# Function of Drg1/Rit42 in p53-dependent Mitotic Spindle Checkpoint

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# Function of Drg1/Rit42 in p53-dependent Mitotic Spindle Checkpoint*

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**Mutations in the *Drg1/RTP/Rit42* gene are commonly identified in hereditary neuropathies of the motor and sensory systems. This gene was also identified as a p53 target gene and a differentiation-related, putative metastatic suppressor gene in human colon and prostate cancer. In this study, we show that the Rit42 protein is a microtubule-associated protein that localizes to the centrosomes and participates in the spindle checkpoint in a p53-dependent manner. When ectopically expressed and exposed to spindle inhibitors, Rit42 inhibited polyploidy in several p53-deficient tumor cell lines and increased the population of cells in mitotic arrest. Blocking endogenous Rit42 expression by small interfering RNA in normal human mammary epithelial cells resulted in the disappearance of astral microtubules, and dividing spindle fiber formation was rarely detected. Moreover, these cells underwent microtubule inhibitor-induced reduplication, leading to a polyploidy state. Our findings imply that Rit42 plays a role in the regulation of microtubule dynamics and the maintenance of euploidy.**

*Drg1/Rit42* was identified previously as a p53 target gene that is up-regulated in response to DNA-damaging agents (1). *Rit42* is also known to be down-regulated during breast, colon, and prostate tumor progression (2–7). It has been shown that the expression of this gene is repressed by c-Myc and the N-Myc/Max complex (8, 9). *Drg1/Rit42* is mutated in hereditary motor and sensory neuropathies (10). The gene is repressed in cellular transformation but up-regulated in growth-arrested differentiating cells and under conditions of cellular stress including hypoxia (11, 12), retinoic acid (13), androgens (3, 14), and nickel compounds (15). Moreover, several studies suggested that Drg1 suppresses colon and prostate cancer metastasis by inducing cell differentiation and reversing the metastatic phenotype (5, 7). *Drg1/Ndrg1/Rit42*−/− mice were successfully generated and exhibited a peripheral neuropathy characterized by demyelination, suggesting that Ndrg1 plays an essential role in the maintenance of myelin sheaths (16).

Although the structural and biochemical properties of Drg1/Rit42 are known, its biological function and the physiological relevance of its role in the cellular context remain elusive. In light of the implication of Rit42 in cell cycle checkpoints and its potential role in p53-mediated tumor suppression, we investigated the involvement of Rit42 in regulation of the mitotic checkpoint. Here, we demonstrate that Rit42 is associated with the microtubule and localizes in centrosomes and spindle esters. We also present evidence that Rit42 acts as an inhibitor of polyploidy in p53-null tumor cells. Thus, we have identified the role of Rit42 in mitosis as that of a potential regulator of spindle organization, which may be one of the components in the spindle checkpoint that ensures the maintenance of diploidy.

## EXPERIMENTAL PROCEDURES

*Cell Culture*—p53+/+ HCT116, p53−/− HCT116, EJ, and DLD1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen). Normal human mammary epithelial cells (hNMECs)[1] were maintained in D Complete medium (17).

*Rit42 Antibody and Western Blotting*—Rabbit antibodies to human Rit42 were raised against synthetic peptides corresponding to amino acid residues 229 to 244 ($^{229}$NAYNSRRDLEIERPMP$^{244}$) of human Rit42/RTP. Cells were lysed in lysis buffer (20 mM Tris, pH 7.4, 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM $Na_3VO_4$, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml aprotonin, and 10 $\mu$g/ml leupeptin). An equal amount of total cellular protein per sample (~15 $\mu$g) was run on a SDS-PAGE. The following antibodies were used for immunoblotting analysis: Rit42 antibody (affinity-purified); $\alpha$-tubulin antibody (Sigma); acetylated $\alpha$-tubulin antibody (Sigma); $\gamma$-tubulin antibody (Sigma); and $\beta$-actin antibody (Sigma).

*Transfection and Adenovirus Expression*—Mammalian expression vectors encoding Rit42 or a RFP-Rit42 fusion have been described (1). LipofectAMINE 2000 was used for transfection studies. An adenovirus expressing Rit42 or GFP was generated, amplified, and titrated. Cells were grown to 50–70% confluence and infected with recombinant adenovirus at a multiplicity of infection of 25–50 for the indicated times. GFP-expressing adenovirus (Ad-GFP) was used as a control.

*siRNA Experiments*—Sense and antisense oligonucleotides corresponding to the Rit42 cDNA sequence $^{210}$AACCTGCTACAACCCCCTC$^{228}$ were purchased from Dharmacon. siRNA against p53 corresponding to the sequence $^{775}$TGAGTACTTCACCAAGATT$^{793}$ was also used. A scrambled siRNA oligonucleotide against Rit42 (5′-AA**G**-**G**TG**ATT**CA**TGGGCA**TC-3′; mutated sequences are shown in boldface) was also made and used as a control. 76N cells were seeded at 30% density the day before transfection and transfected with Nucleofector (program p-22; AMAXA) according to the manufacturer's protocol in the presence of siRNA. siRNA against luciferase (5′-CTGACGCGGAATACTTCGA-3′) was used as additional control.

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[1] The abbreviations used are: hNMEC, normal human mammary epithelial cell; Ad, adenovirus; GFP, green fluorescent protein; mAb, monoclonal antibody; RFP, red fluorescent protein; siRNA, small interfering RNA.

*Immunoflourescence Microscopy*—Cells were fixed with 3% paraformaldehyde and 2% sucrose in phosphate-buffered saline or permeabilized in 0.5% Triton X-100 in phosphate-buffered saline followed by methanol fixation. Endogenous Rit42 localization was visualized with an anti-Rit42 polyclonal antibody following rhodamine Red-X™-conjugated anti-rabbit IgG antibody. Transfected cells were co-stained with the RFP-Rit42 (Red) and γ-tubulin antibody (Sigma), the acetylated α-tubulin antibody (Sigma), or the α-tubulin (Sigma) with Cy2™-conjugated anti-mouse IgG.

*Flow Cytometry*—To profile the cell cycle, cells were exposed to nocodazole (100 ng/ml) for 48 h prior to harvesting. Propidium iodide, a marker of DNA content, was used to prepare for flow cytometry. >4N DNA cell populations were analyzed by FACScan flow cytometry and processed using Cell-Quest software (BD Biosciences).

*Microtubule Binding and Co-sedimentation Assay*—Microtubule assays were performed as described (18, 19). Using 76N cell lysates for the reaction, 5 mg/ml bovine tubulin was stabilized with 20 μM Taxol and GTP. A microtubule-associated protein spin-down assay kit (Cytoskeleton, BK029) was used to perform the reaction. The isolation of centrosome extracts from 76N cells was carried out in accordance with previously described methods (18, 20). For the microtubule co-sedimentation assay, 76N cell lysates were prepared by centrifugation for 30 min at 15,000 rpm at 4 °C. Sucrose gradients (5–40%) were poured as step gradients in lysis buffer. Fifteen fractions were collected and subjected to Western blotting using anti-γ-tubulin antibody and anti-Rit42 antibody.

## RESULTS

*Rit42 Localizes in the Centrosome and Binds to Microtubules in Normal Cells*—To address the function of Drg1/Rit42 in mammalian cell division, we examined its localization in various stages of the cell cycle. We generated an RFP-Rit42 fusion protein expressed it in DLD cells and visualized the localization of the RFP signal. As shown in Fig. 1*A*, when RFP-Rit42-transfected cells were doubly stained with RFP-Rit42 (*red*) and the mAb to γ-tubulin (*green*), RFP-Rit42 was localized in the centrosome. Because γ-tubulin is known as a centrosome marker, these data suggest that the Rit42 protein was localized within the centrosome. Co-distribution of Rit42 with γ-tubulin was confirmed by sucrose gradient sedimentation assays. To perform these tests, centrosome extracts were prepared from 76N normal hNMECs by sucrose density gradient centrifugation as described (20, 21). Immunoblotting analysis using antibodies against Rit42 and γ-tubulin revealed that both Rit42 and γ-tubulin were co-sedimented with centrosome fractions (Fig. 1*B*). To determine whether cellular Rit42 could bind to microtubules, 76N cell lysates were incubated with Taxol-stabilized microtubules and centrifuged to spin down microtubule-associated proteins. The pellet and supernatant were then assayed by Western blotting using Rit42-specific antibodies. As shown in Fig. 1*C*, Rit42 co-sedimented in a microtubule-dependent manner, suggesting an interaction between cellular Rit42 and microtubules.

*Rit42 Suppresses Polyploidy Formation following Spindle Checkpoint Disruption*—In view of the findings of *Rit42* localization in the centrosome and its role as a p53 target gene, we investigated the effects of *Rit42* on the mitotic checkpoint. Following the exposure of three human cancer lines that contained nonfunctional p53 to a spindle checkpoint inhibitor, nocodazole, we ectopically overexpressed Rit42 in the cells and assessed their susceptibility to polyploidy formation following spindle disruption. As expected, control p53-null cells exposed to nocodazole for 20 h continued their cell cycle progression, forming cycling polyploidy cell populations (Fig. 2, *A* and *B*). In contrast, p53-null cells transfected or infected with adenovirus expressing Rit42 (Ad-Rit42) appeared to develop such cell populations in significantly reduced numbers (Fig. 2). HCT116 cells containing wild type p53 did not develop significant polyploid cell populations following nocodazole treatment regardless of Rit42 overexpression. These data suggest that Rit42 can rescue polyploidy induced in p53-null environments and

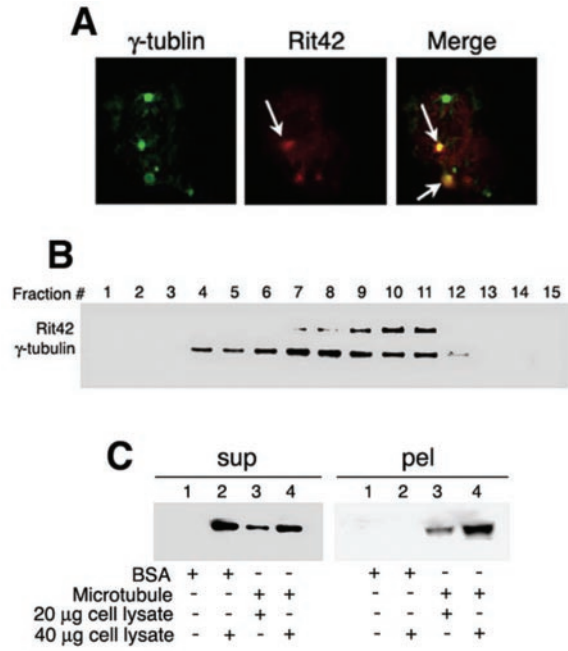


FIG. 1. **Rit42 localizes in the microtubules in DLD cells.** *A*, DLD cells were transfected with an RFP-Rit42 fusion construct. Thirty-six hours later, cells were stained with γ-tubulin mAb and visualized by fluorescence microscopy. The color key is as follows: *green*, γ-tubulin; *red*, RFP-Rit42; *yellow/orange*, merged. *Arrows* represent overlapping images at the centrosome. *B*, Rit42 co-sediments with γ-tubulin. The centrosome extracts were prepared from 76N cells ($2 \times 10^7$ cells) incubated for 2 h in the presence of nocodazole (10 μg/ml) and cytochalasin B (5 μg/ml). Extracts were fractionated by sucrose gradient centrifugation at 14,500 rpm for 1 h, and fractions were analyzed by Western blotting using the anti-Rit42 and anti-γ-tubulin antibodies. *C*, Rit42 binds to microtubules. 76N cell lysates were incubated with Taxol-stabilized microtubules or control buffer and separated into pellets and supernatants. Pellets were washed with microtubule-stabilizing buffer followed by Western blot analysis using Rit42-specific antibodies. *Lane 1*, negative control with bovine serum albumin only; *lane 2*, 40 μg of cell lysates plus bovine serum albumin; *lane 3*, microtubule plus 20 μg of cell lysates; *lane 4*, 40 μg of cell lysates plus microtubule; *sup*, the supernatant fraction; and *pel*, the precipitated fraction.

that it plays a role in the spindle checkpoint during the cell cycle. The proportion of mitotic cells was determined at various time intervals by counting the number of cells after treatment with nocodazole. Compared with p53-null cells infected with Ad-GFP, the percentage of mitotic cells in each of the three cell lines infected with Ad-Rit42 increased up to ~2–3 times within 24 h after nocodazole treatment. No significant effect was seen in p53 wild type HCT116 cells (Fig. 3). These results suggest that Rit42 may compensate for mitotic checkpoint defects resulting from p53-null status.

*Rit42 Inhibition Increases the Number of Cells in the Polyploidy State and Disrupts Spindle Fiber Formation*—To further investigate whether Rit42 contributes to the maintenance of euploidy as well as the regulation of microtubule dynamics, we used small interfering RNA targeting Rit42 to knock down Rit42 expression in normal human mammary epithelial cells (76N) as well as in HCT116 containing wild type p53. Transfection of a siRNA against Rit42 efficiently inhibited endogenous expression of Rit42 in 76N cells, which express Rit42 abundantly (1), whereas control siRNA against luciferase or scrambled siRNA against Rit42 had no effect on the levels of Rit42 mRNA and protein expression (Fig. 4*A*). We next examined whether the knock-down of Rit42 had an effect on the mitotic checkpoint. As shown in Fig. 4*B*, inhibition of Rit42 expression increased polyploid cell populations from ~5 to ~22% following nocodazole treatment. The effects of Rit42

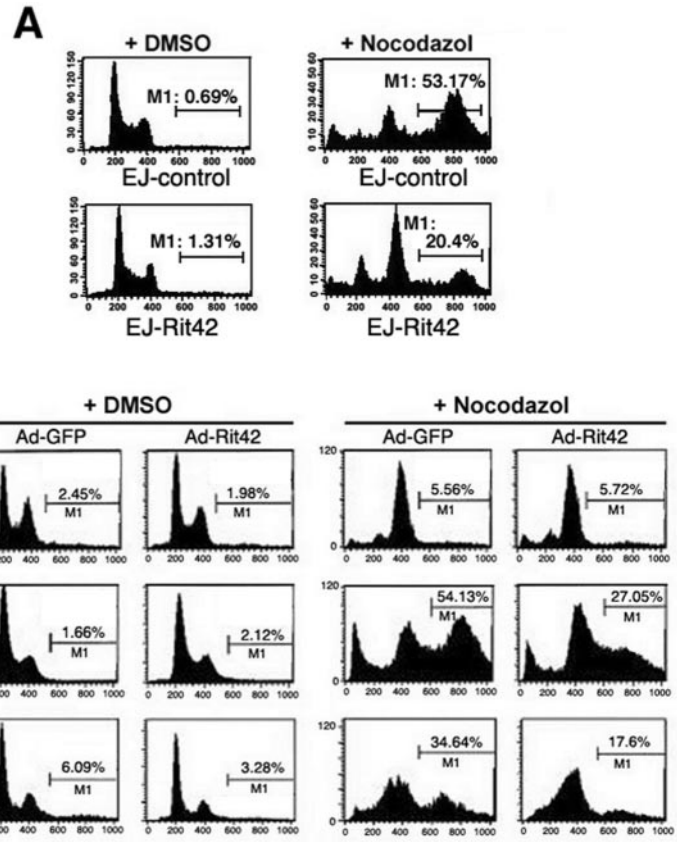


FIG. 2. **Rit42 expression suppresses polyploidy induced by spindle disruption in human cancer cells containing nonfunctional p53.** *A*, EJ cells were transfected with Rit42, and resistant clones were selected under puromycin and pooled. Using stably transfected cells expressing Rit42, cell cycle distributions with or without nocodazole treatment were analyzed by flow cytometry. $Me_2SO$ (*DMSO*) was used as a solvent. *B*, p53+/+ HCT116, p53−/− HCT116, and DLD cells were infected with Ad-GFP or Ad-Rit42 and treated with $Me_2SO$ (*DMSO*) or nocodazole (100 ng/ml) 24 h after infection. The cell cycle distribution was analyzed by flow cytometry. EJ, p53−/− HCT116, and DLD cells, all of which contain nonfunctional p53, showed an increase in polyploid cell populations following nocodazole treatment, which was inhibited by ectopically expressed Rit42.

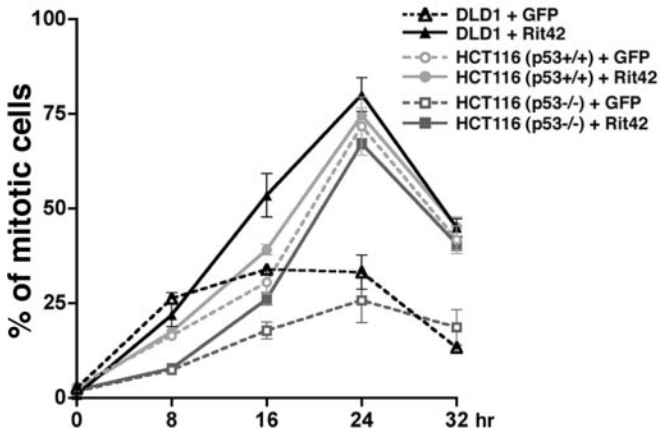


FIG. 3. **Rit42 expression increases the mitotic index during exposure of cell lines to nocodazole.** DLD cells, p53+/+ HCT116, and p53−/− HCT116 cells were infected with Ad-Rit42 or Ad-GFP and treated with nocodazole for up to 32 h. Bromodeoxyuridine was also added to nocodazole-treated cells. Cells were harvested at the indicated time intervals, and at least 200 cells were scored for bromodeoxyuridine staining at each time point. The results shown are representative of cells observed during three independent experiments

suppression were not consistent with cell death, because we tested directly for apoptotic effects by performing a terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling assay, which was negative (data not shown).

Moreover, inhibition of Rit42 caused the disappearance of α-tubulin protein and resulted in dramatic changes in microtubule structure (Fig. 5*A*). Microtubules are important components of the cytoskeleton and carry out a variety of essential functions. Microtubule dynamics is generated by extensive array of reversible post-translational modifications, such as acetylation, phosphorylation, and palmitoylation (22–24). Acetylated tubulin is one of the major characteristics of stabilized microtubule structure and may contribute to regulating microtubule dynamics (23). Thus, we investigated the possibility that Rit42 also influences acetylated α-tubulin accumulation rather than just reduced tubulin synthesis. siRNA against Rit42 resulted in the reduced level of acetylated α-tubulin as well as a decrease of total α-tubulin expression (Fig. 5*B*). In concurrence with these observations, acetylated α-tubulin immunostaining of Rit42 knock-down cells showed a significant decrease in tubulin acetylation, resulting in the failure to form dividing spindle fibers (Fig. 5*B*). These data suggest that Rit42 expression in hNMECs is required to maintain spindle structure during cell division.

To further define the functional relationship of Rit42 to p53, we next examined the effect of Rit42 suppression in comparison with the effect of p53 inhibition during mitotic progression in HCT116 cells. We selectively knocked down Rit42 or p53 and assessed their susceptibility to polyploidy formation following spindle disruption by the exposure of HCT116 cells to nocodazole. Immunoblot analysis showed that the levels of Rit42 or p53 expression were significantly decreased by Rit42- or p53-specific siRNA; in contrast, no significant changes were detected in control scrambled siRNA-transfected HCT116 cells (Fig. 6*A*). The inhibition of p53 expression by p53 siRNA appeared to develop significant polyploid cell population (~36%) following 48 h of nocodazole treatment (Fig. 6*B*). Rit42 suppression by Rit42 siRNA also resulted in a significant increase of polyploidy cell population up to ~26% (Fig. 6*B*). HCT116 cells containing wild type p53 (control siRNA-transfected HCT116 cells) did not develop any significant polyploid cell populations. These data suggest that *Rit42* may play an important role in the p53-mediated euploidy maintenance as a p53 downstream gene.

## DISCUSSION

We show here that Drg1/Rit42 is a microtubule-associated protein and participates in the spindle checkpoint. Overexpres-

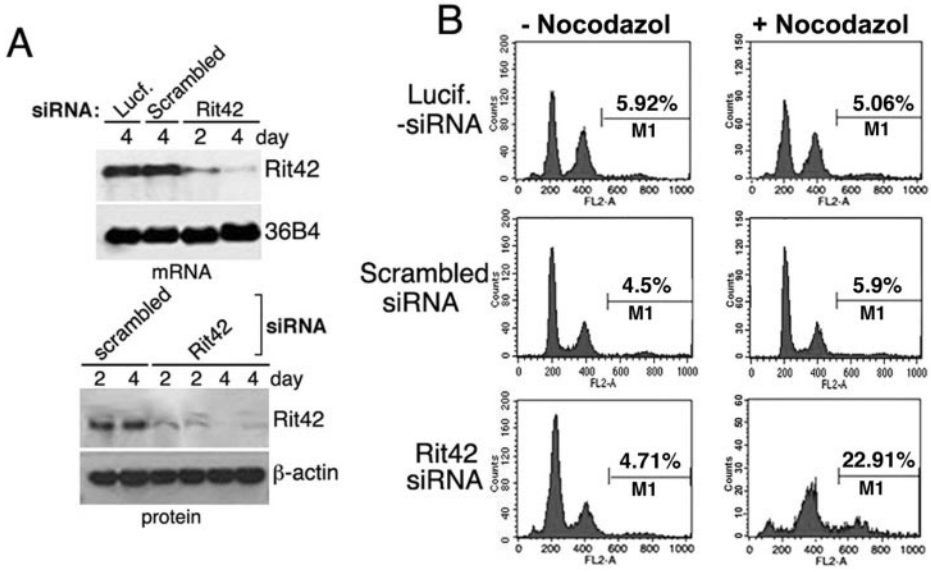


FIG. 4. **The effect of Rit42 inhibition on cell cycle distribution.** *A*, inhibition of Rit42 expression in 76N hNMECs by RNA interference. Cells were transfected with Rit42, scrambled-Rit42, or control (luciferase (*Lucif.*)) siRNA, and cell lysates were extracted for Northern blot and Western blot analyses at the indicated times following transfection. Northern blot was performed sequentially using a $^{32}$P-labeled probe against Rit42 and 36B4 (loading control). Cell lysates were immunoblotted with antibodies against Rit42 and β-actin (loading control). *B*, effect of Rit42 suppression on cell populations in human normal mammary epithelial cells containing wild type p53 (76N). 76N hNMECs were transfected with Rit42 siRNA or control siRNAs, including scrambled Rit42 siRNA and luciferase siRNA treated with nocodazole or $Me_2SO$ and harvested for flow cytometry analysis 48 h later. Note that polyploid cell populations (8N) increased from 4–5% to 22.9% as a result of spindle checkpoint disruption.

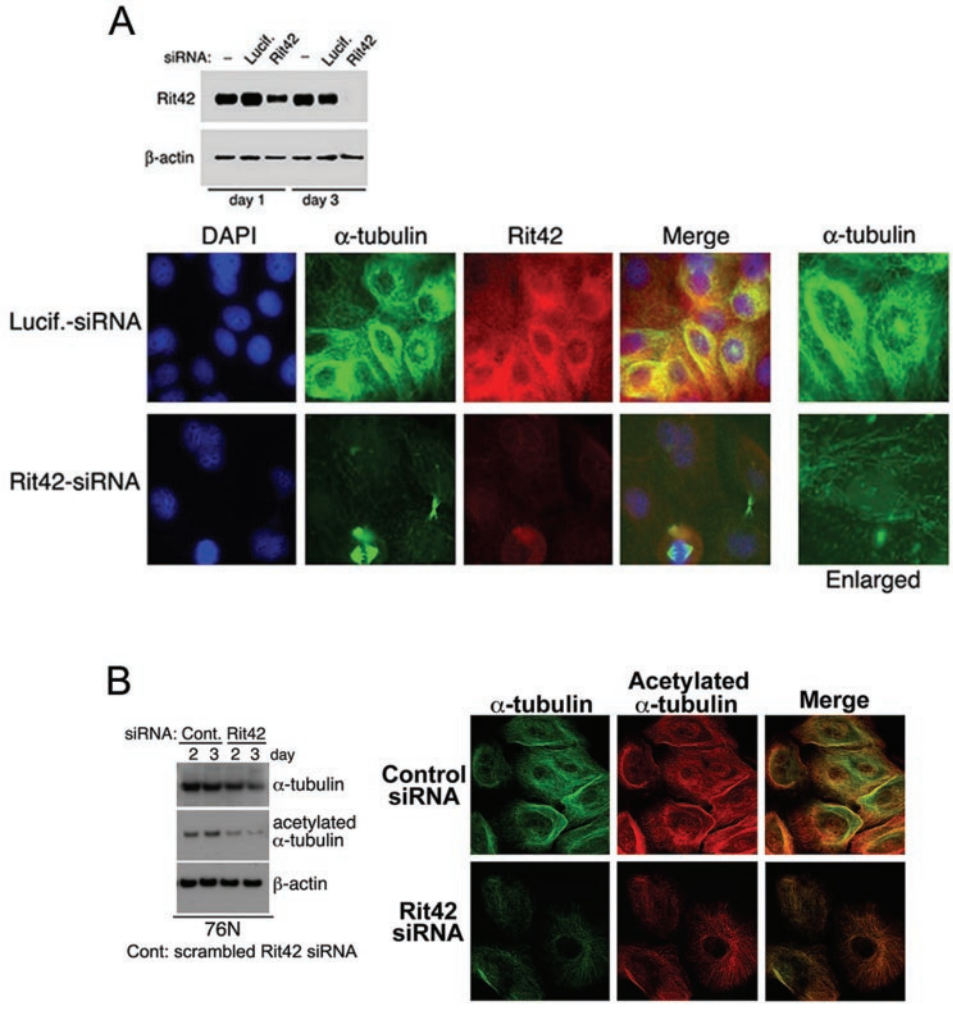


FIG. 5. **Rit42 inhibition leads to decreased accumulation of acetylated α-tubulin and disrupts spindle fiber formation.** *A*, inhibition of Rit42 expression in 76N hNMECs by RNA interference. Cells were transfected with Rit42- or control (luciferase (*Lucif.*)) siRNA, and cell lysates were extracted for Western blot analysis at the indicated times following transfection. Cell lysates were immunoblotted with antibodies against Rit42 and β-actin (loading control). The *lower panel* shows that Rit42 inhibition disrupts spindle fiber formation. 76N cells were transfected with Rit42 siRNA. Forty-eight hours after transfection, cells were co-stained with 4′,6-diamidino-2-phenylindole (*purple*), anti-α-tubulin mAb (*green*), and anti-Rit42 polyclonal antibodies (*red*). Enlarged images are shown in the *far right panels*. *B*, Western blot analysis showing a decreased level of acetylated α-tubulin expression by Rit42 siRNA (*left panel*). 76N hNMECs were transfected with Rit42 siRNA or control siRNA (scrambled Rit42 siRNA (*Cont.*)). Cell lysates were immunoblotted with antibodies against α-tubulin, acetylated α-tubulin, and β-actin (loading control). The *right panels* show immunostaining of acetylated and total α-tubulin. Forty-eight hours after transfection, cells were co-stained with α-tubulin mAb (*green*) and acetylated α-tubulin mAb (*red*). Merged images are also shown.

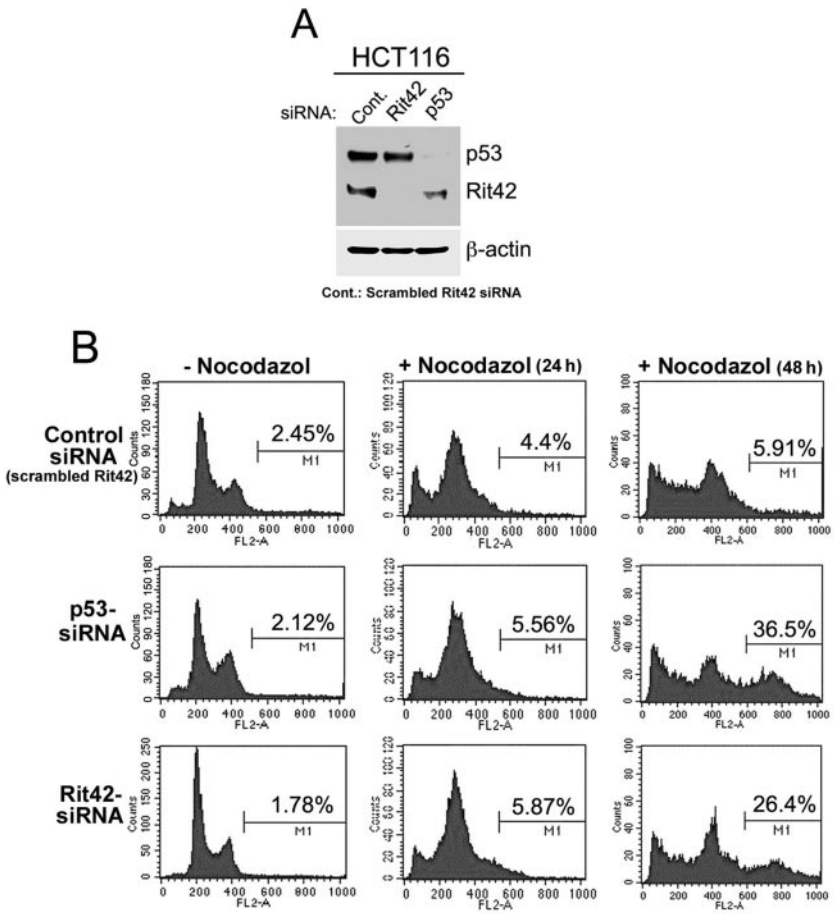


FIG. 6. **Rit42 inhibition increases polyploidy induced by spindle disruption mimicking the effect by p53 inhibition.** *A*, Western blot analysis showing a reduction of Rit42 or p53 expression following Rit42 or p53 siRNA transfection. *Cont.*, control. *B*, flow cytometric analysis after p53 and Rit42 knock-down. HCT116 cells were transfected with control (scrambled Rit42 siRNA), Rit42 siRNA, or p53 siRNA followed by nocodazole treatment and harvested for fluorescence-activated cell sorting analysis at indicated times.

sion studies indicate that Rit42 can inhibit polyploidy development after disruption of the spindle checkpoint, resulting in an increase in the cell population arrested in mitosis in several p53-deficient tumor cell lines. Knock-down experiments of endogenous Rit42 expression using siRNA in normal human mammary epithelial cells resulted in astral microtubule disappearance and barely detectable dividing spindle fiber formation. Cells with suppressed Rit42 expression could undergo microtubule inhibitor-induced reduplication. These findings lead us to suggest that Rit42 localizes in the centrosome and binds to the microtubule so that Rit42 can function as a mitotic checkpoint gene, thereby ensuring cell division fidelity.

The ability of p53 to suppress genomic instability is related to its ability to participate in a mitotic spindle checkpoint (21, 25–27). However, a recent report demonstrates that inactivation of p53 does not lead to the development of aneuploidy (28). Additional studies have also shown that suppression of p53 does not directly cause altered centrosome numbers in any of several mammalian primary cell lines (29). Therefore, during tumor progression, p53 inactivation alone is not sufficient to cause chromosome instability.

Expanding on studies identifying *Rit42* as a p53-responsive gene and an inhibitor of tumor cell growth (1), we offer a novel proposal, namely, that Drg1/Rit42 regulates the process of microtubule assembly and is involved in sensing damage caused by microtubule disruption. In this study, we have shown that Rit42 is localized in the centrosome, specifically at the microtubule matrix where it protects cells from spindle disruption damage. Rit42 may also play a role in the maintenance of p53-mediated fidelity during cell division, because Rit42 rescues p53-null or -mutated cells from mitotic arrest due to spindle damage. Although the mechanism by which Rit42 regulates microtubule dynamics and maintains euploidy remains to be determined, knock-down experiments strongly imply that Rit42 inhibition increases the polyploidy state after nocodazole treatment and also causes the failure to form dividing spindle fibers and the disappearance of astral microtubules. It has been well established that acetylated tubulin is mostly associated with stable microtubular structures and may contribute to the regulation of microtubule dynamics (22–24). Thus, our findings that the elimination of Rit42 impairs acetylated $\alpha$-tubulin accumulation rather than just reduced tubulin synthesis may provide a direction for better understanding the functions of tubulin modification. The results presented here suggest that Rit42 plays a role in the regulation of microtubule dynamics and the maintenance of genomic euploidy. Our findings encourage consideration of Rit42 as a previously unrecognized component involved in maintaining functional microtubule dynamics whose loss may contribute to genomic instability in cancer cells.

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