Polycomb Antagonizes p300/CREB-binding Protein-associated Factor to Silence FOXP3 in a Kruppel-like Factor-dependent Manner

Yuning Xiong, Sahil Khanna, Adrienne L. Grzenda, Olga F. Sarmento, Phyllis A. Svingen, Gwen A. Lomberk, Raul A. Urrutia, and William A. Faubion, Jr.

Background: Epigenetic regulation of the T regulatory cell transcriptional program remains unclear.

Results: Without KLF10, Polycomb permanently silences FOXP3, the master transcription factor of T regulatory cells.

Conclusion: Chromatin remodeling events mediated by KLF10 and Polycomb regulate FOXP3 through a Polycomb response element.

Significance: Polycomb and KLFs may direct a heritable, broadly applicable regulatory circuit within T cell development.

Inducible gene expression underlies the epigenetically inherited differentiation program of most immune cells. We report that the promoter of the FOXP3 gene possesses two distinct functional states: an “off state” mediated by the Polycomb histone methyltransferase complex and a histone acetyltransferase-dependent “on state.” Regulating these states is the presence of a Kruppel-like factor (KLF)-containing Polycomb response element. In the KLF10−/− mouse, the FOXP3 promoter is epigenetically silenced by EZH2 (Enhancer of Zeste 2)-mediated trimethylation of Histone 3 K27; thus, impaired FOXP3 induction and inappropriate adaptive T regulatory cell differentiation results in vitro and in vivo. The epigenetic transmittance of adaptive T regulatory cell deficiency is demonstrated throughout more than 40 generations of mice. These results provide insight into chromatin remodeling events key to phenotypic features of distinct T cell populations.

FOXP3+ T regulatory (Treg) cells may develop outside the thymus, generally in response to TGFβ and antigen where they are critically important in intestinal immunologic homeostasis (so-called adaptive or induced Treg cells) (1–6). Dysregulation of the transcription factor FOXP3, a key driver of the phenotypic features of distinct T cell populations.

The Polycomb group chromatin-modifying protein complex (PcG) is a key chromatin remodeling complex, the role for which in lymphocyte gene regulation and biology remains to be fully understood. As opposed to the dynamic regulation of transcription by the activity of histone acetyltransferases (HATs) and histone deacetylases, PcG proteins lead to permanent silencing through the activity of histone methyltransferases (HMTs) (15). Recent studies indicate that site-specific recruitment of PcG to promoters occurs at least in part through Polycomb response elements. Polycomb response elements (PREs) have been defined extensively in Drosophila, but only recently in humans (16, 17). Interestingly, KLF binding sites are present in most well characterized PREs (16, 18), yet a role for this novel PcG-PRE mechanism in the regulation of T cell biology remains unexplored.

We have extended the recent observation that animals carrying a disruption in a KLF transcription factor (KLF10) are impaired in FOXP3 activation (19, 20) and provide here the first biochemical evidence revealing PcG silencing of FOXP3 as a default state in naïve lymphocytes, a state that must be over-
Polycomb Regulates FOXP3

EXPERIMENTAL PROCEDURES

Bioinformatics Analysis—Gene expression and ChIP-seq data were curated from NCBI Gene Expression Omnibus, data set GSE14254 (21). CpG island prediction was performed using EMBOSS CPGPLOT at default values (22). Alignments constructed with the Geneious Alignment implemented in Geneious 5.4.6 (23), using a 65% similarity matrix and a gap opening and extension penalty of 12 and 3.

Isolation of Primary T Cells—Male mice were used for all experiments related to H3K27 methylation because of concerns regarding random inactivation of the X chromosome in females and the X-linked FOXP3 gene. Murine naïve CD4+ splenocytes were isolated using a combination of magnetic separation kits (Miltenyi Biotec). Sequential use of the CD4+CD25+ regulatory T cell isolation kit and the CD4+CD62L+ T cell isolation kit resulted in naïve FOXP3-negative T cells used for in vitro induction of FOXP3. When using the B6.Cg-FOXP3tm12Tch/j mouse, flow cytometry was performed to sort for FOXP3-expressing cells. The cells were sorted on either a FACS Aria cell sorter running with FACSDiva software (BD Biosciences) or a FACS Vantage SE cell sorter running with CellQuest software (BD Biosciences). Human naïve CD4+ cells were isolated from anonymous healthy blood donors also using a combination of Miltenyi Biotec beads. Once the CD4+ cells were negatively selected, a positive selection of the CD45RA- cells yielded the naïve population of T cells.

Cell Stimulation—In vitro activation of the isolated T cells followed similar conditions among the different cell types. Anti-CD3, OKT3 (eBioscience) for the Jurkat cells, 145-2C11 (BD Biosciences) for the mouse T cells, and UCHT1 (BD Biosciences) for the human T cells was plate-bound at 2 μg/ml. Soluble anti-CD28 (BD Biosciences) at 2 μg/ml plus 100 units/ml IL-2 was added to the cultures throughout the incubation period. Human TGF-β1 recombinant (Austral) at a concentration of 5 ng/ml was used to generate adaptive Treg cells.

RNA Isolation, cDNA Synthesis, and Quantitative Real Time PCR—Total RNA was isolated using the manufacturer protocol in the RNeasy Mini Kit (Qiagen). cDNA was synthesized from 0.5–1 μg of total RNA with random primers using SuperScript® kit III First-Strand (Invitrogen). Two μl reverse transcription products were used for each real time PCR. PCRs were in 20 μl of total volume that contained primers and 10 μl of Express SYBR green ER quantitative PCR Supermaster mixes (Invitrogen).

For semiquantitative RT-PCR, genes of interest were amplified under the following conditions: initial denaturation, 95 °C for 3 min, followed by 34 cycles with denaturation at 95 °C for 30 s, annealing at 55 °C for 60 s, and extension at 72 °C for 60 s. All the PCR products were visualized by running 1.5% agarose gels electrophoresis and ethidium bromide staining for the pictures.

Cloning of the FOXP3 Core Promoter and FOXP3 Core Plus E1 Constructs—The human FOXP3 (NCBI AF235097) core promoter containing −511 bp from the transcription start site was amplified by PCR using FOXP3 promoter sequence-specific primers from position −511 to +176. The genomic DNA extracted from CD4+ T cells of a healthy donor was used as a template. The PCR product was subcloned in the pGL3 basic vector (Promega). Similarly, the FOXP3 core promoter plus the first enhancer (E1) in a continuous fragment containing −511 bp to +2738 was also amplified by PCR and subcloned in the pGL3 basic vector (Promega).

Construction of Flp Cell Line—Flp-In system (Invitrogen) was used for the generation of a stable human FOXP3 core promoter and FOXP3 core +E1 promoter Flp-In Jurkat. Flp-InJurkat cells (Invitrogen) were co-transfected with FOXP3 core or FOXP3 core +E1 in pcDNAs5/FLP recombination target vector and the FLP-recombinase vector (pOG44) (pOG44:FOXP3 core or FOXP3 core +E1/pcDNA5/FLP recombination target ratio = 9:1) resulting in a stable integration of the gene of interest at the FLP recombination target site in the genome. For the selective growth test, individual cells were grown in 24-well plates. The culture medium was supplemented with hygromycin at 250 or 100 μg/ml. An additional PRE deletion mutant was generated through deletion of the FOXP3 core element (−511 to +176, supplemental Fig. S1) from the FOXP3 core and FOXP3 core + E1 constructs and rederivation of Flp-In Jurkat cell lines (Table 1).

ChIP Assays—ChIP assays were performed using a ChIP isolation kit (Millipore). One to two million Jurkat cells, or 1 × 10^5 to 5 × 10^5 murine or human naïve T cells were treated with 1% formaldehyde to cross-link histones to DNA. Fixed cells were sonicated to yield chromatin fragments of 200–1000 bp. Antibodies used in the ChIP assays included H3K27me3, p300, and acetyl-Histone H4 (Millipore) plus PCAF and CBP (Abcam). DNA was recovered by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation with the addition of an inert carrier. Options for critically relevant control samples include total IgG or pre-enriched chromatin (input). We chose to control with pre-enriched chromatin because nonspecific IgG frequently does not control adequately for nonspecific cross-reactivity. Furthermore, the chromatin input generates a more accurate estimation of biases introduced through sonication of chromatin and subsequent PCR (24).

Transfection and Luciferase Assays—Two million FOXP3 core and FOXP3 core +E1 Flp Jurkat cells were transfected using the Amaxa cell line nucleofector kit V for Jurkat cells according to the optimized protocol provided with the kit. Two μg of plasmid DNA for EZH2, Suz12, and EED were used in the nucleofection procedure. Luciferase assays were done follow-
**Polycomb Regulates FOXP3**

### Table 1

**FOXP3 ChIP Primers**

| Primer Name | Primer Sequence |
|-------------|-----------------|
| Primer 1 forward | ATATTGTTCTGACAGGACTAG |
| Primer 1 reverse | GCAGCTCTAGTGCCAGAGTGCTTG |
| Primer 2 forward | CTCTGGAGACAGAGCACTAC |
| Primer 2 reverse | AGCTGGAGATCGCTGAGT |
| Primer 3 forward | AAAACAAAGTAAGAGAGCAAAG |
| Primer 3 reverse | AGACCTCGCTCTTCTAATAATCCAA |
| Primer 4 forward | AACACCCAGCCACTCCAGT |
| Primer 4 reverse | GAGACCAGCAGTGTAGACATA |
| Primer 5 forward | CTCTGACTCTCGGCTCTATCTTC |
| Primer 5 reverse | CCTAAGACACACAGAACTATG |

**Human FOXP3 Core ChIP Primer**

5'CAGATGACTCGTAAAGGGGCAAAG 3'CGATGAGTGTGTGCGCTGATAATC

ing the manufacturer’s recommendations (Promega). For siRNA experiments, 600 nm total siRNA was used (300 nm scrambled + 300 nm targeted, ON-TARGET plus siRNA; Thermo Scientific Dharmacon, Lafayette, CO).

**Adenoviral Transduction**—Naïve T cells isolated from the CAR transgenic Balb/c[Tg]CARdelta1-[Tg]DO11.10 mice were activated for 48 h with either EV or EZH2 at a multiplicity of infection of 250. The cells were activated under the typical stimulation conditions for 7 days and processed through ChIP to determine methylation of H3K27 marks at the FOXP3 core promoter.

**Plasmids and Recombinant Adenovirus**—Standard molecular biology techniques were used to clone full-length EZH2, SUZ12, and EED into pcDNA3.1/His (Invitrogen). All of the constructs were verified by sequencing at the Mayo Clinic Molecular Biology Core Facility. Epitope-tagged (6XHis-Xpress™) EZH2, as well as empty vector (Ad5CMV), was generated as recombinant adenovirus in collaboration with the Gene Transfer Vector Core at the University of Iowa.

**Mouse Strains**—C57BL/6 and B6.Cg-FOXP3<sup>g<sub>me2R/ch</sub></sup> mice ("FOX3-EGFP mice" co-express EGFP and FOXP3 under the control of the endogenous promoter) were purchased from the Jackson Laboratory. The CAR transgenic mouse was obtained through the NIAID Exchange Program of the National Institutes of Health: Balb/c[Tg]CARdelta1-[Tg]DO11.10 mouse line 4285 (25). KLF10<sup>-/-</sup> mice were kindly provided by Thomas C. Spelsberg (Mayo Clinic, Rochester, MN) (26). All of the mice used in experiments were males of 8~20 weeks in age. The mice were age-matched in experiments comparing wild type with KLF10<sup>-/-</sup>. All animal work was done in accordance with the Mayo Clinic Institutional Animal Care and Use Committee.

**DSS Colitis**—The mice were given water supplemented with 3% dextran sulfate sodium salt for 5 days. The water was then replaced with normal drinking water for 3 more days prior to the mice being sacrificed for tissue removal for histology. Flow cytometry was utilized to look at levels of FOXP3 expression within the CD4+ population. Intracellular staining procedures for FOXP3 were followed using the application notes from Alexa Fluor® 488 anti-mouse/rat/human FOXP3 (BioLegend). The mice were weighed daily, and their colon lengths were determined during autopsy. The degree of colitis was quantified using three outcome variables: weight loss, colon histology, and a disease activity index. The disease activity index is an established clinical index of colitis severity encompassing clinical signs of colitis (wasting and hunching of the recipient mouse and the physical characteristics of stool) and an ordinal scale of colonic involvement (thickness and erythema) (27). The histologic activity index (maximal score = 10) includes: ulcer (0–1), crypt abscess (0–3), neutrophilic infiltrate (0–3), and thickening of the lamina propria (0–3) (27).

**Statistical Methodology**—Statistical analyses were performed using JMP version 9.0 (SAS Institute, Cary, NC). Descriptive analyses including means and standard deviations were performed in normally distributed data. t-tests were used to compare means between two groups. Paired t tests were used to compare means between paired samples. A p value of <0.05 was considered as statistically significant.

**RESULTS**

Evidence for the Existence of a KLF-containing PRE-like Motif within Mouse and Human FOXP3 Loci—Brown et al. (16, 18) recently reported that an Sp1/KLF binding site is present in the majority of Drosophila PREs. The mammalian DNA-binding, zinc finger transcription factor Yin Yang 1 (YY1), is the vertebrate orthologue to Drosophila Pho, which is one of the few pieces of Polycomb recruitment machinery conserved from fly to human. An additional binding element for GAGA factor has been demonstrated to cooperate with Pho at the PRE (28). Analysis of the first 1000 bp upstream from the transcription start site within the mouse FOXP3 promoter reveals consensus sequences for YY1, GAGA factor, and Sp1/KLF binding motifs (Fig. 1). Functional PREs tend to be flanked by YY1 binding sites and in association with CpG islands that also potentially influence recruitment of PcG complexes. CpG island analysis reveals the presence of a putative CpG island within ~5 kb from the transcription start site of the mouse FOXP3 promoter (data not shown). Pairwise alignment demonstrates a high degree of conservation between the mouse and human core promoter. Taken together, the combined predictive value of these multiple bioinformatic comparisons (pairwise alignment, cis-regulatory element analysis, and CpG prediction) led us to subsequently test the hypothesis that the PcG complex binds and potentially regulates FOXP3 and Treg adaptive responses.
The PRE-like Module from the FOXP3 Promoter Functionally Recruits the Pcg HMT Pathway—Because Pcg works at the epigenetic level, we created a T cell line with the putative FOXP3-associated PRE module integrated into the genome to test whether this region specifically recruits Pcg proteins and silences reporter expression in this nonepisomal state. Using the FLP-recombinase system (FLP cell line; see “Experimental Procedures”) to allow genomic integration of a luciferase reporter construct, we generated a Jurkat FLP cell line expressing luciferase under the control of the CMV promoter either with (JFOXP3-FLP) or without (J-FLP) the putative FOXP3-associated PRE. The CMV promoter allows a basal level of expression on which to perform repression studies in a similar manner to the widely used Gal4-based reporter, an episomal repression system not optimal for epigenetic studies. Given the established role for a conserved nucleotide sequence downstream of the core promoter in the TGFβ-mediated induction of Treg cells (Enhancer 1) and the known role for KLF10 in this nonepisomal state, we aimed to validate these results—As the overexpression system of Fig. 2 suggested the presence of H3K27me3 marks correlating with the functional outcome of reduced luciferase expression. Fig. 2b demonstrates H3K27me3 marks occupying the FOXP3 core promoter region upon overexpression of the PRC2 complex (2.6-fold change over cells transfected with empty vector; Fig. 2b). Thus, using a human lymphocyte cell line, we demonstrate that the FOXP3 PRE-like module recruits the PRC2 complex and silences FOXP3 gene expression. Together, these experiments demonstrate that the FOXP3 promoter has the functional properties expected of a PRE. Although the mechanistic novelty of a PRE-like module on the inducible FOXP3 promoter is clear, we sought to extend these important observations to primary T cells.

To evaluate the role of the HMT EZH2 in the regulation of FOXP3 expression in primary T cells, we utilized an adenoviral expression system introduced into a mouse line transgenically expressing the adenosival receptor (CAR transgenic mouse; Taconic, model 4285). Naïve CD4+ splenocytes were isolated from the CAR transgenic mouse and infected with EZH2 or control empty virus. In these experiments, EZH2 overexpression leads to increased levels of H3K27 methylation at the FOXP3 core promoter (Fig. 2c). Importantly, primary naïve murine CD4+ lymphocytes transduced with EZH2 do not express FOXP3 upon stimulation when compared with cells transduced with empty vector (7.6% FOXP3+ cells versus 72.4% FOXP3+ cells; Fig. 2d), indicating that recruitment of EZH2 to the FOXP3 core promoter results in specific and persistent silencing of FOXP3 expression. Thus, these data demonstrate that the FOXP3 promoter displays both structural and functional properties similar to the well characterized Drosophila KLF-containing PRE sites including the ancestral relative forkhead.

**Promoter Occupation and Histone-K27 Methylation by PRC2**

*Is the Defining Feature of the Silenced State of the FOXP3 Promoter—*As the overexpression system of Fig. 2 suggested the PRC2 complex to be one mechanism of FOXP3 gene silencing, we aimed to validate these results in vivo using an established
FOX3 reporter mouse. We initially examined the chromatin landscape of the FOX3 gene and the differentiation of adaptive Treg cells for evidence of histone marks that reflect an operational PRC2 methyltransferase system. Primary T cell populations isolated from the spleen of the FOX3-EGFP expressing mouse were sorted for CD62L and FOX3 (on the basis of EGFP expression). Both naïve (CD62L high) and antigen experienced (CD62L low) populations of FOX3-negative T cells displayed an enrichment of H3K27me3 marks on the FOX3 promoter (1.8 ± 0.4 and 2.5 ± 0.7-fold difference in optical

![Image of bar graphs and flow cytometry analysis]

FIGURE 2. PRC2 complex silences the FOX3 core promoter. a, luciferase counts represented relative to empty vector control (pcDNA, open bar) upon transfection of EZH2 alone or the entire PRC2 complex EZH2, Suz12, and EED (E/S/E). Identical analysis in the J-FLP control cell line (right panel). b, J-FOX3-E1 cells transfected with PRC2 demonstrate enhanced H3K27me3 marks at the FOX3 core promoter. Five primer pairs were designed 200 bp apart covering the FOX3 core genomic insert (Pr1–5). The center of the core promoter demonstrated the highest H3K27me3 marks. Cells transfected with empty vector (pcDNA) serve as the relevant control. The results are presented controlled by their own input. c, semiquantitative PCR analysis of the expression of FOX3 from the product of ChIP for H3K27me3 in EZH2 transduced or pcDNA transduced primary mouse naïve T cells. d, flow cytometry analysis of primary T cells transduced with EZH2 (shaded histogram) or empty vector (open histogram) for FOX3 after 7 days of stimulation. The data represent at least three independent experiments.
density over FOXP3-expressing CD62L high and CD62L low cells, respectively; mean/S.D.; Fig. 3a, left panel). These data suggest that H3K27me3 marks the silenced state of the inducible gene FOXP3. To test this model in vitro, we isolated naïve FOXP3− T cells and induced FOXP3 gene transcription. Fourteen days post-induction FOXP3-expressing (EGFP-positive) or FOXP3-negative (EGFP-negative) cells were isolated by FACS. DNA from each cell population was subjected to ChIP assay using specific antibodies against the H3K27me3 mark and the HMT, EZH2. Congruent with the in vivo findings, FOXP3 induction in adaptive Treg cells was associated with minimal H3K27me3 marks, whereas the persistent absence of FOXP3 expression associated with both H3K27me3 marks and the presence of EZH2 (2.1 ± 1.0-fold change in H3K27me3 marks (Fig. 3b) and 1.8 ± 0.5-fold change in EZH2 occupancy (Fig. 3c) as compared with FOXP3-expressing activated cells; mean/ S.D.). These data using optical density were confirmed using quantitative real time PCR as a second methodology of quanti-

FIGURE 3. Histone methylation marks associated with PRC2 complex predict FOXP3 expression in primary CD4+ lymphocytes. a, inset gel, left panel, representative DNA gel for PCR analysis of the expression of FOXP3 in cell fractions post-immunoprecipitation for H3K27 methylation in primary CD4+ splenocytes isolated on the basis of CD62 ligand expression and FOXP3 transcriptional activity (GFP). The accompanying histogram represents the optical density of the bands normalized to CD62L+ GFP+. The histogram on the right demonstrates quantitative real time PCR analysis of the expression of FOXP3 in cell fractions post-immunoprecipitation for H3K27 methylation in primary CD4+ splenocytes isolated on the basis of CD62 ligand expression and FOXP3 transcriptional activity (GFP), relative to CD62L+ GFP+; the results are presented controlled to FOXP3 expression of preimmunoprecipitated sample. The data represent three independent experiments (means and S.D.). b and c, inset gels, left panels, demonstrate representative DNA gels for PCR using FOXP3 specific primers in samples post-precipitation for H3K27 methylation marks (b) or EZH2 (c) in primary CD4+ CD62L+ cells post-activation for 14 days and isolated on the basis of FOXP3 transcriptional activity (GFP). The left panels represent the relative optical density normalized to GFP+. The right panels demonstrate the quantitative real time PCR analysis of the expression of FOXP3 in cell fractions post-immunoprecipitation for H3K27me3 or EZH2; the results are presented controlled to FOXP3 expression of preimmunoprecipitated sample. The data represent three independent experiments (means and S.D.).
fication (Fig. 3, right panels). Thus, PRC2-mediated trimethylation of H3K27, which occurs at the newly identified PRE-like cis-regulatory module, appears to define the silenced state of the FOX3 promoter. These results indicate that PRC2-mediated silencing behaves as a default mechanism for keeping FOX3 silenced until the moment of its induction. To better understand how the FOX3 promoter changes from this silenced to its induced state, we designed subsequent experiments to address the identity of the chromatin pathways capable of reversing the effects of PRC2, initiate FOX3 activation, and facilitate the differentiation of progenitors into immunocompetent Treg cells.

**Antagonism of PRC2 Function by a KLF-PCAF Pathway Marks the Beginning of the Inducible Phase of the FOX3 Promoter.—** Both histone acetylation and H3 K4 methylation, with few exceptions, are associated with gene activation (30, 31). Indeed, bivalent modification with H3 K4 and H3 K7 methylation has been described on promoters of inducible, T cell-specific genes (31). It is not clear how the bivalent state primes genes for activation in a sequence-specific manner. Given the established KLF binding site within the FOX3 promoter and the association of HATs with KLF family members, we investigated whether a KLF-dependent recruitment of HAT(s) antagonize the PRC2-induced silenced state leading to FOX3 activation. Appreciation for the significance of histone acetylation has been advanced through the identification of the GCN5 family of HATs, many of which, particularly CBP, p300, and PCAF (p300/CBP-associated factor), have been described to interact with KLF family members (32, 33). Although histone acetylation events have been described on the activated FOX3 core promoter, mechanistic insight into the sequence-specific recruitment and identity of precise HATs is lacking (34). In Fig. 4a, we demonstrate marked H4 acetylation of the FOX3 core promoter upon activation of murine primary T cells (8-fold induction over unstimulated cells). This acetylation event is associated with the recruitment of PCAF to the FOX3 core promoter (7-fold induction over unstimulated cells; Fig. 4a). Experiments designed to study the additional HAT enzymes CBP and p300 did not support its functional association with this process (Fig. 4a and supplemental Fig. S3). Similar results were demonstrated using primary human naive CD4+ T cells (Fig. 4b and supplemental Fig. S3), indicating that the PCAF-induced activation of the FOX3 core promoter is conserved between mice and humans.

Potential antagonism between the PCAF and PRC2 pathways was further explored in two experimental sets. First, we over-expressed the HMT EZH2 in primary naïve murine T cells and activated the cells to induce FOX3 as in Fig. 2, above. After 5 days of stimulation, in the setting of EZH2 overexpression, the core promoter region of FOX3 fails to recruit PCAF and concomitantly, lacks histone acetylation events (Fig. 4c). Second, we performed overexpression assays of both EZH2 and PCAF in the genome-integrated FLP cell line to directly measure antagonism in this system in an experimental context in which either endogenous PCAF or EZH2 had been silenced by siRNA. These experiments show that EZH2 knockdown leads to enhanced promoter activation in the presence of PCAF (percentage of enhancement = 32.7 ± 7.02%, mean and S.D., respectively; p < 0.05; Fig. 4d) and conversely, knockdown of PCAF leads to enhanced repression (percentage of repression = 26 ± 5.57%, mean and S.D. respectively; p < 0.05; Fig. 4d). Using the genome integrated cell line in which the PRE has been deleted, we demonstrate lack of regulation of either PCAF or EZH2 in this model system (Fig. 4e), further substantiating an antagonistic role for these chromatin modifying proteins on the FOX3 core promoter.

Thus, PCAF, a prominent chromatin-modifying enzyme appears responsible for antagonizing the silencing effects of PRC2 on FOX3. Collectively, this experimental set demonstrates that the region of the FOX3 promoter containing the PRE-like cis-regulatory module utilizes the HMT, EZH2, or the HAT, PCAF, to turn off and on the promoter, imparting characteristic inducibility to this gene. These data then led to additional experiments to define the mechanisms underlying the sequence-specific recruitment of the chromatin-modifying protein complexes to the core FOX3 promoter.

**Role of Kruppel-like Factor 10 in the Regulation of the FOX3 KLF-PRE Domain.—** The presence and chromatin recruitment activity of the FOX3 KLF-PRE module suggested that, similar to Drosophila, human Kruppel-like factor proteins are involved in the functional regulation of these elements. Recent results suggested that KLF10 modification by the E3 ubiquitin ligase itch was important for adaptive Treg differentiation (19). This observation made KLF10 an attractive candidate as a key regulator of the FOX3 PRE function. Subsequently, we tested the role for KLF10 in the epigenetic regulation of FOX3. Using the predicted KLF binding site on the human and mouse core FOX3 promoter as a guide, we designed primer pairs for ChIP-based promoter occupancy assays. The results from these experiments demonstrated that KLF10 readily binds to the KLF-PRE on the core FOX3 promoter in both murine and human primary CD4+ T cells (Fig. 5a). This observation that FOX3 is a direct target for both PRC2 and KLF10 supports the concept that the KLF-PRE domain displays both structural and functional aspects of the PRE.

**FIGURE 4. Activation of the FOX3 core promoter is associated with histone 4 acetylation and the histone acetyltransferase PCAF.** a, quantitative real time PCR analysis of the expression of FOX3 in cell fractions post-immunoprecipitation for histone 3 and histone 4 acetylation states (left panel) and the histone acetyltransferases CBP and PCAF (right panel) in primary murine CD4+ CD62L+ cells post-activation for 18 h; the results are presented controlled to FOX3 expression of preimmunoprecipitated sample, relative to CD4 + 62L + GFP+, inset gel, left panel, representative DNA gel for PCR using FOX3 specific primers in samples post-precipitation for histone acetylation states or HATs. B, DNA gel for PCR using FOX3 specific primers in samples post-precipitation for histone acetylation states or HATs in human CD4+ RA+ peripheral T cells post-activation for 18 h. C, DNA gel for PCR using FOX3 specific primers in samples post-precipitation for histone 4 acetylation and PCAF using DNA isolated from CD4+ splenocytes transduced with EZH2 or empty vector (EV) and stimulated to produce FOX3 for 5 days. The data are representative of three independent experiments. D, EZH2 and PCAF compete for regulatory control of FOX3. Luciferase counts represented relative to siRNA scrambled control (scr-EZH2 + scr-PCAF, open bar) upon transfection of siRNA directed against EZH2 (si-EZH2 + scr-PCAF) or PCAF (scr-EZH2 + siPCAF). The data represent three independent experiments.
functional properties conserved from *Drosophila forkhead* to its human orthologue, FOXP3. Using the KLF10 deficient mouse (KLF10$^{-/-}$), we next determined the importance of KLF10 to the chromatin remodeling events required for FOXP3 promoter activation.

In the resting state, KLF10$^{-/-}$ CD4+ naïve lymphocytes display increased levels of H3K27 trimethylation (3.39 ± 1.5-fold change over naïve wild type lymphocytes, mean/S.D.; Fig. 5b). These data were confirmed using quantitative real time PCR as a second methodology of quantification (Fig. 5b, right panel). Activated KLF10$^{-/-}$ T cells retain H3K27 trimethylation marks (Fig. 5c) and fail to up-regulate FOXP3 (48.2% FOXP3+ versus 20.7% FOXP3+, wild type versus KLF10$^{-/-}$; Fig. 5d). No significant differences were seen in H3K4 methylation between
DISCUSSION

The major finding of this study is the novel definition of a PRE on the core promoter of the inducible gene, FOXP3. The significance of this finding is the functional relevance of KLF-dependent PCI2 recruitment to the FOXP3 core, the develop-
ment of T regulatory cells, and the impact on chronic intestinal inflammation.

A KLF-PRE Exists within Forkhead (Drosophila melanogaster) and Its Mammalian Homologue, FOXP3—The polycomb group proteins PRC1 and PRC2 are members of a gene silencing complex best defined in the long term silencing of the Hox gene cluster of *Drosophila* (17). Most PcG proteins do not possess a DNA-binding domain; thus, it is widely accepted that the DNA-binding protein YY1, which binds in complex to both PRC1 and PRC2 may provide sequence-specific DNA binding activity to PcG complexes (17, 35–37). The importance of the mechanisms of PcG gene silencing is clearly appreciated, yet the gene-specific recruitment events are not well understood. Early studies in *Drosophila* identified the importance of both Kruppel and Polycomb as critically relevant to regulation of the homeotic gene Scr (Sex combs reduced) (38). Subsequent work on a homeotic gene within the bithorax complex *Abdominal-B* provided evidence for a generalized model of gap gene products (Kruppel) promoting stable silencing through Polycomb in homeotic *Drosophila* genes (39). Building upon the recent
observation that the majority of PREs have KLF binding sites, the data provide evidence in support of a conserved mechanism.

Further support for an evolutionarily conserved mechanism for the regulation of FOXP3 by a KLF-PRE domain exists in a publically available ChIP-sequencing (ChIP-seq) database. Wei et al. (21) investigated alterations in gene expression with simultaneous ChIP-seq mapping of histone modifications (H3-K4me3 and H3-K27me3) during the differentiation of CD4+ T cells. FOXP3 is identified among a subset of genes that demonstrate concurrent increased expression with reductions in H3K27 trimethylation. Surprisingly, a number of other FOX proteins appear in the data set in addition to FOXP3, including FOXM1, FOXJ1, FOXP4, and FOXK1, further supporting the concepts of evolutionary-conserved regulation of these genes described in this study (supplemental Fig. S4) (21).

The PRE-like Module from the FOXP3 Promoter Functionally Recruits the PRC2 HMT Pathway—Our experiments demonstrate the presence of both the characteristic histone marks and PRC2 complex members on the silenced core promoter of FOXP3. Furthermore, we demonstrate functional relevance of PRC2 to FOXP3 gene silencing in an overexpression system. The function of PcG members in T cells is poorly understood. There is limited, conflicting data largely focused on cytokine promoters of differentiated T cell phenotypes. Of the limited published data, most focus on a role for PRC1 complex members (primarily BMI-1) on the GATA-3 driven, IL-4 producing, Th2 profile. Although evidence exists demonstrating a role for PRC1 members BMI-1 and Mel-18 in promoting stability of the Th2 phenotype (40–42), more recent data suggest a largely repressive effect maintained by the interaction of PRC1 and an upstream promoter region of the GATA-3 gene (43). These discordant data likely reflect the utmost importance of context-dependent recruitment and the potential for disparate complex function dependent upon precise PcG members.

Antagonism of PRC2 Function by a KLF-PCAF Pathway Marks the Beginning of the Inducible Phase of the FOXP3 Promoter—Histone acetylation events associated with FOXP3 gene activation and the induction of FOXP3 with histone deacetylation inhibitors is well established (29, 44–47); however, definition of particular histone acetyltransferase proteins or the mechanism of site-specific HAT recruitment is unknown. We report the requirement for KLF10 and the recruitment of the HAT, PCAF, to be critically important for FOXP3 expression. Of interest, work in Drosophila has associated histone acetylation to temporal-spatial expression of the Hox gene locus (48, 49). The evolutionary predecessor of PCAF in Drosophila is GCN5, and indeed GCN5 has been linked to Hox gene regulation in murine skeletal development (50). We therefore speculate that the novel mechanistic observation of recruitment of PCAF to the FOXP3 core promoter through KLF-PRE may represent an evolutionarily conserved mechanism of FOXP3 gene regulation.

Disruption of the KLF-PRE Mechanism Results in Failure of Adaptive Treg Cells and Inflammatory Bowel Disease in Vivo—A functional impairment in generation of TGFB-induced adaptive Treg cells has previously been described in the KLF10-deficient animal (19). The data put forth defined a role for the post-translational modification (ubiquitination) of KLF10 by the E3 ubiquitin ligase, ITCH, resulting in a gain of function in the transcriptional activity of the FOXP3 gene (19). The results of this paper extend these observations to the function of KLF10 on FOXP3 transcription at the level of the chromatin. In the absence of KLF10, PCAF is not recruited to the silenced promoter, resulting in a lack of H4 acetylation and a persistent block in FOXP3 gene transcription. The association of KLF proteins with E3 ubiquitin ligases is worthy of further investigation because PcG complexes contain E3 ubiquitin ligase activity (51), and a conserved regulatory domain (R3 domain) of KLF10 consists of WW domains that bind prototypical E3 ubiquitin ligases in hybridization assays.

Conclusion—A KLF-PRE exists within forkhead (D. melano-gaster) and its mammalian homologue, FOXP3. The PRE-like module from the FOXP3 promoter functionally recruits the PRC2 HMT pathway. Antagonism of PRC2 function by the GCN5 orthologue PCAF marks the beginning of the inducible phase of the FOXP3 promoter. Integrating these experimental discoveries with existing literature from Drosophila suggests an evolutionarily conserved mechanism of FOXP3 (Drosophila, Forkhead, E(z)) recruitment antagonized by PCAF (Drosophila, GCN5) in a KLF-dependent (Drosophila, Kruppel) fashion. Disruption of the KLF-PRE mechanism results in failure of adaptive Treg cells and inflammatory bowel disease in vivo. Because the KLF-PRE likely binds additional KLF family members, further characterization of the molecular interactions between PcG, KLF family members, and PCAF may lead to novel therapeutic strategies in human immune-mediated disease.

REFERENCES
1. Curotto de Lafaille, M. A., and Lafaille, J. J. (2009) Natural and adaptive foxp3 + regulatory T cells. More of the same or a division of labor? Immunity 30, 626–635
2. Chen, W., Jin, W., Hardegen, N., Lei, K. J., Ll. L, Marinos, N., McGrady, G., and Wahl, S. M. (2003) Conversion of peripheral CD4+ CD25− naive T cells to CD4+CD25+ regulatory T cells by TGFB induction of transcription factor Foxp3. J. Exp. Med. 198, 1875–1886
3. Li, M. O., Sanjabi, S., and Flavell, R. A. (2006) Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulation T cell-dependent and -independent mechanisms. Immunity 25, 455–471
4. Li, M. O., Wan, Y. Y., and Flavell, R. A. (2007) T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. Immunity 26, 579–591
5. Marie, J. C., Letterio, J. J., Gavin, M., and Rudensky, A. Y. (2005) TGFB-1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. J. Exp. Med. 201, 1061–1067
6. Faria, A. M., and Weiner, H. L. (2005) Oral tolerance. Immunol. Rev. 206, 232–259
7. van der Vliet, H. J., and Nieuwenhuis, E. E. (2007) IPEX as a result of mutations in FOXP3. Clin. Dev. Immunol. 2007, 89017
8. Kleinseck, M. A., Boniface, K., Sadekova, S., Grein, J., Murphy, E. E., Turner, S. P., Raskin, L., Desai, B., Faubion, W. A., de Waal Malefyt, R., Pierce, R. H., McClanahan, T., and Kastelein, R. A. (2009) Circulating and gut-resident human Th17 cells express CD161 and promote intestinal inflammation. J. Exp. Med. 206, 525–534

4 Y. Xiong, S. Knaz, A. L. Grzenda, O. F. Sarmen, P. A. Svingen, G. A. Lomberk, R. A. Urrutia, and W. A. Faubion, Jr., unpublished observations.
Polycistrom Regulates FOXP3

B. S. Powner, F., and Maloy, K. J. (2006) Interleukin-23 drives innate and T cell-mediated intestinal inflammation. J. Exp. Med. 203, 2473–2483

Ghaleb, A. M., Nandan, M. O., Chanchevalap, S., Dalton, W. B., Hisamuddin, I. M., and Yang, W. W. (2005) Krüppel-like factors 4 and 5. The Yin and Yang regulators of cellular proliferation. Cell Res 15, 92–96

Safe, S., and Abdelrahim, M. (2005) Sp transcription factor family and its role in cancer. Eur. J. Cancer 41, 2438–2448

Wu, J., and Lingrel, J. B. (2005) Krüppel-like factor 2, a novel immediate-early transcriptional factor, regulates IL-2 expression in T lymphocyte activation. J. Immunol. 175, 3060–3066

Carlson, C. M., Endrizzi, B. T., Wu, J., Ding, X., Weinreich, M. A., Walsh, E. R., Wani, M. A., Lingrel, J. B., Hoggquist, K. A., and Jameson, S. C. (2006) Kruppel-like factor 2 regulates thymocyte and T-cell migration. Nature 442, 299–302

Zhou, M., McPherson, L., Feng, D., Song, A., Dong, C., Lyu, S. C., Zhou, L., Shi, X., Ahn, Y. T., Wang, D., Clayberger, C., and Kremsky, A. M. (2007) Krüppel-like transcription factor 13 regulates T lymphocyte survival in vivo. J. Immunol. 178, 5496–5504

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R. S., and Zhang, Y. (2002) Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science 298, 1039–1043

Brown, J. L., Grau, D. J., DeVido, S. K., and Kassis, J. A. (2005) An Sp1/KLF binding site is important for the activity of a Polycomb group response element from the Drosophila engrailed gene. Nucleic Acids Res. 33, 5181–5189

Woo, C. J., Kharchenko, P. V., Daheron, L., Park, P. J., and Kingston, R. E. (2011) Functional domains and DNA-binding sequences of histone modifications in human T cells. Proc. Natl. Acad. Sci. U.S.A. 108, 15782–15787

Song, C. Z., Keller, K., Chen, Y., and Stamatoyannopoulos, G. (2003) Functional interplay between CBP and PCAF in acetylation and regulation of transcription factor KLF13 activity. J. Biol. Chem. 282, 207–215

Song, A., Patel, A., Thamatrakoln, K., Liu, C., Feng, D., Clayberger, C., and Krensky, A. M. (2002) Functional domains and DNA-binding sequences of RFLAT-1/KLF13, a Krüppel-like transcription factor of activated T lymphocytes. J. Biol. Chem. 277, 30655–30665

Liu, Z.; Zhang, C., and Sun, J. (2010) Deacetylase inhibitor trichostatin A down-regulates Foxp3 expression and reduces CD4+CD25+ regulatory T cells. Biochem. Biophys. Res. Commun. 400, 409–412

Brown, J. L., Mucci, D., Whiteley, M., Dirk森, M. L., and Kassis, I. A. (1998) The Droso phila Polycomb group gene pleiohomeotic encodes a DNA binding protein with homology to the transcription factor YY1. Mol. Cell 1, 1057–1064

KoopY, C. Y., Hsu, H. C., Shen, M. R., Chang, W. C., and Wang, J. M. (2008) Epigenetic silencing of CCAAT/enhancer-binding protein delta activity by YY1/polycomb group/DNA methyltransferase complex. J. Biol. Chem. 283, 30919–30932

Wilkinson, F. H., Park, K., and Atchison, M. L. (2006) Polycomb recruitment to DNA in vivo by the YY1 REPO domain. Proc. Natl. Acad. Sci. U.S.A. 103, 19296–19301

Riley, P. D., Carroll, S. B., and Scott, M. P. (1997) The expression and regulation of sex combs reduced protein in Drosophila embryos. Genes Dev. 11, 716–730

Casares, F., and Sánchez-Herrero, E. (1995) Regulation of the infraabdominal domains of the bithorax complex of Drosophila by gap genes. Genes Dev. 121, 1855–1866

Hosokawa, H., Kimura, M. Y., Shinnakasu, R., Suzuki, A., Miki, T., Koseki, H., van Lohuizen, M., Yamashita, M., and Nakayama, T. (2006) Regulation of Th2 cell development by Polycomb group gene bmi-1 through the stabilization of GATA3. J. Immunol. 177, 7656–7664

Kimura, M., Koseki, Y., Yamashita, M., Watanabe, N., Shimizu, C., Katsu moto, T., Kitamura, T., Taniguchi, M., Koseki, H., and Nakayama, T. (2001) Regulation of Th2 cell differentiation by mll-18, a mammalian polycomb group gene. Immunity 15, 275–287

Miyazaki, M., Kawamoto, H., Kato, Y., Itoi, M., Miyazaki, K., Masuda, K., Tashiro, S., Ishihara, H., Igarashi, K., Amagai, T., Kanno, R., and Kanno, M. (2005) Polycomb group gene mll-18 regulates early T progenitor expansion by maintaining the expression of Hes-1, a target of the Notch pathway. J. Immunol. 174, 2507–2516

Onodera, A., Yamashita, M., Endo, Y., Kuwahara, M., Tofukuji, S., Hosokawa, H., Kanai, A., Suzuki, Y., and Nakayama, T. (2010) STA6-mediated displacement of polycomb by trithorax complex establishes long-term maintenance of GATA3 expression in T helper type 2 cells. J. Exp. Med. 207, 2493–2506

Lal, G., Zhang, N., van der Touw, W., Ding, Y., Ju, W., Bottinger, E. P., Reid, S. P., Levy, D. E., and Bromberg, J. S. (2009) Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation. J. Immunol. 182, 259–273

Li, B.; Samanta, A., Song, X., Iacono, K. T., Bembas, K., Tao, R., Basu, S., Porrett, P. M., Li, B., Turka, L. A., Olson, E. N., Greene, M. I., Wells, A. D., and Hancock, W. W. (2007) Deacetylase inhibition promotes the generation and function of regulatory T cells. Nat. Med. 13, 1299–1307
47. Wang, L., Tao, R., and Hancock, W. W. (2009) Using histone deacetylase inhibitors to enhance Foxp3+ regulatory T-cell function and induce allograft tolerance. *Immunol. Cell Biol.* 87, 195–202
48. Fujimura, Y., Isono, K., Vidal, M., Endoh, M., Kajita, H., Mizutani-Koseki, Y., Takihara, Y., van Lohuizen, M., Otte, A., Jennewein, T., Deschamps, J., and Koseki, H. (2006) Distinct roles of Polycomb group gene products in transcriptionally repressed and active domains of Hoxb8. *Development* 133, 2371–2381
49. Rastegar, M., Kobrossy, L., Kovacs, E. N., Rambaldi, I., and Featherstone, M. (2004) Sequential histone modifications at Hoxd4 regulatory regions distinguish anterior from posterior embryonic compartments. *Mol. Cell. Biol.* 24, 8090–8103
50. Lin, W., Zhang, Z., Chen, C. H., Behringer, R. R., and Dent, S. Y. (2008) Proper Gcn5 histone acetyltransferase expression is required for normal anteroposterior patterning of the mouse skeleton. *Dev. Growth Differ.* 50, 321–330
51. Wang, H., Wang, L., Erdjument-Bromage, H., Vidal, M., Tempst, P., Jones, R. S., and Zhang, Y. (2004) Role of histone H2A ubiquitination in Polycomb silencing. *Nature* 431, 873–878
52. Yant, S. R., Zhu, W., Millinoff, D., Slightom, J. L., Goodman, M., and Gumucio, D. L. (1995) High affinity YY1 binding motifs. Identification of two core types (ACAT and CCAT) and distribution of potential binding sites within the human beta globin cluster. *Nucleic Acids Res.* 23, 4353–4362
53. Sing, A., Pannell, D., Karaiskakis, A., Sturgeon, K., Djabali, M., Ellis, J., Lipshitz, H. D., and Cordes, S. P. (2009) A vertebrate Polycomb response element governs segmentation of the posterior hindbrain. *Cell* 138, 885–897
54. McConnell, B. B., and Yang, V. W. (2010) Mammalian Krüppel-like factors in health and diseases. *Physiol. Rev.* 90, 1337–1381