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Title: Protein tyrosine phosphatase PTPN21 acts as a negative regulator of ICAM-1 by dephosphorylating IKKβ in TNF-α-stimulated human keratinocytes

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Running Title: Inhibition of IKKβ by PTPN21

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ABSTRACT

Intercellular adhesion molecule-1 (ICAM-1), which is induced by tumor necrosis factor (TNF)-α, contributes to the entry of immune cells into the site of inflammation in the skin. Here, we show that protein tyrosine phosphatase non-receptor type 21 (PTPN21) negatively regulates ICAM-1 expression in human keratinocytes. PTPN21 expression was transiently induced after stimulation with TNF-α. When overexpressed, PTPN21 inhibited the expression of ICAM-1 in HaCaT cells but PTPN21 C1108S, a phosphatase activity-inactive mutant, failed to inhibit ICAM-1 expression. Nuclear factor-κB (NF-κB), a key transcription factor of ICAM-1 gene expression, was inhibited by PTPN21, but not by PTPN21 C1108S. PTPN21 directly dephosphorylated phospho-inhibitor of κB (IκB)-kinase β (IKKβ) at Ser177/181. This dephosphorylation led to the stabilization of IκBα and inhibition of NF-κB activity. Taken together, our results suggest that PTPN21 could be a valuable molecular target for regulation of inflammation in the skin by dephosphorylating p-IKKβ and inhibiting NF-κB signaling.

INTRODUCTION

A critical step in the development of inflammatory skin diseases is the infiltration of various immune cells, such as monocytes, neutrophils, and activated T cells, from the blood into the skin (1). Upon stimulation with proinflammatory agents, including tumor necrosis factor (TNF)-α and interferon (IFN)-γ, keratinocytes in the skin express proinflammatory cytokines, chemokines, and adhesion molecules, like intercellular adhesion molecule-1 (ICAM-1), which contribute to the entry of immune cells from the blood into the site of inflammation in the skin (2, 3).

TNF-α is a major proinflammatory cytokine produced by a number of cells, including keratinocytes (4). Stimulation of keratinocytes with TNF-α leads to activation of nuclear factor-κB (NF-κB) and subsequently increases the expression of adhesion molecules and proinflammatory genes (5, 6). NF-κB activation by TNF-α is due to the activation of the inhibitor of κB (IκB)-kinases (IKKs) complex, an upstream kinase complex of IκBα, which phosphorylates IκBα at Ser32/36, resulting in its ubiquitination and subsequent proteasomal degradation (7). Degradation of IκBα leads to translocation of cytosolic NF-κB complexes into the nucleus, where they activate the transcription of proinflammatory target genes (7).

Protein tyrosine phosphatase non-receptor type 21 (PTPN21), also known as PTPD1, is localized in the cytoplasm and phosphorylated by the proto-oncogene tyrosine-protein kinase (Src) (8, 9). It also upregulates Src by dephosphorylating Src at Tyr527 in response to stimulation by growth factors (10). PTPN21 forms a complex with actin, Src, and focal adhesion kinase (FAK), localizes at focal adhesion sites, and exerts major effects on cell adhesion, scattering, and migration (11). Through the formation of complex with A-kinase anchor protein 121 (AKAP121), PTPN21 plays diverse roles in cellular processes. Efficient

maintenance of mitochondrial membrane potential and ATP oxidative synthesis occur via the PTPN21-Src-AKAP121 complex (12). When not in complex with AKAP121, PTPN21 activates extracellular signal-regulated kinases 1/2- and Elk1-dependent gene transcription by directing epidermal growth factor/Src signaling to the nucleus (10). PTPN21 activates Etk, a member of the Tec family kinases, by specific interaction with Etk, resulting in the activation of Janus kinase 2-independent signal transducer and activator of transcription 3 (13). However, other substrates of PTPN21 involved in the regulation of cellular signal transduction pathways remain to be identified.

Previous studies on the regulation of ICAM-1 expression and skin inflammation focused on the findings of chemical or plant-extracted components and the elucidation of their mechanisms of action. In this study, we investigated PTP candidates that can regulate the expression of ICAM-1 and identified PTPN21 as a potential candidate. We examined the effect of PTPN21 on TNF-α-induced ICAM-1 expression and the role of PTPN21 in the regulation of NF-κB signaling in a human keratinocyte cell line, HaCaT. Our study may provide a basis for the therapeutic use of PTPN21 in inflammatory skin diseases.

RESULTS

TNF-α regulates ICAM-1 and PTPN21 expression in HaCaT cells

TNF-α is one of the most critical cytokines that induce the expression of ICAM-1, which is an important adhesion molecule for the recruitment of various immune cells to inflammatory sites. To identify PTPs that are regulated by TNF-α in keratinocytes, we investigated how PTP gene expression changes upon TNF-α treatment. *ICAM-1* mRNA expression was induced 1 h after TNF-α treatment and remained constant until 3 h. Of the forty-nine PTPs

tested (Supplementary table 1), *PTPN21* expression was induced by TNF-α treatment, whereas other PTPs showed unchanged or decreased expression (Fig. 1A). This implies that the *PTPN21* gene is induced by TNF-α and might be involved in the regulation of *ICAM-1* expression. We then measured the expression profiles of PTPN21 and ICAM-1 proteins at different time points of TNF-α treatment to investigate the relationship between PTPN21 and ICAM-1 expression (Fig. 1B). ICAM-1 expression started to increase at 3 h, showed maximal expression at 12 h, and decreased from 24 h after TNF-α treatment, whereas PTPN21 expression increased until 24 h after TNF-α treatment and thereafter decreased.

PTPN21 inhibits TNF-α-induced ICAM-1 expression in HaCaT cells

As shown in Fig. 1B, a correlation between PTPN21 and ICAM-1 expression upon TNF-α treatment was observed. We, therefore, investigated the effect of PTPN21 on TNF-α-induced ICAM-1 expression in HaCaT cells. HaCaT cells were transfected with either PTPN21 wild type (WT) or a catalytically inactive C1108S mutant expression plasmid, and stimulated with TNF-α prior to mRNA extraction (Fig. 2A). TNF-α treatment increased *ICAM-1* mRNA expression in HaCaT cells. While overexpression of PTPN21 WT inhibited the TNF-α-induced expression of *ICAM-1*, PTPN21 C1108S overexpression failed to do so. Similarly, TNF-α-induced ICAM-1 protein levels were tightly regulated by PTPN21 WT, but not by PTPN21 C1108S (Fig. 2B). These results suggest that PTPN21 WT regulates ICAM-1 gene expression and its phosphatase activity is required for gene regulation.

To confirm that PTPN21 affects the expression levels of ICAM-1, knockdown of *PTPN21* gene expression was performed by transfecting HaCaT cells with PTPN21-targeting siRNAs. By performing siRNA transfection followed by immunoblotting analysis with anti-PTPN21 antibody, siRNA#1 was selected to inhibit the expression of PTPN21 in HaCaT cells

throughout this study (Supplementary Fig. 1). *PTPN21* knockdown enhanced the TNF-α-induced expression of ICAM-1, compared to transfection with non-targeting control siRNA (Fig. 2C). This result suggests that PTPN21 negatively regulates the TNF-α-induced ICAM-1 expression in HaCaT cells.

PTPN21 inhibits TNF-α-induced NF-κB activation in HaCaT cells

TNF-α activates NF-κB signaling to induce ICAM-1 expression (14). To verify that PTPN21-mediated decrease in ICAM-1 expression is a result of NF-κB inhibition, knockdown of endogenous PTPN21 expression in HaCaT cells was performed by transfection with PTPN21 siRNA#1. NF-κB transcriptional activities were then measured using a NF-κB reporter system. As shown in Fig. 3A, in the absence of TNF-α treatment, NF-κB transcriptional activities, which are represented as luciferase activities, were not distinguishable between control siRNA- and PTPN21 siRNA#1-transfected cells. However, *PTPN21* knockdown cells showed a significant increase in NF-κB activity compared to control siRNA-transfected cells upon exposure to TNF-α.

Next, we investigated the effect of PTPN21 WT or C1108S overexpression on TNF-α-induced NF-κB transcriptional activity in HaCaT cells. As shown in Fig. 3B, TNF-α-induced NF-κB activity was reduced by PTPN21 WT, whereas PTPN21 C1108S did not exert any inhibitory effect on NF-κB activity. These results imply that PTPN21 inhibits NF-κB transcriptional activity in a phosphatase activity-dependent manner.

PTPN21 inhibits IκB and IKKs phosphorylation in HaCaT cells by its phosphatase activity

The reporter assay results prompted us to investigate the alterations in NF-κB activation by

silencing PTPN21 in HaCaT cells. *PTPN21* knockdown was achieved by transfecting HaCaT cells with PTPN21-targeting siRNA#1. As shown in Fig. 3C, TNF-α-induced phosphorylation and degradation of IκBα significantly increased in PTPN21 siRNA-transfected cells compared to control siRNA-transfected cells. In addition to the effect on IκBα, IKK phosphorylation was also notably increased in *PTPN21* knockdown cells. These results indicate that PTPN21 inhibits NF-κB transcriptional activity by regulating the phosphorylation of IκBα and IKK.

To elucidate a more concise mechanism of action of PTPN21 in the regulation of NF-κB signaling, the effects of PTPN21 on the phosphorylation of IκBα at Ser32/36 and IKKs (IKKα at Ser176/180 and IKKβ at Ser177/181) were determined by overexpressing PTPN21 WT and C1108S since phosphorylation at those sites is critical for NF-κB activation. Phosphorylation of IκBα and IKKs were induced by TNF-α stimulation in HaCaT cells. TNF-α-induced phosphorylation and subsequent degradation of IκBα were decreased by PTPN21 WT in a dose-dependent manner, whereas PTPN21 C1108S did not influence IκBα phosphorylation and degradation (Fig. 3D). Similar to the result of IκBα phosphorylation, PTPN21 WT led to dephosphorylation of p-IKKα/β, whereas PTPN21 C1108S did not. These results indicate that the regulatory effects of PTPN21 on NF-κB signaling are due to the dephosphorylation of p-IκBα and p-IKKα/β in a phosphatase activity-dependent manner.

To gain more insight into the direct target of PTPN21 for the regulation of NF-κB signaling, we examined the association between PTPN21 and the NF-κB signaling axis, including IκBα and IKKs, which are the key molecules for NF-κB activation and are tightly regulated by phosphorylation in HaCaT cells. Immunoprecipitation of endogenous IκBα or IKKα/β by specific antibodies and subsequent immunoblotting analysis with anti-PTPN21 antibody showed the association of IκBα and IKKα/β with endogenous PTPN21 in HaCaT

cells (Fig. 3E and F). These data indicate that PTPN21 associates with endogenous IκBα and IKKα/β in HaCaT cells.

PTPN21 directly interacts with IKKβ and dephosphorylates p-IKKβ

The results of Fig. 3D-F provoke the question as to what is the direct dephosphorylating target of PTPN21 in the regulation of NF-κB signaling. IKKβ is activated through phosphorylation at Ser177/181 and it subsequently catalyzes the phosphorylation of IκBα at Ser32/36 (15). Therefore, we performed *in vitro* binding assays using recombinant His-PTPN21 protein expressed in and purified from bacteria to find out the direct association of PTPN21 with IκBα and IKKβ. When immunoprecipitated FLAG-IκBα was incubated with recombinant His-PTPN21, no direct interaction between IκBα and PTPN21 WT was detected (Fig. 4A). However, immunoprecipitated FLAG-IKKβ showed an association with His-PTPN21 protein (Fig. 4B). These results indicate that IKKβ might be the direct target of PTPN21 in the regulation of NF-κB signaling.

To clarify whether PTPN21 directly dephosphorylates p-IKKβ, but not p-IκBα, we performed an *in vitro* phosphatase assay using recombinant His-PTPN21 WT or C1108S. Immunoprecipitates from FLAG-tagged IκBα- or IKKβ-transfected HEK 293 cells were extensively washed and then incubated with His-PTPN21 proteins followed by immunoblotting with phospho-specific antibodies. As shown in Fig. 4C, both His-PTPN21 WT and C1108 proteins did not dephosphorylate p-IκBα *in vitro*. However, His-PTPN21 WT decreased the levels of p-IKKβ at Ser177/181, whereas His-PTPN21 C1108S did not (Fig. 4D). These results suggest that PTPN21 directly dephosphorylates p-IKKβ in a phosphatase activity-dependent manner, leading to dephosphorylation of p-IκBα in the cells.

DISCUSSION

Growing evidence indicates that adhesion molecules, such as ICAM-1 on the surface of epidermal keratinocytes, play important roles in the initiation and maintenance of skin inflammation (3, 16). Therefore, elucidating the signaling molecules that regulate ICAM-1 expression as well as the discovery of pharmacological agents inhibiting ICAM-1 expression in keratinocytes are considered crucial for the treatment of inflammatory skin diseases. In this study, we showed that PTPN21 significantly inhibited the expression of ICAM-1 in TNF-α-stimulated HaCaT cells, indicating that PTPN21 could be a potential cellular target for the treatment of inflammatory skin diseases. Further investigations, such as alleviation of TNF-α-induced adhesion of monocytes to HaCaT cells by PTPN21, may be required to verify the consequences of PTPN21-mediated ICAM-1 inhibition.

A major signaling pathway activated by TNF-α is the NF-κB signal transduction pathway, which plays a central role in skin inflammatory responses by inducing the production of adhesion molecules, including ICAM-1 and basal-cell adhesion molecule in epidermal keratinocytes (17). Upon cell stimulation with TNF-α, the cytosolic NF-κB heterodimer is freed from IκBα and translocates into the nucleus, where it activates the transcription of proinflammatory genes (7). Our data and the molecular basis for regulation of NF-κB and ICAM-1 provide a clue that PTPN21 can regulate ICAM-1 expression in HaCaT cells by inhibiting NF-κB signaling.

PTPN21 is one of the class I PTPs, which consist of receptor type and non-receptor type PTPs, and has been reported to exhibit substrate specificity for phosphorylation at Tyr residues (9). However, in this study, PTPN21 dephosphorylated p-IKKβ at Ser177/181. This suggests that PTPN21 has a phosphatase activity towards p-Ser as well as p-Tyr. Protein

tyrosine phosphatase receptor type O, which is known as a Tyr-specific class I PTP, also has an ability to dephosphorylate p-Akt at Ser473 and p-glycogen synthase kinase 3β (GSK3β) at Ser9 (18). Furthermore, phosphatase and tensin homolog (PTEN), a Tyr- and lipid-specific PTP (19), dephosphorylates p-Akt at Ser473 and p-GSK3α/β at Ser21/9 in insulin-like growth factor-II-overexpressing rhabdomyosarcomas cells (20). Our results as well as these reports imply that some receptor type and non-receptor type PTPs have phosphatase activity toward p-Ser as well as p-Tyr.

Using siRNAs targeting various phosphatases, Li et al. reported that a variety of phosphatases, including PTPN21, inhibit NF-κB transcriptional activity in mouse astrocytes (19). The inhibitory effects of some identified phosphatases and their underlying mechanisms of action on NF-κB activation were further elucidated. However, the involvement of PTPN21 in the regulation of NF-κB was not investigated at a molecular level.

In addition, there are several reports that protein phosphatases regulate NF-κB signaling. IKKβ phosphorylation and activation were observed when PP2A was inhibited by ultraviolet-B (UVB) radiation, leading to UVB-induced apoptosis (21, 22). PTEN reduced NF-κB DNA binding to the FAK promoter by inhibiting the PI3K/NF-κB pathway, and subsequently inhibited invasion and metastasis of gastric cancer (23). Furthermore, profilin, a tumor suppressor protein, interacts with PTEN and suppresses NF-κB activity via inhibition of IKK phosphorylation (24). Unlike these phosphatases, no further investigations were available although PTPN21 seemed to be involved in the regulation of NF-κB activity (19). Therefore, the results of our study, which suggest that PTPN21 is a phosphatase activity-dependent regulator of p-IKKα/β, provide valuable insights for understanding PTPN21-mediated NF-κB regulation.

PTPN21 inhibits ICAM-1 expression by inhibiting the NF-κB signal transduction

pathway in HaCaT cells (Fig. 2 and 3). PTPN21 also activates the Src signaling pathway, which has been known to induce ICAM-1 expression and phosphorylation, by dephosphorylating its inhibitory phosphorylation site at Tyr527 (10). These controversial effects of PTPN21 on the NF-κB and Src signaling pathways give rise to a hypothesis that the activation of NF-κB signaling is more dominant for ICAM-1 expression than that for Src in HaCaT cells. This hypothesis can be supported by a previous study, which reported that low-molecular-weight protein tyrosine phosphatase (LMW-PTP) is oncogenic or anti-oncogenic depending on its interaction with different substrates (25). LMW-PTP was revealed to induce cell migration, suggesting that its oncogenic properties are more dominant than its anti-oncogenic properties (25). Similarly, we assume that PTPN21-mediated activation of the NF-κB signaling pathway might be more crucial for regulating ICAM-1 expression compared to PTPN21-mediated activation of the Src signaling pathway.

NF-κB is usually considered as an anti-apoptotic factor since it promotes cell proliferation. The inhibitory effect of PTPN21 on the NF-κB signaling pathway seems controversial when compared to PTPN21-mediated activation of the Src signaling pathway and its promoting effects on cell motility and migration (10, 11). However, NF-κB activation by interleukin-1 leads to NF-κB-dependent secretion of TNF-α, which activates TNF-R1 in an autocrine fashion to enhance UVB-induced apoptosis (26). These data suggest that PTPN21-mediated inhibition of TNF-α-induced NF-κB signaling could have the same cancer-promoting effects as PTPN21-mediated Src activation. Further studies are needed to clarify the consequence of PTPN21-mediated NF-κB inhibition with regard to cancer promotion.

MATERIALS AND METHODS

See the supplementary informations.

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CONFLICTS OF INTEREST

The authors have no conflicting financial interests.

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contributes to interleukin-1-mediated enhancement of ultraviolet B-induced apoptosis. J Biol Chem 280, 15635-15643

FIGURE LEGENDS

Fig. 1. TNF-α treatment regulates PTPN21 expression. (A) After stimulation of HaCaT cells with TNF-α (10 ng/ml) for the indicated time periods (0, 1, and 3 h), total RNA was extracted and cDNA was synthesized. Expression levels of PTPs in each sample were determined by RT-PCR. Expression levels of each PTP, relative to GAPDH levels, were represented as fold changes in comparison with the untreated group. (B) Total cell lysates were prepared after TNF-α (10 ng/m) stimulation for the indicated time periods (0, 3, 6, 12, 24, and 48 h). Immunoblotting analysis was performed using anti-ICAM-1 and anti-PTPN21 antibodies. Each protein level was normalized to endogenous GAPDH levels and the relative values compared to the untreated group were represented as fold changes.

Fig. 2. PTPN21 inhibits ICAM-1 production. (A and B) HaCaT cells were transfected with FLAG-PTPN21 plasmids (WT or C1108S; 1 μg for + or 2 μg for ++) for 48 h. (A) After stimulation with TNF-α (10 ng/ml) for 1 h, total RNA was extracted and cDNA was synthesized. Expression levels of *ICAM-1* and *PTPN21* in each sample were measured by RT-PCR. *ICAM-1* expression levels were normalized to GAPDH levels and the relative values compared to the TNF-α-treated group were represented as fold changes. (B) Total cell lysates were prepared after TNF-α (10 ng/ml) stimulation for 12 h. Immunoblotting analysis was performed using appropriate antibodies. ICAM-1 protein levels were normalized to endogenous GAPDH levels and the relative values compared to the TNF-α-treated group were represented as fold changes. (C) HaCaT cells were transfected with control siRNA (50 nM) or *PTPN21* siRNA#1 (100 nM) for 45 h and were stimulated with TNF-α (10 ng/ml) for 3 h. ICAM-1 protein levels were determined by immunoblotting analysis with appropriate antibodies. ICAM-1 protein levels were normalized to endogenous GAPDH levels and the relative values compared to control siRNA-transfected group were represented as fold

changes.

Fig. 3. PTPN21 inhibits NF-κB activation. (A) HaCaT cells were transfected with pNF-κB-luc reporter plasmid containing a luciferase gene and a gWIZ-GFP plasmid, and subsequently transfected with control siRNA (50 nM) or PTPN21 siRNA#1 (100 nM). After 24 h of incubation, the cells were treated with TNF-α (10 ng/ml) and further incubated for 24 h. The luciferase activities of each group were calculated using GFP fluorescence values as an internal control for transfection efficiency. Luciferase activities relative to control siRNA-transfected group were represented as a bar graph. *$p < 0.05$ relative to the TNF-α-treated group. (B) HaCaT cells were transfected with pNF-κB-luc reporter plasmid containing a luciferase gene and a gWIZ-GFP plasmid, and subsequently transfected with FLAG-PTPN21 WT or C1108S plasmids (1 μg for + or 2 μg for ++). After 24 h of incubation, the cells were treated with TNF-α (10 ng/ml) and further incubated for 24 h. The luciferase activities of each group were calculated using GFP fluorescence values as an internal control for transfection efficiency. Luciferase activities relative to the TNF-α-treated group were represented as a bar graph. *$p < 0.05$ relative to the TNF-α-treated group. (C) HaCaT cells were transfected with control siRNA (50 nM) or *PTPN21* siRNA#1 (100 nM) for 48 h and were stimulated with TNF-α (10 ng/ml) for 10 min. The levels of p-IκBα, IκBα, and p-IKKα/β were determined by immunoblotting analysis with appropriate antibodies. p-IκBα and IκBα levels were normalized to endogenous GAPDH levels, and p-IKKα/β levels were normalized to total IKK levels. The relative values of each protein were represented as fold changes compared to control siRNA-transfected group. (D) HaCaT cells were transfected with FLAG-PTPN21 plasmids (WT or C1108S; 1 μg for + or 2 μg for ++) for 48 h. Total cell lysates were prepared after TNF-α (10 ng/ml) stimulation for 15 min. Immunoblotting

analysis was performed using appropriate antibodies. Relative levels of p-IκBα and IκBα were normalized to endogenous GAPDH levels, and that of p-IKKα/β (p-Ser176/180 of IKKα and p-Ser177/181 of IKKβ) was normalized to total IKKα/β levels. The values relative to the TNF-α-treated group were represented as fold changes. (E and F) Total cell lysates from HaCaT cells were immunoprecipitated with anti-IκBα (E) or anti-IKKα/β (F) antibody. The immunoprecipitates were subjected to immunoblotting analysis with anti-PTPN21 antibody to detect interaction. Endogenous expression of PTPN21, IκBα, and IKKα/β in total cell lysates was confirmed by immunoblotting analysis with each antibody.

Fig. 4. PTPN21 directly dephosphorylates p-IKKβ. (A and B) After transfection with FLAG-tagged IκBα (A) or IKKβ (B), cell lysates were immunoprecipitated with anti-FLAG M2 agarose and then mixed with purified His-PTPN21 WT proteins. After binding, the immunoprecipitates were subjected to immunoblotting analyses with indicated antibodies to detect direct interaction between the immunoprecipitated FLAG-tagged proteins and purified His-PTPN21. (C and D) After transfection with FLAG-tagged IκBα (C) or IKKβ (D), cell lysates were immunoprecipitated with anti-FLAG M2 agarose and extensively washed. The immunoprecipitates were then reacted with purified His-PTPN21 WT or C1108S proteins in PTP reaction buffer. The reaction was stopped by adding sample buffer and the samples were subjected to immunoblotting analyses with indicated antibodies. CIP-treated group was used to detect the disappearance of p-IκBα and p-IKKβ in immunoprecipitates.

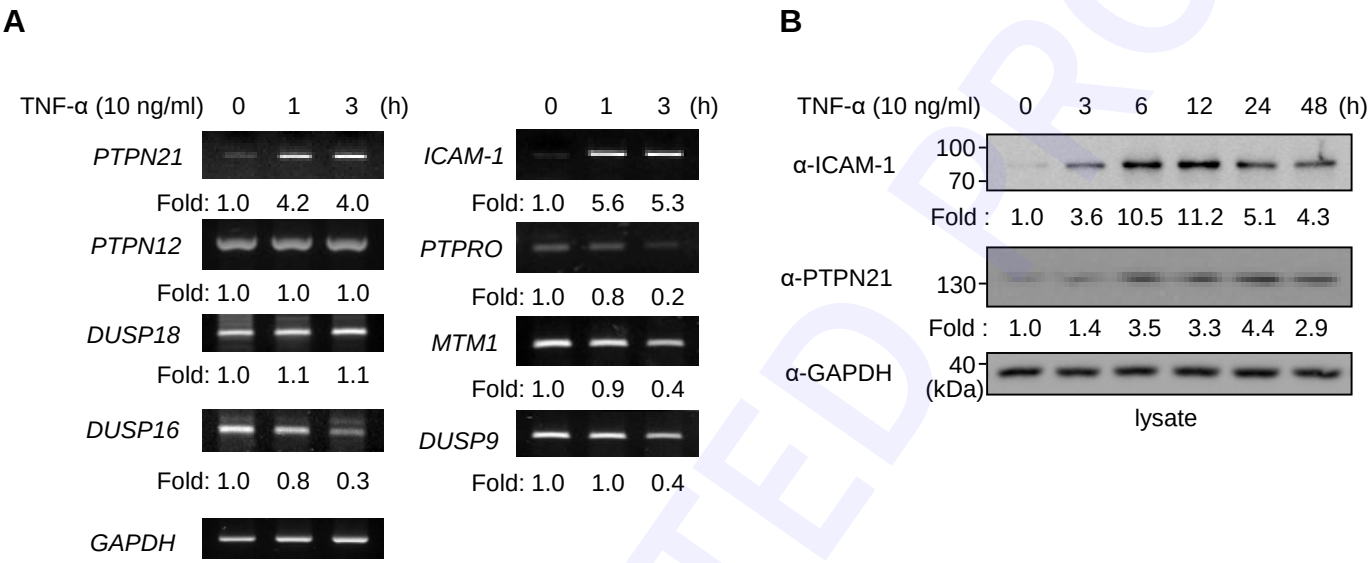


Figure 1

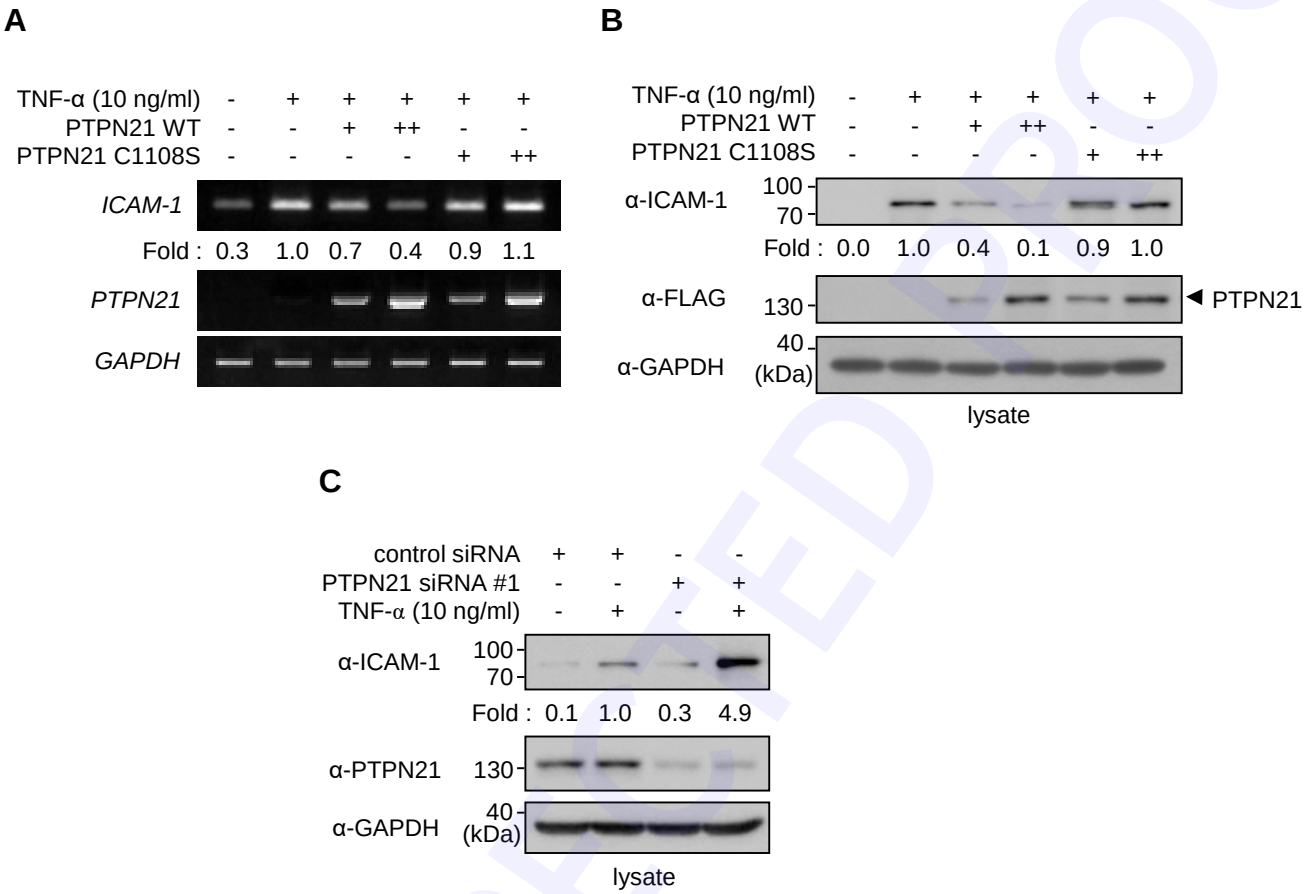


Figure 2

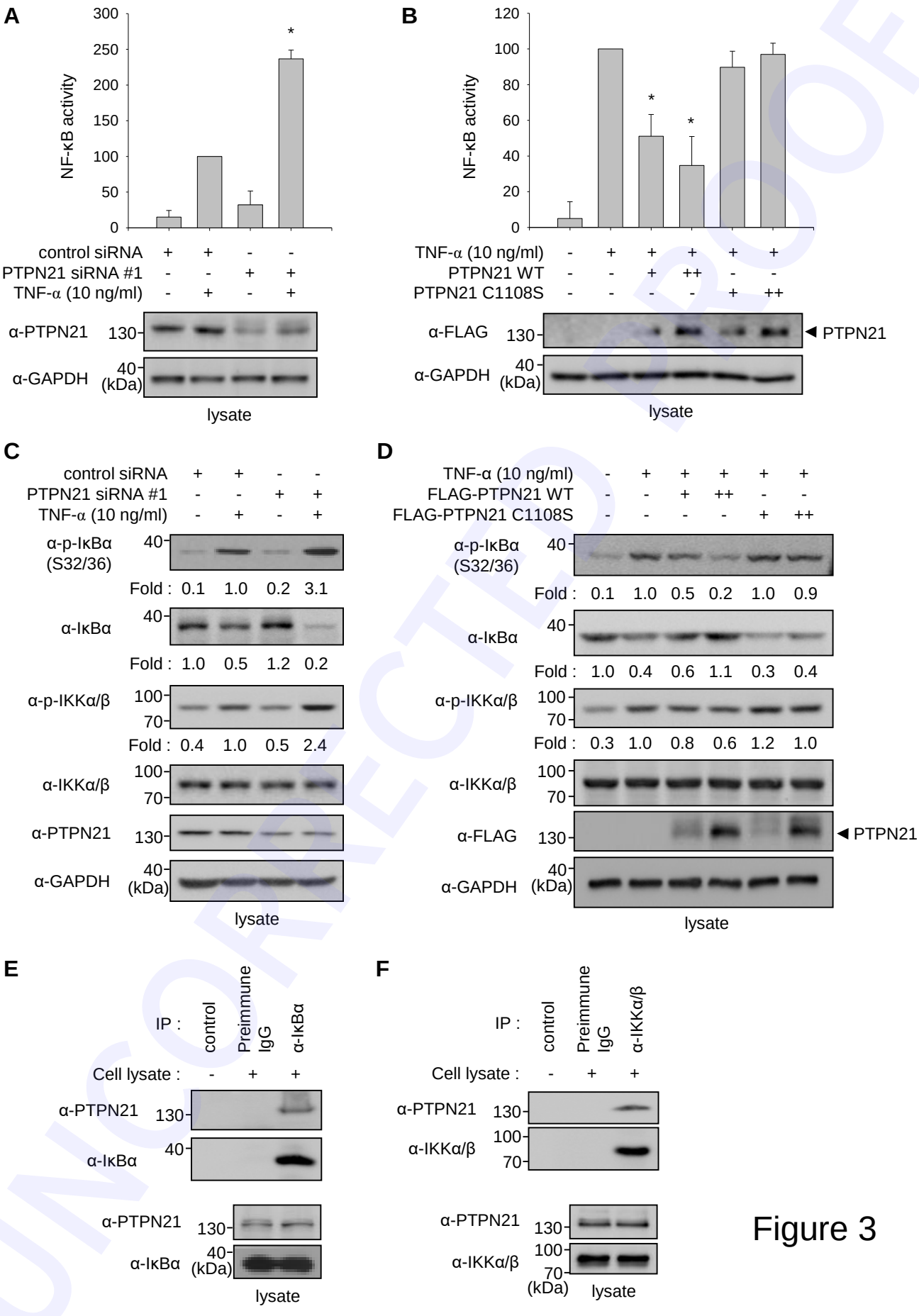


Figure 3

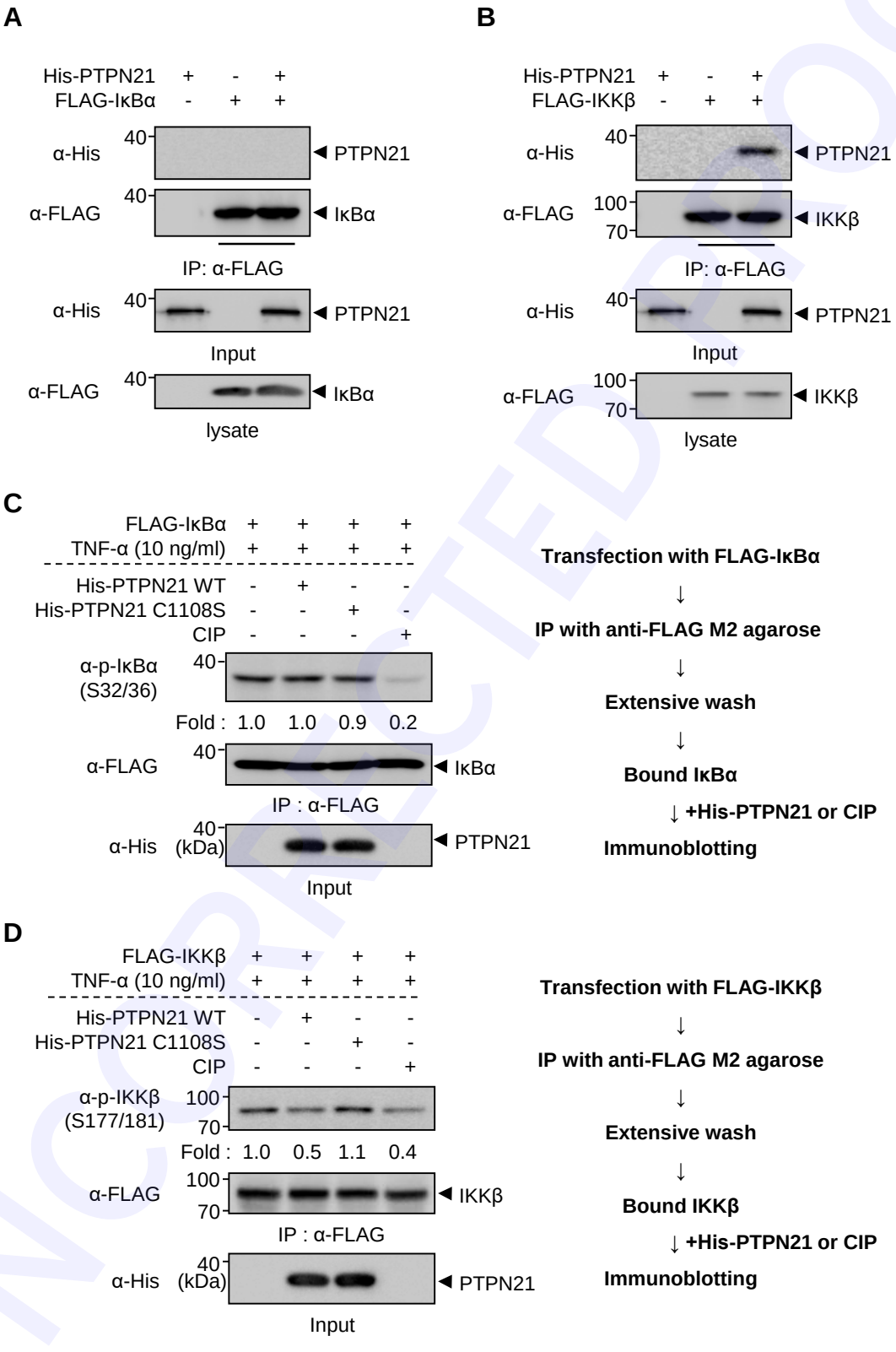
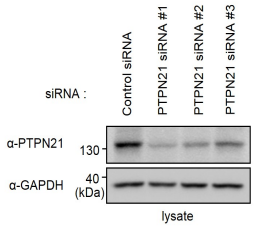


Figure 4

Supplementary Fig. 1



Sup. 1.

PTPs	primer sequence	PTPs	primer sequence
ACP1	Sense: 5'-GAG GGT CTG CAC CGA AAC ATG-3' Antisense: 5'-CTG ACA GCT CTT GGG TCT GGG-3'	DUSP28	Sense: 5'-CAC GCT GTG CGT CAA CGT C-3' Antisense: 5'-GCG GCC GTT CTT GCA GTA G-3'
Cdc14A	Sense: 5'-GAC CCC AGC AGC ACT TCC TG-3' Antisense: 5'-AAG GCA CGT AGT TTG TCT CCC TG-3'	DUSP3	Sense: 5'-GAG GGA GGG CAG GTC CTT CA-3' Antisense: 5'-CCA GGA AGC CAT CGT TGG G-3'
Cdc14B	Sense: 5'-GCC AGC AGC TCC TGG ACA G-3' Antisense: 5'-GGC AGG GGC TGG GTA GAG G-3'	DUSP4	Sense: 5'-CCA CCA TCT GCC TGG CCT AC-3' Antisense: 5'-GAA GAC GAA CTG CGA GGT GG-3'
Cdc25A	Sense: 5'-GGC TTC GTG GAC CTT CTC G-3' Antisense: 5'-GGC CCC AGA CAT GCT CTT C-3'	DUSP5	Sense: 5'-GAG GCA AGG TCC TGG TCC AC-3' Antisense: 5'-GCC TCC CCT TGG CAG GAG-3'
Cdc25C	Sense: 5'-GCC AAC CGT GTC AGG GAA AC-3' Antisense: 5'-GGG TGT CCA AAG GGA CGA TG-3'	DUSP6	Sense: 5'-CTC GGG CTG CTG CTC AAG-3' Antisense: 5'-GCT GGC TGT TGG ACA GCG-3'
DUSP1	Sense: 5'-CCT GTG GAG GAC AAC CAC AAG G-3' Antisense: 5'-GCT GGC CCA TGA AGC TGA AG-3'	DUSP7	Sense: 5'-CCT GCC CTA CCT CTA CCT CGG-3' Antisense: 5'-CAC CAC ACT TCT TGG AGC GG-3'
DUSP10	Sense: 5'-CAC CCC TGA CAT CGA GAA CG-3' Antisense: 5'-GCC TGG CAG TGG ATG AGA AG-3'	DUSP9	Sense: 5'-CCA ATT TGG AGA GCC TGG CC-3' Antisense: 5'-GCT TCT GCA TGA GGT AGG CCA C-3'
DUSP13A	Sense: 5'-CAG CCC ACG ACC TCC CTG-3' Antisense: 5'-GCC TCG GTT GGG GAA GAC C-3'	hSSH-1L	Sense: 5'-CAG GCT GGA GGC CAG CAT C-3' Antisense: 5'-CTC CGG GTC AGG TTG GAG C-3'
DUSP15	Sense: 5'-CCC AAC CCA GGC TTT AGG C-3' Antisense: 5'-CCC TCG GAG GCT GCT GAG-3'	KAP1	Sense: 5'-CGC AGA TGG AGG GAC TCC TG-3' Antisense: 5'-CCG GAT CCT CTT AGG TCT CGC-3'
DUSP16	Sense: 5'-CGG AGC AGA CTC CCG AAA CC-3' Antisense: 5'-CAG GCC AGC AGA CTT CGT GAG-3'	MTM1	Sense: 5'-CAA CAG CCG AAT CCA GTG GAG-3' Antisense: 5'-GAG CTC TAA TGC GGT GCC AGG-3'
DUSP2	Sense: 5'-GTG CCT GGT TCC AGG AGG C-3' Antisense: 5'-CTC AGT GAC ACA CGA CCT GGG-3'	MTMR2	Sense: 5'-GGC CAT GGA GAT AAG AACACAT GC-3' Antisense: 5'-GCG CAT GCT GGC TAC TGG-3'
DUSP22	Sense: 5'-TGG TGA TCG CAT ACA TCA TGA C-3' Antisense: 5'-CAG TCT TCT GAG AAA GGC CCA G-3'	MTMR7	Sense: 5'-CAC AGC CAG ACT CAG GGA ACC-3' Antisense: 5'-CTG ACC TGG ATG GGG TTG TG-3'
DUSP23	Sense: 5'-CGG GCC GAC TAC CTG AAT CC-3' Antisense: 5'-GGT AGT GAG GGT CCA GCA GCA G-3'	PALD1	Sense: 5'-CCA GGG CCG TAC CAC AAC TG-3' Antisense: 5'-GGC GTC ATG GTC TCG CTG-3'

Supplementary Table 1. List of PTP primers used in this study

PTPs	primer sequence	PTPs	primer sequence
PTP1B	Sense: 5'-CCA CAT GGC CTG ACT TTG GAG-3' Antisense: 5'-GGT AGG AGA AGC GCA GCT GG-3'	PTPNR	Sense: 5'-TTA CTG GCC CAT TTC TCT GAA G-3' Antisense: 5'-CTG AGG CAG GAG TGC CAT-3'
PTP4A1	Sense: 5'-GGT GCA CCA CCA TCC AAC C-3' Antisense: 5'-GAA TCT TTG AAA CGC AGC CGC-3'	PTPRB	Sense: 5'-GGT GTG GCC AGA CCA TGG-3' Antisense: 5'-CTG GAC CAT GTG AAC CCT GTG-3'
PTP4A3	Sense: 5'-GGG CTA CCA CTG TGG TGC G-3' Antisense: 5'-GAG CTG CTT GCT GTT GAT GGC-3'	PTPRD	Sense: 5'-CAC CAA GCT GCG TGA AAT GG-3' Antisense: 5'-CCG GAC TTT GGC ACT CCT TG-3'
PTPMT1	Sense: 5'-CCT CCA TGG ATT CAG GGA AGG-3' Antisense: 5'-CTC CCT GGT GTG CTA CAA TCC C-3'	PTPRG	Sense: 5'-CCT TTC GTC CTC CGG GAC C-3' Antisense: 5'-TCA TGC AGA CGC TGC TGT GG-3'
PTPN12	Sense: 5'-GTG ATC ATC CAG CGG GAG G-3' Antisense: 5'-GGC AGG TAG ATG GTC CCA GA-3'	PTPRG	Sense: 5'-CGT GCG GCA AGT CAA GTC C-3' Antisense: 5'-GGC TTC CAG GAT CGC ATC G-3'
PTPN14	Sense: 5'-CAG AGG AGG AGG GTG GAC G-3' Antisense: 5'-GAA CAT CCT CTG CTC CCT GAG G-3'	PTPRO	Sense: 5'-GCC GCC AAC GAC AAA CTC C-3' Antisense: 5'-GGC TCA CTC CAG CCA TGC AG-3'
PTPN18	Sense: 5'-CCA GCT ACA GTA TAT GTC CTG GCC-3' Antisense: 5'-CCT GTA CTG CTC CTC TGT CTG CAC-3'	PTPRQ	Sense: 5'-GGA GAA TGG TGT GGG AAA CCA G-3' Antisense: 5'-GGT GTC ATG TGC CCT GCT TG-3'
PTPN2	Sense: 5'-GGC GCT CTG GCA CCT TCT C-3' Antisense: 5'-CAT CTG CTG CAC CTT CTG AGC-3'	PTPRT	Sense: 5'-GGG ACA AGG ATG TGG CAA GG-3' Antisense: 5'-CCC CAA ACA GAG CCC ACA TC-3'
PTPN21	Sense: 5'-GGT CTA CAG CCA GCC CGA GA-3' Antisense: 5'-GCT CGC TGA CCT CCT GCA G-3'	PTPRZ	Sense: 5'-GCC AAG CGC CAT GCA GT-3' Antisense: 5'-CCC TTG ATC TTT CCA CAG GGA TG-3'
PTPN22	Sense: 5'-CTT CTC CCC CAC CTC CTC TCC-3' Antisense: 5'-CTG CAG GCT TGT TTG GTG GG-3'	SHP-1	Sense: 5'-GGC TGG CTT CTG GGA GGA G-3' Antisense: 5'-CCT GGC TGG CGA TGT AGG TC-3'
PTPN3	Sense: 5'-CGA GGA CGC CAG CCA GTA CTA C-3' Antisense: 5'-CTC CTG ATC ACC AGG GCC AG-3'	STNS	Sense: 5'-CAA GGA AGA CGC CGA GGA C-3' Antisense: 5'-CCT CCC ATT TGT AAG CTC CCA TC-3'
PTPN7	Sense: 5'-CAT CGC CAC GCG AAT TG-3' Antisense: 5'-GTC AGG GGC TGG GTT CCT C-3'		

Supplementary Table 1. List of PTP primers used in this study (continued)

Manuscript Type: Article

Title: Protein tyrosine phosphatase PTPN21 acts as a negative regulator of ICAM-1 by dephosphorylating IKKβ in TNF-α-stimulated human keratinocytes

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Running Title: Inhibition of IKKβ by PTPN21

Keywords: Protein tyrosine phosphatase non-receptor type 21, Skin inflammation, Intercellular adhesion molecule-1, Nuclear factor kappaB, Inhibitor of κBα kinase

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Supplementary information :

Supplementary file 1 : Supplementary information.

Supplementary file 2 : Supplementary figure 1.

Supplementary file 3 : Supplementary table 1.

1. MATERIALS AND METHODS

Cell culture, plasmids, and reagents

The human HaCaT keratinocyte cell line was purchased from Cell Lines Service (Eppelheim, Germany). HEK 293 cells were obtained from ATCC (Manassas, VA, USA). Cells were maintained in Dulbecco′s modified Eagle′s medium (DMEM; GE Healthcare, Milwaukee, WI, USA) containing 10% fetal bovine serum (FBS; GE Healthcare), 50 units/ml penicillin, and 50 μg/ml streptomycin (GIBCO BRL, Grand Island, NY, USA) at 37 °C in humidified air containing 5% CO_2. FLAG-PTPN21 WT was generously provided by Dr. A. Feliciello (Federico II University, Italy). FLAG-PTPN21 C1108S catalytically inactive mutant was generated from FLAG-PTPN21 WT using a QuikChange II Site-Directed Mutagenesis Kit (Agilent Technology, Santa Clara, CA, USA). Rabbit anti-PTPN21 antibody (cat no. ab133812) was purchased from Abcam (Cambridge, U.K.). Rabbit anti-p-IκBα (Ser32/36; cat no. sc-101713), mouse anti-IκBα (cat no. sc-1643), mouse anti-ICAM-1 (cat no. sc-8439), rabbit anti-IKKα/β (cat no. sc-7607), and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; cat no. sc-25778) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-p-IKKα/β (p-Ser176/180 of IKKα and p-Ser177/181 of IKKβ; cat no. 2697) was purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Mouse anti-FLAG antibody was from Sigma-Aldrich (St. Louis, MO,

USA). Recombinant human TNF-α protein was from R&D Systems (Minneapolis, MN, USA). Calf intestine phosphatase (CIP) was purchased from Takara Bio, Inc. (Otsu, Japan). AccuZol reagent and AccuPower PCR Master Mix were from Bioneer (Daejeon, Korea) and TOPscript cDNA synthesis kit was from Enzynomics (Daejeon, Korea).

RNA preparation and cDNA (complementary DNA) synthesis

HaCaT cells were seeded on 6-well plates (2×10^5 cells/well) and incubated at 37 °C overnight. Cells were treated with TNF-α (10 ng/ml) in the absence or presence of PTPN21 plasmids for the indicated time periods according to experimental settings. Total RNA was prepared from cells using Accuzol reagent and reverse-transcribed into cDNA using a TOPscript cDNA synthesis kit as manufacturer's protocols.

Polymerase chain reaction (PCR)

PCR primer sequences of PTPs used in this study are listed in Supplementary Table 1. The primer sequences of ICAM-1 and GAPDH were designed as previously described (27, 28). The PCR was run for 17–25 cycles of 94 °C (30 s), 60 °C (30 s), and 72 °C (30 s) on a Bioer thermal cycler (Bioer Technology Co., Hangzhou, China). After amplification, 10 μl of the PCR products was separated on 1.5% (w/v) agarose gels, which were then stained with ethidium bromide.

Preparation of total cell lysates

HaCaT cells (1×10^6 cells/ 60-mm dish) transfected with PTPN21 plasmids were incubated with TNF-α for the indicated time periods. After incubation, total cell lysates were prepared as previously described (29). Cells were washed 3 times with ice-cold phosphate-buffered saline. Lysis buffer, containing 0.5% IGEPAL CA-630, 0.5% Triton X-100, 150 mM NaCl,

20 mM Tris-HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid, 1% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaF, and 1 mM Na_3VO_4, was added to the cells and incubated for 10 min. The supernatants were collected after centrifugation at 15,814 × *g* for 30 min at 4 °C.

Luciferase activity assay

HaCaT cells (5×10^6 cells) were seeded on 100-mm dishes (70% confluence on the day of transfection) and transfected with pNF-κB-luc cis-reporter plasmids (Agilent Technology) and gWIZ-green fluorescent protein (GFP; internal control for transfection efficiency). Transfected cells were split into 12-well plates, incubated overnight, and then transfected with FLAG-PTPN21 plasmids. After 24 h of incubation, TNF-α (10 ng/ml) was added and cells were incubated for additional 24 h. Luciferase activity assay was performed as described previously (30). Briefly, cells were lysed in cell culture lysis reagent (Promega Corporation, Fitchburg, WI, USA) and luciferase activity was measured using VivoGlo Luciferin (Promega) as a substrate. GFP fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 525 nm.

Immunoblotting analysis

Immunoblotting analysis was carried out as described previously (31). Briefly, aliquots of each boiled sample (20 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. After blocking with 5% non-fat dried milk, each membrane was incubated overnight at 4 °C with primary antibody. Each membrane was then incubated for an additional 1 h with secondary peroxidase-conjugated IgG antibody (1:5,000). The proteins were detected using enhanced chemiluminescence reagent. Protein levels were quantified by scanning the immunoblots and

analyzing them with LabWorks software (UVP Inc., Upland, CA, USA).

Knockdown of PTPN21

For RNA interference of PTPN21, HaCaT cells (5×10^5 cells/ 6-well plate) grown to 40% confluence were transfected with 50 nM of scrambled negative control siRNA or 50–100 nM of PTPN21 siRNAs [#1: 5′-CUC UGU CAG UGG AAU CGA A(dTdT), #2: 5′-GAG AAG AGC UUU AGG UAC U(dTdT), or #3: 5′-GAG AAG AGC UUU AGG UAC U] (Bioneer) using Neon Transfection System (Invitrogen, Carlsbad, CA, USA). The negative control siRNA used was purchased from Bioneer. After 48 h of transfection, cell lysates were prepared and subjected to immunoblotting analysis with an anti-PTPN21 antibody.

Endogenous protein binding assay

Total lysates from HaCaT cells were incubated with mouse anti-IκB antibody, mouse anti-IKKα/β, or normal mouse immunoglobulin G (IgG) for 3 h at 4 °C and then further incubated with protein A/G beads for 1 h at 4 °C. To clear the immunoprecipitates, unbound proteins were discarded from immunoprecipitates by extensive washing (5 times) with lysis buffer. Following that, the cleared immunoprecipitates were mixed with 1× sample loading buffer, boiled at 100 °C for 5 min, and then subjected to immunoblotting analysis.

Purification of the bacterial His-tagged proteins

After *Escherichia coli* BL21 (DE3)RIL was transformed with pET28a-His-PTPN21 (a.a. 839-1174) WT or pET28a-His-PTPN21 C1108S, cells were grown on LB medium containing kanamycin and 0.2 mM isopropyl-β-D-1 thiogalactopyranoside at 18 °C for 16 h. Cells were harvested, resuspended in lysis buffer [50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1 mM PMSF, 4 mM 2-mercaptoethanol, and 5% (v/v) glycerol], and then lysed by sonication. The

cell extracts were centrifuged at 15,814 × *g* for 50 min and the supernatant was subjected to Ni-NTA agarose affinity chromatography. The PTPN21 phosphatase bound to the affinity gel was eluted by imidazole gradient method and frozen at –80 °C in a buffer containing 25 mM Tris-HCl (pH 7.5), 200 mM NaCl, 10 mM 2-mercaptoethanol, and 5% (v/v) glycerol until use in enzyme assay. Phosphatase activities of His-PTPN21 WT and C1108S were measured using the substrate 3-O-methylfluorescein phosphate (OMFP; Sigma-Aldrich). The amount of 3-O-methylfluorescein was determined by the absorbance change at 490 nm or fluorescence change of excitation at 485 nm and emission at 525 nm.

In vitro protein binding assay

HEK 293 cells (5×10^6 cells/ 100-mm dishes) were transfected with FLAG-tagged IκBα or IKKβ expression plasmid (5 μg) for 48 h. Total cell lysates were pulled down with anti-FLAG M2 agarose beads for 3 h and the pulled-down proteins were subjected to extensive washing to purify FLAG-fusion proteins by excluding any bound proteins in the pulled-down complexes. To determine whether PTPN21 directly binds to IκBα or IKKβ, each anti-FLAG bead-bound protein was mixed with His-PTPN21 WT (2 μg) in 1 ml of PTP reaction buffer [100 mM Tris-HCl (pH 7.5), 40 mM NaCl, and 1 mM DTT] and incubated for 3 h at 4 °C. After incubation, the beads were washed with binding buffer 5 times and 1× sample buffer was added and boiled for 5 min at 100 °C. The samples were subjected to immunoblotting analyses using appropriate antibodies.

In vitro phosphatase assays

Each anti-FLAG bead-bound protein was mixed with His-PTPN21 WT or C1108S (0.1 μg) in 20 μl PTP reaction buffer [100 mM Tris-HCl (pH 7.5), 40 mM NaCl, and 1 mM DTT] and reaction mixtures were incubated at 30 °C for 30 min. CIP was used to prove the bands

detected by each antibody, which recognizes specific phosphorylation sites, are phospho-specific bands. Phosphatase reaction was stopped by adding 5× sample buffer. The beads were then resolved on SDS-PAGE and analyzed by immunoblotting using specific antibodies.

Statistical analysis and experimental replicates

The data are represented as the mean ± standard error of the mean (SEM). Differences between experimental conditions were assessed by Student's *t*-test. $p < 0.05$ was considered statistically significant. In all instances, the means of data from three independent experiments were analyzed.

2. SUPPLEMENTARY DATA LEGENDS

Supplementary Fig. 1. Knockdown of PTPN21. After transfection with control or PTPN21 siRNAs (#1, #2, and #3), PTPN21 knockdown was confirmed by immunoblotting using anti-PTPN21 and anti-GAPDH antibodies.

Supplementary Table. 1. List of PTP primers used in this study

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