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E3 Ubiquitin Ligase RNF126 Promotes Cancer Cell Proliferation by Targeting the Tumor Suppressor p21 for Ubiquitin-Mediated Degradation

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Abstract

To identify novel oncogenic E3 ubiquitin ligases as anticancer targets, we screened an E3 ubiquitin ligase siRNA library containing siRNA pools against 555 individual E3s using the sulphorhodamine B assay in the MDA-MB-231 breast cancer cell line and the PC3 prostate cancer cell line. RNF126 was identified and validated as a candidate from this screening. Knockdown of RNF126 dramatically decreased cell viability in these cancer cell lines. Consistently, RNF126 knockdown delayed cell-cycle G_1–S progression and decreased cell proliferation. Using protein array analysis we found that RNF126 silencing increased cell-cycle dependent kinase inhibitor $p21^{cip}$ protein levels in both MDA-MB-231 and PC3. Knockdown of RNF126 stabilized the p21 protein rather than increased *p21* mRNA levels. We showed that RNF126 interacts with p21 and RNF126 overexpression increased p21 protein ubiquitination in an E3 ligase activity-dependent manner. RNF126 knockdown induced loss of cell viability in MDA-MB-231 and PC-3 can be partially rescued by depletion of p21. RNF126 stable knockdown in PC3 inhibited tumor growth in SCID mice. Finally, we found that RNF126 is highly expressed in a subset of breast cancer cell lines and negatively correlated with p21 expression levels. These findings suggest that RNF126 promotes cancer cell proliferation by targeting p21 for ubiquitin-mediated degradation. RNF126 could be a novel therapeutic target in breast and prostate cancers. *Cancer Res; 73(1); 1–10. ©2012 AACR.*

Introduction

Protein ubiquitination regulates a multitude of cancer-related cellular processes, including the cell cycle (1). Protein ubiquitination is typically sequentially mediated by 3 enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) that controls substrate specificity (2). Ubiquitin E3 ligases are considered the next wave of molecules for targeted therapy (3–6). Several specific and effective small molecular inhibitors of Mdm2 have been reported to have substantial antitumor effects *in vivo* (3, 4). A CRL E3 ligase inhibitor MLN4924 is undergoing testing for cancer treatment in clinical trials (5, 6).

E3 ligases are classified into 2 main families: RING (really interesting new gene) finger domain-containing E3s, and homologous to E6-AP COOH terminus (HECT) domain-containing E3s. In mammalian cells there are more than 600 E3s (7) and most of these have not been well characterized in terms of identification of their substrates and functions. RNA interference (RNAi) based screening has been used to identify the TRAF2 E3 ligase as a novel therapeutic target in glioblastoma and lung cancer (8). Here, we describe a functional small interfering RNA library screen to identify E3 ligases that promote cancer cell growth. Ring finger protein 126 (RNF126) was identified as a novel pro-proliferation target. RNF126 and BCA2 E3 ligases share similar protein structures, which contain a RING finger at the C-terminus and a Zn finger near the N-terminus (9). The expression of BCA2 has been shown to correlate with pathologic outcome in invasive breast cancer (10).

$p21^{Waf1/Cip1}$ is an inhibitor of cyclin-dependent kinases and is required for proper cell-cycle progression. Growing evidence suggests that p21 is tightly regulated at the transcriptional and posttranslational levels (11, 12). Several E3 ligases, such as SCF^{skp2} (13), $CRL4^{cdt2}$ (14), and APC/C^{cdc20} (15) target p21 for ubiquitin-mediated degradation at different stages of the cell cycle. In addition, MKRN1 E3 ligase has also been shown to

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promote p21 ubiquitination and degradation (16). Here, we present evidence that the RNF126 E3 ubiquitin ligase interacts with p21, ubiquitinates p21 and promotes p21 degradation.

Materials and Methods

Cell culture and transfection

The breast cancer cell line MDA-MB-231 was maintained in Dulbecco's Modified Eagle's Medium (DMEM), containing 10% FBS, 4.5 g/L glucose, 1 mmol/L sodium pyruvate, 1.5 g/L sodium bicarbonate, 0.1 mmol/L MEM nonessential amino acids, 4 mmol/L L-glutamine, and 1% penicillin/streptomycin (P/S). The colon cancer cell line SW527 (17) was cultured in DMEM, supplemented with 5% FBS, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 1% P/S. The immortalized breast cell line MCF10A, prostate cancer cell line PC3, and human embryonic kidney cell line 293T (HEK293T) were cultured as described previously (18, 19). Lipofectamine 2000 (Invitrogen) was used for transfection of siRNA and plasmid constructs.

E3 ubiquitin ligase siRNA library screening

The siRNAs against 555 E3 ubiquitin ligases were purchased from Applied Biosystems (Ambion Silencer siRNA Library, Catalog Number: 4392425). Screening was carried out in 96-well plates using MDA-MB-231 and PC3 cells. The expression of E3s was knocked down by 10 nmol/L pooled siRNA, containing 3 individual siRNAs for each gene. Cell viabilities were measured by sulphorhodamine B assays. Common hits from both cell lines were validated by secondary screening. Fourteen genes were selected for tertiary screening using 3 individual siRNAs. All experiments were conducted in duplicate.

Antibodies

The anti-RNF126 rabbit polyclonal Ab was generated using a synthesized peptide from the C-terminus of RNF126 (QADDGRDPESRREREH, Panora Biotech). The peptide conjugated to KLH was used as the antigen to produce antibodies (Ab) in rabbits. The anti-$p15^{INK4B}$ rabbit polyclonal (#4822), anti-$p21^{Waf/Cip1}$ (12D1) rabbit monoclonal (#2947), anti-PARP rabbit polyclonal (#9542), anti-Caspase-3 rabbit polyclonal (#9662), and anti-caspase-8 (mouse, #9746) Abs were from Cell Signaling Technology. The anti-$p27^{Kip1}$ monoclonal mouse Ab (610241) is from BD Biosciences. The anti-HA (Y-11) rabbit polyclonal (sc-805) Ab was from Santa Cruz Biotechnology. The anti-GST rabbit monoclonal (G7781), anti-Flag (F3165), and anti-β-actin (AC-15) mouse monoclonal (A5441) Abs were from Sigma-Aldrich.

GST-RNF126 protein purification

The full-length wild-type (WT) and RING domain mutated (C229A/C232A) RNF126 nucleotide sequences were cloned into the pGEX-6P-1 vector using BamHI and EcoRI restriction enzymes (primers shown in Table S1). An overnight culture of *E. coli* transformed with pGEX-6P-1-RNF126 was diluted by 1:100 in 1 L LB media. IPTG (0.1 mmol/L) was added for 3 hours at 26°C to induce GST-RNF126 protein expression. Cells were harvested and resuspended in 50 mL buffer containing 100 mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl, 50 mmol/L EDTA, 2% TritonX-100, 10 μg/mL PMSF, 5 mmol/L DTT, and 1.67 mg/mL lysozyme for 1 hour and sonicated. The lysate was centrifuged for 30 minutes at 13,000 × *g*. The supernatant was incubated with Glutathione Sepharose 4B slurry for 30 minutes at room temperature with shaking. Then the beads were washed 3 times with a total of 50 mL PBS. The GST-RNF126 protein was eluted with the elution buffer (50 mmol/L Tris-HCl, pH 8.0, 10 mmol/L reduced glutathione). The pGEX-6P-1 vector was used as a control to purify the GST protein.

In vitro translation

The p21 protein was translated using the TNT Quick Coupled Transcription/Translation Systems (Promega) *in vitro* following the manufacturer's protocol.

Immunoprecipitation and GST pull-down

For endogenous immunoprecipitation, anti-p21 rabbit monoclonal Ab was used to immunoprecipitate the endogenous p21 protein from MDA-MB-231. Rabbit IgG was used as the negative control. The full length RNF126 gene was cloned into the p3xFlag-myc-cmv-24 vector and expressed in HEK293T cells for detecting the interaction between Flag-RNF126 and endogenous p21. Different RNF126 fragments were subcloned into the pEBG vector. The coimmunoprecipitation and GST pull-down experiments have been described in our previous studies (20, 21).

Ubiquitination assays

The *in vitro* auto-ubiquitination assay of RNF126 was carried out using an ubiquitination kit from Enzo Life Science. The reaction was carried out with 0.75 μL E1, 1.5 μL E2 (UbcH5b), 0.75 μL Mg-ATP buffer, 1.5 μL 10 × ubiquitination buffer, 0.75 μL Ub, 10 μg GST-RNF126 (WT or C229A/C232A mutant), and H_2O in a 15 μL volume at 30°C for 1 hour. The ubiquitinated RNF126 proteins were detected by Western blotting using the anti-Ub Ab. The *in vivo* ubiquitination assay has been described in our previous study (22).

Cell proliferation and cell viability assays

Cell proliferation of MDA-MB-231 and PC3 cells was measured using Click-iT EdU Alexa Fluor 488 and 647 Imaging Kits (Invitrogen), following the manufacturer's instructions, or by the ^{3}H-thymidine incorporation assay as previously described (19, 23). The cell viability was measured by SRB assays (19).

Stable knockdown of RNF126

The pSIH1-H1-puro shRNA vector was used to express RNF126 and luciferase (LUC) shRNAs. Target sequences for RNF126 and LUC are listed in Table S2. MDA-MB-231, SW527, and PC3 cells were infected with lentiviruses. Stable populations were selected using 1 to 2 μg/mL puromycin. The knockdown effect was evaluated by Western blotting.

Tumorigenesis in SCID mice

For PC3 xenograft tumor growth experiment, 12 6-week-old male NOD SCID mice were injected with 5×10^6 PC3-LUCsh or RNF126sh cells subcutaneously. Tumor sizes were measured every week for 7 weeks. Twelve 4- to 5-week-old female NOD SCID mice were purchased from Harlan Laboratories. Mice

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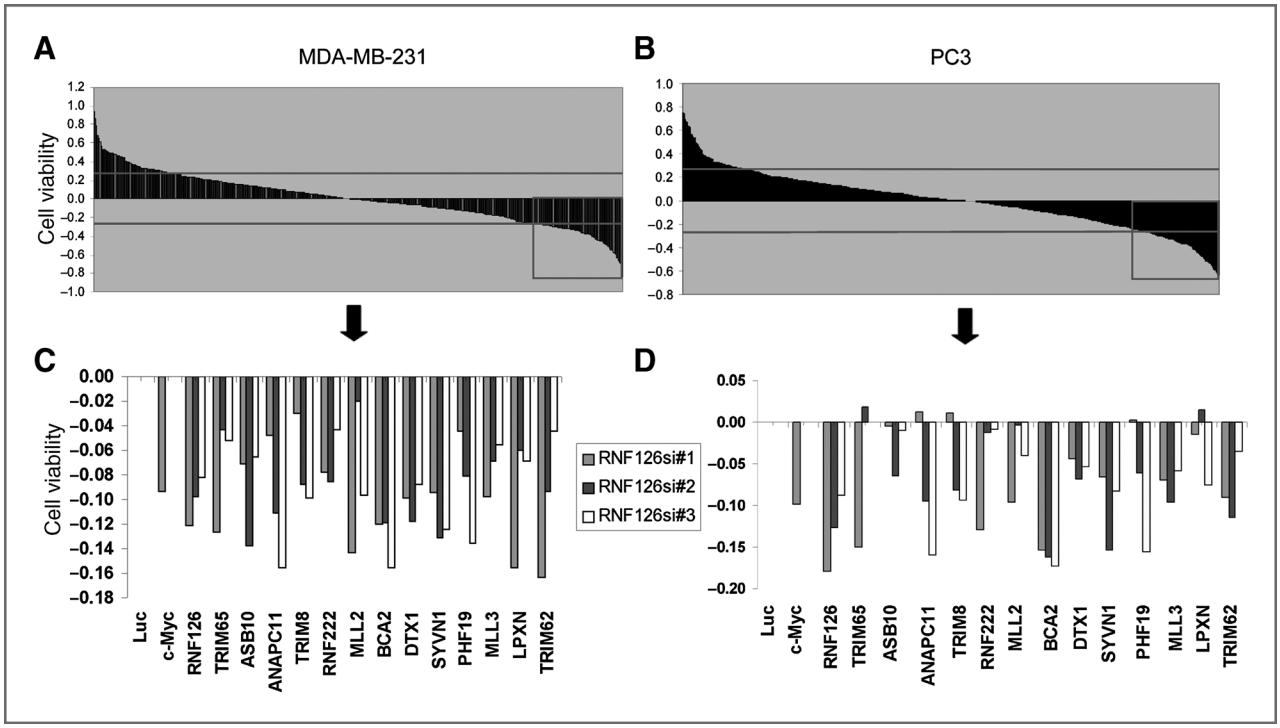

Figure 1. Screening of the E3 ubiquitin ligase siRNA library in MDA-MB-231 and PC3. Pooled siRNAs, which contain 3 different siRNAs for each gene, were transfected into MDA-MB-231 (A) and PC3 (B) cells for 5 days. Cell viability was measured by SRB assay. A total of 555 E3 ubiquitin ligases were screened. Lines represent a standard deviation from the average value. Candidate genes are shown in squares. The tertiary screening results of 14 candidate genes using 3 individual siRNAs for each gene in both MDA-MB-231 (C) and PC3 (D). Myc siRNA and Luc siRNA were used for positive and negative controls.

were randomly and equally distributed into 2 groups. SW527 cells of 2.5×10^6 for each stable population (LUCsh or RNF126sh) were resuspended in PBS and subcutaneously injected into the mice. Tumor size was measured every 3 to 4 days using Vernier calipers once tumors became palpable. Tumor volumes were calculated using the following equation: tumor volume (cm^3) = (length $\times$ width^2)/2. All mice were sacrificed at the end of the experiment and tumors were harvested and weighed.

Statistical analyses

E3 ubiqutin ligase siRNA library screening, qRT-PCR, flow cytometry, DNA synthesis, cell proliferation, and cell viability assays were repeated 2 or 3 times. The data were analyzed as mean $\pm$ standard deviation, and the differences between any 2 groups were compared by Student *t* tests. *P* values of less than 0.05 were considered to be significant.

Results

Identification of RNF126 as a novel target in MDA-MB-231 and PC-3

To identify novel oncogenic E3 ligases for cancer treatment, we screened a siRNA library against 555 E3 ubiquitin ligases by the SRB assay using the MDA-MB-231 breast cancer cell line and the PC-3 prostate cancer cell line. The first round of screening was carried out in duplicate using pooled siRNAs, which contained 3 different siRNAs for each gene (Fig. 1A and B). The common hits from both cell lines, which appear in the squares in Fig. 1, were defined as those that decreased cell viability from the average by more than a standard deviation at day 5. These hits were then confirmed by secondary screening in triplicate in both cell lines. Several well-known E3 ligases, including F-box protein 5 (FBXO5/EMI1; ref. 24) and Tumor susceptibility gene 101 (TSG101; ref. 25), essential for cell-cycle progression, were identified. The confirmed hits were subjected to further validation using 3 individual siRNAs. The Myc siRNA and Luc siRNA were used for positive and negative controls. Results from our siRNA screening yielded 14 novel hits that reproducibly decreased cell viability (Fig. 1C and D).

Among 14 validated E3 genes we selected RNF126 for further investigation because there are no publications for RNF126 to date. Depletion of RNF126 in both MDA-MB-231 and PC3 cells by 3 different siRNAs consistently and significantly decreased cell viability (Fig. 1C and D). RNF126 silencing in other breast cancer cell lines, such as BT20 (Supplementary Fig. S1A) and SUM149PT (Supplementary Fig. S1B), also decreased cell viability, suggesting that RNF126 may be ubiquitously essential for maintaining cancer cell growth.

RNF126 is an E3 ubiquitin ligase

Relative mRNA levels of *RNF126* were investigated in 48 major human tissues. RNF126 is expressed at low levels in the vena cava, but is highly expressed in the liver and testis (Supplementary Fig. S2A). By immune-fluorescence staining, Flag-RNF126 is ubiquitously expressed in both the cytoplasm and the nucleus of PC3 (Supplementary Fig. S2B) and TSU-Pr1 bladder cancer cells (data not shown). The RNF126 protein is a

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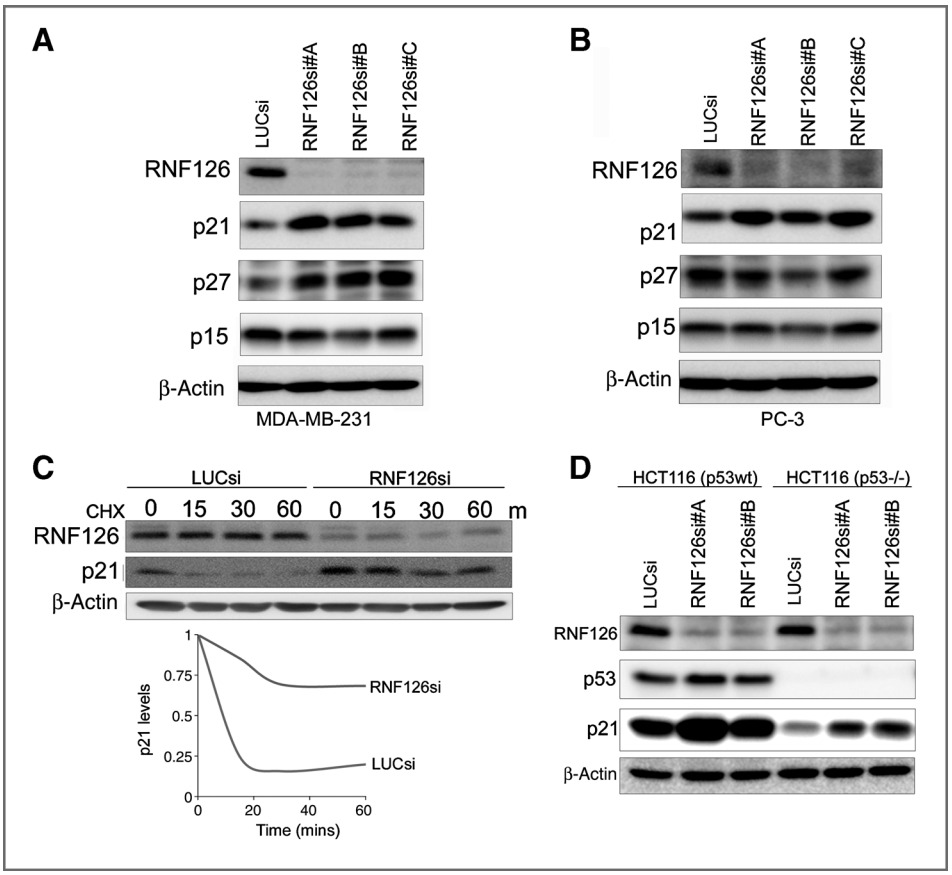

Figure 2. RNF126 knockdown increases p21 protein levels by decreasing p21 protein degradation. RNF126 knockdown by 3 individual siRNAs increased p21 protein levels in both MDA-MB-231 (A) and PC3 (B) cells. p27 was also increased by RNF126 knockdown in MDA-MB-231 (A). There were no increases for p15 by RNF126 knockdown in both cell lines. C, RNF126 knockdown extended p21 protein half-life. The half-lives of p21 were examined by CHX chase assay and Western blotting. Quantitative data is shown below the Western blot panels. D, regulation of p21 by RNF126 is independent of the p53 status. RNF126 was knocked down by 2 different siRNAs in HCT116 (p53 wt and −/−) cells. RNF126 knockdown increased p21 protein levels in both cell lines.

stable protein with a long half-life (>8 hour), as examined by the cycloheximide (CHX) chase assay (Supplementary Fig. S2C). The ubiquitination assay indicated that Flag-RNF126 is strongly ubiquitinated in HEK293T cells (Supplementary Fig. S2D). Self-ubiquitination assays were then carried out *in vitro* using purified recombinant WT GST-RNF126 and GST-RNF126-C229/232A (RINGm) in which the RING domain is mutated (Fig. 4A). RNF126 can ubiquitinate itself in E3 ligase activity dependent manner in the presence of E1, UbcH5b (E2), ATP, and Ub (Fig. 4A). These results show that RNF126 is an E3 ubiquitin ligase.

Depletion of RNF126 increases p21 protein stability

To identify RNF126 substrates we screened an apoptosis antibody array in both MDA-MB-231 and PC-3 because RNF126 knockdown reduced cell viability in both cell lines. As shown in Supplementary Fig. S3A, p21 protein levels were universally upregulated by RNF126si#A in both cell lines. We further validated this result by regular Western blotting using 3 different RNF126 siRNAs. Indeed, p21 was dramatically upregulated by 3 RNF126 siRNAs in both MDA-MB-231 and PC3 cells (Fig. 2A and B). Stable knockdown of RNF126 by 2 different shRNAs in the breast cancer cell lines BT20 and SUM149PT also increased the p21 protein levels (Supplementary Fig. S1C). Another important cyclin-dependent kinase (CDK) inhibitor, p27, was also upregulated in MDA-MB-231 but was not increased in PC3 (Fig. 2A and B). CDK inhibitor p15 protein levels were not increased by RNF126 siRNAs in both cell lines (Fig. 2A and B). These results suggest that RNF126 may specifically target p21 for degradation.

To determine the regulation mechanism by which RNF126 siRNAs increase p21 expression we measured *p21* mRNA levels by qRT-PCR and found that there were no significant changes in both cell lines (Supplementary Fig. S3B). The half-lives of the p21 protein were measured by CHX chase assay in MDA-MB-231. RNF126 depletion dramatically extended p21 protein half-life (Fig. 2C). Similar results were observed in PC3. These results indicate that RNF126 depletion increases p21 protein stability rather than *p21* transcription. In addition, we have shown that the depletion of RNF126 increased the expression of p21 in a p53 independent manner. In the p53 null HCT116 colon cancer cell line, RNF126 siRNAs also upregulated p21 protein levels as they did in HCT116 parental cells (Fig. 2D).

The RNF126 protein interacts with the p21 protein

Flag-RNF126 was overexpressed in HEK293T cells. When Flag-RNF126 was immunoprecipitated by anti-Flag Ab, the endogenous p21 protein could be detected in the protein complex (Fig. 3A). In addition, the protein–protein interaction between endogenous RNF126 and endogenous p21 was detected in MDA-MB-231 (Fig. 3B). These results suggest that RNF126 and p21 proteins interact with each other under physiologic conditions.

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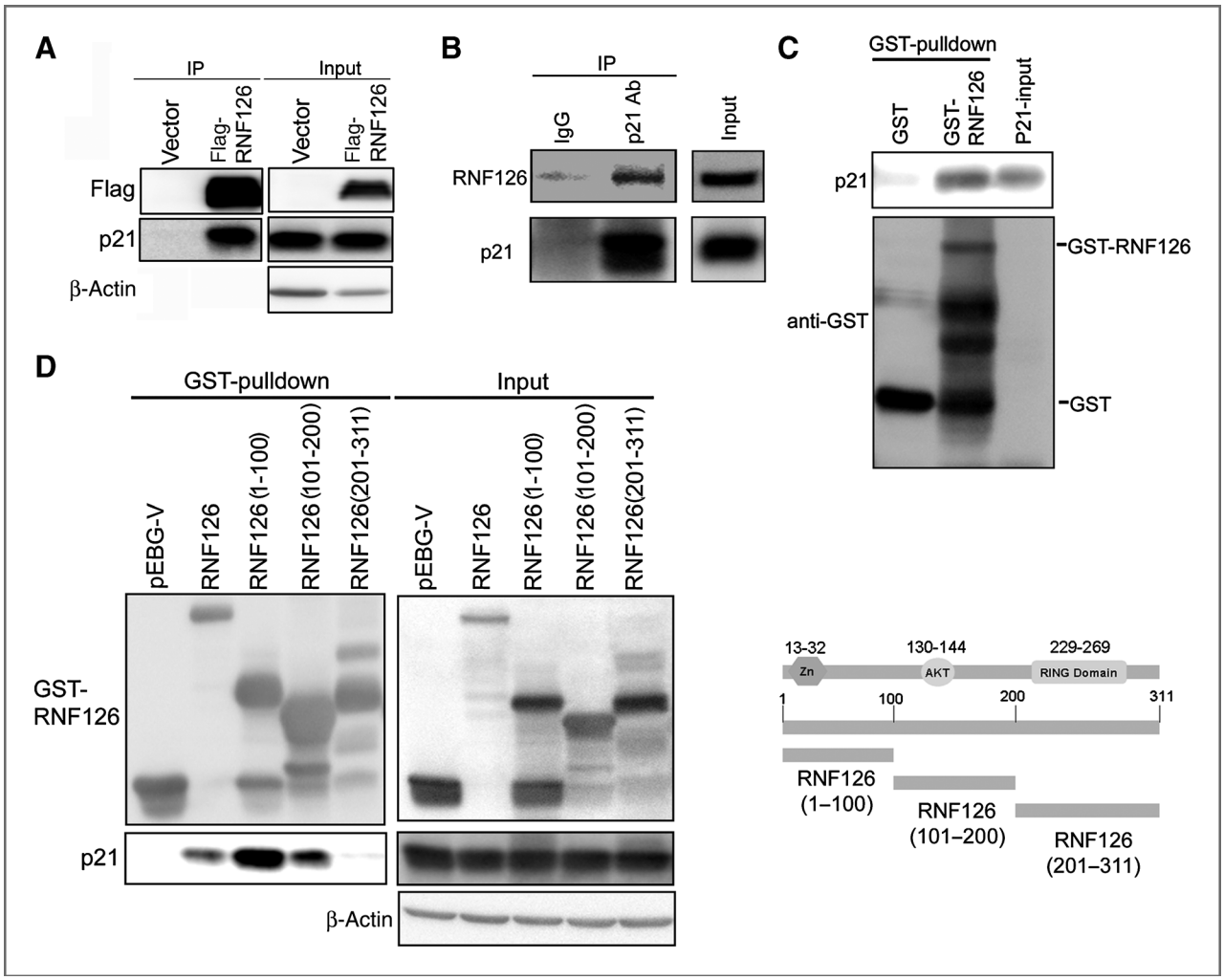

Figure 3. RNF126 and p21 proteins interact with each other. A, p21 was coimmunoprecipitated with Flag-RNF126 in HEK293T cells. 3xFlag-RNF126 and p21 constructs were transfected into HEK293T cells. Two days later, the cells were treated with 20 μmol/L MG132 for 4 hours before harvest. Flag-RNF126 proteins were immunoprecipitated with anti-FLAG M2 beads. The p3xFlag-myc-cmv-24 vector served as the negative control. B, endogenous RNF126 and p21 proteins interact with each other in MDA-MB-231 cells. Rabbit antihuman p21 Ab was used for immunoprecipitation (IP). Rabbit IgG was used as the negative control. C, purified recombinant RNF126 interacts with the *in vitro* translated p21 proteins. The GST-RNF126 and GST proteins were purified from *E. coli*. GST-RNF126 seems to be partially degraded. GST-RNF126 could specifically pull down p21 compared with GST. D, map of p21 interaction domains of RNF126. Different GST fused RNF126 fragments (diagram shown on right side) and p21 were expressed in HEK293T cells. The pEBG vector (GST alone) was used as the negative control. The GST pull-down experiment suggests that p21 interact with the N-terminal of RNF126. Both RNF126 (1 to 100) and RNF126 (101 to 200) could interact with p21.

To test if RNF126 directly interacts with p21, we purified the recombinant GST-RNF126 protein and translated the p21 protein *in vitro*. GST pull-down experiments indicated that GST-RNF126 can interact with p21 whereas the GST control cannot (Fig. 3C). We further mapped the interaction domains of RNF126 by generating 3 truncated RNF126 fragments. As shown in Fig. 3D, both the N-terminus (1 to 100) of RNF126 and the middle part (101 to 200) of RNF126 interacted with p21 whereas the C-terminus (201 to 311) of RNF126 did not.

RNF126 targets p21 protein for ubiquitin-mediated degradation

As described earlier, RNF126 is a RING finger type E3 ubiquitin ligase. To test whether RNF126 ubiquitinates p21, we overexpressed Flag-p21, HA-Ub, and WT RNF126 or catalytic inactive RNF126-RINGm in HEK293T cells and immunoprecipitated Flag-p21 under a denaturing condition. The ubiquitination of p21 was increased by WT RNF126 but not by catalytic inactive RNF126 (Fig. 4B). These results indicate that RNF126 ubiquitinates p21 in an E3 ligase activity-dependent manner. We also investigated if purified GST-RNF126 ubiquitinates *in vitro* translated p21 directly. However, we failed to detect the ubiquitiantion of p21 by GST-RNF126 *in vitro*, suggesting that additional factors, such as p21 modifications, appropriate E2s, or other cofactors/proteins may be required for p21 ubiquitination by RNF126. Nevertheless, the overexpression of RNF126 decreased p21 levels in HEK293T cells in an E3 ligase-dependent manner (Fig.

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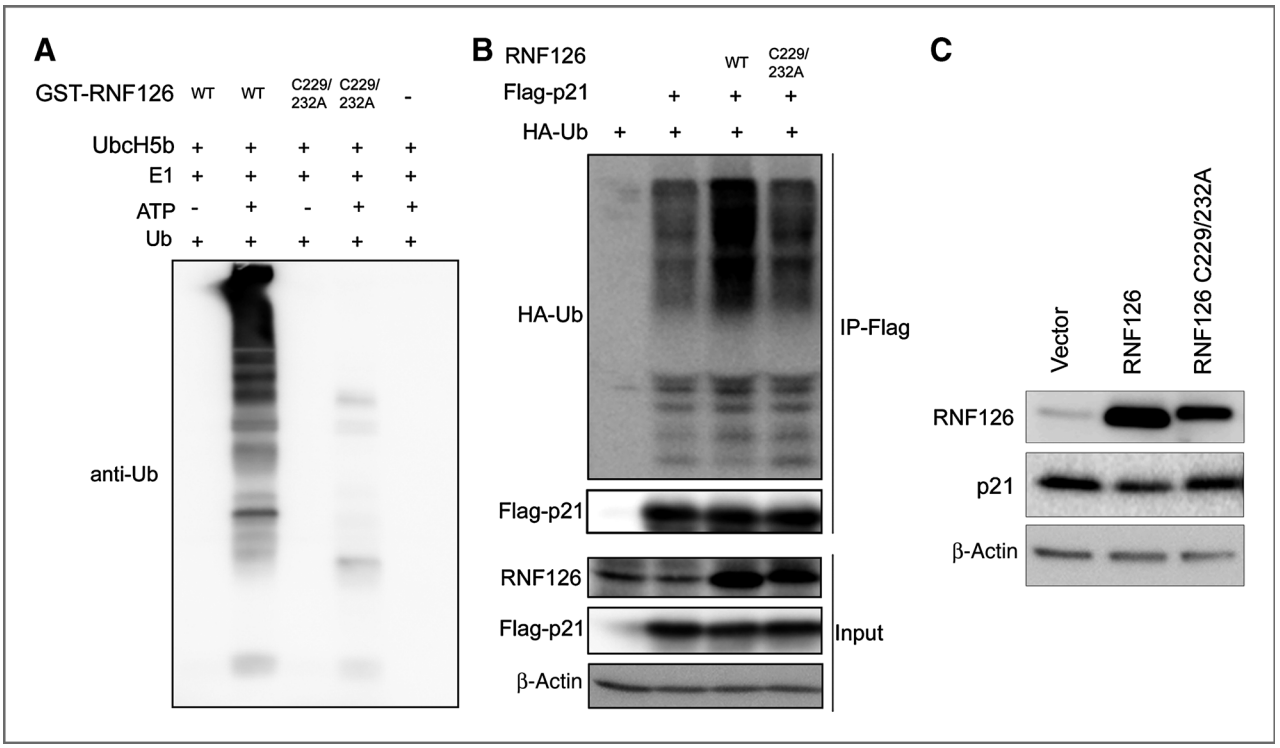

Figure 4. RNF126 promotes ubiqutin-mediated p21 degradation. A, *in vitro* auto-ubiquitination of RNF126 in an E3 ligase activity-dependent manner. The ubiquitination assay was carried out using the purified recombinant wild type or RING finger mutated (C229/232A) RNF126 proteins in the presence of ubiquitin, E1, UbcH5b (E2), and ATP. B, RNF126 ubiquitinates p21 in an E3 ligase activity-dependent manner. Wild-type or RING domain mutated (C229/232A) RNF126, Flag-p21, and HA-Ub were expressed in 22Rv1 cells. MG132 (20 μmol/L) were added to the cells before harvest to prevent p21 degradation. Flag-p21 was immunoprecipitated under a denaturing condition and ubiquitinated p21 was detected by Western blotting using anti-HA Ab. C, RNF126 overexpression decreases the p21 protein level in an E3 ligase activity dependent manner. WT RNF126 or RING finger mutated RNF126 were overexpressed in HEK293T cells. Endogenous p21 protein levels were detected by Western blotting.

4C). These results imply that RNF126 promotes p21 protein ubiquitin-mediated degradation.

RNF126 promotes cancer cell proliferation by inhibiting the expression of p21

It is well known that p21 suppresses cell-cycle progression. To test if RNF126 regulates the cell cycle, we knocked down RNF126 and examined DNA synthesis in the S phase using the Click-iT EdU Alexa Fluor Imaging Kit in MDA-MB-231 and PC3 cells. As shown in Fig. 5A, RNF126 knockdown dramatically reduced the ratio of EdU-positive cells in both cancer cell lines. Moreover, stable knockdown of RNF126 by 2 different shRNAs in the breast cancer cell lines BT20 and SUM149PT significantly decreased the cell growth (Supplementary Fig. S1D). These results suggest that RNF126 is essential for cancer cell proliferation.

To further test whether RNF126 depletion regulates the cell cycle, we knocked down RNF126 in MDA-MB-231 and examined the cell cycle using propidium iodide (PI) staining and flow cytometry. As expected, RNF126 depletion significantly increased the percentage of cells in the G_1 phase and decreased the percentage of cells in the S and G_2–M phases (Supplementary Fig. S4A). Thus, it is likely that RNF126 depletion caused the G_1 cell-cycle arrest and blocked G_1–S transition. To test this hypothesis we synchronized RNF126 stable knockdown MDA-MB-231 and Lucsh control cells in the G_1 phase using a double thymidine block and released the cells by serum stimulation for different time. As a result, a lower percentage of RNF126 silencing cells entered the S phase compared with the control (Supplementary Fig. S4B). RNF126 is likely to target p21 for degradation in the G_1 phase rather than the G_2–M phase (Supplementary Fig. S4C).

Similarly, RNF126 depletion significantly increased the percentage of cells in the G_1 phase and decreased the percentage of cells in the S and G_2–M phases in PC-3 (Fig. 5B). To test if RNF126 functions through p21 we conducted a rescue experiment by knocking down both RNF126 and p21. As shown in Fig. 5B, depletion of both p21 and RNF126 restored the normal cell cycle in PC3. Consistently, p21 silencing partially rescued the RNF126 knockdown-induced cell growth arrest in both MDA-MB-231 and PC3 (Fig. 5C and D). These results indicate that RNF126 promotes cell proliferation at least partially through targeting p21 for degradation in these cancer cells.

Inhibition of RNF126 suppresses tumor growth

To investigate whether RNF126 knockdown suppresses tumor growth *in vivo*, we generated stable RNF126 knockdown PC3 cell population. Consistent with the results in Figs. 2B and 5D, stable depletion of RNF126 increased the p21 protein

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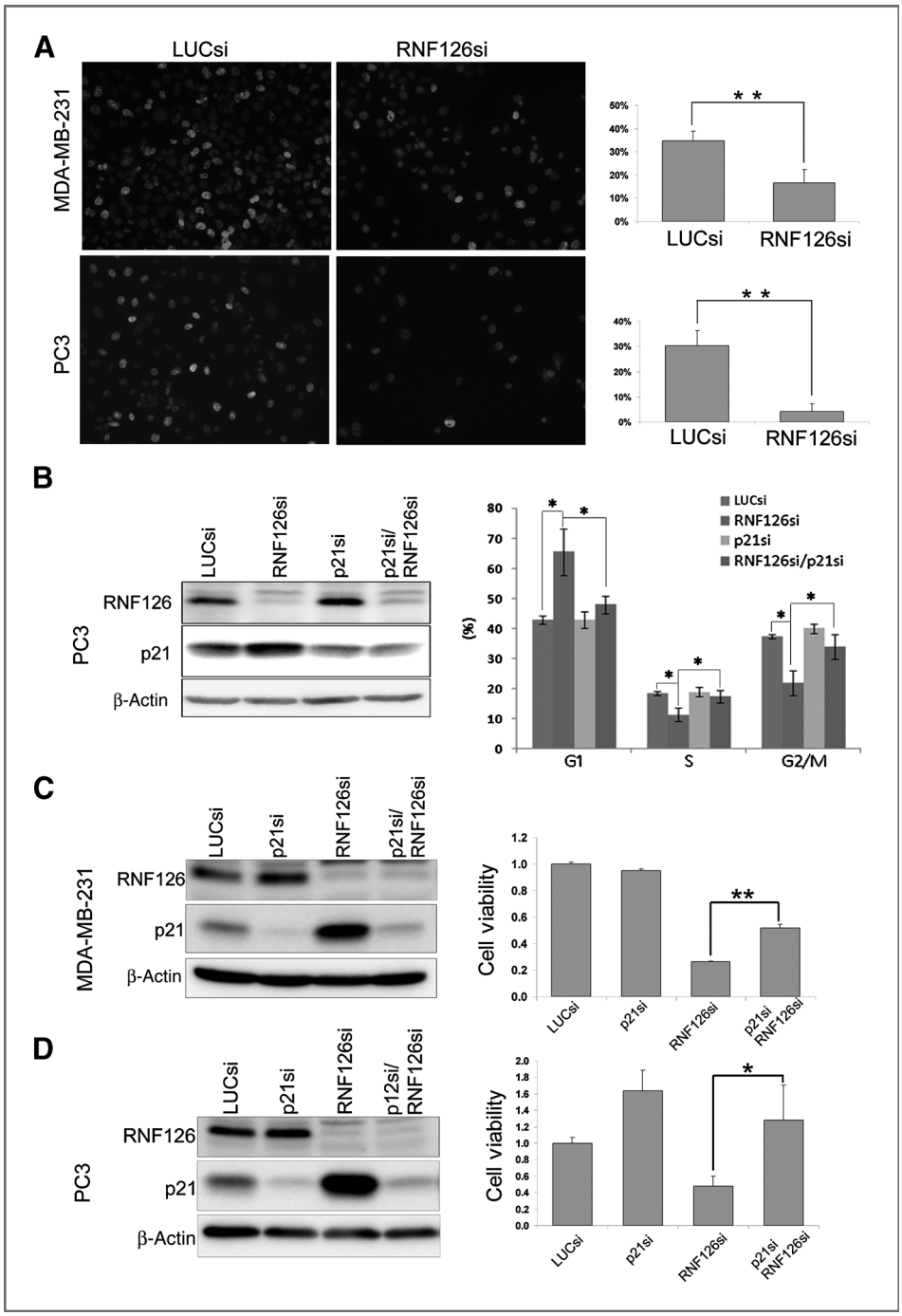

Figure 5. RNF126 knockdown inhibits cancer cell proliferation through upregulating p21 A, RNF126 knockdown inhibited DNA synthesis in both MDA-MB-231 and PC-3. Click-iT EdU Alexa Fluor 488 and 647 Imaging Kits were used to examine cell proliferation. The quantitative results are shown in the right graphs. B, RNF126 knockdown caused G_1 arrest and p21 depletion can rescue the arrest, as examined by PI staining. LUCsi and RNF126si were transfected into PC-3 cells for 2 days and the cell-cycle profile was analyzed by flow cytometry. Average results from triplicate samples are shown on the right side. The Western blot result is shown on the left side. C, RNF126 knockdown-induced growth decrease was partially rescued by p21 knockdown in MDA-MB-231 cells. The knockdown effects are shown on the left and cell viability shown on the right. **, $P < 0.01$. D, RNF126 knockdown-induced growth decrease was rescued by p21 knockdown in PC-3 cells. *, $P < 0.05$.

level compared with the LUCsh control (Fig. 6A). When the PC3-LUCsh and PC3-RNF126sh cells were injected to immunodeficient male SCID mice subcutaneously, the RNF126sh cells grew much more slowly than the LUCsh control cells did (Fig 6B and C). The mean tumor weight from RNF126sh were less than one third of those from LUCsh after 49 day growth *in vivo* (Fig 6D).

In addition, we generated stable RNF126 depleted MDA-MB-231 and SW527 cell populations. Compared with LUCsh control cells, the RNF126sh cells showed higher levels of p21, significantly slower cell growth *in vitro*, and significantly less DNA synthesis in both cell lines (Fig. 6E–G and Supplementary Fig. S5A–C). The anchorage independent growth of SW527-RNF126sh cells was also significantly reduced compared with SW527-LUCsh cells (Supplementary Fig. S5D). Depletion of p21 significantly rescued RNF126 knockdown induced anchorage independent growth inhibition (Supplementary Fig. S5D). When SW527

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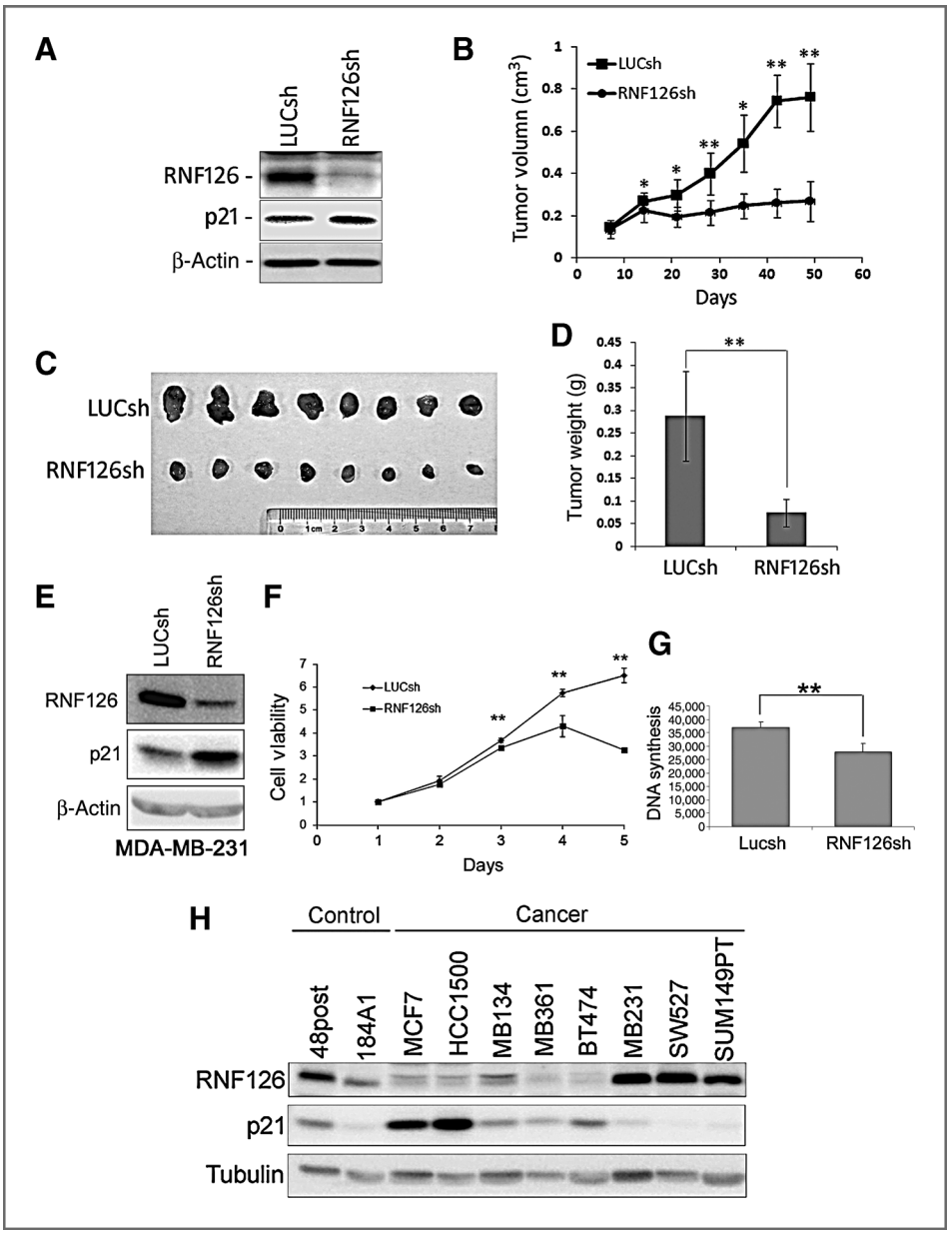

Figure 6. Stable knockdown of RNF126 suppresses tumor growth and RNF126 is overexpressed in a subset of breast cancer cell lines. A, stable knockdown of RNF126 upregulated the p21 protein level in PC-3. B, RNF126 stable knockdown significantly suppressed xenograft growth of PC3 in SCID mice in the course of 49 days. *, $P < 0.05$; **, $P < 0.01$, Student t test. C, PC3-RNF126sh generated smaller xenografts compared with PC-3-LUCsh at day 49. D, RNF126sh stable knockdown significantly reduced tumor weights compared with the LUCsh group ($P < 0.001$, Student t test). E, p21 was upregulated when RNF126 was stably knocked down in MDA-MB-231. F, RNF126 stable knockdown significantly suppressed MDA-MB-231 cell growth in the course of 5 days, as determined by SRB assay. G, RNF126 stable knockdown significantly suppressed DNA synthesis in MDA-MB-231, as determined by ^{3}H-thymidine incorporation assays. H, RNF126 is overexpressed in a subset of breast cancer cell lines. RNF126 protein levels were detected by Western blotting in cell lines. The p21 protein expression pattern is negatively correlated with that of RNF126 (Fig. S6).

LUCsh and RNF126sh cells were injected into the SCID mice, the tumor growth and tumor weight of SW527-RNF126sh was significantly less than those of SW527-LUCsh (Supplementary Fig. S5E and F).

Finally, we examined expression levels of RNF126 and p21 in a couple of breast cell lines by Western blotting. The RNF126 protein was highly expressed in several breast cancer cell lines, including MDA-MB-231 and SUM149PT, compared with the primary (48post) and immortalized (184A1) breast cell lines as well as other cancer cell lines (Fig. 6H). Interestingly, p21 protein levels in these 2 breast cancer cell lines was relatively low (Fig. 6H). There is a negative expression correlation between RNF126 and p21 in these cell lines (Supplementary Fig. S6).

Discussion

E3 ubiquitin ligases are potential therapeutic targets for cancer treatment. RNAi based large-scale screening for E3 ligases is an effective and unbiased approach to identify novel therapeutic targets. We identified RNF126 as a candidate target through a screening of 555 E3 ligases in malignant breast and prostate cancer cell lines. We showed that RNF126 is essential for maintaining cell growth in multiple cancer cell lines. As an uncharacterized RING finger type E3 ubiquitin ligase, RNF126 was found to promote cell-cycle G_1–S progression by targeting the CDK inhibitor p21 protein for ubiquitin-mediated degradation. Importantly, RNF126 is overexpressed in a subset of breast cancers and knockdown of RNF126 significantly inhibited tumor growth. Thus, RNF126 is a

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potential novel therapeutic target in human breast and prostate cancers.

RNF126 is an E3 ligase in which the RING-finger domain is responsible for its auto-ubiquitination activity. Although the RNF126 protein is heavily ubiquitinated in cells, it has a long half-life. Thus, the linkage of the polyubiquitin chains attached to RNF126 is unlikely to be via K48 because the K48-linked polyubiquitin chains are well known to target the substrate for proteasomal degradation. RNF126 is expressed in both the nucleus and cytoplasm. Therefore, the activity of RNF126 may be regulated by ubiquitination and alternative subcellular localization. Further studies are needed to fully understand the regulation of RNF126.

The RNF126 protein shares 46% overall amino acid identity with BCA2, an E3 ligase implicated in human breast cancer (9). RNF126 and BCA2 are very similar in terms of gene structure and protein domains. It has been speculated that both RNF126 and BCA2 have a similar function (9). In our screening, both RNF126 and BCA2 were identified as candidates in MDA-MB-231 and PC-3 (Fig. 1). BCA2 has been shown to be overexpressed in 56% of 945 invasive breast tumors (10) and knockdown of BCA2 in the T47D breast cancer cell line significantly decreased cell proliferation (10). However, the mechanism by which BCA2 promotes breast cell proliferation is unknown. As RNF126 promotes cell proliferation partially through degrading p21, it would be interesting to test if BCA2 also targets p21 for ubiquitin-mediated degradation.

$p21^{cip}$ is well known to execute the function of p53 to arrest the cell cycle in response to DNA damage. The expression of p21 is regulated by gene transcription, mRNA stability, translation, protein stability, and posttranslational modifications (11). Studies show that ubiquitin-dependent degradation of p21 plays an important role in cell-cycle control (12). Several cell cycle-related ubiquitin E3 liagses target p21 for degradation at different stage of the cell cycle including SCF^{SKP2} (26), $CRL4^{CDT2}$ (27) and APC/C^{CDC20} (15). Here, we showed that RNF126 directly interacts with p21, ubiquitinates p21, and promotes p21 degradation in the G_1 phase (Fig. 3–4 and Supplementary Fig. S4). It is also worth noting that RNF126 targets p21 for ubiquitination and degradation in a p53 independent manner (Fig. 2D).

Besides p21, p27 protein levels were increased by RNF126 siRNAs in MDA-MB-231, although this was not the case in PC3 (Fig. 2). RNF126 has been shown to interact with TRAF6 and BAT3 through high-throughput screening (28, 29). BAT3 has been implicated in the inhibition of apoptosis (30). We confirmed that RNF126 interacted with BAT3 by coimmunoprecipitation experiments but RNF126 did not target the BAT3 protein for degradation in several breast cancer cell lines (unpublished results). We are unable to exclude the possibility that RNF126 functions partially through other substrates besides p21.

In addition to RNF126 and BCA2, screening identified several other novel candidate pro-survival E3s including TRIM65, TRIM8, TRIM62, ASB10, ANAPC11, RNF222, MLL2, MLL3, DTX1, SYVN1(HRD1), PHF19, and LPXN. Limited evidence suggests that several of them are oncogenic E3 ligases. For example, SYVN1 has been shown to target p53 and gp78 for degradation (31, 32), LPXN may promote prostate cancer metastasis by serving as a co-activator of androgen receptors (33, 34), and TRIM8 may promote STAT3 and NF-κB activation (35, 36). These candidate E3 ligases warrant further investigation.

In summary, we identified RNF126 as a novel gene essential for cancer cell growth using high throughput RNAi based screening of the E3 ligase library. The RNF126 E3 ligase promotes cell-cycle progression by targeting the CDK inhibitor p21 protein for ubiquitin-mediated degradation. Depletion of RNF126 decreased cell proliferation and tumor growth. These findings suggest that RNF126 could be a potential cancer therapeutic target.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: X. Zhi, D. Zhao, C. Chen
Development of methodology: X. Zhi, D. Zhao
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Zhi, D. Zhao, Z. Wang, C. Wang, W. Chen, R. Liu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Zhi, D. Zhao, R. Liu, C. Chen
Writing, review, and/or revision of the manuscript: X. Zhi, D. Zhao, R. Liu, C. Chen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Zhi, D. Zhao, Z. Zhou
Study supervision: R. Liu, C. Chen

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