Epigenetic regulation of *Kcna3*-encoding Kv1.3 potassium channel by cereblon contributes to regulation of CD4$^+$ T-cell activation

Jung-Ah Kang[a,1], Sang-Heon Park[a,1], Sang Phil Jeong[a], Min-Hee Han[a], Cho-Rong Lee[a], Kwang Min Lee[a], Namhee Kim[a], Mi-Ryoung Song[a], Murim Choi[b], Michael Ye[c], Guhung Jung[d], Won-Woo Lee[e], Soo Hyun Eom[a], Chul-Seung Park[a], and Sung-Gyoo Park[a,f,2]

[a]School of Life Sciences, Gwangju Institute of Science and Technology, Gwangju 61005, Republic of Korea; [b]Department of Biomedical Sciences, College of Medicine, Seoul National University, Seoul 03080, Republic of Korea; [c]School of Liberal Arts and Sciences, Gwangju Institute of Science and Technology, Gwangju 61005, Republic of Korea; [d]Department of Biological Sciences, Seoul National University, Seoul 08826, Republic of Korea; [e]Department of Microbiology and Immunology, College of Medicine, Seoul National University, Seoul 03080, Republic of Korea; and [f]Cell Logistics Research Center, Gwangju Institute of Science and Technology, Gwangju 61005, Republic of Korea

Edited by Kenneth M. Murphy, Washington University, St. Louis, MO, and approved June 14, 2016 (received for review February 2, 2015)

The role of cereblon (CRBN) in T cells is not well understood. We generated mice with a deletion in *Crbn* and found cereblon to be an important antagonist of T-cell activation. In mice lacking CRBN, CD4$^+$ T cells show increased activation and IL-2 production on T-cell receptor stimulation, ultimately resulting in increased potassium flux and calcium-mediated signaling. CRBN restricts T-cell activation via epigenetic modification of *Kcna3*, which encodes the Kv1.3 potassium channel required for robust calcium influx in T cells. CRBN binds directly to conserved DNA elements adjacent to *Kcna3* via a previously uncharacterized DNA-binding motif. Consequently, in the absence of CRBN, the expression of Kv1.3 is derepressed, resulting in increased Kv1.3 expression, potassium flux, and CD4$^+$ T-cell hyperactivation. In addition, experimental autoimmune encephalomyelitis in T-cell–specific *Crbn*-deficient mice was exacerbated by increased T-cell activation via Kv1.3. Thus, CRBN limits CD4$^+$ T-cell activation via epigenetic regulation of Kv1.3 expression.

T-cell activation | CRBN | Kv1.3 | calcium flux | potassium flux

Recent studies have identified cereblon (CRBN) as a thalidomide-binding protein (1, 2). CRBN is expressed in the cytoplasm, nucleus, and peripheral membranes of a wide variety of cells and tissues (1, 3–5). CRBN forms an E3 ubiquitin ligase complex with damaged DNA-binding protein (DDB) 1 and Cul4A, and this complex is involved in limb outgrowth and controls fibroblast growth factor 8 expression in zebrafish and chicks (1). When thalidomide binds to CRBN, it inhibits formation of the E3 ubiquitin ligase complex, which in turn reduces the activity of the ubiquitin ligase and results in defective limb development (1).

Recent work suggests that immunomodulatory drugs (IMiDs) stimulate increased IL-2 production in T cells by binding to CRBN. This binding alters the substrate specificity of CRBN-containing ubiquitin ligase complexes (6, 7) even though IMiDs reduce the overall activity of E3 ubiquitin ligase (1). As a result, binding promotes abnormal degradation of the Ikaros zinc finger proteins 1 and 3 (IKZF1 and IKZF3), which in turn increases IL-2 production in T cells (6–8). However, although the CRBN-mediated effects of IMiDs are known, the physiological role of CRBN in T cells remains unclear.

We deleted the *Crbn* gene from murine T cells to examine the physiological role of CRBN during T-cell activation, with the aim of gaining new insight into the regulation of potassium flux during T-cell signaling. Deletion of *Crbn* from T cells led to IL-2 production and differentiation of CD4$^+$ T cells into Th17 effector cells, as well as worsening of the phenotype associated with experimental autoimmune encephalitis (EAE). CRBN represses T-cell activation by binding to the chromosomal regions adjacent to the *Kcna3* locus, a gene encoding the Kv1.3 potassium channel, which participates in calcium influx in T cells. The binding of CRBN to *Kcna3* leads to epigenetic modification of the *Kcna3* locus and reduces the expression of Kv1.3. Triggering of TCR signaling in CRBN-deficient T cells results in (*i*) increased potassium and calcium flux, (*ii*) increased activation of transcription factor NF-AT, and (*iii*) increased production of IL-2. Our data identify CRBN as an important regulator of T-cell activation, restricting calcium influx via epigenetic repression of *Kcna3*.

Results

CRBN Deficiency Does Not Affect T-Cell Development, but Does Increase T-Cell Activation. Analysis of the relative levels of CRBN transcripts in multiple tissue types using the Novartis BioGPS expression array database (biogps.org) (9) suggested that the expression of CRBN mRNA is higher in lymphoid cells, including CD4$^+$ T cells, than in other cell types (Fig. S1*A*). Thus, we used *Crbn* gene-targeted mice to examine the effect of CRBN deficiency in T-cell development and activation. First, we confirmed the loss of CRBN protein from CD4$^+$ T cells isolated from *Crbn*-deficient (*Crbn*$^{-/-}$) mice (Fig. 1*A*). There were no obvious changes in thymic T-cell development (Fig. 1*B*). In addition, peripheral T-cell and B-cell populations in 6- to 8-wk-old *Crbn*$^{-/-}$ mice were no different from those in 6- to 8-wk-old littermate control (*Crbn*$^{+/-}$) mice (Fig. 1 *C* and *D* and Fig. S2*A*). Moreover, the Foxp3$^+$ CD4$^+$ T-cell population in the spleens of *Crbn*$^{-/-}$ mice was similar to that

Significance

In resting T cells, CRBN normally represses expression of the Kv1.3 potassium channel by regulating histone modifications to prevent hyperactivation of T cells. It does this by controlling recruitment of EZH1 to the potassium channel region of the Kcna3 gene (or locus). However, lack of CRBN causes upregulation of Kv1.3 expression, which in turn increases potassium flux, thereby triggering increased calcium flux during T-cell activation.

Author contributions: S.-G.P. conceived of the study; J.-A.K., S.-H.P., M.-R.S., M.C., G.J., W.-W.L., and S.-G.P. designed research; J.-A.K., S.-H.P., S.P.J., M.-H.H., C.-R.L., N.K., and S.-G.P. performed research; K.M.L., S.H.E., and C.-S.P. contributed new reagents/analytic tools; J.-A.K., S.-H.P., S.P.J., M.-H.H., C.-R.L., N.K., M.Y., and S.-G.P. analyzed data; and J.-A.K., M.Y., and S.-G.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]J.-A.K. and S.-H.P. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: sgpark@gist.ac.kr.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1502166113/-/DCSupplemental.

IMMUNOLOGY AND INFLAMMATION

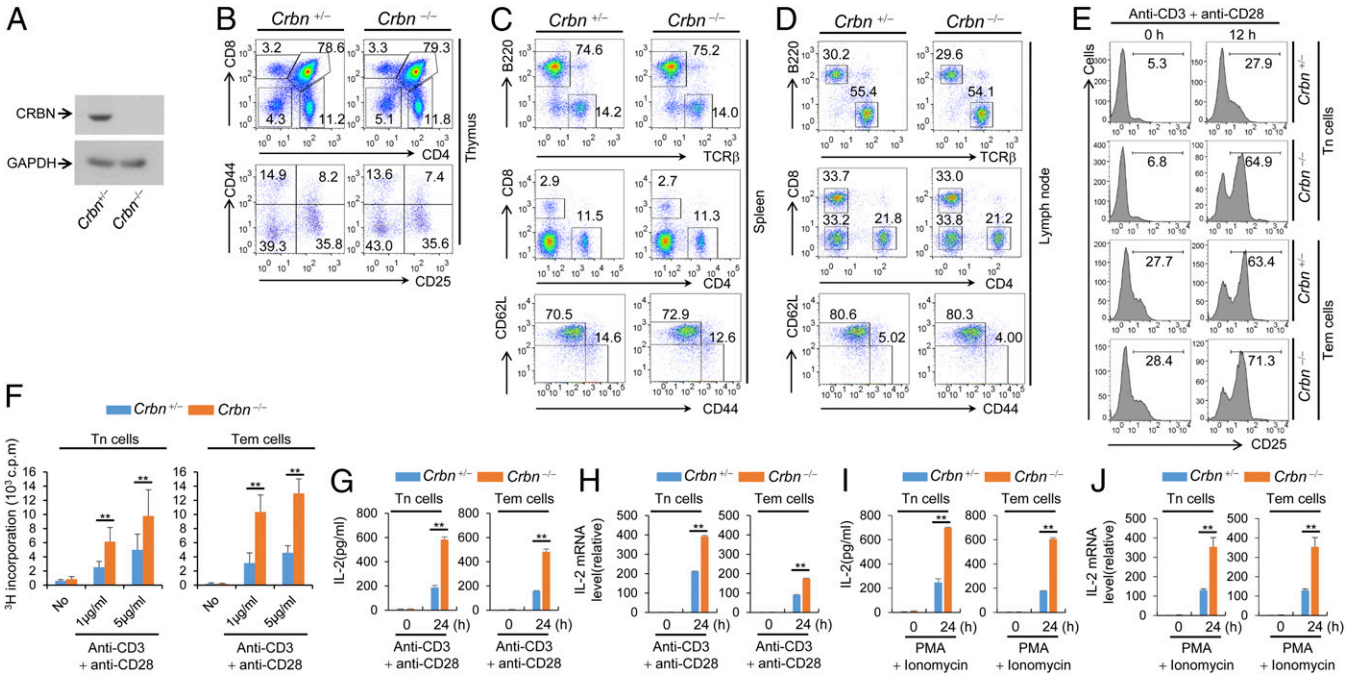

Fig. 1. CRBN deficiency does not affect T-cell development, but does increase T-cell activation. (*A*) Loss of CRBN protein expression in CD4$^+$ T cells from *Crbn*$^{-/-}$ mice was confirmed by immunoblot analysis with an anti-CRBN antibody. (*B*) T-cell development in the thymus was analyzed by flow cytometry. (*C* and *D*) Lymphocytes in the spleen (*C*) and lymph nodes (*D*) were analyzed by flow cytometry. (*E*) CD4$^+$ Tn and Tem cells from *Crbn*$^{-/-}$ mice and *Crbn*$^{+/-}$ mice were stimulated with anti-CD3 and anti-CD28 antibodies for the indicated times, after which cell surface expression of CD25 was analyzed by flow cytometry. (*F*) Proliferation of CD4$^+$ Tn and Tem cells stimulated with anti-CD3 and anti-CD28 antibodies was measured by [^{3}H]thymidine incorporation. (*G–J*) IL-2 secretion and IL-2 mRNA expression in CD4$^+$ Tn and Tem cells stimulated by either anti-CD3 or anti-CD28 antibodies (*G* and *H*) or PMA and ionomycin (*I* and *J*) were analyzed by ELISA and quantitative RT-PCR. Data are representative of two (*A*), four (*B–D*), or three (*E–J*) independent experiments. Results are expressed as the mean ± SD. *P < 0.05; **P < 0.01, unpaired two-tailed Student's *t* test.

in *Crbn*$^{+/-}$ mice, suggesting that CRBN does not affect the selection of T cells during development (Fig. S2*B*). However, when CRBN-deficient CD4$^+$ T cells, including CD4$^+$ naïve T (Tn) cells and effector memory T (Tem) cells, from *Crbn*$^{-/-}$ mice were stimulated with anti-CD3 and anti-CD28 antibodies, both CD4$^+$ Tn and Tem cells expressed more CD25 on the cell surface compared with the control CD4$^+$ Tn and Tem cells from their *Crbn*$^{+/-}$ littermates (Fig. 1*E*). In contrast to T cells, on B cells a loss of CRBN did not affect the activation-induced cell surface expression of CD69 and CD86 (Fig. S2*C*).

Along with the expression of activation markers on the cell surface, anti-CD3– and anti-CD28–stimulated CD4$^+$ Tn and Tem cells from CRBN-deficient mice were more proliferative (Fig. 1*F*) and secreted higher levels of IL-2 compared with CD4$^+$ Tn and Tem cells, respectively, from their littermate control mice (Fig. 1*G*) and showed increased transcription of IL-2 mRNA (Fig. 1*H*). Moreover, stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, which bypasses TCR- and CD28-mediated proximal signaling events, still led to increased IL-2 secretion and increased IL-2 mRNA levels in CRBN-deficient CD4$^+$ Tn and Tem cells (Fig. 1 *I* and *J*). Consistent with these data, CRBN overexpression significantly reduced activation-induced expression of cell surface markers, such as CD69 and CD25, and IL-2 production in Jurkat T cells (Fig. S3 *A* and *B*) and in mouse CD4$^+$ T cells (Fig. S3 *C* and *D*). The increased IL-2 production was not caused by alterations in IKZF1 and IKZF3 protein levels, because CRBN deficiency did not affect these proteins in CD4$^+$ Tn and Tem cells (Fig. S4).

CRBN Regulates the TCR-Induced NF-AT Activation Pathway Through Kv1.3. We examined the role of CRBN in various TCR signaling pathways in CD4$^+$ Tn and Tem cells isolated from *Crbn*$^{-/-}$ and *Crbn*$^{+/-}$ mice. We found that TCR-induced phosphorylation of ERK and JNK was unaffected in CRBN-deficient CD4$^+$ Tn and Tem cells (Fig. 2*A*). The NF-κB pathway in CD4$^+$ T cells also was unaffected, because IκBα degradation (Fig. 2*A*) and NF-κB DNA-binding activity (Fig. 2*B*) were unchanged in CRBN-deficient CD4$^+$ Tn and Tem cells. However, we found it interesting that NF-AT DNA binding (Fig. 2 *B* and *C*) and nuclear localization (Fig. 2*D*) in CRBN-deficient CD4$^+$ Tn and Tem cells were increased after activation, suggesting that loss of CRBN in CD4$^+$ T cells affects primarily the NF-AT activation signaling pathway.

To identify the genes responsible for the increased activation of NF-AT observed in CRBN-deficient CD4$^+$ T cells, we examined gene expression patterns in CD4$^+$ Tn cells from CRBN-deficient mice and their normal littermates. We identified 674 down-regulated genes and 263 up-regulated genes in CRBN-deficient CD4$^+$ Tn cells (Fig. S5*A*). When these differentially expressed genes were categorized, we found that genes related to TCR signaling were affected in the absence of CRBN (Fig. S5*B*). Of these TCR signaling-related genes, *Zap70* and *Kcna3* showed the greatest differences in CRBN-deficient CD4$^+$ Tn cells (Fig. 2*E*). Although Zap70 is involved in most TCR signaling pathways, only NF-AT was specifically affected by CRBN deficiency. Thus, we narrowed our focus to Kv1.3, because this channel protein is involved in potassium flux, which is linked to calcium flux.

Consistent with increased NF-AT activation, CD4$^+$ Tn and Tem cells from *Crbn*$^{-/-}$ mice also had increased calcium flux compared with CD4$^+$ Tn and Tem cells from *Crbn*$^{+/-}$ mice (Fig. 2*F*). This finding suggests that in the absence of CRBN, increased TCR-induced CD4$^+$ T-cell activation results from increased calcium flux and the subsequent increase of NF-AT activation (Fig. 2*G*). In Tem cells, the voltage-dependent Kv1.3 channel is essential for optimal calcium flux during TCR

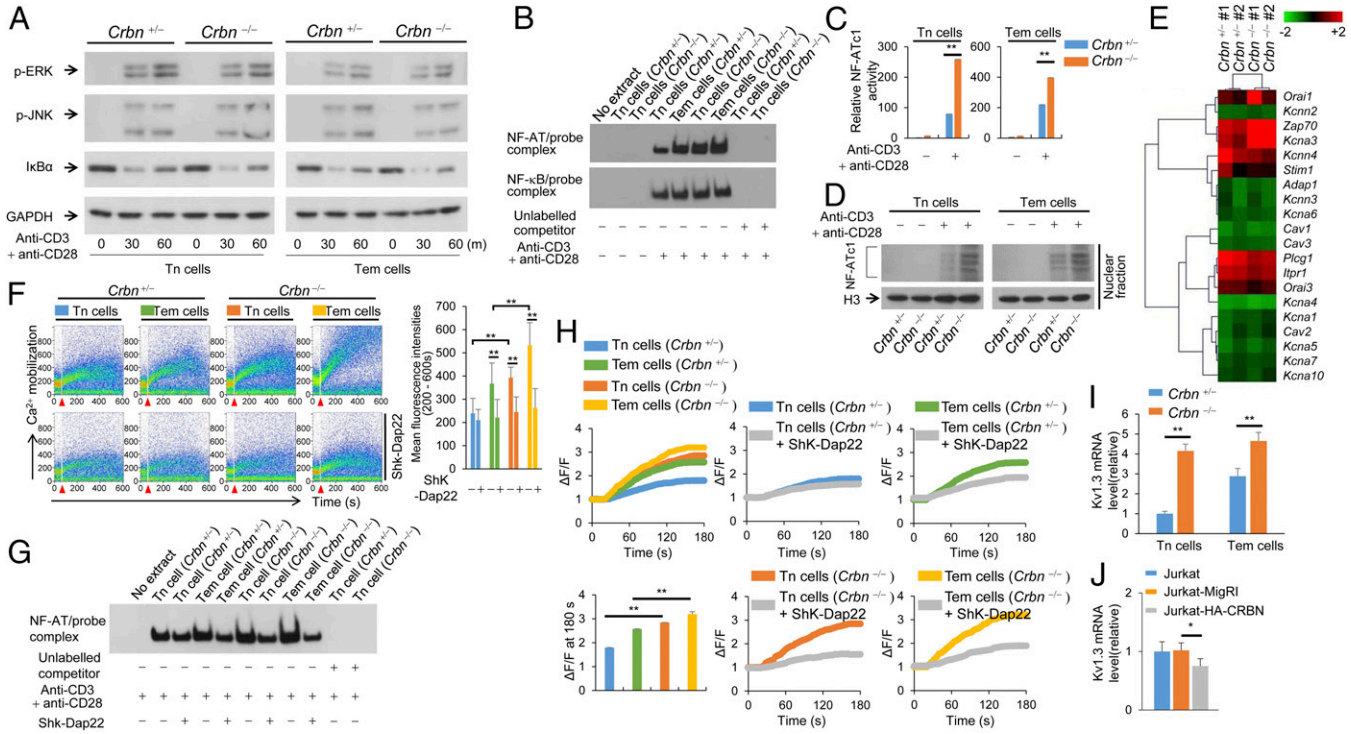

Fig. 2. CRBN deficiency increases NF-AT activation during anti-CD3/CD28–induced T-cell activation. (*A*) Immunoblot analyses were performed to examine ERK and JNK phosphorylation and IκBα degradation in primary CD4$^+$ Tn and Tem cells from the *Crbn*$^{-/-}$ and *Crbn*$^{+/-}$ mice after stimulation with anti-CD3 and anti-CD28 antibodies for the indicated times. (*B*) CD4$^+$ Tn and Tem cells were stimulated with anti-CD3 and anti-CD28 antibodies for 12 h (NF-AT) or 1 h (NF-κB). After activation, the nuclear fractions were isolated, and the DNA-binding activity of NF-AT (*Upper*) or NF-κB (*Lower*) was analyzed by electrophoretic mobility-shift analysis (EMSA). (*C*) The DNA-binding activity of NF-AT was quantified using the TransAM Transcription Factor ELISA Kit (Active Motif) with an NF-AT–specific probe and antibodies. (*D*) Nuclear localization of NF-ATc1 was analyzed by immunoblot analysis. (*E*) Heat map data for TCR signaling-related genes differentially regulated by CRBN deficiency in unstimulated CD4$^+$ Tn cells (CD4$^+$ Tn cells pooled from two mice for each sample). (*F*) Calcium flux in isolated CD4$^+$ Tn and Tem cells was analyzed by Fluo3-AM with or without the addition of Kv1.3 inhibitor (100 nM Shk-Dap22). The arrow indicates activation initiated by the addition of anti-hamster IgG to anti-CD3– and anti-CD28–coated CD4$^+$ T cells. (*G*) CD4$^+$ Tn and Tem cells were stimulated with anti-CD3 and anti-CD28 antibodies for 12 h. After activation in the presence or absence of a Kv1.3 inhibitor (100 nM Shk-Dap22), the nuclear fractions were isolated, and the DNA-binding activity of NF-AT was analyzed by EMSA. (*H*) Potassium flux in isolated CD4$^+$ Tn and Tem cells was analyzed using a FluxOR Thallium Detection Kit in the presence or absence of a Kv1.3 inhibitor (100 nM Shk-Dap22). (*I*) Kv1.3 mRNA levels in CD4$^+$ Tn and Tem cells from *Crbn*$^{-/-}$ and *Crbn*$^{+/-}$ mice were analyzed by quantitative RT-PCR. (*J*) Levels of human Kv1.3 mRNA in CRBN-overexpressing Jurkat T cells and control Jurkat T cells were analyzed by quantitative RT-PCR. Data are representative of three (*A–D* and *G–J*) or four (*F*) independent experiments. Results are expressed as mean ± SD. **P* < 0.05; ***P* < 0.01, unpaired two-tailed Student's *t* test.

stimulation, because these cells express more Kv1.3 compared with Tn cells (10). In addition, calcium flux was reduced in CRBN-deficient CD4$^+$ Tn cells when Shk-Dap22, a specific inhibitor of Kv1.3 (11), was added, whereas calcium flux in control CD4$^+$ Tn cells was barely affected (Fig. 2*F*). Calcium flux was also increased in CD4$^+$ Tem cells deficient in CRBN compared with littermate control CD4$^+$ Tem cells; moreover, Shk-Dap22 reduced calcium flux in CRBN-deficient CD4$^+$ Tem cells to the same degree as in littermate control CD4$^+$ Tem cells. Finally, increased NF-AT activity in activated CRBN-deficient CD4$^+$ Tn and Tem cells, compared with the littermate control CD4$^+$ Tn and Tem cells, was reduced by Shk-Dap22 in a manner similar to that observed with calcium flux (Fig. 2*G*).

To determine whether Kv1.3 function was also affected, we measured potassium mobilization directly. Potassium flux was also greater in CRBN-deficient CD4$^+$ Tn and Tem cells compared with the littermate control CD4$^+$ Tn and Tem cells and, similar to what we observed with calcium flux, this increase in potassium mobilization was reduced when Shk-Dap22 was added (Fig. 2*H*). Moreover, Kv1.3 mRNA expression was higher in CRBN-deficient CD4$^+$ Tn and Tem cells than in littermate control CD4$^+$ Tn and Tem cells (Fig. 2*I*). Thus, CRBN controls Kv1.3 expression in both CD4$^+$ Tn and Tem cells, which affects potassium flux coupled to calcium flux. Consistent with this observation, overexpression of CRBN in Jurakt T cells significantly reduced Kv1.3 mRNA expression (Fig. 2*J*).

CRBN Controls Histone Modification of *Kcna3* Regulatory Regions in CD4$^+$ T Cells. Recent studies have shown that Cul4A, which binds to CRBN, plays a role in histone modification (12–15). Moreover, analysis of the relative levels of Cul4A transcripts in multiple tissue types using the Novartis BioGPS expression array database (9) revealed that, like CRBN, Cul4A is expressed to the greatest extent in lymphoid cells (including CD4$^+$ T cells) compared with other cell types (Fig. S1*B*). This led us to investigate whether CRBN is an epigenetic regulator of the *Kcna3* gene, which encodes Kv1.3. To investigate this possibility, we used chromatin immunoprecipitation (ChIP) analysis to measure the trimethylation of lysine 27 on histone H3 (H3K27me3), which inhibits gene transcription, and the acetylation of lysine 27 on histone H3 (H3K27ac), which activates gene transcription. In the *Kcna3* region of CD4$^+$ T cells from *Crbn*$^{+/-}$ and *Crbn*$^{-/-}$ mice, we analyzed the status of H3K27 modification at five selected regions, including one 5′ upstream region (R1), two ORF regions (R2 and R3), and two 3′ downstream regions (R4 and R5), on *Kcna3* itself (Fig. 3*A*). In addition, we verified that CRBN is recruited to *Kcna3* (Fig. 3*A*), because CRBN regulates the expression of Kv1.3 mRNA (Fig. 2 *I* and *J*).

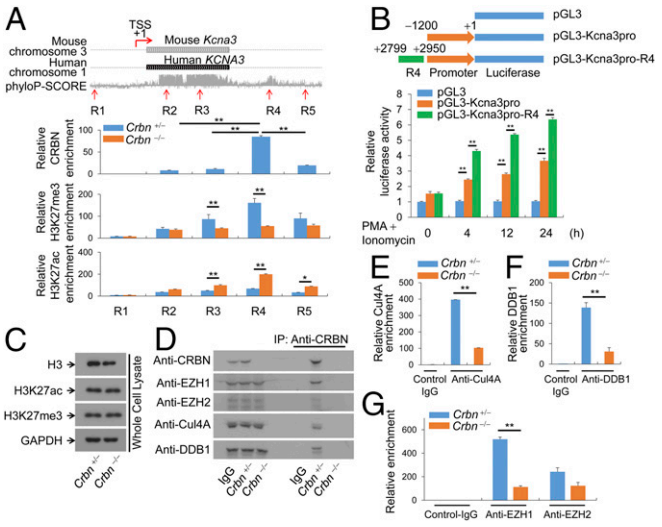

Fig. 3. CRBN regulates histone modification at Kv1.3 regulatory regions. (*A*) The *Kcna3* region in the mouse and human chromosomes. The phyloP-SCORE shows evolutionary conservation of the bases. TSS, transcription start site. Five regions on mouse *Kcna3* are marked as R1, R2, R3, R4, and R5. ChIP was performed with anti-CRBN, anti-H3K27me3, or anti-H3K27ac antibodies, and quantitative PCR analyses for R1–R5 regions were performed. (*B*) Jurkat T cells were transfected with the indicated reporter plasmid and pRenilla. The cells were then activated with PMA and ionomycin. *Renilla* luciferase activity served as a reference to normalize gene expression. (*C*) H3K27me3 and H3K27ac levels in $Crbn^{-/-}$ and $Crbn^{+/-}$ CD4$^+$ T cells were examined by immunoblot analysis with anti-H3K27me3 and anti-H3K27ac antibodies. (*D*) Binding of CRBN to Cul4A, EZH1, EZH2, or DDB1 in nuclear extracts of CD4$^+$ T cells was analyzed by immunoblot analysis. (*E*–*G*) Enrichment of Cul4A (*E*), DDB1 (*F*), or EZH1 and EZH2 (*G*) at *Kcna3* was examined by ChIP using anti-Cul4A, anti-DDB1, or anti-EZH1 and anti-EZH2 antibodies. Chromatin was prepared from CRBN-deficient and littermate control CD4$^+$ T cells. After ChIP, DNA fragments were measured by quantitative RT-PCR. Data are representative of two (*A*) or three (*B*–*G*) independent experiments. Results are expressed as mean ± SD. $*P < 0.05$; $**P < 0.01$, unpaired two-tailed Student's *t* test.

Our results indicate that the CRBN protein is enriched at the R4 region, which is a 3′ downstream conserved region of *Kcna3* (Fig. 3*A*). Interestingly, CRBN deficiency altered the modification of H3K27 in the R4 region of CD4$^+$ T cells (Fig. 3*A*). At regions inside the *Kcna3* ORF, including the R3 region, loss of CRBN significantly reduced H3K27me3 levels, whereas H3K27ac levels increased significantly (Fig. 3*A*). Reporter gene analysis revealed that the R4 region can enhance the *Kcna3* promoter (Fig. 3*B*).

Finally, we performed immunoblot analysis with H3K27ac- and H3K27me3-specific antibodies to determine whether CRBN alters global H3K27 modification. We found no global changes in H3K27 modification in CRBN-deficient CD4$^+$ T cells (Fig. 3*C*), suggesting that CRBN does not regulate the global pattern of histone modifications in CD4$^+$ T cells, similar to what has been reported about the regulation of histone modification by MSI4/FVE (12).

Because H3K27 methylation is mediated by EZH1 or EZH2, we attempted to identify the histone methyltransferase responsible for modifying H3K27me3 at *Kcna3*. We first examined the binding of CRBN to EZH1 or EZH2 in nuclear fractions, and found that CRBN bound to Cul4A, DDB1, and EZH1, but only weakly to EZH2 (Fig. 3*D*). In addition, ChIP analysis showed that Cul4A (Fig. 3*E*) and DDB1 (Fig. 3*F*) were recruited to *Kcna3* in CD4$^+$ T cells; however, the loss of CRBN adversely affected recruitment of Cul4A (Fig. 3*E*) and DDB1 (Fig. 3*F*). In addition, EZH1 was more enriched than EZH2 at the R4 region; however, EZH1 recruitment to *Kcna3* was markedly reduced when CRBN was absent (Fig. 3*G*). Consistent with these results, down-regulation of EZH1 and Cul4A in CD4$^+$ T cells increased Kv1.3 mRNA levels (Fig. S6). In addition, the half-life of the Kv1.3 protein was not altered by CRBN overexpression (Fig. S7).

The C-Terminal Domain of CRBN Is Crucial for CRBN Enrichment on *Kcna3* Chromatin. Analysis of the CRBN amino acid sequence using the Pfam domain library revealed the presence of the LON motif and Yippee, a novel DNA-binding motif (Fig. 4*A*). Moreover, the DNA-binding motif of CRBN is situated in an area in which substrates generally bind to WD40 DDB1/CUL4-associated factors, such as DDB2, to engage DNA (16). Therefore, we tested whether this potential DNA-binding motif of CRBN is required for binding to the *Kcna3* R4 region. In Jurkat T cells, deleting the Yippee-Mis18 motif from CRBN abolished its ability to bind *Kcna3* chromatin (Fig. 4*A*). In addition, to test whether CRBN can directly bind to *Kcna3* DNA, we expressed maltose-binding protein (MBP)-tagged full-length CRBN, the N

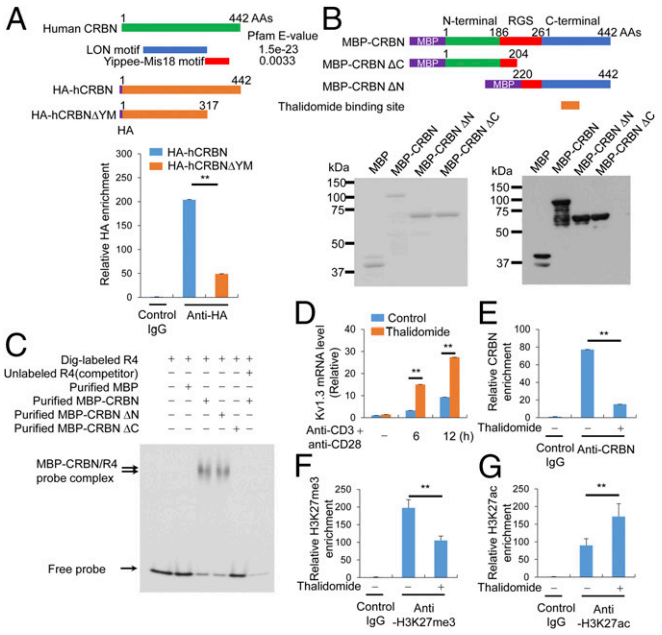

Fig. 4. The C-terminal domain of CRBN is crucial for CRBN enrichment in *Kcna3* chromatin. (*A*) The DNA-binding motif in CRBN was analyzed using a Pfam domain library (*Upper*), and HA-tagged CRBN enrichment on *Kcna3* chromatin in Jurkat T cells was examined by ChIP (*Lower*). (*B*) MBP-CRBN proteins [i.e., full-length, C-terminal region-deleted (amino acids 1–204), and N-terminal region-deleted (amino acids 220–442) CRBN proteins] were expressed in *E. coli* and purified with MBPTrap HP (GE Healthcare Life Sciences). The purified proteins were analyzed by SDS/PAGE and Coomassie blue staining (*Left*) or immunoblot analysis (*Right*) with an anti-MBP monoclonal antibody (New England Biolabs). (*C*) EMSA was performed with the purified MBP-CRBN proteins and a Dig-labeled R4 DNA fragment. Analysis was performed using DIG Gel Shift Kit, 2nd generation (Roche). Unlabeled competition demonstrated that the EMSA probes specifically bound to the MBP-CRBN proteins. (*D*) Kv1.3 levels in CD4$^+$ T cells activated with anti-CD3 and anti-CD28 antibodies were examined by quantitative RT-PCR. Before activation, CD4$^+$ T cells were pretreated with 40 μM thalidomide for 6 h. (*E*) Relative levels of CRBN enrichment on *Kcna3* chromatin from cells treated with or without thalidomide (40 μM) for 6 h were examined in ChIP assays incorporating anti-CRBN antibodies. (*F* and *G*) Relative levels of histone modification on *Kcna3* chromatin in CD4$^+$ T cells were examined in ChIP assays using anti-H3K27me3 (*F*) and anti-H3K27ac (*G*) antibodies after treatment with or without thalidomide for 6 h. Data are representative of two (*A* and *B*) or four (*C*–*G*) independent experiments. Results are expressed as mean ± SD. $*P < 0.05$; $**P < 0.01$, unpaired two-tailed Student's *t* test.

terminus of CRBN, and the C terminus of CRBN in *E. coli* and purified the proteins. The purity of the proteins, as assessed by SDS/PAGE and Coomassie blue staining, was >90% (Fig. 4*B*). The purified proteins were readily detected by immunoblot analysis with anti-MBP antibodies (Fig. 4*B*).

Using these recombinant CRBN constructs, we determined whether CRBN binds directly to the R4 region of *Kcna3* DNA. Full-length CRBN and the C-terminal CRBN fragment containing the Yippee-Mis18 motif, but not the N-terminal region, was able to bind to the *Kcna3* DNA (Fig. 4*C*). Therefore, CRBN directly binds to the *Kcna3* regulatory region R4 via the C-terminal Yippee-Mis18 motif (Fig. 4*C*). The thalidomide-binding region in CRBN is located within the C terminus of CRBN and is essential for CRBN binding to *Kcna3* DNA; therefore, we hypothesized that thalidomide prevents CRBN from binding to *Kcna3*. Consistent with this hypothesis, we found that long-term thalidomide treatment (>6 h) increased activation-induced Kv1.3 expression in $CD4^+$ T cells (Fig. 4*D*) and reduced recruitment of CRBN to *Kcna3* R4 (Fig. 4*E*). Previous reports have shown that thalidomide affects histone modifications (17, 18), and our present results demonstrate that thalidomide alters the epigenetic modifications of the *Kcna3* locus specifically (Fig. 4 *F* and *G*).

***Crbn* Deficiency Exacerbates EAE.** T-cell activation is important for disease progression in the mouse model for EAE, and the Th17 cell population is particularly important. In addition, NF-AT is important for Th17 cell differentiation. We found that $CD4^+$ T cells lacking CRBN have a greater potential for differentiation into Th17 cells in vitro (Fig. 5*A*). Thus, we tested the susceptibility of *Crbn*-deficient mice for developing EAE, which is mediated by Th17 cells. We used T-cell–specific *Crbn*-deficient ($Crbn^{flox/flox}$;*Cd4-Cre*) mice, because CRBN deficiency can affect the activity of other immune cells even though CRBN is preferentially expressed in T cells. In addition, T-cell–specific *Crbn* deficiency does not affect thymic T-cell development (Fig. S8*A*), nor does it alter the peripheral T-cell and B-cell populations in the littermate control ($Crbn^{+/flox}$;*Cd4-Cre*) mice (Fig. S8 *B* and *C*). The absence of CRBN protein expression in $CD4^+$ T cells from $Crbn^{flox/flox}$;*Cd4-Cre* mice was confirmed by immunoblot analysis (Fig. S8*D*). In addition, surface expression of activation markers, such as CD69 and CD25 (Fig. S8*E*), and IL-2 production (Fig. S8 *F*–*I*) were higher in $CD4^+$ T cells from T-cell–specific $Crbn^{flox/flox}$;*Cd4-Cre* mice than in $CD4^+$ T cells from $Crbn^{+/flox}$;*Cd4-Cre* mice. Moreover, consistent with the increased T-cell activation observed in vitro, $Crbn^{flox/flox}$;*Cd4-Cre* mice exhibited exacerbated and sustained EAE symptoms (Fig. 5 *B* and *C*). Mice lacking *Crbn* exhibited increased proliferation of peripheral myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$-specific T cells, as analyzed by [^{3}H]thymidine incorporation after $MOG_{35–55}$ immunization and recall stimulation ex vivo (Fig. 5*D*). The percentages of Th17 and Th1 cells in the periphery and spinal cord of immunized mice were significantly higher in $Crbn^{flox/flox}$;*Cd4-Cre* mice than in $Crbn^{+/flox}$;*Cd4-Cre* mice (Fig. 5 *E* and *F*). In addition, the population of GM-CSF–producing $CD4^+$ T cells was also higher in the spinal cords of $Crbn^{flox/flox}$;*Cd4-Cre* mice than in the spinal cords of $Crbn^{+/flox}$;*Cd4-Cre* mice (Fig. 5*F*). Moreover, T-cell–specific deletion of *Crbn* also increased the production of IL-17A and IFN-γ cytokines by peripheral $MOG_{35–55}$-specific T cells after $MOG_{35–55}$ immunization and recall stimulation ex vivo (Fig. 5*G*). In addition, the EAE symptoms were reduced by the Kv1.3-specific inhibitor Shk-Dap22, and worsening of the EAE symptoms in $Crbn^{flox/flox}$;*Cd4-Cre* mice was halted to levels seen in the EAE-induced $Crbn^{+/flox}$;*Cd4-Cre* mice treated with Shk-Dap22 (Fig. 5 *H* and *I*). $Crbn^{-/-}$ mice exhibited effects similar to those seen in $Crbn^{flox/flox}$;*Cd4-Cre* mice for in vitro Th17 cell differentiation (Fig. S9) and the EAE mouse model (Fig. S10). Thus, CRBN is an important antagonist of T-cell activation and limits disease pathogenesis in a mouse model for T-cell–mediated autoimmunity.

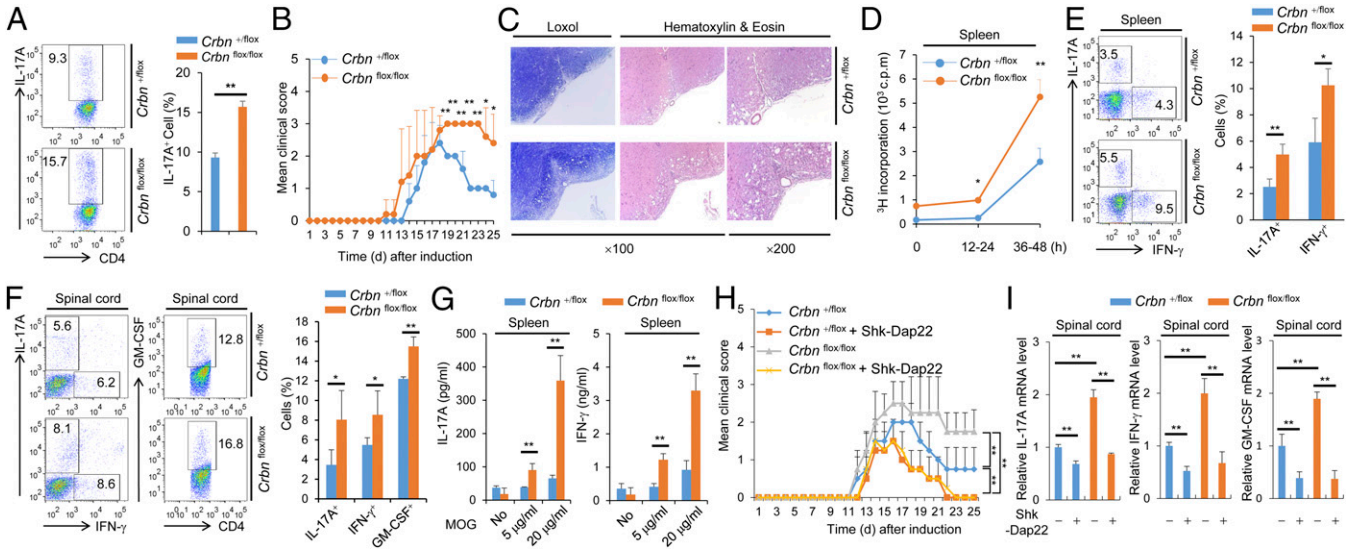

Fig. 5. Exacerbated and sustained EAE symptoms in T-cell–specific $Crbn^{flox/flox}$;*Cd4-Cre* mice. (*A*) Flow cytometry of IL-17A–producing $CD4^+$ T cells among the in vitro-differentiated Th17 cell population within $CD4^+$ T cells from $Crbn^{flox/flox}$;*Cd4-Cre* (flox/flox) or $Crbn^{+/flox}$;*Cd4-Cre* (+/flox) mice. (*B*) Clinical scores for EAE mice ($n = 7$ per group). (*C*) Spinal cord sections. (*D*) Proliferation of splenocytes in response to $MOG_{35–55}$, was measured by [^{3}H]thymidine incorporation, at the indicated time points for 12 h. (*E*) Percentage of $CD4^+$IL-17A$^+$ and $CD4^+$IFN-γ$^+$ T cells in the spleens of mice after EAE induction ($n = 3$ per group). (*F*) Percentages of $CD4^+$IL-17A$^+$, $CD4^+$IFN-γ$^+$, and $CD4^+$GM-CSF$^+$ T cells in the spinal cords of mice after EAE induction ($n = 3$ per group). (*G*) Concentration of cytokines in the supernatant from splenocyte cultures stimulated with $MOG_{35–55}$ peptide ($n = 3$ per group). (*H*) Clinical scores for EAE mice ($n = 8$ per group) with or without Shk-Dap22 treatment. (*I*) Relative mRNA levels of IL-17A, IFN-γ, and GM-CSF in isolated spinal cord mononuclear cells ($n = 3$ per group). Data are representative of five (*A*), three (*B*–*G*), or two (*H* and *I*) independent experiments. Results are expressed as mean ± SD. *$P < 0.05$; **$P < 0.01$, unpaired two-tailed Student's *t* test.

Discussion

Our experiments demonstrate that *Crbn* gene deficiency in $CD4^+$ T cells increases both calcium influx and IL-2 production. In addition, we found that CRBN regulates expression of the Kv1.3 potassium channel by directly binding to the 3′ conserved region of *Kcna3* chromatin, thereby influencing the types of histone modifications of the locus. Moreover, we noticed that Cul4A and DDB1 fail to be recruited to the *Kcna3* chromatin in the absence of CRBN. This finding is significant, because Cul4A and DDB1 are components of the CRBN E3 ubiquitin ligase complex that not only participate in ubiquitin ligase activity, but also are involved in H3K27 modifications, such as H3K27me3 and H3K27ac (12, 15). We found that CRBN is responsible for EZH1 (a histone methyltransferase for H3K27 modification) recruitment to *Kcna3* chromatin. Finally, increased expression of Kv1.3 in T cells lacking CRBN results in increased potassium flux and, subsequently, increased calcium flux, T-cell activation, and IL-2 production in vitro. These changes were associated with increased susceptibility to T-cell–mediated autoimmunity in vivo.

These observations are consistent with reports showing that Kv1.3 blockade reduces both T-cell activation and the susceptibility of mice to T-cell–mediated autoimmune disorders, such as EAE (19, 20). This suggests that CRBN acts as an important controller of calcium influx in T cells by regulating *Kcna3* expression. In addition, prolonged treatment of T cells with thalidomide can affect the expression of Kv1.3 by regulating histone modifications in *Kcna3*, because the thalidomide-binding region in CRBN is located within a newly identified DNA-binding domain. We found that thalidomide treatment blocks recruitment of CRBN to the *Kcna3* locus and affects histone modifications (i.e., H3K27me3 was decreased and H3K27Ac was increased). Interestingly, however, whereas previous reports showed that specific enhancement of calcium signaling ameliorates EAE symptoms (21, 22), our data show that $Crbn^{-/-}$ mice and $Crbn^{flox/flox}$; *Cd4-Cre* mice display exacerbated and sustained EAE symptoms even though potassium flux-coupled calcium flux is increased in the *Crbn*-deficient $CD4^+$ T cells. Other reports have shown that thalidomide treatment relieves EAE symptoms (23) whereas CRBN deficiency increases these symptoms. Apparently, CRBN is capable of "off-target" degradation activity in the presence of IMiDs. Thus, although thalidomide inhibits the DNA-binding ability of CRBN, IMiDs can affect other CRBN functions, such as off-target degradation, which possibly results from differences in CRBN deficiency and thalidomide treatment in the EAE mouse model. Other unidentified targets of IMiDs can also affect these results. Therefore, it is possible that increasing the potassium flux-coupled calcium flux in the *Crbn*-deficient $CD4^+$ T cells creates a different intracellular environment compared with increasing calcium flux only, because cytosolic calcium levels are increased by decreasing the cytosolic potassium concentration during T-cell activation. In addition, unidentified downstream signaling cascade differences between CRBN and other calcium-signaling regulators, such as Golli, may possibly contribute to the aforementioned differences in the EAE mouse model.

In conclusion, our results demonstrate that CRBN plays an important role during T-cell activation. CRBN restricts potassium flux by directly modulating the histone modification of *Kcna3* chromatin, thereby inhibiting the expression of Kv1.3. Finally, the difference in expression of CRBN in T cells could serve to regulate sensitivity to TCR stimulation.

Materials and Methods

Information on mice, cells, antibodies, plasmids, flow cytometry analysis, the nuclear complex coimmunoprecipitation (co-IP) assay, the microarray analysis, quantitative RT-PCR, calcium and potassium flux, ChIP, *Kcna3* sequence analysis, the *Kcna3* gene luciferase assay, measurement of NF-AT– and NF-κB–specific DNA-binding activity, the EAE model, the proliferation assay, and cytokine measurement is provided in *SI Materials and Methods*. All animal experiments were approved by the Gwangju Institute of Science and Technology's Institutional Animal Care and Use Committee.

ACKNOWLEDGMENTS. This work was supported by the Korean Health Technology R&D Project, Republic of Korea Ministry of Health & Welfare (Grants HI14C1466 and HI16C1074), the Silver Health Bio Research Center of Gwangju Institute of Science and Technology, and the National Research Foundation of Korea (Grants NRF-2013R1A1A2010995 and NRF-2016R1A5A1007318).

1. Ito T, et al. (2010) Identification of a primary target of thalidomide teratogenicity. *Science* 327(5971):1345–1350.
2. Lopez-Girona A, et al. (2012) Cereblon is a direct protein target for immunomodulatory and antiproliferative activities of lenalidomide and pomalidomide. *Leukemia* 26(11):2326–2335.
3. Xin W, et al. (2008) Primary function analysis of human mental retardation related gene CRBN. *Mol Biol Rep* 35(2):251–256.
4. Jo S, Lee KH, Song S, Jung YK, Park CS (2005) Identification and functional characterization of cereblon as a binding protein for large-conductance calcium-activated potassium channel in rat brain. *J Neurochem* 94(5):1212–1224.
5. Higgins JJ, et al. (2010) Temporal and spatial mouse brain expression of cereblon, an ionic channel regulator involved in human intelligence. *J Neurogenet* 24(1):18–26.
6. Lu G, et al. (2014) The myeloma drug lenalidomide promotes the cereblon-dependent destruction of Ikaros proteins. *Science* 343(6168):305–309.
7. Krönke J, et al. (2014) Lenalidomide causes selective degradation of IKZF1 and IKZF3 in multiple myeloma cells. *Science* 343(6168):301–305.
8. Gandhi AK, et al. (2014) Immunomodulatory agents lenalidomide and pomalidomide co-stimulate T cells by inducing degradation of T cell repressors Ikaros and Aiolos via modulation of the E3 ubiquitin ligase complex CRL4(CRBN.). *Br J Haematol* 164(6): 811–821.
9. Wu C, et al. (2009) BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources. *Genome Biol* 10(11):R130.
10. Cahalan MD, Chandy KG (2009) The functional network of ion channels in T lymphocytes. *Immunol Rev* 231(1):59–87.
11. Kalman K, et al. (1998) ShK-Dap22, a potent Kv1.3-specific immunosuppressive polypeptide. *J Biol Chem* 273(49):32697–32707.
12. Pazhouhandeh M, Molinier J, Berr A, Genschik P (2011) MSI4/FVE interacts with CUL4-DDB1 and a PRC2-like complex to control epigenetic regulation of flowering time in Arabidopsis. *Proc Natl Acad Sci USA* 108(8):3430–3435.
13. Dumbliauskas E, et al. (2011) The Arabidopsis CUL4-DDB1 complex interacts with MSI1 and is required to maintain MEDEA parental imprinting. *EMBO J* 30(4):731–743.
14. Wang H, et al. (2006) Histone H3 and H4 ubiquitylation by the CUL4-DDB-ROC1 ubiquitin ligase facilitates cellular response to DNA damage. *Mol Cell* 22(3):383–394.
15. Higa LA, et al. (2006) CUL4-DDB1 ubiquitin ligase interacts with multiple WD40-repeat proteins and regulates histone methylation. *Nat Cell Biol* 8(11):1277–1283.
16. Fischer ES, et al. (2014) Structure of the DDB1-CRBN E3 ubiquitin ligase in complex with thalidomide. *Nature* 512(7512):49–53.
17. Aerbajinai W, Zhu J, Gao Z, Chin K, Rodgers GP (2007) Thalidomide induces gamma-globin gene expression through increased reactive oxygen species-mediated p38 MAPK signaling and histone H4 acetylation in adult erythropoiesis. *Blood* 110(8): 2864–2871.
18. Fard AD, et al. (2013) Evaluation of H3 histone methylation and colony formation in erythroid progenitors treated with thalidomide and sodium butyrate. *Laboratory hematology: official publication of the International Society for Laboratory Hematology* 19(1):1-5.
19. Beeton C, et al. (2001) Selective blockade of T lymphocyte K(+) channels ameliorates experimental autoimmune encephalomyelitis, a model for multiple sclerosis. *Proc Natl Acad Sci USA* 98(24):13942–13947.
20. Matheu MP, et al. (2008) Imaging of effector memory T cells during a delayed-type hypersensitivity reaction and suppression by Kv1.3 channel block. *Immunity* 29(4): 602–614.
21. Feng JM, et al. (2006) Golli protein negatively regulates store depletion-induced calcium influx in T cells. *Immunity* 24(6):717–727.
22. Ghosh S, et al. (2010) Hyperactivation of nuclear factor of activated T cells 1 (NFAT1) in T cells attenuates severity of murine autoimmune encephalomyelitis. *Proc Natl Acad Sci USA* 107(34):15169–15174.
23. Corrêa JO, Aarestrup BJ, Aarestrup FM (2010) Effect of thalidomide and pentoxifylline on experimental autoimmune encephalomyelitis (EAE). *Exp Neurol* 226(1):15–23.