Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

Cell Rep. Author manuscript; available in PMC 2018 May 11.

Published in final edited form as:

Cell Rep. 2018 April 17; 23(3): 796–807. doi:10.1016/j.celrep.2018.03.099.

KAP1 Regulates Regulatory T Cell Function and Proliferation in Both Foxp3-Dependent and -Independent Manners

Shigeru Tanaka[1], **Christian Pfleger**[1], **Jen-Feng Lai**[1], **Florence Roan**[1,2], **Shao-Cong Sun**[3], and **Steven F. Ziegler**[1,4,5,*]

[1]Immunology Program, Benaroya Research Institute, 1201 9th Avenue, Seattle, WA 98101-2795, USA

[2]Division of Allergy and Infectious Diseases, University of Washington School of Medicine, Seattle, WA, USA

[3]Department of Immunology, M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA

[4]Department of Immunology, University of Washington School of Medicine, Seattle, WA, USA

Summary

Regulatory T cells (Tregs) are indispensable for the establishment of tolerance of self-antigens in animals. The transcriptional regulator Foxp3 is critical for Treg development and function, controlling the expression of genes important for Tregs through interactions with binding partners. We previously reported KAP1 as a binding partner of FOXP3 in human Tregs, but the mechanisms by which KAP1 affects Treg function were unclear. In this study, we analyzed mice with Treg-specific deletion of KAP1 and found that they develop spontaneous autoimmune disease. KAP1-deficient Tregs failed to induce Foxp3-regulated Treg signature genes. In addition, KAP1-deficient Tregs were less proliferative due to the decreased expression of Slc1a5, whose expression was KAP1 dependent but Foxp3 independent. This reduced expression of Slc1a5 resulted in reduced mTORC1 activation. Thus, our data suggest that KAP1 regulates Treg function in a Foxp3-dependent manner and also controls Treg proliferation in a Foxp3-independent manner.

In Brief

Tanaka et al. demonstrate that KAP1 works together with Foxp3, the master transcription factor of regulatory T cells (Tregs), to induce effector molecules and Treg-specific KAP1-deficient mice

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

*Correspondence: sziegler@benaroyaresearch.org.

[5]Lead Contact

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.03.099.

AUTHOR CONTRIBUTIONS

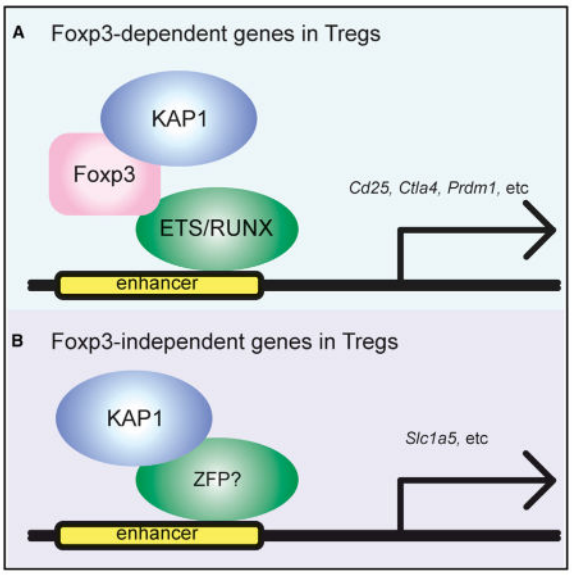
S.T. designed the research, performed most of the experiments, prepared the figures, and wrote the manuscript. C.P. and J.-F.L. analyzed the phenotype of $Treg^{\Delta KAP1}$. F.R. did the histological analysis and edited the manuscript. S.-C.S. provided the $KAP1^{fl/fl}$ mice and edited the manuscript. S.F.Z. designed the research, supervised the experiments, and helped write the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

develop autoimmunity. KAP1 also regulates cell proliferation independent from Foxp3 by inducing a glutamine transporter, Slc1a5.



INTRODUCTION

Regulatory T cells (Tregs) are essential for maintaining immune homeostasis. The development of Tregs is controlled by transcription factor Foxp3, and mutations in the Foxp3 gene result in severe autoimmune disease due to the loss of Tregs (Fontenot et al., 2003; Hori et al., 2003). Although Foxp3 is critical for the development of Tregs, it requires binding partners for its normal function. It was reported that Foxp3 forms multi-protein complexes, and over 300 potential binding proteins were identified (Rudra et al., 2012). For example, Gata-3 binds with Foxp3 in Tregs, and Treg-specific Gata-3-deficient mice develop intestinal inflammation, suggesting that Gata-3 is an important co-factor of Foxp3 (Rudra et al., 2012). Other studies have shown that Eos (Pan et al., 2009), Hdac7 (Li et al., 2007a), and NFAT (Wu et al., 2006) are important partners of Foxp3 and control specific aspects of Foxp3 function. Given that Treg cells that lack any of these partners fail to show normal suppressor function, the transcription program controlled by Foxp3 is tightly regulated, and each of the Foxp3-binding partners is required for Foxp3-dependent establishment of the Treg transcriptional landscape.

KAP1 (also known as TRIM28 or TIF1β) is a member of the tripartite motif proteins and was originally characterized as a chromatin remodeling factor binding to Kruppel family zinc-finger transcription factors (Iyengar and Farnham, 2011). KAP1 has been shown to be critical for heterochromatin formation, recruiting the H3K9 methylase SETDB1 and associating with Mi2, a component of histone deacetylation complex (NuRD) and heterochromatin protein 1 (HP1) (Nielsen et al., 1999; Schultz et al., 2001, 2002). In the T cell compartment of the immune system, KAP1 is highly expressed in $CD4^{+}CD8^{+}$ double-positive cells and contributes to H3K4me3 modification of the T cell receptor α (TCRα) enhancer and promotes recruitment of RAG proteins to the TCRα locus (Zhou et al., 2012).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Global T cell-specific KAP1-deficient mice develop spontaneous autoimmune disease due to enhanced Th17 differentiation (Chikuma et al., 2012). We have shown that KAP1 forms a complex with FOXP3 in human Tregs and that this interaction is mediated by FIK (Huang et al., 2013). Our finding that Tregs that are knocked down for KAP1 or FIK expression display reduced suppressor function demonstrates that KAP1 is an important transcriptional regulator in conjunction with FOXP3. However, the exact mechanisms of how KAP1 affects Treg function remain unclear.

In this study, we analyzed Treg-specific KAP1-deficient mice to address the role of KAP1 in Treg development and function. Treg-specific KAP1 deletion resulted in the development of spontaneous lymphadenopathy and lung inflammation due to impaired Treg proliferation and suppressor function. Epigenetic analysis of Tregs from these mice revealed that KAP1 binds not only to Treg signature gene loci, which were also occupied by Foxp3, but also to loci that were not Foxp3 targets. Among the Foxp3-independent KAP1 target genes, the expression of Slc1a5, a glutamine transporter, was dramatically decreased in KAP1-deficient Tregs, compared to its expression in KAP1-sufficient Tregs. The decreased expression of Slc1a5 led to the diminished activation of mTORC1, followed by impaired cell proliferation and activation. Our results suggest that KAP1 affects the function of Tregs in both Foxp3-dependent and -independent manners.

RESULTS

KAP1 and Foxp3 Form a Complex in Mouse Tregs

We first asked whether mouse KAP1 and Foxp3 interact in Tregs, as these cells lack the human-specific adaptor FIK (Huang et al., 2013). We sorted Tregs from Foxp3-IRES-yellow fluorescent protein (YFP)/Cre mice (Rubtsov et al., 2008), and the cells were cultured for 2 weeks. Roughly 90% of *in vitro*-expanded Tregs still expressed Foxp3 (Figure S1A). Lysates were prepared and subjected to co-immunoprecipitation (coIP) to determine the interaction between KAP1 and Foxp3 in mouse Tregs. To our surprise, KAP1 was immunoprecipitated with anti-Foxp3 antibody, suggesting that Foxp3 and KAP1 form a complex even in the absence of FIK in mouse Tregs (Figure S1B).

Spontaneous Inflammation and Lymphadenopathy in Treg-Specific KAP1-Deficient Mice

Next, we generated Treg-specific KAP1-deficient mice ($Foxp3^{cre}KAP1^{f/f}$; hereinafter referred to as $Treg^{\Delta KAP1}$) by crossing Foxp3-IRES-YFP/Cre mice with KAP1 floxed mice (Cammas et al., 2000). $Treg^{\Delta KAP1}$ mice were viable and fertile but developed spontaneous lymphadenopathy (Figures 1A and 1B) and lung inflammation (Figures 1C and 1D) at 8–12 weeks of age. We also observed increased numbers of immune cells in the colonic lamina propria in $Treg^{\Delta KAP1}$ mice in the absence of any obvious pathology of the mucosal surface (data not shown). In addition to these features, $Treg^{\Delta KAP1}$ mice displayed elevated anti-nuclear antibody levels (Figure S2A), suggesting that $Treg^{\Delta KAP1}$ mice develop a global loss of tolerance. Consistent with the histology and increased cell number in peripheral tissues, $CD4^+$ T cells in $Treg^{\Delta KAP1}$ mice had higher expression of the activation marker CD44 (Figure 1E) and produced more inflammatory cytokines (e.g., interferon (IFN)γ and interleukin (IL)-13) compared to littermate control mice (Figure 1F). Interestingly, we found

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

eosinophilia in the lungs of Treg$^{\Delta KAP1}$ mice (Figure S2B), consistent with the elevated Th2-type cytokine production seen in these mice (Figure 1F). We also found increased IL-17-producing Th17 cells in the lungs of Treg$^{\Delta KAP1}$ mice, but not increased IFNγ-producing Th1 cells or increased numbers of neutrophils (Figures 1F and S2B). In contrast, lamina propria CD4$^+$ T cells mainly produced IFNγ and IL-17 but not IL-13 (data not shown). This indicates that tissue-specific factors in mice with KAP1-deficient Tregs influenced CD4$^+$ T cell differentiation and the cytokine profile in the lungs and the large intestines.

As we also observed that Treg frequency was increased in the spleen, the lung, and the lamina propria of Treg$^{\Delta KAP1}$ mice (Figure 1G; data not shown), we next analyzed thymic Treg differentiation. We observed similar numbers of thymocytes and comparable CD4$^+$ and CD8$^+$ T cell development in the thymus of control and Treg$^{\Delta KAP1}$ mice (Figures S2C and S2D). Intriguingly, the percentage of Foxp3$^+$ cells in the CD4$^+$ population was not distinguishable between Treg$^{\Delta KAP1}$ and littermate control mice (Figure 1H), indicating that T cell development and Treg development in Treg$^{\Delta KAP1}$ mice were not affected. Indeed, despite displaying nearly complete genomic deletion, most thymic Tregs in Treg$^{\Delta KAP1}$ mice still had KAP1 protein (Figures S2E and S2F). In contrast, about 80% of Tregs in the spleen were negative for KAP1 protein (Figure S2E), suggesting that KAP1 in the tTregs was residual protein translated prior to Foxp3 expression. To test this possibility, Tregs from Treg$^{\Delta KAP1}$ and their littermates were sorted and cultured. Tregs from each strain were initially >90% KAP1$^+$; however, at the end of 2 days of culture, 35% of the Tregs from Treg$^{\Delta KAP1}$ mice were KAP1$^+$, while those from control mice remained 90% KAP1$^+$. The percentage of KAP1$^+$ cells in the cultures from Treg$^{\Delta KAP1}$ continued to decrease over time, with a near-complete absence by day 7 of culture (Figure S2G). These results suggest that the turnover of KAP1 is relatively slow and that the deletion of the KAP1 gene in Foxp3$^+$ cells in the thymus does not affect Treg differentiation due to its long half-life. Thus, the autoimmune phenotype observed in Treg$^{\Delta KAP1}$ mice was due to the impaired functionality of KAP1-deficient Tregs in the periphery.

KAP1-Deficient exTreg CD4$^+$ T Cells Are Not Pathogenic

Several studies have shown that Foxp3 expression in Tregs can be shut off in the periphery, generating what are known as exTregs (Komatsu et al., 2014; Zhou et al., 2008, 2009). The exTregs have been shown to be pathogenic in autoimmune settings, because they are auto-reactive and can produce inflammatory cytokines. Of the KAP1-deficient CD4$^+$ T cells in the spleen, approximately one third of the cells were also negative for Foxp3 (Figure S2E). We hypothesized that these KAP1$^-$Foxp3$^-$ T cells are exTregs capable of producing inflammatory cytokines. To address this, these cells in the spleen and the lung were re-stimulated, and cytokine production by KAP1$^-$ and KAP1$^+$ CD4$^+$ T cells was measured. Interestingly, the majority of the cytokine-producing CD4$^+$ T cells were KAP1$^+$, with very few KAP1$^-$ cells producing inflammatory cytokines (Figure S3A). Thus, the exTregs generated in Treg$^{\Delta KAP1}$ mice appeared to not be pro-inflammatory.

We also performed bisulfite sequencing of the *Foxp3* conserved non-coding sequence 2 (CNS2) region from male Treg$^{\Delta KAP1}$ mice, as demethylation of CpG islands in CNS2 is required for continuous *Foxp3* expression (Ohkura et al., 2012; Zheng et al., 2010).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Although we saw a substantial number of KAP1^{-}Foxp3^{-} cells in Treg$^{\Delta KAP1}$ spleens, KAP1-deficient Tregs maintained hypomethylated CpGs in CNS2 (Figure S3B). These results indicate that exTregs are not pathogenic in Treg$^{\Delta KAP1}$ mice and that the instability of Foxp3 expression is independent of CNS2 methylation status.

KAP1 Is Required for Optimal Cell Proliferation

To study the role of KAP1 in Tregs in non-inflammatory conditions, we analyzed female Foxp3$^{cre/wt}$KAP1$^{f/f}$ mice. Due to random X-chromosome inactivation, half of the Tregs in these mice should express KAP1. There was a non-significant trend toward decreased splenic YFP^{+}Foxp3^{+} cells in Foxp3$^{cre/wt}$KAP1$^{+/+}$ females and a marked decrease in the percentage of YFP^{+}Foxp3^{+} (KAP1-deficient Tregs) over YFP^{-}Foxp3^{+} (KAP1-sufficient Tregs) cells in the spleen, but not in the thymus, of Foxp3$^{cre/wt}$KAP1$^{f/f}$ female mice (Figure 2A). As there are generally more Tregs in an inflamed mouse (Zhou et al., 2008), it is possible that the increased number of Tregs seen in Treg$^{\Delta KAP1}$ mice is secondary to inflammation.

We sought to determine the mechanism underlying the competitive disadvantage seen with KAP1-deficient Tregs. We first asked whether apoptosis was involved. To test this, Tregs from Foxp3$^{cre/wt}$KAP1$^{f/f}$ females were stained with fixable viability dye and annexin V. We found that the percentage of apoptotic cells was decreased in Foxp3^{+}YFP^{+}, compared to that in the Foxp3^{+}YFP^{-} population in a competitive setting (Figure 2B), indicating that KAP1-deficient Tregs do not display increased apoptosis relative to their KAP1-sufficient counterparts. Since it has been shown that KAP1 regulates the expression of cell-cycle-related genes in several cell types, including conventional CD4^{+} T cells (Chikuma et al., 2012; Li et al., 2007b; Wang et al., 2005), we tested whether KAP1-deficient Tregs are less proliferative. Foxp3^{+}YFP^{+} cells from Foxp3$^{cre/wt}$KAP1$^{f/f}$ female mice had lower expression of the cell proliferation marker Ki-67 compared to Foxp3^{+}YFP^{-} cells (Figure 2C). We also analyzed cell proliferation *in vitro*. Sorted Tregs from Treg$^{\Delta KAP1}$ mice and their littermates were labeled with CellTrace Violet (CTV). Cells were then cultured for 5 days, and CTV dilution was determined. Consistent with Ki-67 expression in Foxp3$^{cre/wt}$KAP1$^{f/f}$ female mice, KAP1-deficient Tregs were less proliferative compared to KAP1-sufficient Tregs in terms of CTV dilution (Figure 2D). We also cultured Tregs for 4 days and pulsed 10 μM bromodeoxyuridine (BrdU) to determine the cell-cycle progression. As expected, there were significantly fewer percentages of BrdU^{+} cells (cells in S phase) in KAP1-deficient Tregs (Figure 2E). Taken together, KAP1-deficient Tregs are less proliferative both *in vivo* and *in vitro*.

KAP1-Deficient Tregs Have Impaired Suppressor Function

Next, we determined the capacity of KAP1-deficient Tregs to suppress effector T cell proliferation. CFSE-labeled responder CD4^{+} T cells were co-cultured with either KAP1-deficient or -sufficient Tregs in the presence of anti-CD3/CD28 beads for 4 days, and CFSE dilution was analyzed. The ability of KAP1-deficient Tregs to suppress the expansion of responder cells was dramatically diminished as compared to that of KAP1-sufficient Tregs (Figures 3A and 3B). As expected, there was a small but significant difference in the Treg number in the cultures with control or Treg$^{\Delta KAP1}$ (Figure 3C); however, considering that

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Treg$^{\Delta KAP1}$ mice developed the lung inflammation in spite of increased Treg numbers, compared to control mice, the impaired suppressor function of KAP1-deficient Tregs likely involved both impaired cell proliferation and defective suppressor function. We also compared suppressor function in an *in vivo* setting. CD4^{+}CD45RB^{high} T (CD45.1) cells were co-transferred with equal numbers of KAP1-sufficient or -deficient Tregs (CD45.2 cells) into RAG1$^{-/-}$ mice, and the development of the inflammation was examined in the large intestines. Both groups gained weight over time, and there was no difference in body weight change (Figure 3D); however, there was a trend toward an increase in the histology score of the large intestine in RAG1$^{-/-}$ mice receiving KAP1-deficient Tregs (Figure S4A). Interestingly, while the percentages of Tregs and cytokine-producing CD4^{+} T cells were indistinguishable between two groups (Figures S4B and S4C), RAG1$^{-/-}$ mice receiving KAP1-deficient Tregs had more CD45.1^{+} T cells in the lamina propria (Figure 3E). We also observed similar trends for cell numbers in mesenteric lymph nodes and spleen (data not shown). These results indicate that KAP1-deficient Tregs have less potential for controlling lymphocyte number both *in vitro* and *in vivo*.

KAP1 Regulates Treg Signature Genes in a Foxp3-Dependent Manner

To address the mechanism(s) of KAP1 regulation of Treg suppressor function, we analyzed genome-wide histone modification and transcriptional profiles of KAP1-sufficient and -deficient Tregs by chromatin immunoprecipitation sequencing (ChIP-seq) and RNA sequencing (RNA-seq). Since KAP1 has been reported to recruit the H3K9 specific methyltransferase SETDB1 to the genome (Schultz et al., 2002), we focused on the H3K9me3 marks. Interestingly, H3K9me3 marks around transcription start sites (TSSs) in KAP1-deficient Tregs were greatly reduced (Figure 4A). Whereas H3K9me3 marks are thought to be suppressive histone modifications (Zhou et al., 2011), the loss of the H3K9me3 mark around TSSs did not correlate with the change of transcription levels between KAP1-sufficient and -deficient Tregs (Figure 4B). These results suggest that transcriptional regulation by KAP1 in Tregs is not dependent on the H3K9me3 histone modification.

Next, we analyzed differentially expressed genes (DEGs) between KAP1-sufficient and -deficient Tregs to elucidate which molecules affect the impaired suppressor function. Although it has been reported that KAP1 regulates the transcriptional activity of zinc-finger family members (O'Geen et al., 2007) and internal retroviral genes (Rowe et al., 2010, 2013), the enriched gene ontology (GO) terms for DEGs were related to the cell cycle and immune system (Figure S5A). Consistent with the impaired cell proliferation observed in KAP1-deficient Tregs (Figures 2C–2E), numerous cell-cycle-related genes were dysregulated (Figure S5B). In addition to the cell-cycle-related genes, there were several DEGs that are important for Treg suppression ability, such as *Klrg1* (KLRG1), *Prdm1* (Blimp1), *Icos* (ICOS), *S100a4* (S100A4), *Nr4a1* (Nur77), *Plagl1* (PLAGL1), and *Hdac9* (HDAC9) (Figures 4C and 4D) (Beier et al., 2012; Beyersdorf et al., 2007; Cretney et al., 2011; Fu et al., 2012; Herman et al., 2004; Sekiya et al., 2015; Tao et al., 2007). These data indicate that KAP1 plays a pivotal role in establishing the Treg transcriptional signature and that KAP1-mediated transcriptional regulation in Tregs was different from that in other cell types.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Given that KAP1 couples with Foxp3 in Tregs, and that the expression of Treg signature genes was dysregulated in KAP1-deficient Tregs, we next performed ChIP-seq analysis to clarify whether KAP1 is recruited via genomic loci occupied by Foxp3. Nuclear lysates from *in-vitro*-expanded Tregs were immunoprecipitated with anti-KAP1 antibody, and the precipitated chromatin was sequenced. As a reference, we also analyzed Foxp3 ChIP-seq data that were reported previously (Kitagawa et al., 2017). KAP1 binding sites were enriched around TSSs (Figure S6A), which is consistent with the loss of H3K9me3 marks around TSSs in KAP1-deficient Tregs. We found 3,737 genes that were bound by KAP1; among these genes, 2,004 genes (53.6%) were also bound by Foxp3 (Figure 5A). Interestingly, while the GO terms of the genes that were bound by only KAP1 or Foxp3 were not related to the immune system (Figures S6B–S6D), a large part of the enriched GO terms in KAP1/Foxp3 co-occupied genes were related to the immune system/T cell function (Figure 5A), suggesting that the KAP1/Foxp3 complex functions to regulate genes involved in T cell function. For example, the intronic region of *Prdm1* (which encodes Blimp-1) was co-occupied by both KAP1 and Foxp3 (Figure 5B). Previous reports have shown that the Blimp-1/IL-10 axis in Tregs is an important mechanism for Treg suppressor function, especially at mucosal surfaces (Cretney et al., 2011). Since we found that KAP1-deficient Tregs have significantly lower expression of *Prdm1* transcripts by RNA-seq (Figure 4D), we hypothesized that KAP1-deficient Tregs are less suppressive because of the dysregulation of the Blimp-1/IL-10 axis. The protein level of Blimp-1 was slightly but significantly decreased in $Treg^{\Delta KAP1}$ spleens, in agreement with the RNA-seq data (Figure 4D), and the difference was more obvious in the lung (Figure 5C). We also analyzed IL-10 production in Tregs, and the percentage of IL-10-producing Tregs was significantly lower among Tregs from the lungs of $Treg^{\Delta KAP1}$ mice compared to littermate control mice (Figure 5D). We also found reduced IL-10 expression in *in-vitro*-induced Tregs, using naive $CD4^+$ T cells from $Treg^{\Delta KAP1}$ mice (Figure S6E). These results suggest that impaired expression of Blimp-1 followed by decreased IL-10 production is one of the mechanisms contributing to the development of autoimmune disease in $Treg^{\Delta KAP1}$ mice.

While there were several genes that were co-occupied with KAP1 and Foxp3, only a small number of these genes were up- or downregulated in KAP1-deficient Tregs (Figure S6F). To address this point, we analyzed Tregs from female $Foxp3^{cre/wt}KAP1^{f/f}$ mice to determine the protein expression of genes co-bound by KAP1 and Foxp3 that displayed unaltered mRNA expression. We looked at *Ctla4* and *Il2ra*, which are well-known Treg signature genes and were regulated by both KAP1 and Foxp3 (Figure S6G). Interestingly, their protein expression was reduced in the $Foxp3^+YFP^+$ population (KAP1-deficient Tregs), compared to that in the $Foxp3^+YFP^-$ KAP1-sufficient counterpart (Figure S6H). These data suggest that the KAP1/Foxp3 complex affects not only the expression of DEGs that were identified by RNA-seq, but also the expression of Treg signature genes such as *Ctla4* and *Il2ra*.

KAP1 Is Recruited to ETS/RUNX1 Binding Sites

Since KAP1 does not have a DNA binding domain, we next examined KAP1 binding sites to determine what molecules are required to bring KAP1 to the target regions. We performed DNA binding motif analysis of sites identified in the KAP1 ChIP-seq analysis. We found significantly enriched motifs for ETS family molecules (Elf3 and Fli1), CTCFL, and

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

RUNX1. These sites accounted for approximately 60% of KAP1-occupied sites (Figure 5E). Indeed, there is a Fli1/ETS motif in the intron of *Prdm1*, where KAP1 and Foxp3 were recruited (Figure S6I). A previous study showed that ETS and RUNX motifs were more enriched at Foxp3 binding sites than the FKHD domain motif, which is a Foxp3 binding motif (Samstein et al., 2012). Together, these results indicate that KAP1 is recruited to the ETS and RUNX binding sites in Tregs.

KAP1 Affects Cell Proliferation by Regulating Slc1a5 Expression in a Foxp3-Independent Manner

Slc1a5, which encodes a glutamine transporter, was one of the most strikingly downregulated genes in KAP1-deficient Tregs (Figure 4C). The ChIP-seq analysis showed that KAP1, but not Foxp3, bound to the Slc1a5 gene (Figure 6A). A recent study reported that Slc1a5 (also known as ASCT2) was important for glutamine uptake upon TCR stimulation, followed by the activation of mTORC1, which promoted T cell differentiation and proliferation (Nakaya et al., 2014). We hypothesized that decreased levels of Slc1a5 were involved in the impaired cell proliferation seen in KAP1-deficient Tregs. To determine the role of Slc1a5, Treg proliferation was examined in the presence or absence of L-glutamine. While the cells cultured in the presence of 5 mM L-glutamine proliferated well, Tregs cultured in glutamine-free media displayed a profound proliferative deficit (data not shown). We also observed that rapamycin, a mammalian target of rapamycin (mTOR) inhibitor, strongly repressed the proliferation of Tregs (data not shown), indicating that L-glutamine and the activation of mTOR signaling are important for the proliferation of Tregs.

Consistent with the RNA-seq data, the Slc1a5 protein levels were dramatically decreased in KAP1-deficient Tregs (Figure 6B). Importantly, phosphorylation of ribosomal protein S6, which is a downstream mediator of mTORC1 signaling, was reduced in KAP1-deficient Tregs upon anti-CD3/CD28 stimulation (Figure 6C), suggesting that KAP1-deficient Tregs are hypo-respontsive to TCR signaling. To examine whether decreased L-glutamine uptake upon TCR stimulation affects cell activation of KAP1-deficient Tregs, we treated KAP1-sufficient and KAP1-deficient Tregs with L-glutamine, and phosphorylation of S6 was analyzed. Interestingly, KAP1-sufficient Tregs responded to 5 mM L-glutamine with increased levels of pS6, and KAP1-deficient Tregs failed to induce the phosphorylation of S6 (Figure 6D). To better understand whether the decreased Slc1a5 expression is responsible for the impaired cell proliferation observed in KAP1-deficient Tregs, Slc1a5 was retrovirally transduced into Tregs. Interestingly, while KAP1-sufficient Tregs ectopically expressing Slc1a5 proliferated similarly to mock-transduced Tregs, KAP1-deficient Tregs transduced with Slc1a5 proliferated better than mock-transduced KAP1-deficient Tregs (Figure 6E). These results suggest that L-glutamine helps Treg proliferation and that the decreased cell proliferation observed in KAP1-deficient Tregs is partially because of the reduced Slc1a5 expression.

DISCUSSION

Our study demonstrates that the KAP1-Foxp3 interaction is important for Treg function through regulation of the Foxp3-mediated gene transcription program. We also found that

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

KAP1 regulates Treg proliferation/activation independent of its interaction with Foxp3 through control of Slc1a5 expression.

We previously reported that FIK interacts with both FOXP3 and KAP1 in human Tregs (Huang et al., 2013). Because sequences encoding the C terminus of *FIK*, which is critical for FOXP3 binding, are not present in the mouse genome, we initially believed that the interaction between FOXP3 and KAP1 was human specific. However, we found that KAP1 and Foxp3 can interact in mouse Tregs. FIK has a KRAB domain, which is important for the binding with KAP1, and we did not see the molecular interaction between FOXP3 and KAP1 in the absence of FIK in human Jurkat T cells. As the amino acid sequences of Foxp3 and KAP1 are highly conserved between human and mouse (86% and 97%, respectively), it is possible that the interaction between Foxp3 and KAP1 in mouse Tregs is also mediated by a KRAB-domain-containing protein.

Although the physiological relevance of H3K9me3 marks around TSSs is still elusive, this histone modification at promoter regions is critical for transcriptional activity in some cell types. For example, Jmjd2a/c-mediated H3K9me3 marks around TSSs are critical for the maintenance of pluripotency (Pedersen et al., 2016). Amigorena and colleagues reported that H3K9me3 marks around the promoter of lineage-specifying factors are important for helper T cell plasticity (Allan et al., 2012). They found that H3K9me3 marks around the promoter of IFNγ are diminished in SUV39H1 (a H3K9 methyltransferase)-deficient $CD4^+$ Th2 cells and that these SUV39H1-deficient Th2 cells are unstable. In our ChIP-seq data from KAP1-deficient and -sufficient Tregs, in contrast, H3K9me3 marks around TSSs did not show any correlation to transcriptional levels. Since little is known about the role of H3K9me3 marks in Tregs, further studies are needed to understand the role of this chromatin modification in this cell population.

KAP1 deficiency led to the down-modulation of several Treg-specific genes, indicating a role in the positive regulation of the Treg transcriptional signature. KAP1 was originally defined as a transcriptional repressor based on its interaction with histone methyltransferase and histone deacetylase complexes (Iyengar and Farnham, 2011). However, accumulating evidence suggests that KAP1 also works as a transcriptional activator (Li et al., 2017; McNamara et al., 2016; Rambaud et al., 2009; Zhou et al., 2012). Interestingly, in HEK293T cells, the 3′ UTRs of some zinc finger protein (ZNF) family genes displayed KAP1 binding and are associated with SETDB1 and marked with H3K9me3. However, ZNF family genes (and non-ZNF genes) with promoter region binding of KAP1, SETDB1 was not associated, and KAP1 binding correlated with chromatin marks of active transcription (H3K4me3) and a lack of H3K9me3 marks (Iyengar et al., 2011). Our ChIP-seq data also showed that KAP1 was recruited to the TSS, and H3K9me3 marks in these regions were diminished in KAP1-deficient Tregs. However, the lack of H3K9me3 marks in KAP1-deficient Tregs did not correlate with gene expression. These data suggest that KAP1 works primarily as a transcriptional activator when recruited to a gene-proximal regulatory element, while KAP1 binding to 3′ UTRs signals transcriptional repression.

A previous report showed that T cell-specific deletion of KAP1 resulted in increased transforming growth factor (TGF)β3, followed by accelerated Th17 differentiation and

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

autoimmune disease (Chikuma et al., 2012). In addition to dysregulated Th17 inflammation, and consistent with our findings, Tregs from $Lck^{cre}KAP1^{f/f}$ mice also displayed impaired suppressor function *in vivo*. The authors also analyzed $Foxp3^{cre}KAP1^{f/f}$ mice and did not report an inflammatory phenotype, in contrast to the observations in our study. One possible explanation for this discrepancy is the different microbiota in each animal facility, which has been shown to modify the development of autoimmunity (Honda and Littman, 2016). Another explanation may be differences in the Foxp3-Cre lines used in each study. Chikuma et al. used a bacterial artificial chromosome (BAC) transgenic line shown to result in a runted phenotype (Zhou et al., 2008), while our study used a Foxp3 YFP-Cre knockin strain that does not affect the overall phenotype of Cre-expressing mice (Rubtsov et al., 2008). In an adoptive transfer colitis model, while we saw the mild intestinal inflammation in the mice receiving KAP1-deficient Tregs, Chikuma et al. showed severe inflammation in the same disease model. This difference may be due to the composition of Tregs, which were transferred into lymphopenic mice. We observed that the majority of Tregs in the spleen in $Foxp3^{cre}KAP1^{f/f}$ mice were thymic-derived Tregs, based on the expression of Helios (data not shown). Since the inflammation in $LCK^{cre}KAP1^{f/f}$ mice was severe, it is possible that Tregs in these animals contain a decent amount of peripherally induced Tregs that are relatively unstable and less functional. Inflammatory conditions in $LCK^{cre}KAP1^{f/f}$ mice might change the suppression function of Tregs.

We showed that ectopic expression of Slc1a5 improved the ability of KAP1-deficient Tregs to proliferate upon stimulation. These data, along with previous work demonstrating that reconstitution of Slc1a5-deficient T cells with other glutamine transporters failed to rescue proliferation (Nakaya et al., 2014), suggest that Slc1a5 is the critical glutamine receptor in T cells, including Tregs. Interestingly, $CD4^+$ T cells from $LCK^{cre}KAP1^{f/f}$ mice are also defective in proliferation, partially due to reduced IL-2 production (Chikuma et al., 2012). Further study is required to address the exact mechanism to regulate Treg proliferation by KAP1 and the importance of Slc1a5 in Tregs.

In conclusion, the findings in this study advocate that KAP1 is an important transcription factor partner of Foxp3 in mouse Tregs and that it promotes Treg signature gene expression. Moreover, KAP1 is also critical for cell-cycle progression and the proliferation of Tregs, at least partially due to its ability to positively regulate Slc1a5 expression. Thus, KAP1 may be a therapeutic candidate for autoimmune diseases and cancer because of its unique ability to modulate the Foxp3-dependent and -independent gene expression and cell proliferation.

EXPERIMENTAL PROCEDURES

Mice

Foxp3-IRES-YFP/Cre, CD45.1, $RAG1^{-/-}$ mice were purchased from Jackson Laboratory. KAP1 floxed mice were described previously (Zhou et al., 2012). Sex-matched mice aged 8–12 weeks were used. $Foxp3^{cre}KAP1^{+/+}$ mice were used for controls for the most of experiments. All mice were housed in specific pathogen-free facilities, and all experiments were conducted in accordance with the Animal Care and Use Committee at the Benaroya Research Institute.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Flow Cytometry and Sorting

Antibodies for CD3 (2C11), CD4 (RM4-5), CD8a (53-6.7), CD44 (IM7), CD62L (MEL-14), TCR beta chain (H57-597), CD25 (ebio3C7 or PC61), CD45.1 (A20), CD45.2 (104), Ly6G (1A8), Ly6C (HK1.4), B220 (RA3-6B2), major histocompatibility complex (MHC) class II (M5/114.15.2), CD11b (M1/70), CD11c (N418), Siglec-F (E50-2440), Foxp3 (FJK-16 s), IFNγ (XMG1.2), IL-17A (TC11-18H10.1), IL-13 (eBio13A), IL-10 (JES5-16E3), KAP1 (20A1), Blimp-1 (5E7), and CTLA4 (UC10-4B9) were from eBioscience, BioLegend, or BD Biosciences. For YFP staining, rabbit polyclonal antibody to GFP (Thermo Fisher Scientific) was used. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (20 ng/mL), ionomycin (1 μg/mL), and monensin (2 μM) for 5 hr for cytokine staining. Intracellular staining was conducted using Foxp3 intracellular staining reagents from eBioscience. Dead cells were excluded from the analyses using the Zombie NIR Fixable Viability Kit (BioLegend). An apoptosis assay was performed using the Annexin V Apoptosis Detection Kit (eBioscience). $CD4^+$ T cells were isolated using the $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec), and Tregs were further purified by YFP using FACSAria II (BD Biosciences).

In Vitro Treg Expansion

For ChIP and coIP experiments, sorted $CD4^+YFP^+$ cells were cultured with Dynabeads Mouse T-Activator CD3/CD28 (Thermo Fisher Scientific) in the presence of 2,000 U/mL recombinant human IL-2 (R&D Systems) and 0.5 μg/mL rapamycin (Santa Cruz Biotechnology) for 14 days.

Cell-Cycle Analysis

Tregs from $Treg^{\Delta KAP1}$ or control mice were cultured in the presence of Dynabeads Mouse T-Activator CD3/CD28 for 4 days. Cells were then pulsed with BrdU (10 μM, BD Biosciences) and incubated for an additional hour. BrdU was stained by BrdU Flow Kits (BD Biosciences).

Phospho-flow

Cells were stimulated for 30 min in the presence or absence of anti-CD3 (1 μg/mL) and anti-CD28 (2 μg/mL) crosslinked with 10 μg/mL goat anti-hamster immunoglobulin G (IgG) (Sigma Aldrich). Cells were also stimulated with 5 μM L-glutamine (Thermo Fisher Scientific) in glutamine-free RPMI medium (Thermo Fisher Scientific) for 30 min. Phospho-flow was performed as previously described (Srivastava et al., 2014), and anti-pS6 antibody (cupk43k, eBioscience) was used.

In Vitro Suppression Assay

$CD4^+YFP^+$ T cells from $Treg^{\Delta KAP1}$ or control mice were isolated. $CD4^+CD62L^{high}$ cells were also isolated from CD45.1 mice and were labeled with 5-(and -6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen) and cultured with Dynabeads Mouse T-Activator CD3/CD28 for 4 days. Where indicated, KAP1-sufficient or -deficient Tregs were added to the culture at the indicated ratio.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Adoptive Transfer Colitis

Eight-week-old RAG1$^{-/-}$ mice were injected intraperitoneally with CD4^{+} CD45Rb^{high} cells (4×10^5) from CD45.1 mice. Mice were also injected with Tregs from Treg$^{\Delta KAP1}$ or littermate control mice (4×10^5) and weighted twice weekly. Mice were sacrificed at week 8, and the colons were isolated. The distal and proximal parts of the large intestine were subjected to the histological analysis. The rest of the large intestine was prepped as previously described (Eken et al., 2014). For histological analysis, H&E staining of proximal and distal parts of the large intestine were examined and scored in a blinded manner as described previously (Eken et al., 2014).

coIP

Treg nuclear lysate was immunoprecipitated using the Universal Magnetic Co-IP Kit (Active Motif). For the immunoprecipitation, anti-Foxp3 antibody (PA1-45126, Novus Biologicals) or normal rabbit IgG (Cell Signaling Technology) was used. For the immunoblotting, anti-KAP1 antibody (20A1, BioLegend), anti-Foxp3 antibody (150D, BioLegend), and horseradish peroxidase (HRP) goat anti-mouse IgG (Poly4053, BioLegend) were used.

Western Blot

Cells were lysed in RIPA buffer with 1% proteinase inhibitor cocktail (Sigma Aldrich) and subjected to SDS-PAGE. Antibodies used for immunoblotting are as follows: anti-β-actin antibody (2F1-1, BioLegend), anti-SLC1A5 antibody (ab58690, abcam), HRP goat anti-mouse IgG, and HRP donkey anti-rabbit IgG (Ploy4064, BioLegend).

ELISA

Mouse anti-nuclear antibody was measured using the Mouse ANA ELISA Kit (Neo Scientific).

DNA Methylation Assay

Genomic DNA was purified using NucleoSpin Tissue XS (Clontech). After sodium bisulfite treatment using the EpiTect Fast DNA Bisulfite Kit (QIAGEN), bisulfite-converted DNA was amplified by PCR and sub-cloned into the pCR4.1-TOPO Vector (Invitrogen). PCR primers were previously described (Ohkura et al., 2012). Plasmids from 16–32 colonies were purified and sequenced.

RNA-Seq

Total RNA was purified with the NucleoSpin RNA Kit (Clontech). RNA-seq libraries were prepared using the TruSeq RNA Library Prep Kit v2 (Illumina) and sequenced using an Illumina HiSeq 2500. Sequences were mapped to mm10 with TopHat2 (v2.1.1). Tag counts were obtained with HTSeq (v0.7.2), and further analysis was performed using the R package (DESeq2). An adjusted p value <0.05 was considered significant.

ChIP-Seq and ChIP-qPCR

Fresh isolated Tregs (for H3K9me3) and expanded Tregs (for KAP1) were used for ChIP-seq. Cells were cross-linked with 1% formaldehyde for 10 min at room temperature and

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

lysed. Nuclei were collected and sonicated using a Covaris M220 (Covaris). Lysates were incubated with anti-H3K9me3 antibody (ab8898, abcam), anti-KAP1 antibody (1Tb-1A9, Active Motif), or control IgG at 4°C overnight. Protein G beads (Cell Signaling Technology) were added to the lysates and incubated for 2 hr at 4°C. Beads were washed, and antibody/protein/DNA complexes were eluted. Samples were incubated at 65°C overnight to reverse cross-linking, and chromatin was recovered using a PCR purification kit (QIAGEN). ChIP-seq libraries were prepared by using the DNA SMART ChIP-Seq Kit (Clontech), and the libraries were subjected to sequence analysis with an Illumina HiSeq 2500. The obtained sequences (50 bp) were mapped to mm10 using Bowtie 2 (v2.2.9), and uniquely mapped reads were selected. Duplicated reads were discarded using Picard Tools (v2.1.1). For Figure 4B, we counted reads that were aligned between −1 kb and +1 kb of the TSSs using the 'intersect' tool in the bedtools software (v2.26.0). MSCS2 (v2.2.1) was used for the peak calling. For further analyses and visualization, ngsplot (v2.41), ChIPpeakAnno (v3.5), Homer (v4.9), and Integrative Genome Viewer (v2.3.91) were used. Sequences of qPCR primers for ChIP-qPCR are as follows: Prdm1 forward (fwd): CCAGTAGGCCTTT CATGGCT, Prdm1 reverse (rev): TGCTAGTTTCTTGGGGTGCG; Slc1a5 fwd: CCCCTTCTTAAGCCACCAAGA, Slc1a5 rev: AGATGCTGTCATGGGAGGGT.

Retrovirus-Mediated Gene Expression

Slc1a5 cDNA from pCLXSN-HA-Slc1a5 (Nakaya et al., 2014) was sub-cloned into pMSCV-IRES-CFP II (pMIC, Addgene) to create pMIC-Slc1a5. Tregs were stimulated with Dynabeads Mouse T-Activator anti-CD3/CD28 for 2 days and were infected with either pMIC or pMIC-Slc1a5 as previously described (Tanaka et al., 2014). Cells were labeled with CellTrace Far Red (Invitrogen) 3 days after infection and then cultured for an additional 3 days and subjected to the analysis.

In Vitro T Cell Differentiation

$CD4^+CD44^-CD62L^+$ naive T cells were cultured with 3 mg/mL plate-bound anti-CD3e (145-2C11, BioLegend) in the presence of 1 mg/mL anti-CD28 (37.51, BioLegend), 3 ng/mL TGF-β (R&D Systems), 10 ng/mL IL-2, 5 mg/mL anti-IL-4 (11B11, BioLegend), and 5 mg/mL anti-IFN-γ (XMG1.2, BioLegend) for 5 days. Cells were then rested in the larger cell-culture plate for another 3 days and subjected to the analysis.

Statistical Analyses

Data are summarized as means ± SD. The statistical analysis of the results was performed with the unpaired Student's t test and Mann-Whitney U test. For multiple comparison, one-way ANOVA and Turkey's multiple test were used. Two-way ANOVA was used for body weight change over the time, and p values were collected by the Sidak method. $p < 0.05$ was considered significant.

Data and Software Availability

The accession numbers for the ChIP-seq and RNA-seq datasets reported in this paper are Gene Expression Omnibus (GEO): GSE105128 (RNA-seq), GSE105508 (ChIP-seq of KAP1), and GSE105515 (ChIP-seq of H3K9me3).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Vivian Gersuk and Scott Presnell for RNA-seq and ChIP-seq support. S.T. was supported by the Japanese Society of Allergology and the Uehara Memorial Foundation. This project was supported by a grant from the NIH (1R01AI112323 to S.F.Z.).

References

Allan RS, Zueva E, Cammas F, Schreiber HA, Masson V, Belz GT, Roche D, Maison C, Quivy JP, Almouzni G, Amigorena S. An epigenetic silencing pathway controlling T helper 2 cell lineage commitment. Nature. 2012; 487:249–253. [PubMed: 22763435]

Beier UH, Wang L, Han R, Akimova T, Liu Y, Hancock WW. Histone deacetylases 6 and 9 and sirtuin-1 control Foxp3+ regulatory T cell function through shared and isoform-specific mechanisms. Sci Signal. 2012; 5:ra45. [PubMed: 22715468]

Beyersdorf N, Ding X, Tietze JK, Hanke T. Characterization of mouse CD4 T cell subsets defined by expression of KLRG1. Eur J Immunol. 2007; 37:3445–3454. [PubMed: 18034419]

Cammas F, Mark M, Dollé P, Dierich A, Chambon P, Losson R. Mice lacking the transcriptional corepressor TIF1beta are defective in early postimplantation development. Development. 2000; 127:2955–2963. [PubMed: 10851139]

Chikuma S, Suita N, Okazaki IM, Shibayama S, Honjo T. TRIM28 prevents autoinflammatory T cell development in vivo. Nat Immunol. 2012; 13:596–603. [PubMed: 22544392]

Cretney E, Xin A, Shi W, Minnich M, Masson F, Miasari M, Belz GT, Smyth GK, Busslinger M, Nutt SL, Kallies A. The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. Nat Immunol. 2011; 12:304–311. [PubMed: 21378976]

Eken A, Singh AK, Treuting PM, Oukka M. IL-23R+ innate lymphoid cells induce colitis via interleukin-22-dependent mechanism. Mucosal Immunol. 2014; 7:143–154. [PubMed: 23715173]

Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol. 2003; 4:330–336. [PubMed: 12612578]

Fu W, Ergun A, Lu T, Hill JA, Haxhinasto S, Fassett MS, Gazit R, Adoro S, Glimcher L, Chan S, et al. A multiply redundant genetic switch 'locks in' the transcriptional signature of regulatory T cells. Nat Immunol. 2012; 13:972–980. [PubMed: 22961053]

Herman AE, Freeman GJ, Mathis D, Benoist C. CD4+CD25+ T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion. J Exp Med. 2004; 199:1479–1489. [PubMed: 15184501]

Honda K, Littman DR. The microbiota in adaptive immune homeostasis and disease. Nature. 2016; 535:75–84. [PubMed: 27383982]

Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003; 299:1057–1061. [PubMed: 12522256]

Huang C, Martin S, Pfleger C, Du J, Buckner JH, Bluestone JA, Riley JL, Ziegler SF. Cutting Edge: a novel, human-specific interacting protein couples FOXP3 to a chromatin-remodeling complex that contains KAP1/TRIM28. J Immunol. 2013; 190:4470–4473. [PubMed: 23543754]

Iyengar S, Farnham PJ. KAP1 protein: an enigmatic master regulator of the genome. J Biol Chem. 2011; 286:26267–26276. [PubMed: 21652716]

Iyengar S, Ivanov AV, Jin VX, Rauscher FJ 3rd, Farnham PJ. Functional analysis of KAP1 genomic recruitment. Mol Cell Biol. 2011; 31:1833–1847. [PubMed: 21343339]

Kitagawa Y, Ohkura N, Kidani Y, Vandenbon A, Hirota K, Kawakami R, Yasuda K, Motooka D, Nakamura S, Kondo M, et al. Guidance of regulatory T cell development by Satb1-dependent super-enhancer establishment. Nat Immunol. 2017; 18:173–183. [PubMed: 27992401]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Komatsu N, Okamoto K, Sawa S, Nakashima T, Ohhora M, Kodama T, Tanaka S, Bluestone JA, Takayanagi H. Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis. Nat Med. 2014; 20:62–68. [PubMed: 24362934]

Li B, Samanta A, Song X, Iacono KT, Bembas K, Tao R, Basu S, Riley JL, Hancock WW, Shen Y, et al. FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. Proc Natl Acad Sci USA. 2007a; 104:4571–4576. [PubMed: 17360565]

Li X, Lee YK, Jeng JC, Yen Y, Schultz DC, Shih HM, Ann DK. Role for KAP1 serine 824 phosphorylation and sumoylation/desumoylation switch in regulating KAP1-mediated transcriptional repression. J Biol Chem. 2007b; 282:36177–36189. [PubMed: 17942393]

Li J, Xi Y, Li W, McCarthy RL, Stratton SA, Zou W, Li W, Dent SY, Jain AK, Barton MC. TRIM28 interacts with EZH2 and SWI/SNF to activate genes that promote mammosphere formation. Oncogene. 2017; 36:2991–3001. [PubMed: 28068325]

McNamara RP, Reeder JE, McMillan EA, Bacon CW, McCann JL, D'Orso I. KAP1 recruitment of the 7SK snRNP complex to promoters enables transcription elongation by RNA polymerase II. Mol Cell. 2016; 61:39–53. [PubMed: 26725010]

Nakaya M, Xiao Y, Zhou X, Chang JH, Chang M, Cheng X, Blonska M, Lin X, Sun SC. Inflammatory T cell responses rely on amino acid transporter ASCT2 facilitation of glutamine uptake and mTORC1 kinase activation. Immunity. 2014; 40:692–705. [PubMed: 24792914]

Nielsen AL, Ortiz JA, You J, Oulad-Abdelghani M, Khechumian R, Gansmuller A, Chambon P, Losson R. Interaction with members of the heterochromatin protein 1 (HP1) family and histone deacetylation are differentially involved in transcriptional silencing by members of the TIF1 family. EMBO J. 1999; 18:6385–6395. [PubMed: 10562550]

O'Geen H, Squazzo SL, Iyengar S, Blahnik K, Rinn JL, Chang HY, Green R, Farnham PJ. Genome-wide analysis of KAP1 binding suggests autoregulation of KRAB-ZNFs. PLoS Genet. 2007; 3:e89. [PubMed: 17542650]

Ohkura N, Hamaguchi M, Morikawa H, Sugimura K, Tanaka A, Ito Y, Osaki M, Tanaka Y, Yamashita R, Nakano N, et al. T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. Immunity. 2012; 37:785–799. [PubMed: 23123060]

Pan F, Yu H, Dang EV, Barbi J, Pan X, Grosso JF, Jinasena D, Sharma SM, McCadden EM, Getnet D, et al. Eos mediates Foxp3-dependent gene silencing in CD4+ regulatory T cells. Science. 2009; 325:1142–1146. [PubMed: 19696312]

Pedersen MT, Kooistra SM, Radzisheuskaya A, Laugesen A, Johansen JV, Hayward DG, Nilsson J, Agger K, Helin K. Continual removal of H3K9 promoter methylation by Jmjd2 demethylases is vital for ESC self-renewal and early development. EMBO J. 2016; 35:1550–1564. [PubMed: 27266524]

Rambaud J, Desroches J, Balsalobre A, Drouin J. TIF1beta/KAP-1 is a coactivator of the orphan nuclear receptor NGFI-B/Nur77. J Biol Chem. 2009; 284:14147–14156. [PubMed: 19321449]

Rowe HM, Jakobsson J, Mesnard D, Rougemont J, Reynard S, Aktas T, Maillard PV, Layard-Liesching H, Verp S, Marquis J, et al. KAP1 controls endogenous retroviruses in embryonic stem cells. Nature. 2010; 463:237–240. [PubMed: 20075919]

Rowe HM, Friedli M, Offner S, Verp S, Mesnard D, Marquis J, Aktas T, Trono D. De novo DNA methylation of endogenous retroviruses is shaped by KRAB-ZFPs/KAP1 and ESET. Development. 2013; 140:519–529. [PubMed: 23293284]

Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, Treuting P, Siewe L, Roers A, Henderson WR Jr, et al. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity. 2008; 28:546–558. [PubMed: 18387831]

Rudra D, de Roos P, Chaudhry A, Niec RE, Arvey A, Samstein RM, Leslie C, Shaffer SA, Goodlett DR, Rudensky AY. Transcription factor Foxp3 and its protein partners form a complex regulatory network. Nat Immunol. 2012; 13:1010–1019. [PubMed: 22922362]

Samstein RM, Arvey A, Josefowicz SZ, Peng X, Reynolds A, Sandstrom R, Neph S, Sabo P, Kim JM, Liao W, et al. Foxp3 exploits a pre-existent enhancer landscape for regulatory T cell lineage specification. Cell. 2012; 151:153–166. [PubMed: 23021222]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Schultz DC, Friedman JR, Rauscher FJ 3rd. Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD. Genes Dev. 2001; 15:428–443. [PubMed: 11230151]

Schultz DC, Ayyanathan K, Negorev D, Maul GG, Rauscher FJ 3rd. SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. Genes Dev. 2002; 16:919–932. [PubMed: 11959841]

Sekiya T, Kondo T, Shichita T, Morita R, Ichinose H, Yoshimura A. Suppression of Th2 and Tfh immune reactions by Nr4a receptors in mature T reg cells. J Exp Med. 2015; 212:1623–1640. [PubMed: 26304965]

Srivastava S, Koch MA, Pepper M, Campbell DJ. Type I interferons directly inhibit regulatory T cells to allow optimal antiviral T cell responses during acute LCMV infection. J Exp Med. 2014; 211:961–974. [PubMed: 24711580]

Tanaka S, Suto A, Iwamoto T, Kashiwakuma D, Kagami S, Suzuki K, Takatori H, Tamachi T, Hirose K, Onodera A, et al. Sox5 and c-Maf cooperatively induce Th17 cell differentiation via RORγt induction as downstream targets of Stat3. J Exp Med. 2014; 211:1857–1874. [PubMed: 25073789]

Tao R, de Zoeten EF, Ozkaynak E, Chen C, Wang L, Porrett PM, Li B, Turka LA, Olson EN, Greene MI, et al. Deacetylase inhibition promotes the generation and function of regulatory T cells. Nat Med. 2007; 13:1299–1307. [PubMed: 17922010]

Wang C, Ivanov A, Chen L, Fredericks WJ, Seto E, Rauscher FJ 3rd, Chen J. MDM2 interaction with nuclear corepressor KAP1 contributes to p53 inactivation. EMBO J. 2005; 24:3279–3290. [PubMed: 16107876]

Wu Y, Borde M, Heissmeyer V, Feuerer M, Lapan AD, Stroud JC, Bates DL, Guo L, Han A, Ziegler SF, et al. FOXP3 controls regulatory T cell function through cooperation with NFAT. Cell. 2006; 126:375–387. [PubMed: 16873067]

Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. Nature. 2010; 463:808–812. [PubMed: 20072126]

Zhou X, Jeker LT, Fife BT, Zhu S, Anderson MS, McManus MT, Bluestone JA. Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. J Exp Med. 2008; 205:1983–1991. [PubMed: 18725525]

Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martínez-Llordella M, Ashby M, Nakayama M, Rosenthal W, Bluestone JA. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. Nat Immunol. 2009; 10:1000–1007. [PubMed: 19633673]

Zhou VW, Goren A, Bernstein BE. Charting histone modifications and the functional organization of mammalian genomes. Nat Rev Genet. 2011; 12:7–18. [PubMed: 21116306]

Zhou XF, Yu J, Chang M, Zhang M, Zhou D, Cammas F, Sun SC. TRIM28 mediates chromatin modifications at the TCRα enhancer and regulates the development of T and natural killer T cells. Proc Natl Acad Sci USA. 2012; 109:20083–20088. [PubMed: 23169648]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Highlights

- Treg-specific KAP1-deficient mice develop autoimmune disease
- KAP1-Foxp3 complex modulates the expression of effector molecules
- KAP1 regulates cell proliferation partially through the upregulation of Slc1a5

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

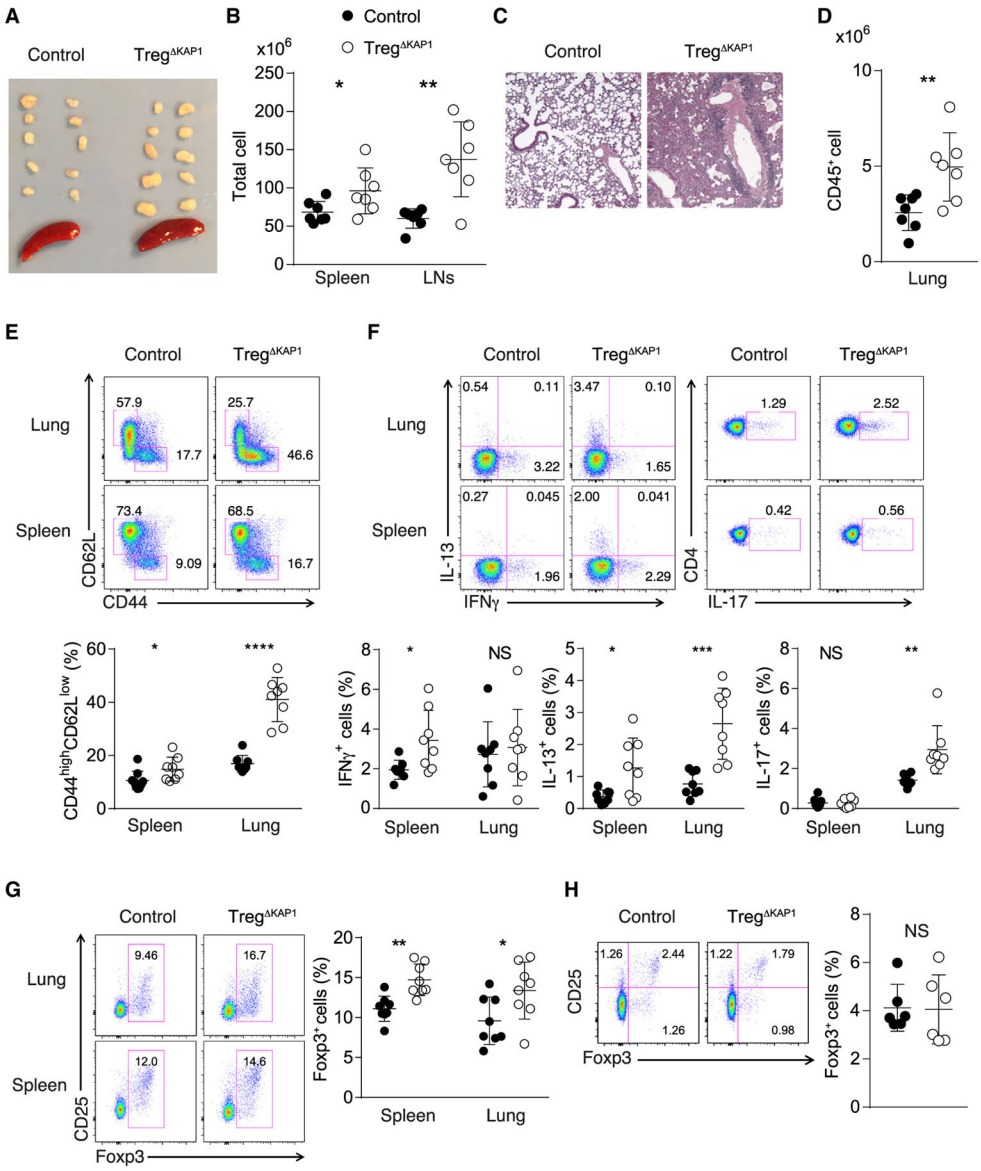


Figure 1. Treg-Specific KAP1-Deficient Mice Develop Autoimmune Disease

(A) Enlarged lymph nodes and spleen in 8-week-old Treg$^{\Delta KAP1}$ mice.

(B) Total cell number in the spleen and the lymph nodes.

(C) Representative lung histology stained with H&E.

(D) Total CD45^{+} cell number in the lung.

(E–G) Flow-cytometric analysis of CD4^{+} T cells from Treg$^{\Delta KAP1}$ and control mice in the spleen and the lung for (E) CD44 and CD62L, (F) IFNγ, IL-13, and IL-17, and (G) Foxp3.

(H) Flow-cytometric analysis of thymic CD4^{+} Foxp3^{+} cells.

Data are expressed as mean ± SD of n = 6–8 mice pooled from 2–3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. NS, not significant. See also Figures S1 and S2.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

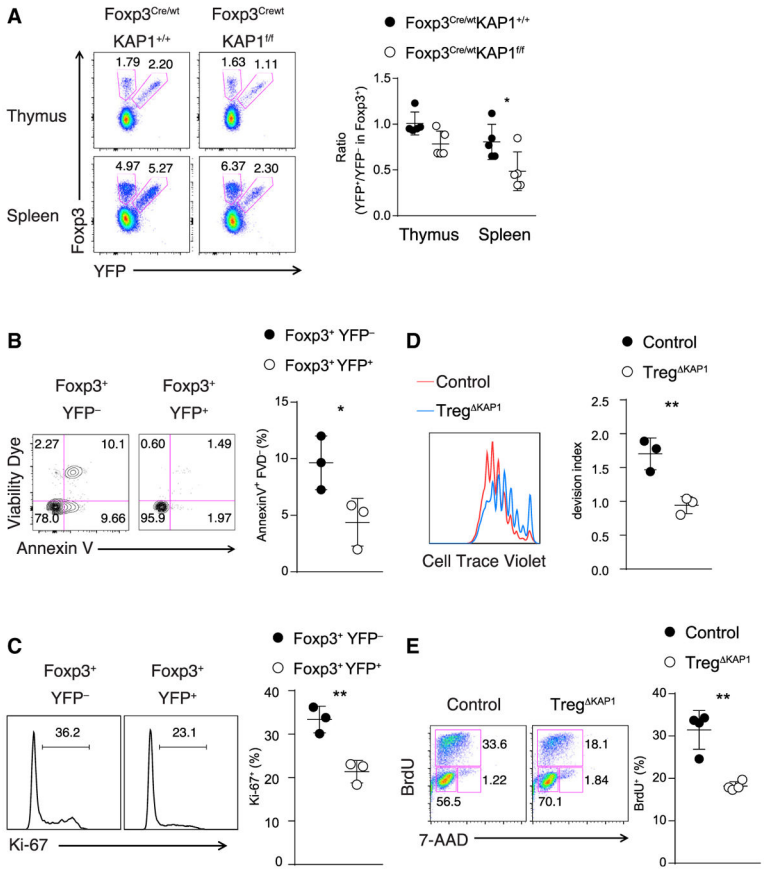


Figure 2. KAP1-Deficient Tregs Are Less Proliferative but Not Apoptotic

(A) Flow-cytometric analysis of CD4$^+$ T cells from Foxp3$^{cre/wt}$KAP1$^{+/+}$ females and Foxp3$^{cre/wt}$KAP1$^{f/f}$ females for Foxp3 and YFP. Data are expressed as mean ± SD of n = 5 mice from 2 independent experiments.

(B) Apoptotic cell analysis. FVD, fixable viability dye.

(C) Flow-cytometric analysis of CD4$^+$ Foxp3$^+$ T cells from Foxp3$^{cre/wt}$KAP1$^{f/f}$ females for Ki-67.

(D) Dilution of CTV of splenic Tregs from Treg$^{\Delta KAP1}$ mice and control mice.

(E) BrdU uptake assay was determined by flow cytometry.

Data shown in (B)–(E) are expressed as mean ± SD of n = 3–4 from 3 independent experiments. *$p < 0.05$, **$p < 0.01$.

See also Figure S3.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

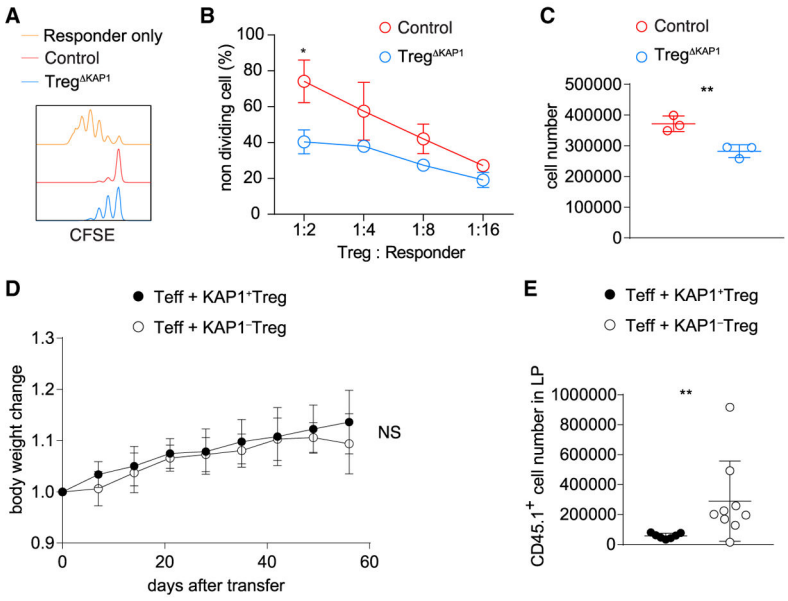


Figure 3. KAP1-Deficient Tregs Are Less Suppressive in Both *In Vitro* and *In Vivo*

(A–C) *In vitro* suppression assay. Tregs from Foxp3^{cre}KAP$^{+/+}$ mice or Foxp3^{cre}KAP$^{f/f}$ mice were co-cultured with CFSE-labeled CD4^{+} CD25^{-} T cells in the presence of anti-CD3/CD28 for 4 days.

(A) The dilution of CFSE was determined by flow cytometry.

(B) Representative histogram of CFSE dilution (Treg:responder T cell ratio = 1:2) and cumulative data (mean ± SD) from 3 independent experiments at the indicated ratio of Tregs to responder T cells.

(C) The cell number of Tregs 4 days after incubation.

(D and E) Adoptive transfer colitis model.

(D) Changes in body weight after cell transfer. Data were expressed as mean ± SEM from 3 independent experiments (n = 7–9).

(E) Total CD45.1^{+} responder cells in the lamina propria.

Data are expressed as mean ± SD from 3 independent experiments (n = 7–9). *p < 0.05, **p < 0.01.

See also Figure S4.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

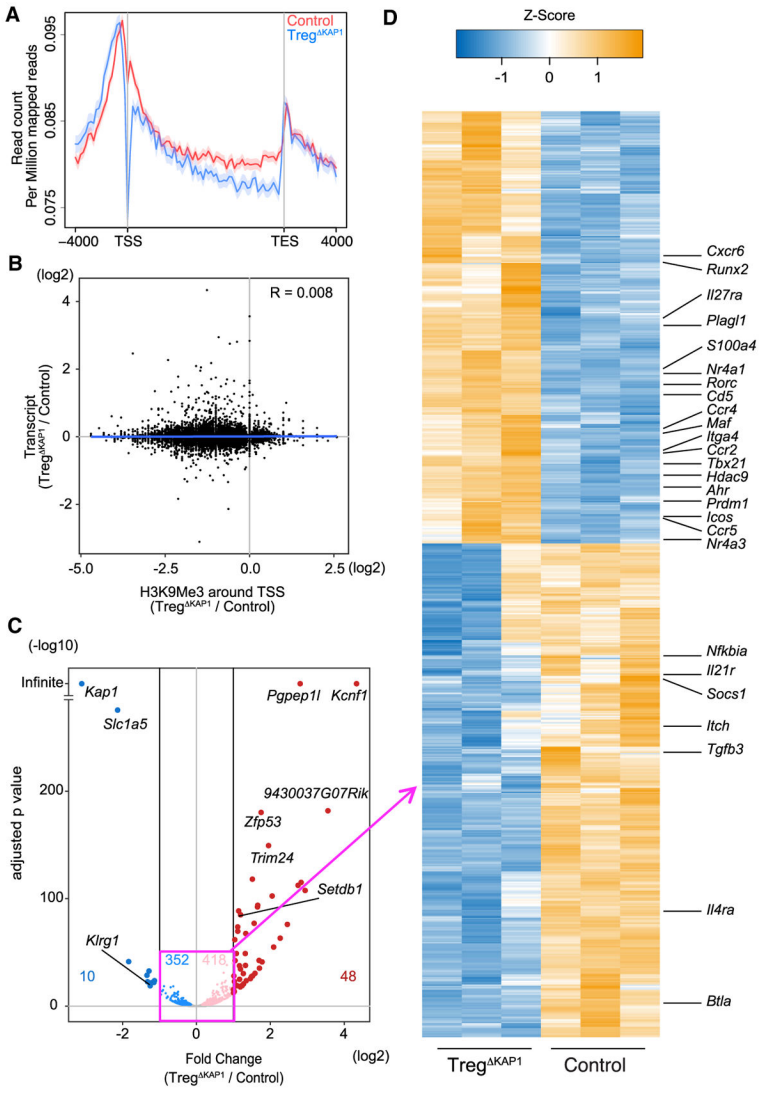


Figure 4. Dysregulated Gene Expression in KAP1-Deficient Tregs

(A) The distribution of H3K9me3 marks along the gene body. Tregs from Treg$^{\Delta KAP1}$ mice and littermate control mice were subjected to ChIP experiment.

(B) Scatterplot of the fold change of transcripts determined by RNA-seq against the fold change of H3K9me3 marks around TSS between KAP1-sufficient and -deficient Tregs. The correlation coefficient was determined by spearman's test.

(C) Changes in gene expression and adjusted p values in KAP1-sufficient Tregs versus KAP1-deficient Tregs as a volcano plot.

(D) Heatmap of 770 differentially expressed genes whose fold change is less than 2 in KAP1-sufficient Tregs versus KAP1-deficient Tregs.

See also Figure S5.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

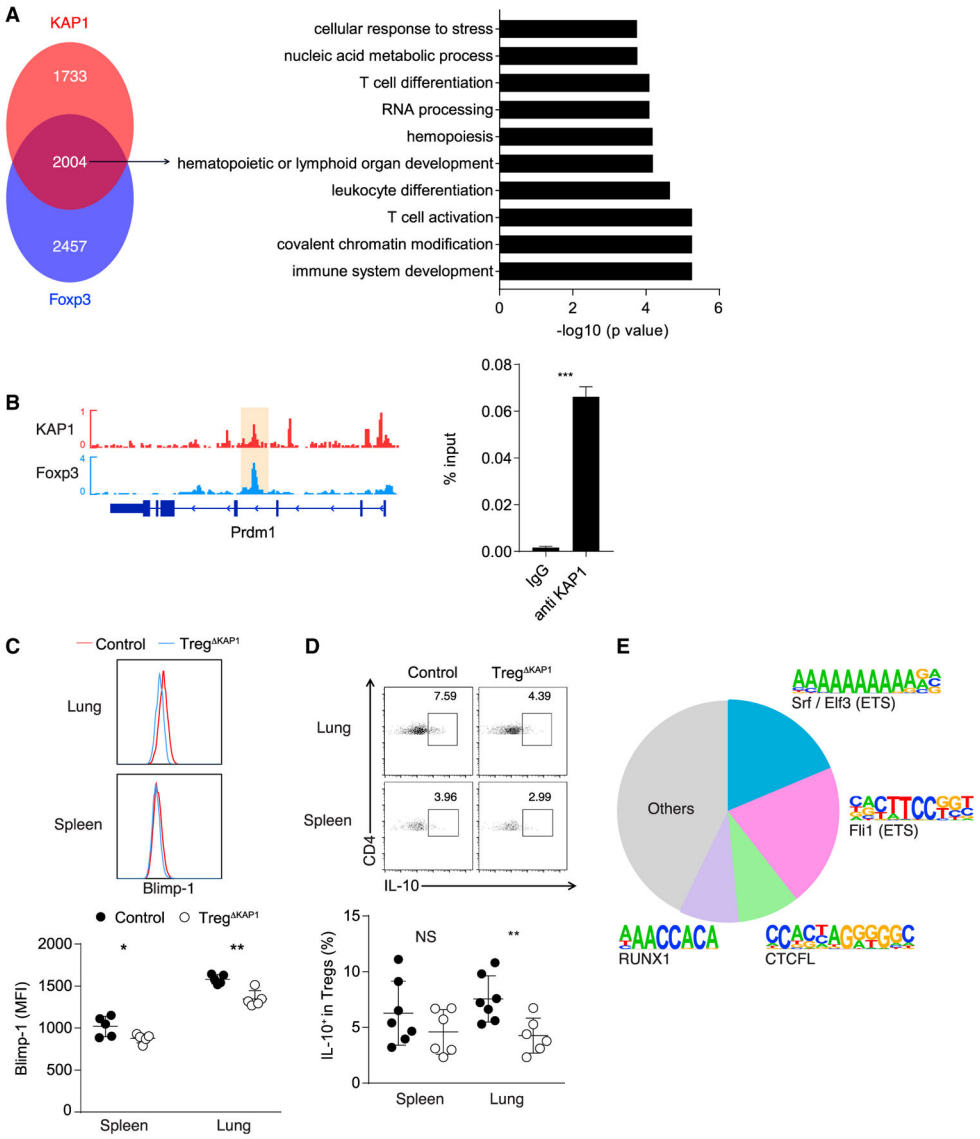


Figure 5. KAP1 Directly Regulates Treg-Associated Gene Expression together with Foxp3

(A) Venn diagram showing the overlap of the genes bound by KAP1 and the genes bound by Foxp3 (left panel). Enriched GO terms of 2,004 genes that were co-occupied with KAP1 and Foxp3 (right panel).

(B) ChIP-seq of KAP1 and Foxp3 tracks at *Prdm1* locus (left panel). Highlighted is the peak called by MACS2. KAP1 binding in the intronic region by ChIP-qPCR is shown in right panel. Data are expressed as mean ± SD of n = 3 from 3 independent experiments.

(C) Representative histogram of Blimp-1 expression in KAP1-sufficient and -deficient Tregs in the spleen and the lung and cumulative MFI of Blimp-1 (lower panel) (n = 5 from 2 independent experiments).

(D) Representative flow plots (gated on Foxp3$^+$ cells) of IL-10 in the spleen and the lung (upper panel) and cumulative data (lower panel) (n = 6–7).

(E) Proportion of KAP1 binding sites that contain the indicated motifs.

Data are expressed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001. NS, not significant.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

See also Figure S6.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

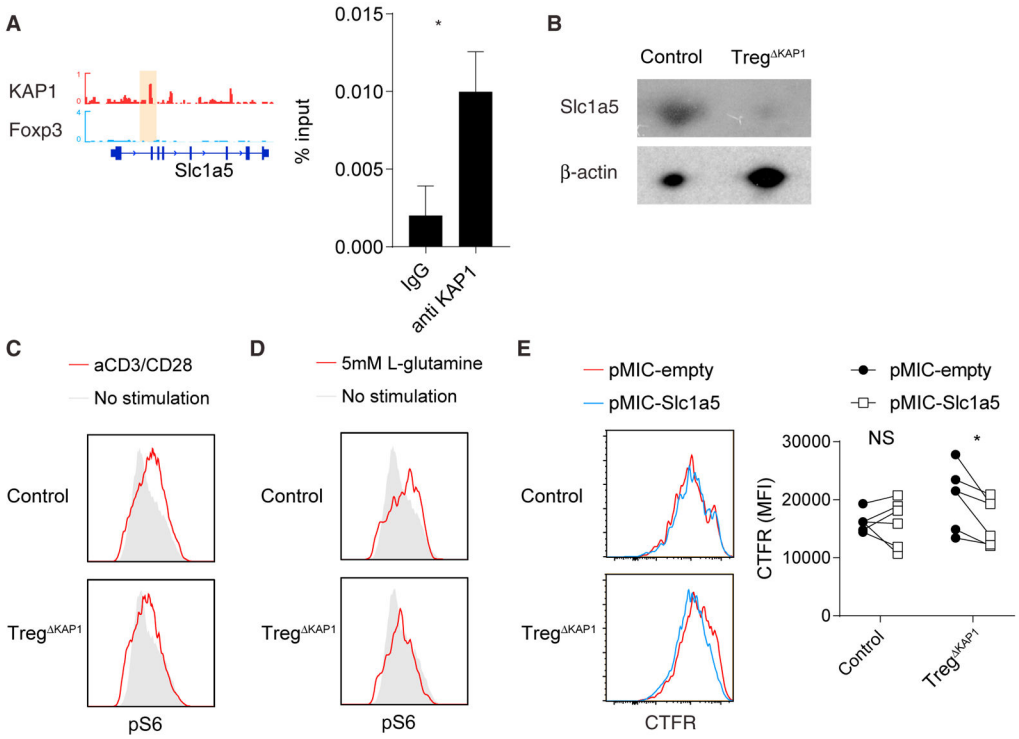


Figure 6. KAP1 Positively Regulates Slc1a5 Expression Followed by mTORC1 Activation in a Foxp3-Independent Manner

(A) ChIP-seq of KAP1 and Foxp3 tracks at *Slc1a5* locus (left). Highlighted is the peak called by MACS2. KAP1 binding in the conserved non-coding sequence of *Slc1a5* by ChIP-qPCR is indicated (right). Data are expressed as mean ± SD of n = 3 from 3 independent experiments. *$p < 0.05$.

(B) Slc1a5 expression in KAP1-sufficient and -deficient Tregs. Data are representative of 3 independent experiments.

(C and D) Phosphorylation of S6 ribosomal protein. Splenocytes from $Treg^{\Delta KAP1}$ mice and control mice were stimulated with (C) anti-CD3/CD28 antibody or (D) 5 mM L-glutamine. Data are representative of 3 independent experiments.

(E) Cell proliferation of Slc1a5-expressing KAP1-deficient Tregs determined by CellTrace Far Red (CTFR) dilution. The histograms (gated on $YFP^{+}CFP^{+}$) are representative of 4 independent experiments, and the cumulative data are also shown (n = 6 each). Data are expressed as mean ± SD. *$p < 0.05$ (paired t test). NS, not significant.