

FOXP3 interacts with hnRNPF to modulate pre-mRNA alternative splicing

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FOXP3 promotes the development and function of regulatory T cells mainly through regulating the transcription of target genes. RNA alternative splicing has been implicated in a wide range of physiological and pathophysiological processes. We report here that FOXP3 associates with heterogeneous nuclear ribonucleoprotein (hnRNP) F through the exon 2–encoded region of FOXP3 and the second quasi-RNA recognition motif (qRRM) of hnRNPF. FOXP3 represses the ability of hnRNPF to bind to its target pre-mRNA and thus modulates RNA alternative splicing. Furthermore, overexpression of mouse hnRNPF in *in vitro*-differentiated regulatory T cells (Tregs) reduced their suppressive function. Thus, our studies identify a novel mechanism by which FOXP3 regulates mRNA alternative splicing to modulate the function of regulatory T cells.

CD4⁺CD25⁺ regulatory T cells (Tregs)³ suppress immune responses to self-antigen and commensal antigens although the immune system mounts defense against pathogens. FOXP3 is a lineage-specific transcription factor for Tregs and its expression is required for the development and function of Tregs. Ablation of the *Foxp3* gene either during Treg differentiation or in mature Tregs abrogates their suppressive function (1). In mice, a spontaneous mutation of *Foxp3* results in scurfy phenotype, which is characterized by lack of Tregs and multiorgan lethal autoimmunity (2). In humans the loss of FOXP3 function caused by mutations attenuates the development and function of Tregs and causes immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome (3, 4).

FOXP3 is an X-linked forkhead/winged helix transcription factor highly conserved between humans and mice. Upon bind-

ing to the promoters of target genes with its C-terminal forkhead domain, FOXP3 either down- or up-regulates their expression (5, 6). FOXP3 can also execute its activity in a forkhead domain-independent manner. In recent years, multiple nuclear proteins have been identified that associate with FOXP3 (7–16). By forming a complex with other nuclear factors, FOXP3 regulates the expression of target genes that do not contain binding sites for its forkhead domain (7).

A hallmark of higher eukaryotic gene expression is the pre-mRNA splicing during transcription. Alternative splicing of multi-exon genes not only regulates the stability, localization, and translation of mature transcript by differential splicing of the 5' or 3' UTRs, but also alters the protein structure, even the ORF of genes, and thus provides an important RNA-based layer of protein regulation and cellular function (reviewed in Ref. 17). In humans, up to 95% of multi-exon genes are estimated to undergo alternative splicing (18). It is predicted that alternative splicing events are overrepresented in nervous and immune systems. For example, genes expressed in immune systems undergo extensive alternative splicing with 60% of primary transcripts alternatively spliced in T or B lymphocytes (19). During T cell receptor (TCR) activation in primary and cultured T cells, over 50 genes with known functions in immunobiology are validated to exhibit robust changes (20% difference) in alternative splicing (17). Although these studies have provided substantive evidence for the importance of alternative splicing in the adaptive immune response, whether alternative splicing is involved in the lineage differentiation and/or function of helper T cells remains to be understood.

In current studies, we identified the association of FOXP3 and heterogeneous nuclear ribonucleoprotein F (hnRNPF). hnRNPF belongs to the hnRNP family that consists of RNA-binding nuclear proteins involved in multiple aspects of RNA metabolism (20). hnRNPF is further grouped into a subfamily with hnRNPH1, hnRNPH2, and hnRNPH3 because of their conserved protein sequence and structure (21). We demonstrate that FOXP3 represses the alternative splicing mediated by hnRNPF whereas increased expression of hnRNPF overcomes the repression of alternative splicing by FOXP3. We further show that overexpression of hnRNPF in induced Tregs (iTregs) reduced their ability to suppress the proliferation of activated CD4 T cells. Taken together, these data suggest a novel function for FOXP3 to modulate pre-mRNA alternative splicing through its interaction with splicing factor hnRNPF.

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This article contains Figs. S1–S3.

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³ The abbreviations used are: Treg, regulatory T cell; qRRM, quasi-RNA recognition motif; FKHD, forkhead domain of FOXP3 protein; Tconv, conventional CD4 T cell; iTreg, induced Treg; CFSE, carboxyfluorescein succinimidyl ester; TCR, T cell receptor; ANOVA, analysis of variance; RIP, RNA immunoprecipitation.

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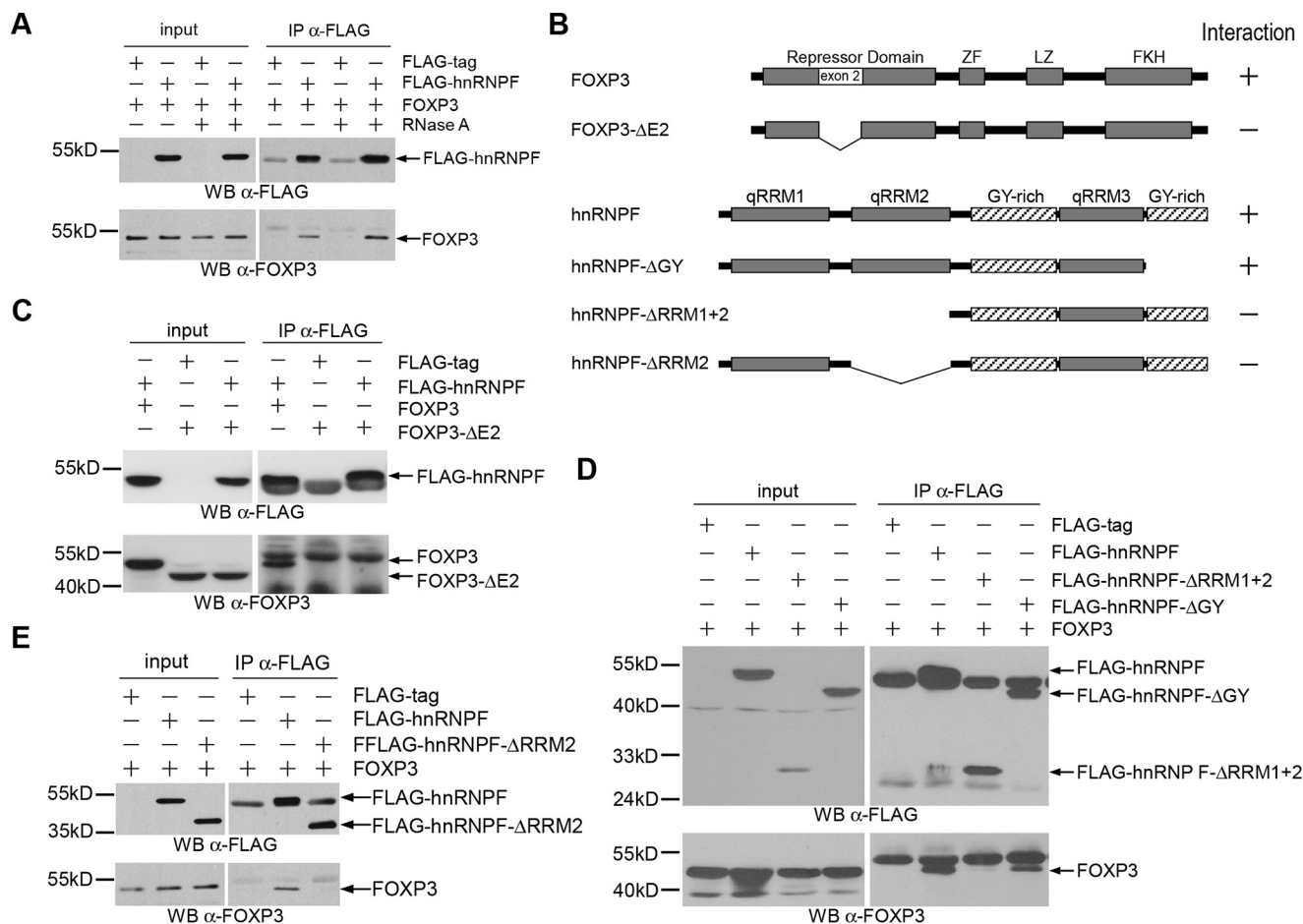


Figure 1. Interaction of FOXP3 and hnRNPF. *A*, co-immunoprecipitation of human FOXP3 and hnRNPF. HEK293T cells were transfected with plasmid of FLAG-tagged human hnRNPF and human FOXP3 followed by immunoprecipitation with anti-FLAG antibody. To determine whether FOXP3 and hnRNPF interaction depends on RNA, some samples were treated with RNase A before immunoprecipitation with anti-FLAG antibody. *B*, constructs used to map the interacting regions in FOXP3 and hnRNPF. The results of co-immunoprecipitation shown in *C* to *E* were also indicated after each construct. *C*, full-length FOXP3, but not FOXP3 lacking exon 2 region (FOXP3- Δ E2), co-immunoprecipitated with full-length hnRNPF. *D*, deletion of the first two quasi-RNA recognition motif (qRRM) abrogated the interaction between hnRNPF and FOXP3. Δ RRM1 + 2: hnRNPF with the first two qRRMs deleted; Δ GY, hnRNPF with the C-terminal GY-rich region deleted. *E*, deletion of the second quasi-RNA recognition motif (Δ RRM2) abrogated the interaction between hnRNPF and FOXP3.

Results

Association of FOXP3 and hnRNPF protein

We carried out yeast two-hybrid screen with a cDNA library of *in vitro*-generated human CD4⁺CD25⁺ Treg cells to understand the mechanism of transcriptional activity by FOXP3 (7). A cDNA clone encoding the C-terminal 2/3 of hnRNPF protein was isolated. To confirm the association of FOXP3 hnRNPF in mammalian cells, we cloned the full-length coding region of human hnRNPF into a vector plasmid to express a FLAG-tag at the N-terminal of hnRNPF protein, and used it in co-immunoprecipitation analysis with full-length FOXP3. FOXP3 was co-immunoprecipitated with FLAG-tagged hnRNPF but not FLAG-tag alone, which indicated the association of these two proteins in mammalian cells (Fig. 1A). This association of FOXP3 with hnRNPF was not RNA-dependent as RNase A treatment did not disrupt their interaction (Fig. 1A).

To determine the interacting region of FOXP3 and hnRNPF proteins, we generated various truncation constructs (Fig. 1B) and transfected HEK293T cells. Whereas full-length FOXP3 co-immunoprecipitated with FLAG-tagged hnRNPF protein, FOXP3 lacking the region encoded by exon 2 did not (Fig. 1C).

Deletion of the C-terminal GY-rich domain of hnRNPF did not disrupt the association with FOXP3 (Fig. 1D). However, an hnRNPF mutant lacking the first two quasi-RNA recognition motifs (qRRM) completely abolished the association with FOXP3, indicating that these two qRRMs were required to bind to FOXP3. Because the fusion hnRNPF protein associated with FOXP3 in the original yeast two-hybrid screen contained the C-terminal half of the second qRRM, the FOXP3-binding site on hnRNPF is likely located in the C-terminal half of the second qRRM (qRRM2). We deleted the qRRM2 of hnRNPF and found that the hnRNPF- Δ RRM2 mutant was localized in the nuclei (Fig. S2A) but failed to co-immunoprecipitate with FOXP3 (Fig. 1E).

hnRNPF belongs to a family that includes three other proteins, hnRNPH1, hnRNPH2, and hnRNPH3 (21). These proteins show high similarity in the qRRM domains. To examine if FOXP3 associates with other family members, we cloned coding regions of hnRNPH1 and hnRNPH3 for co-immunoprecipitation analysis. FOXP3 was co-immunoprecipitated with hnRNPF and H1 but not with hnRNPH3 (Fig. S1A) despite that both proteins were localized in nuclei (Fig. S2A). Although we

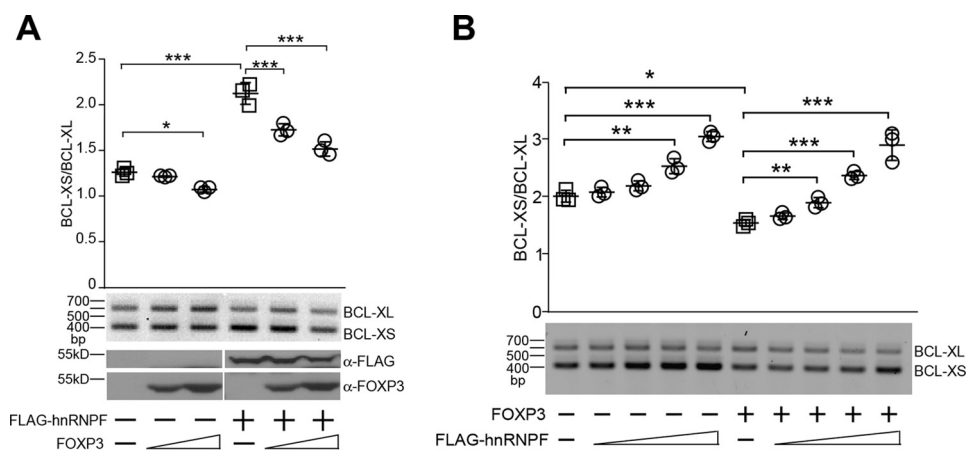


Figure 2. FOXP3 interacts with hnRNPF to modulate alternative splicing of human *BCL-X* minigene. *A*, expression of human hnRNPF increased the ratio of small isoform of *BCL-X* minigene in HEK293T cells, whereas expression of FOXP3 repressed the small isoform expression. *Top panel* shows the ratio of small isoform to large isoform based on RT-PCR results. *Middle panel* shows the gel image of RT-PCR products. *Bottom panel* shows the expression of hnRNPF and FOXP3 detected by Western blotting. *B*, increased expression of hnRNPF repressed the activity of FOXP3 in a dose-dependent manner. Data represent mean \pm S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant; by one-way analysis of variance (ANOVA) with Bonferroni post hoc test.

failed to express hnRNPH2 in the HEK293T cells, the high amino acid identity (96%) between hnRNPH1 and hnRNPH2 suggests that FOXP3 may also interact with hnRNPH2 protein.

FOXP3 modulates alternative splicing by counteracting hnRNPF activity

hnRNPF is an RNA-binding protein and has been reported to modulate the alternative splicing of pre-mRNAs, including the *BCL-X* gene. hnRNPF promotes the expression of the pro-apoptotic isoform *BCL-XS* by binding to G-tracts in the 3' half of exon 2 and facilitating the usage of the alternative 5' splicing site (22, 23). To understand if the association between FOXP3 and hnRNPF is involved in the regulation of pre-mRNA alternative splicing, we made a human *BCL-X* minigene as described in "Experimental procedures" and used it in a minigene splicing assay. HEK293T cells were transfected with a series of plasmids encoding the *BCL-X* minigene, FOXP3, and FLAG-hnRNPF (Fig. 2A). In the absence of FOXP3 and hnRNPF expression constructs, the minigene generated both large transcript (*BCL-XL*) and small transcript (*BCL-XS*). Consistent with the ability of hnRNPF to promote *BCL-XS* expression (22, 23), expression of exogenous hnRNPF increased the ratio of small to large isoforms. By contrast, expression of FOXP3 decreased the ratio of small to large isoforms in a dose-dependent manner, both in the absence and presence of exogenous hnRNPF, which indicates that FOXP3 represses the splicing activity of hnRNPF (Fig. 2A). However, increasing hnRNPF dose can overcome the repression of *BCL-XS* expression by FOXP3 (Fig. 2B). These data suggest that FOXP3 and hnRNPF reciprocally repress each other in regulating *BCL-X* alternative splicing.

Because FOXP3 can associate with other family members such as hnRNPH1 (Fig. S1A), we carried out the minigene assay to determine whether this association also affects hnRNPH1 splicing activity. Whereas exogenous hnRNPH1 increases *BCL-XS* to *BCL-XL* ratio, similar to hnRNPF, expression FOXP3 decreased the ratio (Fig. S1B). These data indicate that FOXP3 binds to hnRNPF/H family members and represses their splicing activity.

Exon 2 region but not the forkhead domain of FOXP3 is required to modulate hnRNPF activity

Because FOXP3 exon 2 region was required for the interaction with hnRNPF (Fig. 1C), we tested whether the exon 2 region was involved in regulating hnRNPF-mediated alternative splicing of *BCL-X* gene. Whereas transfection of full-length FOXP3 significantly reduced the ratio of *BCL-XS* to *BCL-XL* expression in the presence or absence of hnRNPF overexpression, expression of FOXP3 Δ E2 showed no effects on *BCL-X* alternative splicing (Fig. 3A).

FOXP3 is a transcription factor with a functional DNA-binding domain, the forkhead domain (FKH domain). To test whether forkhead domain is involved in regulating RNA alternative splicing, we replaced the forkhead domain of FOXP3 with or without the nuclear localization signal of SV40 large T antigen (FOXP3- Δ FKH+NLS and FOXP3- Δ FKH) (Fig. 3B). Without the nuclear localization signal, the FOXP3- Δ FKH mutant failed to enter cell nuclei (Fig. S2B). Minigene splicing assay was performed with these constructs. Although the cytoplasmic FOXP3- Δ FKH did not shift the *BCL-X* alternative splicing, the mutant with nuclear localization signal (FOXP3- Δ FKH+NLS) was as active as the intact FOXP3 in repressing the splicing of *BCL-XS* isoform in the absence or presence of exogenous hnRNPF, indicating that the nuclear localization but not the forkhead domain *per se* is required for the splice-modifying activity of FOXP3 protein (Fig. 3B). Taken together, the association of FOXP3 with hnRNPF reduced the splicing activity of hnRNPF, and the reduction depends on the exon 2 region that interacts with hnRNPF but not on the DNA-binding forkhead domain of FOXP3.

FOXP3 and hnRNPF interaction suppresses hnRNPF binding to *BCL-X* pre-mRNA

hnRNPF promotes the expression of *BCL-XS* isoform by binding to the G-tracts in exon 2 (22, 23). Mutation in the G-tracts of the *BCL-X* gene abolishes the binding of hnRNPF to *BCL-X* pre-mRNA and the activity of hnRNPF to modulate *BCL-X* alternative splicing. To examine if the splicing of *BCL-X*

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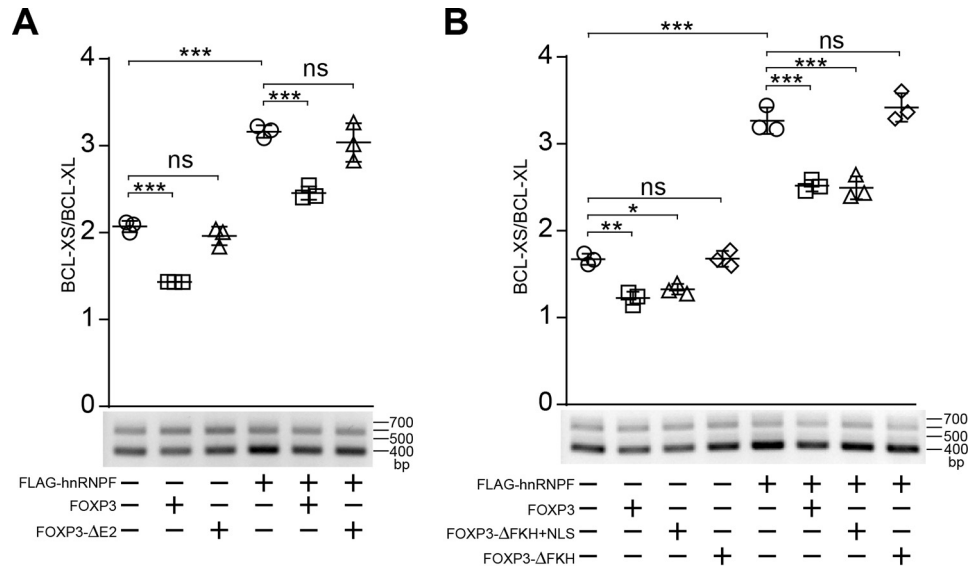


Figure 3. Modulation of hnRNPF-mediated alternative splicing of human *BCL-X* minigene by FOXP3 requires exon 2 region but not DNA-binding forkhead domain. *A*, repression of hnRNPF-mediated *BCL-XS* expression required the exon 2 region of FOXP3. *B*, repression of hnRNPF-mediated *BCL-XS* expression did not rely on the DNA-binding domain of FOXP3, the forkhead domain. FOXP3-ΔFKH+NLS: forkhead domain deleted FOXP3 with a nuclear localization signal from SV40 large T antigen; FOXP3-ΔFKH: forkhead domain deleted FOXP3. Data represent mean ± S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant; by one-way ANOVA with Bonferroni post hoc test.

minigene is regulated through the same mechanism, we mutated the two G-tracts that are crucial for hnRNPF binding. The WT *BCL-X* minigene gave rise to both isoforms and splicing was regulated by both hnRNPF and FOXP3 (Fig. 4A). Mutation of G-tracts in the *BCL-X* minigene abrogated the splicing of small isoform. Next, we knocked down the endogenous expression of hnRNPF and hnRNPH in HEK293T cells with small interfering RNA (siRNA), and then transfected the cells with the human *BCL-X* minigene and FOXP3 plasmid. Western blotting showed the knockdown of both hnRNPF (Fig. 4B) and hnRNPH (data not shown) expression 48 h after siRNA treatment. At this time point, the ability of FOXP3 to modulate *BCL-X* splicing was greatly reduced.

We then carried out RNA precipitation to examine whether association of FOXP3 reduces the binding of hnRNPF to target RNA. HEK293T cells were transfected with FOXP3 or control plasmid. RNA precipitation was performed with mAb that recognizes both hnRNPF and hnRNPH (Fig. 4C). Expression of FOXP3 significantly reduced the amount of *BCL-X* pre-mRNA co-precipitated with hnRNPF/H. Taken together, these data suggest that FOXP3 suppresses the splicing activity of hnRNPF/H by interfering with the binding of hnRNPF/H to its target RNA.

Alternative splicing of hnRNPF/H target gene *Tarbp2* in conventional T cells and Treg cells

To further examine the physiological implications of the interactions between FOXP3 and hnRNPF, we examined alternative splice forms in primary mouse Treg cells. To determine co-expression of FOXP3 and hnRNPF in Tregs, we stained mouse splenocytes with fluorochrome-labeled antibodies against FOXP3 and hnRNPF and examined by fluorescent microscopy. hnRNPF (stained red) was ubiquitously expressed in splenocytes and co-expressed with FOXP3 in Tregs that are stained green (Fig. 5A). The co-expression of hnRNPF and

FOXP3 in Tregs was further supported by FACS analysis on lymphocytes stained with CD4, FOXP3, and hnRNPF antibodies (Fig. 5B). Although expressed in both conventional CD4 T cells and Tregs, hnRNPF expression in Tregs (mean fluorescence intensity = 12,680) was slightly higher than in conventional CD4 T cells (mean fluorescence intensity = 11,050). Using the mAb that recognizes both mouse hnRNPF and hnRNPH, we demonstrated co-immunoprecipitation of hnRNPF/H and FOXP3 in mouse Tregs, suggesting the ability of interaction between these endogenous proteins (Fig. 5C).

Our biochemical analysis in Figs. 1–4 demonstrated that the interaction between human FOXP3 and hnRNPF regulates the splicing of *BCL-X* RNA. Unlike human Tregs that express both *BCL-X* isoforms (Fig. S3), mouse Tregs only express the *BCL-XL* isoform, which prevented us from analyzing the regulation of mouse *BCL-X* isoforms by hnRNPF and FOXP3 in primary mouse Tregs. Because human *TARBP2* was identified as a target of hnRNPH1 by high throughput analysis and RT-PCR (24), we examined the mouse *Tarbp2* sequence and located putative hnRNPF/H binding sites in intron 8 between exons 8 and 9 (Fig. 5D). Based upon the location of this putative binding site, we predict that hnRNPF/H would promote the splicing out of intron 8, whereas FOXP3 should promote the inclusion of the intron to express the long *Tarbp2* mRNA (Fig. 5D diagram). We then cloned exon 7 to exon 9 genomic fragment from mouse *Tarbp2* gene and constructed a minigene to perform minigene assay. As predicted, transfection of hnRNPF significantly increased the ratio of short isoform to long isoform whereas overexpression of FOXP3 significantly decreased this ratio regardless of hnRNPF transfection (Fig. 5D).

To determine endogenous *Tarbp2* isoform expression in Tregs and conventional CD4 T cells (Tconv), we sorted Tregs and Tconv cells from *Foxp3^{eGFP}* mice and carried out RT-PCR on the total RNA with primers matching exon 7 and exon 9 of

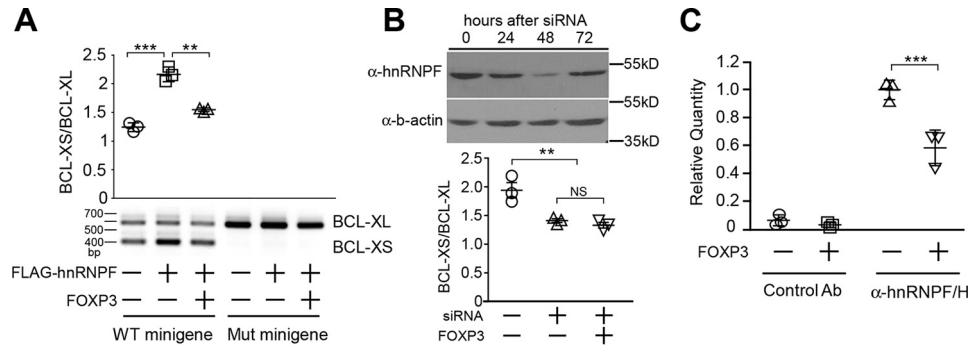


Figure 4. Interaction of FOXP3 with hnRNPF suppresses the binding of hnRNPF to its target *BCL-X* pre-mRNA. *A*, mutations of the G-tracts on *BCL-X* minigene where hnRNPF binding abrogated the splicing of short isoform *BCL-XS*. *B*, knocking down hnRNPF and hnRNPF expression eliminated FOXP3's ability to modulate *BCL-X* alternative splicing. *Top panel*, Western blotting detection of hnRNPF expression in HEK293T cells transfected with siRNAs targeting hnRNPF and hnRNPF. *Bottom panel*, 24 h after siRNA transfection, cells were transfected with plasmids for *BCL-X* minigene and FOXP3. Cells were harvested for RT-PCR 48 h after siRNA transfection when hnRNPF expression was the lowest. *C*, FOXP3 suppressed endogenous hnRNPF/H binding to endogenous *BCL-X* pre-mRNA. RIP assay was carried out as described under "Experimental procedures." Data represent mean \pm S.D. ($n = 3$). **, $p < 0.01$; ***, $p < 0.001$; ns, not significant; by one-way ANOVA with Bonferroni post hoc test.

mouse *Tarbp2*. Tregs expressed lower ratio of short isoform to long isoform when compared with conventional T cells (Fig. 5E). To further determine regulation of *Tarbp2* isoform expression by hnRNPF/H, we knocked down hnRNPF and hnRNPF expression in iTregs with Thy1.1⁺ retrovirus expressing shRNA targeting *hnRNPF* and *hnRNPF*. iTregs with (GFP⁺Thy1.1⁺) or without (GFP⁺Thy1.1⁻) retroviral transduction were FACS purified and *Tarbp2* isoform expression was examined with RT-PCR. Similar to *ex vivo* experiment (Fig. 5E), *Tarbp2* expression in iTregs is shifted toward the long isoform, resulting in lower ratio of short to long isoforms than that in naïve CD4 T cells (Fig. 5F). Whereas the scrambled control shRNA did not change the ratio of the *Tarbp2* isoforms, shRNA-mediated knocking down hnRNPF and hnRNPF expression further increased *Tarbp2* long isoform expression in iTregs (Fig. 5F). These data suggest that hnRNPF/H proteins regulate endogenous gene *Tarbp2* splicing, and FOXP3 may be able to shift the balance of *Tarbp2* isoforms in Tregs. Shifting of isoform expression will likely cause functional changes of TARBP2 because inclusion of intron 8 results in a protein isoform lacking the C-terminal protein-protein interaction domain.

Overexpression of hnRNPF reduced the suppressive activity of iTregs

Because FOXP3 is a master transcription factor for Tregs, alterations in FOXP3 expression will likely result in changes of transcriptional profile and thus development and function of Tregs. To explore whether changes in alternative splicing affect Treg function, we overexpressed hnRNPF in iTregs that would antagonize FOXP3 in regulating pre-mRNA alternative splicing. We transduced CD4 T cells from *Foxp3^{eGFP}* mice with Thy1.1-hnRNPF or control retrovirus during the induction of iTregs. Transduction of retrovirus expressing hnRNPF does not significantly affect iTreg differentiation (Fig. 6A), nor does the expression levels of key Treg phenotypic gene products FOXP3, CD25, and CTLA4 (Fig. 6B). To study the function of iTregs with or without hnRNPF overexpression, the transduced iTregs were sorted by the expression of eGFP and Thy1.1. The purified iTregs were co-cultured with CFSE-labeled naïve CD4

T cells isolated from congenic CD45.1 mice under TCR stimulation. The proliferation of CD45.1⁺ responder CD4⁺ T cells was examined 4 days later. Overexpression of hnRNPF in iTregs significantly reduced their suppression of responder CD4⁺ T cell proliferation when they were co-cultured at 1:1 to 1:4 ratios (Fig. 6C). The difference in suppression between hnRNPF transduced and control transduced iTregs was attenuated by the decreasing ratio of iTregs to responders. These data indicate that overexpression of hnRNPF reduces the ability of FOXP3 to confer suppressive activity even though the expression level of FOXP3 is not impacted.

Discussion

Expression of FOXP3 is essential for Treg cell differentiation irrespective of whether the T cells commit to the Treg lineage in the thymus or in the periphery. Because of its ability to bind to DNA through the FKH domain and to interact with numerous transcription factors and chromatin modifiers (25), the majority of studies on the mechanisms by which FOXP3 governing Treg lineage commitment and maintenance focus on FOXP3-mediated transcriptional regulation. For the first time, our study provides evidence that FOXP3 interacts with and limits hnRNPF and hnRNPF binding to pre-mRNA and modulating alternative splicing of target genes. In the context of already complex transcriptional and epigenetic actions of FOXP3, our finding adds another layer of posttranscriptional regulation of gene expression by FOXP3 in Treg development and function.

FOXP3 forms macromolecular complexes with 361 associated proteins in an unbiased proteomics analysis and 23% of identified FOXP3 protein partners have been implicated in RNA binding and regulation (16). Although hnRNPF was not included in the previous report, the main function of the reported hnRNPL and hnRNPLL proteins, similar to hnRNPF/H family members, is regulation of RNA alternative splicing. The discrepancy in identifying hnRNPF as FOXP3-associated protein in our study and hnRNPL/hnRNPLL as FOXP3 partners could be because of the difference in original material and approach used. While we used a yeast two-hybrid screen with a library constructed with cDNA from human Tregs (7), Rudra *et al.* (16) used mass spectrometric analyses of retrovirus (encoding biotinylated

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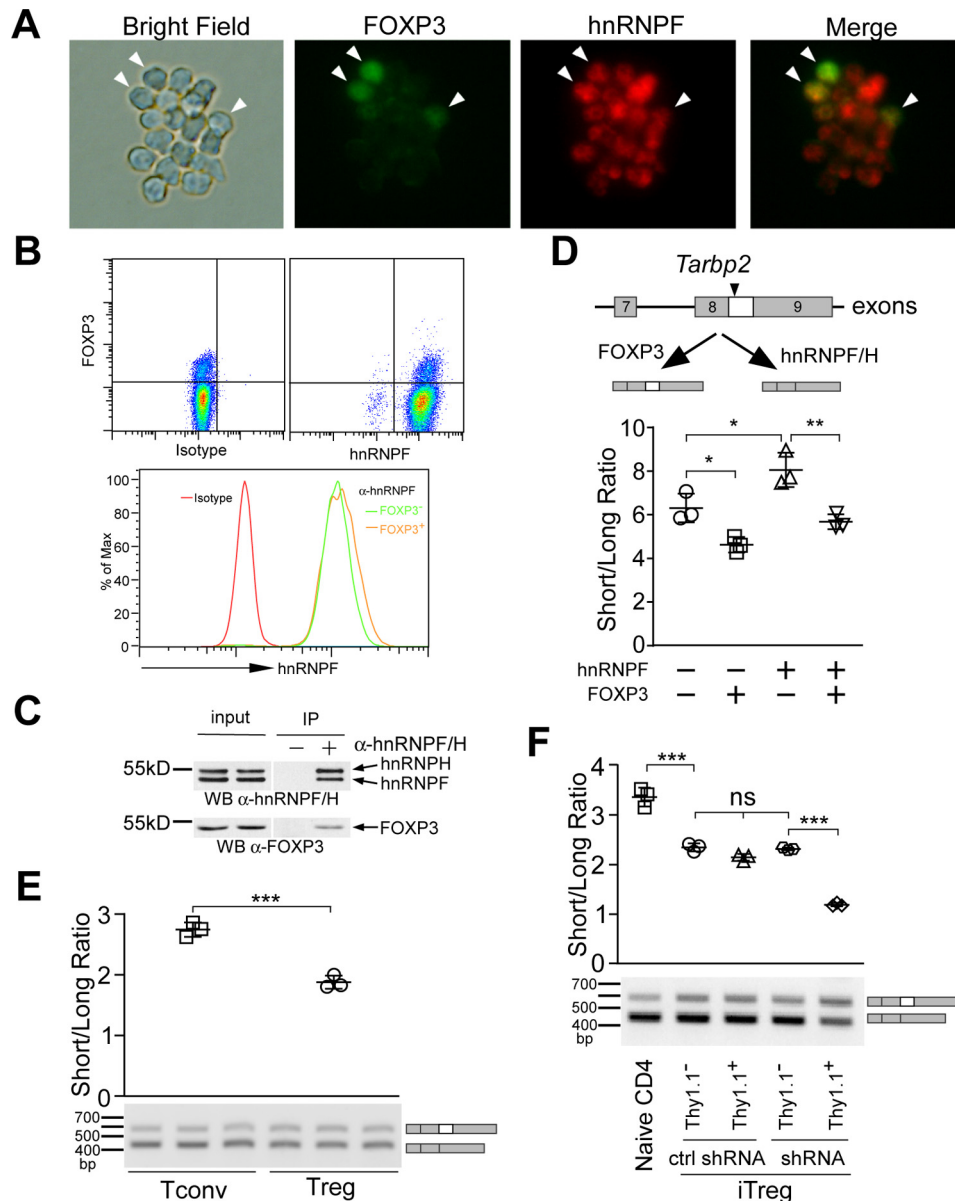


Figure 5. Regulation of alternative splicing of hnRNPF/H target gene *Tarbp2* in mouse Tregs. *A*, co-expression of mouse FOXP3 and hnRNPF. Mouse splenocytes were stained with Texas Red anti-mouse hnRNPF and FITC anti-mouse FOXP3, and analyzed under fluorescent microscope. *B*, FACS analysis of mouse FOXP3 and hnRNPF co-expression was carried out on mouse lymphocytes stained with fluorochrome-labeled antibodies against CD4, FOXP3, and hnRNPF. Cells were gated on CD4⁺ and the expression of FOXP3 and hnRNPF was determined. *C*, co-immunoprecipitation of endogenous FOXP3 and hnRNPF/H in *in vitro* differentiated mouse regulatory T cells. Immunoprecipitation was performed with a mouse mAb (clone 1G11) that recognizes hnRNPF and hnRNPF/H proteins. Normal mouse IgG was added as control (–). *D*, minigene assay demonstrating regulation of *Tarbp2* alternative splicing by FOXP3 and hnRNPF/H. Also shown are a diagram of *Tarbp2* gene structure (exon 7 to exon 9), putative binding site (arrowhead) of hnRNPF/H, and predicted regulation of splicing isoforms by FOXP3 and hnRNPF/H. *E*, expression of *Tarbp2* splicing isoforms in FACS sorted conventional (Tconv) and regulatory (Treg) CD4⁺ T cells. *F*, knocking down hnRNPF/H expression in induced Treg (iTreg) cells further shifted *Tarbp2* expression to the long isoform. iTregs, while differentiating, were transduced with Thy1.1 retrovirus expressing hnRNPF/H targeting shRNA or scrambled control (*ctrl*) shRNA. iTregs (eGFP⁺) transduced (Thy1.1⁺) or nontransduced (Thy1.1⁻) by the retrovirus were sort purified and *Tarbp2* isoform expression was examined by RT-PCR. Data represent mean ± S.D. (*n* = 3). *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001; *ns*, not significant; by one-way ANOVA with Bonferroni post hoc test (*D* and *F*) or two-tailed *t* test (*E*).

FOXP3)-transduced T cell hybridoma Tcli. Nevertheless, the inclusion of multiple splicing factors in the FOXP3 macromolecular complexes in mice supports the idea that FOXP3 is capable of regulating RNA alternative splicing to modulate Treg development and/or function.

Alternative splicing of multi-exon genes often yields protein products with different, even opposite, physiological and cellular functions. For example, the two alternatively spliced isoforms BCL-XS and BCL-XL from the single gene *BCL-X* have

been associated, respectively, with the promotion and the prevention of apoptosis. The balanced ratio of two isoforms is important for cell survival. hnRNPF promotes the production of the pro-apoptotic BCL-XS variant (22, 23, 26). Our results show that FOXP3 interacting with hnRNPF is capable of lowering the BCL-XS/BCL-XL ratio (Fig. 3). In mice, BCL-XL was shown to be required for the development of functional Tregs in lupus-afflicted mice following treatment with a tolerogenic peptide (27), and FOXP3 and BCL-XL cooperatively promoted

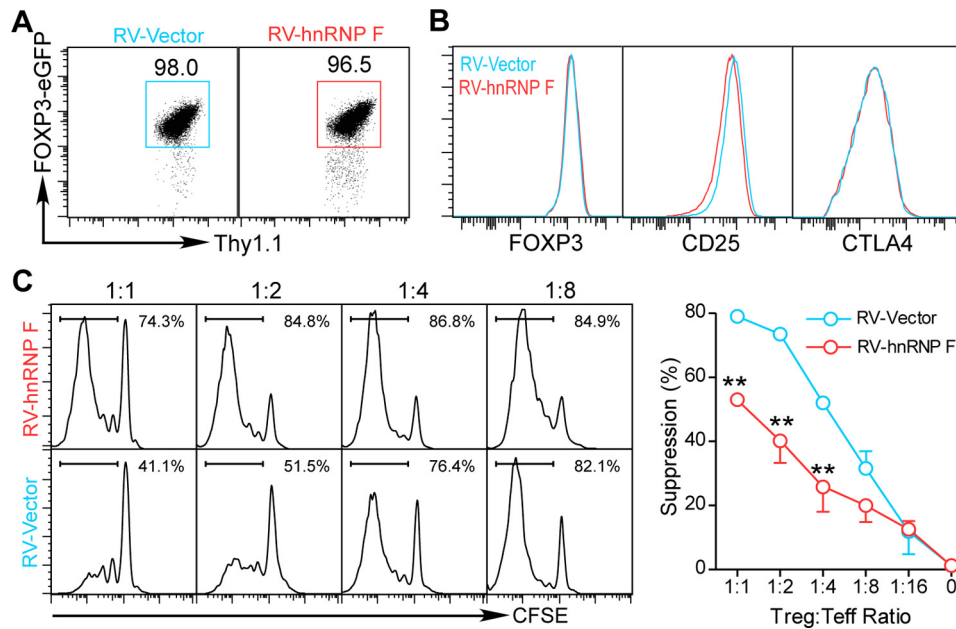


Figure 6. Retrovirus-mediated hnRNPF overexpression reduces suppressive activity of mouse iTregs. Naïve CD4 T cells from CD45.2 *Foxp3^{eGFP}* mice were transduced during induction of iTregs with Th1.1 retroviruses expressing hnRNPF or vector only. **A**, retrovirus-mediated overexpression of hnRNPF did not affect iTreg differentiation (eGFP⁺) in Thy1.1⁺ gated CD4⁺ T cells. **B**, retrovirus-mediated overexpression of hnRNPF did not significantly affect the expression of key Treg genes FOXP3, CD25, and CTLA4. **C**, differences in suppression of CD45.1 responder cell proliferation by iTregs with or without hnRNPF overexpression. Suppressive assay was carried out with sorted iTreg (Thy1.1⁺FOXP3-eGFP⁺) mixed with naïve CD45.1 CD4⁺ T cells as responders in the presence of plate-bound anti-CD3 and soluble anti-CD28. The ratios of iTregs to responders are indicated. Data represent mean \pm S.D. ($n = 3$). **, $p < 0.01$ by two-way ANOVA with Bonferroni post hoc test.

Treg persistence and prevention of arthritis development (28). Whereas mouse Tregs only express the BCL-XL isoform (Fig. S2) and conditional knockout of *Bcl-x* gene, which would eliminate both BCL-XL and BCL-XS, in FOXP3⁺ cells did not affect Treg survival (29), whether changes in the BCL-XS to BCL-XL ratio because of the interaction between FOXP3 and hnRNPF/H would impact human Treg development, function, or survival needs further investigation.

The FKH domain of FOXP3 is important to form homodimers and for DNA binding (30). The FKH domain also directly interacts with nuclear factor of activated T cells, and this interaction was important for transcriptional repression (for example IL-2) and activation (for example CTLA4) by FOXP3 (9, 31). Although critical for Treg differentiation and function, the FKH domain appeared to be dispensable for the transcriptional control of most FOXP3-regulated Treg relevant genes, and deletion of FKH domain only dysregulates the expression of 16% of FOXP3 regulated genes (32). Our study demonstrated that deletion of FKH domain did not abolish the ability to regulate RNA alternative splicing by FOXP3 (Fig. 3B), suggesting DNA recognition and binding are not required for FOXP3 to regulate alternative splicing. In consistency, FOXP3 regulates RNA alternative splicing by limiting hnRNPF/H binding to its target RNA (Fig. 4C).

The N-terminal proline-rich region of FOXP3 protein (exon 1 to exon 3) plays a pivotal role in FOXP3-mediated gene expression control, and 2143 of 2407 FOXP3 target genes are likely regulated through its proline-rich region (32). Within this region, FOXP3 exon 2 interacts and inhibits ROR γ t and ROR α function to suppress Th17 differentiation (33, 34). Our results suggest a novel function of FOXP3 exon 2 that interacts with

splicing factor hnRNPF, and likely hnRNPH1, to regulate mRNA alternative splicing (Fig. 3A).

Experimental procedures

Mice

WT BALB/c mice and *Foxp3^{eGFP}* knockin mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). All animals were housed in specific pathogen-free conditions, and all studies were approved by the Indiana University School of Medicine Animal Care and Use Committee.

Cell lines and transfections

HEK293T cells were maintained in complete DMEM containing 5% FBS and 1% penicillin and streptomycin and were transfected using TransIT-LT1 or TransIT-293. Jurkat cells were maintained and transfected according to the manuals of Cell Line Nucleofactor Kit V (Amaxa).

Antibodies and reagents

The primary antibodies used include rat anti-FLAG (BioLegend), Alexa Fluor 488 anti-FOXP3 (eBioscience), anti-FOXP3 (clone 150D, BioLegend), anti-FOXP3 (poly6238, BioLegend), anti-hnRNPF (Sigma), and anti-hnRNPF/H (clone 1G11, Santa Cruz Biotechnology). Protein AG plus agarose beads were purchased from Santa Cruz Biotechnology.

Plasmids

Human hnRNPF expression plasmid was made by cloning the coding region without start codon into pCMV-Tag2b between EcoRI and XhoI sites. hnRNPF- Δ RRM1 + 2 contains

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coding region of amino acid 201–415. hnRNPF- Δ GY contains amino acid 1–366. Human FOXP3 plasmid was made by cloning the coding region plus 6 nucleotides upstream start coding in pIRES2-eGFP between EcoRI and XbaI. Mouse hnRNPF retroviral plasmid was cloned by inserting the coding region of mouse hnRNPF with 12 nucleotides upstream start codon into vector MSCV-IRES-Thy1.1.

Human *BCL-X* minigene was made by cloning two fragments covering part of exon 2, intron 2, and exon 3 into vector pIRES2-eGFP at BamHI site. The first fragment of human *BCL-X* minigene starts with GTACCGGCGGGCATTTCAGTGAC and ends with CTGTAGGCAAGTTTTGCTCTT; the second fragment starts with GTAACATGGTTTGATTAT and ends with CTCACTCTTCAGTCGGAAATGA. To make the mouse *Tarbp2* minigene, a 925-bp genomic fragment from exon 7 to exon 9 was amplified with primer pair of GTTGTTGTTCGACGCCACCATGGGCACTTCCAAAAGCTGGCA-AAGC and GTTGTGGATCCGCAGTGGGATGCTACTTGCTAC. This genomic fragment was cloned into vector pIRES2-eGFP between Sall and BamHI sites.

Minigene assay

BCL-X minigene assay for alternative splicing was adapted from published methods (22, 23). HEK293T cells were transfected with *BCL-X* minigene and constructs expressing hnRNPF and FOXP3 mutants. 24 h after transfection, RNA was extracted with TRIzol reagent (Invitrogen) and reverse transcribed into cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR was performed on the cDNA with a minigene-specific forward primer (CATTCAGTGACCTGACATCCCA) and a reverse primer that located on pIRES2 vector (ACAGACCTTGCATTCTTTGGC) and resolved on 2% agarose gel. Ethidium bromide-stained gels were captured by Gel Logic 2200 Imaging System (Carestream) and the band intensities of BCL-XS and BCL-XL were quantified by Image J software. Mouse *Tarbp2* minigene assay was performed similarly as described above except that PCR was performed with the forward primer that was used for cloning of the *Tarbp2* minigene. All minigene assays were performed with triplicates and repeated at least twice.

Co-immunoprecipitation

24 h after transfection, Jurkat cells and HEK293T cells were washed with ice-cold PBS and then resuspended in 450 μ l of ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1% Nonidet P-40, 4 mM EDTA, 50 mM NaF, 1 mM Na₃VO₄, 2 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A). The cells were sonicated and centrifuged. The supernatant was transferred to a fresh tube containing 400 μ l of lysis buffer and precleared with protein A/G plus agarose beads (Santa Cruz Biotechnology) for 1 h before 4 μ g of anti-FLAG was added. Immunoprecipitates were collected with protein A/G plus. Western blotting was conducted using rabbit anti-FOXP3, mouse monoclonal anti-FOXP3 (clone 150D), or anti-FLAG antibodies.

To verify interaction of endogenous hnRNPF/H and FOXP3 in mouse regulatory T cells, naive CD4 T cells were differentiated *in vitro* into iTregs (see “iTreg induction and suppressive

function assay” for details). iTregs were processed as described above, precipitated with mouse anti-hnRNPF/H mAb (clone 1G11, Santa Cruz Biotechnology) or normal mouse IgG. Co-immunoprecipitation of hnRNPF/H and FOXP3 were examined by Western blotting using anti-hnRNPF/H and anti-FOXP3 antibodies.

Retrovirus transduction

Retrovirus was generated by transfecting Plat E cells with TransIT-293 and specific retroviral plasmid. Retrovirus was harvested between 48 and 72 h after transfection and was supplemented with 9 μ g/ml of polybrene just before T cell transduction. T cells were transduced on day 2 of TCR stimulation at 2000 rpm for 2 h at room temperature.

iTreg induction and suppressive function assay

Mouse naive CD4 T cells were isolated with magnetic bead sorting as described (35). For iTreg induction, naive CD4 T cells isolated from *Foxp3^{eGFP}* mice were stimulated for 4 to 5 days with 1 μ g/ml plate-bound anti-CD3 ϵ (145–2C11) and 1 μ g/ml soluble anti-CD28 in the presence of 5 ng/ml recombinant human TGF- β 1 and 20 ng/ml recombinant mouse IL-2.

To examine the effect of hnRNPF overexpression on iTreg suppressive function, iTregs were transduced with retrovirus encoding hnRNPF and Thy1.1 marker on day 2 of differentiation. On day 5, transduced iTregs were sorted by the expression of eGFP and retroviral marker Thy1.1. Suppressive function assay was carried out in round bottom 96-well plate that was coated with 2 μ g/ml anti-CD3 ϵ . 2.5×10^4 CFSE-labeled naive CD4 T cells of CD45.1 mice were cultured with sorted iTregs at a series of ratios in the presence of 1.2 μ g/ml soluble anti-CD3 ϵ and 3.5 μ g/ml anti-CD28. 4 days after culture, CFSE intensity was examined for the CD45.1⁺ cells by flow cytometry.

RNA immunoprecipitation

HEK293T cells were transfected with expression plasmid of FOXP3 or vector only. 2 days after transfection, cells were fixed with 1% formaldehyde for 10 min after washing with Dulbecco's PBS (Invitrogen). Fixation was stopped by 0.25 M glycine for 5 min. Cells were then harvested and suspended with nuclear isolation buffer (1.28 M sucrose, 40 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 4% Triton X-100) supplemented with proteinase inhibitor mixture (Roche). Cells were incubated on ice for 20 min with frequent mixing. Nuclei were precipitated by centrifugation at $2500 \times g$ for 15 min. Nuclei were then suspended in RNA immunoprecipitation (RIP) buffer (150 mM KCl, 25 mM Tris, pH 7.4, 5 mM EDTA, 0.5 mM DTT, 0.5% Nonidet P-40, 100 units/ml RNase inhibitor and protease inhibitors), sonicated, and centrifuged at $15,000 \times g$ for 10 min. The supernatant was precleared with salmon sperm DNA blocked Protein A/G plus bead (Santa Cruz Biotechnology) for 30 min at 4 °C. Immunoprecipitation was then performed by adding hnRNPF/H mAb (clone 1G11, Santa Cruz Biotechnology) to the precleared supernatant followed by incubation at 4 °C with rotating overnight. Protein A/G plus bead blocked with salmon sperm DNA was then added and incubated with rotating at 4 °C for 3 h. The beads were then washed with RIP buffer for three times followed by proteinase K digestion. Cross-link was reversed with

0.3 M NaCl at 65 °C for 5 h. Glycogen was added before RNA isolation with TRIzol reagent. *BCL-X* pre-mRNA co-immunoprecipitated with hnRNPF/H was quantified by RT-qPCR.

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