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Protein Phosphatase 1 Abrogates IRF7-Mediated Type I IFN Response In Antiviral Immunity

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Protein phosphatase 1 abrogates IRF7-mediated type I IFN response in antiviral immunity

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Abstract

Interferon (IFN) regulatory factor 7 (IRF7) plays a key role in the production of IFNα in response to viral infection, and phosphorylation at IRF7 C-terminal serine sites is prelude to its function. However, no phosphatases which that may be involved in negatively regulate IRF7 phosphorylation and activity has have not been reported. In this study, we have identified a conserved protein phosphatase 1 (PP1)-binding motif in human and mouse IRF7 proteins, and shown that PP1 physically interacts with IRF7. Exogenous expression of PP1 subunits (PP1α, β or γ) ablates IKKε-stimulated IRF7 phosphorylation and dramatically attenuates IRF7 transcriptional activity. Inhibition of PP1 activity significantly increases IRF7 phosphorylation and IRF7-mediated IFNα production in response to newcastle disease virus (NDV) infection or Toll-like receptor 7 (TLR7) challenge, leading to impaired viral replication. In addition, IFN treatment, TLR challenges and viral infection induce PP1 expression. Our findings disclose for the first time a pivotal role for PP1 in impeding IRF7-mediated IFNα production in host immune responses.

Keywords

PP1; IRF7; TLR7; type I IFN

INTRODUCTION

Interferon (IFN) Regulatory Factor 7 (IRF7) is the “master” regulator of type I IFN (IFN-I) in response to pathogenic infections [1, 2], which activate signaling pathways mediated by specific pathogen-recognition receptors (PRRs) such as Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and the cytosolic receptor cGAMP synthase (cGAS) [2, 3]. Phosphorylation of IRF7 is indicative of its activation, and is prerequisite for its function as

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a transcription factor. Thus, tight regulation of IRF7 phosphorylation and activity is crucial for many fundamental processes involving IFN-I, such as antiviral innate immunity, autoimmune disorders, and cancers [4–6]. The kinases identified for IRF7 phosphorylation include IKKε, TBK1, IRAK1 and IKKα, which phosphorylate IRF7 in a cell type-specific manner [1]. Phosphorylation is a dynamic process in the cell that is in general balanced by kinases and phosphatases. While IKKε was the first kinase identified for IRF7 phosphorylation in 2003 [7], the phosphatases that negatively regulate IRF7 phosphorylation and activity have not been identified thus far.

PP1 and PP2 together account for more than 90% of the protein phosphatase activity in eukaryotes [8, 9]. Recently, the roles of protein phosphatases in antiviral immune response have emerged and drew intense attention. PP1 has been shown to dephosphorylate the RNA sensors, MDA5 and RIG-I, and promote IFN-I production in response to RNA virus infections [10, 11]. Additionally, PP1 interacts with TRAF6, and promotes TRAF6-dependent innate immune responses [12]. PP1 also dephosphorylates Ebola virus VP30 and inhibits its transcription [13]. In contrast, the measles virus V proteins and hepatitis B virus (HBV) X protein can antagonize PP1 activity to facilitate their replication [11, 14]. More recent reports have shown that PP1 negatively regulates IRF3-mediated IFN-I production by inhibiting its phosphorylation at serines 396 and 385 [15], and in conjunction with GADD34, regulates TLR-mediated immune response by inhibiting phosphorylation of TAK1 and eIF2α [16, 17]. Of special note, PP1 and PP2A are positive regulators of Tat-dependent HIV1 transcription, and the ^{35}QVCF^{38} motif of Tat directly interacts with PP1 [18].

In this study, we show that PP1 interacts with IRF7 and potently inhibits IRF7-mediated IFN-I immune responses. We have also shown that TLR challenge or viral infection induces PP1 expression.

RESULTS

IRF7 interacts with PP1

No phosphatases have been identified for IRF7 thus far. Recently, the roles of protein phosphatases, especially PP1, in antiviral immune response have emerged and drawn intense attention [10–17]. To evaluate if PP1 inhibits IRF7 phosphorylation, we first examined the possibility that these two proteins can interact. To this end, we analyzed whether IRF7 protein has potential PP1-binding motifs, the canonical of which has the consensus sequence, R/K-X$_{0–1}$-V/I-X-F/W [9]. The interacting partners identified for PP1, such as NIPP1 [19], PNUTS [20, 21], Bcl2 [22], GADD34 [23], Ikaros [24], Inh3 [25], and BRCA1 [26], all have this binding motif (Fig 1, A). Interestingly, we have identified a conserved sequence, RVLF, matching the canonical PP1-binding motif, in both human and mouse IRF7 proteins. This motif is located at the immediate N-terminus of the DNA-binding domain of IRF7 (Fig 1, A). In addition, there is another potential PP1-binding sequence, ^{408}RVFF^{411}, located in the very end of C-terminus of human IRF7 protein. However, this sequence is not conserved in mouse (Fig 1, A). We then confirmed the protein interaction between IRF7 and PP1 by co-immunoprecipitation (Fig 1, B). The interaction between IRF7 and PP1 is specific since PP2 did not interact with IRF7 (data not shown). To verify IRF7 and PP1

interaction in a biological context, we checked if endogenous IRF7 and PP1 interact in EBV-transformed cells, in which we have previously shown that IRF7 is expressed at significant levels [1]. To this end, IB4, SavIII, and LCL00045, three cell lines transformed with EBV, were subjected to co-IP. Results clearly show that PP1 and IRF7 interact endogenously in EBV-transformed cells (Fig 1, C). Collectively, these results demonstrate that PP1 and IRF7 physically interact *in vitro* and *in vivo*.

PP1 targets IRF7 key activating phosphorylation sites and dampens IRF7 activity

The key phosphorylation sites of IRF7 responsible for its activation include Ser477, -479, -471, and -472 in human IRF7A [27–30]. We show that PP1 targets all the four key phosphorylation sites, and remarkably reduces their phosphorylation stimulated by IKKε. Moreover, all three isoforms of PP1 have the ability to reduce IRF7 phosphorylation. Flag-PP1γ seems to have the weakest effect, which is consistent with its expression level that is also the weakest (Fig 2, A). Ser471/472 phosphorylation is consistently more sensitive to the effect of PP1β, with unknown reason.

We next sought to determine whether PP1 can negatively regulate IRF7 transcriptional activity. To this end, Luciferase reporter assays were performed, and results show that all three PP1 isoforms strikingly reduce IRF7 activity stimulated by IKKε (Fig 2, B). PP1γ has the least effect, in line with its weakest expression level, as shown in Fig 2, A.

To further verify that PP1 targets IRF7 key activating phosphorylation sites, we used the IRF7 mutant, Flag-IRF7(S471D/S472D/S477D/S479D) (designated as Flag-IRF7(4D)), a phosphomimetic mutant that has constitutive activity since these four key serine sites were replaced with aspartic acids [27], for reporter assays. Results show that PP1α dramatically reduces TLR7-stimulated WT IRF7 activity, but did not reduce the activity of IRF7(4D), in 293-TLR7 cells (Fig 2, C).

To assess if PP1 phosphatase activity is required for its inhibition of IRF7, PP1α(T320A) that is constitutively active, and PP1α(T320E) that is a phosphatase-dead mutant [31], were used for promoter-reporter assays. As shown in Fig 2, D, both WT PP1α and PP1α(T320A), but not PP1α(T320E), inhibit IRF7 transactional activity, indicating that the PP1 phosphatase activity is required for PP1 inhibition of IRF7 phosphorylation.

Further, we assessed the effect of PP1 on IRF7 DNA-binding ability. To this end, 293T cells were transfected with indicated plasmids (Fig 2, E). Cells were subjected to ChIP and qPCR analyses after 48 h. Results show that PP1 significantly impairs IKKε-stimulated DNA-binding activity of IRF7, but not of the constitutively active mutantIRF7(4D) (Fig 2, E).

Taken together, these results demonstrated that PP1 targets the key IRF7 phosphorylation sites, dampens its phosphorylation and abolishes its transcriptional activity.

Both IRF7 potential PP1-binding sites contribute to its interaction with PP1

To further determine the importance of the potential PP1-binding sites in IRF7 for their interactions, we transfected a panel of human 2XMyc-IRF7 point mutants of these two potential PP1-binding sites (Fig 3, A), together with Flag-PP1α into 293T cells, and cell

lysates were subjected to co-IP. Results show that mutation in either of these sites did not disable the interaction between IRF7 and PP1α. When both sites were mutated, however, IRF7 lost the ability to interact with PP1 (Fig 3, B), indicating that both sites contribute to their interactions. Of note, mutation of either of these two sites abrogates the ability of IRF7 to transactivate IFNα4 promoter (Fig 3, C), indicating that both of them are essential for IRF7 activation. Thus, both motifs contribute to IRF7 binding to PP1 and are required for IRF7 activation.

Blockage of PP1 activity potentiates IRF7 phosphorylation and IFNα production in viral infection

We further assessed the biological consequences of PP1 blockage of IRF7 activity in viral infection. To this end, the human acute myeloid leukemia cell line U937 (monocyte) was transfected with plasmids expressing nuclear inhibitor of PP1 (NIPP1), or NIPP1-KTK that is defective in its inhibitory activity [32]. Cells were then infected with GFP-NDV (Newcastle Disease Virus). NDV is an ssRNA virus that triggers the TLR7 signaling pathway (in monocytes and dendritic cells) in addition to RIG-I pathway (in mouse embryo fibroblasts), leading to activation of IRF7 for IFN-I production. IRF7 phosphorylation was evaluated by flow cytometry after 6 h and IFNα production in medium was measured by ELISA after 24 h. As expected, IRF7 phosphorylation is significantly higher in cells expressing wild type (WT) NIPP1 (34.0%) compared to that in cells expressing NIPP1-KTK (11.8%) (Fig 4, A and B). Correspondingly, IFNα concentration is significantly increased in the medium of the cells expressing WT NIPP1 (Fig 4, C). We also assessed the production of IL6, which is induced by the TLR7/NFκB signaling axis. Surprisingly, IL6 production is significantly decreased in the medium of the cells expressing WT NIPP1 (Fig 4, D), suggesting that PP1 is a positive regulator of NFκB activation downstream of TLR7 signaling in response to virus infection. Consistent with our findings, a recent report show that PP1γ interacts with TRAF6, and enhances NFκB-mediated inflammatory responses [12]. To ensure the effect on IFNα production is dependent on IRF7, we infected $Irf7^{+/+}$ and $Irf7^{-/-}$ mouse macrophages with NDV, and measured IFNα production. As expected, IFNα production is defective in $Irf7^{-/-}$ macrophages (Fig 4, E), indicating that NDV-triggered IFNα production depends on IRF7. Collectively, our results show that PP1 is a negative regulator of IRF7 phosphorylation and activation, but is likely a positive regulator of TLR7 activation of NFκB, in innate immune responses.

Blockage of PP1 activity impairs viral replication

Since we show that PP1 inhibits IFNα production, we then evaluated if PP1 can affect GFP-NDV replication in U937 cells using microscopy (Fig 4, F), and flow cytometry (Fig 4, G). Results show that viral replication, represented by GFP expression, is significantly inhibited in cells expressing WT NIPP1 than that in cells expressing NIPP1-KTK, indicating that PP1 promotes viral replication in the cells. We also performed the above experiments in the mouse macrophage cell line Raw264.7, and similar results were obtained (data not shown). The evaluation of IRF7 phosphorylation, however, was not feasible in Raw264.7 cells due to the lack of a commercial antibody against mouse phospho-IRF7.

In addition, we also used the PP1-specific inhibitor tautomycin (or DMSO control) to treat Raw264.7 cells before infection with GFP-NDV (or mock). Tautomycin has been widely used for the study of PP1 functions in different contexts [9, 16, 33]. Notably, tautomycin treatment results in a remarkable inhibition in viral replication, as shown by GFP-expressing cells (Fig 5, A–C), and a remarkable increase in IFNα production (Fig 5, D). The specificity of tautomycin on PP1 was verified with the use of the PP1 mutants PP1α(T320A) and PP1α(T320E). Results show that the constitutively active mutant PP1α(T320A) ($p=0.0029$), but neither WT ($p=0.0870$) nor the phosphatase-dead mutant PP1α(T320E) ($p=0.4834$), offsets the effect of tautomycin treatment on IFNα production (Fig 5, E, data for DMSO control is not shown), indicating that tautomycin specifically inhibits PP1 activity.

Taken together, these results (Figs 4 and 5) demonstrate that blockage of PP1 activity enhances IRF7 transcriptional activity and IFNα production, which impairs viral replication.

PP1 expression is induced during immune responses

To further understand the involvement of PP1 in regulation of IRF7-mediated antiviral activity, we examined the possibility that PP1 expression is regulated during antiviral responses. U937 cells were treated with IFNα2 or the TLR3 ligand poly(I:C), or infected with GFP-NDV. Results show that both treatments ($p=0,0266$ for IFNα2 and $p<0.001$ for poly(I:C)) and NDV infection ($p=0.0015$) significantly induce PP1 expression in time-dependent manners, as evaluated by flow cytometry and immunoblotting (Fig 6). The TLR7 ligand gardiquimod also consistently induces PP1 expression although its effect is smaller (data not shown). Poly(I:C) senses TLR3 signaling, and both TLR3 and TLR7 signaling activates IRF7. Thus, we conclude that PP1 is induced during viral infection.

DISCUSSION

Regulation of IRF7 phosphorylation is of paramount importance for controlling antiviral immunity in response to viral infection. IRF7 is required not only for IFN priming at early stage, but also for IFN amplification at later stages when robust IFN-I production depends on a positive regulatory circuit between IRF7 and IFNs [3, 34, 35]. This robust reaction is turned off soon after infection under normal physiological conditions, but excessive production of IFN-I is fatal to the cell. The underlying mechanism for this shutdown is not understood. We show in this study that PP1 targets IRF7 key phosphorylation sites, dampens IRF7 phosphorylation and abolishes its activity, and therefore abolishes IFN-I antiviral responses.

PP1 exerts its function through interaction with a wide variety of regulatory subunits, which function as targeting subunits, substrates and/or inhibitors [8]. These regulatory subunits are generally unrelated, but most (about 70%) possess the RVxF motif, a canonical PP1-binding sequence, or the motif of FxxR/KxR/K [36]. We have here identified IRF7 as a novel PP1-interacting factor, and it is targeted by PP1 for dephosphorylation. As the closest family member to IRF7, a recent report has shown that IRF3 also interacts with PP1, which negatively regulates IRF3 phosphorylation and IRF3-mediated IFN-I production downstream of TLR and RLR signaling pathways in macrophages [15]. Interestingly, we

have also identified a conserved PP1-binding site, RQVF, in IRF3 protein (spanning aa 213–216 of human IRF3, data not shown).

The host innate immune system defends against invading pathogens initially by triggering signaling pathways mediated by the transmembrane receptors TLRs [37], and cytoplasmic receptors that include RLRs [38, 39], NLRs [40], cGAS [41, 42], IFI16 [43], DDX41 [44, 45], DHX9/36 [46, 47], RNA polymerase III [48], TRIM5α [49], ISG56 [50], LRRFIP1 [51], MRE11 [52], amongst others. IRF7 is phosphorylated and activated downstream of many of these innate immune pathways for induction of IFN-I gene expression (especially IFNαs) [1]. Our results are the first to identify PP1 as a phosphatase that targets key activating phosphorylation sites of IRF7, attenuating its activity and blocking the IFN-I response during viral infection. Thus, our study has addressed an important knowledge gap regarding IRF7-mediated IFN-I innate immune response, and has broad significance in antiviral innate immunity.

MATERIALS AND METHODS

Constructs, antibodies, and reagents

Flag-PP1 (α, β and γ) expression plasmids cloned in Flag-CMV4 were provided by Dr. Sherry Winter [53]. Flag-IRF7 expression plasmids and its mutants were described previously [54, 55]. 2XMyc-IRF7 plasmids were generated by subcloning of 2XMyc-IRF7 fragment (by PCR amplification from pcDNA3-IRF7) into pCMV-Tag3B (Stratagene). TLR7 cDNA was cloned from human genome (the human B cell line BJAB) by PCR amplification. Deletion and point mutants were generated by site-directed mutation (Stratagene), and verified by sequencing. PE-conjugated IRF7(p477/479) antibody (clone K47–671) for flow cytometry was purchased from BD Biosciences. The same clone with higher concentration but without conjugation was customized for immunoblotting. Mouse anti-PP1 (clone E-9), mouse anti-PP1α (clone G-4), goat anti-PP1α (clone C19), mouse anti-IRF7 (clone G-8), rabbit anti-IRF7 (clone H-246), goat anti-mouse IgG-HRP, mouse anti-rabbit IgG-HRP, and mouse anti-goat IgG-HRP, were purchased from Santa Cruz. Flag (clone M2) and Myc (clone 9E10) antibodies were from Sigma and Roche, respectively. FITC-, PE-, APC-, and PerCP-eFluor710-conjugated antibodies for flow cytometry were purchased from eBioscience. Gardiquimod (designated as “Gardi” in figure legend) and HIV-1 ssRNA40 and controls were purchased from Invivogen. IFNα2 was purchased from Sigma. Tautomycin was purchased from EMD Millipore.

Cell lines

293 and 293T are human kidney epithelial cell lines, Raw264.7 is mouse macrophage cell line, and U937 is a human acute myeloid leukemic cell line. IB4, SavIII, and LCL00045 are human B cell lines transformed with EBV. Irf7+/+ and Irf7−/− mouse macrophages were gifts from Dr. Kate Fitzgerald. 293-TLR7 stable cell line was generated by transfecting 293 cells with pcDNA3-TLR7 (human), followed by selection with 0.8 mg/ml G418 for two weeks. Epithelial cells and macrophages are cultured with DMEM plus 10% FBS and antibiotics. B cells, U937 cells, and mouse macrophages are cultured with RPMI1640

medium plus 10% FBS and antibiotics. All cell culture supplies were purchased from Life Technologies.

Transfection

293 and 293T cells were transfected with Effectene. U973, Raw264.7, and primary cells were transfected with specific Nucleofector kits (Lonza).

Promoter-reporter assays

293 cells were transfected with expression plasmids as indicated together with IFNα4p-Luc and Renilla as internal transfection control. Empty vector was used to equalize the total amounts of DNA in all transfections. Cells were collected 24 h after transfection. Luciferase activity was measured with equal amounts (10% of total for each sample) of protein lysates with the use of a Dual Luciferase Assay kit (Promega), on a multimode microplate reader (Turner Biosystems). Results are the mean ± standard error (SE) of duplicates for each sample. At least three consistent results were obtained from independent experiments and representative results are shown. The ability of the empty vector controls to activate IFNA4p-Luc was set to 1.

Immunoprecipitation and immunoblotting

293T cells in 60-mm dishes were collected 48 h after transfection. Cells were lysed with NP40 lysis buffer, and cell lysates were subjected to immunoprecipitation with 1.5 μg indicated antibodies for overnight, and then incubated with 40 μl Protein A/G beads (Santa Cruz) for 1 h. After three washes with NP40 lysis buffer, proteins on beads were denatured before separated by SDS-PAGE. Immunoblotting was carried out with the indicated antibodies and signals were detected with an enhanced chemiluminescence (ECL) kit following the manufacturer's protocol (Amersham Pharmacia Biotech).

Chromosome immunoprecipitation (ChIP)

ChIP was performed with the use of ChIP-IT Express Enzymatic kit (Active Motif). Briefly, 293T cells were transfected with Flag-IRF7 or its mutants, IKKε, and PP1α expression plasmids. Cells were harvested 48 h after transfection, and subjected to crosslinking by adding formaldehyde to a final concentration of 1% for 30 min at room temperature with slow rotation. Crosslinking was stopped by adding glycine to a final concentration of 125 mM for 5 min. Shearing and enzymatic digestion of chromatin, IP (with Flag antibody M2, Sigma), and DNA recovery were performed following the manufacturers' instructions. qPCR was performed with the human IFNA4 gene promoter primers: F: 5′-AGCCTTTGAGTGCAGGTG-3′ and R: 5′-TGAACTTCGGCCTCTAGG-3′, and β-actin promoter primers (control): 5′-CCAACAAAGCACTGTGG-3′ (forward) and 5′-GGGCGAAGGCAACGC-3′ (reverse) [56].

Virus infection and TLR7 challenge

GFP-NDV was generously provided by Dr. Christopher Basler with permission from Dr. Peter Palese [57]. GFP-NDV ($2X10^4$ fluorescent focus forming units (FFU)) was added to RAW264.7 and U937 cells and mouse macrophages in serum-free medium, and then

incubated at 37ºC for 1.5 h, before complete medium was replaced. For TLR7 challenge, cells were incubated with Gardiquimod or HIV1 ssRNA40, with indicated concentrations and time-points. Human and mouse IFNα production was measured by ELISA with human and mouse VeriKine IFNα ELISA kits (PBL Assay Science), respectively. IL-6 ELISA kits were from BioLegend, Inc.

Flow cytometry

Flow cytometry was performed as described previously [58], on an AccuriTM C6 flow cytometer (BD) using FlowJo software (Tree Star, Inc). For PP1 expression, cells were fixed in fixation buffer (Biolegend) for 20 min, and then washed before incubated with permeabilization buffer (eBioscience) for 40 min. Cells were then incubated with PP1 primary and secondary antibodies and then further incubated anti-CD4 PerCP-Cy5.5 before flow cytometry analysis. Live cells from PBMCs were gated for pIRF7 or PP1α expression. The gate lines for pIRF7 or PP1α were determined according to corresponding isotope controls. All of the isotype controls were negative.

Statistical analysis

The significance of the difference between control and experimental groups was tested using Student's t-test or one-way ANOVA using Graphpad Prism (version 5). p values were calculated by unpaired Student's *t*-test (between-subjects comparison). $p<0.01$ (**) and $P<0.05$ (*) were considered significant and $p<0.001$ (***) was considered very significant. For comparisons of multiple groups (more than two), data were analyzed by one-way ANOVA and results were adjusted with the Bonferroni's correction. Data are expressed as mean ± standard error (SE) of duplicate samples, and representative results from at least three independent experiments are shown.

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Abbreviations

PP1	Protein phosphatase 1
NIPP1	Nuclear inhibitor of PP1
IFN	Interferon
IRF7	Interferon regulatory factor 7
TLR7	Toll-like receptor 7
NDV	Newcastle disease virus

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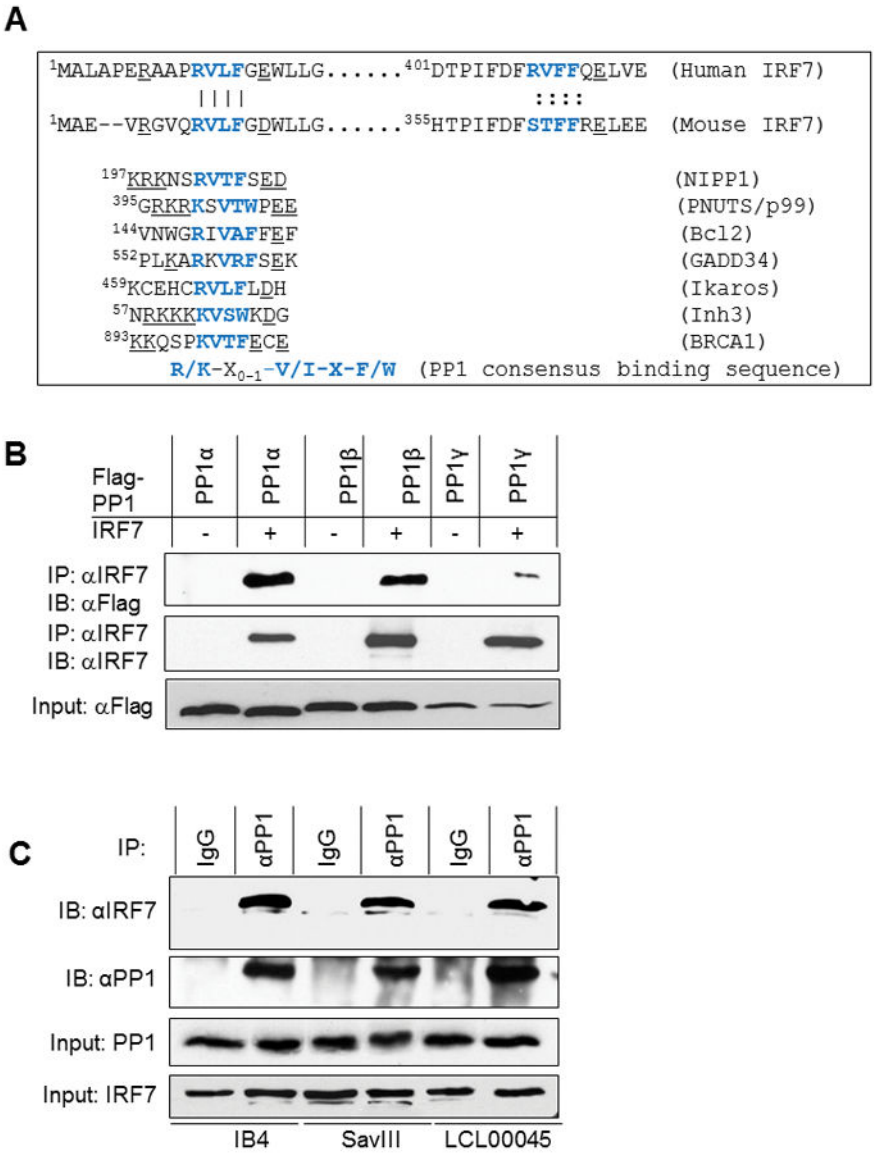

Fig 1. IRF7 interacts with PP1

A. IRF7 protein containsIllustration of IRF7 protein showing the two potential PP1-binding sites, which matches PP1-binding consensus sequence R/K-X$_{0–1}$-V/I-X-F/W. The PP1-binding core sequences are bold and the contributing amino acids (lysine/arginine proximal to, and aspartate/glutamate distal to, the core sequence) are underlined**. B**. 293T cells were transfected with IRF7 and Flag-PP1(α, β, or γ) expression plasmids. Cells were collected for immunoprecipitation with mouse anti-IRF7, and immunoblotting was performed with Flag antibody or rabbit anti-IRF7. 5% input was probed for PP1 expression with Flag antibody. **C**. Cell lysates prepared from EBV-transformed lymphoblastic cell lines IB4, SavIII, and LCL00045 were subjected to IP with mouse anti-PP1 or with mouse IgG control. Immunoblotting was performed with rabbit anti-IRF7. Representative results from three independent experiments are shown.

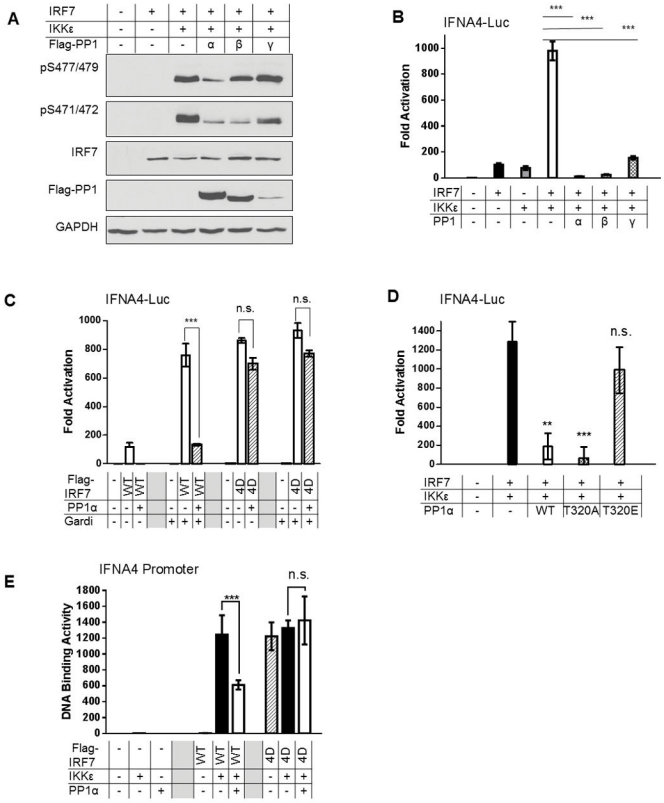

Fig 2. PP1 attenuates IRF7 phosphorylation and transcriptional activity

A. 293T cells were transfected with the plasmids as indicated. Cells were collected and subjected to immunoblotting with anti-IRF7 pS477/479 and pS471/472. **B**. PP1 (α, β, and γ) diminishes IKKε-stimulated IRF7 transcriptional activity (*** $p<0.0001$, one-way ANOVA with post hoc Bonferroni t-test). 293 cells were transfected with IRF7 and Flag-PP1 (α, β, or γ), and IKKε expression plasmids, pGL3/ISRE-Luc, and Renilla. Dual luciferase assays were performed 24 h after transfection. The ability of the vector control to activate the promoter construct was set to 1. **C**. Cells were treated with the TLR7 ligand gardiquimod before subjected to dual luciferase assays (*** $p<0.0001$, unpaired t test). **D**. 293 cells were transfected with IRF7, IKKε, and WT PP1α (or its mutants) expression plasmids, pGL3/ISRE-Luc, and Renilla. Dual luciferase assays were performed 24 h after transfection. WT PP1α, constitutively active mutant PP1α(T320A), phosphatase-dead mutant PP1α(T320E). One-way ANOVA with post hoc Bonferroni t-test was performed. ***$p=0.0010$, **$p=0.0017$, n.s.: non-specific. **E**. 293T cells were transfected with IRF7 or IRF7(4D), IKKε and PP1. Cells were subjected to ChIP after 48 h. DNA pellets were used for qPCR using the IFNA4 promoter primers. (***$p=0.0005$, unpaired t test). Representative results from at least three independent experiments are shown.

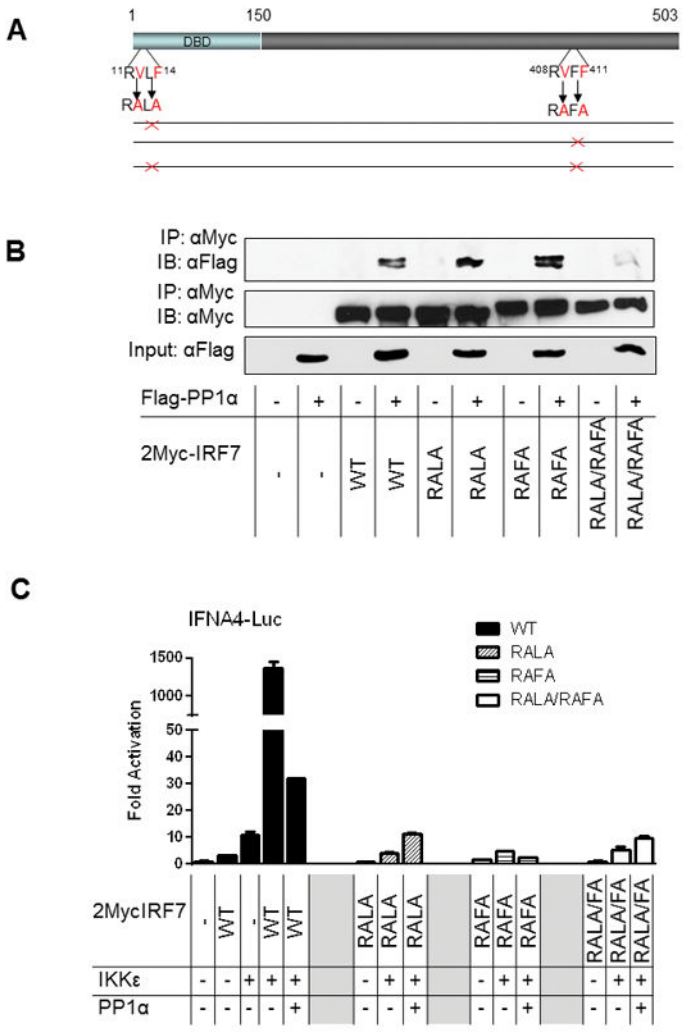

Fig 3. Both PP1-binding motifs on IRF7 are required for its interaction with PP1 and for its activation

A. A diagram showing the potential PP1-binding sites on IRF7 and point mutation. **B**. 293T cells were transfected with Flag-PP1α, 2XMyc-IRF7 or its mutants. Cells were collected for IP with Myc antibody. Immunoblotting was performed with Flag or Myc antibody. **C**. 293 cells were transfected with 2XMyc-IRF7 or its mutants, IKKε, Flag-PP1α, pGL3/ISRE-Luc, and Renilla. Cells were collected 24 h later for dual luciferase assays. Representative results from three independent transfection experiments are shown.

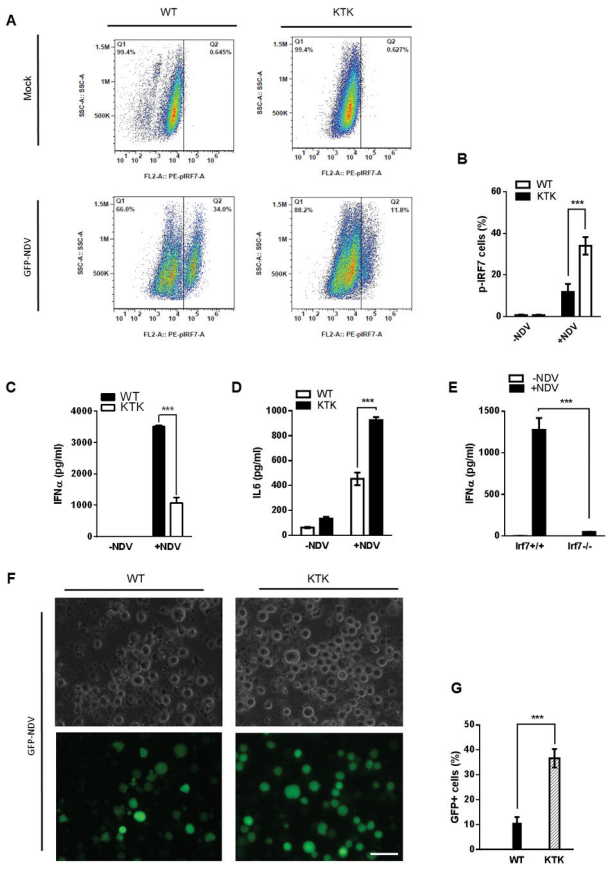

Fig 4. Inhibition of PP1 activity enhances IRF7 phosphorylation and IFNα production, and impairs viral replication in U937 cells

A–D and **F–G**. U937 cells were transfected with Flag-tagged WT-NIPP1 or its mutant NIPP1-KTK. Cells were then infected with GFP-NDV and analysed by flow cytometry for IRF7 phosphorylation (**A–B**). Cell culture medium was tested for (**C**) IFNα and (**D**) IL6 production by ELISA. **E**. $Irf7^{+/+}$ and $Irf7^{-/-}$ mouse macrophages were infected with GFP-NDV. IFNα production was measured by ELISA **F** and **G**. GFP expression in U937 cells was analysed by fluorescence microscopy (**F**), or flow cytometry (**G**), Scale bar=10 μm. ***p<0.0001, unpaired t test. Results are from a single experiment representative of three independent transfection experiments.

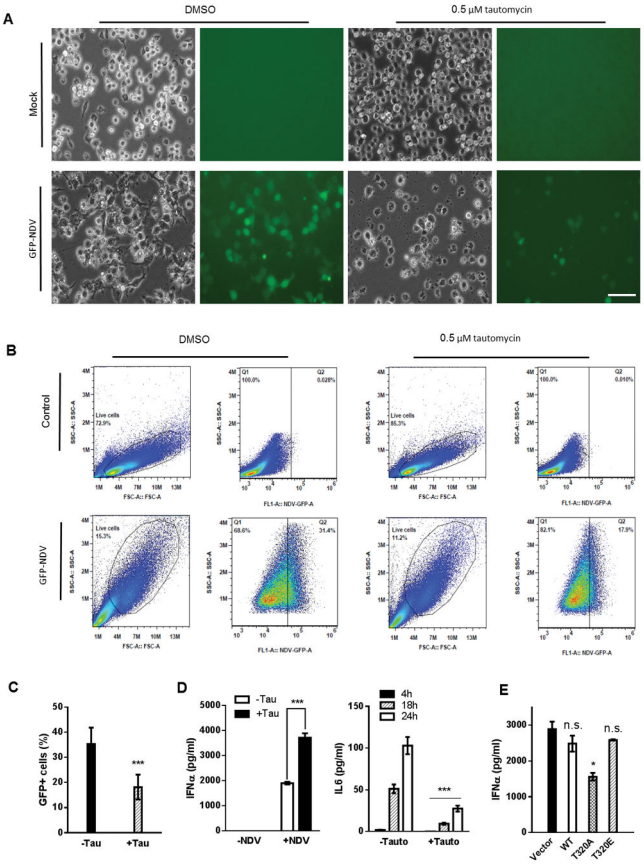

Fig 5. Inhibition of PP1 activity potentiates IFNα production and impairs viral replication in Raw264.7 cells

A–D. Raw264.7 cells were treated with tautomycin (or DMSO control) and then infected with GFP-NDV. GFP expression was evaluated by (**A**) fluorescence microscopy or (**B–C**) flow cytometry. Scale bar=10 μm. **D**. IFNα and IL6 production in the medium of Raw264.7 cells was measured by ELISA and normalized by GFP-expressing cell numbers. ***p<0.0001, unpaired t test. **E**. Raw264.7 cells were transfected with empty vector, WT PP1α or its mutants. Subsequently, cells were treated with tautomycin (or DMSO control) and infected with GFP-NDV as in **A**. One-way ANOVA with post hoc Bonferroni t-test was performed. *p=0.0029. Results are from a single experiment representative of three independent experiments.

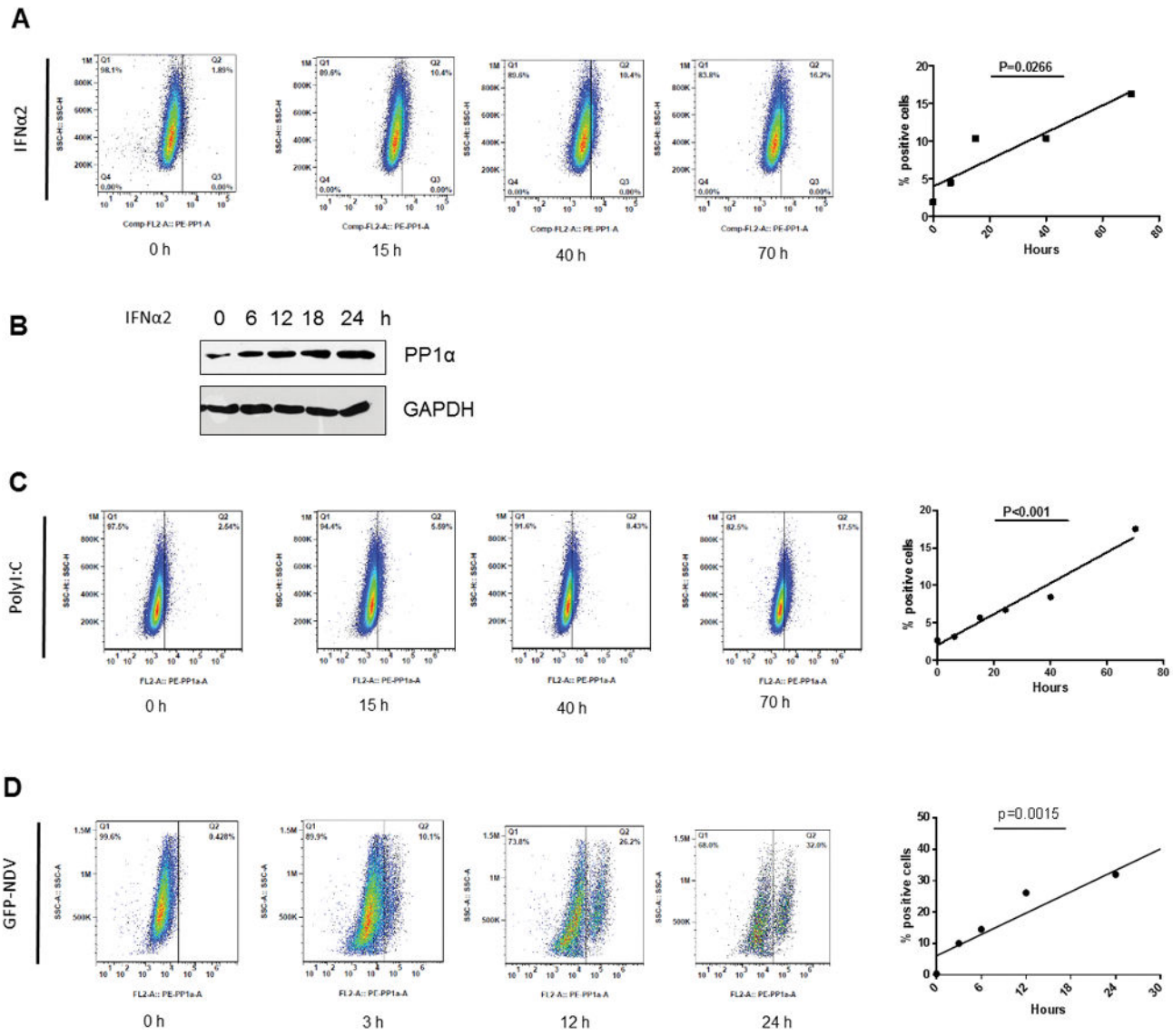

Fig 6. PP1 is induced during antiviral immune responses

U937 cells were treated with IFNα2 (**A** and **B**), or Poly(I:C) (**C**), or GFP-NDV (**D**) at indicated time points. Cells were then fixed and permeabilized before incubation with the mouse anti-PP1α (nd then with the F(ab')2 anti-mouse IgG and analysed by flow cytometry (**A**, **C** and **D**), or subjected to immunoblotting (**B**), for PP1 expression. Linear regression analysis was performed. *p<0.05 (**A**), ***p<0.001 (**C**), **p<0.01 (**D**). Results are from a single experiment representative of three independent experiments.