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A Novel, Human-Specific Interacting Protein Couples FOXP3 to a Chromatin Remodeling Complex that Contains KAP1/TRIM28

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Abstract

Regulatory T cells (Treg) play a pivotal role for the maintenance of immunological self-tolerance. Deficiency or dysfunction of Treg leads to severe autoimmune diseases. While the *forkhead/winged-he*lix family member FOXP3 is critical for Treg differentiation and function, the molecular basis for FOXP3 function remains unclear. Here we identified and characterized a human-specific FOXP3 interacting protein, referred to as FIK (FOXP3-Interacting KRAB-domain containing protein). FIK is highly expressed in Tregs and acts as a bridging molecule to link FOXP3 with the chromatin remodeling scaffold protein KAP1 (TIF-1β/TRIM28). Disruption of the FOXP3-FIK-KAP1 complex in Tregs restored expression of FOXP3-target genes and abrogated the suppressor activity of the Tregs. These data demonstrate a critical role for FIK in regulating FOXP3 activity and Treg function.

Introduction

An important aspect of immune regulation is the establishment and maintenance of tolerance to self-antigens. In the periphery this is largely accomplished by a population of CD4 T cells, referred to as regulatory T cells (Tregs) that express the transcription factor Foxp3 (1). These cells are critical for the suppression of pathological immune responses to both self and foreign antigen, and deficiency or dysfunction of Tregs leads to severe autoimmune disease (2,3). The development and function of Tregs is dependent on the *forkhead*/winged-helix transcription factor FOXP3 (4–6); ablation of Foxp3 in mice, and FOXP3 mutations in humans with IPEX syndrome, leads to the rapid onset of fatal autoimmune lymphoproliferative disease (7). In addition, ectopic expression of Foxp3 in naïve CD4 T cells from humans and mice leads to the generation of T cells with suppressor activity (4,8). While the importance of FOXP3 in Tregs is clear, the molecular mechanism by which it controls gene expression in Tregs is largely unknown. In this report we show that in human

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Tregs a novel interacting protein couples FOXP3 to a repressive chromatin remodeling complex that includes the adaptor KAP1/TRIM28. The interacting protein is found only in human Tregs, and demonstrates a fundamental difference in the mechanism by which FOXP3 regulates transcription in human and mouse regulatory T cells.

Materials and Methods

Yeast 2-hybrid screen

Yeast 2-hybrid screen was performed as described previously (9).

Antibodies

Antibodies were purchased from eBiosciene (San Diego, CA; FOXP3, CD152, IL-2 and IFNγ), Biolegend (San Diego, CA; FOXP3), abcam (Cambridge, UK; FOXP3 and KAP1), BD Pharmingen (San Jose, CA; CD4 and CD25). Anti-human FIK antibody was raised by immunizing rabbit with the peptide SKGERRQKLPRKNP (corresponding to amino acide 99 to 112 of human FIK) conjugated to FKL.

Cell culture and transfection

HEK 293T cells and Jurkat T cells were maintained as previously described (9) HEK 293T cells were transfected by TransIT-LT1 transfection reagent (Mirus, Madison WI). Jurkat T cells were transfected using Amaxa cell line nucleofector kit V (Lonza). CD4 T cells were isolated from peripheral blood mononuclear cells (PBMC) using human naïve CD4 T cells isolation kit II (Miltenyi Biotech, Auburn CA). CD4+CD25− naïve T cells and CD4+CD25+ Treg cells were isolated by cell sorting.

Reverse transcription and Quantitative PCR (qPCR)

Total RNA was isolated using TRIzol reagent and GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St. Louis, MO). Quantitative real-time PCR was performed as described (9) using these primers: human *FOXP3*; forward: 5’-*TGACCAAGGCTTCATCTGTG*-3’; reverse: 5’-*GAGGAACTCTGGGAATGTGC*-3’. Human *FIK*; forward: 5’-*GTTTCCAAGCCAGAGGTGA*-3’; reverse: 5’-*CCCAGATGGTAGCGACAGC*-3’.

Human *IL-2*; forward: 5’-*ATTACAAGAATCCCAAACTC*-3’; reverse: 5’-*ATTGCTGATTAAGTCCCT*-3’. Human *IFN-γ*, forward: 5’-*ACTTCTTTGGCTTAATTCTC*-3’; reverse: 5’-*TCCATTATCCGCTACATC*-3’. Human *GAPDH*; forward: 5’-*GGATTTGGTCGTATTGGG*-3’; reverse: 5’-*GGAAGATGGTGATGGGATT*-3’.

Co-immunoprecipitation(co-IP) and western blot

Co-IP and western blot were performed as described previously (9).

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed according to manufacturer’s instruction (Upstate Biotechnology, Lake Placid NY). The primers used for ChIP are as follows: human *IL-2*

promoter; forward: 5'-*CTCTTGCTCTTGTCCACCAC*-3'; reverse: 5'-*ATCCCTCTTTGTTACATTAGCC*-3'. Human *IFN-γ* promoter; forward: 5'-*CCAGTCCTTGAATGGTGTGAAGT*-3'; reverse: 5'-*TAACTAAGGTTTTGTGGCATTTGGG*-3'.

Retroviral and lentiviral infection

Full-length human *FOXP3* and *FIK* were cloned into retroviral vectors pMIGR1 (*FOXP3*) or pLXSN-NGFR (*FIK*) and packaged as previously described (10). Jurkat T cells were infected by retrovirus encoding FOXP3 and/or FIK by spin infection. Full-length human FIK or dominant negative (dnFIK) was cloned into lentivirus vector pCVL.MND.SceOPT. 2A.GFP (11).

RNA interference

siRNA oligonucleotides specific to human *FOXP3* (siRNA sequence: CACUCAAUGAGAUCUACCA[dT][dT]) and *KAP1* (siRNA sequence: GCUCUACUGGGCCAGCCA A[dT][dT]) (Sigma) were introduced into primary human Tregs by nucleofection using an Amaxa instrument (Lonza).

Treg suppressive assay

$CD4^+CD25^+CD45RA^+$ primary human T cells were sort purified and transduced with lentiviral vectors as previously described (11) or transfected with siRNA as described above. Suppression activity was measured using CFSE dilution assays as previously described (12) or BD FastImmune Regulatory T-Cell Function Kit according to manufacturer's instruction with minor modifications. The percent suppression was calculated using the formula: (1 − number of Teff divisions in suppressed condition/number of Teff divisions in unsuppressed condition) × 100.

Results and Discussion

To gain an understanding as to the molecular basis of FOXP3-mediated transcriptional regulation we performed a yeast 2-hybrid screen using the amino terminal 200 amino acids of FOXP3 as bait and human Treg cDNA as prey. This region was chosen as we had previously shown that it contained sequences required for FOXP3 at act as a transcriptional repressor (13). Among the sequences that bound to this region of FOXP3 were RORα and RORγt, which provided important insights into the role of FOXP3 in the regulation of Treg/Th17 differentiation (9,10). In addition, a clone that encoded a protein of 113 amino acids was isolated, with the first 85 amino acids identical to those in the Krüppel family gene *ZFP90*, while the C-terminal 26 amino acids are unique (Fig. S1A). An examination of the gene structure showed that the mRNA encoding this protein contains the first 3 exons of *ZFP90*, in addition to a unique sequence that is 3' of the *ZFP90* gene, suggesting this mRNA was generated through alternative splicing of the *ZFP90* mRNA (data not shown). The *ZNF90* sequences retained in the mRNA encode a Kruppel-associated box (KRAB) domain was isolated (Fig. S1A). This domain, which is present in about one third of the 300~700 human zinc-finger proteins (ZFPs), is one of the most widely distributed transcriptional repression domains yet identified in mammals (14,15). KRAB domains are

50–75 amino acid sequences consisting of two motifs, referred to as the A and B boxes. The A box recruits a co-repressor complex through association with KAP1/TIF1β/TRIM28 (referred to here as KAP1), while the function of the B box remains unknown (16,17). Based on the presence of a KRAB domain, we will refer to this protein as **F**OXP3-**I**nteracting **K**RAB domain-containing protein (FIK), which becomes the third example of a novel protein generated from differential splicing of a Kruppel-family ZnF gene (18,19). Interestingly, we were unable to find a sequence homologous with the unique C-terminal sequence of *FIK* in the mouse genome, although an ortholog of ZFP90 was found in mouse chromosome 8 (data not shown). Thus, FIK is a human-specific FOXP3 interacting protein. Consistent with its role in Tregs, CD4+CD25+ human T cells expressed the spliced mRNA encoding *FIK* (Fig. 1A), and the ability of FIK to interact with FOXP3 was shown by co-immunoprecipitation from CD4+CD25+ human T cells (Fig. 1B).

To map the interacting domains within FOXP3 and FIK, we constructed a truncation series for each and used co-immunoprecipitation following transient transfection. As shown in Figure S1B, the proline rich N-terminal of FOXP3 especially the amino acid 106–198 is required to interact with FIK. Interestingly, this region of FOXP3 can also interact with Eos to mediate gene silencing in Treg cells (20). In addition, Li et al. (21) showed that this region of FOXP3 was important for its ability to interact with Tip60 and HDACs. Taken together, these data suggest that this region of FOXP3 is critical in regulating its association with repressive chromatin remodeling complexes, possibly through interactions involving FIK. The unique C-terminal region of FIK is necessary and sufficient for interacting with FOXP3 (Fig. S1C).

As mentioned previously, a major feature of FIK is the presence of a KRAB domain (Fig. S2A). As described above, this domain, found primarily in about one third of eukaryotic Kruppel-type C2H2 zinc finger proteins, serves as a binding site for the scaffolding protein KAP1 (16,17). Gene silencing requires the binding of the KRAB domain to the RING-B box-coiled coil (RBCC) domain of the co-repressor KAP1. KAP1 then represses gene transcription by recruiting the histone methyltransferase SETDB1, heterochromatin protein 1 (HP1) and the NuRD histone deacetylase complex (17). The KRAB domain in FIK contains both A box and B box, both of which are required for it to bind to KAP1 (Fig. S2B). These data suggest a model whereby FIK acts as a bridge between FOXP3 and a gene-silencing chromatin remodeling complex that contains KAP1. To test this hypothesis we first asked whether FOXP3, FIK, and KAP1 formed a complex in cells. As shown in Figure 2A, following co-transfection of expression plasmid encoding each protein, immunoprecipitating KAP1 (anti-FLAG), FOXP3 could be co-immunoprecipitated. We also transduced the human T cell line Jurkat (which does not express FOXP3 or FIK, but does express KAP1) with retroviruses encoding these genes to determine their role in regulating IL-2 and IFN-γ expression. After 3 days, cells transduced with both retroviruses were sorted, stimulated with PMA plus ionomycin for 4 hours and IL-2 and IFN-γ expression were analyzed. As shown in Figure 2B, FOXP3 alone can suppress IL-2 and IFN-γ expression, while FIK alone cannot. However, cells transduced with both genes showed a significantly decreased level of IL-2 and IFN-γ expression, consistent with a role for FIK in FOXP3 function.

We next performed ChIP assay to determine whether the FOXP3-FIK-KAP1 complex physically binds to a target gene promoter. Human CD4+CD25+ cells were isolated and expanded in vitro. A ChIP assay was performed using antibodies against FOXP3, FIK, and KAP1. As shown in Figure 2C, we were able to immunoprecipitated all 3 proteins from the same site on the IL-2 promoter. Similar results were obtained using a site from the IFN-γ promoter (Fig. 2D). Taken together, these data demonstrate that FOXP3, FIK and KAP1 can form a complex that is present on the promoters of FOXP3-repressed genes.

Next we investigated if blockade of FOXP3-FIK-KAP1 interaction in Treg cells would affect gene expression and suppressor function in Tregs. We initially attempted to generate siRNAs to target *FIK*, but were unsuccessful in finding sequences that led to demonstrable knockdown of *FIK* mRNA. We next took advantage of the fact that C-terminus of FIK was capable of binding to FOXP3, but that the entire KRAB domain was required for KAP1 interaction (Fig S1 and S2). Using this information we designed a form of FIK that would bind to FOXP3, but not to KAP1 (amino acids 75–113), and thus act as a dominant-negative (dnFIK), competing with endogenous FIK for binding to FOXP3. A lentiviral vector encoding this fragment linked to GFP via a 2A sequence was used to transduce human CD4+CD25+ Tregs. Cells expressing dnFIK showed increased IL-2 and IFN-γ production following stimulation, 2 genes known to be repressed by FOXP3 (Fig. 3A). However, expression of genes that have been shown to be positively regulated by FOXP3 (CTLA-4 and CD25) was unaffected by dnFIK (data not shown).

We next determined whether inhibiting FOXP3-KAP1 interactions affected Treg function. For these studies we again transduced activated human CD4+CD25+ T cells with the dnFIK Lentivirus and sorted GFP+ cells. The infected Tregs were cultured at various ratios with CFSE-labeled PBMC and stimulated with anti-CD3 beads. Proliferation was measured by CFSE dilution of CD8+ T cells. We found that Treg cells expressing dnFIK had a dramatically reduced ability to suppress the proliferation of conventional CD8 T cells (Fig. 3B and 3C), showing that FOXP3-FIK-KAP1 interactions play an important role in human Treg function.

These studies were extended to directly determine the role of KAP1 in human Tregs, targeting *FOXP3* and *KAP1* with siRNAs. Human CD4+CD25+ T cells were expanded in vitro with anti-CD3/CD28 beads plus 300U/ml IL-2 as described (23). Then these expanded Treg cells were treated with siRNA specific to human *FOXP3* and *KAP1*, after 3 days, these cells were further stimulated with PMA plus ionomycin and cytokine expression was assessed. Knockdown of FOXP3 or KAP1 in CD4+CD25+ Treg cells resulted in increased expression of IL-2 and IFN-γ (Fig. 4A and B). Concomitant with this increased expression, the *IL-2* promoter displayed an increased in marks consistent with open chromatin (acetylated histone H4) and a decreased tri-methyl histone H3K9, a mark of closed chromatin (Fig. 4C). Finally, we determined the ability of the *FOXP3*- and *KAP1*-knockdown Tregs to suppress T cell proliferation in vitro. Both cell populations had a dramatic reduction in suppressor function in vitro (Fig. 4D). These data demonstrate that KAP1 is important for FOXP3 and Treg function.

KAP1 is a ubiquitously expressed multifunctional protein that has been shown to be a critical factor for chromatin stability in both humans and mice (17). Recently Chikuma *et al.* reported that mouse T cells require Kap1 for proper cell-cycle progression in response to TCR stimulation (24). In addition, they showed that mice with T cell-specific deletion of KAP1 develop spontaneous autoimmunity (24). However, Tregs from these mice display equivalent suppressor function in vitro as their wild-type counterparts, suggesting that the defect in these animals is in the effector T cell compartment. These data are consistent with our model as mice lack a FIK ortholog, and thus cannot couple Kap1 to Foxp3. Further support comes from an exhaustive analysis of Foxp3 interacting proteins that failed to identify Kap1 (25). Taken together, these data suggest that the mechanism of transcriptional repression regulated by FOXP3 in human and mouse Tregs differs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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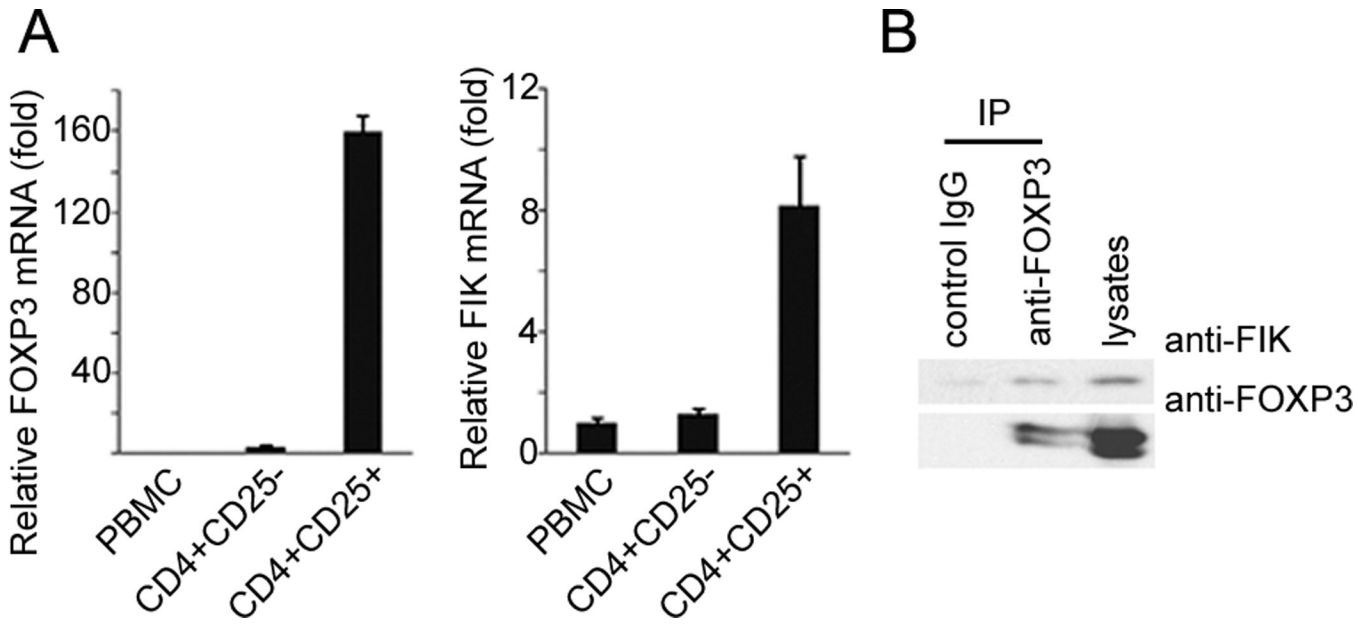

Figure 1. FIK is highly expressed in Treg cells, and physically interacts with FOXP3

A. PBMC were isolated and sorted into CD4+CD25− naïve T cells and CD4+CD25+ Treg cells. Reverse transcription and qPCR were performed as described. Expression of *FOXP3* and *FIK* mRNA relative to control *GAPDH* (in triplicate wells ± SD) was determined. Transcript level in PBMC group was considered as 1. **B.** Cell lysates from expanded CD4+CD25+ Treg cells were subject to co-IP experiment. Anti-human FOXP3 antibody was used as immunoprecipitation antibody. Western blot were performed to detect both Foxp3 and FIK. Data represent three or more independent experiments. Error bars indicate standard deviation.

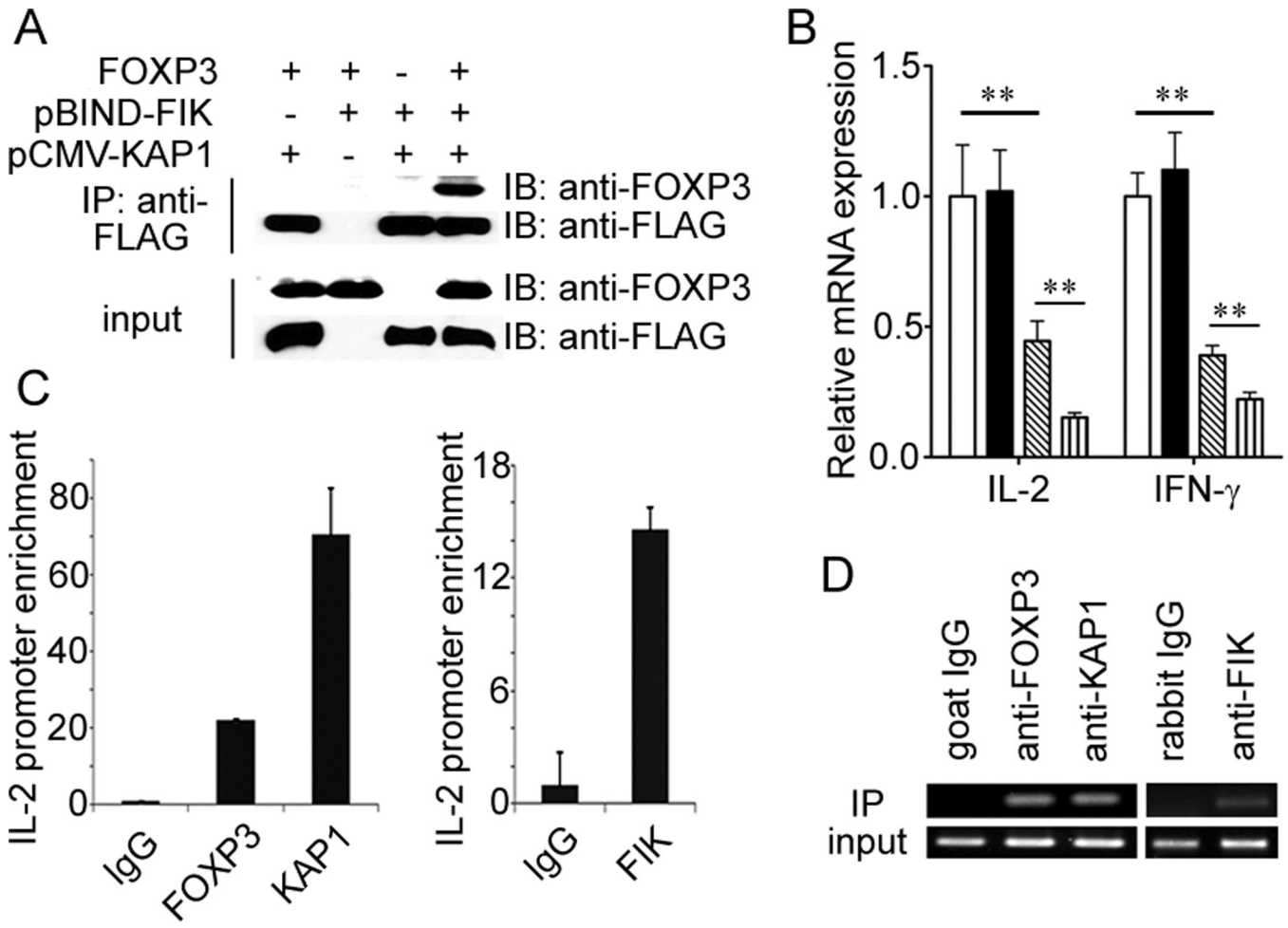

Figure 2. FOXP3, FIK, KAP1 form a transcription suppressive complex in Treg cells

A. HEK 293T cells were transfected with plasmids as indicated. Co-IP experiments were performed as described using anti-FLAG antibody. **B.** Jurkat T cells were infected with retrovirus encoding FOXP3 and FIK as described. After 3 days, double positive cells were sorted and stimulated. *IL-2* and *IFN-γ* transcription relative to *GAPDH* were quantified by qPCR analysis. Open bar: MIGR1+NGFR; Filled bar: MIGR1+FIK; Hashed bar: FOXP3+NGFR; bar represents FOXP3+FIK. Transcript level in control group infected with MIGR1 plus NGFR was set as 1. **C and D.** ChIP assays were performed as described in Materials and Methods. Goat anti-human FOXP3, goat anti-human KAP1 and rabbit anti-human FIK antibodies were used to pull down the protein-DNA complexes, followed by qPCR analysis (**C**) or gel electrophoresis (**D**) using primers specific to human *IL-2* **(C)** and *IFN-γ* **(D)** promoter. Data are shown as mean ± s.d. and represent three independent experiments.

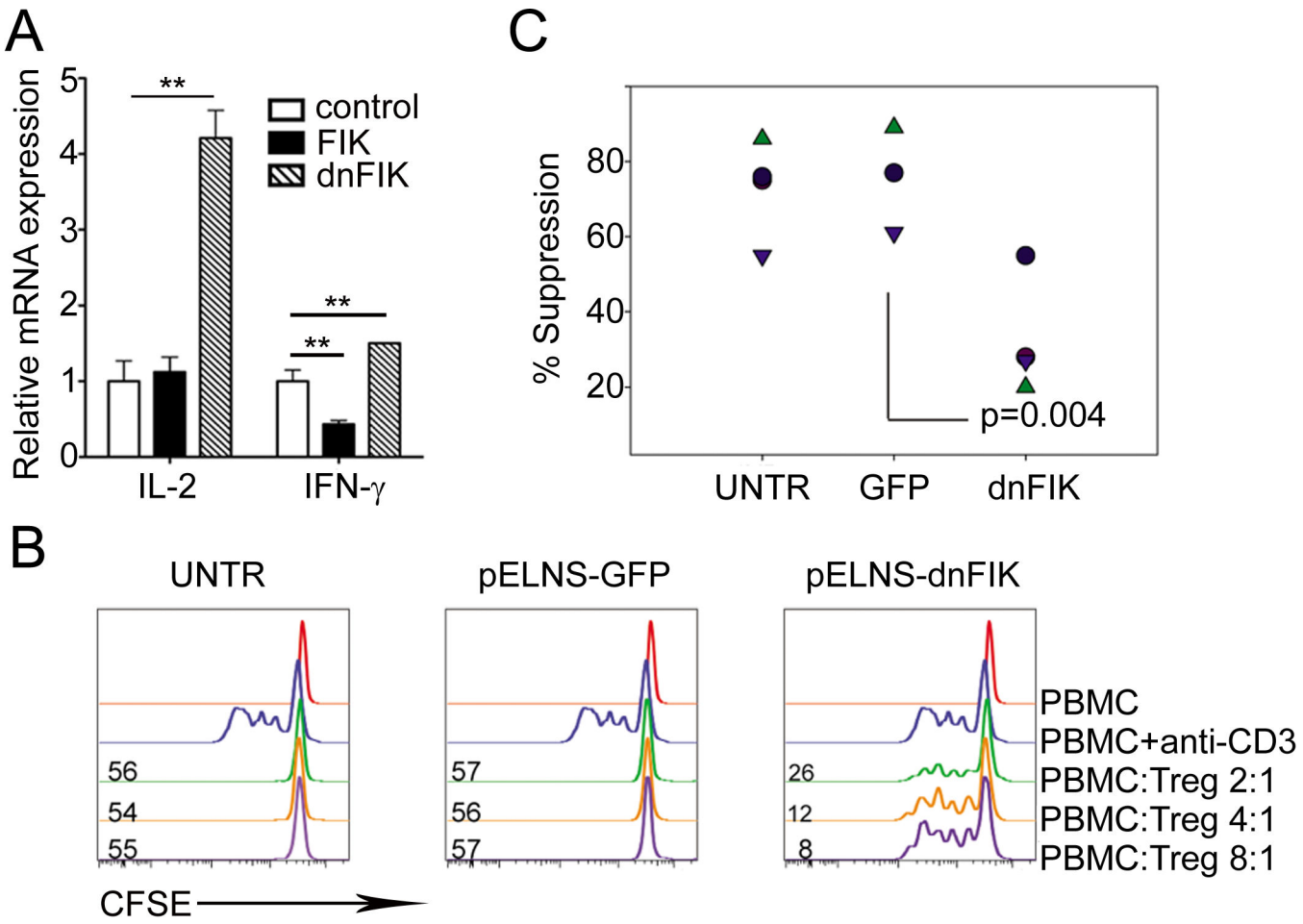

Figure 3. FOXP3-FIK interaction is essential for Treg cells function

CD4+CD25+ Treg cells were maintained in X-VIVO medium with anti-CD3/CD28 beads plus 300U/ml IL-2. These cells were infected by lenti-virus encoding full length FIK or dnFIK or corresponding control virus. GFP+ cells were sorted after 4 days and analyzed. **A.** Sorted GFP+ cells were stimulated with anti-CD3/CD28 beads for 4 hours. Total RNA was reverse transcript and qPCR was performed to determine *IL-2* and *INF-γ* expression. **B.** Sorted GFP+ cells were mixed with CFSE labeled PMBC in the presence of anti-CD3 beads and cultured for 4 days. CFSE dilution of CD8+ cells were accessed by flow cytometry. UNTR represents un-treated Treg cells; pELNS-GFP represents Treg cells infected by control virus and pELNS-dnFIK represents Treg cells infected by virus encoding dnFIK. The digits beside the peak represented suppressive activity of each group. **C.** Summary of four independent suppressive assays. P value between GPF group and dnFIK group is <0.004. Data are shown as mean ± s.d. and represent three independent experiments.

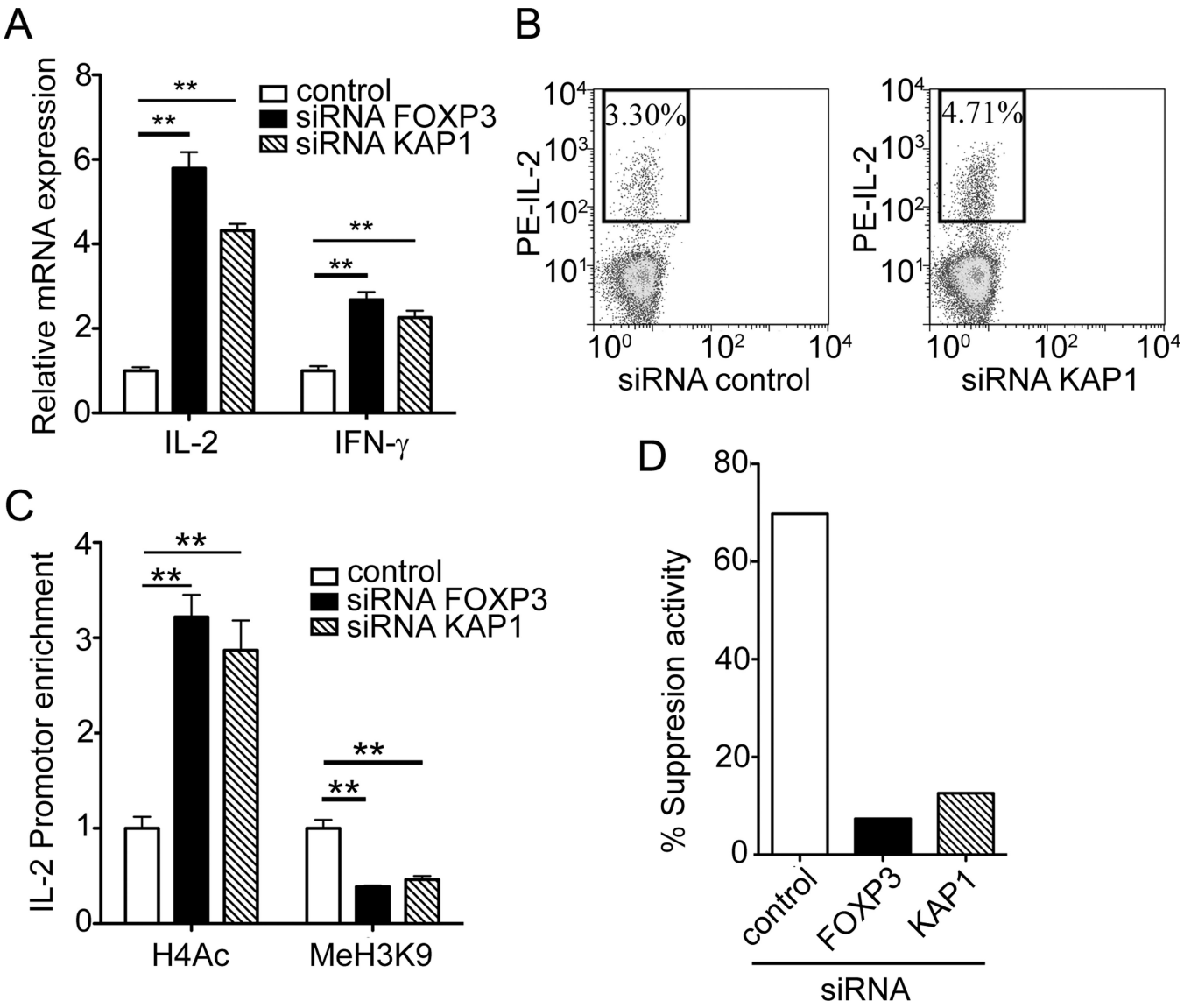

Figure 4. KAP1 is required for FOXP3 function

CD4+CD25+ Treg cells were transfected with siRNA specific to human *FOXP3* or *KAP1* using Amaxa human T cells transfection kit. After 3–4 days, cells were harvested. **A.** *IL-2* and *IFN-γ* expression were determined by qPCR normalized to *GAPDH* expression. **B.** IL-2 expression was also analyzed by intracellular staining. **C.** ChIP assays were performed as described. Rabbit anti-Histone H3K9 (ab8898, Abcam) and rabbit anti-Acetyl-Histone H4 (17–630, Millipore) were used to pull down the protein-DNA complexes, followed by qPCR analysis using primers specific to human *IL-2* promoter. **D.** Suppressive assay was performed using BD FastImmune Regulatory T-Cell Function Kit according to manufacturer's instruction with minor modifications. Data are shown as mean ± s.d. and represent three independent experiments.