

Distinct B subunits of PP2A regulate the NF- κ B signalling pathway through dephosphorylation of IKK β , I κ B α and RelA

Yoshihiro Tsuchiya, Keiko Osaki, Mayu Kanamoto, Yuki Nakao, Ena Takahashi, Toru Higuchi and Hideaki Kamata

Laboratory of Biomedical Chemistry, Department of Molecular Medical Science, Graduate School of Biomedical Science, Hiroshima University, Japan

Correspondence

H. Kamata, Department of Molecular Medical Science, Graduate School of Biomedical Science, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8553, Japan
Fax: +81 (82) 257-5136
Tel: +81 (82) 257-5138
E-mail: hkamata@hiroshima-u.ac.jp

(Received 7 July 2017, revised 4 October 2017, accepted 25 October 2017, available online 3 December 2017)

doi:10.1002/1873-3468.12912

Edited by Kazuhiro Iwai

PP2A is composed of a scaffolding subunit (A), a catalytic subunit (C) and a regulatory subunit (B) that is classified into four families including B, B', B'' and B'''/striatin. Here, we found that a distinct PP2A complex regulates NF- κ B signalling by dephosphorylation of IKK β , I κ B α and RelA/p65. The PP2A core enzyme AC dimer and the holoenzyme AB'''C trimer dephosphorylate IKK β , I κ B α and RelA, whereas the ABC trimer dephosphorylates I κ B α but not IKK β and RelA in cells. In contrast, AB'C and AB''C trimers have little effect on dephosphorylation of these signalling proteins. These results suggest that different forms of PP2A regulate NF- κ B pathway signalling through multiple steps each in a different manner, thereby finely tuning NF- κ B- and IKK β -mediated cellular responses.

Keywords: NF-kappa B; protein phosphatase; protein phosphatase 2 A (PP2A)

Nuclear factor kappa B (NF- κ B) is a critical transcription factor that regulates many cellular and organismal processes including immune and inflammatory responses, cellular growth and cell survival. The NF- κ B signalling pathway is regulated by the phosphorylation of several proteins including inhibitor of kappa B (I κ B) kinase β (IKK β), an inhibitory protein I κ B α and an essential subunit of NF- κ B RelA/p65 [1–3]. Binding of cytokines such as TNF- α to respective receptors leads to the conjugation of ubiquitin to several signalling components and recruits adaptor proteins and kinases [3–6]. These chains of reaction induce IKK β activation through phosphorylation of two critical serine residues, Ser177 and Ser181, at its activation loop [7,8]. IKK β in turn phosphorylates I κ B α at the N-terminal serines, Ser32 and Ser36, which leads to ubiquitination at lysine residues Lys21 and Lys22 and I κ B α degradation by the

ubiquitin-proteasome system. These reactions result in the nuclear translocation of NF- κ B and binding to its cognate κ B sites in the promoters/enhancers of its target genes. IKK β also phosphorylates serine residues, Ser468 and Ser536, in the C-terminal transactivation domain of RelA and promotes gene expression through the association of RelA with a transcriptional coactivator, CREB-binding protein [9–11].

Aberrant activation of NF- κ B is linked to various diseases such as inflammatory disorders and cancer. Thus, there are numerous regulatory mechanisms at multiple levels to ensure the tight control of NF- κ B activity [1–6]. In particular, dephosphorylation of its protein represents the most important mechanism to downregulate NF- κ B activity. There are three families of protein serine/threonine phosphatases: protein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs)

Abbreviations

I κ B, inhibitor of kappa B; IKK β , inhibitor of kappa B kinase β ; IKK β KN, kinase-negative IKK β mutant; NF- κ B, nuclear factor kappa B; PPPs, protein phosphatases.

and DxDxT phosphatases [12,13]. PPP constitutes the largest family, containing several members including protein phosphatase 1 (PP1), PP2A, PP2B and PP5, whereas the PPM family includes PP2C. Several of these, such as PP1 [14], PP2A [7,15,16], PP2C [17] and PP5 [18], are required for dephosphorylation of the activation loop serines in IKK β , which is a critical step to downregulate NF- κ B. In addition, protein phosphatases dephosphorylate downstream signalling proteins as well. Specifically, it has been reported that PP2B dephosphorylates I κ B α [19] and PP2A [20] and PP2C [21] dephosphorylate RelA.

Among these phosphatases, PP2A is the most abundant, constituting approximately 1% of total cellular proteins [22–24]. PP2A is a heterotrimeric complex consisting of a scaffolding (A), regulatory (B) and catalytic (C) subunit. The A and C subunits each are comprised of two possible variants, α and β . The monomeric C subunit is unstable and requires binding to the A subunit to exist as a stable core enzyme AC dimer. The B subunit family consists of four classes including B (B55/PR55), B' (B56/PR61), B'' (PR48/PR72/PR130) and B''' (PR93/PR110)/striatin (Strn). In turn, there are four isoforms of the B class including α , β , γ and δ , five of the B' class including α , β , γ , δ and ϵ , three of the B'' class including α , β and γ , and three isoforms of the B'''/Strn class including Strn, Strn3 and Strn4. These B subunits associate with the AC dimer to form the holoenzyme trimer. Crystal structure analysis revealed that the B subunits bind proximal to the C subunit active site in the holoenzyme trimer and determine the specificity for substrate proteins [25,26].

A previous RNAi screen revealed that PP2A plays an important role in the regulation of NF- κ B signalling and identified the core enzyme subunits, A α , A β , C α and C β , as negative regulators of the NF- κ B signalling pathway in mouse astrocytes [16]. However, this screen did not clarify whether the B subunit is involved in regulation of NF- κ B activity, nor identify the specific B subunits regulating each NF- κ B signalling step. It is plausible that, as cells usually express multiple isoforms of each B family protein, this failure was a result of complementation with other isoforms of the same family following the simple RNAi screen. In this study, we expressed B subunits together with A and C subunits in cells and investigated the role of these subunits on TNF- α -, IKK β - and RelA-induced NF- κ B activation. This assay revealed that distinct B subunits regulate specific steps of NF- κ B signalling. Dephosphorylation of IKK β and RelA is mediated by the AC dimer and AB'''C trimer, whereas dephosphorylation of I κ B α is mediated by the AC dimer, ABC trimer and AB'''C trimer.

Materials and methods

Cell cultures, plasmids and transfection

cDNA-encoding A α , A β , C α , C β , B α , B β , B γ , B δ , B' α , B' β , B' γ , B' δ , B' ϵ , B'' β , Strn and Strn3 subunits of PP2A were amplified from a human cDNA library by PCR. The cDNAs were inserted into pRK-HA and pRK-Flag expression vectors. Expression plasmids encoding IKK β , kinase-negative IKK β mutant (IKK β KN) and I κ B α have been reported previously [27]. Constitutive active IKK β EE mutant of which phosphorylation sites Ser177/181 in the activation loop were exchanged to Glu, the ubiquitination-resistant I κ B α RR mutant of which ubiquitination sites lysine residues Lys21/22 were exchanged to Arg, and the phosphatase-inactive C α D85N mutant of which Asp 85 was exchanged to Gln were generated using the KOD-Plus Mutagenesis kit (TOYOBO). Plasmids were transfected into GP2-293 cells in Opti-MEM (Invitrogen) using Lipofectamine Plus (Invitrogen) following the manufacturer's instructions. GP2-293 cells were obtained from Clontech. Cells were cultured in DMEM supplemented with 10% FBS containing 2 mM L-glutamine at 37 °C.

Luciferase assay

Nuclear factor kappa B activity was estimated by Dual-Luciferase Reporter Assay System (Promega) using pNF- κ B and pRL-TK Luciferase reporter plasmids. After transfection of 0.01 μ g NF- κ B reporter plasmids with or without 0.01 μ g expression plasmids of various PP2A subunits, IKK β , I κ B α and/or RelA into GP2-293 cells in collagen-coated 96-well dishes, cells were incubated in the presence or absence of 50 ng·mL⁻¹ TNF- α for 18 h. After lysing cells with buffer from the assay system, luciferase activity was analysed following the manufacturer's instructions.

Immunoblotting

GP2-293 cells in collagen-coated 12-well dishes were transfected with 0.2 μ g expression plasmids of various PP2A subunits, IKK β , I κ B α and/or RelA. After 24-h transfection, cells were washed with PBS and solubilized with buffer A consisting of 20 mM Tris/Cl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 10 mM MgCl₂, 60 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 mM 4-amidino phenyl methyl sulfonyl fluoride, 50 KIU·mL⁻¹ aprotinin, 20 μ g·mL⁻¹ pepstatin, 20 μ g·mL⁻¹ leupeptin, 2 mM DTT and 1% Triton X-100. After centrifugation at 16 000 $\times g$ for 20 min at 4 °C, the supernatants were used as cell lysates. The cell lysates were subjected to SDS/PAGE and then gel-separated proteins were transferred to PVDF membranes (Millipore) and subjected to immunoblotting using the SuperSignal West Pico Chemiluminescence System (Pierce). Antibodies used were as follows: anti-Flag (M2) (Sigma, F-3165), anti-HA

(Roche, 04-902), anti-I κ B α (Santa Cruz Biotechnology, SC-371), anti-RelA/p65 (Santa Cruz Biotechnology, SC-372), anti-phospho-I κ B α (Cell Signaling Technology, 2859), anti-phospho-RelA/p65(S468) (Cell Signaling Technology, 3039), anti-phospho-RelA/p65(S536) (Cell Signaling Technology, 3033), anti-PP2A (Cell Signaling Technology, 2039), anti-PP2A (Epitomics, 1512-1), anti-IKK β (Cell Signaling Technology, 2684) and anti-phospho-IKK α/β (Cell Signaling Technology, 2697).

***In vitro* dephosphorylation assay by PP2A**

For the dephosphorylation assay of IKK β , GP2-293 cells in 10-cm dishes were transfected with expression plasmids encoding Flag-IKK β , and then phosphorylated IKK β was recovered from cell lysates *via* anti-Flag (M2) agarose beads (Sigma). IKK β proteins were eluted in a buffer containing 20 mM Tris/Cl, pH 7.5, 150 mM NaCl, 2 mM DTT, 1% Triton X-100 and 3 \times Flag peptides (Sigma) from anti-Flag-agarose beads with ultra-free Centrifugal Filter Units (Millipore). For the dephosphorylation assay of I κ B α and RelA, cells were transfected with plasmids encoding Flag-RelA, HA-I κ B α RR and IKK β EE, and then phosphorylated I κ B α RR and RelA protein complexes were purified from cell lysates *via* anti-Flag (M2) agarose beads. PP2A proteins were purified from GP2-293 cells following transfection with plasmids encoding the Flag-tagged A α subunit and HA-tagged C α subunit, together with or without HA-tagged B subunits including B α , B β , B γ , B δ , B' α , B' β , B' γ , B' δ , B' ϵ , B'' β , Strn and Strn3. PP2A core enzyme composed of A α and C α , and PP2A holoenzyme composed of A α , C α and various B subunits were purified from cell lysates *via* anti-Flag (M2) agarose beads with ultra-free Centrifugal Filter Units in the previously described buffer. Phosphorylated IKK β proteins (0.001 μ g) or protein complexes (0.001 μ g) of phosphorylated I κ B α RR and RelA were incubated with purified PP2A (0.001 μ g) for 30 min at 37 $^{\circ}$ C, and then reaction mixtures were subjected to SDS/PAGE. Gel-separated proteins were transferred to PVDF membranes and subjected to immunoblotting using anti-phospho-IKK α/β , anti-phospho-I κ B α and anti-phospho-RelA antibodies, respectively.

Results

The PP2A core enzyme downregulates NF- κ B signalling

PP2A is composed of three subunits scaffolding (A), regulatory (B) and catalytic (C) subunit (Fig. 1A). Cells were transfected with expression plasmids of α and β isoforms of the A and C subunits, and the expression of PP2A proteins were analysed by immunoblotting (Fig. 1B). Then, the effects of expression of these proteins on NF- κ B activity were analysed

by luciferase assay. Expression of the C α and C β subunits and of the C subunits together with A α or A β subunits suppressed TNF- α -induced NF- κ B activation (Fig. 1C) and suppressed IKK β and constitutive active IKK β EE-induced NF- κ B activation (Fig. 1D,E). In contrast, expression of the PP2A did not attenuate RelA-mediated NF- κ B activity (Fig. 1F). These results suggest that PP2A suppresses NF- κ B activity by suppressing I κ B α and/or IKK β phosphorylation. Expression of inactive PP2A core enzyme consisting of the C α D85N mutant did not suppress TNF- α -, IKK β - and IKK β EE-induced NF- κ B activation (Fig. 1G,H).

Distinct PP2A holoenzymes downregulate NF- κ B signalling

To investigate the involvement of the PP2A in regulation of NF- κ B signalling, we constructed expression plasmids encoding each subunit of the B, B'', B''' and B''''/Strn family (Fig. 2A). Among isoforms of B'' and B''''/Strn families, we used expression plasmids encoding B'' β for the B'' family, along with Strn and Strn3 for the B''''/Strn family, as B'' α , B'' γ and B''''/Strn4 hardly express in cells. Cells were transfected with NF- κ B reporter plasmids and plasmids encoding A α , C α and each B subunit, and NF- κ B activity was investigated following TNF- α treatment by luciferase assay. Expression of the AC holoenzyme, the ABC holoenzyme including A(B α)C, A(B β)C, A(B γ)C and A(B δ)C, and the AB''''C holoenzyme including A(Strn)C and A(Strn3)C suppressed TNF- α -induced NF- κ B activity, whereas the AB'C holoenzyme including A(B' α)C, A(B' β)C, A(B' γ)C, A(B' δ)C and A(B' ϵ)C and the AB''C holoenzyme including A(B'' β)C had little effect on TNF- α -induced NF- κ B activation (Fig. 2B). Then, we investigated the effects of distinct B subunits on NF- κ B signalling. NF- κ B activity was analysed in cells transfected with plasmids encoding IKK β or IKK β EE together with A α , C α and each B subunit. A luciferase assay demonstrated that expression of AC, ABC and AB''''C attenuated IKK β - and IKK β EE-induced NF- κ B activity, whereas AB'C and AB''C had little effect on NF- κ B activation (Fig. 2C,D). These results suggest that AC, ABC and AB''''C suppress NF- κ B activity downstream of IKK β .

AC and AB''''C dephosphorylate IKK β and AC, ABC and AB''''C dephosphorylate I κ B α in cells

We next investigated the effects of distinct B subunits on phosphorylation of the IKK β activation loop. Cells were transfected with expression plasmids encoding IKK β and plasmids encoding A α , C α and each B

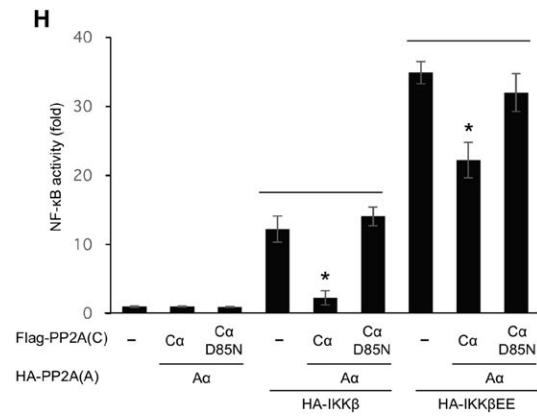
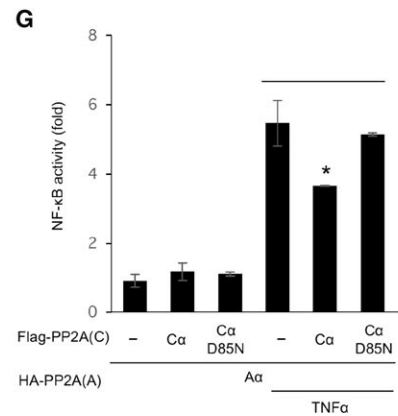
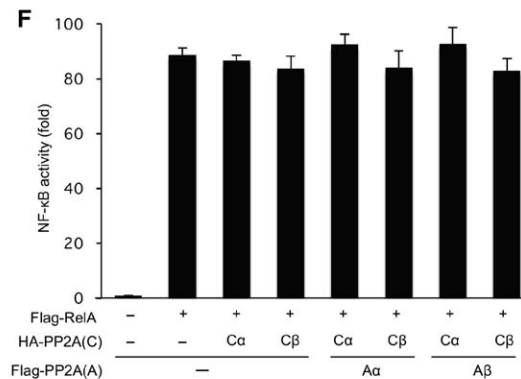
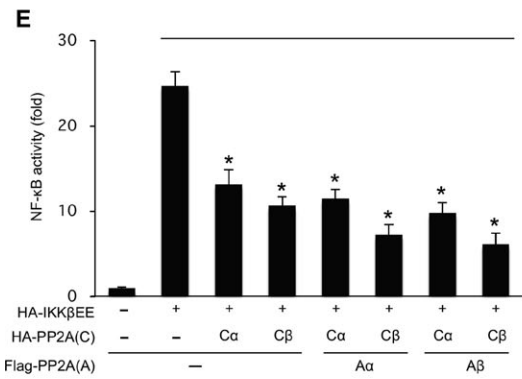
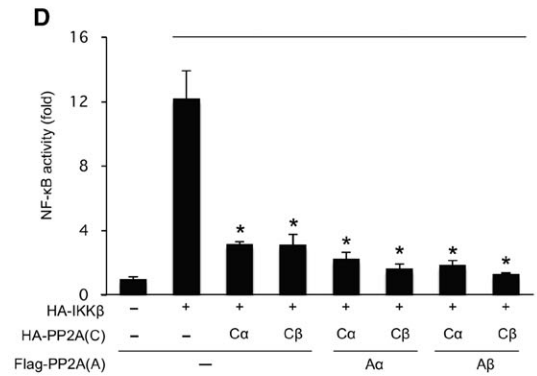
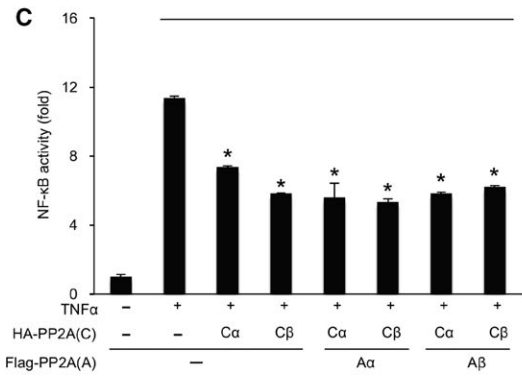
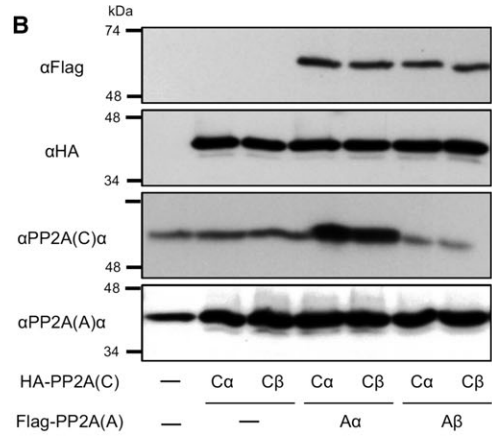
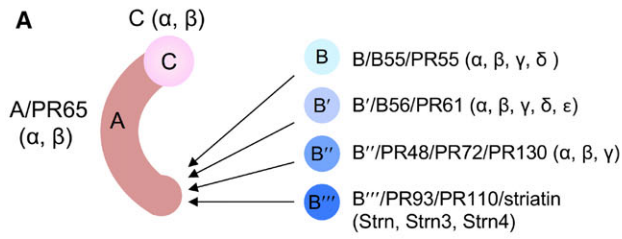


Fig. 1. PP2A core enzymes suppress NF- κ B activity. (A) Schematic model of PP2A core enzyme and holoenzyme. (B) Expression of PP2A subunits in GP2-293 cells. Cells were transfected with plasmids encoding HA-PP2A C α and C β , together with Flag-PP2A A α and A β , and expression of these subunits was analysed by immunoblotting. (C) Cells were transfected with NF- κ B reporter plasmids together with plasmids encoding PP2A core enzyme subunits, then stimulated with TNF- α for 18 h. (D-F) Cells were transfected with NF- κ B reporter plasmids and plasmids encoding PP2A core enzyme subunits together with plasmids encoding HA-IKK β (D), HA-IKK β EE (E) or Flag-RelA (F). (G) Cells were transfected with NF- κ B reporter plasmids and PP2A plasmids encoding C α or C α D85N, and were incubated for 20 h. Then, cells were stimulated with TNF- α for 4 h. (H) Cells were transfected with NF- κ B reporter plasmids and PP2A plasmids encoding C α or C α D85N, together with plasmids encoding HA-IKK β and HA-IKK β EE. Cells were incubated for 18 h, and NF- κ B activity was estimated by luciferase assay. *Statistically significance at $P < 0.01$.

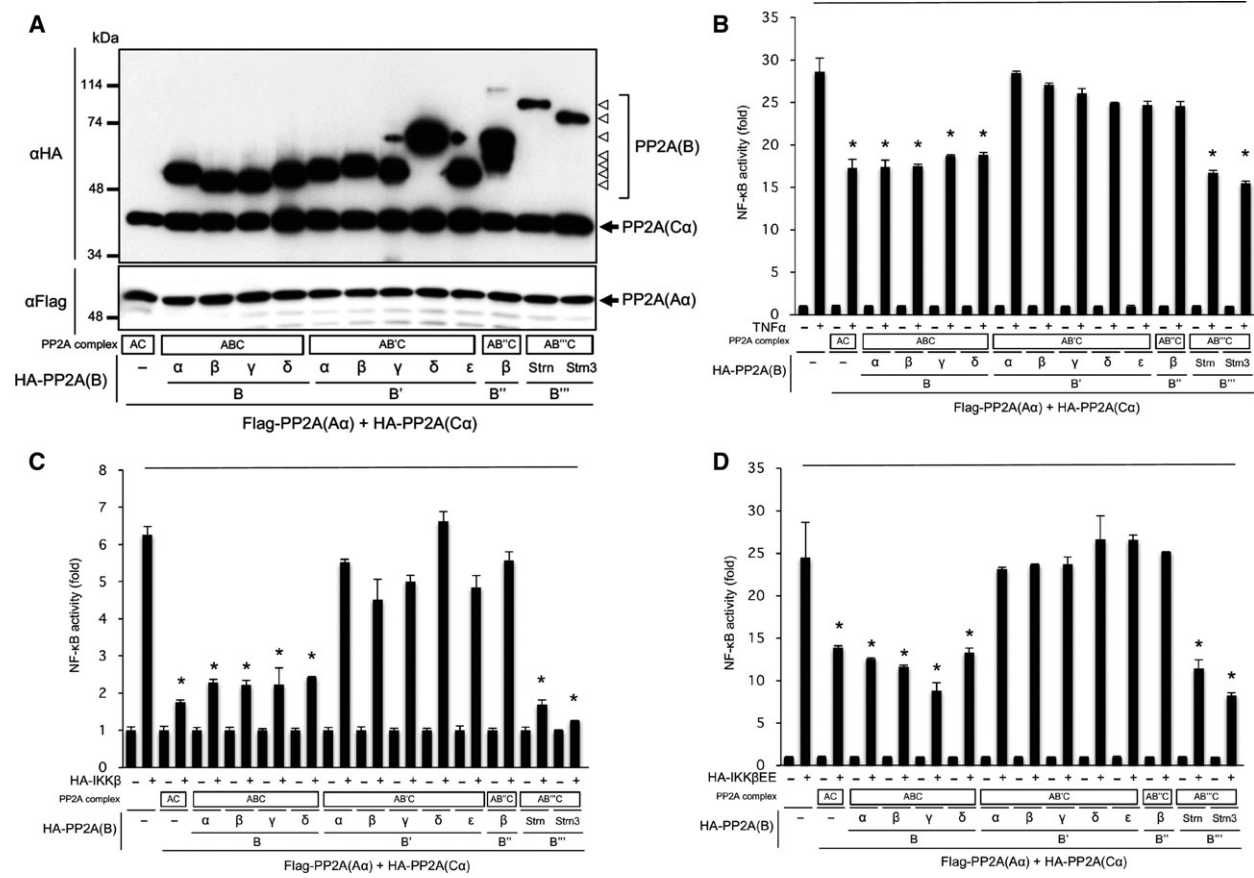


Fig. 2. PP2A core enzyme AC and holoenzymes ABC and AB'''C suppress TNF- α - and IKK β -induced NF- κ B activity. (A) GP2-293 cells were transfected with plasmids encoding PP2A core enzyme subunits A α and C α together with subunits of B, B', B'' and B'''/Strn families. Following incubation for 24 h, cells were lysed and expression of these subunits was analysed by immunoblotting. (B) Cells were transfected with NF- κ B reporter plasmids and plasmids encoding PP2A subunits, and then stimulated with TNF- α for 18 h. NF- κ B activity was estimated by luciferase assay. (C, D) Cells were transfected with NF- κ B reporter plasmids and plasmids encoding PP2A subunits together with plasmids encoding IKK β (D) and IKK β EE (E). Cells were incubated for 18 h, and NF- κ B activity was estimated by luciferase assay. *Statistically significance at $P < 0.01$.

subunit, and phosphorylation of the activation loop was investigated by immunoblotting. Expression of the AC core enzyme markedly suppressed IKK β phosphorylation in a phosphatase activity-dependent manner (Fig. 3A). The AC core enzyme and the AB'''C holoenzyme markedly suppressed IKK β phosphorylation, whereas three types of holoenzyme including

ABC, AB'C and AB''C had little effect (Fig. 3B). These lines of evidence indicate that AC and AB'''C attenuate TNF- α -induced NF- κ B activity, at least in part, through dephosphorylation of IKK β .

To reveal the effects of each B subunit on the NF- κ B/I κ B α complex, we transfected cells with expression plasmids encoding I κ B α RRR, RelA and IKK β EE,

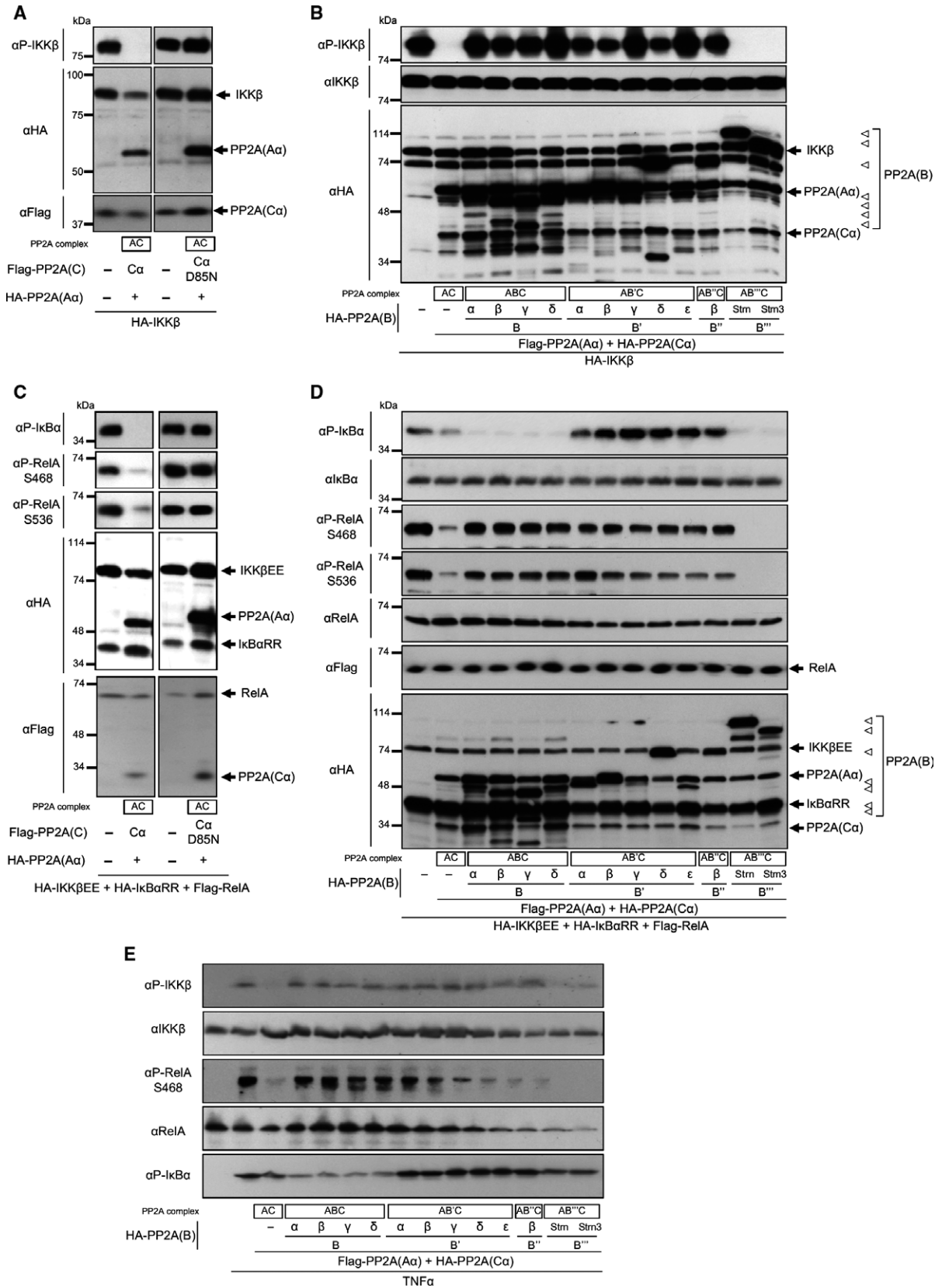


Fig. 3. PP2A enzyme AC and AB'''C suppress IKK β and RelA phosphorylation, and AC, ABC and AB'''C suppress I κ B α phosphorylation *in vivo*. (A) GP2-293 cells were transfected with plasmids encoding IKK β and PP2A plasmids encoding A α and C α or phosphatase-inactive C α D85N. (B) Cells were transfected with plasmids encoding IKK β and PP2A plasmids encoding A α , C α and each subunit of B, B', B'' and B'''/Strn families. (C) Cells were transfected with plasmids encoding IKK β EE, I κ B α RR, RelA and PP2A plasmids encoding A α and C α or C α D85N. (D) Cells were transfected with plasmids encoding IKK β EE, I κ B α RR, RelA and PP2A plasmids encoding A α , C α , and each subunit of B, B', B'' and B'''/Strn families. Following incubation for 24 h, cells were lysed, and phosphorylation of proteins was estimated by immunoblotting. (E) GP2-293 cells were transfected with expression plasmids of PP2A, and incubated for 24 h. Cells were stimulated with TNF- α for 5 min, and phosphorylation of proteins was analysed by immunoblotting.

together with plasmids encoding A α , C α and each B subunit. Then, the effects of PP2A on IKK β EE-mediated phosphorylation of I κ B α RR and RelA were investigated by immunoblotting. Expression of the AC core enzyme suppressed phosphorylation of I κ B α RR and RelA in a phosphatase activity-dependent manner (Fig. 3C). AC, ABC and AB'''C reduced I κ B α phosphorylation efficiently, whereas AB'C and AB''C had little effect on dephosphorylation of I κ B α RR (Fig. 3D). In turn, AC and AB'''C dephosphorylated RelA efficiently, whereas ABC, AB'C and AB''C had little effect on dephosphorylation of RelA. These results suggest that AC, ABC and AB'''C attenuate NF- κ B activity, at least in part, by dephosphorylation of I κ B α .

Then, we investigated the effects of distinct B subunits on phosphorylation of endogenous NF- κ B signalling proteins in TNF- α -stimulated cells. Cells were transfected with expression plasmids of PP2A, and then stimulated with TNF- α . AC and AB'''C suppressed TNF- α -induced phosphorylation of IKK β and RelA, whereas ABC, AB'C and AB''C had little effect (Fig. 3E). AC, ABC and AB'''C reduced TNF- α -induced I κ B α phosphorylation, whereas AB'C and AB''C had little effect. These results suggest that functional features of B subunits have physiological significance in NF- κ B signalling.

PP2A core enzyme and holoenzyme effectively dephosphorylate IKK β , I κ B α and RelA *in vitro*

Dephosphorylation of IKK β , I κ B α RR and RelA was analysed by using purified PP2A enzyme *in vitro*. Expression plasmids encoding Flag-tagged A α , HA-tagged C α and HA-tagged B subunits were transfected in cells, and PP2A complexes of various isoforms of B subunit family were purified from cells by using Flag-agarose beads (Fig. 4A). Phosphorylated IKK β was purified from cells transfected with Flag-IKK β expression plasmid (Fig. 4B). Following incubation of phosphorylated IKK β with various purified PP2A core enzyme and holoenzyme, dephosphorylation of IKK β was analysed by immunoblotting. This assay revealed that purified PP2A

complexes of different B subunits effectively dephosphorylate IKK β , and these complexes showed similar levels of activity (Fig. 4C). Then, we purified phosphorylated complexes of I κ B α RR and RelA from cells transfected with plasmids of Flag-RelA, HA-I κ B α RR and IKK β EE by using Flag-agarose beads (Fig. 4D). Following incubation of phosphorylated I κ B α RR and RelA protein complexes with various purified PP2A core enzyme and holoenzyme, dephosphorylation of I κ B α RR and RelA was analysed by immunoblotting. Purified PP2A complexes of different B subunits effectively dephosphorylate I κ B α RR and RelA (Fig. 4E). These results indicated that, although all PP2A complexes have an ability to dephosphorylate IKK β , I κ B α and RelA *in vitro*, AC and AB'''C preferentially dephosphorylate IKK β and RelA, and AC, ABC and AB'''C preferentially dephosphorylate I κ B α *in vivo*, suggesting that intracellular mechanisms may relate to the preference of AC, ABC and AB'''C to dephosphorylate IKK β , I κ B α and RelA.

Distinct B subunits regulate the binding of PP2A to the substrate proteins *in vivo*

To resolve the discrepancies in the dephosphorylation of IKK β and RelA between *in vitro* and *in vivo*, we investigated intracellular mechanisms of the preference of AC, ABC, AB''C and AB'''C to dephosphorylate NF- κ B signalling proteins. The effects of B subunits on the interaction between PP2A and IKK β were analysed by immunoprecipitation assay. Cells were transfected with plasmids encoding Flag-tagged C α D85N, HA-tagged A α , HA-tagged B subunits and HA-tagged IKK β . PP2A complexes were immunoprecipitated by using Flag-agarose beads from cells, and the association of IKK β proteins to the PP2A complexes was analysed by immunoblotting (Fig. 5). This assay revealed that IKK β associates to PP2A, and B, B' and B'' subunits block the association. These results suggest that the preference of AC, ABC, AB''C and AB'''C to dephosphorylate NF- κ B signalling proteins is regulated by the binding features of B subunits to the substrate proteins in cells.

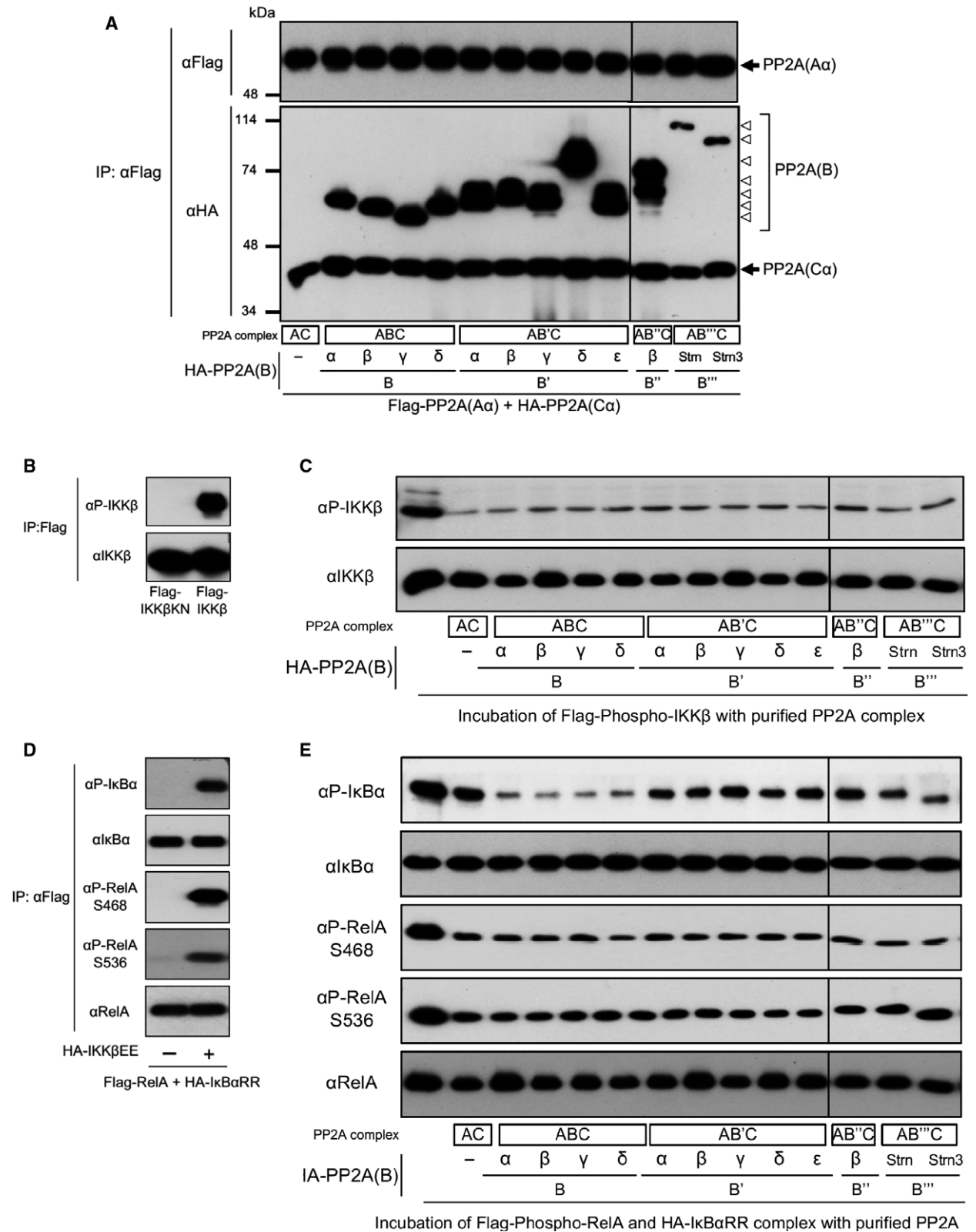


Fig. 4. Dephosphorylation of IKK β , I κ B α and RelA by PP2A *in vitro*. (A) Cells were transfected with plasmids encoding PP2A core enzyme subunits A α and C α together with subunits of B, B', B'' and B'''/Strn families. Following incubation for 24 h after transfection, PP2A enzymes were purified using anti-Flag (M2) agarose beads. Purified proteins (0.01 μ g) were subjected to SDS/PAGE and analysed by immunoblotting. (B) Cells were transfected with plasmids of Flag-IKK β and kinase-inactive Flag-IKK β KN. Phosphorylated IKK β proteins were purified by using anti-Flag (M2) agarose beads. (C) Phosphorylated IKK β was incubated with purified PP2A, and dephosphorylation levels of IKK β were analysed by immunoblotting. (D) Cells were transfected with plasmids encoding RelA, I κ B α RR and IKK β EE. Phosphorylated RelA and I κ B α complexes were purified using anti-Flag (M2) agarose beads. (E) Phosphorylated RelA and I κ B α proteins were incubated with purified PP2A, and dephosphorylation levels of each protein were analysed by immunoblotting.

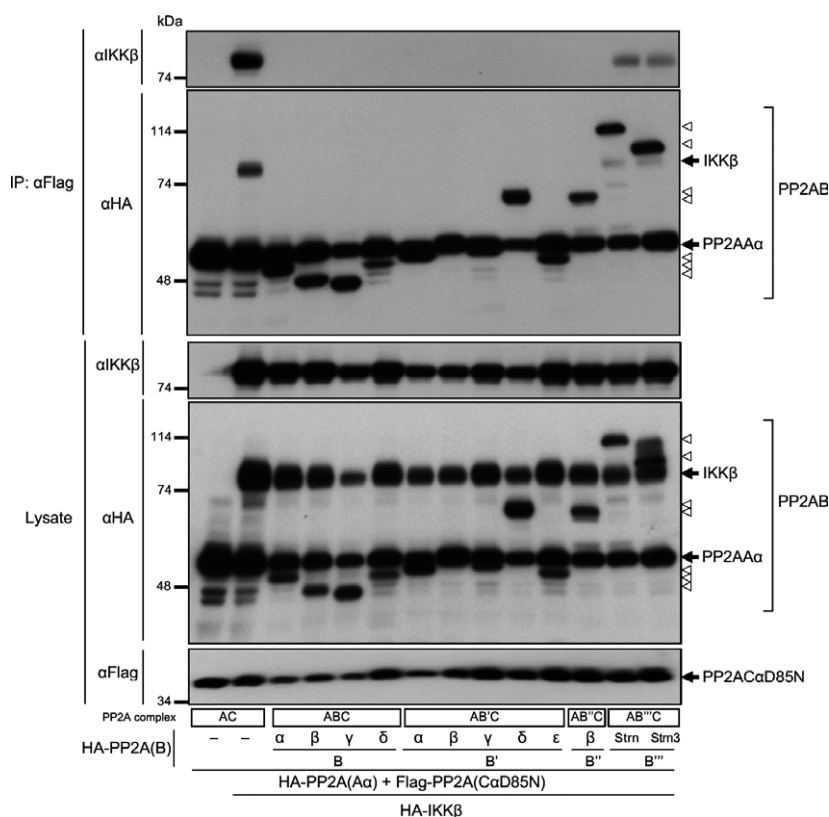


Fig. 5. PP2A B, B' and B'' subunits suppress the association of PP2A AC to IKK β *in vivo*. Cells were transfected with plasmids encoding A α , C α D85N mutant and subunits of B families together with IKK β plasmids. Following incubation for 24 h after transfection, PP2A complexes were immunoprecipitated and the association of IKK β proteins to the PP2A complexes was analysed by immunoblotting.

Discussion

Many studies have revealed important and complicated roles of PP2A in IKK β regulation. For example, it has been reported that PP2A suppresses IKK β activity *in vitro* and *in vivo* [7,15,16]. However, another contradictory study shows that PP2A forms a stable complex with IKK β and promotes its kinase activity *in vivo* [28]. We found here that AC and AB'''C dephosphorylate IKK β in cells, whereas ABC, AB'C and AB''C do not. These results suggest the possibility that ABC, AB'C and AB''C complexes positively regulate IKK β by competing against AC and AB'''C complexes. The data in this study may help to reconcile the seemingly contradictory observations that PP2A both downregulates and promotes IKK β activity. To

confirm the specific regulation by distinct PP2A complexes, we tried to knockdown of multiple isoforms of each B family protein by RNAi. However, RNAi screen in GP2-293 cells did not clarify distinct functions of each isoform of B family protein, because expression levels of B and B''' family proteins are very low in GP2-293 cells, and these cells express multiple isoforms of B' and B'' family proteins.

NF- κ B signalling is not uniformly regulated by PP2A but rather is subjected to specific regulation by distinct PP2A complexes at multiple steps. In contrast to AC and AB'''C, which dephosphorylate IKK β , I κ B α and RelA, ABC dephosphorylates I κ B α but not IKK β and RelA in cells. These results indicate that ABC suppresses NF- κ B activity without inhibition of IKK β . A recent study revealed that IKK β not only

activates NF- κ B through the phosphorylation of I κ B α but also regulates many cellular functions by phosphorylating various proteins in an NF- κ B-independent manner [29]. For example, IKK β regulates autophagy [30], mRNA stability [31], apoptosis [32], angiogenesis [33] and cellular vesicle trafficking [34] by phosphorylating various proteins that are unrelated to I κ B α . Thus, ABC potentially suppresses NF- κ B without inhibiting these NF- κ B-independent functions of IKK β , thereby ensuring that PP2A regulates NF- κ B activity and IKK β -mediated cellular responses independently.

Among the four classes of B subunit families, the B^{'''}/Strn family proteins only facilitate dephosphorylation of three proteins including IKK β , I κ B α and RelA. Members of the B^{'''}/Strn family are evolutionarily conserved and have critical roles in biological processes such as development and cell growth [35,36]. B^{'''}/Strn family proteins form a large complex termed Striatin-interacting phosphatase and kinase (STRIPAK) along with the germinal centre kinase family and many adaptor proteins, and recruit C subunits *via* A subunits of PP2A in the complexes. STRIPAK complexes have a critical role in protein dephosphorylation and act as important regulators of multiple vital signalling pathways, including the Hippo pathway, mitogen-activated protein kinases and cytoskeleton remodelling. Recent studies suggest that the dysregulation of STRIPAK complexes correlates with human diseases including cancer [35–37]. Regulation of NF- κ B signalling pathway by AB^{'''}C may, therefore, be involved in STRIPAK complex-related biological processes and cancer.

PP2A is a confirmed tumour suppressor protein that is genetically altered or functionally inactivated in many cancers [22–24]. Its A and C subunits are reportedly inactivated through several mechanisms including somatic mutation, phosphorylation, methylation, and/or increased expression of PP2A inhibitors, and inactivation of PP2A is linked to cancer progression. Expression of B α , B β and B γ is also decreased in many cancers owing to deletion, point mutation and DNA hypermethylation [38–44]. Furthermore, it has been revealed that IKK β and NF- κ B signalling pathways are linked to inflammation and cancer [45]. Therefore, the regulation of IKK β and NF- κ B signalling by distinct PP2A complexes may be involved in multiple human diseases including cancer, and thus might serve as specific and novel targets for disease therapy.

Acknowledgements

This work was supported, in whole or in part, by Grants-in-Aid from the Japan Society for the Promotion of

Science (JSPS) grant numbers #15H01406, #15H01382, and #23370062.

Author contributions

HK and YT designed and performed the experiments, interpreted the study, and wrote the paper. KO, MK, YN, ET and TH performed the experiments.

References

- Hayden MS and Ghosh S (2008) Shared principles in NF-kappaB signaling. *Cell* **132**, 344–362.
- Vallabhapurapu S and Karin M (2009) Regulation and function of NF-kappaB transcription factors in the immune system. *Annu Rev Immunol* **27**, 693–733.
- Hinz M and Scheidereit C (2014) The IkappaB kinase complex in NF-kappaB regulation and beyond. *EMBO Rep* **15**, 46–61.
- Rieser E, Cordier SM and Walczak H (2013) Linear ubiquitination: a newly discovered regulator of cell signalling. *Trends Biochem Sci* **38**, 94–102.
- Iwai K (2014) Diverse roles of the ubiquitin system in NF-kappaB activation. *Biochim Biophys Acta* **1843**, 129–136.
- Shimizu Y, Taraborrelli L and Walczak H (2015) Linear ubiquitination in immunity. *Immunol Rev* **266**, 190–207.
- DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E and Karin M (1997) A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. *Nature* **388**, 548–554.
- Delhase M, Hayakawa M, Chen Y and Karin M (1999) Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation. *Science* **284**, 309–313.
- Sakurai H, Chiba H, Miyoshi H, Sugita T and Toriumi W (1999) IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain. *J Biol Chem* **274**, 30353–30356.
- Schwabe RF and Sakurai H (2005) IKKbeta phosphorylates p65 at S468 in transactivation domain 2. *FASEB J* **19**, 1758–1760.
- Chen LF, Williams SA, Mu Y, Nakano H, Duerr JM, Buckbinder L and Greene WC (2005) NF-kappaB RelA phosphorylation regulates RelA acetylation. *Mol Cell Biol* **25**, 7966–7975.
- Brautigan DL (2013) Protein Ser/Thr phosphatases—the ugly ducklings of cell signalling. *FEBS J* **280**, 324–345.
- Shi Y (2009) Serine/threonine phosphatases: mechanism through structure. *Cell* **139**, 468–484.
- Li HY, Liu H, Wang CH, Zhang JY, Man JH, Gao YF, Zhang PJ, Li WH, Zhao J, Pan X *et al.* (2008) Deactivation of the kinase IKK by CUEDC2 through

- recruitment of the phosphatase PP1. *Nat Immunol* **9**, 533–541.
- 15 Fu DX, Kuo YL, Liu BY, Jeang KT and Giam CZ (2003) Human T-lymphotropic virus type I tax activates I- κ B kinase by inhibiting I- κ B kinase-associated serine/threonine protein phosphatase 2A. *J Biol Chem* **278**, 1487–1493.
 - 16 Li S, Wang L, Berman MA, Zhang Y and Dorf ME (2006) RNAi screen in mouse astrocytes identifies phosphatases that regulate NF- κ B signaling. *Mol Cell* **24**, 497–509.
 - 17 Prajapati S, Verma U, Yamamoto Y, Kwak YT and Gaynor RB (2004) Protein phosphatase 2C β association with the I κ B kinase complex is involved in regulating NF- κ B activity. *J Biol Chem* **279**, 1739–1746.
 - 18 Chiang CW, Liu WK, Chiang CW and Chou CK (2011) Phosphorylation-dependent association of the G4-1/G5PR regulatory subunit with IKK β negatively modulates NF- κ B activation through recruitment of protein phosphatase 5. *Biochem J* **433**, 187–196.
 - 19 Pons S and Torres-Aleman I (2000) Insulin-like growth factor-I stimulates dephosphorylation of I κ B through the serine phosphatase calcineurin (protein phosphatase 2B). *J Biol Chem* **275**, 38620–38625.
 - 20 Yang J, Fan GH, Wadzinski BE, Sakurai H and Richmond A (2001) Protein phosphatase 2A interacts with and directly dephosphorylates RelA. *J Biol Chem* **276**, 47828–47833.
 - 21 Chew J, Biswas S, Shreeram S, Humaidi M, Wong ET, Dhillion MK, Teo H, Hazra A, Fang CC, López-Collazo E *et al.* (2009) WIP1 phosphatase is a negative regulator of NF- κ B signalling. *Nat Cell Biol* **11**, 659–666.
 - 22 Sangodkar J, Farrington CC, McClinch K, Galsky MD, Kastrinsky DB and Narla G (2016) All roads lead to PP2A: exploiting the therapeutic potential of this phosphatase. *FEBS J* **283**, 1004–1024.
 - 23 Eichhorn PJ, Creghton MP and Bernards R (2009) Protein phosphatase 2A regulatory subunits and cancer. *Biochim Biophys Acta* **1795**, 1–15.
 - 24 Westermarck J and Hahn WC (2008) Multiple pathways regulated by the tumor suppressor PP2A in transformation. *Trends Mol Med* **14**, 152–160.
 - 25 Xing Y, Xu Y, Chen Y, Jeffrey PD, Chao Y, Lin Z, Li Z, Strack S, Stock JB and Shi Y (2006) Structure of protein phosphatase 2A core enzyme bound to tumor-inducing toxins. *Cell* **127**, 341–353.
 - 26 Cho US and Xu W (2007) Crystal structure of a protein phosphatase 2A heterotrimeric holoenzyme. *Nature* **445**, 53–57.
 - 27 Tsuchiya Y, Asano T, Nakayama K, Kato T Jr, Karin M and Kamata H (2010) Nuclear IKK β is an adaptor protein for I κ B α ubiquitination and degradation in UV-induced NF- κ B activation. *Mol Cell* **39**, 570–582.
 - 28 Kray AE, Carter RS, Pennington KN, Gomez RJ, Sanders LE, Llanes JM, Khan WN, Ballard DW and Wadzinski BE (2005) Positive regulation of I κ B kinase signaling by protein serine/threonine phosphatase 2A. *J Biol Chem* **280**, 35974–35982.
 - 29 Chariot A (2009) The NF- κ B-independent functions of IKK subunits in immunity and cancer. *Trends Cell Biol* **19**, 404–413.
 - 30 Criollo A, Senovilla L, Authier H, Maiuri MC, Morselli E, Vitale I, Kepp O, Tasdemir E, Galluzzi L, Shen S *et al.* (2010) The IKK complex contributes to the induction of autophagy. *EMBO J* **29**, 619–631.
 - 31 Iwasaki H, Takeuchi O, Teraguchi S, Matsushita K, Uehata T, Kuniyoshi K, Satoh T, Saitoh T, Matsushita M, Standley DM *et al.* (2011) The I κ B kinase complex regulates the stability of cytokine-encoding mRNA induced by TLR-IL-1R by controlling degradation of regnase-1. *Nat Immunol* **12**, 1167–1175.
 - 32 Yan J, Xiang J, Lin Y, Ma J, Zhang J, Zhang H, Sun J, Danial NN, Liu J and Lin A (2013) Inactivation of BAD by IKK inhibits TNF α -induced apoptosis independently of NF- κ B activation. *Cell* **152**, 304–315.
 - 33 Lee DF, Kuo HP, Chen CT, Hsu JM, Chou CK, Wei Y, Sun HL, Li LY, Ping B, Huang WC *et al.* (2007) IKK β suppression of TSC1 links inflammation and tumor angiogenesis via the mTOR pathway. *Cell* **130**, 440–455.
 - 34 Nair-Gupta P, Baccarini A, Tung N, Seyffer F, Florey O, Huang Y, Banerjee M, Overholtzer M, Roche PA, Tampé R *et al.* (2014) TLR signals induce phagosomal MHC-I delivery from the endosomal recycling compartment to allow cross-presentation. *Cell* **158**, 506–521.
 - 35 Shi Z, Jiao S and Zhou Z (2016) STRIPAK complexes in cell signaling and cancer. *Oncogene* **35**, 4549–4557.
 - 36 Hwang J and Pallas DC (2014) STRIPAK complexes: structure, biological function, and involvement in human diseases. *Int J Biochem Cell Biol* **47**, 118–148.
 - 37 Madsen CD, Hooper S, Tozluoglu M, Bruckbauer A, Fletcher G, Eler JT, Bates PA, Thompson B and Sahai E (2015) STRIPAK components determine mode of cancer cell migration and metastasis. *Nat Cell Biol* **17**, 68–80.
 - 38 Bluemn EG, Spencer ES, Mecham B, Gordon RR, Coleman I, Lewinshtein D, Mostaghel E, Zhang X, Annis J, Grandori C *et al.* (2013) PPP2R2C loss promotes castration-resistance and is associated with increased prostate cancer-specific mortality. *Mol Cancer Res* **11**, 568–578.
 - 39 Cheng Y, Liu W, Kim ST, Sun J, Lu L, Sun J, Zheng SL, Isaacs WB and Xu J (2011) Evaluation of

- PPP2R2A as a prostate cancer susceptibility gene: a comprehensive germline and somatic study. *Cancer Genet* **204**, 375–381.
- 40 Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, Speed D, Lynch AG, Samarajiwa S, Yuan Y *et al.*; METABRIC Group (2012) The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* **486**, 346–352.
- 41 Mosca L, Musto P, Todoerti K, Barbieri M, Agnelli L, Fabris S, Tuana G, Lionetti M, Bonaparte E, Sirchia SM *et al.* (2013) Genome-wide analysis of primary plasma cell leukemia identifies recurrent imbalances associated with changes in transcriptional profiles. *Am J Hematol* **88**, 16–23.
- 42 Muggerud AA, Musto P, Todoerti K, Barbieri M, Agnelli L, Fabris S, Tuana G, Lionetti M, Bonaparte E, Sirchia SM *et al.* (2010) Frequent aberrant DNA methylation of ABCB1, FOXC1, PPP2R2B and PTEN in ductal carcinoma *in situ* and early invasive breast cancer. *Breast Cancer Res* **12**, R3.
- 43 Ruvolo PP, Qui YH, Coombes KR, Zhang N, Ruvolo VR, Borthakur G, Konopleva M, Andreeff M and Kornblau SM (2011) Low expression of PP2A regulatory subunit B55alpha is associated with T308 phosphorylation of AKT and shorter complete remission duration in acute myeloid leukemia patients. *Leukemia* **25**, 1711–1717.
- 44 Tan J, Lee PL, Li Z, Jiang X, Lim YC, Hooi SC and Yu Q (2010) B55beta-associated PP2A complex controls PDK1-directed myc signaling and modulates rapamycin sensitivity in colorectal cancer. *Cancer Cell* **18**, 459–471.
- 45 DiDonato JA, Mercurio F and Karin M (2012) NF-kappaB and the link between inflammation and cancer. *Immunol Rev* **246**, 379–400.