



HOKKAIDO UNIVERSITY

Title	Negative regulation of protein phosphatase 2C β by ISG15 conjugation
Author(s)	Takeuchi, Tomoharu; Kobayashi, Takayasu; Tamura, Shinri et al.
Description	http://www.elsevier.com/wps/find/journaldescription.cws_home/506085/description
Citation	FEBS Letters, 580(18), 4521-4526 https://doi.org/10.1016/j.febslet.2006.07.032
Issue Date	2006-08-07
Doc URL	https://hdl.handle.net/2115/14658
Type	journal article
File Information	FL2006-580-18.pdf



Negative regulation of protein phosphatase 2C β by ISG15 conjugation

Tomoharu Takeuchi^a, Takayasu Kobayashi^b, Shinri Tamura^b, Hideyoshi Yokosawa^{a,*}

^a*Department of Biochemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan*

^b*Department of Biochemistry, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Japan*

*Corresponding author. Fax: +81-11-706-4900.

E-mail address: yoko@pharm.hokudai.ac.jp (H. Yokosawa).

Send correspondence to:

Hideyoshi Yokosawa, Ph.D.,

Department of Biochemistry, Graduate School of Pharmaceutical Sciences,

Hokkaido University, Sapporo 060-0812, Japan.

Tel.: +81-11-706-3754; Fax: +81-11-706-4900.

E-mail: yoko@pharm.hokudai.ac.jp

Abstract

ISG15, an interferon-upregulated ubiquitin-like protein, is covalently conjugated to various cellular proteins (ISGylation). In this study, we found that protein phosphatase 2C β (PP2C β), which functions in the NF- κ B pathway via dephosphorylation of TAK1, was ISGylated, and analysis by NF- κ B luciferase reporter assay revealed that PP2C β activity was suppressed by co-expression of ISG15, UBE1L, and UbcH8. We determined the ISGylation sites of PP2C β and constructed its ISGylation-resistant mutant. In contrast to the wild type, this mutant suppressed the NF- κ B pathway even in the presence of ISG15, UBE1L, and UbcH8. Thus, we propose that ISGylation negatively regulates PP2C β activity.

Keywords: ISG15; Interferon; Ubiquitin; Protein phosphatase; NF- κ B

Abbreviations: ISG15, interferon-stimulated gene 15 kDa; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; PP2C β , protein phosphatase 2C β ; TAK1, TGF- β -activated kinase; TAB1, TAK1-binding protein; NF- κ B, nuclear factor κ B; I κ B, inhibitor of NF- κ B; IKK, I κ B kinase; IFN, interferon

1. Introduction

Interferon (IFN) is a pleiotropic cytokine that has an essential role in cellular antiviral response through inducing interferon-stimulated genes (ISGs) [1]. ISG15, one of the ISGs, is a ubiquitin-like protein that is covalently conjugated to various cellular proteins (ISGylation) upon interferon stimuli [2]. This protein functions as an antiviral protein against Sindbis virus and HIV-1 [3,4] and as a suppressor of the ubiquitin-proteasome system [5]. Although various target proteins for ISGylation have been identified by a proteomic approach [6-8] and a cascade of the protein ISGylation system has become clear [9-14], the biological consequences of ISGylation of target proteins have been studied in only a few cases [15-17].

Protein phosphatase 2C β (PP2C β) is an enzyme that belongs to the PP2C type protein phosphatase family and functions as a monomer [18]. PP2C β dephosphorylates and suppresses TAK1 and IKK, both of which have essential roles in the NF- κ B pathway, an important pathway functioning in innate immunity, adaptive immunity, and cancer [19-22].

We previously reported that ISGylation of UbcH6 and Ubc13 suppresses their ubiquitin E2 enzyme activities [16,17]. In a search for ISGylated proteins, we identified PP2C β as a target protein for ISGylation. In addition, we found that ISGylation of PP2C β suppresses the activity of PP2C β against TAK1-induced NF- κ B activation.

2. Materials and methods

2-1. Cell culture and transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% heat-inactivated calf serum (Hyclone). Transfection was performed according to the standard calcium precipitation protocol.

2-2. Plasmid construction

The mammalian expression plasmids of ISG15, UBE1L, and UbcH8 were generated as described previously [17]. The open-reading frames of human PP2C β , TAK1, and TAB1 were amplified by PCR. Deletion and point mutants of PP2C β were generated by PCR. All constructs were verified by DNA sequencing. To generate mammalian expression plasmids of C-terminally and N-terminally Flag-tagged PP2C β , the PCR fragment was subcloned into pCI-neo-C3Flag and pCI-neo-3Flag vectors, respectively, which had been generated by inserting three repeats of Flag tag sequence into the pCI-neo mammalian expression vector (Promega). To generate the mammalian expression plasmids of TAK1 and TAB1, the PCR fragments were subcloned into the pCI-neo-2S and pCI-neo-5HA vectors that had been generated by inserting oligonucleotides encoding two repeats of S peptide sequence and five repeats of HA tag sequence, respectively, into the pCI-neo mammalian expression vector.

2-3. Immunoprecipitation and Western blotting

HeLa cells that had been transiently transfected with indicated plasmids and cultured for 24-30 h were lysed with RIPA buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride and 5 mM N-ethylmaleimide, and the supernatant of the cell lysate was subjected to immunoprecipitation and then to Western blotting with various antibodies as described previously [16,17] except for anti-PP2C β [19] and anti-I κ B α (BD Biosciences) antibodies. The effect of IFN treatment was analyzed as described previously [17] except for HeLa cells and lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 5 mM N-ethylmaleimide.

2-4. Reporter gene assay

HeLa cells were transfected with the pNF- κ B-Luc reporter plasmid (BD Biosciences) and indicated plasmids, together with pRL-TK (Promega) for normalizing transfection efficiency. Twenty-four h after transfection, cells were lysed and luciferase activity was measured by using a Dual-Luciferase Reporter Assay System (Promega) and an AB-2000 luminescencer-PSN (Atto, Tokyo, Japan). The same experiments were repeated three times.

3. Results and discussion

3-1. PP2C β is modified with ISG15

To identify proteins modified with ISG15, we carried out a proteomic analysis of ISGylated proteins. We expressed Flag-tagged ISG15, S-tagged UBE1L (E1), and S-tagged UbcH8 (E2) in HeLa cells, and ISGylated proteins were isolated by immunoprecipitation with anti-Flag antibody and subjected to peptide mass fingerprinting. We identified several novel ISGylated proteins (data not shown; data to be presented elsewhere). Among them, we focused on PP2C β because PP2C β dephosphorylates and suppresses TAK1 and IKK, kinases both functioning in the NF- κ B pathway [19,20].

To confirm ISGylation of PP2C β , we expressed Flag-tagged PP2C β together with T7-tagged ISG15, S-tagged UBE1L and S-tagged UbcH8 in HeLa cells, and the extract of transfected cells was subjected to immunoprecipitation with anti-Flag tag antibody and then to Western blotting with anti-T7 tag and anti-Flag tag antibodies (Fig. 1). Two bands (the molecular masses of 88 kDa and 110 kDa) with slower mobilities than that of intact PP2C β (67 kDa) were detected by anti-Flag tag antibody in UBE1L- and UbcH8-dependent manners and these bands were also detected by anti-T7 tag antibody: The above difference in molecular masses between modified and intact ones is reasonably thought to be due to ISGylation. These results strongly suggest that PP2C β is covalently modified with ISG15, the conjugation being mediated by UBE1L and UbcH8. It should be noted that ISGylation of

various proteins was detected by over-expression of UBE1L in the presence of ISG15 and was further enhanced by co-expression of UBE1L and UbcH8 (Fig. 1, left panel): These results are consistent with the previous report [11].

Next, to confirm that PP2C β is ISGylated in response to IFN signal, HeLa cells that had expressed Flag-tagged PP2C β were treated with IFN β . The cell extract was subjected to immunoprecipitation with anti-Flag tag antibody and then to Western blotting with anti-Flag tag and anti-ISG15 antibodies (Fig. 2A). In another experiment (Fig. 2B), the extract of HeLa cells that had been treated with IFN β was subjected to immunoprecipitation with anti-PP2C β antibody and then to Western blotting with anti-PP2C β and anti-ISG15 antibodies. In both experiments, two bands due to ISGylated PP2C β were detected only in the case of IFN treatment, indicating that PP2C β is ISGylated in response to IFN signal under physiological conditions. ISGylation of various proteins was also detected only in the case of IFN treatment (Fig. 2A and B, right panels). It should be noted that the molecular mass (67 kDa) of exogenously expressed Flag-tagged PP2C β isoform 1, the longest isoform of human PP2C β [23] and the isoform used in this study, is different from that (42 kDa) of endogenous PP2C β isoform 2, the most abundant isoform in HeLa cells [24].

3-2. PP2C β activity is suppressed by addition of a protein ISGylation system

It has been reported that PP2C β dephosphorylates and suppresses TAK1 [19], which

functions in the NF- κ B pathway together with TAB1 [25]. We therefore carried out an experiment to determine whether a protein ISGylation system modulates PP2C β activity against TAK1/TAB1-induced NF- κ B activation. HeLa cells were co-transfected with plasmids of HA-tagged TAK1 and TAB1, Flag-tagged PP2C β , and ISGylation system (T7-tagged ISG15, S-tagged UBE1L, and S-tagged UbcH8), together with NF- κ B luciferase reporter plasmid, and luciferase activity was measured (Fig. 3). PP2C β suppressed TAK1/TAB1-induced NF- κ B activation in the absence of the ISGylation system but not in the presence of the ISGylation system. It should be noted that TAB1 alone has little effect [25]. These results suggest that ISGylation of PP2C β suppresses the activity of PP2C β against TAK1/TAB1-induced NF- κ B activation.

3-3. PP2C β is modified with ISG15 at least through Lys12 and Lys142

To confirm the above assumption, it is necessary to construct an ISGylation-resistant mutant of PP2C β . Since PP2C β is expected to be ISGylated through at least two Lys residues (see Fig. 1), we first constructed various PP2C β deletion mutants and expressed them together with the above ISGylation system in HeLa cells. The extract of transfected cells was subjected to immunoprecipitation with anti-Flag tag antibody and then to Western blotting with anti-Flag tag and anti-T7 tag antibodies (Fig. 4). PP2C β Δ 296-479 was modified with ISG15 to an extent similar to that in the case of the wild type, while PP2C β Δ 1-295 was not

modified with ISG15 (Fig. 4A) and PP2C β Δ 1-92 and Δ 93-193 seemed to be less modified (Fig. 4B). We hypothesized that one of the Lys residues modified with ISG15 is located in the sequence 1-92, while another is in 93-193. We next constructed various PP2C β mutants in which the Lys residues had been changed to Arg, measured their levels of ISGylation, and found that PP2C β K12R was less modified with ISG15 than was the wild type (data not shown). We finally constructed various PP2C β KR mutants in which the two Lys residues had been changed to Arg and analyzed their levels of ISGylation (Fig. 5). PP2C β K12,142R was barely modified with ISG15 in either the case of N-terminally (Fig. 5A) or C-terminally Flag-tagged PP2C β (Fig. 5B). Thus, we concluded that PP2C β is modified with ISG15 at least through Lys12 and Lys142.

3-4. PP2C β activity is suppressed by ISG15 conjugation

Using the PP2C β K12,142R mutant as an ISGylation-resistant mutant, we performed an NF- κ B luciferase reporter assay as described above (see Fig. 3). As shown in Fig. 6A, in the absence of the ISGylation system, the wild type (Wt) and the K12,142R (KR) mutant suppressed TAK1/TAB1-induced NF- κ B activation, while in the presence of the ISGylation system, the wild type had little suppressive effect as shown above (see Fig. 3), but the K12,142R mutant had a suppressive effect. It should be noted that the ISGylation system alone has little effect (data not shown). These results suggest that ISGylation of PP2C β

suppresses its activity.

Since degradation of I κ B α by the ubiquitin-proteasome system is essential for activation of NF- κ B [26-28], we next carried out an experiment to clarify the effect of ISGylation of PP2C β on I κ B α degradation. We expressed Flag-tagged wild-type PP2C β or its K12,142R mutant, S-tagged TAK1 and TAB1, and the ISGylation system in HeLa cells, and the cells were then treated with cycloheximide (CHX), a protein synthesis inhibitor, for 30 min. The cell extract was subjected to Western blotting with anti-I κ B α , anti-Flag tag, anti-S peptide, and anti-T7 tag antibodies (Fig. 6B). In our assay conditions, in the absence of cycloheximide, the amount of I κ B α protein remained unchanged in all combinations of expression plasmids. However, when the cells had been treated with cycloheximide, the level of I κ B α in the case of wild-type PP2C β was less than that in the case of the K12,142R mutant in the presence of the ISGylation system. It should be noted that the ISGylation system alone has little effect (data not shown). These results imply that ISGylated PP2C β has a less suppressive effect on TAK1/TAB1-induced degradation of I κ B α than the ISGylation-resistant mutant does, supporting the above assumption that ISGylation of PP2C β suppresses its activity.

In conclusion, we found that PP2C β is covalently modified with ISG15 in an IFN-dependent manner and that ISGylation of PP2C β suppresses the activity of PP2C β against TAK1/TAB1-induced I κ B α degradation and NF- κ B activation. It can be inferred that the activity of ISGylated PP2C β is less than that of the original one. The

construction of an *in vitro* assay system will clarify this point and is our future project. Considering that TAK1 is indispensable for cellular responses to various stimuli, including interleukin-1, tumor necrosis factor- α , and a toll-like receptor ligand [25,29], it can be inferred that ISGylation of PP2C β has an effect on various cellular responses.

Acknowledgement: This study was supported in part by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

- [1] Kalvakolanu, D.V. (2003) Alternate interferon signaling pathways. *Pharmacol. Ther.* 100, 1-29.
- [2] Dao, C.T. and Zhang, D.E. (2005) ISG15: a ubiquitin-like enigma. *Front. Biosci.* 10, 2701-2722.
- [3] Lenschow, D.J., Giannakopoulos, N.V., Gunn, L.J., Johnston, C., O'Guin, A.K., Schmidt, R.E., Levine, B. and Virgin IV, H.W. (2005) Identification of interferon-stimulated gene 15 as an antiviral molecule during Sindbis virus infection in vivo. *J. Virol.* 79, 13974-13983.
- [4] Okumura, A., Lu, G., Pitha-Rowe, I. and Pitha, P.M. (2006) Innate antiviral response targets HIV-1 release by the induction of ubiquitin-like protein ISG15. *Proc. Natl.*

- Acad. Sci. USA 103, 1440-1445.
- [5] Desai, S.D., Haas, A.L., Wood, L.M., Tsai, Y.C., Pestka, S., Rubin, E.H., Saleem, A., Nur-E-Kamal, A. and Liu, L.F. (2006) Elevated expression of ISG15 in tumor cells interferes with the ubiquitin/26S proteasome pathway. *Cancer Res.* 66, 921-928.
- [6] Malakhov, M.P., Kim, K.I., Malakhova, O.A., Jacobs, B.S., Borden, E.C. and Zhang, D.E. (2003) High-throughput immunoblotting: Ubiquitin-like protein ISG15 modifies key regulators of signal transduction. *J. Biol. Chem.* 278, 16608-16613.
- [7] Zhao, C., Denison, C., Huibregtse, J.M., Gygi, S. and Krug, R.M. (2005) Human ISG15 conjugation targets both IFN-induced and constitutively expressed proteins functioning in diverse cellular pathways. *Proc. Natl. Acad. Sci. USA* 102, 10200-10205.
- [8] Giannakopoulos, N.V., Luo, J.K., Papov, V., Zou, W., Lenschow, D.J., Jacobs, B.S., Borden, E.C., Li, J., Virgin, H.W. and Zhang, D.E. (2005) Proteomic identification of proteins conjugated to ISG15 in mouse and human cells. *Biochem. Biophys. Res. Commun.* 336, 496-506.
- [9] Yuan, W. and Krug, R.M. (2001) Influenza B virus NS1 protein inhibits conjugation of the interferon (IFN)-induced ubiquitin-like ISG15 protein. *EMBO J.* 20, 362-371.
- [10] Zhao, C., Beaudenon, S.L., Kelley, M.L., Waddell, M.B., Yuan, W., Schulman, B.A., Huibregtse, J.M. and Krug, R.M. (2004) The UbcH8 ubiquitin E2 enzyme is also the

- E2 enzyme for ISG15, an IFN- α/β -induced ubiquitin-like protein. *Proc. Natl. Acad. Sci. USA* 101, 7578-7582.
- [11] Kim, K.I., Giannakopoulos, N.V., Virgin, H.W. and Zhang, D.E. (2004) Interferon-inducible ubiquitin E2, Ubc8, is a conjugating enzyme for protein ISGylation. *Mol. Cell. Biol.* 24, 9592-9600.
- [12] Malakhov, M.P., Malakhova, O.A., Kim, K.I., Ritchie, K.J. and Zhang, D.E. (2002) UBP43 (USP18) specifically removes ISG15 from conjugated proteins. *J. Biol. Chem.* 277, 9976-9981.
- [13] Zou, W. and Zhang, D.E. (2006) The interferon-inducible ubiquitin-protein isopeptide ligase (E3) EFP also functions as an ISG15 E3 ligase. *J. Biol. Chem.* 281, 3989-3994.
- [14] Dastur, A., Beaudenon, S., Kelley, M., Krug, R.M. and Huibregtse, J.M. (2006) Herc5, an interferon-induced HECT E3 enzyme, is required for conjugation of ISG15 in human cells. *J. Biol. Chem.* 281, 4334-4338.
- [15] Zou, W., Papov, V., Malakhova, O., Kim, K.I., Dao, C., Li, J. and Zhang, D.E. (2005) ISG15 modification of ubiquitin E2 Ubc13 disrupts its ability to form thioester bond with ubiquitin. *Biochem. Biophys. Res. Commun.* 336, 61-68.
- [16] Takeuchi, T. and Yokosawa, H. (2005) ISG15 modification of Ubc13 suppresses its ubiquitin-conjugating activity. *Biochem. Biophys. Res. Commun.* 336, 9-13.
- [17] Takeuchi, T., Iwahara, S., Saeki, Y., Sasajima, H. and Yokosawa, H. (2005) Link

- between the ubiquitin conjugation system and the ISG15 conjugation system: ISG15 conjugation to the UbcH6 ubiquitin E2 enzyme. *J. Biochem. (Tokyo)* 138, 711-719.
- [18] Schweighofer, A., Hirt, H. and Meskiene, I. (2004) Plant PP2C phosphatases: emerging functions in stress signaling. *Trends Plant Sci.* 9, 236-243.
- [19] Hanada, M., Ninomiya-Tsuji, J., Komaki, K., Ohnishi, M., Katsura, K., Kanamaru, R., Matsumoto, K. and Tamura, S. (2001) Regulation of the TAK1 signaling pathway by protein phosphatase 2C. *J. Biol. Chem.* 276, 5753-5759.
- [20] Prajapati, S., Verma, U., Yamamoto, Y., Kwak, Y.T. and Gaynor, R.B. (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.
- [21] Hayden, M.S. and Ghosh, S. (2004) Signaling to NF- κ B. *Genes Dev.* 18, 2195-2224.
- [22] Karin, M. and Greten, F.R. (2005) NF- κ B: linking inflammation and immunity to cancer development and progression. *Nat. Rev. Immunol.* 5, 749-759.
- [23] Marley, A.E., Kline, A., Crabtree, G., Sullivan, J.E. and Beri, R.K. (1998) The cloning expression and tissue distribution of human PP2C β . *FEBS Lett.* 431, 121-124.
- [24] Cheng, A., Kaldis, P. and Solomon, M.J. (2000) Dephosphorylation of human cyclin-dependent kinases by protein phosphatase type 2C α and β 2 isoforms. *J. Biol. Chem.* 275, 34744-34749.
- [25] Sato, S., Sanjo, H., Takeda, K., Ninomiya-Tsuji, J., Yamamoto, M., Kawai, T.,

- Matsumoto, K., Takeuchi, O. and Akira, S. (2005) Essential function for the kinase TAK1 in innate and adaptive immune responses. *Nat. Immunol.* 6, 1087-1095.
- [26] Alkalay, I., Yaron, A., Hatzubai, A., Orian, A., Ciechanover, A. and Ben-Neriah, Y. (1995) Stimulation-dependent I κ B α phosphorylation marks the NF- κ B inhibitor for degradation via the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci. USA* 92, 10599-10603.
- [27] Spencer, E., Jiang, J. and Chen, Z.J. (1999) Signal-induced ubiquitination of I κ B α by the F-box protein Slimb/ β -TrCP. *Genes Dev.* 13, 284-294.
- [28] Hatakeyama, S., Kitagawa, M., Nakayama, K., Shirane, M., Matsumoto, M., Hattori, K., Higashi, H., Nakano, H., Okumura, K., Onoe, K., Good, R.A. and Nakayama, K. (1999) Ubiquitin-dependent degradation of I κ B α is mediated by a ubiquitin ligase Skp1/Cul1/F-box protein FWD1. *Proc. Natl. Acad. Sci. USA* 96, 3859-3863.
- [29] Shim, J.H., Xiao, C., Paschal, A.E., Bailey, S.T., Rao, P., Hayden, M.S., Lee, K.Y., Bussey, C., Steckel, M., Tanaka, N., Yamada, G., Akira, S., Matsumoto, K. and Ghosh, S. (2005) TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. *Genes Dev.* 19, 2668-2681.

Figure legends

Fig. 1. PP2C β is covalently modified with ISG15. The extracts of HeLa cells that had been transiently transfected with indicated plasmids were subjected to Western blotting with anti-T7 tag and anti-S peptide antibodies (left panel) or to immunoprecipitation with anti-Flag tag antibody and then to Western blotting with anti-Flag tag and anti-T7 tag antibodies (right panel). The ISGylated PP2C β is indicated by an open arrowhead. Nonspecific bands are indicated by asterisks.

Fig. 2. PP2C β is ISGylated in response to IFN signal. (A) The extract of HeLa cells that had been transiently transfected with Flag-tagged PP2C β expression plasmid, followed by 24-h treatment with IFN β (1,000 units/ml) (Pepro Tech), was subjected to immunoprecipitation with anti-Flag tag antibody and then to Western blotting with anti-Flag tag and anti-ISG15 antibodies. (B) The extract of HeLa cells pretreated with IFN β was subjected to immunoprecipitation with anti-PP2C β antibody and control immunoglobulin (Ig) and then to Western blotting with anti-PP2C β and anti-ISG15 antibodies.

Fig. 3. ISGylation system suppresses PP2C β activity. HeLa cells were transiently transfected with indicated plasmids together with pNF- κ B reporter plasmid and were then lysed, and

luciferase activity was measured. Results are expressed as fold induction in luciferase activity relative to that of control mocked cells that had been transfected with an empty vector. Triple experiments were carried out, and the error bars represent standard deviation. The ISGylation system contains T7-tagged ISG15, S-tagged UBE1L, and S-tagged UbcH8.

Fig. 4. ISGylation of PP2C β deletion mutants. The extracts of HeLa cells expressing the wild type PP2C β and its various deletion mutants, (A) Δ 1-295 and Δ 296-479 and (B) Δ 1-92, Δ 93-193, Δ 194-295, Δ 296-378 and Δ 379-479, in the presence and absence of the ISGylation system were subjected to immunoprecipitation with anti-Flag tag antibody and then to Western blotting with anti-Flag tag and anti-T7 tag antibodies.

Fig. 5. ISGylation of PP2C β KR mutants. The extracts of HeLa cells expressing various KR mutants, N-terminally Flag-tagged (A) and C-terminally Flag-tagged (B), in the presence of the ISGylation system were subjected to immunoprecipitation and then to Western blotting as in Fig. 4.

Fig. 6. ISGylation of PP2C β suppresses its activity. (A) HeLa cells were transiently transfected with indicated plasmids together with pNF- κ B reporter plasmid, and luciferase activity was measured as in Fig. 3. (B) The extracts of HeLa cells that had been transiently

transfected with indicated plasmids and then treated with cycloheximide (CHX) (50 μ g/ml) for 30 min were subjected to Western blotting with anti-I κ B α , anti-Flag tag, anti-S peptide, and anti-T7 tag antibodies. Wt, wild type; KR, K12,142R.

Fig. 1. Takeuchi et al.

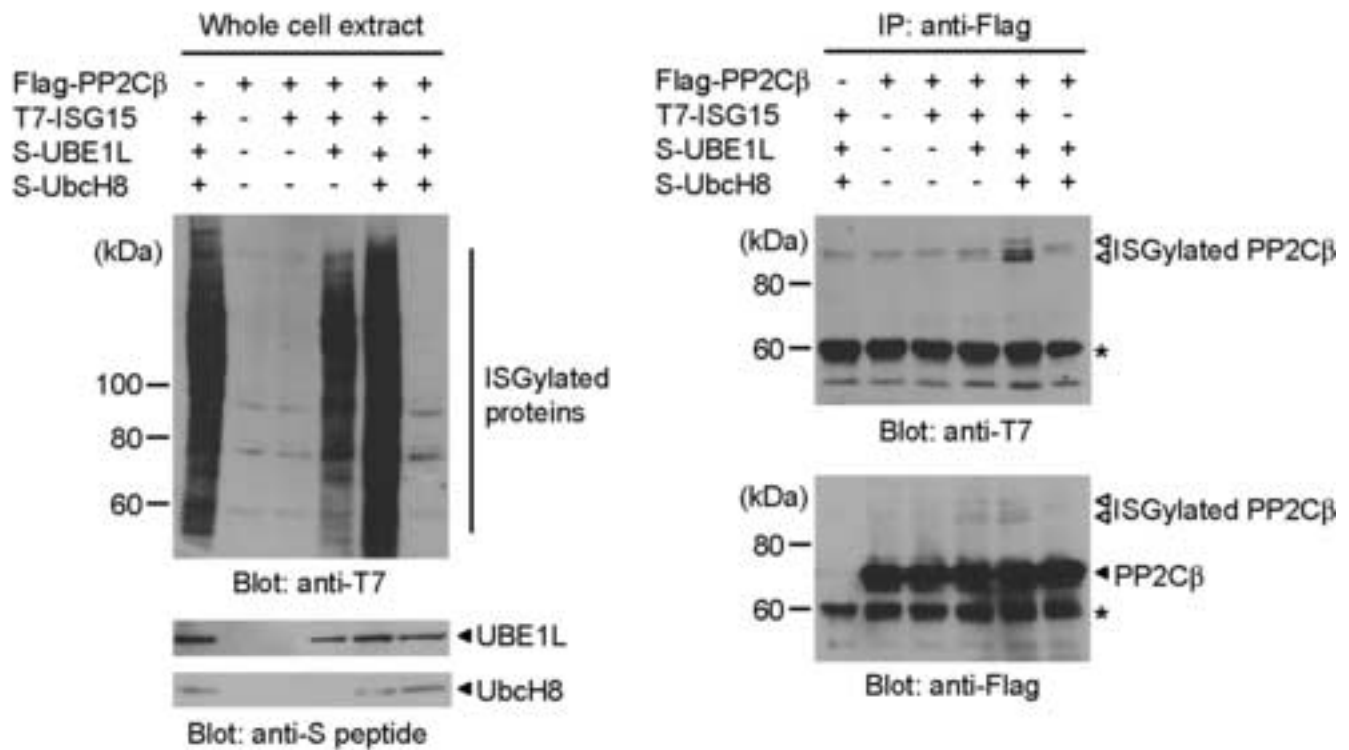


Fig. 2. Takeuchi et al.

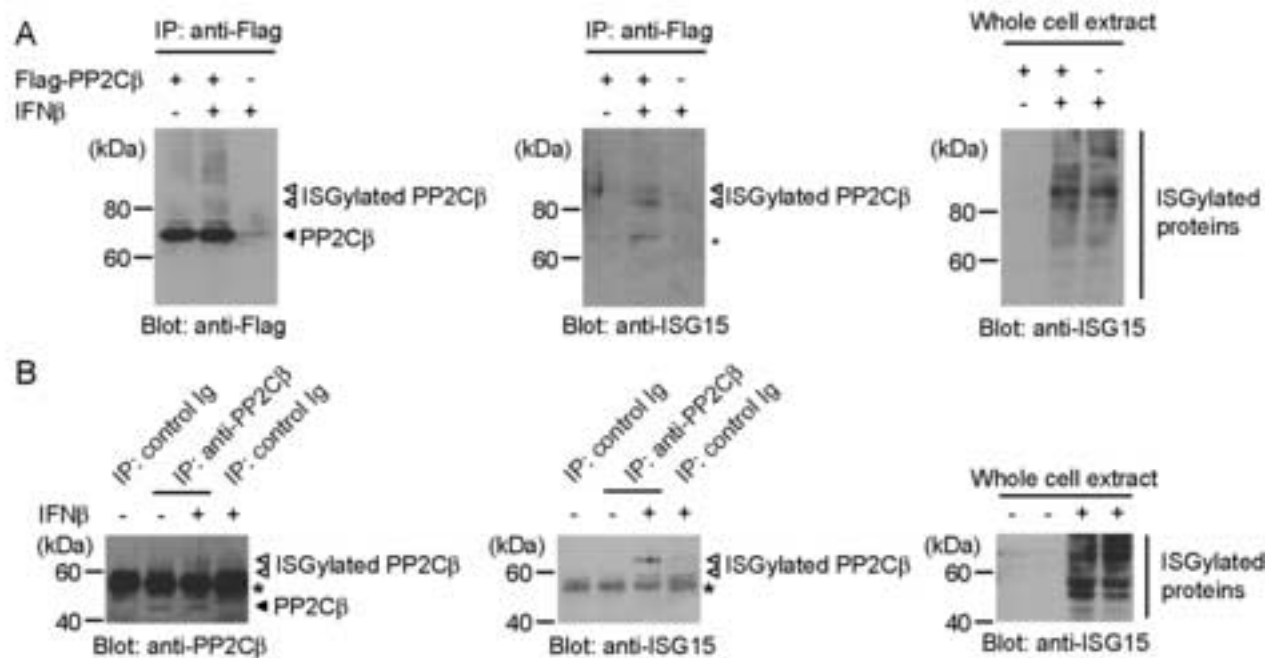
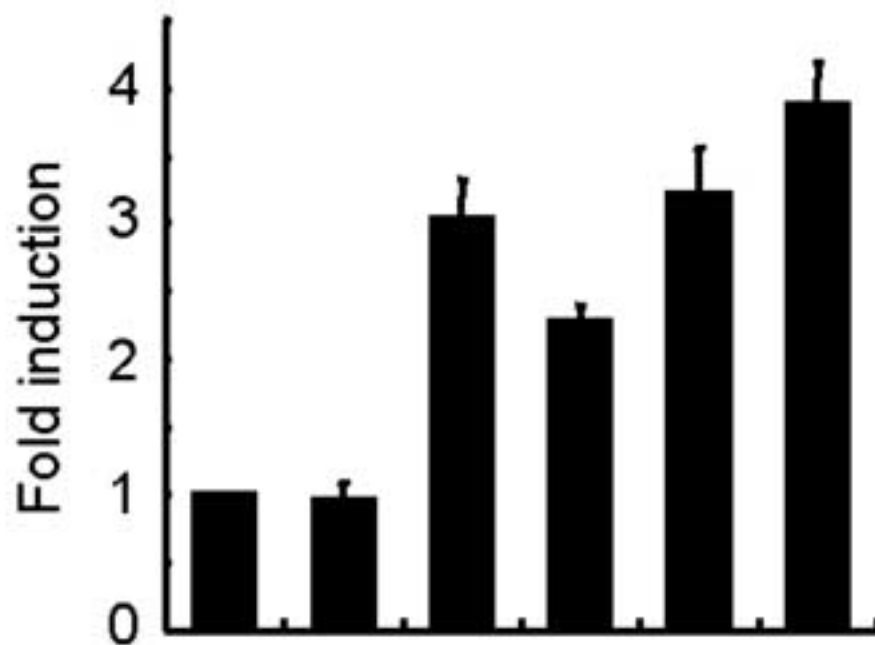


Fig. 3. Takeuchi et al.



HA-TAK1	-	+	+	+	+	+
HA-TAB1	-	-	+	+	+	+
Flag-PP2C β	-	-	-	+	-	+
ISGylation system	-	-	-	-	+	+

Fig. 4. Takeuchi et al.

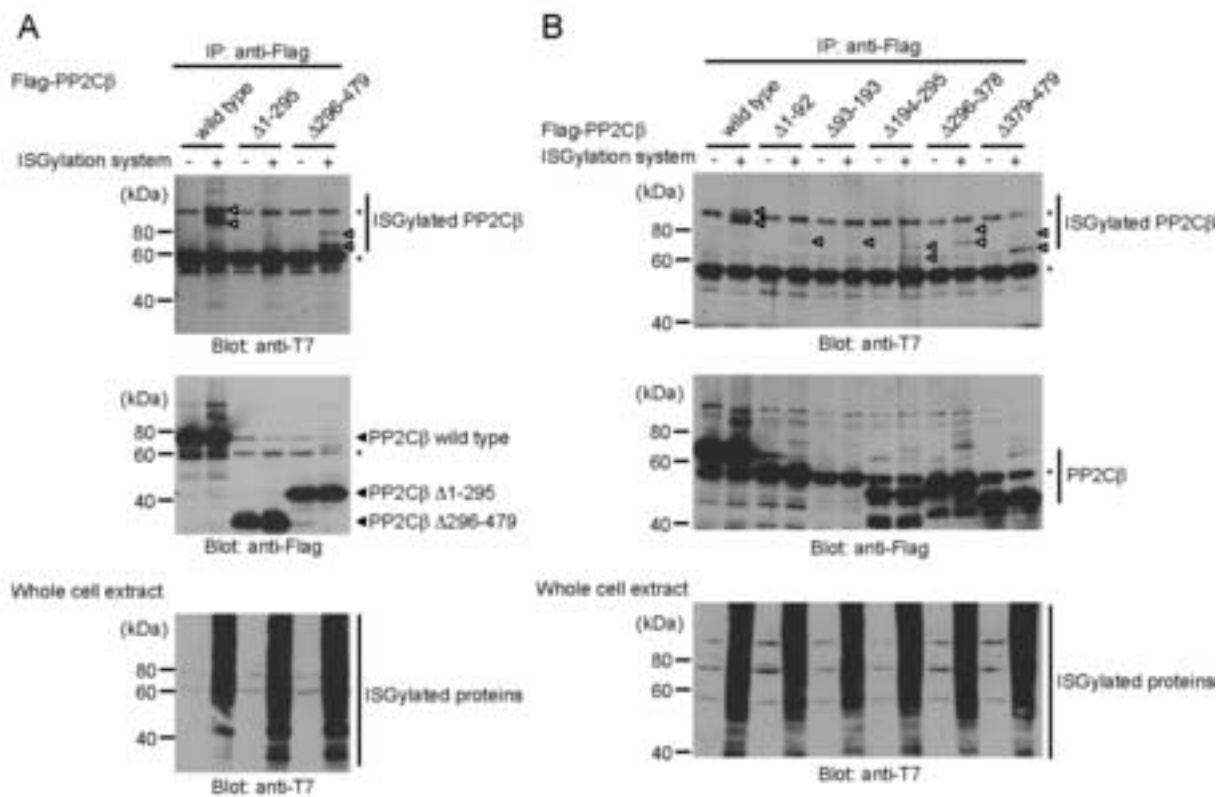


Fig. 5. Takeuchi et al.

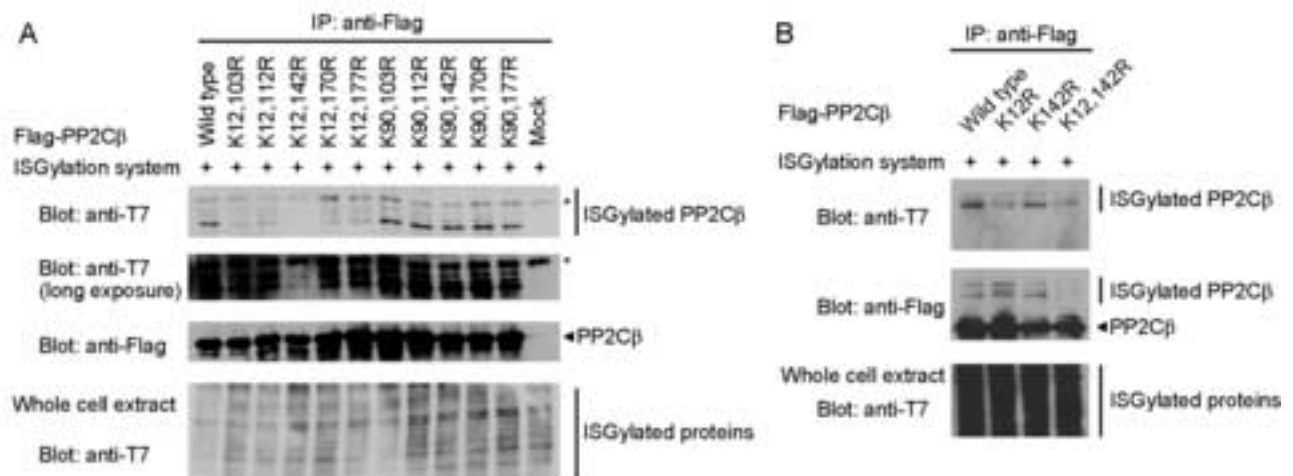
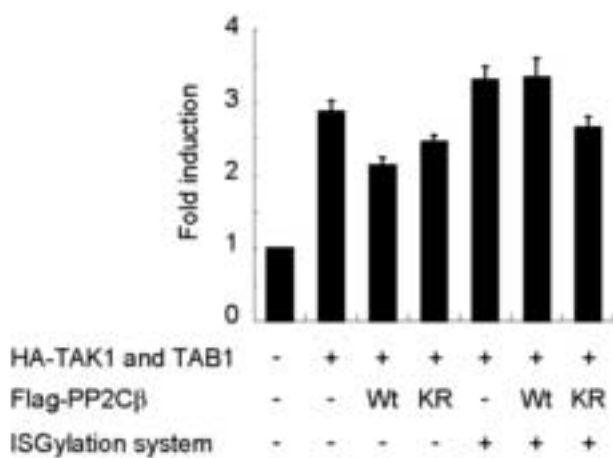


Fig. 6. Takeuchi et al.

A



B

