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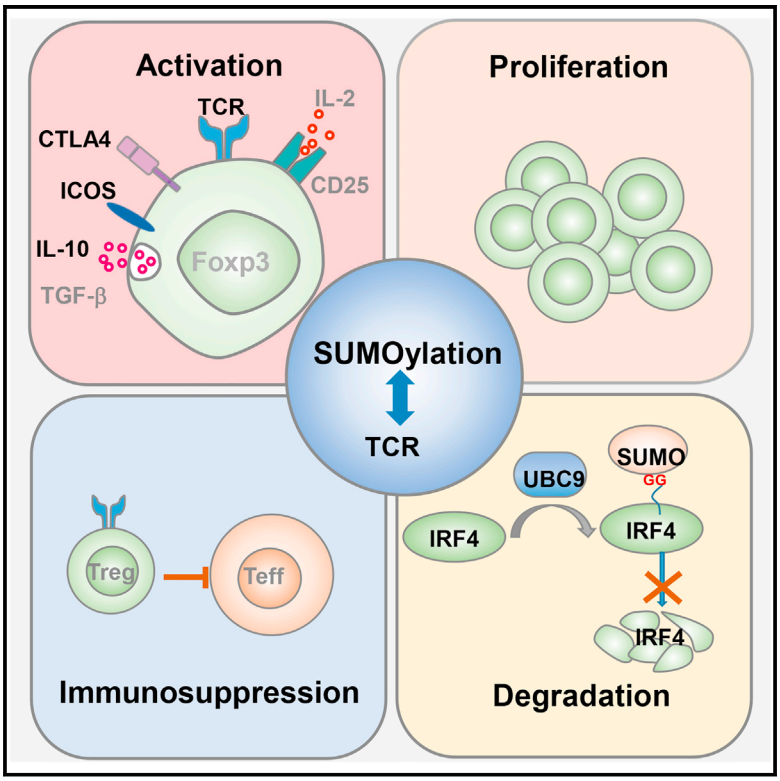
Graphical Abstract



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In Brief

Ding et al. find that UBC9-mediated protein SUMOylation is required for Treg cell homeostasis, proliferation, activation, and suppressive function by sustaining TCR signaling. The authors identify IRF4 as a SUMO target regulated by TCR-enhanced SUMOylation.

Highlights

- *Ubc9* deletion in Treg cells leads to early-onset lethal autoimmune disorders
- *Ubc9* is indispensable for Treg cells homeostasis proliferation, activation, and suppressive function
- TCR-dependent genes in Treg cells are regulated by UBC9-mediated SUMOylation
- SUMOylation promotes IRF4 protein stability and function in response to TCR

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Protein SUMOylation Is Required for Regulatory T Cell Expansion and Function

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SUMMARY

Foxp3-expressing regulatory T (Treg) cells are essential for immune tolerance; however, the molecular mechanisms underlying Treg cell expansion and function are still not well understood. SUMOylation is a protein post-translational modification characterized by covalent attachment of SUMO moieties to lysines. UBC9 is the only E2 conjugating enzyme involved in this process, and loss of UBC9 completely abolishes the SUMOylation pathway. Here, we report that selective deletion of *Ubc9* within the Treg lineage results in fatal early-onset autoimmunity similar to *Foxp3* mutant mice. *Ubc9*-deficient Treg cells exhibit severe defects in TCR-driven homeostatic proliferation, accompanied by impaired activation and compromised suppressor function. Importantly, TCR ligation enhanced SUMOylation of IRF4, a critical regulator of Treg cell function downstream of TCR signals, which regulates its stability in Treg cells. Our data thus have demonstrated an essential role of SUMOylation in the expansion and function of Treg cells.

INTRODUCTION

Regulatory T (Treg) cells are essential in maintaining immune tolerance and preventing autoimmune disorders (Josefowicz et al., 2012; Sakaguchi et al., 2008). Foxp3 is the lineage-specific transcription factor that determines Treg cell differentiation, maintenance, and suppressor function (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003; Williams and Rudensky, 2007). Mutation or deficiency of Foxp3 leads to fatal autoimmunity both in mice and humans due to the absence of Treg cells (Bennett et al., 2001; Brunkow et al., 2001). Constitutive expression of Foxp3 is required for establishing a distinct transcriptional program in Treg cells by reinforcing the expression of Treg signature genes (such as CTLA4, IL10, CD25, and GITR) while repressing expression of genes associated with effector T cells (Fontenot et al., 2005b; Gavin et al., 2007; Marson et al., 2007; Williams and Rudensky, 2007; Zheng et al., 2007). Other transcription factors, such as necrosis factor κB (NF-κB), NFAT, RUNX1, EOS, and IRF4, cooperate with Foxp3 to establish or stabilize the Treg transcriptional program (Long et al., 2009; Ono et al., 2007; Pan et al., 2009; Wu et al., 2006; Zheng et al., 2009). How post-translational modifications regulate these factors in response to environmental stimuli is unclear.

$Foxp3^+$ Treg cells are initially derived from the thymus as a result of a high-avidity interaction between T cell receptor (TCR) and major histocompatibility complex class II (MHC class II) molecules in the presence of CD28 costimulatory signals; common gamma-chain (γc) cytokines, especially interleukin-2 (IL-2), provide an important second signal for Treg cell differentiation (Josefowicz and Rudensky, 2009). After export to the periphery, Treg cells must proliferate and differentiate into effector Tregs to prevent autoimmunity or excessive immune activation. Unlike conventional T cells, Treg cells have a high proliferation rate counterbalanced with frequent apoptosis in vivo (Pierson et al., 2013; Wang et al., 2012a), which is regulated by the costimulatory molecule CD28 and cytokines like IL-2 and IL-7 (Appleman et al., 2000; Fontenot et al., 2005a; Simonetta et al., 2012). A recent study revealed that TCR signaling also plays an important role in peripheral Treg cell homeostasis and function, especially for effector Tregs; inducible ablation of TCR in mature Treg cells results in impaired activation, compromised homeostatic proliferation and decreased suppressor function, independent of FOXP3 expression, Treg signature gene expression or the ability to sense IL-2 (Levine et al., 2014; Vahl et al., 2014). However, the molecular pathways downstream of TCR in peripheral Treg regulation are poorly understood.

SUMOylation is a reversible post-transcriptional modification, involving covalent attachment of a SUMO moiety to a lysine residue in the target protein. SUMOylation is involved in many biological processes including nuclear organization, DNA replication and repair, sub-nuclear localization, transcription, meiosis, and signal transduction (Flotho and Melchior, 2013). E1, E2, and E3 enzymes are involved in SUMOylation, which can be reversed by SUMO-specific proteases (SENPs) (Flotho and Melchior, 2013). As the only SUMO E2 in mammalian cells, *Ubc9* is indispensable for embryonic development at the early post-implantation stage (Nacerddine et al., 2005). In *ROSA26-creERT2xUbc9$^{fl/-}$* mouse model, *Ubc9* deletion

induced by 4-hydroxytamoxifen treatment mainly affects the small intestine leaving other epithelial tissues unaffected (Demarque et al., 2011). In myeloid cells, impaired SUMOylation can promote Toll-like receptor (TLR)-induced production of inflammatory cytokines and massive expression of type I interferon signature genes without affecting the differentiation or survival of bone marrow-derived dendritic cells (Decque et al., 2016). These data indicate the pleiotropic and cell-fate-specific roles of SUMOylation at different developmental stages.

The functional roles of SUMOylation in T cells have not been well studied. SUMO-specific protease 1 (SENP1) is essential for the early development of T and B cells; SENP1 deficiency causes the accumulation of SUMOylated STAT5, thus blocking STAT5 acetylation and subsequent signaling (Van Nguyen et al., 2012). PIAS1, a SUMO E3 ligase, was shown to inhibit Treg cell differentiation by maintaining a repressive chromatin state at Foxp3 promoter, indicating crosstalk between SUMOylation and chromatin modification (Liu et al., 2010). Whether SUMOylation is involved in Treg cell regulation and the physiological consequence of SUMOylation deficiency in Treg cells are unknown.

Here, we generated Treg cell-specific *Ubc9* KO mice (*Foxp3*^{cre} *Ubc9*$^{fl/fl}$) and find they develop fatal early-onset autoimmune disease. *Ubc9*-deficient Treg cells show defects in homeostatic proliferation, impaired activation, and reduced suppressor capability. TCR-dependent gene expression in Treg cells is regulated by UBC9-mediated SUMOylation with IRF4 stability, an important transcription factor downstream of TCR, reduced in the absence of UBC9. Thus, SUMOylation coordinates several key aspects of Treg cell expansion and function.

RESULTS

Loss of *Ubc9* in Treg Cells Leads to Fatal Early-Onset Inflammatory Disorders

To investigate the role of UBC9-mediated SUMOylation in regulatory T cells, we generated mice with Treg cell-specific *Ubc9* ablation by crossing mice harboring a conditional *Ubc9* allele (*Ubc9*^{fl}) with *Foxp3*^{Cre} mice expressing an YFP-Cre fusion protein under the control of endogenous *Foxp3* locus (Rubtsov et al., 2008). *Ubc9* mRNA expression level was reduced by half in CD4$^+$YFP$^+$ Treg cells of *Foxp3*^{cre}*Ubc9*$^{fl/wt}$ male mice and further reduced in those from *Foxp3*^{cre}*Ubc9*$^{fl/fl}$ mice (Figure S1A). We then confirmed *Ubc9* deficiency in CD4$^+$ YFP$^+$ Treg cells at protein level from female *Foxp3*$^{cre/wt}$*Ubc9*$^{fl/fl}$ mice (Figure S1B). All *Foxp3*^{cre}*Ubc9*$^{fl/fl}$ mice developed severe autoimmune diseases at 3 weeks of age, characterized by blepharitis and dermatitis, reduced body size, extensive lymphadenopathy, and splenomegaly (Figures 1A and S1C). CD4$^+$ T cells in *Foxp3*^{cre} *Ubc9*$^{fl/fl}$ mice exhibited activated phenotype (CD44^{hi}CD62L^{lo}) in peripheral lymphoid organs (Figure 1C), with increased expression of activation markers such as CD25, ICOS, CD69, GITR, PD-1, and proliferation indicator Ki67 (Figure S1D). In addition, diseased mice showed massive lymphocyte infiltration in kidney, liver, lung, and salivary gland and succumbed to death at 3–5 weeks (Figures 1D and 1B).

All of these severe symptoms are reminiscent of the phenotypes in *Foxp3*-deficient mice (Fontenot et al., 2003). Loss of *Ubc9* in Treg cells resulted in increased percentage and number of CD4$^+$ T cells expressing interferon (IFN)-γ, IL-4, IL-5, IL-13, and IL-17A as well as CD8$^+$ T cells expressing IFN-γ, although tumor necrosis factor α (TNF-α) or IL-2 production in CD4$^+$ T cells were not elevated (Figures 1E and S1E; data not shown). In addition, *Foxp3*^{cre}*Ubc9*$^{fl/fl}$ mice showed notably increased level of IgG1, IgG2a, IgG2b, and IgG3 as well as anti-dsDNA auto-antibodies in serum compared with *Foxp3*^{cre}*Ubc9*$^{fl/wt}$ littermates (Figure S1F), which was associated with increased percentages of Tfh and GC B cells (Figures S1G and S1H). These data suggest that *Ubc9* expression in Treg cells is required to systematically control Th1, Th2, Th17, effector CD8$^+$ T, and B cell responses.

Reduced Cell Number and Impaired Activation of *Ubc9*-Deficient Treg Cells

In full agreement with the aforementioned severe autoimmune diseases, *Foxp3*^{cre} *Ubc9*$^{fl/fl}$ mice had decreased percentages and numbers of CD4$^+$ Foxp3$^+$ Treg cells in lymph nodes and spleen (Figures 2A and 2B). *Ubc9*-deficient Treg cells showed decreased expression of many activation markers such as CTLA4, ICOS, CD44, PD-1, Ki67, but not CD69, while CD62L and CD25 expression maintained at high levels in lymph nodes (Figure 2C). In contrast, loss of *Ubc9* had little effect on Foxp3, GITR, CD103, and CD127 expression (Figure 2C).

To assess the possibility that some of these phenotypes were caused by the inflammatory signals in diseased mice, we examined Treg cells in 7-day-old *Foxp3*^{cre} *Ubc9*$^{fl/fl}$ male mice, which exhibited minimal inflammation (data not shown). Consistent with the observation in Figure 2B, *Ubc9*-deficient Treg cells showed reduced numbers (Figure S2A) and proliferation (Figure S2B) in the spleen, accompanied with impaired expression of ICOS and PD-1 but largely normal expression of CD44 and CD62L (Figure S2C; data not shown). Thus, in the disease-free environment, *Ubc9*-deficient Treg cells have reduced numbers and defective expression of ICOS and PD-1.

To examine *Ubc9*-deficient Treg cells in a competitive environment, we analyzed CD4$^+$ YFP$^+$ Treg cells in female *Foxp3*$^{cre/wt}$ *Ubc9*$^{fl/fl}$ and *Foxp3*$^{cre/wt}$*Ubc9*$^{fl/wt}$ littermates. Due to X chromosome random inactivation, *Ubc9*-sufficient CD4$^+$YFP$^-$ Treg cells safeguard the mice from autoimmunity in female *Foxp3*$^{cre/wt}$ *Ubc9*$^{fl/fl}$ mice. Comparable CD4$^+$ YFP$^+$ Treg cells were found in the thymus of *Foxp3*$^{cre/wt}$*Ubc9*$^{fl/fl}$ and *Foxp3*$^{cre/wt}$*Ubc9*$^{fl/wt}$ mice (Figure S2D), suggesting that *Ubc9* is not required for initial Treg lineage development. However, *Ubc9*-deficient Treg cells had a competitive disadvantage in the periphery of *Foxp3*$^{cre/wt}$ *Ubc9*$^{fl/fl}$ mice (Figures S2D and S2E), with slightly reduced expression of certain activation markers such as CTLA4 and ICOS in disease-free setting (Figure S2F), similar to that of the inflammatory environment. Inflammation signals were shown to promote local Treg cell proliferation and activation. To address the role of *Ubc9* in inflammatory Tregs, female mice were subjected to keyhole limpet hemocyanin immunization in the presence of CFA. We found immunization augmented the cell number reduction and the activation defect of *Ubc9*-deficient Treg cells in draining lymph nodes after immunization (Figures S2G–S2I). Taken together, these results suggest that loss of *Ubc9* in Treg cells leads to reduced cell numbers and defective

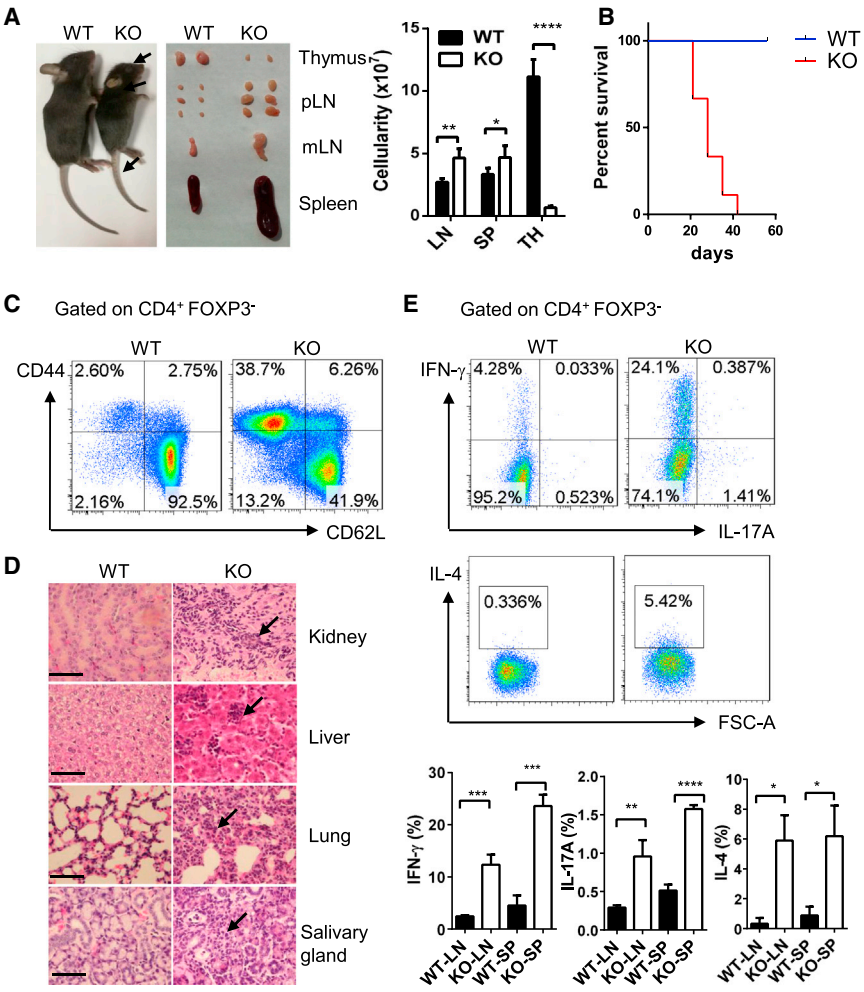

Figure 1. Loss of *Ubc9* in Treg Cells Leads to Fatal Early-Onset Inflammatory Disorders

(A) Left panel: images of 28-day-old male mice, thymus, peripheral lymph nodes, mesenteric lymph node, and spleen. Right panel: total cellularity of the lymph nodes, spleen, and thymus. n = 4–5 per group.

(B) Survival curve of male mice.

(C) Flow cytometry analysis of CD44, CD62L expression in $CD4^+$ $Foxp3^-$ T cells of lymph nodes. n = 3 per group.

(D) Histopathology of kidney, liver, lung, and salivary gland by H&E staining; infiltrated lymphocytes were indicated by black arrow. n = 4 per group. Bars, 50 μM.

(E) Flow cytometry analysis of cytokines produced by $CD4^+$ $Foxp3^-$ T cells of lymph nodes. Lymphocytes were stimulated with PMA and Ionomycin for 5 hr in the presence of Golgi-Plug or Golgi-Stop before staining CD4, Foxp3, and indicated cytokines. n = 4 per group.

In (A)–(E) WT, $Foxp3^{cre}Ubc9^{fl/wt}$; KO, $Foxp3^{cre}Ubc9^{fl/fl}$; LN, lymph nodes; SP, spleen; TH, thymus. In (A) and (C)–(E), 21- to 23-day-old male mice were used. In (A)–(E), values shown are mean ± SD. A representative of three independent experiments is shown. See also Figure S1.

expression of activation markers in a cell-intrinsic manner, and inflammatory signals can further enhance this defect.

Ubc9 Is Indispensable for Treg Cell Homeostatic Proliferation

Treg-specific deletion of *Ubc9* resulted in reduced Treg cells in periphery but not in the thymus, suggesting that *Ubc9* may be required for Treg homing to periphery or their expansion there. To address the homing issue, we transferred CD4SP thymocytes isolated from $Foxp3^{cre/wt}$ $Ubc9^{fl/wt}$ or $Foxp3^{cre/wt}$ $Ubc9^{fl/fl}$ mice to CD45.1 congenic mice and found *Ubc9*-deficient thymic Treg cells could home to lymph nodes and spleen normally (Figure S3A). In lymphopenic environment, Treg cells undergo homeostatic proliferation induced by the combination of cytokines and TCR stimulation (Gavin et al., 2002). To determine the role of *Ubc9* in this process, CellTrace-Violet-labeled CD4SP thymocytes isolated from female $Foxp3^{cre/wt}$ $Ubc9^{fl/fl}$ or $Foxp3^{cre/wt}$ $Ubc9^{fl/wt}$ mice were transferred to $Rag1^{-/-}$ recipient mice. *Ubc9*-deficient $CD4^+YFP^+$ Treg cells failed to proliferate (Figure 3A) or upregulate activation markers such as CTLA4, ICOS, and CD25 (Figure 3B) but behaved normally in apoptosis (Figure S3B). Notably, Foxp3 expression was attenuated in *Ubc9*-deficient Treg cells after homeostatic proliferation (Figure 3C), suggesting that SUMOylation is required for maintaining Foxp3 expression during Treg cell expansion process. The proliferation defect was further confirmed in vitro with anti-CD3 plus anti-CD28 stimulation and could not be rescued by additional IL-2 or IL-7 (Figure 3D). Cell-cycle analysis revealed that *Ubc9*-deficient Treg cells accumulated at G2M phase with tetraploid (4n) cell percentage twice as much as *Ubc9*-sufficient cells (Figure 3E). In addition, *Ubc9*-deficient Treg cells tended to lose Foxp3 expression upon TCR stimulation accompanying cell division (Figures S3C and S3D), which was reminiscent of the phenotypes in CNS2-deficient Treg cells (Feng et al., 2014; Li et al., 2014). Bisulfite sequencing of CNS2 region of purified $CD4^+YFP^+$ Treg cells from male mice showed that *Ubc9*-deficient Treg cells did not fully maintain the hypomethylation state as wild-type (WT) Treg cells do, especially after proliferation (Figure S3E), indicating the requirement of SUMOylation for Treg-specific epigenetic maintenance and thereby Foxp3 stability.

Next, we investigated the signaling pathways potentially contributed to the proliferation defect. Of cytokine signaling pathways, IL-2 and IL-7 are most critical for Treg cell homeostatic proliferation (Gavin et al., 2002; Simonetta et al., 2012). However, phosphorylated STAT5 was comparable in *Ubc9*-deficient and *Ubc9*-sufficient Treg cells both before and after IL-2 or IL-7 stimulation (Figures S4A and S4B). This could be further supported by the high level of CD25 (α chain of IL-2 receptor) and normal level of CD127 (α chain of IL-7 receptor) expression in *Ubc9*-deficient Treg cells (Figure 2C).

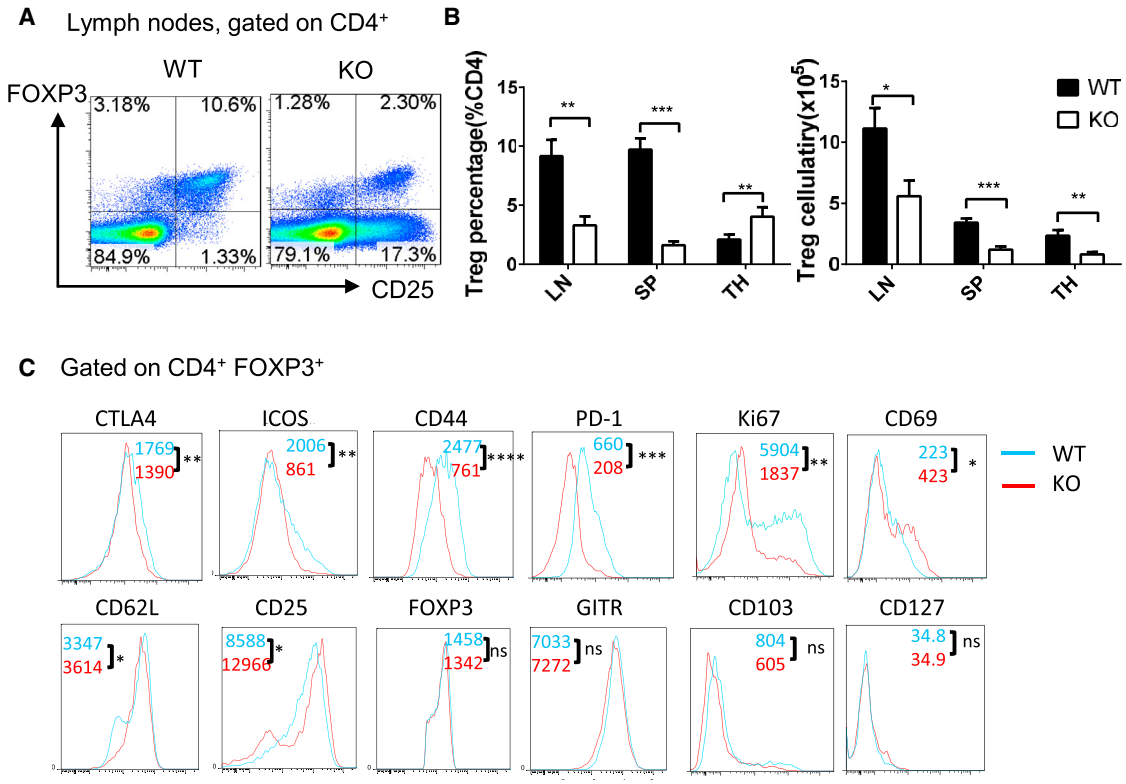

Figure 2. Reduced Cell Number and Impaired Activation of *Ubc9*-Deficient Treg Cells

(A and B) Percentage and cell number of CD4$^+$ Foxp3$^+$ Treg cells of lymph nodes, spleen, and thymus. n = 4–5 per group. Values shown are mean ± SD.

(C) Flow cytometry analysis of indicated molecules on CD4$^+$ Foxp3$^+$ T cells of lymph nodes. n = 3 per group.

In (A)–(C), WT, *Foxp3*^{cre}*Ubc9*$^{fl/wt}$; KO, *Foxp3*^{cre}*Ubc9*$^{fl/fl}$; LN, lymph nodes; SP, spleen; TH, thymus; 21- to 25-day-old male mice were used; a representative of three independent experiments is shown. See also Figure S2.

Based on these data, we reasoned that TCR signaling pathway might be responsible for the proliferation defect of *Ubc9*-deficient Treg cells. We analyzed ERK, NF-κB, NFAT signaling pathways downstream of TCR in *Ubc9*-deficient TH0 cells using creERT2 system (Figure S4I). Moreover, phosphorylated ERK, P38, AKT, S6, and calcium influx were detected directly in *Ubc9*-deficient Treg cells by phosphoflow (Figures S4C–S4H). However, most of the pathways detected were largely intact except AKT and S6. *Ubc9*-deficient Treg cells showed reduced AKT S473 phosphorylation and slight decrease in S6 phosphorylation (Figures S4D and S4E), especially in the inflammatory environment (Figure 3F). mTOR integrates immune signals, environmental cues, and nutrients in T cells and regulates protein translation, cell growth, and survival (Chi, 2012). Treg cells depend on mTORC1 for homeostasis and function, with S6 as the direct target of mTORC1 (Zeng et al., 2013). mTORC2, a regulator downstream of TCR signals (Vahl et al., 2014), can directly phosphorylate AKT at serine 473 and thereby inhibiting constitutively active FoxO1, which can promote effector Treg cell differentiation and migration (Luo et al., 2016). Furthermore, AKT itself is a SUMO target and SUMOylation can promote AKT kinase activity to control cell survival and proliferation (Li et al., 2013). Thus, SUMOylation is required for TCR-mediated Treg cell homeostatic proliferation through regulating mTOR-AKT signaling pathway.

To gain an overview of SUMOylated proteins in T cells, we purified endogenous SUMO2 conjugates in EL4 cell line and analyzed by mass spectrum. Overall, 215 proteins were identified in this experiment, including those reported by published literatures such as TRIM28, RanGAP-1, PML, BHLHE40, and TOP2A (Dawlaty et al., 2008; Li et al., 2007; Mahajan et al., 1997; Shen et al., 2006; Wang et al., 2012b). Unbiased gene ontology (GO) analysis revealed that SUMOylated proteins were most enriched in translation, RNA processing, ribonucleoprotein complex protein biogenesis, ribosome biogenesis, PML body organization, DNA topological change, and so on (Figure S4J), many of which are essential biological process and involved in cell proliferation.

Diminished Expression of Multiple Suppressor Molecules in *Ubc9*-Deficient Treg Cells

In addition to the proliferation defect, impaired expression of activation markers led us to consider whether *Ubc9*-deficient Treg cells are functionally competent. To address this question, we performed in vitro suppression assay. *Ubc9*-deficient Treg cells sorted from healthy *Foxp3*$^{cre/wt}$*Ubc9*$^{fl/fl}$ mice were still functional but slightly less efficient compared with their wild-type

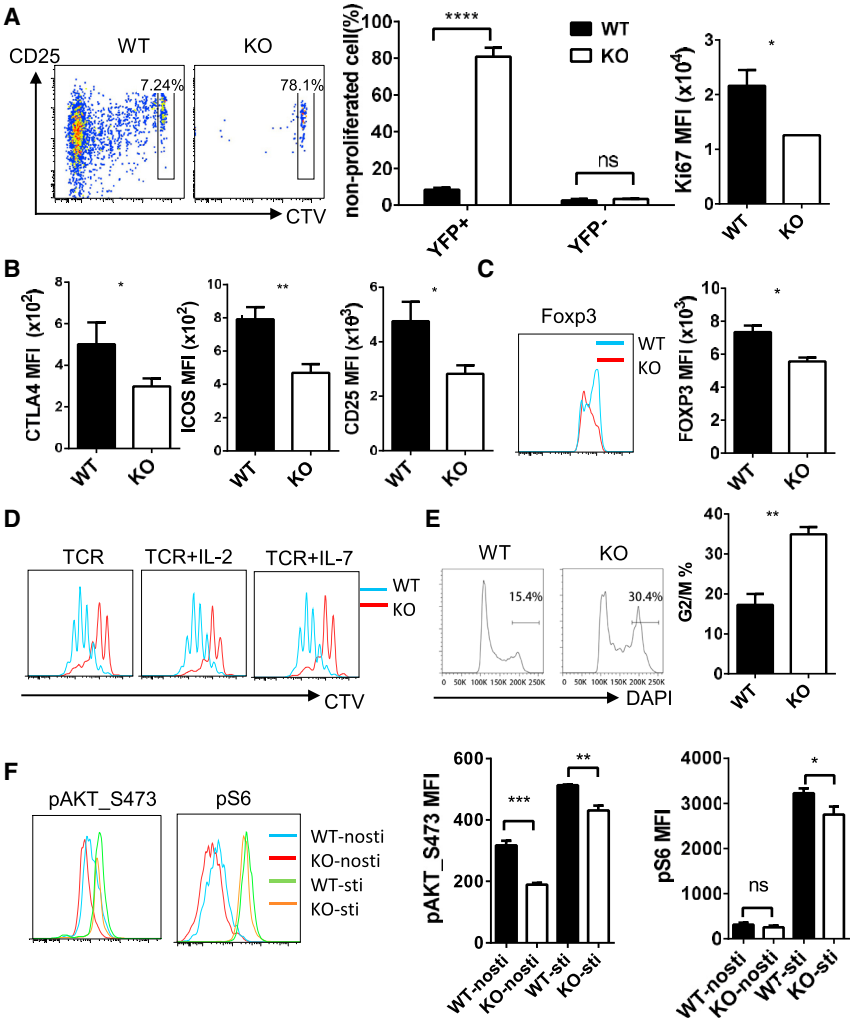

Figure 3. *Ubc9* Is Required for Treg Cell Proliferation

(A–C) CellTrace-Violet-labeled CD4SP thymocytes were transferred into *Rag1*$^{-/-}$ mice, and dye dilution was analyzed 7 days after transfer. n = 3 per group. (A) CellTrace Violet dilution, percentage of non-proliferating cells, and Ki67 MFI after homeostatic proliferation. (B) MFI of CTLA4, ICOS, CD25 of CD4^{+}YFP^{+} Treg cells after homeostatic proliferation. (C) Flow cytometry analysis of Foxp3 expression and Foxp3 MFI. MFI, mean fluorescence intensity.

(D) CellTrace-Violet-labeled CD4^{+} YFP^{+} Treg cells were stimulated by anti-CD3/CD28 for 3 days with or without IL-2 or IL-7; dye dilution was analyzed by flow cytometry.

(E) Flow cytometry analysis of cell cycle of CD4^{+} YFP^{+} Treg cells after anti-CD3/CD28 stimulation by DAPI staining.

(F) CD4^{+} YFP^{+} Treg cells were stimulated with PMA and Ionomycin for 15 min; phosphorylated AKT-S473 and S6 were stained. n = 3 per group; WT, *Foxp3*^{cre}*Ubc9*$^{fl/wt}$; KO, *Foxp3*^{cre}*Ubc9*$^{fl/fl}$.

In (A)–(E), WT, *Foxp3*$^{cre/wt}$*Ubc9*$^{fl/wt}$; KO, *Foxp3*$^{cre/wt}$*Ubc9*$^{fl/fl}$. In (A)–(F), values shown are mean ± SD. ns, no significance. A representative of three independent experiments is shown. See also Figures S3 and S4.

counterparts (Figure 4A). Since reversal of anergy has been suggested to impair Treg function, we also analyzed this possibility and found *Ubc9* KO Treg cells well retained the anergic state as WT (data not shown). Putative suppressor effector molecule like CTLA4 failed to be upregulated in *Ubc9*-deficient Treg cells in this process or by anti-CD3/CD28 plus IL-2 stimulation (Figures 4B and 4C). In addition, transforming growth factor β (TGF-β)-induced iTreg differentiation from female naive T cells was quite normal at day 3 but impaired at day 4 in the absence of *Ubc9* (Figure S5A). Moreover, we examined the suppressor function of YFP^{+} iTreg and found a profound defect without *Ubc9* (Figure S5B). Taken together, partial loss of function and remarkable defect in proliferation may contribute to the fatal early-onset autoimmune disease in *Foxp3*^{cre} *Ubc9*$^{fl/fl}$ mice.

To gain insights into the mechanism underlying defective Treg function upon *Ubc9* deletion, we performed RNA sequencing (RNA-seq) using CD4^{+}YFP^{+} Treg cells sorted from lymph nodes and spleen of *Foxp3*$^{cre/wt}$*Ubc9*$^{fl/wt}$ or *Foxp3*$^{cre/wt}$*Ubc9*$^{fl/fl}$ mice. In total, loss of *Ubc9* affected the expression of 365 genes by at least 1.5-fold ($p < 0.05$) and 149 genes at least 2-fold ($p < 0.01$), and 92 genes were downregulated by 2-fold in *Ubc9*-deficient Treg cells. To get an overview of transcriptome, we performed GO analysis of genes with at least 1.5-fold change using DAVID Bioinformatics Resources 6.7 (https://david.ncifcrf.gov/) (Huang et al., 2009). On one hand, genes upregulated in *Ubc9*-deficient Treg cells were enriched in regulation of transcription, ribosome, RNA metabolic process, translation, and proliferation, which is consistent with other reports that SUMO modification mainly inhibits transcription and actively participates in meiosis (Figure S6A). On the other hand, genes downregulated by *Ubc9* deletion were mainly enriched in immune responses, including cytokine binding and production, chemokine receptor activity, IL-1 receptor activity, and regulation of lymphocyte activation (Figure S6A), indicating that SUMOylation is required for the full immune function of Treg cells.

To further explore the functional defect in *Ubc9*-deficient Treg cells, we analyzed the putative suppressor molecules based on RNA-seq data and further confirmed by RT-PCR (Figures 4D–4F). In agreement with the protein expression detected by flow cytometry, many suppressor molecules in Treg cells were downregulated upon *Ubc9* deletion, including CTLA4, ICOS, CD44, PD-1, LAG3, Granzyme B, Perforin-1, CD38, CD39, and NT5E. In terms of suppressive cytokines (Rubtsov et al., 2008; Shevach, 2009), IL-10 but not TGF-β expression was severely impaired in *Ubc9*-deficient Treg cells (Figures 4D–4F). A recent study also suggested that chemokine receptors are required for Treg cells to migrate to the inflammatory sites to execute their anti-inflammation function

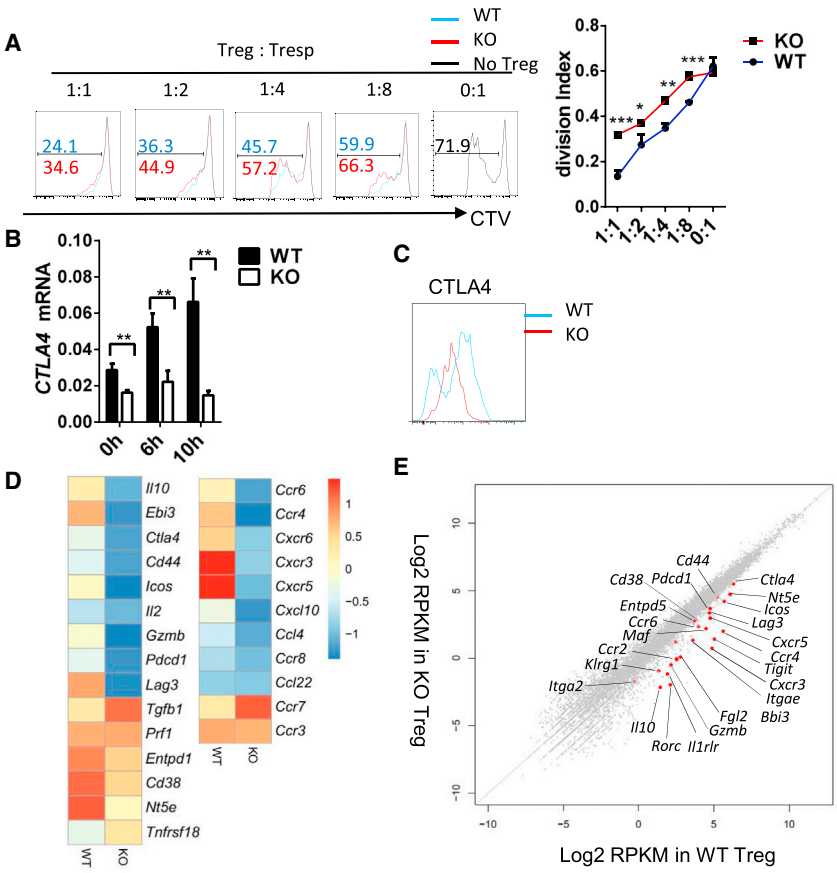

Figure 4. *Ubc9* Is Required for Treg Cell Functional Activity

(A) Peripheral CD4$^+$YFP$^+$ Treg cells sorted form female mice were co-cultured with CellTrace-Violet-labeled wild-type naive T cells (Tresp) in the presence of anti-CD3 and mitomycin-treated splenocytes (APCs) for 84 hr, dye dilution and cell division index were analyzed by FlowJo, and the percentage of proliferated cells was shown. A representative result of two independent experiments is shown.

(B) mRNA expression of CTLA4 in CD4$^+$YFP$^+$ Treg cells after anti-CD3/CD28 and IL-2 stimulation for indicated time. Values shown are mean ± SD.

(C) Flow cytometry analysis of CTLA4 expression on Treg cells in (A).

(D and E) RNA-seq was performed using peripheral CD4$^+$YFP$^+$ Treg cells sorted from healthy female mice. Each sample contained pooled Treg cells from five to ten mice. (D) Expression patterns of potential suppressor molecules and chemokine receptors in Treg cells. Heatmaps depict the absolute value of RPKM (reads per kilobases per million reads, Log2-transformed). (E) Genes expressed in *Ubc9*-sufficient Treg cells (WT) were plotted against those expressed in *Ubc9*-deficient Treg cells (KO); genes shown in (C) are indicated by red dots.

(F and G) qPCR confirmation of relative expression of selected genes shown in (C) and (D). Values shown are mean ± SD. ns, no significance. A representative of two independent experiments is shown.

In (A)–(G), WT, *Foxp3*$^{cre/wt}$*Ubc9*$^{fl/wt}$; KO, *Foxp3*$^{cre/wt}$ *Ubc9*$^{fl/fl}$. 5-week-old female mice were used. See also Figure S5.

Regulation of TCR Signaling Pathway by *Ubc9* in Treg Cells

Unbiased analysis of gene expression profile against public available database at the GSEA server of the Broad Institute (http://software.broadinstitute.org/gsea/index.jsp) (Mootha et al., 2003; Subramanian et al., 2005) revealed that loss of *Ubc9* mainly affected the gene sets of inflammatory response, Foxp3-targeted genes, adhesion molecules, Treg signature genes and activation/effector phenotype (Table S1). In terms of Treg signature genes, more than 32% (104/322) of genes were affected at least 1.5-fold, the majority of which (76/104) were downregulated by *Ubc9* deletion. Among genes whose expression level changed at least 2.5-fold ($p < 0.05$), 21 genes were downregulated, while only one gene was upregulated (Figure 5A), indicating Treg cellular identity is largely maintained. Although Foxp3 expression was normal at steady state, 22% of genes regulated by Foxp3 were affected by *Ubc9* deletion for at least 1.5-fold (Figure 5B), suggesting other factors cooperating with Foxp3 might be regulated by *Ubc9*. Among these *Ubc9* regulated genes, Foxp3-activated genes were mostly downregulated (80%), while Foxp3 repressed genes were mostly upregulated (71%) by *Ubc9* deletion (Figure 5B), suggesting that UBC9-mediated SUMOylation

(Ding et al., 2012). Indeed, *Ubc9*-deficient Treg cells had compromised expression of many chemokine receptors such as CXCR3, CCR6, CXCR5, and CCR2 (Figures 4D, 4E, and 4G). CCR6 regulates the migration of Treg cells to TH17 accumulation sites (Yamazaki et al., 2008), while CXCR3 and CXCR6 expressed by TH1 are also important for Treg cells to control TH1 inflammation (Ding et al., 2012). CXCR5 is highly expressed by Tfr cells, which are involved in restraining B cell response through inhibition of Tfh cell function (Crotty, 2011). Thus, we proposed that diminished expression of suppressor molecules and defective migration to inflammation sites both contribute to the defective suppressive function of *Ubc9*-deficient Treg cells.

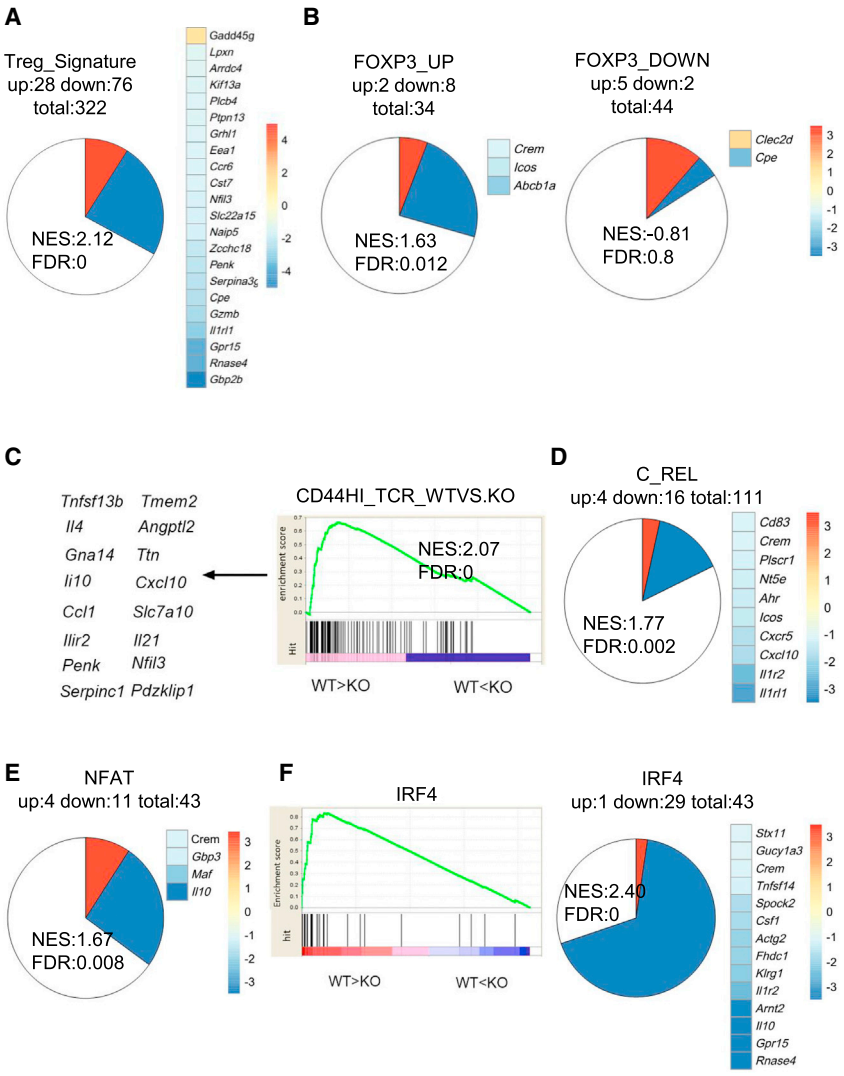

Figure 5. *Ubc9*-Dependent Genes in Treg Cells Are Associated with TCR Signals and Correlated with IRF4 Targets

(A–F) Normalized enrichment scores (NES) and false discovery rate (FDR) were calculated at the GSEA server of the Broad Institute. Gene sets associated with Treg signature (A) and gene sets regulated by Foxp3 (B), TCR (C), c-REL (D), NFAT (E), and IRF4 (F) were analyzed by GSEA using the above RNA-seq results.

For (A), differentially expressed genes with fold change >1.5 are shown in pie (red, upregulated genes in KO, blue, downregulated genes in KO), while genes with fold change >2.5 ($p < 0.05$) are shown in the heatmap. For (C)–(F), differentially expressed genes with fold change >1.5 are shown in pie (red, upregulated genes in KO; blue, downregulated genes in KO), and genes with fold change >2 ($p < 0.05$) are shown in the heatmap. The plots in (C) and (F) show the distribution of the genes ("hits") against the ranked list of genes. Gene list in (C) shows the representative genes with diminished expression in *Ubc*9 KO Treg cells within the gene set regulated by TCR. Heatmaps depict KO to WT fold-change values (Log2-transformed). WT, $Foxp3^{cre/wt}Ubc9^{fl/wt}$; KO, $Foxp3^{cre/wt}Ubc9^{fl/fl}$. See also Figure S6 and Table S1.

facilitates Foxp3 to exert its master regulation function in Treg cells.

Recent studies showed that TCR signals are indispensable for effector Treg cells differentiation, homeostatic proliferation, and function, although not for Foxp3 expression and IL-2 responsiveness (Levine et al., 2014; Vahl et al., 2014). *Ubc9*-deficient Treg cells were quite similar to TCR-ablated Treg cells, suggesting that UBC9-mediated SUMOylation are involved in TCR signaling pathway. To test this, we further analyzed the gene expression profile using gene-set enrichment analysis. Our results showed that TCR-dependent gene expression was significantly disturbed due to *Ubc9* deletion (false discovery rate [FDR] = 0.00) (Figure 5C), and most of which were downregulated in *Ubc9*-deficient Treg cells. Furthermore, we checked a series of transcription factors downstream of TCR signals. Genes targeted by Egr2 or NF-κB were largely unaffected in the absence of *Ubc9* in Treg cells (Figures S6B and S6C). Nevertheless, c-Rel-controlled genes were partially downregulated in *Ubc9*-deficient Treg cells (Figure 5D). More than 30% NFAT-targeted genes were disturbed upon *Ubc9* deletion, although calcium influx was intact in *Ubc9*-deficient Treg cells (Figures 5E and S4H). Interestingly, IRF4-controlled genes had the most significant defect due to *Ubc9* depletion, with 67% of IRF4-dependent genes downregulated at least 1.5-fold, and only one gene was upregulated in *Ubc9*-deficient Treg cells (Figure 5F), consistent with its important role of effector Treg cell differentiation.

Taken together, although *Ubc9*-deficient Treg cells maintain most of the Treg identity, TCR-dependent gene expression landscape largely relies on UBC9-mediated SUMOylation, and these TCR-regulated genes are mainly controlled by c-Rel, NFAT, and, most importantly, IRF4.

SUMOylation Promotes IRF4 Protein Stability and Function in Response to TCR

IRF4 in Treg cells was reported to control TH2 response cooperating with Foxp3; IRF4-deficient Treg cells lost expression of a subset of suppressor molecules such as ICOS, IL10, EBI3, GZMB, and FGL2 (Zheng et al., 2009). In our study, since IRF4-targeted genes were mostly affected by *Ubc9* deletion, we were particularly curious about whether IRF4 could be modified by SUMOylation. To address this question, we overexpressed SUMO1 or SUMO2, UBC9 and IRF4 in 293T cells. We found that IRF4 could be SUMOylated selectively by SUMO2 in 293T cells (Figures 6A and S7A). To identify SUMO-targeted lysine, we applied mutagenesis approach on several lysine residues either predicted by software or implied

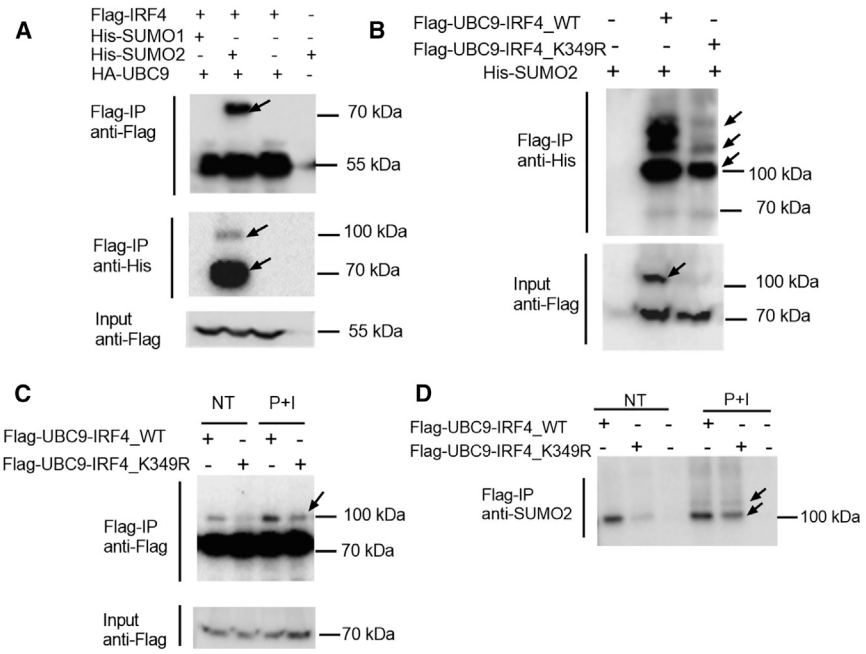

Figure 6. IRF4 Is SUMOylated at K349 in T Cells

(A) 293T cells were transfected with indicated plasmids expressing His-tagged SUMO1 or SUMO2, Flag-tagged IRF4 and HA-tagged UBC9. Flag tag IP was performed in denatured condition; elution was immunoblotted with anti-Flag or anti-His antibodies.

(B) Flag-UBC9 was fused to the N terminus of IRF4; Flag tag IP was performed as in (A).

(C and D) Primary CD4$^+$ T cells were infected with retrovirus expressing Flag-tagged UBC9-IRF4 (IRF4 WT or IRF4 K349R mutant) and stimulated with PMA and Ionomycin for 1 hr. Flag tag IP was performed as in (A); elution was immunoblotted with anti-Flag antibody (C) or anti-SUMO2 antibody (D). For (A)–(D), SUMOylated bands are indicated by arrow. A representative of at least three independent experiments is shown. NT, no treatment. P+I, treated with PMA and Ionomycin. See also Figure S7.

by other IRF4 family members and found K349 was the SUMO-targeted lysine in our case (Figure S7B). Furthermore, we confirmed K349 as the major SUMOylation site using UBC9 fusion-directed SUMOylation (UFDS) method (Figure 6B) (Jakobs et al., 2007).

To test whether IRF4 could be SUMOylated in T cells, UBC9-IRF4 fusion protein was overexpressed in primary CD4$^+$ T cells by retroviral infection, and cells were stimulated with PMA and Ionomycin for 1 hr before collection. Consistent with the results in 293T cells, we found that IRF4 could be SUMOylated in primary T cells, and K349R mutation abolished most of the SUMOylation band (Figures 6C and 6D). More interestingly, IRF4 SUMOylation was enhanced by TCR stimulation (Figures 6C and 6D). Next, to investigate the function of SUMOylation on IRF4 transcriptional activity, we performed a dual-luciferase assay using murine IL-4 promoter in Jukart T cells, in which IRF4 expression was comparable in both groups due to the constitutive expression driven by exogenous promoter. We found that K349R mutation affected IRF4-induced luciferase expression both before and after TCR stimulation (Figure 7A), indicating that SUMOylation is required for IRF4 in order to fully exert its function in T cells.

Flow cytometry data showed that IRF4 expression was reduced at protein level in *Ubc9*-deficient Treg cells (Figure 7B), while IRF4 mRNA expression was not altered, even after anti-CD3, anti-CD28, and IL-2 stimulation (Figure S7C), which suggests that IRF4 stability might be regulated at protein level. To assess this hypothesis, we overexpressed the IRF4-IRES-GFP cassette in primary T cells and stimulated the cells with PMA and Ionomycin in the presence of CHX (cycloheximide) for different time. Compared with WT IRF4, SUMO-deficient IRF4 (IRF4 K349R) showed significantly decreased stability, and this defect can be rescued by MG132 treatment (Figure 7C), indicating that SUMOylation could protect IRF4 from proteasome-mediated protein degradation. Taken together, TCR signals can promote IRF4 protein stability though IRF4 SUMOylation, thus endowing suppressive function in Treg cells.

DISCUSSION

SUMOylation, as critical post-translational modification in eukaryotic cells (Mahajan et al., 1997; Matunis et al., 1996), is not well studied in the immune system. Our current study demonstrated the functional consequence of global SUMOylation ablation in Treg cells. *Ubc9* deficiency in Treg cells led to catastrophic early-onset autoimmunity. We found that *Ubc9* is required for Treg cell homeostatic proliferation, activation, and suppressor function, and loss of *Ubc9* disturbed the gene expression program driven by TCR signaling. Interestingly, we identified TCR-regulated SUMOylation of IRF4, which enhances its stability and activity.

Many post-translational modifications (PTMs) including acetylation, ubiquitination, and phosphorylation can modulate Foxp3 activity by altering DNA binding, transactivation, and protein stability (van Loosdregt and Coffer, 2014). In our case, we were unable to detect Foxp3 SUMOylation in Treg cells. Foxp3 expression is well maintained in *Ubc9*-deficient Treg cells at steady state; only about 20% of Foxp3-targeted genes are moderately influenced by *Ubc9* deletion. However, when stimulated with anti-CD3/CD28, *Ubc9*-deficient Treg cells lost Foxp3 preferentially, underscored by increased CNS2 methylation. This is not likely attributed to reduced IRF4 expression, since IRF4 knockdown did not affect Foxp3 expression (data not shown). Thus, we proposed that maintenance of Treg cell-specific methylation pattern and thereby Foxp3 stability upon TCR stimulation are largely dependent on UBC9-mediated SUMOylation.

IRF4, as a cooperator of Foxp3, is required for Treg cells to control TH2 response (Zheng et al., 2009). IRF4 also plays an

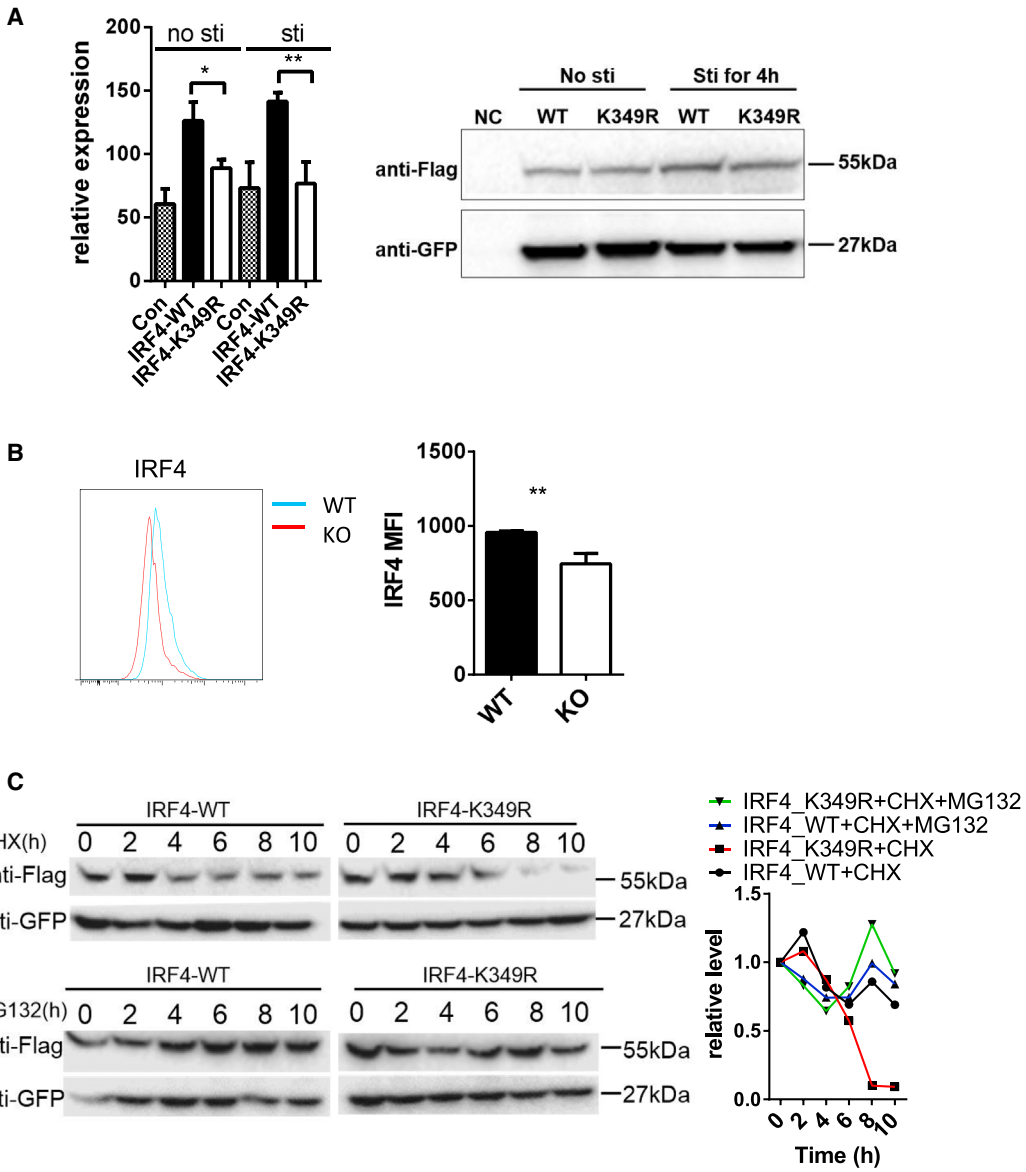

Figure 7. SUMOylation Promotes IRF4 Protein Stability and Function

(A) Left panel: plasmids containing IRF4 WT or IRF4 K349R mutant (GFP was located downstream of IRF4 via IRES) were transfected together with luciferase reporter driven by murine IL-4 promoter into Jurkat cells via electroporation. Renilla vector was used as an inner control and the ratio of luciferase to Renilla was shown. Right panel: western blot of IRF4 expression using anti-Flag antibody.

(B) Flow cytometry analysis of IRF4 protein expression in $CD4^+YFP^+$ Treg cells of *Foxp3*$^{cre/wt}$*Ubc9*$^{fl/wt}$ (WT) and *Foxp3*$^{cre/wt}$*Ubc9*$^{fl/fl}$ (KO) mice.

(C) Primary $CD4^+$ T cells were infected with retrovirus expressing Flag-tagged IRF4 in the presence of CHX (Cycloheximide) for indicated time. MG132 was added to block proteasome degradation. Relative protein content was measured by ImageJ software.

For (A)–(C), a representative of at least three independent experiments is shown. nosti, no stimulation. sti, stimulation with PMA and Ionomycin for 4 hr. See also Figure S7.

important role in the differentiation and function of effector Treg cells (Cretney et al., 2011). Our study revealed that many activation markers and suppressor molecules affected by *Ubc9* deletion are regulated by IRF4. Moreover, IRF4 stability and activity are regulated by TCR and SUMOylation, and IRF4 expression is reduced at protein level in *Ubc9*-deficient Treg cells. *Foxp3*^{cre}*Irf4*$^{fl/fl}$ mice are succumbed to autoimmune symptoms including lymphadenopathy, weight loss, blepharitis, and dermatitis, with notable increase of Th2 cytokines and marginal increase of IL-17A, similar to *Foxp3*^{cre}*Ubc9*$^{fl/fl}$ mice, although the disease in the latter case is much earlier in onset (3–4 weeks versus 6–8 weeks). *Foxp3*^{cre}*Ubc9*$^{fl/fl}$ mice showed more robust production of IFN-γ, which could be explained by the numerical decrease of *Ubc9*-deficient Treg cells and a systematic failure to control immune homeostasis (Dadke et al., 2007). However, IRF4 overexpression failed to rescue the proliferation defect in *Ubc9*

KO Treg cells (data not shown). Since SUMOylation is intensively involved in meiosis, other important players might contribute to this defect. Thus, we consider that IRF4 might be the tip of iceberg in the world of SUMOylation, though it may be associated with the defective differentiation and function in *Ubc9*-deficient Treg cells. In addition, it further supports the concept that Foxp3 is not the sole regulator in Treg cells; signaling-dependent post-translational modifications of its cooperators are needed for Treg cell maturation and function.

Although IL-2 and IL-7 are most important cytokines for Treg cell homeostatic proliferation, their downstream signaling leading to STAT5 phosphorylation was unaffected in *Ubc9*-deficient Treg cells. Moreover, the addition of IL-2 or IL-7 in vitro did not rescue the proliferation defect. This phenomenon correlates well with that seen in TCR-deleted Treg cells (Levine et al., 2014; Vahl et al., 2014), which can further support our hypothesis that *Ubc9* deletion mainly affects the TCR signals. In Treg cells, mTOR inhibition has been shown to induce Foxp3 expression (Haxhinasto et al., 2008); *Mtor*-deficient T cells fail to differentiate into effector T cells but tend to promote Foxp3 expression in response to TCR stimulation (Delgoffe et al., 2009). However, Treg cells also depend on mTORC1 for homeostasis and function induced by TCR and IL-2 (Zeng et al., 2013). mTORC2, a regulator downstream of TCR signals (Vahl et al., 2014), can directly phosphorylate AKT at serine 473 and thereby inhibit constitutively active FoxO1, which can promote effector Treg cell differentiation and migration (Luo et al., 2016). Furthermore, mTOR-AKT inhibition can block Treg cell proliferation (data not shown). In our study, decreased phosphorylation of mTORC1 target S6 and reduced AKT-S473 phosphorylation revealed the attenuated mTOR signals as a result of *Ubc9* deletion. SUMOylation usually functions in a fine-tuned, reversible way; although the reduction is moderate, this decrease in mTOR function might still be important for Treg cell homeostatic proliferation, migration, and function when challenged with strong immune signals.

Previous study suggests that SUMOylation preferentially affects the proliferating cells rather than non-proliferating or terminally differentiated cells (Demarque et al., 2011). Peripheral Treg cells can be functionally divided into two distinct subsets, including central Tregs as the majority of Treg cell population and effector Tregs activated by antigens and proliferate rapidly (Liston and Gray, 2014). Our study revealed that loss of *Ubc9* in Treg cells resulted in impaired proliferation and defective activation, suggesting the failure of effector Treg cell differentiation and maturation. However, it does not necessarily indicate that central Treg cells are not affected by *Ubc9* deletion, since the effector Treg cell defect alone could not explain the significant reduction in total Treg cell number in *Foxp3*^{cre}*Ubc9*$^{fl/fl}$ mice. Actually, central Treg cells exhibit even higher extent of SUMOylation compared with effector Treg cells (data not shown), it is possible that SUMOylation can promote central Treg cell survival and help to keep them in a quiescent state.

In summary, our study has uncovered a critical function of SUMOylation in regulation of Treg cell homeostatic proliferation and function in vivo. This study opens up an avenue for future identification of SUMO targets in immune tolerance and function.

EXPERIMENTAL PROCEDURES

Additional detailed methods can be found in the Supplemental Experimental Procedures.

Mice

Ubc9$^{fl/fl}$ mice were backcrossed to C57BL/6 mice strain for at least six generations. *Foxp3*^{cre} mice were all kept on a C57BL/6 genetic background. Experimental mice were age matched and housed under specific-pathogen free conditions in Animal Facility of Tsinghua University. All animal protocols are approved by governmental and institutional guidelines for animal welfare.

Plasmid Construction

For pRVKM-Flag-UBC9-IRF4 plasmid, mouse *Ubc9* gene was amplified from cDNA of mouse primary T cells, and SalI-Ubc9-XhoI cassette without stop codon was cloned to RV-GFP retrovirus vector (gift of K. Murphy) by XhoI site downstream of 3xFlag tag; mouse IRF4 was clone from cDNA of mouse TH17 cells, and XhoI-IRF4-SnaBI cassette with stop codon was cloned to the downstream of *Ubc9* to generate the *Ubc9-Irf4* fusion gene. K349R mutation was generated by Gibson Assembly Cloning Kit. GFP was located downstream of IRF4 via IRES, which is transcribed with IRF4 but translated independently.

In Vitro Suppressive Assay

2×10^4 Celltrace-Violet-labeled naive $CD4^+$ T cells were co-cultured with different numbers of $CD4^+$ YFP^+ Treg cells in the presence of 5×10^5 mitomycin treated spleenocytes and 2 μg/ml anti-CD3 in round-bottom 96-well plate for 3 days or 4 days. Cell proliferation was detected by Celltrace Violet dilution by flow cytometry.

Statistical Analysis

Data were analyzed by Graph Prism 6.0 software, statistical analysis of the results was performed by unpaired Student's t test as indicated. p values are presented in figure legends where a statistically significant difference was found: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

ACCESSION NUMBERS

The accession number for the RNA-seq data reported in this paper is NCBI GEO: GSE82031.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.06.056.

AUTHOR CONTRIBUTIONS

X.D., A.W., W.J., H.X., and C.D. designed the experiments. X.D. and A.W. performed all the experiments. X.M. analyzed all the bioinformatics data. M.D. and A.D. generated the *Ubc9*$^{fl/fl}$ mice. X.D. and A.W. analyzed the data. X.D. and C.D. wrote the manuscript.

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