Histone deacetylase 5 deacetylates the phosphatase PP2A for positively regulating NF-κB signaling

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Histone deacetylase 5 (HDAC5) has been reported to have a strong regulatory function in the proinflammatory response, but the mechanism is still unknown. Here, we identified HDAC5 as a positive regulator of NF-κB signaling *in vivo*. HDAC5-deficient mice exhibited enhanced survival in response to LPS challenge. Using LPS, TNFα, different kinds of viruses, hydrogen peroxide, or ultraviolet stimulation, we demonstrate that HDAC5-mediated regulation of NF-κB occurs in manners both dependent on and independent of IKK, an upstream kinase in the NF-κB signaling pathway. Deficiency in HDAC5 impaired the phosphorylation of IKKβ, subsequent phosphorylation of the NF-κB inhibitor protein IκBα and NF-κB subunit p65. We also show that the phosphatase PP2A repressed transcriptional activation of NF-κB by decreasing phosphorylation of IKKβ, p65, and IκBα. *In vitro* deacetylation experiments and site-directed mutagenesis experiments indicated that HDAC5 directly deacetylated PP2Ac at Lys136, which resulted in the deactivation of PP2A. Our data add mechanistic insight into the cross talk between epigenetic and posttranslational modifications regulating NF-κB signaling and protein phosphatase activation that mediate survival in response to inflammatory challenges.

Histone deacetylases (HDACs) can regulate gene expression *via* translational modification. HDACs are evolutionarily highly conserved proteins and have emerged as crucial transcriptional corepressors in highly diverse biological systems. Mammalian HDACs have been classified into four classes based on the homology of their catalytic domains, and the class II HDACs are further subdivided into two subclasses, classes IIa (HDAC4, 5, 7, and 9) and IIb (HDAC6 and 10) (1). Some classical HDACs have been attributed key roles in immunity and inflammation (2). It has been reported that HDAC5 promotes inflammatory response through NF-κB activation (3, 4), but the mechanism is unclear.

NF-κB is a critical regulator of diverse cytokine-mediated cellular responses and regulates cell survival, proliferation, apoptosis, and immune responses (5, 6). Normally, NF-κB proteins are inactive, binding to the IκB family. Extracellular stimuli, such as tumor necrosis factor (TNF), interleukin 1 (IL-1), and toll-like receptor (TLR) ligands (7, 8), lead to the phosphorylation and degradation of IκB proteins and also the modifications of NF-κB subunits. The phosphorylation of NF-κB transcription factors was regulated not only by protein serine/threonine kinases but also by protein serine/threonine phosphatases. It had been reported the protein phosphatases (PPs) could regulate the NF-κB activation (9, 10).

The predominant form of PP2A is a heterotrimeric holoenzyme. In the trimeric forms, the core enzyme component is the 36-kDa catalytic subunit (PP2Ac). A 65-kDa regulatory subunit is PP2Aa. PP2Ab could bind to the core enzyme (PP2Ac) and confer substrate specificity to its dephosphorylating activity (11). Treatment with okadaic acid (OA), a specific inhibitor of PP1 and PP2A, induces translocation of NF-κB to the nucleus and activation of NF-κB (12). PP2A could dephosphorylate p65 to reduce NF-κB activity (13, 14). Whether a host factor regulates the function of PP2A remains an open question.

Here, we describe that HDAC5 regulates the phosphorylation of IKKβ and p65 by deacetylating and inactivating PP2A. HDAC5 promotes NF-κB activation in a deacetylation-dependent manner. HDAC5 deficiency suppresses the production of cytokines, such as TNF, both *in vitro* and *in vivo*. HDAC5 contributes to persistent phosphorylation of IKK, p65, and IκBα by disrupting the phosphatase PP2A activity. The acetylation of PP2Ac at Lys136 is crucial for its phosphatase activity. These results provide insights into the posttranslational modification involved in PP2A activity and established a novel function of HDAC5 as a key protein in the positive feedback mechanism to constitutively maintain NF-κB in an active state during stimulation.

Results

HDAC5 acts as a positive regulator of NF-κB signaling

In our previous work, we found that HDAC3 and HDAC4 could functionally regulate type I interferon and

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inflammatory signaling pathway (15–17). We further explored the role of other members of the HDAC family in innate immune signaling pathways. Knockdown of endogenous HDAC5 via shRNA in HEK293T reduced the SeV, IL-1β or TNFα-triggered activation of NF-κB in reporter assays (Fig. 1A). However, knockdown of HDAC5 did not affect the IFNγ-triggered IRF1 promoter activity (Fig. S1A). The production of TNFα or IL-6 in the HDAC5-deficient THP-1 cells or BMDMs was significantly lower compared with the control cells during LPS challenge (Fig. 1, B and C). When we expressed the HDAC5 in the HDAC5 knockdown HEK293T cells, the supplemental HDAC5 could rescue NF-κB reporter's depreciation (Fig. 1D) and the mRNA levels of TNFα, IL-6, and CXCL-10 (Fig. S1B) after TNFα treatment. Collectively, these data suggested that deficiency of HDAC5 negatively regulated NF-κB signaling.

To confirm the contribution of HDAC5 to inflammatory responses, we stimulated HDAC5-overexpressing HEK293T cells with SeV or TNFα. The promoter activity of NF-κB was significantly higher when HDAC5 was overexpressed compared with samples transfected with the empty vector or the HDAC5 deacetylase partial inactive mutant (decreased by 77%, H1006A) (18) (Fig. 1E). As expected, overexpression of HDAC5 did not affect IFNγ-triggered IRF1 promoter activity (Fig. S1C). The production of TNFα or IL-6 was higher in HDAC5-overexpressing THP-1 cells after being stimulated with LPS (Fig. 1F). Then, we constructed HDAC5 mutants that predominantly localized to the cytoplasm (HDAC5-ΔNLS) and nucleus (HDAC5-ΔNES) (Figs. 1G and S1D). The promoter activity of NF-κB was enhanced with the expression of wild-type HDAC5 or cytoplasmic HDAC5 (HDAC5-ΔNLS), but not the nuclear HDAC5 (HDAC5-ΔNES) (Fig. 1H). The TNFα treatment could enhance the HDAC5 deacetylase activity in a time-dependent manner (Fig. 1I). These results suggested that the deacetylation activity and cytoplasm localization of HDAC5 played positive roles in regulating inflammatory signaling.

Pivotal role for HDAC5 in the inflammation reaction in vivo

To further elucidate the importance of HDAC5 in inflammation reaction in vivo, we adopted a strategy to generate Hdac5 flox/flox-Lyz2-Cre mice (Hdac5^fl/fl Lyz2-Cre), which undergo deletion of loxP-flanked HDAC5 alleles (Hdac5^fl/fl) specifically in myeloid cells via Cre recombinase expressed from the myeloid cell-specific gene Lyz2 (Lyz2-Cre). We then challenged these mice with LPS and found that Hdac5^fl/fl Lyz2-Cre mice's mortality rate was lower than that of the Hdac5^fl/fl littermates (Fig. 2A). Meanwhile, Hdac5-deficient mice produced decreased serum TNFα or IL-6 in response to LPS (Fig. 2B). The secretion of TNFα or IL-6 from Hdac5-deficient BMDMs from Hdac5^fl/fl Lyz2-Cre mice was severely impaired compared with the secretion from Hdac5^fl/fl BMDMs stimulated by various inducers, including SeV, VSV, HSV-1, or LPS (Fig. 2C). Therefore, HDAC5 was required for the efficient production of inflammatory cytokines.

HDAC5 regulates IKK-dependent and IKK-independent NF-κB activation

To understand the mechanism by which HDAC5 regulates inflammatory signaling, we examined the effect of HDAC5 knockdown on NF-κB activation mediated by adaptor proteins. Knockdown of HDAC5 significantly inhibited NF-κB reporter activity induced by upstream activators, including TRADD, RIP, MyD88, VISA, TAK1 plus TAB1, TAK1 plus TAB2, TRAF6, and IKKβ, but not p65 (Fig. 3A). These results indicated that HDAC5 affected p65 activation. The phosphorylation of p65 at Ser536 helped transport to the nucleus and provided the transactivation activity. The nuclear localization of p65 was lower in HDAC5-knockdown samples relative to control samples during SeV infection in HEK293T cells. The data demonstrated that the HDAC5-knockout significantly blocked p65 to translocate to the nucleus in THP-1 cells during LPS stimulation (Fig. S2, A and B). In mouse macrophage cell lines (RAW264.7) or BMDMs, the levels of phosphorylated p65 induced by TNFα or LPS were significantly impaired upon HDAC5 silencing (Fig. S3, A and B). The major responses were analyzed in the signaling pathways of different cells challenged with various stimuli to understand how HDAC5 regulates inflammatory signaling. In HDAC5-knockdown HEK293T cells, the phosphorylation of IKKα/β, p65, and IκBα was reduced after SeV or TNFα stimulation (Fig. 3, B and C). This phenomenon was also observed in the HDAC5-knockout THP-1 cells during the LPS challenge (Fig. 3D). Furthermore, phosphorylation of IKKα/β, p65, and IκBα in the Hdac5-deficient BMDMs (Hdac5^fl/fl Lyz2-Cre) was significantly more reduced than in the control counterparts during SeV, VSV, HSV-1, or LPS challenge (Fig. 3E). It had been reported that the IKKβ could phosphorylate the IκBα and p65 (19). These data suggested that HDAC5 could regulate the IKK-dependent NF-κB activation.

Furthermore, because NF-κB could be activated through IKK-independent pathways, such as treatment with hydrogen peroxide (H_2O_2) or ultraviolet irradiation (UV) (20), then we examined whether knockdown HDAC5 inhibited NF-κB activation induced by H_2O_2 or UV. Knockdown of HDAC5 in HEK293T cells blocked the IκBα degradation and the p65 phosphorylation at Ser536 (Fig. 3F). These results collectively indicate that HDAC5 could affect IKK-dependent and IKK-independent NF-κB activation.

HDAC5 mediates IKKβ and p65 phosphorylation via PP2Ac

Next, we investigated the mechanisms underlying HDAC5's activity in regulating NF-κB activation. HDAC5 did not affect the acetylation of p65 at Lys310 (Fig. S4, A and B). HDAC5 had little effect on the interaction between the signaling pathway molecules (Fig. S5A). The in vitro IKK kinase assay indicated that HDAC5 did not directly regulate the kinase activity of IKKα or IKKβ (Fig. S5B). The phosphorylation was regulated by protein serine/threonine kinases and protein serine/threonine phosphatases. It has been reported that the PPs PP1, PP4 and PP2 could repress the activation of NF-κB signaling by decreasing phosphorylation of IKKβ (9, 10, 13, 21). We further
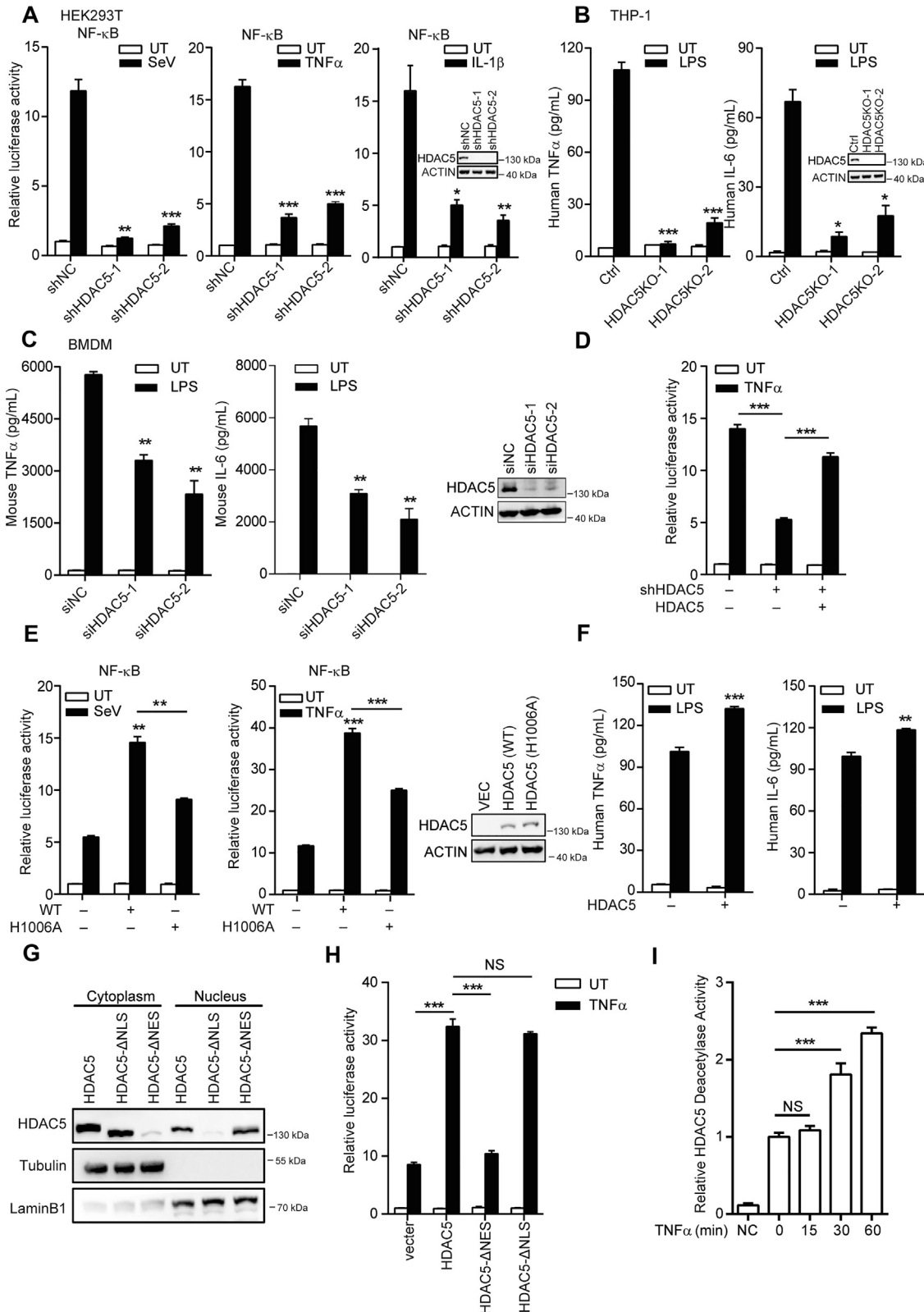
Figure 1. Identification of HDAC5 as a positive regulator of NF-κB signaling. *A*, luciferase activity in shNC, shHDAC5-1, or shHDAC5-2 cell lines (1×10^5), transfected for 36 h with a luciferase reporter for NF-κB (NF-κB-Luc), untreated (UT) or treated with SeV (0.1 MOI), TNF-α (20 ng/ml), or IL-1β (20 ng/ml) for 10 h. The HDAC5 knockdown efficiency in the HEK293T cells was confirmed by immunoblotting analysis. *B* and *C*, ELISA analysis of TNF-α and IL-6 in the supernatant of control, HDAC5 knockout(KO)-1 or HDAC5KO-2 THP-1 cell lines (*B*), or the supernatant of siNC, siHDAC5-1 or siHDAC5-2 of mouse bone-marrow-derived macrophage (BMDM) (*C*) followed by treatment with or without LPS (100 ng/ml) for 8 h. The Western blot showed the deficiency effi-ciency in the THP-1 cells or BMDMs. *D*, luciferase activity in shNC, shHDAC5 or shHDAC5 covering expression of HDAC5 cell lines (1×10^5), transfected for 36 h with a luciferase reporter for NF-κB, untreated (UT) or treated with TNF-α (20 ng/ml) for 10 h. *E*, the NF-κB Luciferase activity in HEK293T cells (1×10^5), cotransfected for 36 h with empty vectors, HDAC5 WT or HDAC5 H1006A together with a luciferase reporter for NF-κB, untreated or treated with TNF-α
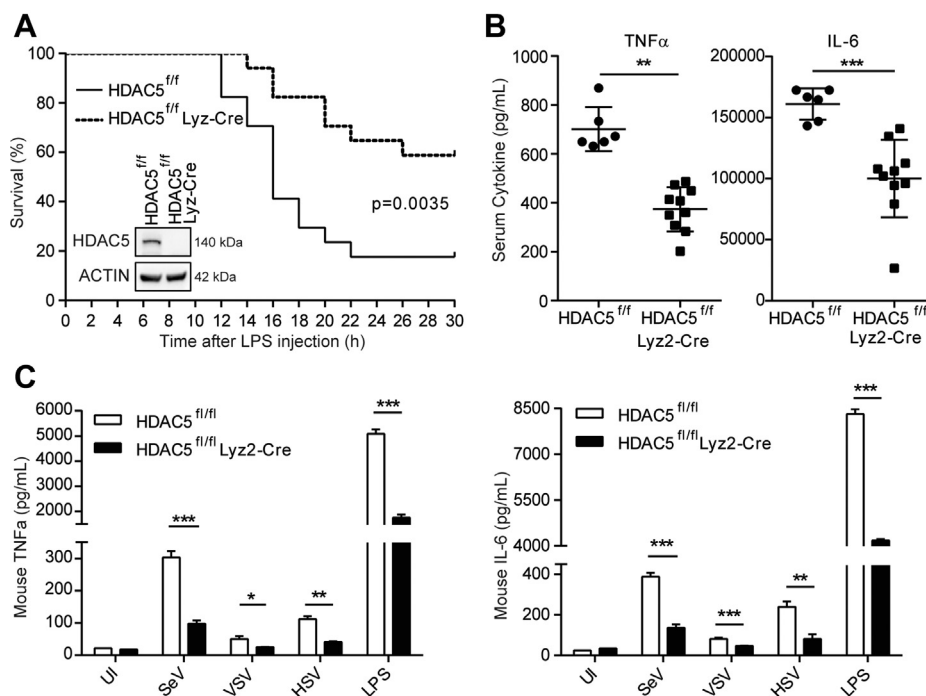
Figure 2. HDAC5 deficiency impairs inflammatory responses *in vivo*. *A*, effects of HDAC5 deficiency on LPS-induced inflammatory death of mice. Age- and sex-matched *Hdac5*^{fl/fl} (n = 17) or *Hdac5*^{fl/fl} Lyz2-Cre mice (n = 17) were intraperitoneally injected with LPS (20 mg/kg). The survival of mice was monitored every 2 h. The log-rank (Mantel–Cox) test was applied to analyze the statistical significance. *B*, effects of HDAC5 deficiency on LPS-induced cytokine production. Age- and sex-matched *Hdac5*^{fl/fl} or *Hdac5*^{fl/fl} Lyz2-Cre mice were intraperitoneally injected with LPS (20 mg/kg) for 3 h, and the concentrations of TNF-α and IL-6 in the sera were determined by ELISA. *C*, ELISA analysis of TNF-α and IL-6 in the supernatant of *Hdac5*^{fl/fl} or *Hdac5*^{fl/fl} Lyz2-Cre BMDMs, infected with SeV, VSV, HSV-1, and LPS. Data are representative of three independent experiments. Graphs show mean± SD. *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Student's *t* test).

analyzed whether HDAC5 regulated NF-κB signaling by targeting PPs. The real-time PCR analysis showed the HDAC5 could not influence the mRNA levels of PPs (Fig. S6*A*) in HDAC5-knockout THP-1 cells with or without LPS stimulation. Immunoprecipitation assays indicated that PP4C, PP1C, and PP2Ac interact with HDAC5 (Fig. 4*A*). As in previous reports, overexpressions of the catalytic subunit PP4C, PP1C, or PP2Ac significantly decrease the IKKβ phosphorylation. HDAC5 rescued the IKKβ phosphorylation only when co-overexpressed with PP2Ac, but not PP4C and PP1C (Fig. 4*B*). Furthermore, HDAC5 could markedly abrogate the dephosphorylation of IKKβ, p65, and IκBα by PP2Ac (Fig. 4*C*), which was further confirmed by NF-κB reporter assays (Fig. 4*D*). When the HDAC5 knockout cells were treated with PP2A inhibitor Cantharidin (10 nM), the phosphorylation of IKKβ and p65 was significantly enhanced (Fig. 4*E*). These results imply that HDAC5 negatively regulates PP2A, the inhibitor of NF-κB signaling, to activate NF-κB signaling.

The exogenous or endogenous coimmunoprecipitation experiments indicated that the PP2Ac could associate with IKKβ, IκBα, or p65, which suggested that these three proteins were the substrate of PP2Ac (Fig. 4, *F* and *G*). Furthermore, the

coimmunoprecipitation assay showed HDAC5, IKKβ, and PP2Ac exist in the same complex. However, HDAC5 did not influence the interaction between IKKβ and PP2Ac (Fig. S6*B*). Finally, the partial enzyme active mutant of HDAC5 (H1006A) or knockout of HDAC5 had little effect on the interaction of IKKβ and PP2Ac (Fig. 6*C*). These results collectively suggested that HDAC5 mediated IKKβ, IκBα, and p65 phosphorylation by regulating PP2Ac activity.

HDAC5 deacetylases PP2Ac to regulate its function

The observation that the deacetylase activity of HDAC5 was required to regulate NF-κB signaling (Fig. 1*E*) indicated that HDAC5-catalyzed deacetylation of lysine residues of PP2Ac might be involved in regulating phosphatase activity. First, endogenous PP2Ac was acetylated in BMDMs, HEK293T, and THP-1 (Fig. 5*A*), and LPS or TNFα stimulation decreased acetylation of PP2Ac (Fig. 5*B*). Knockout of HDAC5 resulted in upregulation of the acetylation of PP2Ac in BMDMs in the absence of LPS challenge; this upregulation was enhanced in the LPS challenge (Fig. 5*C*). The acetylation of PP2Ac was reduced dramatically following HDAC5 overexpression

(20 ng/ml) for 10 h. *F*, ELISA analysis of TNF-α and IL-6 in the supernatant of NC or HDAC5-stable-expressing THP-1 cells, followed by treatment with or without LPS (100 ng/ml) for 8 h. *G*, HEK293T cells (2×10^5) were transiently transfected for 48 h with HA tagged wild-type (WT), HDAC5-ΔNLS, or HDAC5-ΔNES plasmids (100 ng). Nuclear and cytoplasmic separation. The cells were separated into nuclei and cytoplasm and analyzed by Western blot. *H*, reporter analysis of NF-κB in HEK293T cells (1×10^5) transiently transfected for 48 h with vectors, HA tagged HDAC5 (WT), HDAC5 ΔNLS, or HDAC5-NES plasmids (100 ng), followed by treatment with TNF-α (20 ng/ml) for 8 h. *I*, the HDAC5 deacetylase activity was measured by the Deacetylase Activity Detection Kit after treatment with TNF-α (20 ng/ml) for indicated time. NC, negative control in the kit. Data are representative of three independent experiments. Graphs show mean± SD; n = 3. NS, no significant differences; *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Student's *t* test).
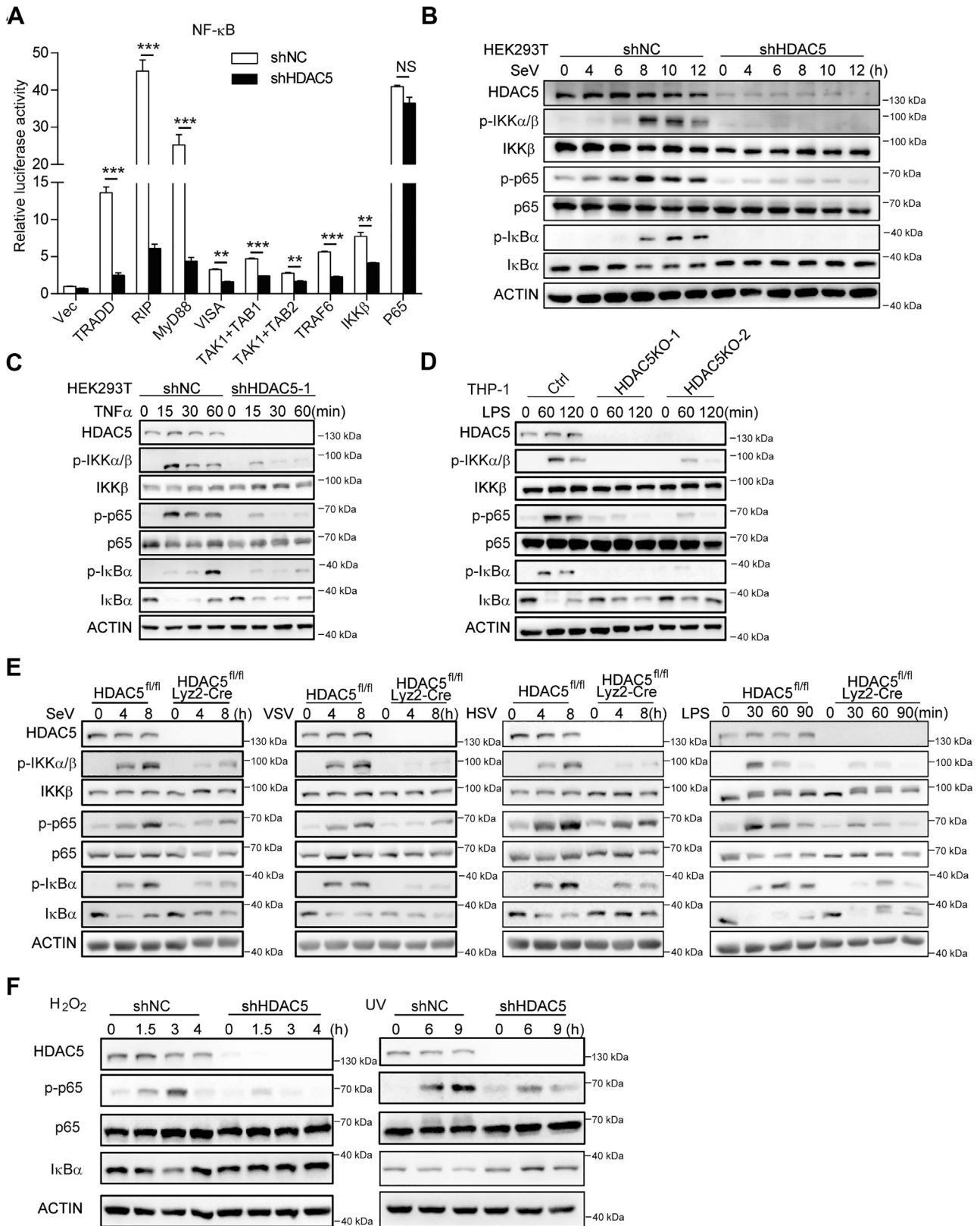
Figure 3. Deficiency of HDAC5 impairs activation of NF-κB. *A*, luciferase activity in shNC or shHDAC5 cell lines (1×10^5), transfected with an NF-κB responsive luciferase reporter plasmid and various constructs. Assessed 1 day after transfection and presented relative to Renilla luciferase activity. Control, pXJ40-HA vector. *B–E*, immunoblot analysis of phosphorylated and total IKKβ, p65 or IκBα in shNC or shHDAC5 HEK293T cell lines (*B* and *C*), control or HDAC5 deficient THP-1 cell lines (HDAC5KO-1 or HDAC5KO-2) (*D*), *Hdac5*^fl/fl or *Hdac5*^fl/flLyz2-Cre BMDMs (*E*), followed by stimulation with indicated stimulus for the indicated times. *F*, immunoblot analysis of lysates of shNC and shHDAC5 HEK293T cells treated with hydrogen peroxide (H_2O_2, 100 nM) or exposed to ultraviolet irradiation (20 J/m^2) for the indicated times. Data are representative of three independent experiments. Graphs show mean ± SD; n = 3. NS, no significant differences; **$p < 0.01$; ***$p < 0.001$ (Student's t test).
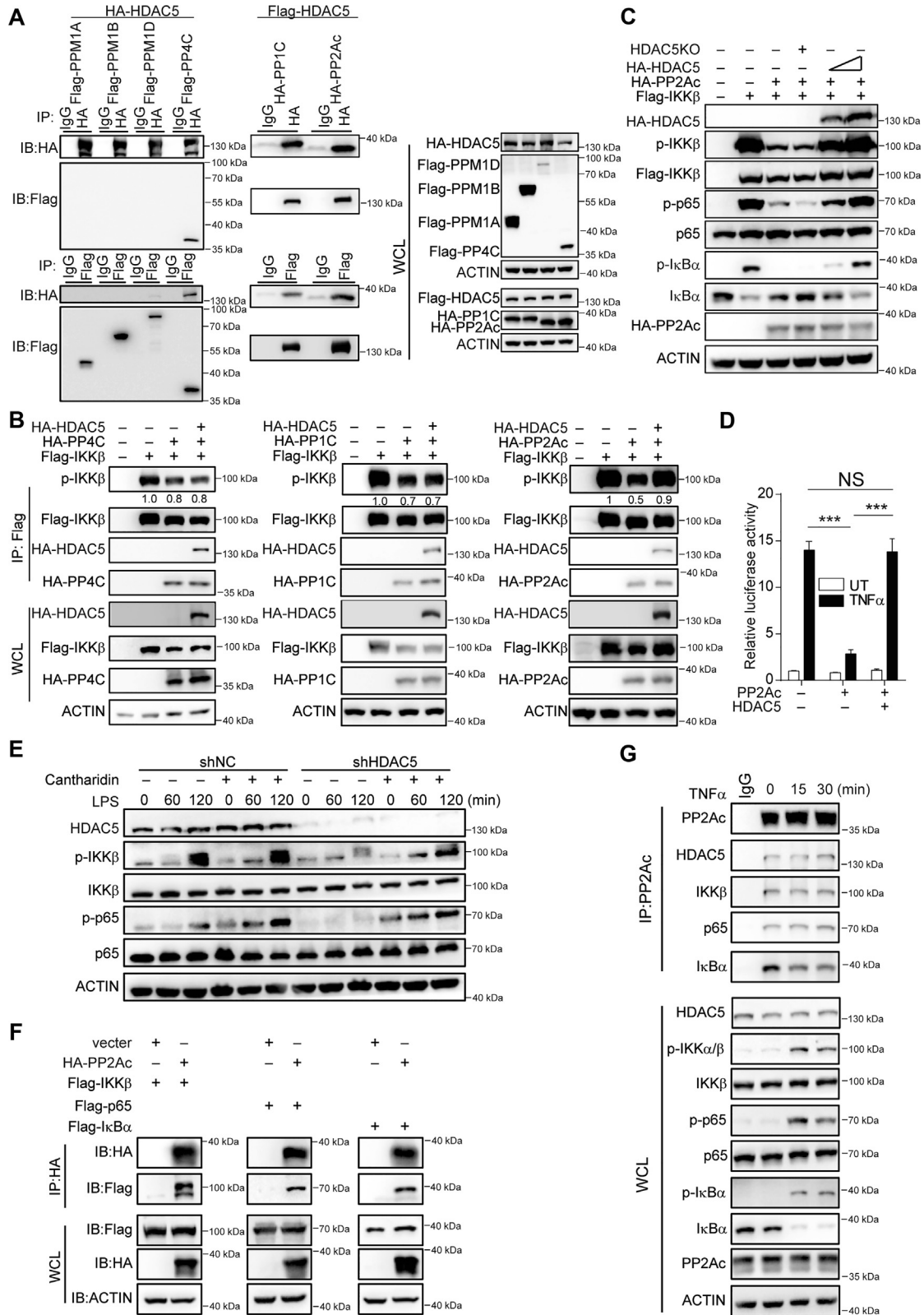
Figure 4. HDAC5 can rescue PP2Ac-mediated dephosphorylation of IKKβ, p65 and IκBα. *A*, the interaction between HDAC5 and Ser/Thr phosphatases. HEK293T cells (1.5×10^6) were cotransfected with the indicated plasmids for 48 h. Co-immunoprecipitation and immunoblotting were performed with the indicated antibodies. *B* and *C*, immunoblot analysis of HEK293T cells or HDAC5-knockout cells transfected with Flag-IKKβ alone or together with various expression plasmids (above lanes). Coimmunoprecipitation and immunoblotting were performed with the indicated antibodies. *D*, luciferase assay of HEK293T cells transfected with an NF-κB luciferase reporter alone or together with various expression constructs (below graph) and incubated for 24 h in the presence or absence of TNFα (20 ng/ml). *E*, shNC or shHDAC5 THP-1 cells were either left untreated or treated with the phosphatase inhibitor Cantharidin, as indicated and then with LPS (100 ng/ml) for the indicated times. Levels of the indicated proteins were assessed by Western blotting. *F*, the interaction between PP2Ac and IKKβ, p65 or IκBα. HEK293T cells (1.5×10^6) were cotransfected with the indicated plasmids (3 μg each) for 48 h.

(Fig. 5D), while the acetylation of PP2Ac was increased when treated with the HDAC5 inhibitor Trichostatin A (TSA) (Fig. 5E). The acetylation of PP2Ac was higher in cells expressing the HDAC5 deacetylase partial inactive mutant (H1006A) than in cells expressing the wild-type HDAC5 (Fig. 5F). Consistently, the H1006A mutant did not inhibit the dephosphorylation of IKKβ, p65, and IκBα mediated by PP2Ac (Fig. 5G). These results indicate that acetylation of PP2Ac is important for its phosphatase activity, and HDAC5 deacetylates PP2Ac to promote the phosphorylation and activation of NF-κB. To determine how PP2Ac interacts with HDAC5, we constructed HDAC5 mutants with deletions in various domains (Fig. 6D). The results manifested that the deacetylate domain of HDAC5 was required for the interaction of PP2Ac with HDAC5 (Fig. 5H). Collectively, these data suggested that HDAC5 could deacetylate PP2Ac to promote the phosphorylation and activation of NF-κB.

The acetylation of PP2Ac at Lys136 is critical for its phosphatase activity

First, HDAC5 did not affect the interaction of trimeric forms of PP2A (data not shown). We sought to determine the functional acetylation sites of PP2Ac. Each identified lysine residue was mutated into arginine (K to R) (to mimic the nonacetylated state of the lysine residue), followed by transient transfection and reporter assays. Only the K136R mutants still retained partial ability to activate the NF-κB luciferase reporter (Fig. 6A). Also, only the K136R mutant resulted in higher phosphorylation of IKKβ and p65 (Fig. 6B). Meanwhile, the K136Q mutant (lysine residues mutated into a glutamine (K to Q) mimic the acetylated state of the lysine residue) reduced the phosphorylation of IKKβ and p65 compared with the K136R mutant, and the enzyme activity mutant D85N was used as a positive control (Fig. 6C). An *in vitro* PP2A phosphatase assay using a PP2A phosphatase assay enzyme system showed a lower optical density value, indicating decreased phosphatase activity in K136R or PP2A and HDAC5 together compared with the K136Q and WT (Fig. 6D). To further investigate the effect of HDAC5 on regulating the phosphatase activity of PP2A, we performed an *in vitro* PP2A phosphatase assay. Immunoblot (IB) analysis showed that PP2Ac but not the PP2Ac-K136R could decrease the phosphorylation of IKKβ at Ser181 or phosphorylation of p65 at Ser536. The addition of WT-HDAC5, but not the H1006A, enhanced the phosphorylation of IKKβ or p65 (Fig. 6E). These results indicated that IKKβ and p65 are substrates of PP2A, and the deacetylase activity of HDAC5 could negatively regulate PP2A phosphatase activity.

As shown in Figure 6F, K136R almost abolished the acetylation of PP2Ac compared with WT and other mutants such as K104R. These results suggested that the Lys136 is the primary acetylation site of PP2Ac. Then, we analyzed Lys136-acetylated

PP2Ac immunoprecipitated from THP-1 during LPS stimulation. We found that the acetylation of PP2Ac at Lys136 gradually decayed, accompanied by p65 poststimulation phosphorylation (Fig. 6G). Therefore, HDAC5 regulated NF-κB activation by modifying the acetylation of PP2Ac, which controlled the phosphatase activity.

Discussion

In this study, the working model is shown in Figure 7. We report that HDAC5 is a novel positive regulator of the NF-κB pathway *in vitro* and *in vivo*. HDAC5 prolongs IKKβ, p65, and IκBα phosphorylation in response to various stimuli, including TNF, IL-1β, or LPS. We have shown that HDAC5 deacetylated the PP2Ac directly to impair its phosphatase activity. More importantly, the acetylation of PP2Ac at Lys136 is important for the phosphatase activity, and deacetylated PP2Ac maintains phosphorylated IKKβ, p65, and IκBα in a default active state. Therefore, these findings give rise to the possibility that HDAC5 functions as a positive effector that regulates NF-κB activation, as in the context of pathological conditions such as inflammation-linked cancer development.

In our hands, knockout of HDAC5 in macrophages was associated with significantly subdued levels of proinflammatory TNFα and IL-6. The finding strengthened this that overexpressing HDAC5 significantly augmented TNFα and IL-6 levels. Supporting the functional impact of HDAC5, a partial inactivating mutation (H1006A) had a little proinflammatory effect. We conclude that the integrity of the HDAC domain is indeed mandatory for the inflammatory regulatory function of HDAC5.

IKK activity was a key step modulating events leading to the NF-κB activation by extracellular stimuli. IKK activity was controlled by kinases and phosphatases to keep balance (9, 22). In response to stimulation with TNF or IL-1, IKK could be activated promptly, but this higher activity decreased to a basal activity shortly after stimulation, where PP played an important role (23). Although a large body of literature was generated on IKK activation and deactivation mechanisms, the molecular regulation machinery involved in deactivation factors such as PP remained to be elucidated. During the PP2A active complex formation, the core enzyme subunit (PP2Ac) is submitted to different posttranslational modifications and associations to ensure the correct and selective formation of different PP2A holoenzymes. Here, we reported that HDAC5 could deacetylate PP2Ac, the core enzyme subunit. We identified Lys136 as the key acetylation site of PP2Ac, which controlled the phosphatase activity of PP2Ac. The K136R PP2Ac mutant showed a limited ability compared with WT PP2Ac or K136Q PP2Ac mutant. The underlying mechanism for this might be that the charge state and structure of PP2Ac were altered by acetylation. Thus, its catalytic activity was also altered. We demonstrated that the acetylation was enhanced

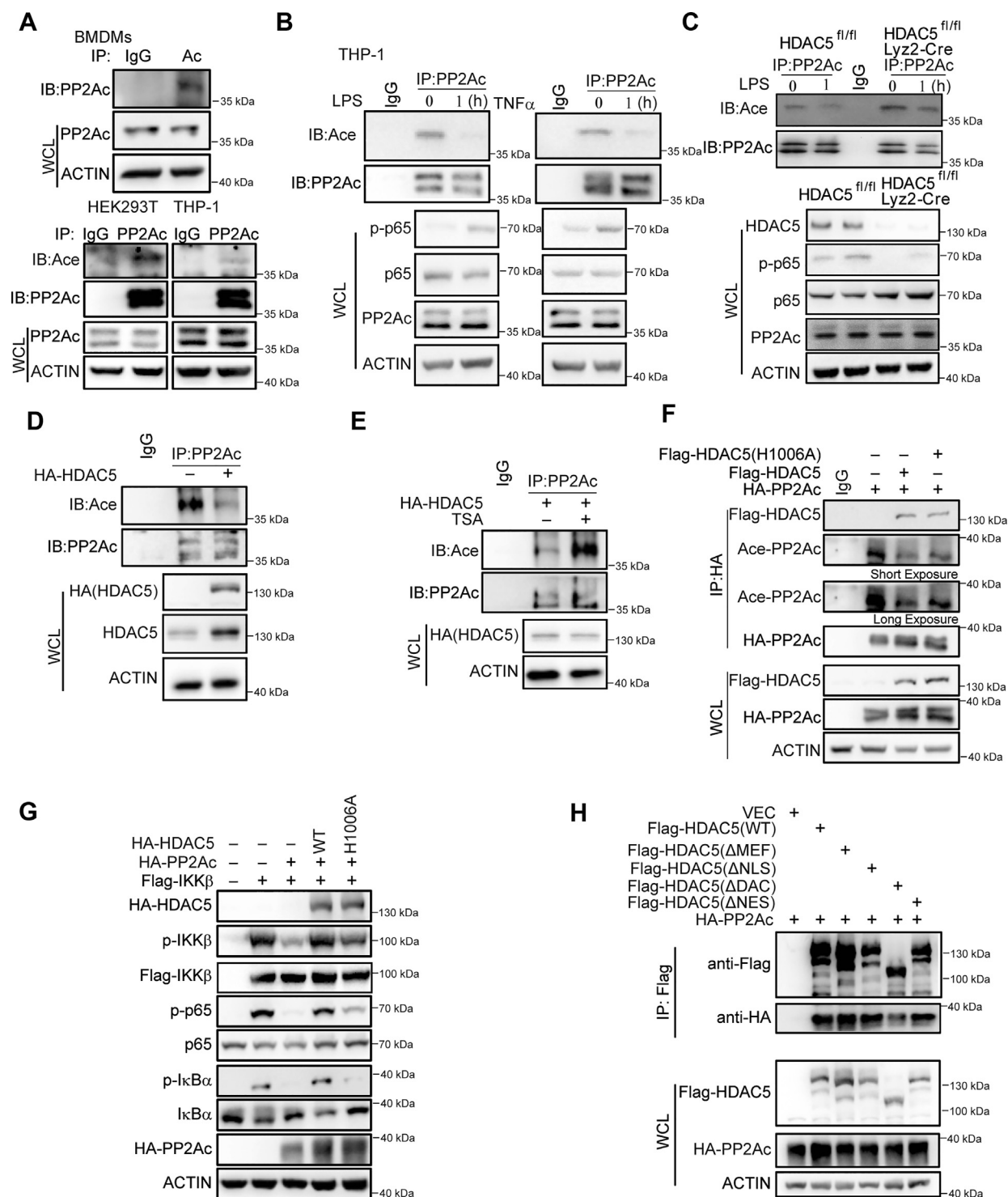# HDAC5 inactivated PP2A to regulate NF-κB signaling

Figure 5. HDAC5 suppresses the PP2Ac phosphatase activity *via* its deacetylase activity. *A*, immunoblot analysis of endogenous total PP2Ac and acetylated PP2Ac (Ac-PP2Ac) in BMDMs, HEK293T, or THP-1 cells, assessed before (10% input) or after (IP) immunoprecipitation with indicated antibody. *B* and *C*, immunoblot analysis of endogenous acetylation of PP2Ac in THP-1 cells (*B*) or BMDMs from *Hdac5*^fl/fl^ or *Hdac5*^fl/fl^ Lyz2-Cre mice (*C*), followed by treatment with LPS for 1 h or TNFα (20 ng/ml), before coimmunoprecipitation (with anti-PP2Ac or IgG as a control) and immunoblot analysis with anti-phosphorylated and total p65. *D*, immunoblot analysis of acetylated PP2Ac (Ac-PP2Ac) in HEK293T cells (1.5 × 10⁶) transiently cotransfected for 48 h with empty vectors or HA-tagged HDAC5 (1 μg) expression plasmids before coimmunoprecipitation (with anti-PP2Ac or IgG as a control) and immunoblot analysis with indicated antibody. *E*, effect of HDAC5 inhibitor (Trichostatin A, TSA, 10 μmol/L) on PP2Ac acetylation. Immunoblot analysis of acetylated PP2Ac in HEK293T cells (1.5 × 10⁶), transiently transfected for 48 h with HA-tagged HDAC5 (3 μg) and treated for 24 h with TSA before immunoprecipitation (with anti-PP2Ac or IgG as a control) and immunoblot analysis indicated antibody. *F*, HEK293T cells (1 × 10⁷) were transfected with the indicated expression plasmids for HA-tagged PP2Ac, Flag-tagged HDAC5 or Flag-tagged HDAC5-H1006A (partial enzyme activity mutants). Immunoprecipitation and immunoblotting analysis were performed as in *D*. *G*, immunoblot analysis of phosphorylated and total IKKβ, p65, or IκBα in HEK293T cells transfected with various expression constructs. *H*, immunoblot analysis of HEK293T cells (1.5 × 10⁶) transiently cotransfected for 48 h with HA-tagged-PP2Ac with vector encoding Flag-tagged wild-type or mutant HDAC5 (above lanes), assessed before (10% input) or after (Co-IP) Coimmunoprecipitation with the indicated antibodies. Data are representative of three independent experiments.

first and then weakened quickly during TNF or LPS stimulation, consistent with the dynamic changes of IKK phosphorylation.

It was also increasingly clear that dephosphorylation is an important step in re-establishing the normal responsiveness of NF-κB/Rel transcription factors after stimulation removal. P65 formed a complex with PP2Ac and HDAC5. Upon stimulation, p65 was phosphorylated by certain protein serine/threonine kinases. Phosphorylated p65 was translocated from the cytoplasm to the nucleus to activate the expression of target genes. Then, the transcription factor may form a complex with PP2A and be dephosphorylated by phosphatase. It appeared that the basal level of dephosphorylation had to be relatively active to maintain a low state of phosphorylation of p65, which was necessary for the normal responsiveness of the transcription factor. Disrupting the phosphorylation/dephosphorylation cycle would result in constitutive phosphorylation and activation of the transcription factor. We found that HDAC5 is involved in the regulation of phosphatase activity. The cytoplasm HDAC5 could regulate the NF-κB activation.

The early studies showed a time-dependent decrease of *Hdac5* mRNA after the onset of LPS stimulation (4). Our results suggested the HDAC5 deacetylase activity could be enhanced during TNFα treatment (Fig. 1*I*). This suggested that HDAC5 regulates the NF-κB activation and is itself being regulated. PP2A widely regulated the activity of key proteins controlling cell growth and survival. These proteins include Akt, Ras/Mapk, Src, mTOR, and Wnt/βCatenin (24–28). HDAC5 could regulate phosphatase activity directly, which suggested that HDAC5 may be involved in regulating these signaling pathways. The significance of the HDAC5-PP2A axis in the physiological process still needs further study.

In conclusion, here, we have shown an important mechanism for regulating the PP2A function. We have identified HDAC5 as a key regulator of NF-κB activation. Moreover, our results suggest that HDAC5 deacetylates PP2Ac at Lys136 to suppress its phosphatase activity and thus maintain the phosphorylation of IKKβ, IκBα, and p65.

Experimental procedures

Reagents and antibodies

Lipopolysaccharide (LPS L2630 Sigma), Recombinant human/mouse TNF-α (210-TA-020/410-MT-010, R&D Systems), Recombinant Human IL–1β (201-LB-005, R&D Systems), and Recombinant human IFN-γ (285-IF-100, R&D Systems) were purchased from the indicated companies. The antibodies used in this study are in Table S1.

Mice

All animal procedures in this investigation conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 85-23, revised in 1996), and the approved regulations were set by the Laboratory Animal Care Committee at Wuhan Institute of Virology, Chinese Academy of Sciences (CAS) (WIVA02201703). Lyz2-Cre mice on the C57BL/6 background were from Model Animal Research Center of Nanjing University. *Hdac5*^{fl/fl} C57BL/6 mice 6–8 weeks of age were generated by Shanghai Biomodel Organism Science & Technology Development Co, Ltd. All of the mice were bred and maintained under specific pathogen-free animal facility in Wuhan Institute of Virology, CAS.

Cells and virus

All cell cultures were maintained in a humidified atmosphere at 37 °C with 5% CO_2. The HEK293T (CRL-3216), THP-1 (TIB-202), Raw264.7 (TIB-71), and Vero (CCL-81) cells were from American Type Culture Collection (ATCC). HEK293T, Raw264.7, and Vero cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 2 mM L-glutamine, nonessential amino acids, 10% fetal bovine serum (FBS) (Invitrogen), and 1% penicillin-streptomycin (Life Technologies). THP-1 cells were cultured in RPMI 1640 (Hyclone) supplemented with 10% FBS (Invitrogen) and 1% penicillin-streptomycin (Life Technologies).

Sendai virus (SeV) and vesicular stomatitis virus VSV-GFP were provided by Prof. Hanzhong Wang, HSV-1 with GFP was provided by Prof Chunfu Zheng. All virus were amplified and titrated as previously described (15, 17). Cells were infected with SeV (1 M.O.I.), VSV (1 M.O.I), or HSV-1 (10 M.O.I.) for the indicated time. Cells were stimulated with LPS (100 ng/ml), TNF-α (20 ng/ml), or IFN-γ (100 ng/ml) for the indicated time.

CRISPR-Cas9 knockout

Double-stranded oligonucleotides corresponding to the target sequences were cloned into the lenti-CRISPR-V2 vector and cotransfected packaging plasmids into HEK293T cells. Forty-eight hours after transfection, the viruses were harvested, ultrafiltrated (0.22 mm filter, Millipore), and used to infect HEK293T or THP-1 cells in the presence of polybrene (8 μg/ml). The infected cells were selected with puromycin (2 μg/ml) for at least 5 days. Human HDAC5 sgRNA targeting sequences were 1# 5′-CAGCCCTGTGGAGCTACGGG-3′ and 2# 5′-AACAGCATGACCACCTGACA-3′.

Isolation of macrophages

BMDMs were isolated from the mouse tibia and femur of wild-type and HDAC5-deficient mice and cultured in 10-cm Petri dish at 37 °C for 6 days. Ten milliliter RPMI medium 1640 (supplemented with 10% FBS, L-glutamine, and 30% L929 supernatant) was added at day 4.

Plasmid constructs and transfection

Mammalian expression plasmids for HA- or Flag-tagged HDAC5 and its catalytic-attenuation/deletion mutants, HA or Flag-tagged PP2Ac and its site-directed mutants, Flag-tagged PPM1A, Flag-tagged PPM1B, Flag-tagged PPM1D, Flag-tagged PP4C, and HA-tagged PP1c were constructed by standard molecular biology techniques. All constructs were confirmed by sequencing and a complete list of primers was
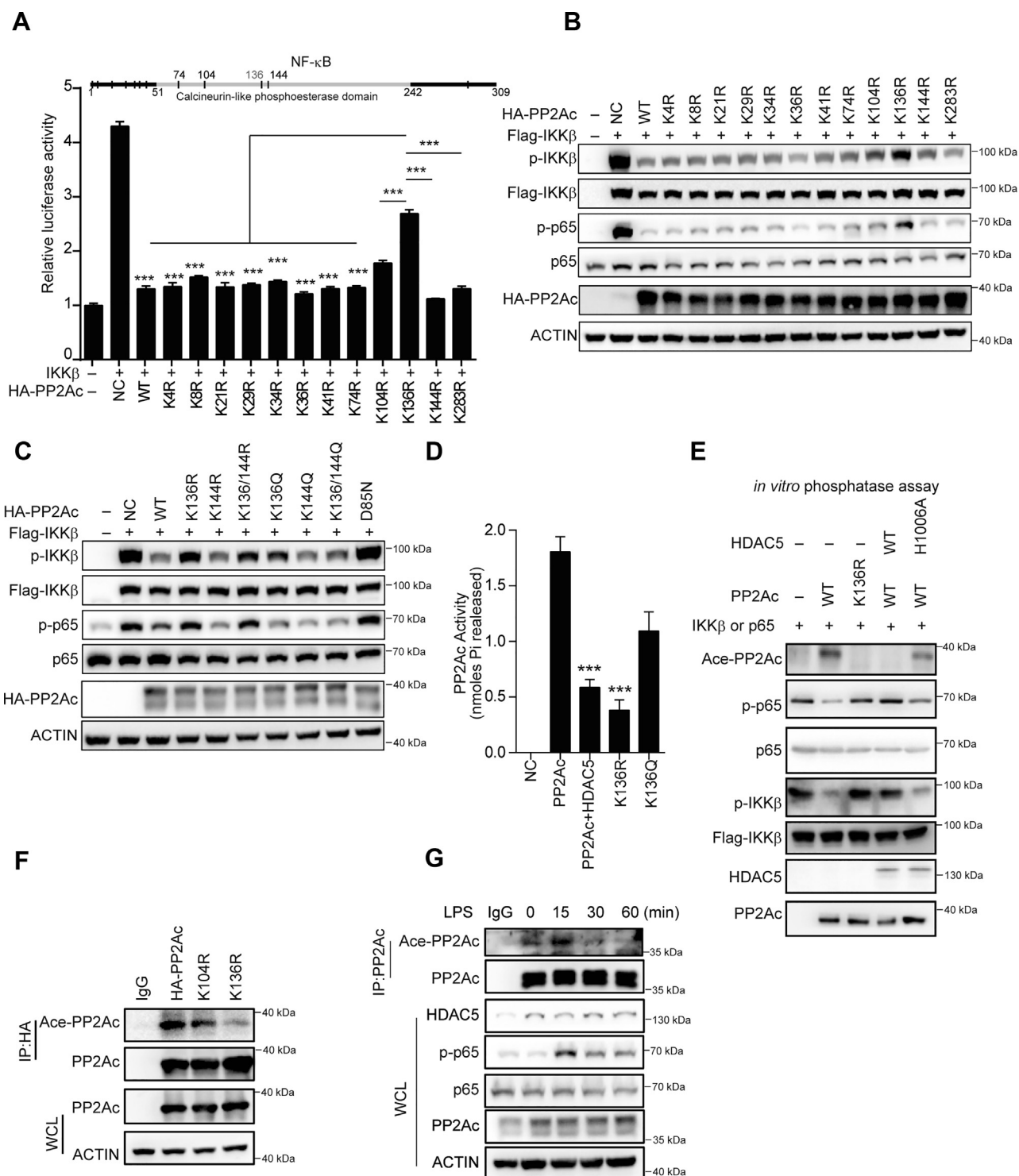
Figure 6. The acetylation at Lys136 is critical for PP2A phosphatase activity. *A*, identification of lysine residues in PP2Ac in HEK293T cells. Luciferase activity of an NF-κB reporter was transfected with empty vector (negative control, NC), IKKβ (WT) or mutants PP2Ac (K4R, K8R, K21R, K29R, K34R, K36R, K41R, K74R, K104R, K136R, K144R and K283R). *B* and *C*, immunoblot analysis of phosphorylated and total IKKβ, p65, or IκBα in HEK293T cells transfected with NC or wild-type PP2Ac (WT) or the indicated mutants. *D*, HEK293T cells (1.5×10^6) were transfected with HA-tagged PP2Ac, HA-tagged PP2Ac, and Flag-tagged HDAC5 or HA-tagged PP2Ac K136R or K136Q separately. The expressed proteins were immunoprecipitated by HA-tag antibodies and incubated with Threonine Phosphopeptide (K-R-pT-I-R-R). Phosphate release was measured using a malachite green colorimetric assay. *E*, *in vitro* phosphatase assay. The immunoprecipitate PP2A and IKKβ or p65 and purified recombinant HDAC5 were performed as indicated for phosphatase assay *in vitro*. Immunoblot was analyzed with antibody to phosphorylated IKKβ and p65, anti-total IKKβ and p65. *F*, immunoblot analysis of HEK293T cells (4×10^5) transiently transfected for 48 h with Flag-tagged wild type (WT) or PP2Ac mutant K136R or K136Q (2 μg each), immunoblot analysis were performed with the indicated antibodies. *G*, immunoblot analysis of endogenous acetylation of PP2Ac in THP-1 cells followed by treatment with LPS for 0 to 60 min, before coimmunoprecipitation (with anti-PP2Ac or IgG as a control) and immunoblot analysis with antiacetylation, antiphosphorylated and total p65. Data are representative of three independent experiments. Graphs show mean ± SD; n = 3. ***$p < 0.001$ (Student's *t* test).
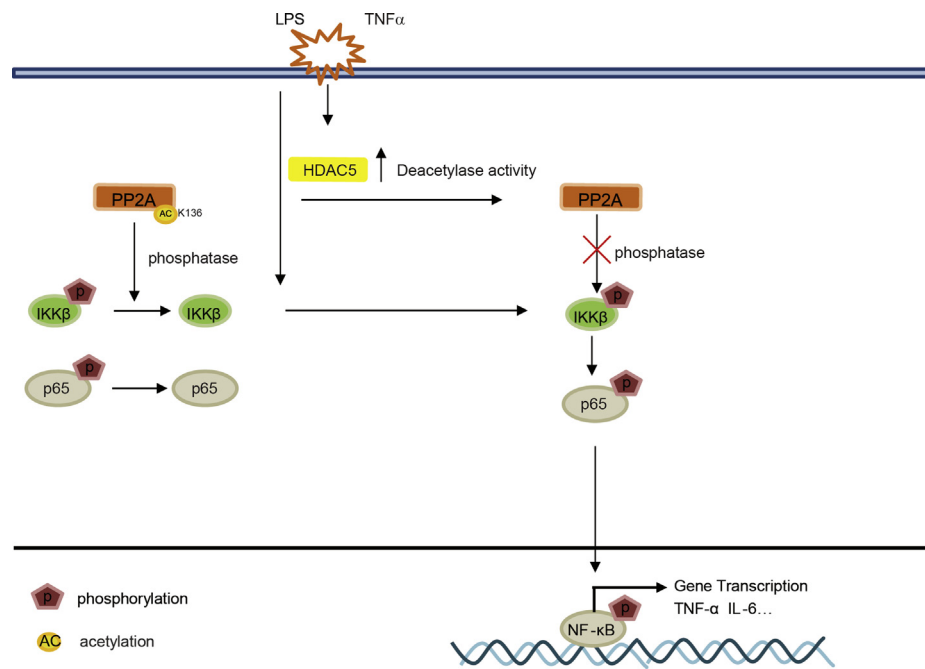
Figure 7. The working model. In the resting state, Lys136-acetylated PP2Ac means abundant phosphatase activity to promote the dephosphorylation of IKKβ and p65, indicating the closure of NF-κB pathway. The LPS or TNFα treatment could enhance the HDAC5 deacetylase activity to reduce the acetylation of PP2A, leading to decreased phosphatase activity. Phosphorylated p65 was translocated from the cytoplasm to the nucleus to activate the expression of target genes.

provided in Table S2. The NF-κB promoter luciferase reporter plasmid was purchased from Clontech. pRL-TK was purchased from Promega. The plasmids for IKKβ, NF-κB p65, IκBα, TAK1, TAB1, TAB2 were constructed by our lab. The IRF1 promoter luciferase reporter plasmid was kindly provided by Prof. Yanyi Wang. Mammalian expression plasmid for VISA was provided by Prof. Hongbing Shu. Plasmids were transiently transfected into HEK293T cells with using Lipofectamine 2000 reagents (Invitrogen) following the manufacturer instructions.

Luciferase assays

HEK293T (1×10^5) in 24-well plates was transfected with NF-κB (10 ng) or IRF1-firefly luciferase reporter (100 ng) and Renilla luciferase reporter (10 ng) together with HDAC5 or empty control plasmid. Twenty-four hours later, the cells were stimulated with virus or cytokines for 10 h, and luciferase activities were measured with Dual-Glo Luciferase Assay System (E1960, Promega). Data were normalized by calculating the ratio between firefly luciferase activity and Renilla luciferase activity.

ELISA

Secreted cytokines in cell culture supernatants or sera from LPS-injected mice were analyzed using human/mouse TNF-α (Biolegend) and human/mouse IL-6 (Biolegend) ELISA kits according to the manufacturer's instructions.

RNA-mediated interference and lentiviral transduction

The siRNAs were transfected using Lipofectamine RNAi-MAX reagent (Invitrogen) at a final concentration of 20 nM according to the manufacturer's instructions. The sequences of siRNAs specific for mouse HDAC5 were #2 5′-GCCUCG GAACCCAACUUAA-3′ and #5 5′-GGGCAAGAUCCUUA CCAAA-3′. The lentiviruses were produced in HEK293T cells by cotransfection of the shNC or shHDAC5 and packaging vectors psPAX2 and pMD2.G (Bought from addgene), (shRNAs were cloned into the lentivirus vector pLKO.1 plasmid). Forty-eight or seventy-two hours posttransfection, the supernatant was collected and applied to infect target cells, followed by cell selection through puromycin (1 μg/ml). Human HDAC5 shRNA targeting sequences were 1# 5′-CC ATCGCTGAGAATGGCTTTA-3′ and 2# 5′-GAC TGTT ATTAGCACCTTTAA-3′.

RNA quantification

Total RNA was extracted with Trizol reagent following the manufacturer's protocols (Invitrogen). Specific mRNAs were quantified by one-step real-time RT-PCR using the QuantiFast SYBR Green RT-PCR kit (Qiagen). The data were normalized to the expression of the β-actin for each individual sample. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative expression changes. The primer sequences for quantitative RT-PCR are provided in Table S3.

Coimmunoprecipitation and immunoblot analysis

For coimmunoprecipitation and immunoblot analysis, cells were lysed with lysis buffer (50 mM Tris, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 150 mM NaCl, 100 μM phenylmethylsulfonyl fluoride [PMSF]), and a protease inhibitor cocktail (Complete Mini, Roche) for 30 min in 4 °C. Cell lysates were centrifuged at 13,000g for 10 min at

4 °C and quantified using the Bradford method (#500-0006, BioRad). For Western blotting, the supernatants were recovered and boiled in 5× loading buffer. For immunoprecipitation, the supernatants were collected and incubated with protein G agarose beads (Millipore) recovered and mixed with specific antibodies for overnight with rotation at 4 °C. Protein G agarose-bound immune complexes were collected by centrifugation at 5000g for 1 min, washed at least five times with IP buffer, and boiled in 2× loading buffer. Samples were separated by SDS-PAGE, electro-transferred to nitrocellulose filter membranes (Millipore), and then blocked for 1 h with 5% nonfat milk solution, followed by blotting with the primary antibodies. The proteins were visualized using suitable HRP-conjugated secondary antibodies (Jackson Immuno Research) and SuperSignal-Femto chemiluminescent substrate (Pierce).

Subcellular fractionation

Nuclear and cytoplasmic extracts were prepared with a nuclear-cytoplasmic extraction kit (Beyotime Biotechnology) according to the manufacturer's instructions.

Immunofluorescence and confocal microscopy

HEK293T cells were plated on glass cover slips and transfected with the indicated plasmids by lipofectamine 2000 (Invitrogen) or stimulated as the indicated time before fixed with 4% paraformaldehyde. Cells were permeabilized with 0.5% Triton X-100 and blocked with 1% normal goat serum. Cells were stained with the indicated primary antibodies and the corresponding secondary antibodies. Nuclei were stained with DAPI. Cells were observed with a Nikon confocal microscope.

In vitro kinase assay

HEK293T cells were transfected with indicated plasmid separately. The expressed proteins were immunoprecipitated by the indicated tag antibodies. The Immune-complexes were washed twice with 1× cell lysis buffer (#9803s, CST) and then twice with 1× kinase buffer (#9802, CST). Kinase reactions were performed by incubation of immune-complexes as indicated with 1× kinase buffer, 10 mM ATP (#9804, CST) and at 30 °C for 90 min in 50 µl reaction mixture. Samples were separated by SDS-PAGE and analyzed by immunoblotting with indicated antibodies.

PP2Ac phosphatase assay kit

HEK293T cells were transfected with indicated plasmid separately. The expressed PP2Ac proteins were immunoprecipitated by the indicated tag antibodies. The immune-complexes were washed three times with TBS, followed by one wash with 500 µl Ser/Thr Assay Buffer (50 mM Tris-HCl, pH 7.0, 0.1 mM CaCl$_2$, Sterilized through a 0.2 m filter). Phosphatase assays were performed in 80 µl reaction containing 750 µM of Threonine Phosphopeptide (K-R-pT-I-R-R) (#12-219, Millipore). Reactions were carried out for 10 min at 30 °C. The released phosphate levels in the reaction mix were measured using a malachite green-based colorimetric phosphate assay kit (Abcam #ab65622), according to the manufacturer's instructions.

In vitro phosphatase assay

HEK293T cells were transfected with PP2Ac or PP2Ac-K136R, HDAC5 or HDAC5-H1006A, and IKKβ or p65 plasmid, respectively. Immunoprecipitations were performed using indicated antibody, after 48 h of transfection. With four washes, PP2Ac, PP2Ac-K136R, IKKβ, p65, HDAC5, or HDAC5-H1006A was immunoprecipitated respectively. The PP2Ac was incubated with HDAC5 or HDAC5-H1006A to complete the deacetylation of PP2Ac. Then the indicated proteins were incubated in phosphatase assay buffer (20 mM tris-HCl, 1 mM EGTA, 5 mM MgCl$_2$, 0.02% 2-mercaptoethanol, BSA [0.2 mg/ml]) at 30 °C for 60 min. Reaction was stopped by addition of 2× SDS loading buffer.

HDAC activity assay

HEK293T cells were stimulated with TNFα for the indicated times. Then the endogenous HDAC5 proteins were immunoprecipitated by the specific antibodies. The immune-complexes were washed three times with IP Buffer, followed by one wash with HDAC Assay Buffer (AAT Bioquest, #13601 Component B). The HDAC activity was measured using a Fluorimetric HDAC Activity Assay Kit (AAT Bioquest, #13601), according to the manufacturer's instructions.

Statistical analysis

Differences between groups were evaluated using the two-tailed, unpaired Student's t test available in the GraphPad Prism 5 software package (GraphPad Software, Inc). Coprecipitation efficiency and fluorescence images were analyzed using ImageJ (NIH). p-values were calculated, and statistical significance was reported as highly significant with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Analytic results are presented as mean±SD

Data availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

H. Z. supervision; J. Chen writing–original draft; J. Chen writing–review and editing.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: CAS, Chinese Academy of Sciences; FBS, fetal bovine serum; HDAC5, histone deacetylase 5; IL-1, interleukin 1; LPS, lipopolysaccharide; PP, protein phosphatase; TLR, toll-like receptor; TNF, tumor necrosis factor.

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