Control of Foxp3 stability through modulation of TET activity

Xiaojing Yue,[1] Sara Trifari,[1] Tarmo Äijö,[1,2] Ageliki Tsagaratou,[1] William A. Pastor,[1] Jorge A. Zepeda-Martínez,[1] Chan-Wang J. Lio,[1] Xiang Li,[1,3] Yun Huang,[1] Pandurangan Vijayanand,[1] Harri Lähdesmäki,[2] and Anjana Rao[1,3,4,5]

[1]Division of Signaling and Gene Expression, La Jolla Institute for Allergy and Immunology, La Jolla, CA 92037
[2]Department of Computer Science, Aalto University School of Science, 00076 Aalto, Finland
[3]Sanford Consortium for Regenerative Medicine, La Jolla, CA 92037
[4]Department of Pharmacology and [5]Moores Cancer Center, University of California, San Diego, La Jolla, CA 92093

Ten-eleven translocation (TET) enzymes oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine and other oxidized methylcytosines, intermediates in DNA demethylation. In this study, we examine the role of TET proteins in regulating Foxp3, a transcription factor essential for the development and function of regulatory T cells (T reg cells), a distinct lineage of CD4$^+$ T cells that prevent autoimmunity and maintain immune homeostasis. We show that during T reg cell development in the thymus, TET proteins mediate the loss of 5mC in T reg cell–specific hypomethylated regions, including *CNS1* and *CNS2*, intronic cis-regulatory elements in the *Foxp3* locus. Similar to *CNS2*-deficient T reg cells, the stability of Foxp3 expression is markedly compromised in T reg cells from *Tet2/Tet3* double-deficient mice. Vitamin C potentiates TET activity and acts through *Tet2/Tet3* to increase the stability of Foxp3 expression in TGF-β–induced T reg cells. Our data suggest that targeting TET enzymes with small molecule activators such as vitamin C might increase induced T reg cell efficacy.

DNA methyltransferases (DNMTs) add a methyl group to cytosine to generate 5-methylcytosine (5mC); in somatic cells, this modification is typically present in the dinucleotide CpG (Ooi et al., 2009). DNA methylation is gradually lost in a replication-dependent manner during several processes of cell lineage specification, including the differentiation of naive T cells into Th2 cells (Lee et al., 2002). The three mammalian members of the ten-eleven translocation (TET) family of Fe(II) and 2-oxoglutarate–dependent dioxygenases, TET1, TET2, and TET3 (Iyer et al., 2009; Tahiliani et al., 2009), successively oxidize 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) in DNA (Tahiliani et al., 2009; He et al., 2011; Ito et al., 2011). All three oxidized methylcytosine species are intermediates in DNA demethylation, the replacement of 5mC with unmodified C (Pastor et al., 2013; Wu and Zhang, 2014).

The X chromosome–encoded transcription factor Foxp3 is essential for the development and function of regulatory T (T reg) cells, a distinct lineage of CD4$^+$ T cells that prevent autoimmunity and maintain immune homeostasis (Sakaguchi et al., 2008; Josefowicz et al., 2012). T reg cells that gain Foxp3 expression at precursor stage in the thymus are termed thymus-derived T reg cells, whereas those that develop extrathymically in vivo are termed peripherally derived T reg cells (Sakaguchi et al., 2008; Josefowicz et al., 2012; Abbas et al., 2013); Foxp3$^+$-induced T reg (iT reg) cells can be generated from naive T cells by stimulation through the T cell receptor in the presence of the inducer TGF-β (Chen et al., 2003; Abbas et al., 2013).

Foxp3 expression during T reg cell differentiation is regulated by three *conserved noncoding sequence* (*CNS*) elements located in the first intron of the *Foxp3* gene, upstream of the first coding exon (Zheng et al., 2010; Feng et al., 2014; Li et al., 2014). Of these, *CNS2* (also known as T reg cell–specific demethylated region; Floess et al., 2007) is unusual in that it controls the stability of Foxp3 expression in a manner linked to the DNA modification status of *CNS2* (Floess et al., 2007; Huehn et al., 2009; Toker and Huehn, 2011; Toker et al., 2013). First, CpG sites in the *Foxp3 CNS2* element are predominantly

Correspondence to Anjana Rao: arao@liai.org

S. Trifari's present address is Sorrento Therapeutics, San Diego, CA 92121.

T. Äijö's present address is Simons Center for Data Analysis, Simons Foundation, NY 10010.

W.A. Pastor's present address is Dept. of Molecular, Cell, and Developmental Biology, University of California, Los Angeles, Los Angeles, CA 90095.

J.A. Zepeda-Martínez's present address is Institute of Molecular Biotechnology, Austrian Academy of Sciences, 1030 Vienna, Austria.

Y. Huang's present address is Center for Epigenetics & Disease Prevention, Institute of Biosciences and Technology, Houston, TX 77030.

P. Vijayanand's present address is Division of Vaccine Discovery, La Jolla Institute for Allergy and Immunology, La Jolla, CA 92037.

Abbreviations used: 5caC, 5-carboxylcytosine; 5fC, 5-formylcytosine; 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; BS, bisulfite; CMS, cytosine-5-methylenesulphonate; DNMT, DNA methyltransferase; DP, double positive; eGFP, enhanced GFP; ESC, embryonic stem cell; IRES, internal ribosome entry site; MFI, mean fluorescent intensity; oxBS, oxidative BS; RA, retinoic acid; SP, single positive; TET, ten-eleven translocation.

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unmethylated (C/5fC/5caC) in T reg cells, but fully methylated (5mC/5hmC) in naive T cells and iT reg cells (Floess et al., 2007; Kim and Leonard, 2007; Polansky et al., 2008; Zheng et al., 2010; Toker et al., 2013). Second, cell division results in the loss of Foxp3 expression (Zheng et al., 2010; Feng et al., 2014; Li et al., 2014), a phenomenon associated with increased DNA methylation at *CNS2* and other regions of the *Foxp3* locus (Feng et al., 2014); this loss of Foxp3 expression is far more pronounced in iT reg cells with methylated *CNS2* than in T reg cells in which *CNS2* is not methylated (Floess et al., 2007). Third, inhibition of DNA methylation by the DNMT inhibitor 5-azacytidine (Kim and Leonard, 2007; Polansky et al., 2008), or genetic deletion of the gene encoding DNMT1 (Josefowicz et al., 2009), eliminated the requirement for TGF-β and promoted Foxp3 expression by naive $CD4^+$ T cells in response to TCR stimulation alone. Fourth, T reg cells from *CNS2*-deleted mice lose Foxp3 expression in a proliferation-, STAT5-, and NFAT-dependent manner after adoptive transfer, and the mice develop autoimmunity relatively late in life (Zheng et al., 2010; Feng et al., 2014; Li et al., 2014).

Here, we examined the role of TET proteins in regulating the stability of Foxp3 expression (i.e., the maintenance of T reg cell identity). We show that Tet2 and Tet3 act redundantly to maintain the demethylated status of several regulatory regions in T reg cells, including *CNS1* and *CNS2* in the *Foxp3* gene. Similar to T reg cells from *CNS2*-deficient animals, T reg cells from *Tet2/Tet3* double-deficient mice show a marked impairment of the stability of Foxp3 expression. Conversely, we show that addition of the TET activator vitamin C during mouse and human iT reg cell differentiation maintains TET enzymatic activity and potentiates the loss of 5mC in *CNS1* and *CNS2*, an effect correlated with a pronounced increase in the stability of Foxp3 expression. Our data indicate that TET proteins regulate the stability of Foxp3 expression by modulating the methylation status of conserved noncoding elements within the *Foxp3* locus as well as potentially other regulatory regions in the T reg cell genome, suggesting that targeting TET enzymes with small molecule activators such as vitamin C might increase iT reg cell efficacy in clinical applications such as transplant rejection and autoimmune disease.

RESULTS

TET proteins mediate the loss of 5mC in T reg cell–specific regulatory regions during T reg cell development

To examine the dynamic changes of DNA modification during T reg cell differentiation, we sorted cells from male Foxp3–internal ribosome entry site (IRES)–enhanced GFP (eGFP) reporter mice at sequential stages of their differentiation toward the T reg cell lineage in the thymus and their final maturation in the periphery. In these mice, a cassette encoding IRES-eGFP had been inserted into the 3′ untranslated region of the *Foxp3* gene, generating a bicistronic region encoding both Foxp3 and eGFP under the control of the Foxp3 promoter (Haribhai et al., 2007). DNA was prepared from the following purified populations: double-positive (DP) thymocytes; $CD4^+CD25^-Foxp3^-$ single-positive (SP; CD4 SP) T cells; $CD25^+Foxp3^-$ (Lio and Hsieh, 2008) and $CD25^-Foxp3^+$ (Tai et al., 2013) cells, both thought to be T reg cell precursors; thymic $CD25^+Foxp3^+$ T reg cells; and peripheral T reg cells (Fig. S1 A). Because bisulfite (BS) sequencing cannot distinguish 5mC and 5hmC (Huang et al., 2010), we used the oxidative BS (oxBS) method, which distinguishes 5mC and 5hmC at single-base resolution by selectively oxidizing 5hmC to 5fC (Booth et al., 2012, 2013). We note that neither BS nor oxBS sequencing (BS-seq and oxBS-seq, respectively) can distinguish unmodified C from 5fC or 5caC (Booth et al., 2015), leading us to avoid the term "DNA demethylation" (which implies complete replacement of 5mC by unmodified C) in favor of "loss of 5mC" (which includes the possibility of conversion of 5mC to 5fC or 5caC). Amplicons spanning 11 CpGs in the *CNS2* element (Fig. 1 A) were sequenced using the MiSeq platform, and the percentage of 5mC, 5hmC, and C/5fC/5caC at each CpG was calculated.

The results were unambiguous. As previously reported for CD4 SP cells (Toker et al., 2013), all CpGs in *Foxp3 CNS2* were methylated in both DP and CD4 SP cells and progressively lost 5mC in T reg cell precursors, with thymic and peripheral T reg cells showing the lowest levels of 5mC (Fig. 1 B). $CD25^-Foxp3^+$ precursor cells displayed a clear loss of 5mC, which was most apparent at CpG motifs 6, 9, 10, and 11. In contrast, 5hmC generally reached its peak levels in either $CD25^-Foxp3^+$ precursor cells or thymic T reg cells (Fig. 1 B). These data suggest that $CD25^+Foxp3^-$ precursors represent an earlier stage of T reg cell differentiation compared with $CD25^-Foxp3^+$ precursors, consistent with previous findings that $CD25^-Foxp3^+$ precursors differentiate more potently than $CD25^+Foxp3^-$ precursors into $CD25^+Foxp3^+$ T reg cells both in vitro and in vivo (Tai et al., 2013).

The appearance of 5hmC at intermediate stages of T reg cell lineage specification pointed to the involvement of TET proteins, the enzymes responsible for generating 5hmC (Tahiliani et al., 2009). To test the importance of TET proteins in T reg cell function, we generated and examined *Tet2*$^{-/-}$*Tet3*$^{fl/fl}$*CD4Cre* and *Tet2*$^{fl/fl}$*Tet3*$^{fl/fl}$*CD4Cre* (here termed *Tet2/3 DKO*) mice. Tet2 and Tet3 were chosen because they are the major TET proteins expressed in differentiated tissues and cell types, including lymph nodes and spleen (Tsagaratou and Rao, 2013), and because of a previous study showing that 5hmC, Tet2, and Tet3 are enriched in thymic T reg cell populations (Toker et al., 2013). The strategies used for conditional disruption of the *Tet2* and *Tet3* genes have been described previously (Ko et al., 2011, 2015; Kang et al., 2015).

To test whether the loss of 5mC in *Foxp3 CNS2* would be impaired in the absence of TET function, we examined the DNA modification status of *Foxp3* regulatory regions in $CD4^+CD25^+$ peripheral T reg cells isolated from young (3–4 wk old) male *Tet2/3 DKO* mice. We first assessed 5mC and 5hmC levels by BS- and oxBS-seq. The 4 CpGs in *CNS1*

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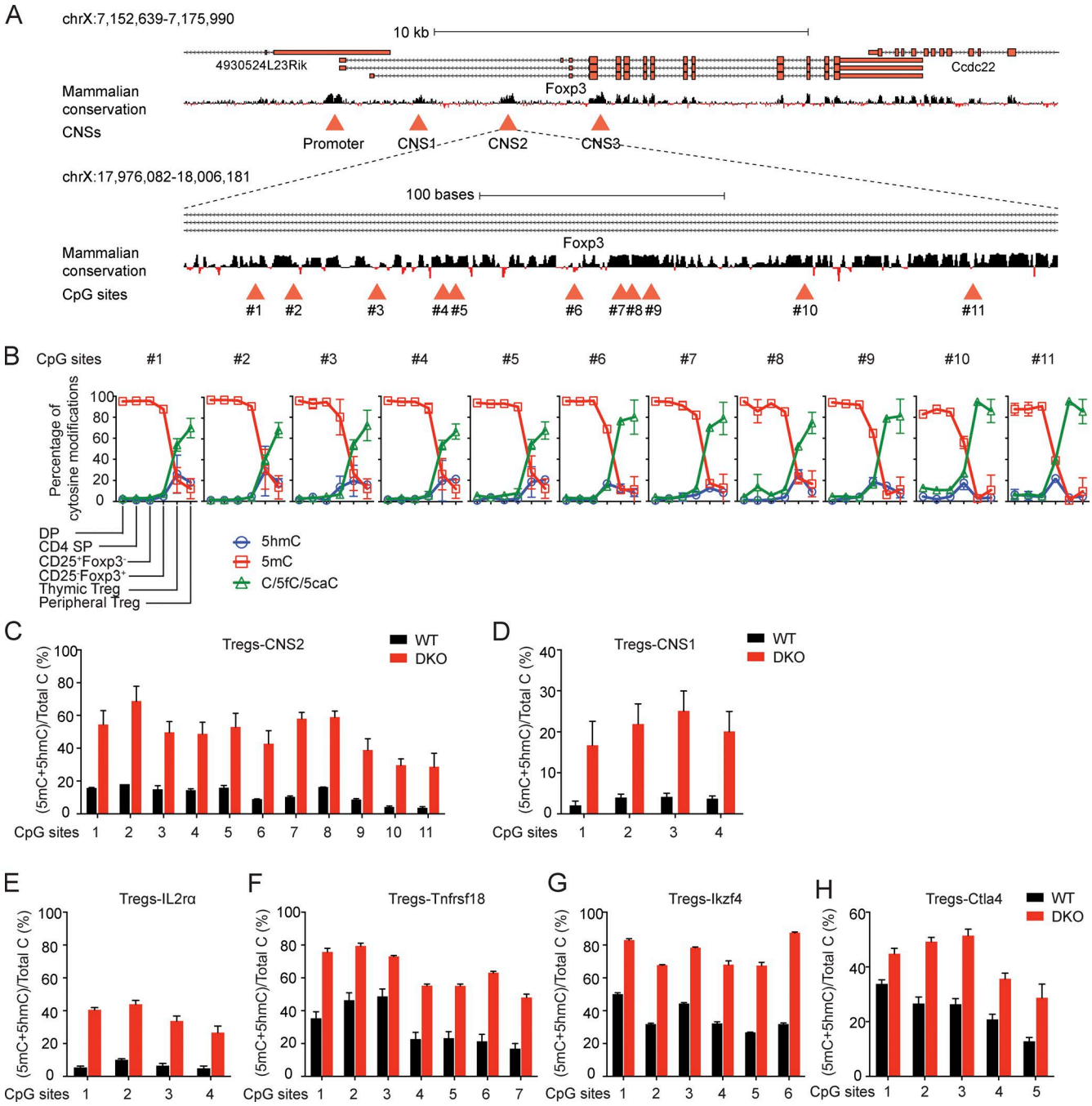

Figure 1. **Double deficiency of *Tet2* and *Tet3* impairs the loss of 5mC.** (A) Schematic representation of the *Foxp3* locus. Top: *Foxp3* transcript variants and mammalian conservation track are shown, and the conserved promoter region and the three conserved noncoding sequences corresponding to the intronic enhancers *CNS1*, *CNS2*, and *CNS3* are highlighted with red triangles. Bottom: 11 CpG sites in the *CNS2* region that were assessed to determine their methylation-hydroxymethylation status are indicated with red triangles. (B) Dynamic changes in 5mC, 5hmC, and C/5fC/5caC in the *Foxp3 CNS2* enhancer during T reg cell lineage specification. The graphs depict the cytosine modification status of the 11 CpGs in the *CNS2* region as determined by BS-seq and oxBS-seq in six different T cell subsets (DP, CD4 SP, precursor T reg cells [CD25$^+$Foxp3$^-$ and CD25$^-$Foxp3$^+$], thymic T reg cells, and peripheral T reg cells) that together capture the sequential steps of differentiation that lead to T reg cell specification. Error bars (which in many cases are too small to be detectable) show mean ± SD of thousands of sequencing reads from two independent experiments. (C–H) Graphs depicting the percentage of 5mC + 5hmC determined by BS-seq in peripheral T reg cells in 11 CpGs in *Foxp3 CNS2* (C), 4 CpGs in *Foxp3 CNS1* (D), 4 CpGs in *Il2ra* intron 1a (E), 7 CpGs in *Tnfrsf18* exon 5 (F), 6 CpGs in *Ikzf4* intron 1b (G), and 5 CpGs in *Ctla4* exon 2 (H). Error bars show mean ± SD of thousands of sequencing reads from three to four independent experiments.

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and the 11 CpGs in *CNS2* were largely unmethylated (<10% 5mC) in WT peripheral T reg cells but showed increased methylation (40–60% 5mC) in peripheral T reg cells from *Tet2/3 DKO* mice (Fig. S1, D and E); they were fully methylated (close to 100% 5mC) in CD4 SP thymocytes from both WT and *Tet2/3 DKO* mice, as expected (Fig. S1, G and H). In contrast, the five CpGs within *CNS3*, and all CpGs within two distinct regions of an upstream CpG island located between −5 and −5.5 kb upstream of the transcription start site, were completely unmethylated (<1% 5mC), both in peripheral T reg cells and in CD4 SP thymocytes from WT mice, and did not show any increase in methylation in *Tet2/3 DKO* mice (Fig. S1, F, I, and J). Thus, the four regulatory regions we examined in the *Foxp3* locus lose 5mC at different developmental stages in the thymus: *CNS1* and *CNS2* lose 5mC after the CD4 SP stage in a process that requires Tet2 and Tet3, whereas *CNS3* and the upstream CpG island lose 5mC before the CD4 SP stage and remain unmethylated in thymic T reg cells, even in the absence of Tet2 and Tet3.

Because $CD4^+CD25^+$ T reg cells cannot readily be distinguished from conventional activated $CD4^+$ T cells, we ensured T reg cell purity by crossing *Tet2/3 DKO* mice with Foxp3-IRES-eGFP reporter mice. By standard BS-seq, peripheral T reg cells from *Tet2/3 DKO* mice showed a 20–50% increase in 5mC + 5hmC levels at *CNS1* and *CNS2* compared with WT T reg cells (Fig. 1, C and D) and a comparable 20–40% increase at several other regulatory regions that undergo T reg cell–specific DNA hypomethylation: *Il2ra* intron 1a, *Tnfrsf18* exon 5, *Ikzf4* intron 1b, and *Ctla4* exon 2 (Fig. 1, E–H; Ohkura et al., 2012).

Notably, the increased DNA methylation required profound TET loss of function. Deletion of Tet2 alone had no effect, and deletion of Tet3 alone had only a mild effect, on the DNA modification status of *CNS1* and *CNS2* in $CD4^+CD25^+$ peripheral T reg cells from male *Tet2*$^{-/-}$ or *Tet3*$^{fl/fl}$*CD4Cre* mice compared with WT mice (Fig. S1, K and L). Together, the data show that Tet2 and Tet3 function redundantly to mediate the loss of 5mC not only at the *Foxp3* locus but also at other regulatory regions during T reg cell development in vivo.

The stability of Foxp3 expression is compromised in the absence of Tet2 and Tet3

Given the established connection between *CNS2* methylation status and T reg cell stability (Floess et al., 2007; Huehn et al., 2009; Zheng et al., 2010; Toker and Huehn, 2011; Toker et al., 2013), we compared the stability of Foxp3 expression in T reg cells from *Tet2/3 DKO* versus WT mice. Young *Tet2/3 DKO* mice (3–5 wk old) exhibited a normal percentage of $Foxp3^+$ cells in the thymus, but a significantly reduced frequency of $Foxp3^+$ cells in the periphery (Fig. 2 A).

We purified WT or *Tet2/3 DKO* T reg cells ($CD45.2^+$ $CD4^+$Foxp3-$eGFP^+$) by FACS and transferred them together with congenically marked WT $CD45.1^+CD4^+$ naive T cells (which serve as a source of IL-2) into *Rag*-deficient recipients (Fig. 2 B). When the recipient mice were examined 5–6 wk later, *Tet2/3 DKO* T reg cells exhibited a marked decrease of Foxp3 expression compared with WT T reg cells in peripheral lymphoid organs (Fig. 2 C). Consistent with loss of Foxp3 expression and consequent decrease of T reg cell function, recipient mice co-injected with naive T cells and *Tet2/3 DKO* T reg cells did not gain weight compared with mice that received WT T reg cells (Fig. 2 D) and exhibited splenomegaly (Fig. 2 E) and mononuclear cell infiltration in the liver (Fig. 2 F, arrowheads). Furthermore, when stimulated with anti-CD3/anti-CD28 in the presence of IL-2, Foxp3-$eGFP^+$ T reg cells from *Tet2/3 DKO* mice progressively lost, whereas WT Foxp3-$eGFP^+$ T reg cells maintained, Foxp3 (eGFP) expression upon cell division (Fig. 2 G). Thus, double deficiency of Tet2 and Tet3 in T cells compromises not only the loss of 5mC at T reg cell–specific regulatory regions, including *CNS1* and *CNS2*, but also the stability of Foxp3 expression subsequent to cell division in peripheral T reg cells.

Vitamin C promotes the loss of 5mC in *CNS1* and *CNS2* during iT reg cell differentiation

Naive T cells can be converted into iT reg cells in vitro by TCR stimulation in the presence of TGF-β (Chen et al., 2003), and this process is potentiated by the addition of retinoic acid (RA; Benson et al., 2007). CpG sites in the *Foxp3 CNS2* element are predominantly unmethylated in T reg cells but uniformly methylated in iT reg cells generated in the presence of TGF-β in vitro (Floess et al., 2007; Kim and Leonard, 2007; Polansky et al., 2008; Zheng et al., 2010; Toker et al., 2013). In cell proliferation and adoptive transfer experiments, Foxp3 expression was lost more rapidly in iT reg cells with methylated *CNS2* compared with T reg cells with unmethylated *CNS2* (Floess et al., 2007; Ohkura et al., 2012), reinforcing the conclusion that the DNA methylation (5mC/5hmC) status of *CNS2* correlates with the stability of Foxp3 expression (Floess et al., 2007; Polansky et al., 2008; Zheng et al., 2010; Toker et al., 2013).

Naturally occurring T reg cells are difficult to isolate because they are present in very small numbers; hence, iT reg cells have been used in the clinic to treat autoimmunity and graft-versus-host disease in humans (Tang and Bluestone, 2013). In this context, the instability of Foxp3 expression in iT reg cells is a serious potential limitation. So far, our data indicated that TET proteins influenced the stability of Foxp3 expression by maintaining low levels of DNA methylation at T reg cell–specific regulatory regions, leading us to ask whether TET activators could be used pharmacologically to maintain Foxp3 expression in iT reg cells. We chose first to test vitamin C, which has been reported to increase TET activity in mouse embryonic stem cells (ESCs; Blaschke et al., 2013); in the context of this study, we have shown that vitamin C enhances the activity of recombinant TET1 in vitro. To extend this observation further, we developed a cell-based assay to examine the activity of each of the three TET proteins in transfected HEK293 cells (Fig. 3 A). The results showed that

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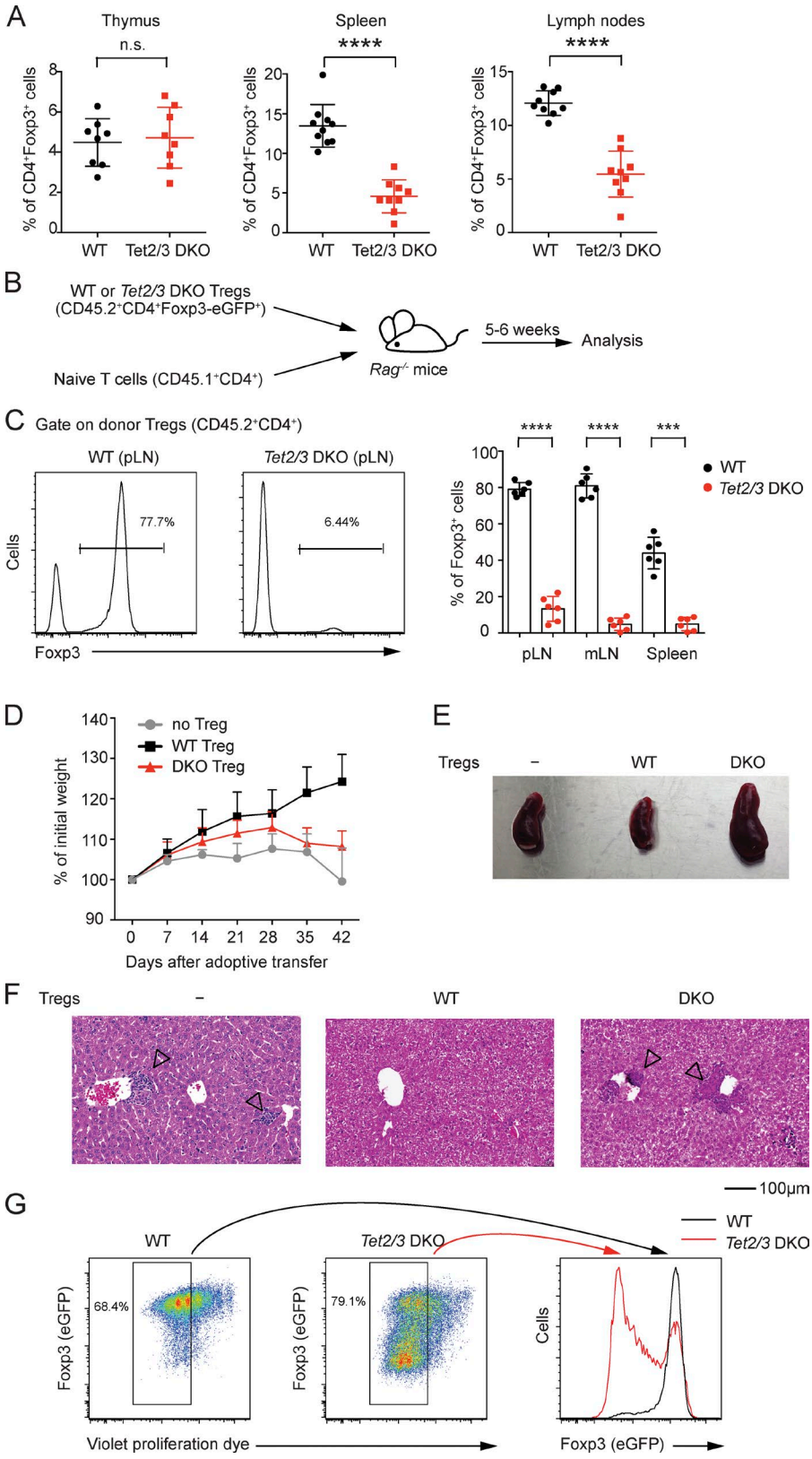

Figure 2. **The stability of T reg cells is significantly compromised with double deficiency of *Tet2* and *Tet3*.** (A) Percentage of Foxp3$^+$ T reg cells among CD4 SP thymocytes or splenic and lymph node CD4$^+$ T cells in *Tet2/3 DKO* mice and littermate controls. n = 8–9. n.s., not significant. (B) Schematic representation of the adoptive transfer experiment for assessing T reg cell stability in vivo. (C) Analysis of peripheral lymphoid organs from *Rag*-deficient mice 5–6 wk after adoptive transfer of peripheral T reg cells from young (3–4 wk old) WT (CD45.2$^+$) or *Tet2/3 DKO* (CD45.2$^+$) reporter mice as a CD4$^+$Foxp3-eGFP$^+$ population together with congenically marked naive T cells (CD45.1$^+$CD4$^+$). Left: Representative histograms of Foxp3$^+$ cells within CD45.2$^+$CD4$^+$ cells. Right: The mean percentage of Foxp3$^+$ cells in the CD45.2$^+$CD4$^+$ cell gate. Each dot represents one mouse. Error bars show mean ± SD from three independent experiments with six mice per group. pLN, peripheral lymph node. mLN, mesenteric lymph node. (D–F) The Foxp3 stability assay using T reg cells sorted from either WT or *Tet2/3 DKO* reporter mice as the CD4$^+$Foxp3-eGFP$^+$ population. The percent change in initial weight after adoptive transfer (D), the picture of spleens (E), and hematoxylin and eosin staining of livers (F) from adoptive transfer without T reg cells or with WT or *Tet2/3 DKO* T reg cells are shown. Arrowheads indicate mononuclear cell infiltration in the liver. Data are representative of three independent experiments. (G) WT or *Tet2/3 DKO* Foxp3-eGFP$^+$ T reg cells were labeled with violet proliferation dye and cultured in vitro for 3 d and then analyzed for Foxp3-eGFP expression by flow cytometry. Left: Representative pseudocolor plots for WT or *Tet2/3 DKO* T reg cells. Right: Histogram overlay of Foxp3-eGFP$^+$ cells from WT and *Tet2/3 DKO* T reg cells within the dividing cells. Data are representative of three independent experiments. ***, $P < 0.001$; ****, $P < 0.0001$ by Student's t test.

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vitamin C potentiated the activity of all three TET proteins; however, the strongest apparent effect was observed in TET3-expressing cells, most likely because these cells showed the lowest level of 5hmC in control conditions in the absence of vitamin C (Fig. 3 A).

Based on these data, we asked whether the addition of vitamin C during iT reg cell differentiation would promote the loss of 5mC and/or stabilize Foxp3 expression in the resulting iT reg cells. To initiate iT reg cell differentiation, naive T cells from Foxp3-IRES-eGFP reporter mice were treated with TGF-β alone (Chen et al., 2003) or with TGF-β + RA (Benson et al., 2007) in the presence or absence of vitamin C. T cell activation under any conditions—including Th1 and Th2 polarizing conditions (Tsagaratou et al., 2014)—resulted in decreased *TET* mRNA expression as well as a strong decrease in overall genomic 5hmC by 5–6 d (Fig. 3, B and C). RA increased *Tet3* mRNA levels, as also observed in mouse ESCs (Koh et al., 2011). Vitamin C did not affect *Tet* mRNA levels and caused a modest increase in *Foxp3* mRNA levels (Fig. 3 B) but clearly augmented TET enzymatic activity, as assessed by anti–cytosine-5-methylenesulphonate (CMS) dot blot (Ko et al., 2010) to measure overall genomic levels of 5hmC (Fig. 3 C). Thus, vitamin C counters the abrupt loss of 5hmC that occurs in activated T cells, presumably by maintaining TET activity during T cell activation and differentiation.

The presence of vitamin C during iT reg cell differentiation resulted in a small but significant increase of the percentage of Foxp3 (eGFP)-expressing cells and also increased the mean fluorescent intensity (MFI) of Foxp3 (eGFP) expression (i.e., the amount of Foxp3 per cell; Fig. 3 D). Moreover, RA and vitamin C both caused a pronounced tightening of the peak of the histogram for Foxp3-eGFP$^+$ cells, which we quantified as a reduced coefficient of variation (Fig. 3 D, right).

When assessed by standard BS-seq, all CpGs in both *CNS1* and *CNS2* were essentially fully modified (5mC + 5hmC) in naive CD4$^+$ T cells from Foxp3-IRES-eGFP reporter mice (Fig. 4, A and B, gray bars). Consistent with previous data (Floess et al., 2007; Kim and Leonard, 2007), iT reg cell differentiation for 6 d with TGF-β or TGF-β and RA led to minimal loss of 5mC + 5hmC at *CNS2*, with the exception of CpGs 10 and 11 at the 3′ end (Fig. 4 D, black and blue bars). In contrast, CpGs in *CNS1*, particularly CpG 1, lost a substantial amount of 5mC + 5hmC under these conditions (Fig. 4 C, black and blue bars). However, when vitamin C was added into the cultures, all *CNS1* and *CNS2* CpGs showed a progressive loss of 5mC + 5hmC that was apparent by day 3 of iT reg cell differentiation (Fig. 5, A and B) and became pronounced by day 6 (Fig. 4, C and D, red and green bars). This effect was specific for iT reg cell differentiation because the addition of vitamin C to T cell cultures activated with anti-CD3 and anti-CD28 in the absence of polarizing cytokines or neutralizing anticytokine antibodies (Th0 conditions) had little or no effect on the loss of 5mC + 5hmC at all CpGs of *CNS1* and *CNS2* (Fig. 4, A and B, black and red bars).

CpGs within other T reg cell–specific regulatory regions—*Il2ra* intron 1a, *Tnfrsf18* exon 5, *Ikzf4* intron 1b, and *Ctla4* exon 2—also displayed loss of 5mC + 5hmC when vitamin C was added into the cultures during iT reg cell differentiation (Fig. 4, E–H). In contrast, CpGs within the upstream CpG island were already completely demethylated (<1% 5mC) in TGF-β–induced iT reg cells, and addition of vitamin C did not affect this demethylated state (Fig. 4 I). Together, these data indicate that vitamin C promotes the loss of 5mC + 5hmC at T reg cell–specific regulatory regions during iT reg cell differentiation. For most subsequent experiments, we focused on *Foxp3 CNS1* and *CNS2*, which exhibited the most significant alteration of methylation in both peripheral T reg cells from *Tet2/3 DKO* mice and in iT reg cells differentiated in the presence of vitamin C.

To capture the dynamic alterations in cytosine modifications in *CNS1* and *CNS2* during iT reg cell differentiation, we performed BS-seq and oxBS-seq with samples collected at 24, 38, 48, and 72 h after activation of naive T cells with TGF-β in the presence or absence of vitamin C (we did not consider RA for these experiments because it did not affect DNA modification status; Fig. 4, C and D, black and blue bars). iT reg cells differentiating in the presence of TGF-β alone showed a progressive but relatively minor loss of 5mC at *CNS2*, <40–50% at 72 h except at CpGs 10 and 11 of *CNS2* (Fig. 5 A, top), whereas iT reg cells induced by TGF-β + vitamin C showed a considerably more rapid loss of 5mC at *CNS2* (Fig. 5 A, bottom). In cells cultured with TGF-β + vitamin C, there was a substantial (40–50%) loss of 5mC at CpGs 10 and 11 of *CNS2* even at 24 h, just barely after initiation of DNA replication in S phase in a small fraction of the cells and well before the occurrence of cell division (not depicted); and at all *CNS2* CpGs, there was <20% residual 5mC at 72 h (Fig. 5 A, bottom). Vitamin C also greatly potentiated the rate of loss of 5mC from the four CpG residues in *CNS1*, especially CpG 1 (Fig. 5 B). In all cases, 5hmC levels were low but clearly detectable, as expected for an intermediate in the conversion of 5mC to 5fC/5caC/C (Fig. 5). Altogether, the addition of the TET activator vitamin C during TGF-β–induced iT reg cell differentiation in vitro promotes the loss of 5mC in *CNS1* and *CNS2* in the *Foxp3* locus, as well as other T reg cell–specific regulatory regions.

Vitamin C acts via TET proteins to induce the loss of 5mC

We asked whether the ability of vitamin C to potentiate the loss of 5mC in *Foxp3 CNS1* and *CNS2* was mediated through TET proteins. *Tet2/3 DKO* mice, in which *Tet3* is deleted using *CD4Cre*, have a decreased frequency of naive T cells (see Materials and methods subsection Mice); hence, for these experiments, we chose to delete *Tet3* inducibly in mice expressing tamoxifen-inducible *ERT2-Cre*. WT and *Tet2*$^{-/-}$ *Tet3*$^{fl/fl}$*ERT2-Cre* mice were injected intraperitoneally with tamoxifen for five consecutive days, after which naive T cells were sorted and cultured in vitro with 4-hydroxytamoxifen (4-OHT) for two more days (Fig. 6 A). Analysis of genomic DNA showed that ~80% of *Tet3* exon 2

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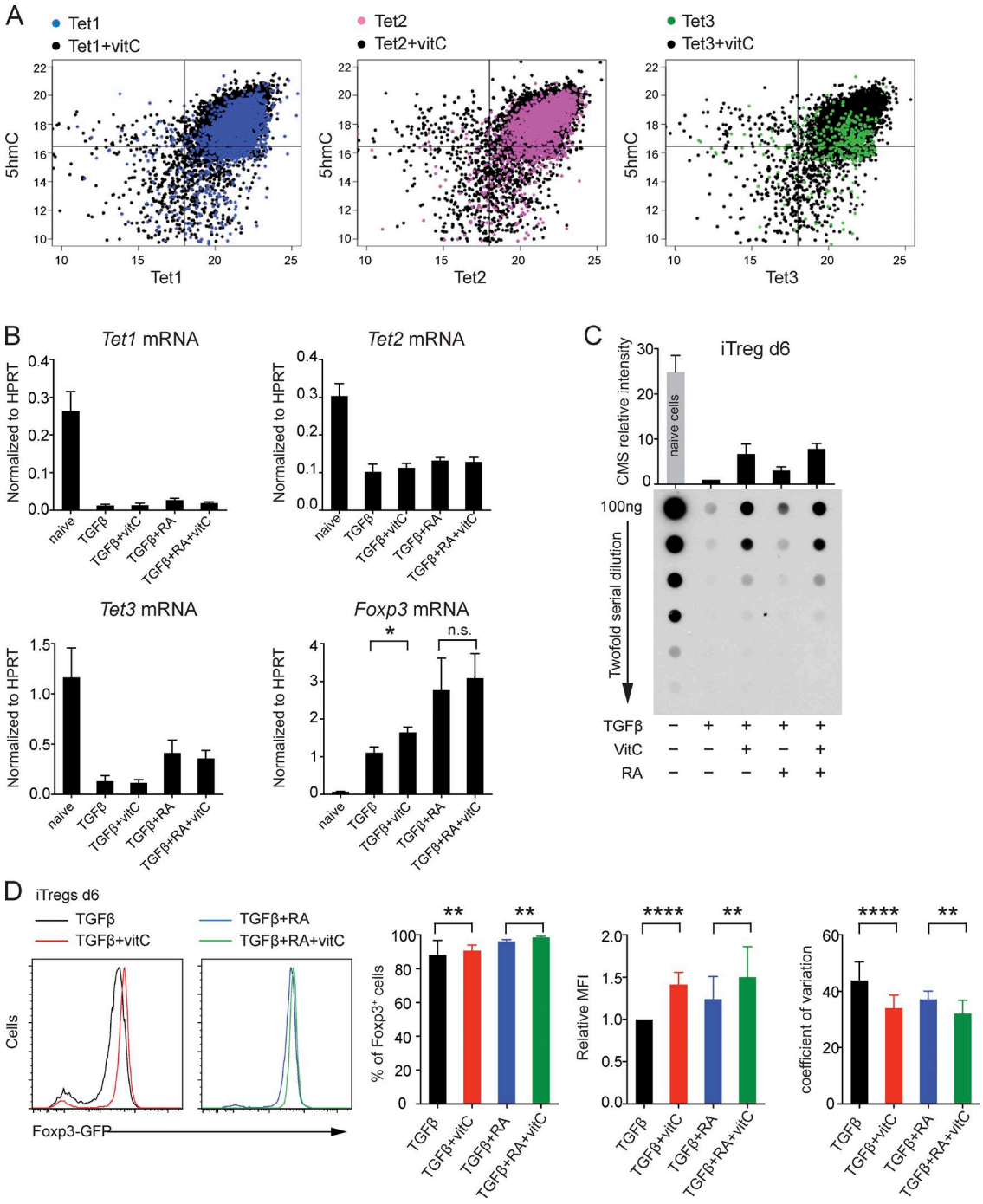

Figure 3. **The effects of vitamin C on TET activity and expression.** (A) The effect of vitamin C on TET activity. HEK293T cells were transfected with HA-tagged TET1–3, cultured in the presence/absence of vitamin C, and then stained and imaged for TET expression (x axis) and 5hmC intensity (y axis). The y/x axis is shown in $\log_2$ scale. Each dot represents a TET-expressing cell, with hundreds of cells analyzed from independent wells. (B) Quantitative real-time PCR analysis of *Tet1*, *Tet2*, *Tet3*, and *Foxp3* mRNA expression levels in naive and iT reg cells differentiated for 3 d in the presence of TGF-β, TGF-β + vitamin C (vitC), TGF-β + RA, or TGF-β + RA + vitamin C. Error bars show mean ± SD from three independent experiments. (C) Genomic DNA from naive and iT reg cells differentiated for 6 d in the presence of TGF-β, TGF-β + vitamin C, TGF-β + RA, or TGF-β + RA + vitamin C was treated with sodium BS to convert 5hmC to CMS. The relative intensity of CMS was quantified by anti-CMS dot blot assay and normalized to the amount of CMS detected in iT reg cells differentiated for 6 d with TGF-β alone. Error bars show mean ± SD from three independent experiments. (D, left) Representative histogram overlay. Right: Graphs for the percentage of Foxp3$^+$ cells, relative MFI (normalized to the MFI in TGF-β condition), and coefficient of variation for iT reg cells differentiated for 6 d in the presence of TGF-β, TGF-β + vitamin C, TGF-β + RA, and TGF-β + RA + vitamin C. Error bars show mean ± SD from more than five independent experiments. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$ by Student's *t* test. n.s., not significant.

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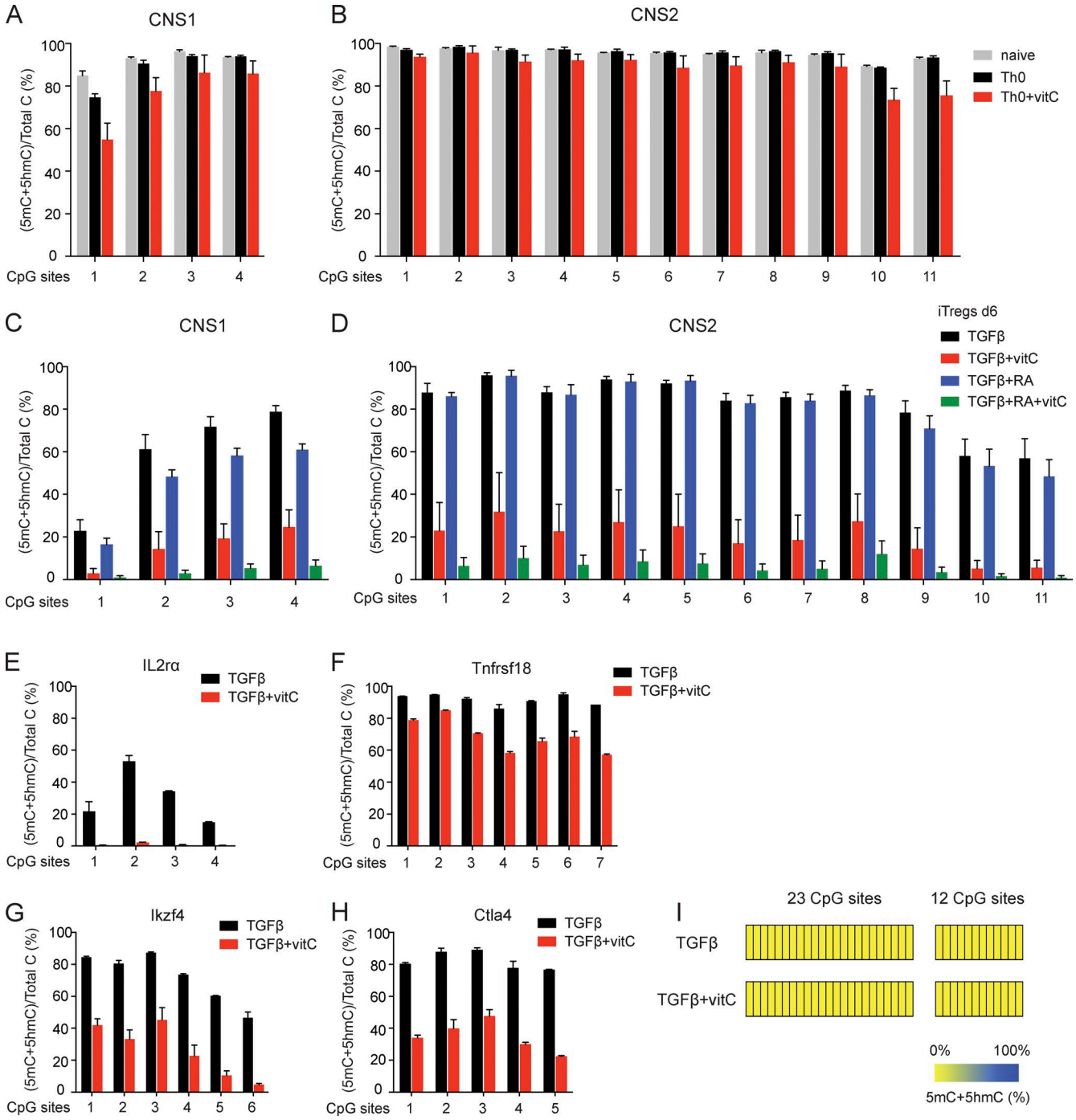

Figure 4. **Supplementation with vitamin C during iT reg cell differentiation leads to the loss of 5mC in T reg cell–specific regulatory regions.** (A and B) BS-seq of the four CpG sites in *Foxp3 CNS1* (A) and the 11 CpG sites in *Foxp3 CNS2* (B). Graphs depict the percentage of (5mC + 5hmC)/total C in naive CD4$^+$ T cells and naive CD4$^+$ T cells activated under Th0 condition without or with vitamin C. Error bars show mean ± SD of thousands of sequencing reads from two to four independent experiments. (C and D) BS-seq of the four CpG sites in *Foxp3 CNS1* (C) and the 11 CpG sites in *Foxp3 CNS2* (D). Graphs depict the percentage of (5mC + 5hmC)/total C in iT reg cells differentiated for 6 d in the presence of TGF-β, TGF-β + vitamin C, TGF-β + RA, and TGF-β + RA + vitamin C. Error bars show mean ± SD of thousands of sequencing reads from four independent experiments. (E–H) BS-seq of four CpGs in *Il2ra* intron 1a (E), seven CpGs in *Tnfrsf18* exon5 (F), six CpGs in *Ikzf4* intron 1b (G), and five CpGs in *Ctla4* exon2 (H). Graphs depict the percentage of (5mC + 5hmC)/total C in iT reg cells differentiated for 6 d in the presence of TGF-β or TGF-β + vitamin C. Error bars show mean ± SD of thousands of sequencing reads from at least two independent experiments. (I) Heat maps depicting the percentage of (5mC + 5hmC)/total C in CpGs within two distinct regions of an upstream CpG island in the *Foxp3* locus in iT reg cells differentiated for 6 d in the presence of TGF-β or TGF-β + vitamin C, as determined by BS-seq. Data show mean of thousands of sequencing reads from at least two independent experiments.

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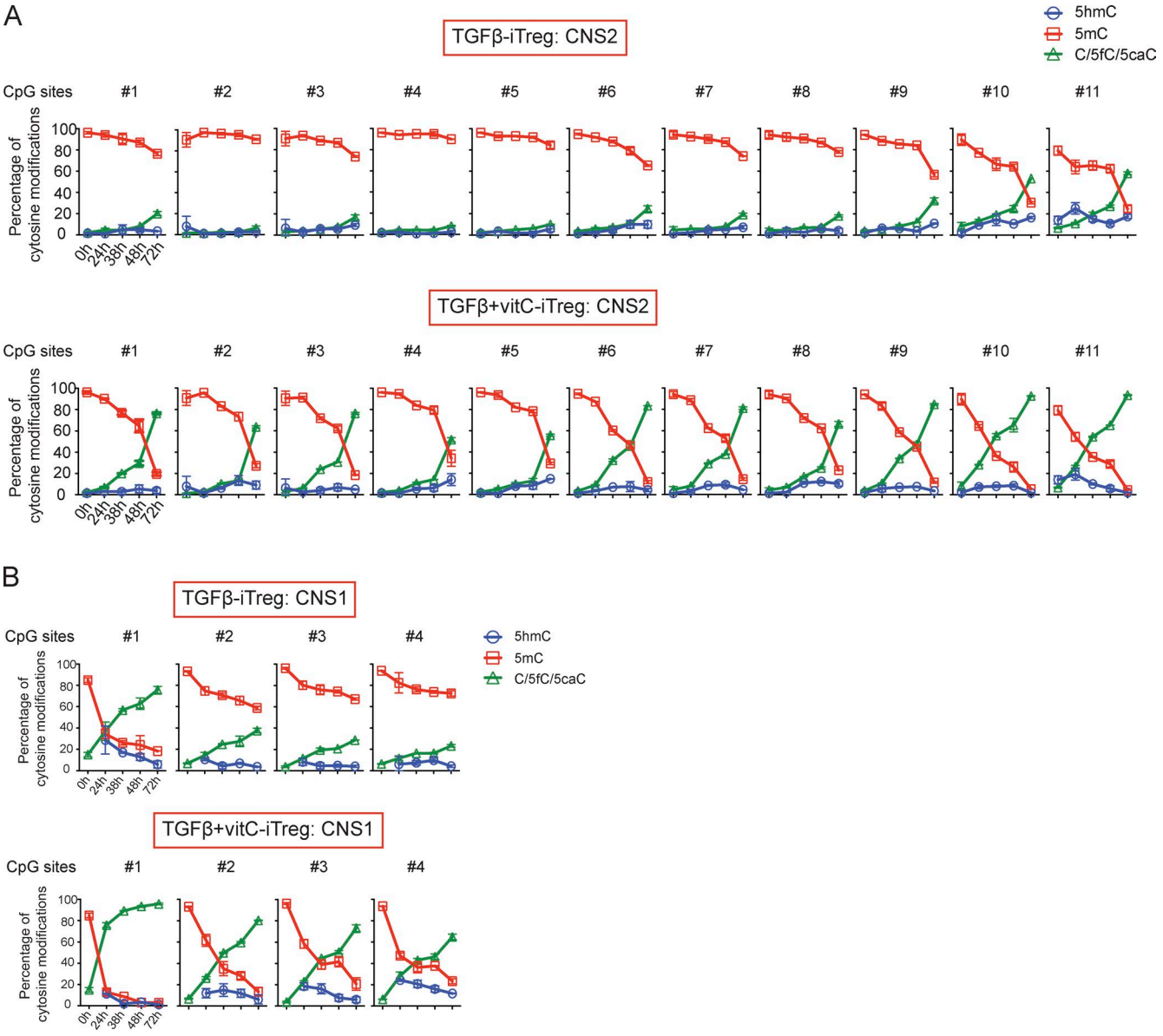

Figure 5. **Dynamic alterations in cytosine modifications in *Foxp3 CNS1* and *CNS2* during iT reg cell differentiation in the presence of vitamin C.** Dynamic changes in 5mC, 5hmC, and C/5fC/5caC in the *Foxp3 CNS2* (A) and *Foxp3 CNS1* (B) during iT reg cell in vitro differentiation. Diagrams depicting the cytosine methylation status of the 11 CpGs in *CNS2* (A) and four CpGs in *CNS1* (B) as determined by BS-seq and oxBS-seq at five different time points (0, 24, 38, 48, and 72 h) that together capture the sequential steps of differentiation that lead to the loss of 5mC in *CNS2* (A) and *CNS1* (B) in the presence of vitamin C. Data at 0 h for *CNS1* locus is BS-seq. Error bars (which in many cases are too small to be detectable) show mean ± SD of thousands of sequencing reads from two independent experiments.

was deleted under these conditions (Fig. 6 B). At the mRNA level, *Tet2* transcripts from the floxed exons were undetectable; consistent with the amount of undeleted *Tet3* exon 2, residual amounts of *Tet3* transcripts (~20%) were observed, and the level of *Tet1* transcripts increased slightly, possibly a result of cellular compensation for the loss of Tet2 and Tet3 (Fig. 6 C).

Naive T cells from tamoxifen-treated mice were differentiated into iT reg cells in the presence of either TGF-β or TGF-β + vitamin C, and the cytosine modification status of Foxp3 *CNS1* and *CNS2* was assessed by standard BS-seq. WT TGF-β–induced iT reg cells remained highly methylated (5mC + 5hmC) at *CNS1* and *CNS2* (Fig. 6, D and E, solid black bars), but vitamin C caused significant loss of this modification (Fig. 6, D and E, solid red bars). *Tet2/3 DKO* iT reg cells induced with TGF-β alone showed higher 5mC + 5hmC levels at *CNS1* and *CNS2* compared with WT iT reg cells, but in this case addition of vitamin C caused only a minor loss or no loss of 5mC + 5hmC (Fig. 6, D and E, com-

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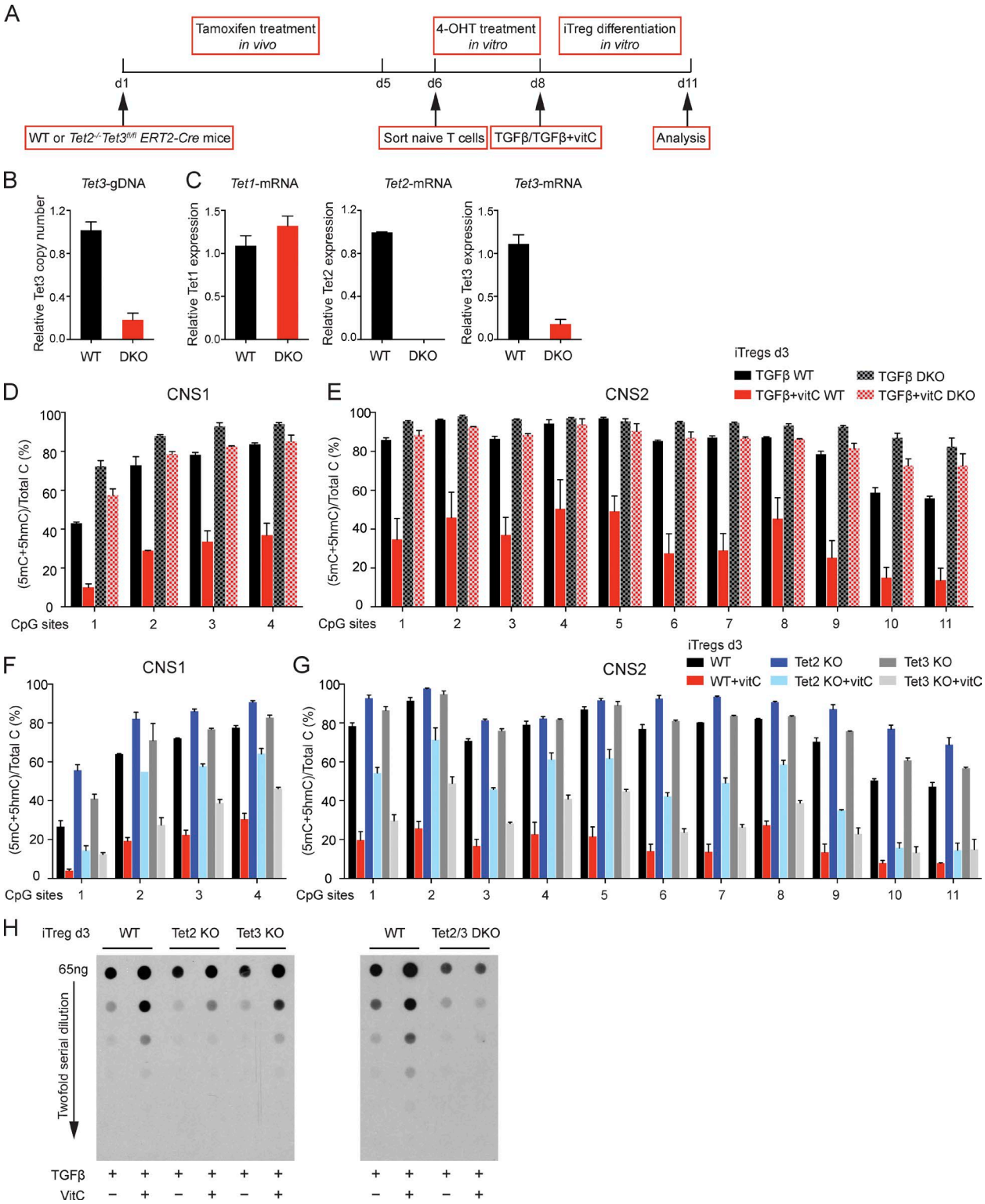

Figure 6. **Vitamin C induces the loss of 5mC in *CNS1* and *CNS2* during iT reg cell differentiation via TET proteins.** (A) Schematic representation of the experimental strategy for *Tet3* deletion using the *ERT2-Cre* system. (B) Quantitative RT-PCR analysis of *Tet3* deletion efficiency at the genomic DNA level. The relative copy number was normalized to GAPDH. (C) Quantitative RT-PCR analysis of *Tet1*, *Tet2*, and *Tet3* mRNA expression levels. The relative expression level was normalized to *HPRT*. Error bars show mean ± SD from three independent experiments. (D and E) BS-seq of the four CpG sites in *Foxp3 CNS1* (D) and 11 CpG sites in *Foxp3 CNS2* (E). Graphs depict the percentage of (5mC + 5hmC)/total C in iT reg cells differentiated for 3 d from WT or *Tet2*$^{-/-}$*Tet3*$^{fl/fl}$ *ERT2-Cre* cells in the presence of TGF-β or TGF-β + vitamin C, in both cases after tamoxifen and 4-OHT treatment as shown in A. (F and G) BS-seq of the

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pare hatched black and red bars); the small decrease may be the result of residual Tet1 or Tet3 expression (Fig. 6, B and C). Thus, Tet2 and Tet3 are major targets of vitamin C in T cells.

To ask which TET protein was the major target of vitamin C in iT reg cells, we repeated these experiments with iT reg cells lacking only Tet2 or Tet3. Vitamin C caused a substantial decrease of 5mC + 5hmC in *CNS1* and *CNS2* in *Tet2 KO* iT reg cells, which retain Tet1 and Tet3 (Fig. 6, F and G, blue bars), as well as a global increase in 5hmC assessed by anti-CMS dot blot (Fig. 6 H). *Tet3 KO* iT reg cells, which express Tet1 and Tet2, showed a more striking decrease of 5mC + 5hmC by vitamin C (Fig. 6, F and G, gray bars) and a more striking increase of 5hmC (Fig. 6 H) than *Tet2 KO* iT reg cells, suggesting a stronger effect of vitamin C on Tet2 compared with Tet3 in this experimental setting. However, the greatest decrease in 5mC + 5hmC and the greatest increase in 5hmC were observed in WT iT reg cells (Fig. 6, F–H), indicating that both Tet2 and Tet3 were targets of vitamin C.

Further attesting to the redundancy between Tet2 and Tet3, the frequency of Foxp3-expressing cells was unchanged in cells with single deletion of Tet2 or Tet3, but was substantially reduced in *Tet2/3 DKO* cells (Fig. S2, A and B). Moreover, vitamin C increased the MFI of Foxp3 (eGFP) expression in WT iT reg cells and in iT reg cells with single deletion of Tet2 or Tet3 (Fig. S2, A and B, right), but not in *Tet2/3 DKO* iT reg cells (Fig. S2 B, right). Together, these data indicate that vitamin C acts through both Tet2 and Tet3 to potentiate the loss of 5mC + 5hmC at T reg cell–specific regulatory regions, as well as to increase the MFI of Foxp3 expression in differentiating iT reg cells.

Vitamin C potentiates the stability of Foxp3 expression in mouse iT reg cells

Given that vitamin C induced the loss of 5mC + 5hmC at T reg cell–specific regulatory regions including *Foxp3 CNS1* and *CNS2* during iT reg cell differentiation, we asked whether iT reg cells induced with TGF-β and vitamin C would display increased stability of Foxp3 expression in vitro and in vivo compared with iT reg cells induced with TGF-β alone. We differentiated purified naive T cells from Foxp3-IRES-eGFP reporter mice into iT reg cells under four conditions: (1) TGF-β alone, (2) TGF-β + vitamin C, (3) TGF-β + RA, and (4) TGF-β + RA + vitamin C. Foxp3-eGFP$^+$ cells were sorted at day 6 and restimulated with anti-CD3 and anti-CD28 in the absence of polarizing cytokines or neutralizing antibodies, and Foxp3 (eGFP) expression was monitored daily (Fig. 7 A). Indeed, iT reg cells generated in the presence of vitamin C lost Foxp3 (eGFP) expression considerably more slowly during restimulation-induced proliferation than corresponding iT reg cells generated using TGF-β or TGF-β + RA alone (Fig. 7 A, left). In addition, iT reg cells generated using vitamin C displayed an approximately twofold increase in the amount of Foxp3 per cell, as assessed by increased MFI (Fig. 7 A, middle). Again, both RA and vitamin C reduced the coefficient of variation, with an additive effect of both agents together (Fig. 7 A, right).

We determined the in vivo stability of Foxp3 expression on iT reg cells induced in the presence of vitamin C by transferring CD45.2$^+$Foxp3-eGFP$^+$ iT reg cells generated under the four conditions together with congenically marked CD45.1$^+$CD4$^+$ naive T cells into *Rag*-deficient recipients (Fig. 7 B). Analysis of the percentage of Foxp3$^+$ cells in peripheral lymphoid organs 4–5 wk after adoptive transfer showed that iT reg cells generated in the presence of vitamin C were more resistant to the loss of Foxp3 and thus were more stable than the corresponding iT reg cells generated in the absence of vitamin C (Fig. 7 C).

Scurfy mice lack T reg cells because they carry a natural frame-shift mutation of *Foxp3* that abrogates Foxp3 protein expression (Brunkow et al., 2001). Transfer of peripheral CD4$^+$ T cells from *scurfy* male mice into *Rag*-deficient recipients leads to expansion of autoreactive *scurfy* CD4$^+$ T cells and severe autoimmune inflammation because of a lack of T reg cell suppression; both features can be suppressed by cotransfer of congenically marked T reg cells (Choi et al., 2010). To examine the in vivo function of iT reg cells generated in the presence of vitamin C, we transferred *scurfy* CD4$^+$ T cells (CD45.1$^+$) into *Rag*-deficient recipient mice together with iT reg cells from CD45.2$^+$ Foxp3-eGFP reporter mice generated in the presence of TGF-β alone, TGF-β + vitamin C, or TGF-β + RA + vitamin C (Fig. 7 D). iT reg cells generated in the presence of TGF-β + vitamin C or TGF-β + RA + vitamin C were superior to iT reg cells induced by TGF-β alone in suppressing *scurfy* CD4$^+$ T cell expansion, as shown by analysis of the percentage of *scurfy* CD4$^+$ T cells in peripheral lymphoid organs 3.5–4 wk after adoptive transfer (Fig. 7 E, top). Moreover, iT reg cells generated in the presence of vitamin C were more resistant to the loss of Foxp3 even under these inflammatory conditions (Fig. 7 E, bottom). In summary, addition of vitamin C during iT reg cell differentiation led to Tet2/3-dependent loss of 5mC in T reg cell–specific regulatory regions and increased the stability of Foxp3 expression by the resulting iT reg cells.

Vitamin C potentiates *CNS2* demethylation and the stability of FOXP3 expression in human iT reg cells

Finally, we asked whether vitamin C also worked via TET proteins to potentiate the loss of 5mC and enhance the sta-

four CpG sites in *Foxp3 CNS1* (F) and the 11 CpG sites in *Foxp3 CNS2* (G). Graphs depict the percentage of (5mC + 5hmC)/total C in iT reg cells differentiated in the presence of TGF-β and TGF-β + vitamin C for 3 d from WT, *Tet2*$^{-/-}$, and *Tet3*$^{fl/fl}$ CD4-Cre naive CD4$^+$ T cells. Error bars show mean ± SD of thousands of sequencing reads from two to three independent experiments. (H) Genomic DNA from WT, *Tet2 KO*, *Tet3 KO*, and *Tet2/3 DKO* naive and iT reg cells differentiated for 3 d in the presence of TGF-β and TGF-β + vitamin C was treated with sodium BS to convert 5hmC to CMS. The relative intensity of CMS was quantified by anti-CMS dot blot assay. Data are representative of three independent experiments.

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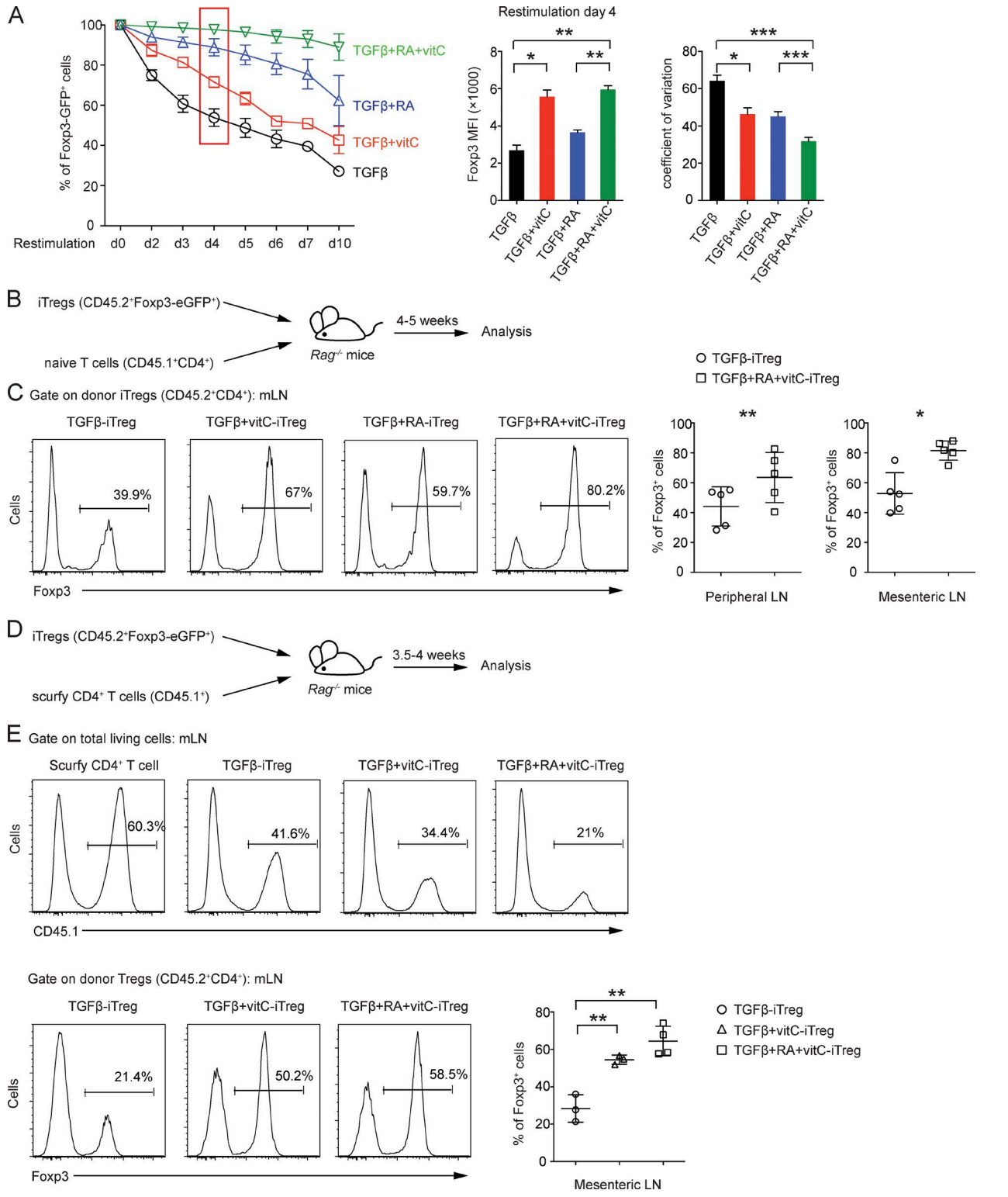

Figure 7. **Supplementation of vitamin C during iT reg cell differentiation enhances the stability of iT reg cells in vitro and in vivo.** (A) Naive CD4+ T cells from Foxp3-IRES-eGFP reporter mice were differentiated into iT reg cells for 6 d in the presence of TGF-β, TGF-β + vitamin C, TGF-β + RA, or TGF-β + RA + vitamin C, and then the Foxp3-eGFP+ population was sorted and restimulated with anti-CD3 and anti-CD28 antibodies. The percentage of Foxp3-eGFP+ cells was monitored daily after restimulation. Right: Geometric MFI and coefficient of variation for Foxp3-eGFP+ cells on day 4 after restimulation. Error bars show mean ± SD from three independent experiments. (B) Schematic representation of the adoptive transfer experiment for assessing iT reg cell stability in vivo. (C, left) Representative histograms of Foxp3+ cells from *Rag*-deficient mice 4–5 wk after adoptive transfer of sorted iT reg cells differentiated for

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bility of FOXP3 expression in human iT reg cells. We sorted naive CD4$^+$ T cells from human peripheral blood, stimulated them with anti-CD3 and anti-CD28 in the presence of TGF-β, TGF-β + RA, TGF-β + vitamin C, or TGF-β + RA + vitamin C, and analyzed FOXP3 expression at different times after stimulation. Similar percentages of FOXP3$^+$ cells were found in all TGF-β–treated cultures on day 4 (Fig. S3 A); by day 12, however, the percentage of FOXP3$^+$ cells declined in iT reg cells stimulated in the presence of TGF-β or TGF-β + RA, but remained high in iT reg cells additionally treated with vitamin C (Figs. 8 A and S3 A). The percentage of FOXP3$^+$ cells and the MFI of FOXP3 expression were similar in iT reg cells generated with TGF-β + RA + vitamin C compared with expanded ex vivo T reg cells (Fig. 8 A, right, compare purple and green histograms). In contrast, human CD4$^+$ T cells activated in the presence of IL-2 only (effectors), which transiently induce low levels of FOXP3 (Allan et al., 2007; Tran et al., 2007; Wang et al., 2007), showed very low FOXP3 expression on day 12 as expected (Fig. 8 A, gray histograms).

To analyze FOXP3 stability in human iT reg cells generated with and without vitamin C, we restimulated iT reg cells previously polarized under the four indicated conditions with anti-CD3 and anti-CD28 in the absence of TGF-β or RA and analyzed FOXP3 expression at different days after restimulation (Figs. 8 C and S3 B). Starting at day 4, the percentage of FOXP3$^+$ cells declined under most conditions but was maintained in iT reg cells previously differentiated with TGF-β + RA + vitamin C (Fig. 8 C, left; and Fig. S3 B). Moreover, at the end of the restimulation period (day 8), the MFI of FOXP3 expression was significantly higher in iT reg cells generated with TGF-β + RA + vitamin C than in the other culture conditions and also displayed a tighter distribution (narrower peak and lower coefficient of variation; Fig. 8 C, middle and right, green histogram and bars). Moreover, as shown for mouse iT reg cells in Figs. 3, 4, and 5, the presence of vitamin C augmented TET enzymatic activity (Fig. S3 C) and potentiated the loss of 5mC at *CNS2* (Figs. 8 B and S3 D) in human iT reg cells collected after 6 d of differentiation.

Finally, we evaluated the ability of vitamin C to potentiate the suppressor activity of human iT reg cells differentiated under the four conditions. iT reg cells differentiated with TGF-β + RA + vitamin C were superior to all the other in vitro–generated iT reg cells in their ability to suppress proliferation of CFSE-labeled heterologous CD8$^+$ T cells (responders) over the course of a 5-d suppression assay in vitro (Fig. 8, D and E). Although this could in part reflect the increased numbers of iT reg cells with increased FOXP3 expression in the TGF-β + RA + vitamin C cultures, the results collectively show that vitamin C results in the loss of 5mC at *FOXP3 CNS2* and promotes the stability of FOXP3 expression in human iT reg cells as it does in mice, and moreover potentiates the suppressor function of human iT reg cells to the levels observed in T reg cells isolated and expanded directly from human peripheral blood.

DISCUSSION

Expression of Foxp3 and establishment of a T reg cell–specific hypomethylation pattern are both indispensable for T reg cell development and function (Ohkura et al., 2012). Here, we implicate TET proteins in these processes. We focused on Tet2 and Tet3 as the major TET proteins expressed in differentiated tissues and cell types, including lymph node and spleen T cells and thymic T reg cell populations (Toker et al., 2013; Tsagaratou and Rao, 2013). By mapping 5mC and 5hmC at a single-base resolution, we show that 5mC is lost progressively during T reg cell development at several T reg cell–specific regulatory regions, including *CNS1* and *CNS2* in the *Foxp3* gene. During this developmental progression, 5hmC levels are low but peak in T reg cell precursors or thymic T reg cells, as expected for a transient intermediate in a TET-mediated process of DNA demethylation (Pastor et al., 2013; Wu and Zhang, 2014). Furthermore, we show that the loss of 5mC from T reg cell–specific regulatory regions is significantly impaired in peripheral T reg cells from *Tet2/3 DKO* mice. Accompanying the loss of 5mC, peripheral T reg cells from *Tet2/3 DKO* mice fail to maintain Foxp3 expression after adoptive transfer in vivo or TCR stimulation in vitro.

The data point to considerable redundancy among the three TET proteins in mediating the loss of 5mC at regulatory regions and the stability of Foxp3 expression in T reg cells. In our hands, single deletion of *Tet2* in peripheral T reg cells did not result in any gain of CpG methylation (5mC + 5hmC) at *Foxp3 CNS1* and *CNS2*; single deletion of *Tet3* resulted in a minimal increase, and double deletion of both *Tet2* and *Tet3* resulted in a more striking increase (compare Fig. 1 [C and D] with Fig. S1 [K and L]). Similarly, although the phenotypes of single Tet1- or Tet2-deficient T reg cells were not investigated, a recent paper reported increased 5mC + 5hmC/total C in *Tet1/2 DKO* T reg cells (Yang et al., 2015), indicating that Tet1 activity also contributes to T reg cell function. Indeed, we find that *Tet1* mRNA is expressed at slightly higher levels in the absence of Tet2 and Tet3 (Fig. 6 C), consistent with the conclusion that all three TET proteins contribute to *CNS2* demethylation and stabilization of Foxp3 expression. Triple

6 d in the presence of TGF-β, TGF-β + vitamin C, TGF-β + RA, or TGF-β + RA + vitamin C. Right: Graphs for the percentage of Foxp3$^+$ cells from peripheral and mesenteric lymph nodes. Data are from five mice per group. (D) Schematic representation of adoptive transfer experiment of *scurfy* CD4$^+$ T cells. (E) Representative histograms of CD45.1$^+$ cells (top) and CD45.2$^+$CD4$^+$Foxp3$^+$ cells (bottom) from *Rag*-deficient mice 3.5–4 wk after adoptive transfer of iT reg cells differentiated in the presence of TGF-β, TGF-β + vitamin C, or TGF-β + RA + vitamin C together with *scurfy* CD4$^+$ T cells. Bottom right: Graph for the percentage of Foxp3$^+$ cells from mesenteric lymph nodes. Data are from three or four mice per group. Error bars show mean ± SD. mLN, mesenteric lymph node. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by Student's *t* test.

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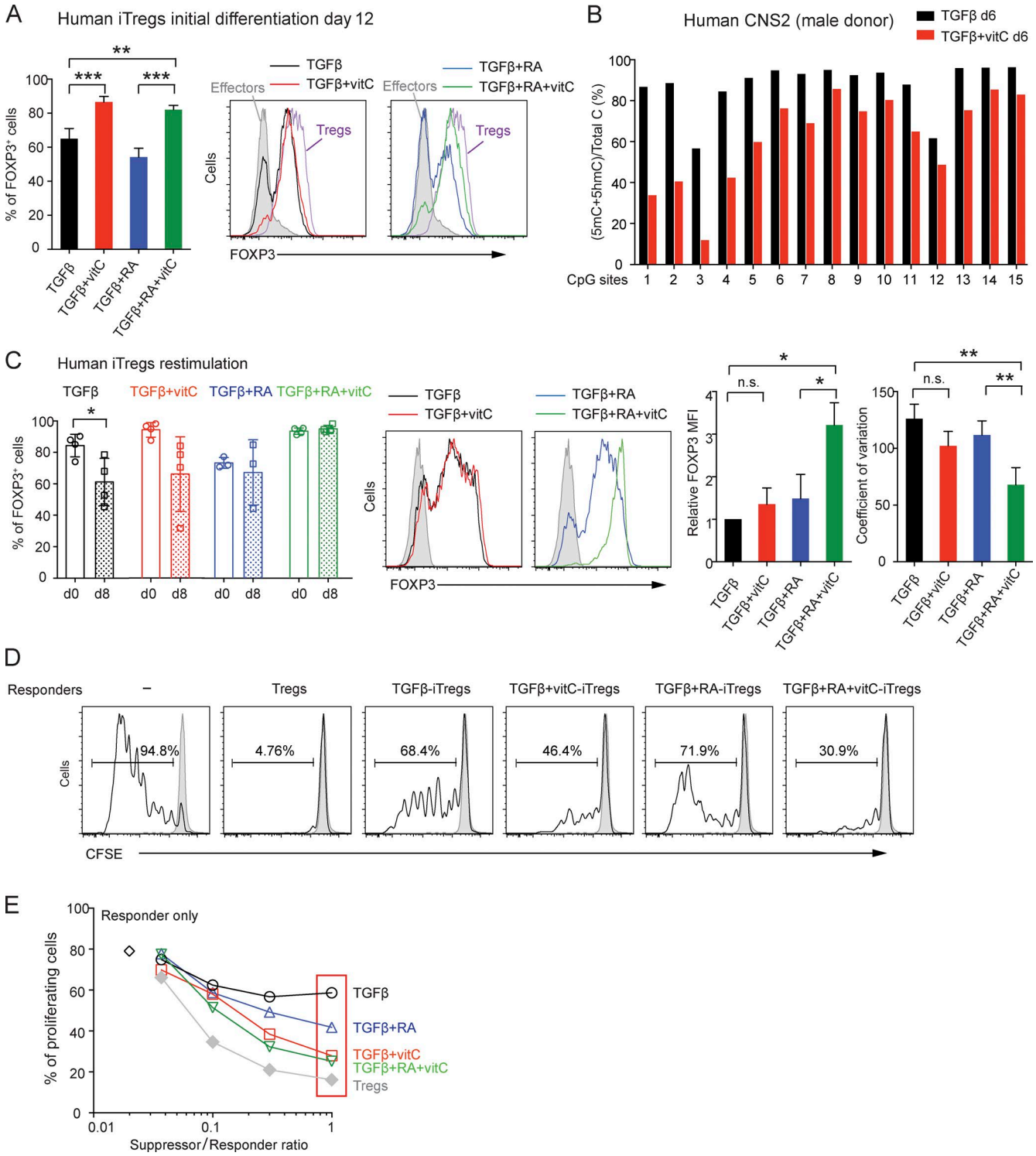

Figure 8. **Vitamin C promotes the loss of 5mC in human *FOXP3 CNS2* and enhances the stability and suppressive function of human iT reg cells.** (A, left) Percentage of Foxp3+ cells in human iT reg cells differentiated from naive CD4+ T cells in the presence of TGF-β, TGF-β + vitamin C, TGF-β + RA, and TGF-β + RA + vitamin C on day 12. Error bars show mean ± SD from three to four independent donors. Right: Representative histogram overlay of Foxp3 expression for human iT reg cells differentiated for 12 d in the presence of TGF-β, TGF-β + vitamin C, TGF-β + RA, and TGF-β + RA + vitamin C. Effector CD4+ T cells (filled gray) and expanded T reg cells (purple) are shown for comparison. (B) BS-seq of 15 CpGs in human *FOXP3 CNS2*. Graph depicts the percentage of (5mC + 5hmC)/total C in human iT reg cells differentiated for 6 d without or with vitamin C from a male donor. (C, left) Analysis of Foxp3 expression in human iT reg cells generated as indicated in the presence of TGF-β, TGF-β + vitamin C, TGF-β + RA, and TGF-β + RA + vitamin C (d0) and

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depletion of all three TET proteins in T reg cells will be needed to confirm this point.

A previous study (Nair and Oh, 2014) reported a surprisingly high increase in the level of 5mC + 5hmC at *CNS2* in T reg cells from mice lacking *Tet2* alone (~90% 5mC + 5hmC/total C). In contrast, we see a negligible increase (Fig. S1, K and L). The discrepancies may reflect differences in genetic background of the mice (we generated *Tet2*-deficient mice on a pure C57BL/6 background, whereas those of Nair and Oh [2014] were on a mixed C57BL/6-129 background) or differences in BS conversion efficiency, time of BS treatment, or BS concentration or potency, which we controlled for by spiking equivalent amounts of λ-DNA fragment into all samples (Table S2). We note that manual sequence analysis of small numbers of clones can result in sampling errors, which can be compounded by poor PCR amplification because of DNA damage resulting from prolonged BS treatment; we minimized these problems by sequencing thousands of amplicons from BS-converted DNA on a MiSeq platform and subjecting the data to rigorous statistical analysis. We also note that TET enzymes are dioxygenases that use molecular oxygen, 2-oxoglutarate (a product of the Krebs cycle), and reduced iron (Fe(II)) as substrates and cofactors (Pastor and Rao, 2013; Huang and Rao, 2014). As a result, TET enzymatic activity is likely to be sensitive to many signals, including hypoxia, metabolic state (2-oxoglutarate levels), Fe^{2+} availability, and the redox environment, as well as vitamin C levels as shown here. If any of these factors were suboptimal in the diet or environment of the mice analyzed by Nair and Oh (2014), it would lead to a larger apparent difference between WT and Tet2-deficient T reg cells.

Vitamin C is a known coactivator for TET proteins in several settings, including mouse ESCs (Blaschke et al., 2013), embryonic fibroblasts (Minor et al., 2013), during induced pluripotent stem cell reprogramming (Chen et al., 2013), and erythroid precursors (Ruiz et al., 2015), and as shown by our laboratory (Blaschke et al., 2013) and others (Yin et al., 2013), it acts directly on recombinant Tet1 and Tet2 in vitro. Here, we show that the presence of vitamin C (ascorbate) during iT reg cell differentiation leads to a marked loss of 5mC at *CNS1* and *CNS2* as judged by BS-seq and oxBS-seq (Fig. 5), as well as increased stability of Foxp3 expression after restimulation in vitro or adoptive transfer in vivo (Fig. 7). BS-seq revealed that the loss of 5mC induced by vitamin C was apparent in WT cells, in iT reg cells lacking Tet3, and to a lesser extent in iT reg cells lacking Tet2, but was completely abrogated in iT reg cells doubly deficient for both Tet2 and Tet3 (Fig. 6, D–H). These data again attest to the strong redundancy between Tet2 and Tet3. Given the likely involvement of Tet1 (Yang et al., 2015), we conclude that all three TET proteins are likely targets of vitamin C and function redundantly to regulate CpG methylation at T reg cell–specific regulatory regions, including *CNS1* and *CNS2* of *Foxp3*.

Together, vitamin C and RA potently stabilized Foxp3 expression in TGF-β–induced iT reg cells (Fig. 7). Vitamin C enhanced TET catalytic activity without altering *Tet* mRNA levels (Fig. 3), consistent with its well-established ability to potentiate directly the activity of many (but not all) Fe(II) and 2-oxoglutarate–dependent dioxygenases (Flashman et al., 2010), including the collagen prolyl hydroxylase, whose defective activity results in scurvy (Loenarz and Schofield, 2008). We previously showed that vitamin C acts directly on recombinant TET1 in vitro to increase its enzymatic activity (Blaschke et al., 2013); here, we have used a cell-based assay and high-throughput automated microscopy to extend this finding to all three TET family proteins (Fig. 3 A). Most likely, vitamin C potentiates TET dioxygenase function by maintaining iron in its reduced Fe(II) form (Du et al., 2012); other less likely scenarios include a postulated role of vitamin C as a direct cofactor of TET (Dickson et al., 2013) or ascorbate-induced localization of TET enzymes to the regulatory regions of T reg cell–specific genes (for which there is no evidence at present). The stabilizing effect of RA on Foxp3 protein expression has been noted before (Benson et al., 2007). RA increased *Tet3* mRNA levels (Fig. 3 B) as also independently observed in mouse ESCs (Koh et al., 2011). However, RA did not affect the CpG methylation status of *CNS1* and *CNS2* (Fig. 4, C and D). Potentially, RA could act through its nuclear receptors to affect *Foxp3* gene transcription or could regulate Foxp3 stability at the protein level as well.

In a previous study, T reg cells lacking *CNS2* were shown to lose Foxp3 upon adoptive transfer into recipient mice, whereas T reg cells lacking *CNS1* did not (Zheng et al., 2010). In contrast, our findings suggest that the stability of Foxp3 expression might be regulated by both these conserved elements as well as potentially other T reg cell–specific regulatory regions in vivo. Notably, *CNS1* CpG 1 and *CNS2* CpGs 10 and 11 displayed a particularly rapid loss of 5mC, both during T reg cell development in the thymus and iT reg cell differentiation in cell culture, suggesting that transcription factors binding in the vicinity of these CpGs might be involved in recruitment of Tet2 and/or Tet3. For instance, the presence of an NFAT binding site near *CNS2* CpGs 10 and 11 (Li et al., 2014) and the rapid loss of 5mC at these CpGs during iT reg

restimulated with anti-CD3 and anti-CD28 antibodies for 8 d (d8). Graphs show results from three independent donors. Middle: Representative histogram overlay of Foxp3 expression for human iT reg cells on day 8 after restimulation. Right: Foxp3 MFI (normalized to the MFI in TGF-β condition) and coefficient of variation analyzed on day 8 after restimulation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by Student's *t* test. n.s., not significant. (D) Proliferation analysis by CFSE dilution in human $CD8^+$ T cells (responders) stimulated with anti-CD3 antibody and cultured with T reg or iT reg cells (suppressors) at a responder/suppressor ratio of 1:0.3. The percentage of proliferating cells (black line) and of resting, unstimulated responders (gray shaded histogram) are shown in each plot. (E) Percentage of proliferating responder cells at different responder/suppressor ratios. Results are representative of three independent donors.

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cell differentiation suggest that NFAT may recruit TET proteins to this region of *CNS2*. A similar activation-dependent mechanism may explain TET recruitment to *CNS1*, which has several consensus NFAT sites (Tone et al., 2008) and displays some loss of 5mC + 5hmC even in T cells activated under Th0 conditions. IL-2 maintains the stability of Foxp3 expression, and STAT5—the downstream transcriptional effector of IL-2 signaling—is known to bind *CNS2* and so may also be involved (Burchill et al., 2007). Finally, factors specific to iT reg cell differentiation (e.g., TGF-β–activated SMAD factors; Tone et al., 2008) must also have a role because naive T cells activated under Th0 conditions failed to lose 5mC in *CNS2* even in the presence of equivalent amounts of IL-2 and vitamin C.

Two recent publications using *CNS2 KO* mice (Feng et al., 2014; Li et al., 2014) have suggested that *CNS2* deficiency stochastically affects the stability of ~50% of mature T reg cells or selectively affects the stability of T reg cells bearing low levels of Foxp3. Regardless of which situation applies, the data suggest that the DNA modification status of *CNS2* is not the sole determinant of T reg cell stability; other cis-elements and mechanisms undoubtedly have a role. The more striking effects observed at *CNS1* and *CNS2* compared with other regulatory regions most likely reflect the relative strength of recruitment of TET proteins to these regions by sequence-specific transcription factors.

From a therapeutic perspective, we have shown that vitamin C increases the stability and suppressive function of human iT reg cells as well, suggesting that manipulation of TET dioxygenase activity using either vitamin C or other more selective TET activators identified in small molecule screens might be a practical strategy for stabilizing iT reg cell function in the clinic.

MATERIALS AND METHODS

Mice. To evaluate the methylation status of the *CNS2* locus of the *Foxp3* gene, we used 4-wk-old male Foxp3-IRES-eGFP reporter mice, which were originally obtained from The Jackson Laboratory and further backcrossed to the B6/C57 background for >10 generations. Congenic mice (strain B6.SJL -Ptprc^{a}Pepc^{b}/BoyJ) were obtained from The Jackson Laboratory. *Tet2*$^{-/-}$*Tet3*$^{fl/fl}$CD4-Cre, *Tet2*$^{fl/fl}$*Tet3*$^{fl/fl}$CD4-Cre mice, and *Tet2*$^{-/-}$*Tet3*$^{fl/fl}$ ERT2-Cre mice were generated in our laboratory using B6/Taconic background "artemis" ESCs. All WT mice used in this study are littermate controls that do not express Cre. All breeding and experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the La Jolla Institute for Allergy and Immunology.

Flow cytometry and FACS. All the antibodies used for flow cytometry analyses and FACS sorting were purchased from eBioscience and BioLegend. For experiments evaluating DNA methylation and hydroxymethylation in the *CNS2* locus, thymocytes were isolated from young (4 wk old) Foxp3-IRES-eGFP male mice. In these mice, a cassette encoding IRES-eGFP was inserted into the 3′ untranslated region of Foxp3 to generate a bicistronic locus encoding both Foxp3 and eGFP under the control of Foxp3 promoter (Haribhai et al., 2007). The thymocytes were stained using CD4 (GK1.5) and CD25 (PC61). Foxp3 expression was indicated by eGFP expression. CD4^{+}CD8^{+} (DP), CD4^{+}CD25^{-}eGFP^{-} cells (CD4 SP), CD4^{+}CD25^{+}eGFP^{-} (precursor T reg cells), CD4^{+}CD25^{-}eGFP^{+} (precursor T reg cells), and CD4^{+}CD25^{+}GFP^{+} (thymic T reg cells) were isolated (Fig. S1 A).

To obtain peripheral T reg cells, spleen and lymph nodes were isolated from young (4 wk old) male Foxp3-GPP reporter mice. Subsequently, CD4^{+} T cells were enriched (Dynabeads untouched mouse CD4 T cells; Invitrogen) and stained with the surface markers CD4 (GK1.5), CD8 (53-6.7), CD62L (MEL-14), CD25 (PC61), and CD44 (IM7). Peripheral T reg cells were isolated as CD4^{+}CD25^{+}eGFP^{+} cells (Fig. S1 A). Post-sort purity was >99%. For Foxp3 intracellular analyses, cells were surface stained and then stained with anti-Foxp3 antibody (eBioscience) using the Foxp3 Fixation/Permeabilization kit (eBioscience) and analyzed by flow cytometry.

In vitro iT reg cell differentiation and restimulation. CD4^{+}CD25^{-}CD62L^{hi}CD44^{lo} (eGFP^{-} for Foxp3-IRES-eGFP reporter mice) naive T cells were FACS sorted from lymph nodes of 4–6-wk-old mice and differentiated into iT reg cells with plate-bound anti-CD3 (clone 2C11) and anti-CD28 (clone 37.51) antibodies at 1 μg/ml in the presence of 2 ng/ml recombinant human TGF-β (PeproTech) and 100 U/ml recombinant human IL-2 (rhIL-2). For the conditions with RA (Sigma-Aldrich) or vitamin C (Sigma-Aldrich), 100 nM RA and 100 μg/ml vitamin C were added into the culture. For the restimulation experiments, iT reg cells differentiated for 6 d were harvested and sorted for eGFP^{+} (Foxp3^{+}) population. Cells were counted and plated at 0.8×10^{6} cells/ml for restimulation with plate-bound anti-CD3 at 50 ng/ml and anti-CD28 at 25 ng/ml, and the cells were then monitored for eGFP expression after restimulation.

In vivo T reg/iT reg cell stability assay. In vivo T reg cell stability assay was performed as described previously (Zheng et al., 2010). In brief, T reg cells were FACS sorted from *Tet2/3 DKO* mice and age-matched WT controls. 10^{5} CD45.2^{+} T reg cells mixed with 10^{6} CD4^{+} naive T cells FACS sorted from CD45.1^{+} congenic mice were injected intravenously into *Rag*-deficient recipients. For iT reg cell stability assay, 4×10^{5} in vitro TGF-β–induced iT reg cells were mixed together with 2×10^{6} CD45.1^{+}CD4^{+} naive T cells and injected intravenously into *Rag*-deficient recipients. 4–5 wk after adoptive transfer, the CD45.2^{+}CD4^{+}Foxp3^{+} cell population was analyzed by flow cytometry.

***Scurfy* CD4^{+} T cell adoptive transfer.** *Scurfy* CD4^{+} T cells were isolated from spleen and peripheral lymph nodes from male *Scurfy* mice and purified using Dynabeads (purity of >98%; Life Technologies). 5×10^{5} CD45.1^{+}CD4^{+} scurfy T cells were injected into *Rag*-deficient mice alone or mixed with 10^{5} in

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vitro–generated iT reg cells (TGF-β alone, TGF-β + vitamin C, and TGF-β + RA + vitamin C). 3.5–4 wk after adoptive transfer, the percentage of $CD45.1^+CD4^+$ cells and $CD45.2^+CD4^+Foxp3^+$ cells were analyzed by flow cytometry.

Tet3 deletion in ERT2-Cre transgenic system. To recombine the *Tet3* floxed allele using the ERT2-Cre transgenic system, tamoxifen (Sigma-Aldrich) was solubilized at 10 mg/ml in corn oil (Sigma-Aldrich) and delivered into either WT control mice or *Tet2*$^{-/-}$ *Tet3*$^{fl/fl}$ ERT2-Cre mice by intraperitoneal injection at 2 mg/mouse/day for five consecutive days. Mice were sacrificed 24 h after the last injection, and naive T cells were FACS purified and treated for 2 d in vitro with 4-OHT (Sigma-Aldrich) to reinforce *Tet3* deletion efficiency. The cells were then washed with cell culture medium and induced into iT reg cells with TGF-β or TGF-β + vitamin C.

Quantitative analysis of CMS levels using dot blots. Genomic DNA samples were treated with sodium BS using the Methylcode kit (Invitrogen). BS-treated DNA samples were then denatured in 0.4 M NaOH and 10-mM EDTA at 95°C for 10 min and neutralized by adding an equal volume of cold 2 M ammonium acetate, pH 7.0. Twofold serial dilutions of the denatured DNA samples were spotted on a nitrocellulose membrane in an assembled Bio-Dot apparatus (Bio-Rad Laboratories) according to the manufacturer's instructions. The membrane was washed with 2× SSC buffer, air dried, vacuum baked at 80°C for 2 h, and then blocked with 5% nonfat milk for 1 h and incubated with anti-CMS antibody (1:3,000) overnight at 4°C. After incubating with HRP-conjugated anti–rabbit IgG secondary antibody, the membrane was visualized by enhanced chemiluminescence.

Analysis of vitamin C effect on TET proteins. HEK293T cells were transfected with hemagglutinin-tagged TET1–3 using Lipofectamine 2000 (Invitrogen) in OptiMEM (Gibco). 6 h later, DNA-Lipofectamine mixture was replaced with DMEM with or without vitamin C (Sigma-Aldrich). 48 h after transfection, cells were fixed with 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature. Subsequently, DNA was denatured with 2 N HCl at room temperature for 30 min and neutralized with 100 mM Tris-HCl buffer, pH 8.5, for 10 min. Cells were then washed and incubated in blocking buffer PBS, 1% BSA, and 0.05% Tween 20 at room temperature for 1 h. Next, mouse anti-5hmC antibody (diluted to 1:500; Active Motif) for 5hmC staining or goat anti-HA antibody (diluted to 1:500; Bethyl) for TET staining was added in blocking buffer at 4°C overnight. The cells were rinsed three times with 0.2% Triton X-100 in PBS and incubated at room temperature for 2 h with donkey anti–mouse Alexa Fluor 555 and donkey anti–goat Alexa Fluor 647 (diluted to 1:1,000; Invitrogen) in blocking buffer, again washed three times with 0.2% Triton X-100 in PBS, and stained with DAPI. Cells were imaged with the Image Express Micro System (Molecular Devices), and data were analyzed with R software.

In vitro human iT reg cell differentiation and restimulation. PBMCs were isolated by Lymphoprep gradient (Cosmo Biosciences) from Leuco reduction system white blood cells obtained from the San Diego Blood Bank from de-identified healthy donors. Per the Institutional Review Board (IRB) of La Jolla Institute (FWA 00000032), work with de-identified human specimens purchased from the blood bank does not meet the regulatory definition of human subject research and does not require IRB approval. $CD4^+$ naive T cells were FACS sorted from PBMCs as a $CD4^+CD45RA^+CCR7^+$ population. Cells were stimulated with Dynabeads human T cell activator CD3/CD28 (Invitrogen) for 4 d at a cell/bead ratio of 1:1 in the presence of 2 ng/ml TGF-β and 100 U/ml rhIL-2. For the conditions with RA or vitamin C, 100-nM RA and 100 μg/ml vitamin C were added into the culture. After the initial 4-d stimulation, the beads were removed and the cells were maintained in complete media (IMDM with 5% FCS, 2% human serum, and β-mercaptoethanol) supplemented with rhIL-2. For the restimulation assay, the cells were subjected to a second round of polarization under the same conditions and restimulated with Dynabeads human T cell activator CD3/CD28 for 2 d at a cell/bead ratio of 1:1 in the presence of rhIL-2 and were analyzed for Foxp3 expression by intracellular staining.

Human T reg cell suppression assay. PBMCs isolated by Lymphoprep gradient from adult peripheral blood or buffy-coats were labeled with CFSE (Invitrogen). In brief, PBMCs were washed with PBS and resuspended in PBS at 10^7 cells/ml. CFSE was added at a final concentration of 5 μM, and cells were incubated for 8 min in the dark with gentle agitation. The reaction was quenched by the addition of an equal volume of FBS, and cells were washed twice in a complete medium. For suppression assays, suppressor T cells (iT reg cells or expanded T reg cells), at day 12 after the first round of stimulation, were rested without IL-2 or any other cytokine or factor for 6 h at 37°C. CFSE-labeled responder cells (PBMCs) were plated at a density of 4×10^4 cells/well in a 96-well U bottom plate, and suppressor cells were added at different suppressor/responder ratios. Soluble anti-CD3 agonistic antibody (OKT3 clone; eBioscience) was added at 100 ng/ml. After 5 d, cells were stained with fixable viability dye, anti-CD4, and anti-CD8 antibodies. Responder $CD8^+$ T cells were gated as live cells (negative for the fixable viability dye), and the percentage of cells that had diluted CFSE (proliferating cells) was evaluated using unstimulated, CFSE-labeled PBMCs kept in culture for the same period of time as reference. Proliferation rate of responder cells, without suppressor cells, was used to calculate the percentage of suppression as follows: 100 − ([% of proliferating cells × 100]/% of proliferating responders alone).

Generation of heat maps. Heat maps were generated using Genesis software (Sturn et al., 2002).

Statistical analysis. Significance was determined by two-tailed Student's *t* tests using Prism 6 (GraphPad Software). Asterisks

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indicate p-values: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; n.s., not significant. The number of mice used in each experiment is indicated in the figure legends. For the methylation analyses in Fig. 1 (C–H; WT vs. *Tet2/3 DKO*), Fig. 4 (C–H; TGF-β vs. TGF-β + vitamin C and TGF-β + RA vs. TGF-β + RA + vitamin C), and Fig. 6 (D–G; TGF-β WT vs. TGF-β + vitamin C WT; Tet2 KO vs. Tet2 KO + vitamin C; and Tet3 KO vs. Tet3 KO + vitamin C), all outcomes are significant with $P < 0.05$, and in many cases, < 0.01 or lower.

BS- and oxBS-seq. The DNA oxidation procedure was done as described previously (Booth et al., 2012, 2013). In brief, up to 1 μg of genomic DNA was ethanol precipitated before oxidation and then filtered through P6 SSC Micro bio-spin column (Bio-Rad Laboratories). DNA was denatured in 0.05 M NaOH for 30 min at 37°C and then snap cooled on ice for 5 min (total of 24 μl). 1 μl of KRuO4 solution (15 mM in 0.05-M NaOH; Sigma-Aldrich) was then added, and the denatured DNA was held on ice for 1 h, with occasional vortexing. The DNA was purified using P6 SSC Micro bio-spin column, and the oxidized and nonoxidized DNA samples were treated in parallel with sodium BS (MethylCode Bisulfite Conversion kit; Invitrogen). PCR primers (Table S1) were designed using Methyl Primer Express software (Life Technologies) or from previous studies (Lal et al., 2009; Ohkura et al., 2012). The PCR amplicons were generated using the PyroMark PCR kit (QIAGEN) and quantified using Quant-iT PicoGreen dsDNA reagent (Invitrogen). To monitor BS conversion efficiency, a 210-bp spike-in control was generated using unmethylated λ-DNA (Promega) as a template and added to the genomic DNA to a final percentage of 0.5% (Table S2). PCR amplicons were then used for library preparation using NEBNext DNA Library Modules for Illumina platform (New England Biolabs, Inc.). The final libraries were quantified using the KAPA library quantification kit for Illumina (KAPA Biosystems) and sequenced on Miseq (300 bp, paired end; Illumina). The data are based on thousands of sequence reads per amplicon, and a rigorous statistical analysis of 5mC, 5hmC, and C/5fC/5caC levels was performed as described in the following two sections.

BS-seq and oxBS-seq data analysis. The BS and oxBS reads were mapped to mouse genome mm9 and λ-phage DNA using the Bismark mapping tool. The mapping was done using the paired end Bowtie2 backend with the following parameter values: –I 0 –X 600 –N 0. For each of the samples, the bismark_methylation_extractor script in the Bismark package was used to extract the number of times each cytosine within the amplicons was converted. These counts were used to calculate the proportions for each cytosine to be nonmethylated (or formylmethylated/carboxylmethylated), methylated, or hydroxymethylated. For details, see the Bismark manual (http://www.bioinformatics.babraham.ac.uk/projects/bismark/Bismark_User_Guide.pdf).

Statistical model for BS-seq and oxBS-seq data. We used a multinomial random variable to model the methylation status of a given cytosine (index omitted for brevity). The proportions or probabilities of the possible states C, 5mC, 5hmC, 5fC, and 5caC are p(C), p(5mC), p(5hmC), p(5fC), and p(5caC) (they sum up to unity), respectively. The cytosine modifications 5fC and 5caC cannot be distinguished from C using the BS- and oxBS-seq approaches, and thus those probabilities are collapsed and denoted as $p(C \cup 5fC \cup 5caC) = p(C) + p(5fC) + p(5caC)$. The unknown parameters are denoted as $\theta = p(C \cup 5fC \cup 5caC)$, p(5mC), and p(5hmC). BS- and oxBS-seq experiments result in convoluted observations of the aforementioned random variable. For example, the probability of sequenced thymine after BS treatment is $p(C \cup 5fC \cup 5caC)$, whereas the probability of observing cytosine is $1 - p(C \cup 5fC \cup 5caC) = p(5mC) + p(5hmC)$. Similarly, the probabilities of observing thymine and cytosine after oxidation and BS treatment are $p(C \cup 5fC \cup 5caC) + p(5hmC)$ and p(5mC), respectively. For a given cytosine, let D_{BS} and D_{oxBS} denote the experimental datasets from the BS- and oxBS-seq experiments, respectively. Moreover, let $N_{BS=C}$ and $N_{BS=T}$ be the number of cytosine and thymine observations in D_{BS}. Similarly, we have $N_{oxBS=C}$ and $N_{oxBS=T}$ for D_{oxBS}. Then the likelihood of the data are $p(D_{BS}, D_{oxBS} | \theta) = p(D_{BS} | \theta) \times p(D_{oxBS} | \theta)$, where $p(D_{BS} | \theta) = p(C \cup 5fC \cup 5caC)^{NBS=T} \times (p(5mC) + p(5hmC))^{NBS=C}$ and $p(D_{oxBS} | \theta) = (p(C \cup 5fC \cup 5caC) + p(5hmC))^{NBS=T} \times p(5mC)^{NBS=C}$. The model is inferred using the Bayesian framework.

Inspired by the Dirichlet-Binomial model, we set a Dirichlet prior for θ, $p(\theta | \alpha)$. A weak prior $p(\theta | \alpha)$ is defined such that the methylation states are equally probable a priori by setting $\alpha = (2, 2, 2)^T$. The posterior distribution of θ cannot be solved analytically with a Dirichlet prior (although it is a conjugate prior for the categorical likelihood) because of the convoluted observations. Therefore, we resort to the Metropolis-Hastings algorithm. A proper proposal distribution for drawing a new parameter sample θ* based on the current one θ_i has to fulfill the unity sum constraint $\Sigma\theta^* = 1$. To enforce this, we choose a Dirichlet distribution as the proposal distribution. The parameter α_{prop} of the Dirichlet proposal distribution $q(\theta^* | \theta_i)$ is the current parameter sample θ_i multiplied by a scalar-β. The scalar-β controls the deviation of the proposal distribution, and thus it can be used to obtain a desired acceptance ratio. Empirically, we set β = 50 to achieve an acceptance ratio varying from 30 to 50%. The Markov Chain Monte Carlo chains were run for 50,000 iterations, which was enough for the chains to converge to their equilibrium distributions. The drawn parameter samples were used to estimate the parameter posterior distributions. The parameter posterior mean was used as an estimate of the proportions of cytosine modifications $p(C) \approx p(C \cup 5fC \cup 5caC)$, p(5mC), and p(5hmC).

Online supplemental material. Fig. S1 shows the gating strategies and the methylation analysis of *Foxp3 CNS* regions in CD4 SP thymocytes and T reg cells isolated from WT, *Tet2/3 DKO* mice, or mice with a single deletion of *Tet2* or *Tet3*. Fig. S2

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shows the percentage and MFI of Foxp3$^+$ cells in iT reg cells differentiated from naive T cells isolated from WT, *Tet2 KO*, *Tet3 KO*, and *Tet2/3 DKO* mice in the presence of TGF-β or TGF-β/vitamin C. Fig. S3 shows the percentage of Foxp3$^+$ cells during a time course analysis of human iT reg cell differentiation and restimulation and the effects of vitamin C on global 5hmC level and methylation changes of *FOXP3 CNS2* in human iT reg cells. Table S1 is a list of primer sequences used for amplicon sequencing in this study. Table S2 is a list of BS conversion efficiency assessed by spike-in control for BS or oxBS in this study. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20151438/DC1.

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The authors declare no competing financial interests.

Author contributions: A. Rao conceived the project and supervised project planning and execution. X. Yue performed BS-seq and oxBS-seq for the *Foxp3* locus, dot-blot assays, and experiments for mouse T reg cells. S. Trifari performed experiments with human T cells. A. Tsagaratou isolated WT and *Tet2/3 DKO* T reg cells and T reg cell precursors in the thymus. W.A. Pastor generated *Tet3*$^{fl/fl}$ mice. J.A. Zepeda-Martínez examined vitamin C effects on TET activity in HEK293T cells under the supervision of Y. Huang and analyzed the data. C.-W.J. Lio suggested and helped with scurfy transfer experiments. X. Li helped with amplicon sequencing. T. Äijö and H. Lähdesmäki performed bioinformatic analyses of BS-seq and oxBS-seq data. P. Vijayanand provided input for experimental design and critically evaluated the manuscript. A. Rao and X. Yue wrote the manuscript with assistance from S. Trifari.

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