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Fas-associated Death Domain (FADD) and the E3 Ubiquitin-Protein Ligase TRIM21 Interact to Negatively Regulate Virus-induced Interferon Production*[S]

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The production of cytokines such as type I interferon (IFN) is an essential component of innate immunity. Insufficient amounts of cytokines lead to host sensitivity to infection, whereas abundant cytokine production can lead to inflammation. A tight regulation of cytokine production is, thus, essential for homeostasis of the immune system. IFN-α production during RNA virus infection is mediated by the master transcription factor IRF7, which is activated upon ubiquitination by TRAF6 and phosphorylation by IKKε and TBK1 kinases. We found that Fas-associated death domain (FADD), first described as an apoptotic protein, is involved in regulating IFN-α production through a novel interaction with TRIM21. TRIM21 is a member of a large family of proteins that can impart ubiquitin modification onto its cellular targets. The interaction between FADD and TRIM21 enhances TRIM21 ubiquitin ligase activity, and together they cooperatively repress IFN-α activation in Sendai virus-infected cells. FADD and TRIM21 can directly ubiquitinate IRF7, affect its phosphorylation status, and interfere with the ubiquitin ligase activity of TRAF6. Conversely, a reduction of FADD and TRIM21 levels leads to higher IFN-α induction, IRF7 phosphorylation, and lower titers of RNA virus of infected cells. We conclude that FADD and TRIM21 together negatively regulate the late IFN-α pathway in response to viral infection.

Fas-associated death domain (FADD)[5] is an adaptor protein known to be crucial for the mammalian cell extrinsic pathway of apoptosis. Ligand engagement of the tumor necrosis factor receptor (TNF-R) family of death receptors, which include Fas, TNF-R1, and TRAIL-R, leads to recruitment of FADD and caspase-8 to form a death-inducing complex (1–4). For Fas, FADD is directly recruited to the cytoplasmic tail and is part of the membrane-associated Fas·FADD·caspase-8 complex. In contrast, TNF-R1 activation leads to the assembly of a cytoplasmic complex that consists of either TRADD·FADD·caspase-8 or RIP1·FADD·caspase-8 (3). FADD contains two domains that facilitate protein-protein interactions; that is, the death-effector domain (DED) and the death domain (5, 6). The FADD DED participates in self-association and binding to procaspase-8, whereas the death domain interacts with the death receptors, TRADD or RIP1 (5, 7–9).

Accumulating evidence also points to a role for FADD in innate immunity. In *Drosophila melanogaster*, FADD is part of the immune deficiency pathway required for the *Drosophila* immune defense against the Gram-negative bacteria (10–12). Immune deficiency, the *Drosophila* equivalent of mammalian RIP1, interacts with *Drosophila* FADD and caspase-8 to initiate the NF-κB pathway, leading to production of Drosomycin, an anti-bacterial peptide. *Drosophila* deficient in FADD expression succumb to infection by Gram-negative bacteria (12). In mice, the absence of FADD or caspase-8 prevents TLR-3/4 (Toll-like receptor)-induced B cell proliferation (13, 14). In human and mouse fibroblasts, FADD was implicated in the interferon (IFN) pathway in response to RNA virus infections (15–18). However, how FADD fits into the RNA viral sensing pathway is not entirely clear. RIG-I, a card-domain-containing RNA helicase, is a cytoplasmic RNA sensor crucial for innate immunity against RNA virus infection (19, 20). RIG-I interacts with the adapter molecule IPS-1/Cardif, which was reported to associate with FADD through TRADD and RIP1 (18, 21). Consistent with this observation, transfection of poly(IC), a synthetic mimetic of viral dsRNA, into FADD-deficient fibroblasts failed to elicit a robust IFN-β promoter response (15–17). However, overexpression of FADD did not stimulate IFN-β promoter activation (21). In addition, virally induced death occurs normally in FADD-deficient MEF cells in contrast to RIG-I- or IPS-1-deficient cells (16). Defects in FADD-deficient cells were only apparent when Type I IFNs were added to the cultures. Although interferons restrict viral replication in wild-type cells, they had no effect in the absence of FADD (15, 16). These data suggest that the major role of FADD in innate immunity is not in the

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[5] The abbreviations used are: FADD, Fas-associated death domain; DED, death-effector domain; SeV, Sendai virus; $TCID_{50}$, 50% tissue culture infective dose.

early phase but during the late phase of the IFN pathway. Interestingly, FADD-deficient cells were reported to exhibit defective late phase IFN-α production and had lower IRF-7 transcription when infected with Sendai virus and vesicular stomatitis virus (15). How FADD impinges upon IRF7 transcription and the secondary IFN response is not clear.

In this paper we report the identification of a novel interaction between TRIM21 and FADD *in vitro* and *in vivo*. TRIM21 (Ro52), a member of the Tripartite motif-containing family (22, 23), is expressed in all the cells but can be up-regulated by interferons (24). Many TRIM molecules have been implicated in anti-viral activities. For example, RIG-I signal transduction requires TRIM25 activity, and in its absence, vesicular stomatitis virus replication is unregulated (25). TRIM5 is a species-specific restricting factor for HIV (26), and the overexpression of TRIM19 can confer resistance to influenza and vesicular stomatitis virus (27). TRIM21 possesses E3 ubiquitin ligase activity similar to many other TRIM members that contain a RING domain. TRIM21 has been reported to ubiquitinate various substrates including itself, the p27 cell cycle inhibitor, IRF3, IRF7, and IRF8 (28–34). Although TRIM21-mediated ubiquitination of p27 and IRF7 leads to degradation, IRF8 activity is enhanced after ubiquitination by TRIM21. Studies in TRIM21-deficient mice suggest that TRIM21 is part of a negative feedback loop for cytokines (35, 36). TRIM21-deficient splenocytes produced increased amounts of inflammatory cytokines, including type I interferons, when stimulated with CpG. We show here that FADD interacts with TRIM21 through its DED and enhances TRIM21 auto-ubiquitination and its ubiquitination activities on IRF7. Co-expression of FADD and TRIM21 represses IRF7 phosphorylation and its transcriptional activities, whereas loss of FADD and TRIM21 leads to moderately higher levels of IFN-α. We concluded that FADD and TRIM21 constitute a negative feedback loop of the late interferon pathway during viral infection.

EXPERIMENTAL PROCEDURES

Plasmids and siRNAs—The expression plasmid pcDNA3-HA-TRIM21 was a gift from Dr. Gemana Meroni. A plasmid expressing a C-terminal FLAG-tagged human FADD (pCI-hFADDFLAG) was generated by ligating an EcoRI-XbaI-digested hFADD-FLAG PCR product in the pCI vector. To generate plasmids expressing human FADD (1–95)-, FADD (96–208)-, FADD (1–83)-, and FADD (1–90)-truncated fragments, pCI-hFADDFLAG was used as a template for PCR amplification. The resulting PCR products were digested with EcoRI and XbaI restriction enzymes and subsequently ligated with the pCI vector. The MSCV2.2-IRF7-FLAG and pcDNA-FLAG-TRAF6 constructs were generous gifts from Dr. Gregory Barton. IRF7 was further cloned into the pCI and pCI-HA vectors. pCI-HAhTRIM39 plasmid construct was also created with the same method using pFLAG-hTRIM39, which was a gift from Dr. Jeese D. Roberts Jr. Plasmid expressing an N-terminal HA-tagged human TRIM20 was kindly provided by Dr. Walter Mothes. To create an N-terminal His-tagged ubiquitin, pcDNA3-HAubiquitin was used in a PCR amplification. The PCR fragment was digested with NheI and KpnI restriction enzymes and subsequently ligated with a pCI-His vector to obtain the pCI-HisUbi construct. FADD mutants were created using QuikChange site-directed mutagenesis (Stratagene). siRNA for human TRIM21 and negative control scramble siRNA were purchased from Qiagen. The scrambled sequence for the negative control is AAT TCT CCG AAC GTG TCA CGT. The target sequence for the human TRIM21 siRNA is AAGCAGGAGTTGGCTGAGAAG. The human FADD siGENOME siRNA (3′-UTR: GAA CUC AAG CUG CGU UUA U) was purchased from Dharmacon.

Cell Culture and Transfection—Transfection with plasmids was performed with Lipofectamine 2000 or TransIT-LT1 (Mirus) as described by the manufacturer's protocol. For siRNA transfections, 293T cells were transfected using Lipofectamine 2000 following the manufacturer's directions. Briefly, all siRNA constructs were at a stock concentration of 20 μM. Transfections for 12-well plates required 2, 3, and 4 μl of the scramble control siRNA, hTRIM21 siRNA, and hFADD siRNA, respectively.

Cell Lysis and Co-immunoprecipitation—Cells were lysed on ice with radioimmune precipitation assay lysis buffer containing 1% Nonidet P-40, 50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1 mM EDTA, and 10% glycerol supplemented with phosphatase and protease inhibitors (1 mM Na_3VO_4 and NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 μg/ml pepstatin,10 μg/ml aprotinin, and leupeptin). To detect ubiquitination, 0.5% sodium deoxycholate and 20 mM *N*-ethylmaleimide were added to the lysis buffer. Immunoprecipitations were performed by incubating cell lysates with the indicated antibody for 2 h and subsequently incubated with Protein A-immobilized beads (Pierce) for 1 h at 4 °C. For endogenous immunoprecipitations, cells were either left uninfected or infected with 50 HA units/ml of Sendai virus. After 20 h, cell pellets were lysed as described above. Approximately 3 mg of the protein extracts were incubated with 5 μg of anti-Ro52 antibody (Santa Cruz) pre-bound to Protein G beads (Pierce) overnight at 4 °C. Alternatively, cell lysates were incubated with anti-FADD antibody (BD Biosciences) for 2 h and then with Protein A beads (Pierce) for 1 h at 4 °C. Immunoprecipitates were washed with buffer, resolved by SDS-PAGE, and analyzed by Western blotting.

To detect ubiquitinated IRF7, cell pellets were resuspended and boiled in 2% SDS, 50 mM Tris-Cl, pH 7.6, and 1 mM DTT. The lysates were diluted 10-fold in a modified radioimmune precipitation assay buffer containing 1% Nonidet P-40, 50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.2% SDS, and 0.5 mM DTT and incubated with anti-HA antibody for 2 h and with Protein A beads for an additional 1 h at 4 °C. Immunoprecipitates were washed 3× with a buffer containing 1% Nonidet P-40, 50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and leupeptin.

Quantitative Real-time PCR—Total RNA was isolated from cell pellets using TRIzol and chloroform extraction and further purified with the RNeasy Mini kit and RNase-Free DNase Set (Qiagen) according to manufacturer's protocols. Oligo$(dT)_{15}$ primer and SuperScript II Reverse Transcriptase

(Invitrogen) were used according to manufacturer's protocols for cDNA synthesis. The following primers were used in quantitative RT-PCR reactions: human IFN-α (forward) CCCGAGGAGGAGTTTGAT, (reverse) TTCCAGGTCATTCAGTTGC (37); human IFN-β (forward) CACGACAGCTCTTTCCATGA, (reverse) AGCCAGTGCTCGATGAATCT; human, GAPDH (forward) AAAATCAAGTGGGGCGATGCT, (reverse) GGGCAGAGATGATGACCCTTT. Quantitative RT-PCR reactions were performed with SYBR Green purchased from the Invitrogen. The PCR results were normalized to GAPDH and analyzed using the comparative Ct method.

Identification of FADD-associated Proteins—293T cells were transfected with pCMV2-FLAG empty vector or FLAG-FADD construct and lysed in radioimmune precipitation assay lysis buffer (0.5% Nonidet P-40, 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, protease inhibitor mixture, 1 mM Na_3VO_4, 1 mM NaF). The lysate was incubated with anti-FLAG antibody/beads for 2 h at 4 °C and washed 3 times with lysis buffer. The immunoprecipitates were mixed with MCF10A cell lysate, incubated 3 h at 4 °C, and washed 6 times with lysis buffer before loading samples on a gel. After electrophoresis, a modified silver stain procedure (38) was carried out to visualize the bands. Briefly, the gel was fixed in 40% ethanol, 10% acetic acid in water overnight. After the overnight incubation, the gel was washed for 10 h in 50% methanol and then distilled water. The gel was subsequently incubated for 1 h in 0.02% (w/v) sodium thiosulfate, washed in water, and incubated in 0.1% (w/v) silver nitrate for 20 min at 4 °C. Vigorous shaking in 0.04% formaldehyde, 2% (w/v) sodium carbonate was used to develop the gel. The developer reagent was removed, and the gel was stored in 1% acetic acid at 4 °C. Excised bands unique to the FADD immunoprecipitates were digested with trypsin and batch-fractionated on a Poros 50 R2 RP micro-tip, and resulting peptide pools were analyzed by matrix-assisted laser-desorption/ionization (MALDI) reflectron time-of-flight mass spectrometry using a BRUKER UltraFlex TOF/TOF instrument (Bruker Daltonics; Bremen, Germany) as described (39, 40). Selected experimental masses (m/z) were taken to search the human segment of a non-redundant protein data base utilizing the Mascot Peptide Mass Fingerprint program (41), Version 2.3.01 for Windows, with a mass accuracy restriction better than 35 ppm and maximum of one missed cleavage site allowed per peptide. Any tentative confirmation (Mascot score $\geq$30) of a Peptide Mass Fingerprint result, thus, obtained was verified by comparing the computer-generated fragment ion series of the predicted tryptic peptide with the experimental MS/MS data. To confirm Peptide Mass Fingerprint results with scores $\leq$40, mass spectrometric sequencing of selected peptides was done by MALDI-TOF/TOF (MS/MS) analysis on the same prepared samples using the UltraFlex instrument in LIFT mode. Fragment ion spectra were taken to search NR using the Mascot MS/MS Ion Search program.

Viruses—Influenza viruses A/WSN/33 strain (H1N1; ATCC) were propagated in Madin-Darby canine kidney cells (ATCC) in minimum Eagle's medium containing 0.125% BSA and 1% HEPES in the presence of 1 μg/ml tosylamide-2 phenyl ethyl chloromethyl ketone-treated trypsin. Sendai virus Cantell strain (SeV) was purchased from Charles River. For infection, HEK 293T cells were transfected using Lipofectamine 2000 with siRNAs. After 24 h, the cells were split into 12-well plates for infection (1 h at 37 °C). The efficacy of the siRNA knockdown was checked by Western blot analysis. The inocula were aspirated after a 1 h absorption, and cells were incubated in DMEM with 1% FBS for the indicated times. After 1 or 2 days of infection, supernatants were taken for viral titers, and cell pellets were used for protein extracts or mRNA isolation. For protein extracts, cell pellets were lysed as described above, and supernatants were collected after centrifugation.

Viral Titers—50% tissue culture infective dose ($TCID_{50}$) was measured as follows; viral supernatant was diluted in 10-fold steps up to 10^{-7}. 25 μl of each dilution were incubated with Madin-Darby canine kidney cells for influenza virus and LLC-MK2 cells for SeV on flat-bottom 96-well plates. After 1 h absorption, 175 μl of media were added to each well. After 9 days of incubation for influenza, 100 μl of the culture supernatant from each well were mixed with 25 μl of 0.75% chicken red blood cells (from Josman, LLC) on v-shape 96-well plates. For Sendai virus, culture supernatants were collected after 4 days of incubation and mixed 1:1 with 0.5% chicken RBC on v-shape 96-well plates. After an additional 1 h incubation, hemagglutinin titers were determined by the presence or absence of fully formed chicken RBC buttons on the bottom of v-shape plate. $TCID_{50}$ titers were then calculated according to the method of Reed and Muench (42).

RESULTS

FADD Interacts with TRIM 21 but Not the Other Closely Related TRIM Family Members—To understand how FADD might play a role in biological processes other than cell death, we attempted to identify any proteins that associated with FADD in non-stimulating conditions. 293T cells were transfected with a FLAG-tagged FADD expression plasmid or an empty control plasmid. Anti-FLAG immunoprecipitates were incubated with cellular extracts *in vitro* to enhance detection of any FADD-associated proteins. After electrophoresis, proteins were visualized by silver staining. Three bands in addition to FADD were detected in immunoprecipitates from FADD-containing lysates but not from control lysates (Fig. 1*A*). These bands were identified as RIP1, TRIM21, and Ezrin by mass spectrometric analysis. A FADD-RIP1 interaction has been described previously under many conditions (18, 43–46). Ezrin is a cytoplasmic peripheral membrane protein that is functionally linked to actin protein (47) but only appeared as a very faint band in the co-immunoprecipitation experiment. In contrast, the amount of TRIM21 co-immunoprecipitated with FADD was equivalent to RIP1. We, thus, focused on this novel interaction.

TRIM21 or Ro52 is a member of a large family consisting of more than 70 TRIM proteins (22, 23), many of which contain a tripartite motif that includes a RING domain, B-boxes, and a coiled-coiled domain. To confirm the interaction between FADD and TRIM21 and explore potential complexes with other TRIM family members, we transfected FLAG-tagged FADD and HA-tagged TRIM21 expression constructs into

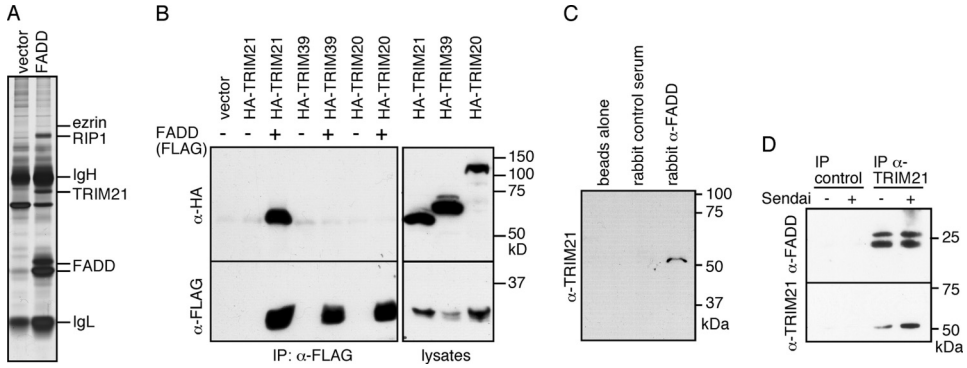

FIGURE 1. **FADD interacts with TRIM21 *in vitro* and *in vivo*.** *A*, 293T cells were transfected with pCMV2-FLAG (vector) or pCMV2-FLAG-FADD (FADD). Cell were lysed and immunoprecipitated with anti-FLAG antibodies. The immunoprecipitates were incubated with additional extracts followed by six washes before running them on the gel. FADD and its co-immunoprecipitated proteins were visualized by silver staining and identified by the mass spectrometric analysis. *B*, HA-TRIM21, HA-TRIM39, HA-TRIM20, or an empty vector were transfected into 293T cells with (+) or without (−) FADD-FLAG. Anti-FLAG antibody was used to immunoprecipitate FADD, and the associating TRIM proteins were detected with an HA-specific antibody in a Western blot analysis. Total cell extracts were immunoblotted as a control for protein expression. *C*, 293T cell extracts were immunoprecipitated with rabbit anti-FADD or control antisera. Western blot analysis of the immunoprecipitates was then performed with anti-TRIM21 specific antibodies. *D*, cell extracts from 293T cells with or without Sendai virus infection were immunoprecipitated (*IP*) with anti-TRIM21 monoclonal antibodies or isotype control antibodies. Western blot analysis of the immunoprecipitates was then performed with anti-FADD or anti-TRIM21 specific antibodies.

293T cells. TRIM39 and TRIM20, two other TRIM proteins that are closely related to TRIM21, were also transfected. FADD was immunoprecipitated with anti-FLAG antibody, and any associating TRIM proteins were examined by Western blotting with anti-HA antibody. FADD complexed with TRIM21, but an association with TRIM39 or TRIM20 was not detected (Fig. 1*B*). To determine whether TRIM21 association with FADD disrupted the interaction between FADD and RIP1 or TRADD (48), we transfected constructs expressing FADD with either RIP1 or TRADD in the presence or absence of TRIM21. As shown in supplemental Fig. 1, FADD was capable of associating with RIP1 or TRADD with TRIM21 present. Interaction between endogenous FADD and TRIM21 was examined using anti-FADD polyclonal antibodies for immunoprecipitations followed by Western blot analysis using a TRIM21-specific antibody. Beads alone or irrelevant rabbit antiserum was used as the control. As shown in Fig. 1*C*, a band with the expected size of TRIM21 was detected after FADD immunoprecipitation but not with beads or control sera. Similarly, the FADD/TRIM21 endogenous association could also be detected by immunoprecipitation with a monoclonal antibody against TRIM21 followed by Western blot analysis with FADD-specific antibodies (Fig. 1*D*). The association of FADD/TRIM21 could also be detected in Sendai virus-infected cells. These results demonstrate that FADD specifically interacts with TRIM21 in a constitutive manner.

FADD Interacts with TRIM 21 through Its DED Domain—To determine the domains involved in the FADD-TRIM21 interaction, various truncated mutants were generated. TRIM21 consists of an N-terminal RING domain followed by a B-box domain, a coiled-coil domain, and a SPRY/B30.2 domain. The B30.2 domain is a known protein-protein interacting region (23). Truncation mutants of TRIM21 were generated without the B30.2 domain (1–333) or without the RING domain (131–475). Co-immunoprecipitation experiments were performed similar to the experiments described previously. Full-length TRIM21 and TRIM21-(131–473) co-immunoprecipitated with FADD, but an interaction was not detected when the B30.2 domain was deleted (Fig. 2*A*). Thus, the TRIM21 B30.2 domain is necessary for the association with FADD.

Conversely, reciprocal experiments analyzed the FADD domain that interacts with TRIM21. FADD truncations were designed to contain different lengths of the DED (FADD 1–95, 1–83, 1–90) or only the death domain (FADD 96–208). These constructs were expressed at similar levels after transfections (Fig. 2*B*, *lower panel*). In addition to full-length FADD, longer truncations of the FADD DED (1–95, 1–90) were capable of interacting with TRIM21 (Fig. 2*B*, *lanes 4*, *5*, and *8*). Neither FADD (96–208) nor FADD (1–83) associated with TRIM21 (Fig. 2*B*, *lanes 6* and *7*). These results suggest that residues 83–95 in the DED are crucial for the FADD/TRIM21 interaction. However, a closer examination of the amino acid composition in this region revealed mostly alanines and glycines, which are unlikely to provide specificity for the interaction. Those residues are also not conserved among species. Thus, it is possible that the amino acids downstream of the DED do not directly contact TRIM21 but help to interact with the anti-FLAG antibody in the immunoprecipitations. To pinpoint the exact residues involved in TRIM21 interaction, amino acids on the surface of the DED were mutated based on the crystal structure of full-length FADD (5, 9). Amino acid substitutions included D2A, V6A, S10A, L28E, D44R, D74A, R78A, D81A, F82A, and E83A. All mutants were expressed at approximately equal amounts (Fig. 2*C*, *lower panels* and data not shown). FADD mutants were immunoprecipitated, and the presence of TRIM21 was assessed by Western blotting. The association between TRIM21 and FADD was alleviated with an aspartic acid to alanine substitution at residue 74 (D74A) (Fig. 2*C*, *lane 7*). All other mutants interacted with TRIM21 (Fig. 2*C* and data not shown). Asp-74 is a key residue in the DED and is conserved among different species (human, mouse, rat, cow, guinea pig, *Xenopus*, chicken). Importantly, the overall structure of D74A mutant FADD has been shown to be intact by NMR studies (9); thus, the inability to bind to TRIM21 is not due to a general

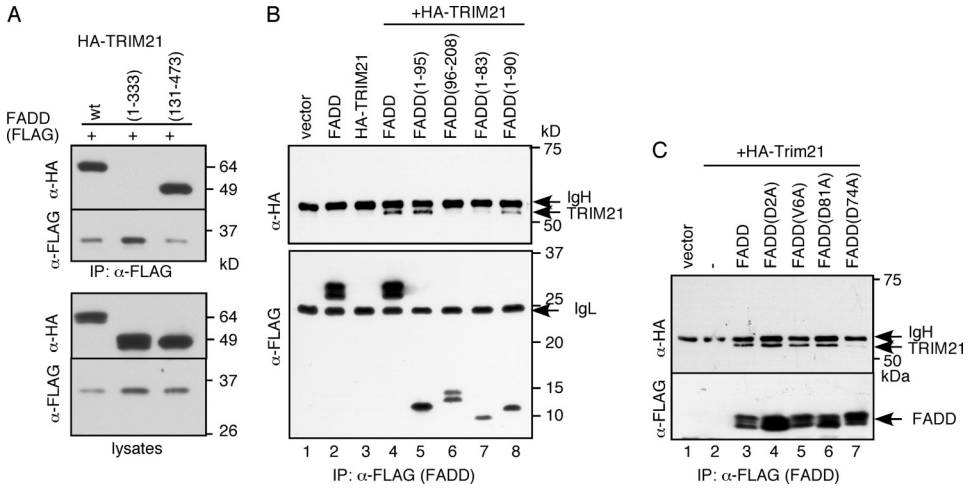

FIGURE 2. **Truncation and mutagenesis studies of FADD/TRIM21 interaction.** *A*, the FADD-FLAG plasmid was co-transfected with wild-type HA-TRIM21 (*wt*), HA-TRIM21-(1–333), or HA-TRIM21-(131–473). Cell lysates were immunoprecipitated (*IP*) with anti-FLAG antibodies, and immunoprecipitates were Western-blotted with either HA- or FLAG-specific antibodies. The corresponding lysates were also Western-blotted as a control. *B*, the indicated plasmids were transfected into 293T followed by immunoprecipitation with FLAG-specific antibodies. The presence of FADD-associated TRIM21 was detected by anti-HA antibodies. *C*, the indicated FLAG-tagged FADD mutants were co-transfected with HA-TRIM21. FADD was immunoprecipitated with anti-FLAG antibodies, and associated TRIM21 was detected using HA-specific antibodies.

problem in protein folding. Extensive FADD DED mutagenesis was previously conducted to reveal residues, specifically Phe-25, Leu-28, and Lys-33, that are important for FADD self-association, CD95 receptor binding, and pro-caspase-8 interaction (8, 9). A complex with TRIM21 was detected with the L28E FADD mutant, indicating that FADD dimerization is not necessary to interact with TRIM21 (data not shown). Although neither D74A nor D44R FADD mutants bind caspase-8 (8), only D74A was defective in associating with TRIM21 (Fig. 2*C* and data not shown). Together, these data indicate that the DED of FADD binds TRIM21 in a distinct fashion from its association with CD95 or caspase-8 observed during extrinsic apoptosis.

FADD Is Not Ubiquitinated by TRIM21 but Enhances TRIM21 Auto-ubiquitination—TRIM21 possesses an E3 ubiquitin ligase activity and can ubiquitinate itself (32). Other substrates regulated by TRIM21 ubiquitination include the cell cycle inhibitor p27 and the interferon transcription factors IRF3 and IRF8 (28–31). Therefore, we considered the possibility that TRIM21 ubiquitinates FADD. To examine this, 293T cells were transfected with various combinations of TRIM21, FLAG-tagged FADD, and HA-tagged ubiquitin. Cells were treated with the proteasome inhibitor MG132 to stabilize ubiquitinated proteins. If FADD is ubiquitinated by TRIM21, high molecular weight bands should be detected after immunoprecipitation of FADD and Western blotting. We did not detect any higher molecular weight species of FADD when co-expressed with TRIM21 and exogenous ubiquitin (Fig. 3*A*, *upper panel*, *lane 5*). Only a 27–28-kDa band, the expected molecular weight of FADD, was detected (Fig. 3*A*). Subsequent immunoblotting with anti-HA antibodies to detect ubiquitinated proteins revealed a high molecular weight smear of more than 50 kDa in samples co-transfected with FADD, TRIM21, and ubiquitin (Fig. 3*A*, *lower panel*, *lane 5*). This higher molecular weight smear is most likely ubiquitinated TRIM21. Because no ubiquitinated species between 25 and 50 kDa were observed, these results suggest that FADD is not ubiquitinated by TRIM21.

Although FADD was not ubiquitinated by TRIM21, we noticed that TRIM21 auto-ubiquitination was increased when co-expressed with FADD. Cells were co-transfected with various combinations of FADD, TRIM21, and ubiquitin expressing plasmids, and then cell lysates were Western-blotted for TRIM21 or FADD. Higher molecular weight species of TRIM21 were observed in cells co-expressing TRIM21 and exogenous ubiquitin (Fig. 3*B*). Furthermore, TRIM21 ubiquitination was significantly enhanced in the presence of wild-type FADD (Fig. 3*B*, *lane 3 versus lane 5*). The increase in ubiquitinated TRIM21 disappeared when D74A mutant FADD was co-expressed instead (Fig. 3*B*, *lane 5 versus lane 9*). To assess whether endogenous FADD contributes to TRIM21 auto-ubiquitination, we performed knockdown experiments with siRNA specific for FADD. Transfection of FADD siRNA significantly reduced the expression of endogenous FADD (Fig. 3*C*, *lanes 4* and *5*). The amounts of ubiquitinated TRIM21 were considerably decreased when the levels of FADD were diminished (Fig. 3*C*, *lane 3 versus lane 4*). Thus, the presence of FADD appears to enhance the E3 ubiquitin ligase activity of TRIM21.

FADD and TRIM21 Synergize to Repress IFN-α but Not IFN-β Activities—Previous reports have demonstrated that overexpression of FADD moderately up-regulated NF-κB and IFN-β activation (15, 49). Using luciferase reporter assays, we also observed a small, albeit reproducible increase in NF-κB or IFN-β activation with FADD overexpression in unstimulated or SeV-stimulated 293T cells (Fig. 4, *A* and *B*). These results are consistent with previous experiments where RIG-I/IPS-1-induced activation of IFN-β and NF-κB was reduced in $FADD^{-/-}$ MEF cells (17), suggesting that FADD positively regulates IFN-β and NF-κB activities in the RIG-I pathway. In contrast, transfection of TRIM21 alone did not lead to increased luciferase activities (Fig. 4, *A* and *B*). Transient trans-

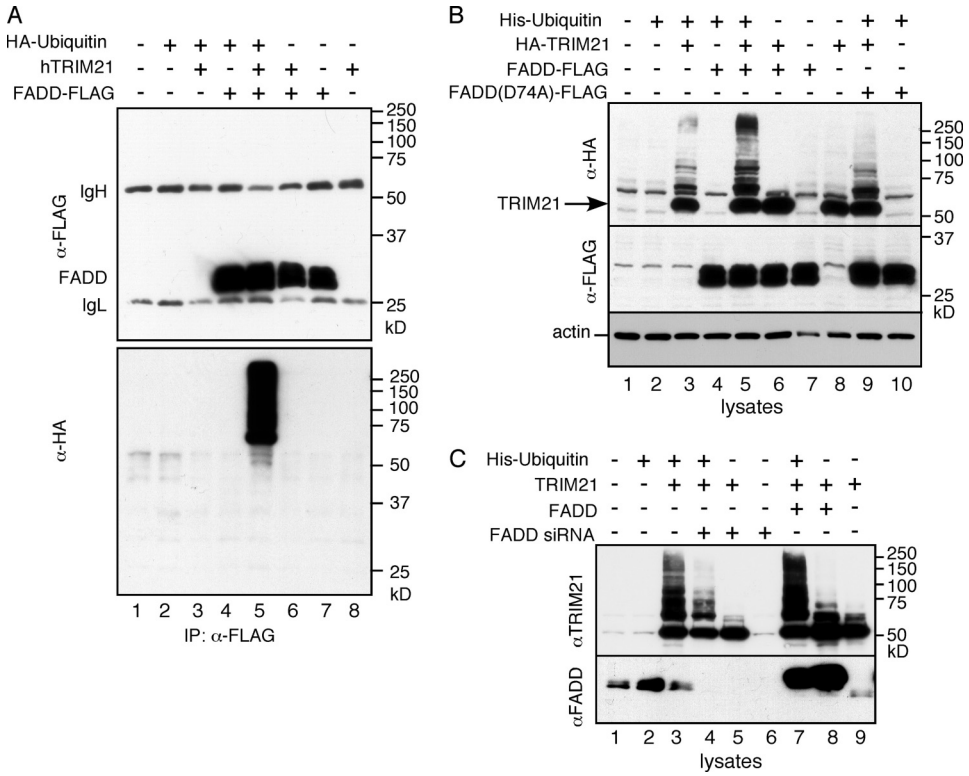

FIGURE 3. **Ubiquitination by TRIM21 is enhanced by FADD.** *A*, 293T cells were transfected with HA-ubiquitin, FADD-FLAG, and TRIM21 in different combinations. FADD was immunoprecipitated from cell lysates using anti-FLAG antibodies, and immunoprecipitates were subsequently immunoblotted with anti-FLAG and anti-HA antibodies. *B*, His-ubiquitin, HA-TRIM21, and FADD-FLAG were transfected into 293T cells as indicated. Total cell extracts were examined by Western blot with anti-HA, anti-FLAG, and anti-β-actin antibodies. *C*, procedures were the same as *B*, except FADD siRNA was transfected where indicated (+).

fections of both TRIM21 and FADD only resulted in a modest increase in IFN-β and NF-κB activation (Fig. 4, *A* and *B*). However, the effect on IFN-β activation disappeared in the presence of SeV infection (Fig. 4*A*).

Regulation of Type I interferon is a two-step process consisting of an early and late phase. During the early phase, IRF3 activation results in IFN-β production. Autocrine stimulation of the interferon-αβ receptor by IFN-β then results in activation of IRF7 and subsequent induction of IFN-α as well as other interferon-responsive genes (50, 51). Because the levels of TRIM21 are regulated by IFN-β (24), we examined the role of FADD and TRIM21 in IRF7-mediated IFN-α luciferase activation. Cells were transiently transfected with IRF7, which is necessary in 293T cells for the activation of IFN-α4. In uninfected cells, a slight inhibition of IFN-α4 activation was observed with FADD overexpression but not with TRIM21 (Fig. 4*C*). SeV infection significantly increased the activation of IFN-α (Fig. 4*C*). Ectopic expression of TRIM21 or FADD individually repressed the SeV-induced IFN-α activity by ~2–3-fold (Fig. 4*C*). However, expressing both FADD and TRIM21 resulted in a complete repression of the IFN-α4-luciferase expression (Fig. 4*C*).

We previously demonstrated that the D74A FADD mutant interacted poorly with TRIM21 (Fig. 2*D*). To examine the effect of mutant FADD on IFN-α activity, similar luciferase experiments were performed with FADD(D74A). Slightly lower amounts of TRIM21 were used to avoid its overwhelming inhibitory effect. In contrast to wild-type FADD, expression of FADD(D74A) alone did not inhibit IFN-α activities in uninfected or Sendai virus-infected cells (Fig. 4*D*). Co-expressing TRIM21 with FADD(D74A) had no suppressive effect on IFN-α in uninfected cells (Fig. 4*D*). In Sendai virus-infected cells, the levels of IFN-α-luciferase activities were lower in cells expressing TRIM21/FADD(D74A) compared with cells expressing FADD(D74A) alone, but the signals were comparable with those with TRIM21 alone transfection (Fig. 4*D*). To assess whether the same trends could be seen in the endogenous IFN-α or IFN-β levels, we performed quantitative RT-PCR assays. Consistent with the luciferase results, combined ectopic expression of FADD and TRIM21 led to a significant decrease of the IFN-α but not IFN-β mRNA levels in Sendai virus-infected cells (Fig. 4*E*). These data indicate that FADD regulates IFN-β and IFN-α differently, and IRF7-mediated activation of IFN-α is negatively regulated by FADD and TRIM21 in a synergistic manner.

IRF7 Ubiquitination and Phosphorylation Are Regulated by TRIM21 and FADD—Previously published data had demonstrated an interaction between TRIM21 and IRF7 (30). The dramatic effect FADD and TRIM21 has on IFN-α activation suggests that both FADD and TRIM21 may complex with IRF7. To investigate the association with IRF7, 293T cells were co-transfected with FADD, TRIM21, and IRF7. Cells were then infected with SeV or left uninfected. FADD was immunoprecipitated with anti-FLAG antibodies, and IRF7 was detected by immunoblotting. IRF7 interacted with FADD exclusively in the presence of TRIM21 (Fig. 5*A*, *lanes 4* and

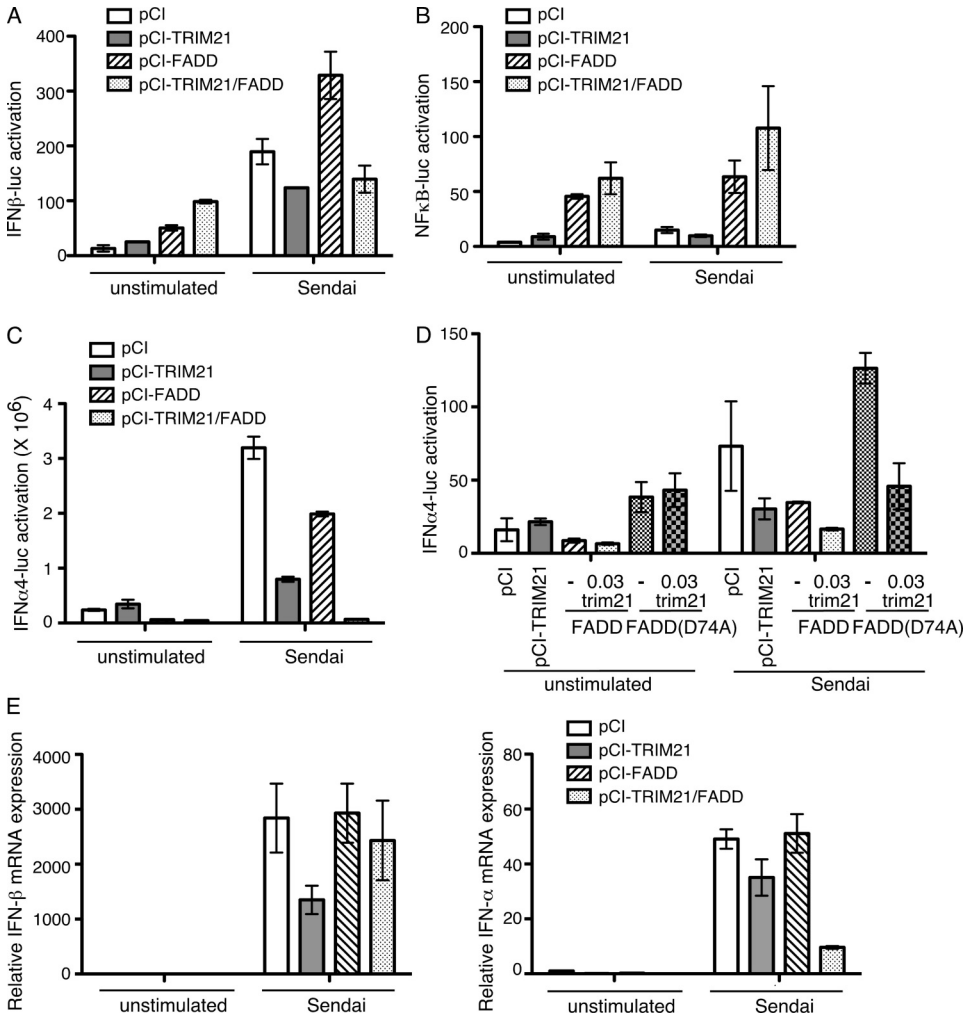

FIGURE 4. **Overexpression of FADD enhances IFN-β and NF-κB but attenuates IFN-α activation when co-transfected with TRIM21.** 293T cells were transfected with an IFN-β-luciferase (*A*) or NF-κB-luciferase (*B*) reporter construct along with 0.3 μg of TRIM21 and/or FADD expression plasmids. Cells were stimulated with 50 HA units/ml SeV 24 h after transfection. Cells were lysed 18 h post-infections, and dual luciferase reporter assays (firefly and renilla) were performed. Samples were normalized with renilla luciferase. *C*, 293T cells were transfected with an IFNα4-luciferase reporter construct with IRF7 and 0.3 μg of TRIM21 and/or FADD expression plasmids. Cells were infected with SeV and lysed. Dual luciferase assays were performed as described in *C*. *D*, procedures were the same as *C*, except mutant FADD(D74A) was used where indicated along with 0.03 μg TRIM21 plasmid where indicated (0.03). All samples were normalized to renilla luciferase. All experiments were performed >3 times with similar results. *E*, *left panel*, 293T cells were transfected with TRIM21 and/or FADD, and expression of the endogenous IFN-β mRNA levels was measured by quantitative RT-PCR. *Right panel*, procedures were the same as the *left panel*, except cells were additionally transfected with IRF7, and IFN-α mRNA levels were measured. All experiments were performed three times with similar results.

10). Interaction between IRF7 and FADD was not detected in cells co-transfected with only IRF7 and FADD (Fig. 5*A*, *lanes 2* and *8*). Interestingly, the IRF7-FADD association increased after SeV infection (Fig. 5*A*, *lane 10*). This enhanced interaction may be due to virus-induced modification of IRF7 or other unknown events. These data suggest that IRF7 can complex with FADD through its interaction with TRIM21.

In the TLR signaling pathway, IRF7 is activated upon TRAF6-mediated ubiquitination and phosphorylation (50, 52). A similar pathway also exists downstream of the RIG-I pathway, although other TRAF proteins might be involved as well (53, 54). To elucidate the effects of FADD and TRIM21 on IRF7 activation, SeV-induced phosphorylation of IRF7 was examined. IRF7 was expressed in 293T cells in combination with TRIM21 and FADD. Cells were subsequently stimulated with SeV, and phospho-IRF7 was assessed from total cell lysates. IRF7 was identified as a 55-kDa band in both uninfected and infected cells (Fig. 5*B*). The appearance of a slower migrating band, corresponding to phosphorylated IRF7, was observed after SeV stimulation (Fig. 5*B*, *lane 1 versus lane 5*). Overexpression of TRIM21 or FADD resulted in lower levels of IRF7 phosphorylation (Fig. 5*B*, *lane 6* and *7*). Phosphorylated IRF7 was almost undetectable when both TRIM21 and FADD were expressed together (Fig. 5, *lane 8*). Because phosphorylation of IRF7 has been shown to correlate with its activation (53, 55), these results are consistent with the effects seen in the IFN-α luciferase assays.

To determine whether TRIM21 and FADD can alter the ubiquitination status of IRF7, the levels of IRF7 ubiquitination were examined. Cells were transfected with HA-tagged IRF7, TRIM21, FADD, and exogenous ubiquitin in different combinations, and IRF7 was subsequently immunoprecipitated from total cell lysates. TRIM21 and ubiquitin expressing plasmids were transfected into cells without IRF7 or FADD as a negative control. The immunoprecipitates were then immu-

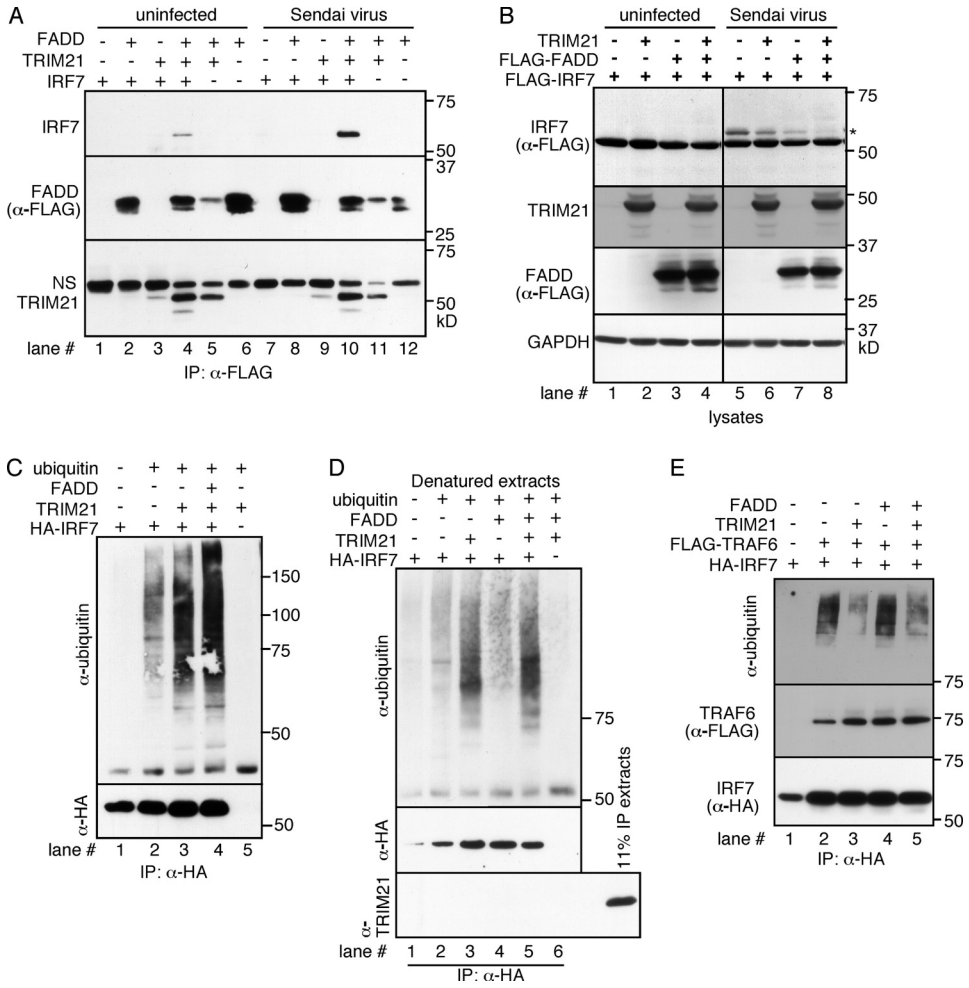

FIGURE 5. **IRF7 associates with TRIM21 and FADD.** *A*, 293T cells were transfected with the indicated expression plasmids. FADD was immunoprecipitated with anti-FLAG antibodies from uninfected or SeV-infected cell lysates. Immunoprecipitates were immunoblotted for IRF7, TRIM21, and FADD. *B*, 293T cells were transfected with IRF7 and FADD and TRIM21 where indicated. 24 h post-transfected, cells were stimulated with 50 HA units/ml SeV for 18–20 h. Whole cell lysates were immunoblotted to detect the levels of phosphorylated IRF7(*), total IRF7, TRIM21, FADD, and GAPDH. *C*, 293T cells were transfected with different combinations of HA-IRF7, ubiquitin, TRIM21, and FLAG-FADD. Cells were lysed, and IRF7 was immunoprecipitated with anti-HA antibodies. Immunoprecipitates were analyzed by Western blotting with anti-ubiquitin and anti-IRF7 antibodies. *D*, procedures were the same *C*, except cells were lysed under denaturing conditions and then diluted for anti-HA immunoprecipitations. For TRIM21 Western blot analysis, whole cell lysates (at 11% of the extracts used for immunoprecipitations (*IP*)) from cells transfected with ubiquitin, TRIM21, and FADD were loaded as a control. *E*, 293T cells were transfected with HA-IRF7, TRAF6-FLAG, FADD-FLAG, and TRIM21. Anti-HA antibodies were used to immunoprecipitate IRF7, and anti-ubiquitin antibodies were used to detect ubiquitinated IRF7.

noblotted for ubiquitinated IRF7. Polyubiquitination of IRF7 was detected as a smear in cells transfected with IRF7 and ubiquitin (Fig. 5*C*, *lane 2*). The addition of TRIM21 intensified IRF7 ubiquitination, and this signal increased further in the presence of both TRIM21 and FADD (Fig. 5*C*, *lane 3* and *4*). No polyubiquitination was observed in TRIM21/ubiquitin-transfected cells (Fig. 5*C*, *lane 5*). To further confirm that the ubiquitin signals detected were ubiquitinated IRF7 and not an associating protein like TRIM21, we performed the same experiments with denatured extracts. As expected, no TRIM21 could be seen in the HA immunoprecipitates (Fig. 5*D*). Western blot analysis with ubiquitin-specific antibodies still showed increased levels of ubiquitinated IRF7 in the presence of TRIM21, which was further enhanced when FADD was added (Fig. 5*D*). These results demonstrate that FADD can enhance ubiquitination activities of TRIM21 with one of its substrates, IRF7. Consistent with a recent paper showing ubiquitination of IRF7 by TRIM21 led to protein degradation (34), we also found that the addition of MG132 proteasome inhibitor led to further enhancement of IRF7 ubiquitination (data not shown).

We were also interested in investigating whether TRIM21 and FADD affected TRAF6-mediated ubiquitination of IRF7 (53). Cells were transfected with IRF7 in combination with TRAF6, TRIM21, and/or FADD. IRF7 was immunoprecipitated and analyzed by Western blotting with ubiquitin antibodies. Consistent with the published data, ubiquitination of IRF7 was observed after the addition of TRAF6 (Fig. 5*E*, *lane 1 versus lane 2*). This ubiquitin activity was greatly reduced in the presence of TRIM21 or both TRIM21 and FADD (Fig. 5*E*, *lanes 3* and *5*). No changes in the levels of IRF7 or TRAF6 were detected in the presence of TRIM21, suggesting that TRIM21 activities did not lead to TRAF6 degradation but instead inhibited TRAF6 activity in an unknown fashion. Collectively, these data indicate that TRIM21 and FADD can directly and indirectly impair IRF7 activation by acting on IRF7

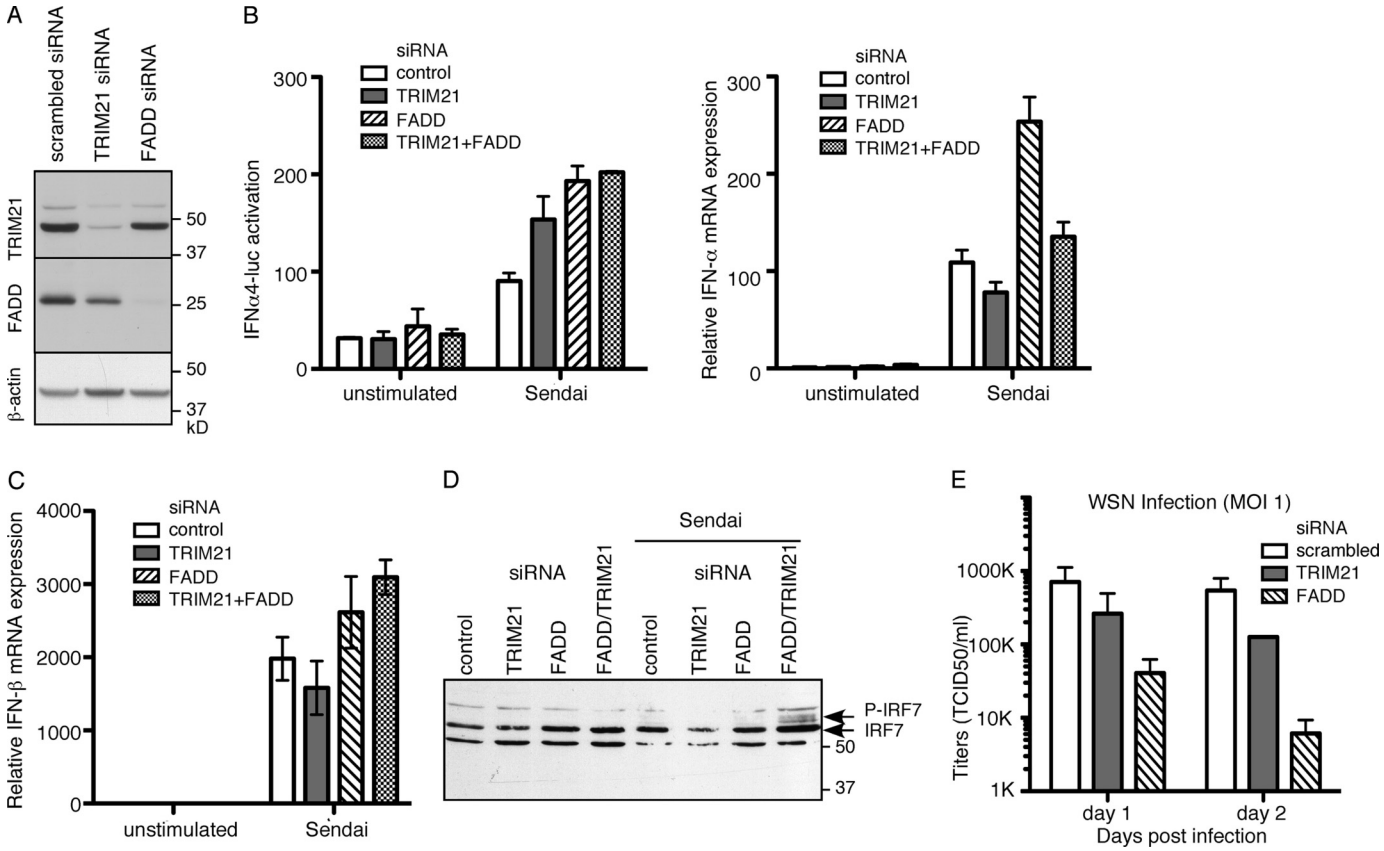

FIGURE 6. **Reduced TRIM21 or FADD expression leads to an increase in IFN-α activation and reduced viral replication.** *A*, 293T cells were transfected with hTRIM21 siRNA, hFADD siRNA, or a control siRNA. Cells were lysed 48 h post-transfection, and extracts were immunoblotted with anti-TRIM21, anti-FADD, and anti-β-actin antibodies. *B*, *left panel*, an IFNα4-luciferase reporter construct was transfected into 293T cells with control siRNA, hTRIM21 siRNA, hFADD siRNA, or a combination of TRIM21 and FADD siRNAs with 50 ng of IRF7 expressing plasmid. After 24 h, cells were stimulated with 50 HA units/ml SeV for 18–20 h. Cells were then lysed and used in a dual luciferase reporter assay as previously described. *Right panel*, procedures were the same as the *left panel*, except IFN-α transcription was measured by quantitative RT-PCR. *Left* and *right panel* experiments were performed >3 and 2 times, respectively, with similar results. *C*, 293T cells were transfected with hTRIM21 siRNA, hFADD siRNA, or a control siRNA. Cells were stimulated with SeV similar to *B*, and IFN-β was measured by quantitative RT-PCR. *D*, procedures were similar to *B*, except extracts were blotted with HA-specific antibodies (for IRF7). *E*, TRIM21, FADD, or TRIM21/FADD knockdown experiments were done by the corresponding siRNAs. After confirmation of protein knockdown by Western blot analysis, cells were infected with influenza WSN at a multiplicity of infection of 1 for 1 or 2 days. Supernatants were harvested, and the $TCID_{50}$/ml of the viruses in the supernatant were calculated by titration on Madin-Darby canine kidney cells. Experiments were performed 3 times with similar results.

and TRAF6. As a result, IRF7 phosphorylation and IFN-α induction are inhibited.

Increased IFN-α Activation and Decreased Viral Replication in FADD or TRIM21 Knockdown Cells—Despite the participation of FADD in multiple aspects of Type I IFN production, we decided to assess the effect of FADD or TRIM21 knockdown on IFN-α up-regulation and viral replication. Others have reported that loss of FADD modestly affects IFN-β during the early phase of IFN-β production (16, 17). In turn, IFN-α production might be lowered, as its activation is dependent on IFN-β expression. To somewhat alleviate this problem, we co-transfected a low amount of IRF7 expression plasmid. IFN-α4 luciferase activity was measured in cells transfected with TRIM21 and/or FADD siRNA. A scramble siRNA construct was used as a control. Transfections with siRNA specific for TRIM21 and FADD resulted in efficient knockdown of the corresponding protein expression (Fig. 6*A*). In SeV-infected cells, a modest but consistent increase in IFN-α4 luciferase activation was observed in the absence of TRIM21 or FADD when compared with control cells (Fig. 6*B*, *left panel*). In addition, a modest increase in IFN-α mRNA was detected in FADD siRNA-transfected cells (Fig. 6*B*, *right panel*). The increase was not seen in TRIM21/FADD double knockdown, perhaps because reduction of both TRIM21 and FADD affects cellular pathways that stress the cells. No statistically significant changes of the endogenous IFN-β mRNA levels were seen in all the knockdown cells (Fig. 6*C*). To detect IRF7 phosphorylation, cells extracts from the described luciferase assay were used in a Western blot analysis. The smaller amounts of IRF7 used in these assays made it hard to detect phosphorylated IRF7 in Sendai virus-stimulated cells. However, phospho-IRF7 was clearly seen in extracts from FADD and TRIM21 double knockdown (Fig. 6*D*, *eighth lane*), suggesting that reduction of both proteins synergistically led to increased IRF7 phosphorylation. Finally, to assess the consequence of FADD or TRIM21 deficiency on viral replication, cells were infected with Sendai virus or influenza WSN RNA virus. Culture supernatants were harvested from infected cells 24 and 48 h post-infection, and viral titers ($TCID_{50}$) were calculated. The results for SeV infections were variable. Either no differences in viral titers or low viral titers were observed in TRIM21 or FADD knock-down cells (data not shown). The

variability might be due to the fact that the SeV C and V proteins are known to inhibit the IFN pathway, but at the same time SeV defective viral particles in the viral stock can induce interferon production (56, 57), leading to unpredictable outcomes. Alternatively, 293T cells might not produce enough IFN-α to affect SeV titers. In contrast, the viral titers for influenza were consistent from experiment to experiment and showed that viral titers decreased in TRIM21 or FADD knockdown cells (Fig. 6*E*). This effect was seen at both 1 and 2 days after infection (Fig. 6*E*). These results are consistent with the idea that TRIM21 and FADD are capable of negatively regulating IFN-α and, thus, are important negative regulators of innate immunity against viral infection.

DISCUSSION

Despite extensive experimentation, the role of FADD in innate immunity remains controversial. Although several groups have found that FADD-deficient MEF cells exhibit reduced IFN-β production and interferon-stimulated regulatory element activation in response to poly(I:C) or dsRNA stimulation (16–18), others did not see this effect in a different cell line (21). In our hands, overexpression of FADD had a modest but consistent positive effect on IFN-β luciferase activation. This effect occurred in the absence or presence of SeV. These data are consistent with the proposed role of FADD in the initial phase of interferon production (18).

We demonstrate here that FADD interacts with TRIM21, an E3 ubiquitin ligase that can ubiquitinate itself, IRF transcription factors, and p27 cell cycle inhibitor (22, 28–31). The death effector domain of FADD associates with the B30.2 domain of TRIM21. The site of interaction overlaps with that of FADD-caspase-8 association during death receptor-induced apoptosis. Thus, FADD might be able to only interact with either TRIM21 or caspase-8 alone but not both at the same time, although we have not tested this directly. Loss of TRIM21 or ectopic expression of TRIM21 had no effect on Fas-induced apoptosis,[6] suggesting that FADD-TRIM21 interaction does not affect the Fas-mediated apoptotic pathway. Interestingly, TRIM21 has been described to associate with and ubiquitinate IRF3. However, two groups have reported conflicting biological consequences of the interaction. One report concluded that TRIM21 is a negative regulator of IFN-β (30), whereas another group claimed that TRIM21 is required for sustained IRF3 activation during viral infection (29). We only saw a small effect of TRIM21 on IFN-β activities in the presence of SeV and, thus, decided not to pursue it further. In contrast to the modest role of FADD and TRIM21 in the initial phase of interferon production, ectopic expression of both FADD and TRIM21 dramatically lowered IFN-α activation, especially after virus infection. The repression correlates with a reduction in phosphorylation levels of IRF7, a transcription factor essential for IFN-α transcription. IRF7 complexes with FADD through TRIM21. We further showed that IRF7 association with FADD was enhanced in the presence SeV. Our results are consistent with a previous report by another group reporting a weak interaction between TRIM21 and IRF7 (30). During the course of our studies, the same group demonstrated that TRIM21 ubiquitinated IRF7 by both lysine 48 and lysine 63 linkages (34). Overexpression of TRIM21 resulted in IRF7 degradation and a dose-dependent repression of IFN-α activation (34). Thus, FADD might enhance IRF7 protein degradation by affecting TRIM21 E3 ligase activity on IRF7. TRIM21 and FADD also additionally affect TRAF6, which is a positive regulator of IRF7 activation. TRAF6 can confer lysine 63 ubiquitination on IRF7, resulting in recruitment of kinases that phosphorylate IRF7 (53, 58). How TRIM21 ubiquitination inhibits TRAF6 enzymatic function has yet to be determined. Together with the results showing FADD enhancement of TRIM21 auto-ubiquitination, these data suggest that TRIM21 ubiquitination of its targeted substrates can be greatly augmented by FADD. However, additional factors must also contribute to the enhancement of TRIM21 activities by FADD. Although TRIM21 associates with FADD constitutively in both uninfected and Sendai virus-infected cells, their repressive activities were greatly amplified when cells were infected with virus. It is, thus, possible that other FADD-interacting protein or an unknown post-transcriptional modification participates in regulation of the TRIM21/FADD molecular complex.

In contrast to the finding that TRIM21 and FADD negatively regulate IRF7 through post-transcriptional modifications, a previously published report indicates that IRF7 transcription is greatly reduced in FADD-deficient cells (15). We did not detect any significant changes in the levels of IRF7 expression that correlated to FADD expression. It is not clear what contributes to this discrepancy. One explanation is that overexpression of IRF7 in our experiments masked the changes in IRF7 expression. Alternatively, different cell lines may have distinct regulatory signaling pathways for IRF7. The reduction of FADD for only a few days in our knockdown experiments may also be insufficient to affect IRF7 transcription.

Two groups have published apparently different phenotypes of TRIM21-deficient mice (35, 36). In one study overt abnormalities in the knock-out mice were not detected, but they reported that the TRIM21-deficient MEF cells produce higher amounts of proinflammatory cytokines when stimulated. The other group found that their TRIM21-deficient mice exhibited systemic autoimmune disease, caused by excessive Th17 cytokine production (35). Thus, despite reporting different phenotypes of TRIM21 knock-out mice, both groups concluded that TRIM21 is a negative regulator of pathways that affect several cytokines. Whether FADD participates in all these TRIM21-regulated signaling pathways requires further experimentation.

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[6] J. A. Young, D. Sermwittayawong, H.-J. Kim, S. Nandu, N. An, H. Erdjument-Bromage, P. Tempst, L. Coscoy, and A. Winoto, unpublished information.

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