A combination of transcription factors, enhancers, and epigenetic marks determines the expression of the key transcription factor FoxP3 in regulatory T cells (Tregs). Adding an additional layer of complexity, the long noncoding RNA (lncRNA) Flicr (Foxp3 long intergenic noncoding RNA) is a negative regulator that tunes Foxp3 expression, resulting in a subset of Tregs with twofold- to fivefold-lower levels of Foxp3 protein. The impact of Flicr is particularly marked in conditions of IL-2 deficiency, and, conversely, IL-2 represses Flicr expression. Flicr neighbors Foxp3 in mouse and human genomes, is specifically expressed in mature Tregs, and acts only in cis. It does not affect DNA methylation, but modifies chromatin accessibility in the conserved noncoding sequence 3 (CNS3)/Accessible region 5 (ARS5) region of Foxp3. Like many lncRNAs, Flicr's molecular effects are subtle, but by curtailting Treg activity, Flicr markedly promotes autoimmune diabetes and, conversely, restrains antiviral responses. This mechanism of Foxp3 control may allow escape from dominant Treg control during infection or cancer, at the cost of heightened autoimmunity.

**Significance**

Regulatory T cells (Tregs) are an essential population of immunoregulatory cells that play a central role in immune tolerance and the control of autoimmune disease, infections, and cancer. The transcription factor FoxP3 is the central orchestrator of Treg differentiation, stability, and function. Here we report the discovery of the noncoding RNA, Flicr, and its fine-tuning of Foxp3 expression through modification of chromatin accessibility, with marked consequences on the progression of autoimmune diabetes. Our findings add an important piece to the puzzle of Treg differentiation and stability, and how their function adapts to physiological circumstances.
**Results**

**Flicr, an IncRNA Specifically Expressed in Tregs.** *Flicr* first caught our attention as an IncRNA detected only in Tregs among the ImmGen compendium of immune cell gene expression (39) (Fig. 1A). This exclusivity was confirmed by RNA sequencing (RNA-Seq) analysis (Fig. S1). Outside of the immune system, *Flicr* was observed only in the testis, at low levels (40) (Fig. 1A). During Treg differentiation, *Flicr* was primarily expressed in testis, at low levels (40) (Fig. 1A). During Treg activation, *Flicr* expression was increased and was also detected in peripheral lymphoid organs and at lower levels in the specific Treg populations found in several nonlymphoid tissues (Fig. 1B). Tissue Tregs tend to have activated phenotypes and, accordingly, Treg populations found in several nonlymphoid tissues (Fig. 1B). Tissue Tregs tend to have activated phenotypes and, accordingly, Treg populations found in several nonlymphoid tissues (Fig. 1B). Tissue Tregs tend to have activated phenotypes and, accordingly, Treg populations found in several nonlymphoid tissues (Fig. 1B). Tissue Tregs tend to have activated phenotypes and, accordingly, Treg populations found in several nonlymphoid tissues (Fig. 1B). Tissue Tregs tend to have activated phenotypes and, accordingly, Treg populations found in several nonlymphoid tissues (Fig. 1B). Tissue Tregs tend to have activated phenotypes and, accordingly, Treg populations found in several nonlymphoid tissues (Fig. 1B). Tissue Tregs tend to have activated phenotypes and, accordingly, Treg populations found in several nonlymphoid tissues (Fig. 1B). Tissue Tregs tend to have activated phenotypes and, accordingly, Treg populations found in several nonlymphoid tissues (Fig. 1B). Tissue Tregs tend to have activated phenotypes and, accordingly, Treg populations found in several nonlymphoid tissues (Fig. 1B).

**Structure and Conservation of the Mouse and Human FLICR.** We combined several types of analyses and external data sources to accurately position *Flicr* transcripts in the mouse genome (Fig. 24), identifying four different isoforms of varying lengths (566, 737, 3,278, and 4,150 bp) that share two exonic elements and an intron, located 1.8 kb upstream of the transcriptional start site (TSS). They are all transcribed from the same sense strand of *Chromatin* (45). *Flicr* was present in Tregs from all peripheral lymphoid organs and at lower levels in the specific Treg populations found in several nonlymphoid tissues (Fig. 1D). Tissue Tregs tend to have activated phenotypes and, accordingly, Treg activation slightly reduced *Flicr* expression in vivo (41) and in vitro (42) (Fig. 1E). In contrast, *Flicr* was not expressed in induced Tregs generated in vitro from naive conventional T cells (Tconv) with TGF-β and IL-2 (43) (Fig. 1F). That *Flicr* expression may require thymic Treg differentiation was consistent with its lower abundance in colonic RORγ+ Helios− Tregs, considered to result from extrathymic differentiation, relative to their thymically derived RORγ+ Helios+ counterparts (44) (Fig. 1G). In keeping with the notion that Foxp3 alone is not sufficient to promote *Flicr* expression, transduction of Foxp3 in CD4+ T cells did not induce *Flicr* (11) (Fig. 1H). However, Foxp3 was necessary for *Flicr* expression, because Treg-like cells, in which Foxp3 is transcriptionally active but encodes a nonfunctional protein, were *Flicr*-negative (45) (Fig. 1I).

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**Fig. 1.** *Flicr* is expressed specifically in Tregs. *Flicr* expression in various contexts. (A and B) ImmGen mouse immune cell (39) or GNF mouse organ microarray (40) (dashed lines: background level). (C) During thymic Treg differentiation. (Left) ImmGen data. (Right) RNA-Seq analysis of CD4+ SP thymocytes sorted as shown; y-axis, transcripts per million (tpm). (D) Tissue Tregs. Each point is an individual mouse. Sp, spleen; Col, colonic lamina propria; Panc, pancreas; Adip, adipose tissue; Muscle, injured muscle at 4 d postcardiotoxin injection. (E) After activation in vitro with anti-CD3/CD28 beads (42) (Left) or in vivo (41) (Right), Rest., resting CD44+CD62L+; Act., activated CD44+CD62L− Tregs; rpmk, reads per kb per million reads. (F) In vitro converted induced Tregs (TCR activation with IL-2 and TGF-β) (43). (G) Ex vivo HeliosRORγ+ colonic peripheral Tregs (44). (H) In Tconv transduced with Foxp3 or control retrovectors (11). (I) In Treg-like cells of mice with Foxp3 inactivation by Gfp insertion (Foxp3ΔGfp) (45).
had computationally predicted low peptide coding potential by codon substitution frequency analysis (PhyloCSF) (47); however, as for most IncRNAs, the possibility of translation into very short peptides cannot be ruled out (Fig. S2C).

IncRNAs are usually poorly conserved, but the region common to mouse Flicr isoforms showed distinct sequence homology to a similar location upstream of human FOXP3 (Fig. 2A) and more generally among placental mammals. Indeed, focused PCR identified three FLICR isoforms in the human FOXP3+ C5/MJ cell line, structures that were confirmed by parsing RNA-Seq from human Tregs (48). The main 5′ ends mapped closer to PPP1R3F than in the mouse, and we did not find FLICR transcripts that overlapped the FOXP3 TSS, but the intron and flanking exons were again found ~2 kb upstream of FOXP3 (Fig. 2A and Fig. S2B). There was no preservation of putative ORFs in the conserved region, with nonsense or frame shift mutations in all possible reading frames (Fig. S2C). As in the mouse, FLICR expression was restricted to Tregs (Fig. 2B; human RNA-Seq) (48). Thus, human and mouse Tregs specifically express an IncRNA of very similar structure and position, situated very close to the Foxp3 locus.

**Flicr Dampens Foxp3 Expression in Tregs.** To determine its function, we generated a Flicr-deficient mouse line by deleting a short segment (263 bp) encompassing exon 2 and the splice junction, the focal point of sequence conservation (Fig. 3A), using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats) to introduce two specific breaks for nonhomologous end-joining in fertilized mouse eggs (49). We purposely kept the deletion very short to avoid affecting Foxp3 enhancer elements, targeting a region devoid of H3K27Ac enhancer, H3K27me3 repressive marks, or CpG islands (Fig. S3A) (50). Indeed, transcriptome analysis showed that Tregs in Flicr-deficient (KO) mice lost Flicr, but not Pp1r3f and Foxp3 transcripts, the latter being slightly increased (Fig. 3B). KO mice developed and grew normally, with no histological evidence of systemic autoimmune disease. Treg differentiation and homeostasis seemed unchanged, with normal Treg proportions in the thymus, spleen, and lymph nodes (Fig. S3B); however, close examination of cytometry profiles revealed a consistent trait (Fig. 3C): whereas CD25hi Tregs from WT mice showed the usual range of FoxP3 expression, with some cells having twofold to fivefold less FoxP3 than the main peak, Flicr-deficient Tregs had a tighter distribution, with significantly fewer FoxP3lo Tregs. Correspondingly, the overall mean fluorescence intensity (MFI) increased slightly (Fig. 3D). This disappearance of the FoxP3lo Tregs was observed in Flicr-deficient mice on the NOD background, where the mutation was initially constructed, and on the autoimmune-resistant B6xNOD background (Fig. S3C).

Although the Flicr KO deletion was relatively small (263 bp) and eschewed regulatory regions identifiable by chromatin marks, we wanted to assess definitively whether these observations could be explained by the deletion of a putative regulatory DNA element. First, the same reduction of FoxP3lo cells was present in an independent Flicr mutant mouse in which only 12 bp at the splice junction of exon 3 were deleted (Fig. 3A and Fig. S3D). Unfortunately, only one progeny could be obtained from this line, precluding statistical analysis and follow-up, but nevertheless this provides confirmatory evidence. Second, we confirmed the results by an independent RNAi approach, transfecting locked nucleic acid (LNA) antisense oligonucleotide to target Flicr in Tregs in culture (34). Here again, a reduction in Foxp3lo cells was observed (Fig. 3E), confirming a direct role of Flicr RNA on FoxP3 expression. Third, if Flicr down-regulates Foxp3 in a subset of cells, then it would be predicted to be overrepresented in Foxp3lo cells. Indeed, more Flicr RNA was detected in cells with lower Foxp3 (Fig. 3F).

To test whether the Foxp3 phenotype of Flicr-deficient mice was Treg-autonomous or resulted from an indirect effect, we
constructed mixed chimeras. A lymphoid Rag-deficient hosts were reconstituted with equal proportions of bone marrow from Flicr WT and KO donors. In this setting, we again observed the “tightening” of Foxp3 expression in Tregs of Flicr KO genotype, relative to WT Tregs in the same hosts (Fig. 4A), indicating a Treg-intrinsic effect. Interestingly, KO Tregs had a competitive advantage over their WT counterparts in the same mice; the Treg-intrinsic effect. Interestingly, KO Tregs had a competitive advantage over their WT counterparts in the same hosts (Fig. 4A), revealing better fitness of Flicr-deficient Tregs than was observed in the full KO mice.

**Flicr Destabilizes Foxp3 Expression in Conditions of Limiting IL-2.** The foregoing results suggested that Flicr affects the stability of Foxp3 expression in some Tregs. For confirmation, we cultured Tregs in conditions of limited IL-2, under which Foxp3 tends to be lost (51). The Foxp3-deficient cells that normally appear in low–IL-2 cultures were essentially absent in Treg cultures from Flicr-deficient mice (Fig. S4A). Thus, the subtle effect observed under steady state was magnified when trophic support became limiting.

These results also suggested that IL-2 and Flicr are diametric opposites with regard to Treg homeostasis. Because IL-2 signaling stabilizes Foxp3 expression by recruiting the transcription factor STAT5b to the Foxp3 locus (52), we hypothesized that it might inhibit Flicr to maintain Foxp3 expression. Indeed, provision of IL-2 lowered Flicr expression in culture (Fig. 5B). Interestingly, a slight accumulation of STAT5b was present in the absence of IL-2 (Fig. 5B). In-
In contrast to the putative enhancer of FoxP3 transcript, Flicr did not complement FoxP3 expression or restore FoxP3lo Tregs even when inserted into Flicr-deficient mice (Figs. 5B and 6C). This suggested that Flicr’s influence on their expression is indirect, via FoxP3. Thus, Flicr’s genomic proximity to Foxp3 is necessary for its mechanism of action. These results show that Flicr is a nuclear IncRNA that acts in cis on Foxp3 expression, and indirectly has a subtle but broader impact on the Treg transcriptome.

Flicr Influences Focal Chromatin Accessibility in the Foxp3 Locus.

Given that Flicr acts in cis, we reasoned that it might influence the Foxp3 epigenetic landscape. Methylation at several CpG clusters was increased in Flicr-deficient Tregs that were cultured in IL-2. Expression of the genes affected by the Flicr deficiency also was not normalized by the Flicr-encoding complementing transgene (Fig. S4B), reinforcing the notion that Flicr’s influence on their expression is indirect, via FoxP3. Thus, Flicr’s genomic proximity to Foxp3 is necessary for its mechanism of action. These results show that Flicr is a nuclear IncRNA that acts in cis on Foxp3 expression, and indirectly has a subtle but broader impact on the Treg transcriptome.

Fig. 4. Cell-intrinsic effect of Flicr on FoxP3 expression. (A) Rag-KO mice were reconstituted with equal proportions of congenically labeled bone marrow cells from Flicr WT (CD45.2) and KO (CD45.1) mice. Shown are representative FACS plots distinguishing CD4+ T cells of different origins for FoxP3 vs. CD25 analysis (Left), FoxP3 MFI (Middle), and proportion of FoxP3lo cells (Right) in Tregs in KO and WT compartments in the same mice. (B, Left) Relative proportions of Flicr KO- vs. Flicr WT-derived Treg and Tconv splenocytes in the same mice. (B, Right) Splenic proportions of Tregs in the WT and KO compartments. All P values are from the paired Student t test.

Fig. 5. Flicr destabilizes FoxP3 expression in vitro and is down-regulated by IL-2. (A) In vitro stability assay. Tregs were cultured for 48 h in limiting IL-2 before cytometry. (Left) Representative FoxP3 vs. CD25 plot. (Middle) FoxP3 MFI and proportion of FoxP3lo cells. (Right) Foxp3 expression (relative to Actin1) by RT-qPCR. Data are pooled from three experiments. (B) Flicr and Foxp3 expression (relative to Actin1) by RT-qPCR in purified Tregs after 2 h in culture with or without IL-2. Data are pooled from two experiments. P values are from ANOVA. (C) STAT5b binding to the FLICR promoter and the CNS2 enhancer in chromatin immunoprecipitation (53).
within the Foxp3 locus, particularly in the CNS2 enhancer region, correlates with the stability of its expression (10, 57). However, comparison of methylation at the Foxp3 locus in Flox WT and KO Tregs showed superimposable profiles, except for a minor alteration within Flicr itself (Fig. 7A). Treg-specific hypomethylation at CNS2 was intact.

We then used assay for transposase-accessible chromatin (ATAC) high-throughput sequencing (ATAC-seq), which probes chromatin openness by its accessibility to the Tn5 transposase, and produces detailed information on the configuration of hypersensitive TSS and enhancer elements (58). Six accessible regions were identifiable across Foxp3 in Tregs: the promoter, the CNS2 and CNS3 enhancers (20), two other regions [hereinafter referred to as accessible regions (AR) 5 and 6], and the 3′ UTR (Fig. 7B). Overall, ATAC-seq profiles were very similar in Flox WT and KO Tregs, but close examination revealed reciprocal shifts at Accessible region 5 (AR5) [less accessible in KO Tregs; P = 0.03 based on the genome-wide variance distribution between replicates (DiffBind)] (Fig. 7B) and CNS3 (more accessible; P = 0.01). These shifts were reproducible in independent experiments, as was the increased accessibility in the 3′ UTR, possibly reflecting altered Pol-II recycling. In contrast, CNS2 profiles did not vary.

Physiological Consequences of Flicr Deletion. FoxP3 stability is needed to maintain Treg homeostasis and prevent autoimmunity (6). An active mechanism that destabilized Foxp3 expression in Tregs might be advantageous as a “back door” to avoid dominant Treg control in some circumstances, but also seems risky. We tested immune function in Flicr-deficient mice in several Treg-dependent settings. A first set of experiments showed identical growth of the MC38 tumor line in Flox WT and KO hosts. We then tested the notion that Flicr might promote autoimmunity by destabilizing Treg function (8, 56), and analyzed the course of autoimmune diabetes in Flicr-deficient NOD females. These mice showed a significantly reduced rate and incidence of overt diabetes (Fig. 8A). This halving of diabetes incidence was reflected in reduced severity of insulitis at age 11 wk (Fig. 8B). In the inflammatory context of these infiltrated islets (Fig. 8C), Tregs showed the same stabilization of Foxp3 levels in the absence of Flicr as they did in lymphoid organs. As reported previously (59), insulitis was inversely correlated with the proportion of Treg in the pancreas (Fig. 8D). Interestingly, the slope of this anticorrelation was steeper in Flicr KO mice than in heterozygous or WT control females (r = −0.91 vs. r = −0.46; P = 0.05), possibly suggesting that Flicr-deficient Tregs are functionally more efficient on a per-cell basis, reminiscent of the superior fitness noted above.

Discussion

Here we report the discovery of the lncRNA Flicr, a negative regulator of Foxp3 expression in Tregs. It appears to act exclusively in cis, but by controlling Foxp3 has wider effects on the specific Treg transcriptome and Treg fitness. Its molecular effects are subtle, and more particularly visible in a subset of Tregs,
but they have a marked impact on the efficacy of peripheral immunologic tolerance. The Foxp3 locus appears to come directly equipped with a counterregulatory mechanism.

Based on its genomic location, expression pattern, and physiological impact, Flicr seems to be one of the lncRNAs that selectively modulates a specific physiological function, here Treg activity. Foxp3 does not merely obey on/off regulation to dictate Treg repressive functions, but can be tuned in response to different environmental cues, particularly in conditions of limiting IL-2 (2, 4, 18, 19). These results show that IL-2 has two means of enhancing Foxp3 expression, directly via activation of the Csn2 enhancer and indirectly by repressing Flicr, the attenuator of Foxp3. Flicr expression is also curtailed in conditions of heightened Treg activation and functionality, in tissue Tregs and after TCR activation. By destabilizing Foxp3, Flicr dampens the Treg signature and may lower Treg stability, allowing stronger antiviral responses but also increasing the risk of autoimmune disease.

Like other lncRNAs in the immune system (60), Flicr has a focused role that matches its expression. In this respect, it contrasts with Rnmp, the impact of which in T lymphocytes seems limited to Th17 cells despite ubiquitous expression (36), but is akin in this respect to Morbid (34) and NeST (35), which have a range of activity conditioned by their restricted expression. From the lack of trans complementation by the Flicr-expressing BAC transgene, we infer that the mild bias that it imparts on other loci, predominantly Treg signature genes, is indirectly due to Foxp3 dampening. Several of these Treg signature genes are related to the different mechanisms through which Treg cells exert their suppressive activity (61). Thus, modification of the stability of Foxp3 expression and shifts in Treg signature genes likely contribute to the down-modulation of Treg function and fitness by Flicr. Importantly, Flicr seems to preferentially impact a subset of Tregs; increased Foxp3 levels are seen not in the main Treg pool, in which mean Foxp3 levels are not noticeably affected, but rather in the Foxp3^{lo} subset. We speculate that in vivo Foxp3^{lo} Tregs are equivalent to those observed in vitro when IL-2 is limiting, and that Flicr may be hastening their shutdown of Foxp3 expression.

Cis-acting lncRNAs have several modes of action (30). The localized human/mouse sequence conservation suggests that it is not akin to lncRNAs, the very transcription of which is regulated by promoter interference, but that the primary or secondary structure of the RNA matters (30), possibly involving the splicing machinery given that the sequence conservation extends into the intron. Like many other lncRNAs, Flicr’s molecular signature is subtle, but ultimately results in a larger shift in the outcome of pancreatic autoimmunity and the progression to overt diabetes. This amplification is congruent with the observation that many of the genetic variants (eQTLs) that condition the propensity to autoimmune disease have only subtle effects on the expression of the gene that they influence. Because much of lymphocyte differentiation is related to engagement by self molecules, the immune system is likely tuned at the edge of autoimmunity, and

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**Fig. 7.** Cis effect of Flicr on Foxp3 chromatin accessibility. (A) CpG methylation profile of the Foxp3 locus in Flicr WT and KO Tregs (bisulfite sequencing). Each dot represents one 5-methylcytosine site; methylation frequency is averaged from two independent experiments. (B) Chromatin accessibility (ATAC sequencing) at the Foxp3 locus in WT and KO Tregs (red and blue lines, respectively). Two independent experiments are shown. *P < 0.05, **P < 0.01, differential peak analysis (Diffbind; negative binomial distribution).

**Fig. 8.** Physiological consequences of modulation of Treg activity by Flicr. (A) Incidence of diabetes in Flicr KO and heterozygous littermate NOD females; P values from the Mantel–Cox log-rank test. (B) Proportion of infiltrated islets in the pancreas of 10-week-old Flicr WT, heterozygous, and KO NOD females. P values are from the Mann–Whitney U test. (C) Proportion of Foxp3^{lo} in CD25^{hi} Tregs in the pancreas of 10-week-old WT, heterozygous, and KO mice. Data are pooled from six experiments; Student t test. (D) Inverse correlation between islet infiltration and the proportion of Tregs among CD4^{+} cells in the pancreas. Lines represent linear regression fits for Flicr WT (blue), heterozygous (green), and KO (red). P values were obtained using Fisher z-transformation.
small changes, here in Treg fitness, can tip the system toward serious autoimmune consequences.

Different mechanisms are involved in controlling the stability of Foxp3 expression. At the epigenetic level, the CNS2 enhancer plays a critical role, reinforced by DNA methylation (10), and by integrating signals from the TCR and the IL-2 receptor (18, 19). However, Flcr does not seem to influence DNA methylation or chromatin accessibility at CNS2, but instead affects chromatin structure in the CNS3/AR5 region. CNS3 previously has been described as a poised enhancer open more generally in the T-cell lineage and important for Treg differentiation (41). Our results suggest this region also has a role in mature Tregs, balanced with AR5. IncRNAs commonly operate within ribonucleoprotein complexes with specific TFs, chromatin modifiers (28), or Hnrnp proteins (37, 60), and we propose that Flcr may target a repressive complex to the CNS3/AR5 region of Foxp3.

The functions of IncRNAs in the immune system are just being elucidated. Through its effects on Tregs, Flcr may be associated with human diseases associated with enhanced Treg activity, like infections or tumors, and its modulation opens avenues to suppress or enhance Treg function.

Materials and Methods

All experimental procedures are described in detail in SI Materials and Methods.

Mice. C57BL/6j mice were obtained from The Jackson Laboratory. NOD/Lt狄O1 and Foxp3-DTR-GFP/ΔN (56), Foxp3-Ires-Thy1.1/86 (62), and Foxp3-IRES-GFP/ΔN (63) mice were maintained in our colony. Foxp3-DTR-GFP/ΔN carries a BAC transgene encompassing 150 kb downstream and 70 kb upstream of the Foxp3 locus. C57BL/6J mice were obtained from The Jackson Laboratory. NOD/Lt狄O1 (reviewed and approved HMS IACUC protocols 02954). Mice.

Mapping of Mouse and Human Flcr Transcripts. Treg RNA was prepared from double-tailed CD4⁺ TCRβ⁺ Thy1.1⁺ Tregs from Foxp3-Ires-Thy1.1⁻/⁻ mice. 3′ and 5′ RACE were performed using the SMARTer RACE cDNA Amplification Kit (Clontech), followed by Sanger sequencing. The 3′ termini were also mapped using RNA-Seq, and 5′ extremities were mapped from the FANTOM CAGE data (46). Exploratory PCR was performed using primers spanning the 4930524L23Rik locus, as shown in Fig. 52A. Isoforms were cloned with primers designed based on the extremities found. RNA from the FOXP3-specific TSS, with a TCR-εGFP-stop insertion between the first and second codons of Foxp3. All experimentation was performed following animal protocol guidelines of Harvard Medical School (reviewed and approved HIMS IACUC protocols 02954).

Flicr KO Mice. To generate Flcr-deficient mice using the CRISPR/Cas9 system, we closely followed the method of Yang et al. (49) with several modifications. Because the active region of Flcr is unknown, we chose to introduce a point mutation using one guide RNA (gRNA) targeting the shared splice donor site or to delete a whole exon (exon 2) using two gRNAs, encompassing the region of highest homology between mouse and human transcripts, but keeping the mutation as small as possible to avoid interfering with Foxp3 enhancer elements. Two mutants were obtained. One founder had a 12-bp deletion deleting the donor splice site of exon 3, but gave only one progeny. Another mutant had a 263-bp deletion spanning Flcr exon 2.

In Vitro Foxp3 Stability. CD4⁺ T cells from spleen and subcutaneous lymph nodes isolated by magnetic negative selection and CD4⁺ TCRβ⁺CD25⁺ (top 50%) Tregs were sorted by flow cytometry, then cultured with anti-CD3/CD28 beads (1:1 bead:cell; Gibco) and IL-2 (Peprotech; 212–12).

Gene Expression Profiling by Microarray. Microarray preparation and analysis were performed in accordance with ImmGen protocols (39). In brief, 50,000 CD4⁺ TCRβ⁺CD25⁺ (top 50%) Tregs and CD25⁻ Tconvs from pooled spleen and subcutaneous lymph nodes from 8-kw-old Flcr WT and KO mice were double-sorted as above. RNA was prepared from TRIzol extracts and used to prepare probes for Affymetrix Mouse Gene 1.0 ST arrays. Gene expression differences were calculated using the Limma and eBayes functions of the limma package (64).

ATAC-seq. ATAC-seq was performed in biological duplicates following the protocol described by Buenrostro et al. (58). In brief, 50,000 CD4⁺ TCRβ⁺CD25⁺ (top 50%) Tregs were sorted and lysed. After transposition and PCR, final bead purification and selection (100–600 bp) were performed twice using 0.6x and 1.6x solid phase reversible immobilization (SPRI) beads. Libraries were paired-end sequenced (40, 50) using a 75-bp kit on an Illumina NextSeq High-Throughput Sequencing System. Reads were filtered for quality and adapter-trimmed before mapping to the mm9 mouse genome. Reads mapping to multiple positions and PCR duplicates were discarded. Nucleosome-free fragment categories (<120 bp) were analyzed, and peaks were called using Homer (65). Data were imported in R, and differential peak analysis was performed using DiffBind (differential peak analysis based on negative binomial distributions) (66).

Methylation Analysis by Bisulfite Treatment and High-Throughput Sequencing. The protocol was adapted from Feng et al. (18). Here, 80,000 CD4⁺ Tregs were sorted as above, and DNA was purified and bisulfite-converted using the EZ DNA Methylation Direct Kit (Zymo Research), amplified by PCR. Illumina adapters were ligated and libraries amplified by a final PCR before sequencing using an Illumina MiSeq system. Reads were trimmed of the adapter aligned to the mm9 genome using Bismark (67). Data were analyzed and visualized using custom R scripts and the BSeq library (68).

Autoimmune Diabetes. For diabetes incidence, mice were screened for diabetes by glucose urinary analysis every week for 30 wk. A positive strip test was confirmed by blood analysis, and animals were considered diabetic with glucose >250 mg/dL on 2 consecutive days.

For histological evaluation, formalin-fixed and paraffin-embedded pancreas was sectioned and stained with hematoxylin and eosin. Four steps sections separated by 100 μm were used to calculate the insulitis score for each islet (0, no infiltration; 1, peri-insulitis; 2, intraislet insulitis), and 100 independent islets were scored for each mouse.

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C57BL/6J mice were obtained from The Jackson Laboratory.

ACK lysis (500 μL of PBS into the heart. Careful inspection ensured removal of visible lymph nodes.

After filtering through a 10-μm cell strainer (Falcon), cold medium (DMEM-2% FBS) was added, and then cells were pelleted by centrifugation at 500 × g for 15 min. ACK lysis (500 μL) was performed for 1 min on ice, followed by filtration through a 50-μm cell strainer, washing for 5 min at 500 × g, and resuspension in 200 μL of PBS (DMEM-2% FBS). Cells were stained on ice for 5–10 min in the dark in FACS buffer (DMEM without phenol red-2% FBS) containing antibodies against CD4 (GK1.5; BioLegend), CD25 (PC61; BioLegend), TCRβ (H57-597; BioLegend), and Foxp3 staining was performed in accordance with the manufacturer’s protocol with an anti-Foxp3 mAb (FJK-16S; eBiosciences). Permeabilization and staining were done at room temperature for 30 min.

Antibody staining was performed for 20 min at 4 °C in PBS with the following antibodies (1/200 dilution unless noted otherwise): CD45 (PB, clone 30-F11; BioLegend), TCRβ (PE-Cy7, clone H57-597; BioLegend), CD25 (FITC, clone PC61; eBiosciences), CD4 (V500, clone RM4.5; BD Biosciences), CD8 (PE, clone 53-6.7; BioLegend), and LiveDead Fixable Near IR (1/1000; Life Technologies). After permeabilization fixation for 2 h on ice, Foxp3 staining (APC, clone FJK-16S; eBiosciences) was performed for 30 min at room temperature in the dark in accordance with the manufacturer’s instructions.

Data were recorded on an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo version 9.3.2. Foxp3 MFI was calculated using a FlowJo internal function. The percentage of Foxp3-low cells was calculated as the proportion of cells with 50% Foxp3 expression compared with the average normalized to CD25 expression, as shown in the gates.

Mapping of Mouse Flicer Transcripts. We performed 5’ and 3’ RACE using the SMARTer RACE cDNA Amplification Kit (Clontech). cDNA was synthesized as described in the kit using 5’ and 3’ RACE CDS primers and SMARTer IA oligo for template switching for 5’ RACE; cDNA ends were then amplified by nested touchdown PCR. The first PCR used Universal Primer A mixed with TD_R1 = TGGCA-GCTGCGTGTCTCTCGATTTGCC for the 5’ ends or TD_F1 = TGCAAAAGGGTGCGTGCGCCATCGCAG F for the 3’ ends; 5 cycles of 94 °C for 30 s and 72 °C for 4 min → 5 cycles of 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 4 min → 25 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 4 min. The second nested PCR used Nested Universal Primer A and TD_R2 = CTGCA-GTGGCACACACACTCTTTGCGA for the 5’ ends or TD_F2 = TTGGTAGACGGAGCCGGTCTGTGGCA for the 3’ ends; 5 cycles of 94 °C for 30 s and 72 °C for 4 min → 5 cycles of 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 4 min → 25 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 4 min. Fragments were purified after agarose gel electrophoresis with the Qiagen Gel Extraction Kit (Qiagen) and cloned in TOPO-TA PCR-2.1 (Invitrogen), and the inserts were sequenced.

Mapping of 3’ termini by RNA-Seq. Because it involves oligo-dT priming and sequencing of 3’ UTR tags, RNA-Seq performed as described by Hashimshony et al. (71) yielded additional information on the polyadenylation site of Flicer transcripts. RNA-Seq was performed as detailed elsewhere (72). In brief, first-strand cDNA synthesis was performed using a barcoded oligonucleotide primer, which included an oligo-dT sequence, a sample barcode, and a 109-mer primer for reverse transcription. Second-strand cDNA synthesis was performed using the NEBNext mRNA Second-Strand Synthesis Module (New England BioLabs). After Agen- court AMPure XP bead size selection and purification, in vitro transcription was performed overnight with the MEGAGshortscript T7 Transcription Kit (Ambion). The amplified RNA was fragmented using the NEBNext Magnesium RNA Fragmentation Module (New England BioLabs), purified with Ampure beads, and treated with phosphatase and T4 polynucleotide kinase before ligation with an Illumina 3’ adapter. Ligated RNA was then reverse-transcribed. P5 and P7 Illumina sequences were added by PCR. Libraries were size-selected and sequenced on an Illumina HiSeq 2500 system.

 Reads were filtered for quality using the FASTX toolkit, version 0.0.13 (fastq_quality_filter -Q -O 33 -q 20 -p 80). Mapping to the mm10 reference genome and transcriptome was performed using Bowtie2 (73), conserving the strand information (tophat -p 2 -library-type fr-firststrand -read-mismatches 5 -read-gap-length 5 -read-edit-dist 5 -no-coverage-search -segment-length 15 -transcriptome-index). Transcripts were mapped to the reference genome, and the reads were counted using HTSeq. The resulting count matrix was then passed through Cufflinks (74) to generate a gene expression matrix. The resulting gene expression matrix was then used to perform a principal component analysis (PCA) and hierarchical clustering (80).
300 bp), and 17 cycles of PCR were performed using Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Fisher Scientific). Libraries were sequenced on an Illumina HiSeq 2000 instrument. Reads were filtered for quality using the FASTX toolkit, version 0.0.13 (fastq_quality_filter -v -Q 33 -q 20 -p 80). Mapping was performed using TopHat2 (73) on the mm9 transcriptome and genome using default parameters.

**Exploratory PCR.** RNA from 5 × 10^6 C57BL/6J GFP+ Tregs from Foxp3-ires-GFP/B6 sorted and prepared as above was used for cDNA synthesis with SuperScript II enzyme (Invitrogen) and oligo(dT) following the manufacturer’s instructions. PCR analyses were performed using primer pairs spanning the 4930524L23Rik locus, as shown in Fig. S2A. The primers were designed to encompass regions identified by RACE and RNA-Seq, as well as putative regions suggested by the ESTs or probing introns between Ppp1r3f and Foxp3.

**Synthesis by PCR.** Primers were designed following the extremities of 3′ and 5′ ends identified by RACE and 3′ RNA-Seq, as diagramed in Fig. S2A. All primer combinations were used, and fragments resulting from successful amplification were cloned into the TOPO-TA pCR-2.1 vector (Invitrogen) and sequenced. Four isoforms of *Flicer* were identified in this manner (Fig. 2A and Fig. S2A).

**Mapping of Human Flicer Transcripts.** Essentially similar techniques were used to map the human *Flicer* transcripts, using RNA prepared from the FOXP3+ C5/MJ HTLV1-transformed cell line (ATCC CRL-8293), cultured in Iscove’s modified Dulbecco’s medium supplemented with 20% FBS and penicillin-streptomycin. **RACE.** We performed 5′ and 3′ RACE using the same protocol as described above. Nested touchdown PCR was performed with the following primers designed in the conserved DNA region between mouse and human:

5′ reaction 1: h_F2 = CACAGAGCTGGCGAGTGGTGGTCCC for PCR1, then h_F5 = TGACCTCAGGAGCACTCTA for PCR2

5′ reaction 2: h_F5 = TGACCTCAGGAGCACTCTA for PCR1, then h_F3 = GGATGAGCACAAGG for PCR2

3′ reaction 3: h_R5 = TCTCAGTACGTGATGTATCTCTG-3′ AATT for PCR1, then h_R4 = GGTGAAAGGGGTATCGT-3′ for PCR2.

**RNA-Seq.** We reanalyzed the human RNA-Seq data published by Bonnal et al. (48), using tophat2 to map the data to the custom-made transcriptome reference derived from Hg19 and supplemented with *Flicer*. After removing PCR duplicates using the Picard tool MarkDuplicates (picard.sourceforge.net), we obtained gene counts using htseq-count (htseq-count -f bam -r pos -s no -t exon -i gene_id -m union) (74) and a custom GTF file supplemented with *Flicer* annotation. Data were normalized to the length of the genes and the library size in fragments per kb per million reads using a custom R script.

**Synthesis by PCR.** Primers were designed following the extremities of 3′ and 5′ ends identified by RACE. All primer combinations were performed by PCR and fragments were cloned into TOPO-TA pCR-2.1 (Invitrogen) and sequenced. Three isoforms of *Flicer* were identified with h_F2 and h_R5.

**Construction of a Flicer KO Mouse.** We closely followed the methods described by Yang et al. (49) to generate *Flicer*-deficient mice using the CRISPR/Cas9 system, with a few modifications. Because the active region of *Flicer* is unknown, we chose to introduce a point mutation at the shared splice donor site (*Flicer* mutant 1) or to delete a whole exon (exon 2) encompassing the region of highest homology between mouse and human transcripts, but keeping the mutation as small as possible to avoid interfering with Foxp3 enhancer elements (*Flicer* mutant 2).

For gRNA selection, we followed the protocol published by Cong et al (75) for the design and cloning of gRNAs (www.genome-engineering.org/crispr/wp-content/uploads/2014/05/CRISPR-Reagent-Description-Rev20140509.pdf), with the following criteria: (i) 20–21 bp complementary to the target region on the sense or antisense strand; (ii) flanked in 3′ with a PAM sequence (NGG); (iii) no off-targets predicted by alignment with bowtie (bowtie -a -best -v 0 -c mm9 gRNA with all four possible PAM sequences). For *Flicer* mutant 1, we used GCCTGTGTGGCTCCTGAAGGC (gRNA_2a) and CAA-AAAATGGGATGTAAGATG (gRNA_2b) flanking exon 2. We cloned the gRNAs in the pX330 vector following the protocol (75). In brief, gRNA oligos were synthesized with BbsI overhangs caccGACAGACTCGTTCAGGCTGAAAGACTGGCTTCAGGCCTG (gRNA_1) and caccGACAGACTCGTTCAGGCTGAAAGACTGGCTTCAGGCCTG (gRNA_2a), and caccGACAGACTCGTTCAGGCTGAAAGACTGGCTTCAGGCCTG (gRNA_2b) annealed; phosphorylated; ligated to the pX330 vector (75) previously digested with BbsI; and gel-purified using the QiaQuick Gel Extraction Kit (Qiagen). DH5alpha bacteria were transformed with the ligation product (30 min on ice, 42°C for 30 s, 30 s on ice) and spread on ampicillin LB agar plates. Clones were hand-picked and sequenced after Mini Prep (Invitrogen).

gRNA was prepared following Yang et al. (49). T7 promoter was added to the gRNAs by PCR using forward primers TAATACGACTCACTATAGG and reverse primers for gRNA_2a, and TAATACGACTCACTATAGG for gRNA_2a and gRNA_2b. The gRNAs were then transcribed in vitro from the PCR amplicons (150 nM final) using the MEGAshortscript T7 Kit (Life Technologies), and the RNA was purified using the MEGAclear Kit (Life Technologies).

**For Flicer mutant 1**, the ssDNA oligonucleotide AGGCCCTCAGCCTTGGTTCCTGGAG for gRNA_1, caccGACAGACTCGTTCAGGCTGAAAGACTGGCTTCAGGCCTG (gRNA_1) and caccGACAGACTCGTTCAGGCTGAAAGACTGGCTTCAGGCCTG (gRNA_2a) were cloned into the TOPO-TA pCR-2.1 vector (Invitrogen) and sequenced. Three isoforms of *Flicer* were identified in this manner (Fig. 2A and Fig. S2A).

**Zygote microinjection.** Here 8– to 10-wk-old NOD females were used as donors of fertilized zygotes. Superovulation was induced by two injections of 2.5 IU of pregnant mare serum gonadotropin/human chorionic gonadotropin given 47.5 h apart, gRNA, Cas9 mRNA, and HR substrate were suspended just before injection in a microinjection buffer (10 μM Tris/0.1 mM EDTA, pH 7.4) at a concentration of 5, 2.5, and 5 ng/μL, respectively. Microinjection was performed into the male pronucleus, and surviving embryos were implanted directly in pseudopregnant NOD females.

Cas9 protein was purchased from TriLink Biotechnologies (L-6325).

**Zygote microinjection.** Here 8– to 10-wk-old NOD females were used as donors of fertilized zygotes. Superovulation was induced by two injections of 2.5 IU of pregnant mare serum gonadotropin/human chorionic gonadotropin given 47.5 h apart, gRNA, Cas9 mRNA, and HR substrate were suspended just before injection in a microinjection buffer (10 μM Tris/0.1 mM EDTA, pH 7.4) at a concentration of 5, 2.5, and 5 ng/μL, respectively. Microinjection was performed into the male pronucleus, and surviving embryos were implanted directly in pseudopregnant NOD females.
The founder female for *Flicr* mutant 1 had a 12-bp deletion with a 17bp insertion containing the BamHI site: CTACCTTCTTCTCCTGGATCTTGAAGGCGTCTTTGGAAG.

This founder gave rise to one mutant male, but no further progery could be obtained.

The founder male for *Flicr* mutant 2 had a 263-bp deletion including exon 2 (mm9 chrX:7154602-7154665): CTCTCCTTCAACTCATCCTCCTCCTT. This founder gave rise to multiple progeny in the expected ratio from Mendelian segregation of the ChrX. Progeny were genotyped by PCR with the following primer combinations: WT PCR using F, AGTTGAAAGTTTCTGCTGAACAGTAATGAT and R, TACAGAGGCAAGCTTACAGC and F, AAGACTGACCTCTTCTCAG and R, AGAGACTGACCTCTCAGTGAAG.

**Bone Marrow Chimera Experiments.** Rag-KO NOD mice were irradiated with 5 Gy and reconstituted with equal proportions of congenically labeled T cell-depleted bone marrow cells from WT (CD45.2) and KO (CD45.1) mice. Bone marrow cells were harvested from both femurs and tibias of NOD CD45.1 (*Flicr* KO) and CD45.2 (WT) mice. After red blood cell lysis with ACK, T cells were depleted as follows. Bone marrow single-cell suspensions were incubated with biotinylated anti-CD3e antibodies for 5 min in MACS buffer, washed, and then incubated with 100 μL of streptavidin beads (Dynabeads Biotin Binder, 11047; Thermo Fisher Scientific) for 30 min. Isolation of the CD3ε population was performed after three magnet incubations for 2 min. A total of 4 million cells (2 million CD45.1 and 2 million CD45.2) were injected i.v. in each mouse. Mice were treated for 2 wk with trimethoprim-sulfamethoxazol and analyzed 10 wk later.

**In Vitro FoxP3 Stability.** Tregs from spleen and subcunataeous lymph nodes were prepared by magnetic negative selection (using PE-conjugated antibodies against CD8α, CD11b, CD11c, CD19, NK1.1, and Ter119 anti-PE MACS microbeads; Miltenyi Biotech); stained with mAbs against CD4, TCRβ, and CD25, with DAPI (1 μg/mL) as a viability dye; and then sorted by flow cytometry on a FACSAria II cell sorter into 500 μL of complete RPMI medium with anti-CD3/CD28 beads (1:1 bead:cell ratio; Dynabeads Mouse T-Activator CD3/CD28; Thermo Fisher Scientific) and 50 U/mL IL-2 (recombinant human IL-2, 212–12; Peprotech).

After 24 h of incubation in complete RPMI supplemented with 100 U/IL-2, FoxP3 expression was assessed by flow cytometry as described above.

**ATAC-seq.** ATAC-seq was performed in biological duplicates following the protocol described by Buenrostro et al. (58). In brief, 50,000 CD4+ TCRβCD25hi (top 50%) Tregs were sorted and lysed. After transposition and PCR, a final bead purification and selection (100–600 bp) was performed twice using 0.6x and 1.6x SPRI beads. Libraries were paired-end sequenced (40, 50) using a 75-bp kit on an Illumina NextSeq high-throughput sequencing system.

Fastq reads were first filtered on quality using sickle 1.2 (sickle pe -f R1.fastq -r R2.fastq -q 30 -f filtered_R1.fastq -r filtered_R2.fastq). Then adapters were trimmed using cutadapt 1.8.1 (76) (cutadapt -a AGATGTGTAAGAGACGACAG -A GTCTCTCTATATAGACGTCT -o clean_R1.fastq -2 clean_R2.fastq). Reads were then mapped against mm9 mouse genome using bowtie2 (77) (bowtie2 -p 2 -x mm9 -X 1000 -fr -1 clean_R1.fastq -2 clean_R2.fastq -S btout2.sam). Reads mapping to more than one location were discarded (using ‘nsm’; mapped reads > mapped_laligmentonly.sam).

Library duplicates were removed using Picard tools 1.130 (java -Xms1024m -jar /opt/picard-1.130/picard.jar MarkDuplicatesWithMateCigar INPUT = mapped_laligment.sorted; bam OUTPUT=S$fexpr.sorted.uniq.bam METRICS_FILE=metrics.txt REMOVE_DUPLICATES=true ASSUME_SORTED=true SKIP PAIRS_WITH_NO_MATE_CIGAR=true). Nuclease-free fragments (<120 bp) were isolated (samtools view -h $sexpr.sorted.uniq.bam | awk ‘/@/ { print $0 » “nuc120.sam” } ‘) (if $(99 <120 && $9 > 120) (print $0 » “nuc120.sam” ))). Finally, peaks were called using Homer 4.6 (findPeaks homer_tagdir_nuc120 -o $sexpr.nuc120_peaks_size250minidist500.txt -region size250-minDist500) (65). Data were imported in R, and differential peak analysis was performed using DiffBind (i.e., differential peak analysis based on negative binomial distributions) (66). DiffBind calculates P value for each peak based on the genome wide distribution of differences (following a negative binomial distributions).

**Methylation Analysis of the Foxp3 Locus by Bisulfite Treatment and Illumina Sequencing.** The protocol was adapted from Feng et al. (18) for Illumina sequencing. A total of 80,000 CD25hi-Tregs were sorted as above, DNA was purified, and bisulfate-converted using the EZ DNA Methylation Direct Kit (Zymo Research). Bisulfate-converted regions were then amplified by PCR. Illumina adapters were ligated and libraries amplified by a final PCR before sequencing on an Illumina MiSeq system.
Adapters. PE_adapter_top: ACACTTTTCCCTACGAGCGC-TCTCCGATC* (phosphorothiate linkage). PE_adapter_bottom: pGATCGGAGGAGCACGTCT (p, 5' phosphate). Oligos were dissolved in low-TE buffer to generate 100 μM stock for each oligo. To generate 200 μL of 15 μM adapters, we added 30 μL of the top and bottom oligos to a 1.5-mL Eppendorf tube with 2 μL of 5 M NaCl and 138 μL of low-TE buffer. The reaction incubated in a 98 °C water bath for 5 min, after which the water bath was turned off and the solution was slowly brought to room temperature. Annealed adapters were stored at −80 °C for 2 y.

Bisulfate conversion was done following the instructions in the EZ DNA Methylation Direct Kit (Zymo Research) and eluted in 22 μL.

Primers: meth_R, TGTAGGTATTAAGGTTGGAGTTG; meth_F, AAAAATTTTTAAAATTAAAAATTTT; meth_promF, AAAAACTACACATATATCAAAACACT; meth_promR, GCTTTCGGTTTTTTGGTATATTAA; meth_P7, and indexing was performed as follows: Reads were trimmed of the adapter using trim_galore www.pnas.org/cgi/content/short/1700946114.

Diffbind was used for ATAC-seq. For correlations, Fisher's exact test was used for the bone marrow chimera experiments. The paired Student *t* test was used for the bone marrow chimera experiments. Difffind was used for ATAC-seq. For correlations, Fisher z-transformation was applied. P < 0.05 was considered significant.
Fig. S2. *Flicr* expression and structure in mice and humans. (A) Map of *Flicr* in the mouse genome (mm9 coordinates). From top to bottom: Refseq annotation; PCR clones of *Flicr* isoforms; EST from the thymus and testis; FANTOM CAGE pile-up reads (46); primers used for exploratory PCRs designed to encompass regions identified by RACE and RNA-Seq but also putative regions suggested by the ESTs or probing introns between *Ppp1r3f* and *Foxp3*; 5′ RACE fragments; 3' RACE fragments and 3' RNA-Seq reads; mammalian conservation (PhyloP) (69). (B) Map of *Flicr* in the human genome (hg19 coordinates). From top to bottom: Refseq annotation; primers used in exploratory PCRs designed to encompass regions identified by RACE but also probing introns between *PPP1R3F* and *FOXP3*; PCR clones of *Flicr* isoforms; 5′ RACE fragments; 3' RACE fragments. (C) Map of *Flicr* in the mouse (blue) and human (green) *FOXP3* loci (mm9 and hg19 coordinates), shown at three different levels of resolution, with PhastCons placental mammal sequence conservation score (69). At the bottom is the alignment of mouse and human transcripts in the conserved region of exons 2 and 3 and translation in the three frames. Although this region is conserved across placental mammals (red bases or amino acids), we did not find any conserved peptide sequences between human and mouse in any of the three frames, arguing against purified selection for a specific peptide sequence. PhyloCSF scores were accordingly low (47).
C  Flicr KO F1 C57/Bl6 X NOD

D  Flicr KO mouse, 2nd mutation (exon 3 splice deletion)
Fig. S4. Flicr is a cis-acting nuclear RNA. (A) Flicr is enriched in the nuclear fraction. Nucleocytoplasmic separation of splenic CD4+ cells was performed by treatment with a hypotonic lysis buffer and centrifugation. Flicr, Xist (nuclear RNA), and Actinß1 (cytoplasmic RNA) were amplified by RT-qPCR. Representative amplification curves for Actinß1, Flicr, and Xist from the nuclear (blue) or cytoplasmic (red) fractions are shown. (B) Flicr transgenic expression does not complement the gene expression differences induced by the absence of Flicr. The plot represents the gene expression fold change between Flicr-WT and KO Tregs on the x-axis vs. the gene expression fold change between BAC transgenic and nontransgenic KO Tregs on the y-axis. Blue and red genes are Flicr-induced and repressed genes, respectively, as gated in Fig. 5A.