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Silencing of TBC1D15 promotes RhoA activation and membrane blebbing

Yuko Takahara · Masao Maeda · Hitoki Hasegawa · Satoko Ito · Toshinori Hyodo · Eri Asano · Masahide Takahashi · Michinari Hamaguchi · Takeshi Senga

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Abstract Membrane blebs are round-shaped dynamic membrane protrusions that occur under many physiological conditions. Membrane bleb production is primarily controlled by actin cytoskeletal rearrangements mediated by RhoA. Tre2–Bub2–Cdc16 (TBC) domain-containing proteins are negative regulators of the Rab family of small GTPases and contain a highly conserved TBC domain. In this report, we show that the expression of TBC1D15 is associated with the activity of RhoA and the production of membrane blebs. Depletion of TBC1D15 induced activation of RhoA and membrane blebbing, which was abolished by the addition of an inhibitor for RhoA signaling. In addition, we show that TBC1D15 is required for the accumulation of RhoA at the equatorial cortex for the ingression of the cytokinetic furrow during cytokinesis. Our results demonstrate a novel role for TBC1D15 in the regulation of RhoA during membrane blebbing and cytokinesis.

Keywords Membrane blebbing · RhoA · TBC1D15 · Rab · Cytokinesis

Y. Takahara · M. Maeda · H. Hasegawa · S. Ito · T. Hyodo · E. Asano · M. Hamaguchi · T. Senga (✉)
Division of Cancer Biology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan
e-mail: tsenga@med.nagoya-u.ac.jp

M. Takahashi
Department of Pathology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

Introduction

Membrane blebs are dynamic, spherical protrusions that are observed under various experimental conditions. Blebs typically expand up to 2 μm from the plasma membrane and retract to the exact position from where they initiated [1, 2]. Blebbing is initiated by the localized detachment of plasma membrane from the cortical cytoskeleton and hydrostatic pressure of the cytoplasm [3–5]. Following bleb formation, actomyosin meshwork is organized at the bleb cortex, and a contractility force is generated to retract the bleb [6]. The dynamics of membrane blebs critically depend on the remodeling of the submembranous actin cytoskeleton. One of the major factors that regulates the remodeling of the actin cytoskeleton during membrane blebbing is the Rho family of small GTPases [7, 8]. Activation of RhoA and its effector kinase, Rho-associated kinase (ROCK), promotes the phosphorylation of myosin light chain, which induces actomyosin contraction [9, 10]. Accumulating studies have shown that the RhoA–ROCK pathway is crucial for membrane blebbing; inhibition of this pathway significantly suppresses membrane bleb production [11–14].

The members of the Rab family of small GTPases are conserved regulators of multiple aspects of intracellular membrane trafficking and dynamics [15, 16]. The association and disassociation of Rab proteins with their effectors are tightly controlled by GDP-GTP exchange (activation) and GTP hydrolysis (inactivation) [17]. Cycling between the active and inactive state is primarily regulated by two distinct protein families. Exchange of GDP for GTP is mediated by guanine nucleotide exchange factors, whereas the hydrolysis of GTP for GDP is stimulated by GTPase activating proteins. The Tre2–Bub2–Cdc16 (TBC) domain-containing proteins are known to be Rab-specific GTPase-

activating proteins [18, 19]. Mutational and structural analyses of the TBC domain have shown that it has a conserved arginine residue, which is essential for its GAP activity [20, 21]. The TBC domain is conserved in a wide range of species, and mutations in some TBC domain-containing proteins are associated with human diseases [22–24]. There are more than 50 proteins with TBC domains in humans, but the functions of only a subset of these proteins have been investigated. TBC1D15, one of the TBC domain-containing proteins, is a widely expressed protein that promotes GTP Rab7 and Rab11 hydrolysis [25]. Recent studies have shown that TBC1D15 is associated with the regulation of mitochondrial morphology and stem cell renewal [26, 27]; however, the exact functions of TBC1D15 remain largely unknown. In this study, we show that TBC1D15 depletion induces the activation of RhoA and the production of membrane blebs. In addition, we show that TBC1D15 is required for the proper localization of RhoA at the equatorial cortex during the ingression of the cytokinetic furrow.

Materials and methods

Cells, antibodies, and chemicals

HeLa cells were maintained in DMEM supplemented with 10 % FBS. Anti-TBC1D15 antibody was generated by injecting 200 μg of GST-TBC1D15 (aa 67–aa 125) mixed with Freund adjuvant (Sigma-Aldrich, Taufkirchen, Germany) into a rabbit four times every 2 weeks. Serum was obtained, and anti-TBC1D15 antibody was purified using an NHS-activated column (GE Healthcare BioSciences, Uppsala, Sweden) coupled with GST-TBC1D15 (aa 67–aa 125). Anti-GST antibody was eliminated using recombinant GST. The antibody for RhoA was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-GFP antibody was purchased from MBL (Nagoya, Japan), and anti-β-actin and anti-β-tubulin antibodies were from Sigma-Aldrich. A Rho kinase inhibitor, Y27632, was obtained from Funakoshi (Osaka, Japan).

DNA constructs

Human TBC1D15, Rab7, and Rab11 were amplified by PCR from a HeLa cDNA library and cloned into a pQCXIP retroviral vector with a GFP tag on the N-terminus (Clontech, Mountain View, CA, USA). GFP-tagged siRNA-resistant TBC1D15 (Mut-TBC1D15), siRNA-resistant GAP-inactive TBC1D15 (Mut-TBC1D15(R417A)), the active form of Rab7 (Q67L), and the active form of Rab11 (Q70L) were generated using PCR-based mutagenesis. Substitution of the amino acids was confirmed by DNA sequencing.

Rho GTPase activity assay

Cells were lysed with pull-down lysis buffer [25 mM Tris–HCl, pH 7.4; 150 mM NaCl; 10 % glycerol; 1 % NP40; 5 mM $MgCl_2$; protease inhibitor cocktail (Roche, Basel, Switzerland); and 1 mM PMSF] and incubated with GST-Rhotekin-RBD fusion protein bound to glutathione-agarose beads for 1 h at 4 °C. The beads were washed with pull-down buffer four times and then subjected to western blot analysis with anti-RhoA antibody to detect active RhoA. Total protein was detected by immunoblotting of whole cell lysates.

siRNA transfection

The sequences of the siRNAs used to suppress TBC1D15 expression were 5′-UAAUCUUCCCGCUCACAACACC CGC-3′ (siRNA #1) and 5′-GAACCAGGAUUUGAAGU CAUCACAA-3′ (siRNA #2), the sequence of the control siRNA targeting luciferase was 5′-CUUACGCUGAGUAC UUCGATT-3′. siRNA #1 and siRNA #2 were obtained from Invitrogen (Carlsbad, CA, USA), and the control siRNA was from Sigma-Aldrich. Cells were transfected with 20 nM of siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Immunofluorescence analysis

Cells were grown on glass coverslips coated with fibronectin, fixed with 10 % TCA (for RhoA staining) or 4 % paraformaldehyde (for β-tubulin staining) for 20 min, permeabilized with 0.5 % Triton X-100 for 3 min, and blocked with phosphate-buffered saline (PBS) containing 7 % fetal bovine serum for 30 min. Cells were incubated with anti-RhoA antibody in PBS for 1 h, washed three times with PBS, incubated with Alexa Fluor 594-labeled secondary antibody in PBS for 1 h, and then analyzed using a fluorescence microscope (BX60; Olympus, Tokyo, Japan).

Generation of stable cell lines

HeLa cell lines that constitutively expressed Wt-TBC1D15, Mut-TBC1D15, Mut-TBC1D15(R417A), Mut-TBC1D15 ΔTBC, or Mut-TBC1D15TBC were established using retroviral infection. pQCXIP vectors encoding each cDNA were transfected to 293T cells in combination with the pVPack-GP and pVPack-Ampho vectors (Stratagene, Tokyo, Japan) using Lipofectamine 2000 (Invitrogen). 48 h after transfection, the supernatants were added to the cells

with 2 μg/ml polybrene (Sigma-Aldrich), and infected cells were selected with 1 μg/ml puromycin for 3 days.

Time-lapse imaging

HeLa cells cultured on glass-bottomed dishes were transfected with siRNA; 48 h later, the cells were monitored using a time-lapse microscope system (LCV110, Olympus). Images were acquired and analyzed using the MetaMorph Imaging System (Universal Imaging, Silicon Valley, CA, USA).

Statistical analysis

To determine the percentage of cells with membrane blebs, 100 cells were evaluated in each experiment. Three independent experiments were performed, and the results were compared using Student's *t* test. The data are presented as the mean ± SD.

Results

While we were searching for genes associated with cytokinesis using a limited siRNA library [28], we noticed that transfection of two different siRNAs targeting TBC1D15 significantly induced membrane blebbing (Fig. 1a). We counted cells with membrane blebs and found that nearly 30 % of TBC1D15-depleted cells showed a robust production of membrane blebs, whereas less than 5 % of control siRNA-transfected cells formed blebs (Fig. 1b). To confirm the efficiency of siRNA at depleting TBC1D15, we generated an antibody that specifically detects the protein. Immunoblotting with the antibody revealed that the transfection of both siRNAs efficiently depleted the TBC1D15 protein (Fig. 1c).

To further confirm that TBC1D15 knockdown promotes membrane blebbing, we performed rescue experiments. We first established HeLa cell lines that constitutively expressed GFP-tagged wild type TBC1D15 (Wt-TBC1D15) or mutant TBC1D15 (Mut-TBC1D15), which contains silent mutations providing resistance to TBC1D15 siRNA #1. Wt-TBC1D15 was sufficiently depleted by the siRNA transfection, whereas the expression of Mut-TBC1D15 was not affected (Fig. 2a). A number of Wt-TBC1D15-expressing cells showed membrane blebs after TBC1D15 siRNA transfection, whereas most of the Mut-TBC1D15-expressing cells had no blebs (Fig. 2b). We counted cells with blebs after TBC1D15 siRNA transfection and found that the number of blebbing cells was significantly reduced in the Mut-TBC1D15 cells compared with the Wt-TBC1D15 cells. These results show that

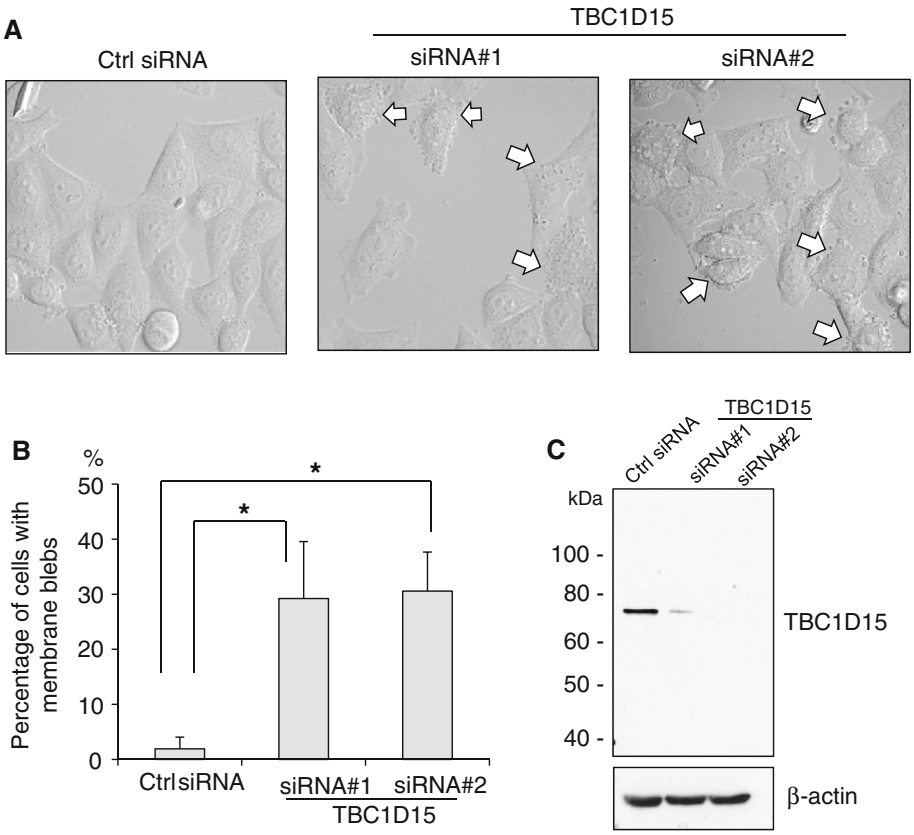

Fig. 1 Depletion of TBC1D15 promotes membrane blebbing. **a** HeLa cells were transfected with the indicated siRNA, and 72 h later, images were taken to visualize cellular morphology. The *arrows* indicate blebbing cells. **b** HeLa cells were treated as in (**a**), and the cells with membrane blebs were counted. The *graph* indicates the percentage of cells with blebs (mean ± SD; $*P < 0.05$). **c** HeLa cells were transfected with siRNA, and 72 h later, the cells were lysed and immunoblotted with anti-TBC1D15 antibody

TBC1D15 is essential for the suppression of membrane blebbing.

Activation of RhoA is one of the crucial factors that promote cell blebbing; thus, we examined the activity of RhoA in the absence of TBC1D15. HeLa cells were transfected with siRNA, and 72 h later, the cells were lysed and incubated with GST-Rhotekin-RBD to affinity precipitate active RhoA. As shown in Fig. 3a, RhoA was activated in TBC1D15 siRNA-transfected cells. We next examined whether the activation of ROCK by RhoA was responsible for the induction of membrane blebbing. HeLa cells were transfected with TBC1D15 siRNA #1, and 72 h later, cells treated with the ROCK inhibitor Y23672 were live-imaged using time-lapse microscopy. Membrane blebbing was significantly suppressed upon the addition of the inhibitor (Fig. 3b). Nearly 30 % of cells formed blebs after TBC1D15 depletion, but after the inhibitor treatment, membrane blebbing was observed in only approximately 5 % of cells. These results clearly indicate that RhoA–ROCK activation was essential for the production of membrane blebs after TBC1D15 knockdown.

The TBC domain of TBC1D15 is known to stimulate the GTP hydrolysis of Rab7 and Rab11 [25]; thus, depletion of TBC1D15 in cells promotes the activation of Rab7 and Rab11. To determine whether the activation of Rab7 or Rab11 was responsible for blebbing, we created constructs expressing the active forms of Rab7 and Rab11 and expressed them in HeLa cells. As shown in Fig. 3c, the expression of the active form of either protein did not promote cell blebbing. We next tested whether the GAP activity of TBC1D15 was required for the suppression of membrane blebs. It has been reported that substitution of the conserved arginine to alanine disrupts the catalytic activity of the TBC domain [20]. We generated siRNA #1-resistant Mut-TBC1D15(R417A), where the conserved arginine is replaced with alanine, and established a HeLa cell line that constitutively expressed Mut-TBC1D15 (R417A) using retroviral infection. Mut-TBC1D15 and Mut-TBC1D15(R417A) cells were transfected with TBC1D15 siRNA #1, and 72 h later, the cells were fixed, and the blebbing cells were counted. If the GAP activity of the TBC domain is required for the suppression of membrane blebbing, the expression of Mut-TBC1D15(R417A) would not inhibit cell blebbing after TBC1D15 depletion. However, Mut-TBC1D15(R417A) expression did suppress membrane blebbing at a level similar to Mut-TBC1D15 expression (Fig. 3d). We also created a HeLa cell line that constitutively expressed Mut-TBC1D15ΔTBC (aa1–aa342), in which the TBC domain was deleted, and examined cell blebbing after siRNA

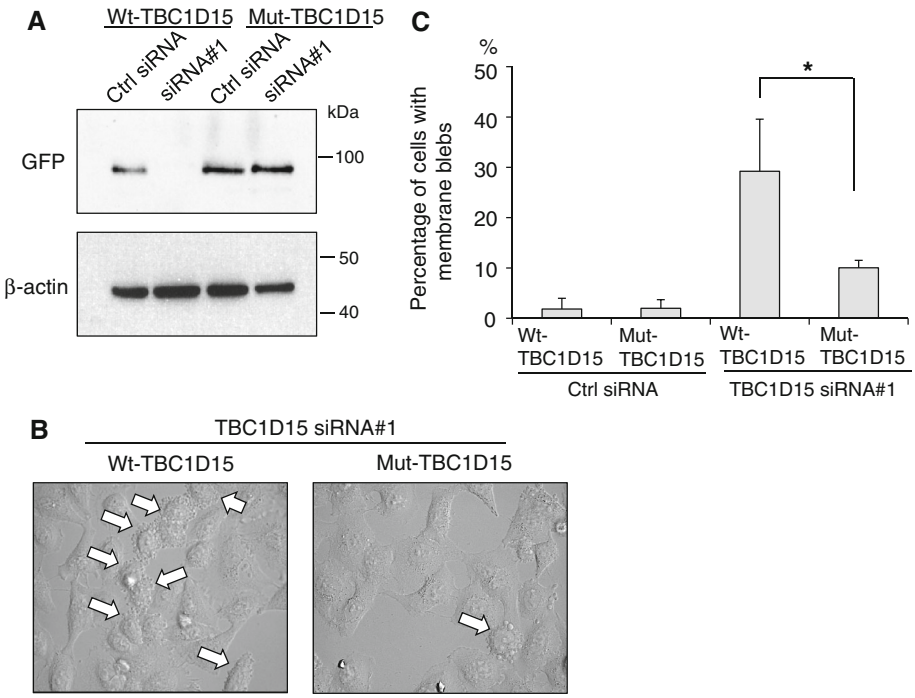

Fig. 2 Expression of siRNA-resistant TBC1D15 inhibits membrane blebbing induced by TBC1D15 depletion. **a** HeLa cells that constitutively expressed GFP-tagged wild type TBC1D15 (Wt-TBC1D15) or siRNA #1-resistant TBC1D15 (Mut-TBC1D15) were established by retroviral infection. Cells were transfected with control siRNA or TBC1D15 siRNA #1, and 72 h later, the cells were lysed and immunoblotted with anti-GFP antibody. **b** Wt-TBC1D15 and Mut-TBC1D15 cells were transfected with TBC1D15 siRNA #1, and 72 h later, images were taken to visualize cellular morphology. The *arrows* indicate blebbing cells. **c** Wt-TBC1D15 and Mut-TBC1D15 cells were transfected control siRNA or TBC1D15 siRNA #1, and 72 h later, the number of cells with membrane blebs was counted. The *graph* indicates the percentage of cells with blebs (mean ± SD; $^*P < 0.05$)

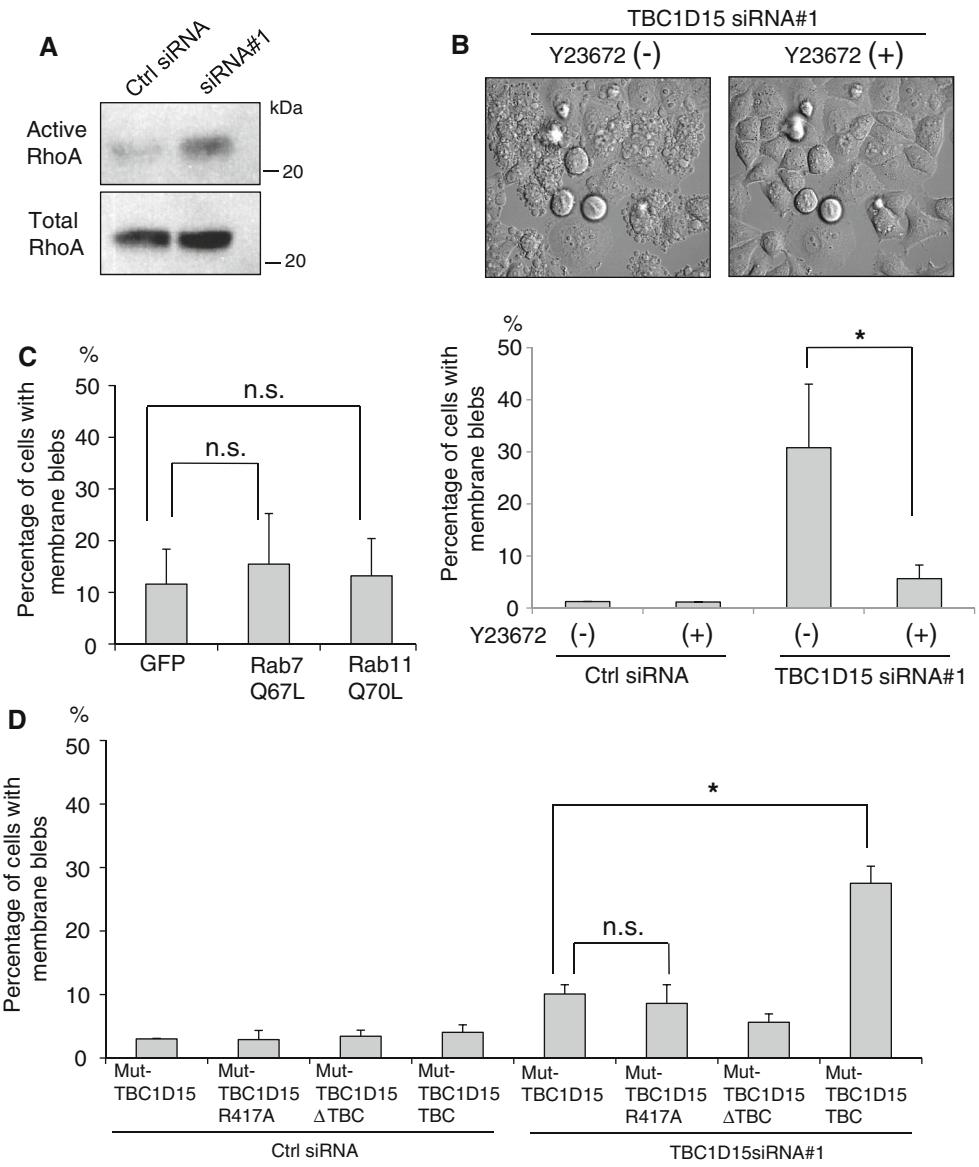

Fig. 3 Rho GTPase is activated by TBC1D15 knockdown. **a** HeLa cells were transfected with control siRNA or TBC1D15 siRNA #1, and 72 h later, the cells were lysed and mixed with GST-Rhotekin-RBD bound to glutathione-agarose beads to precipitate the active form of the Rho GTPase. The immunoprecipitates were subjected to immunoblot analysis with anti-RhoA antibody. **b** HeLa cells were transfected with control siRNA or TBC1D15 siRNA #1, and 48 h later, cells were observed using time-lapse microscopy. Images of cells before and after the addition of Y27632 are shown. The *graph* indicates the percentage of cells with blebs (mean ± SD; $*P < 0.05$). **c** HeLa cells were transfected with plasmids encoding GFP or a GFP-tagged active form of either Rab7 or Rab11, and 48 h later, the cells with membrane blebs were counted. The *graph* indicates the percentage of cells with blebs (mean ± SD; n.s., not significant). **d** HeLa cells that constitutively expressed the indicated protein were transfected with control siRNA or TBC1D15 siRNA #1, and 72 h later, the cells with membrane blebs were counted. The *graph* indicates the percentage of cells with blebs (mean ± SD; n.s., not significant, $*P < 0.05$)

transfection. Consistent with the result of Mut-TBC1D15 (R417A) cells, Mut-TBC1D15ΔTBC expression suppressed membrane blebbing. In contrast, Mut-TBC1D15TBC (aa343–aa691), which has the TBC domain, did not inhibit membrane blebbing. These results show that the TBC domain is not required for the suppression of cell blebbing.

In addition to the production of membrane blebs, we noticed that TBC1D15 depletion induced multinuclear cells. As shown in Fig. 4a, more than 10 % of TBC1D15-depleted cells became multinuclear, whereas only 2 % of the control siRNA-transfected cells were multinuclear (Fig. 4a). Induction of multinuclear cells by TBC1D15 siRNA #1-transfection was significantly reduced by Mut-TBC1D15 expression (Fig. 4b). We used time-lapse microscopy to examine which stage of cell division was disrupted by TBC1D15 knockdown. A number of TBC1D15-depleted cells formed membrane blebs during anaphase and showed aberrant ingression of the cytokinetic furrow (Fig. 4c). Some of these cells failed to complete the ingression of the furrow and eventually became binuclear. RhoA is one of the critical factors for the ingression of the cytokinetic furrow [29, 30]. RhoA is localized at the equatorial cortex during anaphase and promotes formation and ingression of the cytokinetic furrow. We tested whether the localization of RhoA was disrupted in the absence of TBC1D15 during anaphase. RhoA was localized at the equatorial cortex in most of the control siRNA-transfected cells. However, more than 40 % of TBC1D15 siRNA-transfected cells showed aberrant localization of RhoA during anaphase (Fig. 4d). RhoA was either totally displaced from the equatorial cortex or localized on only one side of the equatorial cortex (Fig. 4d). These results indicate that TBC1D15 is required for the

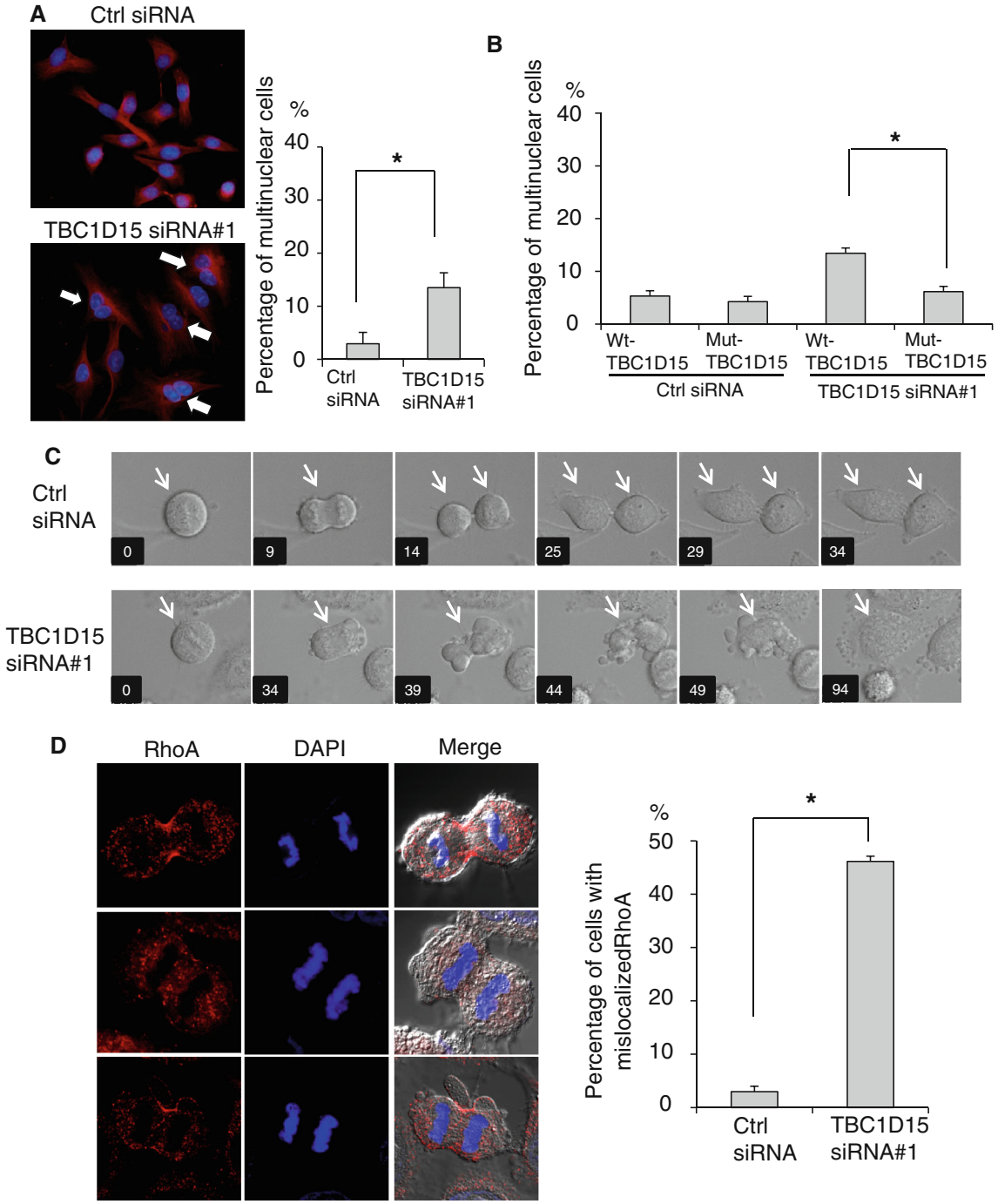

Fig. 4 Depletion of TBC1D15 induces aberrant localization of RhoA and furrow ingression during cytokinesis. **a** HeLa cells were transfected with siRNA, and 72 h later, the cells were fixed and immunostained with anti-β tubulin antibody and DAPI. Representative images are shown, and the *arrows* indicate multinuclear cells. The *graph* indicates the percentage of multinuclear cells (mean ± SD; $*P < 0.05$). **b** Wt-TBC1D15 and Mut-TBC1D15 cells were transfected with control siRNA or TBC1D15 siRNA #1 and 72 h later, cells were fixed and immunostained with anti-β tubulin antibody and DAPI to count multinuclear cells. The *graph* indicates the percentage of multinuclear cells (mean ± SD; $*P < 0.05$). **c** HeLa cells were transfected with siRNA, and 48 h later, cells were monitored by time-lapse microscopy. Representative images of dividing cells are shown. The *arrows* indicate dividing cells. **d** HeLa cells were transfected with siRNA, and 72 h later, the cells were immunostained with anti-RhoA antibody and DAPI. Representative images of cells in anaphase are shown. The *graph* indicates the percentage of cells with mislocalized RhoA in anaphase (mean ± SD; $*P < 0.05$). Fifteen cells in anaphase were evaluated for the localization of RhoA, and three independent experiments were performed

proper localization of RhoA during anaphase for the ingression of the cytokinetic furrow.

Discussion

In this report, we show a novel role for TBC1D15 in the maintenance of the intact morphology of cells. Depletion of TBC1D15 by two different siRNAs clearly induced membrane blebbing. Rescue experiments demonstrated that membrane blebbing after TBC1D15 siRNA transfection was not due to off-target effects of the siRNAs. These results clearly show that TBC1D15 expression is essential for the suppression of cell blebbing. The activation of RhoA and ROCK is known to play a pivotal role in the production of membrane blebs. RhoA was activated by TBC1D15 depletion, and the inhibition of RhoA–ROCK signaling by the inhibitor abolished the induction of membrane blebbing. Thus, RhoA activation is crucial for the promotion of blebbing in the absence of TBC1D15 expression. We speculated that the activation of Rab proteins was associated with RhoA activation after TBC1D15 knockdown because previous studies have shown crosstalk between Rab proteins and Rho family proteins [31]. However, the TBC domain was not associated with the induction of blebbing; therefore, TBC1D15 appears to regulate RhoA independent of the TBC domain and the inactivation of Rab proteins.

We also show that the depletion of TBC1D15 induced aberrant ingression of the cytokinetic furrow during cytokinesis. Once the duplicated chromosomes are separated in anaphase, RhoA accumulates at the cell cortex of the division plane to initiate formation and ingression of the cytokinetic furrow [29]. RhoA activates downstream effectors such as ROCK and MLCK, which subsequently promote the activation of myosin II for the constriction of the cytokinetic furrow [32, 33]. In the absence of TBC1D15 expression, membrane blebs were organized in anaphase, and RhoA was displaced from the equatorial cortex. These results suggest that TBC1D15 is required for the suppression of membrane blebbing and the proper accumulation of RhoA at the equatorial cortex during cytokinesis.

In summary, we have shown that TBC1D15 is essential for the suppression of membrane blebbing. In addition, membrane blebbing after TBC1D15 knockdown was dependent on the activation of RhoA. Future analysis of the mechanism by which TBC1D15 regulates RhoA may reveal novel regulatory mechanisms of RhoA activity and membrane blebbing.

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