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HIPK2 directs cell type–specific regulation of STAT3 transcriptional activity in Th17 cell differentiation

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T helper 17 (Th17) cells are important in adaptive immunity and are also implicated in inflammatory and autoimmune disorders. Th17 cell differentiation from naïve CD4$^+$ T cells is tightly regulated in gene transcription through coordinated activities of the signal-responsive transcription factor STAT3 (signal transducer and activator of transcription 3), the pioneering factors IRF4/BATF, and the Th17-specific transcription factor RORγT, which support Th17 immune functions. Given that STAT3 acts as a master transcription factor in different cell types, whether STAT3 is regulated in a Th17-specific manner has remained a major unanswered question. In this study, we report that in mouse Th17 cells, Stat3 phosphorylation at serine 727, required for its transcriptional activity, is carried out by homeodomain-interacting protein kinase 2 (Hipk2), a nuclear kinase selectively up-regulated in Th17 cells, but not other Th subtypes that have distinct functions in immunity. Unexpectedly, we found that *Hipk2* transcriptional expression is directed by Stat3 itself in Th17 cells and that, upon expression, Hipk2 in turn phosphorylates Stat3 at S727 that potentiates Stat3 activity for transcriptional activation of Th17 signature genes such as *Il17a/f* to ensure productive Th17 cell differentiation. We validated the in vivo function of Hipk2 for Th17 cell development in T cell–induced colitis in mice using *Hipk2*-knockout mice. Our study presents a previously unrecognized mechanism of self-directed cell type–specific regulation of the master transcription factor Stat3 through its own transcriptional target Hipk2 in Th17 cell differentiation, and suggests a therapeutic strategy for developing a targeted therapy for Th17-associated inflammatory disorders.

gene transcription | Th17 cell differentiation | chromatin biology | STAT3

Significance

STAT3 (signal transducer and activator of transcription 3) is a master transcription factor that organizes cellular responses to cytokines and growth factors and is implicated in inflammatory disorders. STAT3 is a well-recognized therapeutic target for human cancer and inflammatory disorders, but how its function is regulated in a cell type–specific manner has been a major outstanding question. We discovered that Stat3 imposes self-directed regulation through controlling transcription of its own regulator homeodomain-interacting protein kinase 2 (*Hipk2*) in a T helper 17 (Th17) cell–specific manner. Our validation of the functional importance of the Stat3–Hipk2 axis in Th17 cell development in the pathogenesis of T cell–induced colitis in mice suggests an approach to therapeutically treat inflammatory bowel diseases that currently lack a safe and effective therapy.

T helper 17 (Th17) cells are critical components of adaptive immunity in host defense against pathogens (1) and function by secreting the characteristic cytokines interleukin (IL)-17A and IL-17F to protect mucosa from bacterial and fungal infection (2). Dysregulation of Th17 cells, however, has been shown to be responsible for the onset and development of inflammatory bowel diseases, multiple sclerosis, and rheumatoid arthritis (2, 3). Lineage-specific differentiation of Th17 cells from naïve CD4$^+$ T cells is tightly regulated in gene transcription that involves the cooperation of the signal-responsive transcription factor STAT3 (signal transducer and activator of transcription 3), the pioneering factors IRF4/BATF, and the Th17 lineage–specific regulator RORγT (4), which together define the gene transcriptional program of Th17 cells and support their functions in immunity (5). This transcriptional program is induced by IL-6 and transforming growth factor β-1 (TGFβ-1) stimulation (6–8) and sustained by an autocrine IL-21 signaling pathway to maintain Th17 cell identity (9). Recent studies from us and others reported that BET (bromodomain and external domain) family proteins BRD2 and BRD4 also participate in transcriptional regulation in Th17 cell differentiation through their distinct functions for chromatin structure organization and transcriptional activation, respectively (10, 11). Notably, the key Th17 regulator STAT3 also functions as an important transcription regulator in many different cell types. However, our current understanding as to whether and how STAT3 is regulated in a Th17-specific manner has remained elusive.

It is known that STAT3 function is tightly regulated by two phosphorylation events at tyrosine 705 (Y705) and serine 727 (S727) (12). The former STAT3 Y705 phosphorylation (STAT3_pY705) is typically carried out by Janus tyrosine kinase family members upon cell-surface receptor activation by cytokines such as IL-6 and IL-21, resulting in STAT3 dimerization through STAT3 SH2 domain binding to pY705 and subsequent nuclear translocation (13). The latter STAT3 S727 phosphorylation (STAT3_pS727), reportedly by members of the mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) families (14–16), is important for

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The authors declare no competing interest.

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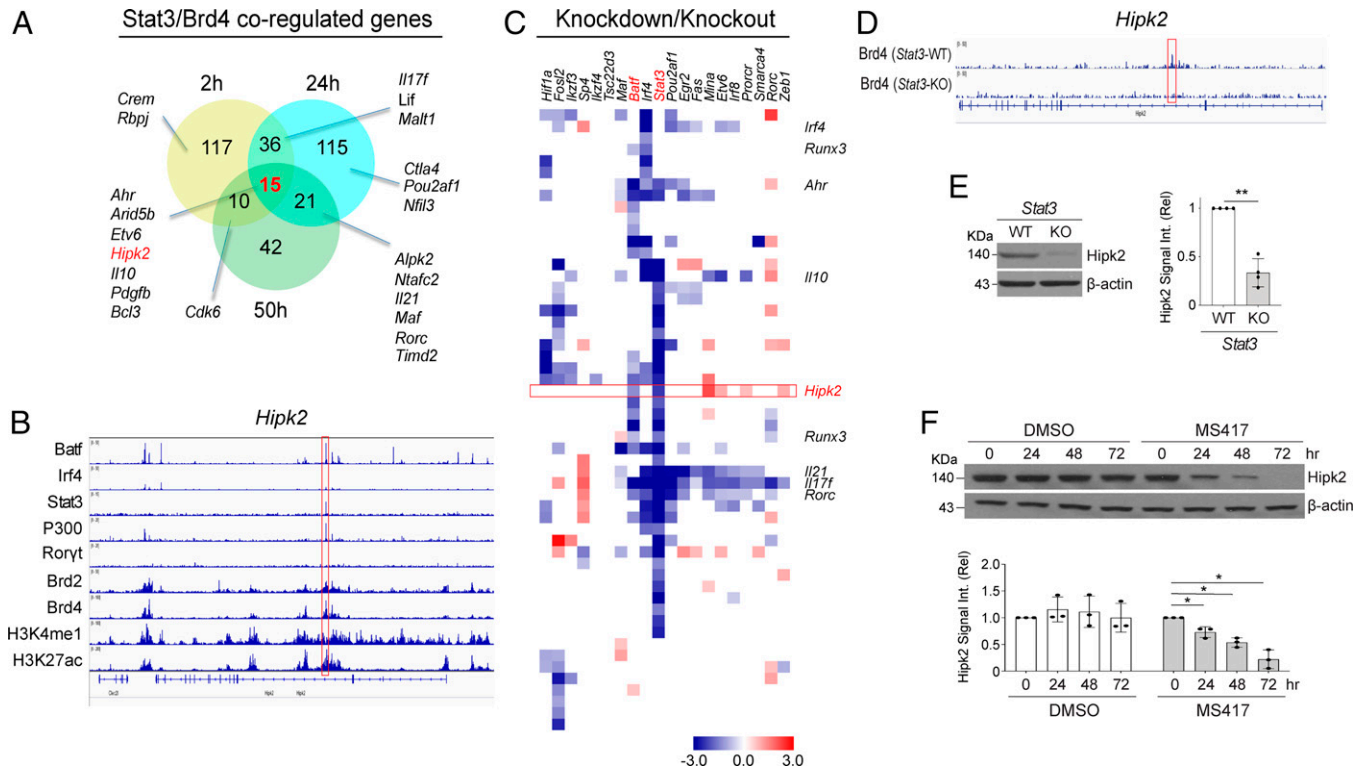

Fig. 1. Regulation of transcriptional expression of *Hipk2* in Th17 cells by Stat3 and Brd4. (*A*) Venn diagram showing Stat3- and Brd4-cobound genes that exhibited decreased transcriptional expression in mouse Th17 cells after treatment of the Brd4 BrD inhibitor MS402 for 2, 24, and 50 h, as indicated. Mouse naïve CD4$^+$ T cells were activated with Th17 conditions (anti-CD3, anti-CD28, IL-6, and TGFβ-1) for 72 h before analysis, unless otherwise indicated. (*B*) ChIP-seq profile of Batf, Irf4, Stat3, p300, RORγT, Brd2, Brd4, H3K4me, and H3K27ac on the *Hipk2* gene locus in mouse Th17 cells. (*C*) Heatmap of mRNA expression of Brd4-regulated genes (*y axis: Irf4*, *Runx3*, *Ahr*, *Il10*, *Hipk2*, *Runx3*, *Il21*, *Il17f*, and *Rorc*) under different KO/knockdown conditions (*x* axis) in Th17 cells. (*D*) ChIP-seq profile of Brd4 on the *Hipk2* gene locus in Stat3-WT (*Stat3$^{fl/fl}$*) and Stat3-KO (*Cd4-Cre;Stat3$^{fl/fl}$*) Th17 cells. (*E*) Protein expression (*Left*) and densitometry analysis (*Right*) of Hipk2 in Stat3-WT and Stat3-KO Th17 cells collected after 72-h ex vivo differentiation of mouse naïve CD4$^+$ T cells. (*F*) Protein expression (*Left*) and densitometry analysis (*Right*) of Hipk2 and β-actin in Th17 cells treated with dimethyl sulfoxide (DMSO) control or Brd4 inhibitor MS417 (125 nM) for 24, 48, and 72 h during the course of 72-h Th17 cell differentiation with DMSO or MS417 being added on day 2, 1, or 0, respectively. Data from four (*E*) or three (*F*) independent experiments are presented as mean ± SD. Statistical analyses were performed using a paired *t* test. *$P < 0.05$, **$P < 0.01$.

STAT3 activity in gene transcription (12). In this study, we discovered that Stat3 phosphorylation at S727 in mouse Th17 cells is carried out by homeodomain-interacting protein kinase 2 (Hipk2), a nuclear kinase (17) whose function has been reported in cancer cells (18, 19), neurons (20, 21), adipose cell differentiation (22), and kidney fibrosis (23) but not in the immune system and particularly T cells. We showed in this study that in mouse T helper subtypes that have distinct functions in immunity (1), differentiated from mouse primary naïve CD4$^+$ T cells from spleen and lymph nodes, Hipk2 is selectively up-regulated in transcription in Th17 cells with low expression in Th2 and almost none in Th1 and regulatory T (Treg) cells. Hipk2 phosphorylates Stat3 at S727 and enhances Stat3 transcriptional activity that is required for lineage-specific differentiation of Th17 cells.

Results and Discussion

Discovery of *Hipk2* as a Stat3/Brd4–Regulated Gene in Th17 Cell Differentiation. To identify new regulators for Th17 cell differentiation, we focused our study on genes whose transcription is regulated by Stat3 and Brd4 in Th17 cells, identified from the available chromatin immunoprecipitation sequencing (ChIP-seq) and RNA-seq data for Stat3 and Brd4 (5, 11) (Dataset S1). We analyzed transcriptional expression changes of such genes in our RNA-seq data of mouse Th17 cells differentiated ex vivo from mouse primary naïve CD4$^+$ T cells isolated from spleen and lymph nodes with and without treatment of MS402, a BRD4 inhibitor (Fig. 1*A*). MS402 targets the first bromodomain (BrD) of BRD4 and selectively inhibits Th17 cell differentiation, with minimal effects on other T helper cell subtypes such as Th1, Th2, and Tregs (24). In addition to known regulators such as *Ikzf3*, *Il21*, and *Rorc*, we discovered that among a set of Stat3/Brd4–coregulated genes, *Hipk2* is expressed as early as 2 h in Th17 cell differentiation (Fig. 1*A* and *SI Appendix*, Fig. S1*A*). This is supported by ChIP-seq data of Th17-specific transcription factors (5, 11), showing that *Hipk2* gene loci are bound by key transcription factors and coregulators including Batf, p300, Stat3, Brd2, and Brd4, and also enriched with histone modifications indicative of active transcription such as histone H3 lysine 4 monomethylation (H3K4me1) and H3 lysine 27 acetylation (H3K27ac) (Fig. 1*B*).

We further analyzed the reported RNA-seq data of Th17 cells generated with knockout (KO) or knockdown of key transcription factors (5) (Dataset S1), which revealed that *Hipk2* expression is positively regulated by Batf and Stat3 and negatively by Mina, Prorc, Zeb1, and Etv6, while not affected by Irf4 and Rorγt (Fig. 1*C*). We found that Stat3 regulates Brd4 binding to *Hipk2* gene loci, as Brd4 occupancy was nearly abolished in Th17 cells derived from *Stat3*-KO (*Cd4-Cre;Stat3$^{fl/fl}$*) mice (25) (Fig. 1*D*). Our Western blotting analysis showed that the protein level of Hipk2 was dramatically reduced in *Stat3*-KO cells (Fig. 1*E*) or by chemical inhibition of Brd4 with the BET BrD inhibitor MS417 (26) in a time- or dose-dependent manner (Fig. 1*F* and *SI Appendix*, Fig. S1*B*). These results indicate that the transcription of *Hipk2* is regulated by Stat3 and Brd4 in Th17 cell differentiation.

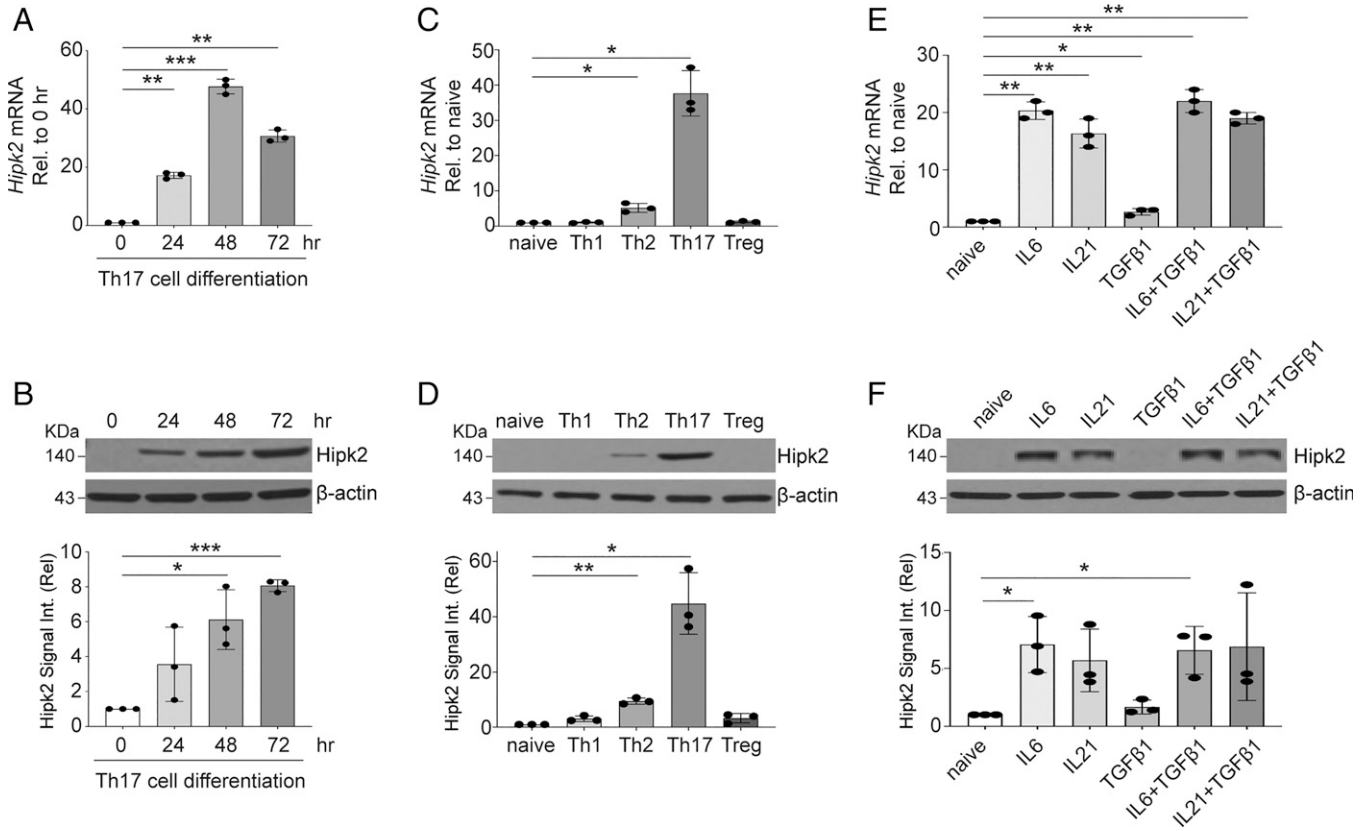

Fig. 2. Hipk2 is selectively expressed during Th17 cell differentiation. (*A* and *B*) *Hipk2* mRNA (*A*) and protein expression (*B*, *Upper*) and densitometry analysis (*B*, *Lower*) as assessed at 0, 24, 48, and 72 h during Th17 cell differentiation (anti-CD3, anti-CD28, IL-6, and TGFβ-1) from mouse primary naïve $CD4^+$ T cells. (*C* and *D*) *Hipk2* mRNA (*C*) and protein expression (*D*, *Upper*) and densitometry analysis (*D*, *Lower*) in mouse primary naïve, Th1 (IL-12 and anti–IL-4), Th2 (IL-4, anti–IL-12, and anti–IFN-γ), Th17 (IL-6 and TGFβ-1), and Treg (TGFβ-1) cells after 72 h of cell differentiation. (*E* and *F*) *Hipk2* mRNA (*E*) and protein expression (*F*, *Upper*) and densitometry analysis (*F*, *Lower*) in mouse primary naïve T cells treated with IL-6, IL-21, TGFβ-1, IL-6+TGFβ-1, or IL-21+TGFβ-1 for 72 h. Data from three independent experiments are presented as mean ± SD. Statistical analyses were performed using a paired *t* test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Hipk2 Is Functionally Important for Th17 Cell Differentiation. Hipk2 expression showed a rapid increase in both messenger RNA (mRNA) and protein levels in ex vivo Th17 cell differentiation, peaking at 48 to 72 h (Fig. 2 *A* and *B*). Hipk2 expression appears to be selective in Th17 cells, as there is only very low expression in Th2 cells, and almost no expression in Th1 or Treg subtypes (Fig. 2 *C* and *D*). Hipk2 expression in Th17 cells was induced by IL-6 or IL-21 but independent of TGFβ-1, as combined treatment of IL-6 or IL-21 with TGFβ-1 did not cause further increase in Hipk2 expression over IL-6 or IL-21 treatment alone (Fig. 2 *E* and *F*). Collectively, these results indicated the IL-6/IL-21–Stat3 pathway as an upstream activator of Hipk2 expression. Notably, Hipk2 kinase activity is dependent on *cis*-autophosphorylation of Y354 and S357 residues in its activation loop (27), a mechanism ensuring that newly synthesized Hipk2 is at least partially active even in the absence of additional signal. Thus, increased Hipk2 expression can possibly lead to an increase of total Hipk2 activity in Th17 cells.

To assess the role of Hipk2 in Th17 cell differentiation, we generated tamoxifen-inducible *Hipk2*-KO mice by crossing *Hipk2*$^{fl/fl}$ mice (28) with transgenic mice expressing Cre recombinase under the control of the Rosa26 promoter (*Rosa26-Cre-ERT2* mice). Using Th17 cells differentiated from naïve $CD4^+$ T cells isolated from *Cre-ERT2-Hipk2*$^{fl/fl}$ mice injected with tamoxifen (*SI Appendix*, *Materials and Methods*), we showed that *Hipk2*-KO resulted in marked down-regulation of transcriptional expression of *Il17a*, *Il17f*, and *Il23r*, but not *Rorc*, in Th17 cells as compared with *Hipk2* wild-type (WT) mice (Fig. 3*A*), and reduction of lineage-specific differentiation of Th17 cells with nearly no effects on differentiation of Th1, Th2, and Treg subtypes (Fig. 3*B* and *SI Appendix*, Fig. S2*A*). We further excluded a possibility that Hipk2 has any effects on early RorγT expression (*SI Appendix*, Fig. S2*B*). In addition, we showed that $CD4^+RorγT^+IL\text{-}17A^+$ but not $CD4^+RorγT^+$ cells are dependent of Hipk2 expression (*SI Appendix*, Fig. S2*C*), further confirming Hipk2 controls IL-17A expression in $RorγT^+$ Th17 cells. Consistent with this observation, protein levels of IL-17A and IL-17F were decreased significantly in the *Hipk2*-KO cells over the WT cells (Fig. 3*C*). Transcriptional expression of lineage-specific transcription factors and cytokines in Th1 (*Tbx21*, *Ifng*), Th2 (*Gata3*, *Il4*), and Treg (*Foxp3*, *Il10*) cells was generally independent of *Hipk2* (Fig. 3*D*). Collectively, these results suggested that Hipk2 is functionally important for lineage-specific differentiation of Th17 cells, but not other Th1, Th2, and Treg subtypes.

Hipk2 Phosphorylates Stat3 at S727 in Th17 Cells. Hipk2 induction through IL-6– and IL-21–dependent signaling pathways is in agreement with the fact that Stat3 is a common downstream transcription factor activated by IL-6 and IL-21 (9), and that Stat3 is important for *Hipk2* expression in Th17 cells (Fig. 1*E*). The function of Stat3 is tightly regulated by phosphorylation at Y705 and S727 (14–16). Unexpectedly, we discovered that *Hipk2*-KO Th17 cells resulted in a marked reduction of Stat3 phosphorylation at S727 (Stat3_pS727) but did not significantly affect Stat3 phosphorylation at Y705 (Stat3_pY705) (Fig. 4*A*). We showed that increase in Stat3_pY705 level preceded Hipk2 expression in Th17 cell

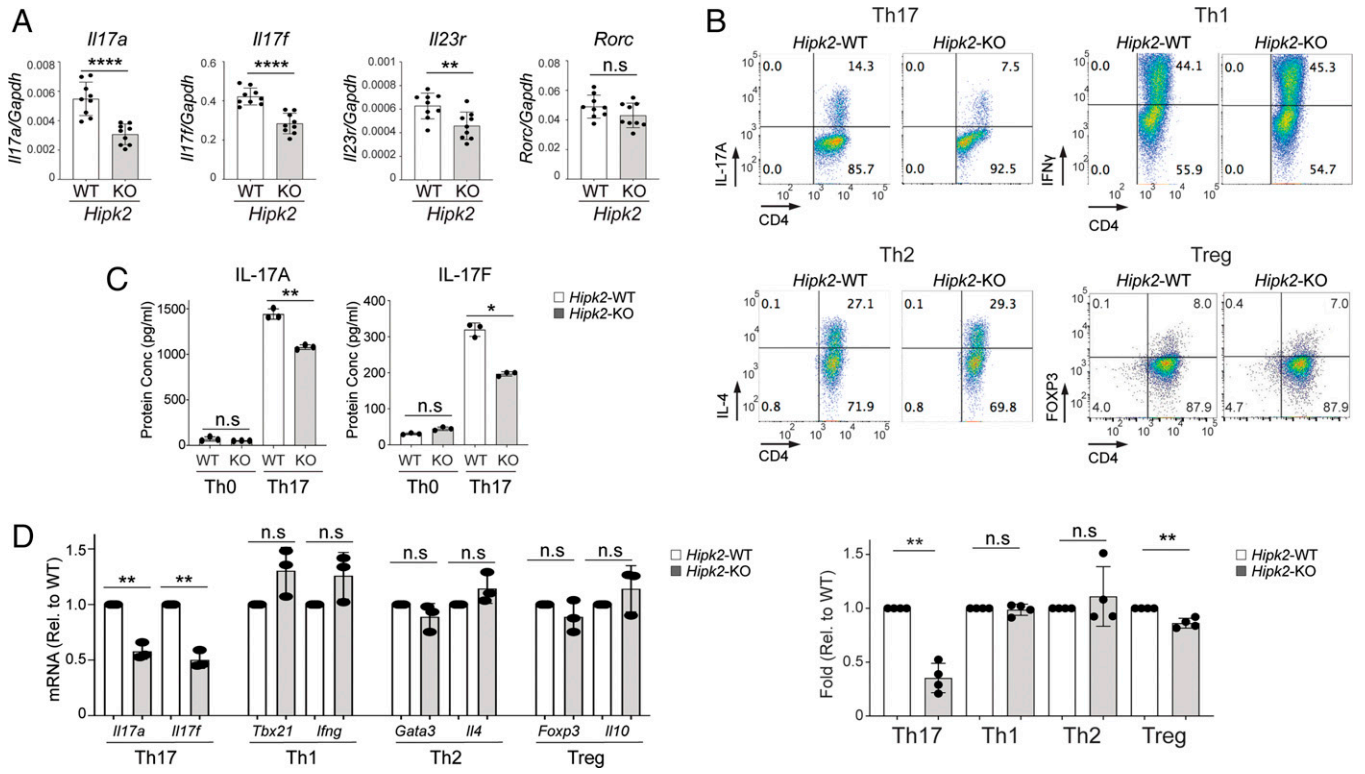

Fig. 3. Hipk2 is functionally important for Th17 cell differentiation. (*A*) mRNA expression of *Il17a*, *Il17f*, *Il23r*, and *Rorc* in Hipk2-WT (*Hipk2*$^{fl/fl}$) and Hipk2-KO (*Cre-ERT2;Hipk2*$^{fl/fl}$) Th17 cells differentiated for 72 h. *Hipk2*$^{fl/fl}$ and *Cre-ERT2;Hipk2*$^{fl/fl}$ naïve T cells were isolated from mice injected with tamoxifen to induce Cre recombination (*SI Appendix, Materials and Methods*). (*B*) Flow cytometry plots (*Upper*) and statistical analysis (*Lower*) of Th17, Th1, Th2, and Treg cells differentiated from Hipk2-WT and Hipk2-KO mouse primary naïve CD4$^+$ T cells for 72 h. (*C*) ELISA analysis of IL-17A and IL-17F in supernatant of Hipk2-WT and Hipk2-KO Th17 cells cultured for 72 h. (*D*) mRNA expression levels of *Il17a* and *Il17f* in Th17 cells, *Tbx21* and *Ifng* in Th1 cells, *Gata3* and *Il4* in Th2 cells, and *Foxp3* and *Il10* in Treg cells, differentiated ex vivo for 72 h from mouse (Hipk2-WT and Hipk2-KO) primary naïve CD4$^+$ T cells. Data from three or more independent experiments (n = 9, *A*; n = 4, *B*; n = 3, *C* and *D*) are presented as mean ± SD. Statistical analyses were performed by unpaired (*A*) and paired (*B–D*) t tests. *P < 0.05, **P < 0.01, ****P < 0.0001; n.s, not significant.

differentiation, while Stat3_pS727–level increase was correlated with Hipk2 expression in a time-dependent manner (Fig. 4*B*). Notably, our RNA-seq data collected at different time points in Th17 cell differentiation demonstrated that *Hipk2* and *Il17a* exhibited a steady increase in transcriptional expression, whereas Dusp2, a dual-specificity protein phosphatase that has been suggested to act as a negative regulator of Th17 cell differentiation through dephosphorylation of Stat3 at both Y705 and S727 sites (29), showed a decrease in expression (*SI Appendix*, Fig. S3*A* and Dataset S2). Moreover, many other kinases showed a reduction (*Dyrk1*, *Mapk8/JNK1*) or remained stable in mRNA expression levels (*Csnk2a1*, *Irak1*, *Mapk1/ERK2*, *Cdk8*, *Prkcd*, *Rhoa*) during Th17 cell differentiation (*SI Appendix*, Fig. S3 *B* and *C*). Taken together, these results suggested a possible functional connection between Hipk2 and Stat3 phosphorylation at S727.

We confirmed with immunoprecipitation experiments that Hipk2 interacts specifically with Stat3 in Th17 cells (Fig. 4*C*). The HIPK2–SMAD2 interaction was reported to be important in kidney fibrosis (23). We found that in Th17 cells the Hipk2–Stat3 interaction appears specific, as no Hipk2–Smad2/3 or Hipk2–p65 interaction was detected (Fig. 4*C*). This observation was supported by the failure of an allosteric HIPK2–SMAD2/3 inhibitor, BT173 (30), to inhibit Th17 cell differentiation (*SI Appendix*, Fig. S4*A*). Moreover, *Hipk2* deficiency reduced the level of Stat3_pS727 but did not affect levels of phosphorylated Smad2/3 (pSmad2/3) (*SI Appendix*, Fig. S4*B*). With ectopic expression of Flag-tagged Stat3 and myc-tagged Hipk2 in HEK293 cells, we further confirmed that Hipk2 interacts with Stat3, resulting in phosphorylation of Stat3_S727 (Fig. 4*D*). Notably, Hipk2 can bind Stat3_S727A but not Stat3_Y705A, and the latter showed very little if any S727 phosphorylation in Stat3 (Fig. 4*D*), indicating that Y705 phosphorylation and dimerization of Stat3 are likely a prerequisite for its phosphorylation at S727 by Hipk2. We also observed that Hipk2 is present in both the cytoplasm and nucleus in Th17 cells (Fig. 4*B*), consistent with a previous study with different cell types (31). Using fractionated Th17 cells, we detected that Hipk2 and Stat3 interaction occurs in both the cytoplasm and nucleus (*SI Appendix*, Fig. S4*C*), agreeing with increased phosphorylation of Stat3_S727 in both fractions during Th17 cell differentiation (Fig. 4*B*). Collectively, from these results, we concluded that Hipk2 interacts with and phosphorylates Stat3 at S727 in the cytoplasm and nucleus in Th17 cells, and that the Stat3–Hipk2 axis is likely a specific node for transcriptional regulation of Th17 cell differentiation.

Stat3–Hipk2 Functions to Regulate Expression of Th17 Signature Genes. To determine the functional role of Stat3–Hipk2 interaction in gene transcription in Th17 cells, we found by ChIP-qPCR that Hipk2 is present together with Stat3_pS727 at Stat3 target gene loci in chromatin including *Il17a* and *Il17f*, but not *Rorc* (Fig. 5*A*). This agrees with our result that *Il17a* and *Il17f* but not *Rorc* expression were decreased in *Hipk2*-KO cells (Fig. 3*A*). Overexpression of Stat3 alone had minimal induction of luciferase activity in a Stat3-reporter assay (Fig. 5*B*). Coexpression of Stat3 and Hipk2, but not kinase-dead Hipk2-K228R, strongly induced luciferase activity, while coexpression of Stat3 phosphorylation site mutant Stat3_Y705A or Stat3_S727A with Hipk2 showed only

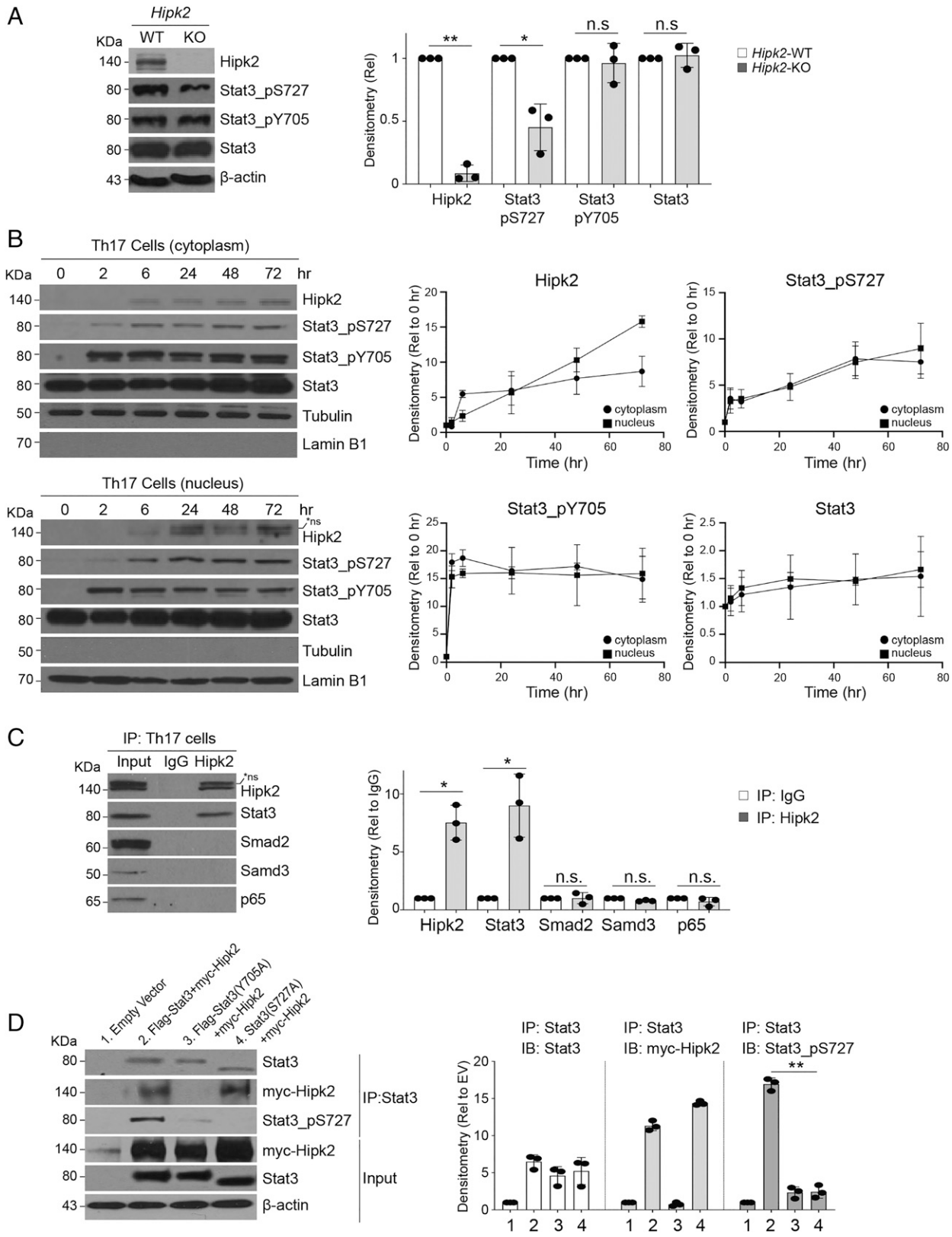

Fig. 4. Hipk2 phosphorylates Stat3 at S727 in Th17 cells. (*A*) Protein expression (*Left*) and densitometry analysis (*Right*) of Hipk2, Stat3_pS727, Stat3_pY705, Stat3, and β-actin in Hipk2-WT (*Hipk2*$^{fl/fl}$) and Hipk2-KO (*Cre-ERT2;Hipk2*$^{fl/fl}$) Th17 cells differentiated for 72 h. (*B*) Protein expression (*Left*) and densitometry analysis (*Right*) of Hipk2, Stat3_pS727, Stat3_pY705, and Stat3 during Th17 cell differentiation of mouse primary naïve CD4^{+} T cells. The cells were collected at different time points (0, 2, 6, 24, 48, and 72 h) and fractionated into cytosolic and nuclear fractions, followed by Western blotting of Hipk2, Stat3_pS727, Stat3_pY705, Stat3, tubulin, and lamin B1. *ns denotes a nonspecific band. (*C*) Th17 cells differentiated for 72 h were assessed for Hipk2–Stat3, Hipk2–Smad2, Hipk2–Smad3, and Hipk2–p65 interactions using immunoprecipitation of Hipk2 followed by Western blotting of Hipk2, Stat3, Smad2, Smad3, and p65 (*Left*) and densitometry analysis (*Right*). (*D*) Analysis of Stat3–Hipk2 interaction in HEK293 cells transiently cotransfected with combinations of myc-Hipk2, flag-Stat3, flag-Stat3(Y705A), and Stat3(S727A). Cell lysates were subjected to immunoprecipitation with Stat3 antibody, followed by Western blotting analysis as indicated (*Left*) and densitometry analysis (*Right*). EV, empty vector; IB, immunoblotting. Data are shown as one representative of three independent experiments presented as mean ± SD. Statistical analyses were performed using a paired *t* test. *$P < 0.05$, **$P < 0.01$; n.s, not significant.

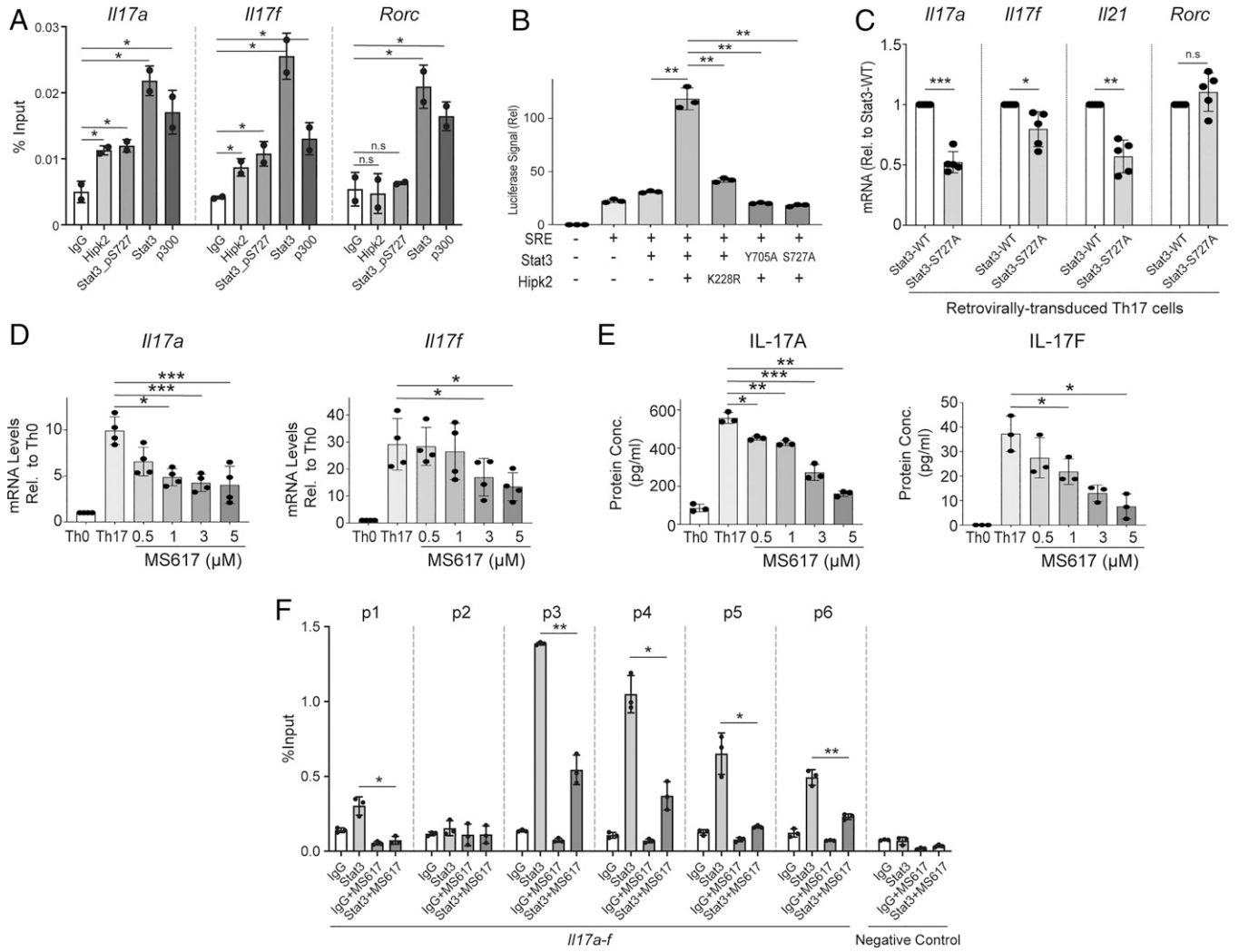

Fig. 5. Hipk2 phosphorylation of Stat3 at Ser727 is required for Th17 signature gene expression. (*A*) ChIP-qPCR analysis of Hipk2, Stat3_pS727, Stat3, and p300 binding at Stat3-binding sites of *Il17a*, *Il17f*, and *Rorc* gene loci in Th17 cells differentiated for 72 h. (*B*) Luciferase reporter assay assessing effects of cotransfection of Hipk2, Hipk2(K228R), Stat3, Stat3(Y705A), or Stat3(S727A) on STAT3-response element (SRE) transcriptional activation in HEK293 cells. (*C*) Naïve CD4$^+$ T cells were retrovirally transduced with virus overexpressing Stat3-WT and Stat3(S727A) (S→A mutation of Stat3_S727), followed by Th17 cell differentiation for 72 h and qPCR analysis of *Il17a*, *Il17f*, *Il21*, and *Rorc*. (*D*) mRNA level of *Il17a* and *Il17f* in Th17 cells differentiated for 72 h and treated with Hipk2 inhibitor MS617 (0.5, 1, 3, and 5 μM) on day 0. (*E*) ELISA analysis of IL-17A and IL-17F in supernatant of Th17 cells differentiated for 72 h and treated with Hipk2 inhibitor MS617 (0.5, 1, 3, and 5 μM) on day 0. (*F*) ChIP-qPCR analysis of Stat3 binding on Stat3-binding sites (p1 to p6) of *Il17a/f* gene loci and gene desert region (negative control) in Th17 cells differentiated for 72 h and treated with Hipk2 inhibitor MS617 (5 μM) on day 0. Data from two (*A*), three (*B*, *E*, and *F*), five (*C*), and four (*D*) independent experiments are presented as mean ± SD. Statistical analyses were performed by unpaired (*A*) and paired (*B*–*F*) *t* tests. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; n.s, not significant.

a basal level of the luciferase activity (Fig. 5*B*). We further established that Hipk2 phosphorylation of Stat3_S727 is required for expression of key Th17 genes, as *Il17a*, *Il17f*, and *Il21* but not *Rorc* expression were decreased in Th17 cells retrovirally transduced with vectors overexpressing Stat3-S727A, as compared with Stat3-WT (Fig. 5*C*).

Since Stat3 and p300 colocalize on the loci of Th17 signature genes *Il17a* and *Il17f* in Th17 cells (Fig. 5*A*), we investigated and found that both Stat3+Hipk2 and Stat3+p300 induced similar levels of luciferase activity, while Stat3+Hipk2+p300 displayed synergistic induction in transcriptional activity (*SI Appendix*, Fig. S5*A*). To further investigate the function of endogenous Hipk2 in Th17 cells, we compared the transcription factor–complex formation in Th17 cells transfected with small interfering RNAs targeting negative control or *Hipk2*. Knockdown of *Hipk2* resulted in decreased Irf4–Stat3 interaction, suggesting that Hipk2 mediates Stat3 chromatin binding, as Irf4 is known as a transcription factor that pioneers the chromatin access of Stat3 in Th17 cells (5) (*SI Appendix*, Fig. S5*B*).

Finally, using a HIPK2 kinase inhibitor, MS617 (32), we showed that chemical inhibition of Hipk2 resulted in a decrease of both mRNA and protein levels of IL-17A and IL-17F in Th17 cells in a dose-dependent manner (Fig. 5 *D* and *E*), which is correlated with reduced Stat3 occupancy on *Il17a/f* gene loci (Fig. 5*F*). Collectively, these results from our genetic and chemical biology studies demonstrated that Stat3 phosphorylation at S727 by Hipk2 potentiates Stat3 function as a major transcription factor to direct transcriptional activation of Th17 signature genes important for lineage-specific differentiation of Th17 cells.

Hipk2 Controls Th17 Cell Development In Vivo. Because Th17 cells have been implicated in the development of inflammatory disorders such as inflammatory bowel diseases (33, 34), we evaluated the in vivo effects of *Hipk2* KO on Th17 cell development in the mouse model of T cell transfer–induced colitis (Fig. 6*A*), which recapitulates the disease conditions of

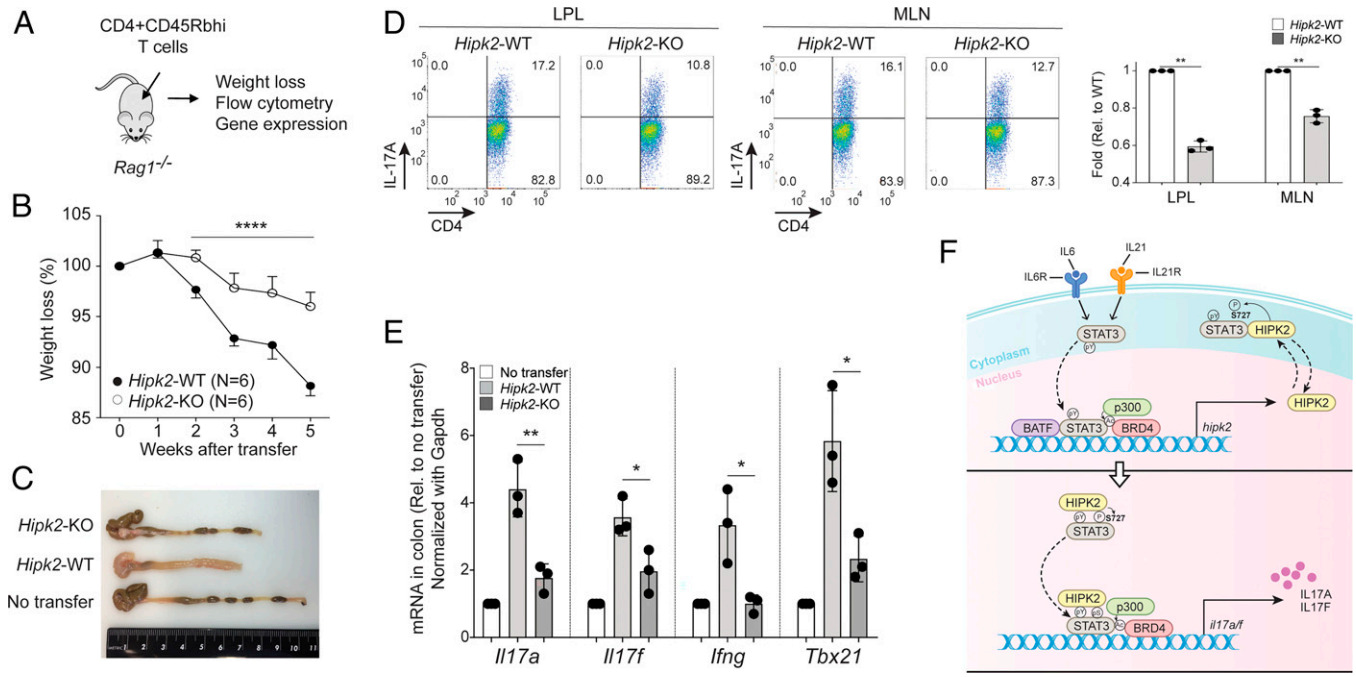

Fig. 6. Hipk2 is required in T cell transfer–induced colitis development in *Rag1*$^{-/-}$ mice. (*A*) Schematic diagram illustrating the T cell transfer–induced colitis mouse model study. (*B*) Body weight of *Rag1*$^{-/-}$ mice injected with Hipk2-WT (*Hipk2*$^{fl/fl}$) or Hipk2-KO (*Cre-ERT2;Hipk2*$^{fl/fl}$) naïve CD4^{+} T cells isolated from mouse spleen and lymph nodes. *Hipk2*$^{fl/fl}$ and *Cre-ERT2;Hipk2*$^{fl/fl}$ naïve T cells were isolated from mice injected with tamoxifen to induce Cre recombination (*SI Appendix, Materials and Methods*). (*C*) Assessment of colon length of *Rag1*$^{-/-}$ mice injected with Hipk2-WT or Hipk2-KO naïve CD4^{+} T cells, or PBS ("no transfer") at the end of the in vivo study (5 wk after T cell transfer). (*D*) Flow cytometry (*Left*) and statistical analysis (*Right*) of Hipk2-WT or Hipk2-KO Th17 cells in lamina propria (LPL) and mesenteric lymph nodes (MLN) at the end of the in vivo study. (*E*) mRNA expression of *Il17a*, *Il17f*, *Ifng*, and *Tbx21* in colon tissue of *Rag1*$^{-/-}$ mice injected with PBS (no transfer), Hipk2-WT, or Hipk2-KO naïve T cells. (*F*) Schematic diagram illustrating transcription regulation of the *Hipk2* gene by Stat3, p300, Batf, and Brd4, and the function of Hipk2 in regulation of Stat3 activity for transcriptional expression of Th17 signature genes such as *Il17a/f* during Th17 cell differentiation. Data (*D* and *E*) from three independent experiments are presented as mean ± SD. Statistical analyses were performed using two-way ANOVA multiple-comparisons (*B*), paired (*D*), and unpaired (*E*) *t* tests. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

inflammatory bowel diseases in humans (35). After reconstitution with naïve CD4^{+}CD45RB^{hi} T cells isolated from spleen and lymph nodes of tamoxifen-treated *Hipk2*-WT and -KO mice, *Rag1*$^{-/-}$ mice injected with WT naïve T cells began losing weight after 3 wk, whereas mice injected with *Hipk2*-KO naïve T cells showed less weight loss (Fig. 6*B*). The WT group mice showed markedly shorter inflamed colon as compared with the KO group (Fig. 6*C*). Furthermore, the WT group mice exhibited more severe inflammatory cell infiltrates and a higher percentage of IL-17–producing CD4^{+} T cells in colon and mesenteric lymph nodes than the *Hipk2*-KO group mice, correlating with expression of inflammatory markers such as *Il17a*, *Il17f*, *Ifng*, and *Tbx21* in the colon tissue (Fig. 6 *D* and *E*). These results indicated that while Hipk2 is not required for Th1 in vitro polarization, Hipk2 expression may affect Th1 differentiation in vivo. Although further study is needed, one possible mechanism to reconcile these results is that Th17 cells function as precursors, which display late developmental plasticity and give rise to interferon γ (IFN-γ)–producing cells contributing to the pathogenesis of colitis (36–38). Overall, our in vivo study supported our findings that Hipk2 plays an important role in the regulation of transcriptional expression of Th17 signature genes such as *Il17a* and *Il17f*, which are critical for Th17 cell development in inflammatory disorders.

In summary, in this study, we discovered that Stat3 works with other key transcription regulators including Batf, Brd4, and p300 to direct transcriptional expression of *Hipk2* selectively in Th17 cells but not other Th1, Th2, and Treg subtypes, and that Hipk2 functions to phosphorylate Stat3 at S727, thereby further potentiating Stat3 function as a major transcription factor for activation of Th17 signature genes such as *Il17a* and *Il17f* to ensure productive lineage-specific differentiation of Th17 cells (Fig. 6*F*). Our study does not exclude a possibility that Hipk2 may play a functional role in Stat3 phosphorylation in IL-6+IL-21–polarized T follicular helper cells (39). Our study highlighted an important cell type–dependent regulatory mechanism for a master transcription factor that is achieved through its direct control of transcriptional expression of its own regulator in a cell type–specific manner. This positive feedback loop endows cells with a powerful yet tightly controlled regulatory mechanism to guide productive cell-type transition from one to another with stimulation of lineage-specific cytokines. We envision that such a self-directed feedback regulatory mechanism is likely operational in different functional contexts in cells, which warrants further investigation. Collectively, our study reports a previously unrecognized self-directed feedback regulatory mechanism of the major transcription factor Stat3 through S727 phosphorylation by its own transcriptional target gene Hipk2 in Th17 cell differentiation. Our study further suggests a therapeutic strategy of selectively targeting Hipk2 kinase activity to control Stat3 transcriptional activity to treat Th17-associated inflammatory and autoimmune disorders including inflammatory bowel diseases that currently lack a safe and effective therapy.

Materials and Methods

Methods and associated references are available in *SI Appendix, Materials and Methods*.

Mice. C57BL/6 WT mice were obtained from The Jackson Laboratory. The generation of *Hipk2*$^{fl/fl}$ mice was described in detail (28). *Hipk2*$^{fl/fl}$ mice were maintained on the C57BL/6J genetic background, and bred with *Rosa26(R26)-Cre-ERT2* mice

(The Jackson Laboratory; 008463) to generate *Cre-ERT2;Hipk2*$^{fl/fl}$ mice. To induce Cre recombination, *Cre-ERT2;Hipk2*$^{fl/fl}$ mice (21 d postnatal) were injected intraperitoneally with tamoxifen (corn oil) at a dose of 100 mg/kg body weight for 5 consecutive days.

Cell Sorting and T Helper Cell Differentiation. CD4$^+$ T cells were isolated from spleen and lymph nodes using anti-CD4 microbeads (Miltenyi Biotec). Naïve CD4$^+$ T cells were activated with plate-bound anti-CD3 (1.5 μM/mL) and anti-CD28 (1.5 μM/mL) plus cytokines, IL-12 (20 ng/mL) and anti–IL-4 (10 μM/mL) for Th1 conditions, IL-4 (20 ng/mL), anti–IL-12 (10 μM/mL), and anti–IFN-γ (10 μM/mL) for Th2 conditions, IL-6 (20 ng/mL) and TGFβ-1 (2.5 ng/mL) for Th17 conditions, and TGFβ-1 (2.5 ng/mL) for Treg conditions. The cells were cultured for 3 d before harvesting for further analysis. All cytokines were purchased from R&D Systems, and neutralizing antibodies were purchased from BD Pharmingen.

Intracellular Staining and Flow Cytometry. Cells were stimulated with phorbol myristate acetate (50 ng/mL; Sigma) and ionomycin (500 ng/mL; Sigma) for 5 h in the presence of brefeldin A (BioLegend) before intracellular staining. Cells were fixed with IC Fixation Buffer (BD Biosciences), incubated with permeabilization buffer, and stained with FITC-CD4, APC-IL-17, APC-IFN-γ, APC-IL-4, and PE-Foxp3 antibodies. Dead cells were excluded using the Live/Dead Fixable Aqua Dead Cell Stain Kit (Invitrogen). Flow cytometry was performed on a FACSCalibur (BD Biosciences) and LSRFortessa (BD Biosciences), and FlowJo (Tree Star) software was used for flow cytometry and analysis.

Enzyme-Linked Immunosorbent Assay. All enzyme-linked immunosorbent assay (ELISA) kits were purchased from eBioscience and experiments were performed according to protocols provided by the manufacturer. Briefly, supernatants of samples were incubated in plates coated with capture antibody. Detection antibody was added after a total of five washes. Avidin–horseradish peroxidase was then added after a total of five washes. Plates were read at 450 nm after addition of substrate solution and stop solution. Concentration of the cytokines in samples was calculated with reference to the absorbance value obtained from the standard curve.

T Cell Transfer–Induced Colitis Study and Histopathology. T-cell transfer-induced mouse model of colitis was performed as previously described (24). Briefly, purified CD4$^+$CD45RB^{hi} T cells from *Hipk2*$^{fl/fl}$ or *Cre-ERT2-Hipk2*$^{fl/fl}$ mice injected with tamoxifen were injected intraperitoneally into *Rag1*$^{-/-}$ recipients (5×10^5 cells per mouse in 200 μL sterile phosphate-buffered saline [PBS] per injection). Mice were weighed every week throughout the course of the experiments. After 5 wk, mice were killed and colon tissues were excised for qPCR, isolation of lamina propria mononuclear cells (40), and flow cytometry analysis.

Statistical Analysis. Statistical analysis was performed using GraphPad Prism 8.

Study Approval. Mouse experiments were approved by the Institutional Animal Care and Use Committees of the Icahn School of Medicine at Mount Sinai.

Data Availability. All study data are included in the article and/or supporting information. The ChIP-seq and RNA-seq data have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo and can be accessed via accession numbers GSE90788, GSE95052, and GSE40918.

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