IFI16 as a Negative Regulator in the Regulation of p53 and p21^{Waf1} *

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IFI16 is a member of the HIN-200 family (hematopoietic interferon-inducible nuclear antigens with 200 amino acid repeats) that contains a DNA binding domain, a transcriptional regulatory domain, and DAPIN/PAAD, a protein domain associated with interferon response. It can function as a transcription repressor and directly binds p53. Although the structural and biochemical properties of IFI16 are known, the physiological relevance of these properties in the cellular context is still elusive. Here we report that the inhibition of endogenous IFI16 expression by small interfering RNA (siRNA) induces p21^{Waf1} mRNA and protein expression through p53 but does not induce pro-apoptotic p53 target genes. This rapid induction of p21 was wild-type p53-dependent and resulted in cell cycle arrest along with a marked reduction of phosphorylated Rb in normally growing cells. We also showed that the repression of IFI16 affects p53 transcriptional activity at the p21 promoter as well as the protein stability of p53 and p21.

Our findings identified a new role for IFI16 in modulating p53 function and its target gene regulation in the control of cell cycle regulation.

The control of cell cycle regulation plays an important role in maintaining normal cellular growth, proliferation, and differentiation, and as a result, the loss of cell cycle control leads to the development of tumors (1). The p53 tumor suppressor gene plays an important role in the regulation of the cell cycle, and it is known that p53 plays a crucial role in the progression of cancer as evidenced by the inactivation or loss of p53 in a majority of human tumors (2, 3). The activity of p53 in controlling cell growth needs to be tightly restrained in unstimulated cells to allow cell proliferation and development (4, 5). p53 protein is positively regulated through a succession of post-translational modifications including acetylation (5, 6). In its inactive form, p53 is bound to the Mdm2 protein, and binding to Mdm2 inhibits the transcriptional activity of p53 (4–6) and promotes the degradation of p53 by the 26 S proteosome (7).

The main effector of p53-mediated cell cycle arrest is the p53 target gene, p21 (4). p21^{Waf1/Cip/ldm1} is an inhibitor of cyclin-dependent kinases which are key regulators of the cell cycle (8, 9). p53 activates p21 (10) transcriptionally, and it has been shown that the inhibition of cell proliferation by p53 is largely because of its ability to transcriptionally activate genes involved in controlling cell fate (2, 3).

Here we describe IFI16, which we found to be involved in the regulation of p21 through p53. IFI16 is a human member of the HIN-200 family of interferon-inducible proteins (11). Members of the HIN-200 family are characteristically induced by interferons and distinctly contain one or more of a conserved 200-amino acid motif. IFI16 was first detected in lymphoid cells (12), but since then, through immunohistochemical staining of different tissues, it was found to be expressed at high levels in highly proliferating cells of various cell types (13). The localization of IFI16 in the nucleus (14) and the ability of IFI16 to bind DNA (15) at its highly basic residues in the N-terminal region (14, 16) are characteristics attributed to transcription factors. IFI16 can function as a transcription repressor and contains a separate DNA binding domain and a transcriptional regulatory domain (17). IFI16 also contains DAPIN/PAAD (domain in apoptosis and interferon response/pyrin A{alpha}M ASC and death domain-like), protein domain associated with apoptosis and interferon response (18, 19), and it directly binds p53 at its first 200-amino acid repeat region (20). Although the structural and biochemical properties of IFI16 are known, the physiological relevance of these properties in the cellular context is not known. However, from the biochemical properties of IFI16, its nuclear localization and ability to bind DNA as well as the expression of IFI16 in transcriptionally active cells with a nucleoli (16), it has been predicted that IFI16 may be a transcriptional factor involved in the regulation of cell differentiation (11). In light of a recent expression pattern study that detected high levels of IFI16 in various tissue types (i.e. tissues of the gastrointestinal tract, digestive organs, reproductive organs, excretory organs) of high proliferative potential (specifically the epithelial lining tissue of many of these organs), it appears that IFI16 is not simply an interferon-response gene (13) and that it is likely to be involved in the broader pathway of general cell differentiation and/or cell cycle regulation in the cell. In this study, we have found that IFI16 transcriptionally and post-transcriptionally regulates the expression of p21 through wild-type p53. We show that the knock-down of endogenous IFI16 protein levels by RNA interference in p53 wild-type cells results in significantly increased expression of p21, which leads to decreased S phase and cell cycle arrest only in a wt-p53-dependent manner. We also show that IFI16-regulated p21 expression is transcriptionally mediated through increased p53 binding to the p21 promoter at the p53 responsive element. Moreover, the inhibition of IFI16 resulted in increased stabilization of both the p53 and p21 protein in the cell. These data demonstrate a functional role of IFI16 as a novel regulatory element in p53-mediated cell cycle regulation.
Inhibition of IFI16 by RNA-mediated interference increases p21/Waf1 expression in a p53-dependent manner. A, effect of IFI16 inhibition on p21/Waf1 expression. U2OS cells were transfected with either control (luciferase) siRNA or IFI16 siRNA, and at the indicated times after transfection, cell lysates were extracted for Western blot analysis. Cell lysates were immunoblotted with antibodies against IFI16, p53, p21, Bax, and β-actin (loading control). B, p21 induction by IFI16 inhibition is wild-type p53-dependent. p53-null Saos-2 cells were transiently transfected with p53 or empty vector (pcDNA3). At the time of transfection with p53, cells were also transfected with control siRNA or IFI16 siRNA. After 48 h of transfection, cell lysates were prepared for Western blot analysis with IFI16, p53, p21, and β-actin antibodies.

**Experimental Procedures**

**Cell Culture**—U2OS and 293T cells were grown in Dulbecco’s modified Eagle’s medium (Biowhittaker) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C.

**siRNA Experiment**—The following IFI16 siRNA sequence was generated by this laboratory and purchased through Dharmacon: 5’-GCUGGUCCUAACACAGUdTdT-3’ (IFI16 nucleotides 1106–1126). U2OS cells were seeded at 30% density the day before transfection. Cells were transfected with OligofectAMINE (Invitrogen) transfection reagent according to the manufacturer’s protocol.

**Fluorescence-activated Cell Sorter Analysis**—Cells were trypsinized, washed with cold phosphate-buffered saline, collected by centrifugation, and then fixed with ice-cold 80% ethanol and precipitated over night at 4°C. After fixation, the cells were resuspended in room temperature phosphate-buffered saline and treated with propidium iodide (50 μg/ml), incubated for 30 min at 37°C, and then analyzed using FACScan (BD Biosciences).

**Reporter Assays**—U2OS and 293T cells were seeded at 90% confluency the day before transfection. LipofectAMINE 2000 (Invitrogen) was used according to the manufacturer’s instructions to transfect the cells. At 48 h after transfection, luciferase assay was performed using the Dual Luciferase Reporter Assay System (Promega) and measured using a DLReady luminometer (Turner Designs).

**Western Blot Analysis**—Cells were lysed in lysis buffer (20 mm Tris, pH 7.4, 5 mm EDTA, 10 mm NaPO₄, 100 mm NaF, 2 mm NaVO₃, 1% Nonidet P-40, 1 mm phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). An equal amount of total cellular protein per sample (15 μg) was run on a SDS-PAGE and transferred to a nitrocellulose membrane (Invitrogen). The following antibodies were used for immunoblotting analysis: anti-IFI16 antibody (1G7 monoclonal, Santa Cruz Biotechnology), anti-p53 antibody (Ab-1, Ab-6 monoclonal, Oncogene), anti-p21 antibody (Ab-1, Oncogene), anti-β-actin antibody (AC-15, Sigma), anti-Bax antibody (N-20 polyclonal, Santa Cruz), anti-MDM2 antibody (Ab-1, Oncogene), anti-p21 antibody (Ab-1, Oncogene), and anti-Noxa (Ab-1 monoclonal, Oncogene). The following antibody was used for chromatin immunoprecipitation: anti-p53 antibody (FL393 polyclonal, Santa Cruz).

**Northern Blot Analysis**—Total RNA was extracted, denatured, and run on a 1% agarose-formaldehyde gel, and then transferred onto a nylon membrane (Bio-Rad). Hybridization was performed with 32P-labeled probes prepared with RadPrime DNA labeling system (Invitrogen).

**Chromatin Immunoprecipitation**—U2OS cells were seeded in p100 dishes at 40% density the day before siRNA transfection. At 72 h after transfection, cells were formaldehyde cross-linked, and chromatin immunoprecipitation was carried out according to the manufacturer’s instructions using the Chromatin Immunoprecipitation Assay Kit (Upstate) with the following specifications/modifications. Immunoprecipitation was carried out at 4°C overnight using 1 μg of anti-p53 FL393 polyclonal antibody (Santa Cruz). The PCR reaction contained 3 μl of immunoprecipitation sample, or 3 μl of input sample, and 50 ng of each primer (5’T-GAAGGGATCCGCGGCGATGTCG-3’, 5’T-CTTCCATCCATC-CCCTCCTC-3’), and 45 μl of PCR Supermix High Fidelity (Invitrogen). After 35 cycles of amplification, the PCR product was run on a 1% agarose gel and visualized with a fluorescence gel imager (Bio-Rad).
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**RESULTS**

Decrease of IFI16 Leads to Increased p21 Expression and Cell Cycle Arrest—To determine the functional role of IFI16 in p53 regulation as well as in cell growth, we used IFI16 siRNA to knock down IFI16 expression in U2OS cells, which endogenously express both IFI16 and wt-p53. We detected a high level of endogenous IFI16 expression in U2OS cells. A 21-nucleotide siRNA was made against the IFI16 mRNA transcript. IFI16 can be detected as three isoforms resulting from differential mRNA splicing of the exon 7 region, and the isoforms are known to homo- and heterodimerize at the dimerization domain located in the amino terminus (11). Accordingly, the siRNA was directed to a region of the mRNA above the splice region to target all three isoforms. U2OS cells were transfected with IFI16 siRNA, and the expression level of p53 and its downstream target genes was examined. The decrease of endogenous IFI16 over time resulted in a marked increase of p53 expression but an even greater induction of the p53 down-stream target genes, p21 (Fig. 1A). In an effort to determine whether p21 induction mediated by the inhibition of IFI16 requires a functionally active p53, p53-null Saos2 cells, which express endogenous IFI16, were co-transfected with wt-p53 or vector alone and IFI16 siRNA or control siRNA (firefly luciferase). Whereas vector alone did not have an effect on p21 expression with or without IFI16 siRNA, wt-p53 expression induced p21 levels, and the suppression of IFI16 significantly enhanced this p53 induction of p21 (Fig. 1B).

To assess the physiological response of the cell to the induction of p21 resulting from the decrease of IFI16, cell cycle analysis by a fluorescence-activated cell sorter was performed. As shown in Fig. 2A, the decrease in endogenous IFI16 resulted in cell cycle arrest at G0/G1 and G2/M stages with an ~20% decrease in S phase. An important regulator in the transition to G1 is the tumor suppressor Rb (8, 22). The phosphorylation of Rb by cyclin-dependent kinases (Cdks) inactivates Rb and causes it to release E2F. p21 inhibits Cdk2 and Cdk4 from phosphorylating Rb (8, 9). To further investigate the role of increased p21 expression because of IFI16 inhibition in the regulation of the cell cycle, we examined the phosphorylation state of Rb. It was observed that the effect of IFI16 reduction on the induction of p21 resulted in the decrease of Rb phosphorylation, which explained the decrease in S phase of IFI16 siRNA-treated cells (Fig. 2B). These data suggest that IFI16 negatively affects p21 expression level.

Elevated p21 Levels in IFI16 Knock-down Cells Are Transcriptionally Regulated—We demonstrated that IFI16 inhibition causes a significant increase in p21 protein levels as well as a minor increase of p53. Next, we examined whether p21 is transcriptionally up-regulated upon the inhibition of IFI16. First, we performed Northern blot analysis to determine the effect of IFI16 inhibition on p21 mRNA (Fig. 3). In U2OS cells with targeted degradation of IFI16 mRNA by RNA interference, there was a corresponding increase in p21 mRNA level; however, when both p53 siRNA and IFI16 siRNA were transfected, no p21 mRNA increase was detected. These data further indicate that the regulation of p21 by IFI16 is a p53-dependent process.

![Figure 3](image-url)

**Fig. 3.** Increased p21 mRNA induction following IFI16 siRNA treatment is dependent on the presence of p53. Total RNA isolated from U2OS cells 72 h after treatment with either control siRNA or IFI16 siRNA and p53 siRNA was analyzed by Northern blot analysis. Note that reduced IFI16 levels did not result in an increase in p21 in the absence of p53.

![Figure 4](image-url)

**Fig. 4.** Effect of IFI16 on the transcriptional activity of the p21 promoter. A, suppression of endogenous IFI16 expression by IFI16 siRNA in U2OS cells results in increased p21 promoter activity. U2OS cells were co-transfected with a luciferase reporter vector containing the full-length p21 promoter, pGL-p21 promoter, and either control siRNA or IFI16 siRNA and then lysed and assayed for luciferase activity 48 h after transfection. Control cells were co-transfected with an empty luciferase reporter vector, pGL-Basic, and either control siRNA or IFI16 siRNA. B, co-transfection of p53 and IFI16B decreases p53-mediated p21 transcriptional activity. 293T cells were co-transfected with the luciferase reporter vector containing the full-length p21 promoter, pGL-p21 promoter, and with pcDNA3-p53 and/or pcDNA3-IFI16B and then lysed and assayed for luciferase activity 48 h after transfection.
To further support that IFI16 regulates p21 expression at the transcriptional level in the presence of p53, we explored the possibility that IFI16 inhibition affected p21 protein accumulation by increasing stability because the transcriptional regulation of p21 in response to IFI16 inhibition could not completely account for the induction of the p21 protein. To address this issue, we examined the protein level by a CHX chase to assess protein stability. At 72 h after IFI16 siRNA transfection, cycloheximide (25 µg/ml) was added to inhibit de novo protein synthesis. Then the steady-state levels of the proteins, p21, p53, mdm2, and PUMA, in U2OS cells were determined at the given time points after the addition of CHX. In control U2OS cells transfected with luciferase siRNA, the half-life of p21 was about 60 min (Fig. 6, A and B). Importantly, the half-life of p21 was significantly increased to ~3 h in IFI16-inhibited cells transfected with IFI16 siRNA (Fig. 6, A and B). Moreover, inhibition of IFI16 increased the stability of p53 and its target, Mdm2, to lesser extents (Fig. 6, A and B). In contrast, the stability of PUMA, p53 upregulated modulator of apoptosis (23), was not greatly influenced in IFI16-inhibited cells (Fig. 6A). The above results indicate that, in addition to transcriptionally regulating p21, IFI16 also regulates p21 by affecting p21 protein stability.
DISCUSSION

The structural and biochemical aspects of IFI16 have been established, but the functional relevance of IFI16 in the cellular context is still unclear. A number of studies have been published regarding the possible cellular functions of HIN-200 proteins, and these studies imply that these proteins play a role in modulating cell growth and differentiation (24–28).

We describe here that the inhibition of endogenous IFI16 expression in the presence of wt-p53 results in a significant increase of p21 mRNA and protein leading to cell cycle arrest, and this regulation of p21 expression is p53-dependent and occurs at the transcriptional and post-translational level. The ChIP data show that when endogenous IFI16 is knocked down, p53 binding at the p53-responsive element on the p21 promoter increases 2-fold. IFI16 is found in the nucleus (14), and it directly binds p53 at the first 200-amino acid repeat region (20). From this we suggest that IFI16 binds to p53 in the nucleus and hinders p53 binding at the p21 promoter such that the removal of IFI16 results in increased p53 binding at the p21 promoter leading to increased transcription of p21. In addition to increased transcription of p21, the inhibition of IFI16 also resulted in increased p21 and p53 protein stability. The longer half-lives of both p21 and p53 after IFI16 knock-down suggest IFI16 may also regulate p53 and p21 through protein degradation. The protein stability of p21 may be regulated by IFI16 through p53 or through an unknown p53-independent pathway. Unlike p53, which requires ubiquitin-dependent degradation, p21 can bind directly to the 20S proteasome without ubiquitination (29). Therefore, IFI16 may directly or indirectly affect p21 protein stability, and this mechanism remains to be elucidated.

It has been predicted that IFI16 may act as a transcriptional repressor that inhibits cell cycle transition from G1 to S phase based on functional studies describing the activity of the mouse HIN-200 family members like p202 and p204 (30). However, in light of recent evidence showing IFI16 expression in highly proliferative tissue (13), it seems that IFI16 may function differently from its mouse homologs. Although the mouse and human HIN-200 family members share biochemical and structural similarities, it is not clear at this time which of the mouse and human family members are functional homologs (28). Previous functional studies of HIN-200 family proteins focused on the results of overexpression studies (30). In the case of IFI16, we have generated tetracycline-inducible IFI16 cell lines but found no significant alterations in the cell cycle after the overexpression of any individual IFI16 isoforms.2 In addition, the forced overexpression of IFI16B through transfection and adenoviral infection in several different cell types did not result in the repression of p53 or p53 target genes and did not demonstrate any change in cellular phenotypes.2 This lack of an overexpression phenotype may be attributed to the functional importance in the expression of the three different isoforms of IFI16 (11). It has been shown that the different isoforms of IFI16 homo- and heterodimerize, which suggests that the conformation of the IFI16 isoforms may be important in the function of the protein (11).

We propose that IFI16 functions as a negative regulator of p53 that participates in p53-mediated transcriptional activation of its downstream target genes involved in cell cycle regulation. Our study raises the possibility that IFI16 expression may provide an additional mechanism to negatively regulate wt-p53 and its target genes in cells in the normal growth condition. It is well established that in normally growing cells, p53 levels are kept low through proteasome-mediated degradation promoted by the ubiquitin ligases, Mdm2 (6) and/or Pirh2 (31). However, when cells are exposed to a stress that leads to DNA damage, p53 becomes rapidly stabilized and functionally activated as a consequence of post-translational modifications (5, 6), and the p53 protein functions as a tran-

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scription factor capable of binding DNA in a sequence-specific manner (4) and transcriptionally regulating genes involved in the cell cycle (2, 3). We have not established the specific mechanism(s) that regulate(s) the IFI16-mediated regulation of p53, but it is possible that the binding of IFI16 to p53, as in the case of the binding of Mdm2 to p53, is necessary for maintaining low levels of p53 in the normal cell.

Although a potential pathway in which IFI16 regulates the stability of the p53 and p21 proteins remains to be determined, cycloheximide chase experiments strongly imply that IFI16 inhibition increases the half-life of p53 and its target genes p21 and Mdm2 but does not have a significant effect on pro-apoptotic p53 target genes. These data also suggest that IFI16 can influence p53 function toward growth arrest via p21 rather than the apoptosis pathway. We predict that IFI16 may target other proteins in forming a complex with the p53 protein, and the identification of additional component(s) in this cell context should provide important insights into the regulation of p53 and its target genes. Our results invite consideration that IFI16 is a novel negative regulator of the p53 tumor suppressor protein.

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