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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. RNAi-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.

Keywords

ZNF668; nucleolus; MDM2; p53; tumor suppression

INTRODUCTION

Human cancer develops as a result of accumulation of mutations in oncogenes and tumor suppressor genes (1). Through genomewide 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 four out of thirty-five breast cancer samples analyzed (11.4%) (2). ZNF668 belongs to the kruppel 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 analysis.

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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 (Figure 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 demonstrated 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 level and the stress-induced p53 level. ZNF668 bound to MDM2 to prevent MDM2-mediated p53 ubiquitination and degradation. Notably, we demonstrated 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 ATCC. U2OS cells were maintained in McCoy's 5A medium (Cellgro) with 10% FBS. MCF7, MCF7-control, and MCF7-p53 knockdown cells were maintained in DMEM medium (Cellgro) with 10% FBS. MCF10A and HMEC cells were grown in MEGM medium with MEGM SingleQuots growth factors (Lonza). Cells were incubated at 37°C in a humidified incubator with 5% CO_2 and transfected with Fugene 6 (Roche), Lipofectamine2000 (Invitrogen) or Oligofectamine (Invitrogen). Cell lines were validated by STR DNA fingerprinting using the AmpFℓSTR Identifiler kit according to manufacturer instructions (Applied Biosystems cat 4322288). The STR profiles were compared to known ATCC fingerprints, to the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (Nucleic Acids Research 37:D925-D932 PMCID: PMC2686526 and to the MD Anderson fingerprint database (22, 23). The STR profiles matched known DNA fingerprints or were unique.

Plasmids

To generate FLAG-ZNF668, V5-ZNF668, and GST-ZNF668 constructs, full-length ZNF668 cDNA was amplified by PCR and subcloned into pCMV5-3 × FLAG vector (Sigma), pLenti4/TO/V5-DEST vector (Invitrogen), and pGEX-4T vector (Addgene). The R556Q (Mutant 1) and A66T (Mutant 2) point mutations were created from 3 × FLAG-ZNF668 using the GeneTailor Site-Directed Mutagenesis System (Invitrogen). The FLAG-tagged ZNF668 deletion mutants ZNF668- Δ 1~ Δ 12 were generated by PCR and

subcloned into 3 × FLAG vector. FLAG-p53 (108038, pcDNA3 FLAG-p53), GST-p53 (10852, p3113 GST-p53), and GST-MDM2 (16237, pGEX-4T MDM2 wild-type) were from Addgene. MDM2 deletion plasmids Δ 58-89, Δ 212-296, Δ 222-437, Δ 296-437, and Δ 9 (RING finger domain deletion) were kind gifts from Dr. Karen Vousdens (The Beatson Institute for Cancer research). The identity of the plasmids was confirmed by sequencing at the MD Anderson Cancer Center DNA Analysis Core Facility.

Antibodies and Reagents

Nucleotides (548-1449) 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-HRP (SC-126) and anti-p21(C-19) were from Santa Cruz Biotechnology. Anti-p53 (DO-1) was from Calbiochem. Anti-phospho-p53–Ser15 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 thrombine 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-L-leucyl-L-leucyl-L-leucine) was from EMD Biosciences and used at 10 μM. Nutlin-3 was from Cayman Chemical and used at 10 μM. The ON-TARGETplus ZNF668 (siRNA 1: GUGCCAGCGACUUGCGCAAUU; siRNA 2: AAGCCAUACCACUGCGAGAUU), non-targeting and HDM2 siRNA smartpool were from Dharmacon. Lentiviral vector-based MISSION shRNAs targeting ZNF668 and control were from Sigma. RNA Interference was performed by using Lipofectamine 2000 (MCF7) or Oligofectamine (U2OS).

Immunoblotting and Immunoprecipitation

Immunoblotting and Immunoprecipitation were performed as described before (24). Cells were extracted in RIPA 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 mM $MgCl_2$, 100 mM KCl, 420 mM NaCl, 0.2 mM EDTA, and 1 mM 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 using glutathione agarose (Sigma). 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 mM Tris-Cl (pH 7.2), 50 mM NaCl, and 0.2% NP-40). Proteins were recovered (2 h at 4°C) with glutathione agarose beads. Beads were washed extensively with washing buffer (100 mM Tris-Cl (pH 8.0), 100 mM NaCl, and 1% Nonidet P-40), eluted with 10 mg/ml reduced glutathione (pH 8.0) and subjected to Western blotting analysis.

In Vitro Proliferation and Soft Agar Assay

In Vitro proliferation assay and soft agar assay were performed 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, six-week-old female nude mice were implanted subcutaneously with 0.72 mg of 17-β-estradiol 60-day release pellets (E2 pellet; Innovative Research of America). Mice were injected in the mammary glands with 2×10^6 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 five 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 human mammary epithelial cells (HMECs). Western blotting analysis revealed a 70- to 80-kD V5-tagged band in all ZNF668-overexpressing cell lines. Through motif analysis, two consensus nuclear localization sequences (R/K-R/K-XR/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 (aa 183-483) and used for Western blotting analysis. 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 small interfering RNA (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, upper two 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, lower two rows), and the nucleolar localization of ZNF668 was confirmed by ZNF668's colocalization with two known nucleolar proteins, nucleostemin (NS) and NPM (Fig. 1C).

To systematically identify proteins involved in ZNF668 function, we carried out immunoaffinity purification followed 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 performed immunoprecipitation/Western blotting analysis and found that ZNF668 was co-precipitated with NPM and NS (Fig. 1D). The interaction between ZNF668 and NPM was further confirmed by reciprocal immunoprecipitation using 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 NS bind to and regulate the protein stability and function of p53 (6, 7, 25, 26). Therefore, we performed immunoprecipitation/Western blotting analysis 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 using ZNF668, p53, and MDM2 antibodies (Fig. 2C),

supporting the physical interactions among these three proteins in cells. *In vitro* GST-protein binding assay also confirmed the interactions among these three 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 co-precipitated 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). Regions aa84-aa190 and aa268-aa367 were particularly important for the interaction between MDM2 and ZNF668: when these two regions were deleted, the MDM2-ZNF668 interaction was abolished. Interestingly, these two regions were also important for the interaction between p53 and ZNF668. Notably, immunofluorescent staining showed that the ZNF668 deletions lacking regions aa84-aa190 and aa268-aa367 were localized exclusively outside the nucleolar region, indicating that these two 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 to wild type ZNF668 (Figure 3C). In our mapping data (Figure 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 didn't 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 co-precipitated 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 (MDM2 Δ9), the interaction between ZNF668 and an MDM2 protein with the central region deleted (MDM2 Δ222-437) was defective (Fig. 3D, 3E and Supplemental Fig. S1). In contrast, we confirmed that p53 could form a complex with wild-type MDM2, MDM2 Δ9, and MDM2 Δ222–437, but not MDM2 Δ58-89 (Supplemental 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 aa212-aa296 significantly reduced the binding between ZNF668 and MDM2, although deletion of aa295-aa417 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

Since 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 level of p53's downstream target p21, but the p53 mRNA levels were not affected. Increased p53 protein expression was also seen when we overexpressed ZNF668 in MCF7 cells and HMECs (Supplemental 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 h to more than 2 h (Fig. 4B and C), suggesting a role of ZNF668 in stabilizing p53 protein. Inversely, knockdown of ZNF668 with siRNA in MCF7 cells resulted in a decrease of the p53 protein half-life from 2 h 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 radiation (50 J/m^2) or γ radiation (10 Gy). Cell lysates were harvested 2 h 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 results were observed in MCF10A and MCF7 cells (Supplemental 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 demonstrated that in ZNF668-knockdown cells, the overall activation status of p53 and p53 downstream response after DNA damage is impaired.

Since *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 using a p53-luciferase reporter showed that the ability of ZNF668 mutants to activate p53 was impaired compared to 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 performed 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 MDM2's function 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 MDM2's binding affinity for its substrates.

ZNF668 suppresses tumorigenicity of human breast cancer cells

Since 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 five of the control mice had developed tumors (Fig. 6D), indicating the ability of ZNF668 to suppress tumorigenicity *in vivo*. Since we found that cancer-derived ZNF668 mutants couldn't stabilize p53 as much as wild type (Fig.4 G & 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 Figure 6E, 6F and 6G, we found that the ability of cancer-derived mutant proteins to inhibit cell proliferation and cell transformation is reduced compared to wild type ZNF668. However, there was still some residual activity in the mutants or non-specific 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 ZNF668's activity in suppressing cellular transformation, we also performed *in vitro* cell proliferation and transformation assays using 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 analysis (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 p53-independent pathways.

DISCUSSION

Our findings identify ZNF668 as a novel nucleolar protein that interacts with known nucleolar proteins NPM and NS. Recent studies have shown that the function of nucleolar proteins is not limited to participating 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 demonstrated a critical role of ZNF668 in p53 protein stabilization through inhibition of p53's negative regulator, MDM2. Our findings further suggest that ZNF668 regulates MDM2 through a direct interaction between ZNF668 and MDM2-p53 complex since ZNF668 interacted with MDM2 and p53 *in vivo* and *in vitro* and since ZNF668 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 212-296 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 (Supplemental Fig. S4). Balancing MDM2 autoubiquination and ubiquination of its substrates such as p53 highly depends on the association between MDM2 with p53 (39, 40). DNA-damage induced phosphorylation of MDM2 results 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 (Figure 5), thus facilitated 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 protein 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 two 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 p53-independent

pathways. In future studies, we will identify the p53-independent ZNF668 targets and dissect the function of ZNF668 in both p53-dependent and p53-independent pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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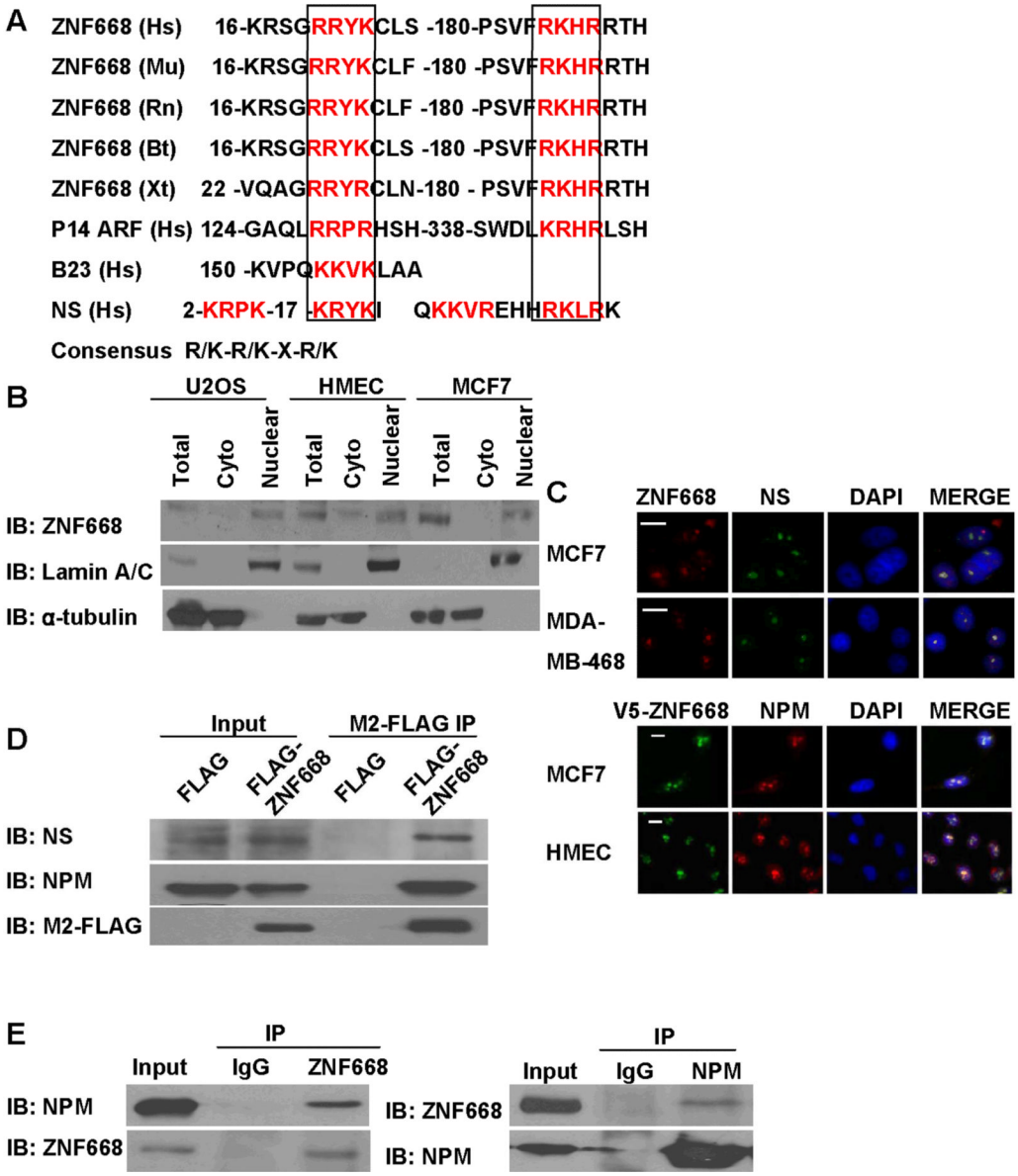

Figure 1.

ZNF668 localizes in the nucleus, accumulates in the nucleolus, and interacts with NPM and NS. A, sequence alignment of ZNF668 in different species. Boxed areas correspond to the consensus nuclear localization sequence. B23 and NS served as positive controls. B, nuclear extracts from U2OS, HMEC and MCF7 were analyzed. Lamin A/C and α-tubulin served as nuclear and cytoplasmic loading markers. C, cells were stained with specific antibodies as indicated. Nuclei were visualized with DAPI. Scale bars: 20 μm. (D) U2OS and (E) MCF7 cell lysates were immunoprecipitated and immunoblotted with antibodies as indicated.

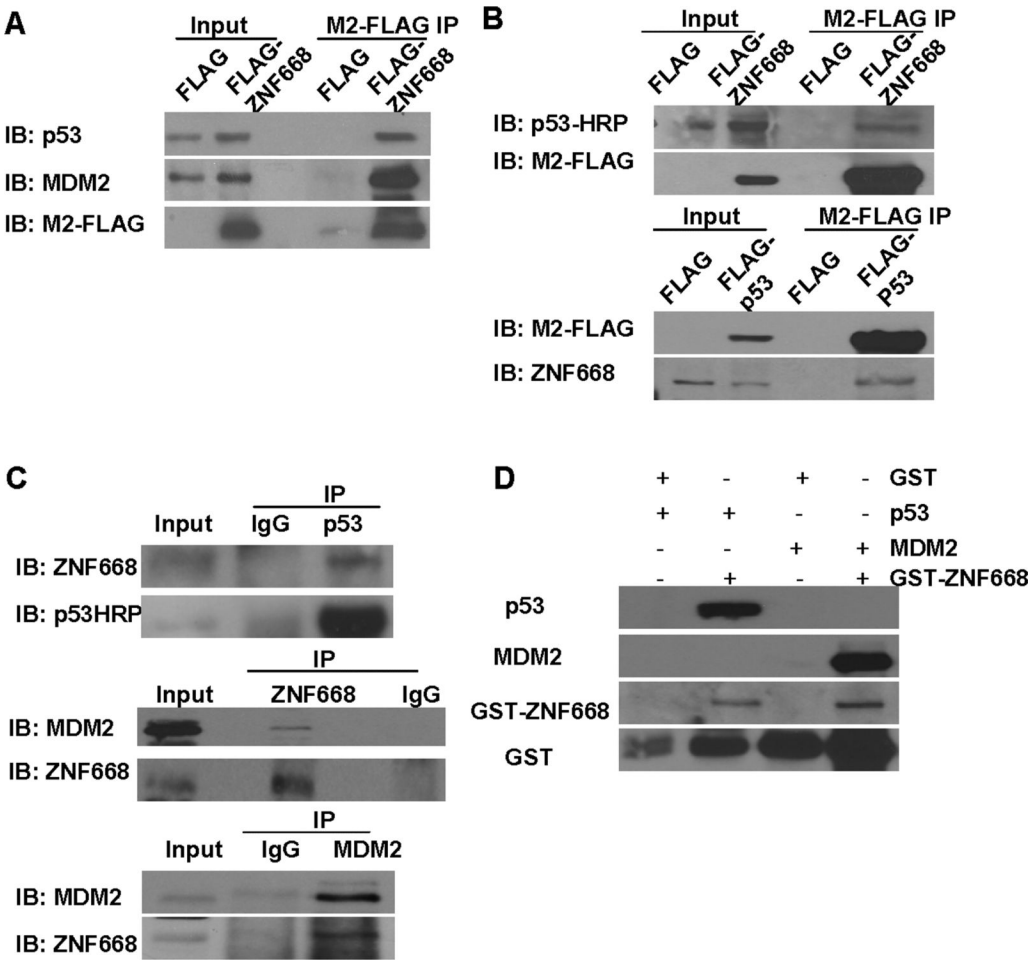

Figure 2.

ZNF668 interacts with MDM2 and p53. A and B, U2OS cells were transfected with control or expression vectors as indicated. 48 h later, cells lysates (3 mg) were immunoprecipitated by M2-FLAG and analyzed. The input was 3% of that used in the immunoprecipitation. 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.

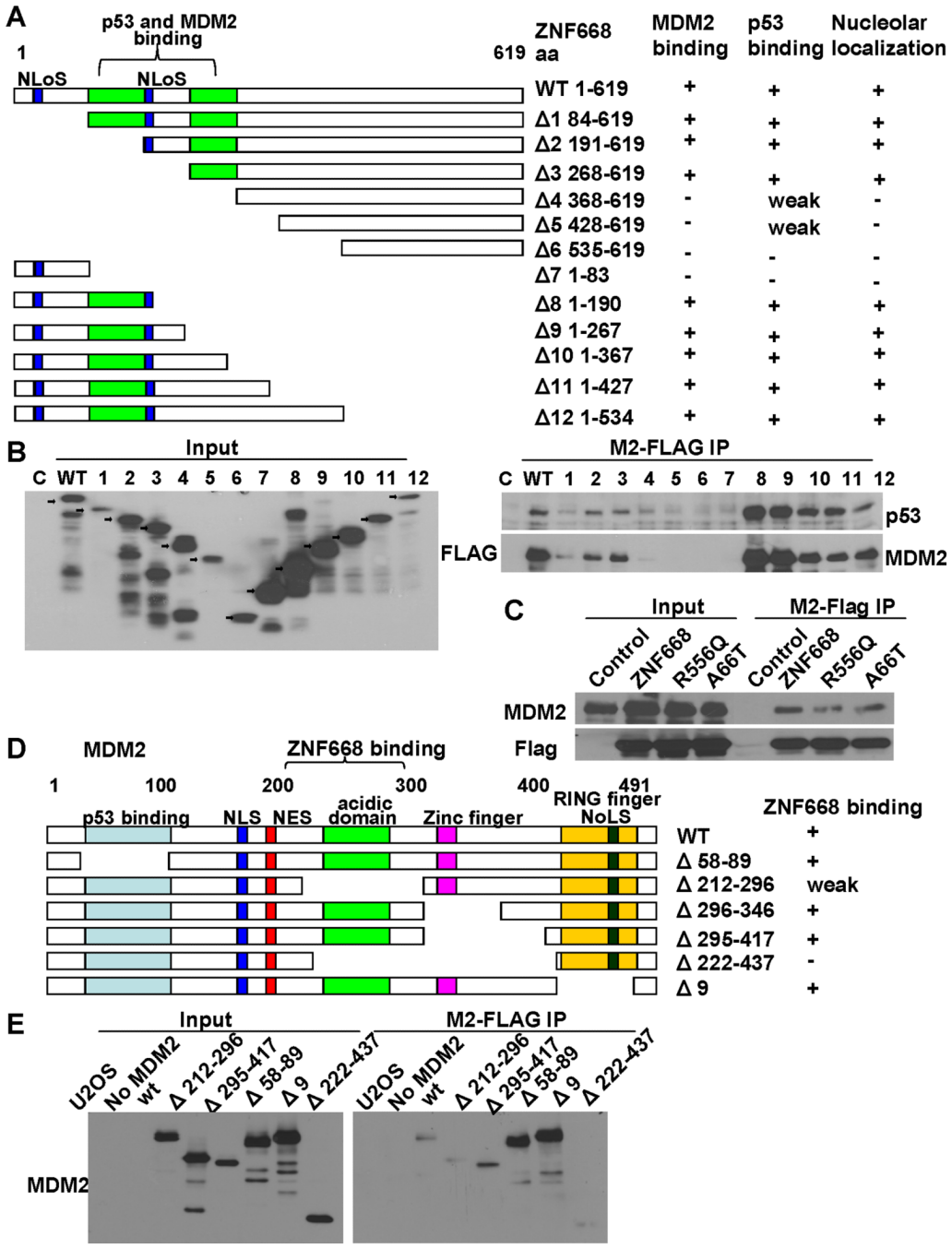

Figure 3.

ZNF668 interacts with MDM2 in the central region. A, cartoon of the ZNF668 protein and ZNF668 deletions. NoLS: nucleolar localization signal. 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. NLS: nuclear localization signal; NES: nuclear export signal. E, U2OS cells with Flag-ZNF668 overexpression were transfected with MDM2 deletion mutants. Cell lysates were immunoprecipitated and analyzed by immunoblotting.

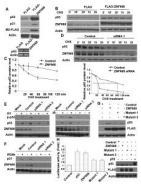

Figure 4.

ZNF668 regulates p53 stability and activity. A, U2OS cells were transfected as indicated. Cell lysates were subjected to immunoblotting (upper panel) or RT-PCR of p53 (lower panel). B, U2OS cells were transfected and treated with cycloheximide (CHX) as indicated. C, quantification (mean of three experiments) of p53 protein levels. The analysis was done using NIH IMAGE software. D, MCF7 cells were transfected with control or ZNF668 siRNA and treated with CHX. Lower panel: Quantification of p53 protein levels. E and F, U2OS cells were transfected with ZNF668 siRNAs and treated with UV (50 J/m^2) or γ radiation (IR; 10 Gy) as indicated. Cell lysates were harvested (E) 2 h later or (F) 24 h 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-LUC reporter vector in the absence or presence of expression vectors. After 48 h, luciferase activities were determined according to the manufacturer's instructions (Promega). Results represent the mean ± SD of three experiments. Western blot analyses were shown.

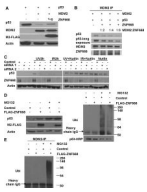

Figure 5.
ZNF668 regulates MDM2-mediated p53 degradation and MDM2 autoubiquitination. A, U2OS cells were transfected with p53 (0.05 μg), MDM2 (1 μg), and ZNF668 (0~1 μg). 48 h later, cell lysates were analyzed. B, purified MDM2 and p53 were mixed and GST-ZNF668 was added at different GST-ZNF668:MDM2 ratios (0:1, 2:1, 4:1, and 8:1). Two-hours later, the protein complex was immunoprecipitated with MDM2 antibody and analyzed. C, U2OS cells were transfected with ZNF668 siRNA and pretreated with or without nutlin. Two-hours later, cells were treated with UV radiation (50 J/m^2) or γ radiation (IR; 10 Gy). Cell lysates were harvested 2 h later and analyzed. D and E, U2OS cells were transfected with Flag-ZNF668 and treated with or without 10 μM MG132 for 6 h. p53 and MDM2 levels were normalized by loading proportionally different amounts of cell extracts for immunoprecipitation with (D) p53 or (E) MDM2 antibody. The protein complex was analyzed by immunoblotting.

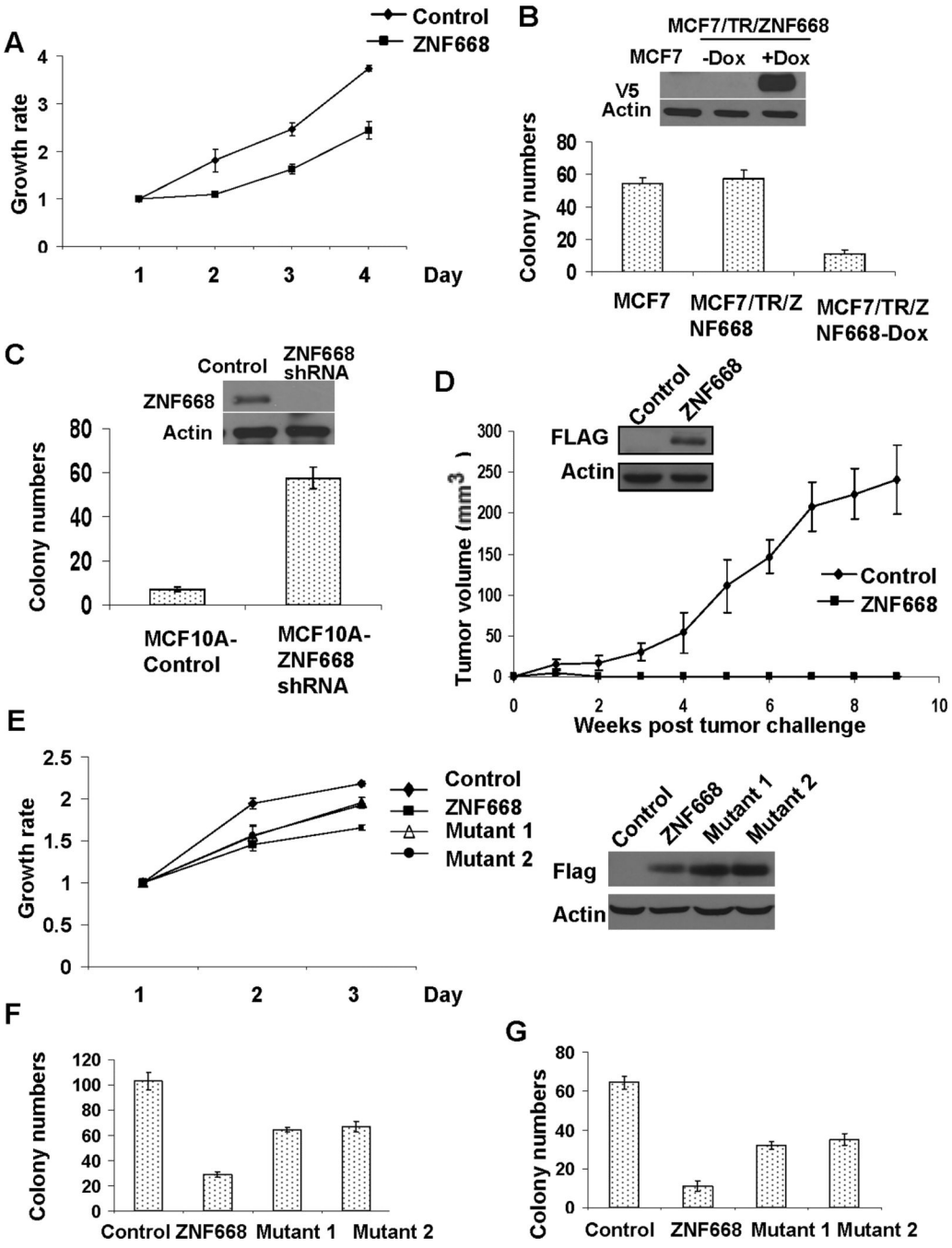

Figure 6.

ZNF668 suppresses tumorigenicity of human breast cancer cells. A, control or ZNF668-overexpressing MCF7 cell proliferation was measured by MTT assay. B, ZNF668-expressing MCF7 and C, ZNF668-knockdown MCF10A cells were seeded in agarose gel. Viable colonies were counted. Western blot analyses of ZNF668 expression level were shown. D, ZNF668-overexpressing or vector-control MCF7 cells were injected into the mammary glands of nude mice and tumor volumes were measured. E-G, Control, ZNF668 or mutants -overexpressing MCF7 cells were seeded. E, cell proliferation was measured by MTT assay. Western blot analyses were shown. Cells were seeded in (F) 6-well plates at a

low cell number and (G) agarose gel. Viable colonies were counted. All of the results represent the mean ± SD of three independent experiments.

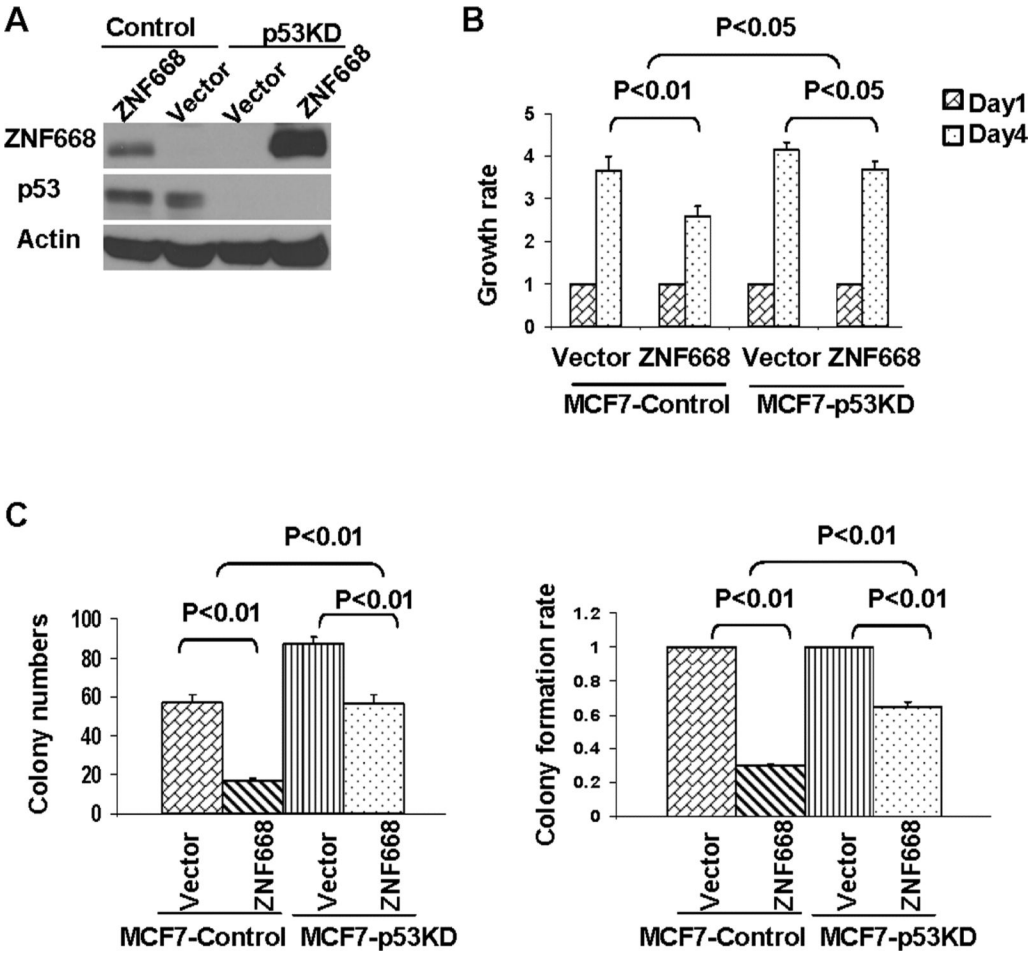

Figure 7.

ZNF668 suppresses transformation phenotype of human breast cancer cells partially through p53. MCF7 control and MCF7-p53-knockdown (KD) cells were transfected with Flag-ZNF668 and selected with G418 for 10 days. A, cell lysates were analyzed by immunoblotting. B, cell proliferation was measured by MTT assay. C, cells were seeded in 0.35% agarose gel. Left: viable colonies in three plates were counted. Right: the colony formation rate was calculated; all soft agar assays were performed in triplicate. Results represent the mean ± SD of three independent experiments. Student's *t*-test.