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Class IIa HDAC4 and HDAC7 cooperatively regulate gene transcription in Th17 cell differentiation

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Class II histone deacetylases (HDACs) are important in regulation of gene transcription during T cell development. However, our understanding of their cell-specific functions is limited. In this study, we reveal that class IIa Hdac4 and Hdac7 (Hdac4/7) are selectively induced in transcription, guiding the lineage-specific differentiation of mouse T-helper 17 (Th17) cells from naive CD4$^+$ T cells. Importantly, Hdac4/7 are functionally dispensable in other Th subtypes. Mechanistically, Hdac4 interacts with the transcription factor (TF) JunB, facilitating the transcriptional activation of Th17 signature genes such as *Il17a/f*. Conversely, Hdac7 collaborates with the TF Aiolos and Smrt/Ncor1-Hdac3 corepressors to repress transcription of Th17 negative regulators, including *Il2*, in Th17 cell differentiation. Inhibiting Hdac4/7 through pharmacological or genetic methods effectively mitigates Th17 cell–mediated intestinal inflammation in a colitis mouse model. Our study uncovers molecular mechanisms where HDAC4 and HDAC7 function distinctively yet cooperatively in regulating ordered gene transcription during Th17 cell differentiation. These findings suggest a potential therapeutic strategy of targeting HDAC4/7 for treating Th17-related inflammatory diseases, such as ulcerative colitis.

gene transcription | HDAC4 and HDAC7 | Th17 cell differentiation | drug discovery

CD4$^+$ T cells are important immune cells in biology and have been implicated in the pathology of autoimmune diseases and cancer (1–5). Effector Th subsets such as Th1, Th2, Th17, and Treg exhibit distinct immune functions through the expression of signature genes (6, 7). Notably, Th17 cells, producing IL-17A and IL-17F, contribute to mucosa protection from bacterial and fungal infections (8, 9). Additionally, Th17 cell development is associated with inflammatory disorders, including multiple sclerosis, rheumatoid arthritis, and inflammatory bowel diseases (IBD) (10–14).

Lineage-specific maturation of Th17 cells from naive CD4$^+$ T cells is governed by cytokines, orchestrating subtype-specific gene transcription through a network of transcription factors (TFs), chromatin regulators, and modifying enzymes that define and maintain cell identity (15). Among these regulators are histone deacetylases (HDACs), recognized for their roles in gene transcription and as drug targets for human inflammatory disorders (16–21). HDACs, comprising class I (HDAC1/2/3/8), class IIa (HDAC4/5/7/9), class IIb (HDAC6/10), and class IV (HDAC11), play a crucial role in regulation of gene transcription in chromatin during T cell development (22–28). Notably, HDAC3 is necessary for efficient transit from double negative stage 4 through positive selection (22). HDAC6 mediates CD8$^+$ T cell functions in skin inflammation (23) and Th17 cell function in acute allograft lung rejection (29), while HDAC3/7/9 collaborate with the transcriptional repressor Foxp3 during Treg cell development (24, 25). Previous studies demonstrate that HDAC inhibition controls inflammation and enhances Treg cells after allogenic hematopoietic cell transplantation in humans (30, 31). The pan-HDAC inhibitor ITF2357 has been shown to decrease the Th17 phenotype and increase Treg cells in systemic lupus erythematosus (SLE) pathogenesis (26), potentially by decreasing IL6-R expression in naive T cells (27). Unlike class I HDACs, class II HDACs have tissue-specific functions and genetic associations with conditions such as lopecia (32), Huntington's diseases (33, 34), glucose homeostasis (35), and emphysema (36). While previous studies reported the anti-inflammatory effects of pan-HDAC inhibitors targeting Th17-related diseases (18, 19, 37), these inhibitors specifically target the deacetylase activity of class I/IIb HDACs but not class IIa HDACs (38). Therefore, the exact role of class IIa HDACs in Th17 cells, especially under inflammatory conditions, remains elusive. A better mechanistic understanding of class II HDACs

Significance

Histone deacetylases (HDACs) play a crucial role in gene transcription. Class I HDACs exhibit ubiquitous expression and broad functions, while class II HDACs are tissue specific. However, our limited understanding of class II HDACs hampers therapeutic development. In this study, we report that class IIa Hdac4 and Hdac7 regulate T-helper 17 (Th17) cell differentiation from mouse primary naive CD4$^+$ T cells but are dispensable in Th1, Th2, and Treg subtypes. Hdac4 facilitates transcription of Th17 genes *Il17a/f*, while Hdac7 represses transcription of Th17 negative regulators such as *Il2*. Disruption of Hdac4/7 function blocks Th17-mediated intestinal inflammation in mice, suggesting a therapeutic strategy of pharmacological inhibition of HDAC4/7 for treating chronic Th17-related inflammatory diseases.

The authors declare no competing interest.

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in biological systems is urgently needed to explore their therapeutic potential for safer and more effective disease treatments.

In this study, we found that class IIa Hdac4 and Hdac7 are selectively up-regulated in gene transcription in Th17 cells, differentiated from mouse primary naive CD4$^+$ T cells, but not Th1, Th2, or Treg subtypes, which have different functions in adaptive immunity. Remarkably, Hdac4 and Hdac7 exhibit distinct functions in the regulation of gene transcription in Th17 cells. Specifically, Hdac4 interacts with the TF JunB to facilitate transcriptional activation of Th17 signature genes, such as *Il17a/f*, while Hdac7 collaborates with the TF Aiolos and Smrt/Ncor1-Hdac3 corepressors to repress transcription of Th17 negative regulators, including *Il2*. Displacement of Hdac4/7 from chromatin by class IIa HDAC inhibitors results in the blockage of Th17 cell differentiation through the transcriptional upregulation of *Il2* and downregulation of *Il17a/f* and blocks the development of pathogenic Th17 cells in T cell transfer–induced colitis in mice.

Results and Discussion

Class IIa HDAC4/7 Are Selectively Up-Regulated in Th17 Cells. We analyzed single-cell RNA-sequencing data in the literature (39) and observed consistently higher expression of *HDAC4* and *HDAC7* in human T cells isolated from uninflamed/inflamed colon tissues of ulcerative colitis patients compared to healthy controls (*SI Appendix,* Fig. S1*A*). To understand the functions of HDAC4 and HDAC7 (HDAC4/7) in Th17 cells, we systematically profiled Hdacs expression during ex vivo lineage-specific differentiation of different Th subtypes from mouse primary naive CD4$^+$ T cells. We conducted Th1, Th2, Th17, and Treg subset lineage-specific differentiation using established protocols, involving the profiling of characteristic TF expression for each subset and cell purity in comparison to Th0 cells (*SI Appendix,* Fig. S1*B*). In contrast to class I Hdacs (*Hdac1*, *2*, *3,* and *8*), we found that class IIa *Hdac4* and *Hdac7* exhibited marked transcriptional upregulation and protein induction exclusively in Th17 cells (Fig. 1 *A* and *B*). The kinetics of Hdac expression during Th17 cell differentiation also differed. While the expression of other Hdacs remained unchanged or slightly increased during Th17 differentiation, Hdac4/7 showed robust upregulation in the cell cytoplasm and nucleus starting 2 and 6 h, respectively, after Th17 cell differentiation (Fig. 1*C* and *SI Appendix,* Fig. S1*C*). The selective transcriptional upregulation of Hdac4/7 in Th17 cells is supported by the co-occupancy of Th17-lineage defining TFs Irf4, Batf, p300, RORγT, and Stat3 at the *Hdac4/7* loci. This co-occupancy is observed along with bromodomain and extraterminal (BET) proteins Brd2 and Brd4, as well as enhancer marks H3K4me1 and H3K27ac (15, 40) (Fig. 1*D*). Their presence is low, if any, on *Hdac1/2/3/8*, *Hdac5/9*, and *Hdac6/10/11* loci (*SI Appendix,* Fig. S1*D*).

To determine the signaling pathways responsible for Hdac4/7 induction in Th17 cells, we differentiated mouse naive CD4$^+$ T cells with IL-6, TGF-β1, or IL-6+TGF-β1 treatment. Hdac4/7 expression was strongly induced by IL-6 and IL-6+TGF-β1 treatment, promoting increased Hdac4/7 expression in both the cytoplasm and nucleus (Fig. 1*E* and *SI Appendix,* Fig. S1*E*). Notably, Hdac4 and Hdac7 were detected in the cytoplasm but not in the nucleus in Th0 cells, while in Th17 cells, they were detected in both compartments (*SI Appendix,* Fig. S1*E*). These data suggest that the nuclear localization of Hdac4/7 is influenced by IL-6-STAT3 signaling. Additionally, Hdac4/7 expression in Th17 cells depends on the TF Stat3, but not Irf4 or Rorc (Fig. 1*F* and *SI Appendix,* Fig. S1*F*). Upregulation of Hdac4 and Hdac7 can also be mediated through IL-21/Stat3 activation, as shown in our previous study, indicating that both IL-6 and IL-21 induce Stat3-directed transcriptional activation of Hipk2, promoting Th17 cell differentiation (41). These results indicate that the IL-6-Stat3 pathway likely induces Hdac4/7 expression in Th17 cells. Notably, MS417, a BET bromodomain (BrD) inhibitor that blocks BET BrD/acetyl-lysine binding (42), disrupts acetylation-mediated association of p65 and Stat3 with BET proteins (43). It effectively down-regulated Hdac4/7 expression in Th17 cells in a dose- and time-dependent manner (Fig. 1*G*). Collectively, these results suggest that HDAC4/7 are up-regulated through the IL-6-Stat3 pathway selectively in Th17 cells compared to other Th subtypes.

Class IIa HDAC-Specific Inhibitor TMP269 Inhibits Th17 Cell Differentiation. We focused our further study on Hdac4/7 in Th17 cell differentiation, as Hdac5 is reportedly dispensable for Th17 cells (44) and *Hdac9* shows minimal expression in Th17 cells (*SI Appendix,* Fig. S1*C*). Individual knockdown of *Hdac4* or *Hdac7* by shRNA in Th17 cells led to a decrease in IL-17A-producing cells and changes in Th17 signature genes (*SI Appendix,* Fig. S2 *A–C*), confirming that Hdac4 and Hdac7 are essential for Th17 cell differentiation. Notably, the combined knockdown of *Hdac4* and *Hdac7* demonstrated an additive inhibitory effect on Th17 cell differentiation compared to individual knockdown of *Hdac4* or *Hdac7* (Fig. 2*A* and *SI Appendix,* Fig. S2*D*). We also assessed the effects of chemical inhibition of Hdacs on Th17 cell differentiation using TMP269, a potent class II HDAC inhibitor with affinity (IC_{50}) of 126 nM, 80 nM, 36 nM, and 19 nM for HDAC4, 5, 7, and 9, respectively, and 20- to 400-fold selectivity over class I HDACs (38). TMP269 dose dependently inhibited both mouse and human Th17 cell differentiation (Fig. 2*B* and *SI Appendix,* Fig. S2 *E–G*) but had minimal effects on Th1, Th2, and Treg subtypes (Fig. 2*C* and *SI Appendix,* Fig. S2*H*). The inhibitory effects of TMP269 on Th17 differentiation were comparable to that by the combined knockdown of *Hdac4* and *Hdac7*, confirming that TMP269's impact results from the inhibition of both Hdac4 and Hdac7. Finally, using CFSE and PI/Annexin V assays, we demonstrated that single or combined knockdown of *Hdac4* and *Hdac7*, or TMP269 treatment, did not significantly induce apoptosis or alter proliferation of Th17 cells compared to control Th17 cells (*SI Appendix,* Fig. S2 *I–L*). Collectively, these results establish that HDAC4/7 play a significant role in the regulation of Th17 cell differentiation, independent of T cell expansion, fitness, or survival.

In a dose-dependent manner, TMP269-mediated Hdac4/7 inhibition resulted in the downregulation of the transcriptional expression of Th17 signature genes *Il17a* and *Il17f* (Fig. 2*D*) and decreased secretion of IL-17A and IL-17F (Fig. 2*E*). TMP269 treatment had no impact on Th17 regulators such as *Ahr* and *Irf4* in mRNA and protein expression. However, it led to a slight increase of mRNA levels of *Rorc* and *Il21* with minimal changes in protein levels (Fig. 2 *D–F*). These results ruled out the possibility that Hdac4/7 promote Th17 cell differentiation through these major positive regulators of Th17 cells. Our RNA-seq study further demonstrated that Hdac4/7 inhibition by TMP269 caused the transcriptional upregulation of approximately 300 genes and downregulation of 1,000 genes in Th17 cells (Fig. 3*A* and Dataset S1). Besides *Il17a* and *Il17f,* pathogenic signature genes like *Il23r, Tgfb3,* and *Il22* (45, 46) were down-regulated, and *Il2* was up-regulated by TMP269 in Th17 cells (Fig. 3 *A* and *B* and *SI Appendix,* Fig. S2*M*). This effect was confirmed with another class IIa HDAC inhibitor, TMP195, an analog of TMP269 (*SI Appendix,* Fig. S2*N*). Additionally, TMP269 inhibited the differentiation of Th17 cells polarized under pathogenic conditions (*SI Appendix,* Fig. S2*O*), causing a decreased expression of pathogenic markers (*Il17a*, *Il23r,*

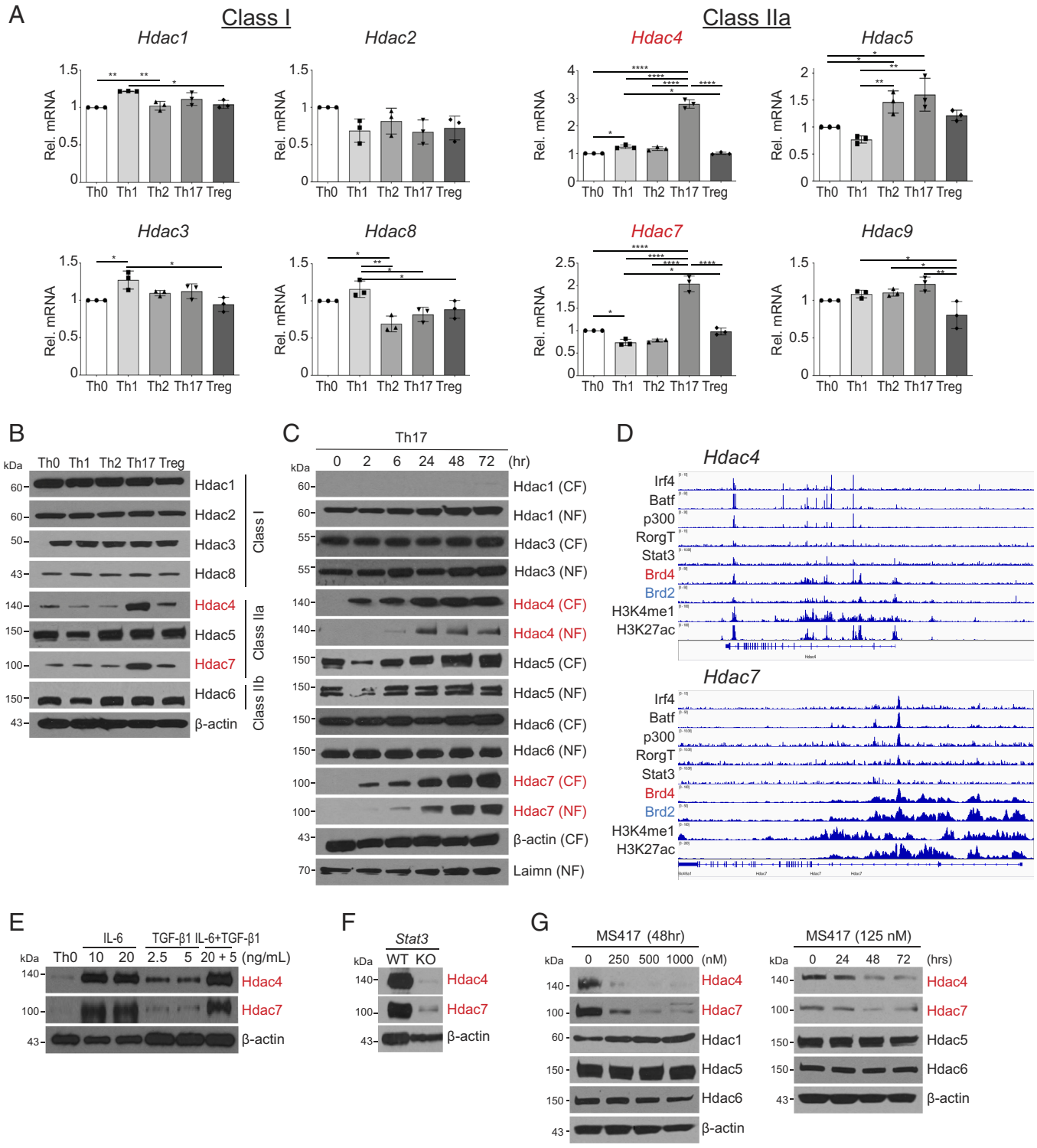

Fig. 1. Class IIa Hdac4/7 are selectively up-regulated in Th17 cells. (*A*) RT-qPCR analysis of class I and class IIa *Hdac* expression in ex vivo differentiated Th subtypes from mouse primary naive CD4$^+$ T cells for 48 h. Hdac expression is normalized with *Gapdh* and presented relative to Th0. n = 3/group. (*B*) Western blotting analysis of class I (Hdac1, 2, 3, and 8), class IIa (Hdac4, 5, and 7), and class IIb (Hdac6) protein expression in Th0 (CD3+CD28), Th1, Th2, Th17, and Treg cells differentiated ex vivo from mouse primary naive CD4$^+$ T cells for 72 h. (*C*) Western blotting analysis of class I (Hdac1 and 3), class IIa (Hdac4, 5, 7), and class IIb (Hdac6) Hdac distribution in cytoplasmic and nuclear fractions of Th17 cells at various time points (0, 2, 6, 24, 48, and 72 h) during ex vivo lineage-specific differentiation from mouse primary naive CD4$^+$ T cells. Note that at 0 h timepoint, the cells are unstimulated naive CD4$^+$ T cells. (*D*) ChIP-seq tracks displaying major TFs/activators/regulators Irf4, Batf, p300, RORγT, Stat3, Brd4, Brd2, and enhancer histone marks H3K4me1 and H3K27ac on *Hdac4* and *Hdac7* gene loci in Th17 cells differentiated from mouse primary naive CD4$^+$ T cells for 72 h. (*E*) Western blotting analysis of Hdac4 and Hdac7 in naive T cells stimulated with CD3+CD28 alone (Th0), or in combination with IL-6, TGF-β1, or IL-6+TGF-β1 (Th17) for 72 h as indicated. (*F*) Western blotting analysis of Hdac4 and Hdac7 in *Stat3*$^{fl/fl}$ wild-type (WT) and CD4-Cre;*Stat3*$^{fl/fl}$ knockout (KO) Th17 cells. (*G*) Western blotting analysis of Hdac1, 4, 5, 6, and 7 in Th17 cells differentiated from mouse primary naive CD4$^+$ T cells for 72 h, treated with BET BrD inhibitor MS417 (0, 250, 500, and 1,000 nM) for 48 h (*Left*), and Hdac4, 5, 6, and 7 in Th17 cells differentiated from mouse primary naive CD4$^+$ T cells for 72 h, treated with low dose of MS417 (125 nM) for 24, 48, and 72 h (*Right*). All western blot data are representative of three independent experiments. qPCR data represent mean ± SD, and each data point represents an independent experiment. Data are analyzed by ANOVA followed by Tukey's multiple comparisons (*A*). *P < 0.05, **P < 0.01, and ****P < 0.0001. Quantification and statistical summary of western blots are provided in Datasets S2 and S3.

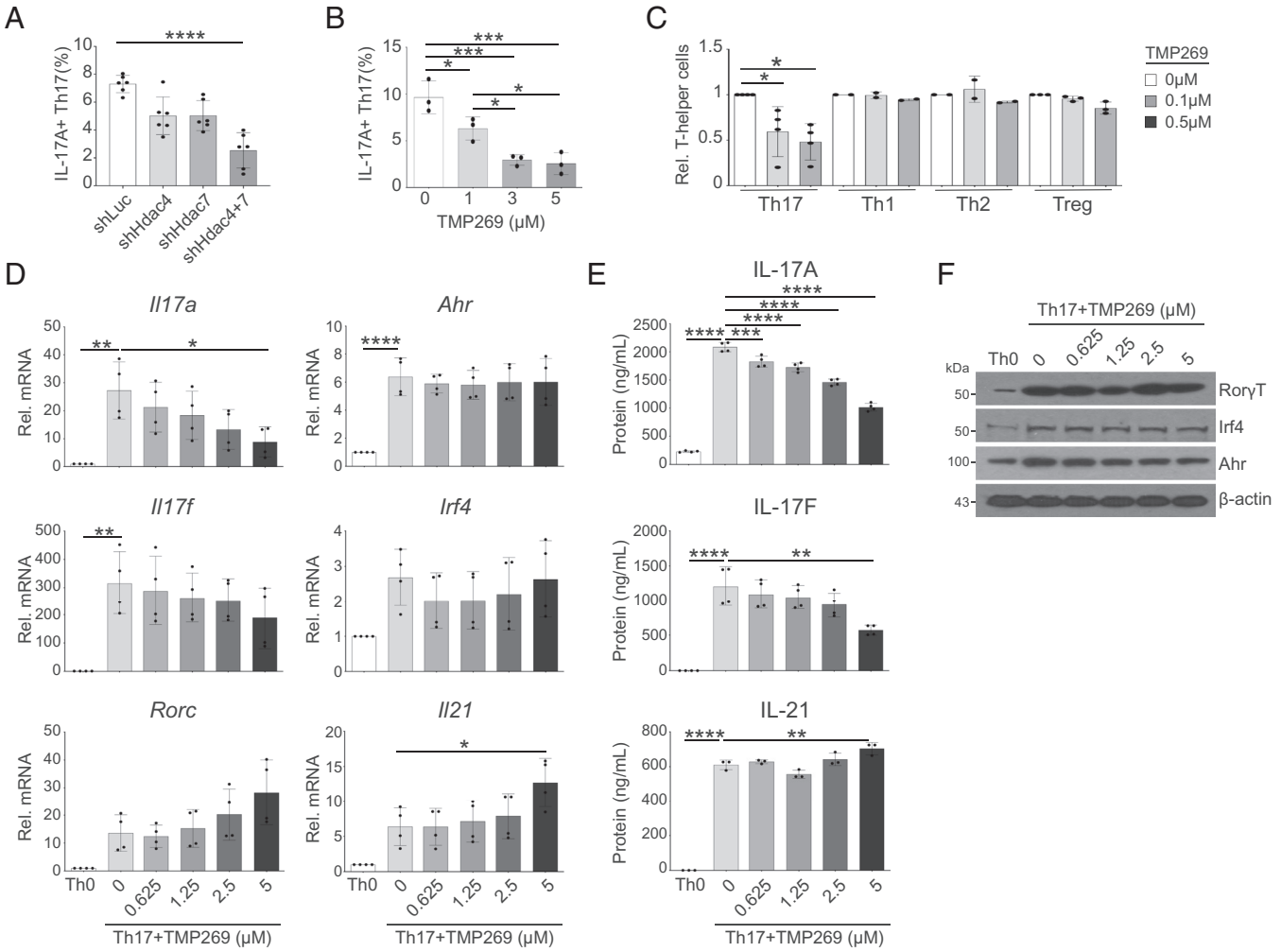

Fig. 2. Class IIa HDAC-specific inhibitor TMP269 inhibits Th17 cell differentiation. (*A*) Flow cytometry analysis of Th17 cells transduced with lentivirus targeting luciferase, Hdac4, Hdac7, or Hdac4+Hdac7. n = 6/group. (*B*) Flow cytometry analysis of Th17 cells differentiated ex vivo for 72 h from mouse primary naive $CD4^+$ T cells with dose-dependent treatment of TMP269 (0, 1, 3, or 5 μM). n = 3/group. (*C*) Flow cytometry analysis of Th17, Th1, Th2, and Treg cell differentiation with or without TMP269 treatment. TMP269 (100 nM or 500 nM) was added daily and Th cells were differentiated ex vivo from mouse primary naive $CD4^+$ T cells for 72 h. Data are presented as relative to Th cells without TMP269 treatment. n = 4,2,2,3/group. (*D*) RT-qPCR analysis of Th17 signature genes *Il17a, Il17f, Ahr, Irf4, Rorc,* and *Il21* in Th17 cells after 48 h differentiation with an increasing dose of TMP269 (0, 0.625, 1.25, 2.5, or 5 μM). Data are presented as relative to Th0 cells. n = 4/group. (*E*) ELISA analysis of cytokines IL-17A, IL-17F, and IL-21 in the supernatant of Th17 cells treated with an increasing dose of TMP269 as indicated. n = 3 to 4/group. (*F*) Western blotting analysis of Ahr, Irf4, and Rorγt of Th17 cells treated with an increasing dose of TMP269 as indicated. All western blot data are representative of three independent experiments. All data represent mean ± SD, and each data point represents an independent experiment. Data are analyzed by ANOVA followed by Tukey's multiple comparisons (*A–E*). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

Il22, and *Tgfb3*) and increased expression of the regulatory marker *Il10* in pathogenic Th17 cells polarized with IL-6+IL-1β+IL-23 (Fig. 3*C*). Given that Hdac4/7 are highly expressed at 72 h in Th17 cells postdifferentiation (Fig. 1*C*), we tested and found that TMP269 also exhibited an inhibitory effect on the maintenance of fully differentiated Th17 cells (Fig. 3*D*). Therefore, Hdac4 and Hdac7 play crucial roles in the regulation of transcriptional expression of genes involved in the differentiation, maintenance, and pathogenicity of Th17 cells.

Our RNA-seq data revealed a set of genes exhibiting marked upregulation in transcription after TMP269 treatment, including *Il2, Gfi1,* and *Tnfsf4* (Fig. 3*A* and *SI Appendix,* Fig. S3*A*), known to have negative effects on Th17 differentiation (47–49). Gene set enrichment analysis further indicated that IL-2RB, AP1, and Nfat pathways were among the top up-regulated pathways in Th17 cells provoked by TMP269, suggesting that Hdac4/7 inhibition induced IL-2/IL-2 receptor activation (*SI Appendix,* Fig. S3*B*). As IL-2 is recognized to conteract Th17 cell differentiation by decreasing *Il17a/f* transcription without affecting *Il21* and *Rorc* (50), our data suggested that the upregulation of IL-2 by Hdac4/7 inhibition likely dampens Th17 cell differentiation. This is supported by our study of Th17 cell differentiation in the presence of neutralizing antibodies against IL-2, IL-4, or IFN-γ, with or without TMP269. Only the anti-IL-2 antibody was able to rescue TMP269 inhibition of *Il17a* and *Il17f* expression (*SI Appendix,* Fig. S3*C*), emphasizing IL-2 as the major target of Hdac4/7. The addition of TMP269 at the 24-h time-point led to a rapid attenuation of *Il2* repression and inhibition of *Il17a* transcription as Th17 cell differentiation proceeded (*SI Appendix,* Fig. S3*D*). These results confirm that the blockade of Th17 cell differentiation by Hdac4/7 inhibition likely involves upregulation of the IL-2 pathway.

As cytokines and TFs of other Th subtypes, such as *Ifng, Tbx21, Il4, Gata3,* and *Foxp3,* typically act as negative regulators of Th17 cells, their expression is repressed in Th17 cell differentiation (6, 48). To assess whether TMP269 induces alternative lineage polarization to inhibit Th17 cell differentiation, we investigated and found that the expression of Th1 (*Ifng* and *Tbx21*), Th2 (*Il4* and *Gata3*), and Treg (*Tigit, Foxp3, Il10, Il2ra, Il2rb, Ctla4, Ikzf2, Ikzf4,* and *Entpd1*) signature genes (51) remained largely unchanged

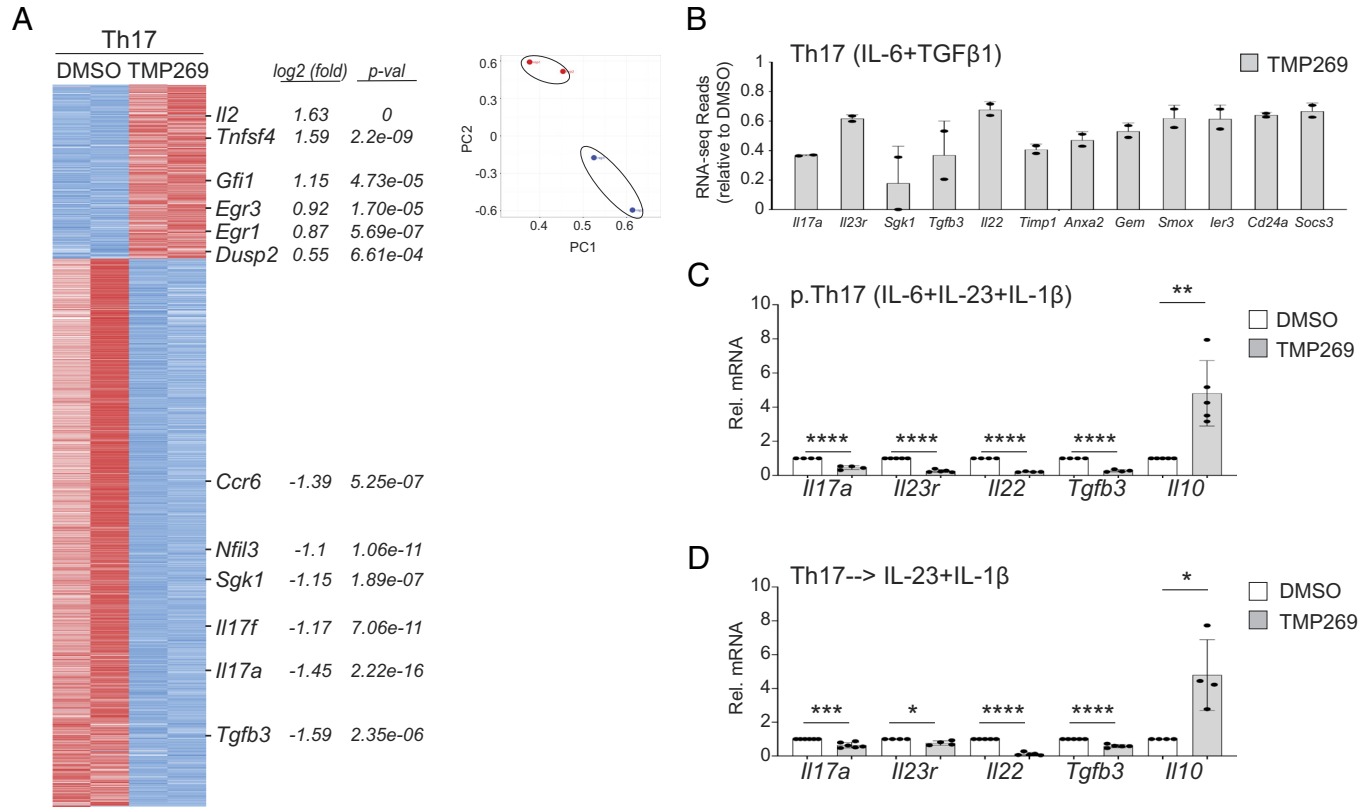

Fig. 3. HDAC4/7 inhibition effects on gene transcription in Th17 cell differentiation. (*A*) RNA-seq analysis of TMP269 effects on gene transcription in Th17 cells. *Left,* Heatmap showing up-/down-regulated genes in Th17 cells with TMP269 treatment. *Right,* PCA of RNA-seq samples with duplicates. Samples are from Th17 cells treated with or without TMP269 (5 μM) for 72 h. (*B*) RNA-seq transcript levels of pathogenic Th17 genes in Th17 cells (IL-6+TGF-β1) treated with TMP269 (5 μM) relative to DMSO control. n = 2/group. (*C*) RT-qPCR analysis of pathogenic genes *Il17a, Il23r, Il22,* and *Tgfb3*, and regulatory gene *Il10* in pathogenic Th17 cells (IL-6+IL-23+IL-1β) treated with or without TMP269 (5 μM) for 72 h. Data are presented relative to DMSO-treated pathogenic Th17 cells. n = 4 to 5/group. (*D*) RT-qPCR analysis of pathogenic genes *Il17a, Il23r, Il22,* and *Tgfb3*, and regulatory gene *Il10* in differentiated Th17 cells (IL-6+TGF-β1), followed by maintenance by IL-23+IL-1β for 72 h, treated with or without TMP269 (5 μM). Data are presented relative to DMSO-treated pathogenic Th17 cells. n = 4 to 6/group. Data represent mean ± SD, and each data point represents an independent experiment. Data are analyzed by the paired Student *t* test (*C* and *D*). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

in Th17 cells treated with TMP269 (*SI Appendix,* Fig. S4*A*), a finding supported by flow cytometry data (*SI Appendix,* Fig. S4*B*). Flow cytometry analysis of Th17 cells treated with shRNA targeting Hdac4 or Hdac7 also showed unaltered populations of IFNγ$^+$IL17A$^-$, IFNγ$^+$IL17A$^+$, IL4$^+$IL17A$^-$, IL4$^+$IL17A$^+$, Foxp3$^+$IL17A$^-$, and Foxp3$^+$IL17A$^+$ cell (*SI Appendix,* Fig. S4*C*). Notably, Th17 cell differentiation was performed for murine cells after MACS isolation without blocking antibodies for IL-12, IFNγ, or IL-4. The presence of immune cell contaminants might possibly attenuate Th17 differentiation, leading to a lower proportion of IL-17A$^+$ cells during Th17 differentiation and the appearance of IFNγ in Th17 cultures (*SI Appendix,* Fig. S4*C*). We further conducted Th17 and Treg cell differentiation with a cocktail of antibodies against IL-12, IFN-γ, and IL4 to induce stronger Th17 and Treg cell differentiation. Consistently, TMP269 inhibited Th17 but not Treg cell differentiation (*SI Appendix,* Fig. S4 *D* and *E*). These results demonstrate that in the presence of TMP269, no aberrant cytokine/TF expression was observed in Th17 cells.

HDAC4 Facilitates Transcription of Th17 Signature Genes in Th17 Cell Differentiation. We utilized transgenic mice to elucidate how Hdac4 and Hdac7 transcriptionally regulate Th17 cell differentiation. We isolated naive CD4$^+$ T cells from *Hdac4*$^{fl/fl}$;CD4-Cre (*Hdac4*-KO) mice, generated and characterized Th1, Th2, Th17, and Treg cells ex vivo. Consistent with our TMP269 data (Fig. 2*C*), *Hdac4*-KO cells exhibited decreased Th17 cell differentiation, while Th1, Th2, or Treg cell differentiation remained mostly unaffected (*SI Appendix,* Fig. S5*A*). Aligned with the results of *Hdac4* shRNA knockdown (*SI Appendix,* Fig. S2*C*), *Hdac4*-KO Th17 cells demonstrated reduced expression of *Il17a*, *Il17f,* and *Il23r*, with *Rorc* and *Il2* showing little changes (Fig. 4 *A*, *Left*). This was corroborated by changes in protein levels of IL-17A, IL-17F, and IL-2 (Fig. 4*B*), emphasizing Hdac4's role in the transcriptional activation of genes crucial for Th17 cells. Furthermore, *Hdac4*-KO Th17 cells displayed reduced expression of pathogenic markers (*Il22* and *Tgfb3*) (*SI Appendix,* Fig. S5*B*) and Th17 cells differentiated under pathogenic condition also showed similar reductions (*SI Appendix,* Fig. S5*C*). However, *Hdac4*-KO did not impact protein levels of Th17-specific TFs Stat3, Irf4, Batf, and RORγT (*SI Appendix,* Fig. S5*D*).

To gain the mechanistic insights into how Hdac4 activates gene transcription in Th17 cells, we conducted ChIP-seq of Hdac4 in Th17 cells, revealing enriched motifs, including Batf and AP-1 TFs Jun and Fos dimers, through HOMER (Hypergeometric Optimization of Motif EnRichment) analysis (*SI Appendix,* Fig. S5*E*). We verified Hdac4-JunB and JunB-Batf interactions through immunoprecipitation (*SI Appendix,* Fig. S5*F*), confirming Hdac4 colocalization with Th17-specific TFs JunB, Irf4, and Batf on chromatin globally and on *cis*-regulatory regions of *Il17a, Il17f,* and *Il23r* in Th17 cells (Fig. 4 *C* and *D* and *SI Appendix,* Fig. S5*G*). Given that AP-1 factors are crucial for Irf4-mediated gene transcription in Th17 cells by interacting with Batf in coordination with Irf4 binding to AP-1/IRF composite elements (AICEs) (52, 53) and that JunB have been shown to promote DNA binding of Irf4, Batf, and Stat3 on *Il17a* locus in Th17 cells (54, 55), our results suggest that Hdac4-JunB

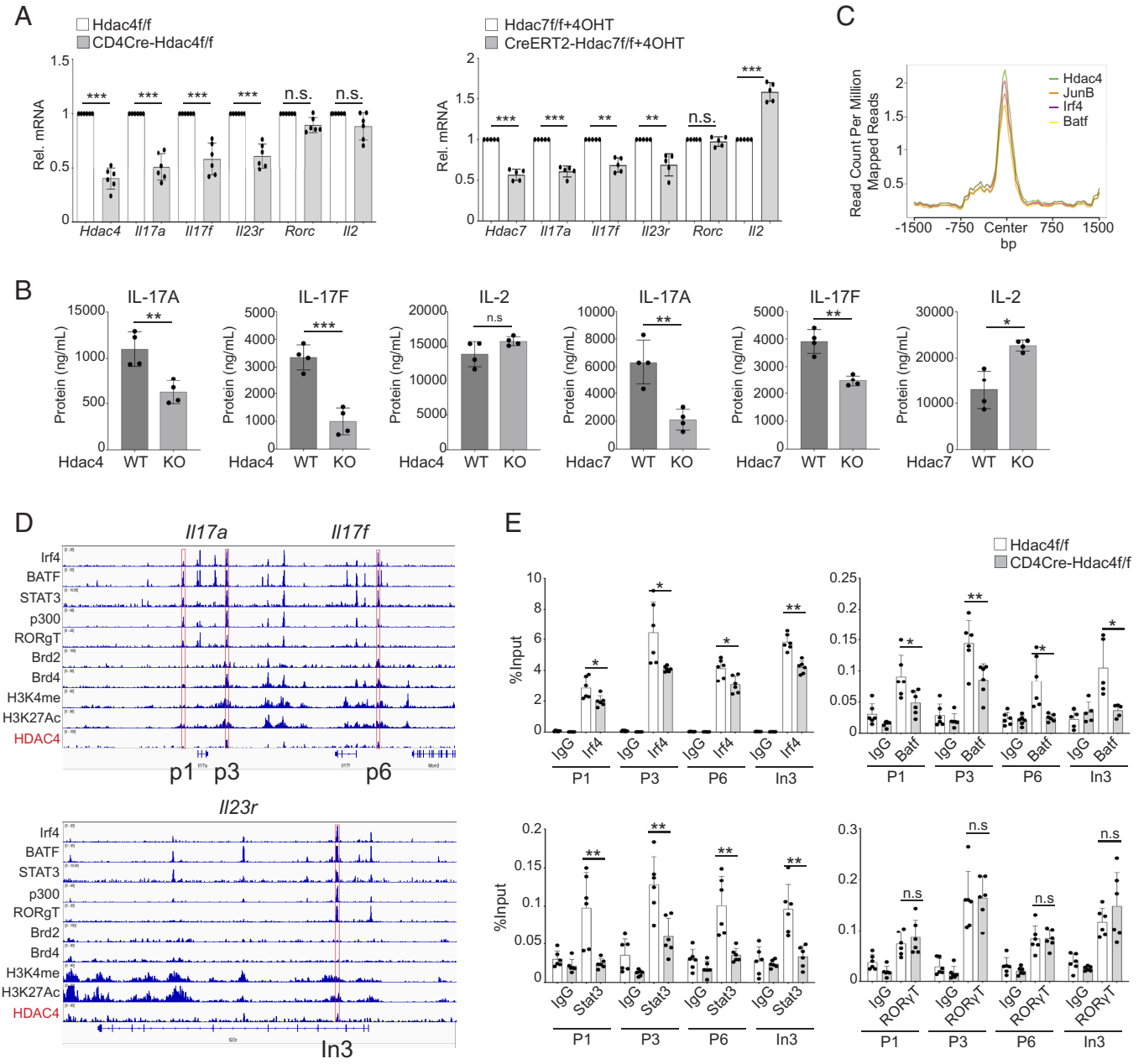

Fig. 4. Hdac4 regulates gene transcription in Th17 cells. (*A*) qPCR analysis of *Hdac4, Il17a, Il17f, Il23r, Rorc,* and *Il2* in *Hdac4*-WT (*Hdac4*$^{fl/fl}$) versus *Hdac4*-KO (*Hdac4*$^{fl/fl}$;CD4-Cre) (*Left*), *Hdac7, Il17a, Il17f, Il23r, Rorc,* and *Il2* in *Hdac7*-WT (*Hdac7*$^{fl/fl}$+4OHT) versus *Hdac7*-KO (*Hdac7*$^{fl/fl}$;CreERT2+4OHT) Th17 cells (*Right*). Data are presented relative to WT cells. n = 5 to 6/group. (*B*) ELISA analysis of cytokines IL-17A, IL-17F, and IL-2 in the supernatant of Hdac4- and Hdac7-WT and -KO Th17 cells. n = 4/group. (*C*) Global ChIP-seq peak analysis of Hdac4, JunB, Irf4, and Batf in Th17 cells. Normalized JunB, Irf4, and Batf ChIP-seq were centered around Hdac4 peak regions. (*D*) ChIP-seq tracks showing occupancy of major TFs/activators/regulators Irf4, Batf, Stat3, p300, RORγT, Brd2, Brd4, H3K4me1, H3K27ac, and Hdac4 on *Il17a-f* and *Il23r* gene loci in Th17 cells differentiated from mouse primary naive CD4$^+$ T cells for 72 h. (*E*) ChIP-qPCR analysis showing occupancy of Irf4, Batf, Stat3, and RORγT on *cis*-regulatory regions of *Il17a-f* and *Il23r* gene loci in *Hdac4*-WT versus *Hdac4*-KO Th17 cells. n = 6/group. Data represent mean ± SD, and each data point represents an independent experiment. Data are analyzed by the paired Student *t* test (*A*, *B*, and *E*). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

could regulate Irf4-Batf binding to chromatin. Indeed, Irf4, Batf, and Stat3 occupancy, but not RORγT, on the *cis*-regulatory regions of *Il17a, Il17f,* and *Il23r* were found to be reduced in *Hdac4*-KO Th17 cells (Fig. 4*E*), or upon TMP269 treatment (*SI Appendix,* Fig. S5*H*). Collectively, these results indicate that HDAC4 activates Th17 gene expression by modulating chromatin occupancy of TFs, and its function is independent of IL-2.

HDAC7 Has Distinct Functions in Transcriptional Repression of Th17-Negative Regulators. Unlike *Hdac4*$^{fl/fl}$;CD4-Cre mice that displayed unaltered development of T cell lineages (56), *Hdac7*$^{fl/fl}$;CD4-Cre mice exhibited impaired thymocyte development (57). To gain insights into HDAC7 function during Th17 differentiation, we isolated naive CD4$^+$ T cells from *Hdac7*$^{fl/fl}$;CreERT2 (*Hdac7*-KO) mice and characterized Th17 cells treated with 4-OHT (250 nM) that induces Cre-recombination ex vivo. *Hdac7*-KO cells demonstrated decreased Th17 cell differentiation, while Th1, Th2 or Treg cell differentiation was largely unaffected (*SI Appendix,* Fig. S6*A*). In *Hdac7*-KO Th17 cells, *Il17a*, *Il17f*, and *Il23r* showed reduced expression, and *Rorc* remained unchanged, whereas *Il2* expression was markedly up-regulated (Fig. 4 *A*, *Right*), aligning with our *Hdac7* shRNA knockdown data (*SI Appendix,* Fig. S2*C*). In addition to *Il2*, we found that Hdac7 but not Hdac4, represses transcription of *Il10,* a gene marker with regulatory properties (45) in pathogenic Th17 cells (*SI Appendix,* Fig. S6 *B* and *C*). Similar to *Hdac4*-KO, *Hdac7*-KO did not affect protein levels

of Th17-specific TFs Stat3, Irf4, Batf, and RORγT (*SI Appendix,* Fig. S6*D*).

Our ChIP-seq study of Hdac7 in Th17 cells revealed distinct global chromatin binding and TFs binding motifs for Hdac7 compared to Hdac4 (*SI Appendix,* Fig. S6*E*). In line with Hdac7's suggested repressive role of gene transcription during thymocyte development (57, 58), we found that in Th17 cells, *Il2*, along with T cell development genes such as *Nr4a1, Egr1, Dusp2, Fos, Asb2,* and *Gadd45b*, were directly targeted by Hdac7 and up-regulated upon TMP269 treatment (*SI Appendix,* Fig. S6*F*). In contrast to *Hdac4*-KO, *Hdac7*-KO in Th17 cells showed no marked effects on Irf4-Batf or RORγT but reduced Stat3 occupancy on the *cis*-regulatory regions of Th17-signature genes (Fig. 5*A*), highlighting the distinct functions of Hdac4 and Hdac7. As Hdac7 showed no interaction with the JunB-Irf4-Batf activator complex (*SI Appendix,* Fig. S5*F*), the loss of Stat3 occupancy on cis-regulatory regions of Th17 genes in *Hdac7*-KO Th17 cells is likely due to derepression of *Il2*. Il2 has been reported to promote Stat5 but displace Stat3 binding to chromatin in Th17 cells (50). In our analysis of Th17 cells transduced with lentivirus targeting luciferase, Hdac4, or Hdac7, with or without neutralizing antibody against IL-2, we demonstrated that IL-2 neutralizing antibody rescued the inhibitory effect of shHdac7 but not shHdac4, ruling out the possibility of Hdac4 regulating Th17 differentiation via IL-2 repression as shown for Hdac7 (*SI Appendix,* Fig. S6*G*). Additionally, the reduction in Th17 cell differentiation resulting from IL-2 upregulation induced by *Hdac7*-KO or TMP269 treatment was rescued by IL-2 neutralizing antibody, suggesting that Hdac7, but not Hdac4, promotes Th17 cell differentiation via IL-2 repression (Fig. 5*B* and *SI Appendix,* Fig. S6*H*). It is noteworthy that TMP269 modestly inhibits Th17 cells under the condition of IL-2 neutralization, further supporting the notion that Hdac4 and Hdac7 have an additive effect in Th17 cell differentiation (Fig. 2*A*).

Class IIa HDACs have been shown to work with the Ncor1-Hdac3 corepressor complex to acquire deacetylase activity (59). Our findings support this notion in the context of Th17 cells, as we observed decreased Ncor1-Hdac3 occupancy and increased histone H3 lysine acetylation (H3Kac) on the *Il2* promoter in *Hdac7*-KO Th17 cells (Fig. 5*C*). Consistently, TMP269 treatment reduced the *Il2* promoter occupancy of Hdac7, Ncor1, and Hdac3, associated with an increased H3Kac level (*SI Appendix,* Fig. S6*I*) and *Il2* expression.

We further characterized the functional interactions of Hdac4 and Hdac7 with JunB and Aiolos, using immunoprecipitation and luciferase assays. Hdac4 interaction with JunB in Th17 cells was confirmed (*SI Appendix,* Fig. S5*F*). While Hdac7 does not interact with Hdac4, or Th17-specific Stat3, Irf4, Batf, RORγT, Hdac7 binds to Aiolos, a TF known for repressive function in Th17 cells (60) and is present at the *Il2* promoter (*SI Appendix,* Figs. S5*F* and S6*J*). Our findings reveal that Hdac4 and Hdac7 interact with JunB and Aiolos through their C-terminal pseudocatalytic domains (Fig. 6*A*), which are required for *Il17a* and *Il2* transcriptional activation and repression, respectively (Fig. 6*B*). Importantly, Hdac4 and Hdac7 cannot substitute their respective transcriptional activator or repressor functions when cooperating with Batf-JunB or Aiolos in controlling the transcription expression of *Il17a* and *Il2* (Fig. 6*C*). Interestingly, the molecular interactions and transcriptional activities of JunB-Hdac4 and Aiolos-Hdac7 can be disrupted by TMP269 (Fig. 6 *D* and *E*). Collectively, we have established distinct functions of Hdac4 and Hdac7, working with TFs JunB and Aiolos, respectively, to facilitate gene transcriptional activation and repression crucial for Th17 cell differentiation.

Class IIa HDAC Inhibition Ameliorates T Cell Transfer–Induced Colitis in Mice. Th17 cells have been implicated in the development of inflammatory disorders, including IBD, rheumatoid arthritis, and multiple sclerosis (10, 13). Accordingly, we assessed the in vivo effects of disrupting Hdac4/7 functions on Th17 cell development using a mouse model of T cell transfer–induced colitis (Fig. 7*A*), which recapitulates disease conditions of IBD in humans. Following the adoptive transfer of naive $CD4^{+}CD45RB^{hi}$ T cells, *Rag1*$^{-/-}$ mice treated with TMP269 intraperitoneally (i.p.) (10 mg/kg, twice per week) for 7 wk (Fig. 7*A*) exhibited milder colitis symptoms

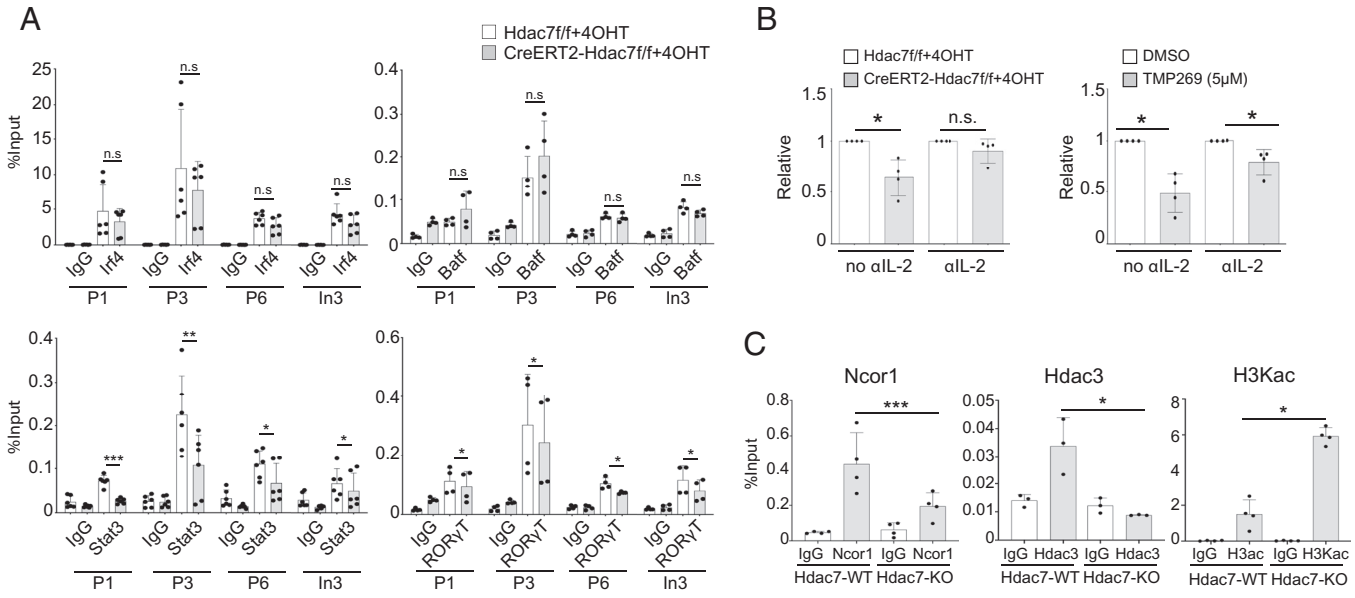

Fig. 5. Hdac7 regulate gene transcription in Th17 cells. (*A*) ChIP-qPCR analysis showing occupancy of Irf4, BatfF, Stat3, and RORγT on *cis*-regulatory regions of *I17a-f* and *Il23r* gene loci in *Hdac7*-WT versus *Hdac7*-KO Th17 cells. n = 4 to 6/group. (*B*) *Left*, Flow cytometry analysis of *Hdac7*-WT versus *Hdac7*-KO Th17 cells, neutralized with or without antibody against IL-2. n = 4 to 6/group. Data are presented relative to WT-Th17 cells. *Right*, Flow cytometry analysis of Th17 versus Th17+TMP269, neutralized with or without antibody against IL-2. Data are presented relative to DMSO-treated Th17 cells. n = 4/group. (*C*) ChIP-qPCR analysis showing occupancy of Ncor1, Hdac3, and H3Kac on the promoter region of the *I12* gene locus in *Hdac7*-WT versus *Hdac7*-KO Th17 cells. n = 4,3,4/group. Data represent mean ± SD, and each data point represents an independent experiment. Data are analyzed by the paired (*A* and *B*) and unpaired (*C*) Student *t* test. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

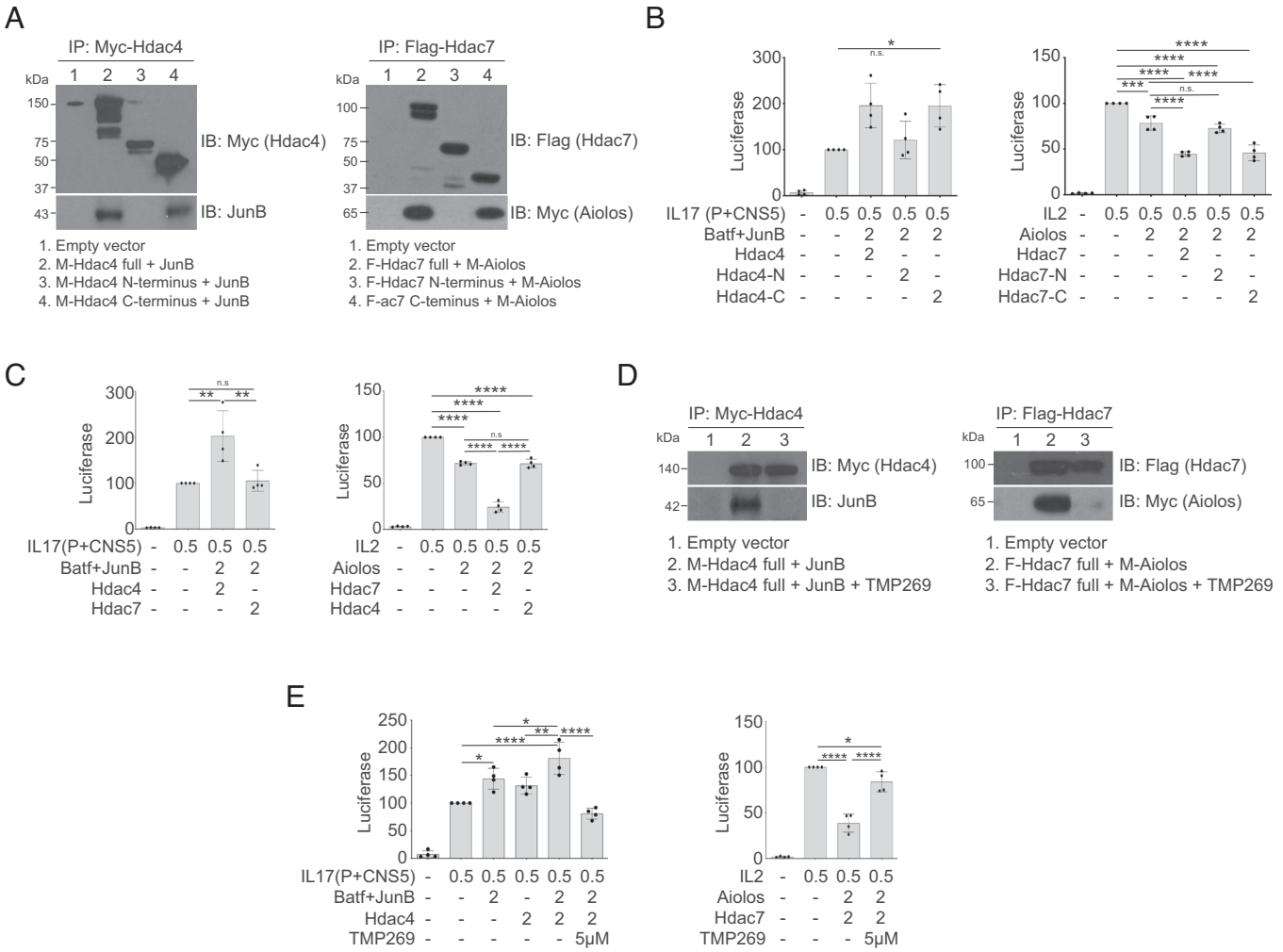

Fig. 6. Hdac4 and Hdac7 interact with JunB and Aiolos via C-terminal domains. (*A*) IP-western analysis of cell lysates probing interactions of Hdac4, Hdac4-N and -C terminus with JunB (*Left*), Hdac7, Hdac7-N and -C terminus with Aiolos (*Right*) in HEK293 cells. (*B*) Luciferase assays probing transcriptional activation of *Il17a* by Hdac4, Hdac4-N and -C terminus with JunB (*Left*), transcriptional repression of *Il2* by Hdac7, Hdac7-N and -C terminus with Aiolos (*Right*) in Jurkat T cells. (*C*) Luciferase assays probing transcriptional activation of *Il17a* by Hdac4 and Hdac7 with JunB (*Left*), transcriptional repression of *Il2* by Hdac4 and Hdac7 with Aiolos (*Right*) in Jurkat T cells. (*D*) IP-western analysis of cell lysates probing interactions of Hdac4 and JunB (*Left*), Hdac7 and Aiolos (*Right*), treated with or without TMP269 in HEK293 cells. (*E*) Luciferase assays probing transcriptional activation of *Il17a* by Hdac4 with JunB (*Left*), transcriptional repression of *Il2* by Hdac7 with Aiolos (*Right*), treated with or without TMP269 in Jurkat T cells. All western blot data are representative of three independent experiments. All luciferase data are presented as relative to IL17 or IL2 reporter alone-transfected cells (2nd lane) and represent mean ± SD. Each data point represents an independent experiment. Data are analyzed by ANOVA followed by Tukey's multiple comparisons. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

compared to control mice, as evidenced by reduced weight loss, colon shortening, and crypt damage (Fig. 7 *B*–*D*). These histological changes were accompanied by a reduction of $CD4^+IL\text{-}17A^+$ and $CD4^+IFN\text{-}\gamma^+$ cell populations in the lamina propria (Fig. 7*E* and *SI Appendix*, Fig. S7*A*). Notably, *Rag1*$^{-/-}$ mice injected with *Hdac4*-KO or *Hdac7*-KO (recombination induced by tamoxifen injection of mice) naive $CD4^+$ T cells developed milder colitis symptoms (Fig. 7 *B*–*D*) and showed a marked reduction of $CD4^+IL\text{-}17A^+$ and $IFN\text{-}\gamma^+IL\text{-}17A^+$ cells in the lamina propria, whereas $IL\text{-}17A^-IFN\text{-}\gamma^+$ cells were much less affected (Fig. 7*E* and *SI Appendix*, Fig. S7 *B* and *C*). Collectively, our in vivo data support our cellular study findings that Hdac4/7 inhibition likely results in the blockade of Th17 and pathogenic Th17 cell development, leading to amelioration of colonic inflammation in the mouse model of colitis.

In this study, we uncovered previously unappreciated transcriptional mechanisms of class IIa HDAC4/7 in the regulation of lineage-specific differentiation of Th17 cells, which are implicated in autoimmune and inflammatory orders (14, 61, 62). While Hdac4's association with Th17 differentiation was noted before (21), the precise mechanism through which Hdac4 and other class IIa Hdacs regulate Th17 cells has remained unclear until this study. We demonstrate that Hdac4 and Hdac7 interact with distinct Th17 TFs to direct transcriptional activation and repression. Specifically, HDAC4 collaborates with Th17 TFs and coactivators (JunB, Irf4/Batf, Stat3, Rorγt, p300, and Brd2/4) to activate Th17 genes (*Il17a/f*), while HDAC7 works with Aiolos and Smrt/Ncor1-Hdac3 corepressors to repress Th17 negative regulators (*Il2*). These interactions ensure the lineage-specific Th17 cell differentiation from naive $CD4^+$ T cells (Fig. 7*F*). The selective induction of Hdac4/7 under Th17-cell polarizing condition explains the lack of T cell transcriptome responsiveness to class II HDAC inhibition by TMP269 observed in a previous study (38). While the N-terminal domains of class IIa HDACs typically bind to TFs and the C-terminal domains recruit NcoR-Hdac3 to repress transcription of genes (59, 63–66), our study reveals a dominant role of the C-terminal domain of Hdac4 and Hdac7 in both binding TFs and mediating transcriptional activation or repression in Th17 cells. This suggests that targeting the C-terminal domain of HDAC4 and HDAC7 using class II HDAC-specific inhibitors, such as TMP269, presents a selective therapeutic option. Accordingly, we demonstrated that pharmacological inhibition of Hdac4/7 by TMP269 effectively attenuates in vivo Th17 cell development in

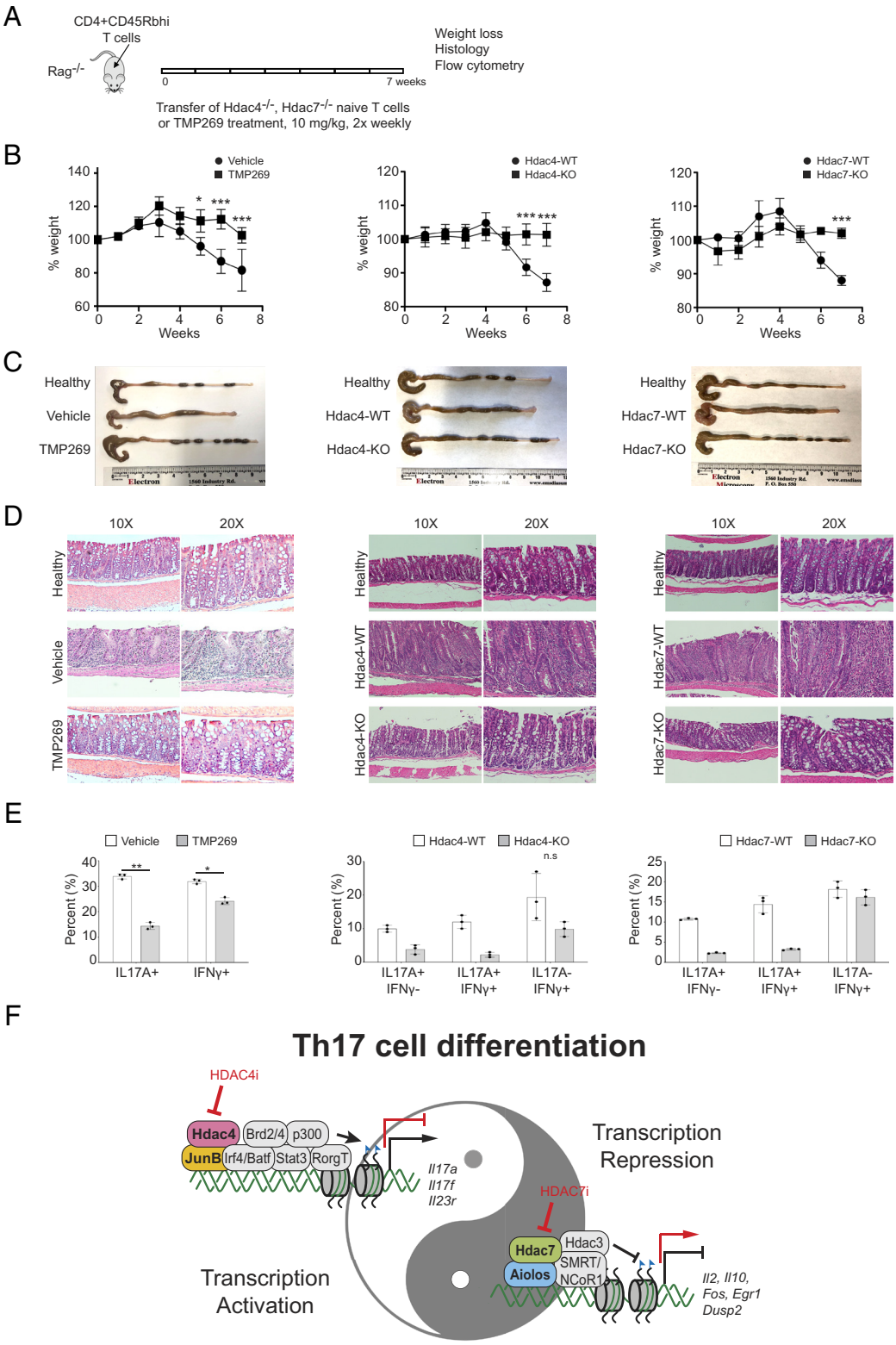

Fig. 7. Class IIa HDAC inhibition ameliorates T cell transfer–induced colitis in mice. (*A*) Schematic diagram illustrating the experimental colitis study. $CD4^+CD45RB^{hi}$ T cells were purified from spleens and lymph nodes of wild-type mice, and 5×10^5 cells were injected (i.p.) into recipient *Rag1*$^{-/-}$ mice. Mice were treated with PBS (control) or TMP269 (10 mg/kg) twice a week starting at week 0. Additionally, $CD4^+CD45RB^{hi}$ T cells were also purified from spleens and lymph nodes of *Hdac4*-WT (*Hdac4*$^{fl/fl}$), *Hdac4*-KO (*Hdac4*$^{fl/fl}$;CD4-Cre), *Hdac7*-WT (*Hdac7*$^{fl/fl}$), and *Hdac7*-KO (*Hdac7*$^{fl/fl}$;CreERT2) mice and injected (i.p.) into recipient *Rag1*$^{-/-}$ mice. Tamoxifen was injected into *Hdac7*-WT and *Hdac7*-KO naive T cells-transferred *Rag1*$^{-/-}$ mice for Hdac7 recombination and gene deletion. Body weight change was monitored weekly, and mice were killed at the end of the experiment for histology analysis. (*B*) Changes in body weight of *Rag1*$^{-/-}$ mice transferred with TMP269 treatment (n = 6 mice/group), *Hdac4*-KO cells (n = 6 to 9 mice/group), and *Hdac7*-KO cells (n = 5 mice/group). (*C*) Changes in colon length of the *Rag1*$^{-/-}$ mice treated with TMP269, transferred with *Hdac4*-KO cells, and *Hdac7*-KO cells. (*D*) H&E staining of colons of the *Rag1*$^{-/-}$ mice treated with TMP269, transferred with *Hdac4*-KO cells, and *Hdac7*-KO cells. (*E*) Flow cytometry analysis of $IL17^+$, $IFN\gamma^+$ $CD4^+$T cells in colons of the *Rag1*$^{-/-}$ mice treated with TMP269 treatment (n = 2 to 3/group) (*Left*), $IL17^+IFN\gamma^-$, $IL17^+IFN\gamma^+$, and $IL17^-IFN\gamma^+$ $CD4^+$T cells in colons of the *Rag1*$^{-/-}$ mice transferred with *Hdac4*-KO cells (n = 3/group) (*Middle*), and *Hdac7*-KO cells (n = 3/group) (*Right*). T cells were isolated and pooled from 2 mice colons for analysis. (*F*) Mechanistic model illustrating the distinct mechanisms of HDAC4 and HDAC7 functions in the regulation of gene transcription during Th17 cell differentiation. Data are analyzed by the ANOVA multiple comparison test (*B*) and unpaired Student *t* test (*E*). All data represent mean ± SD, and each data point represents an independent experiment. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

the mouse colon and the development of T cell transfer–induced colitis in mice, which mimics IBD in humans. Our proof-of-concept study rationalizes pharmacological inhibition of class IIa HDAC4/7 as a therapeutic strategy for treating Th17-related inflammatory and autoimmune diseases.

Materials and Methods

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

Mice. C57BL/6, CD4-Cre, Rosa26Cre-ERT2, *Irf4*$^{-/-}$, *Ror*γ*T*$^{-/-}$, and *Rag1*$^{-/-}$ mice were obtained from Jackson Laboratory. Stat3$^{fl/fl}$, Hdac4$^{fl/fl}$, and Hdac7$^{fl/fl}$ mice were generous gifts from Mark Kaplan (Indiana University, Indianapolis, Indiana) and Eric Olson (University of Texas Southwestern Medical Center, Dallas, Texas). All animals were housed and maintained in a conventional pathogen-free facility at the Icahn School of Medicine at Mount Sinai (ISMMS). The animal study protocols in this study were approved by the Institutional Animal Care and Use Committees of ISMMS.

Cell Sorting and Mouse T-Helper Cell Differentiation. Mouse (male and female, 6 to 8 wk of age) naive CD4$^+$ T cells were purified from mouse spleens and lymph nodes using anti-CD4 microbeads (Miltenyi Biotech). Naive 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-IFNγ (10 μM/mL) for Th2 conditions, IL-6 (20 ng/mL), TGF-β1 (2.5 ng/mL) for Th17 conditions, IL-6 (20 ng/mL), IL-23 (20 ng/mL), TGF-β3 (2.5 ng/mL), and IL-1β (20 ng/mL) for pathogenic Th17 conditions, TGF-β1 (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, and neutralizing antibodies were purchased from BD Pharmingen.

Human Th17 Cell Differentiation. Mononuclear cells were isolated from human blood (deidentified prior to be used in this study) by density gradient centrifugation using Ficoll-Paque (GE, 17-1440-03). The naive CD4$^+$ T cells were further purified using the Miltenyi naive CD4$^+$ T cells sorting kit (Miltenyi, 130-096-533) and were resuspended in fresh serum-free TexMACs™ medium (Miltenyi, 130-097-196) at a concentration of 1 × 10^6 cells/mL of medium. The naive T cells were activated by anti-CD3/CD28 activation beads (Miltenyi, 130-091-441) and differentiated with addition of 30 ng/mL of IL-6, 20 ng/mL of IL-1β, 30 ng/mL of IL-23, 2.25 ng/mL of TGF-β1, 1 μg/mL of anti-IL-4, and 1 μg/mL of anti-IFN-γ. The cells were incubated at 37 °C for 7 d before analysis.

Flow Cytometry Analysis. For cytokine analysis, cells were incubated for 3 h with phorbol PMA (50 ng/mL; Sigma), ionomycin (500 ng/mL; Sigma), and GolgiStop (BD). Intracellular cytokine staining was performed according to the manufacturer's protocol (FoxP3 staining buffer set from eBioscience). LSR II flow cytometer (BD Biosciences) and FlowJo (Tree Star) software were used for flow cytometry and analysis. Dead cells were excluded using the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen).

Proliferation and Apoptosis Assay. Proliferation of T cells was measured by CFSE dilution assay while apoptosis of T cells was measured by staining of PI and annexin V followed by flow cytometry analysis.

Luciferase Reporter Assay. Jurkat cells were transfected with a TransIT-Jurkat transfection reagent (Mirus). The cells were transfected with either IL-17 or IL-2 luciferase reporter construct plasmid (Addgene) along with plasmids encoding WT and/or mutant Hdac4, Hdac7, JunB, and Aiolos plasmids. Transfected cells were incubated for 24 h and then were either stimulated for 4 h with 10 ng/mL phorbol 12-myristate 13-acetate (PMA) and 500 nM ionomycin for IL-17 luciferase assay or stimulated for 6 h with 50 ng/mL PMA and 1 μM ionomycin for IL-2 luciferase assay. Cells were lysed, and firefly luciferase activities were measured with a luciferase assay system (Promega).

T Cell Transfer Colitis Study, Histopathology, and Lamina Propria Lymphocyte Isolation. Purified CD4$^+$CD45RB^{hi} T cells from C57/B6 mice were injected intraperitoneally into *Rag1*$^{-/-}$ recipients (5 × 10^5 cells per mouse in 200 μL sterile PBS per injection). Mice were weighed every week throughout the course of experiment. After 5 to 7 wk, mice were killed, and colon tissues were excised. Tissues were fixed in 10% buffered formalin and paraffin embedded. The sections of tissue samples were stained with hematoxylin and eosin. Lamina propria lymphocytes were isolated by different steps of digestion of colon tissue using collagenase, DNase, and dispase, and the resulting cells are purified using Percoll gradient.

Statistical Analysis. Statistical analysis was performed using GraphPad Prism 9.

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

Data, Materials, and Software Availability. RNA-seq data have been deposited in [GEO database] (GSE144830) (67). Information regarding the reagents and resources utilized for this study is provided in *SI Appendix*, Table S1. All other data are included in the manuscript and/or supporting information.

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