Cell Reports

Pak2-mediated phosphorylation promotes RORγt ubiquitination and inhibits colonic inflammation

Highlights

- Pak2 directly binds and phosphorylates RORγt at S316
- Inhibition of Pak2 in Th17 cells enhances IL-17 expression and colitis severity
- Phosphorylation facilitates binding of Itch to RORγt and promotes its degradation

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In brief

Kathania et al. show that Pak2, a Ser/Thr kinase, associates with RORγt and phosphorylates Ser-316 of RORγt. Deletion of Pak2 in Th17 cells enhances IL-17 expression and colitis severity. Pak2-mediated phosphorylation causes a conformational change resulting in increased ubiquitination of RORγt by the E3 ubiquitin ligase Itch.
Pak2-mediated phosphorylation promotes RORγt ubiquitination and inhibits colonic inflammation

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https://doi.org/10.1016/j.celrep.2022.111345

SUMMARY

Dysregulated interleukin-17 (IL-17) expression and its downstream signaling is strongly linked to inflammatory bowel diseases (IBDs). However, the molecular mechanisms by which the function of RORγt, the transcription factor of IL-17, is regulated remains elusive. By a mass spectrometry-based approach, we identify that Pak2, a serine (S)/threonine (T) kinase, directly associates with RORγt. Pak2 recognizes a conserved KRLS motif within RORγt and phosphorylates the S-316 within this motif. Genetic deletion of Pak2 in Th17 cells reduces RORγt phosphorylation, increases IL-17 expression, and induces severe colitis upon adoptive transfer to Rag1−/− mice. Similarly, reconstitution of RORγt-S316A mutant in Rorc−/− Th17 cells enhances IL-17 expression and colitis severity. Mechanistically, we demonstrate that Pak2-mediated phosphorylation causes a conformational change resulting in exposure of the ubiquitin ligase Itch interacting PPLY motif and degradation of RORγt. Thus, we have uncovered a mechanism by which the activity of RORγt is regulated that can be exploited therapeutically.

INTRODUCTION

Interleukin-17 (IL-17) is a proinflammatory cytokine that is produced by a variety of immune cells, including the Th17 subset of helper CD4+ T cells, δ T cells, and innate lymphoid cells (Honda and Littman, 2016; Kumar et al., 2021b; Zhou and Sonnenberg, 2020). IL-17 signals through the IL-17 receptor, composed of IL-17RA and IL-17RC receptor subunits (Gaffen, 2009). The binding of IL-17 to its receptor activates the target cells, such as epithelial cells, endothelial cells, and fibroblasts, and induces the expression of CXCL1, CXCL2, and CXCL8, which attract myeloid cells such as neutrophils and promote inflammation (Gaffen, 2009). IL-17 is critically essential for host defense against fungal and bacterial infections. However, dysregulated IL-17 expression and downstream signaling are involved in several human diseases, including inflammatory bowel disease (IBD) (McGeachy et al., 2019; Patel and Kucharro, 2015).

The orphan nuclear receptor RORγt is a transcription factor crucial for IL-17 expression (Ivanov et al., 2006; Zhang et al., 2008). RORγt consists of a ligand-independent activation function 1 helix, a DNA-binding domain, a flexible hinge domain, and a C-terminal ligand-binding domain (Kumar et al., 2021b). The zinc finger motifs within the DNA-binding domain recognize the ROR response elements within the IL-17 promoter to induce IL-17 expression (Kumar et al., 2021b). Given the critical role of IL-17 in inflammatory diseases, RORγt has emerged as an attractive target for pharmacological interventions (Beringer et al., 2016). However, a clear understanding of the regulation of RORγt is currently lacking, which is absolutely necessary to target RORγt effectively.

Crosstalk between different posttranslational modifications plays a critical role in regulating the activity of transcription factors (Hunter, 2007). We have demonstrated earlier that the E3 ligase Itch targets RORγt for ubiquitination, and Itch deficiency results in spontaneous rectal prolapse as a result of severe spontaneous colitis (Kathania et al., 2016). Here, we show how Pak2-mediated phosphorylation facilitates RORγt ubiquitination to prevent excessive Th17 response. A clear understanding of this regulatory pathway could lead to the development of safe and highly selective modulators of RORγt as a targeting strategy for inflammatory diseases driven by IL-17.
RESULTS

Pak2 interacts and phosphorylates RORγt

To gain molecular insights into the regulation of RORγt, we immunoprecipitated RORγt from the cell lysate of in vitro-generated Th17 cells and subjected it to mass spectrometry (MS) analysis. Through this approach, we identified Pak2 as a RORγt-binding protein (Figure 1A). To validate the MS results, we transiently transfected 293T cells with plasmids encoding Myc-tagged Pak2 and FLAG-tagged RORγt. Cell lysates were immunoprecipitated with either anti-RORγt antibody and analyzed by western blotting with anti-RORγt and anti-Pak2 antibody.

Figure 1. Pak2 interacts and phosphorylates RORγt

(A) Lysate of Th17 cells was precipitated using anti-RORγt antibody and subjected to high-resolution liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis. A representative LC-MS/MS spectrum corresponding to SDNGELEDKPPAPPVR of Pak2 is shown. Observed b and y ions are indicated. (B) Lysates were prepared from Th17 cells and immunoprecipitated using anti-RORγt and anti-Pak2 antibody analyzed by western blotting with anti-RORγt and anti-Pak2 antibody. (C) Immunoblotting of recombinant Pak2 precipitated with GST or GST-RORγt. Below are input controls for recombinant Pak2. (D) Sequence alignment of RORγt showing the conserved KRLS motif. (E) Pak2 was knocked down in CD4+ T cells using shRNA and cultured under Th17-inducing conditions. The cells were restimulated with anti-CD3 and anti-CD28 antibodies, and the lysate was subjected to immunoprecipitation (IP) with anti-RORγt antibody and analyzed by p-Ser specific antibody. (F) ADP-glow kinase assay result is represented as relative luciferase units (RLUs). (G) CD4+ T cells from Pak2fl/flCD4Cre were reconstituted with either wild-type or kinase-dead mutant of Pak2 and cultured under Th17 condition. Cell lysates were subjected to IP with anti-RORγt antibody and analyzed by p-Ser specific antibody. (H) CD4+ T cells were isolated from Rorcγt−/− mice, reconstituted with either wild-type or RORγt-ΔS316A mutant, and cultured under Th17 condition. Cell lysates were subjected to IP with anti-V5 antibody and analyzed by p-Ser specific antibody. Data are representative of two independent experiments. Data represent means ± SD. *p < 0.05; **p < 0.01; ***p < 0.001.
interaction between RORγt and Pak2 (Figure 1B). Similar results were obtained in CD4+ T cells sorted from cLPLs of mice treated with DSS (Figure S1D). We also performed glutathione S-transferase (GST) pull-down assays using recombinant Pak2 protein. GST-RORγt, but not GST alone, precipitated recombinant Pak2 (Figure 1C), suggesting a direct interaction.

Pak2 is a Serine/Threonine protein kinase that phosphorylates its target proteins on KRXS[S/T] motif (Taglieri et al., 2014). Amino acid sequence analysis revealed a highly conserved KRLS motif in RORγt, which could be potentially phosphorylated (Figure 1D). To test if Pak2 phosphorylates RORγt, we knocked down Pak2 in \textit{in vitro}-generated Th17 cells using short hairpin RNA (shRNA) lentiviral particles and found that inhibiting Pak2 reduced the phosphorylation of RORγt. As seen in Figure 1E, knocking down Pak2 reduced RORγt phosphorylation. Further, we confirmed that Pak2 phosphorylates RORγt by performing an \textit{in vitro} kinase assay. As shown in Figure 1F, incubation of RORγt with wild-type Pak2, but not a Pak2 kinase-dead mutant (Pak2–ΔK278R), resulted in RORγt phosphorylation. Similarly, incubation of the RORγt–ΔS316A mutant with wild-type Pak2 exhibited a defect in phosphorylation. These data suggest that Pak2 phosphorylates RORγt at Serine 316 (S316). To further confirm these results, we reconstituted CD4+ T cells isolated from Pak2f/fCD4Cre mice with either wild-type Pak2 or Pak2–ΔK278R by lentivirus transduction. We found Pak2−/− cells reconstituted with wild-type Pak2 or the Pak2−/− S316A mutant, resulted in RORγt phosphorylation (Figure 1H). These data collectively indicate that Pak2 phosphorylates RORγt at S316 in Th17 cells.

Inhibition of Pak2 enhances IL-17 expression and colitis
To gain insights into the functional consequence of Pak2-mediated phosphorylation of RORγt, we analyzed the expression of Il17a and Il17f mRNA in \textit{in vitro}-generated Th17 cells from wild-type and Pak2f/fCD4Cre mice. Results of real-time PCR experiments showed that Pak2 deficiency enhances the expression of Il17a and Il17f (Figures S2A and S2B). However, no significant difference in Rorc mRNA level was observed (Figure S2C). To gain \textit{in vivo} evidence for Pak2-mediated phosphorylation of RORγt in colonic inflammation, we sorted CD4+ T cells from wild-type and Pak2f/fCD4Cre mice, and the cells were differentiated under Th17 polarizing conditions. The cells were then adoptively transferred into Rag1−/− mice as described before (Lee et al., 2009). The Rag1−/− mice that received Pak2−/− Th17 cells showed more body weight loss, an increased fecal occult blood (FOB) score, increased diarrhea, splenomegaly, reduced colon length, and a higher weight-to-length ratio of the colon compared with the mice that received wild-type Th17 cells (Figures 2A–2E). Colonoscopic examination showed increased inflammation in mice that received Pak2−/− Th17 cells compared with those that received wild-type Th17 cells (Figures 2F and 2G). Similarly, histological analysis of H&E-stained sections showed greater infiltration of inflammatory cells, more crypt damage, and higher clinical scores in mice that received Pak2−/− Th17 cells than wild-type cells (Figures 2H and 2I). Real-time PCR analysis showed higher expression of Il17a and Il17f mRNA in the colonic mucosa of mice that received Pak2−/− Th17 cells (Figures 2M and 2N). Further, flow cytometric analysis (Figure S2D) showed an increased IL-17 expression by the adoptively transferred Pak2−/− Th17 cells in Rag1−/− mice compared with wild-type Th17 cells (Figure 2J). However, no significant changes in interferon gamma (IFN-γ) production (Figures 2K and S2E) was observed. Also, as expected, no change in IL-17 expression by innate lymphoid cells (ILCs) of host Rag1−/− mice was observed (Figure 2L). These data suggest that Pak2-mediated phosphorylation in Th17 cells plays a crucial role in colonic inflammation.

To test if increased IL-17 expression by the adoptively transferred Pak2−/− Th17 cells is potentially due to elevated Rorc mRNA expression, we performed real-time PCR analysis. No change in Rorc mRNA level (Figure 2O) was noted. However, immunoblotting the lysate of cLPLs showed an increased level of RORγt protein in mice that received Pak2−/− Th17 cells compared with wild-type Th17 cells (Figure 2P). These data suggest that Pak2 regulates RORγt protein turnover without affecting RORγt transcription.

Pak2 enhances the ubiquitination of RORγt
Next, we sought to investigate how Pak2 regulates RORγt protein turnover. We have previously shown that Itch targets RORγt for ubiquitination (Kathania et al., 2016). Since phosphorylation can affect protein turnover via ubiquitination (Hunter, 2007), we hypothesized that Pak2-mediated phosphorylation promotes RORγt ubiquitination. To test this hypothesis, we performed a ubiquitination assay by co-expressing either wild-type RORγt, RORγt–ΔS316A kinase mutant, or RORγt–ΔS316D phosphomimetic mutants along with the Itch. Our results showed an increased polyubiquitination of RORγt–ΔS316D (Figure 3A), suggesting that phosphorylation regulates ubiquitination of RORγt. To further confirm that phosphorylation promotes RORγt ubiquitination, we performed \textit{in vitro} ubiquitin assays using recombinant wild-type RORγt or RORγt mutants (RORγt–ΔS316A or RORγt–ΔS316D) along with recombinant Itch. Increased ubiquitination of RORγt–ΔS316D phosphomimetic mutant was observed compared with the RORγt–ΔS-A phospho-null mutant (Figure 3B). Finally, we analyzed ubiquitination of RORγt in \textit{in vitro}-generated Th17 cells from Pak2f/fCD4Cre mice lentivirally transduced with wild-type Pak2 or the Pak2–ΔK278R kinase-dead mutant. We found an increased ubiquitination in the cells transduced with wild-type Pak2 compared with the Pak2–ΔK278R kinase-dead mutant (Figure 3C).

To investigate if phosphorylation-regulated ubiquitination affected RORγt protein turnover, we performed a cycloheximide (CHX) chase experiment. The primary CD4+ T cells from Rorc–/− mice were lentivirally transduced with wild-type RORγt and RORγt–ΔS316D phospho-mimetic mutants. The cells were then treated with CHX, and the level of RORγt was analyzed by immunoblotting. Our results showed a reduced abundance of RORγt protein in RORγt–ΔS316D compared with wild-type RORγt transduced cells (Figure 3D). Similarly, the CHX chase experiment in \textit{in vitro}-generated Th17 cells from Pak2f/fCD4Cre mice reconstituted with wild-type Pak2 or the Pak2–ΔK278R kinase-dead mutant also showed a reduced abundance of RORγt protein in wild-type Pak2 transduced cells compared with the
Figure 2. Pak2 inhibition enhances IL-17 expression and Th17 cell-induced colitis.

Th17 cells were generated in vitro using CD4+ T cells isolated from wild-type and Pak2f/fCD4Cre mice and injected intraperitoneally into Rag1−/− mice (n = 5 animals per group).

(A) Body weight change in percentage.
(B) FOB score.
(C) Diarrhea score.
(D) Representative image of colon and spleen.
(E) Colon weight to length ratio.
(F) Representative colonoscopic images.
(G) Colonoscopic score.
(H) Representative H&E images. Scale bars, 100 µm.
(I) Histology score.
(J and K) cLPLs were stained with anti-CD4, anti-IL-17, and anti-IFN-γ antibodies and analyzed by flow cytometry.
(L) Flow cytometric analysis of IL-17 expression by ILCs in cLPLs.
(M–O) RNA isolated from cLPLs was assayed for the expression of Il17a, Il17f, and Rorc by real-time PCR.
(P) Lysates from CD4+ T cells sorted from cLPLs were analyzed by immunoblotting with anti-RORγt and anti-actin antibodies.

Data are representative of two independent experiments (n = 5). Dots indicate individual mice. Data represent means ± SD. *p < 0.05; **p < 0.01; ***p < 0.001. N.S., not significant.
Pak2-ΔK278R kinase-dead mutant (Figure 3E). These results strongly suggested that phosphorylation increases RORγt protein turnover. Additionally, the mRNA-to-protein ratio of RORγt in Rorc−/− CD4+ T cells reconstituted with wild-type RORγt and RORγt-ΔS316D phospho-mimetic mutant was lower compared with wild-type-RORγt (Figure 3F).
Itch is a HECT type E3 ligase that contains four WW domains (each of which contains two conserved tryptophan residues), which recognizes proline-rich PPXY (P, proline; X, any amino acid; Y, tyrosine) motifs in its substrate proteins (Venuprasad et al., 2015). We showed earlier that Itch binds to the conserved PPLY motif on RORγt via its WW domain (Kathania et al., 2016). To gain molecular insights into the mechanism by which phosphorylation at S316 promotes RORγt ubiquitination, we performed homology modeling of mouse RORγt. In silico analysis of the modeled structure showed that S316 made an H-bond (3.6 Å) with a side-chain amino group of asparagine (N) 253 of the neighboring α-helix and stabilized the ligand-binding domain (LBD) of RORγt, hence there was less accessibility of the PPLY motif (Figure 3G). We then substituted the S316 with phospho-mimetic aspartic acid residues (D) 316, and the energy of the modeled structure was minimized after the substitution mutation. The modeled structure revealed that the H-bond interaction between S316 to N253 is abolished after phosphorylation of serine, suggesting that phosphorylation may provide increased accessibility of the PPLY motif of RORγt to Itch (Figure 3G). To validate the computational analysis, we performed a GST pull-down assay using purified wild-type Itch, GST-RORγt, GST-RORγt-S316A, and GST-RORγt-S316D mutants. Our results showed that Itch association with RORγt-S316D mutant was more prominent compared with GST-RORγt or GST-RORγt-S316A mutant (Figure 3H). These results suggest that phosphorylation enhances the binding affinity of Itch to RORγt.

**Phosphorylation of RORγt inhibits IL-17 expression and colonic inflammation**

To confirm that Pak2-mediated phosphorylation prevents excessive IL-17-mediated inflammation, we reconstituted Rorc−/− CD4+ T cells with wild-type RORγt or RORγt-S316A or RORγt-S316D mutants. Cells were cultured under Th17 polarizing conditions, and expression of IL-17 was measured by real-time PCR. Our results showed an increased Il17a expression in Rorc−/− cells expressing RORγt-S316A mutant compared with wild-type RORγt, whereas significantly reduced Il17a expression was observed in cells expressing RORγt-S316D mutant (Figure 3I). However, no significant difference in Rorc mRNA was observed (Figure S2F).

To investigate the physiological impact of RORγt phosphorylation on the regulation of IL-17-mediated inflammation in vivo, we utilized an adoptive transfer colitis model (Lee et al., 2009). We sorted CD4+CD25− cells from Rorc−/− mice and reconstituted them with either empty vector, wild-type RORγt, or RORγt-S316A or RORγt-S316D mutants. The cells were differentiated under Th17 polarizing conditions for 5 days, and 5 × 10^5 cells were adoptively transferred into Rag1−/− mice (Figure 4A). The mice were monitored for signs of disease, including weight loss, FOB, and diarrhea. Rag1−/− mice that received Th17 cells expressing a phosphorylation-deficient mutant, RORγt-S316A, exhibited greater loss of body weight, higher FOB, diarrhea scores, splenomegaly, reduced colon length, and a higher weight-to-length ratio of the colon compared with host mice that received Th17 cells expressing RORγt-S316D mutant and wild-type RORγt (Figures 4B and 4F). Colonoscopic examination revealed enhanced inflammation in Rag1−/− mice that received Th17 cells expressing RORγt-S316A mutant compared with RORγt-S316D mutant or wild-type RORγt (Figures 4G and 4H). Histological analysis of H&E-stained sections showed greater infiltration of inflammatory cells, more crypt damage, and higher clinical scores for Rag1−/− mice that received RORγt-S316A mutant expressing cells compared with RORγt-S316D mutant or wild-type RORγt cells (Figures 4I and 4J). Rag1−/− mice that received Th17 cells expressing RORγt-S316A mutant had higher expression of Il17a and Il17f mRNA in the colonic mucosa (Figure 4K). Similarly, flow cytometric analysis of cLPLs showed increased IL-17 expression in Rag1−/− mice that received Th17 cells expressing the RORγt-S316A mutant compared with cells expressing the RORγt-S316D mutant or wild-type RORγt. However, there was no change in IFN-γ production by adoptively transferred cells (Figure 4L and 4M). Further expression of IL-17-induced chemokines and MMPs Cxcl1, Ccl2, Ccl7, Ccl20, Mmp1, Mmp2, Mmp3, and Mmp13 were significantly upregulated in Rag1−/− mice that received Th17 cells expressing RORγt-S316A mutation (Figures 3A and 3B). To test the potential difference in Rorc gene expression in adoptively transferred Th17 cells expressing wild-type RORγt and RORγt mutants, we performed real-time PCR experiments using RNA isolated from cLPLs of Rag1−/− mice. As shown in Figure 4K, no significant changes in Rorc mRNA expression were observed.
However, immunoblotting the lysates with anti-V5 antibody showed increased RORγt protein expression of the RORγt-Δ316A mutant, suggesting a defect in turnover of RORγt protein (Figure 4N). Together, these findings suggest that Pak2-mediated phosphorylation of RORγt negatively regulates IL-17 expression and controls colonic inflammation.

**DISCUSSION**

Genome-wide association studies have highlighted the central role of the RORγt:IL-17 pathway in several human inflammatory diseases (Duer et al., 2006; Lock et al., 2002; Parkes et al., 2013). Accordingly, the IL-17-inducing transcription factor RORγt has emerged as an attractive target for pharmacological interventions (Beringer et al., 2016). Our results show that Pak2-mediated phosphorylation of RORγt at S316 inhibits IL-17 expression by promoting ubiquitination of RORγt. Pak2−/− Th17 cells are highly colitogenic due to elevated IL-17 expression. Similarly, transfer of Th17 cells expressing RORγt-Δ316A phosphor mutant resulted in severe colitis, whereas the RORγt-Δ316D mutation that mimics the constitutively phosphorylated form of the RORγt mutant resulted in attenuated colitis in Rag1−/− mice. Mechanistically, we demonstrated that S316 phosphorylation of RORγt enhances its interaction with Itch due to increased exposure of the E3 ligase Itch interacting PLY motif. Our results could be exploited therapeutically to inhibit IL-17-mediated inflammation.

Pak2 was shown to be essential for maintaining regulatory T cell (Treg) stability and their suppressive function, and loss of Pak2, specifically in Tregs, resulted in defective Treg function (O’Hagan et al., 2017). Our data show that Pak2 plays a distinct role in Th17 cells by facilitating RORγt phosphorylation. Adoptive transfer of Pak2-deficient Th17 cells into Rag1−/− mice (which are devoid of T cells and Tregs) resulted in severe colitis. T cells recovered from the lamina propria of the recipient Rag1−/− mice exhibited an increased level of RORγt protein. Similarly, adoptive transfer of Th17 cells expressing the RORγt-Δ316A phospho-null mutant resulted in a defect in RORγt protein turnover and elevated IL-17 expression. These data strongly suggest a distinct function for Pak2 in regulating Th17 cells and Tregs.

IL-17 is produced by a variety of immune cells, including Th17 cells, ILCs, Tc17 cells, natural killer (NK) cells, and neutrophils (Kumar et al., 2021b). The cell-surface receptors involved and the intracellular signaling pathways leading to the secretion of cytokines significantly differ in these cells. However, it remains poorly understood if different immune cells’ regulation of IL-17 expression varies or adopts the same pathways. The data presented here identify a previously unknown mechanism of regulation of the RORγt:IL-17 pathway by Pak2 in Th17 cells. Our assay systems exclude the regulation of other IL-17-producing cells such as NK cells, CD8+ T cells, and ILCs. If Pak2 regulates IL-17 expression in these cells by a similar mechanism remains unknown, which requires further detailed studies, including cell-type-specific knockout mice (e.g., NK cell or ILC-specific Pak2−/− mice) and additional adoptive transfer experiments.

The upstream mechanisms that activate the Pak2-Itch pathway to trigger RORγt degradation in Th17 cells remain unclear. Pak2 is shown to be activated by Cdc42/Rac downstream of Vav following T cell receptor (TCR) stimulation (Ha et al., 2015; Taglieri et al., 2014). Therefore, it is possible that in Th17 cells, Pak2 acts as a feedback mechanism and inhibits IL-17 expression by phosphorylation of RORγt. It is also possible that IL-17 signaling activates Pak2 in Th17 cells to impede the pathogenicity of Th17 cells. Such a phenomenon has been reported recently via the induction of IL-24 (Chong et al., 2020). Additionally, Pak2 is involved in TGF-β signaling (Sato et al., 2013; Wilkes and Leof, 2006; Wilkes et al., 2003, 2005, 2009; Yan et al., 2012), which regulates Th17 development (Korn et al., 2009; Patel and Kuchroo, 2015). Therefore, it is also possible that Pak2 activated via the transforming growth factor β (TGF-β) pathway may trigger RORγt phosphorylation. Further, a detailed investigation of these pathways is essential to fully understand how RORγt protein turnover is regulated during an inflammatory response to prevent chronic inflammation.

The current strategies of targeting IL-17 using antagonistic antibodies (against IL-17 or its receptor) have shown some success in treating psoriasis and ankylosing spondylitis (Beringer et al., 2016); however, clinical trials using this approach led to mixed results in rheumatoid arthritis (RA) (Fragoulis et al., 2016; Genovese et al., 2013; Hueber et al., 2010) and failure in IBDs (Fitzpatrick, 2013; Hueber et al., 2012; Targan et al., 2016). Several recent studies have shown that RORγt function and protein stability are modified by posttranslational mechanisms, including ubiquitination, acetylation, and SUMOylation (Kumar et al., 2021b). A defect in RORγt protein degradation has been reported in tumor-infiltrated Th17 cells of patients with colorectal cancer (Sun et al., 2021). CRNDE-h protein was shown to form a complex with the PLY region of RORγt, and binding of CRNDE-h to RORγt in patients’ Th17 cells prevented ubiquitination and proteasomal degradation of RORγt by impeding its association with the Itch protein (Sun et al., 2021). On the contrary, our results show that Pak2 facilitates the binding of Itch to RORγt and promotes RORγt degradation. A clear understanding of the mechanism that regulates RORγt degradation could lead to alternative avenues to promote RORγt degradation at the site of inflammation. Such a strategy could transiently inhibit IL-17, resulting in therapeutic inhibition of Th17-mediated inflammation without deleterious effects observed in antibody-mediated systemic blocking of IL-17.

**Limitations of the study**

Although our study comprehensively shows that phosphorylation promotes ubiquitination of RORγt by enhancing its interaction with Itch, additional biophysical and cryoelectron microscopy evidence is necessary for the phosphorylation-dependent conformational changes in RORγt. In our in vivo experiments, we have lentivirally expressed RORγt-Δ316A in CD4+ T cells; studies using RORγt-Δ316A point mutation knockin mice will be necessary to determine the broader physiological relevance of our findings. Finally, in this study, we utilized only the Th17-adoptive transfer colitis model to demonstrate that phosphorylation of RORγt regulates Th17 cell-induced colon inflammation. If the Pak2-RORγt-Itch pathway operates in other Th17-dependent disease models needs to be tested.
STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.111345.

ACKNOWLEDGMENTS

The authors thank Dr. Ezra Burstein for the helpful discussions. This work was supported by funds from the National Institutes of Health (R01-DK115668 and R01-AI155786) and Cancer Prevention Research Institute of Texas (RP160577 and RP190527), a translational pilot project grant from the Harold C. Simmons Comprehensive Cancer Center, UT Southwestern Medical Center (23001045) and RP190527), a translational pilot project grant from the Harold C. Simmons Comprehensive Cancer Center, UT Southwestern Medical Center (23001045), and RP190527), a translational pilot project grant from the Harold C. Simmons Comprehensive Cancer Center, UT Southwestern Medical Center (23001045) and RP190527), a translational pilot project grant from the Harold C. Simmons Comprehensive Cancer Center, UT Southwestern Medical Center (23001045).補充実験データと統計解析

AUTHOR CONTRIBUTIONS

M.K., R.K., and T.L.E. performed the experiments, analyzed the data, and helped to prepare the manuscript. V.B. performed MS analysis. A.L.T. and J.C. helped to prepare the manuscript. K.V. conceived the project, designed the experiments, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We worked to ensure sex balance in the selection of non-human subjects. One or more of the authors of this paper self identifies as an underrepresented ethnic minority in science.

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### STAR+METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-c-Myc (9E10)   | Santa Cruz Biotechnology | Cat: sc-40; RRID:AB_2857941 |
| Anti-Pak2           | Santa Cruz Biotechnology | Cat: sc-133113; RRID:AB_1568809 |
| Anti-Flag (M2)      | Sigma Aldrich | Cat: F1804; RRID:AB_262044 |
| Anti-RORγt (FKJS-9) | eBioscience | Cat:14698882; RRID:AB_1834475 |
| Anti-Italic (D2)    | BD Bioscience | Cat: 611198; RRID:AB_398732 |
| Anti-ROTY (D28-835) | BD Bioscience | Cat: 562197; RRID:AB_10894594 |
| Anti-GST (B14)      | Santa Cruz Biotechnology | Cat: sc-138; RRID:AB_627677 |
| Anti-Ahr (B-11)     | Santa Cruz Biotechnology | Cat: sc-74571; RRID:AB_2223948 |
| Anti-Itch (C4)      | Santa Cruz Biotechnology | Cat: sc-47778; RRID:AB_626632 |
| Anti-mouse-HRP      | Amersham Bioscience | Cat: NA931; RRID:AB_772210 |
| Anti-rabbit-HRP     | Cell Signaling Technology | Cat: 7074; RRID:AB_2099233 |
| Clean-BlotTM IP (HRP) | Thermo Fisher Scientific | Cat: 21320; RRID:AB_2864363 |
| VeriBlot IP (HRP)   | Abcam | Cat: 131366; RRID:AB_2892718 |
| Anti-CD45-APC (104) | eBioscience | Cat:17045482; RRID:AB_469400 |
| Anti-IL17-PerCP-Cy5.5 (TC11) | Biolegend | Cat:506920; RRID:AB_961384 |
| Anti-CD4-FITC (GK1.5) | Biolegend | Cat:100406; RRID:AB_312691 |
| Anti-IFN-γ-APC      | BD Bioscience | Cat:554413;RRID:AB_395851 |
| FC-block (2.4G2)    | BD Bioscience | Cat:553142; RRID:AB_394657 |
| Anti-mouse CD3ε     | Biolegend | Cat:100223; RRID:AB_1877072 |
| Anti-mouse IFN-γ     | BioXCell | Cat:BE0054; RRID:AB_1107692 |
| Anti-mouse CD28      | BioXCell | Cat:BE0015; RRID:AB_1107624 |

**Chemicals, peptides, and recombinant proteins**

- Murine IL-2: Peprotech, Cat: 212-12
- Murine IL-6: Peprotech, Cat: 216-16B
- Murine TGF-β1: Peprotech, Cat: 100-21
- Pak2 shRNA(m) lentiviral particle: Santa Cruz Biotechnology, Cat: sc-36184-V
- Live Dead Aqua: Invitrogen, Cat: L34957
- Cytofix/Cytoperm: BD Bioscience, Cat: 55028
- Perm/Wash Buffer: BD Bioscience, Cat: 554723
- Lamina Propria Dissociation Kit (mouse): Miltenyi Biotec, Cat:130097410
- Naïve CD4+ T Cell Isolation Kit (mouse): Miltenyi Biotec, Cat:130104453
- CD4+ T Cell Isolation Kit (mouse): Miltenyi Biotec, Cat:130104454
- Verso cDNA Synthesis Kit: Thermo Fisher Scientific, Cat: AB1453/B
- SYBR GREEN I Master Mix (2X): Roche Diagnostic, Cat: 507203180
- OneTag 2X Master Mix: NEB, Cat: M0482L
- Q5® Site-Directed Mutagenesis Kit: NEB, Cat: E0554S
- QIAGEN Plasmid Plus Maxi Kit: Qiagen, Cat: 12963
- QIAquick Gel Extraction Kit: Qiagen, Cat: 28706
- RNasy Micro Kit: Qiagen, Cat: 74004
- RNasy Mini Kit: Qiagen, Cat: 74104
- Luciferase Assay System: Promega, Cat: E1500
- LS Columns MACS Separation Columns: Miltenyi Biotec, Cat:130042401
- cOmplete™ Proteinase Inhibitor Cocktail: Roche, Cat:4693159001

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact: K Venuprasad (venuprasad.poojary@utsouthwestern.edu).

Materials availability
This work did not generate any unique reagents.

Data and code availability
This paper does not report the original code. All software utilized is freely or commercially available and is listed in the key resources table. All data reported in this paper will be shared by the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
C57BL/6 mice, Rag1<sup>−/−</sup> mice, Rorc<sup>fl/fl</sup> mice, and CD4<sup>cre</sup> mice were purchased from the Jackson Laboratory. Pak2<sup>fl/fl</sup> mice were described before (Kosoff et al., 2013). Equal number of male and female mice were used. All mice were housed in micro isolator cages in the barrier facility of the University of Texas Southwestern Medical Center. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| Phorbol 12-myristate 13-acetate (PMA) | Sigma Aldrich | Cat: P8139 |
| Ionomycin calcium salt | Sigma Aldrich | Cat: I0634 |
| Restore<sup>TM</sup> Western Blot Stripping Buffer | Thermo Fisher Scientific | Cat: 21063 |
| ECL Prime Detection Reagent | GE Healthcare | Cat: RPN2236 |
| Lipofectamine<sup>TM</sup> 2000 | Thermo Fisher Scientific | Cat: 11668019 |
| RPMI 1640 Medium | Thermo Fisher Scientific | Cat: 11875093 |
| HBSS | Thermo Fisher Scientific | Cat: 14175103 |
| Golgi Stop | BD Bioscience | Cat: 554724 |
| Golgi Plug | BD Bioscience | Cat: 555029 |
| RNase-Free DNase Set | Qiagen | Cat: 79254 |
| RBC Lysis Buffer (10X) | Biolegend | Cat: 420301 |
| SYBR Safe DNA Gel Stain | Invitrogen | Cat: S33102 |
| Protein A/G PLUS-Agarose | Santa Cruz Biotechnology | Cat: sc2003 |
| HEK293T | ATCC | Cat: CRL-3216 |
| Mouse: C57BL/6 | Jackson Laboratory | Cat: 000664; RRID:IMSR_JAX:000664 |
| Mouse: Rag1<sup>−/−</sup> | Jackson Laboratory | Cat: 002216; RRID:IMSR_JAX:002216 |
| Mouse: Pak2<sup>fl/fl</sup> | Dr. Jonathan Chernoff |
| Mouse: Rorc<sup>fl/fl</sup> | Jackson Laboratory | Cat: 007571; RRID:IMSR_JAX:007571 |
| Mouse: CD4<sup>cre</sup> | Jackson Laboratory | Cat: 022071; RRID:IMSR_JAX:022071 |
| Plasmid:MIGR-RORγt | Addgene | Cat: 24069 |
| FlowJo v10.5.3 | BD Bioscience | https://www.flowjo.com |
| GraphPad Prism v7 | GraphPad | https://www.graphpad.com |
| Biorender | Biorender | https://biorender.com/ |
| PyMol | Schrodinger | https://pymol.org/2/ |
**Th17 cell-induced colitis**

Naïve CD4+ T cells were isolated from spleen and lymph nodes from wild-type, Pak2/Cd4Cre mice or Rorc--/-- mice using CD4+ T cell isolation kit. Cells were then stained with antibodies against APC-CD25 and FITC-CD4 and were sorted for CD4+CD25+ populations (Kumar et al., 2021a). CD4+CD25+ cells from Rorc--/-- mice (male and female, 7-8 week old) were lentivirally transduced with RORγt and RORγt phospho mutants (ΔS316A and ΔS316D) for 24 h. Transduced cells were then cultured under Th17 inducing conditions for 5 days. Rag1--/-- mice (male and female, 7-8 week old) were injected intra-peritoneally with 5 x 10^5 Th17 cells and monitored for disease severity up to 8 weeks.

**METHOD DETAILS**

**Plasmid construction and cell transfection**

Myc-Itch, HA-Ub (wild-type), and Flag-mRORγt have been described (Kathania et al., 2016). Transient transfection of 293T cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Myc-Pak2 and Myc-Pak2-ΔK278R were created and cloned into pCDNA3.1 between NotI and BamHI sites. The sequences of all clones were verified.

**Lentiviral transduction**

Expression clones were created by Gateway cloning technology according to the manufacturer’s instructions (Invitrogen). Lentiviral expression clones of mouse mRORγt-ΔS316A, mRORγt-ΔS316D, Pak2, and Pak2-ΔK278R were constructed by first subcloning into pENTR-3C entry vector (Invitrogen), and then subcloned to pLenti6.2/N-Lumio/V5-DEST (Invitrogen) via Gateway cloning. The ViraPower lentiviral expression system (Invitrogen) was used for packaging and producing lentiviruses in 293FT cells. The supernatant was harvested after 72 h. CD4+ T cells were transduced using lentiviruses in the presence of 5 μg/mL polybrene (Santa Cruz), and the media was changed after 12 h.

**Protein identification by liquid chromatography (LC)-tandem MS**

The protein samples were processed and analyzed at the Mass Spectrometry Facility of the Department of Pathology at the University of Michigan. Gel slices (10 slices/lane) were destained with 30% methanol for 4 h. Upon reduction (10 mM DTT) and alkylation (65 mM 2-Chloroacetamide) of the cysteines, proteins were digested overnight with sequencing grade, modified trypsin (Promega). The resulting peptides were resolved on a nano-capillary reverse phase column (Acclaim PepMap C18, 2 micron, 25 cm, ThermoScientific) using a 1% acetic acid/acetonitrile gradient at 300 nL/min and directly introduced in to Orbitrap Fusion tribrid mass spectrometer (Thermo Scientific, San Jose CA). MS1 scans were acquired at 120K resolution. Data-dependent high-energy C-trap dissociation MS/MS spectra were acquired with top speed option (3 sec) following each MS1 scan (relative CE ~32%). Proteins were identified by searching the data against the Mus musculus database (24861 entries) using Proteome Discoverer (v1.4, Thermo Scientific). Search parameters included MS1 mass tolerance of 10 ppm and fragment tolerance of 0.2 Da; two missed cleavages were allowed; carbamidimethylation of cysteine was considered fixed modification and oxidation of methionine were considered a potential modification. Percolator algorithm was used for discriminating between correct and incorrect spectrum identification. The false discovery rate (FDR) was calculated at the peptide level and peptides with <1% FDR were retained. Immunoprecipitated proteins were separated by SDS-PAGE. In-gel digestion with trypsin, followed by protein identification using LC-tandem MS, was performed as described elsewhere. Briefly, tryptic peptides were resolved on a nano-LC column (Magic AQ C18; Michrom Bioresources, Auburn, CA) and introduced into an Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA). The Orbitrap was set to collect a high-resolution MS1 (FWHM 30,000@400 m/z), followed by the data-dependent collision-induced dissociation spectra on the “top 9” ions in the linear ion trap. Spectra were searched against a human protein database (UniProt release 2011_05) using the XITandem/TPP software suite. Proteins identified with a Protein Prophet probability Z0.9 false discovery rate o2% were considered for further analysis.

**Real-time PCR analysis**

Total RNA was prepared using RNeasy Mini Kit (Qiagen), followed by cDNA synthesis using Verso cDNA kit (Thermo Scientific). Quantitative real-time PCR was performed on a Mastercycler Realplex2 (Eppendorf). Light Cycler 480 SYBR Green I master reaction mix (Roche) was used in a 20 μL reaction volume. The expression of individual genes was normalized to the expression of actin. Cycling conditions were: 95°C for 2 min, followed by 50 cycles of 95°C for 15 sec, 55°C for 15 sec, and 72°C for 20 sec.

**Th17 cell differentiation**

For in vitro experiments, naïve CD4+ T cells from the spleen and lymph nodes were differentiated into Th17 cells in plates coated with anti-CD3 (1 μg/mL) and anti-CD28 (2 μg/mL). The cells were cultured in the presence of TGF-β (5 ng/mL), IL-6 (20 ng/mL), anti-IL-4 (5 μg/mL) and anti-IFN-γ (5 μg/mL) antibodies for 5 days.
Flow cytometry
Lamina propria lymphocytes were isolated using the Lamina Propria Dissociation Kit (Miltenyi Biotec) following the manufacturer’s instructions. Cells were washed with 1X PBS and then incubated with Fc block (553142, BD Bioscience). Cells were then stained with Live-Dead aqua (L34957, Invitrogen) for live cells and further stained with combinations of antibodies. Antibodies used were Lin-PB (79724, BioLegend), CD45.2-APC (17-0454-81, eBioscience), CD4-FITC (100406, BioLegend), IL-17-PerCP-Cy5.5 (506920, BioLegend), IFN-γ-APC (1554413, BD Bioscience). Data were acquired with a FACSCanto II (BD) and analyzed with FlowJo software (Tree Star).

Immunoprecipitation and immunoblot analysis
Cell lysates were prepared from 293T cells transfected with Myc-Pak2 and Flag-RORγt plasmids after 36 h by homogenizing in NP-40 lysis buffer. Protein estimations were done using the Pierce BCA protein assay kit according to the manufacturer’s protocol. Whole-cell lysates were precleared with 20 µL of Protein A/G plus agarose beads (Santa Cruz) for 1 h at 4°C. Lysates were then incubated with 1 µg of the desired antibody overnight at 4°C followed by a further 1 h of incubation at 4°C with 25 µL of Protein A/G beads. The immunocomplexes were washed five times with lysis buffer and denatured using 4X Laemmli buffer. Further, they were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Blots were visualized using Amersharm ECL plus immunoblot analysis detection system (GE, Chicago, IL) on a Biorad ChemiDoc. For reprobing, membranes were stripped by incubation in a stripping buffer (62.5 mM Tris-HCl, pH 6.7; 100 mM 2-mercaptoethanol and 2% SDS) at 55°C for 45 min and washed thoroughly before reprobing.

In vitro kinase assays
Pak2 kinase activity was assessed using ADP-Glo Kinase Assay Kit (V9101, Promega), following the manufacturer’s instructions. Recombinant RORγt protein (1 µg) was incubated with 100 ng of recombinant Pak2 kinase. The kinase reaction with a final ATP concentration of 50 µM was incubated at room temperature for 2 h. The kinase reaction was then terminated by adding ADP-Glo reagent for 40 min, followed by the addition of kinase detection reagent for 30 min incubation before reading the luminescence on a multimode microplate reader.

Ubiquitination assay
293T cells were transfected with Flag-RORγt, Myc-Itch, and various other constructs as indicated. MG132 was added 6 h before cell lysis. Cells were washed three times with PBS and lysed in NP-40 lysis buffer. Immunoprecipitation was performed using anti-Flag antibody. RORγt-associated ubiquitin was analyzed by immunoblot using antibody against HA. An in vitro ubiquitination assay was performed using the E2-Ubiquitin Conjugation Kit (Abcam, Cambridge, MA) with GST-RORγt, GST-RORγt-ΔS316A or GST-RORγt-ΔS316D protein as substrate and Itch as the E3 ligase following the manufacturer’s protocol.

TUBE pull-down assay
The pull-down of ubiquitinated proteins was performed with GST-tagged TUBE, following the manufacturer’s instructions (Life Sensors). Briefly, for Pan–Ub, cells were collected, washed twice with PBS, and lysed with TUBE buffer supplemented with protease inhibitors (Pierce) and 5 mM NEM. Clarified lysates were incubated for 2 h on equilibrated glutathione Sepharose-GST-TUBE under rotation (Mukherjee et al., 2020). The resin was washed 3 times with TBS-T (200 mM Tris-HCl pH 8.0, 0.15 M NaCl, 0.1% Tween-20) and resolved by SDS-PAGE. RORγt-associated ubiquitin was analyzed by immunoblot using antibody against HA.

Protein expression and purification
RORγt and RORγt mutant (ΔS316A and ΔS316D) were sub-cloned into pGEX-6P1-DEST vector containing GST tag at N-terminal. Further, GST–RORγt and GST–RORγt mutant proteins were individually expressed in RosettaTM (DE3) pLysS E. coli. Cleared lysates were prepared and the soluble fusion proteins were purified on glutathione Sepharose 4B GST-tagged protein purification resin (GE Healthcare Life Sciences) and tested by immunoblot using monoclonal anti-GST (Cell Signaling Technology). GST-Itch protein was purified as shown previously (Paul et al., 2018).

Homology modeling
The protein sequences of mouse RORγt were retrieved from the UniProt database (P51450-2). Homologs to the target mouse RORγt LBD domain sequence were identified by PSI-blast for PDB databank on NCBI. The three-dimensional structure of mouse RORγt LBD domain was generated by SWISS-MODEL using the PDB: 5EJV. The substitution mutation of S316 to D316 was performed in PyMol. H-bond distances between the amino acids were measured in PyMol. Further, the analysis of the modeled structure of the LBD domain of mouse RORγt by AlphaFold (AF-P51450-F1) was done in PyMol.
**Colonoscopic examination**

To measure colitis severity, a high-resolution murine video endoscopic system was used. For monitoring colon inflammation *in vivo*, mice were anesthetized with Ketamine/Xylazine, and endoscopy was performed using a mini-endoscope from Karl-Storz. Grading of colitis scores was performed according to the mouse endoscopic index of colitis severity (MEICS). In brief, MEICS was determined by perianal findings, wall transparency, intestinal bleeding, and focal lesion. Each of these four different parameters of inflammation was given a score from 0 to 3, resulting in a total MEICS ranging from 0 to 12 (Kodani et al., 2013).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Data are represented as mean ± SD. Differences in group survival were analyzed by the Kaplan-Meier test using Prism 8 (GraphPad Software). Statistical significance was determined by unpaired Student’s t-test and one-way ANOVA; *p* < 0.05 was considered statistically significant.