H3 trimethylated on lysine 9. Using real time PCR, we measured the relative enrichment of specific target sequences in equivalent amounts of DNA obtained from unprecipitated and precipitated chromatin. PCR primer pairs targeted 17 locations at the Il4-Ii13 locus, sampling elements known to be functionally (11, 12), structurally (5, 13), or computationally (11, 12) implicated in cytokine regulation (Fig. 2, top, and described in Ref. 6). Although in T4R1 cells control major centromeric repeat sequence displayed enrichment for H3K9me3 (Fig. 2B; see Ref. 14), at the Il4-Ii13 locus no H3K9me3 enrichment was detected (Fig. 2A). Centromeric repeat sequences were not enriched in chromatin precipitated with antibodies to H3K9/14ac (data not shown). Similar results were obtained with antibodies to H3K9me2 (data not shown). These results suggest that the transcripational silence of Il4 and Ii13 in T4-primed cells does not involve H3 lysine 9 methylation.

As the anti-H3K9me antibody used in the previous study may have cross-reacted with H3K27me3 (10, 15), we decided to perform ChIP analysis of T4-primed cells with an antibody highly specific for H3K27me3 (15). T4R1 cells displayed two prominent peaks of H3K27me3 enrichment centered on HSIV and HSS3, respectively (Fig. 2C). T4R2 cells showed, in addition to these peaks, increased H3K27me3 levels across the entire Il4-Ii13 locus, extending at least from HSSV downstream of Il4 to HSSB upstream of Il13 (Fig. 2D). This increase was specific insofar as H3K27me3 was at or below background levels at the G6pd locus in both T4R1 and T4R2 cells (data not shown). These results suggest that the initial report of H3K9me at the Il4-Ii13 locus was likely due to cross-reactivity of antibodies that failed to distinguish between H3 methylation on lysine 9 and lysine 27, residues with very similar sequence contexts (16). We conclude that, during the development of T4 cells, H3K27me3 occurs initially at HSSV and HSS3 and subsequently spreads to surrounding regions of the locus.

To determine whether any aspect of the H3K27me3 pattern was T4-specific, as would be predicted for a histone modification involved in Il4 and Ii13 transcriptional silence, we used the H3K27me3-specific antibody in ChIP analysis of developing T4 cells. In contrast to T4-primed cells, T4R1 and T4R2 cells displayed significantly lower and almost undetectable H3K27me3 levels, respectively (Fig. 3A and B), despite exhibiting strong signals at the control MyoD locus (Fig. 3C). The low level of H3K27me3 that was detectable in one-round T4-primed cells localized predominantly to HSS3 and could not be found in surrounding regions (Fig. 3A). A faint signal was observed at HSSV in T4R1 cells but was no longer detectable in T4R2 cells. As was the case in T4R2 cells, no H3K27me3 enrichment was detected across the entire Il4-Ii13 locus of the T4R2 clone D10.G4 despite strong signals at the control MyoD locus (data not shown).

Repressive H3 Modifications in Naive CD4 T Cells—The occurrence of H3K27me3 at HSSV and HSS3 (HS sites that are shared among T4, T4, and naive CD4 T cells) in T4R1 and T4R2 cells prompted us to investigate whether H3K27me3 might pre-exist in naive CD4 T cells. To test this possibility, we isolated naive CD4 T cells from spleen and lymph nodes of young BALB/c mice and performed quantitative ChIP analysis for H3K27me3. Indeed, H3K27me3 was detected in naive CD4 T cells in a pattern similar to that of T4R1 cells, focused in two peaks at HSSV and HSS3 but absent from surrounding regions of the locus (Fig. 4). Interestingly, the HSS3 peak was significantly higher in naive CD4 T cells than in T4R1-primed cells, suggesting greater involvement of this putative regulatory element early in the development of CD4 effector cells. This result demonstrates that low level Il4 and Ii13 transcriptional permissiveness in naive CD4 T cells is compatible with the occurrence of H3K27me3 at HSS3 (and, perhaps, to a much lower level at HSSV).

EZH2 Binds to HSSV and HSS3 in Naive T4- and T4R-primed Cells—The only known HMT capable in vivo of tri-methylating H3 lysine 27 is the Polycomb family protein EZH2 (17). Apart from the inactive X chromosome in XX female cells, physiological targets of EZH2 binding have only recently been described and have not yet been verified functionally (18–20). To test whether H3 lysine 27 trimethylation at the Il4-Ii13 locus might be catalyzed by EZH2, we asked whether it binds in vivo to the Il4-Ii13 locus. We used an EZH2-specific antibody to perform quantitative ChIP analysis of naive CD4 T cells and T4R2 cells. In T4R2 cells we detected peaks of EZH2 binding that coincided precisely with the locations of maximal H3K27me3 enrichment at HSSV and HSS3 (Fig. 5B). No EZH2 binding was detected in the regions surrounding HSSV and HSS3 (Fig. 5B), where H3K27me3 was detected at lower levels in T4R2 cells (Fig. 2D). A similar binding pattern was also detected in naive CD4 T cells, correlating quantitatively with the increased magnitude of H3K27me3 enrichment at HSS3 (Figs. 4 and 5A). Surprisingly, in T4R2 cells, where H3K27me3 was no longer detectable at the Il4-Ii13 locus, we still detected strong EZH2 binding at HSSV and HSS3 (Figs. 3B and 5C). Together, these results demonstrate that EZH2 binds constitutively to HSSV and HSS3 in naive CD4 T cells and in T4R1 and T4R2 cells. In naive CD4 T cells and T4-primed cells, quantitative and spatial correlations of EZH2 binding and H3K27me3 abundance are consistent with EZH2 being the HMT responsible for H3 lysine 27 trimethylation. The persist-
ence of HS IV- and HS S3-bound EZH2 in TH2 cells where H3K27me3 is no longer detectable is consistent with developmental regulation of EZH2 activity at a post-chromatin binding step or with the involvement of a novel H3 lysine 27-specific HMT. Finally, the TH1-specific appearance of H3K27me3 in the regions surrounding HS IV and HS S3 may indicate that EZH2 can interact transiently with these regions, perhaps processively by tracking or by looping from points of nucleation at HSIV and HSS3 (21).

Repressive H3 Modifications in a Fibroblast Cell Line—The fibroblast cell line NIH3T3 does not express Il4 or Il13 and lacks all known DNase I hypersensitive sites at the Il4-I13 locus, including HSIV and HSIII (5). At the Il4-I13 locus the transcription-associated H3 modification phosphoserine 10, found broadly distributed in TH2-primed cells and the T112 clone D10.G4, does not occur in NIH3T3 (6). Similarly, despite strong signals for each at the control G6pd locus, no H3K9/14ac and H3K4me2 occurred across the Il4-I13 locus of NIH3T3 (Fig. 6, A–C). To assess whether transcriptional silence of Il4 and I13 in a non-T cell lineage would be associated with the same pattern of repressive chromatin modifications detected in TH1-primed CD4 T cells, we analyzed NIH3T3 by quantitative ChIP for the occurrence of H3K9me3. Despite control signals at major centromeric repeat sequences, we detected no H3K9me3 across the Il4-I13 locus (Fig. 6, D and E). Similar results were obtained for H3K27me2 (data not shown). These results were confirmed in primary mouse embryonic fibroblasts (data not shown). Thus, the constitutive transcriptional silence of Il4 and I13 in fibroblast lineage cells appears to involve neither H3K27me3 nor H3K9me3, suggesting lineage-specific epigenetic mechanisms for the maintenance of Il4 and I13 transcriptional silence.

**DISCUSSION**

Knowing the chemical nature of post-translational histone modifications associated with Il4 and I13 gene silencing as well as the responsible catalytic enzymes is important for understanding the mechanisms by which heritable transcriptional states are developmentally specified at the Il4-I13 locus. In this report we exploited antibodies with proven specificity for distinct transcriptional silence-associated H3 modifications in ChIP analyses to demonstrate that the principal H3 modification associated with Th1-dependent silencing is the tri-methylation of lysine 27 and not of lysine 9. The striking localization of H3K27me3 to HSIV and HSS3 (21) suggests that elements containing these sites function either together or separately as transcriptional silencers in Th1 cells.

**FIG. 2.** Silencing H3 modifications at the Il4-I13 locus during Th1 development. At the top is a physical map of the chromosome 11 Kif3a-I14-I13-Rad50 locus. Horizontal black arrows represent full (solid lines) or partial (dotted lines) transcription units. Tall black rectangles represent exons. Arrowheads indicate the location of Th2-specific (filled symbols) and naive/Th1/Th2-shared (open symbols) DNase I hypersensitive sites. Small black rectangles represent regions of high sequence conservation between mouse and human (CNS1 and CNS2). Diamond-encased letters (A–P) represent the names and locations of PCR primer pairs. Dotted vertical lines in each plot bracket the locations of transcription units. Below this are ChIP plots (A, C, and D) depicting fold enrichment for H3K9me3 (panel A, n = 1) and H3K27me2 (panel C, n = 5, and panel D, n = 2) at the Il4-I13 locus in naive CD4 T cells following Th1-priming for one round (panel C) or two rounds (panels A and D). Shown in panel B is the relative abundance of major centromeric repeat sequences in two-round Th1-primed naive CD4 T cell DNA before (Input) and after (IP) ChIP using an antibody directed against H3K9me3. Input and IP PCR products were subjected to 4-fold serial dilutions over a 64-fold range prior to gel electrophoretic separation. The relative intensities of the gel-resolved major centromeric repeat products (showing a typical laddering pattern) reveal a 4–16-fold enrichment of H3K9me3 in the IP versus input samples, as described previously (14).
deletion of one of them (HSIV) prevents efficient silencing of Il4 in TH1-primed cells (23). We also demonstrate that the Polycomb family H3 lysine 27-specific HMT EZH2 binds to HSIV and HSS3, suggesting it is likely to be responsible for trimethylating H3 lysine 27 at the TH2 cytokine locus.

Located in phylogenetically conserved regions of the T_H2 cytokine locus, HSIV and HSS3 are unique in being the only two DNase I hypersensitive sites that occur in T_H1, T_H2, and naive CD4 T cells. Our data demonstrate that they are also the only sites at the T_H2 cytokine locus to display EZH2 binding in all

**FIG. 3. Silencing H3 modifications at the Il4-I13 locus during T_H2 development.** The map at the top is as described in the Fig. 2 legend. Below this map are ChIP plots depicting H3K27me3 enrichment at the Il4-I13 locus (A and B) and the MyoD locus (C) in naive CD4 T cells following T_H2-priming for one round (panels A and C, n = 4) or 2 rounds (panels B and C, n = 2).

**FIG. 4. H3K27me3 at the Il4-I13 locus of naive CD4 T cells.** The map at the top is as described in the Fig. 2 legend. Below this is a ChIP plot depicting H3K27me3 enrichment at the Il4-I13 in naive CD4 T cells (n = 3).
three lineages, suggesting that this binding is the basis for their shared DNase I hypersensitivity. The level of H3K27me3 enrichment at HSS3 and HSIV was much higher in naïve CD4 T cells than in developing TH2 cells, suggesting a progressive loss of H3K27me3 that was essentially complete after two rounds of priming. This loss may be due to the activity of an as yet unidentified H3 lysine 27 demethylase or perhaps progressive dilution of the H3K27me3 mark through successive cell cycle rounds in the absence of maintenance H3 lysine 27 methylation. The pattern in developing TH1 cells was more complex, comprising an increase and decrease at HSIV and HSS3, respectively, such that in TH1R1 cells H3K27me3 remains and appears to have spread into flanking regions. However, interactions between EZH2 and flanking regions must be transient, as EZH2 binding was not detectable. Alternatively, H3K27me3 in flanking regions is dependent upon an unidentified H3 lysine 27-specific HMT.

One possible mechanism for regulating the activity of EZH2 independently of its chromatin binding is through developmental regulation of cofactors capable of influencing the HMT activity of EZH2. Consistent with this possibility, EZH2 is known to occur in at least two distinct multisubunit complexes (PRC2 and PRC3) along with another Polycomb family member, EED (9). Alternative translation initiation generates four

H3K9me may occur only in the mature stage of TH1 development represented by the TH1 clone analyzed by Grogan et al. (10).

In TH1R2 cells, HSIV and HSS3 contain bound EZH2 but no H3 lysine 27 trimethylation. This result is consistent with either EZH2 not being responsible for HSIV and HSS3 H3 lysine 27 trimethylation or with the HMT activity of EZH2 being regulated independently of chromatin binding. We support the latter interpretation. First, in naïve CD4 T cells and in TH1 cells EZH2 is observed to bind specifically at HSIV and HSS3, precisely the locations where peak H3K27me3 enrichment occurs. Second, in naïve CD4 T cells and TH1 cells there is a strong correlation between the degree of EZH2 binding and H3K27me3 enrichment. Finally, following polyclonal stimulation by phorbol 12-myristate 13-acetate plus ionophore, CD4 T lymphocytes harboring a homozygous targeted deletion activating the Ezh2 gene rapidly lose all detectable nuclear H3K27me3, suggesting that EZH2 is responsible for most if not all H3 lysine 27 tri-methylation.3

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distinct EED isoforms that feature differentially truncated amino termini (9). Depending on the isoform of its EED partner, EZH2 has been shown in vitro to switch its substrate specificity from H3 lysine 27 to histone 1 lysine 26 or to become unable to mediate methylation of either substrate (9). Thus, EZH2 that remains bound to HSIV and HS S3 in TH2-primed cells may have acquired a new EED partner that prevents H3 lysine 27 methylation, thereby preventing transcriptional silencing. Alternatively, the TH2-specific absence of H3K27me3 may arise from H1 recruitment shown in in vitro biochemical studies to prevent the H3 lysine 27 methyltransferase activity of EZH2 (9).

In somatic cell lineages, distinct biochemical pathways may execute facultative and constitutive gene silencing. The textbook example of facultative heterochromatin is the inactive X chromosome in female somatic cells. Recent work has demonstrated that the process of X inactivation involves H3 lysine 27 methylation (24). By contrast, constitutive heterochromatin at telomeres and centromeres involves principally H3 lysine 9 methylation (25). Transcriptional silencing of Il4 and Il13 is facultative in CD4 T lymphocytes and constitutive in fibroblasts. From this perspective it is interesting to note that transcriptional silence of Il4 and Il13 was accompanied by H3 lysine 27 methylation in CD4 T lymphocytes but not in fibroblasts (Fig. 6 and data not shown). This difference may be indicative of a more general phenomenon in which, for a given cell lineage, constitutive silencing and facultative silencing are functionally segregated to different biochemical pathways, with the establishment of only the latter (facultative silencing) involving H3 lysine 27 methylation. Consistent with this hypothesis is the absence of H3K27me3 from the constitutively silenced muscle-specific gene MyoD in fibroblast lineage cells (data not shown). The occurrence of H3K27me3 at the MyoD locus of CD4 T cells (Fig. 3E) is not necessarily inconsistent with this hypothesis, as it has been reported that hematopoietic cells can trans-differentiate into muscle (22).

In summary, we have shown that the principal H3 modification associated with TH1-dependent silencing is trimethylation of lysine 27 initially at HSIV and subsequently in regions flanking HSIV and HS S3, consistent with the known role of HS S3 as a transcriptional silencer in TH1 cells. We also demonstrate that the Polycomb family H3 lysine 27-specific HMT binds to HSIV and HS S3, suggesting that it is likely to be responsible for trimethylating H3 lysine 27 at the TH2 cytokine locus and, hence, is an important mediator of Il4 and Il13 gene silencing.

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Madoka Koyanagi, Aurelie Baguet, Joost Martens, Raphael Margueron, Thomas Jenuwein and Mark Bix

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