TBC1D8B, a GTPase-activating protein, is a novel apoptosis inducer

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ABSTRACT
Overexpressed TBC1D8B, a GTPase-activating protein, significantly reduced cultured HCT116 human colon cancer cell number. We tested N-terminal TBC1D8B, which is identical to wild type TBC1D8B from amino acid positions 1 to 427 and possesses a modified sequence from position 428 to 435 (ECGGLFLL) because of the introduction of a premature stop codon at position 436 to narrow down the minimum requirement element. The N-terminal TBC1D8B contains two GRAM domains but not the TBC domain essential for Rab-GTPase activity. The N-terminal TBC1D8B overexpression significantly reduced the cultured HCT116 cell number. When we tested C-terminal TBC1D8B, containing the portion of TBC1D8B absent in the N-terminal TBC1D8B, the cell number reduction was not observed. The N-terminal TBC1D8B overexpression significantly increased the coronin 1B expression and reduced the phosphorylation of serine 51 in eIF2α, respective markers of apoptosis and cell death/survival. Also, caspase 3 and poly ADP-ribose polymerase increased cleavage in suspended cells overexpressing the N-terminal TBC1D8B. Taken together, it is not the TBC domain for Rab-GTPase activity, but amino acids 1 to 435, including the two GRAM domains, that is enough for TBC1D8B to cause spontaneous apoptosis. TBC1D8B could be a potential anticancer therapeutic molecule.

INTRODUCTION
TBC1D8B consists of 1,120 amino acids with domains of diverse types. The N-terminal portion of TBC1D8B contains two glucosyltransferase Rab-like GTPase activators and myotubulurins (GRAM) domains at amino acid positions 145–251 and 286–376, and its C-terminal portion contains the GTPase-activating protein (GAP). The C-terminal (amino acid positions from 491 to 694) is specific for Rab-GTPases. TBC1D8B is a member of a family of more than 40 TBC-proteins, which share the eponymous Tre-2-Bub2-Cdc16 (TBC) domain (Fukuda, 2011). This domain commonly confers a functional role as a GAP specific for Rab-GTPases, the master regulators of vesicular trafficking, including endocytosis. TBC1D9B is closely related to TBC1D8B and has been described as a GAP specific for RAB11A (Gallo et al. 2014), which plays a role in endocytic recycling, autophagy, and exocytosis. Additionally, to its TBC domain-mediated Rab-GAP function, TBC1D8B contains two GRAM domains that allow binding to lipid rafts, which are critical elements for slit diaphragm–signaling in podocytes (Huber et al. 2006). Recently, TBC1D8B loss-of-function mutations were reported to lead to X-linked nephrotic syndrome via defective trafficking pathways (Dorval et al. 2019). Also, mutations in TBC1D8B were
shown to affect RAB11-dependent vesicular trafficking in the pathogenesis of nephrotic syndrome (Kampf et al. 2019).

The deficiency in dipeptidyl peptidase-4 (DPP4) was reported to promote chemoresistance in breast cancer cells (Lis et al. 2020). Therefore, we tried to identify a new mechanism which regulates cancer cell growth by using DPP4 inhibitor as a tool. To explore this, 60-year-old-healthy Japanese man tested Sitagliptin (50 mg/day) as a DPP4 inhibitor for 14 days. We collected plasma samples before Sitagliptin administration started. Fourteen days post-administration, we performed proteomics analysis in plasma samples in order to pick up the potential molecules regulated by DPP4 inhibitor in human.

Of 749 molecules, 23 showed more than 50% increment after Sitagliptin administration. Among the 23 molecules, we reported earlier that FAM19A2/TAFA-2 increased food intake in mice (Okada et al. 2019). On the other hand, of 749 molecules, 22 showed more than 50% reduction. Especially, plasma TBC1D8B level was reduced by almost 70% after DPP4 inhibitor administration. After we tested all of the 22 molecules on cultured tumor cell growth, we discovered that overexpressed TBC1D8B significantly reduced the cultured tumor cell number. Therefore, we decided to focus on TBC1D8B. In fact, TBC1D8B is a relatively new molecule because we could find only 4 studies about TBC1D8B until today, based on a literature search at PubMed.

MATERIALS AND METHODS

Reagents. The live/dead cell staining kit II (PKCA707-3002) was procured from PromoCell (Heidelberg, Germany). The Apoptotic DNA Ladder Isolation Kit (ab65627) for cultured cells was purchased from Abcam (Cambridge, UK). Anti-caspase 3 (9662), anti-phospho-eIF2α (eukaryotic initiation factor 2) (Ser51) (3597), anti-eIF2α (5234), anti-α-tubulin (2144), and poly ADP-ribose polymerase (PARP) (9542) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-FLAG M2 antibody (F1804) was supplied by Sigma-Aldrich (St. Louis, MO, USA). Anti-TBC1D8B antibody (PAS-71263) was purchased from Thermo Fisher Scientific (Waltham, MA, USA), and anti-coronin 1B antibody (sc-271445) from Cosmo Bio (Tokyo, Japan). Anti-Myc Tag Antibody (9E10) (05-419) was purchased from Sigma-Aldrich. Horseradish peroxidase-conjugated anti-rabbit (31460) and anti-mouse (31437) IgG antibodies were acquired from Thermo Fisher Scientific (Waltham, MA, USA). All cell culture media and reagents were purchased from Thermo Fisher Scientific. All other chemicals used in this study were supplied by Sigma-Aldrich. The quantitative polymerase chain reaction (qPCR) probe for the N-terminal TBC1D8B mRNA was purchased from Thermo Fisher Scientific.

Expression vector. Human N-terminal TBC1D8B was purchased from ORIGENE Technology (RC215906; currently commercially not available), which was identified as N-terminal TBC1D8B by sequencing, and full length, wild type TBC1D8B (WT-TBC1D8B) from mouse was also purchased from ORIGENE Technology (MR219347).

Cloning of C-terminal TBC1D8B. PCR was performed to obtain cDNA of C-terminal TBC1D8B using mouse WT-TBC1D8B as a template. The forward primer was GAAAAGCTTATGACAGTTTTCACCCT in length including amino acid positions 436 to 441 and the reverse primer was CGTGGATCCCATCTTGGTTCT. The PCR product was confirmed by sequencing.

Cell culture. The human colon cancer cell line HCT116 (ATCC CCL-247) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HCT116 cells were maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum. The cells were grown to subconfluence, collected, and frozen in liquid nitrogen. They were kept at −80°C until further use. The medium was collected by pipetting and transferred to the 1.5 mL Eppendorf tube to analyze the suspended cells in the culture medium. After that, the samples were centrifuged at 900 rpm at 18°C for 10 min. The supernatant was carefully removed, and the cell pellets were stored at −80°C until further use (the typically collected pellet is shown in Fig. 4B).

Transfection. The HCT116 cells were electroporated with 300 μg of plasmid DNA at 950 μF and 0.2 kV, as described previously (Saito et al. 2003). Under these conditions, 70% of cells were functionally transfected, as determined by in situ staining for β-galactosidase activity.

Evaluation of mRNA levels via qPCR. The N-terminal TBC1D8B mRNA levels were analyzed by real-time qPCR as previously described (Sunaga et al. 2014). The primers and probes were obtained from Applied Biosystems (Tokyo, Japan; Assay ID: Hs02786624_g1). The quantitative analysis was per-
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A. Sequence of amino acid of N-terminal TBC1D8B

MVLKLPVEVLL KNAKLKLWLE RSNDYVYVLQR BBGNYEGGG GLTGGLYGT dsYLAlTAK APFRILHTQF DSYQVYLSCAC GANKREETKH WDYLQSMNIMK TLSSFSDNSED ITNFGQGKIR GLIAEERGKH FAKEDDPKFE

REALKKEFC FGLPEEKEVLV VTYSSCVWKG RVPQCQWYL STNLSFYSISF LGGEKLLI SWDEVSSKLEK TSNVILTSH HVCQCGENHY FSMLFHQNDT YLLMEQLANY AIRRLFDFKET FIDNPYVLYNP LQITKBGLEN RABSEEQNFA FRPLKGEGSLK EVINELFDLWYP FSBEFNLHGKM CISENYICFA SQDGQCSV1 ITRGLIVLAID

KTNDSSSKV SIISKGTAFR FEYKDFEQL VAKLRLRCCGA ASTQVHIDT ELAISSETE PSDKFVQSL

TGQECG GLLFLL* (*STOP CODON)

428 435 436

B. Structure of wild type (WT-TBC1D8B), N-terminal, and C-terminal TBC1D8B

Fig. 1 Structure of the full length (wild type) TBC1D8B (WT-TBC1D8B), the N-terminal TBC1D8B, and the C-terminal TBC1D8B. A. Amino acid sequence of the N-terminal TBC1D8B. Note the difference between the human WT-TBC1D8B and N-terminal TBC1D8B from amino acid 428 because of the premature introduction of a stop codon (*) by frameshift (KTVNTEALM to ECGGLFLL*). B. Structure of the WT-TBC1D8B, the N-terminal TBC1D8B, and the C-terminal TBC1D8B. The N-terminal TBC1D8B contains two GRAM domains (presented by light blue color) but does not possess the TBC domain (presented by yellow color), whereas the C-terminal TBC1D8B contains the TBC domain but neither of the GRAM domains.

Apoptosis DNA ladder method. According to the manufacturer’s instructions, the apoptosis DNA ladder method was performed using the Apoptosis DNA Ladder Assay Kit. Cells were briefly lysed with the Tris-EDTA lysis buffer and mixed with the enzyme solution supplied with the kit. After DNA isolation and purification, the samples were analyzed with agarose gel electrophoresis. The ethidium bromide-stained DNA was visualized by ultraviolet transillumination.

Cell viability assay. Cell viability assay was performed according to the manufacturer’s instructions. Cells were briefly lysed with the Tris-EDTA lysis buffer and mixed with the enzyme solution supplied with the kit. After DNA isolation and purification, the samples were analyzed with agarose gel electrophoresis. The ethidium bromide-stained DNA was visualized by ultraviolet transillumination.

Statistical analyses. All data are expressed as the mean ± standard deviation. Data were analyzed using a one-factor analysis of variance to compare the means among all groups. The Tukey–Kramer multiple comparison procedure from InStat 2.00 was used to determine the statistical differences between

Immunoblotting. The thawed HTC116 cells were gently agitated for 10 min at 4°C with NP-40 lysis buffer containing 25 mM HEPES (pH 7.4), 10% glycerol, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 137 mM sodium chloride, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μM/L aprotinin, 1 μg/mL pepstatin, and 5 μg/mL leupeptin. Insoluble material was separated from the soluble extract by centrifugation. Total supernatant protein was quantified by BCA assay, and samples were normalized by total protein content. Samples were resuspended in sodium dodecyl sulfate (SDS) sample buffer and heated at 100°C for 5 min. The samples were separated by SDS polyacrylamide gel electrophoresis using standard (18 × 16 cm) or large (18 × 24 cm) gels. Then, they were electrophoretically transferred to polyvinylidene difluoride membranes. The samples were immunoblotted with specific primary antibodies, followed by conjugation with an appropriate secondary antibody and visualized.
Confirmation of N-terminal TBC1D8B and WT-TBC1D8B expression

The N-terminal TBC1D8B is neither recognized by the TBC1D8B antibody for western blotting nor by the Myc tag antibody because the stop codon appears before the Myc tag position, which was at the C-terminus end. The qPCR was selected to detect the expression of N-terminal TBC1D8B. We confirmed the presence of a considerable amount of N-terminal TBC1D8B mRNA by electroporation, using a mammalian expression vector (Fig. 2A).

Contrarily, WT-TBC1D8B expression was confirmed by western blotting (Fig. 2B, left panel). A large gel was used to separate exogenously introduced WT-TBC1D8B and endogenous TBC1D8B (Fig. 2B, left panel). However, the signal of WT-TBC1D8B was found to be much weaker than that of endogenous TBC1D8B (Fig. 2A, left panel). We attributed the difference in signal strength to a species difference because WT-TBC1D8B was de-
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Therefore, we considered that the structure involved in the N-terminal TBC1D8B was enough for the WT-TBC1D8B to cause apoptosis.

Effect of N-terminal TBC1D8B on caspase 3 and PARP cleavage

Next, we decided to confirm whether the N-terminal TBC1D8B, as a variant form of TBC1D8B, can induce spontaneous apoptosis in cultured cancer cells without any other apoptotic stimuli. To do this, we studied the effect of N-terminal TBC1D8B on caspase 3 and PARP cleavage status. As shown in Fig. 4A, the suspended cell amounts in the culture medium from the N-terminal TBC1D8B overexpression increased significantly compared to that of control cells represented as pcDNA3.1. Contrarily, the adherent cells in the case of the N-terminal TBC1D8B were slightly more substantial than that of WT-TBC1D8B. C. DNA ladder method. The apoptotic DNA fragmentation was qualitatively analyzed using the DNA Ladder method. Experiments were independently performed in triplicate and representative results are presented.

Effect of various types of TBC1D8B on the HCT116 cell viability

The exogenously introduced WT- and N-terminal TBC1D8B significantly reduced the number of HCT116 cells as compared with controls with pcDNA3.1 plasmid DNA (Fig. 3A). We found no change to C-terminal TBC1D8B. The expression of C-terminal TBC1D8B and WT-TBC1D8B was confirmed by western blot analysis (Fig. 3B).

We determined the effects of overexpressed WT- and N-terminal TBC1D8B on apoptosis to identify the mechanism involved in reducing cell numbers. As depicted in Fig. 3C, both the WT-TBC1D8B and N-terminal TBC1D8B overexpression increased the occurrence of the apoptosis DNA ladder. However, the C-terminal TBC1D8B overexpression did not show the occurrence of the apoptosis DNA ladder. Therefore, we considered that the structure involved in the N-terminal TBC1D8B was enough for the WT-TBC1D8B to cause apoptosis.

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Fig. 4 Effect of the N-terminal TBC1D8B overexpression on caspase 3 and PARP cleavage. 

A. Estimation of the number of suspended and adherent cells in culture. The closed bar represents the cells in which pcDNA3.1 plasmid DNA was introduced. The hatched column represents cells in which the N-terminal TBC1D8B was overexpressed. 

B. Collected suspended cells as a pellet from the culture medium. 

C. The effect of the N-terminal TBC1D8B overexpression in cultured HCT116 cells on caspase 3 cleavage. Uncleaved and cleaved caspase 3 status is shown in the top and bottom panel, respectively. Note that the images are of the same caspase 3 blotting, but the exposure period of the top panel was much shorter than that of the bottom panel. 

D. The effect of the N-terminal TBC1D8B overexpression in the cultured HCT116 cells on the PARP cleavage. Uncleaved and cleaved PARP statuses are shown in the top and bottom panels, respectively. Note that the exposure period of the top panel (10 seconds) was much shorter than that of the bottom panel (10 minutes exposure). Red * represents cleaved PARP in the bottom panel.

Fig. 5 Effect of the N-terminal TBC1D8B overexpression on the coronin 1B expression and the elf2α (S51) phosphorylation. 

A. Effect of the N-terminal TBC1D8B on the coronin 1B expression. The effect of the N-terminal TBC1D8B overexpression on the coronin 1B expression in the cultured HCT116 cells is shown compared with that of control cells with the pcDNA3.1 plasmid DNA. 

B. Effect of the N-terminal TBC1D8B on the elf2α expression and the elf2α (S51) phosphorylation. The effect of the N-terminal TBC1D8B overexpression in the cultured HCT116 cells on the elf2α expression (lower panel) and the elf2α (S51) phosphorylation (upper panel) compared with that of the controls (pcDNA3.1).
shows the collected suspended cells as described in the materials and methods section. The left picture represents cells in which pcDNA3.1 plasmid DNA was introduced, and the right picture represents the cells in which the N-terminal TBC1D8B plasmid DNA was introduced.

Fig. 4C shows decreased uncleaved caspase 3 and a corresponding increase of cleaved caspase 3 in suspended cells overexpressing the N-terminal TBC1D8B compared with that in controls. There was no change of caspase 3 cleavage status in adherent cells compared with that in control cells. Similarly, Fig. 4D shows a reduced signal of uncleaved PARP with a concomitant increase of cleaved PARP in suspended cells overexpressing N-terminal TBC1D8B. In contrast, we found no change in PARP cleavage status in adherent cells. These findings indicate that the N-terminal TBC1D8B can spontaneously induce apoptosis without any other stimulator or inducer of apoptosis.

We focused on two additional markers, coronin 1B and eIF2α to further understand the mechanism by which the N-terminal TBC1D8B induces spontaneous apoptosis in HCT116 cells. First, we examined whether coronin 1B expression was increased in HCT116 cells, overexpressing N-terminal TBC1D8B. Coronin 1B has a known role in caspase 3 cascade activation that leads to apoptosis, and reduction of coronin 1B expression rescues cultured cells from TNF-α-induced apoptosis (Kim et al. 2020). Fig. 5A shows that the overexpression of the N-terminal TBC1D8B dramatically increased the coronin 1B expression in HCT116 cells. As the same membrane was stripped and reused after the coronin 1B detection for eIF2α blotting, the loading control was confirmed by eIF2α blotting (Fig. 5B).

Next, we examined the phosphorylation status of the eIF2α at serine 51 (S51) in the presence or absence of phosphorylated eIF2α (S51). A reduction of eIF2α (S51) phosphorylation was previously reported to have an antitumorigenic effect, although the phosphorylated eIF2α is known to act as either an inducer of cell death or mediator of cell survival in response to distinct forms of stress (Koromilas et al. 2013). Fig. 5B shows that the overexpression of N-terminal TBC1D8B dramatically reduced the eIF2α (S51) phosphorylation in the HCT116 cells compared with that found in cells with the pcDNA3.1 plasmid DNA. Furthermore, we confirmed that neither the electroporation nor the reduction of eIF2α (S51) phosphorylation affected eIF2α expression status (Fig. 5B, lower panel).

DISCUSSION

We had unique access to a cDNA of the N-terminal TBC1D8B obtained from a human cancer tissue. As a result, in the current study, we tested the effect of three different TBC1D8B constructs namely, WT-TBC1D8B, N-terminal TBC1D8B, and C-terminal TBC1D8B, overexpressed in cultured HCT116 tumor cells.

The overexpression of WT-TBC1D8B in the HCT116 cells resulted in an increased number of suspended cells due to apoptosis and a corresponding reduction in the number of adherent live cells in culture. Similarly, the N-terminal TBC1D8B overexpression caused an increase in the number of apoptotic suspended cells and decreased the number of live adherent cells. However, the C-terminal TBC1D8B overexpression did not affect the viability of either suspended or adherent cancer cells in culture. Thus, constituting nearly 40% of the full protein from amino acids 1 to 435, including the two GRAM domains, N-terminal TBC1D8B was sufficient to cause apoptosis.

This finding illustrates a new physiological role of TBC1D8B in apoptosis, in addition to previous findings that TBC1D8B functions as a Rab-GAP by binding to specific Rab proteins, stimulating their GTPase activity (Fukuda, 2011). Furthermore, those cells detached from the culture dish in response to the N-terminal TBC1D8B overexpression showed the increased appearance of the apoptosis DNA ladder and reduced levels of uncleaved caspase 3 and PARP as well as increased levels of cleaved caspase 3 and PARP, whereas the levels of cleaved and uncleaved caspase 3 and PARP were unchanged in the adherent cells exposed to the overexpressed N-terminal TBC1D8B.

These results suggest that overexpression of N-terminal TBC1D8B alone was sufficient to cause apoptosis in the absence of other known inducers. These results also indicate that this protein fragment consisting of amino acids 1 to 435 and including the two GRAM domains, is enough to cause spontaneous apoptosis. Because the overexpressed C-terminal TBC1D8B did not affect the cultured cancer cells, we conclude that the TBC domain and its role as a Rab-GAP are not required for effects of N-terminal TBC1D8B and WT-TBC1D8B on the viability of cultured cancer cells.

Lastly, we investigated how the N-terminal TBC1D8D overexpression in the HCT116 cells caused an increased number of suspended cells due to apoptosis and reduced adherent live cells in cul-
ture. We found that the overexpression of N-terminal TBC1D8B increased the coronin 1B expression and decreased the S51 phosphorylation of eIF2α. Both of them indicate, at least in part, how the N-terminal TBC1D8B overexpression may cause spontaneous apoptosis in the absence of any other apoptotic stimuli. There is evidence that some GRAM domain-containing proteins such as GRAMD1A and GRAMD1B, promote cancer cell growth. Thus, we hypothesize that the overexpressed N-terminal TBC1D8B may suppress GRAMD1A and GRAMD1B by competing through the GRAM domains (Wu et al. 2014; Fu et al. 2016). However, the precise mechanism of how the N-terminal TBC1D8B increases the coronin 1B expression and decreases S51 phosphorylation of eIF2α needs to be elucidated.

In conclusion, we reported a couple of new findings of TBC1D8B. Although the WT-TBC1D8B overexpression causes apoptosis without apoptotic stimuli, the N-terminal TBC1D8B consisting of amino acids 1 to 435, including the two GRAM domains, is enough to cause spontaneous apoptosis. Thus, the TBC domain for Rab-GTPase activity is not necessary for TBC1D8B to induce apoptosis. TBC1D8B was previously reported to associate with nephrotic syndrome (Huber et al. 2006; Dorval et al. 2019), and we opened a new possible angle for TBC1D8B to be a regulator for apoptosis. These results suggested that TBC1D8B could be a potential anticancer therapeutic molecule.

CONFLICT OF INTEREST
The authors declare no conflicts of interest.

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