Supplementary Information

The MicroRNA miR-17 modulates regulatory T cell activity by targeting Foxp3 Co-regulators

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Supplementary Data Figure 1

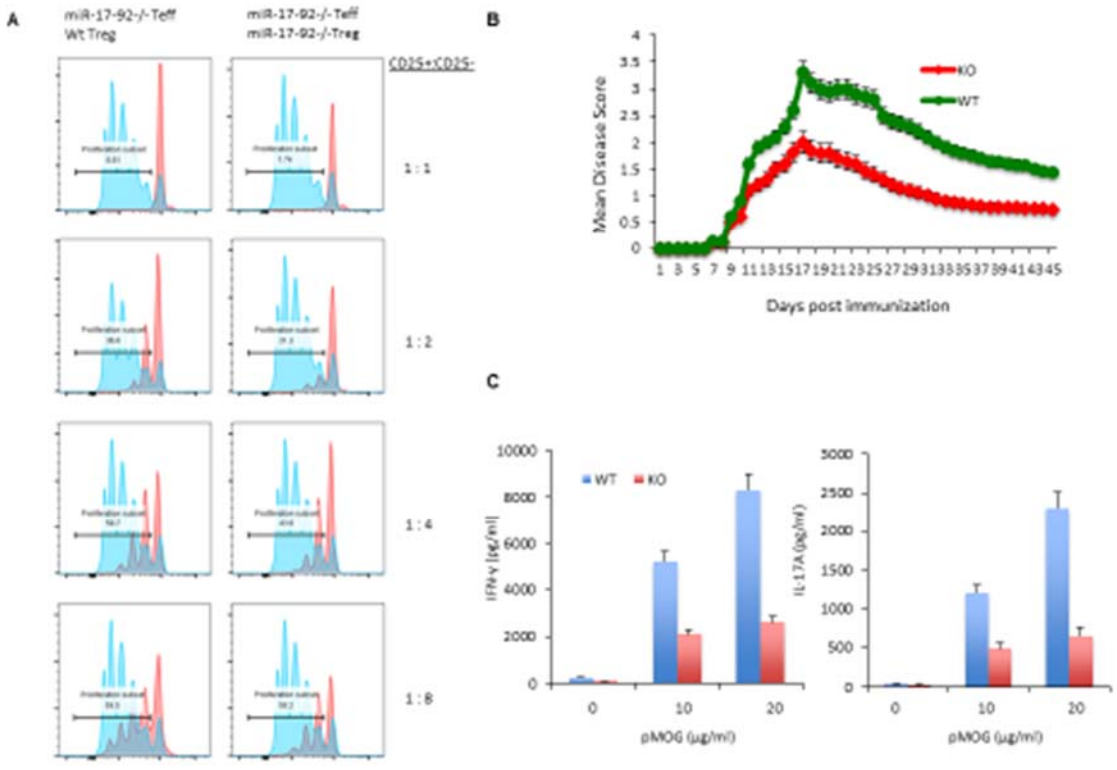

Figure S1, relates to Figures 1 and 2. Foxp3+ Treg specific miR17-92 knock out mice are functionally enhanced compared to wild type Tregs. (A) The ability of Tregs from WT and miR17-92$^{-/-}$ littermates to suppress the proliferation of Teff cells from miR17-92-/- littermates was assessed. Treg cells were mixed with the indicated CFSE labeled Naïve T lymphocytes from CD45.1+ C57BL/6 mice for three days. CFSE dilution was measured by flow cytometry. (B) Wild type (WT) and Foxp3+ specific miR17-92 knockout (miR17-92 Flox/Flox/Foxp3-Cre, KO) mice were injected s.c. with 100 µg MOG $_{35–55}$ in CFA and 250ng Pertussis Toxin i.p. Disease severity was monitored and scored daily. Mean scores for WT and KO mice over time are represented (±SEM; $p < 0.05$, n = 8–10 per group). Shown is the combined results of three individual experiments. (C) On day 45, CNS-infiltrating T cells were recovered. The cells were stimulated with the irradiated autologous antigen presenting cells plus MOG peptide (33-55) for 3 days. The secretion of IFN-γ and IL-17A in the culture supernatant was measured by ELISA. Panels B and C present the mean (± SEM; *$p < 0.05$) of at least three experiments.

Supplementary Data Figure 2

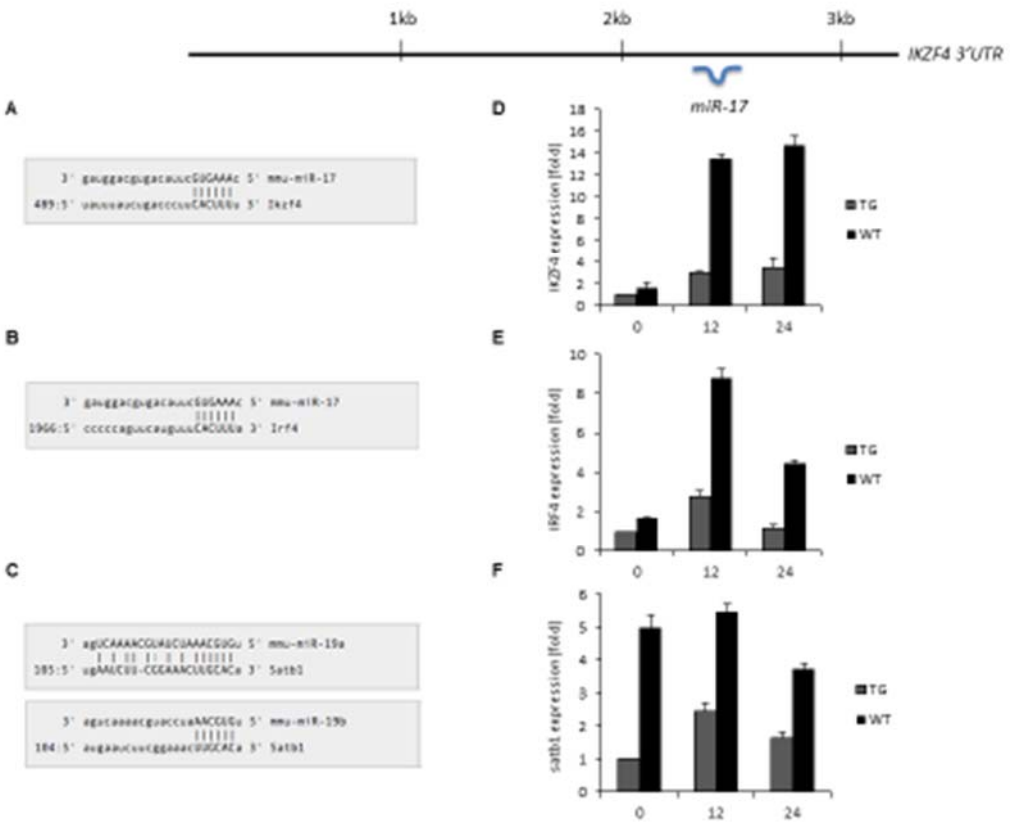

Figure S2, r**elates to Figure 3. Schematic representations of the localization of the miR17 binding sites** in the 3'UTR of Eos (IKZF4) (A), IRF-4 (B), and Satb1 (C). (D-F) qRT-PCR analysis of the expression of IKZF4 (B) IRF-4 (D) and Satb1 (F) mRNA in Treg isolated from miR17-92 transgenic (TG) and wild type (WT) mice over time upon anti-CD3/CD28 stimulation. Shown are mean +/- SEM from 3 independent experiments.

Supplementary Data Figure 3

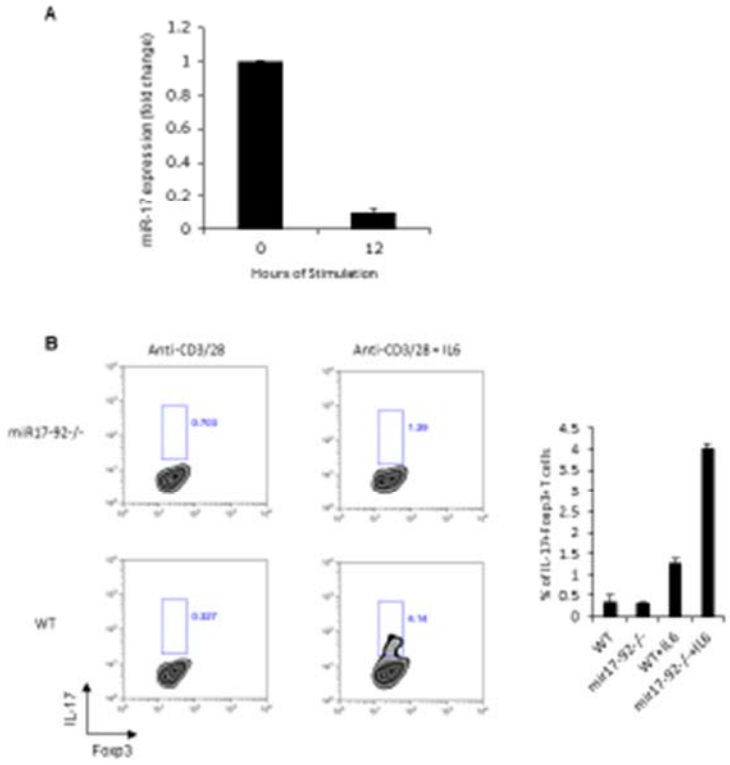

Figure S3, relates to Figure 4. Regulation of miR-17 and Eos by TCR activation IL-6. (A) Tregs were stimulated with anti-CD3/CD28 antibodies for 12 hours, followed by qRT-PCR to determine the expression of miR17. (B) CD4+ Foxp3+ cells from wild type and miR17-92-/- mice crossed onto a GFP-Foxp3 reporter (C57BL/6) background were stimulated with plate-bound anti-CD3 and anti-CD28 in the presence or absence of 20ng/ml recombinant IL-6 for 3 days. T cells were re-stimulated and stained for intracellular IL-17 prior to flow cytometric analysis. Shown are Foxp3+ gated events. Bar graph represents the mean (± SEM) of three trials and representative analyses.

Supplementary Data Figure 4

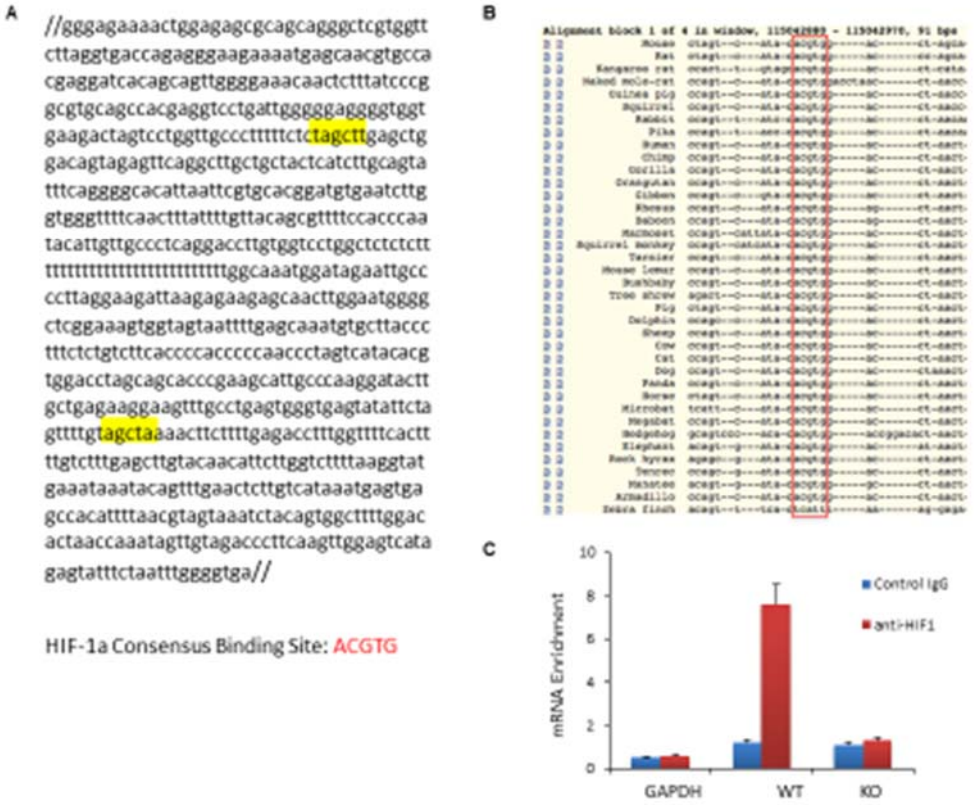

Figure S4, relates to Figure 4. Regulation of miR-17 by HIF-1. (A) Depiction of the miR-17 promoter sequence with potential HIF1α binding sites highlighted in yellow. (B) Identification of a highly conserved HIF1α-binding site in the promoter of the miR17-92 cluster. Alignment of this region was obtained at the UCSC Genome Bioinformatics site. Alignment with several mammalian species reflects the evolutional conservation of this binding site. (C) CHIP assay of HIF1 binds to the promoter of miR17-92 promoter.

Supplementary Data Figure 5

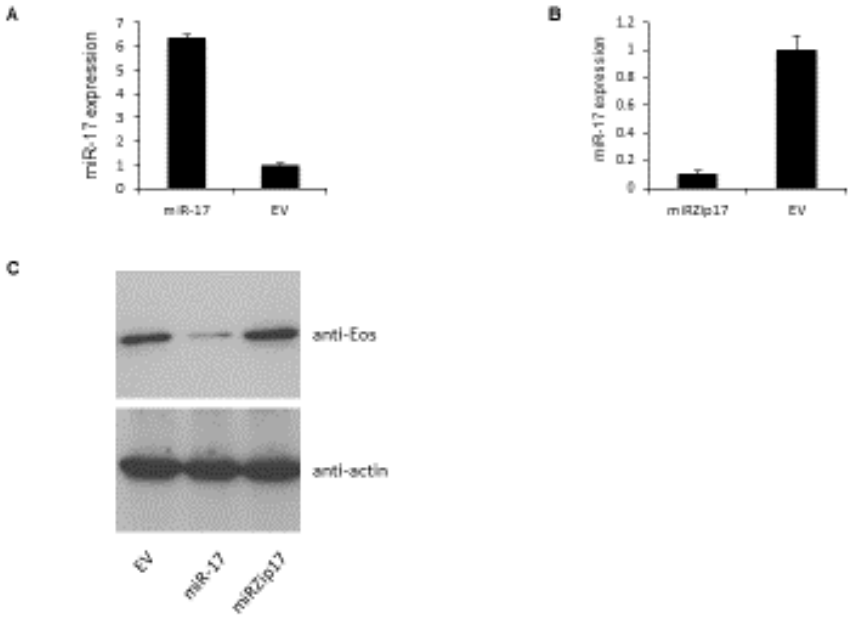

Figure S5, relates to Figure 5. Confirmation of miR-17 and Eos modulation by miR-17 expression and miRZip17 anti-sense constructs. (A,B) miR-17 levels were measured by qRT-PCR from (A) Treg transduced with either a miR-17 or empty vector (EV) construct described in Fig 5A, and (B) either an antisense construct, miRZip17 or an empty vector construct described in Fig 5B. (C) Eos protein was measured by western blot of lysed cells from Tregs transduced with EV, miR-17 or miRzip17carrying viruses.

Supplementary Data Figure 6

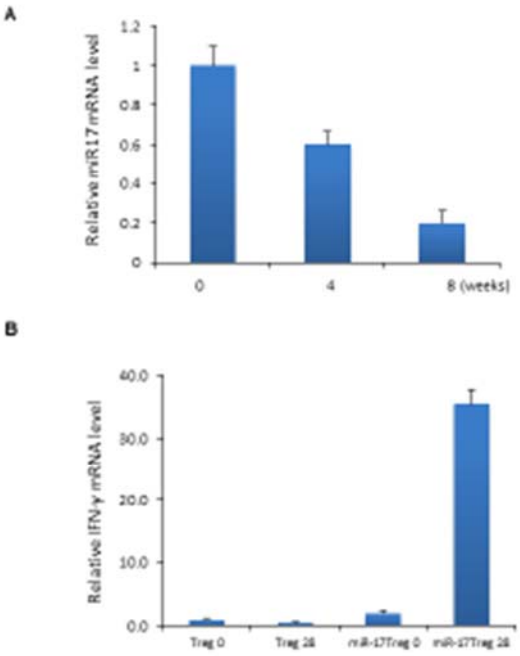

Figure S6, relates to Figure 6. Regulation of miR-17 and IFNγ expression during colitis. (A) Adoptively transferred wild type Tregs were recovered from recipient Rag2-/- mice and levels of miR-17 mRNA in them and pre-transfer Tregs were measured by qRTPCR. (B) Expression of IFNγ mRNA was determined in Tregs recovered from the recipients of control Tregs and those over-expressing miR-17 (described in Figure 6).

Supplementary Materials and Methods

Mice

All animal experiments were performed in specific-pathogen-free, Helicobacter-free facilities in the Johns Hopkins Animal Resource Center following national, state and institutional guidelines. Foxp3 GFP mice were kindly provided by A. Rudensky. miR17-92 flox and transgenic mice were gifts from H. Li. The other mice strains used in this study were purchased from Jackson Laboratory. Animal protocols were approved by the Johns Hopkins Animal Care and Use Committee.

Colitis induction

Naïve $CD4^+CD25^-CD62L^{hi}$ T cells were isolated from C57BL/6 and injected via tail vein (i.v.) into C57BL/6 $RAG2^{-/-}$ immunodeficient recipients ($1x10^6$/mouse). C57BL/6 wild-type EV transduced $CD4^+CD25^+$ Treg or miR-17/Eos transduced Treg cells ($2x10^5$) were co-injected i.v. where indicated. Mice were monitored weekly for wasting disease and mice losing more than 20% of its starting body weight or showing severe signs of disease were sacrificed. Colons were removed from mice 8 weeks after T cell reconstitution and fixed in 10% formalin. Five-micrometer paraffin-embedded sections were cut and stained with haematoxylin and eosin (H&E). CD3+ T cells were visualized using goat anti-CD3 polyclonal antibodies (Santa Cruz) and diaminobenzidine chromogen with haematoxylin as a counter-stain. $Foxp3^+$ cells were visualized with rat anti-Foxp3 clone FJK-16 antibody (eBioscience). Pathology of colon tissue was scored in a blinded fashion, on a scale of 0-5 where a grade of 0 was given when there were no changes observed. Changes associated with other grades were as follows: grade 1, minimal scattered mucosal inflammatory cell infiltrates, with or without minimal epithelial hyperplasia; grade

2, mild scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submucosa and associated with erosions, with mild to moderate epithelial hyperplasia and mild to moderate mucin depletion from goblet cells; grade 3, moderate inflammatory cell infiltrates that were sometimes transmural, with moderate to severe epithelial hyperplasia and mucin depletion; grade 4, marked inflammatory cell infiltrates that were often transmural and associated with crypt abscesses and occasional ulceration, with marked epithelial hyperplasia, mucin depletion; and grade 5, marked transmural inflammation with severe ulceration and loss of intestinal glands.