Differential Coupling of KLF10 to Sin3-HDAC and PCAF Regulates the Inducibility of the FOXP3 Gene


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Running title: Dichotomous HAT/HDAC regulation of FOXP3

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Character count (with spaces): 50,476 (59,200)
Word count: 8,815
Figures: 12

Key words: T regulatory cell, KLF10, PCAF, Sin3, FOXP3

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Grant support: This research is supported by grants from the National Institute of Allergy and Infectious Disease to WAF (NIH NIAID AI89714-R01) and NIDDK52913 and P30DK084567 to RAU. Molecular Modeling experiments were supported by the Epigenomics Translational Program of the Mayo Clinic Center for Individualized Medicine.
Abstract

Inducible gene expression, which requires chromatin remodeling on gene promoters, underlies the epigenetically inherited differentiation program of most immune cells. However, chromatin-mediated mechanisms that underlie these events in T regulatory cells remain to be fully characterized. We here report that inducibility of FOXP3, a key transcription factor for the development of T regulatory cells depends upon Kruppel-like factor 10 (KLF10) interacting with two antagonistic histone-modifying systems. We utilized chromatin immunoprecipitation, genome integrated reporter assays, and functional domain KLF10 mutant proteins, to characterize reciprocal interactions between this transcription factor and either the Sin3-Histone Deacetylase complex or the Histone Acetyltransferase, PCAF. We characterize a Sin3 interacting repressor domain on the N-terminus of KLF10, which works to limit the activating function of this transcription factor. Indeed, inactivation of this Sin3 interacting domain renders KLF10 able to physically associate with PCAF as to induce FOXP3 gene transcription. We show that this biochemical data derived from studying our genome-integrated reporter cell system is recapitulated in primary murine lymphocytes. Collectively, these results advance our understanding of how a single transcription factor, namely KLF10 functions as a toggle to integrate antagonistic signals regulating FOXP3 and thus immune activation.
40Introduction

42 FOXP3+ T regulatory (Treg) cells may develop outside the thymus, generally in response to Transforming Growth Factor (TGF)β and antigen to become critically important in intestinal immunologic homeostasis (adaptive Treg cells) (2, 6, 7, 22, 23, 26). Dysregulation of the transcription factor FOXP3, a key initiator of the Treg differentiation and functional program leads to immune dysregulation in both mice (scurfy mouse) and humans (IPEX-Immune polyendocrinopathy enteritis and X-linked- syndrome) (16, 38). While advances in T lymphocyte biology indicate the importance of TGFβ-induced activated T cells in both the induction (Th17 cells) and regulation (FOXP3+ Treg cells) of intestinal inflammation (3), the precise mechanistic events integrating the TGFβ signal to intracellular pathways that function as inducers or regulators of inflammation are not fully defined.

42 To identify these pathways, we recently characterized a role for a TGFβ-inducible Kruppel-like factor (KLF10) in silencing FOXP3 leading to enhanced colitis susceptibility while providing mechanistic insights into how these functions require novel chromatin coupling events (40). These studies defined the importance of p300/CBP-associated factor (PCAF), a histone acetyltransferase (HAT) recruited by KLF10 to the FOXP3 transcriptional regulatory regions that are critical for the induction of this gene (40). In the experiments reported here, we build upon our previous discovery and demonstrate that KLF10 possesses the dual capacity to either positively or negatively regulate FOXP3 through its differential association with PCAF or the histone deacetylase binding protein Sin3, respectively. Collectively, these results increase our understanding of the chromatin pathways critical for mediating the inducibility of immune genes, such as FOXP3, in which alterations may cause human disease. Since drugs against these pathways are being used in clinical trials for several
diseases, this new knowledge has both significant mechanistic value and potential biomedical relevance.

Materials and Methods

Cloning of the FOXP3 promoter construct and the development of the genome integrated FLP cell line—The cloning strategy has been previously described (40). Briefly, the human FOXP3 (NCBI AF235097) promoter locus including the core promoter (-511 to +176) and the core promoter through the TGFβ-responsive enhancer region (Enhancer 1, -511 to 2738) was amplified by PCR using FOXP3 promoter sequence-specific primers. The genomic DNA extracted from CD4+ T cells of a healthy donor was used as template. The PCR product was subcloned initially into the pGL3 basic vector in frame with the luciferase reporter sequence (Promega, Madison, WI). We subsequently utilized the Flp-In system (Life Technologies, Grand Island, NY) for genome integration of the smaller FOXP3-core and larger FOXP3-core+enhancer 1 reporter constructs. First, we subcloned both FOXP3 promoter constructs with the luciferase reporter into the pcDNA5/FRT vector. Flp-In-Jurkat cells (Life Technologies, Grand Island, NY) were co-transfected with either FOXP3-core or FOXP3 core+E1 in pcDNA5/FRT vector and the FLP-recombinase vector (pOG44) resulting in a stable integration of the gene of interest at the ‘FLP Recombination Target’ (FRT)-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 μg/ml or 100 μg/ml. We subsequently refer to these cell lines as FLP-core or FLP-coreE1.

Mutation- The KLF10 Sin3 interacting domain (SID) mutant was generated by using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). The binding
domain fragment was mutated from GAAG to CCAC. The mutant construct was verified by sequencing.

Human FOXP3 Core ChIP Primer:

5’CAGATGACTCGTAAAGGGCAAAG
3’CGATGAGTGTGTGCAGCCTGATAATC

Chromatin immunoprecipitation (ChIP) assays - ChIP assays were performed using a ChIP isolation kit (EMD Millipore, Billerica, MA). One to two million Jurkat cells or primary lymphocytes were treated with 1% formaldehyde to cross link histones to DNA. Fixed cells were sonicated to yield chromatin fragments of 200 to 1000 bp. Antibodies used in the ChIP assays included Sin3a (#7691, Cell Signaling, Danvers, MA), His Tag (#SC-7270, Santa Cruz Biotechnology Inc, Dallas, TX), HDAC1 (#39534) and HDAC2 (#40967, Active Motif, Carlsbad, CA), acetyl-Histone H4 (#06866, Millipore, Temecula, CA), and PCAF (#Ab12188, Abcam, Cambridge, MA). DNA was recovered by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation with the addition of an inert carrier. All ChIP results are presented controlled to FOXP3 expression of pre-immunoprecipitated sample (input).

Quantitative real-time PCR - Three µL of above recovered DNA were used for each real-time PCR. PCR reactions were in 20µL total volume that contained primers and 10µL Express SYBR greenER qPCR Supermaster mixes (Life Technologies, Grand Island, NY). For control, we utilized pre-enriched chromatin (input).
For semi-quantitative PCR, we amplified cDNA under the following conditions: initial denaturation, 94°C for 2 minutes, followed by 34 cycles with denaturation at 94°C for 30 seconds, annealing at 53°C for 30 seconds, and extension at 72°C for 60 seconds. All the PCR products were visualized by running 1.5% agarose gels electrophoresis and ethidium bromide staining for the pictures.

Transfection and luciferase assays- Two million Jurkat cells were transfected using the Amaxa Cell Line Nucleofector Kit V for Jurkat cells according to the optimized protocol provided with the kit (Lonza, Portsmouth, NH). Two μg of plasmid DNA for pcDNA 3.1, KLF10, KLF10- Sin3 mutation and KLF family members were used in the nucleofection procedure. Luciferase assays were performed following the manufacturer’s recommendations (Promega, Madison, WI). For siRNA experiments, 300nM siRNA was used (ON-TARGET plus siRNA, Thermo Scientific Dharmacon, Lafayette, CO). Cell stimulation conditions include plate bound CD3 (2µg/ml), soluble anti-CD28 (2µg/ml, BD Biosciences, San Jose, CA), human IL2 (100u/ml), and human TGFβ1 (5ng/ml) in complete RPMI media (both cytokines Peprotech, Rocky Hill, NJ).

Primary lymphocyte transduction and stimulation- Naïve lymphocytes (CD4+CD62L+) were isolated using MACS bead magnetic sorting (#130-093-227 Miltenyi Biotech Inc., Auburn, CA) from splenocytes of 4-6 week old mice. Ratio of viral particles to cell was MOI of 150. Cells were exposed for 48 hours to adenoviral particles and subsequently washed prior to stimulation. For induction of FOXP3, cell stimulating conditions included plate bound CD3 (2µg/ml), soluble anti-CD28 (2µg/ml), human IL2 (100u/ml), and human TGFβ1 (5ng/ml) in complete RPMI media.
Immunoprecipitation and western blotting- Two μg of plasmid DNA for KLF10 in pcDNA3.1/His and KLF10 Sin3 mutation in pcDNA3.1/His were transfected into Jurkat cells by nucleofection; and 72 hours later cells were harvested. Protein was extracted from whole cell lysates derived from the harvested cells. The cells were lysed in lysis buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% Glycerol, 1% NP-40 and 2mM EDTA). Protein was subjected to immunoprecipitation with His tag overnight and precipitated by protein A agarose beads. After three washes, we added 50µL lysis buffer and boiled the eluant. The supernatants were run on 4-20% BIO RAD gels. Upon transferral to nitrocellulose, the membrane was incubated with primary antibodies in 5% milk for 1 hour. His tag and Sin3a proteins were detected with 1/25,000 dilution of anti-rabbit-HRP conjugate and Peroxide Solution and Luminol Enhancer Solution detection kit (GE Health Care UK Limited).

For PCAF binding assays: 25 million pelleted Jurkat cells were lysed in 0.5ml MPER lysis buffer (Thermo Scientific, Rockford, IL) supplemented with 0.5M NaCl and Complete Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN), vortexed thoroughly and incubated on ice for 5 minutes. 0.5 ml MPER buffer supplemented with Complete Inhibitor Cocktail without NaCl was then added for immunoprecipitation. Sixty µl of GST-tagged protein was added. Sixty µl of settled and washed glutathione beads (Sigma-Aldrich Corp., St. Louis, MO) were incubated with lysate containing GST-tagged proteins. Reactions were rotated at 4°C for 3hrs. After extensive washing, bound proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and blotted with antibodies to PCAF (ab12188 Abcam, Cambridge, MA). Three different antibodies were utilized to confirm non-cross-reactivity to other HATs (Anti-PCAF #A-4012 Epigentek, Farmingdale, NY and #AP12075b PCAF Antibody-c term, Abgent, San Diego, CA).
Molecular Modeling of the KLF10 SID-Sin3 PHA2 complex with In Silico Mutational Analyses - The three dimensional structure of the KLF10 SID complexed with the Sin3-PAH2 domain was determined using a similar approach previously described for both KLF11 (41) and KLF16 (8). Briefly, we utilized the Sin3a-HBP1 complex (Protein Data Bank code 1s5r) as template for KLF10-Sin3 SID homology-based modeling. After directly obtaining the three-dimensional structure of the Sin3a-PAH2 domain from the Sin3a-HBP1 complex, we modeled the structure of KLF10 SID bound with Sin3a-PAH2 domain (Protein Data Bank code 1G1E) by docking the KLF10 SID to the Sin3a-PAH2 domain to achieve maximal intermolecular interactions between the two partners using AutoDock 3.0.5 (27). In silico mutations were created using the builder module of Discovery Studio 4 (Accelrys, San Diego, CA) and energy minimized as previously described (14). Refinement of the complex involved a 2.0-ns (1-fs time step) molecular dynamics simulation using the CHARMm force field with an assignment of charges using the Momany-Rome method, as described previously (28). Using PROCHECK, the model was structurally verified and evaluated (21). For binding interphase analyses, complexes were energy minimized, and amino acid interactions were checked using the bond monitoring function of Discovery Studio 4 (Accelrys, Sand Diego, CA) using a distance cutoff of 3.4 Å for conventional Hydrogen bonds, 3.8 Å for weak hydrogen bonds, and 4 Å for Salt bridges. The Ramachandran plot for the model showed 96.5% residues in most favored regions; thus indicating that the model displays appropriate stereochemistry.

Chimera Generation and Colitis induction-

To make chimeras, 3-4-week-old RAG-2−/− mice were total body-irradiated (10 Gy) before receiving 5x10⁶ erythrocyte-depleted bone marrow cells that were extracted from KLF10−/− or wt
mice as previously described (9). Mice recovered for two weeks, and flow analysis of peripheral blood demonstrated mature B and T cell populations (data not shown).

Mice were subsequently given water supplemented with 3% dextran sulfate sodium salt for 5 days. The water was then replaced with normal drinking water for 2 more days prior to the mice being sacrificed for tissue removal for histology. The mice were weighed daily. 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) (13).

Statistical Methodology- Statistical analyses were performed using JMP version 9.0 (SAS institute, NC). Descriptive analyses including means and standard errors were performed in normally distributed data using T-tests. For experiments with greater than two experimental groups, the Kruskal Wallis non-parametric, single-sided ANOVA was used, and if variance was found to be significant, the Dunn’s multiple comparison’s test was utilized to demonstrate significant difference between selected data sets. A p-value of <0.05 was considered as statistically significant.

Animal Use- All animal studies have been reviewed and approved by the Mayo IACUC committee.

Results

The TGFβ-inducible Kruppel-like factor (KLF10) represses the core FOXP3 promoter through physical association with the Sin3 corepressor protein
Previous work has underscored a role for KLF10 in the chromatin-mediated silencing of FOXP3, the master transcription factor for Treg cells (40). However, the biochemical mechanisms that explain the functional interaction between these two transcription factors remain poorly understood. Consequently, we began this study utilizing Jurkat cell lines with genomic integration of relevant components of the FOXP3 core promoter in frame with a luciferase reporter construct (FLP Jurkat cells lines, see methods). These cells lines are used to investigate KLF10-mediated transcriptional regulation of both the core promoter (FLP-core) and a larger regulatory region including the downstream TGFβ responsive enhancer (FLP-core E1) (34). Both sequences have been inserted into a single site of the Jurkat T cell line genome using FLP recombinase, a model which circumvents the problems associated with the use of a non-episomal reporter system such as poor resolution of chromatin-mediated events (40). Basal luciferase production in these cell lines under the promotional control of CMV allows studies of transcriptional regulation in a manner similar to the widely used Gal4-based reporter, an episomal reporter system not optimal for epigenetic studies. We have previously demonstrated that the CMV promoter alone does not respond to KLF10-dependent regulatory mechanisms rendering the system appropriately controlled so as to draw useful inferences on how chromatin remodeling induced by this transcription factor affects the activity of the FOXP3 promoter (40).

Previous studies have demonstrated that KLF10 is required to recruit P300/CBP-associated factor (PCAF, KAT2B) to the FOXP3 core promoter for gene induction (40). Congruent with this data, it has been found that in vivo, KLF10 is required for activation of the FOXP3 promoter during generation of adaptive Treg cells using episomal reporter systems (40). However, using improved, genomically integrated reporters, here we found that KLF10 instead significantly repressed this promoter (Figure 1). Using closely related KLF family members (KLF9, 11, 12, 13, and 16) we
demonstrated \textit{FOXP3} repression to be specific to KLF10 in both stimulated and unstimulated FLP-core cell lines (red columns, Figure 1A, B, respectively).

To confirm the direct regulatory role of KLF10 upon the core \textit{FOXP3} promoter we performed chromatin immunoprecipitation assays (ChIP) in Jurkat FLP-core cells. Overexpression of KLF10 resulted in significant binding of KLF10 to the \textit{FOXP3} core promoter (Figure 1C). These experiments reveal that KLF10 functions as a direct transcriptional regulator of \textit{FOXP3}, leading us to subsequently investigate the chromatin-mediated mechanisms underlying this effect.

\textit{Molecular modeling predicts association of KLF10 with the Sin3 co-repressor complex.}

Previous studies have demonstrated that Sin3 interacts with several transcription factors through complexing of its second paired amphipathic helix domain (PAH) with an alpha-helical repression motif present in the target protein which accordingly has been termed the SID (Sin3 Interacting Domain) (28). Extensive biophysical and structural studies have demonstrated that critically located hydrophobic residues which are imbedded in an amphipathic alpha-helix are critical for the interaction of transcription factors with Sin3. Interestingly, bioinformatics analyses using the linear motif searching software ELM (10) demonstrate that amino acids 36 to 51 within KLF10 contain a sequence that conforms to the SID consensus \[FHYM].A[AV].[VAC]L[MV].[MI] previously identified in Sin3-interacting repressor proteins like HBP1 (Figure 2A). Since the NMR structure of the HBP1-SID-Sin3 has been previously solved (32), this finding led us to use this complex to develop the first homology based structural models for the putative KLF10 SID bound to the Sin3 PAH2 domain (4, 32) (Figure 2) so as to further orient our subsequent functional studies using site directed mutagenesis. Note that this protein complex adopts a five-helix-bundle fold where the deep cleft of PAH2 is enriched in hydrophobic amino acids forming the docking pocket for the apolar
upper surface of the KLF10 α-helix (Figure 2B). The side chains of V302, A307, I308, V311, L329, 244L332, Y335, V358, F376 and F379 from all four helices are sequestered from the hydrophilic 246surface to form the hydrophobic pocket of PAH2 thus accommodating the KLF10 SID (Figure 2C).
247Careful inspection of the protein-protein interaction interphase revealed that the KLF10 SID E5 248forms a critical bond with Sin3 H333, KLF10 A6 with Sin3 V311, KLF10 A9 with Sin3 L332, 249KLF10 L10 with Sin3 A307, and KLF10 M13 with Sin3 F379 (Figure 2D). Note KLF10 AA 250sequence numbering in Figure 2 refers to the SID, not the full length KLF10 protein. This 251information prompted us to evaluate the contributions of selected KLF10 SID residues toward the 252Sin3-dependent function by site-directed mutagenesis and immunoprecipitation. We sought to 253disrupt the critical binding between KLF10 E5 with Sin3 H333 and KLF10 A6 with Sin3 V311 by 254proline mutagenesis. The comparative binding interphase analyses depicted in Figure 2D and 2E 255predicts that these mutations would indeed impair Sin3 binding.

Indeed, the binding experiments shown in Figure 3 experimentally confirm this prediction by 257showing that while the wt KLF10 interacts with Sin3 this binding is abrogated by the EA to PP 258mutations (Figure 3A). Importantly, using ChIP assay we determined that mutation of the SID does 259not affect the binding of KLF10 to DNA (Figure 3B). These data indicate that the functional impact 260of this mutation is to selectively abolish chromatin coupling without affecting genomic binding by 261this transcription factor. This observation allows us to utilize the Jurkat FLP cell lines to define the 262role for the KLF10-SID in FOXP3 core promoter transcriptional activity. The KLF10-SID mutant 263(KLF10-SIDmt) was no longer capable of repressing FOXP3 core promoter function (Figure 4A). 264Overexpression of the KLF10-SIDmt in the core-E1 FLP cell line produced equivalent results 265(Figure 4B). Thus, these results identified an important mechanism for repressing FOXP3 through 266the coupling of KLF10 to the co-repressor Sin3, via a defined HBP1-like SID.
Members of the histone deacetylase subfamily I are critical for Sin3-mediated repression of FOXP3 by KLF10

Given the established role for histone acetylation/deacetylation states and the transcriptional activity of FOXP3 (15, 33, 37), we next explored the role for a Sin3-deacetylase complex in this important regulatory process. We performed a series of ChIP assays to characterize the capacity for KLF10 to recruit Sin3 to the FOXP3 core promoter and the resultant effect on the resident histone acetylation state. Upon overexpression of the KLF10 wt construct, we observed a 2.7 fold increase of Sin3 at the FOXP3 core promoter (Figure 5A) with a 0.44 fold change in histone 4 baseline acetylation state (Figure 5B). Moreover, we found that the KLF10-SIDmt, a construct which does not bind Sin3 or repress transcriptional activity (Figure 3A, 4A), failed to induce Sin3 recruitment to the core promoter (Figure 5A) or lead to histone 4 deacetylation (Figure 5B). Concordant with the Sin3-dependent loss of histone 4 acetylation at the FOXP3 core promoter, ChIP assay specific to histone deacetylase 1 (HDAC1) demonstrated a 1.25 fold increase of this enzyme at the core promoter upon expression of the wt KLF10 but not the KLF10-SIDmt (Figure 5C). Thus, this line of experimentation demonstrated that a chromatin-mediated mechanism of FOXP3 silencing through KLF10 requires Sin3 and the co-repressor complex member, HDAC1.

Evidence for the existence of a histone acetyltransferase pathway mediated by PCAF with the ability to antagonize KLF10-Sin3-HDAC-dependent FOXP3 repression

KLF family members can frequently regulate both gene activation and repression (5, 14, 24). Indeed, we have previously identified a key role for PCAF-mediated KLF10 functions in the activation of both murine and human FOXP3 promoters (40). For this purpose, we again utilized the FOXP3
core-E1 promoter genome integrated cell line to directly test the ability of PCAF, KLF10, and Sin3 to competitively regulate gene transcription. Consistent with previous data, overexpression of the wt KLF10 construct significantly represses *FOXP3* core promoter function (black column, Figure 6), and *FOXP3* repression depends upon the SID (KLF10-SIDmt, green column, Figure 6). To characterize the role for PCAF in the *de*-repression evident upon mutation of the SID, we performed identical experiments upon knockdown of PCAF through siRNA methodology. Notably, through an apparent dominant effect of the SID-Sin3-HDAC repressor mechanism, the knockdown of PCAF does not affect KLF10 repression of *FOXP3* gene transcription (middle black column, Figure 6). In contrast, PCAF knockdown significantly abrogates the activity of the *FOXP3* promoter when the interaction of KLF10 with the Sin3-HDAC complex is disrupted by mutation of its SID (red column vs green column, Figure 6).

These data suggest that Sin3 repression is independent of PCAF activity (black columns); however PCAF de-repression depends upon abrogation of Sin3-KLF10 interaction. These data are congruent with our previously published report that PCAF is recruited to the *FOXP3* core promoter in wt murine lymphocytes, but not in KLF10 deficient lymphocytes (40). However, it remains to be established whether the access of PCAF to the *FOXP3* promoter, though facilitated by KLF10, involves a direct protein-protein interaction with this transcription factor or occurs through the recruitment by other proteins. Therefore, we next explored the potential for physical association between KLF10 and PCAF. For this purpose, we generated GST (glutathione S transferase) fusion proteins of either full-length KLF10 or truncation mutants. We initially performed binding assays using purified recombinant PCAF that demonstrated, by western blot analysis, putative interaction in this cell free system to binding domains in both the N- (KLF10 1-210) and C-terminus (KLF10 210-311350), Figure 7A. Extending these results to an *in vivo* system, we repeated GST binding assays
utilizing Jurkat cell lysates. Using this system, we find that differently from other related transcription factors such as KLF16 and KLF13, KLF10 couples to PCAF not through the zinc finger domain but rather via the N-terminal region of the protein (1-210), Figure 7B. Thus, combined, our biochemical and transcriptional assays demonstrated that KLF10 interacts with both PCAF and Sin3, forming complexes that mediate FOXP3 activation and repression, respectively. To confirm physiologic relevance, we subsequently explored this differential interaction in primary murine lymphocytes.

We systematically evaluated the role for KLF10 in primary lymphocytes beginning with the whole animal and culminating in mechanistic single cell experiments. As we have previously demonstrated colitis susceptibility in the KLF10 deficient mouse (39), we began by confirming that the colitis susceptibility transfers with the immune compartment in bone marrow chimera models. Indeed, recipient mice of KLF10 deficient bone marrow demonstrate enhanced colitis as assessed by weight loss, clinical disease activity, and histology in response to dextran sodium sulfate (DSS) when compared to wt bone marrow recipients (Figure 8). Having demonstrated the relevance of KLF10 to intestinal immunoregulation in vivo, we focused more deeply on the function of KLF10 in primary CD4+ lymphocytes.

We subsequently investigated endogenous FOXP3 gene regulation utilizing a C57/BL6 transgenic mouse line. In this mouse line the transgenic expression of the Coxsackievirus and adenovirus receptor (CAR) allows efficient gene transduction with adenoviral packaged expression vectors in resting lymphocytes (36, 40). Wild type KLF10 or KLF10-SIDmt adenoviral constructs were packaged and transduced into resting CD4+ naïve murine lymphocytes. Greater than 5-fold expression of transduced constructs was evident at 6-48 hours (Figure 9). The trend to enhanced KLF10 expression upon mutation of the SID likely relates to the established capacity of wt KLF10
to repress its own gene transcription (18). After a 48-hour resting period, cells were stimulated for 5 days to induce FOXP3 through TCR activation and exogenous TGFβ (see methods). Through a series of ChIP assays, we characterized chromatin changes that occur at the native FOXP3 core promoter upon overexpression of wt or SID binding mutant KLF10. Congruent with the cell line experiments, overexpression of wt KLF10 resulted in 2.4 fold increased recruitment of Sin3 to the native FOXP3 core promoter when compared to the KLF10-SID binding mutant (Figure 10A, B). In the absence of Sin3 binding, overexpression of the KLF10-SID binding mutant led to 1.5 fold increased acetylation of histone 4 when compared to wt KLF10 (Figure 10C, D), and an approximately 7.5 fold increased recruitment of the HAT, PCAF at the native FOXP3 core promoter (Figure 10E, F). We repeated the above assay and performed alternative quantitative methodology in primary lymphocytes to confirm physiologic relevance. By quantitative real time PCR, we demonstrate KLF10 to recruit Sin3 (Figure 10B) and result in loss of histone 4 acetylation at the FOXP3 core promoter in primary T cells transduced with wt KLF10 and activated to induce FOXP3 (Figure 10D). Upon transduction with KLF10-SIDmt, PCAF is recruited to the core promoter with resultant restoration of H4 acetylation (Figure 10D, F).

Importantly, we assessed the functional relevance of these KLF10-dependent chromatin modifications through native FOXP3 transcriptional activity. Five days after adenoviral transduction of wt or SID binding mutant KLF10 into primary murine CD4+ lymphocytes activated to induce FOXP3, mRNA was isolated and quantitative RT-PCR was performed to measure FOXP3 gene transcriptional activity. Upon overexpression of the KLF10-SIDmt compared to wt KLF10, a 7-fold increase in FoxP3 mRNA was evident (Figure 11A).

Subsequently, we assessed the physiologic relevance of these KLF10-dependent events by assay of FOXP3 protein production upon transduction of wt or SIDmt KLF10 constructs. Primary KLF10-
deficient lymphocytes were transduced with KLF10 or KLF10-SIDmt adenovirus and activated for 12 days in conditions to induce FOXP3. Note, as demonstrated previously KLF10 deficient CD4+ lymphocytes produce minimal FOXP3 protein upon TGFβ stimulation (40). The transduction of KLF10-SIDmt, but not wt KLF10, into KLF10-deficient CD4+ T cells leads to partial rescue of FOXP3 protein production (Figure 11B). Taken together, these results demonstrate that KLF10 has the ability to silence or activate the FOXP3 core promoter through either association with the histone deacetylase co-repressor complex, Sin3 or the histone acetyltransferase, PCAF. This observation has biological relevance as several intracellular signaling cascades have the ability to selectively activate or inactive the interaction of many transcription factors with HAT or HDAC pathways which, in the case of KLF10, should function as a physiological switch for the regulation of FOXP3 by this protein (Figure 12).

Discussion

In this report we characterize a novel mechanism of reversible chromatin-dependent silencing of the key immunoregulatory gene FOXP3. We demonstrate coupling of the DNA-binding protein KLF10 with the Sin3/HDAC repressor complex. In the absence of this Sin3 binding event, KLF10 may couple with the HAT, PCAF to facilitate the activation state of FOXP3. While literature describing epigenetic regulation of FOXP3 to date has focused on less reversible DNA methylation events (19, 39), this report is the first to characterize a rapidly reversible, chromatin-dependent pathway integrating key signaling events critical to immunoregulation. As persistent activation of FOXP3 is critical to Treg function (39), the chromatin-dependent events regulating induction or silencing of the FOXP3 promoter locus in humans are critically important. The importance of understanding these mechanisms is evident in the disease-inducing potential of self-reactive “ex-Treg cells”
identified through cell fate mapping as to have extinguished \textit{FOXP3} expression (42); yet mechanistic insight into purported silencing events are lacking.

\textbf{Epigenetic regulation of \textit{FOXP3}}

A consistent feature of Treg cells with stable \textit{FOXP3} expression is relative hypomethylation of the conserved noncoding sequence 2 (CNS2) within intron 1 of the \textit{FOXP3} promoter locus (Treg specific demethylated region, TSDR) (29). The hypomethylated CNS2 region has been demonstrated to bind the transcription factors CREB (cyclic adenosine monophosphate response element binding protein) and Ets-1 (E26-AMV virus oncogene cellular homolog 1) stabilizing expression, and this DNA methylation pattern appears to be most frequently associated with thymus-derived, “natural” Treg cells (20). Comparatively little is known about the chromatin dependent events regulating adaptive Treg cells, the mechanisms of which we address here. Histone acetylation states, altered through the competitive recruitment of HAT and HDAC histone modifying complexes are key mechanisms of chromatin dynamic regulation of inducible genes, particularly within the immune pathways (31). Indeed, recruitment of SMAD proteins to conserved nucleotide sequence 1 has previously been demonstrated to lead to histone acetylation at the core promoter; however further mechanistic details were lacking (34). We have previously deduced indirectly through the study of KLF10 deficient lymphocytes that KLF10 likely plays a role in the recruitment of PCAF to the core promoter and induction of \textit{FOXP3} in adaptive Treg cells (40); yet the direct role for KLF10 to alternatively induce or repress \textit{FOXP3} expression through the regulation of acetylation/deacetylation of regional nucleosomes is the novel discovery outlined in this report. Our data would suggest that Sin3 repressor function appears to be dominant over PCAF activation; however we do not formally exclude the possibility that Sin3 binding normally functions as a rheostat of PCAF induced activation as has been demonstrated for KLF11 and the recruitment of heterochromatin protein 1.
In this system, PCAF titration experiments do not affect FOXP3 gene activation (data not shown) and PCAF knockdown does not effect Sin3-mediated repression; thus we favor the interpretation that Sin3 repressor function is dominant over PCAF activation. One would expect the Sin3-mediated mechanism to be inactivated in order for KLF10 to express PCAF-induced activation. Inactivation may occur through KLF10 post translational modifications downstream of lymphocyte cell signaling cascades in a similar manner to Sin3-binding disruption to KLF11 (12) and KLF16 (8).

**Post-translational modification of KLF10**

Key functionally relevant, post-translational modifications including serine/threonine phosphorylation and ubiquitination of KLF10 have been previously observed (1, 35). Several key pieces of data are important to consider within the context of this report. First, the SID of the closely related family member KLF16 bears a tyrosine residue (Y10) that is a Src-family tyrosine kinase target and upon which phosphorylation clearly regulates function (8). Second, ERK-induced phosphorylation of KLF11, the most closely related family member to KLF10, has been clearly demonstrated to disrupt Sin3 binding and repressor function (11). Finally, specific kinase inhibitor therapy has recently been shown to modulate Treg function (17). Thus, given the key role for TCR dependent Src-family tyrosine kinases in lymphocytes, further experimentation as to functionally relevant phosphorylation events on KLF10 are ongoing.

Perspectives and Significance: Thus, we report a novel mechanism of reversible chromatin-dependent silencing of the key immunoregulatory gene FOXP3. Through differential coupling with the Sin3/HDAC repressor complex or the HAT, PCAF, the DNA-binding protein KLF10 functions as a switch mediating FOXP3 repression or activation, respectively (model, Figure 12). These insights have broad relevance to TGFβ induced regulatory T cells; yet further investigation is
required to draw conclusions on the role for KLF10 in maintenance of FOXP3 expression in stable Treg cells. Further insight into lymphocyte receptor signaling events leading to the modification of key functional domains of KLF10 will likely lead to novel therapeutic targets for induction or repression of $FOXP3$ and Treg cells.
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Figure Legend:

Figure 1: KLF10 represses the core FOXP3 promoter in vitro

Luciferase reporter assays in genome integrated Jurkat cell lines demonstrating repression of core FOXP3 promoter function by KLF10.

1A, B: Relative luciferase units (RLU) upon overexpression of KLF family members into FLP-core cells with (1A) or without (1B) activating conditions (see methods). KLF10 uniquely represses FOXP3 promoter function in both activating and resting conditions (0.63 +/- 0.04 and 0.53 +/- 0.10 RLU, red column Figure 1A, B, respectively). RLUs are normalized to EV control (1.00, white column). The data represent mean/SE of 3 independent experiments with * p=0.035 (A) and p=0.004 (B).

1C: Chromatin immunoprecipitation assay demonstrating binding of KLF10 to the FOXP3 core promoter locus. Inset gel, upper left, demonstrates representative DNA gel for PCR reaction analysis of the expression of FOXP3 in cell fractions post immunoprecipitation for His. Quantitative real time PCR analysis of the expression of FOXP3 in cell fractions post immunoprecipitation for His-tagged KLF10 in FLP-core cells transfected with KLF10-His expression vector demonstrate significant binding of KLF10 to the core promoter (3.81 +/- 0.70 fold change over EV control). Results are presented controlled to FOXP3 expression of pre-immunoprecipitated sample (input). The data is representative of 3 independent experiments with * p=0.04.

Figure 2: Putative structural model for the Sin3-PAH2 and KLF10-SID complex.

Molecular modeling of KLF10 and Sin3 interaction utilizing HBP1 as template performed to predict disruptive domain mutations.
6022A: Sequence alignment of HBP1 SID, putative wt KLF10 SID, and E5P, A6P double KLF10 SID mutant. The established SID HBP1 was used as our modeling template. Consensus of the minimum SID is demonstrated in shaded grey.

6052B: The upper apolar surface of KLF10 SID alpha helix established hydrophobic interactions within the similarly charged pocket created by the 4-helix bundle of the Sin3-PAH2.

6072C: Sequestration of hydrophobic side chains (depicted as brown) away from hydrophilic surface (depicted as blue) to form the hydrophobic pocket which accommodates the KLF10 SID.

6092D: Critical bonds predicted between KLF10SID and Sin3 upon examination of protein-protein interaction interphase. Pairs of interacting residues are labeled with the same color while bonds are indicated by lines.

6122E: Predicted disruption of binding between KLF10 SID E5 with Sin3 H333 and KLF10 A6 with Sin3 V311 by proline mutagenesis. Pairs of interacting residues are labeled with the same color while bonds are indicated by lines. Note the disappearance of lines indicating disruption of bonds upon mutations of E5 and A6 to P.

617Figure 3: Proline mutagenesis of the SID disrupts KLF10-Sin3 protein/protein interaction but not KLF10 DNA binding.

6193A: Immunoblot using antibody specific for Sin3 (top row) or His tag (bottom row) of Jurkat cell lysates post overexpression of His-tagged KLF10 wt (left lane) or KLF10 SID mutant construct (right lane). Note that as predicted in the molecular modeling experiments, wt KLF10 physically interacts with Sin3, and the proline mutagenesis completely disrupts this interaction. The data is representative of 3 independent experiments.
KLF10 or KLF10 SID binding mutant constructs were expressed in Jurkat cells to demonstrate by chromatin immunoprecipitation the preserved competence of DNA binding by the KLF10 SID mutant construct. Inset gel, upper left, demonstrates representative DNA gel for PCR reaction analysis of the expression of FOXP3 in cell fractions post immunoprecipitation for His. Below, quantitative real time PCR analysis of the expression of FOXP3 in cell fractions post immunoprecipitation for His-tagged KLF10 demonstrates significant binding of both KLF10 (black column, 3.63 +/-0.3 fold change) and KLF10-SIDmt (grey column, 4.29+/-0.2 fold change) constructs to the core promoter compared to EV control. Note the ability of the KLF10-SID mutant to bind DNA, equivalent to wt KLF10. The data is representative of 3 independent experiments, p=0.024.

Figure 4: KLF10 represses FOXP3 through association with Sin3. Luciferase reporter assays in genome integrated Jurkat cell lines demonstrating disruption of repressor function upon mutation of the KLF10 SID.

A, B: Relative luciferase counts upon overexpression of KLF10 or KLF10-SIDmt into FLP core (4A) or FLP-core E1 cells (4B), normalized to empty vector control (1.00, white column). Note the established repression of FOXP3 gene transcription by wt KLF10 is abrogated by proline mutagenesis of the KLF10-SID (0.97 +/- 0.05 vs 0.45 +/- 0.07 RLU, 4A; and 1.14 +/- 0.12 vs 0.79 642 +/- 0.03 RLU, 4B). The data represents mean/SE of 6 independent experiments, p=0.003 (4A); p=0.009 (4B).

Figure 5: KLF10 recruits HDAC chromatin modifying complexes to the FOXP3 promoter.
Chromatin immunoprecipitation assay demonstrating that recruitment of the Sin3-HDAC repressor complex to the FOXP3 core promoter locus is dependent upon the KLF10 SID.

A, B, C: Quantitative real time PCR analysis of the expression of FOXP3 in cell fractions post immunoprecipitation for Sin3 (A), H4 polyacetylation (B), or HDAC1 (C) in FLP-core-E1 cells transfected with KLF10 or KLF10-SIDmt expression vectors. Note the capacity of wt KLF10 but not the KLF10-SIDmt protein to recruit Sin3 (2.717 +/- 0.35 vs 1.63 +/- 0.09 fold change, p=0.024) and histone deacetylase HDAC1 (1.25 +/- 0.12 fold change vs 0.43 +/- 0.05 fold change, p=0.024) to the core promoter resulting in loss of baseline histone 4 acetylation state (0.44 +/- 0.15 vs 0.82 +/- 0.14 fold change, p=0.034). The data represents mean/SE of 3 independent experiments. Inset gel, upper left, demonstrates representative DNA gel for PCR reaction analysis of the expression of FOXP3 in cell fractions post immunoprecipitation for Sin3 (A) and poly H4-Ac (B).

Figure 6: De-repression depends upon the HAT, PCAF.

Luciferase reporter assay demonstrates that abrogation of repressor function evident upon mutation of the KLF10 SID is dependent upon the HAT, PCAF.

Relative luciferase counts, normalized to empty vector control (1.00, white column), upon overexpression of KLF10 (black columns) or KLF10-SIDmt (colored columns) into FLP core cells with concomitant knockdown of PCAF (PCAF siRNA) or scramble siRNA (scr) control. KLF10 represses FOXP3 promoter function (0.62 +/- 0.03 RLU, second column from the left) only when the SID domain is intact (1.65 +/- 0.34 RLU KLF10-SIDmt, green column). Note the de-repression evident upon proline mutagenesis of the KLF10-SIDmt (green column) entirely depends upon the expression of PCAF (1.65 +/- 0.34 RLU, green column vs 0.58 +/- 0.13 RLU, red column, p=0.005). The data represents mean/SE of 3 independent experiments.
Figure 7: KLF10 associates with PCAF.

Assays of protein-protein interaction demonstrate physiologically relevant interaction between PCAF and the N-terminus domain of KLF10.

7A: Immunoblot for PCAF in cell free association assay of purified PCAF and GST constructs indicated. Note in this cell free assay PCAF associates with both the N and C terminus of KLF10.

7B: Immunoblot for PCAF using lysates of Jurkat lymphocytes incubated with GST fusion proteins (GST control, GST-KLF10 1-210, or GST-KLF10 210-350). Purified PCAF run on the left lane represents the positive control. Note that in a physiologically relevant system using Jurkat cell lysates, PCAF clearly interacts with the N terminus, but not the C terminus of KLF10.

Figure 8: Rag2⁻/⁻ mice reconstituted with KLF10⁻/⁻ bone marrow exhibit more severe colitis than Rag2⁻/⁻ mice reconstituted with Wild type (wt) bone marrow (BM). Data are presented as mean ± SEM (n=6 mice per group).

8A: Weight loss at the time of sacrifice. The values of body weight are expressed as a percentage of initial body weight on day 0. Rag2⁻/⁻ reconstituted with KLF10⁻/⁻ BM demonstrated significantly enhanced weight loss as compared to mice reconstituted with wt BM (6.89 ± 0.53 vs 3.64 ± 1.31%, p = 0.04).

8B: Macroscopic disease score based on presence of 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) shows Rag2⁻/⁻ reconstituted with KLF10⁻/⁻ BM had significantly enhanced disease activity as compared to mice reconstituted with Wt lymphocytes (4.31 ± 0.38 vs 2.64 ± 0.33, p = 0.01)
The levels of cytokines IL-6, IL-10 were determined in serum using mouse cytometric bead array cytokine assay (BD biosciences, San Jose, CA) and analyzed using FCAP array version 3 software (Soft Flow Hungary Ltd.). Rag2\(^{-/-}\) reconstituted with KLF10\(^{-/-}\) lymphocytes demonstrated higher levels of pro-inflammatory cytokine IL-6 (166.3 ± 41.34 vs 65.65 ± 27.60 pg/ml, \(p = 0.11\)) and lower levels of anti-inflammatory cytokine IL-10 than Rag2\(^{-/-}\) mice reconstituted with Wt bone marrow (0.56 ± 0.56 vs 6.60 ± 3.82 pg/ml, \(p = 0.08\)).

Representative histologic sections of mouse colon upon sacrifice from the two experimental conditions. Note a higher degree of crypt loss and diffuse eosinophilic infiltration in the KLF10\(^{-/-}\) recipient animals compared to WT recipient controls.

Figure 9: Quantitative real time PCR analysis of the expression of KLF10 in primary naïve CD4+ murine lymphocyte cells transduced with KLF10 or KLF10-SIDmt expression vectors. Note that overexpression of KLF10 adenoviral vectors results in 5-10 fold increased expression over the empty adenoviral vector. Results are presented controlled to KLF10 expression of empty vector transduced cells.

Figure 10: KLF10 associates alternatively with Sin3 or PCAF to repress or activate FOXP3 gene transcription in primary naïve CD4+ lymphocytes. Using adenoviral constructs in primary murine lymphocytes, chromatin immunoprecipitation demonstrates recruitment of alternatively Sin 3 or PCAF to the FOXP3 core promoter locus to depend upon the KLF10 SID.

Inset gels, upper left, demonstrate DNA gel for PCR reaction analysis of the expression of FOXP3 in cell fractions post immunoprecipitation for Sin3 (A), poly H4-Ac (C), and PCAF (C) in
primary lymphocytes post transduction with KLF10 or KLF10-SIDmt expression vectors. Histogram represents densitometry units normalized by input for associated gels. Note that recapitulating the data from cell lines, wt KLF10 but not the KLF10-SIDmt protein recruits Sin3 to the core promoter (2.43 +/- 0.18 vs 1.00 +/- 0.1 densitometry units normalized to input) resulting in a decrease of the histone 4 acetylation state (52.45 +/- 4.45 vs 77.95 +/- 7.14 densitometry units normalized to input, 10C). KLF10-SIDmt recruits PCAF to the core promoter (11.43 +/- 1.58 vs 2.51 +/- 1.09 densitometry units normalized to input). The data represents mean/SE of 3 independent experiments.

721B, D, F: Quantitative real time PCR analysis of the expression of FOXP3 in cell fractions post immunoprecipitation for Sin3 (10B), H4 polyacetylation (10D), or PCAF (10F) in primary T cells transfected with EV, KLF10 or KLF10-SIDmt expression vectors. Note the capacity of wt KLF10 but not the KLF10-SIDmt protein to recruit Sin3 (2.93 +/- 0.25 vs 1.23 +/- 0.30 fold change, p=0.04). In the absence of Sin3, the KLF10-SIDmt recruits PCAF (4.56 +/- 1.22 fold change vs 0.51 727 +/- 0.03 fold change) to the core promoter resulting in enhancement of the histone 4 acetylation state (0.27 +/- 0.15 vs 2.59 +/- 1.59 fold change). The data represents mean/SE of 3 independent experiments.

731Figure 11: KLF10 overexpression drives FOXP3 protein production upon disruption of binding with Sin3.

7331A: Quantitative real time PCR analysis of the expression of FoxP3 mRNA in primary CD4+ T cell lysates post transduction with wt KLF10 (white column) or KLF10-SIDmt (black column). Consistent with the chromatin landscape, KLF10-SIDmt results in enhanced native FOXP3 gene transcription in primary cells (7.02 +/- 1.15 vs 0.95 +/- 0.12 fold change, p=0.03, mean/SE). The data
represents mean/SE of 4 independent experiments. Inset gel upper left demonstrates DNA gel for PCR reaction for the expression of Foxp3 in primary CD4+ T cell lysates.

11B: Flow cytometry for FOXP3 protein in primary KLF10-deficient CD4+ T lymphocytes induced with TGFβ post transduction with wt KLF10, KLF10-SIDmt, or EV adenoviral constructs. Note the established block in FOXP3 transduction in the absence of KLF10 (EV, 6.8% left upper blot) and the significant enhancement in FOXP3 protein production upon reconstitution of cells with KLF10-SIDmt but not wt KLF10 (10.7% lower left dot plot vs 1.7% upper right dot plot). The experiment was repeated three times and mean/SE for FOXP3 protein expression in EV transduced (white column), KLF10 transduced (black column), and KLF10-SIDmt transduced (grey column) demonstrate significant enhancement in FOXP3 protein in KLF10-SIDmt transduced but not wt KLF10 transduced cells when compared to EV (1.57 +/-0.07 vs 0.57 +/-0.16, p=0.02).

Figure 12: KLF10 dichotomous regulation of FOXP3

A model depicting how KLF10 functions as a switch integrating cell signaling cascades to alternatively activate or silence the FOXP3 core promoter through interaction with the HAT, PCAF (upper panel) or the Sin3/HDAC complex (lower panel), respectively.
Figure 6
Figure 9
Figure 12

[Diagram showing the interaction between PCAF, KLF10, and HDAC with Sin3 in regulating FOXP3 expression.]

- **PCAF** interacts with **KLF10** near the **FOXP3 CORE PROMOTER** (ON state).
- **HDAC**, **Sin3**, and **KLF10** interact near the **SP1/KLF site** of the **FOXP3 CORE PROMOTER** (OFF state).