ZNF668 Functions as a Tumor Suppressor by Regulating p53 Stability and Function in Breast Cancer

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Abstract

Genome-wide sequencing studies in breast cancer have recently identified frequent mutations in the zinc finger protein 668 (ZNF668), the function of which is undefined. Here, we report that ZNF668 is a nucleolar protein that physically interacts with and regulates p53 and its negative regulator MDM2. Through MDM2 binding, ZNF668 regulated autoubiquitination of MDM2 and its ability to mediate p53 ubiquitination and degradation. ZNF668 deficiency also impaired DNA damage–induced stabilization of p53. RNA interference–mediated knockdown of ZNF668 was sufficient to transform normal mammary epithelial cells. ZNF668 effectively suppressed breast cancer cell proliferation in vitro and tumorigenicity in vivo. Taken together, our studies identify ZNF668 as a novel breast tumor suppressor gene that functions in regulating p53 stability. Cancer Res; 71(20); 6524–34. ©2011 AACR.

Introduction

Human cancer develops as a result of accumulation of mutations in oncogenes and tumor suppressor genes (1). Through genome-wide gene sequencing, many genes that are frequently mutated in breast tumor samples have been discovered (2, 3). Among these mutated genes, a hypothetical protein (FLJ13479) later named zinc finger protein 668 (ZNF668) was identified and validated as one of the highly mutated genes in breast cancer (2, 3). ZNF668 gene mutations were found in 4 of 35 breast cancer samples analyzed (11.4%; ref. 2). ZNF668 belongs to the krüppel C2H2-type zinc finger protein family and contains 16 C2H2-type zinc fingers. So far, the function of ZNF668 has remained entirely unexplored. To understand the role of ZNF668 in breast cancer development, we used a proteomics-based approach to systematically identify ZNF668-binding proteins. A nucleolar protein, nucleophosmin (NPM, B23), was pulled down by ZNF668, and interaction between ZNF668 and NPM was confirmed by immunoprecipitation.

NPM is a nucleolar phosphoprotein constantly shuttling between the nucleus and cytoplasm (4, 5). It is involved in ribosome biogenesis and can function as either an oncogene or a tumor suppressor. Recent studies showed that NPM was involved in regulation of the p53 tumor suppressor protein (6, 7). NPM has been shown to bind to MDM2 and protect p53 from MDM2-mediated degradation. Researchers have also proposed that the nucleolus, where most NPM resides, functions as a cellular stress sensor that integrates a variety of cellular stresses to trigger p53 responses and regulate the role of p53 in tumor suppression (8, 9, 10); the nucleolus plays an important role in p53 stabilization under stress conditions. Indeed, subcellular redistribution of NPM and other nucleolar proteins, such as ARF, has functioned importantly on p53 regulation (5, 7, 11–13). Therefore, the interaction between ZNF668 and NPM and the nucleolar localization of ZNF668 (Fig. 1C–E) suggested that ZNF668 might be involved in p53 regulation.

p53 plays a critical role in preventing damaged cells from transforming into malignant cells (14). In normal cells, p53 level is tightly controlled by its negative regulator, the E3 ubiquitin ligase MDM2 (15–18). Ubiquitination of p53 by MDM2 leads to export of p53 from the nucleus to the cytoplasm followed by proteasomal degradation (19, 20). Under cellular stresses, such as genetic alterations, DNA damage stress, oncogene activation, and hypoxia, dissociation of MDM2 and p53 stabilizes p53 protein, and stabilization of p53 in turn leads to various cellular responses including cell-cycle arrest, DNA damage repair, and apoptosis (21).

In this study, we showed a functional interaction among ZNF668, p53, and MDM2. Overexpression of ZNF668 significantly increased p53 level and stability. Inversely, ZNF668 depletion decreased both the basal p53 and the stress-induced p53 levels. ZNF668 bound to MDM2 to prevent MDM2-mediated p53 ubiquitination and degradation. Notably, we showed the ability of ZNF668 to suppress breast cell transformation in vitro and tumorigenicity in mice. Together, our study identifies ZNF668 as a novel tumor suppressor gene in...
breast cancer and reveals its mechanistic function in regulating the MDM2–p53 interaction.

Materials and Methods

Cell culture and transfection

U2OS cells and breast cancer cells were from American Type Culture Collection. U2OS cells were maintained in McCoy’s 5A medium (Cellgro) with 10% FBS. MCF7, MCF7-control, and MCF7-p53 knockdown cells were maintained in Dulbecco’s Modified Eagle’s Media (DMEM; Cellgro) with 10% FBS. MCF10A and human mammary epithelial cells (HMEC) were grown in MEGM medium with MEGM SingleQuots growth factors (Lonza). Cells were incubated at 37°C in a humidified incubator with 5% CO₂ and transfected with Fugene 6 (Roche), Lipofectamine 2000 (Invitrogen), or Oligofectamine (Invitrogen). Cell lines were stained with specific antibodies as indicated. Nuclei were visualized with DAPI Scale bars, 20 μm. U2OS and MCF7 cell lysates were immunoprecipitated (IP) and immunoblotted (IB) with antibodies as indicated. DAPI, 4',6-diamidino-2-phenylindole; NS, nucleostemin.
Beaton Institute for Cancer Research, Glasgow, UK). The identity of the plasmids was confirmed by sequencing at the MD Anderson Cancer Center DNA Analysis Core Facility.

**Antibodies and reagents**

Nucleotides (548–1,449) were subcloned into pGEX-4T and the protein product was used for immunization for anti-ZNF668 antibody (Proteintech Group, Inc.). Anti-FLAG M2-agarose affinity gel was from Sigma. Anti-p53 (FL-393), anti-MDM2 (SMP14), anti-NPM (C-19), anti-p53 (FL393), anti-p53-agarose (Sigma). Glutathione agarose beads was from Cell Signaling. Anti-ubiquitin (FK2) and an ubiquitinylation kit were from BioMol. Anti-NS (MAB4311) was from Chemicon. Anti-V5 (ab9116) and anti-NPM (ab10530) were from Abcam. A thrombin cleavage capture kit was from Novagen. Cycloheximide was from Sigma. Anti-ubiquitin HRP (SC-126), and anti-p21 (C-19) were from Santa Cruz Biotechnology. Anti-p53 (FL393), anti-p53-agarose affinity gel was from Sigma. Anti-p53 (FL-393), anti-FLAG M2-agarose capture kit was from Novagen. Cycloheximide was from Sigma and used at 50 μg/mL (U2OS cells) or 20 μg/mL (MCF7 cells). MG132 (carbobenzoxy-o-leucyl-l-leucyl-l-leucine) was from EMD Biosciences and used at 10 μmol/L. Nutilin-3 was from Cayman Chemical and used at 10 μmol/L. The ON-TARGET plus ZNF668 (siRNA 1: GUGCCAGCGACUUUCGAUU; siRNA 2: AAGGCCAUACCAGCGGAUU) nontargeting and HDMD2 siRNA SMARTpool were from Dharmacon. Lentiviral vector–based MISSION shRNAs targeting ZNF668 and control were from Sigma. DNA was purified with glutathione agarose (Sigma). Glutathione agarose beads was from Cell Signaling. Anti-ubiquitin (FK2) and an ubiquitinylation kit were from BioMol. Anti-NS (MAB4311) was from Chemicon. Anti-V5 (ab9116) and anti-NPM (ab10530) were from Abcam. A thrombin cleavage capture kit was from Novagen. Cycloheximide was from Sigma and used at 50 μg/mL (U2OS cells) or 20 μg/mL (MCF7 cells). MG132 (carbobenzoxy-o-leucyl-l-leucyl-l-leucine) was from EMD Biosciences and used at 10 μmol/L. Nutilin-3 was from Cayman Chemical and used at 10 μmol/L. The ON-TARGET plus ZNF668 (siRNA 1: GUGCCAGCGACUUUCGAUU; siRNA 2: AAGGCCAUACCAGCGGAUU) nontargeting and HDMD2 siRNA SMARTpool were from Dharmacon. Lentiviral vector–based MISSION shRNAs targeting ZNF668 and control were from Sigma. RNA interference was carried out by Lipofectamine 2000 (MCF7) or Oligofectamine (U2OS).

**Immunoblotting and immunoprecipitation**

Immunoblotting and immunoprecipitation were carried out as described before (24). Cells were extracted in radioimmunoprecipitation assay buffer and immunoprecipitated with specific antibodies. The immunocomplexes were collected on Protein A/G plus conjugated agarose beads (Santa Cruz Biotechnology). The nuclear extracts were prepared in 20 mmol/L HEPES (pH = 7.9), 1.5 mmol/L MgCl2, 100 mmol/L KCl, 420 mmol/L NaCl, 0.2 mmol/L EDTA, and 1 mmol/L dithiothreitol.

**In vitro GST protein–binding assay**

The GST-ZNF668, GST-MDM2, and GST-p53 fusion proteins were expressed in *Escherichia coli* strain BL21 and purified with glutathione agarose (Sigma). Glutathione S-transferase (GST) fusion proteins harboring p53 and MDM2 were cleaved and purified with a thrombin cleavage capture kit. Purified proteins were incubated in 300 μL of binding buffer [25 mmol/L Tris-HCl (pH = 7.5), 50 mmol/L NaCl, and 0.2% NP-40]. Proteins were recovered (2 hours at 4°C) with glutathione agarose beads. Beads were washed extensively with washing buffer [100 mmol/L Tris-HCl (pH = 8.0), 100 mmol/L NaCl, and 1% Nonidet P-40], eluted with 10 mg/mL reduced glutathione (pH = 8.0) and subjected to Western blotting.

**In vitro proliferation and soft agar assay**

**In vitro** proliferation assay and soft agar assay were conducted as described before (24). To measure cell proliferation, cells were plated in 96-well plates, and MTT substrate (2 mg/mL) was added into the culture medium. Four hours later, the optical density was measured spectrophotometrically at 490 nm. For soft agar assay, cells were resuspended in DMEM containing 0.35% agarose (ISC BioExpress GenePure LE) and 10% FBS and seeded onto a coating of 0.5% agarose in DMEM containing 10% FBS. Colonies were counted 2 to 4 weeks later.

**Tumor growth in nude mice**

All animal studies were conducted in compliance with animal protocols approved by the MD Anderson Institutional Animal Care and Use Committee. Before injection of MCF7 cells, 6-week-old female nude mice were implanted subcutaneously with 0.72 mg of 17β-estradiol 60-day release pellets (E2 pellet; Innovative Research of America). Mice were injected in the mammary glands with 2 × 106 cells from various cell lines in 100 μL of PBS. After 1 week, tumors were measured every 3 days. Each cell line was tested in 5 different animals. Volume was calculated as $W^2 \times L \times 0.52$.

**Results**

**ZNF668 localizes in the nucleus, accumulates in the nucleolus, and interacts with NPM and nucleostemin**

V5-tagged ZNF668 construct was induced to ectopically express ZNF668 protein in different cell lines including MCF7 and MCF10A and HMECs. Western blotting revealed a 70- to 80-kD V5-tagged band in all ZNF668-overexpressing cell lines. Through motif analysis, 2 consensus nuclear localization sequences (R/K-R/K-X-R/K) were detected in the N-terminal of ZNF668 protein (Fig. 1A), suggesting that ZNF668 is a nuclear protein. A rabbit polyclonal antibody was raised against a GST-ZNF668 fusion protein (183–483 amino acids) and used for Western blotting. This ZNF668 antibody also detected a band between 70 and 80 kD with its expression enriched in the nuclear fraction (Fig. 1B). The specificity of our ZNF668 antibody was verified by siRNA knockdown as well as overexpression of V5-tagged ZNF668 (data not shown).

Immunofluorescence analysis revealed specific nuclear staining for ZNF668 with strong signals in one or more nucleolus-like nodular structures in both MCF7 and MDA-MB-468 cells (Fig. 1C, top 2 rows). The specificity of the staining pattern was supported by V5 antibody staining in MCF7 cells and HMECs with overexpression of V5-tagged ZNF668 (Fig. 1C, bottom 2 rows), and the nucleolar localization of ZNF668 was confirmed by colocalization of ZNF668 with 2 known nucleolar proteins, nucleostemin and NPM (Fig. 1C).

To systematically identify proteins involved in ZNF668 function, we carried out immunopurification–immunoblotting by mass spectrometry. We found that NPM was one of the major ZNF668-associated proteins (data not shown). To validate the mass spectrometry result, we conducted immunoprecipitation-Western blotting and found that ZNF668 was coprecipitated with NPM and nucleostemin (Fig. 1D). The interaction between ZNF668 and NPM was further confirmed by reciprocal immunoprecipitation with ZNF668 or NPM antibody (Fig. 1E). These results were consistent with results on immunofluorescent staining (Fig. 1C) and strongly...
suggested that ZNF668 localizes in the nucleus, accumulates in the nucleolus, and interacts with nucleolar proteins.

**ZNF668 interacts with MDM2 and p53**

It has been shown that NPM and nucleostemin bind to and regulate the protein stability and function of p53 (6, 7, 25, 26). Therefore, we conducted immunoprecipitation/Western blotting to test whether ZNF668 also interacted with p53. We found that ZNF668 interacted with p53 when ZNF668 was ectopically expressed in U2OS human osteosarcoma cells (Fig. 2A and B). Interestingly, ZNF668 also interacted with the negative regulator of p53, MDM2 (Fig. 2A and B). The interactions between ZNF668, p53, and MDM2 were further confirmed by reciprocal immunoprecipitation with ZNF668, p53, and MDM2 antibodies (Fig. 2C), supporting the physical interactions among these 3 proteins in cells. *In vitro* GST–protein–binding assay also confirmed the interactions among these 3 proteins (Fig. 2D).

To map the binding domain on ZNF668, we expressed FLAG-tagged wild-type ZNF668 and ZNF668 mutants lacking different parts of amino acid sequences in U2OS cells (Fig. 3). We found that both MDM2 and p53 could be coprecipitated with wild-type ZNF668, but their binding to ZNF668 with N-terminal deletions was much weaker than their binding to ZNF668 with C-terminal deletions (Fig. 3B). Amino acid regions 84 to 190 and 268 to 367 were particularly important for the interaction between MDM2 and ZNF668: When these 2 regions were deleted, the MDM2-ZNF668 interaction was abolished. Interestingly, these 2 regions were also important for the interaction between p53 and ZNF668. Notably, immunofluorescent staining showed that the ZNF668 deletions lacking amino acid regions 84 to 190 and 268 to 367 were localized exclusively outside the nucleolar region, indicating that these 2 regions contain the nucleolar localization signals for ZNF668 (Fig. 3A). These results identified the interaction domain of ZNF668 required for the interaction between MDM2, p53, and ZNF668 and indicated the importance of the nucleolar localization of ZNF668 for its interactions with MDM2 and p53.

To examine whether the cancer-derived ZNF668 mutants have impaired interaction with MDM2, FLAG-tagged ZNF668 vectors that harbored the identified mutations (R556Q and A66T) were constructed through site-directed mutagenesis. The interaction between MDM2 and ZNF668 mutants was reduced compared with wild-type ZNF668 (Fig. 3C). In our mapping data (Fig. 3A), we identified the regions that are essential for ZNF668 and MDM2 interaction. Surprisingly, these ZNF668 mutants are not located at the essential binding regions. These results, indeed, are consistent with the notion that the ZNF668 mutants did not completely abolish their interactions with MDM2. We suspect that these mutations on ZNF668 may affect the formation of proper protein structure that, in turn, displays certain impact on protein–protein interactions.

Conversely, we also sought to map the MDM2 domains that are required for ZNF668 binding. We expressed MDM2 deletion proteins in U2OS cells that constantly expressed FLAG-ZNF668. MDM2 has been shown to bind to p53 through the N-terminal domain (17). Although FLAG-tagged ZNF668 was coprecipitated with wild-type MDM2, an MDM2 protein carrying a p53-binding domain deletion (MDM2 Δ58–89), and an MDM2 protein carrying a C-terminal RING finger deletion

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**Figure 2.** ZNF668 interacts with MDM2 and p53. A and B, U2OS cells were transfected with control or expression vectors as indicated. Forty-eight hours later, cells lysates (3 mg) were immunoprecipitated by M2-FLAG and analyzed. The input was 3% of that used in the immunoprecipitation (IP). C, MCF10A cell lysates were immunoprecipitated with antibodies as indicated. The input was 5% of that used in the immunoprecipitation. D, cleaved MDM2 or p53 protein was mixed with GST-ZNF668 and subjected to glutathione beads pull-down. The protein complexes were analyzed by immunoblotting (IB). HRP, horseradish peroxidase.
(MDM2 Δ 9), the interaction between ZNF668 and an MDM2 protein with the central region deleted (MDM2 Δ 222–437) was defective (Fig. 3D and E and Supplementary Fig. S1). In contrast, we confirmed that p53 could form a complex with wild-type MDM2, MDM2 Δ 9, and MDM2 Δ 222–437 but not MDM2 Δ 58–89 (Supplementary Fig. S1). These results indicated that p53 and ZNF668 bound to different regions on MDM2. Further analysis of mutations within the central region of MDM2 showed that deletion of amino acids 212 to 296 significantly reduced the binding between ZNF668 and MDM2, although deletion of amino acids 295 to 417 did not prevent their interaction (Fig. 3E). Interestingly, a similar region of MDM2 is required for binding to ARF and ribosomal protein L11, suggesting a common mechanism by which different molecules modulate the function of MDM2 (27, 28).

**ZNF668 regulates p53 stability and activity**

Because ZNF668 binds to MDM2 and p53, we next sought to determine whether there is a causal relationship between ZNF668 status and p53 protein levels. As shown in Fig. 4A, when FLAG-tagged ZNF668 was ectopically expressed in U2OS cells, we found increased p53 protein level as well as increased

**Figure 3.** ZNF668 interacts with MDM2 in the central region. A, cartoon of the ZNF668 protein and ZNF668 deletions. B and C, U2OS cells were transfected as indicated. Cells lysates (3 mg) were immunoprecipitated and analyzed. D, cartoon of the MDM2 protein and MDM2 mutants. E, U2OS cells with Flag-ZNF668 overexpression were transfected with MDM2 deletion mutants. Cell lysates were immunoprecipitated (IP) and analyzed by immunoblotting. NLS, nucleolar localization signal; NES: nuclear export signal; WT, wild-type; C, control.
level of the downstream target (p21) of p53, but the p53 mRNA levels were not affected. Increased p53 protein expression was also seen when we overexpressed ZNF668 in MCF7 cells and HMECs (Supplementary Fig. S2).

To determine whether the increase in the p53 level was regulated at the posttranslational level, we treated U2OS cells with cycloheximide to block de novo protein synthesis. In these cells, overexpression of ZNF668 increased the half-life of p53 protein from 1 hour to more than 2 hours (Fig. 4B and C), suggesting a role of ZNF668 in stabilizing p53 protein. Interestingly, knockdown of ZNF668 with siRNA in MCF7 cells resulted in a decrease of the p53 protein half-life from 2 hours to 30 minutes (Fig. 4D).

To test whether ZNF668 regulates p53 activity, we transfected MCF10A, MCF7, and U2OS cells with siRNA targeting ZNF668 and then treated the cells with UV (50 J/m²) or γ radiation (10 Gy). Cell lysates were harvested 2 hours later, and the changes in ZNF668 and p53 were analyzed. In U2OS cells, depletion of ZNF668 significantly reduced both stress-induced levels of p53 and p53 basal levels (Fig. 4E). Similar

Figure 4. ZNF668 regulates p53 stability and activity. A, U2OS cells were transfected as indicated. Cell lysates were subjected to immunoblotting (top) or reverse transcriptase PCR of p53 (bottom). B, U2OS cells were transfected and treated with cycloheximide (CHX) as indicated. C, quantification (mean of 3 experiments) of p53 protein levels. The analysis was done using NIH ImageJ software. D, MCF7 cells were transfected with control or ZNF668 siRNA and treated with CHX. Bottom, quantification of p53 protein levels. E and F, U2OS cells were transfected with ZNF668 siRNAs and treated with UV (50 J/m²) or γ radiation (IR; 10 Gy) as indicated. Cell lysates were harvested (E) 2 hours later or (F) 24 hours later and analyzed by immunoblotting. G, U2OS cells were transfected with wild-type or mutant Flag-tagged ZNF668. Forty-eight hours later, cell lysates were analyzed. H, U2OS cells were transfected with the pG13-luciferase reporter vector in the absence or presence of expression vectors. After 48 hours, luciferase activities were determined according to the manufacturer’s instructions (Promega). Results represent the mean ± SD of 3 experiments. Western blot analyses are shown.
results were observed in MCF10A and MCF7 cells (Supplementary Fig. S3). The reduction in the level of p53 in response to DNA damage was paralleled by a reduction in phosphorylation of Ser15 of p53 (Fig. 4E), a marker of stress-induced p53 activation. Furthermore, we observed a significant reduction in the expression of the p53 transcriptional target p21 (Fig. 4F). These results showed that in ZNF668-knockdown cells, the overall activation status of p53 and p53 downstream response after DNA damage is impaired.

Because ZNF668 has been shown to be one of the mutated genes in breast cancer, we next tested whether cancer-derived ZNF668 mutants can regulate p53 activity as wild-type ZNF668 protein does. Importantly, we found that mutant ZNF668 could not stabilize p53 as much as wild-type ZNF668 did (Fig. 4G). We also tested whether wild-type and mutant ZNF668 were similar in terms of regulating p53-dependent transcriptional activity. Analysis of p53 function with a p53-luciferase reporter showed that the ability of ZNF668 mutants to activate p53 was impaired compared with that of wild-type ZNF668 (Fig. 4H). These results indicated that ZNF668 mutations in patients may lead to impaired p53 activation, similarly to how p53 mutations can lead to impaired p53 activation.

**ZNF668 facilitates p53 stabilization by disrupting MDM2-mediated ubiquitination and degradation**

To determine whether ZNF668 regulates p53 protein stability through the MDM2-mediated proteasome pathway, we first tested whether ZNF668 could counteract the effect of MDM2 on p53. We expressed p53, MDM2, and ZNF668 in U2OS cells. MDM2 significantly decreased the p53 protein level, which could be restored, at least in part, by simultaneous expression of ZNF668 (Fig. 5A).

To test whether ZNF668 affects the interaction between MDM2 and p53, we conducted an in vitro binding assay. Purified GST-ZNF668, MDM2, and p53 proteins were mixed and pulled down with MDM2 antibody. We found that addition of purified ZNF668 protein decreased MDM2–p53 binding in a dose-dependent manner (Fig. 5B). These data indicated that ZNF668 disrupts the interaction between MDM2 and p53.

The presence of nutlin, which inhibits MDM2–p53 interaction, stabilizes and activates p53 (29, 30). We found that nutlin treatment of ZNF668-knockdown cells reversed the effect of ZNF668 knockdown on DNA damage–induced p53 activation (Fig. 5C). These results further supported the MDM2–p53 interaction as the key target of ZNF668-mediated p53 stabilization.

**MDM2 regulates p53 protein turnover through its E3 activity**

To test whether ZNF668 blocks MDM2-mediated p53 ubiquitination and degradation, cells were treated with the proteasome inhibitor MG132. As expected, MG132 treatment abolished the effect of ZNF668 on p53 level (Fig. 5D, left). Moreover, we detected decreased ubiquitination of p53 when ZNF668 was overexpressed (Fig. 5D, right), suggesting a role of ZNF668 in counteracting MDM2-mediated p53 ubiquitination. Interestingly, we also found that ZNF668 facilitated MDM2 autoubiquitination (Fig. 5E). This observation was consistent with previous findings that L11 differentially regulates MDM2 and p53 ubiquitination (31).

We speculate that in addition to directly blocking MDM2–p53 interaction (Fig. 5B), ZNF668 might also alter function of MDM2 by inducing autoubiquitination of MDM2 molecules. The dysregulated ubiquitination of MDM2 might further facilitate the stabilization of p53 by altering MDM2 E3 ligase activity or binding affinity of MDM2 for its substrates.

**ZNF668 suppresses tumorigenicity of human breast cancer cells**

Because we found that ZNF668 regulates the stability of p53 protein, we posited that ZNF668 might itself function as a tumor suppressor gene. To test this possibility, we first examined the proliferation of cells ectopically expressing ZNF668. Overexpression of ZNF668 repressed proliferation of MCF7 cells (Fig. 6A) and their ability to grow in soft agar (Fig. 6B). In contrast, knockdown of ZNF668 increased soft agar colony formation in nontumorigenic MCF10A cells (Fig. 6C).

Given that ZNF668 effectively suppressed in vitro cellular transformation, we next tested whether ZNF668 suppressed tumorigenicity in vivo. Mice were injected in the mammary glands with ZNF668-overexpressing or vector control MCF7 cells and monitored weekly for tumor formation. By week 8, all 10 mice injected with ZNF668-overexpressing clones remained tumor free, whereas all 5 of the control mice had developed tumors (Fig. 6D), indicating the ability of ZNF668 to suppress tumorigenicity in vivo.

Because we found that cancer-derived ZNF668 mutants could not stabilize p53 as much as wild-type (Fig. 4G and H), we sought to investigate their effects on cell proliferation and transformation. We overexpressed the cancer-derived ZNF668 mutants in MCF7 cells. As shown in Fig. 6E–G, we found that the ability of cancer-derived mutant proteins to inhibit cell proliferation and cell transformation is reduced compared with wild-type ZNF668. However, there was still some residual activity in the mutants or nonspecific effects due to overexpression. These data further support the previously unknown function of ZNF668 in tumor suppression.

**ZNF668 suppresses tumorigenicity in both p53-dependent and -independent manners**

To determine whether p53 is the only target mediating the activity of ZNF668 in suppressing cellular transformation, we also conducted in vitro cell proliferation and transformation assays with MCF7-p53–knockdown cells. Both MCF7-control and MCF7-p53–knockdown cells were transfected with FLAG-tagged ZNF668, and cells were selected with G418 for 10 days. Overexpression of ZNF668 was confirmed by Western blotting (Fig. 7A). ZNF668 suppressed the proliferation and transformation phenotype (Fig. 7B and C) of MCF7-p53–knockdown cells, but to a lesser extent than it suppressed the proliferation and transformation phenotype of MCF7-control cells, indicating that ZNF668 could suppress cell transformation through both p53-dependent and -independent pathways.
Discussion

Our findings identify ZNF668 as a novel nucleolar protein that interacts with known nucleolar proteins NPM and nucleostemin. Recent studies have shown that the function of nucleolar proteins is not limited to participation in ribosomal biogenesis. Indeed, the nucleolus appears to be the site for convergence of the p53 pathway through regulation of different nucleolar proteins under various types of cellular stress (6, 25, 28, 31, 32). Thus, the nucleolar localization of ZNF668 and its interaction with known nucleolar proteins suggested to us a potential role of ZNF668 in p53 regulation.

By forward and reverse genetic approaches, we clearly showed a critical role of ZNF668 in p53 protein stabilization through inhibition of negative regulator of p53, MDM2. Our findings further suggest that ZNF668 regulates MDM2 through a direct interaction between ZNF668 and MDM2–p53 complex because ZNF668 interacted with MDM2 and p53 in vivo and in vitro and interfered with the MDM2–p53 interaction. It has been shown that physical interaction between MDM2 and p53 is a prerequisite for MDM2 to be able to ubiquitinate p53 (15, 18, 33). Therefore, our results strongly support direct protein–protein interactions as a mechanism by which ZNF668 regulates p53 and...
MDM2-mediated p53 ubiquitination in vivo. Our findings do not, however, exclude the possibility that ZNF668 could also regulate p53 through other pathways. For example, p53 can be regulated at the posttranslational level through modifications such as phosphorylation and acetylation. ZNF668 could serve as a platform for assembly of complexes needed for p53 posttranslational modifications in response to cellular stress and thereby promote p53 activation and stabilization. Future experiments will be needed to determine whether other mechanisms besides direct protein–protein interactions may also be involved in ZNF668-mediated p53 regulation.

Recent reports indicate that the central acidic domain of MDM2 is important for controlling p53 activity (34, 35). Indeed, this domain was previously shown to be required for p53 ubiquitination and degradation (36, 37). We found in the current study that deletion of residues 212 to 296 of MDM2 attenuated the interaction between ZNF668 and MDM2, indicating a mechanism by which ZNF668 could attenuate MDM2-mediated ubiquitination and degradation of p53. Indeed, it has previously been suggested that the ubiquitination of p53 is a stepwise process accompanied by a conformational alteration (38). Therefore, it is possible that binding of ZNF668 to the central domain of MDM2 induces a conformational change in both MDM2 and p53 that suppresses the ubiquitination of p53 but facilitates the autoubiquitination of MDM2 (Supplementary Fig. S4).

Balancing MDM2 autoubiquitination and ubiquitination of its substrates, such as p53, highly depends on the association between MDM2 and p53 (39, 40). DNA damage–induced
phosphorylation of MDM2 results in its dissociation with modified p53 and leads to accelerated MDM2 autoubiquitination and consequently, p53 stabilization and activation (33, 39). Our data indicated that ZNF668 could block the interaction between MDM2 and p53 (Fig. 5), thus facilitating MDM2 autoubiquitination and p53 stabilization. Interestingly, it has been shown that L11 and ARF (31, 40–42) can also facilitate autoubiquitination of MDM2 by preventing the recruitment of ubiquitinated MDM2 to the proteasome, either through potential adaptor proteins or by concealing MDM2-binding sites in the proteasome (40, 42), thus inhibiting the postubiquitination pathway. It is therefore tempting to speculate that many p53 regulators, such as ARF and ZNF668, stabilize p53 both by directly interfering with MDM2–p53 interaction and by regulating MDM2 posttranslational modifications, such as autoubiquitination, to alter MDM2 E3 ligase activity or binding affinity toward its substrates.

Our studies also identified ZNF668 as a potential tumor suppressor in breast cancer. ZNF668 was previously identified as one of the highly mutated genes in breast cancer. However, the implications of the mutation of ZNF668 in breast cancer development are entirely unknown. Our studies strongly indicate p53 to be an important target of ZNF668. Indeed, the 2 ZNF668 mutants that we tested clearly had impaired ability to stabilize p53 further support this notion. Of course, we cannot rule out impacts of ZNF668 on proteins other than p53. Indeed, the fact that ZNF668 also suppressed cell transformation in p53-mutated cells (Fig. 7), albeit to a lesser degree than in cells with wild-type p53, indicates that ZNF668 can suppress cell transformation through both p53-dependent and -independent pathways. In future studies, we will identify the p53-independent ZNF668 targets and dissect the function of ZNF668 in both p53-dependent and -independent pathways.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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