Tripartite Motif Protein 32 Facilitates Cell Growth and Migration via Degradation of Abl-Interactor 2

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Abstract

***Tripartite motif protein 32* (*TRIM32*) mRNA has been reported to be highly expressed in human head and neck squamous cell carcinoma, but the involvement of TRIM32 in carcinogenesis has not been fully elucidated. In this study, we found by using yeast two-hybrid screening that TRIM32 binds to Abl-interactor 2 (Abi2), which is known as a tumor suppressor and a cell migration inhibitor, and we showed that TRIM32 mediates the ubiquitination of Abi2. Overexpression of TRIM32 promoted degradation of Abi2, resulting in enhancement of cell growth, transforming activity, and cell motility, whereas a dominant-negative mutant of TRIM32 lacking the RING domain inhibited the degradation of Abi2. In addition, we found that TRIM32 suppresses apoptosis induced by *cis*-diamminedichloroplatinum (II) in HEp2 cell lines. These findings suggest that *TRIM32* is a novel oncogene that promotes tumor growth, metastasis, and resistance to anticancer drugs.** [Cancer Res 2008;68(14):5572–80]

Introduction

Ubiquitination is a versatile posttranslational modification mechanism used by eukaryotic cells mainly to control protein levels through proteasome-mediated proteolysis. Ubiquitin conjugation is achieved by several enzymes that act in concert, including a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3; refs. 1, 2). E3 is thought to be the component of the ubiquitin conjugation system that is most directly responsible for substrate recognition. Enzymes belonging to class E3 that have thus far been identified include members of the HECT (homologous to E6-AP carboxyl terminus), RING-finger, and U-box families of proteins (3–5).

Tripartite motif (TRIM) proteins are characterized by the presence of a RING finger, one or two zinc-binding motifs named B-boxes, and an associated coiled-coil region (6). Most TRIM proteins have been reported to have a role in the ubiquitination process, for example, TRIM25 (EFP) ubiquitinates 14-3-3σ (7), estrogen receptor α (8), and retinoic acid–inducible gene I (9). Furthermore, several TRIM family members are involved in various cellular processes, such as transcriptional regulation, cell growth, apoptosis, development, and oncogenesis (10–12).

The TRIM family protein TRIM32 was identified as a protein that interacts with the activation domain of lentiviral Tat proteins (activators of viral transcription; ref. 13). Each TRIM family member is characterized by a specific carboxyl-terminal domain (6). TRIM32 contains six repeats of the NHL (NCL-1, HT2A, and LIN-41) motif, which is likely to mediate protein-protein interactions (14). Point mutations of human TRIM32 have been reported in two autosomal recessive genetic disorders: limb-girdle muscular dystrophy type 2H, which is a myopathy with a primary or predominant involvement of the pelvic or shoulder girdle musculature (15), and Bardet-Biedl syndrome, which is characterized by obesity, pigmentary retinopathy, polydactyly, renal abnormalities, learning disabilities, and hypogenitalism (16). Moreover, it has been reported that *TRIM32* is highly expressed in the occipital lobe of patients with Alzheimer's disease compared with that in healthy controls (17). It has been shown that TRIM32 has a ubiquitin ligase activity for actin or Piasy (small ubiquitin-like modifier ligase; ref. 18). TRIM32 ubiquitinates actin *in vitro* down-regulates endogenous actin in HEK293 cells and interacts directly with myosin, but not with actin (19). TRIM32 regulates UVB-induced keratinocyte apoptosis through induction of nuclear factor-κB by promoting the degradation of Piasy (18). Furthermore, it has been shown that TRIM32 protein expression is elevated in a mouse skin carcinogenesis model and in human skin squamous cell carcinoma and that *TRIM32* mRNA expression is elevated in human head and neck squamous cell carcinoma (20, 21). However, the role of TRIM32 in the progression of carcinogenesis has not been fully clarified.

Abl-interactor 2 (Abi2) has been identified as a protein that specifically binds to both the src homology 3 (SH3) and carboxy-terminal proline-rich sequences of Abl, which has been shown to be one of nonreceptor tyrosine kinases involved in the regulation of intercellular signals (22). Abi2 promotes Abl-mediated phosphorylation of Cdc2 and inactivation of Cdc2 kinase activity, leading to suppression of cell growth (23). Coexpression of a truncated form of Abi2 with c-Abl activates the oncogenic potential of c-Abl, suggesting that the full-length Abi2 functions as a tumor suppressor (22). Furthermore, Abi family members have been reported to affect cell motility. Abi1 promotes cell motility (24), whereas NESH (Abi3) suppresses cell motility and metastatic dissemination of malignant cells (25, 26). Deletion of the SH3 domain and carboxy-terminal proline-rich sequences in Bcr-Abl prevents Abi2-mediated degradation and inhibits cell migration, suggesting that the Abi2 negatively regulates cell motility (27).

In this study, with the aim of elucidating the molecular function of TRIM32 in carcinogenesis, we identified Abi2 as a novel TRIM32-binding protein by using yeast two-hybrid screening. TRIM32 enhanced the ubiquitination and degradation of Abi2. We also found that overexpression of TRIM32 promoted cell growth, transforming activity and cell motility. In addition, we found that TRIM32 suppressed apoptosis induced by *cis*-diamminedichloroplatinum (II) (cDDP) in HEp2 cell lines, originating from human laryngeal squamous cell carcinoma. Our results provide evidence

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-07-6231

for a direct role of TRIM32 in carcinogenesis, cell motility, and resistance to anticancer drugs.

Materials and Methods

Cell culture. HEK293T, Neuro2a, and HEp2 and its cDDP-resistant variant (HEp2/cDDP) cell lines were cultured under an atmosphere of 5% CO_2 at 37°C in DMEM (Sigma Chemical Co.) supplemented with 10% fetal bovine serum (Invitrogen). HEp2 and HEp2/cDDP cells were provided by Nippon Kayaku Co. NIH 3T3 cells were cultured under the same conditions in DMEM supplemented with 10% calf serum (CS; Camblex).

Human tumor collection. Patients with head and neck squamous cell carcinoma who gave informed consent were selected for this study. Tumor and surrounding uninvolved mucosa samples in the same patient were removed during surgery and subjected to immunoblot analysis and immunostaining with anti-TRIM32 antibody (1:1,000 dilution, mouse polyclonal, Abnova), anti-cytokeratin 1/10 (0.2 μg/mL; LH1, Santa Cruz Biotechnology), anti–cytokeratin 15 (0.2 μg/mL; LHK15, Santa Cruz), anti-basic cytokeratin (0.1 μg/mL; AE3, Santa Cruz), anti-Abi2 (0.4 μg/mL; P-20, Santa Cruz), and anti–phosphorylated histone H3 (Ser^{10}; 5 μg/mL; rabbit polyclonal, Millipore).

Cloning of cDNAs and plasmid construction. Human *TRIM32* and *Abi2* cDNAs were amplified from HeLa cDNA by the PCR with BlendTaq (Takara) using the following primers: 5′-GCAATGGCTGCAGCAG-CAGCTTCT-3′ (*TRIM32*-sense), 5′-CCCCTATGGGGTGGAATATCTTCT-3′ (*TRIM32*-antisense), 5′-GCCATGGCGGAGCTGCAGATGCTG-3′ (*Abi2*-sense), and 5′-TGCCTACTCAGAATAATGCATGAT-3′ (*Abi2*-antisense). The amplified fragments were subcloned into pBluescript II SK^+ (Stratagene). FLAG-tagged, HA-tagged, or Myc-tagged *TRIM32* and *Abi2* cDNAs were then subcloned into pCR, pCGN, and pcDNA3 (Invitrogen) for expression in eukaryotic cells, pET30a (Novagen) for the production of His_6-tagged fusion protein, pGEX-6P1 (Amersham Bioscience) for the production of glutathione *S*-transferase (GST)–tagged fusion protein, and pBTM116 and pACT2 (Clontech) for a yeast two-hybrid system. Deletion mutants of *TRIM32* cDNA containing amino acids 65-653, 163-653, 386-653, and 1-309 were amplified by PCR and subcloned.

Yeast two-hybrid screening. Complementary DNA encoding the full-length of human *TRIM32* was fused in-frame to the nucleotide sequence for the LexA domain (BD) in the yeast two-hybrid vector pBTM116. To screen for proteins that interact with TRIM32, we transfected yeast strain L40 (Invitrogen) stably expressing the corresponding pBTM116 vector with a human HeLa cDNA library (Clontech).

Transfection, immunoprecipitation, and immunoblot analysis. HEK293T cells were transfected by the calcium phosphate method and lysed in a solution containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Nonidet P-40, leupeptin (10 μg/mL), 1 mmol/L phenylmethylsulfonyl fluoride, 400 μmol/L Na_3VO_4, 400 μmol/L EDTA, 10 mmol/L NaF, and 10 mmol/L sodium PPi. The cell lysates were centrifuged at 16,000 × *g* for 10 min at 4°C, and the resulting supernatant was incubated with antibodies for 2 h at 4°C. Protein A or G-sepharose (Amersham Pharmacia Biotech) that had equilibrated with the same solution was added to the mixture, which was then rotated for 1 h at 4°C. The resin was separated by centrifugation, washed five times with ice-cold lysis buffer, and then boiled in SDS sample buffer. For an *in vivo* ubiquitination assay, the cells were lysed in a solution containing 50 mmol/L Tris-HCl (pH 7.4), 0.1% SDS, 150 mmol/L NaCl, 1% Nonidet P-40, leupeptin (10 μg/mL), 1 mmol/L phenylmethylsulfonyl fluoride, 400 μmol/L Na_3VO_4, 400 μmol/L EDTA, 10 mmol/L NaF, and 10 mmol/L sodium PPi. Immunoblot analysis was performed with the following primary antibodies: anti–c-Myc (1 μg/mL; 9E10, Covance), anti-FLAG (1 μg/mL; M5, Sigma), anti-HA (1 μg/mL; HA.11, Covance), anti-HA (1 μg/mL; Y11, Santa Cruz), anti-GST (0.1 μg/mL; B-14, Santa Cruz), anti-TRIM32 (mouse polyclonal, Abnova), anti-ubiquitin (1 μg/mL; P4D1, Santa Cruz), anti–α-tubulin (1 μg/mL; TU-01, ZYMED), anti-Hsp90 (1 μg/mL; 68, Transduction Laboratories), and anti–glyceraldehyde-3-phosphate dehydrogenase (1 μg/mL; 6C5, Ambion). Immune complexes were detected with horseradish peroxidase–conjugated antibodies to mouse or rabbit IgG (1:10,000 dilution, Promega) or goat IgG (1:5,000 dilution, Santa Cruz) and an enhanced chemiluminescence system (Amersham).

Recombinant proteins. GST-tagged TRIM32 was expressed in XL-1 Blue cells and then purified by reduced glutathione–sepharose beads (Roche). His_6-FLAG-tagged Abi2 was expressed in *Escherichia coli* strain BL21 (DE3; Invitrogen) and then purified by using ProBond metal affinity beads (Invitrogen).

***In vitro* pull-down assay.** GST-TRIM32 (0.25 μg) and His_6-FLAG-Abi2 (0.5 μg) were mixed in 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, and 1% Nonidet P-40 for 1 h at 4°C. The binding mixtures were incubated with anti-GST antibody for 1 h at 4°C. Protein G-sepharose was added to the mixture, and then the mixture was rotated for 1 h at 4°C. The resin was separated by centrifugation, washed five times with buffer, and then boiled in SDS sample buffer. Immunoblot analysis was performed with anti-FLAG antibody.

Pulse-chase analysis with cycloheximide. Transiently transfected HEK293T cells were cultured with cycloheximide at the concentration of 50 μg/mL and then incubated for various times. Cell lysates were then subjected to SDS-PAGE and immunoblot analysis with an antibody to FLAG.

Retroviral infection. Retroviral expression vectors for FLAG-TRIM32 wild-type (WT), FLAG-TRIM32 deletion mutant of RING-finger domain (ΔRING), or FLAG-Abi2 was constructed with pMX-puro. Retroviral expression vector for TRIM32(WT) or c-Src(Y527F) was constructed with pMX-hyg. Retroviral expression vectors were kindly provided by Dr. Kitamura (University of Tokyo). For retrovirus-mediated gene expression, Neuro2A and NIH 3T3 cells were infected with retroviruses produced by Plat-E packaging cells and HEp2 cells were infected with retroviruses produced by Plat-A packaging cells. Cells were then cultured in the presence of puromycin (2 μg/mL) or hygromycin B (0.2 mg/mL; Sigma).

RNA interference. The small interfering RNA (siRNA) oligonucleotide sequences specific for mouse and human *TRIM32* mRNA were designed by Silencer Predesigned siRNA (Ambion) and corresponded to nucleotides 808 to 826 and 150 to 168, respectively. siRNAs were transfected by siPORT *NeoFX* (Ambion) into NIH 3T3, HEp2, and HEp2/cDDP cells (final concentration, 10 nmol/L). After 48 h, the resulting cell lines were checked by real-time quantitative PCR and immunoblot analysis.

Real-time quantitative PCR. Total RNA (1 μg) isolated from various cell lines using Isogen (Wako) was subjected to reverse transcription with ReverTraAce (Toyobo). The resulting cDNA was subjected to real-time quantitative PCR by TaqMan gene expression assays following the manufacturer's instructions (Applied Biosystems). The assays were performed with a mouse or human *TRIM32*-specific TaqMan probe and primers in an ABI-PRISM 7000 Sequence Detection System (Applied Biosystems). 18S rRNA was selected as an internal control to normalize the expression levels. Each sample was tested in triplicate.

Cell cycle analysis. NIH 3T3 cell lines were incubated in DMEM with 0.1% CS for 24 h for serum starvation. The cells released from serum starvation were harvested at indicated times and suspended in a solution containing 20 mmol/L HEPES, 160 mmol/L NaCl, 1 mmol/L EGTA, and 0.04% digitonin. The cells were incubated at 37°C for 1 h in a solution with RNase A (100 μg/mL; Novagen) and propidium iodide (20 μg/mL) and then analyzed with a FACSCalibur flow cytometer and Cell Quest software (Becton Dickinson).

Sulforhodamine B assay. Cells were seeded at a density of 2,000 per well in a 96-well plate and then fixed with 10% trichloroacetic acid at indicated times. The cells were stained with 0.4% sulforhodamine B (SRB) solution, washed using 1% acetic acid, and then solubilized with 10 mmol/L Tris base. Absorbances were measured by using a plate reader at 570 nm (28, 29).

Colony formation assay. For the colony formation assay, 1×10^5 cells were plated in 60-mm dishes containing 0.4% soft agar and cultured for 2 wk. The numbers of colonies with a diameter of >0.1 mm in randomized areas (1 cm^2) were counted.

Wound healing assay. A six-well plate was coated overnight with 20 μg/mL fibronectin (Asahi Techno Glass) in PBS, and then cells were seeded (3×10^5 per well) in DMEM with 0.1% CS overnight. The injured line

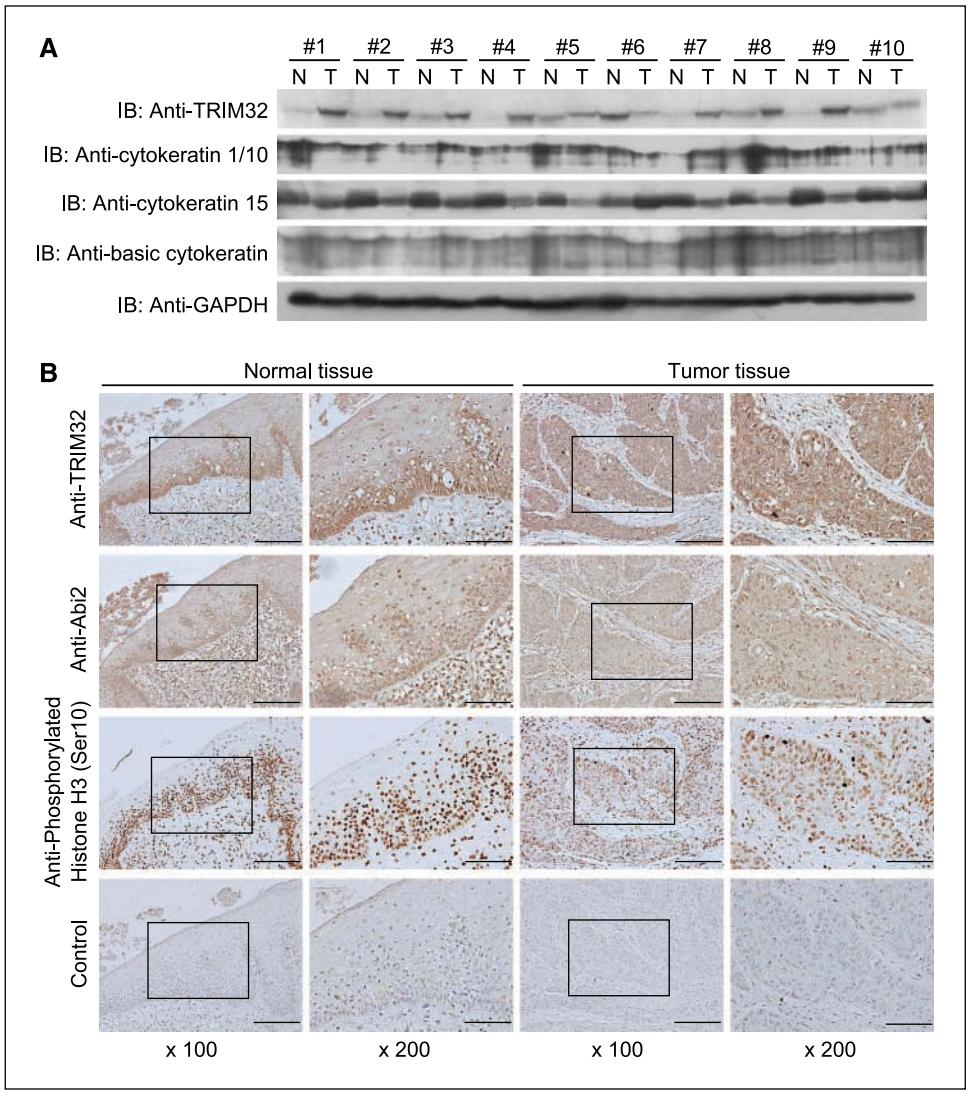

Figure 1. Elevated expression of TRIM32 in human head and neck squamous cell carcinoma. *A,* the tissue lysates from 10 pairs of tumor (*T*) and normal tissues (*N*) were subjected to immunoblot analysis with anti-TRIM32 antibody. *B,* normal and tumor tissues in the same patient with tongue squamous cell carcinoma were stained with anti-TRIM32, anti-Abi2, and anti–phosphorylated specific histone H3 (Ser^{10}) antibodies or with preimmune antibody (*Control*). Micrographs were obtained at 100× magnification (*scale bar,* 200 μm) and each black rectangle was magnified at 200× (*scale bar,* 100 μm).

was made with a cell scraper of 1 mm in width on the confluent monolayer of cells and photographed at indicated times.

Transwell migration assay. Twelve-well polycarbonate transwell migration chambers with 8-μm pores (Corning) were coated overnight on the underside with 20 μg/mL fibronectin in PBS. The lower compartment was filled with DMEM containing 0.1% CS. Cells were cultured in DMEM with 0.1% CS for 24 h and then placed at a density of 1×10^5 cells in the upper part of the transwell plate and incubated for 4 h in a serum-starved condition. The cells on the upper surface of the insert were carefully removed with cotton pads. The cells on the lower surface were fixed with 3.7% formaldehyde and stained with Hoechst 33258. The number of cells that had migrated was counted under a fluorescence microscope.

Apoptosis assay. Assessment of apoptosis was performed by measurement of sub-G_1 peak and detection of cleaved caspase-3. For sub-G_1 peak, HEp2 cells were incubated with or without cDDP (25 μmol/L) for 24 h. The cells were treated with propidium iodide and then sub-G_1 peak was analyzed with a FACSCalibur flow cytometer. For cleaved caspase-3, HEp2 cells were incubated with cDDP (25 μmol/L) for various times. Cell lysates were then subjected to immunoblot analysis with anti–caspase-3 (1:1,000 dilution; 3G2, Cell Signaling) and anti–cleaved caspase-3 (Asp^{175}; 1:1,000 dilution; 5A1, Cell Signaling) antibodies.

Statistical analysis. The unpaired Student's *t* test was used to determine the statistical significance of experimental data.

Results

Elevated expression of TRIM32 in human head and neck squamous cell carcinoma. It has been reported that *TRIM32* mRNA expression was elevated in human head and neck squamous cell carcinoma. Therefore, we examined the expression level of TRIM32 protein in tumor samples that had been surgically excised. Immunoblot analysis with anti-TRIM32 antibody showed that TRIM32 protein levels were considerably elevated in 70% of tumor tissues (except for samples 5, 6, and 10) compared with the levels in normal tissues in the same patient (Fig. 1*A*). To determine whether overexpression of TRIM32 antigen activity depends on the volume of epithelial components in the samples, the same cell lysates were immunoblotted with antibodies to cytokeratin 1/10 and cytokeratin 15, which have frequently been found in squamous epithelium and squamous cell carcinoma, and an antibody to basic cytokeratin. Whereas the expression levels of cytokeratin 15 were elevated in normal tissues except for sample 6, those of cytokeratin 1/10 and basic cytokeratin showed no difference between tumor and normal tissues (Fig. 1*A*). These results suggest that overexpression of TRIM32 in tumor tissues is independent of the abundance of epithelial components. We next performed

immunohistochemical analysis using squamous cell carcinoma tissues and normal tissues of the human tongue (Fig. 1*B*). TRIM32 proteins were detected within the cytoplasm in tumor cells but also in normal epidermal cells near the basement membrane. We also stained the same sample with anti–phosphorylated histone H3 antibody as a mitosis marker. Both tumor cells and normal epidermal cells were stained, corresponding to the results obtained by using the anti-TRIM32 antibody. These results show that the expression of TRIM32 is elevated in human head and neck carcinoma but also in normal epidermal cells during the mitotic phase.

TRIM32 interacts with Abi2. To identify proteins that interact with TRIM32, we screened the pACT2 HeLa cDNA library by using pBTM116-hTRIM32 plasmid as a bait. Thirty-five positive clones were isolated on Leu-Trp-His–deficient medium from 1.36×10^6 transformants. Three clones (clones 10, 18, and 26) of the positive clones have sequence identities with the human *Abi2* cDNA (National Center for Biotechnology Information accession number BT009920). We tested whether TRIM32 physically interacts with Abi2 *in vivo* and *in vitro*. We transfected expression vectors encoding FLAG-TRIM32 and/or HA-Abi2 in HEK293T cells. Cell lysates were subjected to immunoprecipitation with an antibody to HA or FLAG, and the resulting precipitates were subjected to immunoblot analysis with an antibody to FLAG or HA, respectively. FLAG-TRIM32 was selectively coprecipitated by anti-HA antibody, and HA-Abi2 was also coprecipitated by anti-FLAG antibody (Fig. 2*A*). We also verified the interaction between endogenous TRIM32 and endogenous Abi2 by immunoprecipitation (Fig. 2*B*) and the colocalization by immunofluorescent staining (Supplementary Fig. S1). Furthermore, recombinant GST-TRIM32 and His_6-FLAG-Abi2 were used in an *in vitro* pull-down assay and the assay indicated that GST-TRIM32 specifically binds to His_6-FLAG-Abi2 *in vitro* (Fig. 2*C*). To further confirm the binding region of TRIM32 to Abi2, we constructed several deletion mutants of TRIM32 and compared their abilities to bind to Abi2 (Fig. 2*D, left*). An *in vivo* binding assay showed that deletion of the NHL domain abolishes the binding to Abi2, indicating that the NHL domain of TRIM32 is required for interaction with Abi2 (Fig. 2*D, right*).

TRIM32 mediates ubiquitination and degradation of Abi2. To elucidate whether TRIM32 ubiquitinates Abi2, we performed an *in vivo* ubiquitination assay. Expression vectors encoding Myc-Abi2, FLAG-TRIM32, and HA-ubiquitin were transfected in HEK293T cells, and their lysates were subjected to immunoprecipitation with an antibody to Myc and then to immunoblot analysis with an antibody to HA. Although Myc-Abi2 was slightly ubiquitinated even without overexpression of TRIM32, overexpression of TRIM32 considerably enhanced polyubiquitination of Myc-Abi2 (Fig. 3*A*). We next examined the possible effect of TRIM32 on the degradation of Abi2 *in vivo*. Pulse-chase analysis revealed that the expression of WT TRIM32 promoted the degradation of Abi2, whereas TRIM32(ΔRING) delayed it (Fig. 3*B*). To determine whether TRIM32 enhances the degradation of Abi2 through the ubiquitin-proteasome pathway, we examined the stability of endogenous Abi2 by overexpression of TRIM32 with a proteasome inhibitor. Neuro2a cells infected with a retrovirus vector encoding FLAG-TRIM32(WT) were incubated with or without a proteasome inhibitor, LLnL. Overexpression of TRIM32(WT) diminished expression of endogenous Abi2, but the expression of Abi2 was restored by LLnL (Fig. 3*C*). Furthermore, we examined the stability of endogenous Abi2 by RNA interference of *TRIM32*. To knockdown endogenous *TRIM32*, siRNA specific for mouse *TRIM32* (si-TRIM32) or nontargeting siRNA as a control (si-control) was transfected into NIH 3T3 cells, resulting in silencing of *TRIM32* at both mRNA and protein levels (Fig. 3*D*). The expression level of

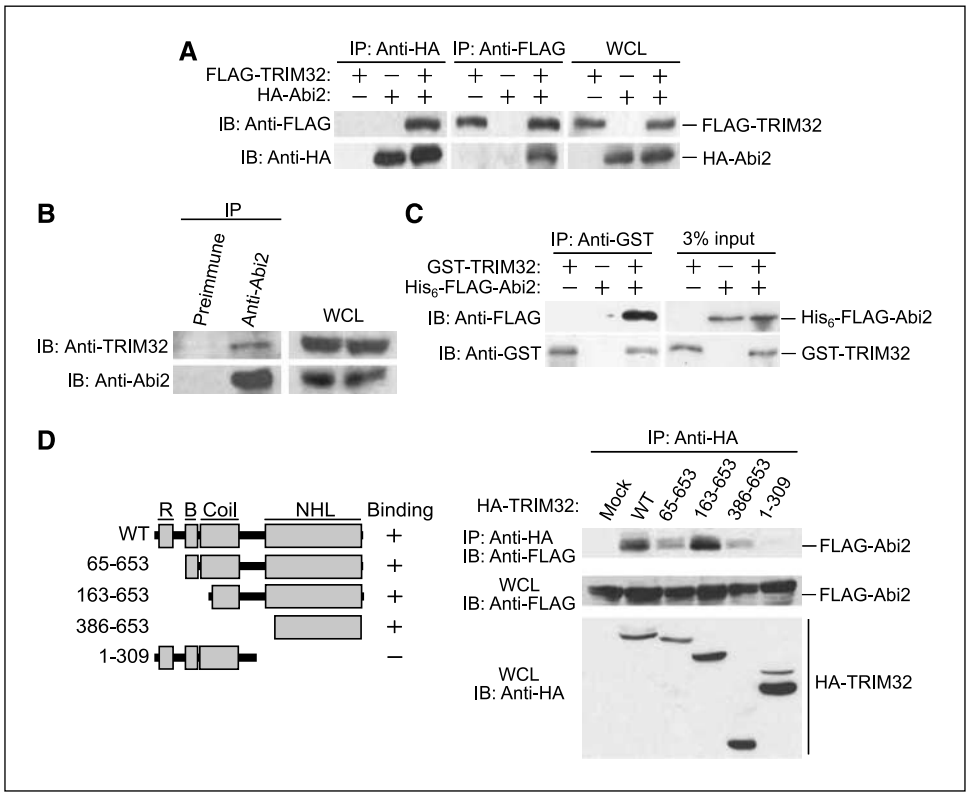

Figure 2. TRIM32 interacts with Abi2. *A, in vivo* binding assay between TRIM32 and Abi2. FLAG-TRIM32 and HA-Abi2 expression vectors were transfected into HEK293T cells. Cell lysates (*WCL*) were immunoprecipitated with anti-HA or anti-FLAG antibody and immunoblotted with anti-FLAG and anti-HA antibodies. *B,* interaction between endogenous TRIM32 and Abi2 in Neuro2a cells. Cell lysates were immunoprecipitated with anti-Abi2 antibody and then immunoblotted with anti-TRIM32 antibody. *C, in vitro* pull-down assay between TRIM32 and Abi2. Recombinant GST-TRIM32 and His_6-FLAG-Abi2 were mixed in combination as indicated. The reaction mixtures were immunoprecipitated with anti-GST antibody and then immunoblotted with anti-FLAG antibody. *D,* specific interaction of the NHL domain in TRIM32 with Abi2. Schematic representation of TRIM32 deletion mutants is shown (*left*). Protein motifs are indicated. *R,* Ring-finger domain; *B,* B-box domain; *Coil,* coiled-coil domain; *NHL,* NHL domain. HEK293T cells were transfected with vectors for FLAG-Abi2 and either WT TRIM32 or deleted mutants tagged with the HA epitope. Cell lysates were immunoprecipitated with anti-HA antibody and then immunoblotted with anti-FLAG antibody.

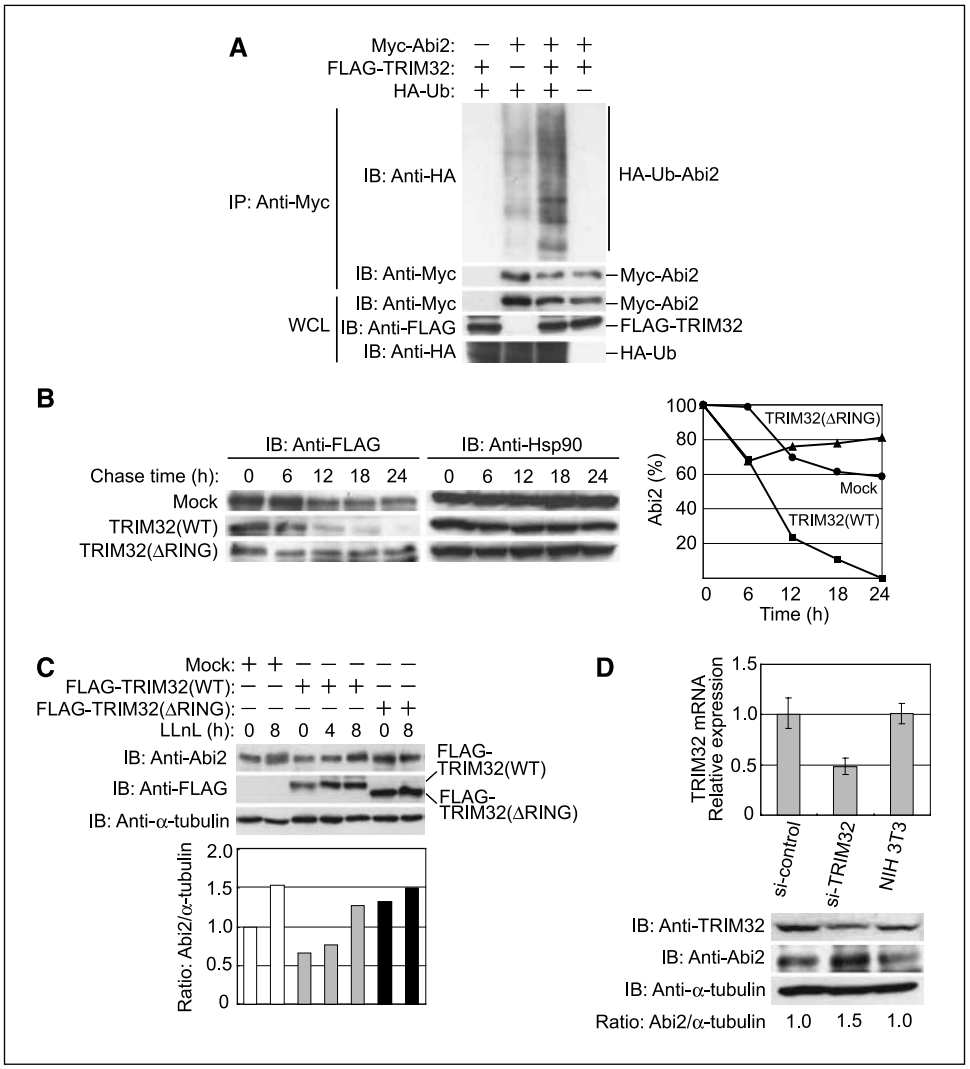

Figure 3. TRIM32 mediates ubiquitination and degradation of Abi2. *A, in vivo* ubiquitination assay for Abi2 by TRIM32. Expression vectors for Myc-Abi2, FLAG-TRIM32, and HA-ubiquitin were transfected into HEK293T cells, and cell lysates were immunoprecipitated with anti-Myc antibody and then anti-HA immunoblot analysis was performed to detect the ubiquitination of Abi2. *B,* pulse-chase analysis of Abi2 with TRIM32. HEK293T cells were transfected with expression vectors for FLAG-Abi2 and HA-TRIM32(WT), HA-TRIM32(ΔRING), or an empty vector (Mock). Forty-eight hours after transfection, the cells were cultured in the presence of cycloheximide (50 μg/mL) for the indicated times. Cell lysates were then subjected to immunoblot analysis with anti-FLAG and anti-Hsp90 antibodies (*left*). Intensity of the FLAG-Abi2 bands was normalized by that of the corresponding Hsp90 bands and then expressed as a percentage of the normalized value for time 0 (*right*). *C,* down-regulation of endogenous Abi2 by TRIM32. Neuro2a cells infected with a retrovirus encoding FLAG-TRIM32(WT), FLAG-TRIM32(ΔRING), or Mock were incubated with or without LLnL (10 μmol/L) for 4 or 8 h, and cell lysates were subjected to immunoblot analysis with anti-Abi2 antibody. The intensity of Abi2 bands was normalized by that of the corresponding α-tubulin bands, and ratio of Mock at time zero was defined as 1. *D,* stabilization of endogenous Abi2 by RNA interference for *TRIM32*. The *TRIM32* mRNAs or proteins derived from NIH 3T3 cells transfected with siRNA specific for mouse *TRIM32* (si-TRIM32) or with nontargeting siRNA as a control (si-control) and from nontransfected cells were measured by real-time quantitative PCR or immunoblot analysis. Cell lysates were also immunoblotted with anti-Abi2 and anti–α-tubulin antibodies. The intensity of Abi2 bands was normalized by that of the corresponding α-tubulin bands.

endogenous Abi2 was elevated in NIH 3T3 cells transfected with si-TRIM32 compared with the level in cells transfected with si-control or nontransfected cells. These results suggest that TRIM32 mediates ubiquitination of Abi2 and thereby promotes its degradation depending on the ubiquitin-proteasome system.

TRIM32 affects cell growth and cell cycle. Abi2 has been reported to inhibit cell growth. Therefore, we examined the effect of TRIM32 on cell growth. We infected retroviruses encoding FLAG-TRIM32(WT), FLAG-TRIM32(ΔRING), or an empty vector (Mock) into NIH 3T3 cells and then counted the cell numbers at indicated times. Overexpression of TRIM32(WT) markedly increased growth rate compared with that of Mock, whereas overexpression of TRIM32(ΔRING) decreased growth rate (Fig. 4*A, left*) and si-TRIM32 attenuated the cell growth more slowly than did si-control (Fig. 4*A, right*). The effect of TRIM32 on cell proliferation was also confirmed by SRB assay (Supplementary Fig. S2). To further confirm that TRIM32 affects the cell cycle, we synchronized the cells at G_0-G_1 phase by serum starvation and analyzed the time for progression to S phase. Flow cytometric analysis showed that NIH 3T3 cells expressing TRIM32(WT) progressed to S phase more rapidly than Mock, whereas TRIM32(ΔRING) slightly delayed the progression to S phase (Fig. 4*B*). Moreover, the cells were synchronized at the G_1-S boundary using aphidicolin after serum starvation for 24 h and then released. Flow cytometric analysis showed that TRIM32(WT) accelerated the cell cycle, whereas TRIM32(ΔRING) decelerated it (Supplementary Fig. S3).

We also examined whether TRIM32 modulates Abi2-mediated cell growth arrest. We infected NIH 3T3 cells with retroviruses encoding FLAG-Abi2 and with a retrovirus vector encoding TRIM32. The expression of Abi2 in the cells stably expressing Abi2 and TRIM32 was reduced compared with that in cells expressing only Abi2, and overexpression of TRIM32 attenuated the suppression of cell growth by Abi2 (Supplementary Fig. S4 and Fig. 4*C*).

Furthermore, to determine whether TRIM32 affects cell transformation of overexpressed c-Src, we established NIH 3T3 cell lines stably expressing the active form of c-Src(Y529F) by retroviral infection. The resulting cell line was further infected with recombinant retroviruses encoding FLAG-tagged TRIM32(WT) or TRIM32(ΔRING). These NIH 3T3 cell lines were then assayed for their ability to form colonies in soft agar to evaluate their ability to undergo anchorage-independent growth. The cells that had not been infected c-Src(Y529F) formed few colonies, whereas cells expressing c-Src(Y529F) formed many colonies. The combination of c-Src(Y529F) and TRIM32(WT) increased the ability of anchorage-independent growth (Fig. 4*D*). These findings suggest

that TRIM32 functions as a positive regulator for anchorage-independent growth in collaboration with c-Src.

TRIM32 induces NIH 3T3 cell motility. Abi family proteins have been reported to mediate cell motility. Thus, we examined whether TRIM32 affects cell motility by degradation of Abi2. First, we performed a wound healing assay using several stably transfected NIH 3T3 cell lines. As shown in Fig. 4*A*, TRIM32 increased cell proliferation rates. Therefore, NIH 3T3 cell lines were cultured under the condition of serum starvation to arrest the cells at G_0-G_1 phase to rule out the effect of cell proliferation. TRIM32(WT) facilitated repair of cell injury more rapidly than Mock, whereas TRIM32(ΔRING) and si-TRIM32 suppressed cell injury repair (Fig. 5*A* and *B*). To further confirm the effect of TRIM32 on cell motility, we performed a transwell migration assay. NIH 3T3 cell lines were precultured in DMEM with 0.1% CS and then incubated in the top of the chamber for 4 h. The migration assay clarified that TRIM32(WT) increases the number of cells migrating compared with Mock but that TRIM32(ΔRING) and si-TRIM32 do not (Fig. 5*C* and *D*). These results indicate that TRIM32 enhances NIH 3T3 cell motility and that the RING-finger domain of TRIM32 is indispensable for activation of cell motility.

TRIM32 suppresses apoptosis induced by cDDP. The sensitivities of HEp2 and the cDDP-resistant variant (HEp2/cDDP) to various concentrations of cDDP were determined by SRB assay and are plotted in the survival curve shown in Fig. 6*A* (*left*). HEp2/cDDP cells showed strong resistance to cDDP compared with the resistance of Hep2 cells. To determine the amount of endogenous *TRIM32* in these cell lines, we performed real-time quantitative PCR with a human *TRIM32*-specific probe and immunoblot analysis with an antibody to TRIM32 (Fig. 6*A, right*). The mRNA and protein expression levels of endogenous *TRIM32* in HEp2/cDDP cells were ~2.0 and 1.7 times higher, respectively, than those in HEp2 cells. We next infected Hep2 cells with retroviruses encoding FLAG-TRIM32(WT), TRIM32(ΔRING) or the corresponding empty vector (Mock). The resulting cells were incubated in the presence of cDDP, and then the number of surviving cells was determined by SRB assay. The results indicated that TRIM32(WT) increased the ratio of surviving cells, whereas TRIM32(ΔRING) decreased the ratio (Fig. 6*B*). We also performed the same assay using HEp2 and HEp2/cDDP cells transfected with si-TRIM32 or si-control and found that knockdown of *TRIM32* decreased cell survival (Supplementary Fig. S5 and Fig. 6*B*). To

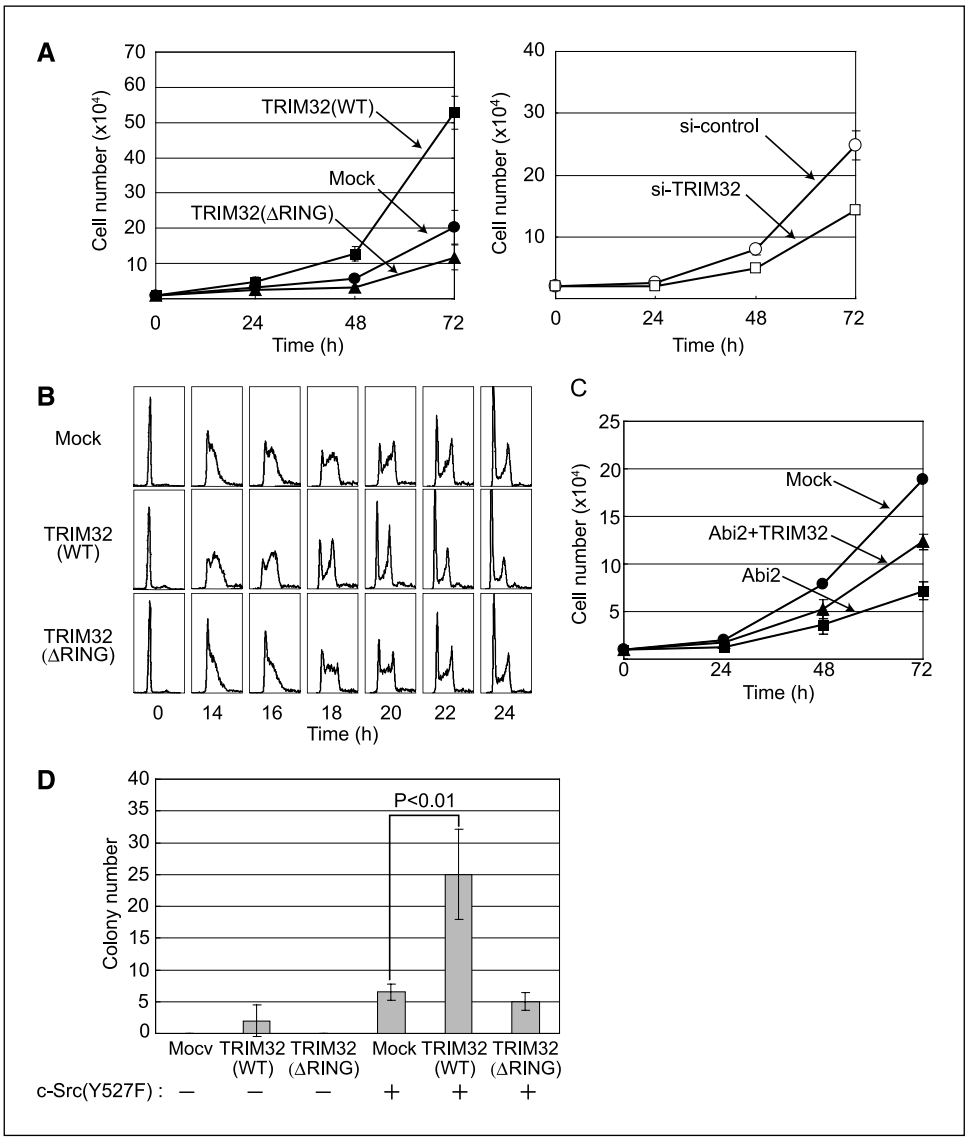

Figure 4. TRIM32 affects cell growth and cell cycle. *A,* acceleration of cell proliferation by TRIM32. NIH 3T3 cells infected with a retrovirus encoding FLAG-TRIM32(WT), FLAG-TRIM32(ΔRING), or the corresponding empty vector (Mock) were seeded at 1×10^4 cells in six-well plates and harvested for determination of cell number at indicated times (*left*). The same experiment was performed using NIH 3T3 cells transfected with siRNA specific for mouse *TRIM32* (si-TRIM32) or nontargeting siRNA as a control (si-control; *right*). *Points,* means of values from three independent experiments; *bars,* SD. *B,* TRIM32 facilitates cell cycle reentry. NIH 3T3 cells infected as above were incubated in DMEM with 0.1% CS for 24 h for serum starvation. The cells released from serum starvation were harvested at indicated times and then analyzed using a flowcytometer. *C,* TRIM32-mediated inhibition of suppression of cell proliferation by Abi2. NIH 3T3 cells were infected with a retrovirus encoding FLAG-Abi2 or the corresponding empty vector (Mock), and then some of the cells overexpressing FLAG-Abi2 were additionally infected with a retrovirus encoding TRIM32. The cells were counted by the same procedure as that described in *A*. *D,* colony formation assay of NIH 3T3 cell lines in soft agar. NIH 3T3 cell lines indicated in *A* were further infected with a retrovirus for c-Src(Y527F) and then seeded at 1×10^5 cells in 60-mm dishes containing 0.4% soft agar and cultured for 2 wk. The numbers of colonies with a diameter of >0.1 mm in randomized areas (1 cm^2) were counted. *Columns,* means of values from three independent experiments; *bars,* SD. *P* values for the indicated comparisons were determined by Student's *t* test.

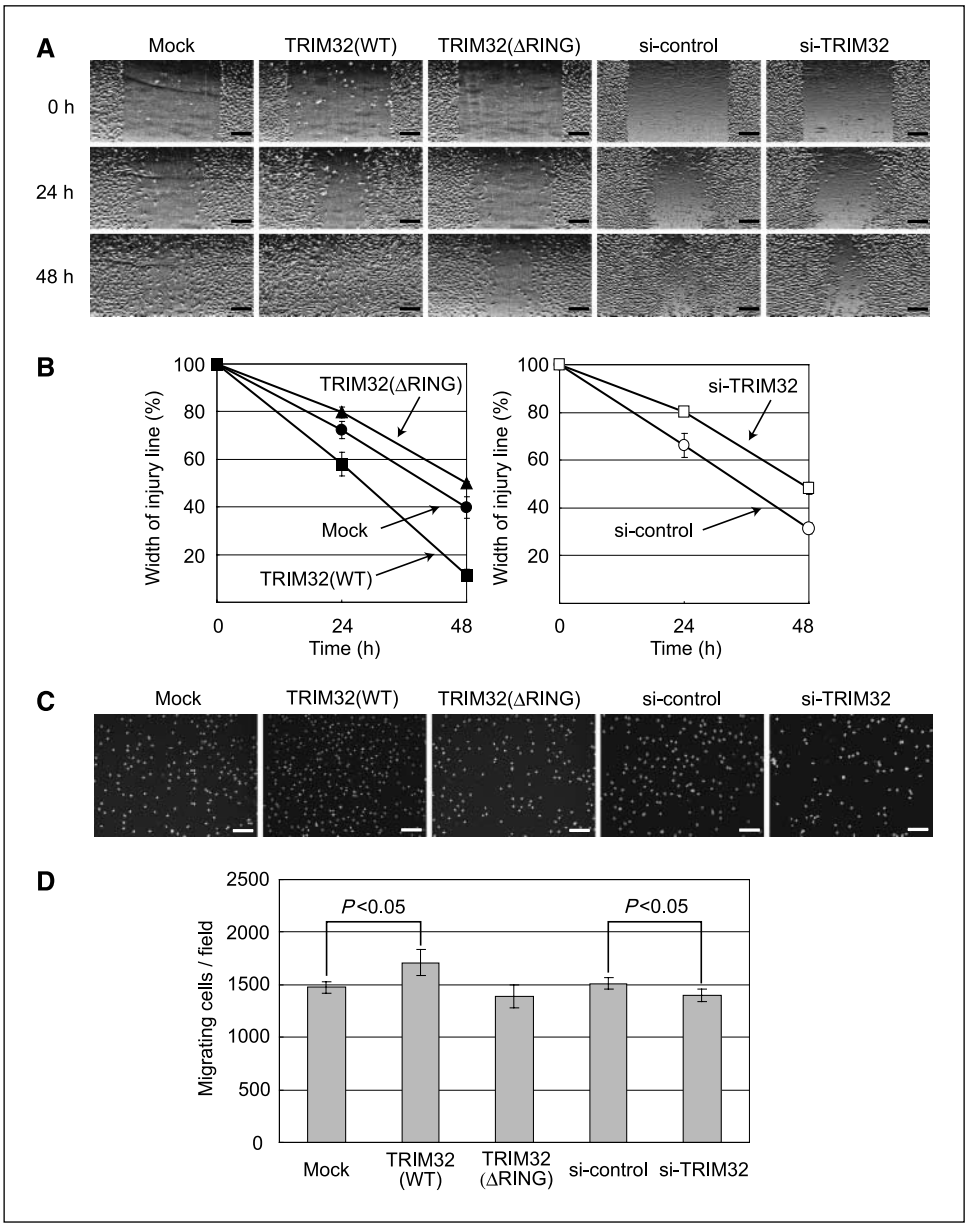

Figure 5. TRIM32 enhances NIH 3T3 cell motility. *A,* wound healing assay. NIH 3T3 cell lines indicated in Fig. 4*A* were cultured in wells coated with 20 μg/mL fibronectin in DMEM with 0.1% CS for 24 h and then an injured line was made on the confluent monolayer of cells. Cells were then incubated in serum-free medium and photographed at indicated times. *Scale bar,* 200 μm. *B,* the width of the injury line was measured at three random positions and then averaged. *Points,* means of values from three independent experiments; *bars,* SD. *P* values for the indicated comparisons were determined by Student's *t* test. *C,* transwell migration assay. NIH 3T3 cell lines precultured in DMEM with 0.1% CS for 24 h were seeded at 1×10^5 cells into the top of the chamber coated with 20 μg/mL fibronectin and incubated for 4 h. Cells that had migrated were fixed in formaldehyde and stained with Hoechst 33258. *Scale bar,* 100 μm. *D,* the number of cells that had migrated was counted within nine fields per insert using a fluorescence microscope. *Columns,* means of values from three independent experiments; *bars,* SD. *P* values for the indicated comparisons were determined by Student's *t* test.

further confirm the suppression of apoptosis by TRIM32, we measured sub-G_1 peak in the cell cycle and cleaved caspase-3. TRIM32(WT) decreased sub-G_1 peak and the expression of cleaved caspase-3 compared with Mock, whereas TRIM32(ΔRING) increased them (Fig. 6*C* and *D*). These results suggest that TRIM32 is likely to have an effect on resistance to anticancer drugs.

Discussion

TRIM32 has been reported to protect keratinocytes against UVB-induced apoptosis by the degradation of Piasy and to play a role in skin carcinogenesis by increasing cell survival (18). However, head and neck squamous cell carcinoma commonly occurs in a mucosal lesion of the nasal cavity, oral cavity, tongue, pharynx, and larynx, which are not exposed to UVB. In the present study, we identified Abi2 as a protein that binds to TRIM32, suggesting a novel mechanism of TRIM32 in carcinogenesis in human head and neck squamous cell carcinoma. We focused on Abi2 among candidates detected by yeast-two hybrid screening because it has been reported that Abi2 suppresses cell growth (23) and that a truncated form accelerates the tumorigenesis (22). Theses previous reports suggest that Abi2 functions as a tumor suppressor. Furthermore, it has been reported that Bcr-Abl elicits ubiquitin-dependent degradation of Abi2 and that a deletion mutant of Bcr-Abl to prevent Abi2 degradation impairs leukemogenesis (27). We showed that overexpression of TRIM32 promoted cell growth and transforming activity, whereas a mutant of TRIM32 lacking the RING-finger domain prolonged the half-life of Abi2 and suppressed these functions. These results suggest that TRIM32 functions as an oncogene via ubiquitination and degradation of Abi2. It has been suggested that, whereas Abi2 functions as an adapter protein to bridge c-Abl and particular substrates (23, 30), Abi2 also regulates the tertiary structure of c-Abl by forming a complex and stabilizes the inactive form of c-Abl or blocks access to the substrates of c-Abl

tyrosine kinase (22, 31). According to this hypothesis, when TRIM32 is overexpressed in cells, the free and active forms of c-Abl may increase due to degradation of Abi2 and thereby promote cell proliferation and mediate carcinogenesis. However, a colony formation assay showed that TRIM32 failed to increase the transforming activity by itself and needed other stimulators, such as active c-Src for transformation (Fig. 4*D*). Furthermore, TRIM32 expression was elevated both in tumor tissues and in normal cells at the mitotic phase (Fig. 1*B*). These findings suggest that overexpression of TRIM32 accelerates cell cycle progression and enhances cell mitosis and cell growth but that TRIM32 is insufficient in carcinogenesis. TRIM32 probably functions as a coactivator in collaboration with other oncogenes in carcinogenesis. However, the possibility of cell type–specific effects cannot be ruled out, although the above studies suggest that the oncogenic role of TRIM32 might be through effects on cell cycle stimulation or transformation, at least in fibroblasts. The connection to cancer, particularly human head and neck squamous cell carcinoma remains to be elucidated. Further study is also needed to determine the relation between expression of TRIM32 and differentiation in tumors.

Abi2 also has been reported to mediate cell motility (27). Other Abi family proteins, including Abi1 and Abi3 (NESH), have been reported to be regulators of cell motility (24–26). Abi1 and Abi3 are localized at the edge of lamellipodial protrusions and form a complex with WAVE2. Abi1 promotes cell motility, whereas Abi3 suppresses cell motility. Abi2 has been reported to localize predominantly in the cytosol (26) and impair cell motility on the basis of results showing that a deletion mutant of Bcr-Abl lacking the ability to degrade Abi2 prevented cell migration (27). We also found that Abi2 was localized in the cytosol of HEp2 cells (Supplementary Fig. S1) and that TRIM32 enhanced cell migration, supporting the results of previous studies regarding Abi2. TRIM32 might induce cell motility via the degradation of Abi2 and mediate the invasion of tumor cells and distant metastasis. These findings

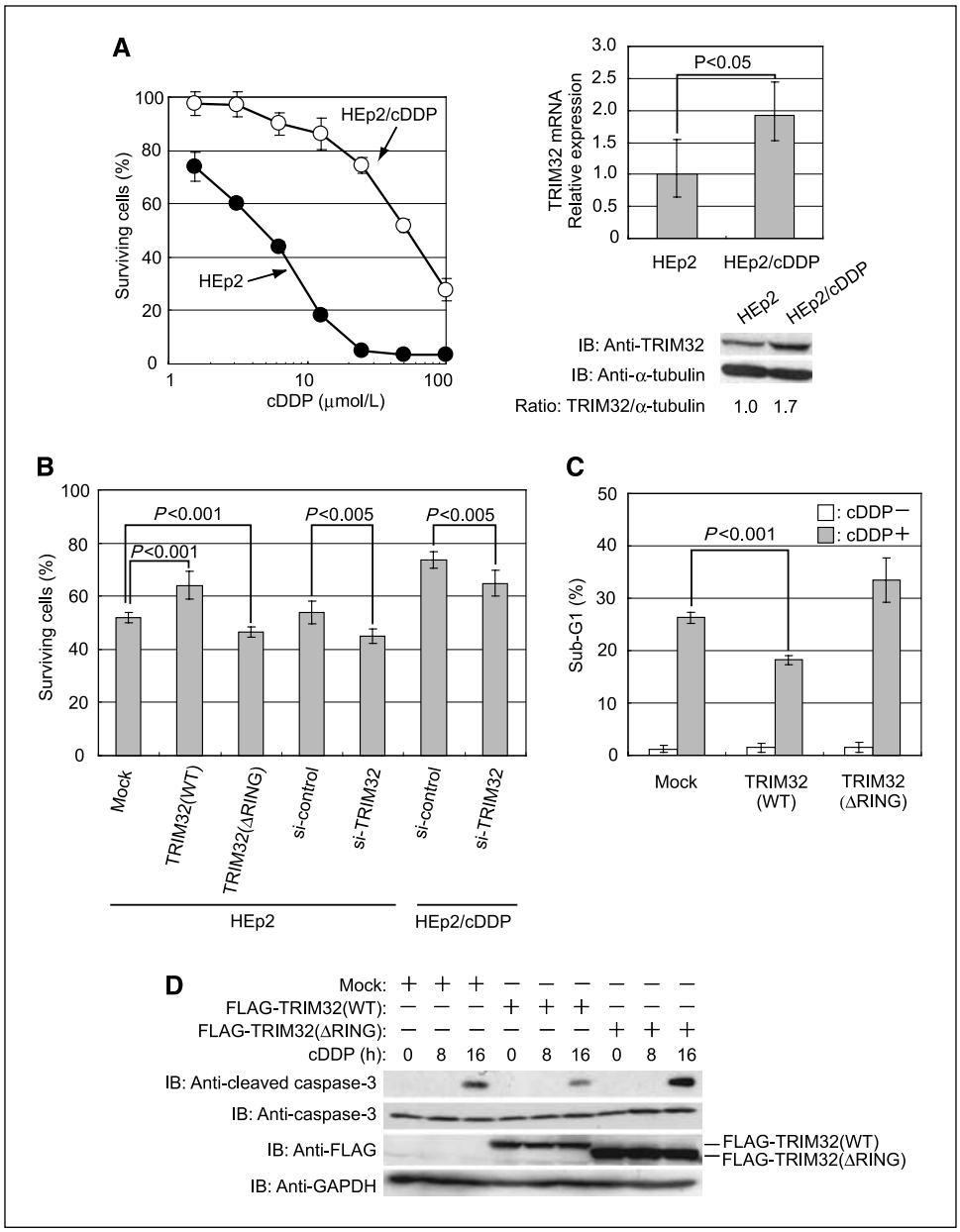

Figure 6. TRIM32 suppresses apoptosis induced by cDDP. *A,* effects of cDDP on cell survival of HEp2 and its cDDP-resistant variant (HEp2/cDDP). Cells were plated on 96-well plates and then incubated in the absence or presence of various concentrations of cDDP for 48 h. Cell survival was estimated using an SRB assay. *Points,* means of values from six independent experiments (*left*); *bars,* SD. Endogenous *TRIM32* expression in HEp2 and Hep2/cDDP cells. Cell lysates were subjected to real-time quantitative PCR with a human *TRIM32*-specific probe and to immunoblot analysis with antibodies to TRIM32 and to α-tubulin. The intensity of TRIM32 bands was normalized by that of the corresponding α-tubulin bands (*right*). *B,* SRB assay. HEp2 cells infected with a retrovirus encoding FLAG-TRIM32(WT), FLAG-TRIM32(ΔRING) or the corresponding empty vector (Mock) were plated on 96-well plates and then incubated with cDDP (5 μmol/L) for 48 h. HEp2 and HEp2/cDDP cells transfected with siRNA specific for human *TRIM32* (si-TRIM32) or nontargeting siRNA as a control (si-control) were also incubated with cDDP (HEp2, 5 μmol/L; HEp2/cDDP, 20 μmol/L). Cell survival was estimated using SRB assay. *Columns,* means of values from six independent experiments; *bars,* SD. *P* values for the indicated comparisons were determined by Student's *t* test. *C,* measurement of sub-G_1 peak. HEp2 cell lines indicated in *B* were incubated with cDDP (25 μmol/L) for 24 h. The cells were treated with propidium iodide, and then sub-G_1 peak was analyzed with a FACSCalibur flow cytometer. *Columns,* means of values from three independent experiments; *bars,* SD. *P* values for the indicated comparisons were determined by Student's *t* test. *D,* detection of cleaved caspase-3. HEp2 cell lines indicated in *B* were incubated with cDDP (25 μmol/L) for various times. Cell lysates were then subjected to immunoblot analysis with an antibody to cleaved caspase-3.

may contribute new information for elucidation of the activities and possible roles in cancer of TRIM32. However, in this study, we did not determine whether the effects of TRIM32 on cell cycle, cell motility, and apoptosis directly depend on the change in activities of Abi2. Further studies are needed to clarify the functional interaction between TRIM32 and Abi2.

Moreover, our study showed that TRIM32 inhibits apoptosis induced by cDDP in HEp2 cells. Development acquired resistance to cDDP occurs commonly in patients with relapse and is a major clinical problem. The mechanism by which cancer cells become resistant to cDDP is not completely understood, although various possibilities have been suggested. For example, it is likely that cDDP is inactivated by thiol-containing molecules, such as glutathione and metallothionein, which are abundant in cells. An increasing cellular thiol content and an elevated activity of GST, which is responsible for the conjugation of cDDP to the thiol molecules, have been reported in many cDDP-resistant cell lines (32–34). As another possible mechanism, up-regulation of the ATP-binding cassette transporter family called efflux pumps, which decrease the accumulation of drugs, has been reported (35). In addition, increased DNA repair, due to an increased level of repair enzymes or DNA-binding proteins recognizing damaged DNA, has been reported in several cDDP-resistant cell lines (36, 37). Differential gene expression in cDDP-sensitive and cDDP-resistant cells has been investigated in recent studies to analyze the molecular mechanism involved in cDDP resistance (38). Our results suggest that TRIM32 contributes to the acquirement of resistance to cDDP. In conclusion, TRIM32 is a novel mediator affecting tumor growth, metastasis, and resistance to cDDP, and results of further studies on TRIM32 may be useful for establishing new chemotherapy for head and neck carcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 11/13/2007; revised 4/30/2008; accepted 5/14/2008.

Grant support: Ministry of Education, Culture, Sports, Science and Technology grants 18013001 and 18390079 and Sagawa Foundation for Promotion of Cancer Research (S. Hatakeyama).

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We thank T. Kitamura for the plasmids and cell lines.

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Cancer Res 2008;68:5572-5580.