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The tumor suppressor PTEN has a critical role in antiviral innate immunity

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The gene encoding PTEN is one of the most frequently mutated tumor suppressor–encoding genes in human cancer. While PTEN's function in tumor suppression is well established, its relationship to anti-microbial immunity remains unknown. Here we found a pivotal role for PTEN in the induction of type I interferon, the hallmark of antiviral innate immunity, that was independent of the pathway of the kinases PI(3)K and Akt. PTEN controlled the import of IRF3, a master transcription factor responsible for IFN-β production, into the nucleus. We further identified a PTEN-controlled negative phosphorylation site at Ser97 of IRF3 and found that release from this negative regulation via the phosphatase activity of PTEN was essential for the activation of IRF3 and its import into the nucleus. Our study identifies crosstalk between PTEN and IRF3 in tumor suppression and innate immunity.

The gene encoding PTEN is one of the most frequently mutated tumor suppressor–encoding genes. By antagonizing signaling via phosphatidylinositol 3-kinase (PI(3)K) and the kinase Akt through its lipid-phosphatase activity, PTEN governs myriad cellular processes and serves a central role in tumor suppression[1–3]. Although its role in tumor suppression is well established, it is unknown whether PTEN is involved in anti-microbial immunity.

Detection of microbial pathogens is the first step in eliciting innate immune responses through a set of pattern-recognition receptors in the host that recognize microbe-associated molecular patterns[4]. For example, receptors of the RLR family recognize viral double-stranded RNA or single-stranded RNA in most cells invaded by viruses[5,6]; membrane-associated Toll-like receptors (TLRs) recognize viral double-stranded RNA or single-stranded RNA internalized into the endosomes of specified cells[7]. The activation of pattern-recognition receptors triggers downstream signaling cascades that lead to rapid production of type I interferons and proinflammatory cytokines[8].

Interferon-regulatory factor 3 (IRF3) acts as a master transcription factor responsible for the induction of the gene encoding interferon-β (IFN-β) and is essential for the establishment of innate immunity[9]. After a pathogen is detected, IRF3 is phosphorylated on several phosphorylation acceptor clusters and undergoes a conformational change and homo-dimerization, which leads to its translocation to the nucleus and its association with the interferon-stimulated response elements of target genes[10,11]. The essential role of IRF3 in the induction of the gene encoding IFN-β suggests that it must be properly regulated and 'finely tuned' to determine the exact nature of the final immune response to an invading virus[12–16]. IRF3 is phosphorylated by the kinases TBK1 and IKKε[17,18], but the phosphatases responsible for the dephosphorylation of IRF3 have remained elusive.

Here we report a previously unknown function for PTEN in antiviral immunity. We found that PTEN's phosphatase activity positively regulated the import of IRF3 into the nucleus and innate immune responses. Our results reveal an additional layer of the regulation of IRF3 by PTEN and might explain in part the enhanced susceptibility of oncogenically transformed cells to viral infection.

RESULTS

The involvement of PTEN in type I interferon production

In an expression cloning screen, we found that PTEN potentiated virus-induced activation of the promoter of the gene encoding IFN-β (*IFNB1*). Exogenous expression of PTEN enhanced the expression of endogenous *IFNB1* induced by Sendai virus (SeV) (**Fig. 1a**) and by the synthetic RNA duplex poly(I:C) (**Supplementary Fig. 1a**). The promoter of *IFNB1* was stimulated in a dose-dependent manner (**Supplementary Fig. 1b**). PTEN expression promoted the activation of promoters containing interferon-stimulated response elements (**Supplementary Fig. 1c**) and enhanced the expression of interferon-stimulated genes but not the interferon-unresponsive genes *IL6* and *IL8* (**Supplementary Fig. 1d,e**). When endogenous PTEN was knocked down through the use of small interfering RNA (siRNA) in HEK293 human embryonic kidney cells, SeV-induced activation of *IFNB1* was reduced, whereas expression of a PTEN variant resistant to RNA-mediated interference in cells treated with PTEN-specific siRNA restored the activation of *IFNB1* (**Fig. 1b**).

To confirm the contribution of PTEN to the induction of *IFNB1*, we infected *Pten*$^{-/-}$ mouse embryonic fibroblasts (MEFs) with SeV. The production of IFN-β in *Pten*$^{-/-}$ MEFs was over tenfold lower than that in *Pten*$^{+/+}$ MEFs, and the production of IFN-α was completely abolished during infection with SeV (**Fig. 1c**). In contrast, deletion

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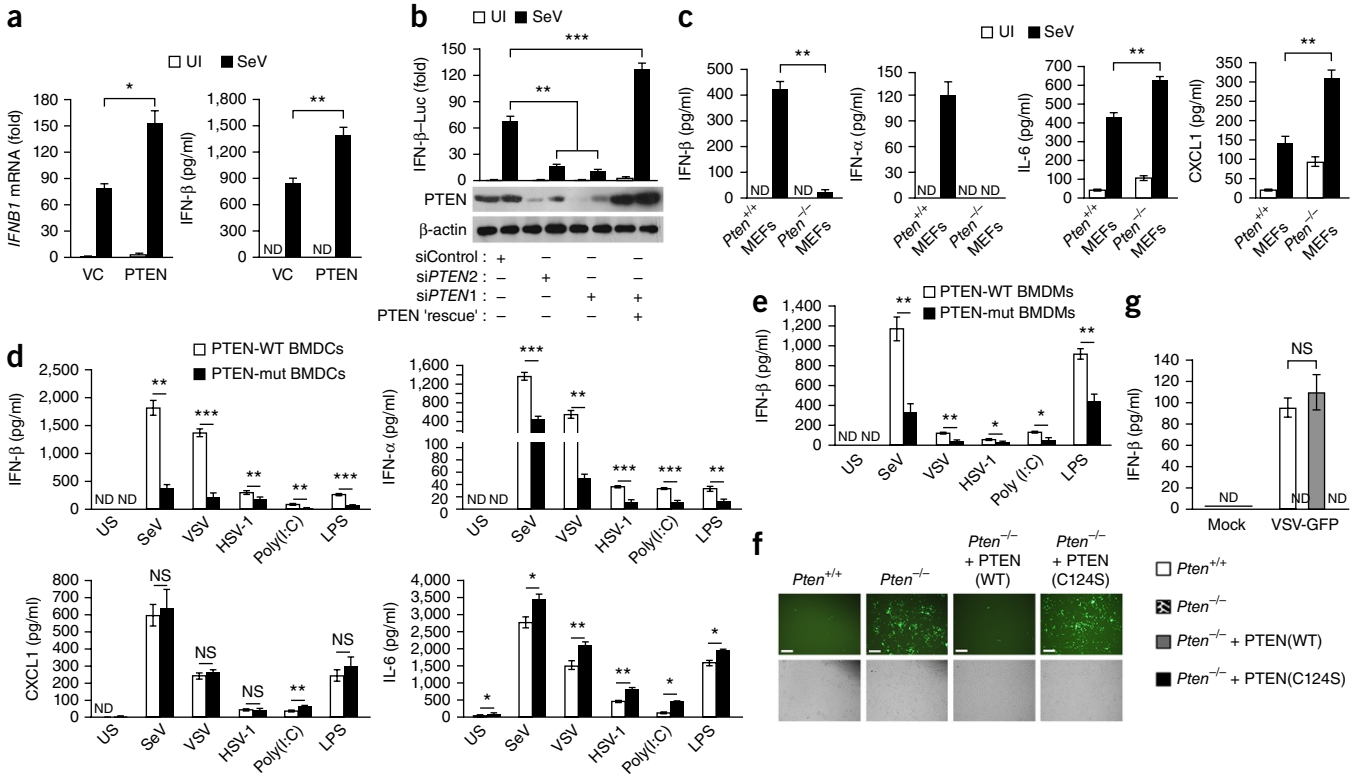

Figure 1 PTEN is involved in virus-induced production of type I interferon. (**a**) Quantitative RT-PCR analysis of *IFNB1* mRNA (left) and enzyme-linked immunosorbent assay (ELISA) of IFN-β (right) in HEK293 cells (2×10^5) transfected for 24 h with vector control (VC) or plasmid for the expression of PTEN, then left uninfected (UI) or infected for another 8 h with SeV (50 hemagglutination units per ml). (**b**) Luciferase assay analyzing *IFNB1* promoter activity (top) and immunoblot analysis of PTEN and β-actin (loading control throughout) (below) in HEK293 cells (2×10^5) transfected for 36 h with a plasmid encoding an IFN-β firefly luciferase reporter (IFN-β–Luc), along with plasmid encoding non-targeting control siRNA (siControl) or siRNA targeting PTEN (si*PTEN2* or si*PTEN1*) (1 μg each), then transfected for another 12 h with empty vector (–; bottom row) or cDNA encoding PTEN (+; bottom row) and left uninfected (UI) or infected for 8 h with SeV; luciferase reporter activity (top) is normalized to that of renilla luciferase. (**c**) ELISA of IFN-β, IFN-α, IL-6 and CXCL1 in *Pten*$^{+/+}$ and *Pten*$^{-/-}$ MEFs left uninfected or infected for 8 h with SeV. (**d**) ELISA of cytokines (as in **c**) in culture medium of PTEN-wild-type (PTEN-WT) or PTEN mutant (PTEN-mut) BMDCs (1×10^5) induced with the cytokine GM-CSF and left unstimulated (US) or stimulated for 10 h with SeV, VSV, herpes simplex virus type 1 (HSV-1), poly(I:C) or LPS. (**e**) ELISA of IFN-β in bone marrow–derived macrophages (BMDMs) treated as in **d**. (**f**,**g**) Microscopy analyzing VSV replication (**f**) and ELISA of IFN-β in culture medium (**g**) of *Pten*$^{+/+}$ and *Pten*$^{-/-}$ MEFs, or *Pten*$^{-/-}$ MEFs reconstituted with cDNA encoding wild-type PTEN(WT) or PTEN(C124S), all infected for 36 h with VSV-GFP (multiplicity of infection, 0.01). Scale bars (**f**), 100 μm. ND, not detectable. NS, not significant ($P > 0.05$); $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (unpaired *t*-test (**a**,**c**,**d**,**e**,**g**) or one-way analysis of variance (ANOVA) with *post hoc* Bonferroni *t*-test (**b**)). Data are from three independent experiments (**a**, **b** (top), **c**–**e**,**g**; mean and s.d. of three independent biological replicates per group) or are representative of three independent experiments (**b** (bottom), **f**).

of PTEN did not reduce the production of proinflammatory factors such as IL-6 and CXCL1 (**Fig. 1c**). These results indicated specific involvement of PTEN in the expression of type I interferons.

We next investigated whether PTEN was required for virus-induced type I interferon responses in cells of the immune system. The secretion of IFN-α and IFN-β from PTEN-mutant bone marrow–derived dendritic cells (BMDCs) from mice described below was severely impaired compared with their secretion from PTEN-wild-type BMDCs under the condition of stimulation by various inducers, including RNA virus, DNA virus, poly(I:C) or bacterial lipopolysaccharide (LPS) (**Fig. 1d**). In parallel experiments, the production of IL-6 and CXCL1 protein was not reduced in PTEN-mutant BMDCs relative to their production in PTEN-wild-type BMDCs (**Fig. 1d**). We obtained similar results with bone marrow–derived macrophages (**Fig. 1e**). Together these results suggested a role for PTEN in virus-induced induction of type I interferon in various cell types.

Deficiency in PTEN disrupts antiviral responses

We next investigated whether PTEN contributed to antiviral responses during viral infection. When PTEN was knocked down by siRNA, the replication of vesicular stomatitis virus (VSV) encoding a green fluorescent protein (GFP) reporter (VSV-GFP) increased, as indicated by enhanced GFP expression (**Supplementary Fig. 2a**), a result confirmed by plaque assay (**Supplementary Fig. 2b**). *Pten*$^{-/-}$ MEFs were more vulnerable to challenge with VSV-GFP than were *Pten*$^{+/+}$ MEFs, and reintroduction of PTEN into *Pten*$^{-/-}$ MEFs restored the antiviral activity, but reintroduction of the phosphatase-deficient PTEN mutant PTEN(C124S) did not (**Fig. 1f**). The higher titer of VSV-GFP in *Pten*$^{-/-}$ MEFs correlated with less production of IFN-β (**Fig. 1g**). These data also suggested that the phosphatase activity of PTEN was required for its role in antiviral activity. Furthermore, we assessed the antiviral activity in PTEN-deficient 786-O human renal cancer cells and PC-3 human prostate cancer cells. The reintroduction of PTEN induced antiviral activity and reduced the titers of VSV, while markedly higher titers of VSV were produced by PTEN-deficient 786-O cells or PTEN-deficient786-O cells reconstituted with PTEN(C124S) (**Supplementary Fig. 2c**). Similarly, the induction of cell death by VSV was significantly higher in PTEN-deficient PC-3 cells and in PTEN-deficient PC-3 cells reconstituted with PTEN(C124S) than in PTEN-deficient PC-3 cells reconstituted with PTEN itself

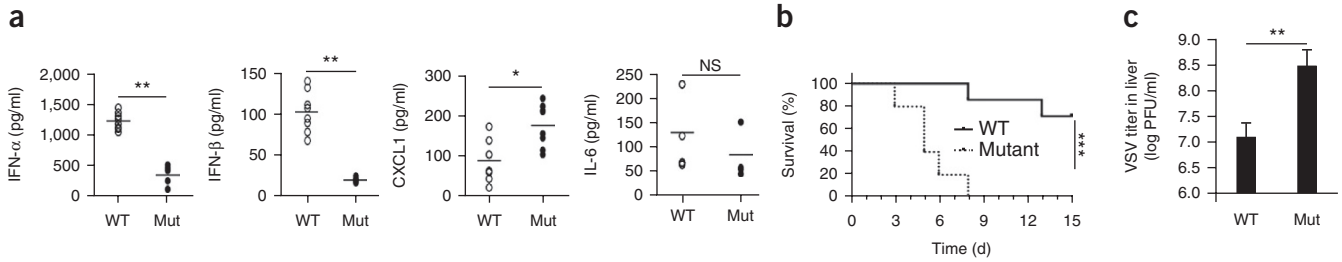

Figure 2 Pivotal role for PTEN in the induction of type I interferon and antiviral response *in vivo*. (**a**) ELISA of IFN-β, IFN-α, CXCL1 and IL-6 in serum from PTEN-wild-type (WT) mice ($n = 8$) and PTEN-mutant (Mut) mice ($n = 8$) infected intraperitoneally for 8 h with VSV (1×10^7 plaque-forming units (PFU) per mouse). Each symbol represents an individual mouse; small horizontal lines indicate the mean. (**b**) Survival (Kaplan-Meier curve) of PTEN-wild-type mice ($n = 7$) and PTEN-mutant mice ($n = 5$) infected intraperitoneally with a high dose of VSV (5×10^7 PFU per mouse) and monitored for 15 d. (**c**) Plaque assay of VSV in livers of PTEN-wild-type mice ($n = 5$) and PTEN-mutant mice ($n = 5$) 48 h after intraperitoneal infection with VSV (1×10^7 PFU per mouse). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (unpaired *t*-test (**a**,**c**) or long-rank test (**b**)). Data are representative of three experiments (**a**) or two experiments (**b**,**c**; mean and s.d. of five independent biological replicates per group in each in **c**).

(**Supplementary Fig. 2d**). These results suggested that PTEN deficiency abrogated the cellular antiviral activity and indicated a critical role for PTEN in antiviral immunity.

Pivotal role for PTEN in the antiviral response *in vivo*

Since germline knockout of the gene encoding PTEN is embryonically lethal[19], we adopted a strategy to generate mice with tamoxifen-inducible conditional knockout of PTEN through the deletion of *lox*P-flanked *Pten* alleles ($Pten^{LoxP/LoxP}$) by a tamoxifen-inducible Cre recombinase with a mutant estrogen receptor ligand-binding domain (CreER) under the control of the promoter of the ubiquitously expressed gene encoding ubiquitin C (*Ubc*) ($Pten^{LoxP/LoxP}$ *Ubc*-CreER$^+$ mice) (**Supplementary Fig. 3a**,**b**). Injection of tamoxifen into 'pre-mutant' (before tamoxifen treatment) $Pten^{LoxP/LoxP}$ *Ubc*-CreER$^+$ mice resulted in excision of the *lox*P-flanked exon and, consequently, in the knockout of PTEN. However, the mice with tamoxifen-induced recombination were usually mosaic, with varying fractions of co-existing PTEN-wild-type cells and PTEN-knockout cells (**Supplementary Fig. 3c**). Thus, we called the mosaic mice 'PTEN-mutant' mice because PTEN was not deleted from all cells.

To determine the role of PTEN in antiviral defense *in vivo*, we investigated innate antiviral immune responses in PTEN-wild-type and PTEN-mutant mice (mutant line 1). The production of IFN-α and IFN-β (collectively called 'IFN-α/β' here) was severely impaired in

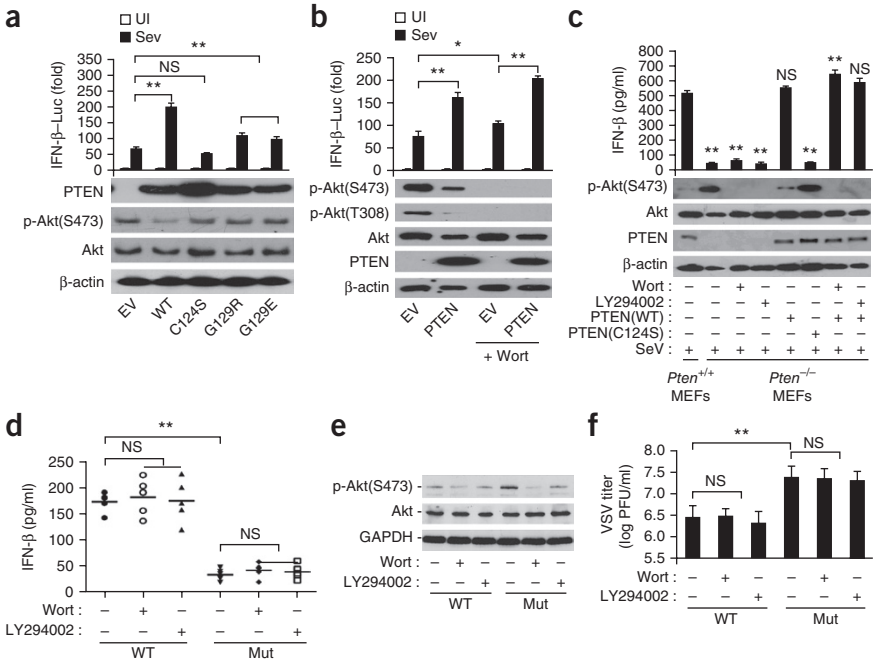

Figure 3 PTEN promotes the induction of *IFNB1* in manner dependent on the phosphatase activity of PTEN and independent of the Akt pathway. (**a**) Luciferase assay (as in **Fig. 1b**) (top) and immunoblot analysis of total PTEN, Akt phosphorylated at Ser473 (p-Akt(S473)) and total AKt (below) in HEK293 cells transfected for 24 h to express empty vector (EV) or vector encoding wild-type PTEN (WT) or PTEN(C124S), PTEN(G129R) or PTEN(G129E) (below lanes), along with an IFN-β reporter plasmid, and left uninfected or infected for 10 h with SeV. (**b**) Luciferase assay (as in **Fig. 1b**) (top) and immunoblot analysis of Akt phosphorylated at Ser473 (p-Akt(S473)) or Tyr308 (p-Akt(T308)) and total AKt and PTEN (below) in HEK293 cells transfected for 24 h with empty vector or plasmid encoding PTEN, along with an IFN-β reporter plasmid, and left untreated (left half) or treated for 2 h with 1 μM wortmannin (+ Wort) and also left uninfected or infected for 10 h with SeV. (**c**) ELISA of IFN-β (top) and immunoblot analysis of Akt phosphorylated at Ser473 and total Akt and PTEN (below) in $Pten^{+/+}$ MEFs, unreconstituted $Pten^{-/-}$ MEFs (–), and $Pten^{-/-}$ MEFs reconstituted with cDNA encoding wild-type PTEN or PTEN(C124S) (below lanes), left untreated (–) or treated (+) for 2 h with wortmannin (1 μM) or LY294002 (5 μM) and then infected for 10 h with SeV. (**d**–**f**) ELISA of IFN-β in serum (**d**), immunoblot analysis of Akt phosphorylated at Ser473 and total Akt in liver (**e**) and plaque assay of VSV in liver (**f**) of PTEN-wild-type mice ($n = 5$) and PTEN-mutant mice ($n = 5$) left untreated or treated intravenously with wortmannin (0.7 mg per kg body weight) or LY294002 (75 mg per kg body weight) for 0.5 h before intraperitoneal infection with VSV (1×10^7 PFU per mouse), assessed 6 h (**d**,**e**) or 24 h (**f**) after infection. Each symbol (**d**) represents an individual mouse; small horizontal lines indicate the mean. $^*P < 0.05$ and $^{**}P < 0.01$ (one-way ANOVA (**a**,**c**) or two-way ANOVA with *post hoc* Bonferroni *t*-test (**b**,**d**,**f**)). Data are from three experiments (**a**–**c**, top; mean and s.d. of three independent biological replicates per group in each) or are representative of three (**a**–**c**, bottom) or two (**d**–**f**) independent experiments (in **f**, mean and s.d. of four independent biological replicates per group in each).

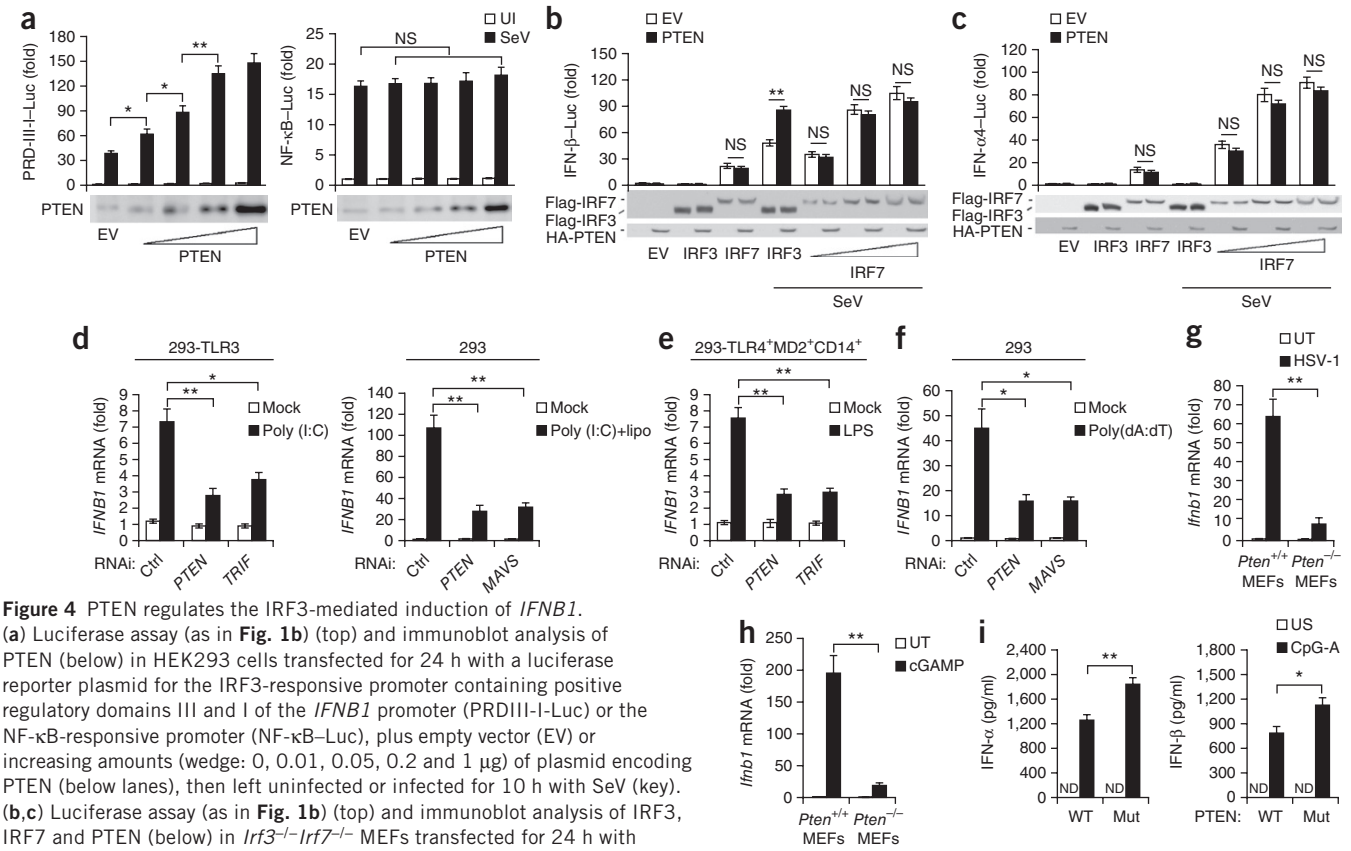

Figure 4 PTEN regulates the IRF3-mediated induction of *IFNB1*. (**a**) Luciferase assay (as in **Fig. 1b**) (top) and immunoblot analysis of PTEN (below) in HEK293 cells transfected for 24 h with a luciferase reporter plasmid for the IRF3-responsive promoter containing positive regulatory domains III and I of the *IFNB1* promoter (PRDIII-I-Luc) or the NF-κB-responsive promoter (NF-κB–Luc), plus empty vector (EV) or increasing amounts (wedge: 0, 0.01, 0.05, 0.2 and 1 μg) of plasmid encoding PTEN (below lanes), then left uninfected or infected for 10 h with SeV (key). (**b**,**c**) Luciferase assay (as in **Fig. 1b**) (top) and immunoblot analysis of IRF3, IRF7 and PTEN (below) in *Irf3*$^{-/-}$*Irf7*$^{-/-}$ MEFs transfected for 24 h with luciferase reporter plasmid for *Ifnb1* (IFN-β–Luc; **b**) or *Ifna4* (IFN-α4–Luc; **c**), together with empty vector or plasmid encoding Flag-tagged IRF3 (single amount) or IRF7 (increasing amounts (wedge), for infected conditions) (below lanes), plus empty vector or vector encoding hemagglutinin (HA)-tagged PTEN, then left uninfected (left half) or infected for 10 h with SeV (right half). (**d**–**f**) Quantitative RT-PCR analysis of *IFNB1* mRNA in HEK293 cells expressing TLR3 (293-TLR3) or HEK293 cells (**d**), HEK293 cells expressing TLR4, MD2, and CD14 (293-TLR4^{+}MD2^{+}CD14^{+}) (**e**) or HEK293 cells (**f**) transfected for 36 h with plasmid encoding non-targeting control siRNA (Ctrl) or siRNA targeting *PTEN TRIF* or *MAVS* (below graphs), then left untreated or treated with poly(I:C) (10 μg/ml) or LPS (10 μg/ml), or transfected with poly(I:C) (1 μg/ml) or poly(dA:dT) (1 μg/ml) for another 10 h; results were normalized to those of the control gene *GAPDH* and are presented relative to those of untreated cells transfected with control siRNA. (**g**,**h**) Quantitative RT-PCR analysis of *Ifnb1* mRNA in *Pten*$^{+/+}$ and *Pten*$^{-/-}$ MEFs (1×10^5) left untreated (UT) or infected with herpes simplex virus type 1 (multiplicity of infection, 0.01) or transfected with 3′3′-cyclic GMP-AMP (cGAMP) (500 ng) for 10 h; results were normalized to those of the control gene *Gapdh* and are presented relative to those of untreated *Pten*$^{+/+}$ MEFs. (**i**) ELISA of IFN-β in BMDCs derived from PTEN-wild-type and PTEN-mutant mice with the cytokine Flt3L, then left unstimulated (US) or stimulated for 10 h with CpG-A DNA (ODN 1585) (10 μg/ml). $^{*}P < 0.05$ and $^{**}P < 0.01$ (one-way ANOVA with *post hoc* Bonferroni *t*-test (**a**,**d**,**e**,**f**) or unpaired *t*-test (**b**,**c**,**g**,**h**,**i**)). Data are from three experiments (**a**–**c**, top, **d**–**i**; mean and s.d. of three independent biological replicates per group in each) or are representative of three independent experiments (**a**–**c**, bottom).

PTEN-mutant mice compared with its production in PTEN-wild-type mice, following infection with VSV (**Fig. 2a**). In parallel experiments, the serum concentration of the pro-inflammatory factors IL-6 and CXCL1 was not lower in PTEN-mutant mice than in PTEN-wild-type mice (**Fig. 2a**). We also compared the survival rates of PTEN-wild-type and PTEN-mutant mice after inoculation of VSV. The results indicated that PTEN-mutant mice were more susceptible to VSV-triggered mortality than were their PTEN-wild-type counterparts (**Fig. 2b**). In accordance with those results, VSV titers in the livers of PTEN-mutant mice were higher than those in their PTEN-wild-type counterparts (**Fig. 2c**). Together these data indicated that PTEN was critical for the induction of type I interferon and antiviral immune responses *in vivo*.

Akt-independent role of PTEN in IFN-β production

We first investigated whether the PTEN-mediated induction of *IFNB1* depended on its phosphatase activity in an *IFNB1* promoter–driven luciferase reporter system. The best characterized PTEN mutants are C124S, G129R and G129E; the first has complete ablation of phosphatase activity, and the other two have some residual phosphatase activity[20]. Compared with the activation of *IFNB1* achieved with an empty vector control, wild-type PTEN enhanced the activation of *IFNB1*, but PTEN(C124S) failed to do so (**Fig. 3a**). In contrast, the expression of PTEN(G129R) or PTEN(G129E) induced subtle increases in *IFNB1* reporter activity (**Fig. 3a**). These results indicated that promotion of *IFNB1* induction by PTEN was dependent on its phosphatase activity. We further assessed other phosphatases, including those that are structurally related to PTEN, such as VHR, PTP1B and Cdc25A, and one that antagonizes the PI(3)K-Akt pathway, SHIP-1. All these phosphatases failed to substantially increase the *IFNB1* reporter activity (**Supplementary Fig. 3d**). These results suggested that the phosphatase activity of PTEN was specifically involved in SeV-triggered activation of *IFNB1*.

The main biochemical function of PTEN in tumor suppression is its dephosphorylation of phosphatidylinositol-(3,4,5)-trisphosphate and thus its antagonizing of the PI(3)K-Akt pathway[21,22]. Therefore, we investigated whether PTEN promoted activation of *IFNB1* through this pathway by using the PI(3)K-specific inhibitors wortmannin and LY294002. In the presence of wortmannin or LY294002, PTEN still markedly

enhanced the activation of *IFNB1* induced by infection with SeV (**Fig. 3b** and **Supplementary Fig. 3e**). Consistent with that, neither a dominant-negative form of Akt nor a constitutively active form of Akt was able to offset the effect of PTEN on SeV-induced activation of *IFNB1* (**Supplementary Fig. 3f**). In *Pten*$^{-/-}$ MEFs, reconstitution with PTEN restored the SeV-triggered production of IFN-β, but treatment with wortmannin did not (**Fig. 3c**). We obtained similar results with other cell types (**Supplementary Fig. 3g**). We further confirmed those results in a mouse model. Neither activation of the PI(3)K-Akt pathway by the constitutively active form of Akt nor its inhibition by the dominant-negative form of Akt or inhibitors of the Akt pathway affected VSV replication or VSV-triggered induction of *Ifnb1* (**Supplementary Fig. 3h**,**i**). Treatment with wortmannin or LY294002 diminished the activation of Akt in PTEN-mutant mice relative to that in PTEN-wild-type mice, but these inhibitors did not significantly affect IFN-β production or VSV replication in PTEN-wild-type or PTEN-mutant mice (**Fig. 3d**–**f**). Collectively, these results suggested that the role of PTEN in the induction of type I interferon and antiviral function was dependent on its phosphatase activity but was independent of the Akt pathway.

PTEN regulates IRF3-mediated pathway

We next determined which pathways of *IFNB1* activation were regulated by PTEN. Transcriptional activation of the *IFNB1* promoter requires cooperative and coordinative activation of the transcription factors IRF3 and NF-κB[23]. In reporter assays, expression of PTEN enhanced the activation of the IRF3-responsive promoter that contains positive regulatory domains III and I of *IFNB1* promoter, but not the NF-κB-responsive promoter (**Fig. 4a**). IRF3 is responsible for the early phase of the induction of IFN-α/β, whereas IRF7 is involved in the late phase[24]. PTEN expression enhanced the IRF3-mediated activation of *Ifnb1* but not the IRF7-mediated activation of *Ifna4* or *Ifnb1* in *Irf3*$^{-/-}$*Irf7*$^{-/-}$ MEFs (**Fig. 4b**,**c**). These findings suggested that PTEN promoted the induction of *IFNB1* through IRF3 but not through NF-κB or IRF7. In *Pten*$^{-/-}$ MEFs, deletion of PTEN resulted in impaired transcription of IRF3-dependent genes, including *Isg15*, *Ifit1*, *Cxcl10* and *Ccl5*, but failed to reduce the induction of NF-κB-dependent and IRF3-independent genes, including *Cxcl1*, *Il6*, *Nfkbia* and *Nfkb1* (**Supplementary Fig. 4a**). Similarly, knockdown of PTEN via siRNA did not influence the activation of NF-κB-responsive promoters induced by poly(I:C) or LPS (**Supplementary Fig. 4b**). These data reinforced the conclusion that PTEN was involved in IRF3-mediated innate immune responses.

We next investigated whether PTEN was involved in the IRF3-mediated activation of *IFNB1* induced by various microbe-associated molecular patterns and signaling pathways. Knockdown or deletion of PTEN markedly reduced the IRF3-dependent transcription of *IFNB1*

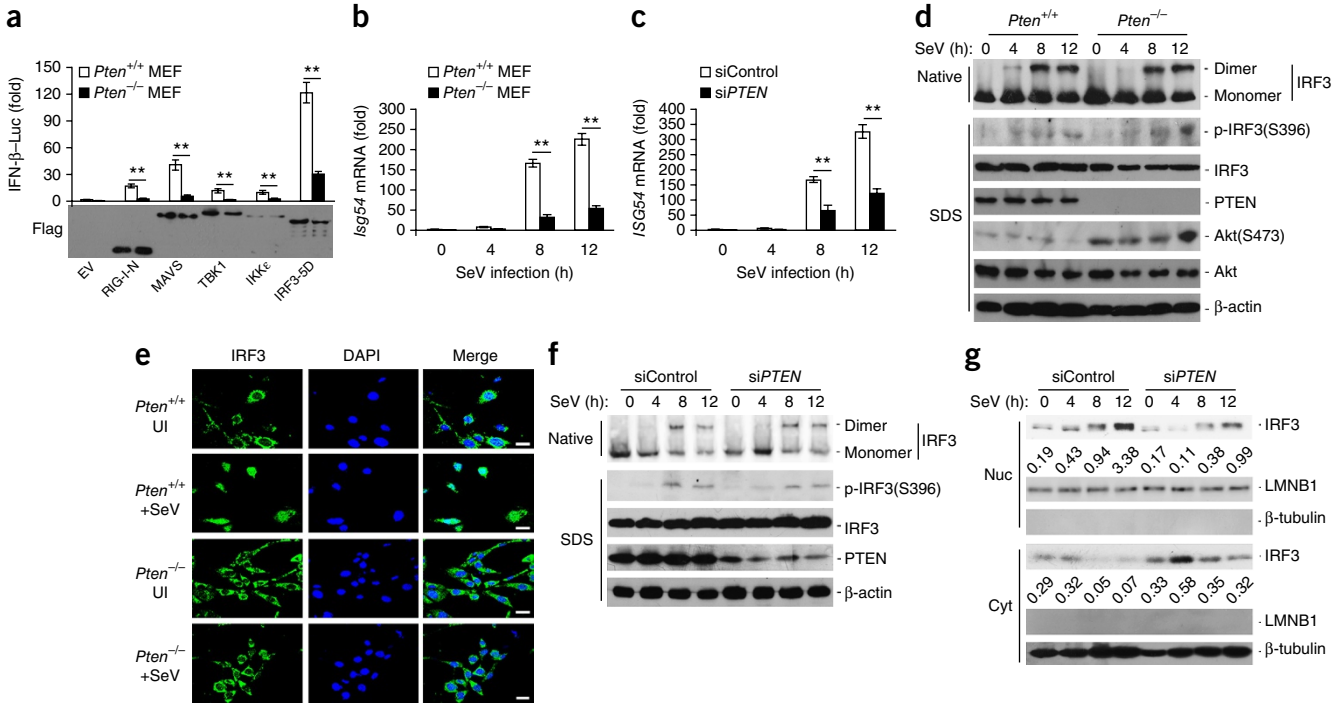

Figure 5 PTEN controls the import of IRF3 into the nucleus. (**a**) Luciferase assay (as in **Fig. 1b**) (top) and immunoblot analysis (below) of *Pten*$^{+/+}$ and *Pten*$^{-/-}$ MEFs transfected for 20 h with empty vector or vector containing a plasmid encoding Flag-tagged RIG-I (amino terminus (RIG-1-N); amino acids 1–200), MAVS, TBK1 and IKKε or IRF3-5D (bottom). (**b**) Quantitative RT-PCR analysis of *Isg54* mRNA in *Pten*$^{+/+}$ MEFs and *Pten*$^{-/-}$ MEFs infected for 0–12 h (horizontal axis) with SeV. (**c**) Quantitative RT-PCR analysis of *ISG54* mRNA in HEK293 cells transfected for 36 h with plasmid encoding non-targeting control siRNA (siControl) or siRNA targeting *PTEN* (si*PTEN*), then infected for 0–12 h (horizontal axis) with SeV. (**d**) Immunoblot analysis of IRF3 in dimer or monomer form (top) or of IRF3 phosphorylated at Ser396 (p-IRF3(S396)), total IRF3 and PTEN, Akt phosphorylated at Ser473 (p-Akt(S473)) and total Akt (below) in *Pten*$^{+/+}$ MEFs and *Pten*$^{-/-}$ MEFs infected for 0–12 h (above lanes) with SeV, followed by native PAGE (top) or SDS-PAGE (below). (**e**) Immunofluorescence microscopy of *Pten*$^{+/+}$ and *Pten*$^{-/-}$ MEFs left uninfected or infected for 12 h with SeV (left margin), stained with antibody to IRF3 (green) and the DNA-binding dye DAPI (blue). Scale bars, 10 μm. (**f**) Immunoblot analysis (as in **d**) of HEK293 cells transfected for 36 h with siRNA-encoding plasmid as in **c** and infected with SeV as in **d**. (**g**) Immunoblot analysis of HEK293 cells transfected for 36 h with siRNA-encoding plasmid as in **c** and infected with SeV as in **d**, followed by nucleus-cytoplasm extraction (5% of cytoplasmic extracts and 10% of nuclear extracts separated by SDS-PAGE). Numbers under lanes indicate IRF3 band intensity, normalized to that of LMNB1 or β-tubulin. $^{**}P < 0.01$ (unpaired *t*-test (**a**) or two-way ANOVA with *post hoc* Bonferroni *t*-test (**b**,**c**)). Data are from three experiments (**a**, top, **b**,**c**; mean and s.d. of three independent biological replicates per group in each) or are representative of three independent experiments (**a**, bottom, **d**–**g**).

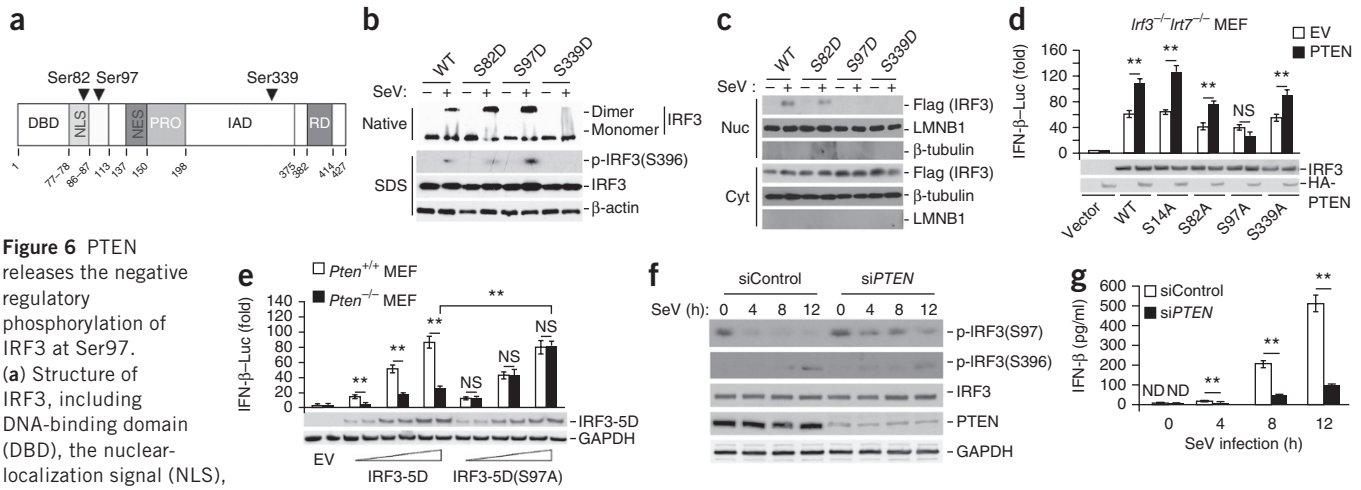

Figure 6 PTEN releases the negative regulatory phosphorylation of IRF3 at Ser97. (**a**) Structure of IRF3, including DNA-binding domain (DBD), the nuclear-localization signal (NLS), the nuclear-export signal (NES), the proline domain (PRO), the IRF3-associated domain (IAD) and the repressor domain (RD); above, location of Ser82, Ser97 and Ser339; below, amino acid positions. (**b**) Immunoblot analysis (as in **Fig. 5d**) of *Irf3*$^{-/-}$*Irf7*$^{-/-}$ MEFs transfected with plasmid encoding wild-type IRF3 (WT) or mutant IRF3 with the S82D, S97D or S339D substitution (top), then left uninfected (–) or infected (+) for 8 h with SeV. (**c**) Immunoblot analysis of HEK293 cells transfected and infected as in **b**, followed by nucleus-cytoplasm extraction (and SDS-PAGE of the extracts). (**d**) Luciferase assay (as in **Fig. 1b**) (top) and immunoblot analysis of IRF3 and HA-tagged PTEN (below) in *Irf3*$^{-/-}$*Irf7*$^{-/-}$ MEFs transfected with empty vector or vector encoding HA-tagged PTEN (key) and with empty vector or with plasmid encoding wild-type IRF3 (WT) or mutant IRF3 with the S14A, S82A, S97A or S339A substitution (below lanes), then infected with SeV. (**e**) Luciferase assay (as in **Fig. 1b**) (top) and immunoblot analysis of IRF3-5D or GAPDH (loading control) (below) in *Pten*$^{+/+}$ MEFs and *Pten*$^{-/-}$ MEFs transfected for 20 h with empty vector or vector encoding increasing amounts (wedges: 50, 200 or 400 ng each) of IRF3-5D or IRF3-5D with the S97A substitution (IRF3-5D(S97A)). (**f**,**g**) Immunoblot analysis of IRF3 phosphorylated at Ser97 (p-IRF3(S97)) or Ser396 (p-IRF3(S396)) and total IRF3, PTEN and GAPDH (**f**) and ELISA of IFN-β (**g**) in HEK293 cells stably expressing control siRNA or PTEN-specific siRNA and infected for 0–12 h with SeV. $^{**}P < 0.01$ (unpaired *t*-test (**d**) or two-way ANOVA with *post hoc* Bonferroni *t*-test (**e**)). Data are from three experiments (**d**,**e**, top, **g** ; mean and s.d. of three independent biological replicates per group in each) or are representative of three independent experiments (**b**,**c**; **d**,**e**, bottom; **f**).

triggered by intracellular or extracellular poly(I:C) (**Fig. 4d**), LPS (**Fig. 4e**), synthetic B-form double-stranded DNA poly(dA:dT) (**Fig. 4f**), herpes simplex virus type 1 (**Fig. 4g**) or cGAMP[25] (**Fig. 4h**). However, knockout of PTEN failed to inhibit IRF7-dependent expression of type I interferon in BMDCs induced by the cytokine Flt3L (**Fig. 4i**). These results suggested that PTEN might be universally involved in IRF3-dependent innate immune responses.

PTEN controls the import of IRF3 into the nucleus

We then investigated whether PTEN targeted IRF3 itself or its upstream activation steps. Deletion of PTEN severely inhibited *Ifnb1* reporter activity induced by all upstream activators (RIG-I, MAVS, TBK1 and IKKε) and the constitutively active phosphorylation mimetic IRF3-5D[16] (**Fig. 5a**). These data indicated that PTEN exerted its function at the level of IRF3. Transcription of the IFN-β-responsive gene *Isg54* (**Fig. 5b**) was considerably reduced in cells in which PTEN was knocked out or knocked down relative to its transcription in their PTEN-sufficient counterparts, after infection with SeV (**Fig. 5b**,**c**). Knockout of PTEN did not affect the dimerization of IRF3 or its phosphorylation at Ser396 (**Fig. 5d**). However, import of IRF3 into the nucleus after challenge with SeV was significantly inhibited in *Pten*$^{-/-}$ MEFs relative to that in *Pten*$^{+/+}$ MEFs and, accordingly, more IRF3 was retained in the cytoplasm of *Pten*$^{-/-}$ MEFs after infection with SeV (**Fig. 5e**). We obtained the same results for cells in which PTEN was knocked down (**Fig. 5f**,**g**). These data demonstrated a critical role for PTEN in the import of IRF3 into the nucleus.

PTEN releases the negative phosphorylation at Ser97 of IRF3

As the role of PTEN in the induction of *IFNB1* was dependent on PTEN's phosphatase activity, we hypothesized that PTEN controlled the import of IRF3 into the nucleus by antagonizing a previously unknown negative regulatory phosphorylation of IRF3. To systematically search for the phosphorylation sites on IRF3 that might negatively regulate IRF3's activity, we individually replaced each conserved serine or threonine residue of IRF3 with alanine (phosphorylation-defective mutants) or with aspartic acid (phosphorylation-mimetic mutants). We introduced each of these mutants into *Irf3*$^{-/-}$*Irf7*$^{-/-}$ MEFs to assess their ability to activate the *Ifnb1* promoter. The effects of such substitution at Ser385, Ser386, Ser396 and Ser398 on the activation of IRF3 (**Supplementary Fig. 5a**) were consistent with published reports[14,15], which indicated that our screening system was efficient and reliable. We found that introduction of any of the three phosphorylation-mimetic IRF3 mutants, S82D, S97D or S339D, abolished the activation of *Ifnb1*, whereas introduction of the corresponding phosphorylation-defective mutants resulted in retention of the function of inducing *Ifnb1* activation (**Supplementary Fig. 5a**).

Among the three putative negative phosphorylation sites of IRF3, Ser82 and Ser97 are located in close proximity to the nuclear-localization signal[26,27], whereas Ser339 resides in the IRF3-associated domain (**Fig. 6a**). Dimerization, carboxy-terminal phosphorylation (at Ser396) and translocation to the nucleus are the three main events in the activation of IRF3. The S339D substitution of IRF3 abrogated all three events, but the S82D substitution did not influence any of these steps (**Fig. 6b**,**c**). Ser339 is reported to be a negative regulatory phosphorylation site, and its phosphorylation leads to ubiquitination-mediated degradation of IRF3 (ref. 13). The S97D substitution did not affect the dimerization or the phosphorylation of Ser396 but inhibited the import of IRF3 into the nucleus (**Fig. 6b**,**c**), a result confirmed by immunocytochemistry (**Supplementary Fig. 5b**). Thus, the S97D substitution generated phenotypes similar to that of the cells with PTEN deficiency. We expected that the three phosphorylation-defective mutants (S82A, S97A and S339A) would escape the control of negative

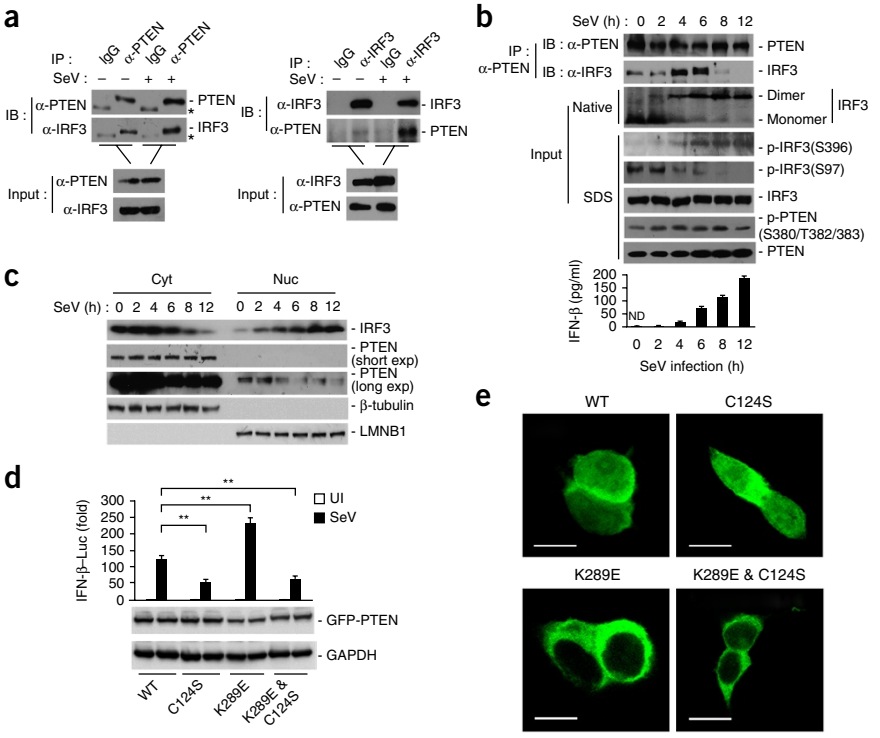

Figure 7 Virus-induced dynamic interaction of PTEN and IRF3 in the cytoplasm. (**a**) Immunoassay of lysates of HEK293 cells (5×10^6) left uninfected or infected for 4 h with SeV, followed by immunoprecipitation (IP) with immunoglobulin G (IgG), as a control, or with antibody to (α-) PTEN or IRF3 (above blots) and immunoblot analysis with antibody to PTEN or IRF3 (left margins); below (Input), immunoblot analysis of the samples above (diagonal lines) without immunoprecipitation. *, heavy chain of immunoglobulin G. (**b**) Immunoassay of lysates of HEK293 cells (2×10^7) infected for 0–12 h (above lanes) with SeV, followed by immunoprecipitation with antibody to PTEN, then native PAGE or SDS-PAGE of the immunoprecipitates and input samples and subsequent immunoblot analysis. Bottom, ELISA of IFN-β in the culture medium. (**c**) Immunoassay as in **b**, but with nucleus-cytoplasm extraction (5% of cytoplasmic extracts and 10% of nuclear extracts used for SDS-PAGE). Exp (right margin), exposure (of blot to film). (**d**,**e**) Luciferase assay (as in **Fig. 1b**) (top) and immunoblot analysis (below) (**d**) and microscopy (**e**) of HEK293 cells transfected for 24 h with plasmids encoding GFP-tagged wild-type PTEN or mutant PTEN with the C124S or K289E substitution, or both substitutions, then left uninfected (**d**) or infected for 8 h with SeV (**d**,**e**). Scale bars (**e**), 10 μm. $^{**}P < 0.01$ (one-way ANOVA with *post hoc* Bonferroni *t*-test; **d**). Data are from three experiments (**b** (bottom), **d**, top; mean and s.d. of three independent biological replicates per group in each) or are representative of three independent experiments (**a**, **b** (top), **c**, **d** (bottom), **e**).

phosphorylation; we then determined which mutant was not affected by PTEN. Ectopic expression of PTEN was still able to enhance the activation of *Ifnb1* mediated by the S82A and S339A IRF3 mutants, but not that mediated by the S97A mutant (**Fig. 6d**). In cells in which PTEN was knocked out, activation of *Ifnb1* induced by IRF3-5D was inhibited, while the interferon responses mediated by IRF3-5D with the S97A substitution were similar in PTEN-wild-type cells and cells in which PTEN was knocked out (**Fig. 6e**). The S97A substitution diminished the import of IRF3 into the nucleus but not the dimerization of IRF3 (**Supplementary Fig. 5c**,**d**), which might account for the finding that IRF3(S97A) activated comparatively less *Ifnb1* than did wild-type IRF3 in the presence of PTEN (**Fig. 6d**,**e**). Together these results indicated that Ser97 of IRF3 might be the negative phosphorylation site targeted by the phosphatase activity of PTEN. Among members of the IRF family, IRF7 and IRF3 are most homologous, and Ser97 is well conserved between IRF3 and IRF7 (Ser112 in IRF7). Ser112 of IRF7 also acted as a putative negative phosphorylation site (**Supplementary Fig. 5e**,**f**). However, both wild-type IRF7 and IRF7(S112A) substantially enhanced the activation of *Ifnb1* both in the presence of PTEN and in its absence (**Supplementary Fig. 5g**), in contrast to the result obtained with IRF3 and IRF3(S97A) (**Fig. 6d**,**e**); this indicated that Ser112 of IRF7 was not the target of the phosphatase activity of PTEN. Of note, knockout of PTEN further enhanced IRF7-mediated expression of interferon (**Supplementary Fig. 5g**); this phenomenon was consistent with published observations that activation of the pathway that includes PI(3)K, Akt and the kinase complex mTORC1 by loss of PTEN can increase IRF7-mediated production of type I interferon[28,29].

To determine whether Ser97 of IRF3 is phosphorylated *in vivo*, we analyzed the phosphorylated peptide of IRF3 by mass spectrometry. Phosphorylated Ser97 could be readily identified (**Supplementary Fig. 6a**,**b**). The abundance of phosphorylated Ser97 (ratio of phosphorylated Ser97 to total Ser97) was about 18% (**Supplementary Fig. 6c**). After treatment of the sample with recombinant glutathione *S*-transferase–tagged PTEN, the abundance of phosphorylated Ser97 was reduced to 1.45%, whereas the abundance of phosphorylation of other sites did not decrease to such an extent (**Supplementary Fig. 6d**). We next used a synthetic IRF3 peptide containing phosphorylated Ser97 as an immunogen to generate a rabbit polyclonal antibody that specifically detected phosphorylation of Ser97 in IRF3 (**Supplementary Fig. 6e.f**). Nuclear-extraction assays showed that IRF3 with phosphorylation of Ser97 was detected only in the cytoplasm (**Supplementary Fig. 6g**).

The recombinant PTEN, but not its C124S mutant, had lipid and serine-threonine phosphatase activity *in vitro* (**Supplementary Fig. 7a–c**), and PTEN was able to specifically dephosphorylate IRF3 at Ser97 (**Supplementary Fig. 7d–f**), consistent with the mass spectrometry analysis. PTEN showed markedly greater phosphatase activity toward IRF3 from SeV-infected cells than that from 'resting' cells (**Supplementary Fig. 7e**). The phosphorylation of Ser97 was much greater in cells in which PTEN was knocked down than in their PTEN-sufficient counterparts during the course of infection with SeV (**Fig. 6f**). Accordingly, the secretion of IFN-β by wild-type cells was much greater than that of cells in which PTEN was knocked down, at corresponding time points of infection with SeV (**Fig. 6g**). We obtained the same results with PTEN-deficient PC-3 cells (**Supplementary Fig. 7g**,**h**). Together these data suggested that PTEN was able to dephosphorylate IRF3 at Ser97 and was consequently able to promote the import of IRF3 into the nucleus.

Dynamic interactions of PTEN and IRF3 in the cytoplasm

We further found that endogenous PTEN was able to interact with endogenous IRF3 in uninfected HEK293 cells and that this interaction

was enhanced by infection with SeV (**Fig. 7a**). We also analyzed the interaction at various time points after infection with SeV. Endogenous IRF3 was retained mainly in the cytoplasm of resting HEK293 cells, and endogenous PTEN was associated with a small amount of IRF3; after infection with SeV, at 2–6 h, as IRF3 dimerized and Ser396 was phosphorylated, the interaction between PTEN and IRF3 increased, and at 8–12 h, as most of IRF3 was imported into the nucleus, the interaction decreased rapidly and finally disappeared (**Fig. 7b**,**c**). The phosphorylation of IRF3 at Ser97 diminished during infection with SeV, whereas the dimerization and the phosphorylation of Ser396 increased (**Fig. 7b**,**c**). Published reports have revealed that ubiquitination of PTEN at Lys289 regulates its import into the nucleus, and the K289E substitution detains PTEN in the cytoplasm[30]. Consistent with those findings, the activation of *IFNB1* mediated by the PTEN(K289E) mutant was much greater than that mediated by wild-type PTEN (**Fig. 7d**,**e**). These data suggested that PTEN interacted with IRF3 in the cytoplasm before the import of IRF3 into the nucleus and that this interaction was dynamic during infection with SeV.

DISCUSSION

The phosphatase PTEN has been studied extensively since the discovery of its function in tumor suppression[31]. Unlike its tumor-suppressive function, here we have reported a previously unknown function for PTEN associated with host defense against viral invasion. We demonstrated that PTEN functioned as a protein phosphatase by dephosphorylating and thereby activating IRF3, the master controller of the production of type I interferon. Therefore, our results provide a new and direct link between tumor suppression and antiviral innate immunity.

PTEN is a negative regulator of the PI(3)K-Akt pathway. Whether PI(3)K-Akt pathway has a role in the regulation of innate immunity is controversial[32]. Notably, our data showed that PTEN promoted SeV-induced production of IFN-β mainly in a PI(3)K-Akt–independent manner and that the contribution of the PI(3)K-Akt pathway to the production of IFN-β was marginal. These findings not only revealed a previously unknown PI(3)K-Akt–independent function for PTEN but also indicated the complexity and diversity of PTEN-mediated cellular regulation.

The phosphatase-dependent role of PTEN in the induction of *IFNB1* indicated the existence of potential substrate of the phosphatase activity of PTEN involved in innate immunity. Unexpectedly, we identified IRF3 as a substrate of the phosphatase activity of PTEN and found the target phosphorylation site at Ser97 of IRF3. The activation of IRF3 by phosphorylation has been studied extensively[33], but activation by dephosphorylation has not been reported before, to our knowledge. Therefore, our study has revealed a previously unknown regulatory mechanism of antiviral innate immunity that acts by inactivating IRF3 via phosphorylation and by activating IRF3 via dephosphorylation.

It is well known that the activation of IRF3 before its import into the nucleus consists of two essential steps: phosphorylation and dimerization. Here we found that dephosphorylation of IRF3 at Ser97 was critical for its import into the nucleus, which indicated that the mechanism of IRF3 activation is much more complicated than previously understood. The regulation of nuclear-cytoplasmic trafficking by phosphorylation is emerging as an important step in regulating the accessibility of various proteins to the nucleus[34]. On the basis of our studies, we propose that the activation of IRF3 and its import into the nucleus might be determined by at least three steps: carboxy-terminal phosphorylation, dimerization, and amino-terminal dephosphorylation. Our studies have shown that the negative and positive regulation of the activation of IRF3 by phosphorylation and PTEN-mediated dephosphorylation represents a sophisticated mechanism for controlling the activity of the master transcription factor in innate immunity.

PTEN is among the most commonly mutated or deleted tumor suppressors in sporadic cancer[35]. We demonstrated that cancer cells deficient in the phosphatase activity of PTEN had 'crippled' production of type I interferon and antiviral responses and thus they might represent optimal targets for interferon-sensitive oncolytic viruses. Our study also demonstrated that PTEN deficiency in mice led to reduced production of type I interferon and increased susceptibility to VSV, whereas the PI(3)K-Akt pathway did not exert a significant effect on the induction of type I interferon or propagation of VSV. Those results have been confirmed by a published report showing that deficiency in PTEN in prostate cancer cells leads to robust viral growth and cell lysis in mouse model, although that study did not address a direct role for PTEN in antiviral immunity[36]. Therefore, our work provides a theoretical basis for the screening and clinical treatment of PTEN-null tumors by oncolytic viruses such as VSV.

Type I interferons have multiple potential effects on tumor cells, including the promotion of apoptosis, inhibition of proliferation and tumor angiogenesis, and activation of innate and adaptive immune responses[37–39]. We are tempted to speculate that ablation of the production of type I interferon by loss of PTEN might further contribute to tumor development. Overall, we propose that PTEN might exert antitumor activity in part through the promotion of type I interferon responses, and thus we have expanded PTEN's repertoire as a tumor suppressor via antagonizing PI(3)K-Akt signaling pathway. Together our findings have revealed crosstalk between the tumor suppression by PTEN and its effect on antiviral immunity and might benefit the development of efficient therapeutic interventions for human cancer and viral infection.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.L. and D.G. designed the experiments and wrote the manuscript.; S.L., M.Z., R.P., T.F., Y.-Y.C. and C.-M.L. performed experimental work; S.L., M.Z., S.C., X.Z., L.G., Y.C., E.J. and D.G. analyzed the data; C.-Q.L., Y.Y. and H.-B.S. provided experimental material; and D.G. conceived of and supervised the study.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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