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Negative Regulation of IRF7 Activation by Activating Transcription Factor 4 Suggests a Cross-Regulation between the IFN Responses and the Cellular Integrated Stress Responses

Qiming Liang,* Hongying Deng,* Chiao-Wang Sun,[†] Tim M. Townes,[†] and Fanxiu Zhu*

Cells react to viral infection by exhibiting IFN-based innate immune responses and integrated stress responses, but little is known about the interrelationships between the two. In this study, we report a linkage between these two host-protective cellular mechanisms. We found that IFN regulatory factor (IRF)7, the master regulator of type I IFN gene expression, interacts with activating transcription factor (ATF)4, a key component of the integrated stress responses whose translation is induced by viral infection and various stresses. We have demonstrated that IRF7 upregulates ATF4 activity and expression, whereas ATF4 in return inhibits IRF7 activation, suggesting a cross-regulation between the IFN response and the cellular integrated stress response that controls host innate immune defense against viral infection. ***The Journal of Immunology*****, 2011, 186: 1001–1010.**

Cells react to viral infections by exhibiting innate immune responses. Central to the host innate antiviral responses is production of type I IFN, which is regulated by members of the IFN regulatory factor (IRF) family of transcription factors (1–7). Among the nine members in mammalian cells, two closely related ones, IRF3 and IRF7, have been implicated as the main regulators of type I IFN gene expression elicited by viruses (2, 4, 8–10). Although IRF3 is expressed ubiquitously and constitutively, IRF7 is expressed at low levels in most cells, but its expression is upregulated by viral infections. Despite low expression, through a positive feedback loop, IRF7 plays a dominant role in regulation of IFN induction, as evidenced by the abrogation of IFN production in most cell types of $Irf7^{-/-}$ but not in $Irf3^{-/-}$ mice (8, 9).

Host cells sense viral infection with pathogen recognition receptors such as membrane-bound TLRs, cytosolic retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerization domain protein-like receptors, and less characterized DNARs DAI and AIM2 (11–13). Recognition of viral pathogen-associated molecular patterns such as viral RNAs or DNAs by the pathogen recognition receptors triggers signaling cascades, ultimately leading to the activation of IRF3 and IRF7 that involves phosphorylation and nuclear accumulation of the two factors. Activation of ubiquitous IRF3 and the pre-existing low level of IRF7 triggers initial induction of IFN-β and a subset of IFN-α, followed by a positive feedback loop that allows efficient production of IFN-β and all forms of IFN-α during viral infection (9, 14, 15). The secreted IFNs bind to receptors, activate the JAK–STAT pathway, and ultimately induce expression of hundreds of IFN-stimulated genes (ISGs), including dsRNA-dependent protein kinase (PKR), RNase L, a subset of TLRs (TLR3, TLR7) and RLRs (RIG-I), and IRF7 (16). The collective effects of these ISGs allow cells to establish an antiviral state. For example, the PKR phosphorylates eukaryotic initiation factor 2α (eIF2α), leading to global translation suppression and thus inhibition of viral replication (17, 18).

In mammalian cells, various metabolic and environmental stresses, such as viral infection, perturbation of endoplasmic reticulum (ER) homeostasis (unfolded protein responses), nutrient deprivation, and reactive oxygen species, induce complex cellular responses that cause phosphorylation of eIF2α (19–21). Although phosphorylation of eIF2α results in global translational suppression, it specifically increases translation of activating transcription factor (ATF)4 through ribosomal leaky scanning of the mini open reading frames (ORFs) in the 5′-untranslated region (UTR) of the mRNA (19, 22, 23). ATF4 belongs to the ATF/CREB family of basic region/leucine zipper transcription factors. It has been reported to function as either a transcription activator or a repressor (24). Accumulation of ATF4 induces expression of genes involved in amino acid metabolism and transport, mitochondrial function, redox chemistry, and others that ensure supply of amino acids for protein synthesis and facilitate recovery from stress (19, 20). ATF4 is therefore thought to play a central role in cellular stress responses by initiating a feedback regulation loop to ensure the transient nature of protein synthesis inhibition (25–27).

Although the induction of type I IFN plays a key role in the control of viral infection, massive IFN production lasts only a few hours. The hosts have evolved elaborate negative regulation mechanisms to ensure that the protective response does not become excessive (28–31), but how the IFN induction process is terminated remains

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Abbreviations used in this article: ATF, activating transcription factor; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; HA, hemagglutinin; ID, inhibitory domain; IKK, IκB kinase; IRF, IFN regulatory factor; IRFE, IFN regulatory factor-binding element; ISG, IFN-stimulated gene; ISRE, IFN-sensitive response element; KSHV, Kaposi's sarcoma-associated herpesvirus; MEF, mouse embryonic fibroblast; ORF, open reading frame; PERK, PKR-like ER kinase; PKR, dsRNA-dependent protein kinase; poly(I:C), polyinosinic-polycytidylic acid; RIG-I, retinoic acid-inducible gene I; RLR, retinoic acid-inducible gene I-like receptor; RT, room temperature; si, small interfering; TBK1, TANK-binding kinase 1; UTR, untranslated region; VSV, vesicular stomatitis virus.

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unclear. In this study, we identified ATF4, whose expression is induced by viral infections and various stresses, as a binding partner and negative regulator of IRF7. Further studies revealed that cross-regulation of the IFN response and the cellular integrated stress response mediated by IRF7 and ATF4, respectively, is critical in controlling IFN induction during viral infection.

Materials and Methods

Cells and reagents

HEK293T cells and HeLa cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics at 37°C under 6% CO_2. 2ftGH and its derivative U3A and U4A cell lines, gifts from Dr. George Stark, were cultured in DMEM supplemented with additional 1 mM sodium pyruvate. Wild-type and ATF4$^{-/-}$ mouse embryonic fibroblasts (MEFs) were cultured similarly in DMEM supplemented with additional 55 μM 2-ME and 1 mM nonessential amino acids instead (20). The mouse anti-Flag, anti-hemagglutinin (HA), anti-lamin A, anti–β-actin, and rabbit anti–vesicular stomatitis virus (VSV)-G Abs were purchased from Sigma-Aldrich; rabbit anti-IRF7, anti-IRF3, anti-ISG15, and anti-GST Abs were purchased from Santa Cruz Biotechnology; rabbit anti-PKR, anti–PKR-like ER kinase (PERK), anti-eIF2α, and anti–phospho-eIF2α (Ser^{51}) Abs were purchased from Cell Signaling Technology; mouse anti-eGFP Ab was purchased from Clontech; mouse anti-pIRF7 (Ser^{477}/Ser^{479}) Ab was purchased from BD Biosciences; rabbit anti-pPERK (Thr^{981}) Ab was purchased from BioLegend; and rabbit anti-pPKR (Thr^{451}) Ab was purchased from BioSource International. Rabbit anti-ATF4 Ab was a gift from Dr. Michael Kilberg at the University of Florida. Mouse anti-ISG56 Ab was provided by Dr. Ganes Sen at the Cleveland Clinic Foundation. EZview red anti-Flag M2 affinity gel beads, 3× Flag peptides, DL-homocysteine, tunicamycin, and thapsigargin were purchased from Sigma-Aldrich.

Plasmids

A 3× Flag-tagged full-length IRF7 and its truncation mutants were cloned by insertion of PCR-amplified fragments into pCMV-TAG3 (Stratagene). A 3× HA-tagged ATF4 was cloned by PCR into pKH3 vector. GST-ATF4 constructs of aa 1–351 (full-length), 1–127, 1–90, 127–271, 271–351, and 271–306 were cloned by PCR into pEBG vector (provided by Dr. Yi Zhou, Florida State University). pHIV7 vector and pHIV7-GFP were provided by Dr. Hengli Tang, Florida State University. pHIV7-HA-ATF4 (mouse) was generated by cloning PCR amplified fragment into pHIV7 vector (32).

Human IRF7 promoter sequence (1.7 kb) was cloned from genomic DNA into pGL3 basic vector by PCR (5′-AGCTAGTCTGGAAGTTCTTCTTC-3′, 5′-GAGCCAAGGCCATTGCTCTTC-3′). Luciferase reporter plasmids pGL3-huIFN-α1, pGL3-huIFN-β, and pGL-muIFN-α6 have been described previously (33); pCHOP-luc (C/EBP-LUC) was provided by Dr. Nikki Holbrook, National Institute on Aging, National Institutes of Health (34). pGL3-ATF4 5′-UTR was kindly provided by Dr. D. Ron, New York University (22).

Yeast two-hybrid screening

A yeast two-hybrid screening was performed essentially as described previously (33). The IRF7 bait plasmid was generated by cloning of the IRF7 internal inhibitory domain (ID) aa 283–466 into pAS2-1 (Clontech) in frame with GAL4 DNA-binding domain. The yeast strain Y190 carrying the plasmid pAS2-1-IRF7 ID was used to screen a human lymphocyte Matchmaker cDNA library (Clontech). About one million clones were screened and plated on $Leu^-Trp^-His^-$ plus 25 mM 3-amino-1,2,4-triazole plates. The His^+LacZ^+ colonies were selected for sequencing and further analyses.

Immunoprecipitation

Forty-eight hours after transfection, HEK293T cells were washed with cold PBS and lysed with whole-cell lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, 40 mM β-gylcerophosphate, 1 mM sodium fluoride, 10% glycerol, 5 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 mM benzamidine, and 1 mM PMSF). Cell lysates were centrifuged at 10,000 × g for 10 min at 4°C and incubated with EZview red anti-Flag M2 beads for 4 h or overnight at 4°C. After washing with lysis buffer and TBS (50 mM Tris-HCl [pH 7.4], 150 mM NaCl), proteins were eluted by incubation with 150 μg/ml 3× Flag peptide in TBS for 1 h at 4°C.

GST pull-down assay

Flag-tagged IRF7 and GST-tagged ATF4 full-length and various truncation mutants were expressed in HEK293T cells by transient transfection of the corresponding expression plasmids. GST and GST-fusion proteins were purified with glutathione Sepharose beads (GE Healthcare). Equal amounts of GST and GST-tagged protein-bound glutathione beads were incubated with lysates of Flag-IRF7–expressing cells in 0.5 ml volume and rotated at 4°C for 2 h. The beads were washed twice with whole-cell lysis buffer and three times with TBS, eluted with SDS-PAGE sample buffer, and then analyzed by immunoblotting.

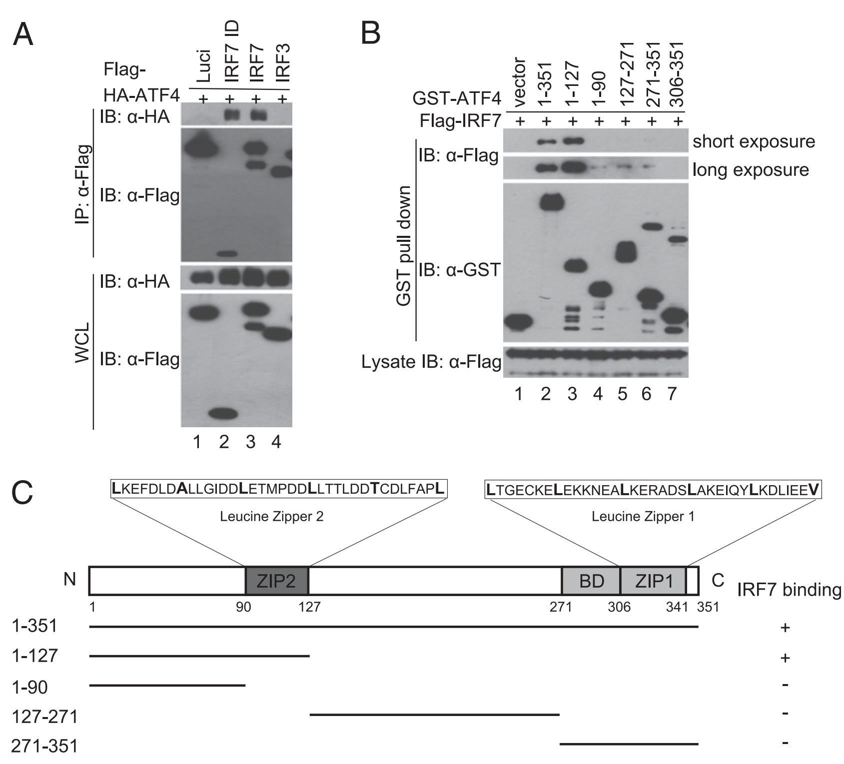

FIGURE 1. Association of ATF4 with IRF7. *A*, ATF4 interacts with IRF7. Flag-tagged IRF7, IRF7 ID, IRF3, and luciferase were cotransfected with HA-tagged ATF4 expression vectors into HEK293T cells. Lysates of the transfected cells were immunoprecipitated with anti-Flag M2 affinity beads. The immunoprecipitation complexes and whole-cell lysates were analyzed by Western blot with Abs as indicated. *B*, ATF4 interacts with IRF7 mainly through the ZIP2 domain. Flag-tagged IRF7 and GST-tagged ATF4 full-length and various truncation mutants were expressed in HEK293T cells. The GST-ATF4 fusion proteins were purified with glutathione beads. After washing and blocking, the bound beads were incubated with lysates of Flag-IRF7–expressing cells. After extensive washes, the bound proteins were eluted and analyzed by immunoblotting with anti-Flag and anti-GST Abs. *C*, Schematic presentation of full-length ATF4 and its mutants. BD, basic amino acid domain; IP, immunoprecipitation.

RNA isolation and RT-PCR

Total RNA was isolated from cells with the RNeasy Plus Mini Kit (Qiagen). First-strand cDNA was synthesized with the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen) according to the protocols recommended by the manufacturer. The following pairs of primers were used for RT-PCR: mouse IFN-α (consensus primers annealing with all IFN-α subtypes) sense, 5′-ATGGCTAGRCTCTGTGCTTTCCT-3′, antisense, 5′-AGGGCTCTCCA-GAYTTCTGCTCTG-3′; mouse IFN-β sense, 5′-CATCAACTATAAGCA-GCTCCA-3′, antisense, 5′-TTCAAGTGGAGAGCAGTTGAG-3′; mouse IRF7 sense, 5′-CAGCGAGTGCTGTTTGGAGAC-3′, antisense, 5′-AAG-TTCGTACACCTTATGCGG-3′; mouse IRF3 sense, 5′-CCAGGTCTTC-CAGCAGACACT-3′, antisense, 5′-TAGGCTGGCTGTTGGAGATGT-3′; mouse ISG56 sense, 5′-ACAGCTACCACCTTTACAGC-3′, antisense, 5′-TTAACGTCACAGAGGTGAGC-3′; mouse β-actin sense, 5′-GGACTC-CTATGTGGGTGACGAGG-3′, antisense, 5′-GGGAGAGCATAGCCCT-CGTAGAT-3′.

RNA interference

Hairpin-forming oligonucleotides were designed and cloned into RNAi-Ready pSIREN-Retro-Q vector (Clontech). Target sequences for IRF7 and ATF4 were small interfering (si)IRF7, 5′-CCA AGA GCT GGT GGA ATT C-3′ and siATF4, 5′-CAC TGA AGG AGA TAG GAA G-3′. Packaging of retroviruses and stable cell-line selection were performed as described previously (35).

Retrovirus

Cells stably expressing HA-ATF4 were established according to standard lentivirus expression protocols (36).

Plaque assay

Standard plaque assays were used to determine the titers of VSV as described previously (37). Briefly, HeLa or Vero cells were infected by exposure to 10-fold serially diluted VSVs for 1 h. The inoculum was then replaced with DMEM containing 1% methylcellulose. Twenty-four hours postinfection, the infected cells were fixed in 5% formaldehyde and stained with 0.1% crystal violet. All samples were assayed in duplicate, and the averages are presented.

Luciferase reporter assays

Luciferase assays were performed as previously described (33). Briefly, HEK293T cells or MEFs seeded in 24-well plates were transfected by luciferase reporter and pRL-TK internal control plasmids with Lipofectamine

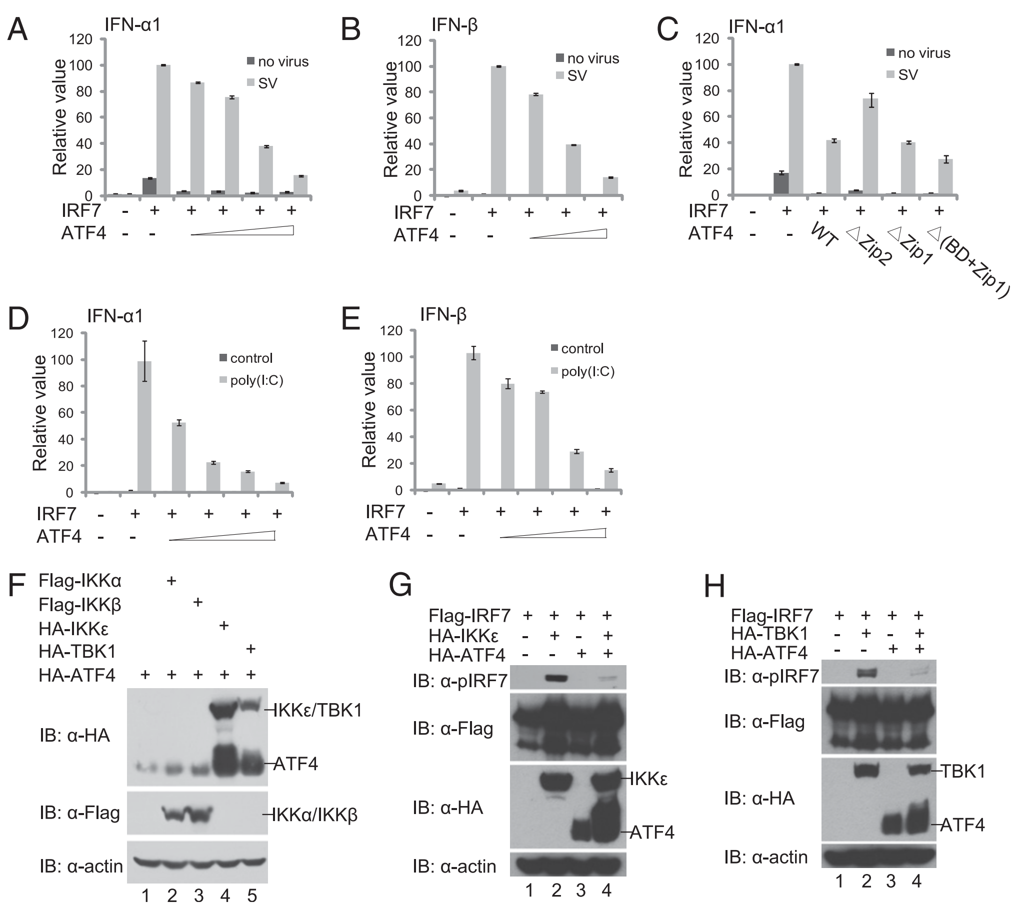

FIGURE 2. ATF4 inhibits IRF7 transactivation activity. *A* and *B*, ATF4 inhibits IRF7-induced IFN-α1 and IFN-β promoter activities in a dose-dependent manner. HEK293T cells were transfected with 100 ng IFN-α1 (*A*) or IFN-β (*B*) luciferase reporter and increasing amounts (50, 100, 250, 500 ng) of ATF4-expressing plasmids as indicated. Eight hours after transfection, cells were infected with Sendai virus or left untreated as controls. Dual luciferase assay were performed at 24 h after transfection. The relative luciferase activity was expressed as arbitrary units by normalizing firefly luciferase activity to *Renilla* luciferase activity. Data represent the average of three independent experiments and error bars represent SD. *C*, Deletion of the Zip2 domain impairs the inhibition of IRF7 by ATF4. HEK293T cells were transfected the IFN-α1 luciferase reporter and plasmids expressing wild-type ATF4 or its mutants. Dual luciferase assays were performed similarly to those described above. *D* and *E*, ATF4 inhibits IRF7 transactivation activities induced by poly(I:C). HEK293T cells were transfected with 100 ng IFN-α1 (*D*) or 100 ng IFN-β (*E*) luciferase reporter, 1 μg/ml poly(I:C), and increasing amounts of ATF4-expressing plasmids (50, 100, 250, 500 ng) as indicated. Dual luciferase assay were performed at 24 h after transfection. *F*, Phosphorylation of ATF4 by IKKε/TBK1 but not IKKα/IKKβ. HEK293T cells were transfected with HA-ATF4 plus IKKα, IKKβ, IKKε, or TBK1 expression plasmids at the mass ratio of 10:1. Forty-eight hours after transfection, cell lysates were analyzed by immunoblotting with Abs as indicated. *G* and *H*, ATF4 inhibits IRF7 phosphorylation by IKKε and TBK1. HEK293T cells were transfected with Flag-IRF7, HA-ATF4 plus HA-IKKε (*G*), or TBK1 (*H*) at the mass ratio of 10:20:1. Forty-eight hours after transfection, cell lysates were analyzed by immunoblotting with anti-pIRF7 (Ser^{477}/Ser^{479}) phosphorylation-specific Ab and other Abs as indicated. SV, Sendai virus.

2000 transfection reagent (Invitrogen). Eight hours after transfection, cells were infected with 80 HA U Sendai viruses per well. Dual luciferase assays were performed 24 h after transfection. The relative luciferase activity was expressed as arbitrary units by normalizing firefly luciferase activity to *Renilla* luciferase activity. Data represent the average of three independent experiments and error bars represent SD.

For IRF7 promoter reporter assay, HeLa cells or MEFs in 24-well plates were transfected with a 10:1 ratio of the reporter and pRL-TK with Effectene reagents (Qiagen). Thirty-six hours after transfection, dual luciferase assays were performed.

IFN ELISA

Human and murine IFNs were measured using commercial ELISA kits according to the manufacturer's protocols (PBL Biomedical Laboratories). Briefly, 100 μl diluted samples and the standards of known concentrations were added to each well and incubated for 1 h at room temperature (RT). The wells were washed and then incubated with 100 μl Ab solution at RT (murine IFN-α for 24 h, human IFN-α for 1 h). After three washes, each well was incubated with 100 μl HRP solution at RT for 1 h. After incubation, the wells were washed three times, then incubated with 100 μl tetramethylbenzidine substrate solution for 15 min at RT in the dark. Finally, 100 μl stop solution was added to each well and mixed by gentle swirling. The absorbance at 450 nm was measured within 5 min. The amounts of IFNs were determined by comparison with the standard curve.

Results

ATF4 binds to IRF7

The ID of IRF7 is a binding target of several viral proteins, including Kaposi's sarcoma-associated herpesvirus (KSHV) ORF45, that suppress IRF7 activation (33, 38–42). This domain seems responsible for IRF7 homodimerization and heterodimerization with IRF3 and might be involved in interactions with other cellular proteins (43). To elucidate the mechanisms underlying IRF7 activation and its inactivation by KSHV ORF45, we performed a yeast two hybrid screening with the IRF7 ID (aa 283–466) as a bait to search for cellular proteins associated with it. The screening yielded three positive clones encoding different truncated forms of ATF4. The interaction between ATF4 and IRF7 was confirmed by coimmunoprecipitation assays. As shown in Fig. 1*A*, HA-ATF4 was coimmnuoprecipitated with Flag-tagged full-length IRF7 (*lane 3*) and the ID fragment (*lane 2*). The interaction was specific because ATF4 was not coprecipitated with luciferase (*lane 1*) or IRF3 (*lane 4*).

We next mapped the regions of ATF4 that bind to IRF7 with GST pull-down assays. As shown in Fig. 1*B*, the N-terminal aa 1–127

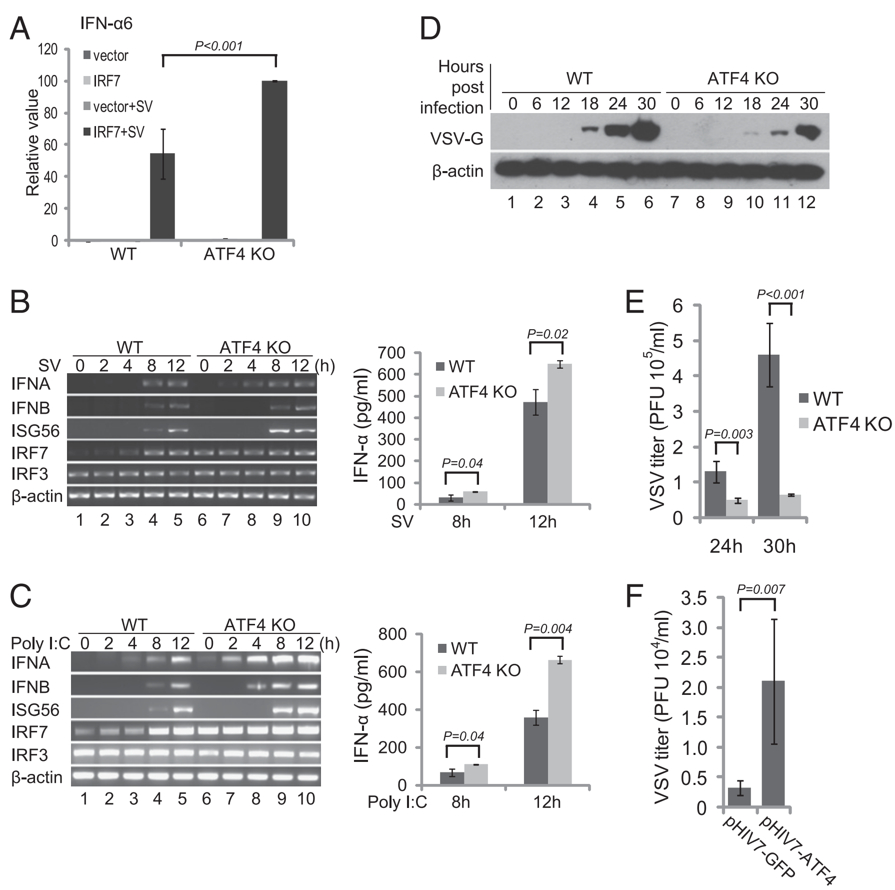

FIGURE 3. Knockout of ATF4 potentiates IRF7 activation and IFN production. *A*, Knockout of ATF4 potentiates IRF7 transactivation activity. The wild-type and ATF4$^{-/-}$ MEFs were transfected with mouse 200 ng IFN-α6 luciferase reporter plasmids. Cells were infected with Sendai virus, and dual luciferase assays were conducted as described in Fig. 1. *B* and *C*, Knockout of ATF4 potentiates type I IFN induction. Wild-type and ATF4$^{-/-}$ MEFs were treated with Sendai virus (*B*) or poly(I:C) (*C*). Total RNAs were isolated at the times indicated, and the levels of IFN-α, IFN-β, IRF7, IRF3, ISG56, and β-actin mRNA were determined by RT-PCR (*left panels*). Secreted IFN-α in the medium collected at 8 and 12 h was measured by ELISA (*right panels*). *D* and *E*, Knockout of ATF4 suppresses VSV infection. Wild-type and ATF4$^{-/-}$ MEFs were infected with VSV at a multiplicity of infection of 0.25. Cell lysates and culture medium were collected at the indicated times postinfection. The lysates were analyzed by Western blot for detection of VSV glycoprotein G; β-actin acted as a loading control (*D*). The titers in the culture medium at 24 and 30 h postinfection were determined by plaque assay (*E*). *F*, Expression of ATF4 in MEFs potentiates VSV replication. The ATF4$^{-/-}$ MEFs were transduced with lentivirus vectors expressing ATF4 or GFP as a control. The transduced MEFs were infected with VSV and viral titers were determined 20 h postinfection. Data in *A–C*, *E*, and *F* represent the average of at least three independent experiments and error bars represent SD. The *p* values represent significant differences (Student *t* test) between wild-type and ATF4$^{-/-}$ MEFs.

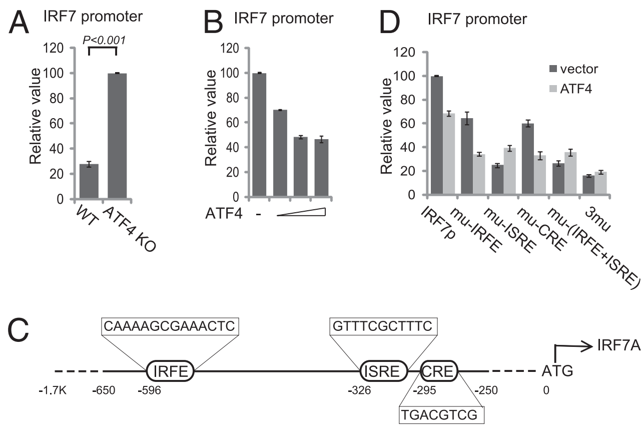

FIGURE 4. ATF4 inhibits transcription of IRF7. *A*, Loss of ATF4 potentiates IRF7 promoter activity. The wild-type and ATF4$^{-/-}$ MEFs were transfected with 200 ng IRF7 promoter reporter plasmid. Luciferase assays were performed 36 h after transfection. *B*, ATF4 inhibits IRF7 promoter in a dose-dependent manner. HeLa cells were transfected with 200 ng IRF7 promoter reporter and increasing amounts (50, 100, 250 ng) of ATF4-expressing plasmids. Luciferase assays were performed as described as above. *C*, Schematic presentation of human IRF7 promoter. IRFE, ISRE, and CRE/ATF are putative regulatory elements in IRF7 promoter. *D*, HeLa cells were transfected with 200 ng IRF7 promoter reporter or the mutant constructs as depicted in *C*. Luciferase assays were performed as described as above. Mutation of the ISRE (a known IRF7 binding site and positive regulatory element) but not the CRE/ATF (putative ATF4 binding site) abolishes the inhibition of IRF7 promoter by ATF4. Data represent the average of at least three independent experiments and error bars represent SD.

fragment of ATF4 bound to IRF7 as well as the full-length one did. Further truncation of the 37-aa ZIP2 domain drastically reduced the binding (compare *lane 4* to *lane 3*). The C-terminal fragments without the ZIP2 domain bound to IRF7 weakly (aa 1–90, 127–271, and 271–351) or did not bind at all (aa 306–351). These results suggest that ATF4 interacts with IRF7 mainly through the ZIP2 region (aa 90–127).

ATF4 inhibits the activation of IRF7

Upon viral infection, IRF7 undergoes virus-induced serine phosphorylation in its C-terminal region that stimulates protein dimerization, nuclear translocation, and cooperation with other transcriptional coactivators to induce robust expression of type I IFN genes (43). To investigate the consequence of ATF4 interaction on IRF7 function, we determined whether ATF4 affects IRF7 transactivation activity. In transient luciferase reporter assays, expression of ATF4 inhibited IRF7-induced IFN-α1 and IFN-β promoter activities triggered by Sendai virus infection in a dose-dependent manner (Fig. 2*A*, 2*B*), whereas deletion of ZIP2 domain of ATF4 impaired its inhibitory activity (Fig. 2*C*). Moreover, ATF4 also inhibits IRF7-mediated reporter activities induced by polyinosinic-polycytidylic acid [poly(I:C)] (Fig. 2*D*, 2E) or components in the TLR or RLR signaling pathways such as MAVS, TRIF, RIG-I, TANK-binding kinase 1 (TBK1), and IκB kinase (IKK)ε (data not shown), suggesting that ATF4 inhibits IRF7 directly. These experiments demonstrate that ATF4 inhibited IRF7 activation.

TBK1 and IKKε, two IKK-related kinases, have been shown to phosphorylate IRF7 primarily on the residues Ser^{477}/Ser^{479}, which are critical for IRF7 activation (44–46). We noticed a significant mobility shift of ATF4 on SDS-PAGE when it was coexpressed with TBK1 or IKKε, suggesting that ATF4 is phosphorylated by TBK1 and IKKε (Fig. 2*F*). Phosphorylation of ATF4 by TBK1 and IKKε seems to be specific because no obvious mobility shift of ATF4 was observed when IKKα or IKKβ was coexpressed (Fig. 2*F*). When ATF4 and IRF7 were coexpressed, ATF4 inhibited IRF7 phosphorylation of Ser^{477}/Ser^{479} by TBK1 and IKKε (Fig. 2*G*, 2*H*,

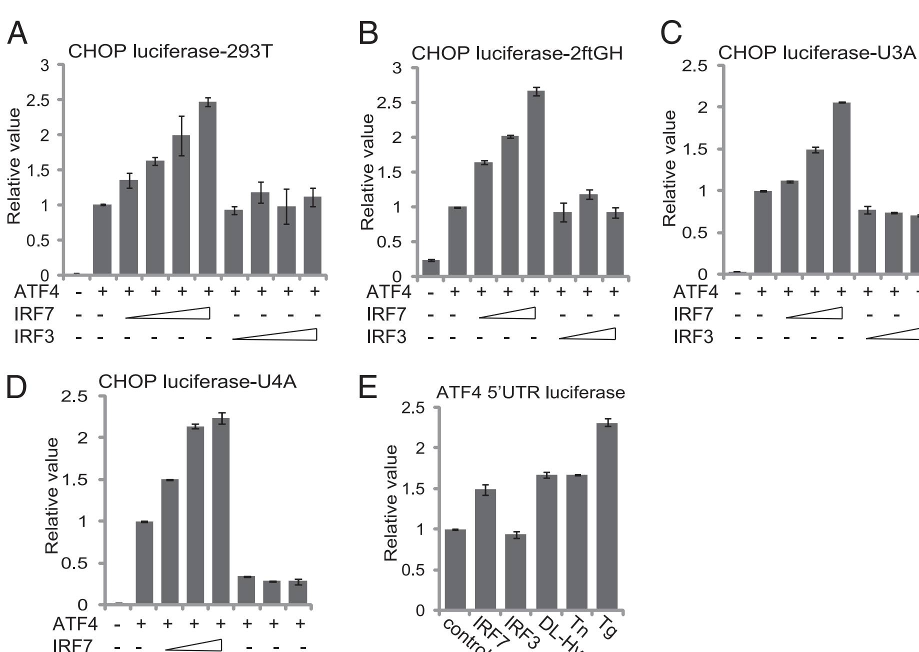

FIGURE 5. IRF7 regulates ATF4 expression and activity. *A*, IRF7 but not IRF3 increases ATF4 transactivation activity. HEK293T cells were transfected with 100 ng ATF4-responsive CHOP promoter reporter and increasing amounts (50, 100, 250, and 500 ng) of IRF7 or IRF3 expression vectors as indicated. Dual luciferase assays were performed 24 h after transfection. *B–D*, IRF7 can increase ATF4 transactivation activity when the IFN circuit is disrupted. The above experiment was repeated in the parental 2ftGH (*B*) and its derivatives, U3A (STAT1^{-}) (*C*) and U4A (JAK1^{-}) (*D*) cells. *E*, IRF7 upregulates translation of ATF4. MEFs were transfected with 200 ng ATF4 5′-UTR luciferase reporter and 200 ng IRF7 or IRF3 expression plasmids as indicated. Stress inducers DL-homocysteine, tunicamycin, and thapsigargin were added 20 h after transfection. A dual-luciferase assay was performed 24 h after transfection. Data represent the average of at least three independent experiments and error bars represent SD. DL-Hyc, DL-homocysteine; Tg, thapsigargin, Tn, tunicamycin.

compare *lane 4* to *lane 2*). Taken together, these data suggest that ATF4 negatively regulates the activation of IRF7 and thus suppresses induction of IFN-α and IFN-β gene expression.

Knockout of ATF4 potentiates IRF7 activation and IFN induction

To determine whether ATF4 is involved in regulation of IRF7 under physiological conditions, we examined the effect of knockout of ATF4 on IRF7 activation and IFN induction. Transient luciferase reporter assays showed that the transactivation activity of IRF7 was higher in the ATF4$^{-/-}$ than in wild-type MEFs, suggesting that ATF4 is a negative regulator of IRF7 (Fig. 3*A*). The RT-PCR assays revealed that the levels of IFN-α, IFN-β, and ISG56 mRNAs induced by Sendai virus or poly(I:C) was greater in ATF4$^{-/-}$ than in wild-type MEFs (Fig. 3*B*, 3*C*, *left panels*). The differences of IFN-α at protein level were confirmed by ELISA assays (Fig. 3*B*, 3*C*, *right panels*), indicating that ATF4 negatively regulates IRF7 activation and IFN induction. Interestingly, the basal level of IRF7 mRNA was higher in ATF4$^{-/-}$ than in wild-type MEFs, whereas the level of IRF3 mRNA remained the same (Fig. 3*B*, 3*C*, *left panels*, compare *lane 6* to *lane 1*). These data suggest that ATF4 not only suppresses IRF7 activity but also IRF7 transcription. Therefore, lack of ATF4 leads to an increase of type I IFN production.

We next determined whether the increased type I IFN production caused by loss of ATF4 affects the susceptibility of cells to viral infection. We chose VSV because it is sensitive to the antiviral actions of type I IFNs. We infected both wild-type and ATF4$^{-/-}$ MEFs with VSV and examined expression of viral proteins by Western blot at various times postinfection. Western blots revealed that expression level of VSV-G protein was higher in the wild-type than in ATF4$^{-/-}$ MEFs at each time point (Fig. 3*D*). Plaque assays revealed that loss of ATF4 reduced VSV titers by >10-fold (Fig. 3*E*). Moreover, ectopic expression of ATF4 but not GFP increased the susceptibility of the ATF4$^{-/-}$ MEFs to VSV infection, confirming that loss of ATF4 contributed to reduced VSV infection in ATF4$^{-/-}$ MEFs (Fig. 3*F*). Collectively, these data suggest that ATF4 is a negative regulator of IRF7 activation and IFN induction.

ATF4 inhibits transcription of IRF7

The level of IRF7 mRNA was lower in wild-type than in ATF4$^{-/-}$ MEFs, suggesting that ATF4 also regulates IRF7 at the level of transcription. When we cloned IRF7 promoter into pGL3 plasmid to generate a luciferase reporter and transfected it into wild-type and ATF4$^{-/-}$ MEFs, IRF7 promoter activity was lower in the wild-type than in ATF4$^{-/-}$ MEFs (Fig. 4*A*). Moreover, overexpression of

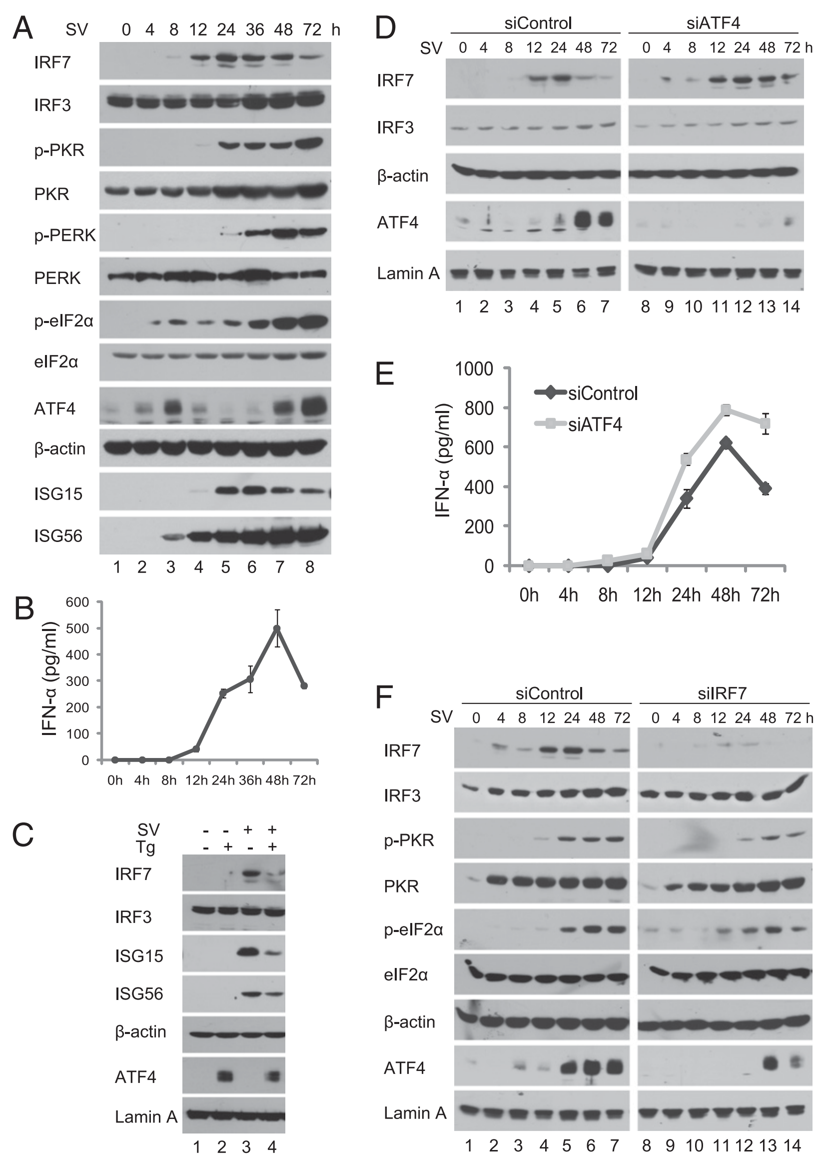

FIGURE 6. Cross-regulation between IFN and the cellular integrated stress responses. *A* and *B*, Expression kinetics of the major components of IFN and stress responses during Sendai virus infection. A549 cells were infected with Sendai virus in triplicate (160 HA U/ml). At the indicated times postinfection, whole-cell lysates or nuclear extract (for detection of ATF4) were prepared and analyzed by immunoblotting with indicated Abs (*A*). IFNs in the culture medium were measured by ELISA (*B*). *C*, Activation of the integrated stress response impairs IRF7-induced expression of IFNs. A549 cells were infected with Sendai virus for 4 h and then treated with thapsigargin for another 4 h. Whole-cell lysates and nuclear extracts were prepared and subjected to immunoblotting with the indicated Abs. *D* and *E*, Knockdown of ATF4 by siRNA potentiates expression of IRF7 and IFN-α. A549 cells stably transduced with siATF4 or siControl were infected with Sendai virus for time as indicated. Whole-cells lysates and nuclear extract were analyzed by immunoblotting with specified Abs (*D*). IFNs in the culture medium were measured by ELISA (*E*). *F*, Knockdown of IRF7 by siRNA reduces virus-induced cellular integrated stress responses. A549 cells stably transduced by siIRF7 or siControl were infected with Sendai virus. Whole-cell lysates and nuclear extract were analyzed by immunoblotting with the indicated Abs.

ATF4 inhibited IRF7 promoter activity in a dose-dependent manner (Fig. 4*B*).

Several studies have shown that IRF7 positively regulates its own promoter through binding to an IFN-sensitive response elements (ISRE) and an IRF-binding element (IRFE) (47, 48). Inspection of the promoter sequence also revealed a consensus ATF/cAMP response element (CRE) element in IRF7 promoter (Fig. 4*C*). We wanted to determine whether ATF4 regulates transcription of IRF7 through direct binding to ATF/CRE element or through interfering with IRF7 activation and thus blocking the feedback activation loop. When the wild-type IRF7 promoter was used, ATF4 reduced reporter activity (Fig. 4*D*). As expected, mutation of IRFE and especially ISRE dramatically reduced the reporter activity, confirming that the self-regulation of its own promoter is mainly through the ISRE site (47, 48). Mutation of ATF/CRE also reduced the reporter activity, suggesting that direct binding of ATF4 to the CRE contributed little to the negative regulation of IRF7 promoter by ATF4. Negative regulation of IRF7 promoter by ATF4 was abolished only when the ISRE, the chief IRF7-positive regulation site, was mutated, suggesting that ATF4 regulated IRF7 promoter mainly though interfering IRF7 activation.

IRF7 regulates ATF4 expression and activity

ATF4 is the key regulator of cellular responses to various stresses, including viral infection and IFN signaling. Because IRF7 is induced by viral infection and triggers IFN induction, we next asked whether IRF7 affects expression and function of ATF4. In reporter assays, IRF7 but not IRF3 enhanced ATF4 transactivation activity (Fig. 5*A*). To determine whether IFN circuit is required for the enhancement, we repeated the assays in 2ftGH and its derivative cells in which the IFN signaling circuit is disrupted because of mutations in STAT1 (U3A) or JAK1 (U4A). IRF7 but not IRF3 increased the ATF4 activity in 2ftGH and its derivative cells, suggesting that IRF7 directly upregulates ATF4 activity in addition to the well-established IFN/eIF2α-dependent mechanisms (Fig. 5*B–D*).

A unique feature of the 5′-UTR of ATF4 permits more efficient translation when phosphorylation of eIF2α causes global translation suppression in response to various stresses, including viral infection and IFN treatment (22, 23). Expression of IRF7 increased the ATF4 5′-UTR–driven luciferase reporter, whereas expression of IRF3 did not (Fig. 5*E*). The level of increase caused by IRF7 was significant and comparable to that caused by treatments with various stress-inducing agents, such as DL-homocysteine, tunicamycin, and thapsigargin. These results suggest that IRF7 regulates ATF4 expression and function.

Crossregulation between IFN responses and integrated stress responses

Viral infection induces IFNs and ISGs including IRF7 as well as integrated stress responses that result in phosphorylation of eIF2α and subsequent global translation suppression but an increase of ATF4 translation. To seek the interrelationship between IFN and integrated stress responses, we wanted to determine the expression kinetics of key components in both pathways during virus infection. Because IRF7 in MEFs is hardly detectable by Western blot, we screened a number of cell lines and found that A549 cells, a human lung adenocarcinoma epithelial cell line, effectively expressed both IRF7 and ATF4. As shown in Fig. 6*A*, IRF7 was induced by Sendai virus infection and became detectable 8 h postinfection. Its expression increased over time, peaked at 24 h, and then decreased. In contrast, IRF3 was expressed constitutively as expected. The kinetics of ISG15 and ISG56 expression were largely correlated with that of IFN-α (Fig. 6*B*). Phosphorylation of eIF2α first appeared 4 h postinfection and increased slightly until 8 h postinfection. It remained at a low level until 24 h postinfection and then increased significantly 36 h postinfection and thereafter. The late increase of eIF2α phosphorylation was coincident with phosphorylation of both PKR and PERK, suggesting that these kinases are activated during Sendai virus infection. Interestingly, ATF4 appeared to peak twice. The first peak occurred at 8 h postinfection, and a late dramatic increase occurred at 48 and 72 h postinfection, when IRF7 expression began to decrease (Fig. 6*A*). These results confirm a reverse correlation between ATF4 and IRF7 supporting a negative regulation of IRF7 and IFN induction by ATF4.

We next determined whether artificial induction of integrated cellular stress responses affects IRF7 activation and IFN expression. We first infected A549 cells with Sendai virus and then treated the cells with stress-inducing agents for 4 h and examined expression of IRF7 and ISGs by immunoblotting. As shown in Fig. 6*C*, immunoblotting revealed that ER stress inducer thapsigargin reduced expression of IRF7, ISG15, and ISG56 that is correlated with increased expression of ATF4 in A549 cells (Fig. 6*C*). Curiously, thapsigargin triggered a lower level of ATF4 induction in Sendai virus-infected cells than in mock-infected cells (Fig. 6*C*, compare *lane 4* to *lane 2*); a similar observation was recently reported elsewhere (49). These experiments suggest that integrated stresses negatively regulate IFN induction.

To investigate the interrelationship between stress and IFN responses further, we knocked down expression of the key components in A549 cells by lentivirus vector-mediated siRNAs. Consistent with previous results in ATF4$^{-/-}$ MEFs, knockdown of ATF4 in A549 cells potentiated expression of IRF7 and increased duration of its expression (Fig. 6*D*, compare *lanes 11–14* to *lanes 4–7*). Consequently, expression of IFN-α was increased by knockdown of ATF4 (Fig. 6*E*). Furthermore, knockdown of IRF7 decreased and delayed phosphorylation of PKR, reduced the level of eIF2α phosphorylation, and resulted in delayed and lower expression of ATF4 (Fig. 6*F*). These data confirmed the role of ATF4 in regulating IRF7 and IFN expression and suggested that IRF7 and the IFN-PKR-eIF2α signaling cascades effectively regulate ATF4 expression (Fig. 6*F*). Taken together, these results suggest that IRF7 and ATF4 link the IFN-based innate immune response to the integrated stress response (Fig. 7).

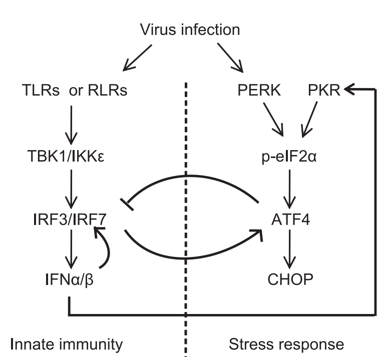

FIGURE 7. Schematic diagram of cross-regulation between IFN and integrated stress responses. Viral infection induces type I IFN expression and also activates multiple eIF2α kinases, mainly PKR and PERK. Phosphorylation of eIF2α and activation of IRF7 itself increases the translation of ATF4. The increased expression level of ATF4 protein induces stress-response genes to help cell recovery but inhibits the expression and transactivation of IRF7 to terminate IFN signaling. As a result, this negative feedback loop allows host cells to terminate the IFN responses effectively.

Discussion

ATF4 interacts with and negatively regulates IRF7

We have identified ATF4 as a binding partner and negative regulator of IRF7. We demonstrated that overexpression of ATF4 inhibits IRF7 activation, whereas knockout of ATF4 potentiates IRF7 transactivation activity, increases IFN production, and suppresses VSV replication. ATF4 interacts specifically with IRF7 but not IRF3, mainly through the second atypical leucine zipper domain (ZIP2). The ZIP2-dependent interaction is specific to IRF7 because ATF4 interacts with most other proteins through the typical ZIP1 domain, such as Zhangfei (50), mitosin/CENP-F (51), GABA (B) receptor (52), RNA polymerase 2 subunit RPB3 (53), ZIP kinase (54), and HTLV1 transactivator Tax (55). This specific interaction is required for ATF4 to inhibit IRF7 transactivation activity because deletion of ZIP2 domain impairs the inhibition. Although the detailed inhibitory mechanisms remain unclear, we found that ATF4 inhibits phosphorylation of IRF7 by TBK1 and IKKε. Additionally, ATF4 inhibits transcription of IRF7 by interfering with IRF7 activation and thus disrupting the positive feedback loop. Because ATF4 down- and upregulates expression of many cellular genes, other inhibitory mechanisms may also be involved. For example, ATF4 is known to upregulate expression of 4E-BPs, negative regulators of cap-dependent translation (56). Interestingly, knockout of 4E-BPs causes a significant upregulation of IRF7 (57), indicating that 4E-BPs inhibit IRF7 translation specifically. Taken together, these observations suggest that ATF4 also downregulates IRF7 translation through induction of 4E-BP. Therefore, ATF4 inhibits IRF7 activation through direct protein–protein interaction and also through indirect suppression of IRF7 transcription and translation.

Negative regulation of IFN responses

Although the innate immune response is an indispensable defense against invasion by pathogens, the host must limit it, because its excessive and prolonged activation would be harmful or even fatal to the host (58). IFN induction is negatively regulated by a number of factors, such as A20 (28), NLRX1 (29), SIKE (59), and Pin1 (60), that target various components in the in RLR- and TLR-induced antiviral signaling. IRF activation is also negatively regulated by postactivation attenuation mechanisms. For example, IRF3 is also subject to ubiquitination-dependent proteasomal degradation (61), whereas both IRF3 and IRF7 are silenced by SUMOylation (42, 62). Furthermore, certain ISGs, such as ISG56, originally thought to be involved in establishment of antiviral state, actually downregulate host antiviral responses, presumably as a mechanism for termination of IFN response (63).

Cross-regulation of IFN and stress responses

During the course of viral infection, translation of ATF4 is augmented as a result of phosphorylation of eIF2α by a group of kinases, including PKR, PERK, and GCN2. These kinases are activated by dsRNA, ER stress, and amino acid deprivation, respectively, each of which often occurs during a viral infection (64, 65). Accumulation of ATF4 not only induces genes facilitating cellular recovery but also suppresses IRF7 activation, disrupts the IFN/IRF7 positive feedback loop, and thus subsequently terminates the IFN circuit. We have demonstrated that further increase of ATF4 expression by stress-inducing agents during viral infection reduces IFN induction and IRF7 activation, that reduction of ATF4 expression by siRNA results in higher levels and longer duration of IFN induction, but that ablation of IRF7 expression reduces the level of eIF2α phosphorylation and ATF4 expression. Collectively, these data suggest a linkage between IFN and stress responses through cross-regulation by IRF7 and ATF4, the two critical regulators of these two pathways. On the basis of these data, we propose the model outlined in Fig. 7. Viral infection induces IFN expression and also activates multiple eIF2α kinases, mainly PKR, PERK, and other possible kinases. Phosphorylation of eIF2α and activation of IRF7 itself increase translation of ATF4. The increased expression level of ATF4 protein induces stress-response genes to help the cell recover but inhibits the expression and transactivation of IRF7 to terminate IFN signaling. As a result, this negative feedback loop enables host cells to terminate the IFN production and response effectively.

The ATF4/IRF7-mediated cross regulation is supported by a recent report showing that increased oxidative stress caused by aging impairs IRF7 activity, whereas reduction of the stress by antioxidant agents increases it and IFN responses (66). Because of the complex natures of the two pathways, the interrelationships between the IFN-based innate immune and integrated stress responses may not be limited to ATF4 and IRF7. For example, ATF3, a downstream target gene of ATF4 in response to ER stresses, has been identified as a negative regulator of TLR signaling (67, 68); activated PERK induced by ER stresses during viral infection promotes phosphorylation-dependent ubiquitination and degradation of IFNAR1 and thus attenuates type I IFN signaling and antiviral defenses (69).

Role of ATF4 in viral replication

Our studies revealed a novel role of ATF4 in negative regulation of IFN-based innate immune responses and thus as a potential cellular factor for better viral replications. Reovirus has been shown to induce and to benefit from an integrated cellular stress response and to require ATF4 for its replication, although the role of innate immunity was not examined (70). Influenza virus has been shown to replicate less efficiently in eIF2αS51A MEFs, in which ATF4 expression cannot be induced (71). Human CMV is known to activate and modulate unfolded protein response and consequently to induce ATF4 expression (72, 73). We also found that KSHV ORF45 increases inhibition of IRF7 by ATF4 (Q. Liang and F. Zhu, unpublished observations). These observations suggest that the cross-regulation of IFN and integrated stress responses by IRF7 and ATF4 are modulated by viruses as a strategy to defeat the IFN antiviral response.

In summary, we demonstrate for the first time, to our knowledge, the physical and functional interactions between IRF7 and ATF4 and revealed a novel mechanism of cross-regulation between the IFN and the cellular integrated stress responses.

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Disclosures

The authors have no financial conflicts of interest.

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