Published in final edited form as:
Cell Signal. 2012 November ; 24(11): 2197–2204. doi:10.1016/j.cellsig.2012.06.017.

PPM1B negatively regulates antiviral response via dephosphorylating TBK1

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Abstract

The production of type I interferon must be tightly regulated and aberrant production of type I interferon is harmful or even fatal to the host. TBK1 phosphorylation at Ser172 plays an essential role in TBK1-mediated antiviral response. However, how TBK1 activity is negatively regulated remains poorly understood. Using a functional genomics approach, we have identified PPM1B as a TBK1 phosphatase. PPM1B dephosphorylates TBK1 in *vivo* and in *vitro*. PPM1B wild-type but not its phosphatase-deficient R179G mutant inhibits TBK1-mediated antiviral response and facilitates VSV replication in the cells. Viral infection induces association of PPM1B with TBK1 in a transient fashion in the cells. Conversely, suppression of PPM1B expression enhances virus-induced IRF3 phosphorylation and IFNβ production. Our study identifies a previously unrecognized role for PPM1B in the negative regulation of antiviral response by acting as a TBK1 phosphatase.

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Keywords

PPM1B; TBK1; phosphatase; antiviral response

Introduction

The innate immune system acts as the first line of defense against invasion by microbial pathogens, including viruses, bacteria, and parasites [1–4]. The detection of pathogens occurs through the germline-encoded pattern recognition receptors (PRRs), which at least includes Toll-like receptor (TLR) family, retinoic acid inducible gene I (RIG-I)-like receptor (RLR) family, Nod-like receptor family and C-type lectin receptor (CTR) family [5–9]. Upon PRRs recognize invading viruses, they initiate a series of signaling events leading to robust production of type I interferons (IFNs) and proinflammatory cytokines [10, 11]. Type I IFNs further activate downstream signaling pathways that lead to transcriptional induction of a wide range of antiviral genes to cooperatively elicit cellular antiviral response through various mechanisms [10, 12]. Although type I interferon is required for viral clearance, the production of type I interferon must be tightly regulated because aberrant production of type I interferon plays a pathological role in autoimmune disorders or could be fatal to the host [13, 14].

Activation of RIG-I by double- and single-stranded RNAs or certain viruses leads to its conformational change and then it is recruited to the mitochondrial adaptor protein MAVS (also known as VISA, IPS-1, and Cardif) [15–20]. MAVS is also associated with a scaffolding protein STING (also known as MITA) to recruit the kinase TBK1 to the MAVS-associated signaling complex [21, 22]. The C terminus of STING further recruits IRF3 and facilitates the phosphorylation of IRF3 by TBK1. Phosphorylated IRF3 translocates to the nuclear to induce type I IFNs production to initiate the antiviral response [23].

TBK1 is a serine/threonine kinase that has been shown to play an essential role in mediating antiviral response [24–26]. Upon viral infection, GSK3β binds with TBK1 and facilitates TBK1 auto-phosphorylation at Ser172 within its kinase activation loop [27]. The activated TBK1 then phosphorylates IRF3/7 and leads to IRF3/7 nuclear translocation as well as IRF3/7-dependent IFN-α/β and RANTES gene expression [28]. However, how the activated TBK1 is negatively regulated by its phosphatase(s) is poorly understood. To our knowledge, there are two phosphatases (SHP2 and SHIP1) have been reported to negatively regulate INFβ production by targeting on TBK1 [29, 30]. SHP-2 inhibits INFβ production by a phosphatase activity-independent mechanism [30]. SHIP1 is a member of the inositol polyphosphate-5-phosphatase (INPP5) family [29]. In this report, we used a functional genomics approach to identify the TBK1 phosphatase(s) by screening a library of serine/threonine phosphatases whose overexpression inhibits TBK1-mediated IRF3 phosphorylation and IFNβ gene expression. Here we present evidence that PPM1B/PP2Cβ functions as a TBK1 phosphatase that dephosphorylates TBK1 at serine172 and terminate TBK1-mediated IRF3 activation and IFNβ gene expression.

Experimental procedures

Cell culture and transfection

HEK293T and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 mg/ml) and glutamine (2 mM). HEK293T and HeLa cells were transfected with FuGene 6 (Roche) and FuGene HD (Roche) respectively according to the manufacturer's recommendation.

Plasmids Construction

Human serine/threonine phosphatase Expression Library was constructed as previously described [31]. The full-length open reading frame of the wild-type PPM1B was subcloned in frame into mammalian expression vector pcDNA3.1 with an N-terminal 3Myc tag (Invitrogen). The PPM1B (R179G) mutant expression constructs were generated by site-directed PCR mutagenesis (Stratagene) and verified by DNA sequencing. Mammalian expression vector for FLAG-TBK1, FLAG-MAVS and FLAG-RIG-I-Card was obtained from Dr. Paul Chiao (The University of Texas MD Anderson Cancer Center, TX). The retrovirus packing vector Pegpam 3e and RDF vectors were obtained from Dr. Gianpietro Dotti (Baylor College of Medicine). The IFNβ dependent *firefly* luciferase reporter plasmid and pCMV promoter-dependent *Renilla* luciferase reporter plasmid were purchased from Clontech (Mountain View, California). For bacterial expression of PPM1B proteins, cDNAs encoding PPM1B wild-type (PPM1B-wt) and phosphatase-deficient R179G mutant (PPM1B-R179G) were subcloned into pRSET vector (Invitrogen) to generate His-tagged fusion proteins. A pSuper-retro vector (Oligoengine) was used to generate shRNA plasmids for PPM1B. For PPM1B, target sequences were 5′-AATGCAGGAAAGCCATACTGA-3′ (sh-PPM1B-1), 5′-AACTTCTGGAGGAGATGCTGA-3′ (shPPM1B-2); Sequences for sh-Control is: 5′-CTGGCATCGGTGTGGATGA-3′. The authenticity of these plasmids was confirmed by sequencing.

Antibodies and reagents

Antibodies against HA- and Myc-epitope were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-β-actin antibody was from Sigma-Aldrich Co. (St. Louis, MO). Antibodies against Phospho-IRF3 (Ser396) and IRF3 were from Cell Signaling Technology, Inc. (Danvers, MA). Anti-phospho-TBK1 (Ser172) was from BD Biosciences, Inc. Antibody against PPM1B was from Bethyl Laboratories, Inc. (Montgomery, TX). SeV was purchased from Charlers River. FuGene 6 and FuGene HD transfection reagents were from Roche (Alameda, CA). Cell culture media were obtained from Invitrogen (Carlsbad, CA). Nitrocellulose membrane was obtained from Bio-Rad (Hercules, CA).

Luciferase reporter gene assays

The luciferase reporter gene assay was performed using a dual luciferase reporter assay system (Promega, Madison, WI) as described previously [32]. Briefly, targeted cells were transiently cotransfected with specific vectors and an IFNβ-dependent *firefly* luciferase reporter construct as well as a *Renilla* luciferase control construct. Cellular extracts were prepared 36 hrs post-transfection and the luciferase activities were determined. Relative IFNβ luciferase activity was normalized to *Renilla* luciferase activity. Data are presented as the mean ± standard deviation.

Quantitative reverse transcription PCR (qRT-PCR) analyses

Total RNAs were prepared using TriZol reagent (Invitrogen) from HeLa sh-Control and sh-PPM1B cells. qRT-PCR was carried out by using 100 ng of total RNA. A volume of 10 μl of 2x QuantiTect SYBR Green RT-PCR Master Mix (Qiagen), 0.2 μl QuantiTect RT Mix (Qiagen), 1 μl of 10 μM forward and reverse primers, and 6.8 μl of RNase-free Water were added to each sample for analysis by absolute quantification. qRT-PCR was performed in 96-well plates with the DNA Engine OpticonTM System (MJ Research). The mRNA levels of target genes in the samples were normalized against β-actin. Each target gene was measured in triplicate. The primers were designed by using the Primer3.0 software and are as follows: IFNβ: 5′-CACACAGACAGCCACTCACC-3′ and 5′-TTTTCTGCCAGTGCCTCTTT-3′; β-actin: 5′-ACCGCGAGAAGATGACCCAG-3′ and 5′-TTAATGTCACGCACGATTTCCC-3′.

Generation of stable HeLa cells expressing shRNA targeting PPM1B

The pSuper- PPM1B retroviral construct was transfected into HEK293T cells with retrovirus packing vector Pegpam 3e and RDF vector using FuGene 6 transfection reagent. Viral supernatants were collected after 48 and 72 hours. HeLa cells were incubated with virus-containing medium in the presence of 4 mg/ml polybrene (Sigma Aldrich). Stable cell lines were established after 10 days of puromycin (2 μg/ml) selection and knockdown efficiency of PPM1B was confirmed by Western blotting.

Immunoblotting and immunoprecipitation

Cells were harvested in ice-cold PBS (pH 7.4) and spun down. The pellets were dissolved in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% IGEPAL, 0.25% Na-deoxycholate, 1 mM PMSF, 1 mM DTT, 10 g/ml aprotinin, 10 g/ml leupeptin, 1 mM Benzamidine, 20 mM disodium p-nitrophenylphosphate (pNPP), 0.1 mM sodium orthovanadate (OV), 10 mM sodium fluoride (NaF), phosphatase inhibitor cocktail A and B (Sigma Aldrich)). The cell lysates were either subjected directly to 10% SDS-PAGE for immunoblotting analysis or immunoprecipitated for 3 hrs with the indicated antibodies. Protein complexes were immunoprecipitated with protein A -agarose (Santa Cruz Biotechnology) for 3 hrs, then washed three times with wash buffer containing 20 mM HEPES (pH 7.4), 50 mM NaCl, 2.5 mM MgCl2, 0.1 mM EDTA, and 0.05% Triton X-100. For immunoblotting, the immunoprecipitates or 10% whole cell lysates (WCL) were resolved on SDS-PAGE and transferred to nitrocellulose membranes. The membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG using the ECL-Plus Western blotting system (GE Healthcare Bio-sciences Corp., USA) according to the manufacturer's instruction.

Purification of His-PPM1B fusion proteins

The bacterial expression plasmids (His-PPM1B-wt and His-PPM1B-R179G) were transformed into E. coli BL-21 strain (Invitrogen), and then the bacteria were grown in Luria broth at 37°C to an A600=0.6 before induction with 0.1 mM isopropyl β-d-thiogalactoside (IPTG) for 4 hrs at 30°C. Bacteria were pelleted and lysed with His extraction buffer (50 mM Tris–HCl, pH 8.5, 100 mM NaCl, 1 mM DTT, 5mg/ml lysozyme, and 1 mM PMSF) 45 min on ice. The bacteria were sonicated at 4°C in 1% Sarcosyl (Sigma Aldrich), and after which Triton X-100 (1%), 5ug/ml DNase, and 5ug/ml RNase (Roche) were added. The lysates were centrifuged at 15,000×g and the supernatants containing His-tagged fusion proteins were collected. A total of 150 μl His-Select TM Nickel Affinity gel (Sigma) was incubated with each bacterial lysate supernatant at 4°C overnight. The beads were washed three times in extraction buffer containing 0.5% Triton X-100, one time in extraction buffer containing 0.1% Triton X-100. Proteins were eluted in elution buffer (250mM imidazole, 50mM Tris-HCl (pH 8.0), 10% glycerol, 300mM NaCl) and dialyzed in dialyzing buffer (20mM Hepes (pH 7.9), 150mM KCl, 0.2mM EDTA, 20% glycerol). The protein concentrations were determined with a Bradford Protein Assay (Bio-Rad) and proteins were subjected to SDS-PAGE and visualized by Coomassie Blue staining.

In vitro Phosphatase Assays

HEK293T cells seeded onto 10 cm dishes were transfected with the FLAG-TBK1 expression plasmid. The FLAG-TBK1 proteins were immunoprecipitated from cell extracts with anti-FLAG antibody. After washing beads three times with the wash buffer, the immunoprecipitated FLAG-TBK1 were then incubated with or without recombinant His-PPM1B wild-type or phosphatase-deficient R179G mutant proteins in phosphatase buffer (250 mM imidazole, pH 7.2, 1 mM EGTA, 25 mM MgCl2, 0.1% 2-mercaptoethanol, 0.1%

BSA) at 30°C for 30 min. The phosphatase reactions were then terminated by boiling the samples in protein sample buffer and proteins were separated by 10% SDS-PAGE. The levels of FLAG-TBK1 phosphorylation were measured by immunoblotting analysis with antibody against phospho-TBK1.

Results

PPM1B inhibits TBK1-induced IRF3 activation and IFNβ gene expression

Phosphorylation of TBK1 at Ser172 within the kinase activation loop is essential for virus infection-induced type I interferon production [27]. We hypothesized that the potential TBK1 phosphatase targeting this site might be a member of protein serine/threonine phosphatase family. We then generated a library of mammalian expression vectors that encode 23 protein serine/threonine phosphatases (catalytic subunits if it is multimeric) including 11 PPPs and 12 PPMs. We used an IFNβ luciferase reporter assay to assess the effect of overexpression of each phosphatase on TBK1-induced IFNβ expression. In this screen, as shown in Figure 1A, PPM1B almost completely abolished TBK1-induced IFNβ reporter gene expression whereas other phosphatases had either no or less effect. To validate our results from this reporter screening assay, we chose the phosphatases with some degree of inhibition on TBK1-induced IFNβ including PPM1B on the status of TBK1-induced IRF3 phosphorylation at Ser396 that is essential for IRF3 activation and IRF3-dependent IFNα/β gene expression. Consistent with our reporter screening assay, overexpression of PPM1B abolished TBK1-induced-phosphorylation of IRF3 at Ser396 (Figure 1B). Taken together, we identified that PPM1B is the only member in our phosphatase library that can significantly inhibit both TBK1-induced IRF3 activation and IFNβ gene expression.

PPM1B phosphatase activity is required for its inhibitory effect on RIG-I-CARD-, MAVS- and TBK1-induced IRF3 activation and IFNβ gene expression

To understand the molecular mechanism of PPM1B function in antiviral response, we test whether PPM1B also inhibit TBK1 upstream molecules-induced IRF3 activation and IFNβ gene expression, and whether its inhibitory effect depends on its phosphatase activity. Overexpression of PPM1B wild-type but not its phosphatase-deficient mutant (R179G) strongly inhibited the RIG-I-CARD, MAVS and TBK1-mediated IFNβ luciferase reporter activities (Figure 2A, 2B, 2C). Consistently, we found that co-overexpression of PPM1B wild-type but not its R179G mutant inhibited RIG-I-CARD, MAVS and TBK1-induced IRF3 phosphorylation at Ser396 (Figure 2D, 2E, 2F). Furthermore, co-overexpression of PPM1B wild-type but not its R179G mutant inhibited RIG-I-CARD, MAVS and TBK1-induced IFNβ production (Figure 2G, 2H, 2I). Together, these results suggest that PPM1B targets TBK1 to inhibit antiviral response.

PPM1B binds to the phosphorylated TBK1 and acts as a TBK1 Phosphatase

To further explore the functional relationship between PPM1B and TBK1, we first examined association of PPM1B with TBK1 and found that SeV infection induced association of PPM1B with TBK1 (Figure 3A). This result suggests that PPM1B is induced to interact with the activated TBK1 and subsequently dephosphorylate TBK1. Indeed, co-overexpression of PPM1B wild-type but not its phosphatase-deficient mutant inhibited TBK1 phosphorylation at Ser172 (Figure 3B). To get the direct evidence that PPM1B is a TBK1 phosphatase, we performed in *vitro* de-phospharylation assay. In this assay, FLAG-TBK1 was overexpressed in HEK293T cells, and phosphorylated FLAG-TBK1 was immunoprecipitated from cell extracts with anti-FLAG antibody and incubated with recombinant His-PPM1B wild-type and phosphatase-deficient R179G mutant proteins. The phosphorylation level of FLAG-TBK1 was much lower after co-incubation with His-PPM1B wild-type protein, but not

phosphatase-deficient R179G mutant (Figure 3C). These results demonstrate that PPM1B inhibits TBK1-mediated antiviral signaling by directly dephosphorylating TBK1 at Ser172.

PPM1B inhibits antiviral signaling

To further dissect the role of PPM1B in antiviral response, we explored whether PPM1B could inhibit SeV-triggered antiviral response. We first examined the effect of PPM1B overexpression on SeV-induced IFNβ promoter activity. In these assays, we found that overexpression of PPM1B wild-type but not its phosphotase-deficeint R179G mutant strongly inhibited SeV-induced IFNβ reporter gene expression (Figure 4A). Consistently, PPM1B wild-type but not its phosphotase-deficeint R179G mutant strongly inhibited SeV-induced IRF3 phosphorylation and IFNβ gene expression in HEK293T cells (Figure 4B, 4C). Furthermore, overexpression of PPM1B wild-type but not its phosphotase-deficeint R179G mutant rendered cells remarkably sensitive to viral infection and increased the level of VSV-eGFP-positive cells (Figure 4D). Taken together, these results indicate that PPM1B inhibits antiviral signaling through acting as a TBK1 phosphatase.

Suppression of PPM1B expression enhances antiviral signaling

Because PPM1B acts as a TBK1 phosphatase to inhibit antiviral response, we reasoned that knockdown of PPM1B would increase TBK1 phosphorylation and antiviral response. To test this hypothesis, we knocked down PPM1B expression by two PPM1B-specific shRNA in HEK293T cells. We found that specific knockdown of PPM1B markedly enhanced TBK1- and SeV- triggered IFNβ reporter activity (Figure 5A and 5B). To further confirm the results obtained from transient transfection assay, we then generated PPM1B stable knockdown HeLa cell lines using a retroviral transduction system and analyzed the effect of PPM1B knockdown on the SeV-induced TBK1 and IRF3 -Control and sh-PPM1B stable expression were then treated with SeV for the different time periods as indicated and subsequently lysed. We found that knockdown of PPM1B expression caused the enhanced phosphorylation of TBK1 and IRF3 in response to SeV infection (Figure 5C and 5D). Consistent with these results, IFNβ production was increased in PPM1B knockdown cells compared to control cells (Figure 5E). Taken together, these results suggest that specific knockdown of PPM1B increases virus-induced TBK1 phosphorylation and antiviral response.

Discussion

The innate immune system acts as the first line of defense against invasion by microbial pathogens. To avoid self-damage, the activated immune system must be tightly regulated negatively. TBK1 is a serine/threonine kinase that plays an essential role in mediating antiviral response [24–26]. Recent report also showed TBK1 phosphorylation at serine 172 plays an essential role in antiviral response [27]. However, how TBK1 phosphorylation is negatively regulated by phosphatase(s) is unclear. Using a functional genomics approach, we identified PPM1B as a TBK1 phosphatase that binds to and dephosphorylates TBK1 in *vivo* and in *vitro*. PPM1B negatively regulates antiviral response by acting as a TBK1 phosphatase.

To our knowledge, there are two phosphatase (SHP2 and SHIP1) have been reported to negatively regulate IFNβ production by targeting on TBK1 [29, 30]. The Src homology 2 domain-containing protein tyrosine phosphatase 2 (SHP-2) inhibited INFβ productions by a phosphatase activity-independent mechanism [29, 30]. C-terminal domain of SHP-2 directly bound with the kinase domain of TBK1 and thus inhibits INFβ production [30]. SHIP1 is a member of the inositol polyphosphate-5-phosphatase (INPP5) family that specifically hydrolyzes the 5-phosphate of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) to

produce PtdIns(3,4)P2 [29]. Although the two phosphates SHP2 and SHIP1 target TBK1 to negatively regulate IFNβ production, it is unlikely that the SHP2 and SHIP1 act as TBK1 phosphatase. Here we provide several lines of evidence to show that PPM1B is a TBK1 phosphatase: (1) PPM1B physiologically binds to TBK1 in an inducible manner by virus infection; (2) PPM1B dephosphorylates TBK1 in *vitro* and *in vivo*; (3) knockdown of PPM1B expression enhances virus-induced TBK1 phosphorylation and IFNβ expression.

Protein serine/threonine phosphatases in the human genome are mainly composed of two structurally distinct families: PPP and PPM/PP2C [33, 34]. The PPM family is a group of monomeric metal-ion-dependent phosphatases including PPM1A, PPM1B, PPM1C, PPM1D/wip1, PPM1E, PPM1F, PPM1G, PPM1H, PPM1J, PPM1L, PPM1K, PPM1M, PHLPP, PPTC7, and PPM2C. Protein phosphatase 1B (PPM1B) is a member of the PPM/PP2C family of Ser/Thr protein phosphatases that in humans is encoded by the *PPM1B* gene. We and other group have demonstrated that PPM1B associated with IKK and acts as IKK phosphatases to negatively regulates TNFα-induced NF-κB activity [31]. TBK1 is an IKK related kinase. Together, our results suggest that PPM1B may act as a major phosphatase to negatively regulate the activation of IKK and IKK related kinases to restrict these kinases-mediated diverse signal transduction pathways.

PPM1A and PPM1B proteins share 76% amino acid sequence identity [35, 36]. One of our interesting finding is that PPM1B but not PPM1A strongly inhibits TBK1-induced IFNβ production and IRF3 phosphorylation. These results suggest that although PPM1A and PPM1B shares high sequence identity, their physiologic function might be different. Genetic disruption of the mouse protein PPM1B gene leads to early pre-implantation lethality [37]. However, mice lacking PPM1A developed normally suggested that PPM1A and PPM1B do have non-redundant function [32].

Our data demonstrate that PPM1B plays a physiologically important role in the negative regulation of antiviral response. Based on the experimental data shown here and reported by others, we propose a model to illustrate how PPM1B could negatively control type I interferon signaling (Figure 6). Upon virus infection, pattern recognition receptors recognize viral RNAs and further activate STING which subsequently recruits IRF3 and TBK1. Virus infection induces GSK3β binding with TBK1 and facilitates TBK1 phosphorylation and activation. Once activated, TBK1 interacts with PPM1B and is rapidly dephosphorylated by bound PPM1B to terminate TBK1 activation and TBK1-mediated antiviral signaling.

Acknowledgments

This work was supported, in whole or in part, by the NIH/NINDS grant 1R01NS072420-01 (to J.Y.), the Virginia & L E Simmons Family Foundation Collaborative Research Fund (to J.Y.)

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Highlights

1. PPM1B inhibits TBK1-mediated antiviral response.
2. PPM1B dephosphorylates TBK1 *in vivo* and *in vitro*.
3. PPM1B inhibits antiviral response and facilitates VSV replication in the cells.
4. Suppression of PPM1B enhances virus-induced IRF3 phosphorylation and IFNβ production.

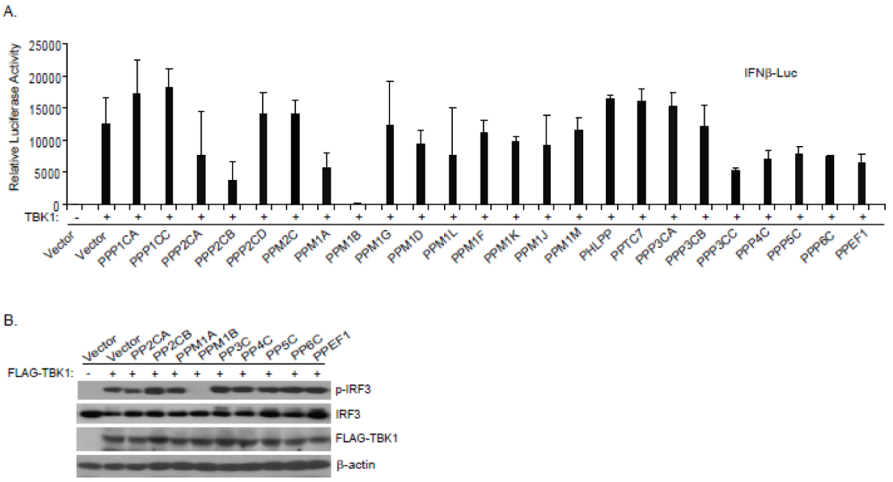

Figure 1.

PPM1B inhibits TBK1-mediated IFNβ activation. A. The effect of serine/threonine phosphatases and the catalytic subunit of polymeric phosphatases on the TBK1-induced IFNβ activation. HEK293T cells were transiently transfected with FLAG-TBK1 expression plasmid and an IFNβ *firefly* luciferase reporter plasmid along with empty vector or different phosphatase plasmids. *Renilla* luciferase plasmid was used to normalize all luciferase assays. Luciferase activity was measured 36h after transfection. (B) The effect of candidate serine/threonine phosphatases on the TBK1-induced IRF3 phosphorylation. HEK293T cells were transfected with FLAG-TBK1 and different phosphatase expression plasmids as indicated, cell extracts from which were analyzed by immunoblotting with the anti-phospho-IRF3, anti-IRF3, anti-FLAG and anti-β-actin antibodies.

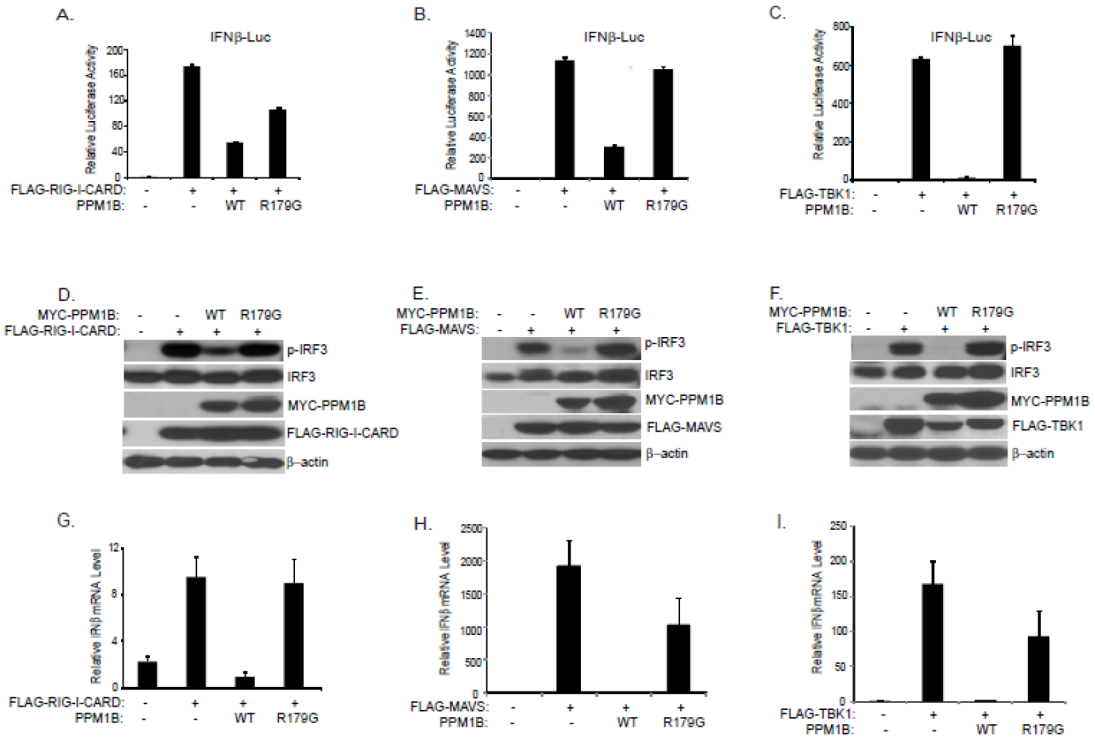

Figure 2.

The phosphatase activity of PPM1B is required for its inhibitory effect on TBK1- and its upstream molecules-mediated IFNβ activation. (A, B, C) The phosphatase activity of PPM1B is required for its inhibitory effect on TBK1- and its upstream molecules-induced IFNβ luciferase reporter activity. HEK293T cells were co-transfected with IFNβ reporter plasmid and FLAG-RIG-I-CARD (A), FLAG-MAVS (B), FLAG-TBK1 (C) along with PPM1B wild-type or R179G mutant plasmids. The relative luciferase activity was measured at 36 h after transfection. (D, E, F) The phosphatase activity of PPM1B is required for its inhibitory effect on TBK1- and its upstream molecules-induced IRF3 phosphorylation. HEK293T cells were transfected with FLAG-RIG-I-CARD (D), FLAG-MAVS (E), FLAG-TBK1 (F) along with PPM1B wild-type or R179G mutant plasmids. Cell extracts from which were analyzed by immunoblotting with anti-phospho-IRF3, anti-IRF3, anti-FLAG, anti-MYC and anti-β-actin antibodies. (G, H, I) The phosphatase activity of PPM1B is required for its inhibitory effect on TBK1- and its upstream molecules-induced IFNβ production. HEK293T cells were transfected with FLAG-RIG-I-CARD (G), FLAG-MAVS (H), FLAG-TBK1 (I) and along with PPM1B wild-type or R179G mutant plasmids. IFNβ mRNA level was determined by quantitative PCR.

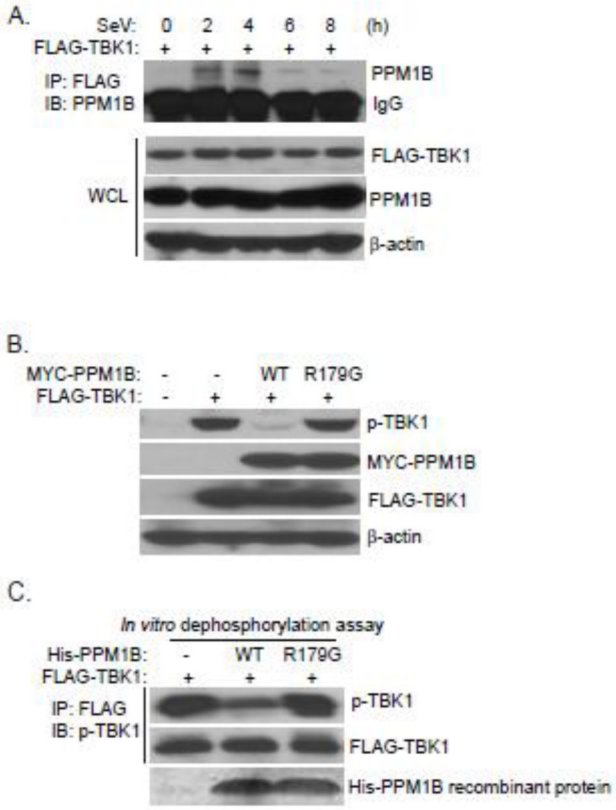

Figure 3.

PPM1B binds to and dephosphorylates TBK1. (A) SeV induces the association of TBK1 with PPM1B. FLAG-TBK1 was transfected into HeLa cells for 36 h, and then cells were either untreated or treated with SeV for the indicated times. The cell lysates were immunoprecipitated with the anti-FLAG antibody, then immunoblotted with the anti-PPM1B antibody. The whole cell lysates (WCL) were immunoblotted with the anti-FLAG and anti-PPM1B antibodies. (B) PPM1B inhibits TBK1 phoshorylation in *vivo*. Cell extracts were prepared from HEK293T cells transfected with FLAG-TBK1 and Myc-tagged PPM1B wild-type or R179G mutant, and immunoblotted with anti-phospho-TBK1, anti-FLAG, anti-MYC and antiβ-actin antibodies. (C) Recombinant PPM1B dephosphorylates TBK1 in *vitro*. HEK293T cells were transfected with FLAG-TBK1, then lysed in the lysis buffer containing PMSF. FLAG-TBK1 proteins in the cell lysates were immunoprecipitated with anti-FLAG antibodies and co-incubated with purified recombinant His-PPM1B-WT or R179G mutant for 1 h in phosphatase reaction buffer. The reaction mix was then analyzed by immunoblotting with the anti-phospho-TBK1 antibodies.

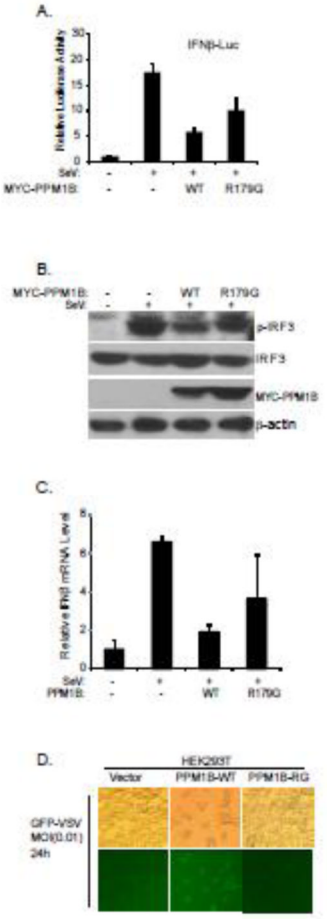

Figure 4.

PPM1B inhibits antiviral response. (A) PPM1B inhibits SeV-induced IFNβ luciferase reporter activity. HEK293T cells were co-transfected with indicated plasmids. 24 h after transfection, cells were left uninfected or infected with SeV for another 12 h, and then the IFNβ reporter activities were determined. (B) PPM1B inhibits SeV-induced IRF3 phosphorylation. HEK293T cells were co-transfected with indicated plasmids. 24 h after transfection, cells were left uninfected or infected with SeV for 8 h. Cell lysates were subjected to western blot with indicated antibodies. (C) PPM1B inhibits SeV-induced IFNβ production. HEK293T cells were co-transfected with indicated plasmids. 24 h after transfection, cells were left uninfected or infected with SeV for 8 h. IFNβ mRNA level was determined by quantitative PCR. (D) PPM1B overexpression impairs the resistance of HEK293T cells to VSV-induced cell death. HEK293T cells were transfected with empty vector, PPM1B wild-type or R179G mutant. After 48h, cells were infected with VSV-EGFP (moi=0.01) for another 48 h, and GFP positive cells were visualized by fluorescent microscope.

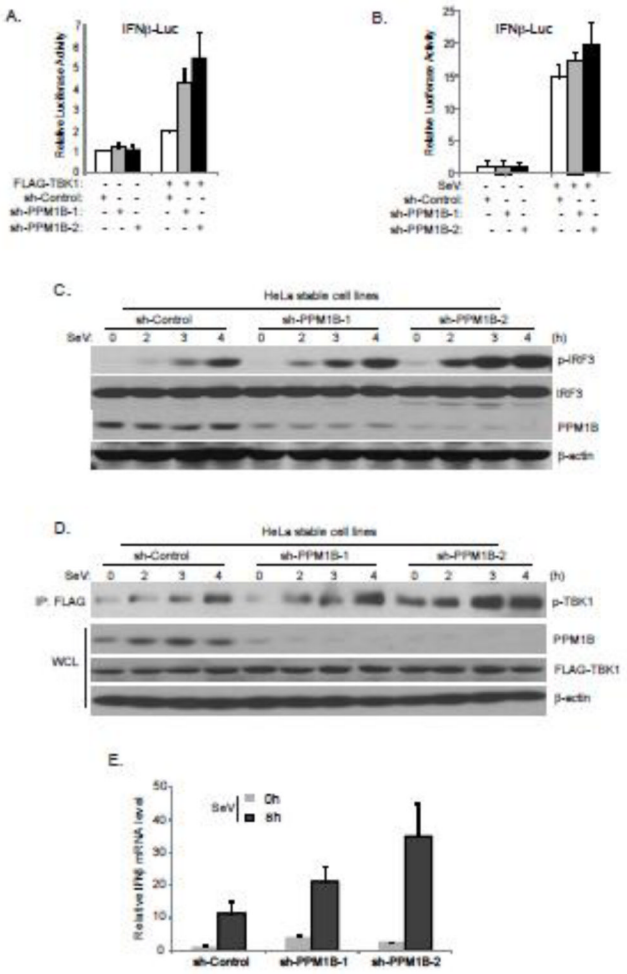

Figure 5.

Knockdown of PPM1B expression enhances TBK1-mediated IFNβ activation. (A) HEK293T cells were co-transfected with IFNβ luciferase reporter plasmid and FLAG-TBK1 along with sh-Control or sh-PPM1B plasmids. The relative luciferase activity was measured at 36 h after transfection. (B) HEK293T cells were co-transfected with IFNβ luciferase reporter plasmid along with sh-Control or sh-PPM1B plasmids. Transfected cells were either untreated or treated with SeV for 20 h. The relative luciferase activity was measured after SeV treatment. (C) Suppression of PPM1B expression enhances the SeV-induced IRF3 phosphorylation. HeLa cells with stable expression of sh-Control or sh-PPM1B were either untreated or treated with SeV for the time periods as indicated. Cell extracts were immunoblotted with the anti-phospho-IRF3 antibody, subsequently, with anti-IRF3 and anti-PPM1B antibodies. (D) Suppression of PPM1B expression enhances the SeV-induced TBK1 phosphorylation. FLAG-TBK1 was transfected into HeLa cells stably expressing sh-control or sh-PPM1B, then cells were infected with SeV for indicated times. The cell lysates were immunoblotted with the anti-phospho-TBK1 antibody, followed with the anti-FLAG and anti-PPM1B antibodies. (E) HeLa cells with stable expression of sh-Control or sh-PPM1B were either untreated or treated with SeV for 8h. Total RNAs from these cells were extracted, and then quantitative RT-PCR was employed to determine mRNA levels of IFNβ.

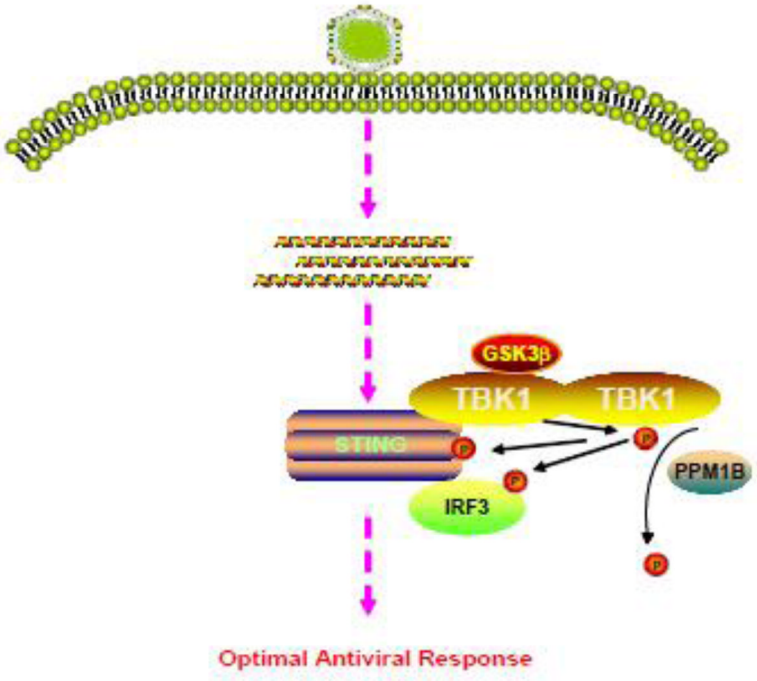

Figure 6.

A working model for the role of PPM1B in the negative regulation of antiviral response. Upon virus infection, pattern recognition receptors (PPRs) recognize viral RNAs and further activate STING which subsequently recruits IRF3 and TBK1. Virus infection induced GSK3β binding with TBK1 and facilitates TBK1 phosphorylation and activation. Activated TBK1 then phosphorylates IRF3 and induces antiviral response. To generate an optimal antiviral response, TBK1 phosphorylation was negatively regulated by PPM1B to prevent excessive antiviral response.