Flicr, a long noncoding RNA, modulates Foxp3 expression and autoimmunity

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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 (AR5) region of *Foxp3*. Like many lncRNAs, *Flicr*'s molecular effects are subtle, but by curtailing 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.

autoimmunity | regulatory T cells | gene regulation | long noncoding RNA | Foxp3

Regulatory T cells (Tregs), a subset of $CD4^+$ T cells determined by the transcription factor (TF) FoxP3, are fundamental actors in maintaining immune homeostasis, by dampening activation of several immunocyte lineages (1, 2). In their absence, humans and mice develop devastating autoimmune and lymphoproliferative pathologies (3). Although beneficial in preventing runaway immune activation, supporting tolerance of symbiotic microbes, and restraining tissue destruction in autoimmune lesions, their regulatory activity can also be deleterious, for example, by inhibiting antitumor (4) or antiviral responses (2, 5).

Phenotypic stability is important for Tregs (1, 6–8), particularly as their antigen receptors tend to show reactivity to self-antigens. This stability, and the stability of *Foxp3* expression, result from a combination of factors (9): DNA hypomethylation at conserved enhancer elements of *Foxp3* and other key loci (10) or self-reinforcing TF networks (11). This stability is bolstered by signals from IL-2, the key trophic cytokine for Tregs (2, 7, 9, 12, 13). At the center of this network lies FoxP3, the lineage-defining TF that is essential for Treg differentiation, maintenance, and function (1, 14–17). Several enhancer elements, conserved across vertebrates, ensure correct *Foxp3* expression, one of them [conserved noncoding sequence 2 (CNS2)] being important for the stability of *Foxp3* expression (18–20) during cell division and in proinflammatory milieus.

Deep transcriptome analyses have revealed the existence of an abundant class of long noncoding RNAs (lncRNAs) (21–24). LncRNAs do not encode proteins and are longer (>200 bp) than other noncoding transcripts like microRNAs. This family represents up to 20% of mammalian transcriptomes, which also applies in Tregs (25), and their expression is more tissue-specific than that of proteins. Evolving rapidly, they are poorly conserved evolutionarily, and only ∼10% of human lncRNAs have a clear homolog in mice (26). lncRNAs seem to be mostly involved in regulating gene expression through several mechanisms, which are conditioned by expression levels (typically low), subcellular localization (nuclear or cytoplasmic) and position in the genome (23). Some nuclear lncRNAs act in *cis* and activate or repress genes in their immediate vicinity; others have longer-range *cis* (e.g., *Xist* inactivates the entire X chromosome) (27) or *trans* action (*Hotair* and *Hox* genes) (28–30). Cytoplasmic lncRNAs also can interfere with translation, have catalytic activity, or act as miRNA sponges (31). In general, lncRNAs are thought to be modulators of expression patterns dictated by transcription regulators, although in some instances they have a dominant impact, as in the allelic inactivation of one copy of the X chromosome driven by *Xist*.

Relatively few lncRNAs specific to the immune system have been studied in detail in vivo, and their cumulative impact on immune functions remains largely unknown (32). Some lncRNAs are dynamically expressed during development (33), and others are important for granulocyte homeostasis (e.g., *Morrbid* regulates myeloid cell lifespan) (34) or immune effector functions (e.g., *Rmrp* and Th17 cell function, *NeST* and IFNγ expression in T cells) (35, 36). Consequently, lncRNAs influence the immunopathology of infections (*NeST*, *Morrbid*), endotoxic shock (*lincRNA-EPS*) (37), or gut inflammatory diseases (*Rmrp*, *linc-13*) (36, 38).

While analyzing lncRNA expression in Treg profiling data, we noticed the transcript *4930524L23Rik*. Its Treg-specific expression and genomic localization, partially overlapping *Foxp3*, were too striking to ignore. Here we report the structure, expression, and function of this lncRNA, hereinafter called *Flicr* (*Foxp3* long intergenic noncoding RNA). This lncRNA is present across mammalian species with clear stretches of sequence conservation. *Flicr* modulates *Foxp3* expression, most visibly in a subset of Tregs. This subtle fine-tuning has important consequences for autoimmune disease, thus subtly modulating the Janus-faced dominant suppressive function of Tregs.

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.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession nos. GSE97048 and GSE97085).

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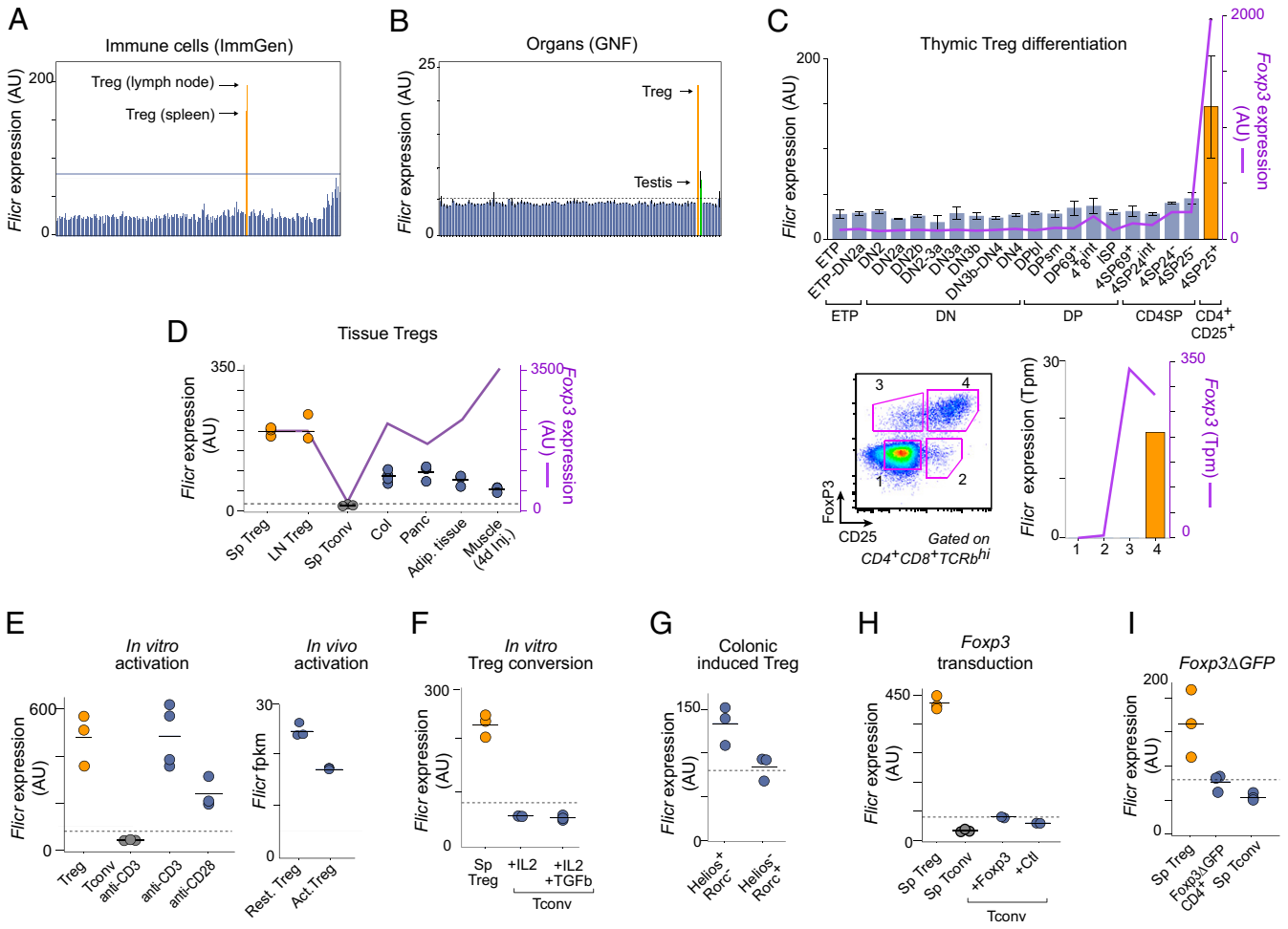

Fig. 1. *Flicr* is expressed specifically in Tregs. *Flicr* expression in various contexts. (*A* and *B*) ImmGen mouse immunocyte (39) or GNF mouse organ microarray compendia (40) (dashed lines: background level). (*C*) During thymic Treg differentiation. (*Left*) ImmGen data. (*Right*) RNA-Seq analysis 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. tissue, visceral 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 $CD44^{lo}CD62L^{hi}$; Act., activated $CD44^{hi}CD62L^{lo}$ Tregs; rpkm, reads per kb per million reads. (*F*) In vitro converted induced Tregs (TCR activation with IL-2 and TGF-β) (43). (*G*) Ex vivo $Helios^-ROR\gamma^+$ colonic peripheral Tregs (44). (*H*) In Tconvs transduced with *Foxp3* or control retrovectors (11). (*I*) In Treg-like cells of mice with *Foxp3* inactivation by *Gfp* insertion (*Foxp3*Δ*Gfp*) (45).

Results

***Flicr*, an lncRNA Specifically Expressed in Tregs.** *Flicr* first caught our attention as an lncRNA detected only in Tregs among the ImmGen compendium of immunocyte gene expression (39) (Fig. 1*A*). 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. 1*B*). During Treg differentiation, it appeared concomitantly with *Foxp3* but only in the final $CD25^{hi}$ stage (Fig. 1*C*). *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. 1*D*). Tissue Tregs tend to have activated phenotypes and, accordingly, Treg activation slightly reduced *Flicr* expression in vivo (41) and in vitro (42) (Fig. 1*E*). In contrast, *Flicr* was not expressed in induced Tregs generated in vitro from naïve conventional T cells (Tconvs) with TGF-β and IL-2 (43) (Fig. 1*F*). That *Flicr* expression may require thymic Treg differentiation was consistent with its lower abundance in colonic $ROR\gamma^+$ $Helios^-$ Tregs, considered to result from extrathymic differentiation, relative to their thymically derived $ROR\gamma^-$ $Helios^+$ counterparts (44) (Fig. 1*G*). 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. 1*H*). 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. 1*I*).

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. 2*A*), 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 *Foxp3* transcriptional start site (TSS). They are all transcribed from the same sense strand of *Chr*X as *Foxp3*, and the two longest ones overlap the *Foxp3* TSS. A 5′ rapid amplification of cDNA ends (RACE) analysis mapped several closely spaced 5′ ends of *Flicr* transcripts, concordant with CAGE data from the FANTOM Consortium (46) (Fig. S2*A*). However, we could not find evidence in Tregs for transcripts initiating further upstream and corresponding to the *4930524L23Rik* or *Ppp1r3fos* expressed sequence tags (ESTs) previously isolated from testis and thymus. A combination of 3′ RACE and polyA-tagged RNA-Seq data identified three main polyadenylation sites (Fig. 2*A* and Fig. S2*A*). These transcripts contained no open reading-frame (ORF) longer than 143 aa, and

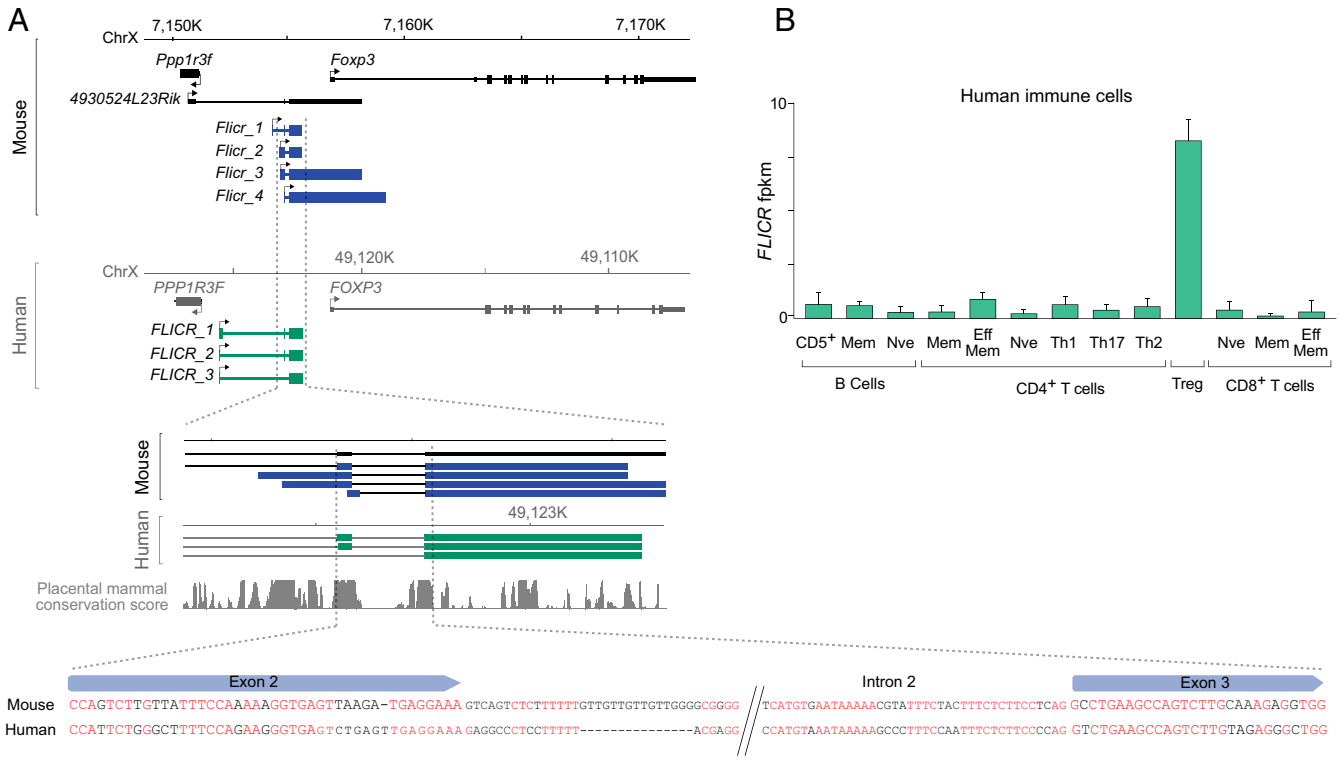

Fig. 2. *Flicr* is a conserved long noncoding RNA expressed in human Tregs. (*A*) Map of *Flicr* in the mouse (blue) and human (green) *FOXP3* loci (mm9 and hg19 coordinates), as deduced from the evidence shown in Fig. S2, shown at different levels of resolution, with PhastCons (69) placental mammal sequence conservation score. (*B*) *FLICR* expression by RNA-Seq in human lymphocyte populations (48). fpkm, fragments per kilobase per million.

had computationally predicted low peptide coding potential by codon substitution frequency analysis (PhyloCSF) (47); however, as for most lncRNAs, the possibility of translation into very short peptides cannot be ruled out (Fig. S2*C*).

lncRNAs 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. 2*A*) 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. 2*A* and Fig. S2*B*). There was no preservation of putative ORFs in the conserved region, with nonsense or frame shift mutations in all possible reading frames (Fig. S2*C*). As in the mouse, *FLICR* expression was restricted to Tregs (Fig. 2*B*; human RNA-Seq) (48). Thus, human and mouse Tregs specifically express an lncRNA 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. 3*A*), 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. S3*A*) (50). Indeed, transcriptome analysis showed that Tregs in *Flicr*-deficient (KO) mice lost *Flicr*, but not *Ppp1r3f* and *Foxp3* transcripts, the latter being slightly increased (Fig. 3*B*). 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. S3*B*); however, close examination of cytometry profiles revealed a consistent trait (Fig. 3*C*): whereas CD25^{hi} 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 FoxP3^{lo} Tregs. Correspondingly, the overall mean fluorescence intensity (MFI) increased slightly (Fig. 3*D*). This disappearance of the FoxP3^{lo} 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. S3*C*).

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 FoxP3^{lo} cells was present in an independent *Flicr* mutant mouse in which only 12 bp at the splice junction of exon 3 were deleted (Fig. 3*A* and Fig. S3*D*). 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 FoxP3^{lo} cells was observed (Fig. 3*E*), 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 FoxP3^{lo} cells. Indeed, more *Flicr* RNA was detected in cells with lower FoxP3 (Fig. 3*F*).

To test whether the FoxP3 phenotype of *Flicr*-deficient mice was Treg-autonomous or resulted from an indirect effect, we

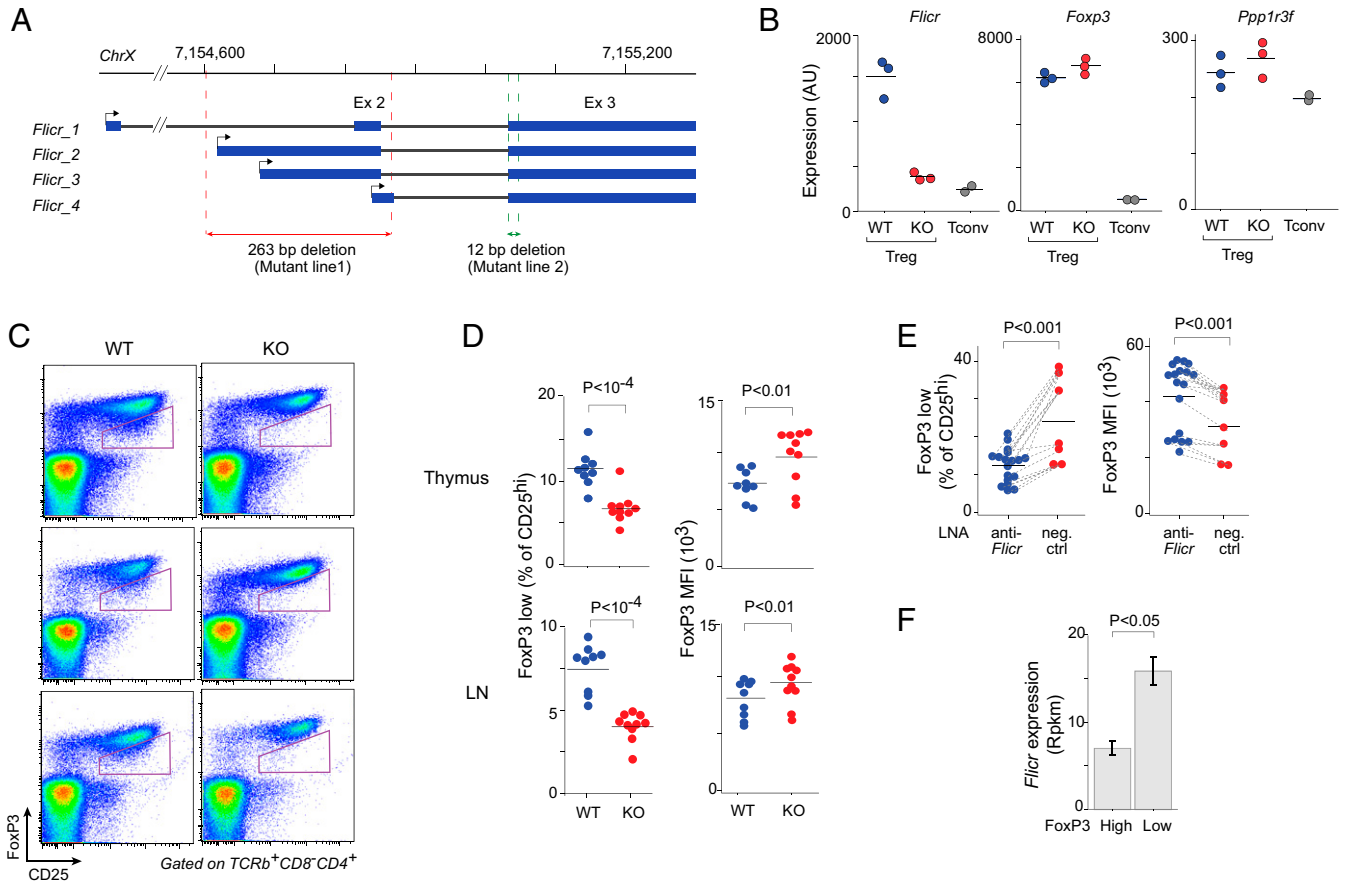

Fig. 3. *Flicr* KO and its action on FoxP3 expression. (*A*) Position of the 263-bp deletion of *Flicr* exon 2 (red) and of the second independent 12-bp deletion of the donor spliced site of exon 3 (green). (*B*) *Flicr*, *Foxp3*, and *Ppp1r3f* expression in *Flicr* WT and KO splenic Tregs and Tconvs (microarray, using only values from array probes mapping outside the deletion). (*C*) FoxP3 vs. CD25 expression in gated splenic $CD4^+$ T cells from in WT and KO mice. Representative plots from four experiments are shown. (*D*) FoxP3 MFI and proportion of $FoxP3^{lo}$ cells (per gating at left) in thymic and lymphoid $CD4^+$ T cells from in WT and KO mice, compiled from four experiments. (*E*) RNAi. FoxP3 MFI and proportion of $FoxP3^{lo}$ cells in activated $CD4^+$ T cells transfected with anti-*Flicr* or control LNAs. Data are compiled from three experiments, one of which had lower staining intensity for technical reasons. (*F*) *Flicr* expression in $FoxP3^{high}$ and $FoxP3^{low}$ in RNA-Seq data (19). *P* values from ANOVA (*D* and *E*) or *t* test (*F*).

constructed mixed chimeras. Alymphoid *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. 4*A*), indicating a Treg-intrinsic effect. Interestingly, KO Tregs had a competitive advantage over their WT counterparts in the same mice; the ratio of cells of KO vs. WT origin was consistently higher in Tregs than in $CD4^+$ Tconvs in the same mice (Fig. 4*B*, *Left*), which resulted in a higher proportion of Tregs in the KO pool (Fig. 4*B*), 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 $FoxP3^{lo}$ cells that normally appear in low–IL-2 cultures were essentially absent in Treg cultures from *Flicr*-deficient mice (Fig. 5*A*). 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. 5*B*). Interestingly, a slight accumulation of STAT5b was present in the *FLICR* promoter region in a ChIP sequencing analysis of human Tregs (53) (Fig. 5*C*), suggesting a pathway by which IL-2 might repress *Flicr* directly. Thus, *Flicr* appeared to be an active mechanism of *Foxp3* destabilization, which IL-2 counteracts.

***Flicr* Acts only in *Cis*.** PCR quantitation after nuclear-cytoplasmic fractionation showed that *Flicr*, like *Xist*, resides primarily in the nucleus (Fig. S4*A*), suggesting that it might regulate transcription. We asked whether *Flicr* had broader effects in Tregs beyond its *cis* action on the neighboring *Foxp3* gene. A comparison of *Flicr* WT and KO Tregs showed a very limited impact on gene expression profiles (from no changes to a twofold increase; Fig. 6*A*). Signature genes overexpressed in Tregs were biased toward underexpression in WT cells ($P = 2 \times 10^{-5}$), and the transcripts most affected by the deletion of *Flicr* were Treg signature genes (Fig. 6*B*). Similarly, FoxP3-binding genomic targets (ChIP sequencing data from ref. 54) were mostly underexpressed in WT Tregs compared with KO Tregs (Fig. 6*C*). These findings suggest that many/most of *Flicr*'s effects result from lowering or destabilization of *Foxp3* expression.

Given its position, *Flicr* seemed likely to influence *Foxp3* transcription through a local *cis* effect, as is the case for several

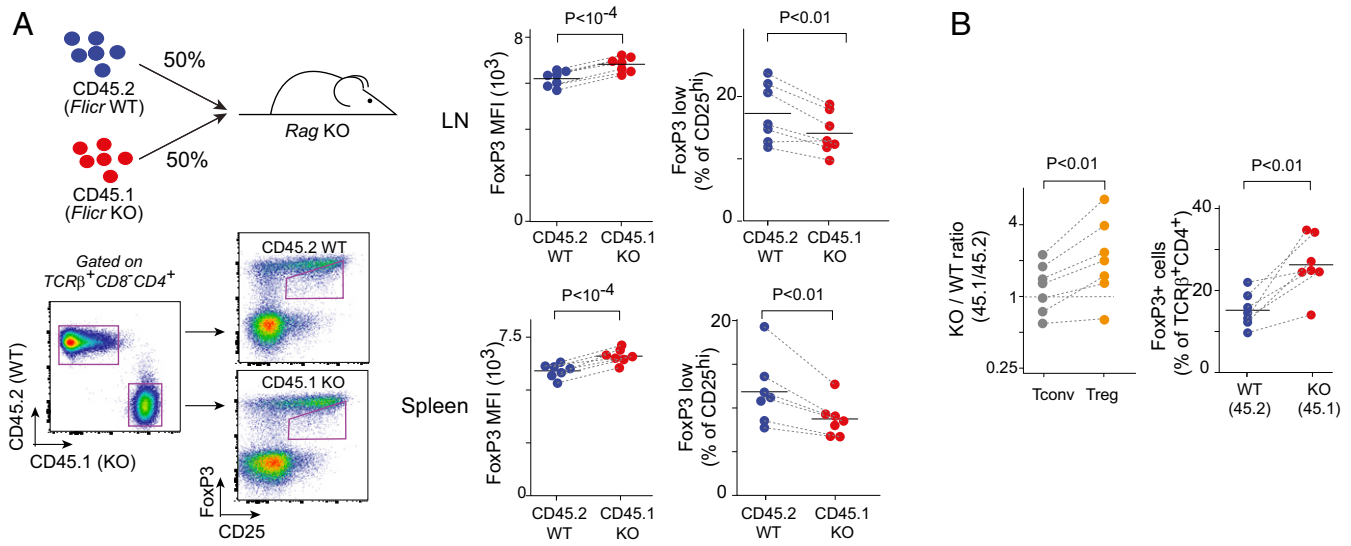

Fig. 4. Cell-intrinsic effect of *Flicr* on FoxP3 expression. (*A*) *Rag*-KO mice were reconstituted with equal proportions of congenically labeled bone marrow cells from *Flicr* WT (CD45.2) and KO (CD45.1) mice. Shown are representative FACS plots distinguishing CD4$^+$ T cells of different origins for FoxP3 vs. CD25 analysis (*Left*), FoxP3 MFI (*Middle*), and proportion of FoxP3^{lo} 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.

lncRNAs (27, 30, 55). We tested this hypothesis by attempting to complement *Flicr* KO mice with a BAC transgene that includes a WT *Flicr*, together with an insertionally inactivated *Foxp3* (56) (Fig. 6*D*). Although the transgene-encoded *Flicr* was expressed at the same level as the endogenous one (Fig. S4*B*), it did not restore FoxP3^{lo} Tregs in vivo (Fig. 6*E*) or the sensitivity to low IL-2 supply in vitro (Fig. 6*F*). Expression of the genes affected by the *Flicr* deficiency also was not normalized by the *Flicr*-encoding complementing transgene (Fig. S4*B*), 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 lncRNA 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

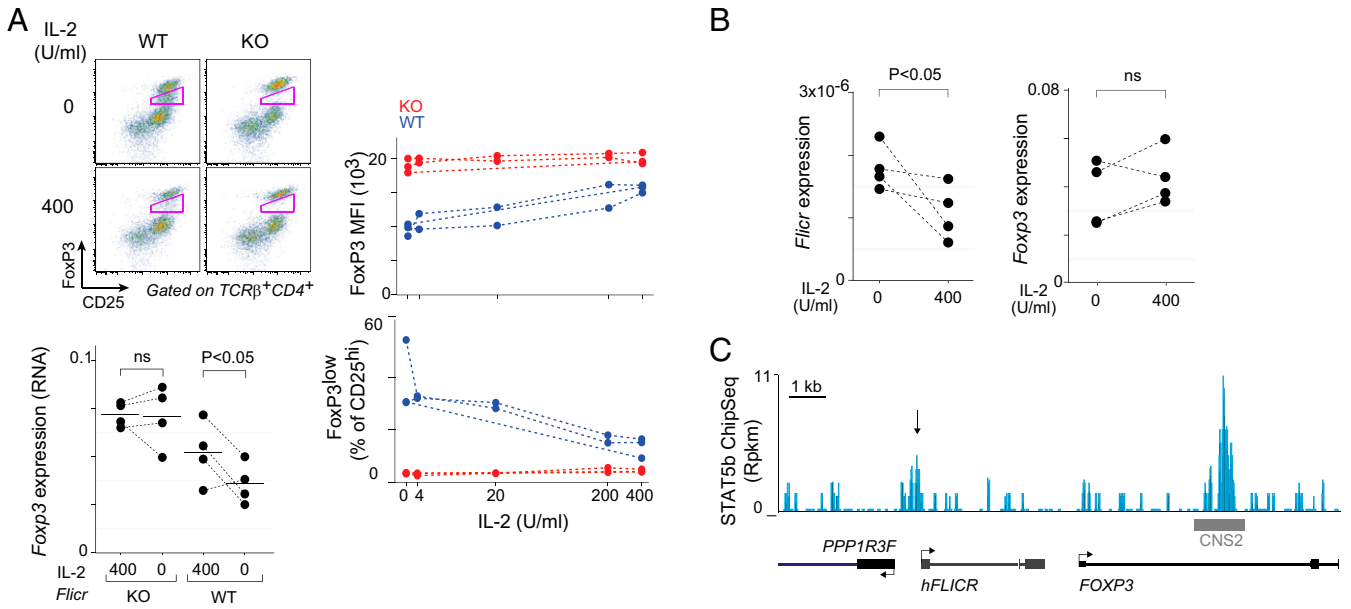

Fig. 5. *Flicr* destabilizes FoxP3 expression in vitro and is down-regulated by IL-2. (*A*) In vitro stability assay. Tregs were cultured for 48 h in limiting IL-2 before cytometry. (*Left*) Representative FoxP3 vs. CD25 plot. (*Middle*) FoxP3 MFI and proportion of FoxP3^{lo} cells. (*Right*) *Foxp3* expression (relative to *Acting1*) by RT-qPCR. Data are pooled from three experiments. (*B*) *Flicr* and *Foxp3* expression (relative to *Acting1*) 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).

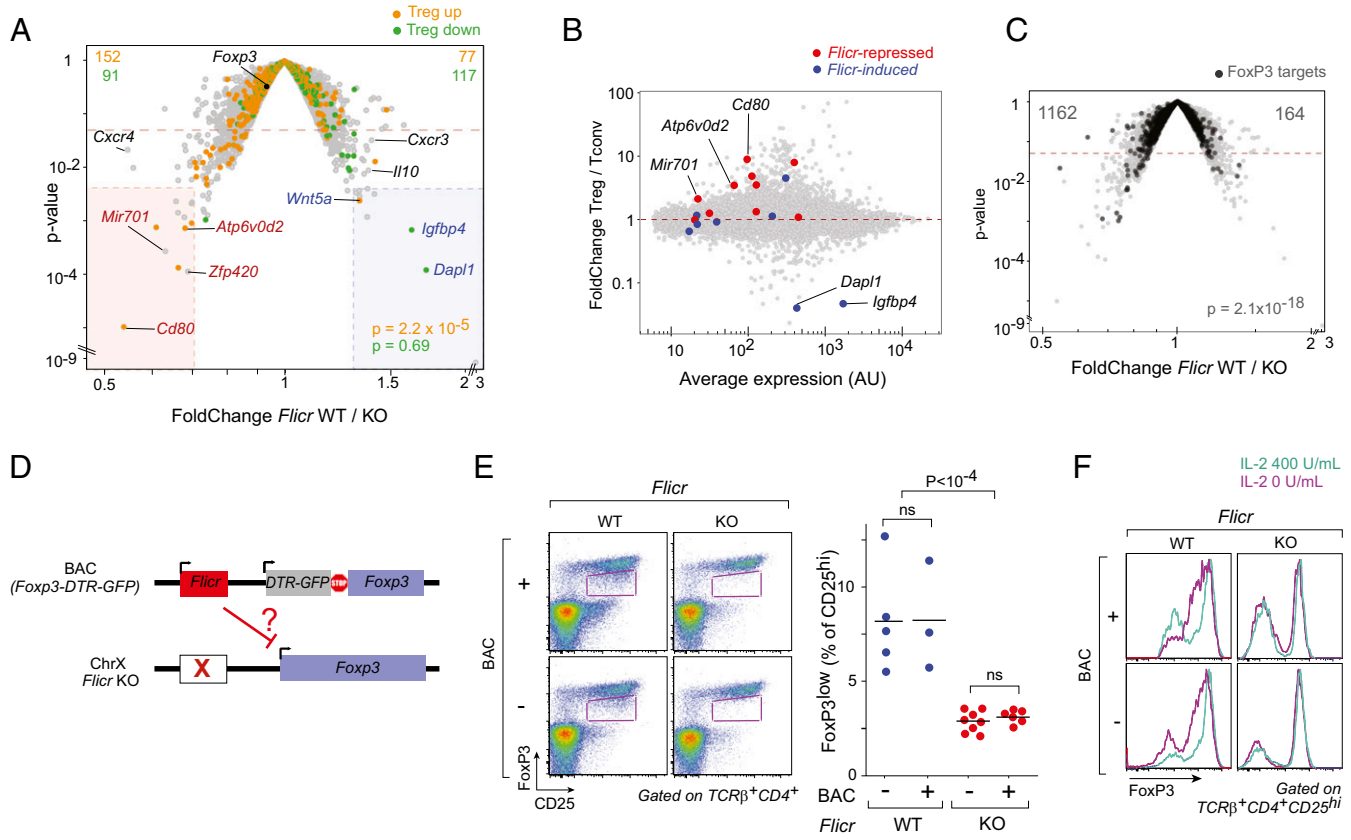

Fig. 6. Impact of *Flicr* on FoxP3 regulated genes. (*A*) Comparison of gene expression profiles of *Flicr*-WT and -KO Tregs (microarray). Up- and down-regulated Treg signature genes (70) are highlighted. *P* values are from the χ^2 test. (*B*) Expression/fold change plot of normal Treg vs. Tconv profiles (70) highlighted with transcripts up- or down-expressed in WT vs. KO (as gated in *A*). (*C*) Same volcano plot as in *A*, with black highlights indicating genes that bind FoxP3 in Treg chromatin (54). (*D*) Complementation experiment design. *Flicr* KO mice were crossed with mice carrying a BAC transgene encompassing WT *Flicr* and an insertionally inactivated *Foxp3* (56), tested for a *trans* effect on endogenous *Foxp3*. (*E*) FoxP3 protein expression in $CD4^+$ splenocytes from *Flicr* WT and KO mice, with or without genetic complementation by the *Flicr* BAC transgene. Shown are representative plots and results compiled from five experiments. *P* values are from ANOVA. (*F*) In vitro stability assay, as shown in Fig. 4*A*, in Tregs from the same mice as in *E*. A representative plot of five experiments is shown.

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 *Flicr* WT and KO Tregs showed superimposable profiles, except for a minor alteration within *Flicr* itself (Fig. 7*A*). 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. 7*B*). Overall, ATAC-seq profiles were very similar in *Flicr* 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. 7*B*) 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 *Flicr* 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. 8*A*). This halving of diabetes incidence was reflected in reduced severity of insulitis at age 11 wk (Fig. 8*B*). In the inflammatory context of these infiltrated islets (Fig. 8*C*), 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. 8*D*). Interestingly, the slope of this anticorrelation was steeper in *Flicr* KO mice than in heterozygous or WT control littermates ($r = -0.91$ 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,

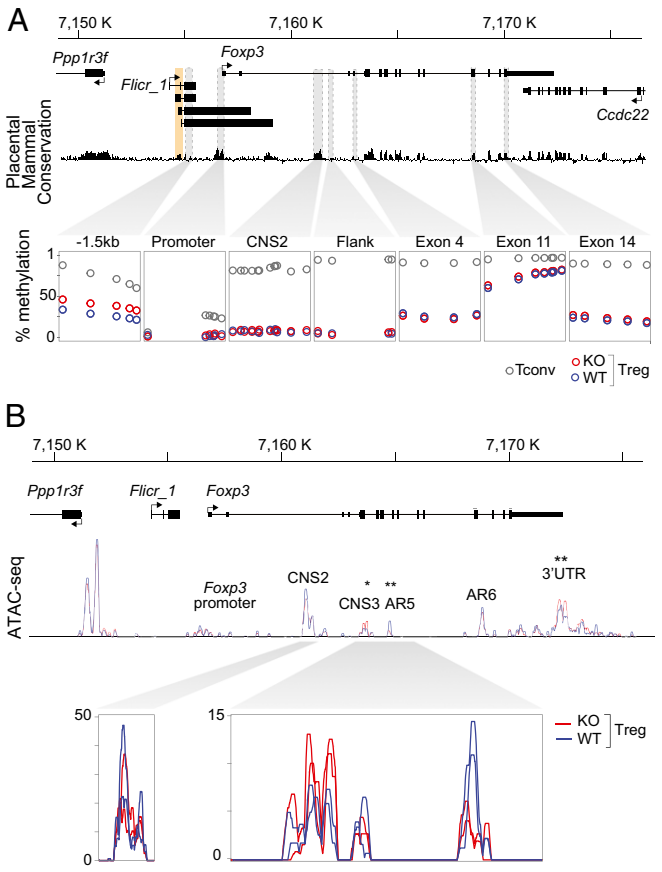

Fig. 7. *Cis* effect of *Flicr* on *Foxp3* chromatin accessibility. (*A*) CpG methylation profile of the *Foxp3* locus in *Flicr* WT and KO Tregs (bisulfite sequencing). Each dot represents one 5-methylcytosine site; methylation frequency is averaged from two independent experiments. (*B*) Chromatin 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).

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 *Cns2* 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 as 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 *Rmrp*, the impact of which in T lymphocytes seems limited to Th17 cells despite ubiquitous expression (36), but is akin in this respect to *Morrbid* (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 regulatory 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

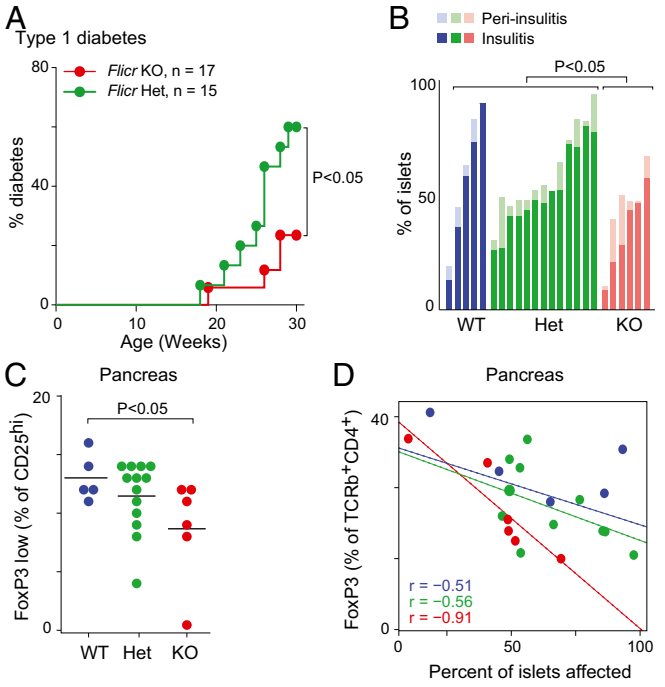

Fig. 8. Physiological consequences of modulation of Treg activity by *Flicr*. (*A*) Incidence of diabetes in *Flicr* KO and heterozygous littermate NOD females; *P* values from the Mantel–Cox log-rank test. (*B*) Proportion of infiltrated islets in the pancreas of 10-wk-old *Flicr* WT, heterozygous, 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-wk-old WT, heterozygous, and KO mice. Data are pooled from six experiments; Student *t* test. (*D*) Inverse correlation between islet infiltration and the proportions of Tregs among CD45^{+} 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, *Flicr* 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. lncRNAs commonly operate within ribonucleoprotein complexes with specific TFs (36), chromatin modifiers (28), or Hnrnp proteins (37, 60), and we propose that *Flicr* may target a repressive complex to the CNS3/AR5 region of *Foxp3*.

The functions of lncRNAs in the immune system are just being elucidated. Through its effects on Tregs, *FLICR* 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/DOI and Foxp3-DTR-GFP/N (56), Foxp3-Ires-Thy1.1/B6 (62), and Foxp3-IRES-GFP/B6 (63) mice were maintained in our colony. Foxp3-DTR-GFP/N carries a BAC transgene encompassing 150 kb downstream and 70 kb upstream of the *Foxp3* TSS, with a DTR-eGFP-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 HMS IACUC protocols 02954).

Mapping of Mouse and Human *Flicr* Transcripts. Treg RNA was prepared from double-sorted $CD4^+$ $TCR\beta^+$ $Thy1.1^+$ Tregs from Foxp3-Ires-Thy1.1/B6 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. S2*A*. Isoforms were cloned with primers designed based on the extremities found. RNA from the $FOXP3^+$ C5/MJ HTLV1-transformed cells [American Type Culture Collection (ATCC); CRL-8293] was used to map human *FLICR* (3′ and 5′ RACE), and confirmed by Treg RNA-Seq data (48).

***Flicr* KO Mice.** To generate *Flicr*-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 *Flicr* 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 spliced site of exon 3, but gave only one progeny. Another mutant had a 263-bp deletion spanning *Flicr* exon 2.

In Vitro FoxP3 Stability. $CD4^+$ T cells from spleen and subcutaneous lymph nodes isolated by magnetic negative selection and $CD4^+TCR\beta^+CD25^{hi}$ (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 $CD4^+TCR\beta^+CD25^{hi}$ (top 50%) Tregs and $CD25^-$ Tconvs isolated from pooled spleen and subcutaneous lymph nodes from 8-wk-old *Flicr* 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 lmFit 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 $CD4^+TCR\beta^+CD25^{hi}$ (top 50%) Tregs were sorted and lysed. After transposition and PCR, final bead purification and selection (100–600 bp) were performed twice using 0.6× and 1.6× 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 fragments (<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 $CD25^{hi}$ 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 BiSeq library (68).

Autoimmune Diabetes. For diabetes incidence, mice were screened for diabetes by glucose urinalysis 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 cells (0, no infiltration; 1, peri-insulitis; 2, intraislet insulitis), and 100 independent islets were scored for each mouse.

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