Supporting Online Material

Materials and Methods

Mice

Wild-type C57BL/6J mice were bred in the SPF barrier facility at the University of Texas Southwestern Medical Center. *Nfil3*$^{-/-}$ mice were imported from the University of Iowa (*15*) and maintained in the SPF barrier facility at UT Southwestern. *Rag1*$^{-/-}$ mice (B6.129S7-Rag1^{tm1Mom}/J) were purchased from the Jackson Laboratory, Bar Harbor, Maine. *Rev-erbα*$^{-/-}$ mice (B6.129S2-Nr1d1^{tm1Schb}/Cnrm) (*21*) were obtained from the European Mutant Mouse Archive (EMMA). The mice from EMMA had been backcrossed to C57BL/6J for at least 6 generations, and we further backcrossed them to C57BL/6J for a total of more than 7 generations. *Rev-erbβ*$^{-/-}$ (B6;129P2-Nr1d2^{tm1Dgen}) mice were purchased from Deltagen, San Mateo, California. *Rev-erbα*$^{+/-}$ and *Rev-erbβ*$^{+/-}$ mice were crossed to generate *Rev-erbα/β* $^{-/-}$ double knockout mice. *Clock* $^{\Delta 19/\Delta 19}$ mice were created as described (*23, 28*). All mouse strains, if not specified otherwise, were maintained under 12-h light and 12-h dark conditions in the SPF barrier facility at UT Southwestern. All mice used throughout this study were 8 weeks old, except for the mice in the light cycle perturbation experiments shown in Fig. 4G, which were 6 weeks old. Germ-free C57BL/6J mice were raised in gnotobiotic isolators as described previously (*1*). Experiments were performed according to protocols approved by the Institutional Animal Care and Use Committees of the UT Southwestern Medical Center.

Lamina propria lymphocyte isolation and analysis

Lamina propria lymphocytes were isolated as previously described (*7*). 1~2 x 10^6 cells were subjected to nuclear staining with eBiosciences Mouse Regulatory T Cell Staining Kit #3 per manufacturer's instruction. CD4-FITC (RM4-5), Foxp3-PECy5 (FJK-16s) and RORγ-PE (AFKJS-9) were used to detect Th17 and T_{reg} cells. For intracellular cytokine staining, 1~2 x 10^6 cells were stimulated with 50 ng/ml phorbol myristate acetate (PMA), 1 mM ionomycin and 1 mg/ml brefeldin A (Sigma) for 4 hr. Cells were

then fixed and permeabilized with BD Cytofix/Cytoperm solution for 30 min, followed by staining with CD3ε-FITC (500A2), IFNγ-PE (XMG1.2), CD4-PerCP (RM4-5) and IL-17A-APC (TC11-18H10.1). Flow cytometry was performed with a FACSCalibur (BD Biosciences) and data were analyzed with FlowJo software (Tree Star).

Microbiota adoptive transfer

The small intestine, cecum and colon were dissected out from conventionally raised mice and homogenized in ice-cold sterile phosphate buffered saline solution (PBS). The homogenate was spread in the cages as well as on the fur of germ-free wild-type mice (*1*). Ex-germ-free mice that received microbiota from wild-type and *Nfil3*$^{-/-}$ mice were maintained in separate gnotobiotic isolators for 6 weeks. Lamina propria lymphocytes were isolated and analyzed as mentioned above.

T cell adoptive transfer

Adoptive transfer of T cells was performed as previously published (*13*). Briefly, total CD4^{+} T cells were isolated from the spleens of wild-type and *Nfil3*$^{-/-}$ mice by negative selection with the Dynal® Mouse CD4 Negative Isolation Kit (Invitrogen). Cells were stained with CD4-FITC (RM4-5) and CD45RB-APC (C363.16A) and CD4^{+} CD45RB^{hi} cells were sorted out with a MoFlo cell sorter (Beckman Coulter). 4 x 10^{5} purified CD4^{+} CD45RB^{hi} cells were transferred into *Rag1*$^{-/-}$ mice through the tail vein. Lamina propria lymphocytes were isolated and analyzed 4 weeks post transfer.

Lentiviral transduction experiments

The *egfp* sequence was amplified from pEGFP-C1 (Clontech) by PCR and cloned into pCDH-CMV-MCS-EF1-Puro (SBI) to generate vector pXY81. *Nfil3* cDNA was cloned from total intestinal cDNA by PCR and inserted downstream of *egfp* in pXY81 to create vector pXY82. HEK293T cells were transfected with either pXY81 or pXY82 in combination with packaging plasmids pVSV-G and

pCMVAR9. Lentiviral particles were purified from cell culture supernatant by poly(ethelene glycol)-8000 precipitation (Sigma), which was subsequently removed by dialysis.

Naïve $CD4^+$ T cells were purified from the spleen of wild-type mice using the $CD4^+CD62L^+$ T Cell Isolation Kit II (Miltenyi Biotec), following the manufacturer's instructions. 2 x 10^5 purified naïve T cells were cultured under Th17-polarizing conditions in one well of a 96-well plate with plate-bound anti-CD3ε (145-2C11) and anti-CD28 (37.51), 10 μg/ml anti-IFNγ (XMG1.2), 10 μg/ml anti-IL-4 (11B11), 20 ng/ml recombinant murine IL-6 and 5 ng/ml recombinant human TGF-β1 (Peprotech) as described (*29*). Lentiviral transduction was performed by spinoculation at 1200*g* for 1 hr at 32°C on the day of polarization. Th17 cells were analyzed by intracellular cytokine staining 4 days later.

***In vitro* differentiation of Th17 cells**

Naïve $CD4^+$ T cells were purified from mouse spleen using the $CD4^+CD62L^+$ T Cell Isolation Kit II (Miltenyi Biotec), as described above and 95% purities were routinely obtained. To culture cells under Th17 polarizing conditions (*12*), 96-well plates were coated with 5μg/mL anti-hamster antibody (MP Bio) overnight at 4°C. After plates were washed three times with sterile PBS, 2 x10^5 cells were added to each well in the presence of 0.25 μg/mL anti-CD3ε, 1 μg/mL anti-CD28, 5 μg/ml anti-IFNγ, 5 μg/ml anti-IL-4, 20 ng/ml recombinant murine IL-6 and 1 ng/ml recombinant human TGF-β1. Cells were analyzed 72hrs later by intracellular staining of IL-17A after PMA/ionomycin/brefeldin A treatment.

Chromatin immunoprecipitation (ChIP) assays

Total $CD4^+$ T cells were purified from the spleen and lymph nodes of wild-type mice by negative selection with the Mouse CD4 Negative Isolation Kit (Invitrogen). Cells were crosslinked in PBS with 1% formaldehyde for 10 min at room temperature. Crosslinking was terminated by adding 1/5 volume of 1 M glycine and incubating for another 10 min.

Fixed cells were used for chromatin immunoprecipitation (ChIP) with the Magna ChIP assay kit (Millipore) according to the manufacturer's instructions, except that Magna protein A beads were saturated with BSA and salmon sperm DNA before use. Chromatin from approximately 5 x 10^6 cells was used for each immunoprecipitation reaction in combination with 5 μg of goat anti-NFIL3 (Santa Cruz Biotechnology, C-18) or total goat IgG antibody and 20 μl of Magna protein A beads. The *Rorγt* promoter was detected using SYBR green-based real-time PCR. Abundance of the *Rorγt* promoter in precipitated DNA was determined as the percentage of total input DNA. Enrichment of the DNA fragment was calculated as the ratio of its abundance in anti-NFIL3-immunoprecipitated DNA to that in total goat IgG-immunoprecipitated DNA.

To examine REV-ERBα binding to the *Nfil3* locus, ChIP was performed as above except that total rabbit IgG and rabbit anti-REV-ERBα antibody (Sigma, HPA007935) were used during pull-down and primers specifically targeting *Nfil3* were used during PCR detection of ChIP DNA.

Electrophoretic mobility shift assays (EMSA)

To prepare protein for EMSA, HEK293T cells were transfected with either an empty vector or an NFIL3-encoding plasmid. Cells were harvested 2 days after transfection and nuclear extract was prepared with nuclear extraction buffer containing 20 mM Tris pH 8.0, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 25% glycerol and 1X protease inhibitor cocktail (Roche). Protein concentration of nuclear extracts was determined by the Bradford assay and adjusted to 1 mg/ml with nuclear extraction buffer.

EMSA assays were performed as previously described (*30*), with modifications. DNA oligos were synthesized according to the *Rorγt* promoter sequences encompassing the putative NFIL3-binding site (WT probe: 5'-GATCTTGAGCAGGTTACTTAATCTCTCTGT-3'; Mutant probe: 5'-GATCTTGAGCAGGCGGCGGCGTCTCTCTGT-3'). Probes were terminally labeled with ^{32}P by T4 polynucleotide kinase (PNK) and α-^{32}P-ATP (PerkinElmer). 8000 cpm of labeled probes were mixed with 4 μg of nuclear extract in EMSA buffer containing 20 mM HEPES pH 7.9, 50 mM KCl, 1 mM EDTA, 2

mM DTT, 2 mM $MgCl_2$, 0.25 mg/mL BSA, 5% glycerol, 50 μg/mL poly(dI-dC). For competition with cold probes, 3 pmol of cold probe was added to the reaction. For supershifting, 100 ng of anti-NFIL3 antibody was used. Reactions were incubated at 30°C for 45 min and loaded onto a 3.5% polyacrylamide gel. Autoradiography was carried out with a storage phosphor screen and images were scanned with a Storm scanner (GE healthcare).

***Rorγt* reporter assays**

A 1018 bp (from -1013 to +5) fragment of the *Rorγt* promoter was amplified from C57BL/6J genomic DNA by PCR and cloned into pGL3-Basic (Promega) to generate the WT reporter. The putative NFIL3-binding site in the WT reporter was mutated from GTTACTTAA to GTTACTTTA to generate the mutant reporter. Jurkat T cells were transfected with 300 ng of reporter plasmid (WT or mutant), 400 ng of an empty vector (pXY47) or an NFIL3-encoding plasmid (pXY52), 300 ng of P1P2N-HIF1α (a kind gift of Dr. Kevin Gardner at UT Southwestern Medical Center) and 50 ng of pCMV-Renilla-Luc, followed by stimulation with 12.5 ng/ml PMA and 0.25 mM ionomycin for 16 hr. Luciferase activities were determined by the Dual-Glo Luciferase Assay kit (Promega) and measured with a SpectraMax M5e plate reader (Molecular Devices). Firefly luciferase activities were first normalized against Renilla luciferase activities and then referenced to the values obtained from cells transfected with pXY47.

Light cycle perturbation experiments

Six week old C57BL/6J male mice were housed in ventilated, light-tight cabinets on a 12 hr-light, 12 hr-dark cycle (Phenome Technologies). After acclimation for 3 days, light cycles were changed for mice in treatment groups while they remained the same for mice in the control group. On day 1, lights were turned on 6 hr earlier than they were previously (6 hr phase advance). The light cycles were maintained for 4 days and changed in the same way again on day 5. After changing light cycles four times, the mice

were maintained on a normal light cycle for 4 days. Mouse intestines were then harvested and lamina propria lymphocytes were isolated and analyzed.

DSS treatment

Age- and sex-matched C57BL/6J mice were maintained under normal light cycles or subjected to perturbed light cycles as described above. After the treatment period, mice were provided 3% DSS (wt/vol) in drinking water. 200 μg mouse IL-17A neutralizing antibody (BioXCell, clone 17F3) or isotype control antibody (BioXCell, clone MOPC-21) in 100 μL PBS was delivered by intraperitoneal injection on day 0, 2 and 4. Mouse weight was monitored daily. All mice were sacrificed on day 5.

Real-Time RT-PCR

$CD4^+$ T Cells were purified by MACS sorting as described before and RNA was isolated with Arcturus PicoPure RNA isolation kit (Life Technologies) per manufacturer's instruction. 200 ng of total RNA was used for reverse transcription with M-MLV reverse transcriptase (Life Technologies). cDNA product was diluted 10-fold with H_2O and 2 uL was used in each real-time PCR reaction with Platinum SYBR Green qPCR SuperMix (Life Technologies). Primer sequences are described in Table S1.

Statistical analysis

Comparisons between groups were done by two-tailed Student's t-test, one-way ANOVA or two-way ANOVA as specified in each experiment.

Supporting References

28. M.H. Vitaterna et al., Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. *Science* **264**, 719 (1994).

29. W. Hu, T. D. Troutman, R. Edukulla, C. Pasare, Priming microenvironments dictate cytokine requirements for T helper 17 cell lineage commitment. *Immunity* **35**, 1010 (2011).

30. J. W. Huh *et al.*, Multivalent di-nucleosome recognition enables the Rpd3S histone deacetylase complex to tolerate decreased H3K36 methylation levels. *EMBO J.* **31**, 3564 (2012).

Table S1: Primers used in this study.

Primer	Sequences	Description
RORγt-ChIPF1	5'-AAGTGCCAGGAGACGGGCCA-3'	Detection of the mouse *Rorγt* promoter by ChIP-PCR
RORγt-ChIPR1	5'-ACAAGACTGCTAGTCTGGGACACA-3'	
Nfil3-ChIPF1	5'-GCGATGAAACGTGGGCACCG-3'	Detection of the mouse *Nfil3* gene locus by ChIP-PCR
Nfil3-ChIPR1	5'-CGTCACAATGGCTCGTCCGGG-3'	
Nfil3-RTF	5'-CTTTCAGGACTACCAGACATCCAA-3'	Detection of mouse *Nfil3* expression by RT-PCR
Nfil3-RTR	5'-GATGCAACTTCCGGCTACCA-3'	
Rorγt-RTF	5'-TTCACCCCACCTCCACTG-3'	Detection of mouse *Rorγt* expression by RT-PCR
Rorγt-RTR	5'-GTGCAGGAGTAGGCCACATT-3'	
Gapdh-F	5'-TGGCAAAGTGGAGATTGTTGCC-3'	Detection of mouse *Gapdh* expression by RT-PCR
Gapdh-R	5'-AAGATGGTGATGGGCTTCCCG-3'	

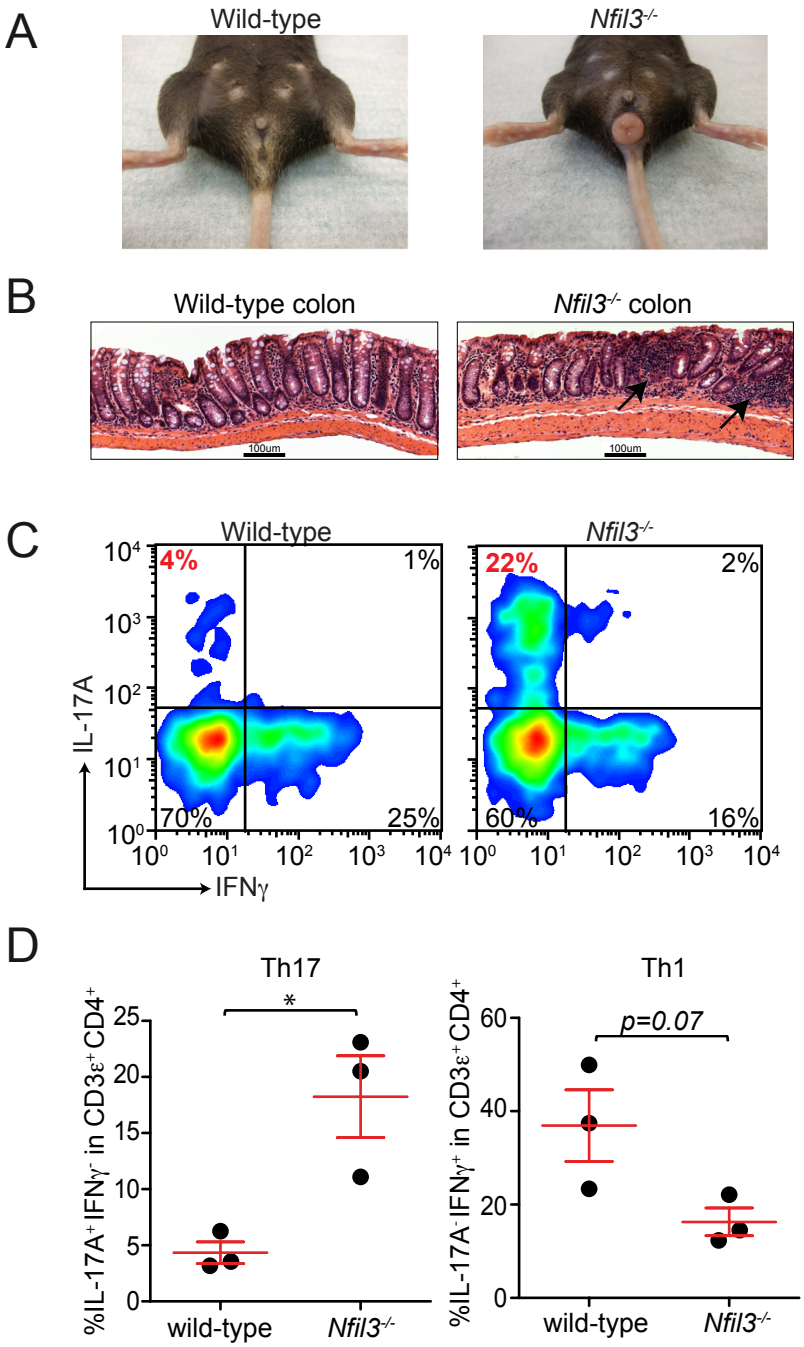

Figure S1: *Nfil3*$^{-/-}$ mice exhibit spontaneous colitis coincident with elevated Th17 cell frequencies. (A) Rectal prolapse, one hallmark of murine sponteneous colitis, was observed in 10% of *Nfil3*$^{-/-}$ mice aged 6 to 9 weeks. **(B)** Hematoxylin and eosin staining revealed more immune cell infiltration in the colons of *Nfil3*$^{-/-}$ mice (indicated by arrows). **(C,D)** Elevated Th17 frequencies were detected in the colons of *Nfil3*$^{-/-}$ mice at 8 weeks of age. Representative FACS plots are shown in **(C)** and data from the wild-type and *Nfil3*$^{-/-}$ groups are summarized in **(D)**. Statistical comparisons between groups were carried out with two-tailed student's t-test. *,$p<0.05$.

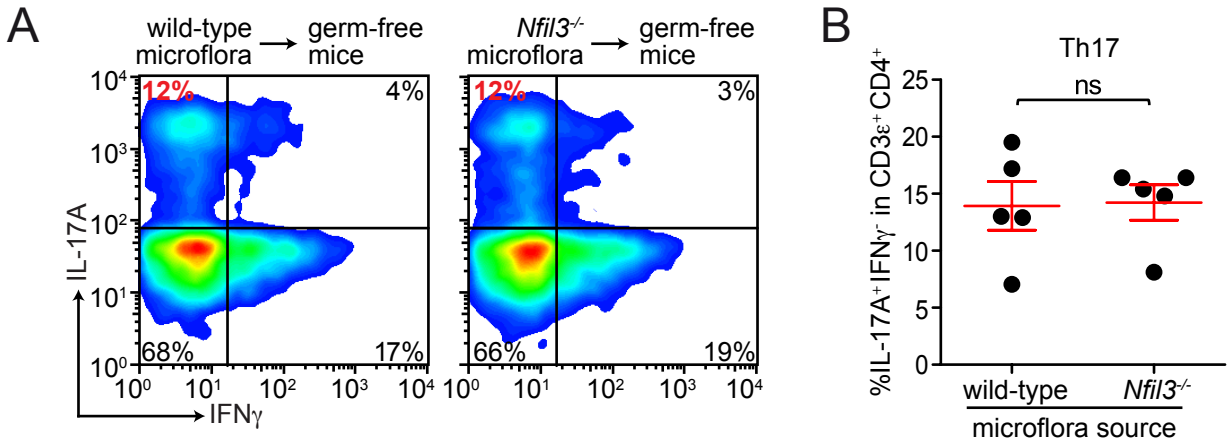

Figure S2: Increased Th17 cell frequencies in *Nfil3*$^{-/-}$ mice are not transferrable to wild-type germ-free mice by microflora transplantation. Microflora from wild-type or co-housed *Nfil3*$^{-/-}$ mice were transplanted into wild-type germ-free recipients and lamina propria lymphocytes were analyzed 6 weeks later. Representative FACS plots are shown in **(A)** and data from multiple mice are shown in **(B)**. Statistical analysis was performed with two-tailed student's t-test. ns, not significant.

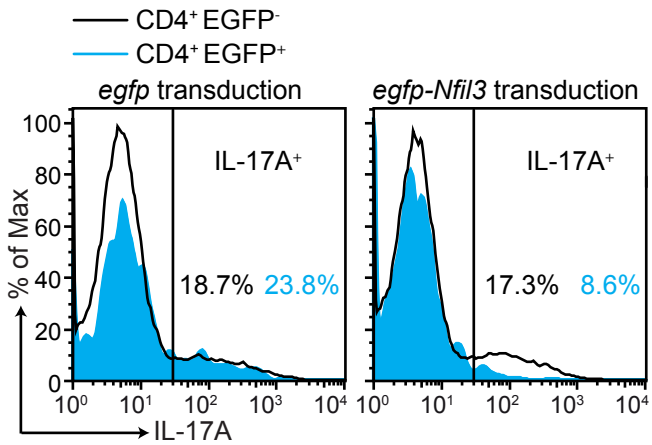

Figure S3: Histogram of Th17 *in vitro* polarization after lentiviral transduction. Experiments were set up as described for Fig. 1F and representative histograms are shown here. Results are summarized in Fig. 1F.

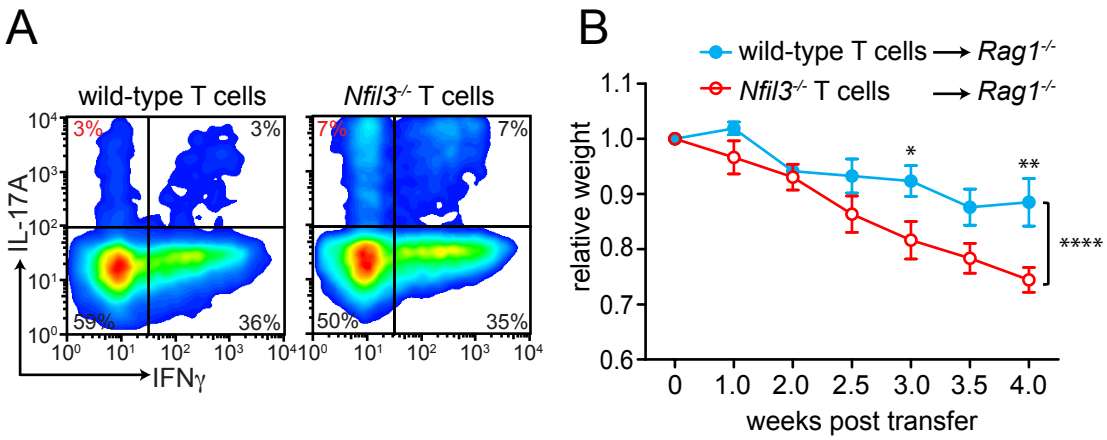

Figure S4: Body weights of *Rag1*$^{-/-}$ mice receiving wild-type or *Nfil3*$^{-/-}$ naive T cells. 400,000 wild-type or *Nfil3*$^{-/-}$ naive T cells were injected into *Rag1*$^{-/-}$ mice through the tail vein. (**A**) Intestinal Th17 cell frequencies were analyzed in mice receiving wild-type and *Nfil3*$^{-/-}$ T cell by intracellular staining for IL-17A and IFNγ. Representative flow cytometry plots are shown and combined data from multiple mice are shown in Fig. 1G. (**B**) Mouse weights were monitored for one month and normalized against the initial weight (Day 0). Statistical analysis was performed with two-way ANOVA with Bonferroni post-tests. *,$p<0.05$, **, $p<0.01$, ****, $p<0.0001$.

Figure S5: The *Rorγt* promoter contains a NFIL3 binding consensus sequence. (A) Sequences of the mouse and human *Rorγt* promoter regions encompassing a putative NFIL3 binding site (red) were aligned. The position that does not agree with the known NFIL3 consensus sequence is underlined.

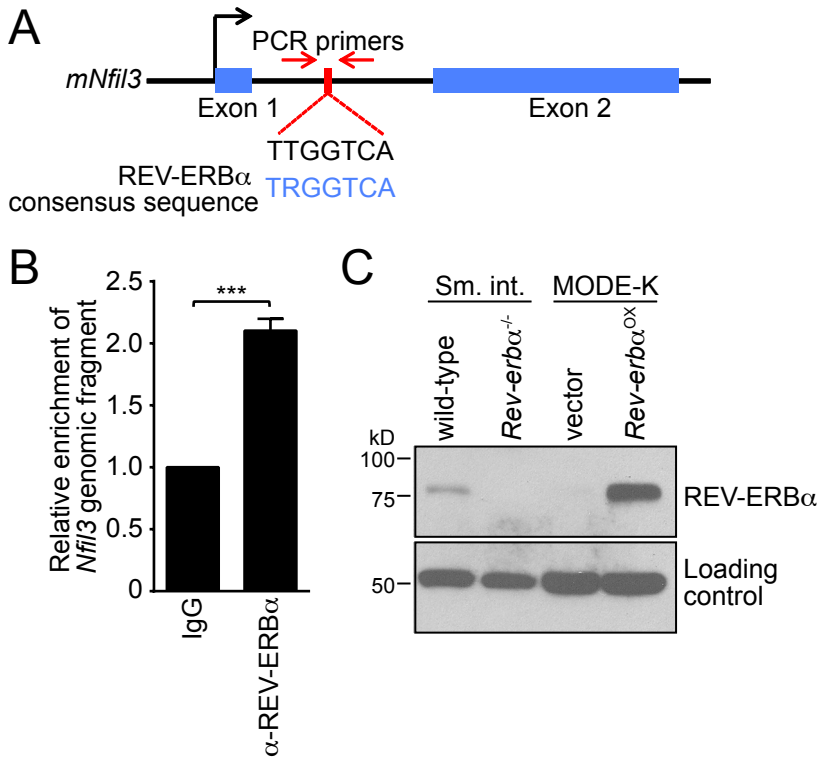

Figure S6: REV-ERBα binds directly to the *Nfil3* gene locus in CD4⁺ T cells. (A) Schematic presentation of the REV-ERBα binding site in the *Nfil3* gene locus and primers designed for ChIP-PCR. **(B)** REV-ERBα binds to the *Nfil3* gene locus in CD4⁺ T cells as demonstrated by ChIP with a REV-ERBα-specific antibody. Data were pooled from five independent experiments. **(C)** The specificity of REV-ERBα antibody was examined by Western blotting of small intestinal protein extracts from wild-type and *Rev-erbα*$^{-/-}$ mice (left) and MODE-K cells transfected with empty vector or a REV-ERBα-encoding plasmid (right). ***: $p<0.001$.

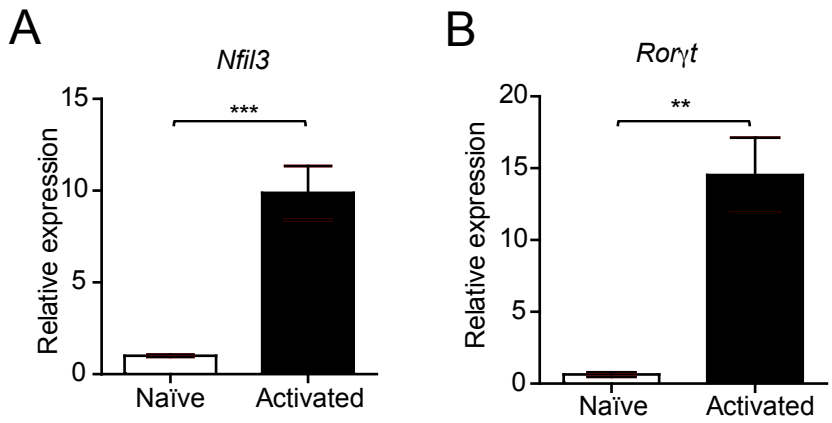

Figure S7: *Nfil3* expression is higher in activated T helper cells. Naïve and activated T helper cells were purified by FACS sorting. Expression of *Nfil3* (**A**) and *Rorγt* (**B**) were determined by real-time PCR. This is attributable to the fact that activated T cells show a global increase in transcriptional activity relative to naïve T cells (*22*). Statistical analysis was performed with two-tailed student's t-test. ***. $p<0.001$; **, $p<0.01$

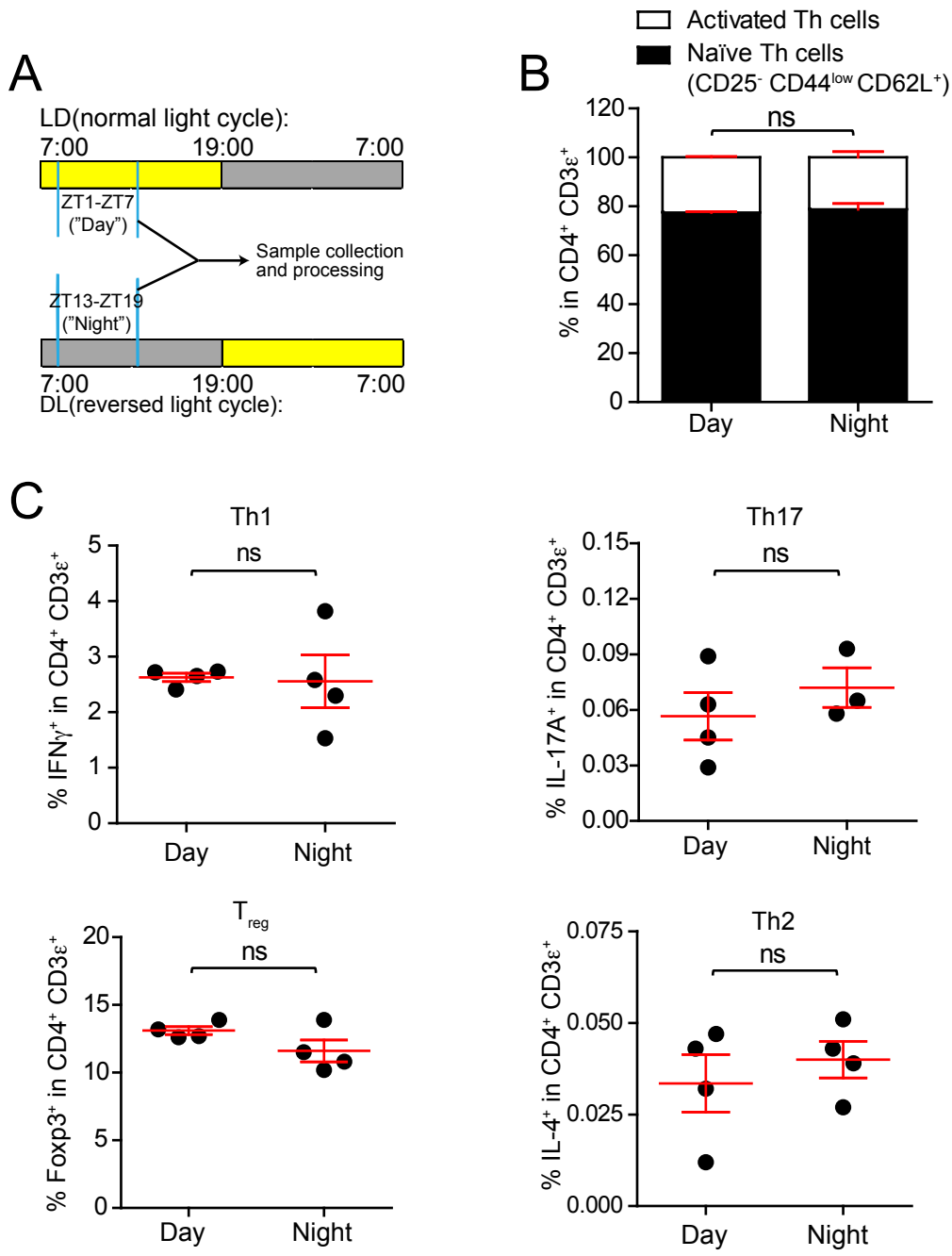

Figure S8: Comparison of splenic T helper cell composition during day and night. (A) Experimental setup for diurnal studies. Two groups of mice were maintained under opposite light cycles and analyzed at the same time. Samples processed during Zeitgeber Time (ZT)1-ZT7 are labeled as "Day" and those during ZT13-ZT19 as "Night". **(B)** Splenic T helper cells from mice subjected to opposite light cycles were analyzed by surface staining for CD25, CD44 and CD62L. **(C)** T helper subtypes were examined by cytokine staining after PMA/ionomycin stimulation (Th1, Th17, Th2) or nuclear staining for Foxp3 (T_{reg}). ns, not significant. Statistical analysis was performed with two-tailed student's t-test.

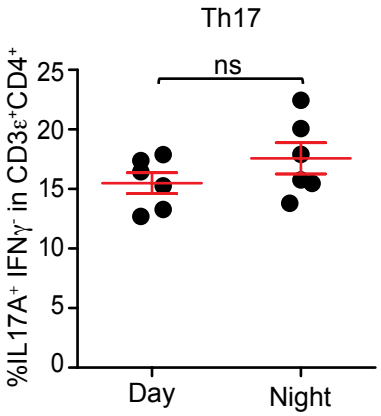

Figure S9: Small intestinal Th17 cell frequencies do not vary significantly across the day-night cycle. Wild-type mice were sacrificed during the day (ZT4) or at night (ZT16) and small intestinal lamina propria lymphocytes were examined by cytokine staining. Statistical analysis was performed with two-tailed student's t-test. ns, not significant.

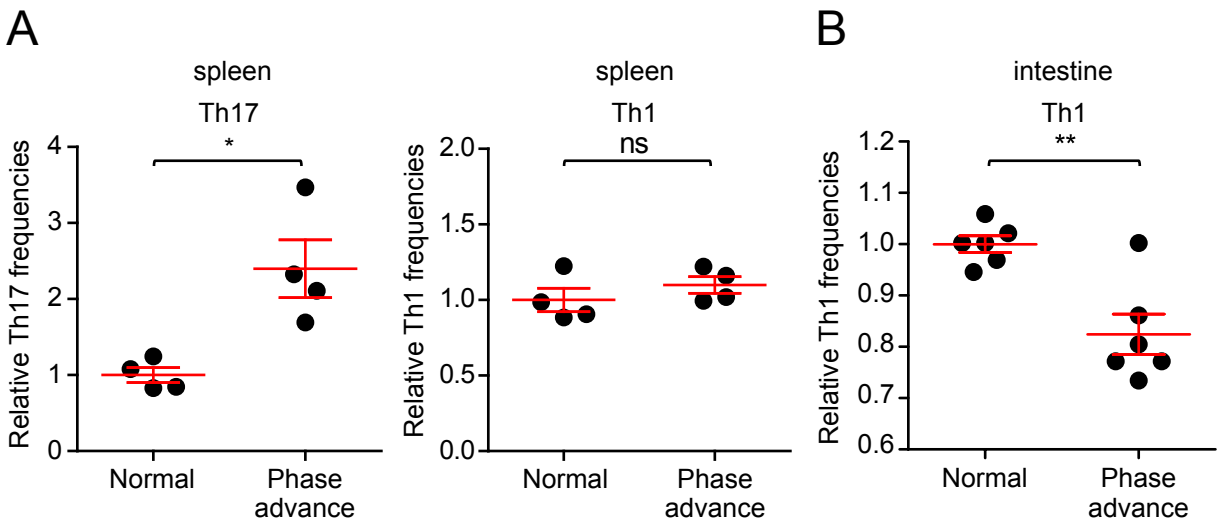

Figure S10: Th17 and Th1 cell frequencies in mice subjected to chronic light cycle perturbation. Mice were treated as described in Methods and splenic (**A**) and intestinal lamina propria (**B**) T cells were analyzed for cytokine production. (**A**) Splenic Th17 cell frequencies increased in mice subjected to light cycle phase advance as described in Fig. 4F while no difference was observed in splenic Th1 cell frequencies. (**B**) In contrast to spleen, intestinal Th1 cell frequencies decreased in mice subjected to chronic light cycle perturbation. Statistical analysis was performed with two-tailed student's t-test. *, $p<0.05$; **, $p<0.01$; ns, not significant.

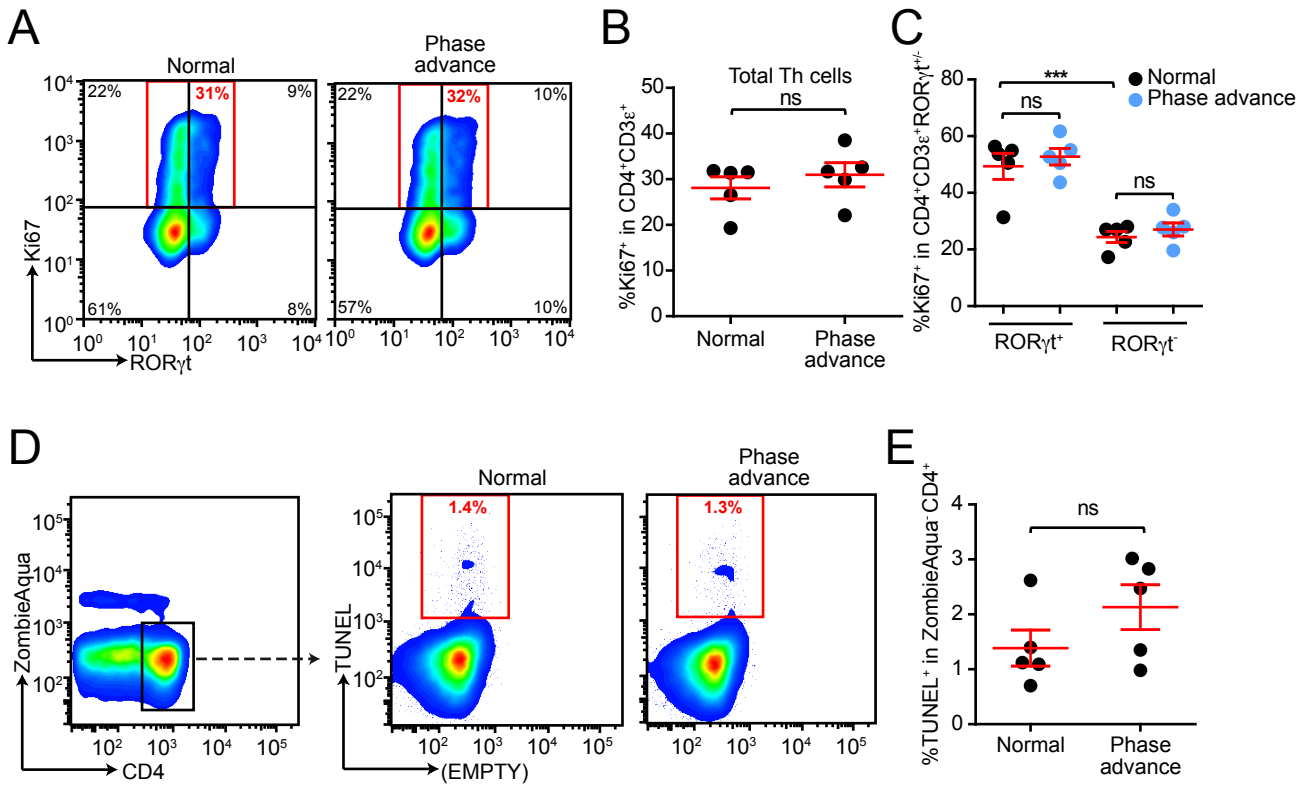

Figure S11: Chronic light cycle disturbance does not impact proliferation or apoptosis of intestinal Th cells. (**A-C**) Proliferation of intestinal Th cells was analyzed by nuclear staining of Ki67 and RORγt. Representative flow plots are shown in (**A**). Percentages of Ki67$^+$ cells in total T helper cells are summarized in (**B**) and those in RORγt$^+$ and RORγt$^-$ fractions are shown in (**C**). (**D,E**) Apoptosis of intestinal T cells was analyzed by TUNEL staining. The gating strategy and representative flow plots are shown in (**D**). Percentages of TUNEL$^+$ cells in total T helper cells are summarized in (**E**). Statistical analysis was performed with two-tailed student's t-test. ***, $p<0.001$; ns, not significant.

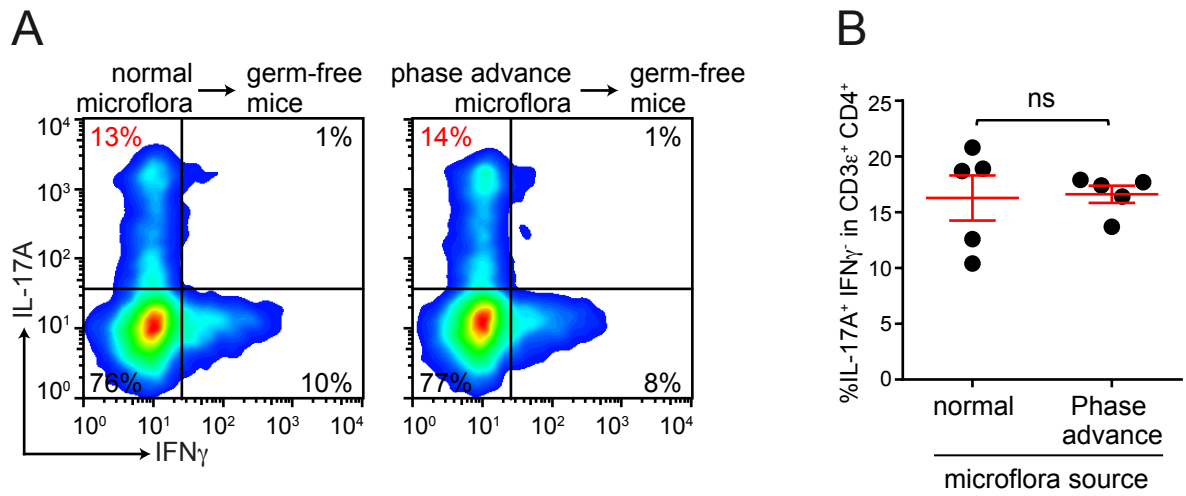

Figure S12: Increased Th17 cell frequencies in mice under perturbed light cycles are not transferrable to germ-free mice by microflora transplantation. Microflora from mice subjected to normal or perturbed light cycles were transplanted into wild-type germ-free mice by oral gavage. Intestinal Th17 cells were examined 6 weeks later. Representative flow plots are shown in **(A)** and data from multiple mice are summarized in **(B)**. Statistical analysis was performed with two-tailed student's t-test. ns, not significant.