Supplemental information

The costimulatory receptor OX40 inhibits interleukin-17 expression through activation of repressive chromatin remodeling pathways

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SUPPLEMENTAL FIGURS

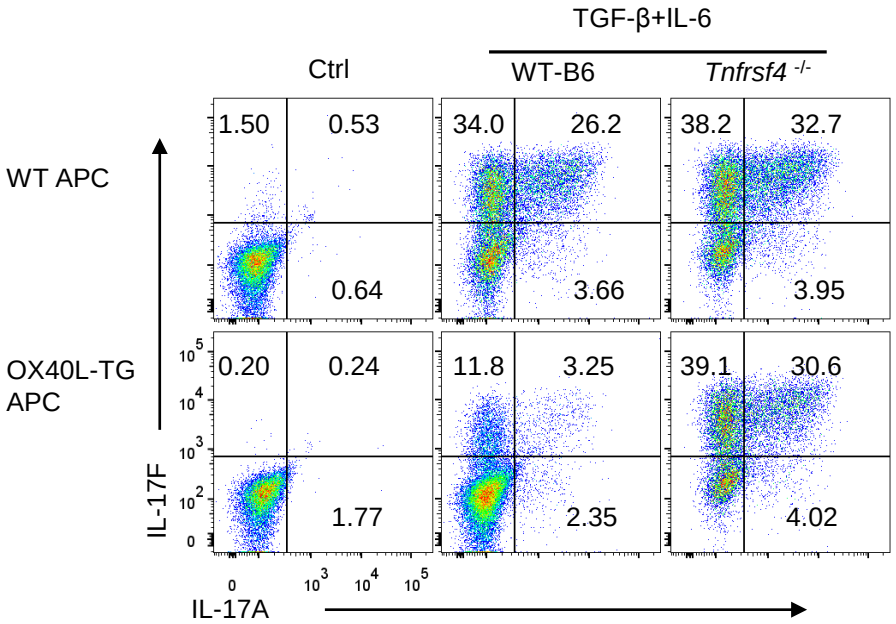

Figure S1 (related to Figure 1). OX40 inhibits expression of IL-17A and IL-17F.

Naive CD4^{+} T cells were FACS sorted from WT B6 and *Tnfrsf4* $^{-/-}$ *foxp3*gfp reporter mice and activated with anti-CD3 plus WT APCs or OX40L-TG APCs under Th17-polarizing conditions (TGF-β and IL-6) for 3 days. Cells were stained with both anti-IL-17A and anti-IL-17F Abs, and induction of IL-17A and IL-17F producing cells was determined by flow cytometry and shown. T cells activated without polarizing cytokines were included as controls (Ctrl). Data are representative of at least five independent experiments.

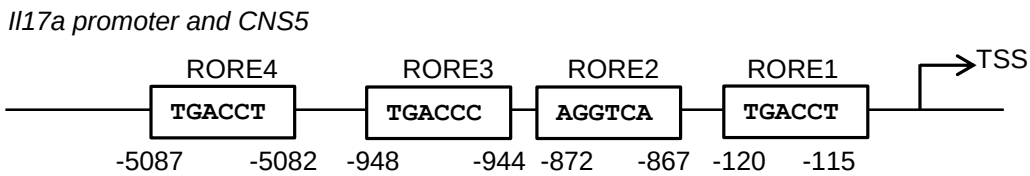

Figure S2 (related to Figure 2) . The diagram shows the RORγt binding sites at *Il17a* promoter and CNS5 regions.
The consensus RORγt binding elements (RORE) in the mouse *Il17a* promoter and CNS5 regions are shown in boxes. The numbers below the diagrams indicate positions relative to the transcription start site (TSS).

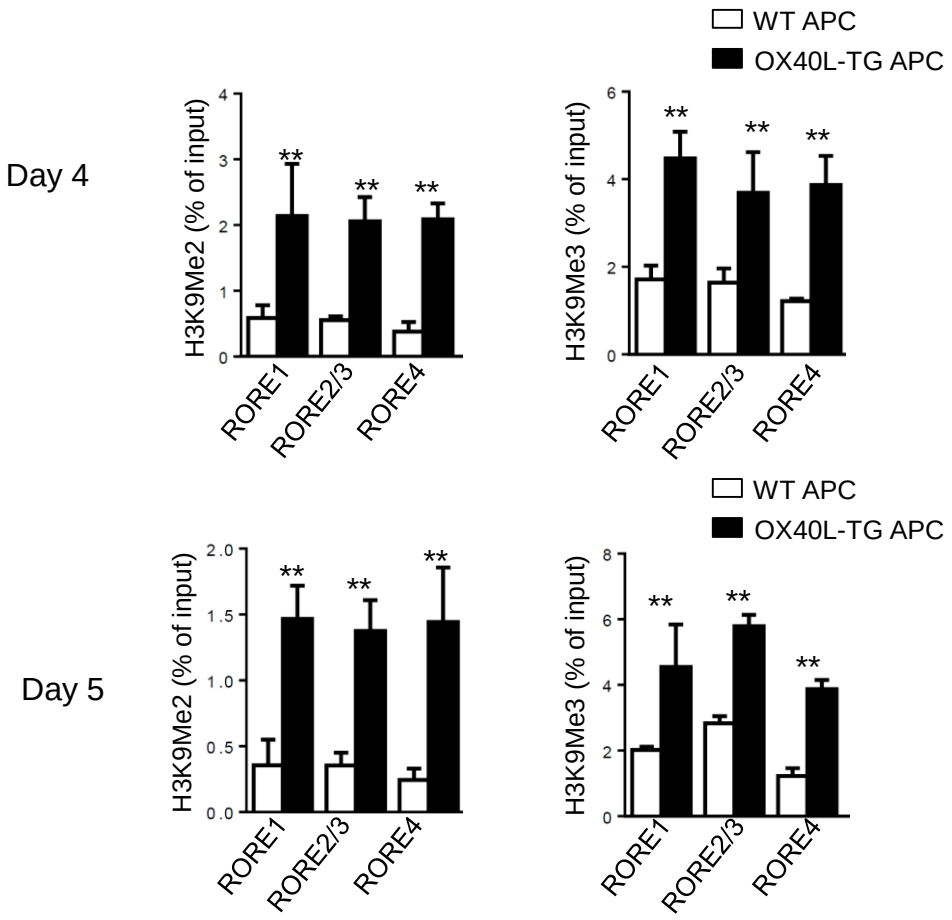

Figure S3 (related to Figure 2). OX40 stimulation induces hyper-methylation of H3K9 at *Il17a* locus for a prolonged period of time. Naïve CD4$^+$ T cells from WT B6 mice were activated under Th17-polarizing conditions with or without OX40 stimulation for 4 and 5 days. ChIP data of H3K9me2 (left) and H3K9Me3 (right) at the *Il17a* promoter (RORE1, RORE2/3) and CNS5 (RORE4) regions were shown. Data are presented as mean ± SD (n=3). ** p <0.01.

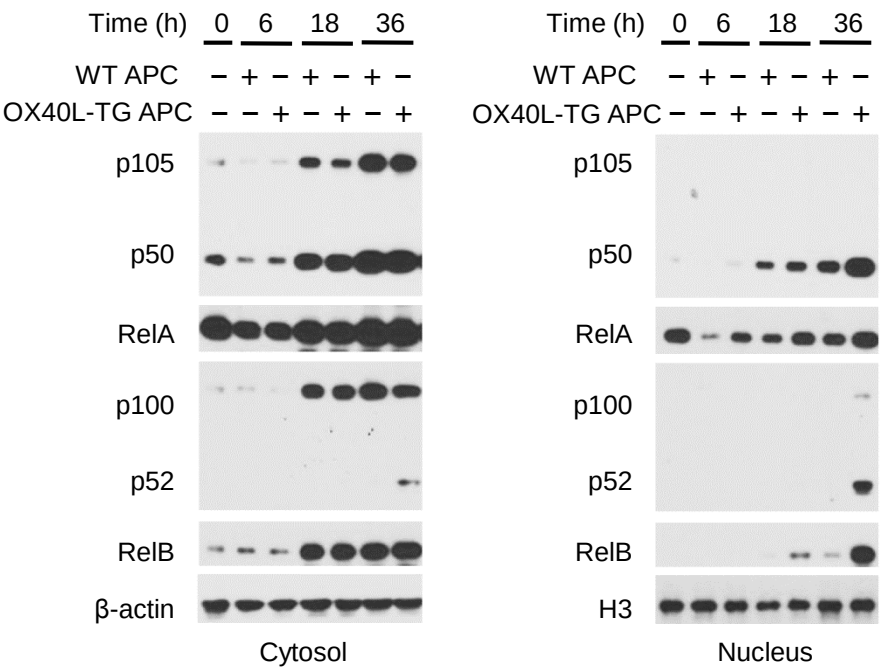

Figure S4 (related to Figure 3) . OX40 activates both canonical (RelA/p50) and the non-canonical (RelB/p52) NF-kB pathways.
Naive $CD4^+$ T cells were activated with anti-CD3 plus WT APCs or OX40L-TG APCs for 0–36 h under Th17-polarizing conditions. Immunoblot analysis on the induction of canonical (RelA, p50) and non-canonical (RelB, p52) NF-κB pathways in the cytosol (left) as well as the nucleus (right) was performed and shown. β-actin and histone 3 (H3) were used as loading controls, respectively. Data are representative of one of three independent experiments.

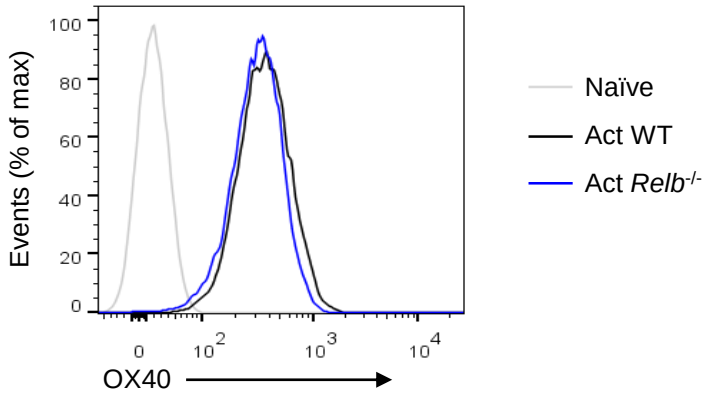

Figure S5 (related to Figure 3) . WT and *Relb*$^{-/-}$ CD4+ T cells express comparable levels of OX40 on the cell surface upon activation.

The FACS plot shows OX40 expression by Wt CD4+ T cells and *Relb*$^{-/-}$ CD4+ T cells activated for 2 days under Th17-polarizing conditions (Act). Naïve unstimulated CD4+ T cells were used as a staining control. Similar pattern of OX40 expression was observed on day 3 and day 4 following activation.

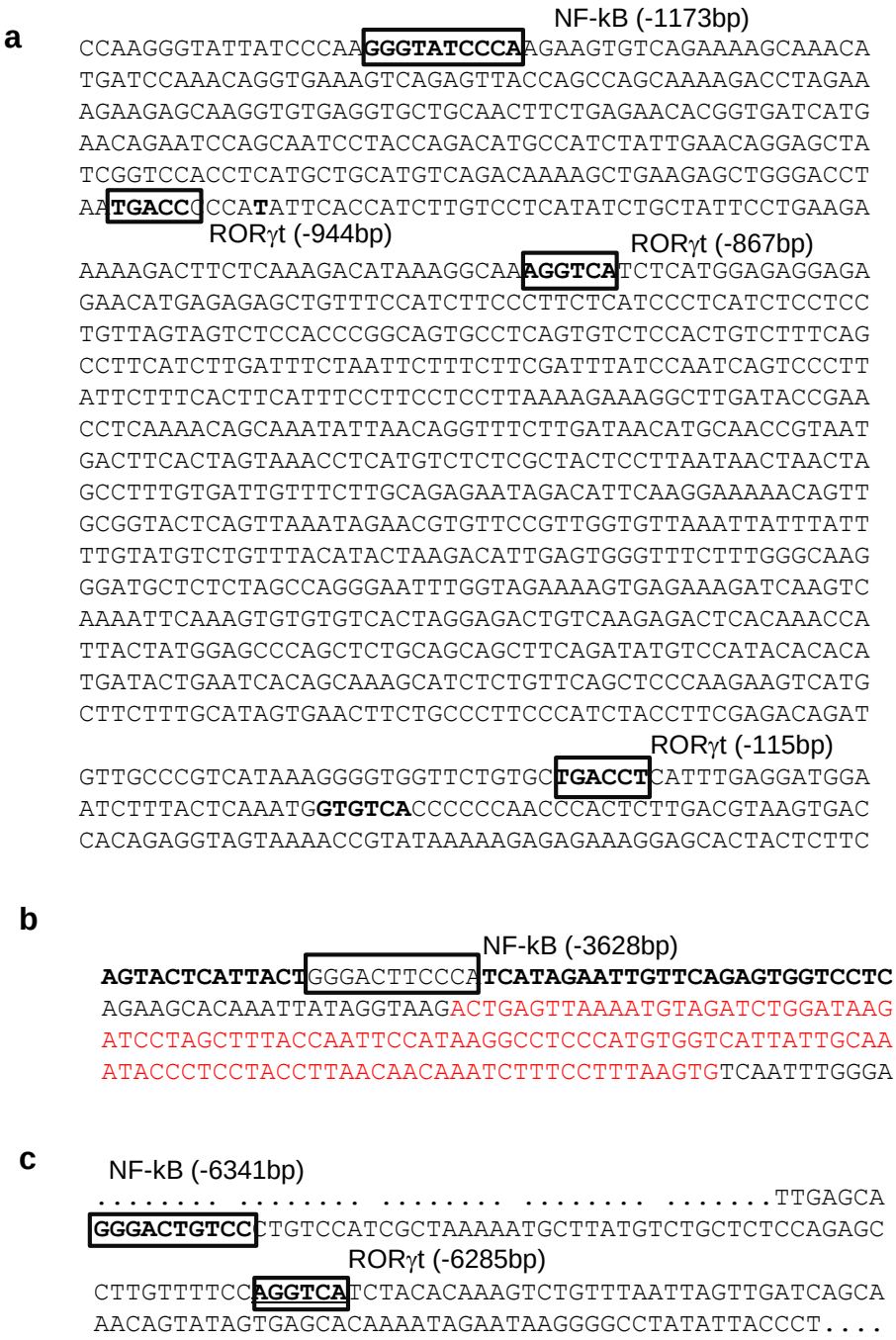

Figure S6 (related to Figure 5) . NF-kB binding sites upstream of the *Il17a* locus. (**a**) NF-kB and RORγt binding sites in the promoter region (~1.2kb) of mouse *Il17a* are shown in boxes. The numbers indicate positions relative to the transcription start site. (**b**) the CNS4 region of the mouse *Il17a* is shown in red, and the position of a NF-kB consensus binding site is shown in a box. (**c**) The locations of NF-κB and RORγt binding sites in the CNS6 region of the mouse *Il17a* are shown. The CNS4 and CNS6 alignments were made using rVista 2.0 software based on mouse vs. human sequences.

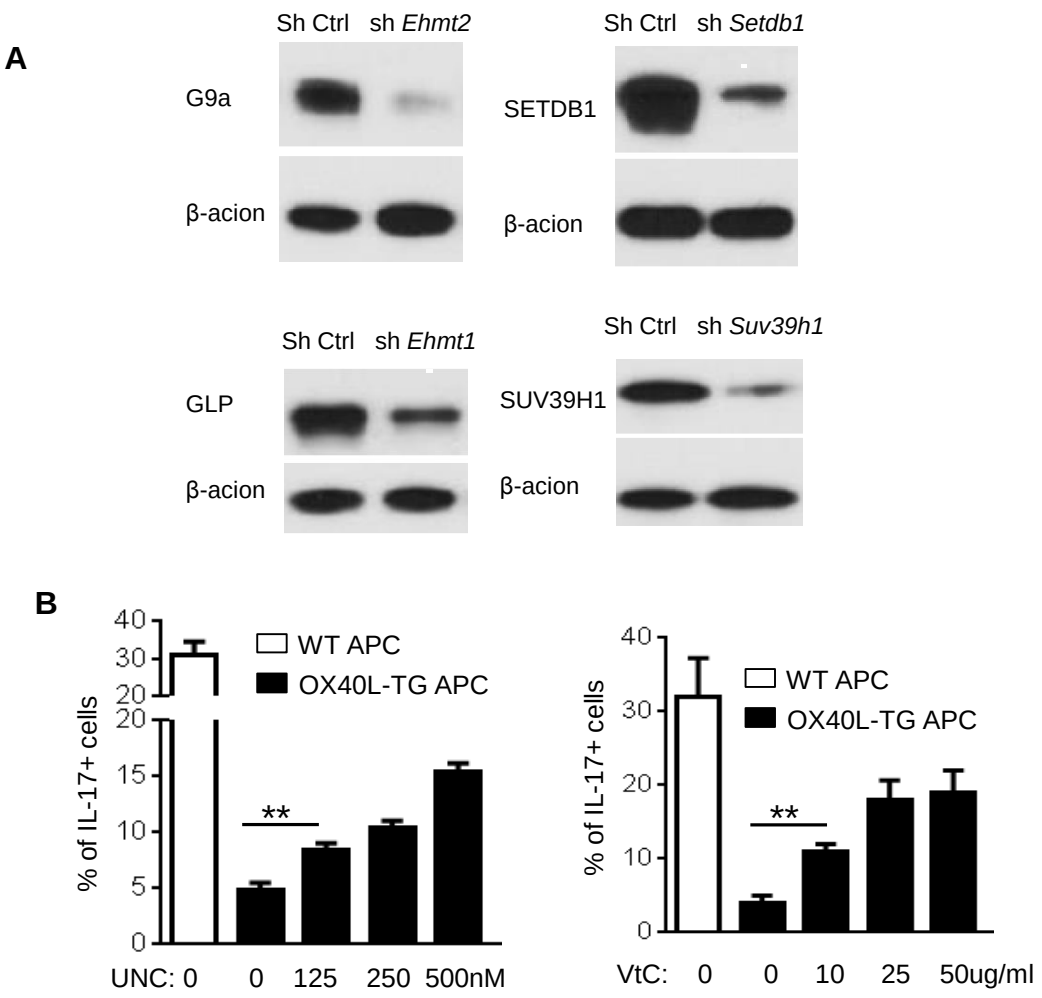

Figure S7 (related to Figure 5 and Figure 6) . RNA knockdown efficiency and pharmacological inhibitors of histone methyltransferases in IL-17 expression.

(A). *Emht1 (Glp)*, *Emht2 (G9a)*, *Setdb1*, and *Suv39h1* specific small hairpin inhibitory RNA (shRNA) was transduced into activated $CD4^+$ T cells using retroviral mediated gene transfer approach. The cells were further cultured under Th17-polarizing conditions for 96 hrs. The transduced T cells were FACS sorted based on GFP expression and assessed for expression of G9a, GLP, SETDB1 and Suvs9h1 by immunoblotting assay. Data are representative of two independent experiments. (B). G9a inhibitor UNC0646 (UNC) or Vitamin C (VtC) alone partially rescued OX40-mediated suppression of Th17 cells. Naive CD4+ T cells were activated with anti-CD3 plus WT APCs or OX40L-TG APCs under Th17-polarizing conditions for 3 days in the presence of different concentrations of UNC (left) or (VtC (right). Induction of IL-17 producing Th17 cells was determined and shown. Data shown are Mean$\pm$ SD of triplicate assays.

Table S1 (relative to Figure 1). Whole genomic analysis of gene expression by RNA-seq in Th17 cells with or without OX40L engagement.

Table S2 (related to the experimental procedures): ChIP-qPCR primers used in this study

	FORWARD	REVERSE
RORE-1	GCAGCAGCTTCAGATATGTCC	TGAGGTCAGCACAGAACCAC
ROER-2/3	CTGAAGAGCTGGGACCTAATG	GCTCTCTCATGTTCTCTCCTCTC
ROER-4	CCGTTTAGACTTGAAACCCAGTC	GTACCTATGTGTTAGGAGGCGC
kBBE-1	GCAATCAGAGGTGTGTGTGA	GCTGGCTGGTAACTCTGACTT
kBBE-2	CCTTCCACCACAAATTCCAGA	ATTTGTGCTTCTGAGGACCACTC
kBBE-3	CAATGCAACAATATGTAAGCCAAA	GCTCTGGAGAGCAGACATAAGC

Table S3 (related to the experimental procedures): shRNA oligos for each target gene

Gens	Targets sequence
Ehmt2	Forward: CGAGAGAGTTCATAGCTCTTTCAAGAGAAGAGCTATGAACTCTCTCGGAAAAA Reverse: TTTTTTCCGAGAGAGTTCATAGCTCTTCTCTTGAAAGAGCTATGAACTCTCTCGG
Ehmt1	Forward: GAATGCAAATGTGGGACAACGTCAAGAGCGTTGTCCCACATTTGCATTCTTTTT Reverse: TTTTTTGCCGACTTCTGCCTGAGTTTACTCTTGATAAACTCAGGCAGAAGTCGGC
Setdb1	Forward: GCCTTGATCTTCCATGTCATTTCAAGAGAATGACATGGAAGATCAAGGCTTTTTT Reverse: AAAAAAGCCTTGATCTTCCATGTCATTCTCTTGAAATGACATGGAAGATCAAGGC
Suvs9h1	Forward: GAATGCAAATGTGGGACAACGTCAAGAGCGTTGTCCCACATTTGCATTCTTTTTT Reverse: AAAAAAGAATGCAAATGTGGGACAACGCTCTTGACGTTGTCCCACATTTGCATTC

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Polarization of naive CD4$^+$ T cells *in vitro*.

Naive CD4$^+$ T cells (CD62L$^+$CD44$^-$Foxp3$^-$) were sorted from *foxp3gfp* reporter mice by flow cytometry, and stimulated with mAb to CD3e (2 μg/ml; 2C11; eBioscience) plus equal numbers of syngeneic APCs in 96-well tissue-culture plates (Sigma-Aldrich) at 1×10^5 cells per well. APCs were prepared by depletion of T cells from total spleen cells with PE–anti-CD3 (2C11; Miltenyi Biotec) and anti-PE microbeads (Miltenyi Biotec), followed by a brief treatment with mitomycin C (50 μg/ml; Sigma-Aldrich) before each experiment. For the induction of Th17 cells in vitro, cells were activated in the presence of 3 ng/ml human TGF-β1 and 30 ng/ml mouse IL-6. All recombinant cytokines were from R&D Systems. In some cases, cells were plated as above in the presence of 2-Phospho-L-ascorbic acid trisodium salt, vitamin C (Sigma-Aldrich), and/or G9a inhibitor UNC0646 (Calbiochem) at the concentrations as indicated. CD4$^+$ T cells cultured for 1–5 days under Th17 polarizing conditions were collected and assessed by intracellular IL-17 staining, CHIP, Immunoblot, and quantitative real-time RT-PCR.

Intracellular staining.

For cytokine staining, CD4$^+$ T cells activated under various polarizing conditions were restimulated for 4hr with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (550 ng/ml; Sigma-Aldrich) in the presence of GolgiStop (BD PharMingen). Cells were fixed and made permeable with Cytofix/Cytoperm solution (BD PharMingen) and were stained with fluorochrome-conjugated anti-IL-17A (TC11; BioLegend) and IL-17F (9D3.1C8, BioLegend). Anti-RORγt (B2D; eBioscience) was used to assess the expression of RORγt in naive and activated CD4$^+$ T cells according to the manufacturers' instructions. All samples were acquired with LSRII (Beckton Dickinson) and data were analyzed with FlowJo v10 software (Tree Star).

Analysis of Th17 cell induction *in vivo*

For induction of Th17 cell *in vivo*, 1×10^6 of CD45.1$^+$ CD4$^+$ CD25$^-$ OT-II T cells (CD4$^+$ CD25$^+$ selection kit, Miltenyi Biotec) were transferred intravenously into CD45.2$^+$ wild-type B6 recipient mice 1 day before immunization with 100 mg OVA (323-339) in 100 ml complete Freund's adjuvant (CFA containing 50 mg heat-killed Mycobacterium tuberculosis H37Ra) (Difco, MI) subcutaneously in the hind flanks. Then the mice were received 3 doses (0.25mg, i.p.) of anti-OX40 agonist anybody (OX86; BioXCell, Lebanon, NH) or control IgG every days starting at day 0 after OVA immunization. Phenotype of transferred CD45.1$^+$ OT-II T cells in draining LN cells was assessed by flow cytometry and gating on live CD4$^+$ cells.

Quantitative RT-PCR.

Cellular RNA was extracted with RNeasy mini kit (Qiagen) and was reverse-transcribed into cDNA by the ABI PRISM TaqMan reverse transcription method. Expression of genes of interest was assessed by Simplex RT-PCR with probes labeled with 5-carboxyfluorescein and VIC

fluorescent dye (Applied BioSystems). For all analyses, *Gapdh* was included as a housekeeping gene for comparison. All primer and probe sets were from Applied BioSystems. Transcription of target genes was calculated according to the $2^{-\Delta CT}$ method as described by the manufacturer (ABI PRISM 7700 user bulletin; Applied BioSystems) and was presented in arbitrary units as reported (Xiao et al., 2012b).

Immunoblot analysis.

Protein extracts were resolved by SDS-PAGE, transferred onto an Immunobilon membrane, and analyzed by immunoblot with the following specific antibodies: anti-SUV39H1 (S8316, Sigma-Aldrich); anti-G9a (ab31874), anti-GLP (ab41969), anti-SETDB1 (ab107225), anti-p105/50 (ab32360), anti-RelA (ab16502), anti-RORα,(ab60134; all from Abcam); and anti-RelB (4922), anti-Histone H3 (4499), anti-β-actin (12262), anti-BATF (8638), anti-p100/52 (4882), anti-c-Rel (4727; all from Cell Signaling Technology). Anti-RORγt (B2D) was from eBioscience. Horseradish peroxidase–linked antibody to mouse immunoglobulin G (7076; Cell Signaling Technology), horseradish peroxidase–linked antibody to rabbit immunoglobulin G (7074; Cell Signaling Technology) and horseradish peroxidase–linked antibody to goat immunoglobulin G (sc-2768; Santa Cruz) were used as secondary antibodies. The expression of target molecules was detected by a chemiluminescence method as reported (Xiao et al., 2012a).

Retrovirus-mediated gene expression.

The cDNA fragments encoding mouse G9a, RelA, p50, RelB (full length or various RelB mutants), p52, cRel, and RORγt were amplified by PCR and further cloned into the pMYs-IRES-EGFP retroviral vector (Cell Biolabs). Retroviral particles were prepared by transfection of these vectors into packaging Plat-E cells according to the manufacturer's recommendations (Cell Biolabs). For T cell transduction, naive $CD4^+$ T cells were first activated for 24 h with anti-CD3/APC, followed by incubation with freshly prepared retroviral particles by centrifugation for 2 h at 780*g* and 32°C in the presence of 10 µg/ml polybrene (Sigma-Aldrich). After centrifugation, cells were cultured for 6 h at 32°C, followed by culture of the transduced T cells under various polarizing conditions for additional 3 d in complete 1640 medium at 37°C. The polarization of $CD4^+$ T cells into Th17 cells was assessed by intracellular staining (Xiao et al., 2012a).