Tumour suppressor death-associated protein kinase targets cytoplasmic HIF-1α for Th17 suppression

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Death-associated protein kinase (DAPK) is a tumour suppressor. Here we show that DAPK also inhibits T helper 17 (Th17) and prevents Th17-mediated pathology in a mouse model of autoimmunity. We demonstrate that DAPK specifically downregulates hypoxia-inducible factor 1α (HIF-1α). In contrast to the predominant nuclear localization of HIF-1α in many cell types, HIF-1α is located in both the cytoplasm and nucleus in T cells, allowing for a cytosolic DAPK–HIF-1α interaction. DAPK also binds prolyl hydroxylase domain protein 2 (PHD2) and increases HIF-1α-PHD2 association. DAPK thereby promotes the proline hydroxylation and proteasome degradation of HIF-1α. Consequently, DAPK deficiency leads to excess HIF-1α accumulation, enhanced IL-17 expression and exacerbated experimental autoimmune encephalomyelitis. Additional knockout of HIF-1α restores the normal differentiation of Dapk−/− Th17 cells and prevents experimental autoimmune encephalomyelitis development. Our results reveal a mechanism involving DAPK-mediated degradation of cytoplasmic HIF-1α, and suggest that raising DAPK levels could be used for treatment of Th17-associated inflammatory diseases.
Upon activation, the T helper 17 (Th17) subset of immune cells plays critical roles in modulating tissue inflammation and combating microbial infections. However, due to their inflammatory nature, Th17 cells also contribute to autoimmune diseases. Experimental autoimmune encephalomyelitis (EAE) is a well-studied mouse model for multiple sclerosis that is also mediated by Th17 (refs 4–6). Th17 cells differ from the Th1 and Th2 lineages in secretion of interleukin (IL)-17 (refs 7,8), which induces inflammatory gene expression in target cells and leads to pathogenesis in the EAE model. Transforming growth factor (TGF)-β is critical for the commitment to the Th17 lineage. Transforming growth factor β (TGF)-β acts synergistically with the STAT3-activating cytokines, IL-6, IL-21 and IL-23, to promote RORγt expression and Th17 differentiation. The Th17-specific transcription factor RORγt acts together with RORα and STAT3 (ref. 17) to induce full Th17 cell differentiation.

Hypoxia-inducible factor-1α (HIF-1α) is an oxygen tension sensor widely expressed in different cell types, including Th17 cells. In the presence of O2, HIF-1α is hydroxylated at Pro402 and Pro564 by prolyl hydroxylase domain proteins 2 (PHD2)/PHD3, followed by ubiquitination by the von Hippel–Lindau (VHL)-containing E3 complex that promotes proteasomal degradation. At low oxygen tension, HIF-1α is stabilized by inactivation of PHD2/PHD3 (refs 18–22). Once stabilized, HIF-1α activates the expression of target genes involved in hypoxic responses. HIF-1α is also upregulated by inflammatory cytokines in normoxic conditions.

The Hif1α transcript is constitutively expressed in T lymphocytes, and the HIF-1α protein is detected after T-cell receptor (TCR) stimulation under hypoxic conditions. HIF-1α is highly expressed in Th17 cells, priming at physiological oxygen tension in the presence of inflammatory cytokines. HIF-1α plays a prominent role in Th17 cell differentiation by activating the transcription of Rorc (RORγt), and it helps recruit CBP/p300 to the RORγt transcription complex but does not directly bind to the IL-17 promoter. Additionally, HIF-1α increases glycolysis by inducing the expression of glycolytic enzymes, which further contributes to Th17 development. HIF-1α also contributes to the survival of Th17 cells by coordination with Notch to enhance Bcl-2 expression. In contrast, targeted degradation of HIF-1α by miR-210 negatively regulates Th17 differentiation.

HIF-1α promotes carcinogenesis and is a prominent cancer target. Various HIF-1α inhibitors have been identified and are currently being studied for their efficacy in cancer therapy. Presumably, HIF-1α inhibitors could also be used for treatment of Th17-mediated inflammatory diseases. However, HIF-1α is essential for oxygen homeostasis, and curtailment of the protective effects of HIF-1α by HIF-1α inhibitors may limit their application.

Death-associated protein kinase (DAPK) is a multi-domain serine/threonine kinase regulated by calcium. DAPK belongs to the DAPK family, which also contains DAPK-related protein 1 and zipper-interacting protein kinase (also called DAPK3), both of which share 80% identity in their kinase domains with DAPK. The DAPK family also contains two distantly related kinases: DAPK-related apoptosis inducing kinase 1 and 2 (DRK1 and DRK2). DAPK family members are pro-apoptotic proteins and function as tumour suppressors, and are specifically downregulated in many types of cancer. DAPK participates in a wide variety of cellular events—including apoptosis, autophagy, membrane blebbing and stress fibre formation—that contribute to its tumour suppressor functions. In T lymphocytes, DAPK inhibits T-cell activation by suppressing TCR-induced nuclear factor (NF)-κB activation. DAPK is induced by TGF-β (ref. 43), and is present in the early precursors of Th17, but the role of DAPK in Th17 immune cells is unclear.

In the present study, we found that DAPK negatively regulates Th17 differentiation. DAPK deficiency leads to preferential Th17 differentiation and exacerbated EAE induction. During the differentiation of Th17, the presence of DAPK is accompanied by downregulation of HIF-1α. We further found that, in contrast to the exclusive nuclear localization of HIF-1α in most other cells, HIF-1α is located in both the cytoplasm and nucleus of T cells, allowing the cytosolic binding of DAPK and subsequent HIF-1α degradation. Our results illustrate a novel mechanism of Th17 regulation by downregulating cytoplasmic HIF-1α, and suggest the therapeutic potential of increasing DAPK levels in Th17-mediated inflammatory diseases.

**Results**

**DAPK inhibits T-cell activation and attenuates EAE.** Our previous studies showed that expression of the dominant negative mutant of DAPK, [K42A]DAPK, leads to increased IL-2 production and NF-κB activation in T-cell lines. Extending these studies, we examined T cells from the Dapk−/− mouse. T-cell development was not affected by DAPK deficiency, as illustrated by the normal thymic and peripheral T-cell populations in the Dapk−/− mouse (Supplementary Fig. 2a). This was associated with increased IL-2 and interferon (IFN)-γ production in naive Dapk−/− T cells (Supplementary Fig. 2b). An increase in IL-17 was also observed in Dapk−/− T cells (Supplementary Fig. 2c). In contrast, the production of IL-4 in primary T cells was not affected by DAPK deficiency (Supplementary Fig. 2d). A prominent increase in NF-κB activation, indicated by the enhanced nuclear translocation of p65, was also observed in Dapk−/− T cells stimulated through the TCR (Supplementary Fig. 2e).

We further examined whether the enhanced T-cell activation in Dapk−/− mice led to increased susceptibility to autoimmune diseases, using EAE as our model. After priming with myelin oligodendrocyte glycoprotein (MOG) peptide, disease onset was earlier in Dapk−/− mice than their WT littermates (Fig. 1a). Additionally, the severity of encephalomyelitis was higher in Dapk−/− mice than their WT counterparts. This was correlated with an increase in mononuclear cell infiltration and demyelination in spinal cords from primed Dapk−/− mice (Fig. 1b,c). We also isolated T cells from the primed mice and determined their reactivity towards antigen. Consistent with exacerbated EAE generation, the response to MOG peptide in Dapk−/− T cells was nearly double that of control T cells (Fig. 1d).

We further used transgenic mice with T-cell-specific expression of [ΔCAM]DAPK, the constitutively active form of DAPK, to analyse EAE induction. T-cell development was normal in [ΔCAM]DAPK-transgenic mice (Supplementary Fig. 3a,b). We increased the dose of MOG peptide for a more profound EAE induction. [ΔCAM]DAPK-transgenic mice (Supplementary Fig. 3a,b). We increased the dose of MOG peptide for a more profound EAE induction. We also isolated T cells from the primed mice and determined their reactivity towards antigen. Consistent with exacerbated EAE generation, the response to MOG peptide in [ΔCAM]DAPK−/− T cells was nearly double that of control T cells (Fig. 1d).

We further used transgenic mice with T-cell-specific expression of [ΔCAM]DAPK, the constitutively active form of DAPK, to analyse EAE induction. T-cell development was normal in [ΔCAM]DAPK-transgenic mice (Supplementary Fig. 3a,b). We increased the dose of MOG peptide for a more profound EAE induction. We also isolated T cells from the primed mice and determined their reactivity towards antigen. Consistent with exacerbated EAE generation, the response to MOG peptide in [ΔCAM]DAPK−/− T cells was nearly double that of control T cells (Fig. 1d). Together, these results demonstrate that DAPK deficiency enhanced EAE generation, whereas T-cell-specific expression of active DAPK suppressed EAE induction.
suggestion that DAPK in T cells negatively regulates EAE pathogenesis.

Both Th17 and Th1 contribute to the development of EAE and multiple sclerosis. Notably, IL-17 production, but not IFN-γ generation, was increased in MOG-primed Dapk−/− T cells (Fig. 1e), suggesting a preferential expansion of DAPK-deficient Th17 cells. We examined the expression of IFN-γ and IL-17 in mononuclear cells infiltrated into the spinal cords of EAE-induced mice. During the peak of EAE induction, IFN-γ + cells were more abundant than IL-17 + cells in spinal cords from WT mice (Supplementary Fig. 4a). Conversely, more IL-17 + cells than IFN-γ + cells were detected in the spinal cords from Dapk−/− mice with encephalomyelitis (Supplementary Fig. 4a). Quantitation shows an increased IL-17 + :IFN-γ + cells ratio in EAE-inducing Dapk−/− mice (Supplementary Fig. 4b).

We further examined the induction of EAE in Rag1−/− mice through adoptive transfer of 2D2 T cells. The adoptive transfer of MOG-specific 2D2 cells that were differentiated into Th17 cells allows for assessment of the effect of DAPK on the induction of antigen-specific and Th17-specific EAE. Dapk−/− 2D2 Th17 cells triggered earlier disease onset and induced more severe encephalomyelitis than WT 2D2 Th17 cells (Fig. 1f). In contrast, the EAE onset and disease severity induced by Dapk−/− 2D2 Th17 cells and WT 2D2 Th17 cells were similar (Fig. 1g). Together, these results suggest that DAPK deficiency leads to an increase in encephalitogenic Th17 cells, but not encephalitogenic Th1 cells.

**Preferential Th17 differentiation from Dapk−/− T cells.** We found that expression of DAPK was higher in Th17 cells than Th1 cells (Fig. 2a), consistent with the report that DAPK is induced by TGF-β. Therefore, we examined differentiation of Th1 and Th17 cells from Dapk−/− T cells. Naïve CD4 + T cells from control and Dapk−/− T cells were stimulated by

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**Figure 1 | DAPK deficiency increases T-cell activation and EAE exacerbation.** (a) EAE induction in wild-type (WT) and Dapk−/− mice. DAPK knockout and WT control mice were immunized with 200 μg of myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 emulsified in CFA, followed by intraperitoneal injection of 200 ng pertussis toxin at day 0 and day 2. The progression of disease was monitored. Values are mean ± s.d. n = 10 for each group. (b, c) Increased mononuclear cell infiltration and demyelination in spinal cords from primed Dapk−/− mice. Spinal cords from WT and Dapk−/− mice were isolated 11 days after MOG immunization, and fixed and frozen sections were obtained. Tissue sections were stained with hematoxylin and eosin (H&E) (b) and luxol fast blue (LFB) (c). Red scale bar, 400 μm; black scale bar, 80 μm. Photos are representative of three mice in each group. (d) Enhanced T-cell response to MOG (33–55) in Dapk−/− mice. Splenic CD4 T cells were harvested 9 days after immunization. Cells were stimulated with irradiated autologous presenting cells plus MOG peptide, and the incorporation of thymidine was determined 72 h later. (e) Increased IL-17 production due to MOG (33–55) stimulation in Dapk−/− T cells. The secretion of IFN-γ and IL-17 was quantitated in T cells from d. Data (d,e) are mean ± s.d. (n = 3), and are representative of three independent experiments. (f) 2D2 Dapk−/− Th17 cells induced exacerbated EAE in Rag1−/− mice. CD4 + T cells from 2D2 or 2D2 Dapk−/− mice were differentiated into Th17 cells in vitro. 2D2 or 2D2 Dapk−/− Th17 cells were re-stimulated and transferred intravenously to Rag1−/− mice, followed by intraperitoneal administration of pertussis toxin. Mice were monitored for clinical signs of paralysis and recorded daily. (g) Comparable EAE generation by 2D2 WT Th1 cells and 2D2 Dapk−/− Th17 cells in Rag1−/− mice. 2D2 or 2D2 Dapk−/− Th17 cells were transferred to Rag1−/− mice, and clinical signs were monitored, as described in f. Values are mean ± s.e.m. (f,g). *P < 0.05, **P < 0.01, ***P < 0.001 for unpaired t-test. NS, not significant.
Figure 2 | Enhanced Th17 development in Dapk−/− T cells. (a) Increased DAPK levels in Th17 cells. WT naive CD4 (CD4+CD25−CD44−CD62L+) T cells were subjected to differentiation into Th1 and Th17 cells for 3 days. The levels of DAPK were determined. (b) Increased IL-17 production in Dapk−/− Th17 cells. WT and Dapk−/− Th17 cells differentiated for 5 days were re-stimulated with TPA/A23187 for 4 h in the presence of monensin during the last 5 h, and the expression of IL-17 and IFN-γ were analysed by intracellular staining. The numbers represent the percentages of cells positive (left). The levels of phospho-STAT3 and total STAT3 were examined by western blotting. Data (a-c,i) are representative of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 for unpaired t-test.
CD3/CD28 in the presence of TGF-β and IL-6 to induce Th17 differentiation. Differentiation into Th17 was significantly increased for \( \text{Dapk}^{-/-} \) T cells, with the IL-17-producing population significantly larger in \( \text{Dapk}^{-/-} \) Th17 cells relative to the WT (Fig. 2b, left panel), as also illustrated by an elevated mean fluorescence intensity (MFI; Fig. 2b, right panel). This was also confirmed by ELISA, which showed a threefold increase in IL-17 secretion by \( \text{Dapk}^{-/-} \) cells compared with control Th17 cells (Fig. 2c). In addition, for Th17 cells induced by IL-21 and TGF-β, IL-17 production also increased approximately threefold for \( \text{Dapk}^{-/-} \) Th17 cells (Supplementary Fig. 5a). A similar extent of increase in IL-17 expression was found for \( \text{Dapk}^{-/-} \) Th17 cells induced by IL-1β, IL-6 and IL-23 (Supplementary Fig. 5b). However, no effect was found on DAPK deficiency in Th17 cells induced by IL-1β, IL-6 and IL-23 (ref. 45; Supplementary Fig. 5c), suggesting that regulation of Th17 by DAPK depends on priming with TGF-β. In parallel experiments, T-cell-specific transgenic expression of \( \Delta \text{CAM} \) DAPK attenuated Th17 differentiation mediated by TGF-β and IL-6 (Fig. 2d,e). Together, these results suggest that DAPK inhibits TGF-β-dependent Th17 development.

Elevated RORγt and RORα in \( \text{Dapk}^{-/-} \) Th17 cells. We then determined whether the increased production of IL-17 correlated with enhanced induction of the transcription of \( \text{Il}17 \) genes. Quantitative PCR confirmed that the expression of \( \text{Il}17a \) and \( \text{Il}17f \) was elevated in DAPK-deficient Th17 cells relative to the WT Th17 cells (Fig. 2f), implying that enhanced IL-17 generation could be partly attributed to increased expression of their mRNAs. Induction of \( \text{Il}21 \) and \( \text{Il}23r \)—the cytokine and receptor preferentially expressed in Th17 cells, respectively—were also increased in \( \text{Dapk}^{-/-} \) Th17 cells. In addition, levels of \( \text{Rorc} \) and \( \text{Rora} \)—the master transcription factors determining the expression of IL-17—were twofold higher in DAPK-null Th17 cells compared with control Th17 cells (Fig. 2f), which correlated with the elevated protein levels of RORγt in \( \text{Dapk}^{-/-} \) Th17 cells (Fig. 2h). As a control, in the activated Th0 cells, \( \text{Il}17a, \text{Il}17f, \text{Il}21, \text{Il}23r \) and \( \text{Il}23c \) were also confirmed by ELISA, which showed a threefold increase in IL-17 secretion by \( \text{Dapk}^{-/-} \) cells compared with control Th17 cells (Fig. 2b, left panel), as also illustrated by an elevated mean fluorescence intensity (MFI; Fig. 2b, right panel). This was also confirmed by ELISA, which showed a threefold increase in IL-17 secretion by \( \text{Dapk}^{-/-} \) cells compared with control Th17 cells (Fig. 2c). In addition, for Th17 cells induced by IL-21 and TGF-β, IL-17 production also increased approximately threefold for \( \text{Dapk}^{-/-} \) Th17 cells (Supplementary Fig. 5a). A similar extent of increase in IL-17 expression was found for \( \text{Dapk}^{-/-} \) Th17 cells induced by IL-1β, IL-6 and IL-23 (Supplementary Fig. 5b). However, no effect was found on DAPK deficiency in Th17 cells induced by IL-1β, IL-6 and IL-23 (ref. 45; Supplementary Fig. 5c), suggesting that regulation of Th17 by DAPK depends on priming with TGF-β. In parallel experiments, T-cell-specific transgenic expression of \( \Delta \text{CAM} \) DAPK attenuated Th17 differentiation mediated by TGF-β and IL-6 (Fig. 2d,e). Together, these results suggest that DAPK inhibits TGF-β-dependent Th17 development.

**Figure 3** | DAPK promotes HIF-1α degradation and DAPK deficiency increases HIF-1α levels in T cells. (a) HIF-1α deficiency impairs Th17 differentiation. WT and Hif1a−/− Th17 cells were re-stimulated and expression of IL-17A and IFN-γ determined. (b) Enhanced HIF-1α protein levels in \( \text{Dapk}^{-/-} \) Th17 cells. HIF-1α protein contents were determined in Th17 cells differentiated for 3 days (left). HIF-1α levels were quantified and normalized to tubulin (right). Mean ± s.e.m., n = 3. *P < 0.01 for unpaired t-test. (c) DAPK-knockout does not affect HIF-1α mRNA induction during Th17 differentiation. The quantities of Hif1α transcript were determined in WT and \( \text{Dapk}^{-/-} \) Th17 cells in the course of Th17 differentiation. Mean ± s.e.m., n = 3. (d) DAPK reduces HIF-1α protein stability. HEK293T cells were transfected with DAPK and HIF-1α, treated with CHX (50 ng ml⁻¹) 48 h later, and the levels of HIF-1α and DAPK were measured. HIF-1α levels were quantified. (f) DAPK deficiency increases HIF-1α protein stability in T cells. WT and \( \text{Dapk}^{-/-} \) CD4⁺ T cells were differentiated into Th17 cells for 2 days, followed by CHX (25 ng ml⁻¹) treatment. The levels of HIF-1α protein were determined by immunobLOTS and were quantified. (g) DAPK-induced HIF-1α degradation is inhibited by MG132. HEK293T cells were transfected with DAPK and HIF-1α. Forty-eight hours later, cells were treated with 10 μM MG132, 200 mM NH4Cl, 100 μM leupeptin, or 100 μM chloroquine for 6 h. The HIF-1α levels were determined. (h) DAPK-induced HIF-1α degradation is proteasome-dependent. WT and \( \text{Dapk}^{-/-} \) CD4⁺ T cells were differentiated into Th17 cells for 2 days, followed by MG132 (5 μM) treatment for 2 h. The levels of HIF-1α were determined. (i) DAPK deficiency increases the induction of HIF-1α protein in T cells. WT and \( \text{Dapk}^{-/-} \) T cells were activated with CD3/CD28 under normoxic conditions, and the levels of HIF-1α at the indicated time points were determined. Data are representative of three (a,e,i) or two (d) independent experiments.
**Il23r, Rorc and Rora were absent (Fig. 2f). In addition, the expression of Il17a, Il17f, Rorc and Il21 was inhibited in ΔCAM/DAPK-transgenic Th17 cells (Fig. 2g). Together, these results suggest that DAPK suppresses Th17 differentiation by inhibiting the induction of RORγt and RORα.

In parallel experiments, T cells were primed with IL-12 for Th1 differentiation. Supplementary Fig. 5d illustrates that the development into Th1 cells was comparable between WT and Dapk0/0 T cells. Consistent with this result, the expression of Tbx21 (T-bet) and Ifng was not affected by the deficiency of DAPK (Supplementary Fig. 5e). Similarly, differentiation into Th1 cells was not altered by the ΔCAM/DAPK transgene (Supplementary Fig. 5f).

Since DAPK targets Rorc expression, we further examined whether the transcription factors regulating RORγt expression were modulated by DAPK. STAT3 is known to activate the Rorc promoter and promote Th17 cell development. Figure 2i demonstrates that the levels of STAT3 were comparable between WT and Dapk0/0 T cells. IL-6-triggered STAT3 phosphorylation was also similar between control and Dapk0/0 T cells (Fig. 2i). Similarly, IL-21-triggered STAT3 phosphorylation was comparable between WT and Dapk0/0 T cells (Supplementary Fig. 5g). Therefore, DAPK deficiency does not appear to interfere with STAT3 expression and activation.

We also determined whether activation of the IL-17 promoter by RORγt and RORα was regulated by DAPK. To exclude interference from endogenous DAPK, DAPK was knocked down in 293T cells by siRNA targeted to its 5′UTR (Supplementary Fig. 6a). IL-17P-Luc was activated in 293T cells by co-transfection with RORγt (Supplementary Fig. 6b). Co-expression of RORγt with DAPK, the dominant negative DAPK [K42A]DAPK or ΔCAM/DAPK all inhibited RORγt-directed Il17l promoter activation. Similarly, RORα-mediated IL-17P-Luc activation was inhibited by co-expression of RORα with DAPK. [K42A]DAPK or ΔCAM/DAPK (Supplementary Fig. 6c). Therefore, DAPK likely inhibits Th17 development at two sequential stages, that is, expression of both RORγt and RORα as well as RORγt/RORα-mediated IL-17 expression.

**DAPK downregulates HIF-1α in Th17 differentiation.** It has been previously shown that HIF-1α promotes Th17 differentiation. Consistent with this observation, diminished Th17 differentiation was found in Hif1a−/− T cells (Fig. 3a). HIF-1α is known for its participation in carcinogenesis and is known for its predominant nuclear localization, as shown in hypoxic HeLa cells (Fig. 4a). HIF-1α was induced in T cells through prolonged TCR stimulation (Fig. 3i), followed by immunoblotting to determine its localization. Some HIF-1α was present in the cytosolic fraction of activated T cells (Fig. 4b). Similarly, the presence of HIF-1α was detected in the cytoplasmic fraction of Th17 cells (Fig. 4c). The cytosolic localization of HIF-1α in Th17 cells, but not in HeLa cells, was further confirmed by confocal microscopy (Fig. 4d). In addition, DAPK co-localized with HIF-1α in the cytoplasm of hypoxic Jurkat cells (Fig. 4e), as well as in the cytosol of normoxic Th17 and Th0 cells (Fig. 4f).

Since HIF-1α and DAPK co-localized to the cytoplasm, we looked for (and found, see Fig. 4g) their direct interaction by co-immunoprecipitation. The cytokine-binding domain of DAPK was required for the association with HIF-1α. The association of DAPK with endogenous HIF-1α was also observed in hypoxic T cells (Fig. 4i). Together, these results suggest that DAPK interacts with HIF-1α in the cytosol of T cells, and DAPK may regulate the protein stability of HIF-1α through this direct binding. We also examined whether the kinase domain of DAPK participates in the downregulation of HIF-1α. An N-terminal DAPK fragment containing the kinase domain and ankyrin repeats weakly reduced HIF-1α expression, while a C-terminal DAPK segment from the cytokine binding to death domain effectively promoted HIF-1α degradation (Supplementary Fig. 9b). In addition, we found that HIF-1α is not a kinase substrate of DAPK since recombinant HIF-1α was not phosphorylated by DAPK in vitro (Supplementary Fig. 9c,d). Therefore, even though DAPK binds HIF-1α and induces HIF-1α protein instability, the kinase domain of DAPK appears to be dispensable in HIF-1α downregulation.

We next examined the effect of DAPK on the stability of HIF-1α. Cycloheximide-induced HIF-1α protein instability was enhanced by the presence of DAPK in 293T cells (Fig. 3e), while HIF-1α protein stability was increased in DAPK-deficient Th17 cells (Fig. 3f), suggesting that DAPK promotes HIF-1α protein destabilization. DAPK-induced HIF-1α protein downregulation was inhibited by MG132 in 293T cells, but not by NH4Cl, leupeptin or chloroquine (Fig. 3g). In addition, MG132 treatment increased HIF-1α to similar levels in WT and Dapk0/0 Th17 cells (Fig. 3h). These results suggest the involvement of the proteasome degradation pathway in DAPK-mediated HIF-1α downregulation in Th17 cells.

In a separate experiment, we examined whether HIF-1α protein expression could be induced by prolonged TCR activation in T cells under normoxic conditions (Fig. 3i). CD3-induced HIF-1α protein expression was much higher in Dapk−/− T cells than WT T cells (Fig. 3i), further illustrating the ability of DAPK to downregulate HIF-1α protein.

HIF-1α has also been shown to antagonize Foxp3 and inhibit regulatory T cells (Treg). The development and in vitro suppressive activity of natural regulatory cells (nTreg) were not affected in Dapk−/− nTregs (Supplementary Fig. 8a,b). However, the differentiation of induced regulatory T cells (iTreg) from CD4+ CD25− T cells was moderately impaired in Dapk−/− T cells (Supplementary Fig. 8c). The in vitro suppressive activity of Dapk−/− iTregs was also reduced relative to WT iTregs (Supplementary Fig. 8d).

DAPK interacts with cytoplasmic HIF-1α in T cells. To isolate the mechanism underlying DAPK-dependent HIF-1α degradation, we next examined whether there was a direct interaction between DAPK and HIF-1α. DAPK is a cytosolic protein whereas HIF-1α is known for its predominant nuclear localization, as shown in hypoxic HeLa cells (Fig. 4a). HIF-1α was induced in T cells through prolonged TCR stimulation (Fig. 3i), followed by immunoblotting to determine its localization. Some HIF-1α was present in the cytosolic fraction of activated T cells (Fig. 4b). Similarly, the presence of HIF-1α was detected in the cytoplasmic fraction of Th17 cells (Fig. 4c). The cytosolic localization of HIF-1α in Th17 cells, but not in HeLa cells, was further confirmed by confocal microscopy (Fig. 4d). In addition, DAPK co-localized with HIF-1α in the cytoplasm of hypoxic Jurkat cells (Fig. 4e), as well as in the cytosol of normoxic Th17 and Th0 cells (Fig. 4f).
DAPK induces PHD2-mediated HIF-1α degradation. The inclusion of ubiquitin enhanced the ability of DAPK to downregulate HIF-1α (Supplementary Fig. 10a), illustrating the involvement of the ubiquitin-dependent degradation process. The stability of HIF-1α protein is regulated by proline hydroxylation, a step necessary for its subsequent binding to VHL protein leading to ubiquitination and proteasome degradation. Therefore, we sought to identify the degradation pathways responsible for DAPK-regulated HIF-1α stability. Deletion of the HIF-1α oxygen-dependent degradation domain containing the proline residues necessary for hydroxylation conferred resistance to DAPK-induced degradation (Fig. 5a). Similarly, inhibition of PHD1/2, which hydroxylates HIF-1α protein, by deferoxamine mesylate (DFX) restored the expression levels of HIF-1α protein in DAPK-expressing T cells and in 293T cells (Fig. 5b; Supplementary Fig. 10b). This result was consistent with the decreased hydroxylation of HIF-1α protein at proline 564 in DAPK−/− T cells (Fig. 5c) and the increased proline 564 hydroxylation in DAPK-overexpressing Jurkat cells (Fig. 5d). Mutation of the hydroxylation sites Pro402 and Pro564 on HIF-1α into alanine (2PA) conferred resistance of HIF-1α protein to DAPK-induced degradation in T cells and in HEK293T cells.
after transfection. (T cells. Naive WT and determined. (a) PHD2 co-localized with HIF-1 in T cells (Fig. 6a). Co-localization of DAPK with PHD2 was observed in Th17 cells by confocal microscopy (Fig. 6b). In addition, PHD2 co-localized with HIF-1 in the nucleus and cytoplasm of Th17 cells (Fig. 6c). We also identified the N-terminal part (aa 1–180) of PHD2 as the DAPK-interacting region (Fig. 6d). Addition of recombinant DAPK protein enhanced the interaction of GST-HIF-1α and PHD2 in an in vitro binding assay (Fig. 6e). Therefore, by binding to both HIF-1α and PHD2, a possible role for DAPK is to enhance the association of PHD2 with HIF-1α and increase HIF-1α degradation.

**HIF-1α knockout inhibits Dapk−/− Th17 cell differentiation.** We hypothesized that if the excess generation of IL-17 in Dapk−/− T cells is due to enhanced HIF-1α-mediated Th17 differentiation, the additional deletion of HIF-1α should reduce IL-17 production to normal levels in Dapk−/− T cells. We generated mice with Dapk−/−/Hif1a−/− by crossing mice with T-cell-specific deletion of HIF-1α (Cd4Cre×Hif1afl/fl) with Dapk−/− mice. Deletion of HIF-1α and/or DAPK in T cells from each genotype was confirmed by immunoblotting (Fig. 7a). In agreement with our hypothesis, we found that while Dapk−/− T cells exhibited enhanced differentiation of naive CD4+ T cells into Th17 cells, Dapk−/−/Hif1a−/− T cells displayed levels similar to WT T cells (Fig. 7b). Knockout of HIF-1α also decreased the levels of RORγt in Dapk−/− Th17 cells (Fig. 7c). Therefore, elevated HIF-1α levels partly account for the increased IL-17 expression in Dapk−/− T cells. We further examined whether selective deletion of HIF-1α in T cells attenuated the exacerbated EAE generation in Dapk−/− mice. The severe EAE disease scores in Dapk−/− mice were largely attenuated by T-cell-selective deletion of HIF-1α (Fig. 7d), supporting that DAPK acts upstream of HIF-1α in EAE development. T-cell-specific deletion of HIF-1α did not affect the development and suppressive activity of nTreg in Dapk−/− mice (Supplementary Fig. 11a,b). The reduced development of Dapk−/− iTregs was restored by HIF-1α knockout (Supplementary Fig. 11c), yet the impaired...
suppressivity was not corrected in Dapk−/− mice during peak EAE induction (Fig. 1a–c). We found that the ratio of IL-17-expressing cells to normal Treg functioning, in contrast to a prominent role of DAPK-HIF-1α antagonism in Th17 cells.

Discussion
In this study, we identified a negative role of DAPK in Th17 development. In Dapk−/− T cells, generation of IL-17A, IL-17F, IL-21 and IL-23R were highly elevated. We further found that the levels of RORγt and RORα—the transcription factors dictating IL-17 expression—were elevated in DAPK-deficient Th17 cells; even though activation of the Rorc inducer STAT3 was similar between WT and Dapk−/− T cells (Fig. 2i; Supplementary Fig. 5g). Instead, increases in the expression of RORγt and RORα could be partly attributed to elevated levels of HIF-1α protein. In addition, phosphorylation of mTOR, p70 S6 kinase, 4E-BP1 and ribosomal S6 were comparable between WT and Dapk−/− Th17 cells (Supplementary Fig. 7), suggesting that the increased levels of HIF-1α in Dapk−/− Th17 cells are not caused by increased mTOR signalling. We further found that DAPK promoted the destabilization of HIF-1α protein in a proteasome-dependent manner (Fig. 3). DAPK expression is induced by TGF-β (ref. 43), which is commonly used for Th17 differentiation10,11. This may partly explain why DAPK levels were higher in Th17 cells than Th1 cells (Fig. 2a). The lower DAPK expression in Th1 cells likely correlated with the weak effect of DAPK deficiency in Th1 cells. Even though naive Dapk−/− T cells displayed increased production of IFN-γ (Supplementary Fig. 2b), IFN-γ expression was not enhanced in re-stimulated DAPK-deficient T cells, including T cells optimized for Th0 and Th1 differentiation (Supplementary Figs 2d and 5d). In addition, IFN-γ production was comparable in MOG-primed T cells from WT and Dapk−/− mice (Fig. 1e). Similarly, transgenic expression of DAPK did not affect Th1 development (Supplementary Fig. 5f).

DAPK deficiency led to enhanced sensitivity to EAE induction (Fig. 1a–c). We found that the ratio of IL-17-expressing cells to IFN-γ-expressing cells was significantly increased in spinal cords from Dapk−/− mice during peak EAE induction (Supplementary Fig. 4), suggesting enhanced spinal cord infiltration of Dapk−/− Th17 cells. We also observed that transfer of Dapk−/− 2D2 Th17 cells induced highly exacerbated EAE in comparison to WT Th17 cells (Fig. 1f). DAPK-deficient Th17 cells thus displayed increased encephalitogenic activity relative to WT Th17 cells. In contrast, EAE induction by Dapk−/− 2D2 Th1 cells was similar to that induced by WT 2D2 Th1 cells (Fig. 1g). Therefore, DAPK deficiency correlates with preferential induction of pathogenic Th17.

We have previously shown that DAPK specifically suppresses TCR-induced NF-κB activation42, as confirmed here by the elevated RelA nuclear translocation in Dapk−/− T cells (Supplementary Fig. 2e). NF-κB activation in T cells is required for the generation of autoimmune encephalomyelitis; mice deficient in NF-κB1 (p50) are resistant to the induction of EAE50, whereas PKCθ deficiency prevents T-cell activation and
the pathogenesis of EAE\textsuperscript{51}, NF-κB has been shown to directly activate the \textit{Hif1a} promoter\textsuperscript{52}. In the present study, we found that induction of \textit{Hif1a} transcripts during Th17 differentiation was not affected by \textit{Dapk} deficiency (Fig. 3c), indicating that enhanced NF-κB activation in \textit{Dapk}\textsuperscript{-/-} T cells did not lead to a further increase in \textit{Hif1a} expression. We speculate that the levels of NF-κB activation in WT T cells were already sufficient for optimal transcriptional activation of \textit{Hif1a} mRNA.

A previous study of DRAK2 showed that deletion of the gene \textit{Stk17b2}, which encodes DRAK2, results in complete resistance to EAE induction\textsuperscript{35}. DRAK2 knockout is accompanied by increased expression of \textit{Stk17b2}, encoding DRAK2, which negatively regulates Treg differentiation\textsuperscript{27,49}. Despite that differentiation and functioning of iTreg from \textit{Dapk}\textsuperscript{-/-} T cells were impaired (Supplementary Fig. 8c,d), knockout of HIF-1α did not restore functioning of \textit{Dapk}\textsuperscript{-/-} iTregs (Supplementary Fig. 11d), suggesting a minor role of DAPK–HIF-1α antagonism in Treg cells. Therefore, the ability of DAPK to inhibit HIF-1α predominantly regulates Th17 cells, and impaired Treg function do not seem to contribute to the enhanced Th17 development and severe EAE disease observed in \textit{Dapk}\textsuperscript{-/-} mice. Why Th17 and Tregs display different susceptibility to regulation by the DAPK–HIF-1α interaction is currently being investigated.

Recent studies have revealed that hypoxia promotes the expression of miR-103/107 that target DAPK for downregulation in cancer cells\textsuperscript{53}. HIF-1α induces the expression of \textit{Klhl20}, which coordinates with Cul3 for ubiquitination and proteasomal degradation of DAPK in several cancer cell lines\textsuperscript{24,55}. Together with the results from the present study, we propose a mutual antagonism between HIF-1α and DAPK, modulated by specific environmental cues, may determine the pathophysiological status of Th17 cells.

![Figure 7](image-url)
DAPK is a tumour suppressor known to be downregulated in many types of cancers. Our results illustrate that DAPK is not only a tumour suppressor, but also plays a role in the prevention of Th17-mediated autoimmune diseases like EAE. DAPK suppresses tumour formation and metastasis through its promotion of apoptosis, induction of autophagy and regulation of cell motility. In the present study, we have demonstrated that DAPK inhibits EAE development by inducing HIF-1α degradation and inhibiting pro-inflammatory Th17 cell differentiation. Notably, HIF-1α is also known for its prominent role in tumorigenesis, with levels of HIF-1α correlating with poor prognoses for various cancer types. Even though HIF-1α is exclusively localized in the nucleus of most cancer cells, it will be interesting to examine whether the ability of DAPK to promote HIF-1α degradation in the cytoplasm also contributes to the tumour suppressing function of DAPK in select cancer types. We found that HIF-1α was present in the cytoplasm of T-cell leukemia Jurkat cells and was subjected to DAPK regulation (Fig. 4e and 5e). Consequently, DAPK likely exhibits a tumour suppressor effect in T leukemia cells by directly binding to the cytosolic HIF-1α to prompt HIF-1α downregulation. Th17 cells contribute to autoimmune diseases—including multiple sclerosis, rheumatoid arthritis, inflammatory bowel diseases, psoriasis and autoimmune diabetes—and Th17 is a target for immunotherapy in these inflammatory diseases. In addition to promoting Th17 differentiation, HIF-1α contributes to the inflammatory activities of different innate immune cells including macrophages and neutrophils. Therefore, targeted HIF-1α degradation by DAPK upregulation is a potential novel approach in the treatment of Th17-associated inflammatory diseases.

The critical role of HIF-1α in carcinogenesis and metastases has led to the identification of drugs that inhibit HIF-1α (refs 18,19,21,32). Conceivably, HIF-1α inhibitors are also potential therapeutic agents for HIF-1α-mediated diseases—including pulmonary arterial hypertension, hereditary erythrocytosis, obstructive sleep apnea, ocular neovascularization and traumatic shock—as well as Th17-mediated inflammatory diseases. However, HIF-1α is critical for oxygen homeostasis, and is protective in coronary artery disease, peripheral artery disease. However, HIF-1α-mediated diseases—

**Methods**

**Reagents**

Calcium ionophore (A23187), phorbol 12-myristate 13-acetate (TPA), MC123ione, ammonium chloride (NH₄Cl), leupeptin, cycloheximide (CHX), DFX, anti-DAPK (D2178, DAPK-55, western blot (WB) 1:1,000, immunofluorescence (IF) 1:50), anti-HA (H3633, HA-7, WB 1:1,000, immunoprecipitation (IP) 1 μg per test), and anti-FLAG (F1804, M2, WB 1:1,000, IF 1:400) were purchased from Sigma (St Louis, MO, USA). Anti-phospho-S6K (sc-23, 1:1,000), anti-phospho-HIF-1α (sc-55541, B-2, WB 1:500) and anti-p65 (sc-372G, C-20, WB 1:200) were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA). Anti-RORγt (14–6981, B2D, WB 1:1,000), anti-CD4-PE-Cy7 (1:1,000), recombinant mouse IFN-γ (505707, R4-6A2, WB 1:6,000, IF 1:200), LEAF anti-mouse IgG (A11034, IF 1:500) and Alexa Flour555-labelled goat anti-rabbit IgG (A21428, goat anti-mouse IgG (A21422, IF 1:500), Alexa Flour488-labelled goat anti-rabbit IgG (14–6981, WB 1:6,000, IF 1:200) were purchased from Bethyl Laboratories (Cambridge, UK). Anti-mouse CD3 (2C11), anti-mouse CD28 (37.51), anti-mouse IL-2, and anti-mouse CD4 (RIL174.2) were purified in our laboratory. Recombinant mouse IL-12 and IL-21, mouse IL-17A ELISA kit, and mouse IL-4 ELISA kit were purchased from R&D (Minneapolis, MN, USA). Recombinant mouse Il-2 and human TGF-β were purchased from PeproTech (Rocky Hill, NJ, USA). Anti-HIF-1α (A300-286A, 1 μg per test) was purchased from R&D (Burlington, Massachusetts, TX, USA). Mouse anti-Lamin B1 (33–2000, L-5, WB 1:1,000), Alexa Flour488-labelled goat anti-mouse IgG (A11001, WB 1:1,000, Alexa Flour555-labelled goat anti-mouse IgG (A2122, WB 1:1,000), Alexa Flour488-labelled goat anti-rabbit IgG (A11034, IF 1:500) and Alexa Flour555-labelled goat anti-rabbit IgG (A21248, IF 1:500) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). siRNA control (sc-37680v10, 100 pmol) was purchased from Dharmacon (Lafayette, CO, USA). siRNA control (sc-37680v10, 100 pmol) was purchased from Dharmacon (Lafayette, CO, USA). Anti-mTOR (2972, WB 1:1,000), anti-4E-BP1 (9452, WB 1:1,000), anti-PHD2 (sc-18, WB 1:6,000) and anti-tubulin (05–661, AA2, WB 1:6,000) were purchased from Sigma (St Louis, MO, USA). Anti-CD4-FITC (100501, RM4-5, FCM 1:100), anti-CD8α-PE (17–0621, MEL-14, FCM 1:100), recombinant mouse IL-2 and mouse IL-12 and IL-21, mouse IL-17A ELISA kit, and mouse IL-4 ELISA kit were purchased from R&D (Minneapolis, MN, USA). Recombinant mouse IL-2 and human TGF-β were purchased from PeproTech (Rocky Hill, NJ, USA). Anti-HIF-1α (A300-286A, 1 μg per test) was purchased from R&D (Burlington, Massachusetts, TX, USA). Mouse anti-Lamin B1 (33–2000, L-5, WB 1:1,000), Alexa Flour488-labelled goat anti-mouse IgG (A11001, WB 1:1,000, Alexa Flour555-labelled goat anti-mouse IgG (A2122, WB 1:1,000), Alexa Flour488-labelled goat anti-rabbit IgG (A11034, IF 1:500) and Alexa Flour555-labelled goat anti-rabbit IgG (A21248, IF 1:500) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). siRNA control (sc-37680v10, 100 pmol) was purchased from Dharmacon (Lafayette, CO, USA).

**Cell cultures**

HEK293T (ATCC CRL-3126), HeLa (ATCC CCL-2) and Jurkat T lymphoma (clone E6-1, ATCC TIB-152) were obtained from ATCC. Cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (Invitrogen Life Technology), 10 mM glutamine, 100 μM 1,100 penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol. Induction of T-helper cells and iTregs

**Mice**

Production of Dapk (−/−) mice and p37.1 [ACAM]DAPK transgenic mice (both in a C57BL/6 background) were previously described. B6.Dcr1−/−; Hif1αm(+/−) (Hif1αmα) mice, Rag1−/− (mice in C57BL/6 background), and C57BL/6-Tg(Tcr2D22, Tcrb2D21)+/Kuch (2D2 TCRmε) transgenic mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). C57/Cre mice (in C57BL/6 background) were purchased from Taconic Farms (Hudson, NY, USA). Mice were maintained in the SPF mouse facility of the Institute of Molecular Biology, Academia Sinica. All mouse experiments were performed using 8–16-week-old mice. For in vivo experiments, sex-matched male and female mice were used. Donor and recipient mice were also sex-matched in T cells transfer assay. Because mouse experiments are limited by the availability of the knockout mice and their litters, most mouse experiments were not blinded. All mouse experiments were conducted with approval from the Institutional Animal Care & Utilization Committee, Academia Sinica.

**Induction of T-helper cells and iTregs**

Mice were isolated from spleen and peripheral lymph nodes by negative selection using an anti-mouse Ig panning
method. Total CD4+ T cells were purified by positive selection using a rat anti-mouse CD4+ antibody (RM172-4) panning method. Naïve CD4 T cells (CD4+CD45RBhiCD62Lhi) were then sorted by FACSVantage SE (BD Bioscience). Naïve CD4 T cells were then activated by 2 μg ml−1 plate-bound anti-CD3 (2C11) plus 1 μg ml−1 anti-CD28 (37.51). IL-2 (20 U ml−1) was added for Th0 differentiation; IL-12 (10 ng ml−1), IL-2 (5 ng ml−1) and anti-IL-4 (10 μg ml−1) were added to induce Th1 development; and TGF-β (2 ng ml−1), IL-6 (5 ng ml−1), anti-IL-6 (5 μg ml−1), and anti-Th2 (5 μg ml−1) were added to induce Th2 differentiation. Th17 cells were also induced by replacing IL-6 with IL-21 (100 ng ml−1) in the same mixture. After 3-5 days, Th1 and Th17 cells were re-stimulated with anti-CD3/CD28 or TPA/12-0-13 and IL-1β for further activation, and intracellular content of IL-17 and IFN-γ was determined after another 3 h. For mRNA expression, cells were harvested 3 h after TPA/12-0-13 stimulation and RNA was prepared. For ELISA, cell culture supernatants were collected 24 h after TPA/12-0-13 activation. Induced Treg (iTreg) cells were generated from CD4+CD25+ T cells and activated by IL-2 (20 ng ml−1), TGF-β (50 ng ml−1) and plate-bound anti-CD3 (5 μg ml−1) plus anti-CD28 (1 μg ml−1) for 1-5 days.

EAE induction. DAPK[ACAM]-transgenic, DAPK-knockout, and their WT littermate control mice were immunized subcutaneously with 200 μg of MOG peptide 35–55 (Kelowna, Taipei, Taiwan) emulsified in CFA containing 400 μg Mycobacterium tuberculos (Sigma), followed by intraperitoneal injection of 200 ng pertussis toxin (List Labs, Campbell, CA, USA) at day 0 and day 2. Mice were monitored daily for clinical signs and then clinically scored. TTC, GAC CAG CAC AT-3 and reverse, 5′-GGG AAG AAG CAG CCA TTG-3′. The non-classical (atypical) EAE was detected by X-ray film. Western blot images have been cropped for presentation. Full size images are presented in Supplementary Figs 12–17.

Histology on spinal cord sections. Mice were perfused with phosphate buffer saline (PBS) followed by 4% (v/v) paraformaldehyde (PFA) in PBS. Spinal cords were isolated and fixed with 4% (v/v) PFA in PBS overnight at 4 °C. Spinal cords were dehydrated with 30% sucrose in PBS for 48 h at 4 °C. Spinal cords were embedded in optimal cutting temperature compound and serial cryosections (30 μm thick) were acquired. Tissue sections were stained with hematoxylin & eosin or luxol fast blue. Images were obtained on a Zeiss Axioscope Diaphot microscope (Jena, Germany) with an objective lens of Plan-Apochromat 63 ×/1.4 Oil DIC M27 at room temperature. DAPI-bound DNA was excited at 405 nm and collected at 420 to 475 nm. Alexa Fluor 488 dye was excited at 488 nm and collected at 500 to 550 nm. Alexa Fluor 555 dye was excited at 555 nm and collected at 568 to 629 nm. The pinhole was at 53 μm, with a 9.8 μm section.

Nuclear extract preparation. Cells were harvested and lysed in cytoplasmic extract (CE) buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl and 3 mM MgCl2) containing 0.1% (primary T cells) or 0.2% NP-40 (for HEla cells) for 3 min on ice. Nuclear extracts were generated from CD4+CD25+ T cells and activated by IL-2 (20 ng ml−1), TGF-β (5 ng ml−1) and plate-bound anti-CD3 (5 μg ml−1) plus anti-CD28 (1 μg ml−1) for 1-5 days.

In vitro binding assays. pGEX-4T3-HIF-1α (GST-HIF-1α) was transformed in BL21 (DE3) Escherichia coli, and recombinant GST-HIF-1α fusion proteins purified on a glutathione Sepharose 4B column. RSK5-DAPK (DAPK-FLAG) and pcDNA4-PHD2 (FLAG-PHD2) were transfected into HEK293T cells. Transfected

Quantitative PCR. T cells were harvested at the time indicated and total RNA was isolated using Trizol (Invitrogen). cDNAs were prepared and analysed for the expression of the gene of interest by real-time PCR using a SYBR-Green PCR master mix kit (Roche). The expression of each gene was normalized to the expression of β-actin. The sequences of the primers were: Rodc, forward, 5′-GGG AGT TTG CTT TGG GGTG-3′ and reverse, 5′-GGG AGT TAT CAT ACTG CAG CAC G-3′; Rora, forward, 5′-GGG AGT TGT CAC ACC TCT CTT-3′ and reverse, 5′-GGC TAC CAT ACC TTG-3′; Il7r, forward, 5′-GGC TAC CAT ACC AAC AG-3′ and reverse, 5′-GTC AGG AAG CAG CCA TTTG-3′; Tbx21, forward, 5′-GTC AGG AAG CAG CCA TTA CAC-3′ and reverse, 5′-GGC TGC CCT TTC TCA TAG GAA TC-3′; Il2rb, forward, 5′-GGC TAA ATG TGG GAC GAA GG-3′ and reverse, 5′-GTA CGT ATG AGC GAC GAA GGA-3′; Acb, forward, 5′-GCC CCC CTG AAC CCT AA-3′ and reverse, 5′-GAC AGC ACA GCC TGG AT-3′; Tn2b1, forward, 5′-CGT GGA GGT GAA TGA TGG-3′ and reverse, 5′-GGC TGC CAT AAC ATG GAT-3′; Il23r, forward, 5′-GAC GTA AAC ATA AGC GT-3′ and reverse, 5′-GCC CAT GGA CAA GGA GAA-3′ and reverse, 5′-GCC AGT GAT GAG CAA CTA CAT CATA-3′.

Nuclear extract preparation. Cells were harvested and lysed in cytoplasmic extract (CE) buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl and 3 mM MgCl2) containing 0.1% (primary T cells) or 0.2% NP-40 (for HEla cells) for 3 min on ice. Nuclear extracts were generated from CD4+CD25+ T cells and activated by IL-2 (20 ng ml−1), TGF-β (5 ng ml−1) and plate-bound anti-CD3 (5 μg ml−1) plus anti-CD28 (1 μg ml−1) for 1-5 days.

In vitro binding assays. pGEX-4T3-HIF-1α (GST-HIF-1α) was transformed in BL21 (DE3) Escherichia coli, and recombinant GST-HIF-1α fusion proteins purified on a glutathione Sepharose 4B column. RSK5-DAPK (DAPK-FLAG) and pcDNA4-PHD2 (FLAG-PHD2) were transfected into HEK293T cells. Transfected
cells were collected and lysed with WCE buffer (25 mM PIPES pH 7.7, 300 mM NaCl, 0.1% Triton X-100, 1.5 mM MgCl₂, 2 mM EDTA, 0.1 mM Na₃VO₄, 50 mM NaF and 0.5 mM DTT). Recombinant FLAG fusion proteins were purified with anti-FLAG M2 agarose. Purified recombinant proteins were incubated in PBS at 4°C for 4 h, and then incubated with anti-GST antibody overnight at 4°C. Samples were incubated with protein G magnetic beads (GE) at 4°C for 1 h. The immune complexes were washed with WCE buffer and examined by western blot.

DAPK kinase assay. DAPK kinase assays were performed as previously described. Flag-proteins were isolated by anti-FLAG from SF21 insect cells and incubated with 5 μg GST-HIF1α or GST-MLC in kinase reaction buffer (50 mM HEPES pH 7.5, 8 mM MgCl₂, 2 mM MnCl₂, 0.5 mM CaCl₂, 0.1 mg/ml bovine serum albumin (BSA)), 1 μM bovine CaM (Sigma), 50 μM ATP, 10 μCi [γ-32P]ATP) at 25°C for 15 min. The reactions were stopped by adding SDS sample buffer, and resolved on SDS-PAGE. The phosphorylation was detected by autoradiography.

Statistics. Our data were randomly collected but were not blinded. We did not exclude any data in this study. Our data mostly meet the assumption of the tests (normal distribution). Microsoft Office Excel and Prism 5.0 (GraphPad software) were used for data analysis. Unpaired two-tailed Student t-tests were used to compare results from between two groups. Data were presented as mean with standard deviation (s.d.) or standard error of the mean (s.e.m.), as indicated in the figure legend.

Data availability. All relevant data are available from the authors upon request.

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Author contributions

T.-F.C., Y.-T.C, W.-C.H, P.-Y.C., H.-Y.L., and S.-T.M. performed experiments, T.-S.H., S.-C.M., R.-H.C., A.K. contributed to generation of key materials and constructs, and M.-Z.L. conceived of, designed and supervised the research and wrote the manuscript.

Additional information

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