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Loss of p53-regulatory protein IFI16 induces NBS1 leading to activation of p53-mediated checkpint by phosphorylation of p53 SER37

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TABLE OF CONTENTS

1. ABSTRACT

Our previous results that IFI16 is involved in p53 transcription activity under conditions of ionizing radiation (IR), and that the protein is frequently lost in human breast cancer cell lines and breast adenocarcinoma tissues suggesting that IFI16 plays a crucial role in controlling cell growth. Here, we show that loss of IFI16 by RNA interference in cell culture causes elevated phosphorylation of p53 Ser37 and accumulated NBS1 (nibrin) and p21WAF1, leading to growth retardation. Consistent with these observations, doxycyclin-induced NBS1 caused accumulation of p21WAF1 and increased phosphorylation of p53 Ser37, leading to cell cycle arrest in G1 phase. Wortmannin treatment was found to decrease p53 Ser37 phosphorylation in NBS-induced cells. These results suggest that loss of IFI16 activates p53 checkpoint through NBS1-DNA-PKcs pathway.

2. INTRODUCTION

The p53 tumor suppressor is a critical component of cellular mechanisms that respond to certain stresses to preserve genomic integrity by arresting cell cycle progression or by inducing apoptosis (1,2). The p53 protein is mutated in many human tumors (3) and expression of a functional wild-type p53 gene in cells with missing or mutant p53 genes arrests their growth (4,5). From the biochemical characterization(s) of p53, it has been well documented that p53 binds to DNA (6-8), and its N-terminal region functions as a transactivation domain when coupled to a heterologous DNA binding domain (9-11).

Several phosphorylation sites have been mapped within the N-terminal human and/or murine p53. Importantly, phosphorylation of p53 *in vivo* was shown to be regulated by DNA damage. The first site that was shown to be inducibly phosphorylated after DNA damage was

Ser15, a substrate for DNA-PKcs, DNA-activated protein kinase (12,13). Recently, another kinase ATM has also been shown to phosphorylate this site (14,15). Importantly, the activity of ATM is significantly increased under conditions of DNA damage, but there are still several kinases that can phosphorylate DNA-damage responsible sites of p53. For example, although phosphorylation of Ser15 is delayed in ATM-mutated cells, this site of p53 eventually becomes phosphorylated in these cells (13). It was recently shown that both Ser33 and Ser37 are also DNA-damage inducible (16). Biochemical studies identified that DNA-PKcs phosphorylates, at least, Ser37 (17,18). Shieh et al. demonstrated that phosphorylation of p53 by DNA-PKcs in vitro both markedly reduces the ability of p53 to bind to Mdm2 and causes a significant alteration in conformation of the N-terminus (12).

Recruitment of ATM, ATR and DNA-PKcs to the sites of damaged DNA is a principal and critical step in their activation and functions in checkpoint signaling (19,20). Recent studies identified conserved carboxy-terminal motifs in human NBS1, ATRIP and Ku80 proteins that are required for their interaction with ATM, ATR and DNA-PKcs, respectively (21). These motifs are also essential for ATM-, ATR- and DNA-PKcs-mediated signaling events that activate cell cycle checkpoint and DNA repair. Although these results suggest a model that a protein complex containing this specific motif may serve as an activator of these kinases, specificity of functional interaction among NBS1, ATRIP, Ku80 and these kinases remains to be clear.

IFI16 was identified as a target of interferon α and γ (22). Recently, our laboratory found that IFI16 is involved in DNA-damage pathway mediated by BRCA1, breast cancer tumor suppressor protein, and that this protein in required for the maximal activation of p53 induced by IR treatment (23,24). Interestingly, it has been shown that expression of IFI16 is frequently decreased in many human breast adenocarcinoma (24). These results strongly suggest that IFI16 plays an important role in cancer development. In the present studies, we found that loss of IFI16 significantly retards cell growth possibly through DNA-PKcs-mediated phosphorylation of p53 Ser37. Biochemical analysis revealed that NBS1 is increased in IFI16-knockdown cells. Doxycyclin-inducible system demonstrated increased phosphorylation of p53 Ser37 and accumulation of p21WAF1 when NBS1 was induced. These studies suggest that loss of IFI16 results in the activation of cell cycle checkpoint and that mammary tumors losing IFI16 expression may be caused by disruption of this checkpoint pathway.

3. MATERIALS AND METHODS

3.1. Cell Culture

U2OS cells were obtained from ATCC. Doxycycline-regulatable NBS1 in U2OS human cells was established by following described protocols (Clontech). Cells were cultured in DMEM-10% fetal bovine serum (FBS). NBS1 was induced in the presence of doxycyclin (Sigma, 50 ng/ml). Ionizing radiation was administrated using a MARK2 IRRADIATOR (J. L. Stephen & Associate). IFI16-siRNA transfection was performed as described previously (24). For the growth retardation analysis, $1x10^5$ cells were transfected with scrambled or IFI16-specific siRNA and treated with ionizing radiation after 24 hrs. Three independent experiments were performed and numbers of cells were counted every 24 hrs for 7 days.

3.1. Western Blotting Analysis

Cell extracts were prepared in EBC buffer (50mM Tris, pH 8, 120mM NaCl, 0.5% Nonidet P-40 [NP-40]), with the addition of 100mM NaF, 0.2mM sodium orthovanadate, 100μg/ml polymethylsulfonyl fluoride (PMSF), 2μg/ml aprotinin, and 2μg/ml leupeptin. Fifty micrograms of whole cell extract were loaded per lane by 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Transfer to nitrocellulose was performed using a semidry transfer method (TRANS-BLOT, BIO-RAD), in 25mM Tris base, 192mM glycine, 10% methanol (for 1.5h at 15V). After blocking with 1% nonfat dried milk in PBS/0.05% Tween 20, the primary antibody was used in PBS/1% nonfat dried milk, for 1h at room temperature. The secondary antibody was peroxidase-conjugated goat anti-mouse or rabbit IgG (H+L, Jackson Immunoresearch), at 1:10,000 in 1% nonfat milk/TBS-T. Signals were developed by ECL (Amersham). Antibodies used in these studies are: IFI16 (Santa Cruz); ATM (GeneTex); ATMSer1981 (Rockland); p53 and p21WAF1 (Calbiochem); phospho-specific p53Ser15 and p53Ser37 (Cell Signaling); NBS1 and NBS1 Ser343 (Novus); Rad50, Mre11, c-Myc, DNA-PKcs, Ku70, Ku86, tubulin and actin (Santa Cruz).

3.2. Plasmids and Luciferase Assay

HA-tagged NBS1 cDNA was subcloned into Tet-On vector (Clontech). For luciferase assay, cells at ~70% confluence in 60mm plates were transfected using FuGENE (Roche), 1μg of myc reporter gene plasmid (a king gift from Dr. R. Eisenman, Seattle), 0.5μg of pCMV-β-gal plasmid. After 48h, transfected cells were resuspended in 50μl of 0.25M Tris (pH 7.8), subjected to three freeze/thaw cycles, and centrifuged at 12,000rpm for 5min to obtain supernatants for measurements of luciferase and β-gal activity as described previously (24).

3.3. Flow cytometry

U2OS cells were transfected with control (mixed) or IFI16-specific siRNA as described above, and cell cycle profile was determined almost every 24 h until 150 h after transfection. For IR treatment, transfected cells were irradiated (5Gy) 24 h after transfection. Similarly, subconfluent U2OS-tet-NBS1 cells were seeded on 10cm plates and treated with 50 ng/ml of doxycyclin for 24, 48, 72 and 96 h and induction of NBS1 was confirmed by westernblot analysis. Propidium iodide analyses were performed after 24 and 72 h of doxycyclin treatment using FACSCalibur (Beckton and Dickinson, Bedford, Massachusetts).

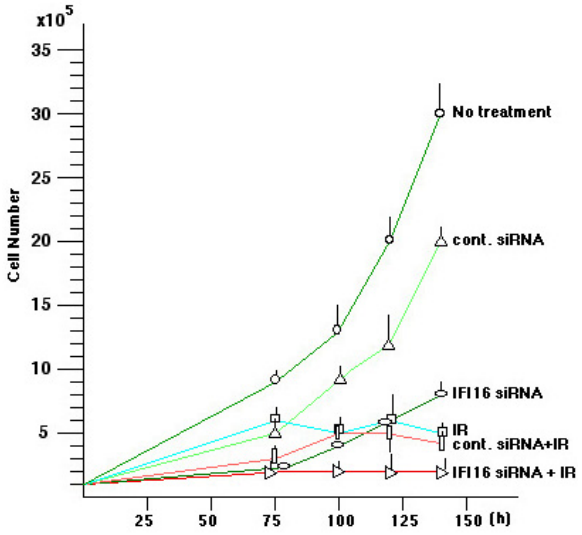

Figure 1. Growth retardation of IFI16-depleted cells. U2OS cells ($1x10^5$) were transfected with scrambled (control) or IFI16-specific siRNA. Cells were treated with ionizing radiation (5Gy) next day. Numbers of cells were counted every 24 h from three independent experiments.

3.4. *In vitro* kinase assay

After immunoprecipitation of DNA-PK from cell lysates, kinase assays were conducted in 20 μl of kinase buffer (25 mM HEPES [pH 7.9], 50 mM KCl, 10 mM $MgCl_2$, 20% glycerol, 1 mM DTT) in the presence of 15 μCi of [γ-^{32}P]ATP, 8 μM ATP and 150-200 ng of purified GST-p53 and 10 μg/ml of DNA fragments generated from HpaII digested pBlueScript as described previously (12). Samples were then subjected to SDS-PAGE. A gel was dried and autoradiographed.

4. RESULTS AND DISCUSSION

4.1. Loss of IFI16 arrests the cell growth

We have previously demonstrated that IFI16 plays a crucial role in cell cycle checkpoint under conditions of DNA damage (23,24). In particular, expression of IFI16 was found to be frequently decreased in many human breast cancer cell lines and breast cancer tissues (24), suggesting that loss of IFI16 is closely associated with cell transformation. To further understand the physiological role of the protein, IFI16 was knocked down by RNA interference (siRNA) in human osteosarcoma cell line, U2OS. Twenty four hours after transfection of cells (time = 0) with control or IFI16-siRNA, cells were treated with IR (5Gy) and maintained for the following 6 days to study cell growth. As shown in Figure1, depletion of IFI16 resulted in retardation of cell growth. IR treatment alone also decreased cell growth of cells transfected with control siRNA, and IFI16-siRNA cells were similarly arrested when treated with IR. These results were further studied by FACS analysis. Twenty four hr after transfection of either control or IFI16 siRNA, both cells showed normal distribution of the cell cycle (Figure 2, a and c). After treatment of these cells with IR (5Gy), cells started to show accumulation of G2/M phase at 24 hr (Figure 2, b and d). Interestingly, transfection of IFI16 siRNA resulted in accumulation of G1 phase in 72 h, but no significant increase in sub G1 fraction, supporting the growth retardation is due to growth inhibition, but apoptosis, by p21WAF1 (see below results). No morphological changes were observed in both control and IFI16 siRNA cells. No obvious changes of the cell cycle profile were observed in cells transfected with control siRNA (not shown).

4.2. Phosphorylation of p53 Ser37 and p21WAF1 are increased in IFI16-depleted cells.

Biochemical studies were performed to study whether DNA-damage associated proteins are involved in this growth retardation of IFI16-siRNA cells. U2OS cells were transfected with IFI16-siRNA and treated with IR (5Gy) after 48 h. About 85% reduction of IFI16 was achieved. Cell lysates were prepared at 0, 0.5, 1, 2, 4, 6 and 12 h after IR treatment. Because we observed growth inhibition of cells depleted for IFI16, we examined whether these cells express growth-inhibitory proteins. Among the proteins studied, we found that a cdk (cyclin dependent kinase)-inhibitor p21WAF1 is highly expressed when IFI16 is knocked down and is further induced after IR damage (Figure 3). Since p21WAF1 is a well-characterized target of p53, we next studied the biochemical analysis of p53. We have previously demonstrated that phosphorylation of p53 Ser15 was similarly induced after IR damage in both control and IFI16-siRNA cells, although protein levels of p53 and Ser15 phosphorylation were decreased after 6 h in IFI16-siRNA cells while control cells maintained high levels (ref. 24, Figure 3). From the current studies, phosphorylation of p53 Ser37 was found to be strongly induced and maintained for 12 h in IFI16-siRNA cells after IR treatment compared to the control cells (Figure 3). It has been demonstrated previously that DNA-PKcs is responsible for phosphorylation of p53 Ser37 (17,18). We found that basal levels of DNA-PKcs are higher in IFI16-siRNA cells than those in the control cells. Increased levels of DNA-PKcs were confirmed from several independent experiments, and the results of two independent experiments are shown as exp.1 and 2. Levels of ATM were similar between them (ref. 24, Figure 3). Increased DNA-PK activity was confirmed by *in vitro* kinase assay using GST-p53 as a substrate. Although similar amount of DNA-PK was immunoprecipitated from U2OS cells transfected with control or IFI16 siRNA, GST-p53 was efficiently phosphorylated in IFI16-depleted cells (Figure 4). These results support that DNA-PK is more activated in IFI16 siRNA cells.

4.3. NBS1 is increased in IFI16-depleted cells

We further analyzed DNA damage-associated proteins in control and IFI16-depleted cells under conditions of IR damage. Accumulating evidence have demonstrated that Mre11-Rad50-NBS1 (MRN) complex is required for activation of ATM in response to DNA damage and that this complex also functions in an amplification circuit with ATM (25-27). We studied levels of Mre11, Rad50 and NBS1 after IR treatment in control and IFI16-siRNA cells. Levels of NBS1 were markedly

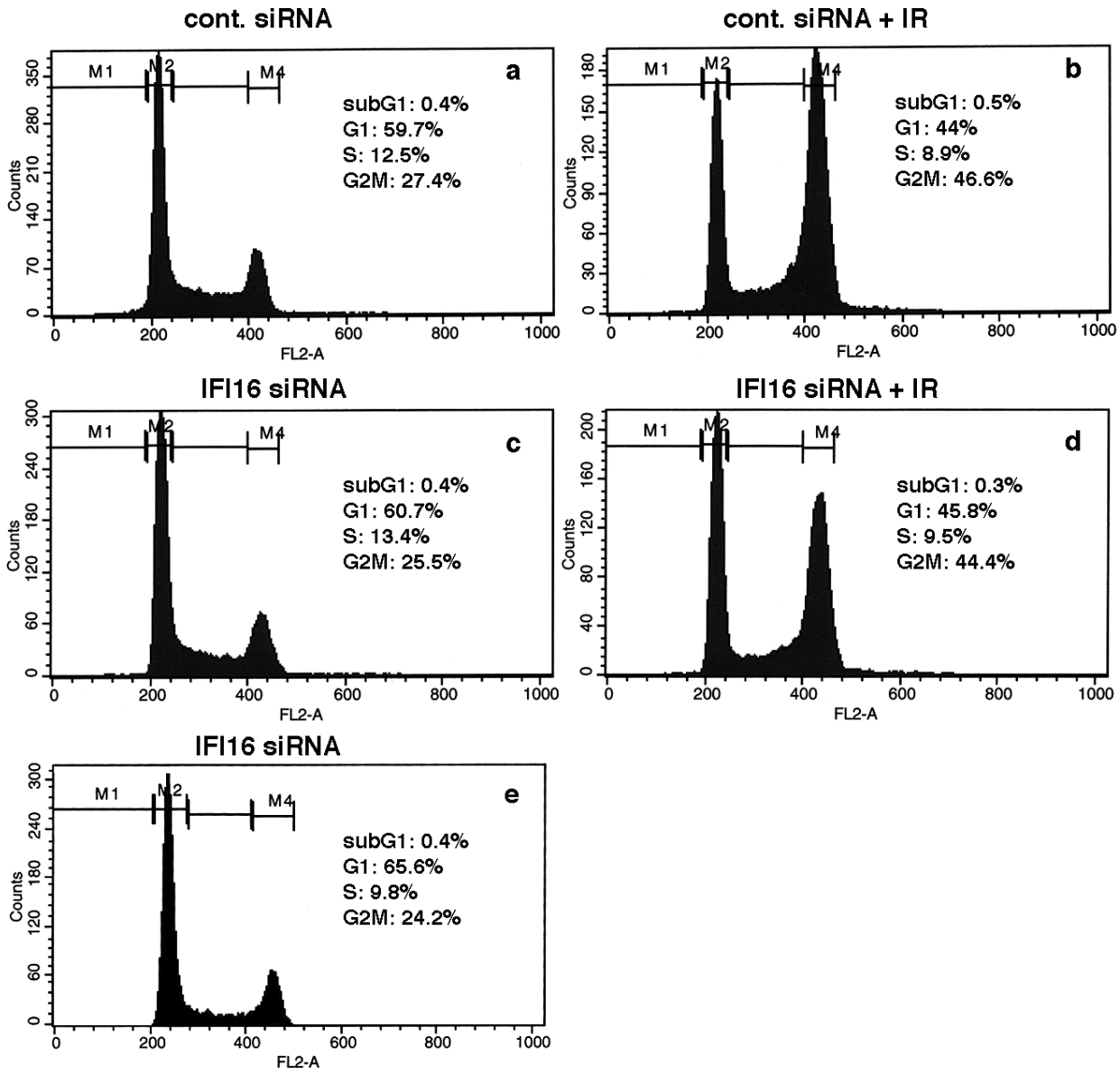

Figure 2. Cells were transfected with control or IFI16 siRNA for 24 h and the cell cycle profile was analyzed by FACS (a and c). Cells were then treated with IR (5Gy) and FACS analysis was performed after 24 h (b and d). Cells transfected with IFI16 siRNA was maintained for 72 h and cell cycle was studied (e).

increased when IFI16 was knocked down and was maintained throughout the time course (Figure 5). No significant changes of Mre11 and Rad50 were observed. It has been well documented that Ser343 of NBS1 is phosphorylated under conditions of DNA damage (28-31). Interestingly, this phosphorylation was similarly observed in both control and IFI16-siRNA cells.

It has been shown that c-myc transcription factor can induce the expression of NBS1 by direct binding to the intron 1 of the NBS1 gene (32). We found that levels of c-myc are increased in IFI16-siRNA cells. Consistent with these results, c-myc reporter gene that contains c-myc binding sites subcloned upstream of the luciferase gene was activated up to 3 folds in IFI16-siRNA cells (Figure 6). It remains unclear how loss of IFI16 results in increased levels of c-myc, but these results suggest that increased expression of NBS1 in IFI16-depleted cells is, at least in part, due to activation of c-myc.

4.4. NBS1 induces p53 Ser37 phosphorylation, p21WAF and growth inhibition

We next investigated whether increased NBS1 can directly regulate cell growth. We generated U2OS cell lines in which NBS1 is inducibly expressed when cells are treated with doxycyclin. HA-tagged NBS1 was detected in24 h after doxycyclin treatment (Figure 7). When NBS1

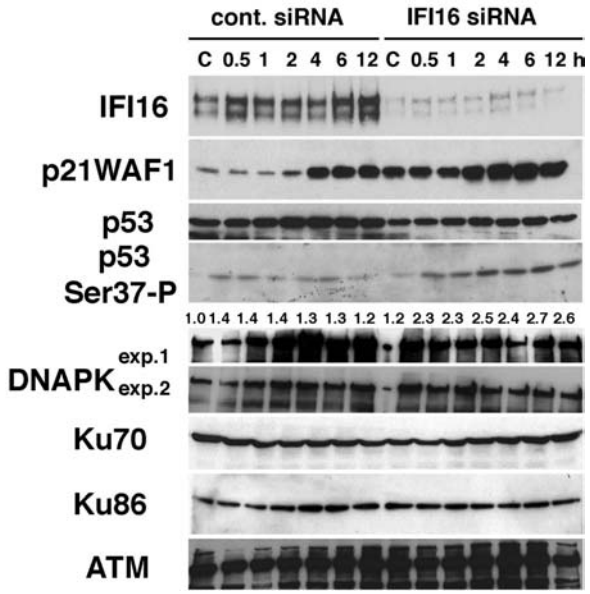

Figure 3. Loss of IFI16 causes differential regulation of levels of proteins involved in cell cycle progression. Accumulation of p21WAF1, p53 Ser37 phosphorylation and increased levels of DNA-PKcs. U2OS cells were transfected with scrambled or IFI16-specific siRNA as described previously (24). Two independent results showing increased levels of DNA-PKcs are shown. Cells were treated with ionizing radiation (5Gy) and indicated proteins were immunoblotted. Relative levels of p53 Ser37 phosphorylation were normalized with levels of ATM.

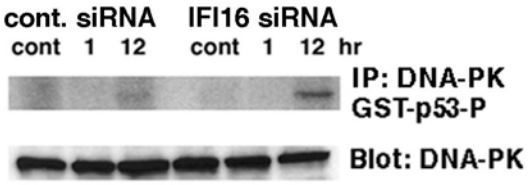

Figure 4. DNA-PK was immunoprecipitated from cells after 1 or 12 hrs of transfection of control or IFI16-siRNA. *In vitro* kinase assay was performed using GST-p53 as a substrate. Immunoprecipitation of DNA-PK was confirmed by immunoblot.

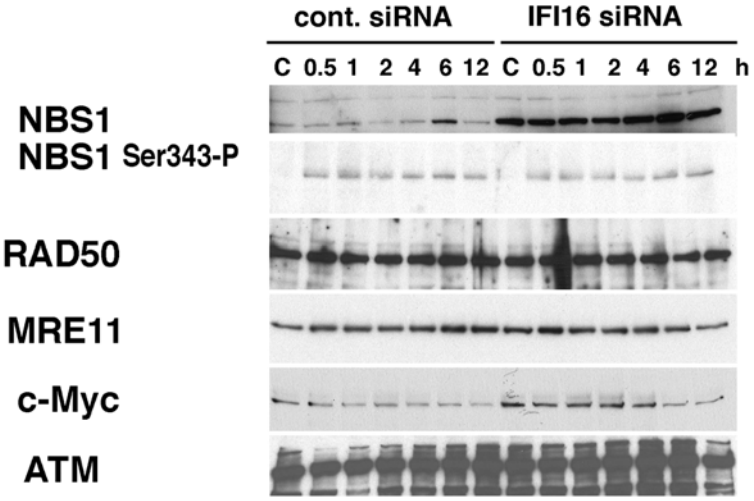

Figure 5. Loss of IFI16 causes increased NBS1 and c-Myc, but not NBS1 Ser343 phosphorylation. Protein levels of MRN complex were studies in IFI16-depleted cells. ATM was used as a loading control.

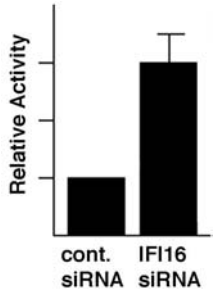

Figure 6. Transcription activity of c-Myc is elevated in IFI16-depleted cells.

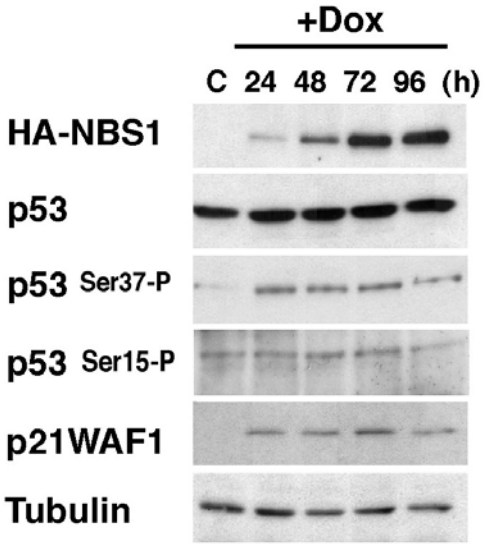

Figure 7. Induction of NBS1 induces p53 Ser37 phosphorylation. Doxycycline-regulatable NBS1 in U2OS cells were induced by doxycyclin (50 ng/ml). After 24, 48, 72 and 96 h, phosphorylation of p53Ser37 and induction of p21WAF1 was studied by immunoblot.

was induced, phosphorylation of p53 Ser37 was concomitantly increased in 24 h without DNA damage and levels of p21WAF1 was also increased. No significant increase of p53 Ser15 phosphorylation was detected in these cells. Consistent with high levels of p21WAF1, cell cycle profiles after treatment with doxycyclin demonstrated that numbers of cells in G1 phase of cell cycle was increased from 61.9% to 72.7% (Figure 8). These results indicate that NBS1 can potentially inhibit cell growth, which is perhaps due to the induction of p21WAF1. We also studied whether potential feedback loop regulates IFI16 from induced NBS1, but no increase of endogenous IFI16 was detected (not shown).

4.5. p53 Ser37 is phosphorylated by kinase(s) that can be inhibited by wortmannin in NBS1-induced cells

On the basis of recent studies concerning activation of ATM family of protein kinases by MRN complex consisting of Mre11, Rad50 and NBS1, we tested whether NBS1-induced phosphorylation of p53 Ser37 is inhibited by wortmannin, an inhibitor of ATM family of kinases including DNA-PKcs. NBS1 was induced by doxycyclin using the same protocol used above in the presence of wortmannin for 24 h. Cells were then treated with IR (5Gy) and cell lysates were prepared after 1 h. Phosphorylation of p53 Ser37 was immunoblotted with phospho-specific antibody (Figure 9). Increased phosphorylation by IR was partially blocked in the presence of wortmannin (lanes 2 and 4). Phosphorylation of p53 Ser37 was also increased by induction of NBS1 (lane 5) and was further enhanced by IR treatment (lane 6). NBS1-induced phosphorylation of p53 Ser37 was blocked by treatment with wortmannin (lane 7). These results support a model that NBS1-induced phosphorylation of p53 Ser37 is mediated by ATM family of kinases. Our previous results demonstrated that there are no significant difference regarding the kinetics of ATM activation indicated by Ser1981 phosphorylation of the protein between control and IFI16-depletde cells (24). We studied whether ATR is responsible for the phosphorylation of p53 Ser37 in NBS1-induced cells by knockdown with ATR siRNA, however, p53 Ser37 was induced in NBS1-expressing cells (data not shown).

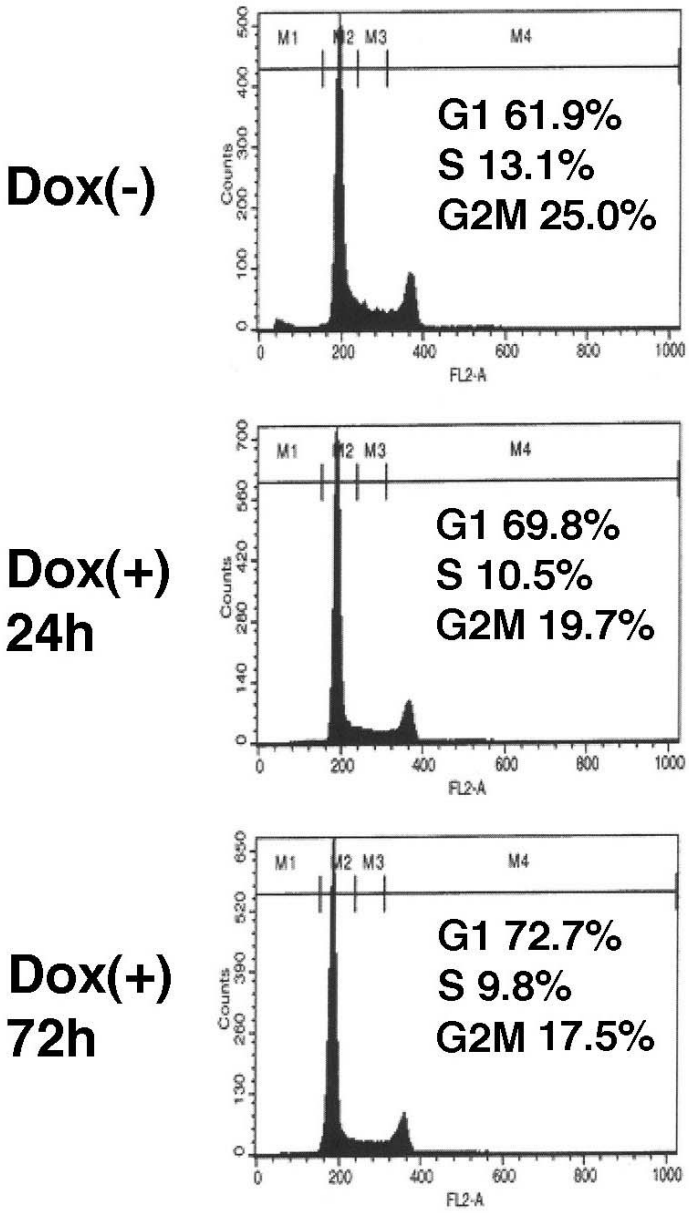

Figure 8. Cell cycle profile of doxycyclin-treated cells was studied with propidium iodide.

We have shown here that loss of IFI16 causes growth arrest by inducing p21WAF1. Biochemical analysis revealed that NBS1 protein is induced when IFI16 is depleted. We found that increased levels of NBS1 result in p53 Ser37 phosphorylation, possibly through DNA-PKcs. We have demonstrated before that phosphorylation of p53 Ser15 is similarly induced in both control and IFI16-siRNA cells although protein levels pf p53 and phosphorylation of Ser15 were decreased earlier than those in control cells (24). These results suggest that increased phosphorylation of p53 Ser37 in IFI16-siRNA cells results in more potent transcription activity of p53 in these cells. However, it is also possible that unknown transcription factors are activated in IFI16-reduced cells, which may be responsible for high levels of p21WAF1. We discovered that expression of c-myc is higher in IFI16-depleted cells than control cells, although it is unclear how loss of IFI16 increases levels of c-myc. Interestingly, it has been shown previously that elevated c-myc induces NBS1 expression through its direct binding to the NBS1 gene (32). Thus, it is possible that c-myc activates the NBS1 expression in IFI16-reduced cells.

Recently, the C-terminal amino acid sequence of NBS1 was shown to be required for the activation of ATM (21). In these studies, homologous motifs were also found in ATRIP and Ku80, which are essential for activation of ATR and DNA-PKcs, respectively. Given our results described above and the similarity of the amino acid sequences found in NBS1, ATRIP and Ku80, NBS1 may

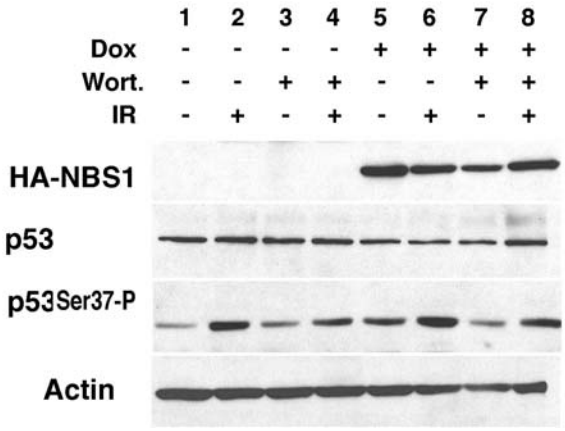

Figure 9. Wortmannin treatment blocked phosphorylation of p53Ser37 in NBS1-induced cells. NBS1 was induced by doxycyclin in the presence of wortmannin (0.1 μM). After two days, cells were further treated with ionizing radiation (5Gy) and p53Ser37 phosphorylation was studied.

be able to specifically activated DNA-PKcs when IFI16 is lost. Our findings suggest that IFI16-negative breast cancer cells may have escaped from DNA damage checkpoint by NBS1-DNA-PKcs pathway.

5. ACKNOWLEDGEMENT

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Abbreviations: ATM: ataxia telangiectasia mutated; NBS1: Nijmegen breakage syndrome 1; DNA-PK: DNA dependent kinase; siRNA: small interfering RNA; ATR: ATM related kinase

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