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## Induction of p53, p21 and apoptosis by silencing the NF90/NF45 complex in human papilloma virus-transformed cervical carcinoma cells

Raghavendra A. Shamanna<sup>1,2</sup>, Mainul Hoque<sup>1</sup>, Tsafi Pe'ery<sup>2,3</sup>, and Michael B. Mathews<sup>1,2</sup>

<sup>1</sup>Departments of Biochemistry & Molecular Biology, UMDNJ-Newark, New Jersey

<sup>2</sup>Graduate School of Biomedical Sciences, UMDNJ-Newark, New Jersey

<sup>3</sup>Medicine, New Jersey Medical School, UMDNJ-Newark, New Jersey

### Abstract

The heterodimeric nuclear factor 90/nuclear factor 45 complex (NF90/NF45) binds nucleic acids and is a multifunctional regulator of gene expression. Here we report that depletion of NF90/NF45 restores the expression of the p53 and p21 proteins in cervical carcinoma cells infected with high-risk human papillomaviruses (HPV). Knockdown of either NF90 or NF45 by RNA interference led to greatly elevated levels of p53 and p21 proteins in HPV-derived HeLa and SiHa cells, but not in other cancerous or normal cell lines. In HeLa cells, p21 mRNA increased concomitantly but the level of p53 mRNA was unaffected. RNA interference directed against p53 prevented the induction of both proteins. These results indicated that the up-regulation of p21 is due to p53-dependent transcription, whereas p53 is regulated post-transcriptionally. Proteasome-mediated turnover of p53 is accelerated by the HPV E6 and cellular E6AP proteins. We therefore examined the hypothesis that this pathway is regulated by NF90/NF45. Indeed, depletion of NF90 attenuated the expression of E6 RNA and inhibited transcription from the HPV early promoter, revealing a new role for NF90/NF45 in HPV gene expression. The transcription inhibition was largely independent of the reduction of P-TEFb levels caused by NF90 depletion. Consistent with p53 derepression, NF90/NF45-depleted HeLa cells displayed elevated PARP cleavage and susceptibility to camptothecin-induced apoptosis. We conclude that high-risk strains of HPV utilize the cellular NF90/NF45 complex for viral E6 expression in infected cervical carcinoma cell lines. Interference with NF90/NF45 function could assist in controlling cervical carcinoma.

### Keywords

Apoptosis; human papillomavirus; transcription; p53; p21; NF90/NF45

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\*Corresponding Author: Michael B. Mathews, UMDNJ-New Jersey Medical School, PO Box 1709, Newark, New Jersey 07101-1709, USA, mathews@umdnj.edu, Ph: (01)973-972-4411, Fax: (01)973-972-5594.

## Introduction

The cellular proteins p53 and p21 are crucial regulators of cell proliferation and genome stability (1-4). p21 is a cyclin-dependent kinase inhibitor that plays a pivotal role in cell cycle control. Largely regulated at the transcriptional level, it is induced by p53 as well as by p53-independent mechanisms (5). p53 is a potent tumor suppressor that activates p21 transcription in response to stress signals (6, 7) via two highly conserved p53-responsive promoter elements that bind p53 directly (5). p21 transcription is also induced by transcription factors such as SP1 and SP3 in response to external and internal stimuli. Increased levels of p21 bring about cell cycle arrest and senescence.

The p53 gene, *TP53*, is regulated at multiple levels, including by factors that stabilize the protein and prevent its degradation (3, 6, 8). Normally p53 is a short-lived protein, sometimes undetectable in cells except under stress conditions when it accumulates in the nucleus and functions as a transcription factor. Approximately 50% of primary tumors contain *TP53* mutations, most of which lead to inactivation of the protein's DNA binding function and allow continued cell proliferation. p53 can bind to MDM2, which interacts with its activation domain and prevents it from activating downstream genes. MDM2 also functions as a ubiquitin-protein ligase targeting p53 for degradation by the proteasome (3, 6, 7, 9, 10).

Viral infection is a stressful event that induces p53 production (11, 12). Many DNA viruses that have the potential to cause cell transformation encode proteins that bind and inactivate p53 (6). The SV40 T-antigen and adenovirus E1B-55K proteins sequester p53 in non-functional complexes, whereas the human papilloma virus (HPV) E6 protein directs p53 to the proteasome for degradation (6, 13). HPV is a small non-enveloped dsDNA virus that establishes productive infections in stratified epithelium of skin and mucous membranes. High-risk HPVs, notably HPV-16 and -18, are implicated in the development of invasive cervical carcinomas. In most primary cervical carcinomas and cell lines established from them, such as HeLa and SiHa, HPV DNA is integrated into the cell genome and expresses HPV E6 and E7 proteins. These viral proteins are responsible for the malignant transformation of fibroblasts and keratinocytes (14-17).

Nuclear factor 90 (NF90) is a nucleic acid binding protein that forms a heterodimeric complex with nuclear factor 45 (NF45) (18). These proteins are products of the interleukin enhancer-binding factor-3 and -2 genes, *ILF3* and *ILF2*, both of which are widespread in vertebrates. The NF90/NF45 core complex forms larger complexes together with NF110, another *ILF3* isoform, and several additional proteins, and regulates cellular gene expression at several levels. It has been implicated in DNA metabolism (19, 20), transcription (21, 22), translation (23, 24), RNA export (25, 26) and microRNA biogenesis (27), and in the replication and gene expression of a number of viruses (28-34). NF90 contains two dsRNA-binding domains and an RGG motif with nucleic acid binding capability; a bipartite nuclear localization signal and a nuclear export signal which promote nucleo-cytoplasmic shuttling; and a DZF domain encompassing a NF45 homology domain necessary for NF45 binding (18, 35, 36). NF90 is largely nuclear during interphase (37) and its phosphorylation correlates with nucleo-cytoplasmic translocation (38-40). The stability of NF90 and NF110

is regulated by NF45, a shorter-lived protein that also participates in multiple cellular functions (18, 19, 22, 41).

In mice, knockout of the *ILF3* gene results in prenatal (42) or perinatal (43) lethality due to muscle degeneration, apoptosis and respiratory failure (43). *ILF3*<sup>-/-</sup> mice exhibited an ~50% decrease in p21 RNA and a corresponding reduction in p21 protein in neonatal skeletal muscle (43). Interactions between NF90 and the p21 3'UTR suggested regulation via mRNA stability (43), consistent with other reports of NF90-mRNA 3'UTR interactions (23, 24, 44-48). In HeLa cells, depletion of either NF90 or NF45 by RNA interference slows cell growth and leads to a multinucleated phenotype (18, 19). These observations led us to examine the effect of NF90/NF45 knockdown on p21 expression in HeLa cells.

In contrast to findings with *ILF3*<sup>-/-</sup> mice, we observed that depletion of NF90 or NF45 *increased* the expression of p21. Up-regulation occurred at both the protein and mRNA levels. Concomitantly the level of p53 protein increased dramatically although p53 mRNA levels were unchanged. We found that the increase in p21 expression is dependent on p53, and that the elevation of p53 expression in response to NF90/NF45 depletion is restricted to HPV-infected cervical carcinoma cells. These findings suggested that NF90/NF45 may be necessary for p53 destruction mediated by HPV E6. Accordingly, NF90/NF45 depletion reduced the level of E6 RNA and transcription driven by the HPV early promoter. Increased p53 can drive malignant cells to apoptosis especially if they are subjected to genotoxic stress (49). Correspondingly, NF90/NF45-depleted HeLa cells displayed an increase in apoptotic markers and in apoptosis resulting from treatment with the topoisomerase I inhibitor and anticancer drug camptothecin. Our data show that NF90/NF45 is required for effective transcription of the HPV E6 gene. The mechanism is partly, but not chiefly, dependent on the positive transcription elongation factor P-TEFb. Stabilizing p53 by inhibiting E6 production can sensitize even highly resistant malignant cells to apoptotic cell death caused by genotoxic drugs.

## Results

### Induction of p21 by NF90/NF45 depletion

Long-term depletion of the NF90/NF45 complex in HeLa cells leads to a multinucleated cell phenotype (18). We considered the possibility that p21 might be involved in this process because reduction in the level of p21 is associated with formation of multinucleated cells (50) and p21 expression is reduced in *ILF3*<sup>-/-</sup> mice (43). To examine the role of NF90 and NF45 in p21 regulation, we transfected HeLa cells with siRNA specific for NF90 (D3) or NF45 (D5) or with control siRNA (C). RT-PCR and immunoblotting analyses showed that the siRNAs specifically depleted their target RNAs (Fig. 1a) and proteins (Fig. 1b). In addition, NF45 was substantially depleted in D3-treated cells and NF90 was partially depleted in D5-treated cells (Fig. 1b). This co-regulation results from the mutual stabilization of the proteins in a heterodimeric NF90/NF45 complex (18). Remarkably, NF90/NF45 depletion by either D3 or D5 increased the level of p21 RNA and protein by up to 7 fold (Figs. 1a,b and 2a,b). Knockdown of the NF110 isoform did not alter p21 expression, however (Supplementary Fig. S1). Permanent cell lines depleted for NF90 and NF45 also exhibited p21 over-expression (data not shown). These observations imply that

NF90/NF45 exerts a negative effect on p21 gene expression in HeLa cells, contrary to results obtained with *ILF3*<sup>-/-</sup> mice lacking both NF90 and NF110.

### NF90/NF45 regulates p53 in HeLa cells

p21 is under transcriptional regulation by p53, which is present at a low level in HeLa cells as a result of accelerated protein turnover (6, 13). We therefore examined the expression of p53 in NF90/NF45-depleted cells. Concomitant with the induction of p21, treatment with D3 or D5 dramatically increased the level of p53 by 13-18 fold (Fig. 2a). This action was not accompanied by an increase in p53 RNA, however (Fig. 2b). We also monitored the levels of several other RNAs encoding proteins involved in mitosis, particularly in abscission, cell architecture and the generation of multinucleated cells (50-53). No change was seen in the levels of RNAs encoding aurora B, matrin-3, ORC-6 or survivin, or the RNA component of telomerase, TERC (Fig. 2b). Similar results were obtained with D6, another siRNA that targets NF45 (Fig. 2b). As a further control, we conducted a “rescue” experiment. Expression of siRNA-resistant-NF90b (Omni-NF90b<sup>D3R</sup>), together with overexpression of NF45, suppressed the induction of p53 by D3 by about 50% (Fig. 2c). Neither alone was sufficient, indicating that the proteins are functioning as a complex. Taken together, these results show that depletion of the NF90/NF45 complex induces the expression of p21 RNA and protein, and of p53 protein.

Transcription of the p21 gene is modulated by p53 and by other factors. To determine whether the observed induction of p21 is due to the elevated level of p53, we simultaneously depleted cells for p53 and NF90/NF45. Immunoblotting showed that siRNA targeting p53 effectively abrogated p53 accumulation and eliminated the induction of p21 by either D3 or D5 (Fig. 2d; compare lanes 5 and 6 with lanes 2 and 3, respectively). Similar results were observed at the RNA level (data not shown). We conclude that NF90/NF45 exerts negative posttranscriptional control over p53 in HeLa cells, with a consequent secondary effect on p21 transcription.

### Cell-type specificity

To assess the generality of this regulation, we evaluated the effect of NF90/NF45 on p21 and p53 levels in a panel of cell types. Cells were derived from cervical carcinoma (SiHa and C33A), osteosarcoma (U2OS), normal lung (WI38), and colon carcinoma (HCT 116). Immunoblotting showed that D3 depleted NF90 and NF45 in all cases, albeit to varying degrees, but only in SiHa cells were p21 and p53 induced (Fig. 3). Like HeLa cells, SiHa cells are derived from human cervix via transformation by high-risk human papillomaviruses (HPV-18 in HeLa and HPV-16 in SiHa). Notably, neither p53 nor p21 was induced in C33A cervical carcinoma cells, which are negative for HPV infection. These results implicate HPV in the NF90/NF45-mediated regulation of p21 and p53. Consistent with the p53 requirement for p21 induction observed above (Fig. 2d), NF90 knockdown by D3 failed to increase the level of p21 in p53<sup>-/-</sup> HCT 116 cells.

### NF90/NF45 regulates the expression of HPV E6

It is well established that HPV infection induces the rapid turnover of cellular p53 through interactions with the viral E6 oncogene and the cellular ubiquitin-protein ligase E6AP (54,

55). The E6 protein forms a complex with E6AP, also known as UBE3A, altering its substrate specificity and targeting p53 for degradation in the proteasome (Fig. 4a). We therefore examined the effect of NF90/NF45 depletion on E6 and E6AP. RT-PCR analysis showed that D3 siRNA reduced the expression of viral E6 RNA by ~90% but had no effect on E6AP RNA (Fig. 4b, left panel). We were unable to visualize E6 protein by immunoblotting, but found E6AP protein to be unaffected by NF90 knockdown (Fig. 4b, right panel).

The reduced level of E6 RNA could be due to decreased transcription of the HPV genome (Fig. 4c), increased RNA degradation, or both. NF90 interacts with several mRNAs, including those encoding the HPV early proteins E6 and E7 (Supplementary Fig. S2), and it plays a role in RNA decay (23, 38, 40, 44, 46, 47, 56). To monitor RNA decay, RNA was isolated from control and D3-treated HeLa cells at varying times after inhibition of transcription by actinomycin D. Analysis by northern blotting resolved two bands containing E6 RNA, which had roughly equivalent intensity at time zero (Fig. 4d). Comparable to RT-PCR results (Fig. 4b), D3 reduced the overall level of E6 RNA by ~66% in untreated cells (Fig. 4d; compare C and D3 at t=0 h). The upper band decayed somewhat faster than the lower band but the turnover rate was the same in control and D3-treated cells ( $t_{1/2} \sim 3$  h; Fig. 4d,e). These data indicate that NF90 does not significantly affect the stability of viral RNA.

To monitor transcription from the HPV promoter, we measured the effect of NF90/NF45 depletion on the expression of a firefly luciferase reporter driven by the HPV-18 upstream regulatory region (URR) responsible for E6 RNA synthesis (Fig. 4c). Renilla luciferase directed by the Rous sarcoma virus (RSV) promoter, which is not influenced by NF90 (21, 57), served as transfection control. At all concentrations tested, firefly luciferase expression was compromised in HeLa cells transfected with D3 or D5 siRNA by 60-70% when compared to control cells (Fig. 4f). No significant inhibition was observed in HPV-negative C33A cells (data not shown). The attenuated expression in NF90/NF45-depleted HeLa cells corresponds to the reduced level of E6/E7 RNA in these cells, indicating that HPV transcription is impaired. We therefore conclude that NF90/NF45 up-regulates the activity of the HPV early promoter.

### Involvement of P-TEFb

The cellular transcription factor P-TEFb, composed of cyclin T1 and CDK9, is important for HPV E6/E7 expression (58). Furthermore, recent data from our lab showed that its cyclin T1 subunit is regulated by NF90 (24). These findings suggested that P-TEFb may be involved in the regulation of E6 expression by NF90/NF45. To evaluate this possibility, we first examined the effects of siRNA-mediated cyclin T1 depletion. Immunoblotting showed that cyclin T1 siRNA reduced the level of cyclin T1 and consequently of CDK9 (24), but not of NF90, by 72 h post-transfection (Fig. 5a). Notably, both p53 and p21 proteins were induced, compatible with a role for P-TEFb in HPV transcription (58). As expected (24), D3 also down-regulated cyclin T1 and CDK9 although to a slightly lesser degree. These observations are consistent with the hypothesis that P-TEFb mediates the action of NF90/NF45 on the HPV promoter, but do not prove it.

We next conducted qRT-PCR analysis to compare the effects of NF90 and cyclin T1 depletion on E6 RNA at different time points. The results confirmed that D3 inhibited E6 RNA expression (by ~30% at 48 h and ~60% at 72 h), but cyclin T1 siRNA had no measurable effect on E6 RNA at 48 h and reduced it only slightly (by ~18%) at 72 h (Fig. 5b). These kinetic data imply that NF90 exerts a separate action on the HPV promoter, in addition to a relatively modest action mediated via cyclin T1.

A prediction of this scenario is that cyclin T1 over-expression should only partially rescue the inhibition of E6 expression brought about by D3. Transfection of cells with a vector expressing hemagglutinin (HA)-tagged cyclin T1 that is resistant to NF90 depletion (24) increased the total levels of cyclin T1 by ~2 fold, exceeding its level in untreated cells (Fig. 5c). The levels of NF90 and NF45 were unchanged (Fig. 5c), and cyclin T1 over-expression did not increase the level of NF90 RNA (Fig. 5d). The level of E6 RNA was also unaffected by cyclin T1 over-expression in control siRNA-treated cells (Fig. 5d). In D3-treated cells, however, E6 RNA increased to a small but significant extent (by 15%;  $P = 0.028$ ). We therefore conclude that NF90/NF45 regulates HPV transcription by both P-TEFb-dependent and P-TEFb-independent mechanisms, and that the latter mechanism predominates under these conditions.

### NF90/NF45 depletion enhances drug-induced apoptosis

p53 is responsible for critical cell functions including apoptosis, which is suppressed in HPV-transformed cell lines. Since *TP53* is wild-type in these cells (59) reactivation of p53 expression sensitizes them to apoptosis induced by anticancer drugs such as cisplatin and camptothecin (60, 61). Because p53 expression is markedly increased in NF90/NF45-depleted HeLa cells, we examined their susceptibility to apoptosis. Inspection under the microscope disclosed changes in the overall morphology of cells in D3-treated cultures compared to controls (Fig. 6a). Many cells were rounded and refractory, exhibiting features such as nuclear condensation and nuclear fragmentation that are characteristic of apoptosis, while few such cells were seen in cultures transfected with control siRNA. Treatment with 10  $\mu$ M camptothecin, a topoisomerase I inhibitor that induces apoptosis (60), markedly increased the number of rounded cells in control cultures and especially in D3-treated cultures (Fig. 6a).

Cleavage of poly ADP-ribose polymerase (PARP) by activated caspase-3 and caspase-7 occurs as a late event in the apoptotic pathway, generating 24-kDa and 89-kDa PARP fragments. D3 induced PARP cleavage, detected by immunoblotting for the 89-kDa fragment (Fig. 6b, compare lanes 1 and 2), and similar results were obtained with D5 (data not shown). Cleavage increased in D3-treated cells in the presence of low concentrations of camptothecin (lanes 3-6), and became detectable in control cells as well as D3-treated cells at higher drug concentrations ( $>0.5 \mu$ M; lanes 7-12). D3 cells contained more 89-kDa PARP than control cells irrespective of drug concentration, and the level of cleaved PARP invariably correlated with the induction of p53 (Fig. 6b). These data indicate a synergistic activation of the cleavage pathway by the drug and NF90/NF45 depletion.

The induction of PARP cleavage was also examined by flow cytometry (Fig. 6c). Cells were incubated in the presence or absence of camptothecin, then immunostained with



phycoerythrin (PE)-labeled antibody specific for the 89-kDa PARP fragment. D3 increased the staining for cleaved PARP only slightly on its own, presumably because a small increase in cleaved PARP in a large number of cells is undetectable above the fluorescence background. In camptothecin-treated cells D3 increased the intensity of staining as well as the proportion of strongly staining cells (from ~9% to ~24%; Fig. 6c). Similar results were obtained with Annexin V, which detects phosphatidylserine on the outer cell membrane, an early marker for apoptosis (62). In the absence of camptothecin ~2.5% of the cells were Annexin V-positive; in the presence of camptothecin, ~7.5% of control cells and ~17% of D3-treated cells were positive for this marker (Fig. 6d). These results show that D3 sensitized cells to camptothecin-induced apoptosis by ~3 fold, indicating that NF90/NF45 depletion induces both p53 and apoptosis in cervical cancer cell lines.

## Discussion

Results presented here show that the NF90/NF45 complex plays an important role in HPV early gene expression and hence in viral control over cell fate (apoptosis) via the key cellular regulators p53 and p21. High-risk HPVs exploit the NF90/NF45 complex to transcribe their E6 oncogene; the E6 protein, together with E6AP, induces degradation of p53; and functions mediated by p53 are suppressed, including p21 expression and apoptosis (Fig. 7). NF90/NF45 is a multifunctional adaptor complex that interacts with DNA, RNA and protein ligands and affects several cellular pathways. We therefore speculate that NF90/NF45 integrates signals from multiple sources and facilitates HPV-induced oncogenesis.

### NF90/NF45 functions in transcription

Our analysis indicates that NF90/NF45 regulates HPV E6 transcription predominantly through a P-TEFb-independent mechanism, and to a lesser extent via P-TEFb. NF90 and NF45 participate in gene expression pathways at many steps, including transcription. Initially purified as DNA binding proteins, they interact with the ARRE element of the interleukin-2 promoter and stimulate its transcription (63). Subsequently, the NF90/NF45 complex has been shown to regulate the activity of several cellular and viral promoters (21) through direct interactions with specific promoter sequences (22, 47, 64, 65). NF90/NF45 can also act indirectly, via other proteins that modulate chromatin structure and transcription (20, 24, 57, 66-69). Efficient transcription by RNA polymerase II depends on its phosphorylation by P-TEFb (70), and NF90 regulates P-TEFb by enhancing expression of the cyclin T1 subunit of this elongation factor (24). First shown to be required for processive transcription of the HIV-1 genome (71), P-TEFb has recently been implicated in the regulation of HPV gene expression. It is recruited to the HPV genome through interactions with the Brd4 protein that are modulated by the viral E2 product (58).

While the precise action of NF90/NF45 at the HPV promoter has yet to be identified, one possible mechanism involves the multifunctional factor Yin Yang-1 (YY1) for which there are binding sites in the HPV URR (72-74). YY1 acts as a repressor or activator depending on the promoter context (67, 72, 73, 75, 76) and serves to recruit the histone deacetylase complex and protein arginine methyltransferase 1 (PRMT1). *ILF3*-derived proteins interact with PRMT1, enhance its methylase activity (77, 78), and favor YY1-PRMT1 interactions

that stimulate transcription (67), although the data for NF90 itself are not unequivocal. The HPV URR also contains sites for several other factors, however, and NF90 engages in numerous interactions with transcriptional activators and co-activators, so further studies are needed to characterize the contributions of these sites and factors to the mechanism of NF90 action at the HPV promoter.

### Regulation of p53, p21 and apoptosis

Depletion of NF90 or NF45 induces the expression of p21 RNA and protein in HPV-transformed cells, whereas depletion of NF110 was ineffective (Fig. S1). NF90 and NF110 differ substantially at their C termini and in their interactions with several ligands (18, 20, 79-81). Accordingly, different consequences ensue from specific knockdown of the two *ILF3* isoforms, either singly or in combination (18). For example, NF90 depletion leads to reduced DNA synthesis and a slow-growth phenotype whereas NF110 knockdown does not (18). Such differential effects could well account for the discrepancy between our observations and those obtained with homozygous knockout mice which lack both NF90 and NF110 (43). p21 is expressed during terminal differentiation (82, 83) and its expression was impaired in *ILF3* knockout pups with profound abnormalities in muscle development (43). Although p21 can be regulated in a variety of ways, our observations indicate that NF90/NF45 affects p21 transcription mediated by p53 in HeLa cells.

p53 is a pivotal regulator of cellular activities including proliferation (1, 84). Functional loss of p53, due to protein degradation or to *TP53* mutations, is a frequent event in cancer development (6) and a number of viruses escape cell cycle regulation by targeting p53 (59, 85-87). In cells infected with HPV, a leading cause of cervical cancer, the viral oncoprotein E6 binds to (wild-type) p53 and induces its degradation via E6AP-mediated polyubiquitination (13, 54, 55, 88). Our results demonstrate the role of NF90/NF45 in suppressing p53 expression in cell lines originating from HPV-infected tumors. Inhibition of E6 expression by NF90/NF45 knockdown represses the degradation of p53, and restores cell functions including apoptosis and the p53-dependent transcription of genes such as p21 (58, 89). p53 is required for efficient activation of apoptosis following irradiation and treatment with chemotherapeutic agents (90). Activation of p53-dependent apoptosis induces *Bax* expression, resulting in cytochrome c release from mitochondria and caspase-3 activation (91-93). Activated caspase-3 executes apoptosis by inducing cleavage of cellular substrates including PARP (93) and NF90/NF45 depletion in HeLa cells resulted in the accumulation of p53 and the 89-kDa cleavage product of PARP. Expression of p53 sensitizes cells to drug-induced apoptosis (49); correspondingly, camptothecin amplified the effect of NF90/NF45 depletion on PARP cleavage, indicating activation of p53-dependent apoptosis.

HeLa cells are highly resistant to apoptosis and expression of p53 is not sufficient to kill them (94). Correspondingly, p53 induction by NF90/NF45 knockdown did not elicit a detectable increase in apoptosis as measured by flow cytometric analysis of PARP cleavage or Annexin V staining. Although this could reflect a characteristic of single-cell assays, which require a threshold level for detection, it is also consistent with the need for a second signal such as that provided by camptothecin. Similarly, p53 induction by E6 knockdown elicited little cell death unless complemented by the drug celecoxib (94). It is also notable



that the slow-growth phenotype of HeLa cells depleted for NF90/NF45 (18) is not rescued by p53 knockdown (Fig. S3), consistent with the pleiotropic functions of NF90 and NF45 in gene expression and cell metabolism.

### NF90/NF45 and virus infection

Our data appear to be the first to establish a connection between NF90/NF45 and HPV gene expression. Human NF90/NF45 participates in numerous biological processes that impinge on virus infection, including the cell cycle (18, 95) and antiviral defense mechanisms (39, 96-98), and it plays a role in the life cycle of several viruses (28, 29, 34, 99). In transient expression assays, NF90 in association with NF45 inhibited transcription from the adenovirus-2 major late promoter, but activated transcription from the cytomegalovirus immediate early promoter (21) as with the HPV early promoter (Fig. 4f). Interactions of NF90 and NF45 with viral proteins that affect viral replication have been observed in influenza virus H5N1, Ebola virus and infectious bursal disease virus (32-34). NF90 is present in protein complexes binding to the non-translated regions (NTRs) of bovine viral diarrhea virus (BVDV), hepatitis C virus (HCV), rhinovirus, and dengue virus, where it functions in viral replication and gene expression (28, 29, 100, 101). Interactions with BVDV and HCV NTRs induce the formation of circular genome structures essential for translation and replication. NF90/NF45 binds to the dengue virus 3' NTR, contributing to the production of infectious virus particles, and it also interacts with hepatitis B virus transcripts (102) and with adenovirus VA RNA (103). By analogy, the interaction of NF90 with E6/E7 RNA (Fig. S2) could imply its involvement in post-transcriptional processes, such as the regulation of HPV gene expression, that await definition.

In conclusion, NF90/NF45 is a multi-domain complex that participates in numerous cellular processes and is harnessed by many viruses to promote their replication. Our findings have revealed a novel role for this complex in HPV-induced oncogenesis and p53-mediated apoptosis. Specifically, the NF90/NF45 complex acts as a positive regulator of HPV E6 expression, and NF90/NF45 depletion results in the accumulation of active p53. Targeting the function of NF90/NF45 could therefore be exploited as a means to combat viral infection and cancer.

## Materials and Methods

### Cell culture and transient expression assays

Cell lines C33A, HeLa, SiHa, U2OS and WI38 lines were obtained from ATCC and HCT116 cells from Dr. Betsy Barnes (UMDNJ-NJMS). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Sigma) and 1% penicillin/streptomycin (Invitrogen). Transfection was performed with  $1.5\text{-}2.5 \times 10^5$  cells using 30 or 50 nM of small interfering RNA (siRNA) and INTERFERin (Polyplus-transfection). At 48 h or 72 h posttransfection, cells were washed with phosphate buffered saline (PBS; Cellgro) and harvested by trypsinization. siRNA sequences used for knockdown experiments were control (C): On-TARGET<sup>plus</sup> Non-Targeting siRNA (Ambion); D3 (NF90) and D5 (NF45) (18); D6 (NF45), 5'-ACTTTGATATCCAAATGGAGT-3'; p53, 5'-GACTCCAGTGGTAATCTACT-3'. For

over-expression studies, at 8 h post siRNA transfection, cells were transfected with 1 µg of pcDNA3.1-siRNA-resistant-NF90b (Omni-NF90b<sup>D3R</sup>), pcDNA3.1-NF45 or pcDNA3.0-HA-cyclin T1 (24) using jetPEI (Polyplus-transfection).

### Western blotting

Cell extract was prepared (18) and analyzed by immunoblotting (19) using antibodies directed against NF90 (DRBP76; BD Biosciences), NF45 (Everest), p21 (Ab-1; Calbiochem, and C-19; Santa Cruz), p53 (Ab-6; Calbiochem), E6AP (E4; Santa Cruz), cyclin T1 (T18; Santa Cruz), CDK9 (C-20; Santa Cruz), 89-kDa N terminus PARP (BD Biosciences), full-length PARP-1/2 (H-250; Santa Cruz), tubulin (Sigma), and actin (Santa Cruz).

### Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated using RNeasy RNA isolation kit (Qiagen). Complementary DNA (cDNA) synthesis and PCR were performed in a single reaction tube with 50 ng of RNA using the Qiagen OneStep RT-PCR kit (Qiagen) with annealing at 62°C for 1 min. Amplified DNA was resolved by electrophoresis in a 1.5% agarose gel. Primer sequences were as follows. NF90: forward primer (FP) 5'GATGGCAATTCATTCGAGGC3', reverse primer (RP) 5'GCAAGAGCAGCGTAGGCCTTC3'; NF45: FP 5'GAATCAGGACCTGGCTCCCA3', RP 5'GGTCAAGGATCCAGGGTGTG3'; p21: FP 5'GTCAGTTCCTTGTGGAGCCG3', RP 5'CTCCAGTGGTGTCTCGGTG3'; p53: FP 5'GTTTCCGTCTGGGCTTCTTG3', RP 5'ACGCAAATTCCTTCCACTCGG3'; E6AP: FP 5'CGCATGTACAGTGAACGAAGA3', RP 5'CTTTGGAAACACCTCCCTCA3'; HPV6: FP 5'CCAGAAACCGTTGAATCCAG3', RP 5'GTTGGAGTCGTTCTGTCGT3'; Cyclin T1: FP 5'ATGGAGGGAGAGAGGAAGAAC3', RP 5'CTACCTTGATGACATGTTCCA3'; AuroraB: FP 5'TCCGGAAAGAGCCTGTCACC3', RP 5'CACAGACCAGCCGAAGTCAG3'; Matrin-3: FP 5'GATGCAATGGCAATGGTTGACC3', RP 5'TCGTCTCCCACTGAACTGCC3'; ORC6: FP 5'GGACAGCAGGTCGACAGAG3', RP 5'AGCCCACTGCCAGACTTAG3'; Survivin: FP 5'GAACTGGCCCTTCTTGAGG3', RP 5'GTGGCACCAGGGAATAAACCC3'; TERC: FP 5'CGCGCTGTTTTTCTCGCTGAC3', RP 5'GACTCGCTCCGTTCTCTTC3'; GAPDH: FP 5'CTGGTAAAGTGGATATTGTTG3', RP 5'GAGGCTGTTGTCATACTTCTC3'.

### Quantitative real-time PCR (qRT-PCR)

cDNA was synthesized from 2 µg of RNA hybridized with 0.5 µg of oligo dT (15-mer; Sigma). Reverse transcription was performed at 37°C for 1 h in the presence or absence of Moloney murine leukemia virus reverse transcriptase (Promega). qPCR was performed in duplicate 25 µl reactions on 1/8<sup>th</sup> of the cDNA using 0.3 µM primers and Maxima SYBR Green/ROX (Fermentas) PCR master mix in StepOnePlus Real-Time PCR system (Applied Biosystems).

### RNA turnover assay

At 72 h after siRNA transfection, cells were treated with 0.5 µg/ml of actinomycin D and total cellular RNA was isolated at intervals. Denatured RNA (10 µg) was subjected to formaldehyde-agarose gel electrophoresis, transferred to GeneScreenPlus hybridization membrane and crosslinked to the membrane by UV irradiation (UVXL-1000, Fischer Scientific). The immobilized RNA was hybridized with random-prime labeled E6 probe at 42°C and radioactive signals were detected by imaging (Typhoon 8600 scanner, Molecular Dynamics) and autoradiography.

### Luciferase assays

HeLa cells ( $2 \times 10^5$ ) transfected with C, D3 and D5 siRNA were transfected 48 h later with 200 or 400 ng of pGL3-HPV18URR-Firefly luciferase (104) and 200 ng of RSV-Renilla luciferase (21) using jetPEI transfection reagent. Luciferase expression was analyzed 24 h after transfection, using the Dual-Luciferase Reporter assay system (Promega).

### Flow cytometry

At 72 h post siRNA transfection,  $2 \times 10^5$  cells were treated with 10 µM camptothecin (MP Biomedicals) or left untreated for 14 h. The cells were harvested by trypsinization and washed twice with ice-cold PBS at (1000x g, 5 min, 4°C). For the analysis of PARP cleavage, cells were incubated with phycoerythrin (PE)-labeled mouse anti-cleaved PARP (89-kDa) antibody (BD Biosciences). For analysis of apoptosis-specific phosphatidylserine, cells were incubated with fluorescein isothiocyanate (FITC) conjugated Annexin V (BD Biosciences) and propidium iodide (PI; BD Biosciences). Stained cells were analyzed by FACSCalibur (BD Biosciences).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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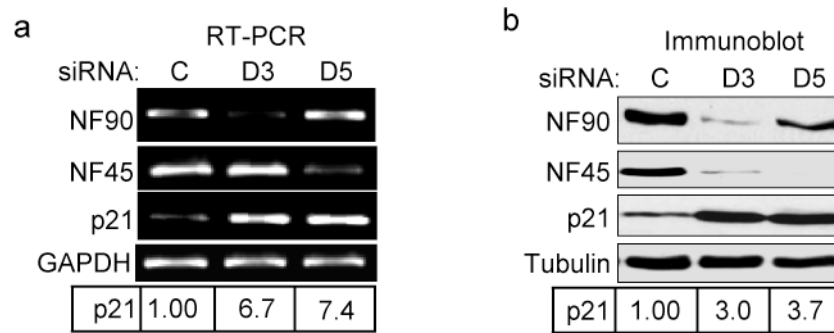
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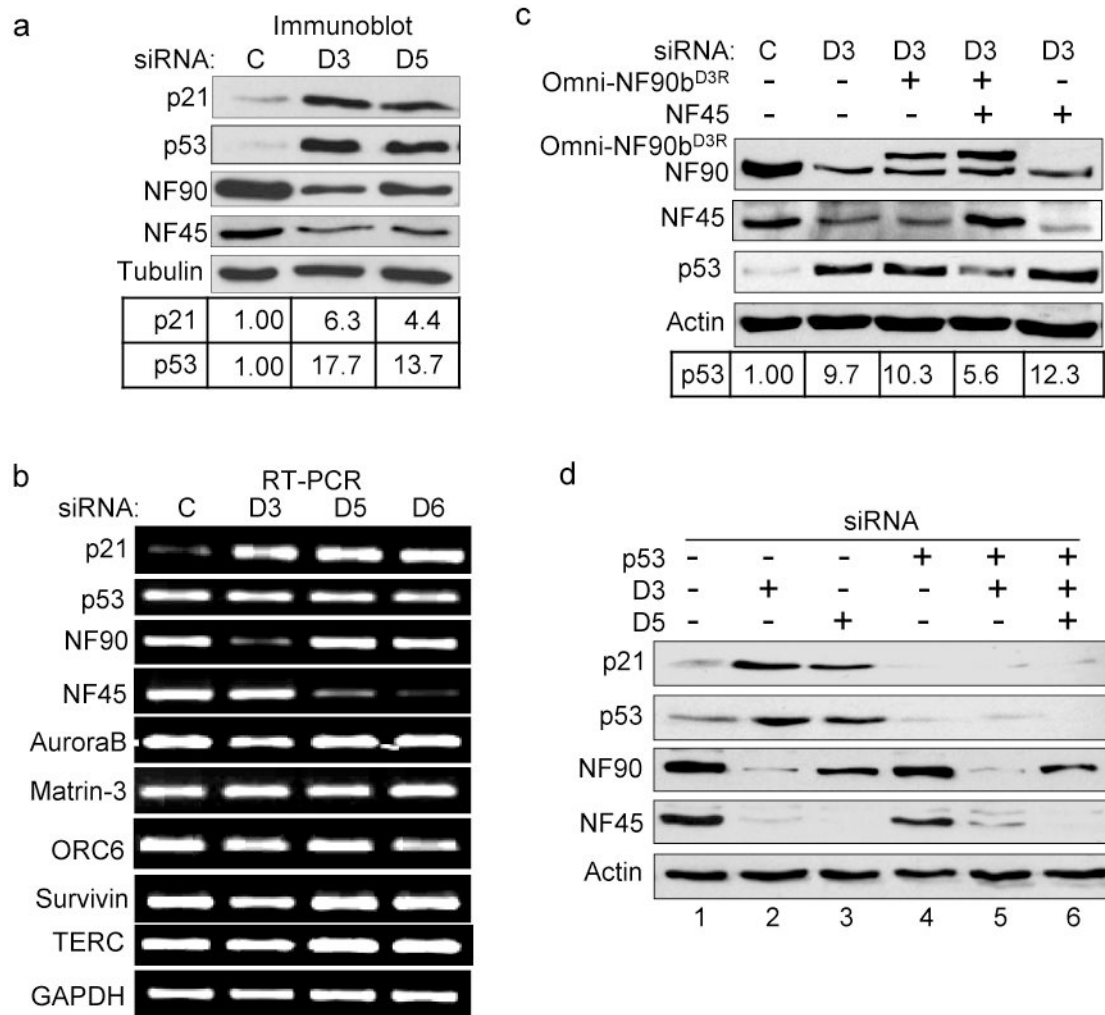
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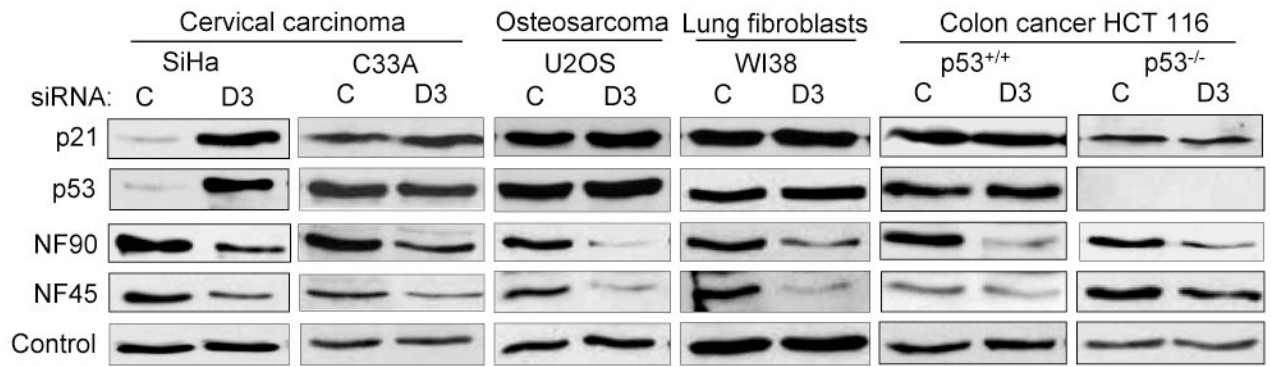
**Fig. 1. Knockdown of NF90 or NF45 causes p21 up-regulation**

HeLa cells were transfected with siRNA directed against NF90 (D3) or NF45 (D5) or control sequence (C). (a) RT-PCR amplified products indicating levels of NF90, NF45, p21 and GAPDH RNA. (b) Immunoblots showing induction of p21 protein in NF90 and NF45 knockdown cells compared to tubulin. Relative expression of p21 RNA and protein in the experiments shown was normalized to GAPDH and tubulin, respectively.



**Fig. 2. Induction of p53 and p21 by NF90/NF45 depletion in HeLa cells**

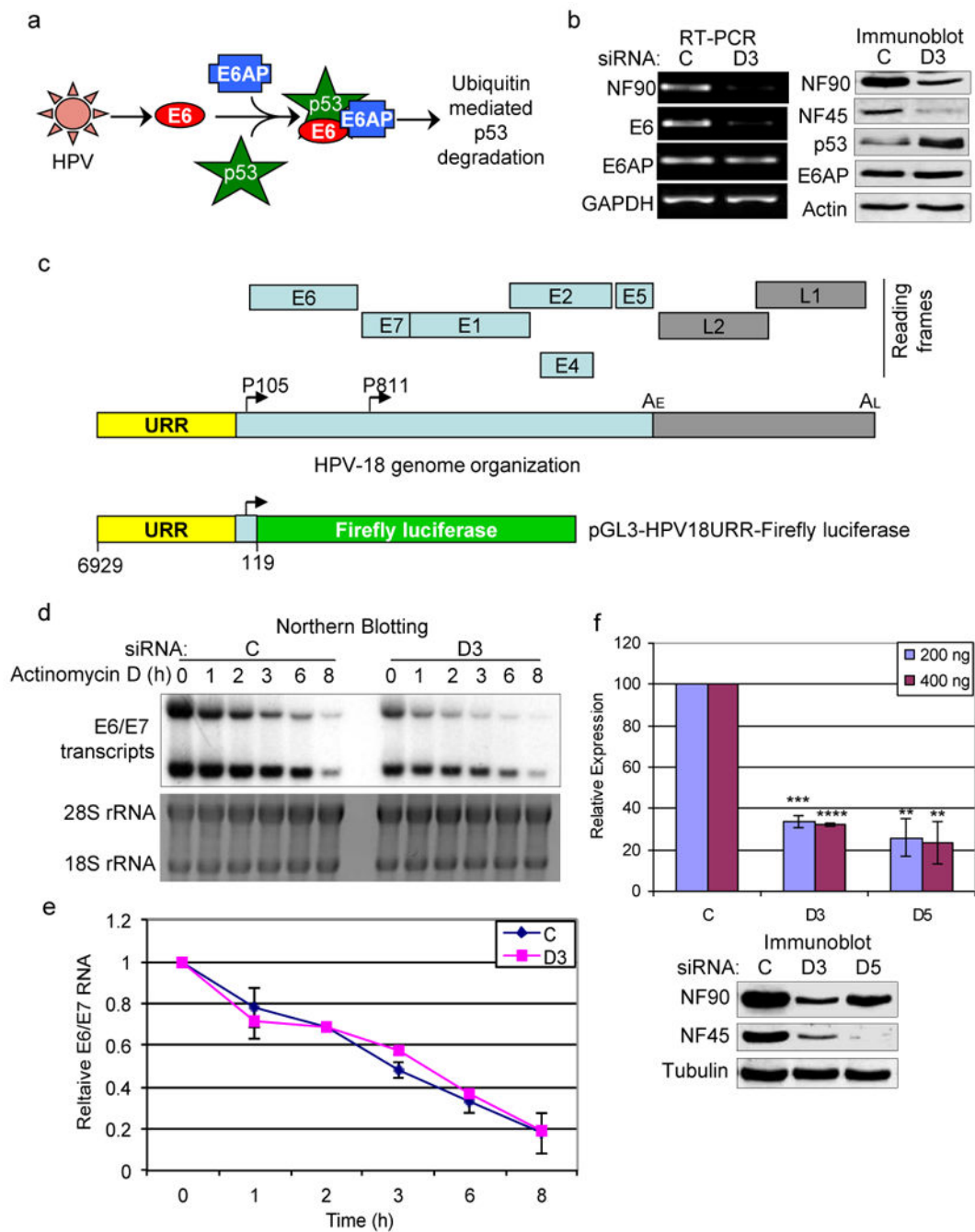
(a) Immunoblots showing the induction of p21 and p53 proteins in NF90 and NF45 knockdown cells. Relative expression of p21 and p53 proteins were normalized to tubulin. (b) RT-PCR analysis showing RNA levels of cellular genes in siRNA-transfected cells in NF90 and NF45 knockdown cells (D6 is a NF45-specific siRNA). (c) Expression of p53 depends on NF90/NF45. Immunoblots from cells transfected with siRNA to deplete the NF90/NF45 complex, in the absence or presence of plasmids expressing siRNA-resistant-NF90b (Omni-NF90b<sup>D3R</sup>) and over-expressing NF45. Relative expression of p53 protein was normalized to actin. (d) p53 regulates the expression of p21 in NF90/NF45-depleted cells. Immunoblots show that p53-specific siRNA prevents the induction of both p53 and p21 in cells transfected with D3 or D5.



**Fig. 3. Regulation of p21 and p53 expression by NF90/NF45 in HPV-infected cells**

Immunoblots showing the induction of p21 and p53 in cervical carcinoma cells derived from HPV16 infection (SiHa) – but not in non-HPV cervical carcinoma cells (C33A), non-cervical carcinoma cells (U2OS, HCT116) or normal lung fibroblasts (WI38) – depleted for NF90/NF45 complex by D3 transfection.





**Fig. 4. NF90 regulates HPV E6 oncogene expression**

(a) Schematic showing HPV-mediated p53 degradation via E6 and E6AP. (b) Knockdown of NF90 suppresses the expression of HPV18 E6 RNA in HeLa cells. Left panel: RT-PCR indicating the levels of viral E6 and cellular E6AP RNA in siRNA-transfected cells. Right panel: immunoblots showing the expression of proteins. (c) Diagrams of HPV18 genome organization (linear form) and open reading frames (top) (105) and of pGL3-HPV18URR-Firefly luciferase reporter construct (bottom). URR, upstream regulatory region; P105, early promoter; P811, late promoter; A<sub>E</sub>, early polyadenylation site; A<sub>L</sub>, late polyadenylation site;

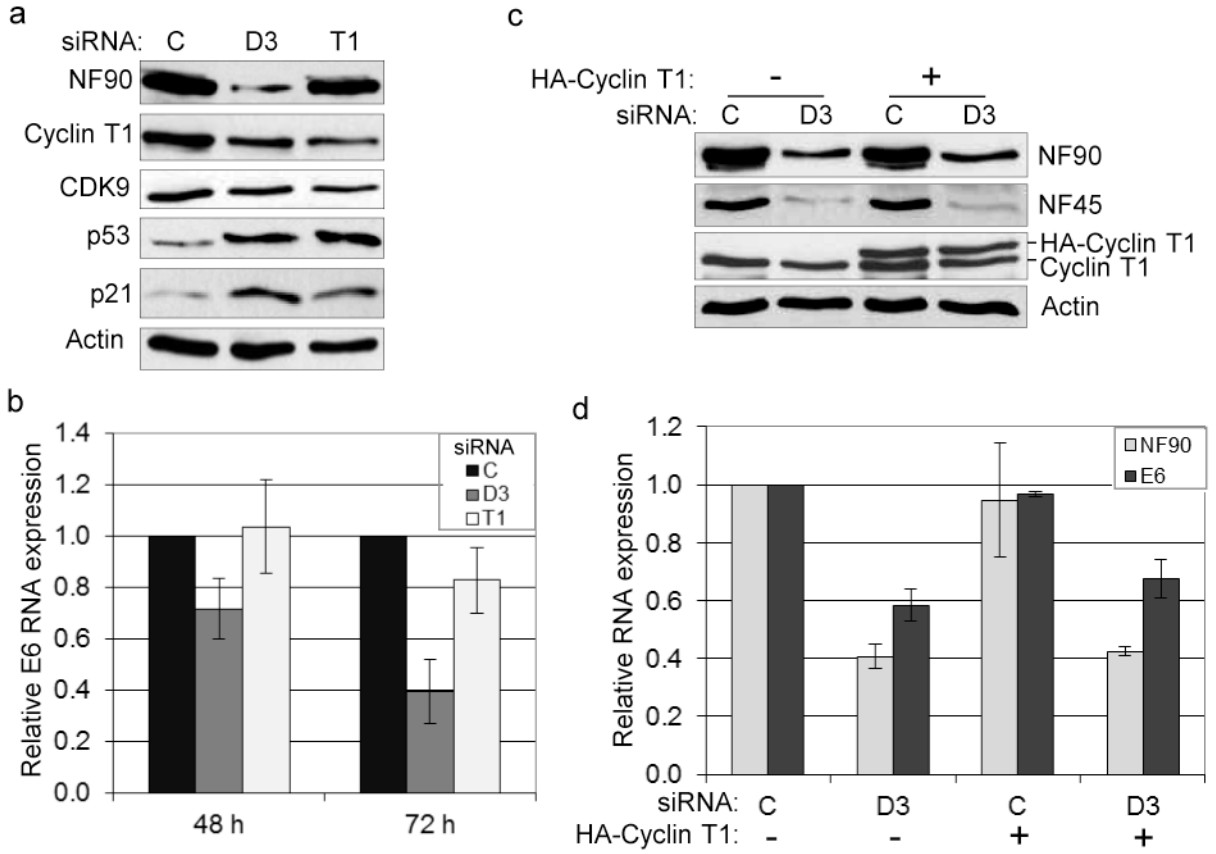
E1, E2, E4, E5, E6 and E7: early genes; L1 and L2, late genes. (d) NF90/NF45 depletion does not destabilize E6 RNA. Top panel: northern blot showing E6/E7 RNA in siRNA-transfected HeLa cells treated with actinomycin D (0.5 µg/ml) compared to untreated cells (0 h time point). Lower panel: 18S and 28S ribosomal RNA stained with ethidium bromide. (e) RNA half-life estimation. Quantitation of E6/E7 transcripts from two independent northern blotting experiments as in panel (d). (f) Depletion of NF90/NF45 inhibits the HPV18 early promoter. Expression of Firefly luciferase from pGL3-HPV18URR-Firefly luciferase plasmid normalized to *Renilla* luciferase expression in HeLa cells transfected with C, D3 and D5 siRNA (n=4 independent experiments). Standard deviations and *P* values are relative to respective controls: \*\*, < 0.01; \*\*\*, < 0.001; \*\*\*\*, < 0.0001. Similar results were obtained with 600 ng reporter plasmid. Immunoblot analysis for a representative experiment is shown.

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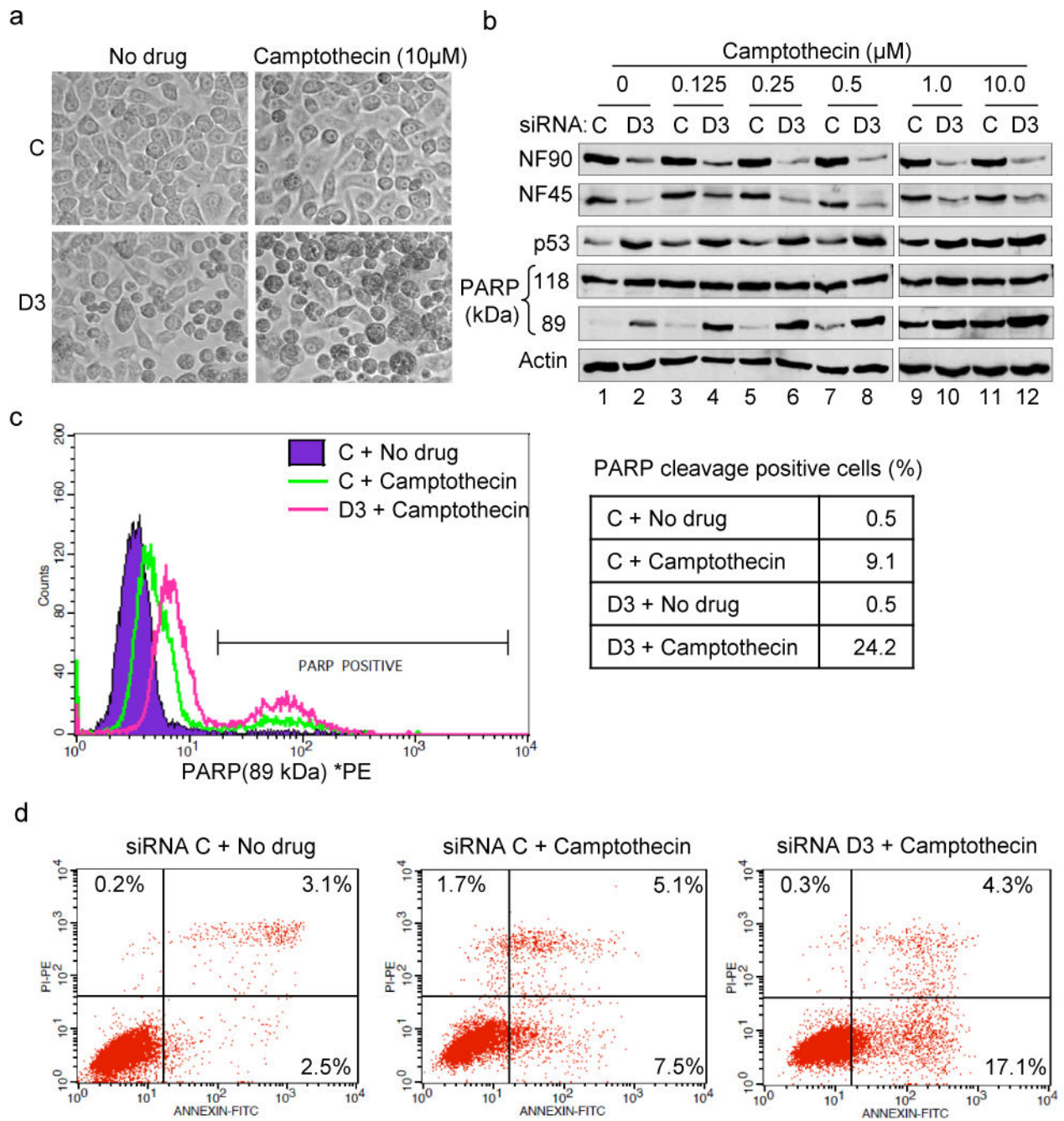
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**Fig. 5. Regulation of E6 expression by P-TEFb-dependent and -independent mechanisms**

(a) Immunoblots showing P-TEFb protein levels (cyclin T1 and CDK9) in HeLa cells treated with C, D3 and T1 (cyclin T1) siRNAs at 72 h post transfection. (b) qRT-PCR analysis of E6 RNA in cells treated as in panel (a) (n=3 independent experiments). (c) Expression of HA-cyclin T1 in NF90 knockdown cells. Immunoblots show the expression of NF90, NF45 and cyclin T1 in HeLa cells transfected with siRNA and pcDNA3.0 HA-cyclin T1. (d) qRT-PCR analysis of E6 RNA in cells transfected with siRNA and pcDNA3.0 HA-cyclin T1 as in panel (c) (n=2 independent experiments).



**Fig. 6. Depletion of NF90/NF45 sensitizes cells to camptothecin-induced apoptosis**

(a) Morphology of HeLa cells treated with siRNA and camptothecin. Phase-contrast images were observed with 20X objective. (b) Knockdown of NF90/NF45 enhances camptothecin-induced PARP cleavage and p53 induction. Immunoblot analysis of proteins in siRNA-transfected-HeLa cells treated with increasing concentration of camptothecin for 14 h. (c) *In vivo* PARP cleavage in cells treated with siRNA and camptothecin as in panel (a). Cells were stained with phycoerythrin (PE)-labeled PARP (89 kDa) antibody and analyzed by flow cytometry. Percentage of cells in each culture that were PARP-positive was the mean

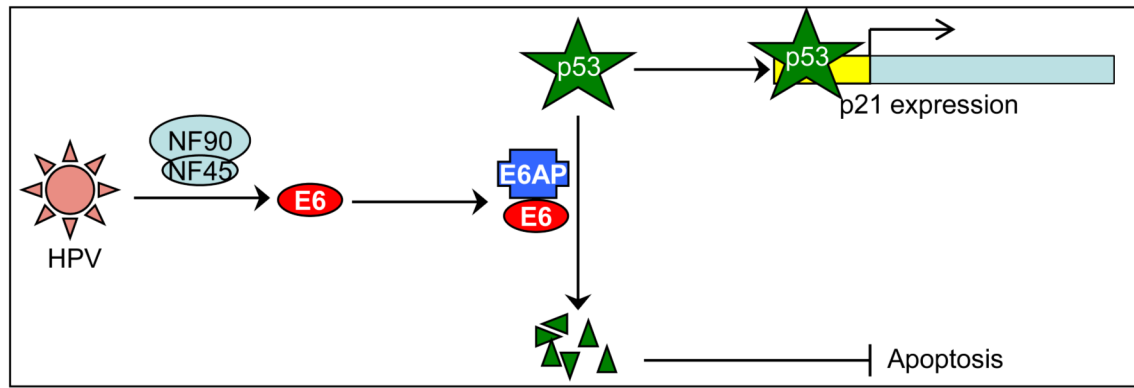
of triplicate analyses. Essentially superimposable profiles were obtained with control and D3-treated cultures in the absence of camptothecin. (d) NF90 depletion enhances camptothecin-induced early apoptosis. Cells treated with siRNA and camptothecin as in panel (a) were stained with fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide. Dot plots represent the cells as analyzed by flow cytometry. Control and D3-treated cultures gave indistinguishable plots in the absence of camptothecin. Percentages of cells in each quadrant (top left, necrosis; top right, late apoptosis; bottom right, early apoptosis) are mean values of triplicate analyses.

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**Fig. 7. Model for NF90/NF45-dependent p53 regulation in HPV-infected cells**

The NF90/NF45 complex mediates expression of the E6 oncoprotein from the viral genome. With E6AP, the viral E6 protein induces degradation of p53 by the ubiquitin-proteasome degradation pathway. Degradation of p53 results in the suppression of apoptosis and reduced p21 expression.