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III. 研究成果の刊行物・別刷（抜粋）

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Negative regulation of interferon-regulatory factor 3–dependent innate antiviral response by the prolyl isomerase Pin1

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Recognition of double-stranded RNA activates interferon-regulatory factor 3 (IRF3)–dependent expression of antiviral factors. Although the molecular mechanisms underlying the activation of IRF3 have been studied, the mechanisms by which IRF3 activity is reduced have not. Here we report that activation of IRF3 is negatively regulated by the peptidyl-prolyl isomerase Pin1. After stimulation by double-stranded RNA, induced phosphorylation of the Ser339–Pro340 motif of IRF3 led to its interaction with Pin1 and finally polyubiquitination and then proteasome-dependent degradation of IRF3. Suppression of Pin1 by RNA interference or genetic deletion resulted in enhanced IRF-3-dependent production of interferon-β, with consequent reduction of virus replication. These results elucidate a previously unknown mechanism for controlling innate antiviral responses by negatively regulating IRF3 activity via Pin1.

The innate immune response is an important, evolutionarily conserved mechanism that protects the host from both viral and microbial infections[1–3]. Increasing evidence has shown the importance of pattern-recognition receptors in immune responses after viral and microbial infection by invading pathogens[3]. Toll-like receptor 3 (TLR3) detects extracellular viral double-stranded RNA (dsRNA) internalized into the endosomes, whereas retinoic acid–inducible gene I (RIG-I), a DExD/H box RNA helicase containing a caspase-recruitment domain, detects intracellular viral dsRNA[3–5]. TLR4, in contrast, recognizes microbial components such as bacterial lipopolysaccharide (LPS)[6]. Engagement of any of those receptors triggers rapid production of type I interferon (IFN-αβ) and thus establishes the innate immune status against infectious agents[3,5,7].

Interferon-regulatory factor 3 (IRF3), a ubiquitously expressed transcription factor, is responsible for the primary induction of IFN-β and is important in the establishment of innate immunity in response to either viral or microbial infection[1–3]. After the detection of pathogens, IRF3 is phosphorylated on multiple phosphorylation acceptor (phospho-acceptor) sites, forms homodimers and then translocates to the nucleus, where it binds to the interferon stimulation–response elements of target genes, as well as the positive regulatory domain III-I in the IFN-β promoter[1–3]. The mechanisms underlying the phosphorylation-induced activation of IRF3 have been the subject of many extensive studies[8–16]. The substitution of alanine for either the Ser385 or Ser386 residue of IRF3 abolishes its activation[8,9]. Additionally, phosphorylation of Ser386 on IRF3 is induced by TLR3 engagement and by viral infection and only for IRF3 dimers[9]. The importance of five critical serine or threonine residues of IRF3 (Ser396, Ser398, Ser402, Thr404 and Ser405) for its activation has been demonstrated[10,11]. Notably, the substitution of alanine for all five amino acids abrogates the function of IRF3 to activate transcription, whereas aspartic acid substitutions result in a constitutively active protein. Those published data demonstrate that phosphorylation of both C-terminal phospho-accepter clusters (Ser385-Ser386 and Ser396-Ser398-Ser402-Thr404-Ser405) is important for the activation of IRF3. Other studies have also shown that two IκB kinase (IKK)–like kinases, TBK1-NAK and IKK-*i*–IKKε, are required for the activation of IRF3 by inducing the phosphorylation of its two C-terminal phospho-acceptor clusters and thus are essential in the expression of type I interferon[14–17]. Phosphorylation-dependent post-translational modifications of IRF3 are therefore crucial for regulating the function of IRF3.

Pin1 is a peptidyl-prolyl isomerase that via its WW domain (with two conserved tryptophan residues) specifically recognizes phosphorylated serine or threonine residues followed by proline and then catalyzes a conformational change of the bound substrate in a

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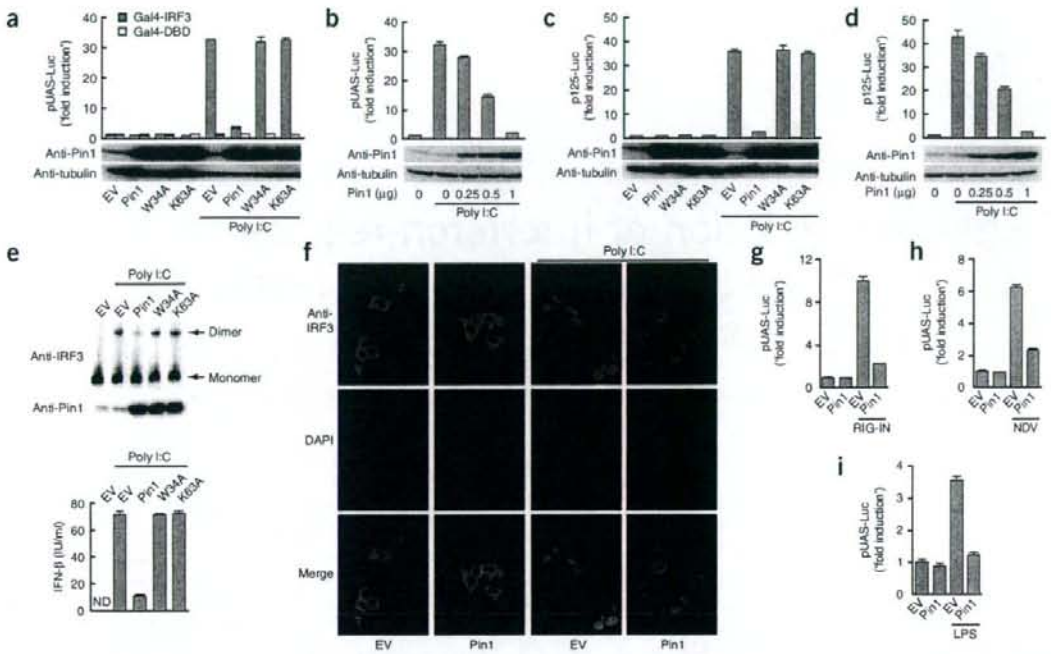

Figure 1 Pin1 suppresses IRF3-dependent transcriptional activation. (**a–d**) Luciferase assay (above) and immunoblot (below) of lysates from 293-TLR3 cells transfected with pEF1-lacZ and either the Gal4-site luciferase reporter pUAS-Luc (**a**,**b**) or the IFN-β promoter reporter p125-Luc (**c**,**d**), transiently expressing empty vector (EV), wild-type Pin1 or Pin1 mutants and either Gal4-IRF3 or Gal4-DBD (control; **a**,**c**) or transiently expressing various amounts of wild-type Pin1 (below lanes; **b**,**d**), and then stimulated with poly(I)•poly(C) (poly I:C). Luciferase activity is normalized to β-galactosidase activity; results are means ± s.d. from three separate transfections. (**e**) Immunoblots of endogenous IRF3 dimer detected by native PAGE (top) and Pin1 expression detected by SDS-PAGE (middle) in 293-TLR3 cells transiently expressing empty vector, wild-type Pin1 or Pin1 mutants and left unstimulated (far left) or stimulated for 3 h with poly(I)•poly(C). Bottom, ELISA of IFN-β secreted into the culture supernatant. ND, not detected. (**f**) Immunohistochemistry to detect endogenous IRF3 (green) in 293-TLR3neo' cells stably expressing Pin1 with or without poly(I)•poly(C) stimulation. Cell nuclei are blue (DAPI, 4,6-diamidino-2-phenylindole). Original magnification, ×40. (**g**) Luciferase assay of lysates prepared from 293 cells at 36 h after transfection with pEF1-lacZ and pUAS-Luc and expressing Pin1 and Gal4-IRF3 with or without the RIG-I N-terminal caspase-recruitment domain (RIG-IN). (**h**,**i**) Luciferase assay of lysates from 293 cells (**h**) or U373-CD14 cells (**i**) transfected with pEF1-lacZ and pUAS-Luc, transiently expressing Pin1 and Gal4-IRF3, and then infected with NDV (**h**) or treated with LPS (**i**). Data are representative of two independent experiments.

phosphorylation-dependent way[18,19]. By that mechanism, Pin1 has been shown to regulate the stability and or localization of its substrates during transcriptional activation, cell cycle progression and cell death, and deregulated expression or loss of function of Pin1 leads to the progression of important human diseases such as cancer and Alzheimer disease[18,20–28]. However, a regulatory function for Pin1 in the host defense against infectious agents and associated signal transduction pathways has not been reported before to our knowledge. Published findings showing that post-translational modification of IRF3 by phosphorylation controls the IRF3 activity prompted us to assess the involvement of Pin1 in regulating IRF3 signaling.

RESULTS

Pin1 suppresses IRF3-dependent transcriptional activation

A well characterized stimulation for IRF3 activation is TLR3 engagement with the synthetic dsRNA poly(I)•poly(C)[7,14,29,30]. We therefore used that mode of activating IRF3 to investigate possible involvement of Pin1 in regulating the activity of IRF3. Reporter gene assays using a yeast transcription factor Gal4–IRF3 fusion protein showed that exogenous expression of wild-type Pin1 inhibited poly(I)•poly(C)-induced IRF3-dependent transcriptional activation in a dose-dependent way, but expression of its WW domain mutant (W34A) or peptidyl-prolyl isomerase domain mutant (K63A) did not (**Fig. 1a**,**b**). That result indicated that functional WW and peptidyl-prolyl isomerase domains of Pin1 are required for the regulation of IRF3 signaling. Similarly, exogenous expression of wild-type Pin1 suppressed TLR3-mediated, IRF3-dependent activation of the IFN-β promoter and reduced IFN-β secretion in culture supernatants (**Fig. 1c–e**). We next addressed whether Pin1 affects the dimerization and nuclear localization of endogenous IRF3 that normally occurs after poly(I)•poly(C) stimulation. Native gel electrophoresis and immunoblot of cell lysates after poly(I)•poly(C) stimulation showed that exogenous Pin1 expression produced a considerable reduction in the activated dimer form of IRF3 (**Fig. 1e**). Immunocytochemical analyses confirmed that Pin1 overexpression decreased the amount of nuclear IRF3 (**Fig. 1f**).

We next assessed if exogenous Pin1 expression would inhibit IRF3 signaling induced by RIG-I-mediated detection of RNA virus infection or by LPS stimulation of TLR4. In agreement with published results[5], we found that IRF3-dependent transcriptional activation by the N-terminal caspase-recruitment domain of RIG-I was inhibited by exogenously expressed Pin1 (**Fig. 1g**). In addition, expression of Pin1 inhibited Newcastle disease virus (NDV)–induced IRF3-dependent

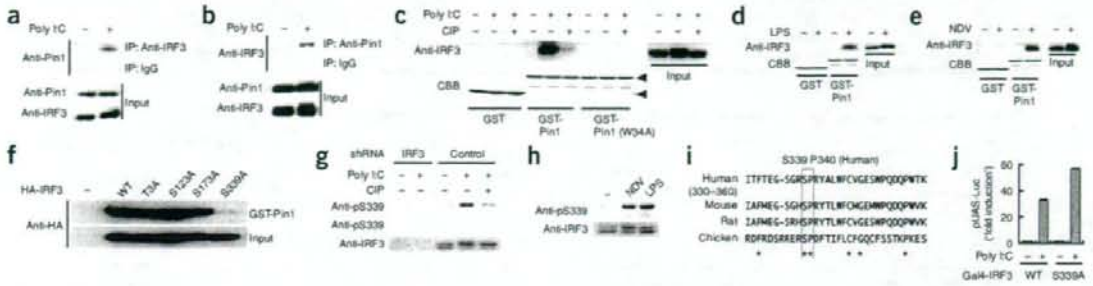

Figure 2 Pin1 interacts with IRF3 through its phosphorylated Ser339–Pro340 motif. (**a**,**b**) Immunoprecipitation (IP) and immunoblot of lysates from 293-TLR3 cells stimulated for 2 h with poly(I)•poly(C), analyzed with either polyclonal anti-IRF3 and anti-Pin1 (**a**) or anti-Pin1 and anti-IRF3 (**b**) or control immunoglobulin G (IgG). (**c**) PAGE and immunoblot with anti-IRF3 of affinity-purified lysates from 293-TLR3 cells stimulated for 2 h with poly(I)•poly(C) or left untreated. Lysates were left untreated or were treated with calf intestinal alkaline phosphatase (CIP), followed by incubation with GST, GST-Pin1 or GST-Pin1 W34A. Recombinant GST proteins (arrowheads) are visualized by Coomassie brilliant blue staining (CBB). (**d**,**e**) GST-Pin1 affinity assay of lysates from U373-CD14 cells stimulated for 2 h with LPS (**d**) or from 293 cells infected for 12 h with NDV or left untreated (**e**). (**f**) GST-Pin1 affinity assay of lysates from 293-TLR3 cells transfected with hemagglutinin-tagged wild-type IRF3 (WT) or various IRF3 mutants, stimulated with poly(I)•poly(C). (**g**) Immunoblot of lysates from U373-CD14 cells infected with a retroviral IRF3-specific or control shRNA construct and selected with puromycin. Cell pools were stimulated with poly(I)•poly(C), then lysates were treated with calf intestinal alkaline phosphatase and were analyzed by PAGE and immunoblot with anti–phospho-Ser339 (Anti-pS339), preincubated with phosphorylated (middle) or unphosphorylated (top) Ser339 peptide. Bottom, immunoblot with anti-IRF3. (**h**) PAGE and immunoblot of lysates from U373-CD14 cells treated with NDV or LPS, analyzed with anti–phospho-Ser339. (**i**) Amino acid alignment of the C-terminal regions of human, mouse, rat and chicken IRF3. *, amino acid residues conserved among the four species; boxed amino acid residues correspond to human Ser339 and Pro340. (**j**) Luciferase assay of lysates from 293-TLR3 cells transfected with pEF1-lacZ and pUAS-Luc and expressing either wild-type Gal4-IRF3 (WT) or the Gal4-IRF3 S339A mutant (S339A), stimulated for 9 h with poly(I)•poly(C). Data are representative of two independent experiments.

transcriptional activation, which is mediated by RIG-I (ref. 5; **Fig. 1h**). Finally, expression of Pin1 also suppressed TLR4-mediated IRF3-dependent transcriptional activation (**Fig. 1i**). These results strongly suggested that Pin1 is a negative regulator of a transcriptional activator of IFN-β and acts on a mediator shared by three independent pathways of IRF3 activation, indicating direct negative regulation of IRF3 itself.

Pin1 interacts with IRF3 in a phosphorylation-dependent way

Because Pin1 has been reported to regulate a subset of transcription factors[18], we determined if it physically interacts with IRF3. Immunoprecipitation followed by immunoblot analysis showed that poly(I)•poly(C) stimulation induced interaction of endogenous Pin1 with endogenous IRF3 *in vivo* (**Fig. 2a**,**b**). Glutathione *S*-transferase (GST) affinity assays further demonstrated an *in vitro* interaction between purified GST-Pin1 and endogenous IRF3 (**Fig. 2c**), and pretreatment of cell lysates with calf intestinal alkaline phosphatase abolished the interaction, indicating that phosphorylation is a prerequisite for the Pin1-IRF3 interaction. Consistent with that, the WW domain mutant of Pin1 (W34A) failed to interact with IRF3. LPS stimulation and NDV infection also induced the interaction of Pin1 with IRF3 (**Fig. 2d**,**e**).

We next sought to identify the specific Ser-Pro or Thr-Pro residues of IRF3 targeted by Pin1. The phospho-accepter residues required for IRF3 activation do not contain the Ser-Pro or Thr-Pro motif required for interaction with Pin1 (refs. 8–16). There are five Ser-Pro or Thr-Pro sites in human IRF3, and four of those (Thr3, Ser 123, Ser 173 and Ser339) are conserved between human and mouse. We analyzed mutants with site-directed substitution of each potential Pin1-binding site of IRF3 (substitution of alanine for serine or threonine) for Pin1 binding after poly(I)•poly(C) treatment; only the S339A substitution substantially disrupted the interaction between IRF3 and Pin1 (**Fig. 2f**). Immunoblot analysis with antibodies raised against an IRF3 peptide phosphorylated at Ser339 (anti–phospho-Ser339) demonstrated that this Ser residue of endogenous IRF3 was phosphorylated after stimulation with poly(I)•poly(C), LPS or NDV (**Fig. 2g**,**h**). Preincubation of anti–phospho-Ser339 with the phosphorylated peptide blocked detection of phosphorylated IRF3, but preincubation with the unphosphorylated peptide did not, establishing the specificity of the antibody (**Fig. 2g**). Suppression of endogenous IRF3 expression by RNA interference or by treatment of cell lysates with alkaline phosphatase diminished the immunoreactivity of anti–phospho-Ser339, indicating that it recognizes phosphorylated IRF3 (**Fig. 2g**). Notably, the amino acid residues corresponding to Ser339-Pro340 in human IRF3 are highly conserved among other species, such as mouse, rat and chicken (**Fig. 2i**), suggesting the importance of this Ser-Pro motif. At present, the kinase responsible for Ser339 phosphorylation remains unknown. Although phosphorylation at Ser396, which is reported to be mediated by TBK1 and IKK-*i*[9,15,16], reached a peak 1 h after stimulation with poly(I)•poly(C) or LPS, phosphorylation at Ser339 was detected several hours later (**Supplementary Fig. 1** online). Loss-of-function experiments using an RNA-interference strategy demonstrated involvement of TBK1 and IKK-*i* in the phosphorylation of Ser339 as well as Ser396 after poly(I)•poly(C) stimulation (**Supplementary Fig. 2** online). However, we do not have evidence for direct phosphorylation of Ser339 by these IKK-related kinases and cannot exclude the possibility that phosphorylation of the C-terminal phospho-accepter clusters by TBK1 and IKK-*i* somehow facilitates subsequent phosphorylation of Ser339 by another kinase. We confirmed the biological consequences of Ser339 phosphorylation by reporter gene assay. The S339A substitution augmented the activation of the reporter gene by poly(I)•poly(C) stimulation (**Fig. 2j**). These results collectively suggest that phosphorylation of IRF3 Ser339 and subsequent interaction with Pin1 are important for the negative regulation of IRF3 signaling.

Pin1 destabilizes activated IRF3

The interaction of Pin1 and IRF3 leading to suppression of IRF3 activity prompted us to examine whether Pin1 regulates IRF3

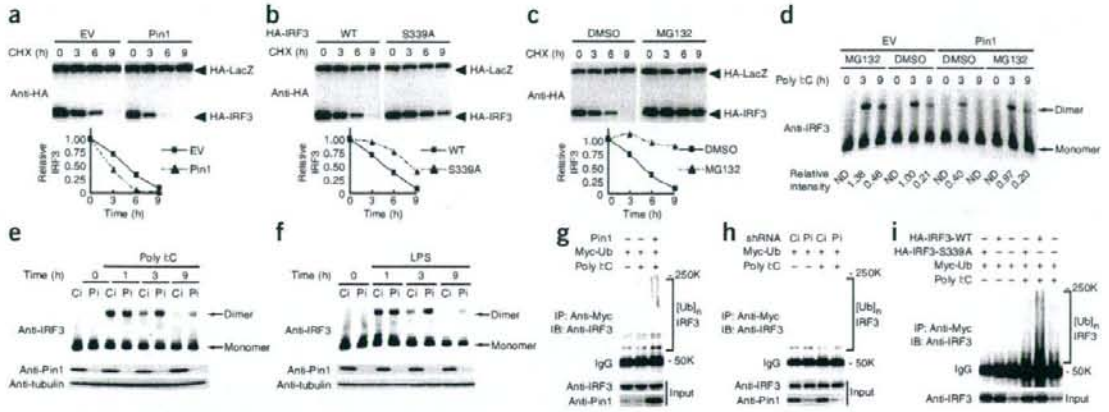

Figure 3 Pin1 regulates the stability of IRF3. (**a**) Immunoblot of lysates from 293-TLR3 cells transiently expressing Pin1, HA-lacZ and HA-IRF3, stimulated with poly(I)•poly(C) and then cultured in the presence of cycloheximide (CHX; time, above lanes). Expression of HA-IRF3 is normalized to that of HA-lacZ (graphed below). (**b**) Immunoblot of lysates from 293-TLR3 cells transiently expressing wild-type HA-IRF3 (WT) and HA-lacZ or HA-IRF3 S339A (S339A) and HA-lacZ and then treated as described in **a**. (**c**) Immunoblot of lysates from 293-TLR3 cells transiently expressing HA-IRF3 and HA-lacZ and then stimulated with poly(I)•poly(C) in the presence of cycloheximide and either DMSO (dimethyl sulfoxide) or MG132 (10 μM). (**d**) Native PAGE and immunoblot with anti-IRF3 of lysates from 293-TLR3 cells transiently expressing Pin1 and stimulated with poly(I)•poly(C) (time, above lanes) in the presence or absence (DMSO) of 10 μM MG132. Bottom, relative amount of IRF3 dimer. (**e**,**f**) Native PAGE and immunoblot with anti-IRF3 of lysates from U373-CD14 cells infected with a retroviral control (Ci) or Pin1-specific (Pi) shRNA construct and stimulated (time, above lanes) with poly(I)•poly(C) (**e**) or LPS (**f**). (**g**) Immunoprecipitation with anti-Myc and immunoblot (IB) with anti-IRF3 of whole-cell lysates from 293-TLR3 cells expressing Myc-tagged ubiquitin (Myc-Ub) and stimulated with poly(I)•poly(C) in the presence of MG132. $[Ub]_n$, polyubiquitination. (**h**) Immunoprecipitation with anti-Myc and immunoblot with anti-IRF3 of lysates from 293 cells infected with retroviral control or Pin1-specific shRNA, transiently expressing human TLR3 and Myc-tagged ubiquitin and stimulated with poly(I)•poly(C) in the presence of MG132. (**i**) Immunoprecipitation with anti-Myc and immunoblot with anti-IRF3 of whole-cell lysates from 293-TLR3 cells transiently expressing various proteins (above lanes) and stimulated with poly(I)•poly(C) in the presence of MG132. Data are representative of two independent experiments.

protein stability. Because IRF3 is highly stable in the steady state[31] (data not shown), we induced IRF3 activation by stimulation with poly(I)•poly(C). After activation with poly(I)•poly(C), IRF3 degraded more rapidly in 293 cell cultures stably expressing human TLR3 ('293-TLR3' cells) expressing exogenous Pin1 (**Fig. 3a**), and the IRF3 S339A mutant, which was defective in Pin1 binding, had a slower turnover than that of wild-type IRF3 (**Fig. 3b**). In the presence of the proteasome inhibitor MG132, IRF3 demonstrated considerably increased stability (**Fig. 3c**), suggesting that a proteasome-dependent mechanism underlies the negative regulation of IRF3. Our finding that Pin1 reduced the homodimer of IRF3 (**Fig. 1e**), in combination with these data demonstrating that turnover of IRF3 may involve a proteasome-dependent process, led us to determine how MG132 and/or Pin1 influences the status of the activated dimer form of IRF3 induced by poly(I)•poly(C) stimulation. Treatment with MG132 substantially increased the amount of IRF3 dimer, whereas the addition of exogenous Pin1 expression reduced the amount of IRF3 dimer (**Fig. 3d**). The addition of MG132 reversed the suppressive effects of Pin1 on IRF3 (**Fig. 3d**).

To verify the function of endogenous Pin1 in IRF3 signaling, we generated a short hairpin RNA (shRNA) expression construct capable of 'knocking down' Pin1 expression. Stable expression of Pin1-specific shRNA effectively reduced the amount of endogenous Pin1 but did not alter the expression of α-tubulin (**Fig. 3e,f**). Native PAGE coupled with immunoblot analysis showed that the suppression of endogenous Pin1 expression greatly increased IRF3 dimers induced by poly(I)•poly(C) or LPS at later time points but not at 1 h after stimulation (**Fig. 3e,f**). These results indicated that Pin1 'preferentially' promotes proteasome-dependent degradation of activated IRF3.

Ubiquitination-dependent degradation of transcription factors is an important mechanism for the termination of transcriptional activation[32]. Because we had found that proteasome inhibition delayed the degradation of IRF3 (**Fig. 3c**), we determined whether IRF3 is ubiquitinated. Immunoprecipitation of ubiquitin followed by immunoblot analysis for IRF3 demonstrated that polyubiquitination of IRF3 was induced by poly(I)•poly(C) stimulation (**Fig. 3g**) and that polyubiquitination was augmented by Pin1 expression and abrogated by expression of Pin1-specific shRNA (**Fig. 3g,h**). Consistent with the finding that Pin1 facilitated IRF3 degradation after poly(I)•poly(C) stimulation (**Fig. 3a**), the S339A mutant of IRF3 was less susceptible to polyubiquitination than was wild-type protein (**Fig. 3i**). These results indicated that Pin1 regulates ubiquitination and proteasome-mediated proteolysis of IRF3.

Endogenous Pin1 regulates TLR3-mediated IFN-β production

We next investigated the function of endogenous Pin1 in dsRNA-induced, IRF3-dependent transcriptional activation. As anticipated, expression of Pin1-specific shRNA enhanced IRF3-dependent transcriptional activation and thus enhanced activation of the IFN-β promoter triggered by the engagement of TLR3 (**Fig. 4a,b**). In contrast, expression of Pin1-specific shRNA did not affect TLR3-mediated activation of NF-κB, a transcription factor also involved in IFN-β production (**Supplementary Fig. 3** online). Moreover, expression of a second Pin1-specific shRNA also augmented IRF3-dependent reporter gene activation, and complementation with mouse Pin1, which does not contain the human Pin1 shRNA target sequence, reversed the effects on IRF3-dependent transcriptional activation (**Supplementary Fig. 4** online). To investigate the temporal regulation of IRF3-dependent transcription by Pin1, we used a

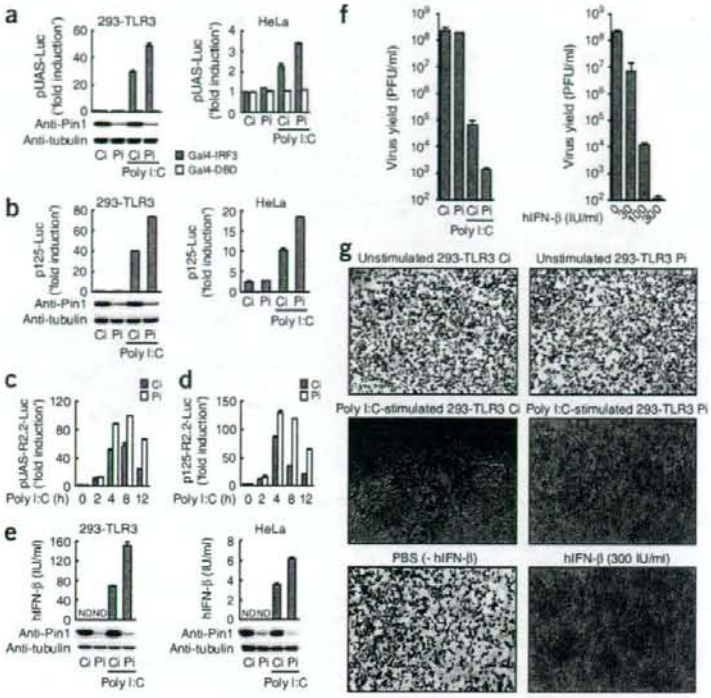

Figure 4 Endogenous Pin1 negatively regulates TLR3-mediated IRF3-dependent transcriptional activation and IFN-β production. (**a–d**) Luciferase assay of lysates from 293-TLR3 cells (**a–d**) and HeLa cells (**a,b**, right) transfected with pEF1-lacZ and pUAS-Luc (**a**), p125-Luc (**b**), pUAS-R2.2-Luc (**c**) or p125-R2.2-Luc (**d**) and transiently expressing control or Pin1-specific shRNA (**a–d**) and Gal4-IRF3 (**a,c**). Cells were stimulated with poly(I)•poly(C) for 9 h (**a,b**) or for various times (horizontal axes; **c,d**); relative luciferase activities are presented as in **Figure 1**. Expression of Pin1 in 293-TLR3 cells was verified by immunoblot (**a**, **b**, bottom). (**e**) ELISA of human IFN-β (hIFN-β) secretion from either 293-TLR3 cells transiently expressing control or Pin1 shRNA (left) or HeLa cells infected with retroviruses expressing either control or Pin1 shRNA (right), analyzed after stimulation for 12 h with poly(I)•poly(C). Data are means ± s.d. from three separate samples. (**f**) VSV production (in plaque-forming units (PFU)/ml) 24 h after infection of Vero cells previously treated with supernatants from 293-TLR3 cells transiently expressing control or Pin1 shRNA and stimulated for 12 h with poly(I)•poly(C) (left) or with various concentrations of human IFN-β (right). (**g**) Phase-contrast micrographs of VSV-infected Vero cells. Dead cells (round shape) are detached from the culture dish. Original magnification, ×20. Data are representative of two independent experiments.

rapid-response luciferase reporter gene. Real-time reporter gene assays showed that suppression of endogenous Pin1 expression substantially prolonged both IRF3-dependent transcription and IFN-β promoter activation after poly(I)•poly(C) stimulation (**Fig. 4c,d**). Consistent with the inhibitory effects of Pin1 on the IFN-β promoter, expression of Pin1-specific shRNA but not that of control shRNA increased the production of IFN-β induced by poly(I)•poly(C) (**Fig. 4e**). Because exogenous expression of Pin1 greatly reduced the production of a potent antiviral secreted factor (IFN-β) from poly(I)•poly(C)-stimulated 293-TLR3 cells (**Supplementary Fig. 5** online), we assessed if endogenous Pin1 was sufficient to induce the same regulation. Culture supernatants of poly(I)•poly(C)-stimulated 293-TLR3 cells expressing Pin1-specific shRNA had approximately 50 times more antiviral activity than that of supernatant of cells expressing control shRNA (**Fig. 4f**); moreover, this amount of secreted antiviral factor was sufficient to fully protect fresh cells from productive infection by vesicular stomatitis virus (VSV; **Fig. 4g**). These results indicated that Pin1 is involved in terminating IRF3 signaling triggered after TLR3 engagement with activating ligand and thus Pin1 regulates the expression of IRF3 target genes relevant to the innate antiviral response. However, unlike other negative regulators of TLR signaling whose expression is upregulated by stimulation[3], the expression of Pin1 protein was not substantially different after stimulation with poly(I)•poly(C) or IFN-β (**Supplementary Fig. 6** online).

Pin1 deficiency enhances IFN-β production induced by dsRNA

To assess the involvement of Pin1 in dsRNA-induced innate antiviral response in immune competent cells, we prepared bone marrow–derived macrophages from *Pin1*$^{-/-}$ or *Pin1*$^{+/+}$ mice. After being stimulated with poly(I)•poly(C), macrophages from *Pin1*$^{-/-}$ mice secreted more IFN-β than did wild-type cells (**Fig. 5a**). Those findings prompted us to determine if Pin1 also limits IFN-β induction *in vivo*. The amount of IFN-β produced after intraperitoneal injection of poly(I)•poly(C) was much greater in sera of *Pin1*$^{-/-}$ mice than in sera of similarly treated wild-type mice (**Fig. 5b**). These results provided

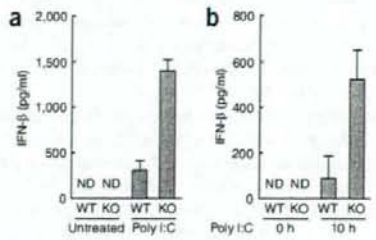

Figure 5 Pin1 deficiency enhances IFN-β production in response to dsRNA. (**a**) ELISA of IFN-β production from bone marrow–derived macrophages prepared from *Pin1*$^{+/+}$ (WT) or *Pin1*$^{-/-}$ (KO) mice; cells were left untreated or were stimulated for 8 h with poly(I)•poly(C) (25 μg/ml). Data are means ± s.d. of three independent experiments. (**b**) ELISA of IFN-β production in sera from *Pin1*$^{+/+}$ or *Pin1*$^{-/-}$ mice ($n = 3$ per group) injected intraperitoneally with 5 μg poly(I)•poly(C) per gram body weight. Sera were collected from each mouse before or 10 h after treatment. Error bars represent s.d.

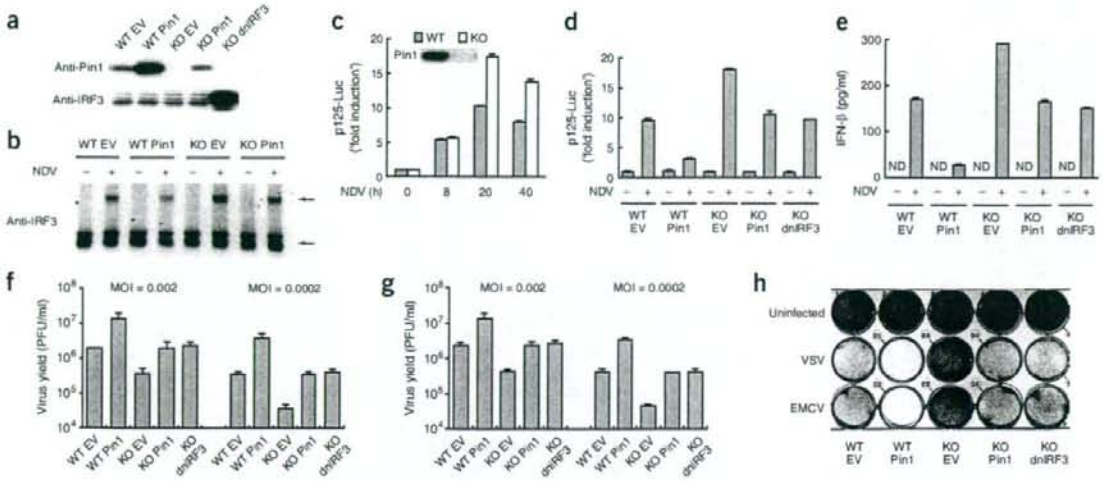

Figure 6 Pin1 regulates RIG-I-mediated IRF3 signaling and innate antiviral response. (**a**) Immunoblot with anti-Pin1 or anti-IRF3 of lysates from MEFs isolated from *Pin1*$^{+/+}$ (WT) or *Pin1*$^{-/-}$ (KO) mice infected with retroviruses expressing mouse Pin1 (WT-Pin1 or KO-Pin1) or hemagglutinin-tagged dominant negative IRF3 (KO dnIRF3) and selected with blasticidin S. (**b**) Native PAGE and immunoblot with anti-IRF3 of lysates from the MEFs described in **a**, infected for 30 h with NDV. Arrows (right margin) indicate IRF3 dimer (top) and monomer (bottom). (**c**) Luciferase assay of lysates from *Pin1*$^{+/+}$ or *Pin1*$^{-/-}$ MEFs transfected with p125-Luc and pEF1-lacZ and then infected with NDV (times, horizontal axis). Inset, immunoblot for Pin1. (**d**) Luciferase assay of lysates from the MEFs in **a**, transfected with p125-Luc and pEF1-lacZ and then infected for 20 h with NDV. (**e**) ELISA of IFN-β produced from the MEFs in **a**, infected for 40 h with NDV. (**f**,**g**) Virus production from the MEFs in **a**, infected with VSV (**f**) or EMCV (**g**) at a multiplicity of infection (MOI) of 0.002 (left) or 0.0002 (right). Virus yield in the supernatants at 20 h after infection was determined by plaque assay. (**h**) Amido black staining of the MEFs in **a**, infected for 20 h with VSV (MOI, 0.002) or EMCV (MOI, 0.002) but not killed; cells were fixed and then stained. Data are representative of two independent experiments.

genetic evidence that Pin1 regulates dsRNA-induced IFN-β production both *in vivo* and *in vitro*.

Pin1 regulates RIG-I-dependent antiviral cellular responses

To assess the involvement of Pin1 in the RIG-I-dependent antiviral cellular response, we used mouse embryonic fibroblasts (MEFs) isolated from *Pin1*$^{-/-}$ mice (**Fig. 6a**). Native PAGE coupled with immunoblot analysis showed that loss of Pin1 expression considerably increased the amount of IRF3 dimer (**Fig. 6b**). Complementation of *Pin1*$^{-/-}$ MEFs with expression of wild-type Pin1 normalized the IRF3 response to NDV infection, confirming that deregulated IRF3 activation in *Pin1*$^{-/-}$ MEFs was due to the loss of Pin1 expression. Reporter gene assays showed that the NDV-induced activation of the IFN-β promoter was enhanced at later time points in *Pin1*$^{-/-}$ MEFs compared with that of wild-type MEFs, an effect that was reversed by complementation with either wild-type Pin1 or a dominant negative mutant of IRF3 (**Fig. 6c,d**). Consistent with that finding, *Pin1*$^{-/-}$ MEFs demonstrated increased production of IFN-β after NDV infection (**Fig. 6e**). To demonstrate the specificity of Pin1 on IRF3 activation, we also evaluated NF-κB activation in *Pin1*$^{-/-}$ MEFs after NDV infection and found that it was only marginally affected by the absence of Pin1 (**Supplementary Fig. 3**). Finally, we noted that NDV infection weakly and transiently increased Pin1 protein expression (**Supplementary Fig. 6**).

We next sought to determine if Pin1 regulated replication of VSV and encephalomyocarditis virus (EMCV), as RIG-I-mediated IRF3 signaling is critical in restricting replication of these RNA viruses[5]. Consistent with the suppressive effects of Pin1 on RIG-I-mediated IRF3 signaling (**Fig. 1g,h**), exogenous expression of Pin1 increased the production of infectious VSV or EMCV in the culture supernatants of infected MEFs, whereas loss of Pin1 expression decreased it (**Fig. 6f,g**). Furthermore, the reduction of virus yield in *Pin1*$^{-/-}$ MEFs was reversed by the expression of a dominant negative form of IRF3. In agreement with those results, increased expression of Pin1 augmented virus-mediated cell killing, whereas loss of Pin1 expression protected cells (**Fig. 6h**). These results provide biological evidence that Pin1 acts as a negative regulator of RIG-I-mediated IRF3 signaling and thereby modulates the innate antiviral cellular response.

DISCUSSION

Studies have identified functions for Pin1 in a variety of pathological conditions[18]. Pin1 prevents the accumulation of hyperphosphorylated tau protein in the brain and thus protects neuronal cells from age-dependent degeneration[18,25]. However, increased expression of Pin1 has often been detected in many different neoplasms, such as breast cancer, and is also involved in the malignant transformation of cancer cells[18,24,33]. Here we have demonstrated that Pin1 is involved in the termination of IRF3-dependent transcriptional activation, which thereby provides negative regulation of the innate antiviral response against infection by RNA viruses. The results presented here suggest that increased expression of Pin1 suppresses the innate antiviral response and thus may allow the replication and persistence of infectious agents. If true, such a possibility could partly explain why cancer cells are more susceptible to lytic infection by VSV[34].

We have also demonstrated that the genetic absence or RNA interference-mediated suppression of Pin1 expression enhanced poly(I)•poly(C)-induced activation of IRF3 and consequent production of IFN-β. Because activation of IRF3 and production of IFN-β are known to mediate TLR-mediated toxicity[35,36] and because ectopic expression of a constitutively active IRF3 mutant induces cell death[37],

IRF3 activation and 'deactivation' must be strictly controlled. Pin1-mediated post-translational regulation of IRF3, therefore, is likely to be important in determining the exact nature of the final immune responses against viruses or microbes.

TBK1-NAK and IKK-*i*–IKKε have been identified as IRF3 kinases responsible for the phosphorylation of the two C-terminal phospho-accepter clusters (Ser385-Ser386 and Ser396-Ser398-Ser402-Thr404-Ser405) and are indispensable for IRF3 activation[14–17]. Our results here have demonstrated that Ser339 is another important phosphorylation target for IRF3 regulation that governs the termination of IRF3-dependent transcriptional activation. Of note, the Pin1 target motif of human IRF3 is conserved in human, monkey, mouse and rat IRF7, another member of the IRF family. As IRF7 is a 'master regulator' for type I interferon expression induced by TLR7 and TLR9 stimulation[38], Pin1 might act as a critical regulator of type I interferon production by modulating both IRF3 and IRF7 signaling. Further studies are needed to fully elucidate the functions of Pin1 in the regulation of type I interferon production.

Here we have demonstrated activation-induced destabilization of IRF3 through direct engagement with Pin1. In general, degradation of transcription factors is one of the principal mechanisms that reduce or terminate transcriptional activation. This type of mechanism occurs not only during immune responses but also in many other biological phenomena. An example of this appeared in a study demonstrating that the turnover of c-Myc is under strict control by phosphorylation-dependent post-translational modification and that c-Myc deregulation results in the induction of oncogenic transformation of fibroblasts[28,39]. Phosphorylation at Ser62 of c-Myc is required for its stabilization, a phosphorylation event that is also a prerequisite for subsequent phosphorylation at Thr58 by glycogen synthase kinase-3β, which in turn leads to ubiquitination and proteasome-dependent degradation of the protein. Thus, sequential phosphorylation events are critical for regulation of the c-Myc-induced proliferation signals[28,39]. Consistent with that, substitution of Thr58 with alanine renders c-Myc more stable and oncogenic[28]. Notably, Pin1 is recruited to the phosphorylated Thr58-Pro59 motif of c-Myc and is critical in the phosphorylation-induced destabilization of c-Myc[28]. Because Pin1 is recruited to phosphorylated IRF3 after stimulation, it would be useful to determine if phosphorylation of the two clusters essential for activation of IRF3, but which do not mediate Pin1 binding, alters IRF3 stability and the efficiency of IRF3 interaction via the Pin1-binding motif on IRF3. Phosphorylation of the C-terminal phospho-accepter cluster of IRF3 is a prerequisite for MG132-sensitive reduction of IRF3 in a human cell line after infection with Sendai virus[10], although the underlying molecular mechanism was not clarified in that study. Although Pin1 regulates the protein stability of many transcription factors, it does not directly catalyze the ubiquitination or subsequent proteasome-dependent degradation of its substrates. Therefore, the identification of an IRF3-specific ubiquitin ligase or conjugating enzyme would facilitate fuller understanding of the regulation of IRF3 signaling.

METHODS

Reagents. Monoclonal anti-α-tubulin and anti-hemagglutinin (HA-7) were purchased from Sigma. Polyclonal anti-Pin1 and monoclonal anti-Myc were purchased from Cell Signaling Technology. Monoclonal anti-Pin1 was purchased from R&D Systems. Polyclonal anti-IRF3 (FL-425) was purchased from Santa Cruz Biotechnology. Polyclonal antibody to human IRF3 phosphorylated at Ser339 was elicited by immunization of a rabbit with the phosphorylated peptide N-CEGSGR(pS)PRY-C. Poly(I)•poly(C) was purchased from Amersham. Calf intestinal alkaline phosphatase was purchased from Takara Shuzo. MG132 was purchased from the Peptide Institute. Human IFN-β and the human IFN-β enzyme-linked immunosorbent assay (ELISA) kit were purchased from FujiRebio. The mouse IFN-β ELISA kit was purchased from PBL Biomedical Laboratories. All other reagents were purchased from Sigma unless indicated otherwise. Polyclonal anti-IRF3 has been described[8]. NDV, EMCV and VSV were prepared as described[5].

Plasmids. The expression constructs pFLAG-CMV1-hTLR3, pMX-puro, pEF1-lacZ and p125-Luc (IFN-β promoter with a luciferase (Luc) reporter) were donated by K. Fitzgerald (University of Massachusetts, Worcester, Massachusetts), T. Kitamura (University of Tokyo, Tokyo, Japan), S. Memet (Institut Pasteur, Paris, France) and T. Taniguchi (University of Tokyo, Tokyo, Japan), respectively. The pcDNA3-Pin1, pcDNA3-Pin1 W34A, pcDNA3-Pin1 K63A, pGEX-Pin1, pCMV-Myc-Ub, pEF-BOS hemagglutinin-tagged IRF3 (HA-IRF3), pEF-BOS dominant negative HA-IRF3 (58-427), pGal4-DBD, pGal4-IRF3, pUAS-Luc, pHCMV-VSV-G, pMRX-IRES-puro and pMRX-IRES-bsr constructs have been described[5,8,20,26,40,41]. The pEF-myc-cyto and pcDNA3 plasmids were purchased from Invitrogen. Construction details for the other plasmids are in the **Supplementary Methods** online.

Cells and mice. Neomycin-resistant 293-TLR3neo^r, Plat-E and U373-CD14 cells have been described[9,29,42]. The 293-TLR3 cultures were established after infection with retrovirus produced from pMX-Flag-hTLR3-puro. *Pin1*$^{-/-}$ mice have been described[20,25,26,43], as have MEFs isolated from *Pin1*$^{+/+}$ and *Pin1*$^{-/-}$ mice[26].

Reporter assays and retrovirus preparations. Cells were transfected using the FuGene6 transfection reagent (Roche) according to the manufacturer's instructions. The culture supernatant of the packaging cell line Plat-E, cotransfected with a retroviral vector and pHCMV-VSV-G, was filtered and used for infection[40]. Reporter assays were done as described[30]. Firefly luciferase activity was normalized to β-galactosidase activity.

Preparation of bone marrow–derived macrophages. Bone marrow–derived macrophages isolated from 12-week-old *Pin1*$^{+/+}$ or *Pin1*$^{-/-}$ mice were differentiated *in vitro* for 6 d. Macrophages were then left unstimulated or stimulated with poly(I)•poly(C). Secreted IFN-β was measured with the mouse IFN-β ELISA kit according to the manufacturer's guidelines.

Injection of poly(I)•poly(C) into mice. Poly(I)•poly(C) (5 μg per gram of body weight) was injected into the peritonea of 12-week-old *Pin1*$^{+/+}$ or *Pin1*$^{-/-}$ mice. Blood was drawn from the tail vein before and 10 h after treatment. Collected sera were immediately frozen. IFN-β in sera was measured with the mouse IFN-β ELISA kit according to the manufacturer's guidelines.

Immunocytochemistry and nuclear staining. Immunocytochemistry and nuclear staining with fluorescent dyes were done as described[8,26].

Immunoblot, immunoprecipitation and GST affinity assays. Immunoprecipitation and GST affinity assays were done as described[26,30]. For the ubiquitination assay, cells either treated or left untreated were suspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% (volume/volume) glycerol and 1% (volume/volume) Nonidet-P40) supplemented with 0.5 mM phenyl methyl sulfonylfluoride, 3 μg/ml of leupeptin, 10 μM MG132, 10 μM MG115, 5 mM NaF and 1 mM Na_3VO_4. Cell lysates were incubated for 2 h with anti-Myc and then were incubated for 1 h with protein G–Sepharose. Beads were washed four times with lysis buffer. SDS-PAGE and native PAGE coupled with immunoblot analysis were done as described[26,30].

Protein stability assay. Protein stability was analyzed as described[26]. After 293-TLR3 cells were transfected with the HA-IRF3 expression plasmid together with pcDNA3-HA-lacZ, they were subjected to various treatments. After the addition of cycloheximide, cells were collected at various time points. Hemagglutinin-tagged proteins were detected by immunoblot and were semiquantified with NIH image software.

Virus yield titration. MEFs or Vero cells were infected with VSV or EMCV. Virus yield in culture supernatants was determined by plaque assay as described[5].

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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