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Interferon-Inducible Protein IFIXα1 Functions as a Negative Regulator of HDM2

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The 200-amino-acid repeat (HIN-200) gene family with the hematopoietic interferon (IFN)-inducible nuclear protein encodes highly homologous proteins involved in cell growth, differentiation, autoimmunity, and tumor suppression. IFIX is the newest member of the human HIN-200 family and is often downregulated in breast tumors and breast cancer cell lines. The expression of the longest isoform of IFIX gene products, IFIXα1, is associated with growth inhibition, suppression of transformation, and tumorigenesis. However, the mechanism underlying the tumor suppression activity of IFIXα1 is not well understood. Here, we show that IFIXα1 downregulates HDM2, a principal negative regulator of p53, at the posttranslational level. IFIXα1 destabilizes HDM2 protein and promotes its ubiquitination. The E3 ligase activity of HDM2 appears to be required for this IFIXα1 effect. Importantly, HDM2 downregulation is required for the IFIXα1-mediated increase of p53 protein levels, transcriptional activity, and nuclear localization, suggesting that IFIXα1 positively regulates p53 by acting as a negative regulator of HDM2. We found that IFIXα1 interacts with HDM2. Interestingly, the signature motif of the HIN-200 gene family, i.e., the 200-amino-acid HIN domain of IFIXα1, is sufficient not only for binding HDM2 but also for downregulating it, leading to p53 activation. Finally, we show that IFIX mediates HDM2 downregulation in an IFN-inducible system. Together, these results suggest that IFIXα1 functions as a tumor suppressor by repressing HDM2 function.

Interferons (IFNs) play an essential role in innate and adaptive immunity and the host defense system against viral, bacterial, and parasitic infections (49). Also, IFNs have been used as therapeutic agents for treating human solid and hematologic malignancies, such as hairy cell leukemia, chronic myelogenous leukemia, follicular (non-Hodgkin's) lymphoma, and malignant melanoma (34, 81). Although the mechanism of the IFN-induced antitumor activity is poorly understood, it is believed that the IFN-inducible proteins may be critical for executing tumor suppression (48). Indeed, IFN-inducible genes, such as those for RNase L (73), IFN regulatory factor 1 (67), and the double-stranded RNA-regulated serine/threonine protein kinase (PKR) (39), have been implicated in tumor suppression.

The IFN-inducible HIN-200 gene family encodes a class of proteins that share a 200-amino-acid (HIN) signature motif of type a and/or type b. Four human (IFI16, MNDA, AIM2, and IFIX) and five mouse (p202a, p202b, p203, p204, and p205 [or D3]) HIN-200 family proteins have been identified (2, 54). HIN-200 genes are located at chromosome 1q21-23 as a gene cluster in both mouse and human genomes. Most HIN-200 proteins possess two major protein domains. First, the N-terminal region of HIN-200 proteins contains a highly helical pyrin domain (PYD) (36), which belongs to the death domain-containing protein superfamily involved in apoptosis and inflammation (52, 66, 75). Second, the C-terminal HIN domain consists of two consecutive oligonucleotide/oligosaccharide-binding folds (1). The oligonucleotide/oligosaccharide-binding fold-containing proteins are involved in a variety of biological processes, including DNA replication, DNA recombination, DNA repair, and telomere maintenance (6, 78). However, the role of HIN-200 proteins in these biological processes is poorly understood.

The observations that HIN-200 proteins interact with several cellular regulators involved in cell cycle control, differentiation, and apoptosis suggest that the physiological role of HIN-200 proteins is beyond the IFN system (2, 54). Moreover, the observation that HIN-200, e.g., IFI16, is widely expressed in normal human tissues, including endothelial and epithelial cells, further supports this notion (27, 65, 83). Therefore, it is not surprising that loss or reduced expression of HIN-200 genes is associated with human cancers (3, 17–19, 26, 46, 61, 64, 80). These studies suggest that HIN-200 proteins may play a role in tumor suppression.

The mouse double-minute gene 2 (*mdm2*) encodes an oncoprotein (22, 24). Consistently, HDM2, the human homologue of mdm2, is found frequently overexpressed in human cancers, including about 50% of breast cancers (33, 55–57, 71). A recent report also showed that HDM2 overexpression in tumors is associated with poor prognosis (59). These results underscore the pivotal involvement of HDM2 in tumorigenesis. Therefore, HDM2 has been an important target for developing cancer therapeutics (25, 45, 72).

The RING finger domain of HDM2 possesses an intrinsic E3 ubiquitin ligase activity (42). Ubiquitinated proteins are targeted for proteasome-mediated degradation. The best-known substrate of the E3 ligase activity of HDM2 is the p53 tumor suppressor protein. HDM2 binds to the N terminus of

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p53. This interaction allows HDM2 to inhibit p53 in two ways: (i) blocking the ability of p53 to activate transcription of its target genes by binding to the N-terminal transactivation domain of p53 (11, 62) and (ii) mediating ubiquitination of p53, leading to degradation by proteasome (30, 31, 47). Interestingly, HDM2 is also a substrate of its own E3 ligase activity (23, 37). The intricate regulation of p53 and HDM2 is further demonstrated by the fact that HDM2 is also p53 responsive (4, 63). Thus, these two molecules link together in a negative feedback loop for the purpose of keeping the cellular p53 at low levels in the absence of stress. The p53-HDM2 autoregulatory loop is vital as demonstrated by the rescue of embryonic lethality of mdm2-null mice in a p53-null background (44, 60). Therefore, a defective autoregulatory loop caused by mutations, DNA damage, or oncogenic insult has a profound impact on tumorigenesis (16).

We recently identified IFIX as a novel member of the human HIN-200 family (18). The IFIX transcriptional unit expresses at least six IFIX isoforms. IFIX proteins are primarily nuclear and possess a single type a HIN motif. Importantly, the expression of IFIX is reduced in the majority of breast tumors and breast cancer cell lines. Therefore, IFIX may function as a putative tumor suppressor. Consistently, the expression of IFIXα1, the longest IFIX isoform, leads to suppression of growth and transformation in vitro and tumorigenicity and tumor growth in vivo (18). The growth inhibitory activity of IFIXα1 is associated with the induction of $p21^{CIP1}$, a key cyclin-dependent kinase inhibitor (18). However, the mechanism of the IFIXα1 tumor suppressor activity has not been well elucidated. In this report, we show a novel interaction between IFIXα1 and HDM2 which leads to destabilization of HDM2. Consequently, p53 is stabilized and activated. The novel cross talk between the IFN-IFIX signaling pathway and the HDM2-p53 autoregulatory loop may contribute in part to the IFN-induced antitumor activity in certain cancers.

MATERIALS AND METHODS

Cell lines, plasmids, and reagents. MCF-7 and its FLAG-tagged IFIXα1 derivatives, X-1 and X-2, as well as MDA-MB-468 and its FLAG-tagged IFIXα1 derivatives, X-1 and X-2, have been described previously (18). The corresponding control cell lines are the pooled stable clones transfected with the empty vector (pCMV-Tag2B [FLAG]; Stratagene, La Jolla, CA) (18). H1299, HCT116 and its p53-null derivative, HCT116 ($p53^{-/-}$) (8), 293 and its large-T derivative, 293T, and $p53^{-/-}$ and $p53^{-/-}$ $mdm2^{-/-}$ double-knockout (DKO) mouse embryonic fibroblasts (MEFs) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Raji cells treated with or without IFN-α (2,000 U/ml) and IFN-γ (1,000 U/ml) (Sigma, St. Louis, MO), respectively, were grown in RPMI medium containing 10% or 0.2% FCS for the time indicated in the figure legends. MG132 (10 μM) (Sigma) treatment was performed for 5 to 6 h prior to harvest. The enhanced green fluorescent protein (EGFP)-tagged IFIXα1 (EGFP-IFIXα1) and IFIXβ1 (EGFP-IFIXβ1) have been previously described (18). The IFIX-N was generated using PCR primer set 5′-CCGGATCCTTAGAGATGGCAAATAACTAC (forward; containing a BamHI site) and 5′-CGGGATCCCTCAGTTGAGGAAGTGTTGG (reverse; containing a BamHI site) to amplify a 579-bp region corresponding to amino acids 1 to 193 of IFIXα1. The IFIX-HIN was generated using PCR primer set 5′-CGGAATTCCAGACCTCATCATCAGCTCC (forward; containing an EcoRI site) and 5′-CGGGATCCTTACTGGATGAAACTATGCATTTC (reverse; containing a BamHI site) to amplify a 654-bp region corresponding to amino acids 179 to 397 of IFIXα1. Both FLAG-tagged or EGFP-tagged IFIX-N and IFIX-HIN were generated as described before (18). GFP-p53 (gift from G. Wahl) (76), CMV-HDM2 (gift from Y. Zhang) (40), and the HDM2 mutants, i.e., HDM2(Δ150–230) and HDM2(1–441), have been previously described (41). Plasmid DNA transfection was performed by using FuGENE 6 transfection reagent (Roche, Indianapolis, IN) according to the manufacturer's instructions. To isolate the GFP-positive cells, at 48 h after transfection, cells transfected with EGFP-IFIXα1, EGFP-HIN, or EGFP empty vector were sorted out by BD FACSAria cell sorting system (Palo Alto, CA).

siRNA transfection. Electroporation was used to transfect small inhibitory RNAs (siRNAs) into cells. The IFIX siRNA, i.e., ^{689}GGAGTAAGATGTCC AAAGA^{707} in exon 4 of IFIXα1 (Dharmacon, Lafayette, CO), was used for transfection. The results in Fig. 8 are from a mixture of four IFIX siRNAs corresponding to amino acid sequences 689 to 707, 1,190 to 1,209, 1,246 to 1,265, and 1,486 to 1,504 of IFIXα1 cDNA (Dharmacon). The nonspecific control siRNA is 5′-TAGCGACTAAACACATCAATT(dT)-3′ (Dharmacon). Briefly, cells were suspended in the electroporation buffer (120 nM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 6 mM glucose, 25 mM HEPES [pH 7.6], 2 mM EGTA, and 5 mM $MgCl_2$). The siRNAs (100 nM) were then added to the cell suspension, followed by electroporation using Nucleofector (Amaxa Biosystems, Koeln, Germany).

Coimmunoprecipitation, Western blotting, and antibodies. Protein lysates (0.5 to 1.5 μg) were prepared using radioimmunoprecipitation assay B lysis buffer as described previously (84). The following antibodies (2 to 4 mg) were used in coimmunoprecipitation (co-IP): anti-HDM2 (D-7 for IP; and D-7 and N-20 for Western blotting [WB]; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-FLAG (Sigma, M2 for co-IP and M5 for WB), and anti-GFP (Santa Cruz Biotech). Immune complexes were recovered using 30 μl of protein G (for monoclonal antibodies) or protein A (for rabbit polyclonal antibodies) agarose (Roche) overnight at 4°C. Immune complex was then washed with phosphate-buffered saline four to six times at 4°C, followed by centrifugation and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Proteins were then transferred to a nitrocellulose membrane and probed with antibodies as indicated in the figure legends. Other antibodies used in the WB include anti-procyclic acidic repetitive protein (BD Biosciences, Palo Alto, CA), anti-α-tubulin (Sigma), anti-PKR (Santa Cruz Biotech.), anti-IFI16 (Santa Cruz Biotech), anti-p53 (NeoMarker, Fremont, CA), and anti-$p21^{CIP1}$ (Santa Cruz Biotech). The peptide-purified anti-IFIX antibodies (recognizing α, β, and γ isoforms) are rabbit polyclonal antibodies against two overlapping peptides that correspond to the sequence ^{195}LKPLANRHATASKNIFREDPIIA^{217} in the N-terminal domain of IFIXα1 (Bethyl Laboratories, Inc., Montgomery, TX) (18). The peptide-purified anti-IFIXα antibodies (recognizing α1 and α2 isoforms) are rabbit polyclonal antibodies against a synthetic peptide, ^{468}FRITSPTVAPPLSS DTSTNRHPAVP^{492}, which corresponds to the C-terminal 25-amino-acid region of IFIXα1 (18) (Bethyl Laboratories). Detection was achieved by incubating the secondary goat anti-rabbit or -mouse antibodies coupled with horseradish peroxidase (1:5,000) (Pierce), followed by use of the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Northern blot analysis. Northern blot analysis was performed as previously described (84).

Luciferase assays. Cells transfected with the luciferase reporter gene, PG13-LUC (a firefly luciferase gene under the control of 13 p53 responsive elements) (79), MG15-LUC (a corresponding construct with mutated p53 responsive elements), and pRL-TK (for normalizing transfection efficiency) (Promega) were harvested to measure luciferase activity using the Dual-Luciferase reporter assay system (Promega, Madison, WI) and an illuminometer (TD-20/20; Promega).

Chromatin immunoprecipitation (ChIP) assay. The modified protocol was based on that previously published (53). Briefly, IFIXα1 stable MCF-7 cell lines and the vector control cell lines, X-1, X-2, and V, were fixed by 1% formaldehyde for 10 min before cell lysis. Cell lysates were subsequently sonicated, followed by centrifugation. The "input" (4% of the supernatant) was used in PCR as a positive control. The supernatant was then precleared using mouse immunoglobulin G (10 μg) for 1 h at 4°C. Protein G-agarose beads (50 μl) (Roche) were added to the supernatant and incubated for 2 h at 4°C. After centrifugation, the supernatant was then used for immunoprecipitation using anti-p53 antibody (2 μg) (NeoMarker) or an irrelevant antibody, e.g., anti-GFP antibody (2 μg) (Santa Cruz Biotech), and incubated overnight at 4°C. The protein/DNA complex was subsequently incubated with protein G-agarose beads for 2 h at 4°C. The immune complex was collected by centrifugation and then washed seven times with the following for 10 min each: twice with 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate; once with 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1% NP-40, and 0.1% SDS; twice with 50 mM Tris-HCl (pH 8.0), 250 mM LiCl, 1% NP-40, 0.5% sodium deoxycholate, and 1 mM EDTA; and twice with TE [10 mM Tris-HCl, 1 mM EDTA] (pH 8.0) buffer. The immune complex was suspended in TE buffer with 0.25% SDS, protease K (250 μg/ml), and RNase A (50 μg/ml) (Sigma) and incubated at 37°C for 4 h and at 65°C overnight. The DNA was then extracted with phenol-chloroform and precipitated with ethanol in the presence of glycogen (20 μg) as a carrier. The

precipitate was used as a template for PCR amplification. The primers that specifically amplify a 320-bp region located approximately 0.7 to 1.0 kb upstream of the human $p21^{CIP1}$ promoter are 5′-AAACCATCTGCAAATGAGGG (forward) and 5′-GAACCAATCTCCCTACACC (reverse). PCR was performed under the following conditions: 35 cycles at 94°C for 40 seconds, 56°C for 1 min, and 72°C for 40 seconds.

Mobility shift assay. Nuclear extracts isolated from MCF-7 vector control, the IFIXα1 stable cell lines (X-1 and X-2), and MCF-7 cells treated with or without UV light (20 J/m^2) were incubated with ^{32}P-labeled oligonucleotide containing p53 binding sites (p53 Nushift kit; Active Motif, Carlsbad, CA) in a binding reaction according to the manufacturer's instructions. The binding reaction was run on a 5% native polyacrylamide gel. Supershift assay was performed using the nuclear extract isolated from MCF-7 (X-1) cells incubated with or without the anti-p53 antibody provided by the p53 Nushift kit according to the manufacturer's protocol.

Cycloheximide-chase assay. Cells were cultured in six-well plates overnight. To measure the p53 turnover rate, MCF-7 vector control and the IFIXα1 stable cell lines (X-1 and X-2) were treated with cycloheximide (CHX; 100 μg/ml) (Sigma) for the indicated time, followed by Western blotting using anti-p53 antibody. To measure the turnover rate, H1299 cells were cotransfected with HDM2 or the HDM2(1–441) or HDM2(C464A) (0.7 μg) mutants and IFIXα1 or the vector control, pCMV-Tag2B (FLAG) (1.3 μg) (Stratagene, La Jolla, CA). Twenty-four hours after transfection, cells were treated with CHX (100 μg/ml) for the indicated time, followed by Western blotting using anti-HDM2 or anti-IFIXα antibody.

RESULTS

IFIXα1 downregulates HDM2. We have previously shown that IFIXα1 expression is associated with growth inhibition, suppressed transformation, and tumorigenicity (18). In searching for the mechanism underlying this phenomenon, we found that HDM2 protein levels were greatly reduced in the IFIXα1-expressing MDA-MB-468 cells (which express the p53 mutant), i.e., X-1 and X-2 (18), compared with those in the vector (Fig. 1A). To test whether IFIXα1 expression is the cause for HDM2 reduction, we performed an IFIXα1 knockdown experiment on a low IFIXα1 expression cell line, i.e., MDA-MB-468 (X-1). As shown in Fig. 1B, IFIX siRNA, a small inhibitory RNA specific for IFIX (Dharmacon), but not the control siRNA transfection rescued the reduced HDM2 level in MDA-MB-468 (X-1). This result indicates that IFIXα1 may regulate the level of HDM2. However, IFIXα1 expression has little effect on the HDM2 mRNA levels (Fig. 1C). Consistently, HDM2 reduction was also observed in two other cell lines, i.e., H1299 (a human nonsmall cell lung carcinoma cell line) (Fig. 2B) and 293 (a human embryonic kidney cell line) (data not shown), in a transient transfection system. These results suggest that IFIXα1 regulates HDM2 protein, but not mRNA, level.

IFIXα1 destabilizes HDM2 protein. Next, we wanted to determine whether IFIXα1 regulates the stability of HDM2. To test this possibility, H1299 cells were cotransfected with HDM2 and FLAG-tagged IFIXα1 (or the empty FLAG vector), followed by a CHX (a protein synthesis inhibitor)-chase assay. As shown in Fig. 1D and E, the half-life of HDM2 was around 13 min in the presence of exogenous IFIXα1 but was 23 min in the absence of IFIXα1. This result suggests that IFIXα1 enhances the turnover rate of HDM2 in cells.

IFIXα1 promotes the ubiquitination of HDM2. To determine whether the IFIXα1-mediated HDM2 destabilization and degradation depends on the proteasome machinery, the IFIXα1 stable MDA-MB-468 cell lines and the vector control cells were treated with a proteasome inhibitor, MG132. As shown in Fig. 2A, MG132 treatment completely restored the HDM2 level in the IFIXα1 stable cells compared with no treatment (Fig. 1A). A similar observation was observed in the MG132-treated H1299 cells transiently transfected with EGFP-IFIXα1 (Fig. 2B). These results clearly indicate that proteasome machinery is required for the IFIXα1-mediated HDM2 downregulation.

Since ubiquitinated proteins are targeted for proteasome-mediated degradation (35) and HDM2 is ubiquitinated by its own E3 ubiquitin ligase activity (23, 38), it is possible that IFIXα1 may destabilize HDM2 by promoting its ubiquitination. To test this possibility, we determined the effect of IFIXα1 on the levels of ubiquitinated HDM2. H1299 cells were cotransfected with HDM2, HA-tagged ubiqutin, and increasing amounts of IFIXα1, followed by IP using anti-HDM2 antibody and Western blotting using anti-HA, anti-HDM2, and anti-IFIXα antibodies. As shown in Fig. 2C (left panel), IFIXα1 increases the levels of ubiquitinated HDM2 in a dose-dependent manner. The results obtained from two independent experiments are presented (Fig. 2D). Interestingly, the presence of IFIXα1 in the HDM2 immunocomplex suggests that IFIXα1 interacts with HDM2 (Fig. 2C, lower panel). The high molecular mass (>150 kDa) of HDM2 suggests that IFIXα1 may promote polyubiquitination of HDM2. Since the polyubiquitin chains formed at the lysine (K)-48 residue of ubiquitin are critical for proteasome-mediated proteolysis (10), we cotransfected H1299 cells with HDM2, IFIXα1, and a myc-tagged ubiquitin mutant, i.e., Ub-K48R (which is unable to form polyubiquitin chains). As shown in Fig. 2C (right panel), IFIXα1 failed to induce the high-molecular-weight ubiquitinated HDM2. These results suggest that IFIXα1 promotes polyubiquitination of HDM2 and are consistent with the observations that IFIXα1 destabilizes HDM2 through proteasome-mediated degradation (Fig. 1D and E and 2A and B).

Since HDM2 is the substrate of its own E3 ligase activity (23, 38), it raises a possibility that IFIXα1 may destabilize HDM2 through the E3 ligase activity of HDM2. To test this possibility, we first performed a CHX-chase experiment to determine the effect of IFIXα1 on the turnover rate of the RING finger deletion mutant, i.e., 1–441 (in which E3 ligase domain is deleted) (41). As expected, the 1–441 mutant is very stable (Fig. 2E, left panel) compared with the wild-type HDM2 (Fig. 1D, left panel). However, IFIXα1 has no effect on the turnover rate of the 1–441 mutant (Fig. 2E, right panel). This result suggests that the RING finger domain of HDM2 is required for the IFIXα1-mediated destabilization. The cysteine residue at position 464 (C464) located in the RING finger domain of HDM2 is important for self-ubiquitination (23, 38). Therefore, mutation of C464 to alanine (C464A) abolishes the E3 ligase activity of HDM2. To specifically test whether E3 ligase activity is required for IFIXα1 to destabilize HDM2, we performed a CHX-chase assay to determine the IFIXα1 effect on the stability of the C464A mutant. As shown in Fig. 2F (left panel), the C464A mutant is relatively stable compared with the wild-type HDM2 (Fig. 1D, left panel). Importantly, IFIXα1 has no effect on the turnover rate of C464A mutant (Fig. 2F, right panel). These data strongly suggest that the E3 ligase activity is required for the IFIXα1-mediated HDM2 destabilization and also rule out the possibility that IFIXα1 may interact with an unknown E3 ligase protein that *trans* ubiquitinates HDM2.

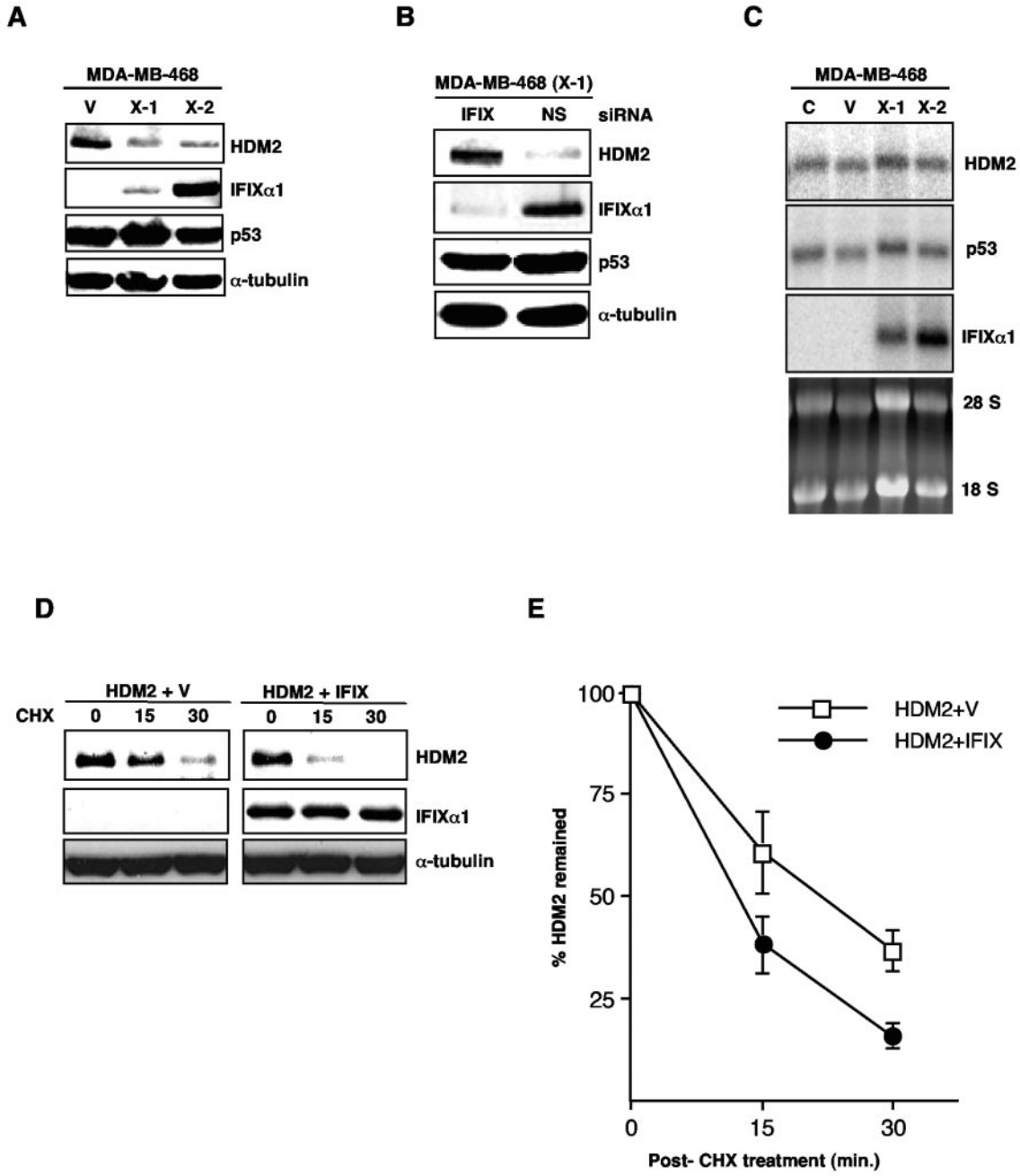

FIG. 1. IFIXα1 destabilizes HDM2. (A) Inverse relationship between IFIXα1 and HDM2 expression. Cell lysates isolated from IFIXα1 stable MDA-MB-468 cell lines (X-1 and X-2) and the vector control cell lines (V) were analyzed by Western blotting using antibodies against HDM2, p53, IFIXα, and α-tubulin. (B) IFIXα1 is responsible for HDM2 downregulation. MDA-MB-468 (X-1) cells were transfected with IFIX siRNA (100 nM) or NS siRNA (100 nM). Forty-eight hours after transfection, the expression levels of HDM2, IFIXα1, p53, and α-tubulin were analyzed by Western blotting. (C) IFIXα1 has little effect on the steady-state mRNA levels of HDM2 in MDA-MB-468 cells. Total RNA (10 μg) isolated from the parental MDA-MB-468 (C), the IFIX stable cell lines (X-1 and X-2), and the empty vector transfected cells (V) was analyzed by Northern blotting using HDM2, p53, or IFIXα1 cDNA as a probe. The 18S and 28S rRNAs are shown as loading controls. (D) IFIXα1 destabilizes HDM2. H1299 cells were cotransfected with HDM2 (0.7 μg) and the empty vector (V) (1.3 μg) or FLAG-IFIXα1 (IFIXα1) (1.3 μg). Twenty-four hours posttransfection, cells were treated with CHX (100 μg/ml). Cell lysates were isolated at 0, 15, and 30 min after CHX treatment for Western blotting using antibodies against HDM2, IFIXα, and α-tubulin. A representative experiment is shown. (E) The amount of HDM2 protein at zero time point was arbitrarily set at 100%. The percentage of HDM2 protein remaining was determined using Bio-Rad software. The results obtained from three independent experiments are shown.

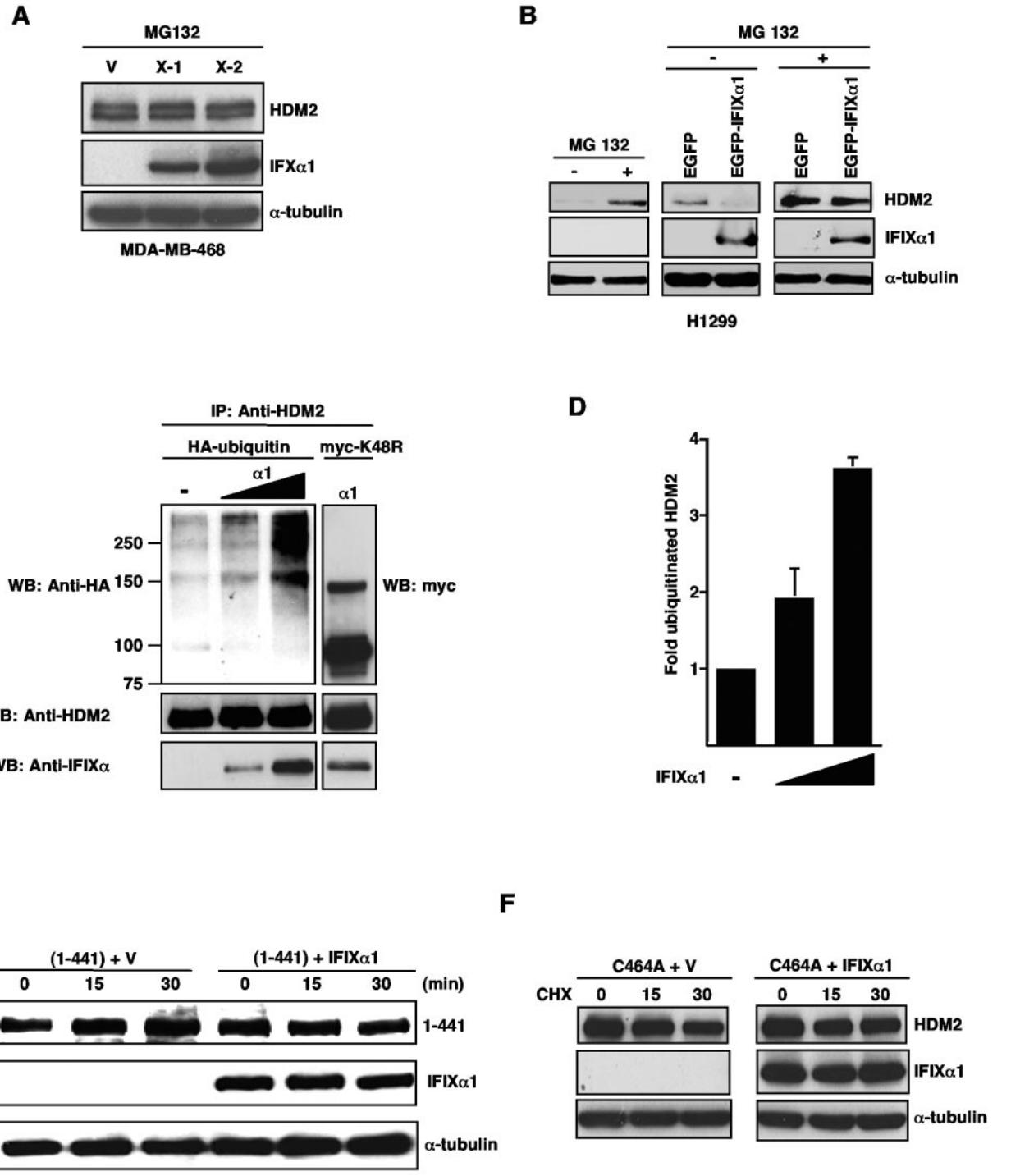

FIG. 2. IFIXα1 promotes the ubiquitination of HDM2. (A) IFIXα1 downregulates HDM2 through proteasome-mediated degradation. The IFIX stable MDA-MB-468 cell lines (X-1 and X-2) and the vector control cells (V) were treated with a proteasome inhibitor, MG132 (10 μM), for 6 h prior to harvest. Cell lysates were analyzed by Western blotting using anti-HDM2, anti-IFIXα, and anti-α-tubulin. (B) H1299 cells were transfected with EGFP-tagged IFIXα1 (EGFP-IFIXα1) or EGFP vector. The GFP-positive cells with (+) or without (−) MG132 treatment were collected by FACS, followed by Western blotting using antibodies against HDM2, IFIXα, and α-tubulin. (C) IFIXα1 induces the polyubiquitination of HDM2. H1299 cells were cotransfected with HDM2 (1.5 μg), hemagglutinin (HA)-ubiquitin (1 μg) (left panel) or myc-tagged ubiquitin (K48R) (1 μg) (right panel), and IFIXα1 (α1) (0 [−], 1.5, and 3.5 μg, left panel; 3.5 μg, right panel). Cells were treated with MG132 (10 μM) 6 h prior to harvest at 48 h posttransfection. Cell lysates were immunoprecipitated using anti-HDM2 antibody and analyzed by Western blotting using anti-HA, anti-HDM2, anti-myc, or anti-IFIXα antibody. (D) The increase of the ubiquitinated HDM2 levels (*n*-fold) was calculated based on two independent experiments using Bio-Rad software. (E and F) The E3 ligase activity is required for the IFIXα1-mediated HDM2 destabilization. The HDM2(1–441) (E) or HDM2(C464A) (F) (0.7 μg) mutant was cotransfected into H1299 cells with the empty vector (V) (1.3 μg) or FLAG-IFIXα1 (IFIXα1) (1.3 μg). CHX-chase assay and Western blotting were carried out as described in the legend to Fig. 1D.

The IFIX effect on HDM2 and p53 autoregulatory loop. HDM2 and p53 form an autoregulatory loop in which HDM2 destabilizes p53 and p53 activates HDM2 transcription. IFIXα1 drastically downregulates HDM2 in p53-deficient cells, such as MDA-MB-468, H1299, and 293 (Fig. 1A and 2B; data not shown). However, little change in the HDM2 protein levels was observed in IFIXα1 stable MCF-7 cells (expressing wild-type p53) (Fig. 3A, top panel). The induction of p53 (Fig. 3A) leads to the increase of HDM2 mRNA levels in these cells (Fig. 3C, top panel). These results support the notion that IFIXα1 cross talks with the HDM2-p53 autoregulatory loop by promoting HDM2 degradation, leading to p53 stabilization. The elevated p53 levels, in turn, increase the HDM2 levels. The net result of these two opposing effects may be responsible for the apparent little change in the HDM2 levels in cells expressing IFIXα1. To further confirm this result, we employed HCT116 (a human colorectal carcinoma cell line) and its p53-null derivative, HCT116($p53^{-/-}$), in which both p53 alleles were deleted by homologous recombination (8). Both cell lines were transfected with either EGFP-IFIXα1 or EGFP control vector. Cell lysates isolated from the GFP-positive cells were analyzed by Western blotting. As shown in Fig. 3B (left panel), similar to that observed in the IFIXα1 stable MCF-7 cells (Fig. 3A), IFIXα1 expression increases p53 levels but has little effect on HDM2 levels in HCT116 cells. In contrast, IFIXα1 expression resulted in a drastic reduction of HDM2 in HCT116($p53^{-/-}$) cells (Fig. 3B, right panel) as observed in other p53-deficient cells (Fig. 1A and 2B).

IFIXα1 increases p53 protein stability. IFIXα1 destabilizes HDM2, resulting in p53 induction in the IFIXα1 stable MCF-7 cell lines, X-1 and X-2, compared with that in the vector control cells (Fig. 3A). Importantly, there is no change in the p53 mRNA levels regardless of IFIXα1 expression (Fig. 3C), supporting the idea that IFIXα1 downregulates HDM2, leading to p53 stabilization. Indeed, we show that the p53 protein turnover rate is significantly increased in X-1 and X-2 cells (>30 min) compared with that in the control cells (<15 min) (Fig. 3D). This result is consistent with the idea that IFIXα1 stabilizes p53 protein by destabilizing HDM2.

The causative effect of IFIXα1 on p53 induction was further confirmed by an IFIXα1 knockdown experiment using IFIX siRNA. As shown in Fig. 3E, the IFIX siRNA transfection specifically reduces p53 protein levels in a low-IFIXα1-expressing MCF-7 cell line, X-1, compared with transfection with the nonspecific scramble control (NS) siRNA. As expected, p53 reduction by IFIX siRNA correlates with decreased expression of a p53 transcriptional target, $p21^{CIP1}$ (Fig. 3F). These results indicate that IFIXα1 can stabilize and activate p53 in the IFIXα1 MCF-7 stable cell lines.

IFIXα1 activates the p53-mediated transcription. IFIXα1 induces p53 and increases the steady-state mRNA levels of p53 target genes, e.g., the HDM2 gene (Fig. 3C), and $p21^{CIP1}$ (18) expression, suggesting that IFIXα1 induces p53-mediated transcriptional activity. To test that possibility, we transfected H1299 with PG13-LUC, a luciferase reporter construct containing multiple p53 binding sites (79), or MG15-LUC, a corresponding construct containing mutated p53 binding sites. Since H1299 is p53 null (86), it is necessary to cotransfect a p53 expression vector to observe the increase of p53-mediated transcription. We found that IFIXα1 readily enhanced the p53-mediated transcriptional activity of PG13-LUC (but not MG15-LUC) in a dose-dependent manner (Fig. 4A). This result indicates that IFIXα1 indeed activates the p53-mediated transcription. We then tested whether IFIXα1 also increases p53 DNA binding. We employed a mobility shift assay in which the ^{32}P-labeled oligonucleotides containing p53 DNA binding sites were incubated with the nuclear extracts isolated from the IFIXα1 stable MCF-7 cell lines (X-1 and X-2) and the vector control cells. The p53/DNA complex was resolved by a native gel electrophoresis. As shown in Fig. 4B (left panel), the p53 DNA binding activity is strongly enhanced in X-1 and X-2 cells compared with that in the control cells. The p53 DNA binding activity induced by DNA-damaging agents, such as UV light, serves as a positive control. The protein/DNA complex is p53 specific since incubation with anti-p53 antibody in the X-1 nuclear extract diminishes this complex. The increase of p53 DNA binding correlates well with an increase of p53 in the nuclear extracts isolated from the X-1 and X-2 cells (Fig. 4B, right panel).

In keeping with the activation of p53 by IFIXα1 (Fig. 4A), we found that IFIXα1 also activates a luciferase reporter gene driven by a $p21^{CIP1}$ promoter (21) in the wild-type p53-expressing cell lines, e.g., MCF-7 and HCT116 (data not shown). The induction of $p21^{CIP1}$ promoter activity was similar to that observed with either IFN-γ (29) or another HIN-200 protein, IFI16 (85). This result indicates that IFIXα1 can transcriptionally activate the $p21^{CIP1}$ gene. Consistently, IFIXα1 enhanced the binding of p53 to the endogenous $p21^{CIP1}$ promoter in the IFIXα1 stable MCF-7 cell lines (X-1 and X-2) compared with the basal levels of p53 binding in the vector control cells, using a ChIP assay (Fig. 4C). These results suggest that IFIXα1 activates $p21^{CIP1}$ transcription by upregulating p53 protein levels and transcriptional activity.

HDM2 downregulation is required for the IFIXα1-mediated p53 induction and activation. Next, we wanted to determine whether the activation of p53 by IFIXα1 is through repression of MDM2. To do so, we cotransfected a $p53^{-/-}$ MEF and a $p53^{-/-}$ $mdm2^{-/-}$ DKO MEF with GFP-tagged p53 and increasing amounts of IFIXα1. The exogenous p53 is induced by IFIXα1 in the $p53^{-/-}$ MEF in a dose-dependent manner, presumably caused by increasing p53 stability in these cells (Fig. 5A, left panel). As expected, p53 is more stable in the DKO MEF (Fig. 5A, right panel). However, IFIXα1 has no effect on the p53 levels in these cells (Fig. 5A, right panel). Consistent with these observations, IFIXα1 activates p53-mediated transcription in a dose-dependent manner in the $p53^{-/-}$ MEF but not in the DKO MEF (Fig. 5B). Notably, p53 drastically enhances p53-mediated transcription in the DKO MEF in the absence of IFIXα1. It is likely due to the higher p53 levels in the DKO MEF than those in the $p53^{-/-}$ MEF (Fig. 5A). Together, these results support the idea that IFIXα1 induces p53 levels and its transcriptional activity by targeting primarily HDM2.

IFIXα1 promotes p53 nuclear accumulation. Since nuclear p53 is active in transcriptional activation, it is conceivable that increased p53-mediated transcription should correlate with increased p53 nuclear localization. Indeed, p53 is predominantly nuclear and colocalized with IFIXα1 in the IFIXα1 stable MCF-7 cell line, X-2 (data not shown) (18). To further confirm this observation, we transiently transfected a vector expressing

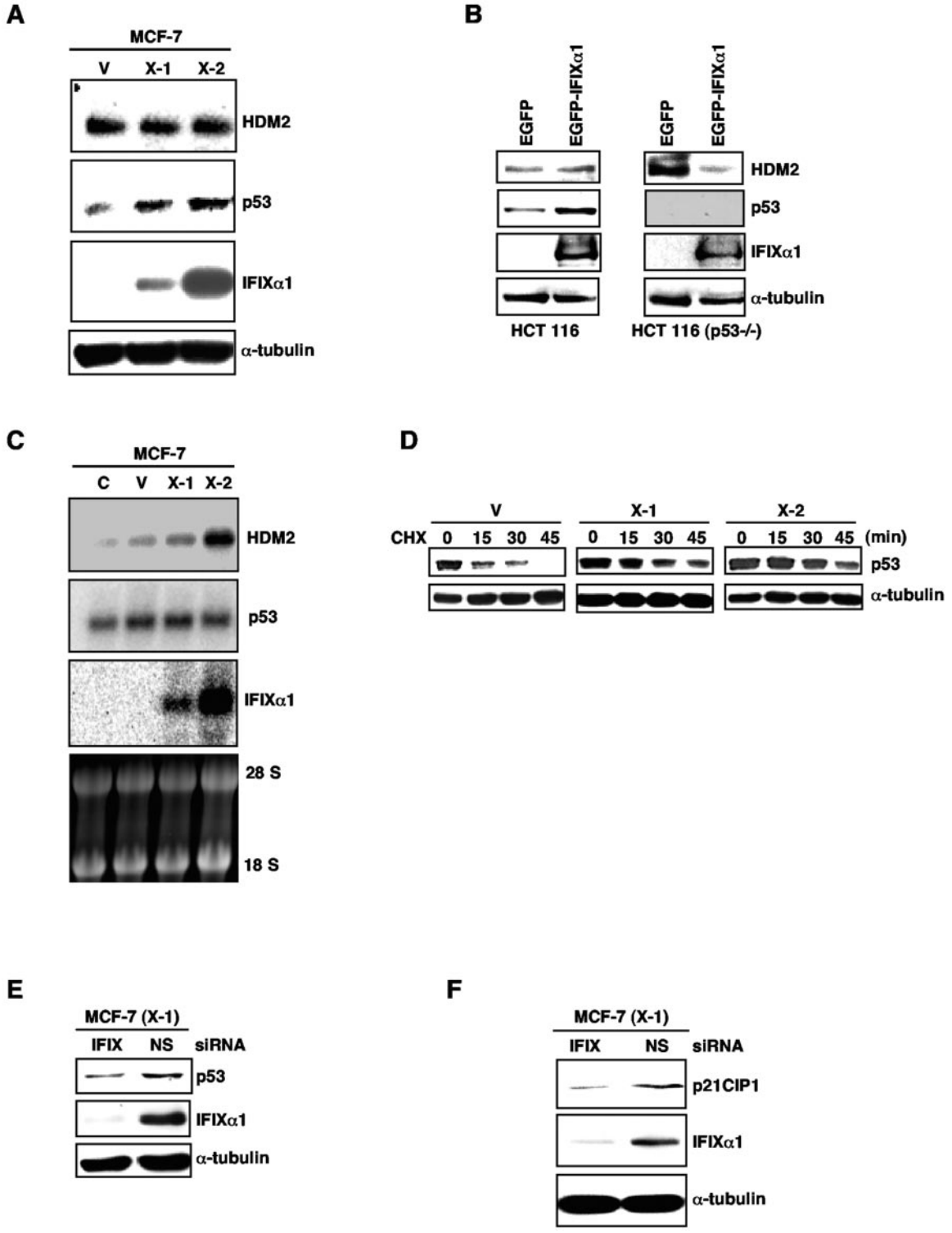

FIG. 3. IFIXα1 stabilizes p53 protein. (A) IFIXα1 exerts different effects on the p53 and HDM2 levels in p53-expressing cells. Total cell lysates isolated from the IFIXα1 stable MCF-7 cell lines (X-1 and X-2) and the vector control (V) cell lines were analyzed by Western blotting using antibodies against HDM2, p53, IFIXα, and α-tubulin. (B) The p53 status influences the IFIXα1 effect on HDM2 levels. HCT116 and HCT116($p53^{-/-}$) cells were transfected with EGFP vector or EGFP-IFIXα1. Forty-eight hours after transfection, the GFP-positive cells were collected using FACS. Cell lysates were analyzed by Western blotting using antibodies against HDM2, p53, IFIXα, and α-tubulin. (C) IFIXα1 induces the steady-state HDM2 mRNA level but has little effect on p53 mRNA levels in MCF-7 cells. Total RNA (10 μg) isolated from the parental MCF-7 (C) and the stable cell lines transfected with the empty vector (V) or IFIXα1 expression vector (X-1 and X-2) was analyzed by Northern blotting using HDM2, p53, or IFIXα1 cDNA as a probe. The 18S and 28S rRNAs are shown as loading controls. (D) IFIXα1 increases p53 protein stability. The IFIXα1 stable MCF-7 (X-1 and X-2) and the vector control (V) cells were treated with CHX (100 μg /ml) for the time indicated. Cell lysates were analyzed for the expression of p53 and α-tubulin. (E and F) Depletion of IFIXα1 reduces p53 and $p21^{CIP1}$ expression levels. The IFIXα1 stable MCF-7 cell line, X-1, was transfected with siRNA specific to IFIXα (IFIX) (100 nM) or NS siRNA (100 nM). Forty-eight hours after transfection, the expression levels of p53, IFIXα1, $p21^{CIP1}$, and α-tubulin were analyzed by Western blotting.

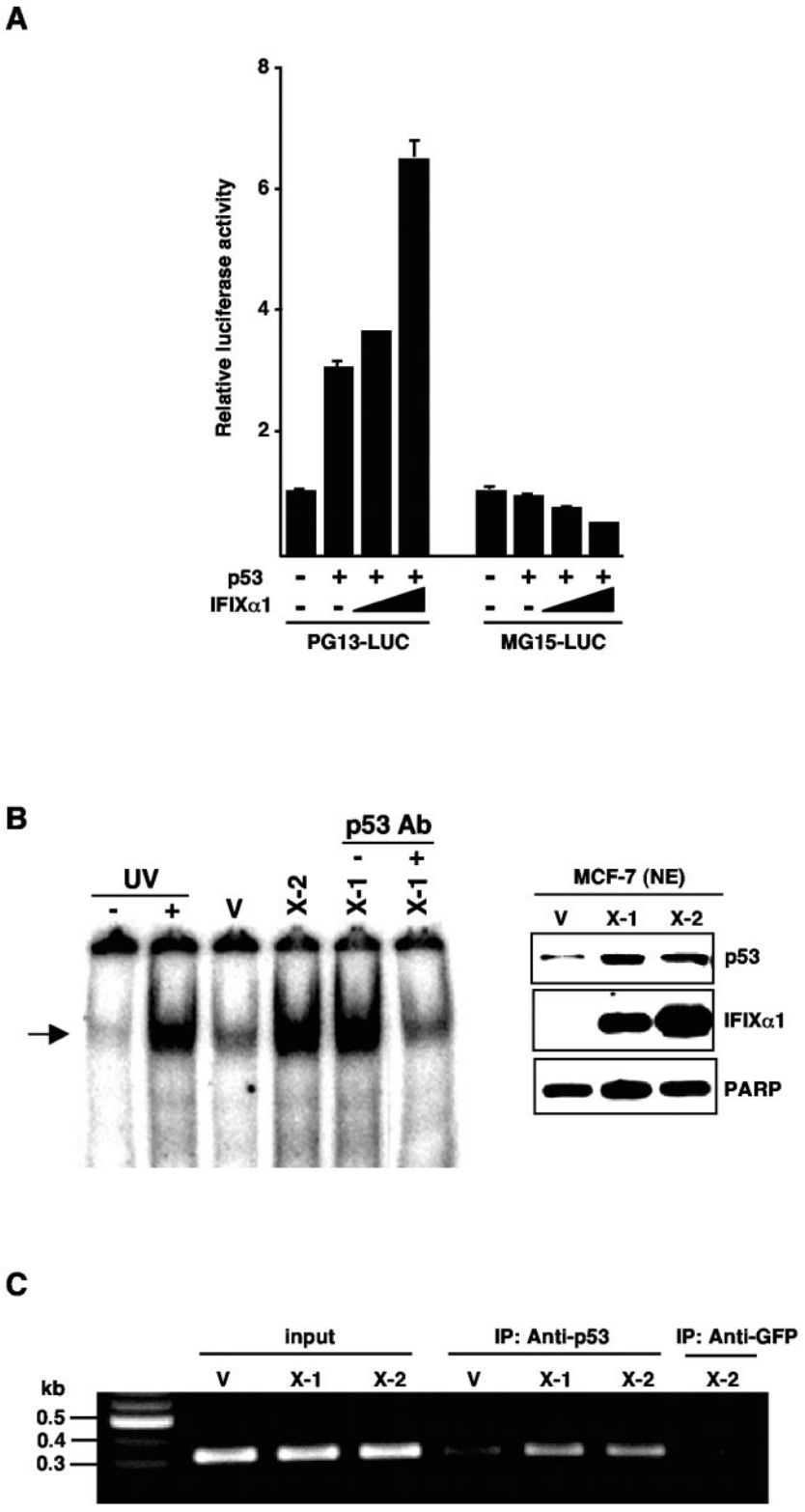

FIG. 4. IFIXα1 activates p53-mediated transcription. (A) IFIXα1 augments the p53-mediated transcriptional activity. H1299 cells were transfected with 0.3 μg of PG13-LUC or MG15-LUC with or without p53 (0.01 μg) and IFIXα1 (0.845 and 1.69 μg). pRL-TK (0.05 μg) was cotransfected to normalize transfection efficiency. Cells were harvested 24 h after transfection, and the luciferase activity was measured using a dual luciferase assay (Promega). The relative luciferase activity was obtained by setting the normalized activity of PG13-LUC or MG15-LUC at 1. (B) IFIXα1 enhances p53 DNA binding activity. Nuclear extract (7.5 μg) was incubated with ^{32}P-labeled oligonucleotide containing p53 binding sites prior to electrophoretic mobility shift assay according to the manufacturer's instructions (p53 Nushift kit; Geneka) (left panel). MCF-7 cells treated with (+) or without (−) UV light (20 J/m^2) serve as a positive control. Nuclear extract isolated from X-1 cells was incubated with (+) or

GFP-tagged p53 fusion protein (GFP-p53) (76) into the IFIXα1 stable cell lines or the vector control cells. Twenty-four hours after transfection, we determined the percentage of GFP-positive cells with p53 localized in both cytoplasmic and nuclear compartments or primarily the nuclear compartment. As shown in Fig. 5C, the majority of GFP-p53 is localized in the nuclear compartment of the IFIXα1 stable cell lines, X-1 and X-2, compared with the vector control cells. These data indicate that IFIXα1 promotes p53 nuclear localization. Since HDM2 is the target of IFIXα1 to induce p53 (Fig. 5A), it is possible that HDM2 may also be the target for the IFIXα1-mediated p53 nuclear accumulation. To test this possibility, we cotransfected GFP-p53, HDM2 (or the C464A mutant), and IFIXα1 (or empty vector control) into H1299 (which is p53 null and expresses very low levels of HDM2). Consistent with the results shown in Fig. 5C, IFIXα1 increases the nuclear localization of p53 in cells transfected with HDM2 compared with empty vector transfection (Fig. 5D). In contrast, the C464A mutant is sufficient to increase p53 nuclear localization because the C464A mutant is defective in p53 nuclear export (7, 28). Importantly, IFIXα1 has no effect on p53 nuclear localization in cells transfected with the C464A mutant (Fig. 5D). Since IFIXα1 cannot destabilize the C464A mutant (Fig. 2F), this result suggests that HDM2 downregulation is required for IFIXα1 to induce p53 nuclear localization.

IFIXα1 interacts with HDM2. The interaction between IFIXα1 and HDM2 was detected by a co-IP experiment (Fig. 2C). To confirm this interaction, 293T cells were transfected with HDM2 and EGFP vector, EGFP-IFIXα1, or EGFP-IFIXβ1. The protein-protein interaction was examined by IP using anti-HDM2 antibody and followed by Western blotting with either anti-GFP or anti-HDM2 antibody. Clear interactions between HDM2 and IFIXα1 and between HDM2 and IFIXβ1 were detected (Fig. 6A). A reciprocal experiment using anti-GFP antibody to pull down EGFP-IFIXα1 and EGFP-IFIXβ1 further confirmed the presence of these complexes (Fig. 6B). These data strongly suggest that HDM2 interacts with IFIXα1 or IFIXβ1. We have attempted to investigate a possible interaction between HDM2 and the smallest IFIX isoform, IFIXγ1 (18). However, we found that IFIXγ1 could be extracted using only SDS-containing lysis buffer (data not shown). Therefore, it is not feasible to detect such an interaction using the standard IP protocol. The interaction between IFIXα1 and HDM2 was also observed in cells cotransfected with FLAG-tagged IFIXα1 and HDM2 into 293T cells (data not shown). Consistently, we also found the interaction between IFIXα1 and HDM2 in the IFIXα1 stable MCF-7 cell lines, X-1 and X-2, but not in the vector control cells (Fig. 6C). Together, these results strongly indicate that IFIXα1 interacts with HDM2.

The apparent lack of HDM2 reduction by IFIXα1 or IFIXβ1 transfection in 293T cells compared with the empty vector transfection (Fig. 6A and B) is likely due to the constitutive expression of the HDM2 gene driven by a cytomegalovirus promoter. In essence, it resembles the comparable levels of the endogenous HDM2 in the p53-expressing MCF-7 cell lines with or without IFIXα1 (Fig. 3A and 6C [right panel]).

IFIXα1 binds to amino acid region 150 to 230 of HDM2. To map the HDM2 region binding to IFIXα1, we cotransfected 293T cells with EGFP-IFIXα1 and HDM2 mutants, e.g., RING finger deletion (1–441) and deletion of amino acids 150 to 230 deletion (Δ150–230) (Fig. 6G) (41), followed by IP/Western blotting. Like the wild-type HDM2 (Fig. 6A-B), the HDM2(1–441) mutant can readily interact with IFIXα1 (Fig. 6D). Since the anti-HDM2 antibody used in IP recognizes that the epitope resides within amino acid region 150 to 230 of HDM2, it is therefore not suitable for immunoprecipitating HDM2(Δ150–230). Instead, we cotransfected 293T cells with either EGFP-IFIXα1 or FLAG-IFIXα1 and HDM2 or HDM2(Δ150–230), followed by IP with anti-GFP antibody (Fig. 6E) or anti-FLAG antibody (Fig. 6F), respectively. The HDM2 and IFIXα1 interaction serves as a positive control. While the expression levels of HDM2(Δ150–230) and IFIXα1 are detectable by direct Western blotting, there is no HDM2(Δ150–230) protein present in the IFIXα1 immunocomplex. This result suggests that amino acid region 150 to 230 of HDM2 is required for IFIXα1 binding (Fig. 6G).

The HIN region of IFIXα1 interacts with HDM2. IFIXα1 differs from IFIXβ1 at the C-terminal sequence (18). The observation that both isoforms interact with HDM2 (Fig. 6A and B) suggests that the C-terminal sequence of IFIXα1 is dispensable for HDM2 binding. To map the HDM2 binding domain of IFIXα1, we generated deletion mutants that express either the N-terminal PYD domain (IFIX-N) or the HIN domain (IFIX-HIN). Although IFIX-N is clearly localized in the nucleus, IFIX-HIN, which lacks the putative nuclear localization signal (NLS) in the N-terminal domain (18), localizes in both nuclear and cytoplasmic compartments. Howbeit, nuclear localization appears dominant (data not shown). To determine their ability to bind HDM2, we cotransfected 293T cells with HDM2 and EGFP, EGFP-tagged IFIXα1, IFIX-N, or IFIX-HIN, followed by IP using anti-GFP (Fig. 6H, left panel) or anti-HDM2 (Fig. 6H, right panel) antibody and Western blotting with either anti-HDM2 or anti-GFP antibody. These results show that IFIX-HIN, but not IFIX-N, is sufficient to bind HDM2 (Fig. 6I).

The HIN domain is sufficient to downregulate HDM2 and induce p53. The observation that IFIX-HIN is sufficient to bind HDM2 (Fig. 6H) prompted us to test whether IFIX-HIN regulates HDM2 expression. We transfected 293 cells with EGFP-tagged IFIX-HIN or EGFP empty vector, followed by fluorescence-activated cell sorter (FACS) analysis to enrich the GFP-positive cells. The HDM2 protein levels of the GFP-positive cells were analyzed by Western blotting. As shown in Fig. 7A, IFIX-HIN transfection is sufficient to reduce the endogenous HDM2 levels compared to transfection with EGFP

without (−) the anti-p53 antibody (Ab) in the binding reactions to indicate the specific p53/DNA complex (arrow). The nuclear extracts used in the mobility shift assay were analyzed for the expression levels of p53 and IFIXα1 by Western blotting (right panel). (C) Increased p53 binding to the $p21^{CIP1}$ promoter in IFIXα1 stable cells. A ChIP assay was performed in the vector control (V) and the IFIXα1 stable MCF-7 (X-1 and X-2) cells. The primer pair that specifically amplifies a 320-bp region of $p21^{CIP1}$ promoter was used to analyze the DNA immunoprecipitated by either anti-p53 antibody or a control anti-GFP antibody. The input DNA used for the ChIP assay was likewise amplified to indicate equal loading.

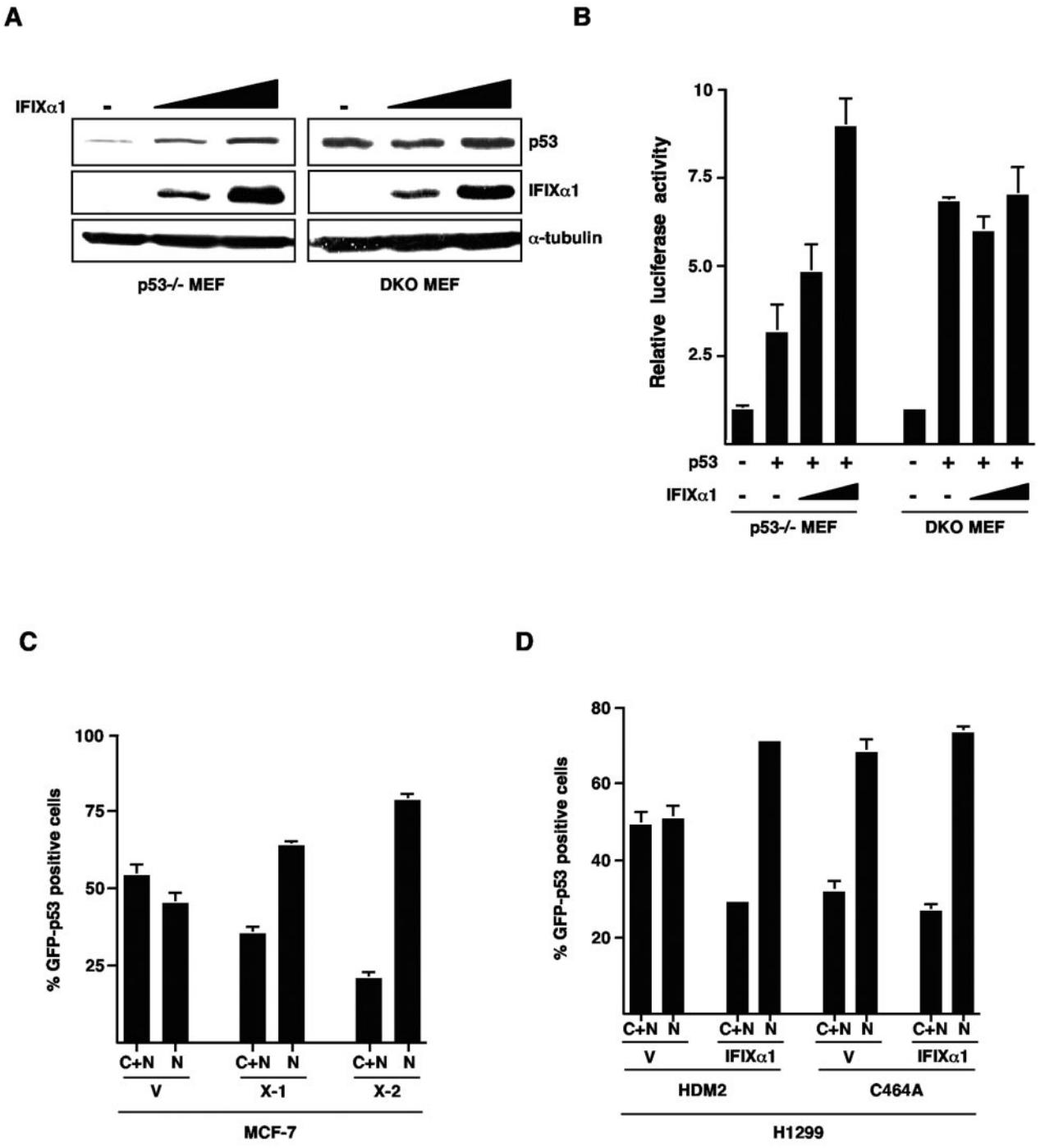

FIG. 5. mdm2 is required for p53 induction and nuclear accumulation by IFIXα1. (A) mdm2 is required for the increased p53 protein expression by IFIXα1. The $p53^{-/-}$ MEF or DKO MEF was transfected with GFP-p53 (0.1 μg) and increasing amounts of IFIXα1 (0, 1, and 2 μg). Twenty-four hours posttransfection, cell lysates were analyzed by Western blotting using antibodies against p53, IFIXα1, and α-tubulin. (B) mdm2 is required for the p53-mediated transcriptional activation by IFIXα1. PG13-LUC (0.3 μg) was cotransfected with p53 (0.01 μg) with or without increasing amounts of IFIXα1 (0.845 μg and 1.69 μg) into the $p53^{-/-}$ MEF or DKO MEF. pRL-TK (0.05 μg) was cotransfected to normalize transfection efficiency. Cells were harvested 24 h after transfection, and the luciferase activity was measured using a dual luciferase assay. The relative luciferase activity was obtained by setting the normalized activity of PG13-LUC alone at 1. (C) IFIXα1 promotes p53 nuclear localization. GFP-p53 was transfected into the IFIXα1 stable MCF-7 (X-1 and X-2) and the vector control (V) cells. Twenty-four hours after transfection, the number of cells in which GFP-p53 localized in both nucleus and cytoplasm (C+N) or predominantly in the nucleus (N) was counted. Average results were obtained from two independent experiments. V (C+N, 54.55% ± 3.45%; N, 45.45% ± 3.45%); X-1 (C+N, 36.1% ± 1.9%; N, 63.9% ± 1.9%); and X-2 (C+N, 21.15% ± 1.85%; N, 78.85% ± 1.85%). (D) IFIXα1 has little effect on p53 nuclear localization in the presence of the HDM2(C464A) mutant. GFP-p53 (0.25 μg) was cotransfected into the H1299 cells with HDM2 (0.75 μg) and IFIXα1 or empty vector (1.25 μg). Twenty-four hours after transfection, the number of cells in which GFP-p53 localized in both nucleus and cytoplasm or predominantly in the nucleus was counted. Average results were obtained from two independent experiments: V and HDM2 (C+N, 49% ± 3.4%; N, 51% ± 3.4%); IFIXα1 and HDM2 (C+N, 29% ± 0.4%; N, 71% ± 0.4%); V and C464A (C+N, 31.7% ± 3.1%; N, 68.3% ± 3.1%); and IFIXα1 and C464A (C+N, 27% ± 1.6%; N, 73% ± 1.6%). On average, more than 80 GFP-positive cells were counted in each transfection experiment.

vector. As expected, MG132 treatment stabilizes HDM2 expression. However, unlike those of IFIXα1 (Fig. 2B) and IFIX-N (data not shown), which are stable proteins, IFIX-HIN expression levels increase with MG132 treatment (Fig. 7A). This result suggests that IFIX-HIN is a relatively unstable protein and is susceptible to degradation by proteasome machinery.

The observation that IFIX-HIN is sufficient to bind and to downregulate HDM2 suggests that it may be sufficient to induce p53. To test this possibility, we cotransfected H1299 cells with p53 and increasing amounts of IFIXα1, IFIX-HIN, or IFIX-N. As expected, we observed a dose-dependent increase of p53 in cells transfected with IFIXα1 (Fig. 7B). The endogenous $p21^{CIP1}$ is also increased in a dose-dependent manner, indicating that the p53-mediated transcription is activated (Fig. 7B). Interestingly, like IFIXα1, IFIX-HIN is able to induce both p53 and $p21^{CIP1}$ (Fig. 7C). In contrast, the HDM2 binding-deficient mutant, IFIX-N (Fig. 6H), has no effect on p53 or $p21^{CIP1}$ (Fig. 7D). This result is further confirmed by a Northern blot analysis in which the p53-induced $p21^{CIP1}$ mRNA levels are increased by IFIXα1 and IFIX-HIN but not IFIX-N (Fig. 7E). Together, these data suggest that the HIN domain of IFIXα1 is sufficient to downregulate HDM2, leading to p53 induction, which in turn activates the p53-mediated transcription, e.g., $p21^{CIP1}$. Our previous observation that IFIXγ1, which lacks the HIN domain, was unable to induce $p21^{CIP1}$ (18) supports the requirement of the HIN domain for p53 induction.

IFIX mediates the HDM2 downregulation by IFN-α. IFIXα1 is an IFN-inducible protein (18). We tested whether the regulation of HDM2 by IFIXα1 can be observed in the IFN-inducible system. We then examined the effect of IFN on HDM2 expression in Raji cells (a human Burkitt's lymphoma cell line in which p53 is mutated) (20) because IFIXα1 expression can be readily induced by IFN-α in these cells (18). Interestingly, we observed a biphasic effect on the HDM2 protein levels in response to the IFN-α treatment (Fig. 8A). In particular, the HDM2 levels gradually increase between 0 and 48 h and decrease sharply at 72 h of treatment. Notably, the endogenous IFIXα (which may include α1 and α2 isoforms) protein expression becomes detectable at 48 h and persists through 72 h of treatment. As a positive control for the IFN-α responsiveness, the same membrane was probed with an antibody against a known IFN-inducible protein, PKR (39). As expected, the PKR levels increase in response to IFN-α treatment, indicating that the IFN pathway is activated (Fig. 8A). This result shows that IFN indeed regulates HDM2 expression. In particular, IFN downregulates HDM2 at 72 h of treatment.

To examine the role of IFIX in the IFN-α-mediated HDM2 downregulation, we looked for a condition in which IFIX, but not other HIN-200 proteins, e.g., IFI16, MNDA, and AIM2, could be readily induced by IFN-α. Under the normal condition with 10% FCS, we found that the expression levels of both IFIXα and IFI16 are induced by IFN-α (Fig. 8B). The expression of MNDA was not detectable regardless of IFN-α treatment (data not shown). The expression of AIM2 is not clear under this condition because the anti-AIM2 antibody is not available. Interestingly, we found that IFIXα but not IFI16 could be induced by IFN-α in low serum with 0.2% FCS (Fig. 8C, left panel). Importantly, the IFN-α-induced IFIXα expression remains correlated with HDM2 downregulation under this condition. Thus, the low serum condition provides us a unique opportunity to determine the role of IFIXα in IFN-α-induced HDM2 downregulation. To ensure the specificity of IFIX siRNA, a mixture of four IFIX siRNAs (see Materials and Methods) was transfected into Raji cells, followed by IFN-α treatment. We show that IFIX siRNAs transfection reduces IFIXα but that it has little effect on IFI16 expression (Fig. 8C, right panel). Importantly, IFIXα knockdown by IFIX siRNAs increases the expression level of HDM2 compared to transfection with NS siRNA (Fig. 8C, right panel). This result indicates that IFIXα plays an essential role in the IFN-α-mediated HDM2 downregulation.

To test whether the interaction between IFIXα and HDM2 also occurs in the IFN-inducible system, we performed IP/Western blot analysis on Raji cells treated with IFN-α for 48 h (at this time point, HDM2 levels are not reduced) (Fig. 8A). As shown in Fig. 8D, a physiological interaction between IFIXα and HDM2 was detected in these cells. The induction of IFIXα by IFN-α correlates well with an increase of interaction between IFIXα and HDM2 (Fig. 8D).

Discussion

In this report, we present evidence suggesting that IFIXα1 functions as a negative regulator of HDM2. Consequently, IFIXα1 positively regulates p53 by stabilizing p53, leading to an increase of p53-dependent transcription and nuclear accumulation. Importantly, these IFIXα1 effects on p53 require HDM2 downregulation, suggesting that HDM2 is the primary target of IFIXα1.

IFIXα1 interacts with HDM2 and promotes its ubiquitination and degradation (Fig. 1, 2, and 6). The mechanism by which IFIXα1 promotes HDM2 ubiquitination is not clear. HIN-200 proteins are not known to possess enzymatic activity. It has been postulated that these proteins may function as nuclear scaffolds to modulate gene transcription through interaction with other proteins (12). Therefore, it is possible that IFIXα1, when binding to HDM2, may simultaneously compromise the binding of certain HDM2 interacting proteins that negatively regulate the ubiquitination of HDM2, e.g., p14ARF (70, 82), MDMX (32, 74), TSG101 (50), and HAUSP (51). Thus, it is conceivable that IFIXα1 may disrupt these interactions and restore the E3 ligase activity of HDM2, resulting in an increase of ubiquitination. In addition, posttranslational modifications, such as sumoylation, acetylation, and phosphorylation, are also known to regulate the E3 ligase activity of HDM2 (9, 58). It is possible that IFIXα1 may alter certain modifications of HDM2 and tip the balance to favoring ubiquitination. HDM2 is known to be a nucleocytoplasmic shuttling protein (68). Moreover, since IFIXα1 binds to HDM2 through amino acid region 150 to 230, which contains nuclear export signals/NLSs (Fig. 6G), it is also possible that IFIXα1 may regulate HDM2 ubiquitination and degradation by altering the cellular localization of HDM2.

Emerging evidence has suggested a cross talk between the IFN signaling pathway and the p53 tumor suppressor pathway. For example, it was shown that IFN-α/β transcriptionally activates p53 (77). In contrast, certain IFN-inducible proteins were shown to regulate p53 posttranscriptionally. For instance,

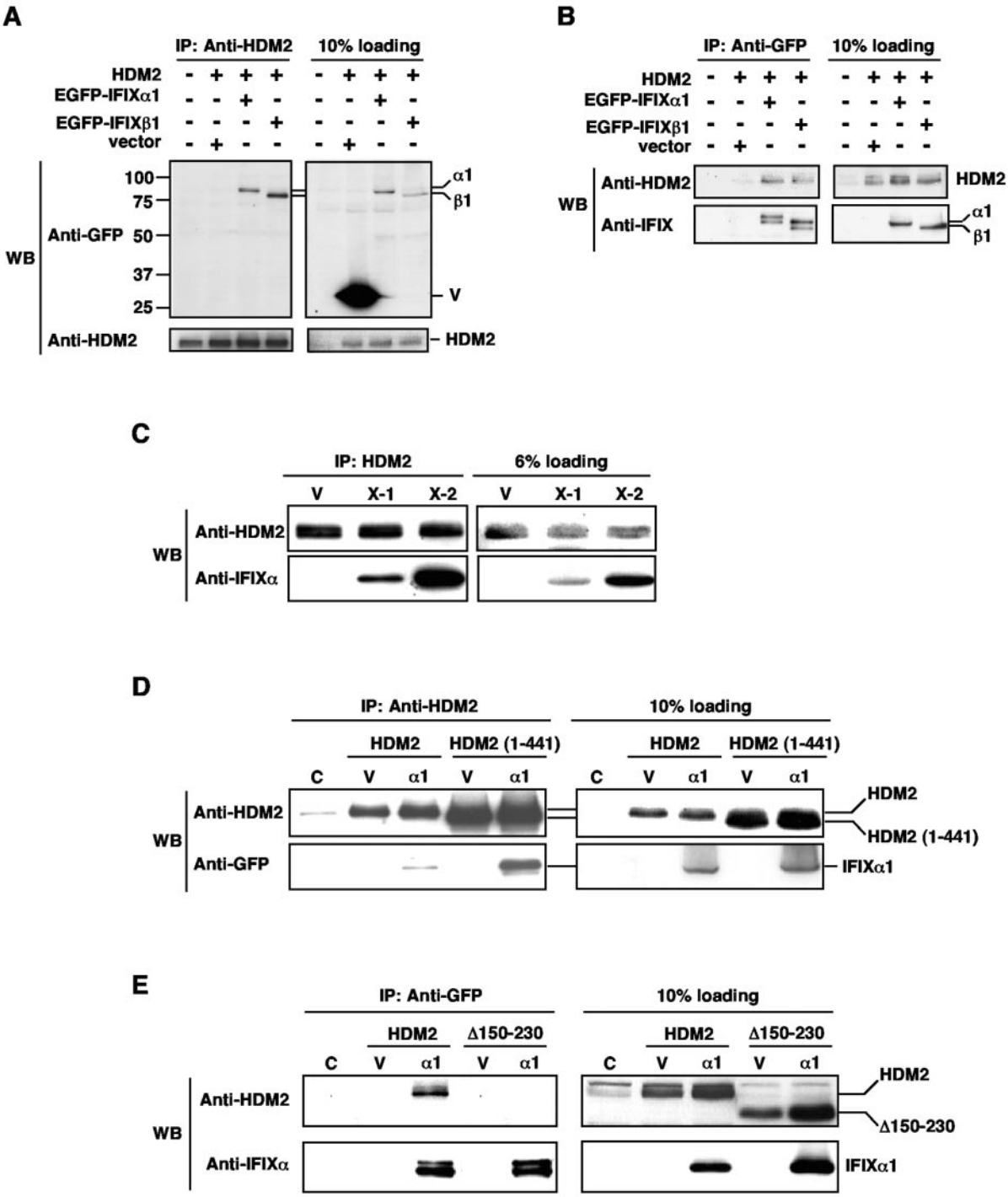

FIG. 6. IFIXα1 interacts with HDM2. (A) HDM2 interacts with IFIXα1 and IFIXβ1. 293T cells were cotransfected with HDM2 (2.5 μg) and EGFP vector (vector) (2.5 μg), EGFP-tagged IFIXα1 (α1) (2.5 μg), or IFIXβ1 (β1) (2.5 μg). Forty-eight hours posttransfection, cell lysates (500 μg) were immunoprecipitated with an anti-HDM2 antibody, and Western blotting was performed using an anti-GFP or anti-HDM2 antibody. (B) A reciprocal experiment that used anti-GFP antibody for IP and Western blotting with anti-IFIX or anti-HDM2 antibodies. (C) IFIXα1 interacts with HDM2 in the IFIXα1 stable cell lines. Cell lysates (600 μg) isolated from the IFIXα1 stable MCF-7 cell lines (X-1 and X-2) or the empty vector cells (V) were immunoprecipitated using anti-HDM2 antibody and analyzed by Western blotting with anti-IFIXα or anti-HDM2 antibody. (D) The HDM2(1–441) mutant interacts with IFIXα1. 293T cells were transfected with HDM2 or the HDM2(1–441) mutant and EGFP-IFIXα1 (α1) or EGFP empty vector (V), followed by IP with anti-HDM2 antibody and Western blotting with either anti-HDM2 or anti-GFP antibody. (E and F) Amino acid region 150 to 230 of HDM2 interacts with IFIXα1. 293T cells were cotransfected with HDM2 or the HDM2(Δ150–230) mutant and EGFP-IFIXα1 (α1) or EGFP empty vector (E) or FLAG-IFIXα1 (α1) or FLAG empty vector (F), followed by IP/Western blotting with the indicated antibodies. An arrowhead indicates the HDM2 band (F). The untransfected 293T cells served as controls (C). (G) A summary of IFIXα1 binding by HDM2 and the HDM2(Δ150–230) and HDM2(1–441) mutants. (H and I) The HIN domain of IFIXα1 interacts with HDM2. (H) 293T cells transfected with HDM2 and EGFP empty vector, EGFP-IFIXα1, EGFP-IFIX-N (N), or EGFP-IFIX-HIN (HIN), followed by IP with anti-GFP antibody (left panel) or anti-HDM2 antibody (right panel) and Western blotting with anti-HDM2 or anti-GFP antibody. Untransfected 293T cells served as controls. (I) A summary of HDM2 binding by IFIXα1, IFIXβ1, IFIX-N, and IFIX-HIN.

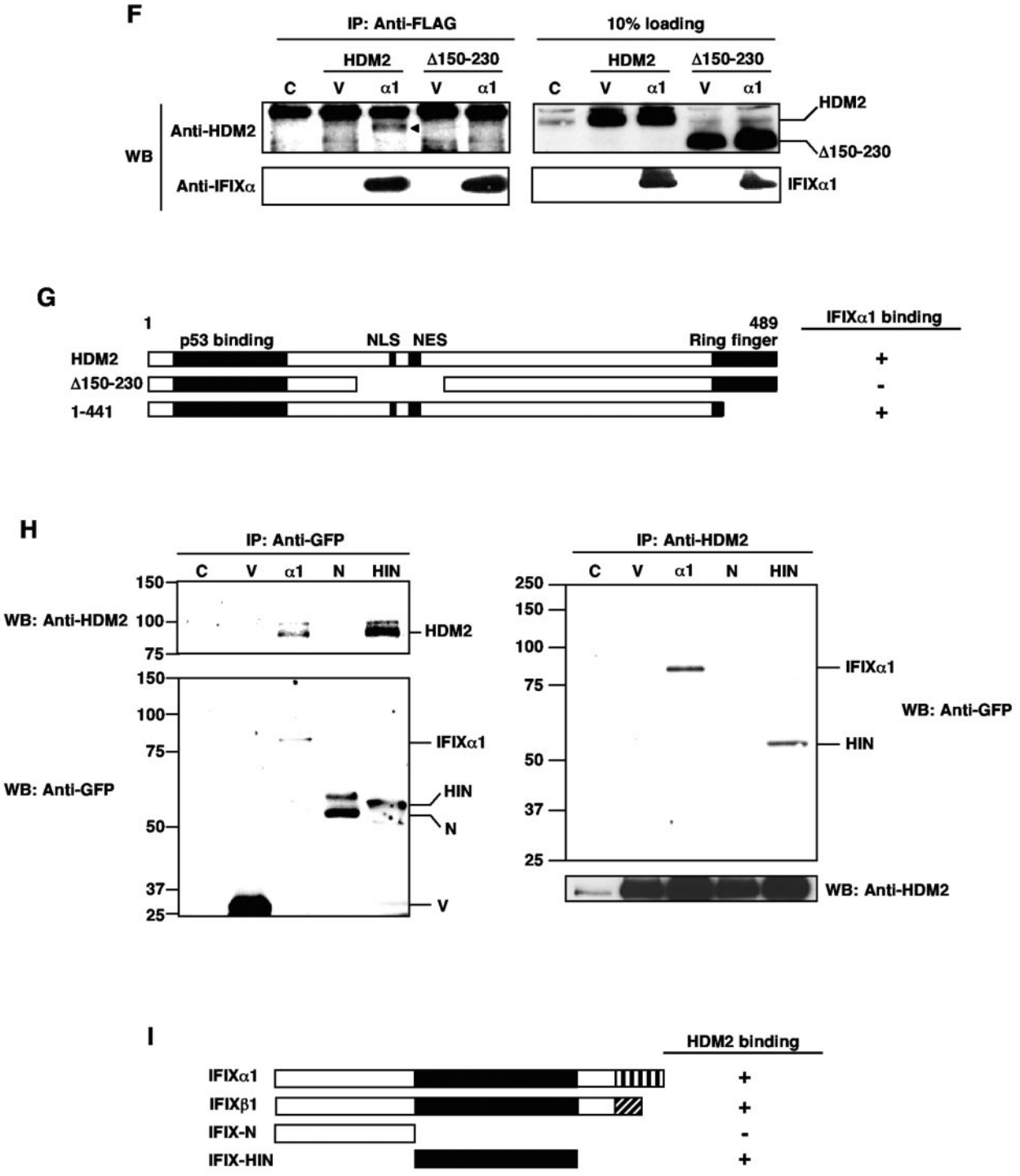

FIG. 6—*Continued.*

PKR not only activates p53 transcription (87) but also binds to the C terminus of p53 and phosphorylates serine 392 (14). In turn, PKR upregulates p53-mediated transcription (13). IFI16 binds to p53 and augments the p53-mediated transcriptional activation (26, 43). This interaction between IFI16 and p53 may contribute to the ability of IFI16 to sensitize cells to p53-dependent apoptosis induced by γ-irradiation (26). Another example is p202a, which inhibits p53-mediated transcriptional activity by presumably being a component of the p53 protein complex through binding to p53 binding protein 1 (15).

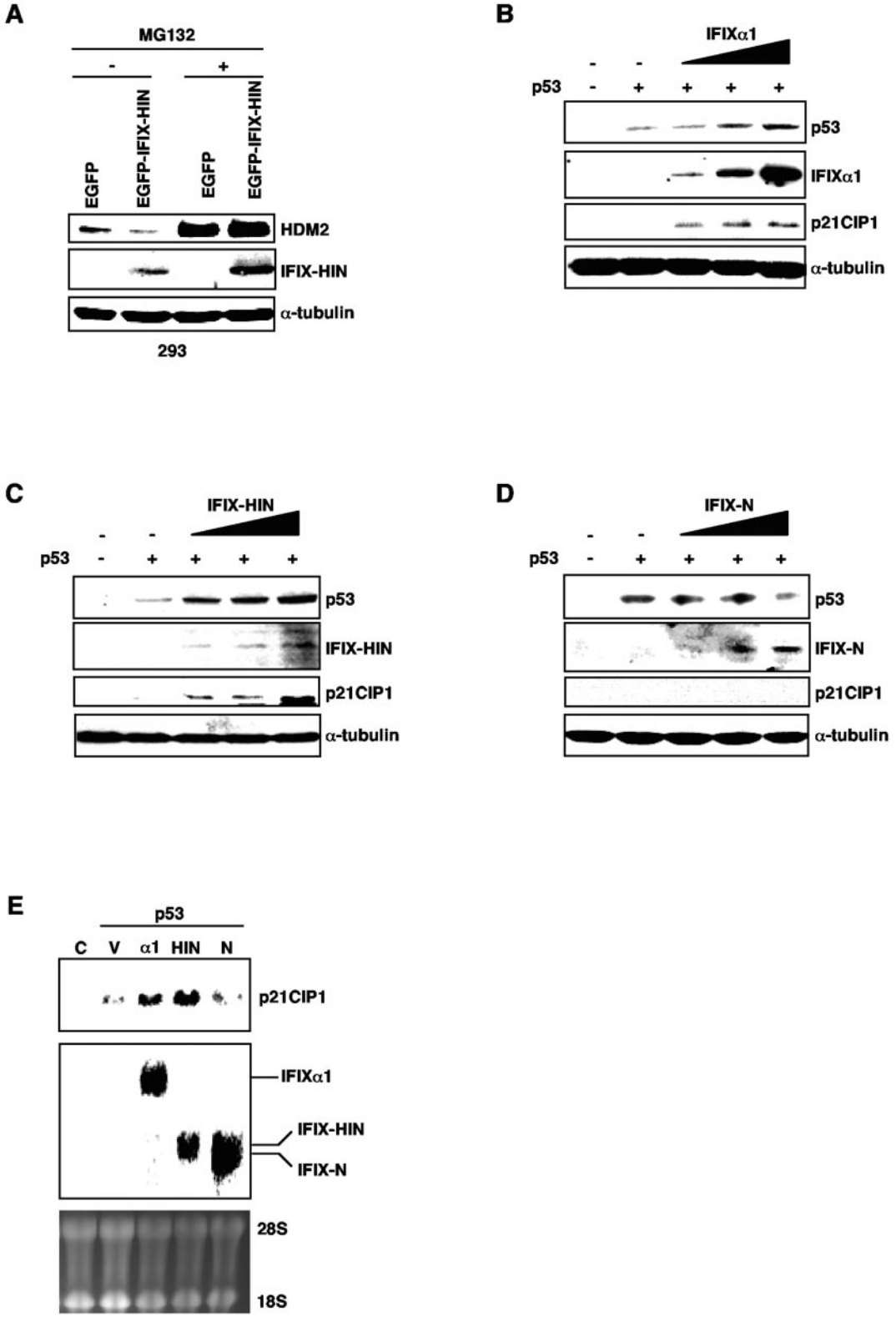

FIG. 7. IFIX-HIN is sufficient to downregulate HDM2. (A) IFIX-HIN downregulates HDM2 expression. 293T cells were transfected with EGFP empty vector (EGFP) or EGFP-IFIX-HIN. MG132 (10 μM) treatment started at 5 h before harvest. Cell lysates isolated from the GFP-positive cells were analyzed by Western blotting using antibodies against HDM2, EGFP, and α-tubulin. (B to D) IFIX-HIN induces p53 and $p21^{CIP1}$. H1299 cells were transfected with p53 (0.1 μg) and increasing amounts (0.5, 1.0, and 1.8 μg) of the FLAG-tagged IFIXα1 (B), IFIX-HIN (C), or IFIX-N (D), followed by Western blotting using antibodies against p53, IFIXα (B), FLAG (C and D), $p21^{CIP1}$, and α-tubulin at 24 h posttransfection. (E) IFIXα1 induces $p21^{CIP1}$ mRNA expression. H1299 cells were cotransfected with p53 (0.5 μg) and 5.5 μg of FLAG-tagged empty vector (V), IFIXα1 (α1), IFIX-HIN (HIN), or IFIX-N (N). At 24 h posttransfection, total RNA (10 μg) isolated from these cells was analyzed by Northern blotting using $p21^{CIP1}$ or IFIX cDNA as a probe. The 18S and 28S rRNAs served as loading controls.

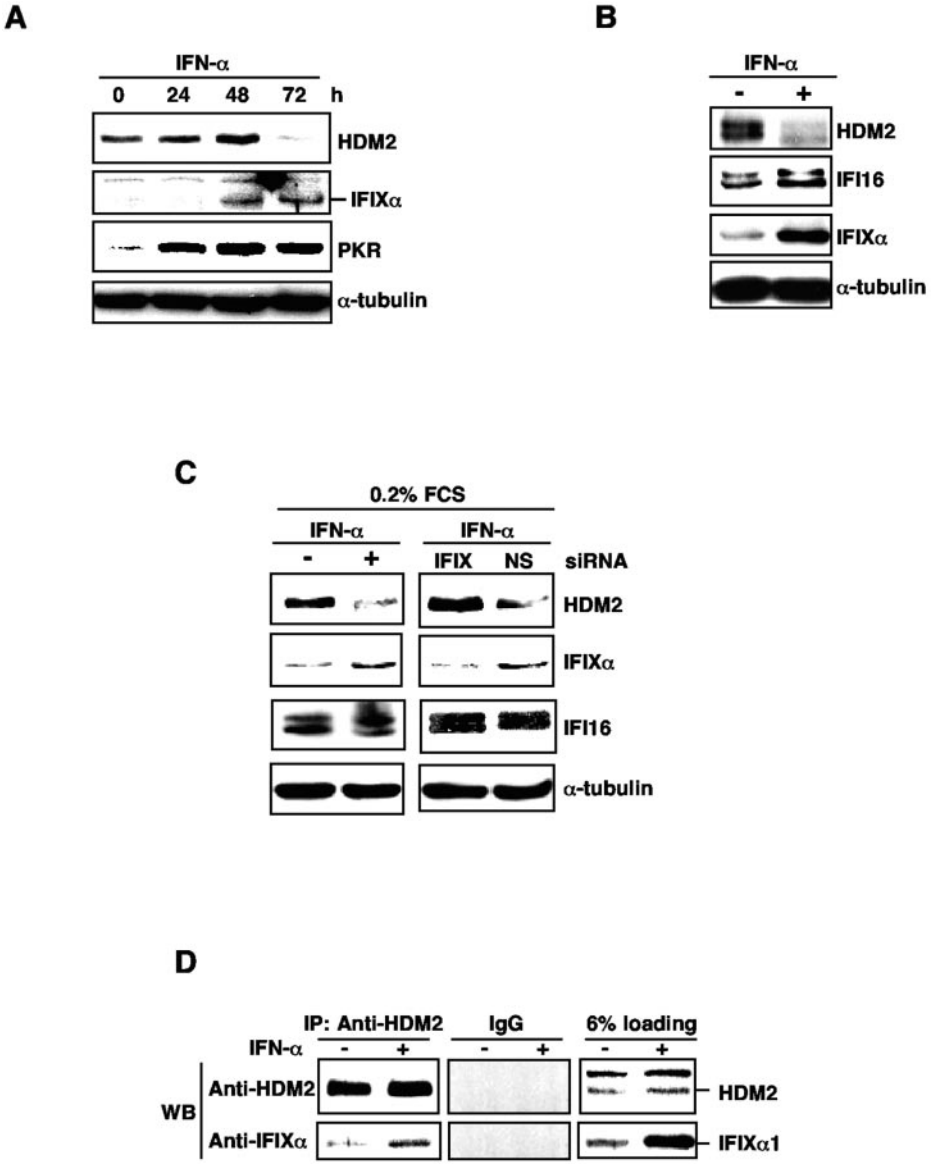

FIG. 8. IFIXα1 mediates the IFN-α-induced HDM2 downregulation. (A) IFN-α treatment reduces the HDM2 protein levels. Raji cells were treated with or without IFN-α (2,000 U/ml) for the indicated times (0, 24, 48, and 72 h), followed by Western blotting using the antibodies against HDM2, IFIXα, PKR, and α-tubulin. (B) IFN-α induces the expression of both IFIXα1 and IFI16 proteins. Raji cells were treated with or without IFN-α (2,000 U/ml) for 72 h, followed by Western blotting using the antibodies against HDM2, IFI16, IFIXα, and α-tubulin. (C) IFIX siRNA transfection reverses the IFN-α-mediated downregulation of HDM2. Raji cells growing in 0.2% FCS DMEM/F12 medium with or without IFN-α treatment (2,000 U/ml) for 72 h were analyzed by Western blotting using antibodies against HDM2, IFIXα, IFI16, and α-tubulin (left panel). The protein expression was likewise analyzed in the Raji cells transfected with either IFIX siRNA (100 nM) or the NS siRNA (100 nM) in 0.2% FCS DMEM/F12 medium, followed by IFN-α treatment (2,000 U/ml) for 72 h (right panel). (D) IFN-α treatment increased the IFIXα and HDM2 interaction. Cell lysates (800 μg) isolated from Raji cells treated with (+) or without (−) IFN-α (2,000 U/ml) for 48 h, followed by IP with anti-HDM2 or immunoglobulin G antibody and Western blotting with anti-HDM2 and anti-IFIXα antibodies.

Although IFIXα1 colocalizes with p53 in the nucleus (data not shown), we have not detected a physical association between IFIXα1 and p53 despite intensive efforts to look for such interaction (data not shown). Additional experiments, such as gel filtration analysis, are needed to verify this observation. However, it remains possible that the interactions between IFIXα1 and HDM2 and between p53 and HDM2 may exist as mutually exclusive complexes. If this hypothesis is proven valid, it may suggest that, unlike that between PKR, IFI16, and p202a, the interaction between IFIXα1 and p53 may not be necessary for the IFIXα1-induced p53 stabilization and transcriptional activation. Rather, our data suggest that HDM2 but not p53 is the primary target of IFIXα1. This conclusion is supported by the observation that ectopic p53 can be stabilized and activated by IFIXα1 only in the $p53^{-/-}$ MEF but not in the DKO MEF (Fig. 5A and B).

The nuclear localization of IFIX-N is somewhat expected since a putative NLS, ^{134}LGPQKRKK, resides within the N-

terminal region of IFIXα1 (18). However, unlike IFIXγ1, which forms nuclear specks (18), IFX-N localizes throughout the nucleus (data not shown). This result suggests that the unique C-terminal 52-amino-acid region of IFIXγ1 may be responsible for the nuclear speck localization. On the other hand, the attenuated nuclear localization of IFIX-HIN (data not shown) suggests that the N-terminal NLS is required for the exclusive nuclear localization of IFIXα1. It also suggests that other NLSs may exist to direct IFIX-HIN to the nucleus. Two highly charged regions, e.g., ^{259}LKRKFIKKR and ^{306}RRAKKIPK, reside within the HIN domain and may represent such NLSs. Alternatively, it is possible that IFIX-HIN may be shuttled into the nucleus by interacting with unknown nuclear protein.

We mapped the HDM2 binding region to the HIN domain of IFIXα1 (Fig. 6H). Remarkably, like IFIXα1, IFIX-HIN is sufficient to downregulate HDM2 and to induce p53 and p21^{CIP1} (Fig. 7C and E). This result is consistent with our previous finding that p21^{CIP1} induction was observed in cells expressing IFIXα1 and IFIXβ1 but not IFIXγ1, which lacks the HIN domain (18). Thus, the N-terminal PYD domain and the C-terminal sequence of IFIXα1 appear to be dispensable for downregulating HDM2. It is thus possible that IFIX-HIN may be sufficient to destabilize HDM2. Although experiments have been performed to test this possibility, the instability of IFIX-HIN protein (Fig. 7A) has become a challenge in this effort. Perhaps an exclusively nucleus-localized IFIX-HIN, by tagging its own NLS or a heterologous NLS, may help to solve the stability issue. Nevertheless, given that IFIX-HIN is the signature motif of HIN-200 proteins, it is possible that other HIN-200 family proteins may possess a similar activity to destabilize HDM2.

We have previously shown that IFIXα1 induced p21^{CIP1} in cells with or without wild-type p53 (18). Although we show in this study that IFIXα1 induces p21^{CIP1} through p53 upregulation (Fig. 3E and F, 4C, and 7B and E), the p53-independent mechanism remains to be elucidated. Interestingly, recent reports showed that HDM2 can interact directly with p21^{CIP1} protein and promotes its degradation (41, 88). Therefore, one possible p53-independent posttranslational mechanism underlying p21^{CIP1} induction may be through HDM2 downregulation by IFIXα1.

In addition to their role in innate and adaptive immunity (5), IFNs also possess proapoptosis, antiangiogenesis, and antiproliferation activities, which have been the basis for using IFNs to treat human malignancies (34, 69). The antitumor activity of IFN is likely attributed to the tumor suppressor functions of certain IFN-inducible proteins (34, 48). HIN-200 genes have been implicated as tumor suppressors due to their loss or reduced expression in certain human malignancies (for recent reviews, see references 2 and 54). IFIXα1, a novel member of the human HIN-200 gene family, is downregulated in breast cancer, and its expression is associated with growth inhibition and tumor suppression (18). Here, we present a mechanism for the IFIXα1-mediated antitumor activity. Our data show that IFIXα1 destabilizes HDM2. IFIXα1 does so by binding to HDM2 and promoting its ubiquitination and degradation. Consequently, p53 is stabilized and the p53-responsive gene products, such as p21^{CIP1}, are activated, leading to growth inhibition. Therefore, the cross talk between the IFN-IFIXα1 pathway and the HDM2-p53 pathway may contribute in part to the overall IFN-mediated antitumor activity in certain human cancers.

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