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Characterization of TRIM31, Upregulated in Gastric Adenocarcinoma, as a Novel RBCC Protein

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ABSTRACT

To explore the molecules associated with gastric adenocarcinoma, we used the gene expression profile database of various human tissues and identified TRIM31 upregulated in both patients with chronic gastritis and stomach cancer. TRIM31 is a new member of RBCC proteins composed of RING finger, B-box and coiled-coil domains. We characterized TRIM31 biochemically and found it possess properties in common with other RBCC proteins, such as occurrence of alternative splicing transcripts, in vitro autoubiquitylating activity and a tendency to homo-oligomerize. The primary localization site of TRIM31 is the cytoplasm but some fraction is potentially associated with the mitochondria. TRIM31 overexpression suppresses colony formation of HCT116 cells while knockdown of its expression with short interfering RNAs (siRNAs) consistently tends to enhance growth of AsPC-1 cells slightly. Thus, TRIM31 is a characteristic RBCC protein with the ability to regulate cell proliferation negatively and may be a potential biomarker of gastric cancer as it is overexpressed from the early stage of gastric carcinogenesis. J. Cell. Biochem. 105: 1081–1091, 2008.

KEY WORDS: BIOMARKER; RBCC; STOMACH CANCER; TRIM31; UBIQUITIN LIGASE

Gastric adenocarcinoma is the second most common cause of cancer-related deaths in the world [Hohenberger and Gretschel, 2003]. Gastric cancer involves a transition from normal mucosa to gastritis, which eventually leads to adenocarcinoma [Fox and Wang, 2007]. Despite advances in diagnostic and treatment technologies that have brought about excellent long-term survival for patients with early gastric cancer, prognosis of advanced gastric cancer still remains poor [Matysiak-Budnik and Megraud, 2006]. Therefore, the patients would benefit from diagnosis of the cancer at an earlier stage before its dissemination and establishment of more effective therapies specifically designed for stomach cancer. In this situation, we have decided to seek critical molecules participating in initiation and/or progression of the cancer as possible anti-cancer drug targets or valuable biomarkers. To this end, we attempted to delineate alterations in the gene expression pattern entailed in the stomach carcinogenesis by using the commercially available BioExpress database comprising the gene expression profiles of normal and diseased human tissue samples [Shen-Ong et al., 2003]. We have already applied the database to explore novel genes upregulated in colorectal adenocarcinoma and non-small cell lung cancer [Sugiura et al., 2004, 2007b, 2008a,b].

Through a genome-wide search for genes overexpressed in gastric adenocarcinoma, we found out TRIM31 upregulated in the cancer. It has been reported that TRIM31 gene is induced by treatment of MCF-7 cells with retinoids [Dokmanovic et al., 2002]; however, no further characterization of the gene product has been described so far. TRIM31 belongs to a subfamily of RING finger domain-containing proteins called the tripartite motif family (TRIM) or RBCC for RING, B-box, and coiled-coil [Meroni and Diez-Roux, 2005]. RBCC proteins contribute to a broad range of biological processes and, as a consequence, when altered, are implicated in several pathological conditions such as immunological and developmental disorders, tumorigenesis and retroviral protective activity [Meroni and Diez-Roux, 2005]. Here we present the first biochemical characterization of TRIM31 as a member of the RBCC protein family.

MATERIALS AND METHODS

DATA MINING OF THE BIOEXPRESS DATABASE

We identified the genes upregulated in human stomach adenocarcinoma by using the BioExpress database (Gene Logic Inc., Gaithersburg, MA) as described previously [Sugiura et al., 2004, 2007b]. Briefly, sample sets were created from 71 normal and 39 adenocarcinoma samples of stomach on the BioExpress database. No further information on the stage or histological classification of stomach cancer was not available from the BioExpress. We

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performed Fold Change analysis with the database tool to find out the genes expressed at least twofold greater in stomach cancers than in normal stomach tissues. Sample sets were also prepared from 41 chronic gastritis samples, 59 pancreatic adenocarcinoma samples and other normal tissue samples consisting of more than 30 samples.

CLONING OF TRIM31 cDNA

The entire coding region of the human TRIM31 cDNA was cloned by PCR with the primers 5′-CCGGAATTCCACCATGGCCAGTGGGCAGTTTGT-3′ and 5′-ATAAGAATGCGGCCGCGCTTGAAGGAACCTCACAAA-3′ based on the deposited TRIM31 sequence (GenBank accession no. NM_007028*)* using human XG colon adenocarcinoma QUICK-Clone cDNA (BD Bioscience Clontech) as a template. PCR products were cloned into pEF1/V5HisB vector (Invitrogen) at the *Eco*RI and *Not*I sites (pEF1/V5HisB-TRIM31). The *Eco*RI–*Not*I fragment was further transferred to pCI-neo5′FLAG (pCI-neo5′-FLAG-TRIM31) and pCI-neo3′FLAG (pCI-neo3′FLAG-TRIM31). pCI-neo5′FLAG and pCI-neo3′FLAG were in-house derivatives of pCI-neo vector (Promega) with a FLAG tag at the 5′ and 3′ termini, respectively. Double point mutant TRIM31 (Cys31Ser/His33Glu) was made as follows. Two types of PCR were performed with 5′-CCAGAGTCGATGGTGACAGG-3′ and 5′-CCGGAATTCCACCATGGCCAGTGGGCAGTTTGT-3′ as one set of primers and 5′-GCAGAATTTCTGCCCTCAAT-3′ and 5′-ATAAGAATGCGGCCGCGCTTGAAGGAACCTCACAAA-3′ as the other set using pEF1/V5HisB-TRIM31 as a template. The former and the latter PCR products thus obtained were blunted, phosphorylated, and digested with *Eco*RI and *Not*I, respectively, followed by tripartite ligation with pEF1/V5HisB vector digested with *Eco*RI and *Not*I for the construction of pEF1/V5HisB-TRIM31(M) that contained the TRIM31 sequence incorporating double mutations. The *Eco*RI–*Not*I fragment of this vector was subcloned into pCI-neo3′FLAG (pCI-neo3′FLAG-TRIM31(M)).

RT-PCR ANALYSIS OF TRIM31 DIFFERENTIAL EXPRESSION

Total RNAs (63 years old male; patient #2) and cDNAs (66 years old male; patient #1) derived from matched human stomach cancer and adjacent normal stomach tissues were purchased from BioChain (Brussels, Belgium). Total RNAs (0.5 μg) were transformed into cDNAs as described previously [Sugiura et al., 2004]. We prepared two primer sets, variants common primers (5′-AATTATCAGGGGCAGATTCA-3′ (409–428 of the nucleotide sequence of TRIM31α relative to the initiation ATG as 1) and 5′-TCGTGTCTTGATTTTGCTTC-3′ (832–851)) and TRIM31α-specific primers (5′-GATATCAAAGTCGTCTTGT-3′ (745–763) and 5′-TCCTTGACCAGAAATCTCAT-3′ (1,142–1,161)). PCR conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, and finally 72°C for 3 min, with Ex Taq HS (Takara Bio, Kyoto, Japan) as polymerase. The PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide.

SUBCELLULAR LOCALIZATION OF TRIM31

The MGFP sequence with stop codon was released from phMGFP (Promega) by digestion with *Nhe*I and *Xba*I and ligated with pCI3′HA vector [Sugiura et al., 2007a] at the *Nhe*I site (pCI-hMGFP). Direction of the insert of the resulting vector was confirmed by restriction analysis. The TRIM31 sequence was amplified from pEF1/V5HisB-TRIM31 with 5′-CGGAATTCCACCATGGCCAGTGGGCAGTTTGT-3′ and 5′-CCGCTCGAGGCTTGAAGGAACCTCACAAA-3′ as primers. The PCR product was treated with *Eco*RI and ligated with pCI-hMGFP at *Eco*RI and *Eco*RV sites. The resultant vector (pCI-hMGFP-TRIM31) expresses TRIM31 C-terminally fused with MGFP. One microgram of this vector was transfected into COS-1 cells grown in 6-well plates using FuGENE6 (Roche Diagnosis) as per the manufacturer. After 20 h, the cells were stained with MitoTracker Orange CMTMRos (1:2,000 dilution; Molecular Probes, Eugene, OR) at room temperature for 15 min. Subsequently they were fixed with 10% formalin in PBS and treated with Hoechst 33248 (Dojindo laboratories, Kumamoto, Japan) for nuclei staining. The images were captured by means of an inverted fluorescence microscope IX70 (Olympus, Tokyo, Japan) [Sugiura et al., 2007, 2008b].

We also conducted immunofluorescence staining for TRIM31 localization. COS-7 cells grown on coverslips were transfected with pCI-neo5′FLAG-TRIM31. After 20 h, the transfected cells were stained with MitoTracker and fixed as above. Then, the cells were permeabilized with 0.1% TritonX-100 in PBS for 5 min and blocked with 5% fetal calf serum (FCS) in PBS for 30 min, after which the cells were incubated with anti-FLAG antibody (1:5,000; Sigma) at 4°C overnight. Next day the cells were stained with anti-mouse IgG Alexa488 (1:2,000; Molecular Probes) and 100 ng/ml Hoechst 33342 (Wako, Osaka, Japan). The cells were monitored as described above for the MGFP imaging.

SUBCELLULAR FRACTIONATION

pCI-neo3′FLAG-TRIM31 vector (2 μg) was transfected into 293 cells cultured in 6-cm plates. At 48 h post-transfection, the cells were harvested for fractionation by the previous method [Sugiura et al., 2004, 2008b]. The equivalent amount of both fractions was analyzed by anti-TRIM31 immunoblot.

PREPARATION OF PROTEINS FOR UBIQUITYLATION ASSAY

For expression of TRIM31 fused with maltose-binding protein (MBP), PCR was carried out using the TRIM31 sequence as a template and 5′-CCGGAATTCATGGCCAGTGGGCAGTTTGTGAA-3′ and 5′-CCCAAGCTTTTAGCTTGAAGGAACCTCAC-3′ as primers. The *Eco*RI–*Hin*dIII fragment of the PCR product was inserted into pMAL-cX2 plasmid (New England Biolabs, NEB) (pMAL-cX2-TRIM31). pMAL-cX2-TRIM31(M) was constructed by replacing the *Eco*RI–*Bam*HI fragment of pMAL-cX2-TRIM31 with the corresponding fragment of pEF1/V5HisB-TRIM31(M) covering the mutated sites of TRIM31(M). The identity of the construct was confirmed by DNA sequencing.

Escherichia coli strain BL21(DE3) cells (Invitrogen) transformed with the respective plasmid were grown exponentially at 37°C in LB medium containing 100 μg/ml ampicillin and 0.2% glucose to A_{600} of ~0.6 and induced with 0.1 mM isopropyl β-D-thiogalactoside. Culture was continued overnight at 25°C. After harvest, the cells were subjected to three rounds of freeze/thawing and sonicated in 20 mM Tris–HCl, pH 8.0 containing 0.2 M NaCl, 0.05 mM $ZnCl_2$ and protease inhibitor cocktail (Roche Diagnosis). After centrifugation, MBP-TRIM31 fusion proteins were purified from the supernatant with amylose column according to the supplier's manual (NEB).

cDNAs of E2 (UbcH5b, UbcH5c, UbcH7, and UbcH8) and ubiquitin were cloned by PCR and inserted into pTrcHisA (GE Healthcare) [Sugiura et al., 2008b]. His-tagged proteins expressed in *E. coli* were purified as described previously [Sugiura et al., 2007a].

IN VITRO UBIQUITYLATION ASSAY

A typical 20 μl reaction contained the following purified components: 2.5 mM ATP: 100 ng His-tagged ubiquitin; 50 ng rabbit E1 (Calbiochem, San Diego, CA): 500 ng His-tagged E2: 50 ng wild-type MBP-TRIM31, mutant MBP-TRIM31 or MBP. Reactions were carried out in 110 mM Tris–HCl, pH 8.0 supplemented with 3 mM $MgCl_2$, 1.2 mM dithiothreitol and 10% DMSO for 2 h at 37°C and stopped by the addition of SDS sample buffer. After heating at 100°C for 5 min, proteins were resolved on 4–20% SDS gels and visualized by ant-ubiquitin (1:1,000; Santa Cruz) immunoblotting. In some reactions, *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN, Carbiochem) was added at a concentration of 2.5 mM.

COLONY FORMATION ASSAY

The experimental procedure was essentially the same as the previous one [Sugiura et al., 2004, 2007b]. Subconfluent HCT116 cells cultured in 24-well dishes were transfected with 0.4 μg pCI-neo3′FLAG or pCI-neo3′FLAG-TRIM31 [Sugiura et al., 2004, 2007b]. Next day, the cells were trypsinized and 1/60 aliquots were replated in 10-cm plates. After selection with G418 (Promega) at 500 μg/ml for 3 weeks, the surviving colonies were fixed with 3% formaldehyde in PBS, followed by staining with 0.2% crystal violet. Colony number was measured by NIH image analyzer (Scion corporation, Frederick, MD) [Sugiura et al., 2007b].

EFFECTS OF TRIM31 TARGETED SIRNAS ON CELL GROWTH

Duplexed Stealth siRNAs were purchased from Invitorgen. The siRNA sequences used were: (a) 5′-GAGGAUGAUGGGAAGUUCCUCUGUU-3′, (b) 5′-CCCAUAAUGUCAGCUUGAUCGAAGA-3′, and (c) 5′-GAGCAGAUCCAAGUCUUGCAGCAAA-3′. Stealth RNAi negative control (High GC, Invitrogen) was used as a control. AsPC-1 cells were seeded in 12-well plates at a density of 4×10^4 cells. Next day, the cells were transfected with 50 nM siRNAs using siPORT Amine (Ambion, Austin, TX). Six days after transfection, cell numbers were measured via WST-8 (Kishida Chemicals, Osaka, Japan). Significant level ($P < 0.05$) was evaluated using the one-way ANOVA. Knockdown effects were monitored by anti-TRIM31 immunoblot.

MISCELLANEOUS

Cells except for AsPC-1 were grown in DMEM (Invitrogen) containing 10% FCS. RPMI1640 (Invitrogen) was used for AsPC-1 cells. All the PCR products were sequenced with ABI3100 for fidelity confirmation. SDS gels were obtained from Daiichi Chemicals (Tokyo, Japan) or DRC Co., Ltd. (Tokyo, Japan). Rabbit polyclonal antibody specific for TRIM31 was raised against a synthetic peptide DRKKDENRFFKS corresponding to the amino acid residues 300–311 of human TRIM31 by Scrum Company (Tokyo, Japan). Anti-TRIM31 antibody was affinity-purified from serum obtained from week 11 breeds and used at a dilution of 1:500.

RESULTS

EXPLORATION FOR GENES OVEREXPRESSED IN HUMAN GASTRIC ADENOCARCINOMA

To search for molecular targets or biomarkers of gastric adenocarcinoma, we resorted to the BioExpress gene expression database that is based on the Affymetrix microarray [Shen-Ong et al., 2003]. The expression profile of 71 normal stomach tissue samples were compared with that of 39 samples of patients with stomach adenocarcinoma. The comprehensive search led us to identify one probe set 215444_s_at whose expression intensity was increased 3.2-fold in stomach cancers compared with normal stomach tissues ($P = 1.6 \times 10^{-9}$) (Fig. 1A, left). With the aid of the database we also examined samples of patients with chronic gastritis for the expression of the 215444_s_at probe set and found that its expression was upregulated in these patients too (Fig. 1A, left). Interestingly, the remarkable expression of the probe set 215444_s_at was limited to digestive tissues such as stomach, colon and small intestine among normal tissues (Fig. 1B).

The gene corresponding to 215444_s_at (http://www.affymetrix.com/index.affix) turned out to be TRIM31, a novel member of the RBCC family proteins [Dokmanovic et al., 2002]. We noticed two sorts of the TRIM31 transcript sequences registered in GenBank, accession no. NM_007028 (TRIM31α) and NM_052816 (TRIM31β). The latter transcript originating from alternative splicing is a truncated version of the former due to the occurrence of an in-frame stop codon (Fig. 2A,B). To validate the BioExpress data mining we carried out RT-PCR using samples of the two patients with stomach cancer. Both samples were derived from cancerous and adjacent normal stomach tissues of the same patients. To discern between the possible variant transcripts (TRIM31α and β), we designed two sets of primers, one specific for α type transcript and the other one common to both types of the transcripts. As shown in the left panel of Figure 1C, TRIM31α expression was appreciably augmented in the tumor samples of patients #1 with both primers. No expression of TRIM31β was detected in either of the patient #1 samples as there appeared no band around its expected size (375 bp) with common primers. The patient #2 samples also revealed an increase in TRIM31α expression in the tumor sample compared with the normal tissue sample. In this case, irrespective of the primers used, both tissue samples gave rise to a shorter band in addition to a band likely corresponding to TRIM31α (Fig. 1C, right). To identify these PCR products definitely, we cloned and sequenced four bands from tumor samples of patient #2, marked in Figure 1C. We confirmed that the longer bands represented TRIM31α (data not shown), as expected, while shorter bands stemmed from a novel transcript variant of TRIM31, indicating no expression of TRIM31β in patient #2 as well. We designated this new isoform as TRIM31γ, which was found to be generated by different usage of the TRIM31 gene exons according to the BLAT program analysis [Kent, 2002] (Fig. 2A). One splice site common to TRIM31β and γ does not conform to a GT-AG rule [Shapiro and Senapathy, 1987].

EXPRESSION OF TRIM31

Because TRIM31α appears to be a main transcript among three variants documented thus far and contains all the RBCC motifs

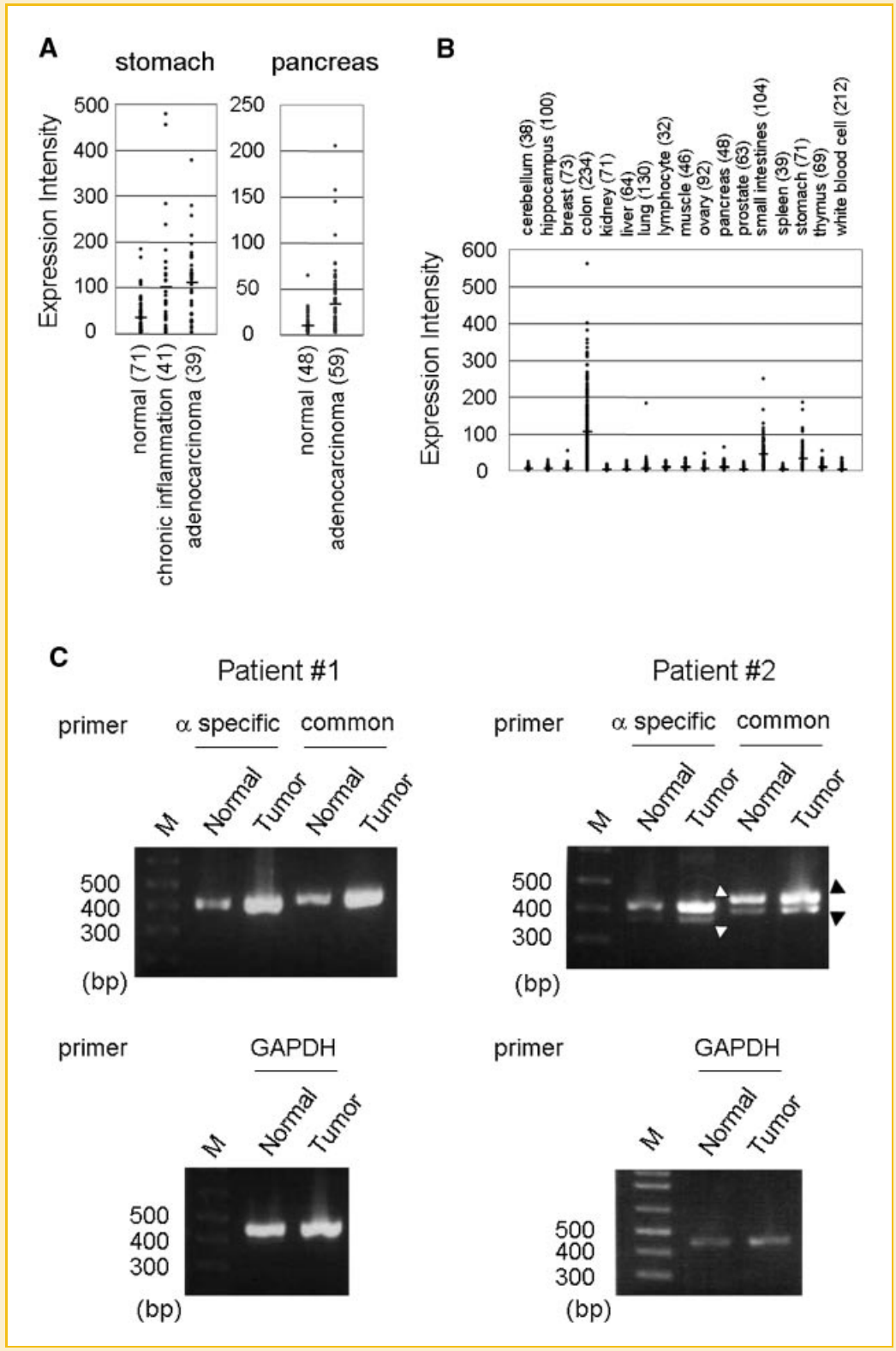

Fig. 1. Expression patterns of TRIM31. A: Expression profile of TRIM31 (probe set 215444_s_at) in human stomach and pancreatic tissues. Samples are normal, chronically inflamed and adenocarcinoma tissues for stomach (left) and normal and adenocarcinoma tissues for pancreas (right). Dots represent the intensity values of individual samples and bars, the average values of the samples. The number in the parenthesis depicts the sample number. B: Expression profile of TRIM31 in various human normal tissues. The graph is drawn as for (A). C: RT-PCR analysis of TRIM31 expression in matched human normal and tumor tissues of stomach from two patients. RT-PCR was performed with primers specific for TRIM31α, common to TRIM31 variants (upper), and specific for GAPDH (lower) as control. The expected sizes of the TRIM31 variants band were 417 bp (α; α specific primers), 443 bp (α; common primers) and 375 bp (β; common primers) and that of GAPDH, 452 bp. The bands of patient #2, of which sequence was determined, are marked by open and closed arrowheads.

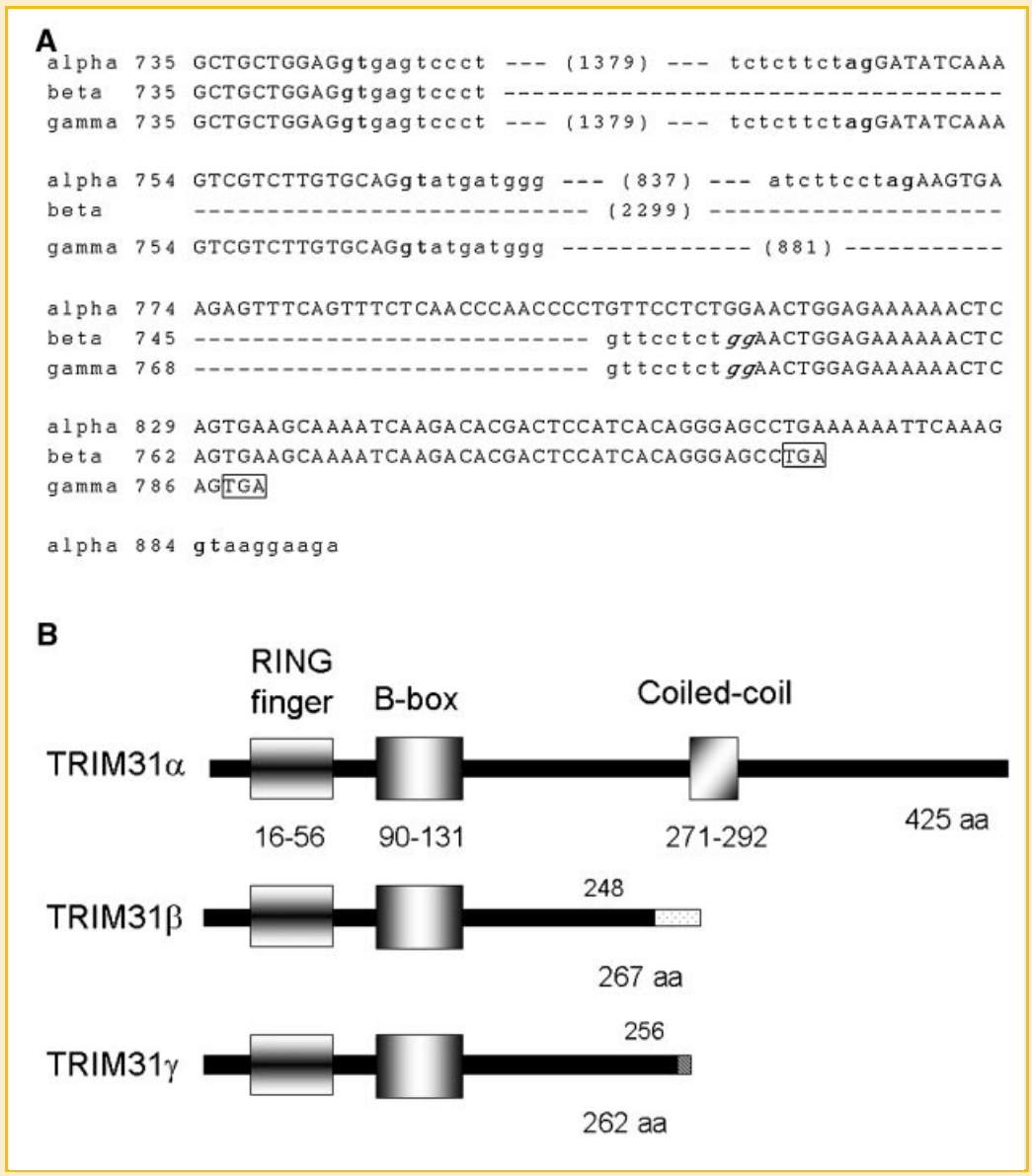

Fig. 2. Distinct exon usages by TRIM31 transcripts. A: Uppercase letters denote exonic sequences while lowercase letters, intronic sequences. Numbers in the parenthesis depict the size of the intron sequence omitted for clarity. Splice donor and acceptor dinucleotides are in bold and unusual acceptor gg are in both bold and italic. In-frame stop codons of TRIM31β and γ are boxed. Numbers on the left refer to the position of nucleotide in the cDNA sequence relative to the ATG initiation codon (1). B: Schematic structure of TRIM31 variant proteins. Three motif regions (RING finger, B-box, and coiled-coil domains) defined by SMART program (http://smart.embl-heidelberg.de/) are indicated. Dotted and striped rectangles of TRIM31β and γ, respectively, show the out-of-frame segments generated by alternative splicing. Numbers above the rectangles of TRIM31β and γ indicate the amino acid residue numbers common to TRIM31α. Nucleotide sequence data of TRIM31γ are available in the DDBJ/EMBL/GenBank databases under the accession no. AB303153.

(Fig. 2B), we focused on this isofrom and hereafter refer to it as TRIM31. To characterize TRIM31, we prepared rabbit polyclonal antibody specific for the gene product. We performed western blot analysis of lysates of 293 cells transfected with empty or C-terminally FLAG-tagged TRIM31 (TRIM31-FLAG) expression vector. As Figure 3A illustrates, the antibody detected a main band with a molecular mass of about 50 kDa, in a good agreement with an expected size of TRIM31, exclusively in the TRIM31-transfected 293 cell lysate. An additional band migrating faster should be a degradation product of TRIM31 rather than its truncated variant, in view of the molecular mass and specificity of the antibody reactive to amino acid residues 300–311 of TRIM31 that exist only in an α type transcript (Fig. 2B). Thus, the polyclonal antibody is able to recognize the TRIM31 protein specifically. Using the antibody, we explored human cell lines expressing endogenous TRIM31 at a level detectable on immunoblot. The search led to the identification of pancreatic adenocarcinoma AsPC-1 showing a band around 50 kDa (Fig. 3B, left), slightly smaller than TRIM31-FLAG produced by

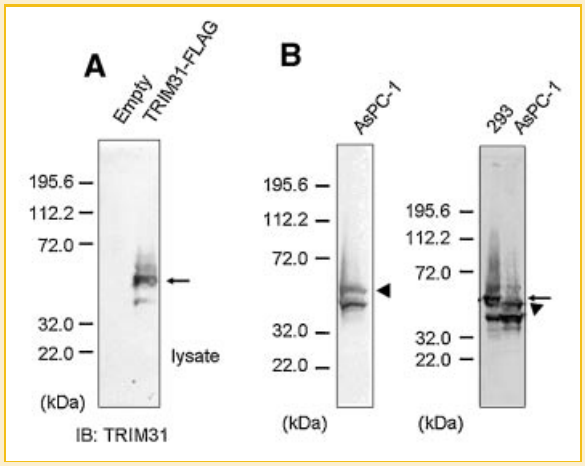

Fig. 3. Exogenous and endogenous expression of TRIM31. Arrows mark the position of FLAG-tagged TRIM31 and closed arrowheads, that of endogenous TRIM31. Smears frequently appearing at a high molecular weight region on TRIM31 immunoblot are probably attributed to poor solubility of TRIM31. A: Specificity of polyclonal anti-TRIM31 antibody. Two hundred ninety-three cells transfected with TRIM31 expression vector were harvested and lysed in PBS containing 1% Triton X-100 and protease inhibitor cocktail. Cell lysates were subjected to anti-TRIM31 immunoblot. B: Left, AsPC-1 cells endogenously express TRIM31. AsPC-1 cell lysates were immunoblotted using anti-TRIM31 antibody. Right, lysates of TRIM31-FLAG-expressing 293 cells and AsPC-1 cells were processed for western blot with anti-TRIM31 antibody.

293 cells (Fig. 3B, right). The difference in the mobility could be attributed to the presence of FLAG tag. We define AsPC-1 as a cell line consistently producing TRIM31 at a high level. Expression of TRIM31 in the cultured pancreatic tumor cells was not unexpected because the BioExpress indicated an elevation of the probe set 215444_s_at expression in pancreatic adenocarcinomas, compared with normal pancreatic tissues (Fig. 1A, right).

UBIQUITIN LIGASE ACTIVITY OF TRIM31

The presence of the RING finger domain in TRIM31 encouraged us to test bacterially expressed TRIM31 fused with MBP for ubiquitin ligase activity in a cell-free system. Autoubiquitylation activity of TRIM31 was assayed by incubating the fusion protein with E1, different recombinant E2s, ATP and ubiquitin, followed by western blot with anti-ubiquitin antibody. The presence of UbcH5b or UbcH5c as a source of E2 yielded a characteristic laddering pattern that results from polyubiquitylation and formation of high molecular weight ubiquitin conjugates (Fig. 4A). The UbcH5 family functions productively, at least in vitro, with a wide range of E3s while the use of UbcH7 or UbcH8 as an E2 for the ligase reaction is limited [Lorick et al., 2005]. Mutations in the RING finger domain abolished the autoubiquitylation activity and, furthermore, inclusion of TPEN, a metal chelating inhibitor, in the reaction impaired the activity (Fig. 4B). These results demonstrate the requirement of the RING finger domain of TRIM31 for E3 activity. In conclusion, TRIM31 possesses E2-selective ubiquitin ligase activity that is dependent on the RING finger domain.

OLIGOMERIZATION OF TRIM31

The RBCC proteins are known to homo-interact through their coiled-coil domain [Peng et al., 2002]. To investigate the potential oligomerization of TRIM31, we co-expressed TRIM31-FLAG and TRIM31 C-terminally fused with monster green fluorescence protein (TRIM31-MGFP) in 293 cells. The immunoprecipitate prepared with anti-FLAG beads was found to contain TRIM31-MGFP (Fig. 5, left), implicating that TRIM31-FLAG forms an oligomer with TRIM31-MGFP in intact cells. This oligomerization is not the RING finger domain-dependent because TRIM31-MGFP was co-immunoprecipitated with RING finger mutant of TRIM31-FLAG as well (Fig. 5, right). Thus, TRIM31 exists in an oligomer state probably through a coiled-coil domain, in agreement with other reported RBCC proteins [Meroni and Diez-Roux, 2005].

LOCALIZATION OF TRIM31

Because RING finger proteins are distributed in various cellular compartments to act specifically in diverse events [Saurin et al., 1996], we attempted to determine its subcellular localization for dissection of the TRIM31 function. First, we transfected COS-1 cells with TRIM31-MGFP vector for direct monitoring of its signal (Fig. 6A, top). The transfected cells showed the fluorescent signal mostly in the cytoplasm with occasional cytoplasmic body pattern typical of RBCC proteins [Reymond et al., 2001]. Because the punctuated signal is reminiscent of the mitochondrial localization pattern, we stained the transfected cells simultaneously with a mitochondria specific fluorescent dye, MitoTracker. The expression of TRIM31-MGFP appeared to overlap, at least partially, with the MitoTracker signal (Fig. 6A, top). We subsequently expressed N-terminally FLAG-tagged TRIM31 in COS-7 cells and obtained a similar staining pattern to the MGFP signal by indirect immunofluorescence analysis (Fig. 6A, bottom). These results support the idea that TRIM31 resides predominantly in the cytoplasm and is partially associated with the mitochondria. To further ascertain the results obtained with the microscopic examinations, we next employed the biochemical method for subcellular fractionation. The cytosolic and mitochondrial fractions of 293 cells expressing TRIM31-FLAG were prepared by differential centrifugation and subjected to western blot analysis using anti-TRIM31 antibody. TRIM31 was detected in both fractions (Fig. 6B, left), however, its amount in the mitochondrial fraction varied considerably from experiment to experiment (data not shown). We also analyzed the localization of endogenous TRIM31 of AsPC-1 cells in the same manner and found that TRIM31 was enriched in the cytosolic fraction with a minority of the protein in the mitochondrial fraction (Fig. 6B, right). Combined with the above data, we conclude that the majority of TRIM31 localizes in the cytoplasm but a fraction is potentially associated with the mitochondria as well.

EFFECTS OF TRIM31 OVEREXPRESSION AND KNOCKDOWN ON CELL GROWTH

Because TRIM31 expression is increased from the early stage of gastric carcinogenesis where both proliferation and apoptosis of stomach epithelial cells are exaggerated [Jones et al., 1997], we were

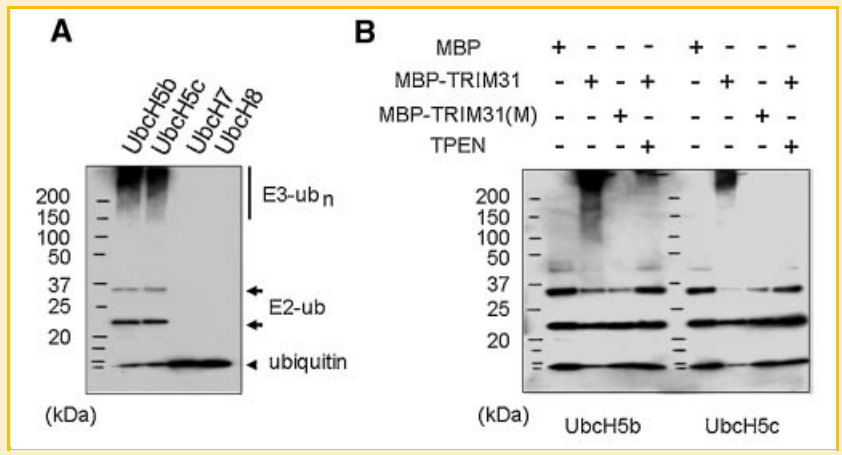

Fig. 4. TRIM31 exhibits ubiquitin ligase activity. A: E2-selective ubiquitin ligase activity of TRIM31. Autoubiquitylation of TRIM31 was detected by anti-ubiquitin immunoblot. B: TRIM31 E3 ligase activity requires the RING finger domain. The reaction was conducted with ubiquitin, E1, E2 (UbcH5b or UbcH5c) and E3 (MBP, MBP-TRIM31 or MBP-TRIM31(M)), as indicated. In some reactions, TPEN was added as a metal chelating inhibitor. After the reaction, the samples were processed as in (A).

interested to test the effects of overexpression of TRIM31 on cell growth. After transfection of a plasmid, cells were selected in the medium with G418, allowing for the colony formation 3 weeks later. Colony formation of HCT116 cells was alleviated upon transfection with the wild-type TRIM31 gene (Fig. 7A).

Conversely, we knocked down TRIM31 expression in AsPC-1 cells with specific siRNAs to examine the involvement of TRIM31 in cell proliferation. Figure 7B shows the feasibility of all the siRNAs in repressing TRIM31 expression; however, its knockdown did not retard the growth of AsPC-1 cells at all (Fig. 7C). On the contrary, there was a reproducible trend that the cell number was slightly increased by a reduction in TRIM31 expression although the growth-stimulatory effects were not statistically significant.

DISCUSSION

The pathogenesis of stomach cancer includes a sequence of events that begins with *H. pylori*-induced chronic gastritis [Matysiak-Budnik and Megraud, 2006]. To uncover the mechanism of the gastric carcinogenesis, we need to delineate the individual molecules involved in this process. We herein found that TRIM31 is upregulated in stomach tumors by the use of the BioExpress database and substantiated the data mining results by RT-PCR using the matched samples of patients with stomach tumor. The differential expression of TRIM31 between normal and tumor stomach tissues was highly significant ($P = 1.6 \times 10^{-9}$) with a large sample number in the BioExpress data (110 in total). To

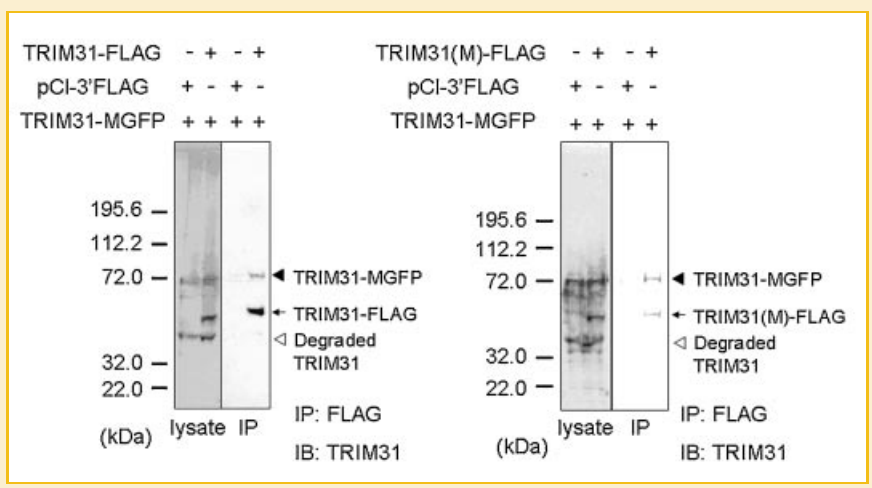

Fig. 5. Oligomerization of TRIM31. 293 cells in 6-cm plates were transfected with expression vectors as indicated. After 48 h, cell lysates and immunoprecipitates prepared with anti-FLAG antibody beads were analyzed on anti-TRIM31 immunoblot. Arrows indicate FLAG-tagged TRIM31 whereas closed arrowheads, TRIM31-MGFP. Open arrowhead depicts degraded TRIM31 or TRIM31(M).

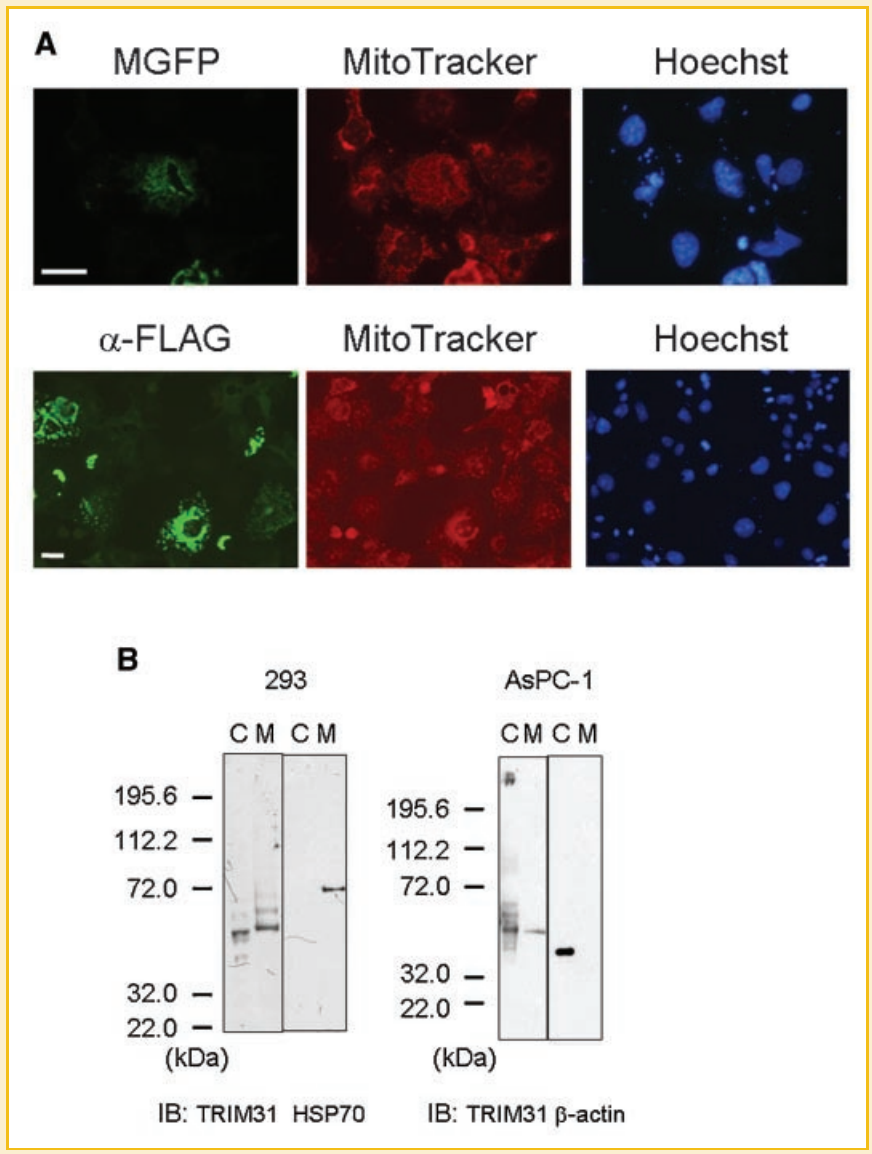

Fig. 6. Localization of TRIM31. A: Intracellular distribution of TRIM31. COS-1 or COS-7 cells were transfected with TRIM31-MGFP (top) or FLAG-TRIM31 (bottom) expression vector, respectively and stained with MitoTracker and Hoechst 33248. FLAG-TRIM31 expression was visualized by immunofluorescence with anti-FLAG antibody. Bars, 25 μm. B: Cytosolic (C) and mitochondrial (M) fractions were isolated from TRIM31-FLAG-expressing 293 (left) or AsPC-1 (right) cells, followed by anti-TRIM31 western blot analysis. The membranes were reprobed with anti-mitochondrial Hsp70 (mitochondrial marker) antibody (Affinity Bioreagent, Golden, CO) (left) or β-actin (cytoplasmic marker) antibody (Sigma) (right), respectively.

further confirm the results, we explored the microarray data of TRIM31 expression in human stomach tissues in the public database and found that of 8 normal and 22 cancer tissues of stomach, three cancerous, but no normal, tissues exhibited TRIM31 expression (GDS1210 of NCBI GEO profile), strengthening our idea that TRIM31 is upregulated in stomach cancer. Taken together, we conclude that the expression of TRIM31 is increased in gastric tumor. The BioExpress data indicated a low level TRIM31 expression in the normal stomach tissues while its expression was not detected in the GEO profile. This inconsistency is probably because of the difference in the detection limit of the method or the amount of RNA employed for the experiments. We searched for human gastric cancer cell lines with a high TRIM31 expression by RT-PCR analysis but no cell line showed a high expression level (data not shown). The discrepancy between the results with cancer tissues (Fig. 1C) and cell lines may be explained by the sensitivity of stomach cancer cells to the alterations in the culture environment. For instance, most gastric cancer cell lines lost the expression of WNT5A mRNA unlike primary gastric cancer [Saitoh et al., 2002]. Hence, we employed pancreatic adenocarcinoma AsPC-1 cells for molecular characterization of endogenous TRIM31, which is supposed to be physiologically relevant since pancreatic cancer tissues displayed overexpression of TRIM31 (Fig. 1A, right). No other type of cancer showed a greater than 2-fold increase in the expression of TRIM31 from the analysis of the BioExpress data.

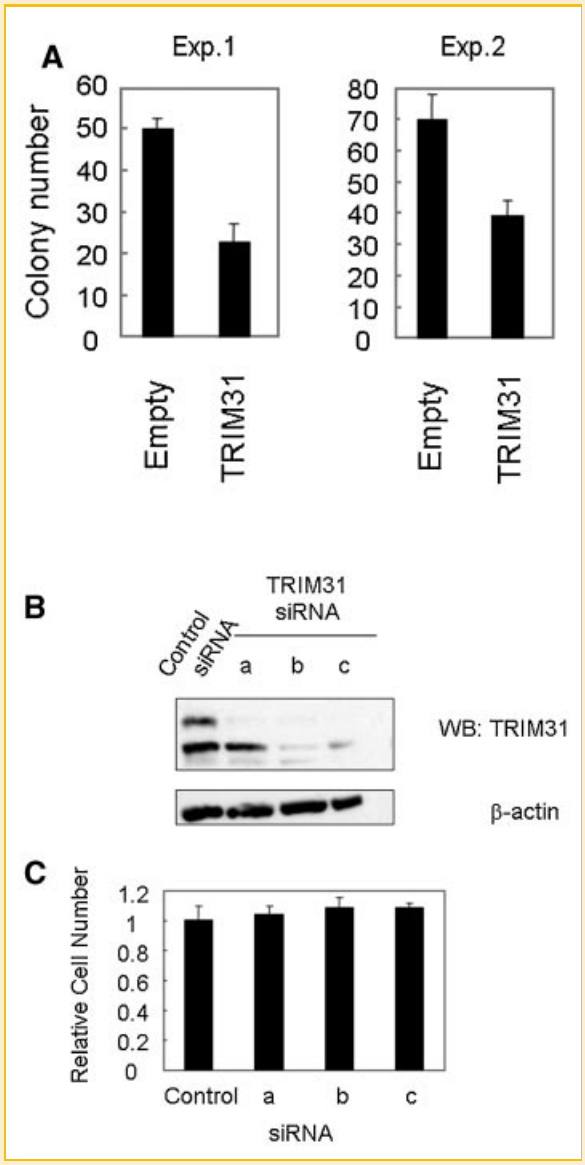

Fig. 7. Effects of overexpression and knockdown of TRIM31 on cell growth. A: After transfection with empty or TRIM31 expression vector, HCT116 cells were grown in selective medium for 3 weeks and stained with crystal violet. The experiments were done in duplicate twice and the mean colony number is indicated with standard deviation. B: TRIM31-specific siRNAs knock down expression of TRIM31. Cell lysates were prepared in PBS containing 1% Triton X and protease inhibitor cocktail for immunoblot with anti-TRIM31 antibody. The membrane was reprobed with anti-β-actin antibody for specificity of siRNAs. C: Cultures were prepared as in (B) and the cell number was assessed with WST-8 assay. Results are presented as the mean cell number relative to that of the control culture with standard deviation for the experiment carried out in triplicate. The experiments were repeated three times with similar results.

TRIM31 belongs to the RBCC family, which is characterized by the presence of the RING finger, B box and coiled-coil domains and connected to various pathological conditions [Meroni and Diez-Roux, 2005]. In this study TRIM31 displayed several features in common with other RBCC members. First, TRIM genes are frequently expressed as multiple alternative splicing forms [Meroni and Diez-Roux, 2005]. Indeed, TRIM31β, a C-terminally truncated version of complete TRIM31, TRIM31α, had been registered in the public database. During RT-PCR analysis of the patient samples, we additionally discovered a novel transcript of TRIM31 (TRIM31γ), which also produces a TRIM31 variant protein truncated at the C-terminus. It is conceivable that different biological roles are assigned to each form, as exemplified by TRIM19/PML isoforms [Jensen et al., 2001] since both TRIM31β and γ retain the RING finger domain but lack the coiled-coil domain.

Second, the presence of the RING finger domain in RBCC proteins suggests their role as an E3 ubiquitin ligase [Lorick et al., 1999] and several members have been demonstrated to exhibit the activity [Meroni and Diez-Roux, 2005]. We proved that TRIM31 is a RING finger-dependent ubiquitin ligase that requires selective E2s by performing in vitro ligase assay where all the components (E1, E2, and E3) were purified. It is reasonable that TRIM31 largely located in the cytoplasm would function as an E3 ligase in concert with Ubch5b and Ubch5c that also work in the cytoplasm [Gonen et al., 1999]. Although the exploration of the substrate of the TRIM31 ubiquitylating activity would aid in delineating its fundamental role, our attempt to identify TRIM31-ineteracting proteins, as its candidate substrates, using the yeast two-hybrid system was unsuccessful so far.

Lastly, the RBCC family members are prone to oligomerize mainly through the coiled-coil domain [Peng et al., 2002]. In the systematic study of the RBCC members TRIM31 was shown to homo-oligomerize via interaction-mating techniques, an extension of the yeast two-hybrid system [Reymond et al., 2001]. Here the co-expression of TRIM31-MGFP and TRIM31-FLAG in 293 cells allowed us to directly demonstrate that TRIM31 exists in an oligomeric state in mammalian cells. The dispensability of the RING finger domain for this oligomerization, as shown by the RING finger mutant of TRIM31, is consistent with the notion that the coiled-coil domain is basically responsible for oligomerization of TRIM proteins [Peng et al., 2002]. The high order molecular weight structures of RBCC proteins arising from oligomerization are considered to define their cellular compartments [Reymond et al., 2001]. A combination of microscopic observations and biochemical fractionation experiments presented evidence that TRIM31 resides primarily in the cytoplasm with a fraction potentially associated with the mitochondria. As the amino acid sequence of TRIM31 reveals no mitochondrial target sequence [Claros and Vincens, 1996], its mitochondrial distribution may occur through oligomerization-dependent binding of the protein to the mitochondrial outer membrane [Eskes et al., 2000; Suzuki et al., 2005]. In this regard the secondary structure prediction algorism (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/npsa/npsa_sopm.html) predicted the presence of a long α-helical segment at the C-terminus of TRIM31 (amino acids 398–420). This helical conformation might be important for directing this protein to the mitochondrial outer membrane [Suzuki et al., 2005].

RBCC proteins are either positively or negatively linked to cell growth. For example, TRIM25/Efp and TRIM32 promote cell growth or tumorigenesis [Urano et al., 2002; Horn et al., 2004] while TRIM19/PML and TRIM35/MAIR repress cell proliferation and cause apoptosis [Mu et al., 1994; Kimura et al., 2003]. TRIM31 expression

is already increased in chronic gastritis (Fig. 1A) where both apoptosis and proliferation are dysregulated [Jones et al., 1997]. Meanwhile, TRIM31 is induced by retinoids with growth inhibitory activity [Dokmanovic et al., 2002]. These facts raise a question whether TRIM31 would stimulate or arrest cell growth. Overexpression of TRIM31 inhibited colony formation of HCT116 cells substantially (Fig. 7A), in accordance with induction of TRIM31 by growth-suppressive retinoids [Dokmanovic et al., 2002]. Knockdown of TRIM31 in AsPC-1 cells by specific siRNAs failed to regress cell growth but exhibited a consistent tendency to cause a slight increase in the cell number (Fig. 7B). Considering the moderate inhibition of colony formation by overexpression of TRIM31, the weak stimulating effect of its knockdown on cell growth was not surprising because, in general, the degree of cell growth enhancement by the knockdown of growth suppressors is not so remarkable even though their overexpression exerts a drastic anti-proliferative effect [Yang et al., 2004; Xiao et al., 2005]. Presumably multiple inhibitory proteins form the balance between cell growth arrest and proliferation. In any case, TRIM31 can be recognized as a growth suppressor; nonetheless, its expression is upregulated from the early stage of tumor generation. As expression of TRIM31 had no influence on colony formation of HeLa cells (data not shown), it is possible that the effect of TRIM31 is cell context-dependent and variable for distinct precursor cells during tumor development. In fact, the effects of oncogenes and tumor suppressors depend on the types of cell [Weinstein, 2000]. Alternatively, the restriction of TRIM31 expression in digestive tissues (Fig. 1B) where rapid turnover of epithelial cells occurs [Hall et al., 1994] may implicate the maintenance of the balance between proliferation and apoptosis of cells as its biological function. Naturally, the normal function of TRIM31 in these digestive tissues would also be the formation of the homeostatic feedback loop regulating cell growth. It is also known that p16/INK4, a tumor or growth suppressor, is overexpressed in cervical and prostate cancers. These cancers may have the distinct mechanism inactivating the senescence program elicited by the tumor suppressor [Bulten et al., 2006; Feng et al., 2007]. Likewise, TRIM31 could be involved in gastric carcinogenesis in a similar manner.

In summary, we demonstrated that TRIM31 is upregulated in gastric adenocarcinoma. TRIM31 has distinguishing properties of the RBCC protein family, including ubiquitin ligase activity. TRIM31 is capable of suppressing cell growth although its precise role remains to be known. TRIM31 may be a potential biomarker of stomach cancer since it appears to be overexpressed from the pre-cancerous stage.

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