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Ring Finger Protein 149 Is an E3 Ubiquitin Ligase Active on Wild-type v-Raf Murine Sarcoma Viral Oncogene Homolog B1 (BRAF)*[S]

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Background: BRAF is a downstream effector kinase of Ras.
Results: RNF149, a RING finger domain-containing E3 ubiquitin ligase, is one of the several proteins shown to interact with wild-type BRAF by tandem affinity purification.
Conclusion: RNF149 induces ubiquitination and subsequent proteasomal degradation of wild-type but not mutant BRAF.
Significance: This is the first ubiquitin ligase shown to degrade wild-type BRAF in a proteasome-dependent manner.

Members of the RAF family (ARAF, BRAF, and CRAF/*RAF*-1) are involved in a variety of cellular activities, including growth, survival, differentiation, and transformation. An oncogene encodes BRAF, the function of which is linked to MEK activation. BRAF is the most effective *RAF* kinase in terms of induction of MEK/ERK activity. However, the mechanisms involved in BRAF regulation remain unclear. In the present work, we used a tandem affinity purification approach to show that RNF149 (RING finger protein 149) interacts with wild-type BRAF. The latter protein is a RING domain-containing E3 ubiquitin ligase involved in control of gene transcription, translation, cytoskeletal organization, cell adhesion, and epithelial development. We showed that RNF149 bound directly to the C-terminal kinase-containing domain of wild-type BRAF and induced ubiquitination, followed by proteasome-dependent degradation, of the latter protein. Functionally, RNF149 attenuated the increase in cell growth induced by wild-type BRAF. However, RNF149 did not bind to mutant BRAF or induce ubiquitination thereof. Thus, we show that RNF149 is an E3 ubiquitin ligase active on wild-type BRAF.

Members of the *RAF* serine/threonine kinase family, which consists of three isoforms, ARAF, BRAF, *RAF*-1 (CRAF), are characteristic downstream effector kinases of *RAS* (1, 2). Of the three *RAF* kinases, BRAF[3] binds most avidly to *RAS* (3, 4) and is the most active in phosphorylating the dual-specificity kinase, MEK (5). A functional analysis has revealed that somatic mutations in the catalytic domain of BRAF significantly increase BRAF kinase activity and enhance subsequent induction of cellular transformation (6). Mutations of *BRAF* are found in ~30% of malignant melanomas, 7–10% of colorectal cancer, and 20% of ovarian carcinoma; they also occur with moderate to low frequency in liver, pancreas, stomach, and non-small cell lung cancer. Thus, somatic mutations in *BRAF* and attendant increases in activity are associated with tumorigenesis and cancer progression, but are extremely rare for the other two members of the Raf family (6, 7). Conversely, the loss of BRAF function has been shown to disrupt ERK activation, and BRAF-deficient mice die during midgestation due to induction of endothelial cell apoptosis (8).

Wild-type and mutant BRAF use different mechanisms for activation of the MEK-ERK pathway (9, 10). Wild-type BRAF activates the MEK-ERK pathway in a RAS-dependent manner, whereas mutant BRAF induces activation in RAS-independent manner. Thus, as an effector of RAS, BRAF alternatively regulates downstream signaling pathway depending on the *RAS* genotype. However, BRAF inhibitory mechanisms are not clear.

RNF149 (RING finger protein 149) is a 400-amino acid protein located on chromosome 2 (2q11.2) that contains a RING finger domain and a specialized type of zinc finger of 40–60 residues that binds two atoms of zinc. It has been previously reported that *RNF149* is mutated in some human breast, ovarian, and colorectal cancers. However, the function of RNF149 has remained unclear.

In this investigation, using a tandem affinity purification approach, we first identified RNF149 as a wild-type BRAF-interacting protein. This finding led us to investigate the effect of RNF149 on wild-type BRAF. We report that RNF149 induces ubiquitination of wild-type BRAF and promotes its proteasome-dependent degradation.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and Transfection—The human colon cancer cell lines, HCT-116 and RKO, and the human embryonic kidney line, 293T, were cultured in 5% CO_2 at 37 °C in

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[3] The abbreviations used are: BRAF, v-Raf murine sarcoma viral oncogene homolog B1; DDK, DYKDDDDK epitope; RNF149, RING finger protein 149.

high-glucose Dulbecco's modified Eagle's medium (DMEM; GIBCO). Other colon cancer cell lines (DLD-1, SW480, SW620, Colo205, and LoVo) were cultured in RPMI 1640 medium (Sigma). All media were supplemented with 10% fetal bovine serum (FBS; Thermo Scientific). GFP-tagged wild-type *BRAF* and DDK-tagged *RNF149* plasmids were purchased from Origene (Rockville, MD). The mutant constructs $BRAF^{V600E}$ and $RNF149^{H289A}$, in which Val-600 was replaced with glutamine and His-289 was replaced with alanine, respectively, were created using the PCR-based QuikChange site-directed mutagenesis kit (Stratagene). Lipofectamine 2000 (Invitrogen) was used for transient transfections. Simvastatin was obtained from Sigma.

Tandem Affinity Purification—Wild-type BRAF-associated proteins were isolated by using tandem affinity purification (11). Briefly, colon cancer cells that express endogenous wild-type BRAF were treated with simvastatin, and then the decreased level of wild-type BRAF was confirmed by Western blot analysis using anti-BRAF antibody. Cells treated with simvastatin following MG132, a proteasome inhibitor, were lysed with radioimmunepreciptation assay lysis buffer containing a protease and phosphatase inhibitor mixture (Sigma). After removal of cell debris by centrifugation, crude cell lysates were immunoprecipitated with anti-BRAF antibody. The eluates were incubated with protein A-agarose beads (Santa Cruz Biotechnology) for 2 h at 4 °C. The proteins bound to S-protein_ agarose beads were resolved by SDS-PAGE and visualized by Coomassie Blue staining. The identities of eluted proteins were revealed by mass spectrometry analysis (11).

RNA Interference—Human colon cancer cells and 293T cells were transiently transfected with siRNA (150 pmol/60-mm dish) targeting *RNF149* (I, 5′-CAC UGA AGU GGC ACC AAC AdTdT-3′; II, 5′-GAA GUU UGG CUU GAA CUA AdTdT-3′; and III, 5′-AGU GUG UUG CUU CAG ACU AdTdT-3′) or *BRAF* (5′-AAG UGG CAU GGU GAU GUG GCA-3′) using Lipofectamine 2000. Scrambled siRNA (5′-GCG CAU UCC AGC UUA CGU A-3′) was used as a control. siRNAs were purchased from Bioneer, Inc.

Colorimetric Assay—Cell proliferation was evaluated colorimetrically using a colony forming assay kit (CBA-135; Cell Biolabs, Inc., San Diego, CA). Cells (2×10^4) were seeded on a soft agar plate and cultured for 10 days. Colonies were then stained with a solution provided in the assay kit and measured with a spectrophotometer at 570 nm.

Western Blot Analysis—Western blot analysis was performed by preparing cell lysates using radioimmunepreciptation assay lysis buffer containing a protease and phosphatase inhibitor mixture (Sigma). Protein concentrations were determined using the Bradford assay, and 20 μg of total cellular protein per sample was resolved by SDS-PAGE and transferred to an Immobilon-PVDF membrane (Millipore). Membranes were blocked using 5% nonfat dry milk in TBS-T buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) and probed with anti-BRAF, anti-ubiquitin, anti-GFP, anti-γ-tubulin (Santa Cruz Biotechnology), anti-DDK (Origene), or anti-RNF149 (Sigma) antibodies. Primary antibodies were detected using horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies, as appropriate and enhanced chemiluminescence reagents (Amersham Biosciences).

Immunoprecipitation and in Vivo Ubiquitination Assays—Cells were treated with simvastatin for 18 h and then treated with the proteasome inhibitor MG132 for 6 h. Total cell lysates from clarified supernatants containing an equal amount of protein (300 μg) were subjected to immunoprecipitation analysis. Endogenous BRAF and BRAF V600E were analyzed by immunoprecipitating with an anti-BRAF antibody for 24 h at 4 °C, followed by the addition of 20 μl of protein A-agarose beads (Santa Cruz Biotechnology) and incubation for an additional 2 h at 4 °C. Immunoprecipitates were washed five times with Nonidet P-40 lysis buffer and boiled in 20 μl of 2× SDS sample buffer. Ubiquitin adducts were detected by immunoblot analysis using an anti-ubiquitin antibody.

RESULTS

Simvastatin Induces the Ubiquitination of Wild-type BRAF, but Not Mutant BRAF—Recently, it was reported that simvastatin can overcome cetuximab resistance in a preclinical model of colorectal cancer with mutant KRAS (12). Interestingly, in this report, the level of wild-type BRAF protein, but not mutant BRAF, was significantly decreased after exposure to simvastatin. Because protein stability is controlled in part by the ubiquitin-proteasome pathway (13), we used the proteasome inhibitor, MG132, to determine whether the degradation of wild-type BRAF protein in response to simvastatin is proteasome-dependent. For this purpose, we selected multiple colon cancer cell lines with different BRAF genotypes. MG132 treatment of cells expressing wild-type BRAF (DLD-1, HCT116, and SW480) significantly increased BRAF protein levels (Fig. 1*A*), whereas the level of BRAF protein in RKO cells, which express mutant BRAF, was not affected by simvastatin and/or MG132 treatment (Fig. 1*A*). Consistent with these data, endogenous wild-type BRAF was ubiquitinated in response to simvastatin, but mutant BRAF was not (Fig. 1*B*). These results indicate that simvastatin induces the ubiquitination and subsequent proteasome-mediated degradation of wild-type BRAF, but not mutant BRAF.

RNF149 Interacts with Wild-type BRAF—The ubiquitination of wild-type BRAF by treatment with simvastatin implies that simvastatin may induce an E3 ubiquitin ligase for wild-type BRAF. In an attempt to identify the E3 ubiquitin ligase for wild-type BRAF, we prepared lysates from simvastatin-treated cells expressing wild-type *BRAF* and immunoprecipitated them with an anti-BRAF antibody. Among the various wild-type BRAF-interacting proteins identified by tandem affinity purification followed by mass spectrometry analysis was RNF149 (supplemental Fig. S1), a RING finger domain-containing E3 ubiquitin ligase. On the basis of these results, we first examined whether simvastatin can induce the expression of RNF149. Expression of RNF149 in cells with wild-type BRAF was gradually increased in response to simvastatin (Fig. 2*A*), but was unchanged in cells with mutant BRAF (Fig. 2*B*). To further validate our tandem affinity purification results, we examined the interaction of endogenous wild-type BRAF and RNF149 in cells. Immunoprecipitation and Western blotting analyses revealed that both RNF149-bound wild-type BRAF and wild-type BRAF-bound RNF149 were detected in wild-type BRAF cells, but not in RKO cells that express mutant BRAF (Fig. 2*C*). We next investigated the interaction between exogenously

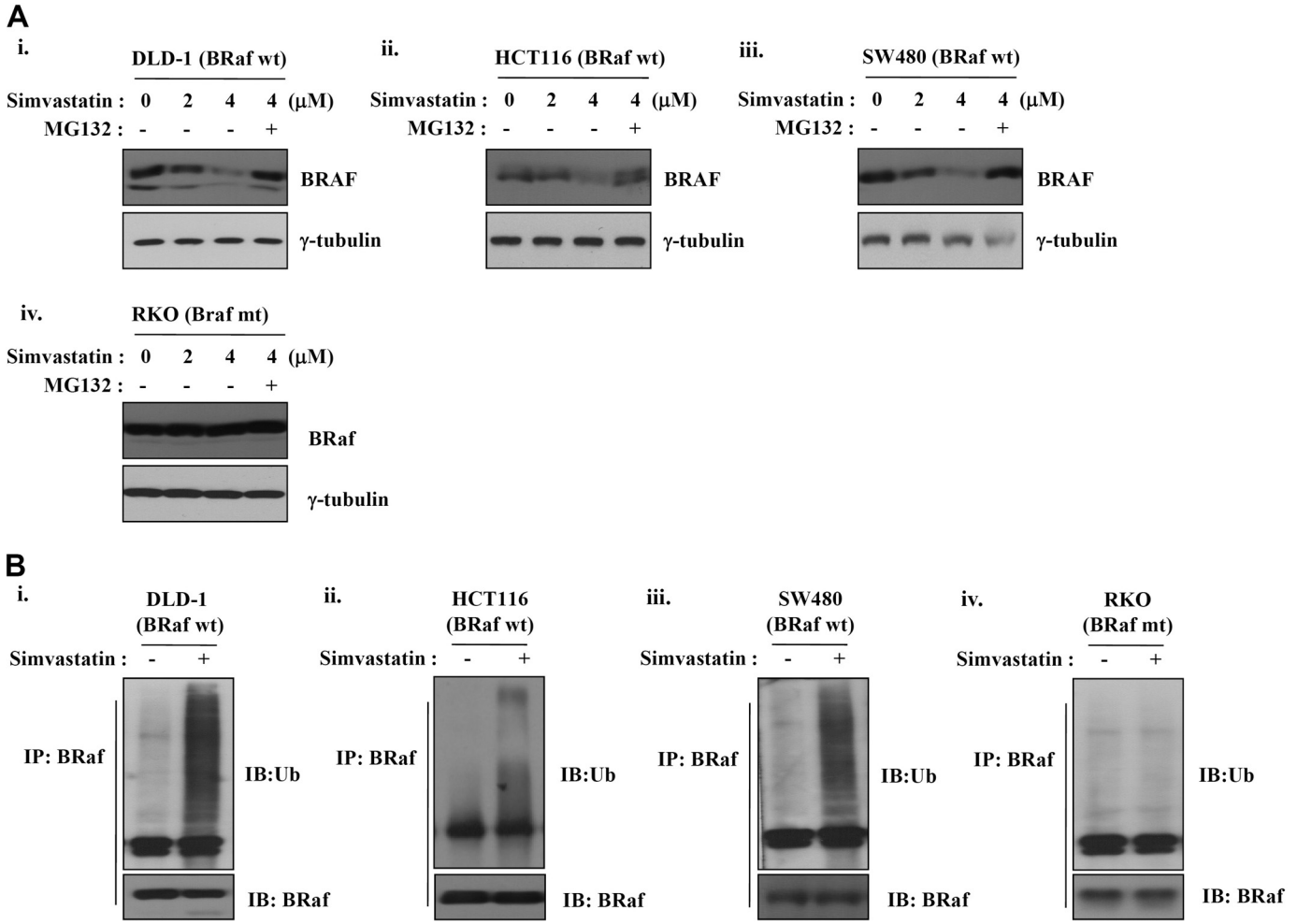

FIGURE 1. **Simvastatin induces ubiquitination and proteasome-mediated degradation of wild-type BRAF, but not mutant BRAF.** *A*, DLD-1 (*i*), HCT116 (*ii*), SW480 (*iii*), and RKO cells (*iv*) were treated with the indicated concentrations of simvastatin for 18 h and then incubated with or without the proteasome inhibitor MG132 (20 μM) for 6 h. Cell lysates were prepared and analyzed by immunoblotting using anti-BRAF and anti-γ-tubulin antibodies. *B*, DLD-1 (*i*), HCT116 (*ii*), SW480 (*iii*), and RKO cells (*iv*) were treated with simvastatin (1 μM) for 18 h and then treated with MG132. Cell lysates were immunoprecipitated (*IP*) with an anti-BRAF antibody and immunoblotted with an anti-ubiquitin antibody.

expressed wild-type BRAF and RNF149. In 293T cells co-transfected with DDK-tagged RNF149 and GFP-tagged wild-type BRAF expression constructs, DDK-RNF149 was readily detected in GFP-wild-type BRAF immunoprecipitates (Fig. 2*D*). Collectively, these results suggest that RNF149 binds to wild-type BRAF. To extend these results, we attempted to map the wild-type BRAF domains involved in direct binding to RNF149. For this, we used a mutant construct of wild-type BRAF lacking the kinase domain (WT BRAF ΔK) and a wild-type BRAF construct containing the kinase domain (WT BRAF K) (Fig. 2*E*). After co-transfecting 293T cells with either construct or DDK-tagged *RNF149*, we assessed DDK-tagged RNF149 in GFP-WT BRAF ΔK and GFP-WT BRAF K immunoprecipitates. Interestingly, RNF149 was detected in GFP-WT BRAF K immunoprecipitates, but not in immunoprecipitates of GFP-WT BRAF ΔK (Fig. 2*E*). In addition, RNF149 did not bind to the kinase domain of mutant BRAF (GFP-mt BRAF K, Fig. 2*F*), suggesting that RNF149 specially interacts with the kinase domain-containing C terminus of wild-type BRAF.

RNF149 Controls the Stability of Wild-type BRAF Protein—Because RNF149 was associated with wild-type BRAF, but not mutant BRAF, we constructed a GFP-tagged construct of the *BRAF* V600E mutant to further confirm the relationship between RNF149 and BRAF (Fig. 3). First, it was examined whether RNF149 influence endogenous wild-type and mutant BRAF. The protein level of wild-type BRAF in HCT116 cells transfected with DDK-tagged RNF149 in dose-dependent manner was gradually decreased, but not in RNF149-expressing RKO cells (Fig. 3*A*). To confirm this, after co-transfecting 293T cells with DDK-tagged *RNF149* and GFP-tagged wild-type *BRAF* or V600E *BRAF*, we assessed the levels of wild-type BRAF and V600E BRAF proteins. Whereas the levels of ectopically expressed wild-type BRAF were gradually decreased in cells co-transfected with *RNF149*, the expression of V600E *BRAF* was not affected by *RNF149* (Fig. 3*B*). Consistent with this, RNF149 induced the ubiquitination of wild-type BRAF, but not V600E BRAF (Fig. 3*B*). In addition, RNF149 also significantly decreased wild-type BRAF expression levels in melanoma cells (supplemental Fig. S2). Next, we further explored the role of RNF149 in regulating the turnover of wild-type BRAF by treating cells with the protein synthesis inhibitor cycloheximide and measuring changes in exogenously expressed wild-type BRAF

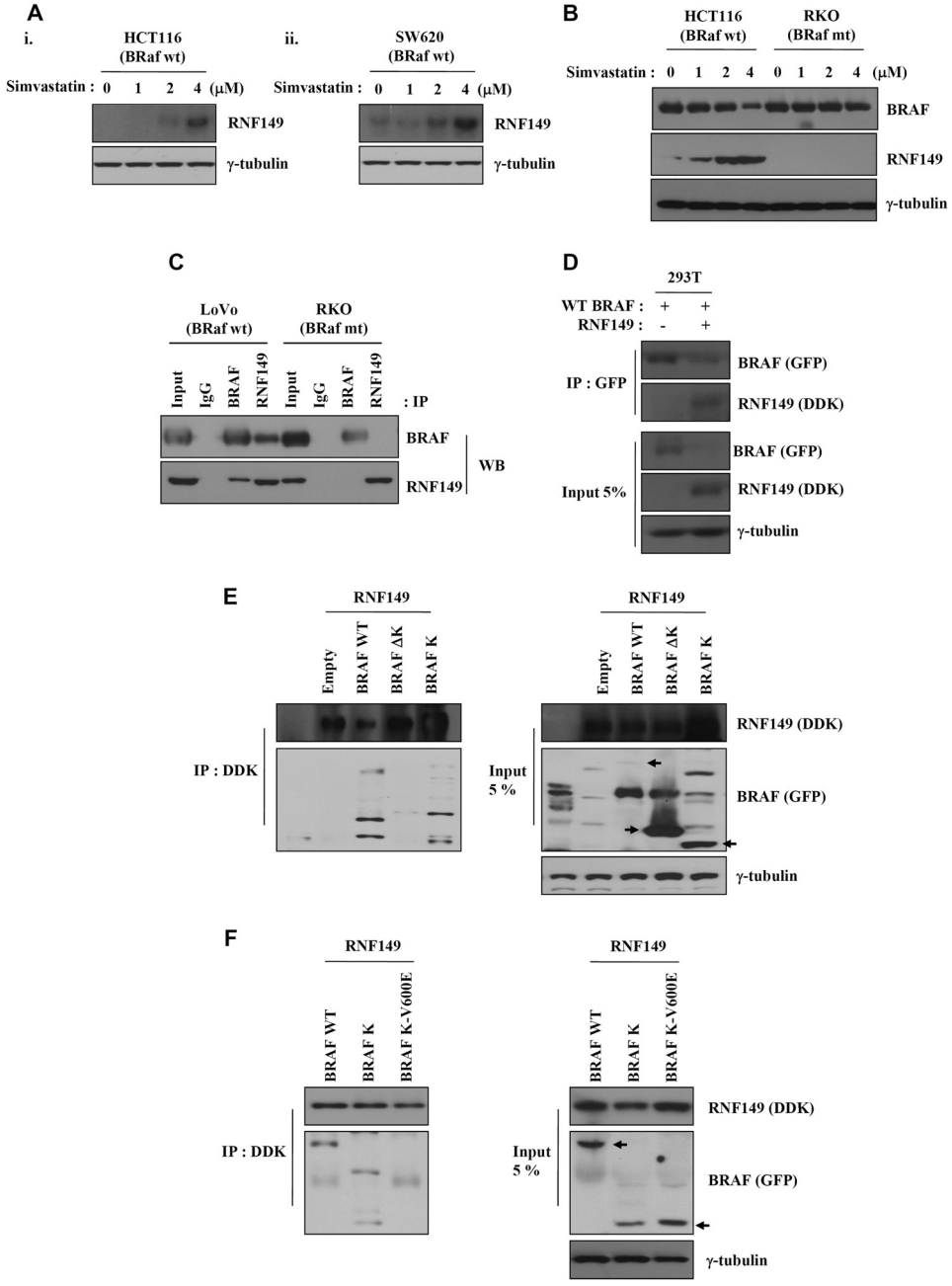

FIGURE 2. **Simvastatin activates the E3 ubiquitin ligase RNF149, which interacts with BRAF.** *A*, HCT116 (*i*) and SW620 cells (*ii*) were treated with the indicated concentrations of simvastatin for 24 h, after which cell lysates were immunoblotted with an anti-RNF149 antibody. *B*, HCT116 and RKO cells were treated with simvastatin at the indicated doses. Expression levels of endogenous BRAF and RNF149 were analyzed by immunoblotting using anti-BRAF and anti-RNF149 antibodies, respectively. γ-Tubulin levels were used as loading controls. *C*, MG132-treated LoVo and RKO cell lysates (500 μg of protein) were immunoprecipitated (*IP*) with anti-RNF149, anti-BRAF, or anti-mouse IgG antibodies, and precipitates were analyzed by Western blot (*WB*) analysis. *D*, 293T cells were transfected with GFP-tagged wild-type *BRAF* with or without co-transfected DDK-tagged *RNF149* for 18 h. Cell lysates were immunoprecipitated with an anti-GFP antibody and immunoblotted with anti-GFP or anti-DDK antibodies. *E*, 293T cells were co-transfected with RNF149 and wild-type BRAF (BRAF KD) or BRAF lacking the kinase domain (BRAF ΔKD) for 18 h. Cell lysates were then immunoprecipitated with an anti-BRAF antibody and analyzed by Western blotting using an antibody against RNF149. *F*, 293T cells were co-transfected with RNF149 and wild-type BRAF or wild-type BRAF KD or BRAF V600E mutant KD for 18 h and then cell lysates prepared for immunoprecipitation with anti-DDK antibody. Binding of RNF149 and WT or mutant BRAF KD was confirmed by Western blotting using an GFP antibody.

or V600E BRAF protein levels over time. In a cycloheximide chase experiment, exogenous expression of DDK-tagged *RNF149* led to diminished BRAF levels in cells co-transfected with GFP-tagged wild-type *BRAF*, but not in those co-transfected with V600E *BRAF* (Fig. 3*C*). Moreover, the expression of endogenous wild-type BRAF, but not endogenous mutant BRAF, was significantly decreased by ectopic expression of RNF149 (Fig. 3*D*). In contrast, knockdown of *RNF149* using siRNA resulted in stabilization of endogenous wild-type BRAF protein compared with that observed in cells treated with scrambled siRNA (Fig. 3, *E* and *F*). These findings indicate that the stability of wild-type BRAF, but not mutant BRAF, is regulated by RNF149, suggesting that wild-type BRAF may be a substrate of RNF149.

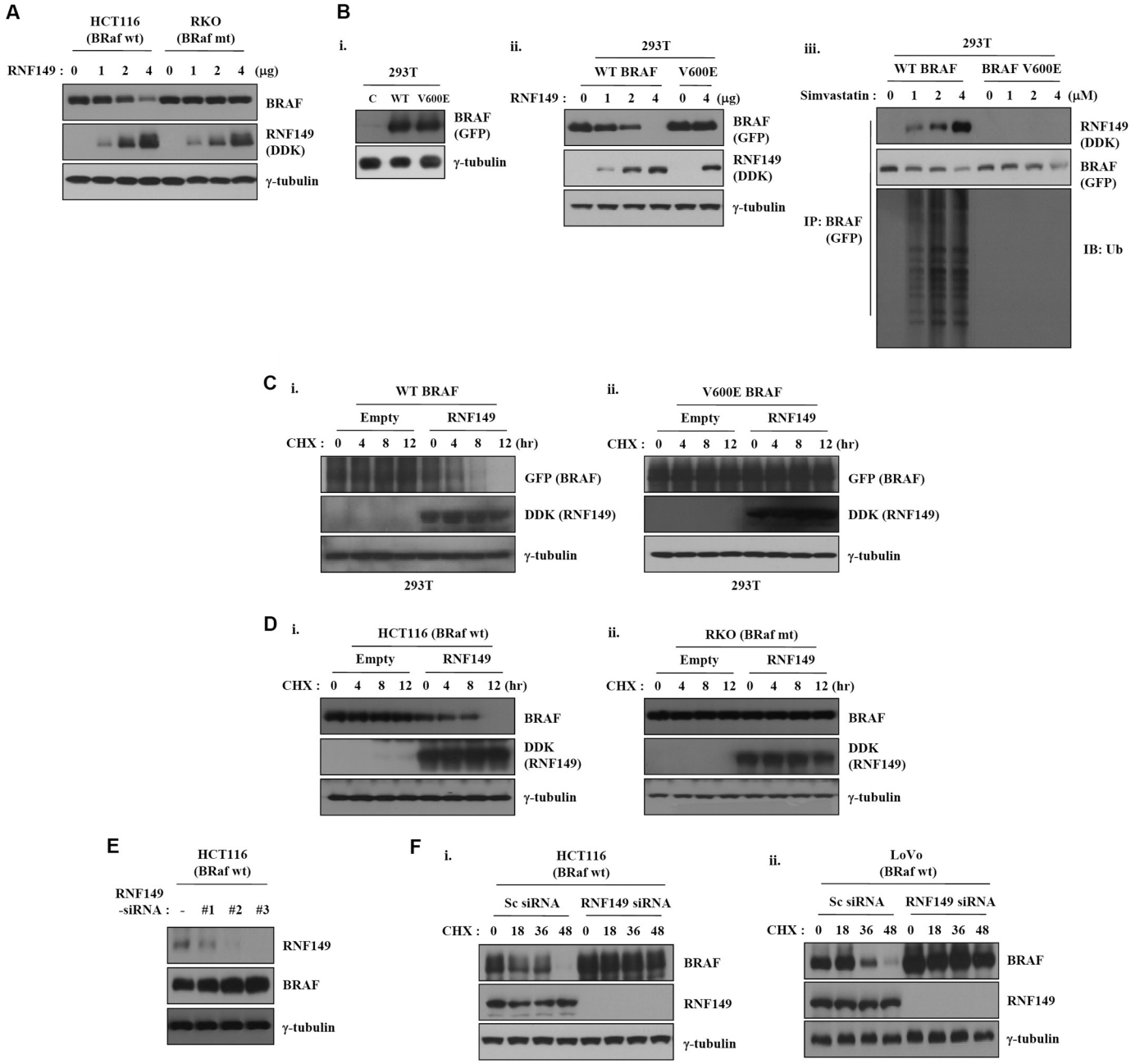

FIGURE 3. **RNF149 induces proteasomal degradation of wild-type BRAF, but not mutant BRAF.** *A*, HCT116 and RKO cells were transfected with DDK-tagged *RNF149* at the indicated doses and analyzed by Western blotting using anti-BRAF and anti-DDK (RNF149) antibodies. γ-Tubulin levels were used as loading controls. *B*, *i*, 293T cells were transfected with GFP-tagged wild-type *BRAF* or GFP-tagged V600E *BRAF* for 18 h. BRAF expression levels in 293T cells were determined by immunoblotting with anti-GFP antibody. *ii*, 293T cells were co-transfected with DDK-tagged RNF149 at the indicated amounts and GFP-tagged wild-type BRAF or V600E BRAF for 18 h. BRAF and RNF149 expression levels were determined by immunoblotting with anti-GFP and anti-DDK antibodies, respectively. γ-Tubulin levels were used as loading controls. *iii*, 293T cells were transfected with DDK-tagged RNF149 and GFP-tagged wild-type BRAF or V600E BRAF and then treated with simvastatin at the indicated doses. Cell lysates were immunoprecipitated with an anti-GFP antibody, and precipitates were immunoblotted with anti-DDK, anti-GFP, or anti-ubiquitin antibodies. *C*, 293T cells were co-transfected with DDK-tagged RNF149 and GFP-tagged wild-type BRAF (*i*) or V600E BRAF (*ii*) expression plasmids and then treated with the protein synthesis inhibitor cycloheximide (*CHX*, 50 μg/ml) at the indicated times. Wild-type (*i*) and V600E BRAF (*ii*) expression levels were determined by immunoblotting with an anti-GFP antibody. RNF149 expression levels were determined by immunoblotting with anti-DDK antibody, respectively. *D*, HCT116 and RKO cells were transfected with DDK-tagged RNF149 and then treated cycloheximide (50 μg/ml) at the indicated times. Expression levels of endogenous BRAF and ectopic RNF149 were analyzed by Western blotting using anti-BRAF and anti-DDK antibodies, respectively. *E*, HCT116 cells were transiently transfected with scrambled siRNA (control) or 3 RNF149-siRNA for 48 h, and then RNF149 and BRAF expression levels were analyzed by immunoblotting with anti-RNF149 and anti-BRAF antibodies. γ-Tubulin levels were used as loading controls. *F*, HCT116 (*i*) and LoVo (*ii*) cells were transfected with scrambled siRNA or RNF149 siRNA for 48 h and then treated with or without cycloheximide at the indicated times. Cell lysates were prepared and analyzed by immunoblotting using anti-BRAF and anti-RNF149 antibodies. γ-Tubulin levels were used as loading controls.

RNF149 Functions as an E3 Ubiquitin Ligase for Wild-type BRAF—To confirm that RNF149 ubiquitinates and promotes proteasomal degradation of wild-type BRAF, we first co-transfected 293T cells with GFP-tagged wild-type BRAF with DDK-tagged RNF149 in the presence/absence of the proteasome inhibitor MG132. Ectopic expression of wild-type BRAF was

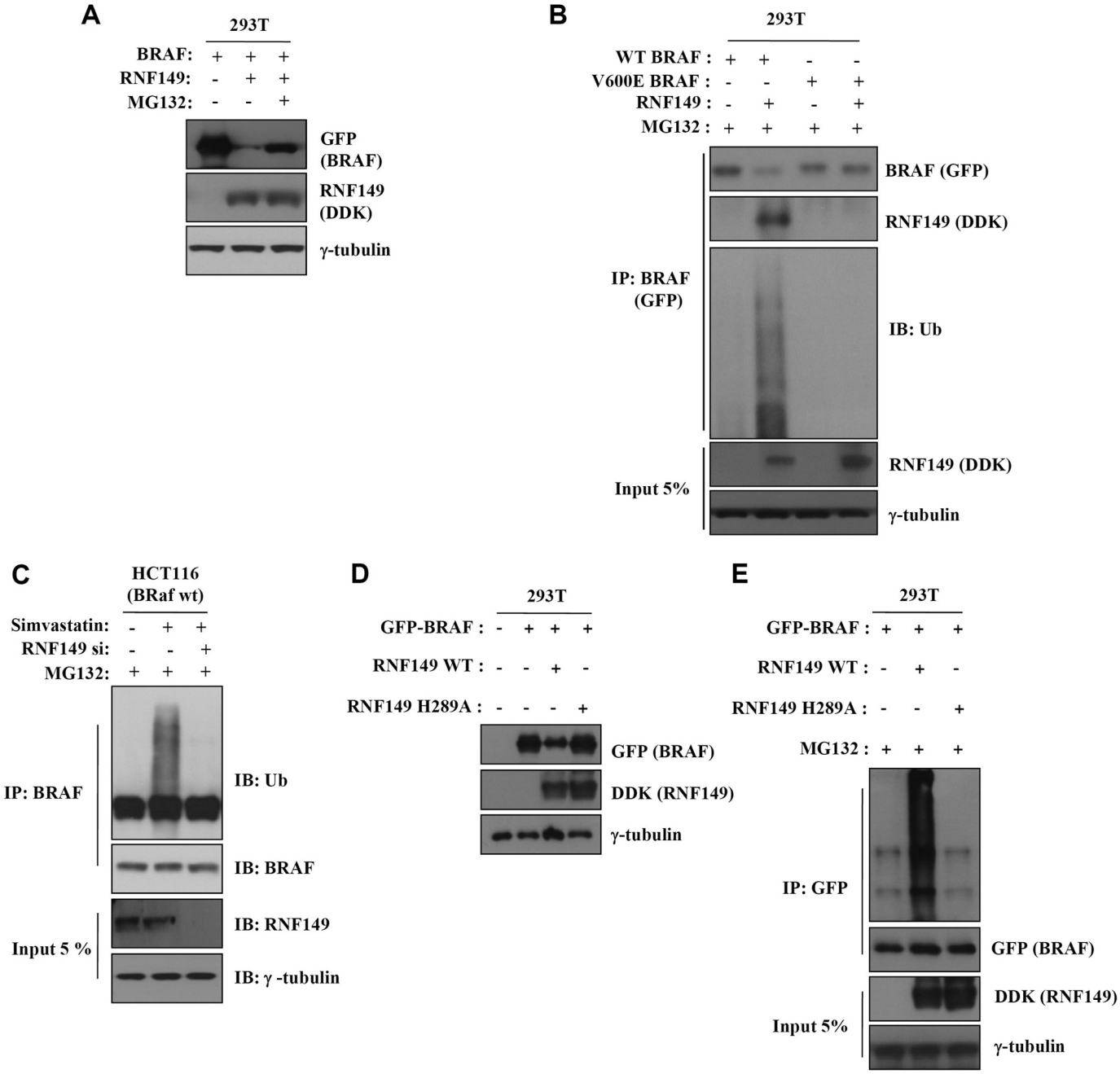

FIGURE 4. **RNF149 negatively regulates MAPK through ubiquitination of wild-type BRAF.** *A*, 293T cells were transfected with *BRAF* or *RNF149* plasmids and then treated with MG132 for 6 h. *B*, 293T cells were co-transfected with *RNF149* and wild-type *BRAF* or V600E *BRAF* for 18 h and then treated with MG132 for 6 h. Cell lysates were immunoprecipitated (*IP*) with an anti-GFP antibody. *C*, HCT116 cells were transfected with RNF149 siRNA for 48 h and treated with simvastatin (1 μM) for 18 h. Cell lysates were immunoprecipitated with an anti-BRAF antibody, and precipitates were analyzed by Western blotting (*IB*) using an anti-ubiquitin (*Ub*) antibody. *D*, 293T cells were co-transfected with GFP-tagged wild-type *BRAF* and wild-type *RNF149* or DDK-tagged *RNF149* H289A for 18 h. Expression levels of BRAF and RNF149 were analyzed by Western blotting using anti-GFP and anti-DDK antibodies. *E*, ubiquitination of BRAF was analyzed by Western blotting using anti-GFP antibody after cell lysates were immunoprecipitation with anti-GFP. Expression levels of transfected RNF149 were detected by immunoblotting using anti-DDK antibody. γ-Tubulin levels were used as loading controls.

significantly increased in cells transfected with RNF149 and MG132 compared with cells transfected with RNF149 only (Fig. 4*A*). To further determine whether RNF149 ubiquitinates wild-type BRAF, we performed intracellular ubiquitination assays (Fig. 4*B*). The higher molecular mass, ubiquitinated form of GFP-tagged BRAF was detected after ectopic expression of RNF149 in cells exogenously expressing GFP-wild-type BRAF, whereas exogenously expressed GFP-tagged V600E BRAF was not ubiquitinated (Fig. 4*B*). Consistent with this, siRNA-mediated knockdown of *RNF149* eliminated ubiquitination of endogenous wild-type *BRAF*, whereas scrambled siRNA had no effect (Fig. 4*C*). To further confirm the function of RNF149 as an E3 ubiquitin ligase for wild-type BRAF, we constructed a RING mutant plasmid of RNF149 (RNF149-$R_{h/a}$) that has no E3 ligase activity. It has been shown that the histidine residue (amino acid 289) in the RING domain is well con-

served among RING domains of E3 ligases and is essential for accepting ubiquitin from E2 (14). Consistent with the functional importance of this residue, we found that wild-type BRAF was not ubiquitinated in cells transfected with RNF149-$R_{h/a}$ (Fig. 4, *D* and *E*). Taken together, these results suggest that RNF149 functions as an E3 ubiquitin ligase for wild-type BRAF, strongly inducing wild-type BRAF ubiquitination and subsequent proteasome-mediated degradation.

RNF149 Affects Cancer Cell Survival through Suppression of Wild-type BRAF—As shown in Figs. 1 and 2, simvastatin resulted in ubiquitination of wild-type BRAF only through induction of RNF149. Based on these results, it was examined whether simvastatin treatment specifically affects cell death-dependent on RNF149 expression in cells expressing wild-type or mutant BRAF. Cell death was significantly increased in HCT116 cells (wild-type BRAF) expressing RNF149 after treatment with simvastatin, but not in RKO cells (mutant BRAF) expressing RNF149 (Fig. 5*A*). In addition, the level of wild-type BRAF protein was clearly decreased dependent on expression of RNF149, whereas the level of mutant BRAF protein was not decreased. Reversely, RNF149 silencing using small interference RNA (siRNA) rendered HCT116 cells (wild-type BRAF) resistant to simvastatin (supplemental Fig. S3*A*). However, knockdown of RNF149 did not decrease cell death induced by simvastatin in RKO cells that express mutant *BRAF* (supplemental Fig. S3*B*). To further confirm, we analyzed the effects of *RNF149* silencing using siRNA in CoLo205 cells that express mutant BRAF and endogenous RNF149. Knockdown of *RNF149* also did not decrease cell death induced by simvastatin in CoLo205 cells (supplemental Fig. S3*C*), indicating that simvastatin specifically affects cell death through suppression of wild-type BRAF by RNF149 in cells expressing wild-type BRAF.

Also, BRAF plays a role in cell survival by positively regulating the MEK-ERK pathway. Hence, RNF149, as an E3 ligase and a negative regulator of wild-type BRAF, might function as a negative regulator of the MEK-ERK pathway. To test this possibility, we used AZD6244, a specific inhibitor of MEK. RNF149-expressing cells showed an increased rate of cell death compared with control (vector-transfected) cells after exposure to AZD6244; similar results were obtained in cells expressing *BRAF*-siRNA (Fig. 5*B*). Soft agar colony-formation assays performed to confirm the inhibitory effect of RNF149 on cell survival showed that colony formation was dramatically decreased among RNF149-expressing cells after exposure to AZD6244 (Fig. 5*C*). Thus, repression of wild-type BRAF in cells that express RNF149 results in an increase in cell death and a decrease in colony formation. Collectively, these results suggest that RNF149 might act through suppression of BRAF to regulate the MEK-ERK pathway negatively.

DISCUSSION

Using a wide variety of approaches, recent studies have elucidated mechanisms by which BRAF regulates downstream signaling pathways, showing that BRAF action on the MEK-ERK pathway varies depending on the RAS genotype (15). Thus, the RAS-RAF-MEK-ERK signaling pathway is well established. However, BRAF inhibitory mechanisms have not been fully elucidated. In this study, we present evidence that RNF149 function is necessary and sufficient for ubiquitination of wild-type BRAF, but not mutant BRAF. Recent reports have shown that simvastatin induces a decrease in wild-type BRAF expression in colon cancer cells with mutant KRAS, but does not affect the expression of mutant BRAF in cells with wild-type KRAS (12). Clinical trials are also underway to evaluate the therapeutic efficacy of simvastatin in combination with anti-EGFR (epidermal growth factor receptor) monoclonal antibodies (cetuximab or panitumumab) in patients with wild-type *BRAF*/mutant *KRAS* (government clinical trials NCT01190462, NCT01281761, and NCT01110785).

On the basis of this report, which implies that simvastatin may induce a negative regulator of wild-type BRAF, we focused on decreases in wild-type BRAF expression after exposure to simvastatin. For this, we first analyzed the mechanism underlying the induction of wild-type BRAF degradation by simvastatin. As shown in Figs. 1 and 2, we subsequently identified RNF149 as a wild-type BRAF-interacting protein. These findings are significant because they suggest that RNF149 is a negative regulator of wild-type BRAF expression. Also, we confirmed how simvastatin increases RNF149. Simvastatin induced expression of *RNF149* mRNA in cells expressing wild-type *BRAF*, but not in cells expressing mutant *BRAF* (supplemental Fig. S4*A*). Consistently, treatment of actinomycin D resulted in a decrease of simvastatin-induced RNF149 expression (supplemental Fig. S4*B*). In addition, induction of RNF149 protein by treating cells with the protein synthesis inhibitor cycloheximide following simvastatin treatment was less increased than in cells treated with dimethyl sulfoxide (supplemental Fig. S4*C*), implying that the increase of RNF149 expression by simvastatin is both RNA- and protein synthesis-dependent. Future research will be focused on a detailed mechanism or unknown molecules that can indirectly/directly regulate RNF149 expression dependent on *BRAF* genotype.

The RING finger domain-containing protein RNF149 is known to function as an E3 ubiquitin ligase. Here, we showed that RNF149 expression affects the stability of wild-type BRAF protein in various colon cancer cells, 293T cells, and melanoma cells (supplemental Fig. S2), demonstrating that RNF149 significantly decreased wild-type BRAF expression levels. More importantly, RNF149 physically interacts with wild-type BRAF to induce its ubiquitination and promote its subsequent proteasomal degradation. Interestingly, it was reported previously that SPRY2, an inhibitor homologous to SPRY4, which was shown to suppress *RAS*/ERK signaling via direct binding to Raf-1 and interacts with wild-type BRAF, but not with mutant BRAF (16). Reversely, melanoma cells were highly sensitive to PLX4032 (vemurafenib) with IC_{50} in the nM range (60 ~ 450 nM), cells that express wild-type BRAF were resistant, with IC_{50} >2 μM (17, 18), implying that the interaction may be disrupted by mutations in BRAF. Thus, the mechanisms that negatively regulate mutant BRAF protein remain largely unexplored and are a subject of ongoing studies in our laboratory.

We also revealed that RNF149 acts as a negative regulator of the MEK-ERK pathway through repression of BRAF. The role of RNF149 in cancer development and progression, and in cel-

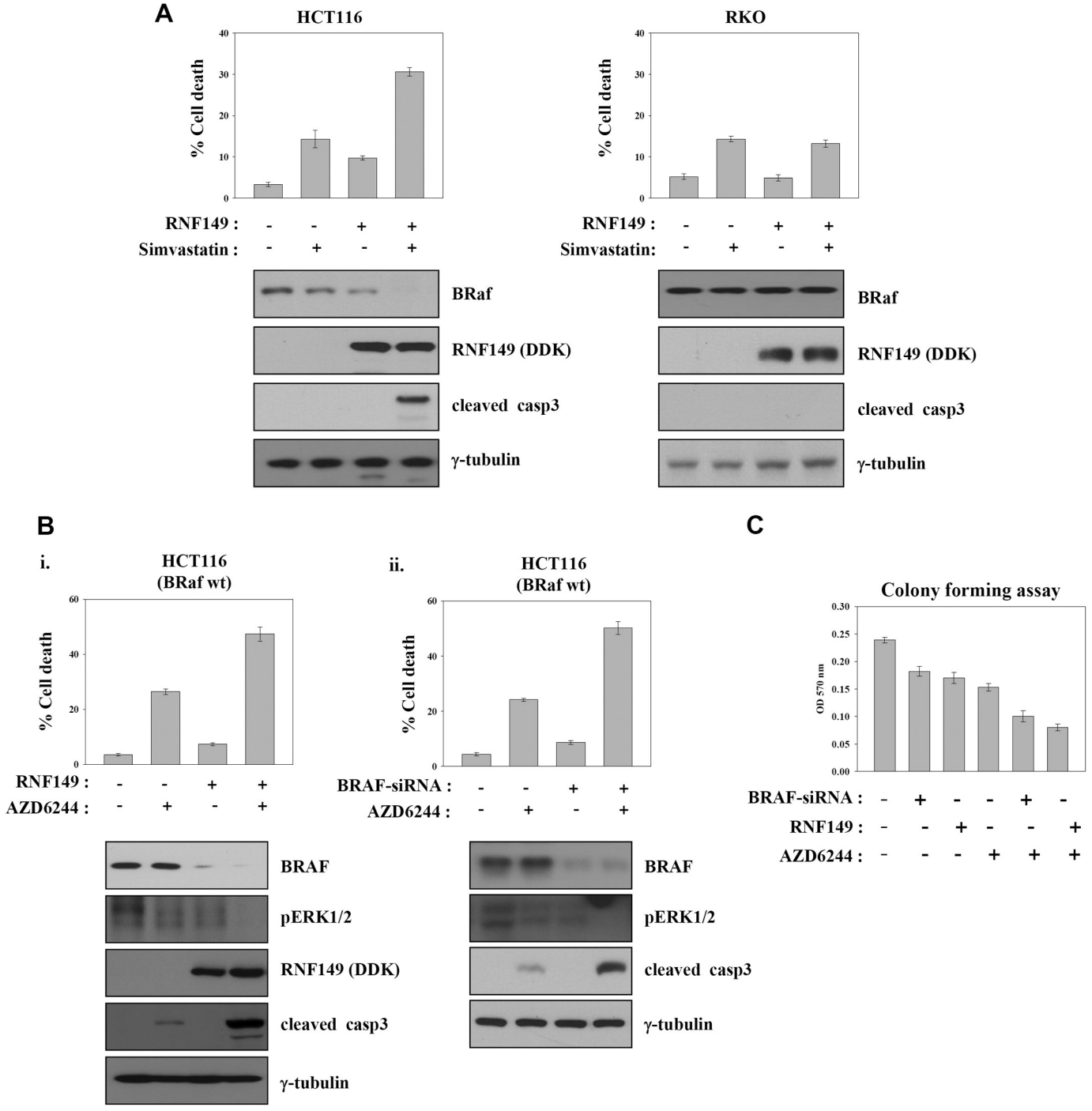

FIGURE 5. **RNF149 affects cancer cell survival through suppression of wild-type BRAF.** *A*, HCT116 and RKO cells were transfected with *RNF149* plasmid and then treated with simvastatin (1 μM) for 48 h. Cell death was determined using the trypan blue exclusion method. Cell lysates were analyzed by Western blotting using anti-BRAF, DDK, cleaved caspase-3 and γ-tubulin antibodies. *B*, HCT116 cells were transfected with an *RNF149* expression plasmid (*i*) or BRAF siRNA (*ii*) for 18 or 48 h, respectively, and then treated with AZD6244 (1 μM) for 24 h. Cell death was measured using trypan blue exclusion assays. Cell lysates were prepared and analyzed by immunoblotting using antibodies against BRAF, pERK, DDK, cleaved caspase 3, and γ-tubulin. Values represent the means ± SD (*error bars*) of three independent experiments. *C*, exogenous RNF149 expression and MEK inhibitor treatment synergistically inhibit cell proliferation only in cells harboring wild-type BRAF (see "Experimental Procedures"). Values represent the means ± SDs of three independent experiments.

lular responses, including cell growth, differentiation, and cell death, however, were not addressed here; the mechanism by which simvastatin increase RNF149 still remains unclear; thus, further studies will be required. Although we clearly established RNF149 as an E3 ligase of wild-type BRAF, our results do not rule out the possibility that RNF149 has additional substrates that might be involved in tumorigenesis. These findings, which collectively indicate that RNF149 functions as an E3 ubiquitin ligase for wild-type BRAF and thus plays a role in the regulation of wild-type BRAF, suggests that therapeutic approaches designed to control RNF149 expression may represent attractive cancer prevention strategies.

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